

# 1807 De-esterified pectins in the cell walls of cotton fibers: a study of fiber mutants

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**Abbreviations:** CDTA, trans-1,2-diaminocyclo-hexane-N,N,N',N'-tetra-acetic acid; DP, Delta and Pine Land; DAA, days after anthesis; IEF, isoelectric focusing; PME, pectin methylesterase; PMEI, pectin methylesterase inhibitor; SDS, sodium dodecyl sulfat; 2-D PAGE, two dimensional polyacrylamide gel electrophoresis.

## ABSTRACT

In the wild-type cotton (DP 5690), the cell walls of elongating cotton fibers are bilayered, with the outer layer enriched in de-esterified homogalacturonan (HGA), and an inner layer enriched in xyloglucans and cellulose. This bilayer is conspicuously absent in the cell walls of the ovule epidermal cells and in the fiber of the short fiber mutants Ligon lintless 1 and Ligon lintless 2. The highly-reduced quantity of de-esterified HGA in the Ligon lintless lines does not appear to be due to decreased pectin methylesterase (PME) activity, however. The use of a mild CDTA extraction of intact ovules (3 days after anthesis) releases similar amounts of PME activity from both normal and short fiber lines. Comparison of protein profiles derived from the CDTA extracts of each line indicates that a few differences occur between the “enriched cell wall” proteins of the wild-type and the Ligon lintless lines. Very few proteins are detectable in the fiberless SL 1-7-1 ovule profiles. The largest protein spot in the 2D PAGE profiles is an acidic protein (pI 4.4) with a molecular weight of 17 kDa and is unique to the Ligon lintless lines. Heterologous expression was used to produce a putative cotton PME1 protein in *Pichia pastoris*. These data indicate that a decrease in the levels of de-esterified HGA in the Ligon lintless lines are not caused by a decrease in PME, but by a decrease in PME activity by an environmental or enzymatic mechanism *in muro*.

**Keywords:** Fiber initiation; *Gossypium hirsutum*; JIM5; Ovule; Pectin; Trichomes.

## Introduction

Pectins consist of a group of complex carbohydrates rich in galacturonic acid which are categorized into three groups including homogalacturonan (HGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II). HGA is a homopolymer consisting of approximately 100-200 residues of (1 → 4)- $\alpha$ -D-galacturonic acid which can exist in various degrees of methylation (Ridley et al., 2001). Two monoclonal antibodies, JIM5 and JIM 7, bind to either highly-unesterified or highly-methylesterified HGAs, respectively, and are valuable tools in the evaluation of wall modification during cell development (Willats et al., 2001b). RG-I is a heterogenous compound consisting of approximately 100 repeats of the disaccharide (1 → 2)- $\alpha$ -L-rhamnose-(1 → 4)- $\alpha$ -D-galacturonic acid with many of the rhamnose residues C-4 substituted by predominantly neutral carbohydrate residues (Albersheim et al., 1996; O'Neill et al., 1990). RG-II has a highly conserved domain consisting of approximately 9 residue HGA backbone substituted with 4 conserved heteropolymeric side chains. These pectins are localized in the primary cell walls of all plants; however, no direct evidence exists as to whether RG-I and RG-II are attached to HGA *in muro* (Ridley et al., 2001; Willats et al., 2001a).

Pectins are versatile, multifunctional components of the cell wall. These complex carbohydrates have been implicated in numerous cellular functions during plant growth and development including a critical role in cell expansion and deposition of other cell wall components, such as cellulose (Chanliand and Gidley, 1999; Cosgrove, 2000; Goldberg et al., 1986; Kim and Carpita, 1992); in the regulation of extracellular pH and cell wall porosity (Baron-Epel et al., 1988; Demarty et al., 1984; Jarvis, 1984; Yu et al., 2002); in cellular adhesion (Knox, 1992); and as a source of oligosaccharides that can signal a defense response in plants

(Darvill et al., 1992; Dumville and Fry, 2000).

The cotton fiber originating from the ovule epidermis has become a model system for the study of cell development and elongation (Kim and Triplett, 2001). These fibers are unique in that they are unicellular trichomes which initiate growth between 0 to 4 days after anthesis (DAA) and elongate to approximately 2 to 3 cm (Stewart, 1975; Kim and Triplett, 2001). The presence of pectin in mature cotton fiber has been reported for years (Anderson and Kerr, 1938; Whistler et al., 1940). However, their presence in fibers has been little more than a distraction as emphasis in cotton cell wall research has focused mainly on the deposition and characterization of cellulose. Additional evidence for a role of pectin in fiber initiation comes from studies of boron deficiency in cotton ovule culture. Under these culture conditions, cotton ovules had either a severe reduction or complete elimination of fiber production, depending on the phytohormone treatment used in culture (Birnbaum et al., 1974). Presently, RG II is identified as the only boron binding compound in the cell (Kobayashi et al., 1996; O'Neill et al., 1996). Willats et al. (2001a) suggests that the presence of boron in RG II dimers may account for the plants requirement for boron. However, this is still a tentative proposal with the numerous affects that boron has on plant development (Läuchli, 2002).

Vaughn and Turley (1999) reported that during early fiber development, the cell wall has a bilayered ultrastructure. Immunolocalization with anti-PGA, JIM5 and JIM7 in 1 DAA fibers demonstrated the existence of a bilayered cell wall. This bilayer consisted of a xyloglucan-cellulose sub-layer ensheathed by a second layer enriched in de-esterified pectins (Vaughn and Turley, 1999). The non-elongating epidermal cells adjacent to the fibers, the epidermal ovule cells of the fiberless line SL 1-7-1, and fibers of the short-fiber mutants Ligon lintless 1 and

Ligon lintless 2 (fibers approximately 10% the length of normal fibers) have a greatly reduced de-esterified pectin layer (Vaughn and Turley, 1999; Turley and Vaughn, unpublished). The lack of de-esterified HGA in epidermal and slowly elongating fiber cells could indicate a role for these complex carbohydrates in cotton fiber elongation.

In this report, we evaluate pectin methylesterase (PME) which enzymatically controls the de-esterification of methylesterified HGA. We used relatively mild treatments to delicately extract cell wall proteins including PME using a CDTA/sorbitol solution from intact cells. The mild CDTA extraction procedure removes calcium from the cell wall, allowing the partial release of de-esterified homogalacturonans and cell wall proteins. The specific activity of PME was easily determined with this method. The total extracted protein was then used to evaluate cell wall proteins from wildtype and mutant fiber using 2-D PAGE. Numerous small molecular weight proteins were observed between 15 to 20 kDa which were unique to the Ligon lintless lines. We report on small candidate proteins, including a secreted PME inhibitor found in cotton, which may be involved in pectin biosynthesis/modification.

## **MATERIALS AND METHODS**

### **Plant Material**

Four inbred lines of cotton (*Gossypium hirsutum* L.), DP 5690 (PVP 9100116), SL 1-7-1 (PI 528807), Ligon lintless 1 and Ligon lintless 2 were grown in the field and greenhouse in multiple years between 2000 and 2007. DP 5690 was obtained from Delta and Pine Land Company, Scott, MS, USA, and used as the wildtype line. The SL 1-7-1 and Ligon lintless 1 lines were a gift from Dr. Reiner Kloth and Ligon lintless 2 was a gift from Dr. James “Mac”

Stewart. Plots were five meters in length and initially over-seeded. After the plants reached the first true leaf stage, they were thinned to approximately 6.5 plants m<sup>-2</sup>. For the two field experiments (2000, 2003), each line was grown in two plots and sub-sampled on a regular basis to extract sufficient quantities of enriched cell wall protein. Flowers were tagged on the day of anthesis and ovules were harvested at 3 DAA and 7 DAA. Weeds and insects were managed using standard agronomic practices for the Mississippi delta. For the greenhouse experiment, 20 plants of each line were grown in 10 pots. Plants were fertilized and insects were managed on a regular basis. Flowers were tagged, ovules harvested, and protein extracted as above.

### **Protein Extraction, 2D PAGE, and PME activity**

Ovules, from 3 and 7 DAA, were harvested by carefully removing the capsule wall and delicately nudging the ovules out of the capsule with blunt forceps. These ovules were submerged in 20 mL of a 100 mM CDTA/30% sorbitol solution (pH 7.3) with 1 mM benzamidine, 1 mM iodoacetamide and 1mM PMSF for 15 min from the time the last ovule was incorporated into the solution at 24 EC with gentle agitation. Ovules and large debris were removed by filtering the solution through one layer of Miracloth. A 200 :1 aliquot was removed for determination of protein and PME activity. Enriched cell wall protein in the remaining solution was precipitated by the addition of 3 volumes of 100% ethanol to the suspension and precipitated overnight at -20EC. Precipitated protein was collected by centrifugation for 15 min at 12,000g at 4EC. The sample was then prepared for 2D PAGE by phenol extraction, precipitation in ammonium acetate/methanol and resuspended in IEF solution as described by Turley and Ferguson (1996). Protein, 75 :g, was loaded on each IEF gel and focused for 1 h at

200 V, 1 h at 500 V, and 16 h at 700 V. The second-dimension gels consisted of 9 to 16 % acrylamide gradient gels which were polymerized with 1,4-dimethylpiperazine (17 mM) and ammonium persulfate (1.8 mM) to reduce background staining that occurs during silver stain. Protein concentrations were measured by the Bradford method (BioRad Lab., Hercules, CA, USA) with gamma-globulin as the standard. Before applying samples to IEF gels, ampholytes (pH 5 to 7) were added to make a 2 % final concentration. PME activity was measured using the gel diffusion assay described by Downie et al. (1998) with 0.1% (w/v) 90% methylesterified pectin.

### **Cloning of PME Inhibitor**

A consensus full length clone for a pectin methylesterase inhibitor was identified from a normalized library derived from DE 119 cotton. The PMEI region from EST accession DW485740 was amplified from a normalized cDNA library from DES 119 obtained from Dr. Earl Taliercio using two PCR primers (Integrated DNA Technologies, Skokie, IL) designed for sense 5'-GGAGCGAGGAATTCCATCGTTGCATCCTGCAAAGCCAC-3' and the anti-sense 5'-GCAGCGACCGGTACCCTAAGGCTTCTCAGTAGGTATAGC-3' strands. Amplification using PCR with 92EC/3 min, 35 cycles of 92EC/30 sec and 53EC/1 min, then 70EC/30 min. The PCR was performed using the DyNAzyme EXT DNA Polymerase kit (New England Biolabs, Beverly, MA) and generated 594 bp product with unique *Eco*RI (5') and (3') *Kpn*I restriction sites (underlined) for directional insertion and ligation in frame with the  $\nabla$ -factor secretion sequence in the expression vector pPICZ $\nabla$ A (Invitrogen, Carlsbad, CA). The PCR product was TA cloned into a TOPO TA pCR<sup>®</sup>2.1 TOPO vector (Invitrogen, Carlsbad, CA) and positive transformants selected with blue/white screening. Plasmid DNA was isolated using the standard

alkali lysis and the precipitation by polyethylene glycol protocols (Sambrook et al., 1989). The PMEI clone was sequenced in both directions and the protein sequence was deduced and analyzed using ExPASy Proteomic tools Translate, ProtParam, MW, pI, and Titration curve (<http://us.expasy.org/tools/>). The PMEI cDNA was evaluated for rare codon usage patterns in *Pichia pastoris* ([http://molbiol.ru/eng/scripts/01\\_11.html](http://molbiol.ru/eng/scripts/01_11.html)).

Once the sequence was confirmed, the PMEI coding region was excised from the TOPO plasmid by *EcoRI/KpnI* restriction digestion (pPICZ $\nabla$ A was processed at the same time), purified by electrophoresis on 1% (w/v) agarose gels, gel extracted using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA), and ligated in frame with the  $\nabla$ -factor secretion sequence in the expression vector pPICZ $\nabla$ A. The recombinant plasmids were transformed into TOP10 electrocomp<sup>Tm</sup> cells (Invitrogen, Carlsbad, CA) and screened for inserts in selected colonies using PCR. Three plasmids with the PMEI gene were purified by the standard alkali lysis and precipitation by polyethylene glycol protocols as described by Sambrook et al., (1989), cut with restriction enzyme *PmeI*, then transformed into *P. pastoris* X-33 cells by electroporation using a Gene Pulser (BioRad, Hercules, CA). *Pichia pastoris* X-33 cells were prepared for transformation by the method of Lin-Cereghino et al. (2005).

### **Heterologous Expression of Cotton PMEI in *Pichia pastoris***

Six colonies were tested for each transformation event and the heterologous expression of PMEI in *P. pastoris* was evaluated. Each colony was suspended in 50 mL of BMG media (100 mM potassium phosphate [pH 6.0], 1.34% YNB, 4 X 10<sup>-5</sup> % biotin, and 1% glycerol) and grown overnight in a shaking incubator (240 rpm) at 30EC in sterile 250 mL baffled flasks. When

OD<sub>600</sub> reached 2.5 to 3.5, the suspensions were poured into sterile Oak Ridge tubes and centrifuged at 3000g for 5 min in a SA-600 rotor (Sorvall) at 4EC. The supernatants were decanted, and the pellets were resuspended in BMM media (100 mM potassium phosphate pH 6.0, 1.34% YNB, 4 X 10<sup>-5</sup> % biotin and 1% methanol) and grown for 96 h in a shaking incubator (240 rpm) at 30EC in sterile 250 mL baffled flasks. Fifteen mL of each sample was collected in 30 mL corex tubes and centrifuged at 5000g for 10 min in a SA-600 rotor (Sorvall) at 4EC. The supernatants were poured into Centriprep YM-10 tubes and concentrated overnight in a SA-600 rotor (Sorvall) at 2500g at 4EC. After the samples were concentrated to 0.7 to 1 mL volumes, they were placed in 1.5 mL microfuge tubes (Bio-Rad Lab., Hercules, CA) and aliquots were removed and evaluated with SDS-PAGE. Single colonies with the highest level of PME1 excretion were replated on YPD zeocin plates and grown for two days.

The process was then scaled up using a liter of BMG, inoculated with the transformed *P. pastoris*, then equally divided into three, two liter baffled flasks and grown overnight at 30EC. Four sterile 250 mL Nalgene centrifugation bottles (Nalge Co., Rochester, New York) were filled with the yeast solution, and the yeast were pelleted at 3000g for 10 min at 4EC in a GSA-9847 rotor. The four pellets were resuspended in 1 L of BMM, then equally divided between three, two-liter baffled flasks and grown for 96 h at 30EC. Four 250 mL Nalgene centrifugation bottles (Nalge Co., Rochester, New York) were filled with the media, and the yeast was pelleted at 3000g for 10 min at 4EC in a GSA-9847 rotor. The supernatant was sterilized by passing through a 1000mL Filter System (Corning, Corning, NY) and then concentrated in Centriprep YM-10 tubes in a SA-600 rotor (Sorvall) at 2500g at 4EC. Protein concentrations were

measured by the Bradford method (Bio-Rad Lab., Hercules, CA) with gamma-globulin as the standard. SDS-PAGE was performed as described by Turley and Ferguson (1996) in 12% gels.

## **RESULTS AND DISCUSSION**

### **Measuring PME Activity from Cotton Fiber/Ovules**

It was decided to assay extractable PME to measure the general levels of activity from the cell walls of DP 5690, SL 1-7-1, Ligon lintless 1 and Ligon lintless 2. The technique was designed to allow the extraction of cell wall proteins with minimal damage to the cell. Care was taken not to damage the ovules during removal from the capsule and then gently agitate while in the CDTA/sorbitol solution. Cell breakage would decrease the measurable specific activity of PME. Even though significant differences exist in the JIM5 labeling of de-esterified HGAs in the cell walls (data not shown) of these mutant lines, PME activities did not reflect this difference (Table 1). Both Ligon lintless lines unexpectedly had higher specific activities than the DP 5690 (fibers with the highest level of de-esterified HGA). The SL 1-7-1 fiberless line had very low levels of PME, in some cases a tenth of the specific activity of the Ligon lintless lines. One explanation for the lower specific activity of PME from the DP 5690 line is that the higher de-esterification might indicate a higher degree of calcium bridge formation between the HGAs. The CDTA/sorbitol extraction would then release more de-esterified pectin and protein into the solution.

Attempts have been made to directly quantify PME without much success. We procured four monoclonal antibodies developed against tomato PME (from the Complex Carbohydrate Research Center, University of Georgia, Athens), unfortunately, no antigenicity was found for

the cotton fiber PME. Also attempts have been made to produce cotton PME in *P. pastoris* eukaryotic expression system. We have been successful in transforming the cotton PME into *P. pastoris*, however, no significant expression or activity has ever been found from these transformation events. In our more recent attempts, we have modified the codon usage at 6 positions in the cotton PME to “improve” the translation of the cotton gene in *P. pastoris* without success. Efforts continue to heterologously express cotton PME in both the *P. pastoris* and *E. coli* expression systems.

### **2D PAGE of Enriched Cell Wall Proteins**

Comparisons of the 2D PAGE indicated obvious similarities and differences between the protein profiles of these four lines. Differences are marked by arrows on the gels. The protein profiles from the short lint Ligon lintless lines are nearly identical for expressed protein even though these plants are genetically different. Equal quantities of protein were loaded from DP 5690, Ligon lintless 1, Ligon lintless 2 and SL 1-71; however, only the SL 1-7-1 gel appears relatively devoid of protein (Figure 1). These gels have been replicated with protein isolated from field grown and greenhouse grown ovules with no apparent differences in protein profiles. Also protein profiles for enriched cell wall extracts from 7 DAA ovules were compared and the results were nearly identical with the protein profiles for 3 DAA for each line (data not shown).

Numerous differences exists between the protein profiles of DP 5690 verses the Ligon lintless lines. The major protein in the Ligon lintless that is lacking from DP 5690 lines has a molecular weight of approximately 17 kDa and a *pI* of 4.5. Other proteins are also apparent with the Ligon lintless lines having unique proteins at 15 kDa and 43 kDa. DP 5690 has unique proteins at approximately 66 kDa and 90 kDa (Figure 1). Future work will be to sequence and

characterize the function of each of these proteins from the cell wall.

Our lab has been interested in the pectin methylesterase inhibitor proteins for the past few years. In this project, they could be invaluable in the interpretation of the de-esterified HGA data. If PME was inhibited, HGA would remain methylesterified, which is what is apparently occurring in the fiber of the Ligon lintless lines. PMEI proteins are usually small molecular weight proteins ranging from approximately 3 to 18 kDa (Giovane et al., 2004). Coincidentally, two of the unique proteins to the Ligon lintless lines are in this molecular weight range. This is not proof that PMEI proteins are actually functioning in the fiber, however, it is one of our working hypotheses.

### **PMEI Protein Expression and Purification**

Production of a cotton PMEI protein from EST accession DW485740 was begun by using PCR to produce a full length coding region determined to be 516 bp in length and encoded a protein with a deduced amino acid sequences of 172 residues. The protein had a molecular weight of 18.7 kDa and a theoretical *pI* of 9.7. This cDNA was subcloned into the yeast expression vector pPICZ $\forall$ A in frame with the  $\forall$ -factor secretion sequence and transformed into *P. pastoris*. The heterologously expressed PMEI protein was determined to approximately 18 kDa by SDS-PAGE and was the predominant protein in the media (Figure 2, lanes B and C). The other four bands were likely yeast proteins as we have seen these same bands in the expression of other heterologous proteins in *P. pastoris*. A control *P. pastoris*, created by transformation with a pPICZ $\forall$ A plasmid without an insert and grown at the same time as PMEI, did not produce the 18 kDa protein (Figure 2, lane A). PMEI was not tested for activity before the writing of this paper. Future work will be to produce antibodies to this PMEI for use in

Western blotting of SDS-PAGE of proteins from these four lines.

The presence of de-esterified HGA in actively growing cell walls is somewhat of an enigma. Usually, cell elongation ceases as the abundance of the more acidic, de-esterified HGA increases (Goldberg et al. 1996; Jarvis 1984; Kim and Carpita 1992; McCann and Roberts 1994; Willats et al 2001b). In addition, a bilayer structure with an outer layer enriched in highly de-esterified HGA has been identified in non-elongating flax hypocotyls (Jauneau et al. 1997) and in non-elongating pea epicotyls (Fujino and Itoh 1998) similar to the epidermal cells of the cotton ovule.

Our working hypotheses for the presence of de-esterified HGA in the fiber include the de-esterified HGA form calcium bridges which give added strength to a rapidly expanding wall, or these de-esterified HGA molecules interact and provide a matrix for the binding of surface waxes (Vaughn et al. 2007). Other functions may also exist for these HGAs. Identifying the mechanism of inhibition which may be responsible for the lowering of de-esterified HGA levels in the Ligon lintless lines should give us added insight into both fiber physiology and elongation.

## References

- Anderson, D., and T. Kerr. Growth and structure of cotton fiber. *Ind. Eng Chem.* 30:48-54.
- Baron-Epel, O., Gharyl, P.K., and Graig, J.W.T. 1988. Pectins as mediators of wall porosity in soybean cells. *Planta* 175:389-395.
- Birnbaum, E.H., Beasley, C.A., and Dugger, W.M. 1974. Boron deficiency in unfertilized cotton (*Gossypium hirsutum* L.) ovules grown *in vitro*. *Plant Physiol.* 54:931-935.
- Chanliand, E., and Gidley, M.J. 1999. *In vitro* synthesis and properties of pectin/*Acetobacter xylinus* cellulose composites. *Plant J.* 20:25-35.

- Cosgrove, D.J. 2000. Expansive growth of plant cell walls. *Plant Physiol. Biochem.* 38:109-124.
- Darvill, A., Augur, C., Bergmann, C., Carlson, R.W., Cheong, J.J., Eberhard, S., Hahn, M.G., Lo, V.M., Marfa, V., Meyer, B. 1992. Oligosacchairs-oligosaccharides that regulate growth, development and defense responses in plants. *Glycobiology* 2:181-198.
- Demarty, M., Morvan, C., and Thellier, M. 1984. Calcium and the cell wall. *Plant Cell Environ.* 7:441- 448.
- Downie, B., Dirk, L.M.A., Hadfield, K.A., Wilkins, T.A., Bennett, A.B., and Bradford, K.J. 1998. A gel diffusion assay for quantification of pectin methylesterase activity. *Anal. Biochem.* 264:149-157.
- Dumville, J.C., and Fry, S.C. 2000. Uronic acid-containing oligosaccharins: their biosynthesis, degradation and signaling roles in non-diseased plant tissues. *Plant Physiol. Biochem.* 38:125-140.
- Fujino, T., and Itoh, T. 1998. Changes in pectin structure during cell elongation in pea (*Pisum sativa*) and its implications for cell wall architecture. *Plant Cell Physiol.* 39: 1315-1323.
- Giovane, A., Servillo, L., Balestrieri, C., Railola, A., D'Avino, R., Tamburrini, M., Ciardiello, M.A., and Camardella, L. 2004. Pectin methylesterase inhibitor. *Biochim. Biophys. Acta* 1696:245-252.
- Goldberg, R., Morvan, C., Janeau, A., and Jarvis, M.C. 1996. Methyl-esterification, de-esterification and gelation of pectins in the primary cell wall. In: *Pectins and Pectinases*. J Visser AGJ Vosagen (eds), pp 151-172. Wageningen, The Netherlands
- Jauneau, A., Quentin, M., and Driouch, A. 1997. Micro-heterogeniety of pectins and calcium distribution in epidermal and cortical parenchyma cell walls of flax hypocotyls.

- Protoplasma 198:9-19.
- Jarvis, M.C. 1984. Structure and properties of pectin gels in plant cell walls. *Plant Cell Environ.* 7:153-164.
- Kim, H., and Triplett, B. 2001. Cotton fiber growth in planta and *in vitro*. Models for plant cell elongation and cell wall biogenesis. *Plant Physiol.* 127:1361-1366.
- Kim, J.B., and Carpita, N.C. 1992. Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiol.* 98:646-653.
- Knox, J.P. 1992. Cell adhesion, cell separation and plant morphogenesis. *Plant J.* 2:137-141.
- Kobayashi, M., Matoh, T., and Azuma, J. 1996. Two chains of rhamnogalacturonan II are cross linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiol.* 110:1017-1020.
- Läuchli, A. 2002. Functions of Boron in higher plants: recent advances and open questions. *Plant Biol.* 4:190-192.
- Lin-Cereghino, J., Wong, W.W., Xiong, S., Giang, W., Luong, L.T., Vu, J., Johnson, S.D. and Lin-Cereghino, G.P. 2005. Condensed protocol for competent cell preparation and transformation of the methylotropic yeast *Pichia pastoris*. *BioTechniques* 38:44-48.
- McCann, M.C., and Roberts, K. 1994. Changes in cell wall architecture during cell elongation. *J. Exp. Bot.* 45:1683-1691.
- O'Neill, M.A., Warrenfeltz, D., Kates, K., Pellerin, P., Doci, T., and Darvill, A.G. 1996. Rhamnogalacturonan-II, a pectic polysaccharide in the cell walls of growing plant cells, forms a dimer that is covalently-linked by a borate ester. *J. Biol. Chem.* 271:22923-22930.
- Ridley, B.L., O'Neill, M.A., and Mohnen, D. 2001. Pectins: structure, biosynthesis, and

- oligogalacturonide-related signaling. *Phytochemistry* 57:929-967.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Edition (Cold Spring Harbor Laboratory Press, New York), pp. 1.38-1.41.
- Stewart, J.M. 1975. Fiber initiation on the cotton ovule (*Gossypium hirsutum*). *Amer. J. Bot.* 62: 723-730.
- Turley RB and Ferguson DL, 1996. Changes in ovule proteins during early fiber development in a normal and a fiberless line of cotton (*Gossypium hirsutum* L.) *J Plant Physiol* 149:695-702.
- Vaughn, K.C., Talbot, M.J., Offler, C.E., and McCurdy D.W. 2007. Wall ingrowth in epidermal transfer cells of *Vicia faba* cotyledons are modified primary wall marked by localized accumulations of Arabinogalactan proteins. *Plant cell Physiol.* 48:159-168.
- Vaughn, K., and Turley, R.B. 1999. Cotton fiber walls contain a pectin sheath. *Protoplasma* 209:226-237.
- Willats, W.G.T., McCartney, L., Mackie, W., and Knox, P. 2001a. Pectin: cell biology and prospects for functional analysis. *Plant Mol. Biol.* 47:9-27.
- Willats, W.G.T., Orfila, C., Limberg, G., Buchholt, H.C., van Alebeek, G.-J.W.M., Voragen, A.G.J., Marcus, S.E., Christensen, T.M.I.E., Mikkelsen, J.D., Murray, B.S., and Knox, J.P. 2001b. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. *J. Biol. Chem.* 276:19404-19413.
- Whistler, R.L., Martin, A.R., and Conrad, C.M. 1940. Pectin substance of cotton fibers in relation to growth. *J. Res. National Burea Standards* 25:305-308.
- Yu, Q., Hlavacka, A., Match, T., Volkmann, D., Menzel, D., Goldbach, H.E., and Bulu ka, F.

2002. Short term boron deprivation inhibits endocytosis of the cell wall pectins in meristematic cells of maize and wheat root apices. *Plant Physiol.* 130:415-421.

Table 1. Specific activity of pectin methylesterase (PME) was determined from CDTA/sorbitol extracts of intact ovules from DP 5690, SL 1-7-1, Ligon lintless 1 and Ligon lintless 2.

Activities are reported in micro-units (1 unit of PME liberates 1 micro-equivalent of acid/min at pH 7.5, 30EC) per mg protein

Cotton Variety	3 DPA	7 DPA
DP 5690	3.5 $\nabla$ 0.3	11.0 $\nabla$ 0.6
SL 1-7-1	1.7 $\nabla$ 0.0	3.9 $\nabla$ 0.5
Ligon lintless 1	14.7 $\nabla$ 0.8	15.2 $\nabla$ 1.4
Ligon lintless 2	19.8 $\nabla$ 0.8	39.8 $\nabla$ 4.1

Figure 1. 2D PAGE of “enriched cell wall” proteins isolated from 3 days after anthesis ovules from DP 5690, SL 1-7-1, Ligon lintless 1 and Ligon lintless 2. Red arrows indicate proteins which are up-regulated in the Ligon lintless lines. Blue arrows indicate proteins which are up-regulated in the DP 5690 line (also present in Ligon lintless 2).

Figure 2. SDS-PAGE of heterologous expressed cotton PMEI protein in *Pichia pastoris*. Lane A, control lane of *Pichia pastoris* grown without the PMEI insert. Lane B, PMEI protein (arrow). Lane C, PMEI protein passed through a 10 DG column.