MOLECULAR PHENOTYPING OF
CELL WALL DEVELOPMENT IN THE
ARABIDOPSIS THALIANA PRIMARY STEM

by

HARDY HALL

M.Sc., The University of British Columbia, Canada 2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

March 2012

© Hardy Hall, 2012
Abstract
Plant cell walls are sophisticated, dynamic structures that play a vital role in coordinating the directional growth of plant tissues. The rapid elongation of the inflorescence stem in the model plant *Arabidopsis thaliana* is accompanied by radical changes in cell wall structure and chemistry, but the study of the underlying mechanisms has been hampered by difficulties in sampling discrete developmental states along the developing stem. I have created a novel sampling approach that allows me to sample stem tissues representing specific and distinct developmental phases (elongation rate increase, maximum growth rate, and growth cessation) from individual plants, by use of time-lapse imagery and computational analysis of growth kinematic profiles. This high-resolution growth context enables the harvest of pooled, developmentally-matched samples that I then used for transcriptome profiling of growth-characterized stem segments, and for immunohistochemical analysis of growth-associated cell wall epitopes in specific cell types within stem cross-sections. The resulting transcript profiles have identified dozens of genes, both known and novel, whose expression is coupled to these growth transitions. Unique cell type-specific epitope patterns were observed during the most rapid phase of elongation, providing evidence for structural divergence of cell walls among these cell types despite a common developmental environment of diffuse elongation. This study has thus produced two global perspectives of the cellular events accompanying the transition from early differentiation through maximum anisotropic cell expansion to growth cessation and secondary cell wall maturation, in a single organ. The results collectively provide important hypothesis-generating insights anchored to a precise developmental scaffold of cell wall differentiation established through growth kinematic profiling.
Preface

Chapter 2: Growth kinematic profiling

The novel approaches to growth profiling and harvesting presented in this Chapter arose from discussion with my supervisor and committee members on the challenges of placing molecular data in adequate developmental context. I performed the experiments with technical assistance from Noriko Tanaka, and carried out the data analysis independently. New Phytologist accepted this chapter on December 9th, 2011 for publication as “Developmentally equivalent tissue sampling based on growth kinematic profiling of Arabidopsis inflorescence stems” (DOI: 10.1111/j.1469-8137.2012.04060.x). I share authorship on this manuscript with my supervisor, Professor Brian Ellis.

Chapter 3: Stage-specific gene expression profiling

I performed the growth profiling and expression profiling independently. I employed Rick White (Department of Statistics, UBC) for experimental design consultation and preliminary statistical analysis, and performed the remaining analyses independently. This work is being prepared with Chapter 4 (Epitope profiling) as a single manuscript, myself as primary author.

Chapter 4: Cell type-specific epitope profiling

Preliminary work in optimizing the high-throughput immunolabelling approach was assisted by student Qing ling (Crystal) Cheung, and resulted in submission of the Directed Studies (Biol 448) report “Using antibodies to detect fibre-specific cell wall constituents in Arabidopsis thaliana stems”. Colin MacLeod provided additional technical assistance for the remaining immunolabelling lab work. I performed all statistical analyses, consulting with Rick White throughout the experimentation and analysis phases.

Appendix D: Cell type-specific epitope profiling

Experimentation and analyses are accredited as for Chapter 3, with the exception of assistance with laser microdissection from volunteers Zhixia (Stella) Cao, Qing ling (Crystal) Cheung, and Florence Leung.
## Table of contents

Abstract .......................................................................................................................... ii  
Preface ........................................................................................................................... iii  
Table of contents .......................................................................................................... iv  
List of tables ................................................................................................................. vii  
List of figures .............................................................................................................. viii  
List of other resources ............................................................................................... x  
Lists of symbols and abbreviations ........................................................................... xi  
Acknowledgements .................................................................................................... xiii  
Dedication .................................................................................................................... xv  

**Chapter 1. General introduction** ................................................................................. 1  
1.1 General introduction .............................................................................................. 1  
1.2 Expansion anisotropy .......................................................................................... 1  
  1.2.1 Deposition of wall material ........................................................................... 2  
  1.2.2 Cellulose deposition and directionality of expansion ................................... 3  
  1.2.3 Microtubule influence on cellulose properties ............................................ 4  
1.3 Mechanical modulation of anisotropic expansion by non-cellulosic wall  
  constituents and their modifiers ............................................................................ 5  
  1.3.1 Hemicellulose .............................................................................................. 6  
  1.3.2 Expansins .................................................................................................. 8  
  1.3.3 Pectins ..................................................................................................... 8  
  1.3.4 Glycoproteins ......................................................................................... 10  
  1.3.5 Lignin .................................................................................................... 12  
1.4 Regulation of differentiation ............................................................................... 14  
  1.4.1 Regulation of vascular cell fate ................................................................. 14  
  1.4.2 Regulation of cell polarity, expansion, and secondary cell wall  
  formation ........................................................................................................... 16  
1.5 Systems biology for cell wall expansion ............................................................. 17  
  1.5.1 Model systems for study of directional cell expansion .............................. 18  
  1.5.2 Defining the gradient of primary stem anisotropic expansion ................. 19  
1.6 Kinematic analysis for characterization of the expansive growth field in  
  root and shoot ....................................................................................................... 20  
1.7 Problem statement ............................................................................................. 22  
1.8 Research/thesis objectives ................................................................................. 22  

**Chapter 2. Developmentally equivalent tissue sampling based on  
  growth kinematic profiling of Arabidopsis primary stems** ..................................... 24  
2.1 Introduction ........................................................................................................... 24  
2.2 Results ................................................................................................................ 28  
  2.2.1 Synthetic optical markers (tags) allow precise tracking of  
  inflorescence elongation ....................................................................................... 28  
  2.2.2 Calculation of relative growth rates from tag movements ....................... 33  

iv
2.2.3 The position of growth stage transitions is widely variable among individual plants ........................................................................................................... 38

2.2.4 Growth kinematic profiles facilitate isolation of distinct stages of cell length.................................................................................................................. 41

2.3 Discussion ....................................................................................................... 43

2.4 Materials and methods ................................................................................ 48

2.4.1 Plant material and growth conditions ......................................................... 48

2.4.2 Growth imaging and image processing ....................................................... 48

2.4.3 Time-lapse series analysis .......................................................................... 50

2.4.4 Growth kinematic profiling ........................................................................ 50

2.4.5 Tissue harvesting for microscopic analysis ............................................... 51

2.4.6 Morphometric analysis............................................................................... 51

Chapter 3. Stage-specific transcriptome profiling across the cell wall expansion continuum in the Arabidopsis primary stem .................................................. 53

3.1 Introduction ..................................................................................................... 53

3.2 Results ........................................................................................................... 57

3.2.1 Sampling approach to stage-specific transcriptomic analysis of cell wall expansion ........................................................................................................... 57

3.2.2 Examination of differential expression between stages ............................... 58

3.2.3 Stage-specific, whole-genome co-expression analysis .................................. 74

3.2.4 Stage-specific co-expression analysis of cell wall-related genes ................... 79

3.2.5 Assessment of commonality between the published transcriptome studies of primary stem development ................................................................. 82

3.3 Discussion ..................................................................................................... 91

3.3.1 ‘Young’ stage .............................................................................................. 91

3.3.2 ‘Maximum growth-rate’ stage .................................................................... 94

3.3.3 ‘Cessation’ stage ......................................................................................... 98

3.3.4 ‘Old’ stage .................................................................................................. 100

3.3.5 Comparison to other transcriptomic studies of inflorescence stems .......... 104

3.4 Experimental procedures ......................................................................... 106

3.4.1 Plant growth, imaging, preservation, and growth kinematic profiling .......... 106

3.4.2 RNA processing ........................................................................................ 106

3.4.3 Reverse transcription and labelling ............................................................. 107

3.4.4 Array hybridization .................................................................................. 108

3.4.5 Microarray scanning and spot quantification ............................................. 109

3.4.6 Microarray data analysis .......................................................................... 109

Chapter 4. Epitope profiling across the cell wall expansion continuum in the Arabidopsis primary stem ................................................................. 111

4.1 Introduction .................................................................................................. 111

4.2 Results ......................................................................................................... 115

4.2.1 High throughput immunological survey for stage-specific epitope profiling ................................................................. 115

4.2.2. Assessment of developmental equivalence among biological replicates ................................................................. 119

4.2.3 Epitope abundance change across development ........................................ 121
List of tables

Table 3.1 Top 40 genes with the greatest differential expression between YNG and MGR stages................................................................. 64
Table 3.2 Top 40 genes with the greatest differential expression between MGR and CSS stages........................................................................ 68
Table 3.3 Top 40 genes with greatest differential expressions between cessation and old tissues (continued)....................................................... 72
Table 3.4 Inter-study grouping co-expression sets................................................................................................................................... 84
Table B.1 Raw and processed data for stage-specific (whole-segment) microarray experiment.............................................................................. 182
Table B.2 AtCoeCis reports for enrichment of gene ontology (GO) terms for top 40 genes most different between YNG and MGR stages........................................................................................................... 182
Table B.3 AtCoeCis reports for enrichment of gene ontology (GO) terms for top 40 genes most different between MGR and CSS stages........................................................................................................... 182
Table B.4 AtCoeCis reports for enrichment of gene ontology (GO) terms for top 40 genes most different between CSS and OLD stages........................................................................................................... 182
Table B.5 AtCoeCis reports for enrichment of gene ontology (GO) terms for [sub] clusters of genes with stage-specific expression........................................................................................................... 182
Table B.6 AtCoeCis reports for enrichment of gene ontology (GO) terms for stage-specific clusters of co-expressed cell wall-related genes........................................................................................................... 182
Table B.7 AtCoeCis reports for enrichment of gene ontology (GO) terms ....................................................................................................... 182
Table C.1 Summary of information for antibodies used in this study ........................................................................................................ 183
Table C.2. Antibodies with greatest change in abundance from young to maximum growth-rate stages....................................................................... 183
Table C.3 Antibodies with greatest change in abundance from Young to Maximum growth-rate stages........................................................................... 183
List of figures
Figure 2.1 Images of a plant within growth chamber, .......................................................... 29
Figure 2.2 Illustration of segment length determination in tagged plants. ....................... 31
Figure 2.3 XY coordinates of tag/plant intersection points derived from feature tracking dataset. ............................................................................................................. 32
Figure 2.4 Three representative growth profiles ............................................................. 38
Figure 2.5 Relationships between GKP-identified stem growth stages, the stem apex, and overall stem height ........................................................................................ 41
Figure 2.6 Summary of mean cell lengths (um) for cortex and endodermal cell types ........................................................................................................................... 42
Figure 2.7 Workflow for growth kinematic profile-guided harvesting of inflorescences ...................................................................................................................... 47
Figure 3.1 Representative growth profiling and harvesting ........................................... 58
Figure 3.2 Complete factorial block design employed for examination of transcriptional change among four developmental stages. .............................................. 60
Figure 3.3 Hierarchical clustering of 4635 differentially expressed genes ..................... 76
Figure 3.4 Schematic of comparison of co-expression datasets for four transcriptome studies of Arabidopsis primary stem development. ................................. 86
Figure 3.5 Pair-wise comparison of commonality among co-expression sets .................. 87
Figure 3.6 Summary of likeness of co-expression sets .................................................. 90
Figure 4.1 Representative developmental series for fluorescence intensity scoring .......................................................................................................................... 118
Figure 4.2 Dendrogram of immunofluorescence similarity of biological replicates at three stages ............................................................................................................. 127
Figure 4.3 Cluster analysis in YNG segments of relative fluorescence intensities associated with 55 antibodies ....................................................................................... 129
Figure 4.4 Cluster analysis in MGR segments of relative fluorescence intensities associated with 55 antibodies ................................................................................. 131
Figure 4.5 Cluster analysis in CSS segments of relative fluorescence intensities associated with 55 antibodies ............................................................................... 134
Figure 4.6 Immunofluorescent labelling of YNG, MGR, and CSS tissue with antibodies identified in clustering as specific to inner walls of rapidly expanding fibres ............................................................................................................ 136
Figure 4.7 Immunofluorescent labelling of YNG, MGR, and CSS tissue with antibodies identified in clustering as abundant within endodermis at MGR and CSS. ........................................................................................................... 139
Figure 4.8 Immunofluorescent labelling of YNG, MGR, and CSS tissue with antibodies identified in clustering as specific to xylem and IFF region at CSS ............ 139
Figure A.1 Electrostatically-attached PVDF disks as an alternative optical marker system. .................................................................................................................. 181
Figure A.2 Effect of smoothing length measurements on growth kinematic profiling. ........................................................................................................................ 181
Figure A.3 Surface plots of REGRs and LOWESS-predicted growth kinematic profiles for 24 plants. ........................................................................................................................ 181
Figure A.4 Tagged plants shown placed within plant-specific growth restraints and chamber........................................................................................................................ 181
Figure A.5 Illustrations of growth imaging chamber. ........................................................................................................................ 181
Figure B.1 Surface plots of relative elongation growth rates and LOWESS-predicted growth kinematic profiles .............................................................................. 182
Figure B.2 Gene ontology (GO) SLIM term enrichment analysis for clusters depicted in Figure 3.3. ................................................................................................................... 182
Figure B.3 Boxplots depicting distribution of estimates of relative gene expression (fold-change) of each developmental stage ................................................................................. 182
Figure B.4 Hierarchical clustering of 742 cell wall-related genes .......................................................................................................................... 182
Figure B.5 Hierarchical clustering of pectin-related genes .......................................................................................................................... 182
Figure B.6 Hierarchical clustering of cell wall-associated transcription factors .......................................................................................................................... 182
Figure C.1A-C Growth profiles of plants used in this study (A-C) and 96-well confocal imaging (D) ................................................................................................................... 183
Figures C.2.1- C.2.58. Antibody-specific confocal panels for biological replicates (folder with 58 images) .......................................................................................................................... 183
Figure C.3. Layout for 96-well imaging runs (Sets1-6) for all developmental stages .......................................................................................................................... 183
Figure C.4.1-3.4.6 96-well montages depicting region of interest (ROI) selection (folder with six images) .......................................................................................................................... 183
Figure C.5 Immunofluorescent labelling of YNG and MGR tissue for antibodies associated with most dramatic intensity changes in six or more tissues from YNG to MGR (i.e. ubiquitous). .......................................................................................................................... 183
Figure C.6. Immunofluorescent labelling of YNG and MGR stages for antibodies associated with marked intensity increases in 5 tissues from YNG to MGR .......................................................................................................................... 183
Figure C.7. Immunofluorescent labelling of YNG and MGR stages for antibodies associated with concurrent and opposing intensity among tissues from YNG to MGR .......................................................................................................................... 183
Figure C.8 Immunofluorescent labelling of MGR and CSS tissue for antibodies associated with ubiquitous decline from MGR to CSS .......................................................................................................................... 183
Figure C.9 Immunofluorescent labelling of YNG, MGR and CSS tissue for antibodies associated with intensities localized to the epidermis and parenchyma at CSS .......................................................................................................................... 183
Figure C.10 Schematic representation of calculation of \( I_{rel} \) scores for hierarchical clustering. .......................................................................................................................... 183
List of other resources

Script A.1 Growth kinematic profiling analysis from ImageJ feature tracking data........ 181

Video A.1 Tagging of 1 complete plant with ~15 paper rectangles for growth analysis within the imaging chamber .................................................................................................................. 181

Video A.2 Video depicting the process of feature tracking plants in ImageJ ............... 181

Video A.3 Video depicting the harvesting of segments bounded by tags.................... 181

Video A.4 Video rendering of an image series of cell outlines used to calculate cell length. ........................................................................................................................................ 181
### Lists of symbols and abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>Δ-I_n</td>
<td>change in mean fluorescence intensities</td>
</tr>
<tr>
<td>AG</td>
<td>arabinogalactan side-chain</td>
</tr>
<tr>
<td>AGP</td>
<td>arabinogalactan protein</td>
</tr>
<tr>
<td>alpha-NAA</td>
<td>alpha-naphthalene acetic acid</td>
</tr>
<tr>
<td>ARF</td>
<td>auxin response factor</td>
</tr>
<tr>
<td>AtCoeCis</td>
<td><em>Arabidopsis</em> co-expression cis-regulatory elements algorithm</td>
</tr>
<tr>
<td>BR</td>
<td>brassinosteroid</td>
</tr>
<tr>
<td>CAZy</td>
<td>carbohydrate active enzyme</td>
</tr>
<tr>
<td>CBM</td>
<td>carbohydrate binding module</td>
</tr>
<tr>
<td>CCRC-M#</td>
<td>‘Complex Carbohydrate Research Centre’ monoclonal Ab</td>
</tr>
<tr>
<td>CE</td>
<td>carbohydrate esterases</td>
</tr>
<tr>
<td>CESA</td>
<td>cellulose synthase</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CMF</td>
<td>cellulose microfibril</td>
</tr>
<tr>
<td>CMT</td>
<td>cortical microtubule</td>
</tr>
<tr>
<td>Col-0</td>
<td>Columbia</td>
</tr>
<tr>
<td>CSS</td>
<td>cessation stage</td>
</tr>
<tr>
<td>Eh</td>
<td>Ehlting et al., 2005</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GA</td>
<td>gibberellic acid</td>
</tr>
<tr>
<td>GAX</td>
<td>glucuronylarabinoxylans, xylan</td>
</tr>
<tr>
<td>GH</td>
<td>glycoside hydrolases</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>GO SLIM</td>
<td>top-level ‘Gene Ontology Consortium’ functional annotation</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidyl inositides</td>
</tr>
<tr>
<td>GRP</td>
<td>glycine-rich protein</td>
</tr>
<tr>
<td>GT</td>
<td>glycosyltransferases</td>
</tr>
<tr>
<td>Ha</td>
<td>Chapter 3 expression dataset</td>
</tr>
<tr>
<td>HCL</td>
<td>hierarchical clustering</td>
</tr>
<tr>
<td>HD-ZIP</td>
<td>class III homeodomain-leucine zippers</td>
</tr>
<tr>
<td>HE</td>
<td>‘High Early’ expression trajectory</td>
</tr>
<tr>
<td>HGA or HG</td>
<td>homogalacturonan</td>
</tr>
<tr>
<td>HL</td>
<td>‘High Late’ expression trajectory</td>
</tr>
<tr>
<td>HM</td>
<td>‘High Middle’ expression trajectory</td>
</tr>
<tr>
<td>HRGP</td>
<td>hydroxyproline-rich glycoprotein</td>
</tr>
<tr>
<td>IAA</td>
<td>auxin/indole-3-acetic acid</td>
</tr>
<tr>
<td>I_n</td>
<td>mean fluorescence gray value intensity</td>
</tr>
<tr>
<td>IFF</td>
<td>interfascicular fibre</td>
</tr>
<tr>
<td>Im</td>
<td>Imoto et al., 2005</td>
</tr>
<tr>
<td>I_rel</td>
<td>relative fluorescence intensity scores</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>JIM#</td>
<td>John Innes monoclonal Ab</td>
</tr>
<tr>
<td>LM</td>
<td>‘Low Middle’ expression trajectory Ab</td>
</tr>
<tr>
<td>LM#</td>
<td>Leads monoclonal</td>
</tr>
<tr>
<td>LOWESS</td>
<td>locally-weighted linear regression</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>LTP</td>
<td>lipid transfer protein</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MFA</td>
<td>microfibril angle</td>
</tr>
<tr>
<td>MGR</td>
<td>maximum growth-rate stage</td>
</tr>
<tr>
<td>MIAME</td>
<td>minimum information about microarray experiments</td>
</tr>
<tr>
<td>OLD</td>
<td>old, most-mature growth stage</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>PL</td>
<td>polysaccharide lyases</td>
</tr>
<tr>
<td>PME</td>
<td>pectin methylesterase</td>
</tr>
<tr>
<td>PMEI</td>
<td>pectin methylesterase inhibitor</td>
</tr>
<tr>
<td>PRP</td>
<td>proline-rich protein</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>Q-Q</td>
<td>quantile-quantile</td>
</tr>
<tr>
<td>REGR</td>
<td>relative elemental growth rate</td>
</tr>
<tr>
<td>RG-I</td>
<td>rhamnogalacturonan-I</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>TAIR</td>
<td>‘The Arabidopsis Information Resource’</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>VSN</td>
<td>variance stabilization normalization</td>
</tr>
<tr>
<td>XTH</td>
<td>xyloglucan endotransglycosylase/hydrolase</td>
</tr>
<tr>
<td>XyG</td>
<td>xyloglucan</td>
</tr>
<tr>
<td>YNG</td>
<td>young growth stage</td>
</tr>
</tbody>
</table>
Acknowledgements
This doctoral dissertation follows a common convention in voicing accomplishments and perspectives in the first-person, thus reflecting the material and philosophical contributions I, individually, have made to science. Without exception, this first-person perspective is built upon the significant contributions from talented researchers and the generous support from my dear friends and family. It is my great fortune that many of these great people fall into both categories.

I would like to first thank my supervisor, Professor Brian Ellis, for his unwavering support and guidance in my professional development. His thoughtful, measured interventions have helped me exceed my personal expectations and develop my strengths. One of his most significant contributions was to assemble an extraordinary committee of Professors Carl Douglas (also co-supervisor, Botany), Geoffrey Wasteneys, Shawn Mansfield, and Lacey Samuels. Their guidance and encouragement have greatly enriched my program, while their expert reviews added tremendous value to this dissertation.

The ambitious program that evolved from committee meetings would not have been possible without the contributions of undergraduate volunteers Stella Cao, Florence Leung, and Crystal Cheung, while Colin MacLeod’s microscopy skills proved critical in accomplishing the immunoprofiling work in a timely manner.

Fortifying this academic network were fellow members of the Ellis lab, especially Dr. Mathias Schuetz and Dr. Apurv Bhargava for their signaling and development perspectives.

I also had many useful discussions with current or former members of the Botany Department at UBC. On development, these included Prof. Jürgen Ehlting (formerly Treenomix, GenomeBC), Dr. Michael Friedmann (Douglas Lab), Dr. Minako Kaneda (formerly Samuels Lab), and Dr. Miki Fujita (formerly Wasteneys Lab). On microscopy, I was greatly assisted by Kevin Hodgson, Derrick Horne, Garnet Martens, and Dr. Eiko Kawamura of the Bioimaging Facility at UBC, while Randy Deane of the Michael Smith Laboratories provided invaluable technical consultation for growth imaging and tissue handling systems. Of course, these interactions were greatly facilitated by the constant help of Botany department administrators Veronica Oxtoby and Judy Heyes as well as Michael Smith Laboratory staff, Darlene Crowe and Pal Baines.
I would also like to thank Prof. Jörg Bohlman and members of his lab for their cooperation, consultation and equipment loan in transcriptomic endeavours. In addition, I benefitted from the valuable perspectives of researchers outside of UBC most notably Prof. Thomas Berleth (University of Toronto), Dr. Rodger Beatson (BCIT, Burnaby), Prof. Herman Höfte (INRA, Versailles), and Prof. Edgar Spalding (University of Wisconsin).

As any doctor of philosophy can tell you, conducting independent research places extraordinary demands upon the individual. In my case, I cannot imagine how I could have succeeded in completing this dissertation without encouragement and guidance from my personal support network of colleagues, friends and family. Though surely an incomplete list, I would especially like to thank colleagues/friends Dr. Marcus Samuel, Dr. Clarice Souza, Dr. Jin Suk Lee, Dr. Eiko Kawamura, Dr. Chris Ambrose, Dr. Mathias Schuetz, Dr. Albert Cairo, and Dr. Zhenhua Yong. Outside the academic realm, I received special encouragement from Johannes von Steffenelli. Owed the greatest of thanks are my wonderfully supportive family who have at many times gracefully taken a backseat to my research obligations; Brigitte, Robert, Tim, Delyth, Margot, Heidi, and Bert. Finally, greatest honours go to my dear wife Noriko for her tremendous sacrifice in my evolution from a blue-collar labourer to a true scientist.
Dedication

In loving memory of my brother, David - Icarus of purest intention
Chapter 1. General introduction

1.1 General introduction

Plants are not amorphous blobs. Many plant cells arise from meristems in isotropic form, and then undergo a radical morphological transformation throughout the process of their differentiation to assume a form suited to their role in the life of the intricately structured plant. This thesis endeavors to build upon a wealth of biochemical and molecular study that has been superimposed over the last forty years on a previous century of anatomical discovery.

In this introductory Chapter (1), I first review the processes that establish anisotropic cell wall expansion, which involves the organized deposition and interaction of materials that define the mechanical properties of the cell wall. I then explore the means by which these basic interactions are modulated, and the underlying regulatory mechanisms for such modifications.

A significant challenge to complete understanding of cell wall expansion and formation processes is the high level of sophistication inherent to cell walls at all stages of a cell’s life. The discovery-driven research presented in this thesis attempts to employ an elegant approach to dissection of the cell wall expansion continuum. To prepare the reader, I present a review of the principal challenges to modern cell wall examination, with emphasis on the utility of growth kinematic profiling for systematic discovery of new, important cell wall formation mechanisms.

1.2 Expansion anisotropy

Plant cells may be described as thin-walled pressure vessels owing to the turgor-induced stress imposed on the resistant primary wall (Geitmann & Ortega, 2009). The
primary cell wall resists uncontrolled relaxation and breakage in the face of such strain through the mechanical properties of the cell wall. Directional expansion is enabled through the localized relaxation of this stress, enacted by regional modifications to the mechanical properties of the cell walls, perpendicular to the axis of anisotropic expansion. Key among these modifications are 1) deposition of new cell wall material to maintain cell wall thickness/composition, and 2) modification to the existing interconnections among wall components that result in cell wall deformation (i.e. strain) to stress.

1.2.1 Deposition of wall material
As the cell wall expands, existing materials become stretched over a wider area and new material must be deposited to maintain the original cell wall thickness. Central to both the deposition of cell wall glycans/proteoglycans and their modifiers (proteins) is trafficking of these materials by the cytoskeleton. Cell wall modifying enzymes such as carbohydrate esterases (CE), glycoside hydrolases (GH), glycosyltransferases (GT), and polysaccharide lyases (PL) are synthesized in the endoplasmic reticulum (ER) and then trafficked directly to the plasma membrane via vesicles (reviewed in Buchanan et al., 2000). Structural proteins synthesized in the ER, such as hydroxyproline-rich glycoproteins (ex. extensin) and arabinogalactan proteins, are modified with glycan components in the Golgi and transported along with hemicelluloses and methyl-esterified pectins via trans-Golgi network (TGN)-derived vesicles (Buchanan et al., 2000). It has also been proposed that the high turgor (0.5mPa) within plant cells may forcibly extrude vesicle-bound cell wall constituents into the expanding cell wall in order to more rapidly counter the thinning effect of wall relaxation (Szymanski & Cosgrove, 2009).
Study of the initiation and elongation of tip-growing trichomes and diffusely growing, inter-digitating (interlocking) pavement cells indicates coordinate action of both actin- and microtubule-facilitated trafficking (reviewed in Smith & Oppenheimer, 2005) and (Sieberer et al., 2005). In both tip-growth and inter-digitation models, actin polymerization is associated with bulging points while microtubules are peripheral in regions of restricted, directional expansion. As MTs are largely peripherally located as cortical microtubules (CMTs), vesicles derived from the ER or TGN are likely transported to the plasma membrane along actin microfilaments and then microtubule motors along CMTs to the bulge (Smith & Oppenheimer, 2005). Abnormal trichome branching in the mutant xik-1, specific to the Arabidopsis class XI myosin XIK, exhibited shortened root hairs, providing some of the only evidence so far provided to support a role for motor proteins in directional cell wall expansion in plants (Ojangu et al., 2007).

1.2.2 Cellulose deposition and directionality of expansion
Cellulose microfibrils (CMFs) are large polymers assembled from closely associated (1→4)-ß-D-glucan chains produced by individual CELLULOSE SYNTHASE proteins (CESAs) which are themselves arranged in a larger (rosette) complex of CESA molecules (reviewed in Doblin et al., 2002; Hematy & Hofte, 2007). The CMFs are long and have the ability form crystalline domains [through hydrogen bonding], although they often appear physically separated within the cell wall matrix. It has long been held that the directional extensibility of primary cell walls is largely established through oriented deposition of CMFs in one or more laminae of the expanding primary wall. That is, the angle between the net orientation of CMFs and the long-axis of the cells, the microfibril angle (MFA) (Donaldson, 2001) would dictate the rate of expansion; the so-called ‘degree of alignment hypothesis’ (Green, 1965).
In such a model, long, inelastic CMFs constrain expansion along their axes in a localized region such that elongation occurs perpendicular to their axis of orientation. In support of this model, mutation of primary wall-associated CESA6 (procuste-1) resulted in a 4-fold reduction in cell elongation in Arabidopsis hypocotyls with only a 28% reduction in cell wall biomass (MacKinnon et al., 2006). Reduced expansion was attributed to reduced lateral orientation of CMFs in the inner walls of epidermal cells, presumably reflecting disruption throughout the hypocotyls. However, outer surfaces of epidermal cells in many species do not fit Green’s model. One possible explanation for this discrepancy is that the correlation between CMF orientation and directional growth may only be evident in cells that bear the majority of strain of the elongating organ (reviewed in Baskin, 2005). While the orientation of CMFs in the primary wall does not always strictly conform to Green’s ‘degree of alignment hypothesis’, CMFs do exhibit increasingly longitudinal orientation (higher MFA) among sequentially-deposited cell wall layers (S1-S3) in the thickening secondary cell wall.

1.2.3 Microtubule influence on cellulose properties
Microtubules appear to play an important role in controlling anisotropic expansion, presumably through their influence on cellulose deposition or CESA activity. Microtubule destabilization through various means has been shown to disrupt anisotropic cell expansion and cause root swelling. Constitutive reduction in stabilization of microtubule severing activity through mutation of a katanin-encoding gene (FRAGILE FIBER 2, FRA2) resulted in altered CMF orientation (Burk et al., 2001; Burk & Ye, 2002). CESA complexes (CSCs) have also been observed to align with MTs and move bi-directionally, suggestive of ongoing mediation/tethering of CESA
complexes by microtubules in addition to a role for microtubules in delivering CESA complexes to the plasmalemma (Gutierrez et al., 2009; Paredez et al., 2006). However, complete removal of MTs does not appear to have a direct effect on CSC movement (Paredez et al., 2006). In another study, altered MT dynamics and organization was not found to affect CMF arrangement (Sugimoto et al., 2000), but instead appeared to affect the degree of cellulose crystallinity, which in turn could alter cross-linking to adjacent CMFs and other cell wall polymers such as hemicelluloses (Fujita et al., 2011). Authors postulate that cortical microtubules may influence plasma membrane fluidity affecting the CSC velocity, and/or may be involved in the secretion of non-cellulose wall components that may affect the degree of crystallinity. Roudier et al. (2005) (Roudier et al., 2005) showed that the deposition pattern of glycosylphosphatidyl inositides (GPI)-anchored COBRA protein is microtubule- dependent. Clearly, the form and/or function of microtubules appear to affect CESA behaviour, and thus CMF deposition and wall extensibility, through a number of possible routes.

1.3 Mechanical modulation of anisotropic expansion by non-cellulosic wall constituents and their modifiers

While CMF orientation and crystallinity likely establish the basis for anisotropic cell wall expansion, it is unlikely that they are sufficient in themselves to control the rate and extent of anisotropic cell wall expansion. While no accurate empirical measures of CMF lengths exist, they are likely closer to the length of microtubules (~10\(\mu\)m) (Kawamura et al., 2006) than cell circumference (>100\(\mu\)m), so individual CMFs cannot themselves restrain cell wall expansion. Instead, lateral strain must be countered by interactions between CMFs and other wall polymers. Few, if any, cell wall constituents appear to be exclusive to either expanding or formerly-expanding regions but do change in proportion. Many appear to be modified in specific ways in a spatiotemporally-specific manner to
regulate the degree of wall plasticity. It is therefore convenient to address each of the major cell wall constituents and their modifiers separately, to consider their effects on cell wall mechanical properties, and hence, anisotropic cell wall expansion.

1.3.1 Hemicellulose
Current models of anisotropic expansion propose that cross-linking glycan (hemicellulose) tethers connecting adjacent, or intersecting, CMFs may restrict both lateral creep and longitudinal separation of parallel CMFs (reviewed in Geitmann & Ortega, 2009). The central role of hemicelluloses in tethering CMFs is thought to be the role of two main classes of these polymers; xyloglucans (XyGs) with (1→4)β-D-glucan backbones which predominate in elongating walls, and glucuronylarabinoxylans (GAXs, or just ‘xylans’) with (1→4)β-D-xylan backbones, which predominate in non-elongating, secondary cell walls (York & O'Neill, 2008). Less abundant in most angiosperms is a class of hemicelluloses with (1→4)β-disacharide unit structures consisting of mannose and varying proportions of galactose (York & O'Neill, 2008). As a result of these backbone differences, side-chain modifications for these hemicelluloses are very different in nature. Such structural differences among cross-linking xylans imply the existence of very different biosynthetic pathways, yet these pathways remain largely unexplored and few of the putative glycosyltransferases involved have yet to be functionally characterized (Brown et al., 2007; Brown et al., 2009; Caffall & Mohnen, 2009; Zabotina et al., 2008).

It is interesting that the backbones of these molecules differ in such a fundamental manner, suggesting that they do in fact behave differently in a biologically-significant way. However, there is a lack of detailed understanding of the impact of these differing chemical structures on their proposed role in cellulose microfibril adhesion. More detailed knowledge is needed of the regionalization within the cell wall expansion
developmental continuum of the genes (and their encoded proteins) involved in regulating the biosynthesis, transport and deposition of these hemicelluloses if we are to understand the functional basis of their structural diversification. In broad terms, the observation that there is a shift in the proportion from xyloglucan in primary walls to xylans in secondary walls implies that such a substitution facilitates significant change in cell wall mechanics.

One of the better-characterized aspects of hemicellulose functionality is their enzymatic modification through hydrolytic breakage and subsequent reductive repair of these breaks, processes that allow adjustment of CMF arrangement under strain. In particular, a large number of xyloglucan endotransglycosylase/hydrolases (XTHs) encoded in the Arabidopsis genome appear to carry out a dual function of cutting and annealing xyloglucan chains. Modifications of xyloglucans by XTHs through combined hydrolysis and polymerization action, likely represent the main mechanism of regulating directional cell expansion by controlling the degree of creep and separation in CMFs tethered by xyloglucans. ENDOXYLOGLUCAN TRANSGLYCOSYLASE A1 (EXGT-A1) expression appears coincident with elongation, and suppression of EXGT-A1 expression in several mutants (acl1,3-5) results in greatly reduced leaf expansion and internode elongation (Akamatsu et al., 1999). Interestingly, a subcellular ‘fibrillar’ pattern of an XTH was observed in tobacco (roots and cultures), oriented transverse to the axis of elongation (Vissenberg et al., 2001). This orientation was parallel to MT and CMF orientation, suggesting that XTHs work directly along CMF boundaries. However, neither the relative abundance of these hemicellulose polymers nor the activity of their genes/proteins, has been definitively established in the context of the developmental continuum of cell wall expansion.
1.3.2 Expansins
Other cell wall constituents may also play critical roles in mediating cell wall expansion, likely through alteration of cellulose/hemicellulose interactions. The ‘acid growth hypothesis’, which emerged from early observations that elongating cell walls were acidified through auxin-mediated H+-export to the apoplast (Taiz & Zeiger, 2006) postulates the active participation of expansin family proteins. These proteins possess a catalytic domain, which is present in glycoside hydrolase family 45 (GH45), although the evidence suggests that expansin-mediated slippage in cellulose microfibrils is a non-enzymatic process (reviewed in Sampedro & Cosgrove, 2005). It is noteworthy that while the ‘degree of alignment hypothesis’ asserts that the elongation occurs perpendicular to the axis of net CMF deposition, many elongating cells exhibit CMFs that are oriented longitudinally to the direction of growth (reviewed in Baskin, 2005). It is therefore possible that longitudinal CMF creep mediated through the action of expansins plays a large role in controlling the rate of expansion along the long-axis.

1.3.3 Pectins
Pectins form the bulk of the extracellular matrix in primary cell walls within which other cell wall polymers are suspended. These are highly hydrophilic polysaccharides with putative functions in mediating cell-cell adhesion along the middle lamella, as well as in modifying the behaviour of other cell wall constituents through alterations in matrix porosity and ion availability. In angiosperms such as Arabidopsis, many pectins take the form of un-branched chains; either a class of highly methyl-esterified chains of (1→4)\(\alpha\)-D-galacturonic acid (homogalacturonan, ‘HGA’) or a separate class of \(\alpha\)-D-xylosyl-substituted HGAs (xylogalacturonan). In addition, angiosperm cell walls are dominated by one type of highly branched pectin, rhamnogalacturonan-I (RG-I), which possesses a \(\alpha\rightarrow2\)\(\alpha\)-D-Rha-(1→4)\(\alpha\)-D-GalA-(1→) backbone decorated with 5-arabinose, or 4-
galactose residues that are potentially decorated with arabinose sugars forming Type I arabinogalactans (Buchanan et al., 2000). Recent study established that a large number of (1→4)α-D-galacturonosyltransferase (GAUT) genes, involved in the formation of the HG backbone, are encoded in the Arabidopsis genome (Caffall & Mohnen, 2009). Furthermore, it appears that GAUT1 and GAUT7 form the catalytic core of a multi-protein complex within the Golgi to synthesize HG (Atmodjo et al., 2011).

It has been postulated that the mechanical properties of cell walls may be altered through the activity of pectin methyl-esterases (PMEs) that strip HG of methyl-ester groups at the C-6 carbons of the HG backbone. Mutational analysis in Arabidopsis has shown that the degree of pectin esterification can affect the extent of cell wall extensibility (Derbyshire et al., 2007), a pattern that was confirmed through over-expression analysis of the poplar pectin methylesterase, PttPME1 (Siedlecka et al., 2008). RNAi silencing of PttPME1 was also shown to reduce the degree of intrusive growth of poplar fibers (Mellerowicz et al., 2008). The increased polymer density that is created by PME-induced ‘egg box’ gelling could generate physical resistance to expansion, or modify the behaviour of other cell wall modifiers through changes in pectin porosity. However, the physical consequence of the proliferation of putative ‘egg-box’ structures on anisotropic cell wall expansion has not been rigorously studied.

One interesting and potentially relevant observation is the apparent localization of de-esterification within regions of cells in expanding tissue. Immunological study of the distribution of de-esterified pectins within pea (Pisum sativum L. cv. Avola) revealed punctate localization of these polymers within intercellular spaces of the stem parenchyma, which may indicate a role for PMEs in cell-cell adhesion (Willats et al., 2001) rather than cell expansion.
Another pectin, RG-I, is also thought to work in conjunction with HG-calcium complexes, based on evidence that these each appear to mediate cell-cell adhesion in *Lycopersicon esculentum* (tomato) and stomatal opening in *Commelina communis* (Asiatic day flower) (Caffall & Mohnen, 2009). Pectins such as RG-I and HG are also known to covalently cross-link extensively with each other, which, from a technical perspective, creates a significant challenge in isolating purified pectic polymers (Caffall & Mohnen, 2009). The functionality of Arabidopsis pectins remains largely unexplored at the molecular level, beyond the participation of PME activity.

### 1.3.4 Glycoproteins
In addition to the large number of cell wall-targeted enzymatic proteins, four classes of glycoproteins (commonly referred to as ‘structural’ proteins), including hydroxyproline-rich glycoproteins (HRGPs), proline-rich proteins (PRPs), glycine-rich proteins (GRPs) and arabinogalactan proteins (AGPs). Among these, the AGPs have been most intensively studied.

It has long been known that AGPs affect cell expansion through the strong [reversible] inhibitory effect on such expansion induced by a class of AGP-specific stains, the β-Yariv reagents, which specifically target [as-yet-unspecified regions of] AGPs (reviewed in Seifert & Roberts, 2007). Mutational interference with galactosylation and fucosylation reactions can also lead to loss of anisotropic cell expansion in roots, as revealed by the phenotypes of *udp-glucose 4-epimerase (uge4)* and *murus 1 (mur1)* mutants, respectively (reviewed in Seifert & Roberts, 2007). This indicates the degree to which AG side-branch galactosyl- and fucosyl- modifications perform a wall-stabilizing role in cell expansion.

Since the protein portions of many AGPs are covalently bonded to GPls that anchor the proteins in cellular membranes, their functionality can be dramatically altered by
cleavage of this linkage. For instance, point mutation of protein interaction domain in \textit{FASCICLIN-LIKE AGP4} (\textit{AtFLA4}) (reviewed in Seifert & Roberts, 2007) presumably disturbing GPI-anchor function resulted in a root bulging phenotype. By contrast, [transient] overexpression of a GPI-anchor-containing AGP from cucumber increased stem elongation in tobacco (reviewed in Seifert & Roberts, 2007).

The developmental timing and location of the expression of the same AGPs also appears to coincide with the process of secondary cell wall formation. For example, \textit{AtFLA11} expression appears to be localized to Arabidopsis tissues forming significantly thickened secondary cell walls at the time of that thickening (Shinsaku \textit{et al.}, 2005). \textit{AtFLA11} and \textit{AtFLA12} were also identified in a regression analysis of public datasets to co-express with secondary cell wall associated \textit{CESAs} (4, 7, & 8) (Persson \textit{et al.}, 2005), and study of mutation of these Arabidopsis genes reveals significant alterations to structural integrity of Arabidopsis stems, perhaps driven by reduced cellulose deposition (MacMillan \textit{et al.}, 2010). An analysis of the functional redundancy of \textit{AtFLA11} and \textit{AtFLA12} has yet to be published. The precise roles of AGPs, whether purely mechanical or signaling, have yet to be elucidated, although extensive [tightly-regulated] cross-linking with other cell wall constituents is a likely mode of action (Seifert & Roberts, 2007).

Also extensively studied are the HRGPs, which share common backbone features with the AGPs but differ markedly from them in structure. While AGPs are more appropriately termed proteoglycans, due to the small proportion of protein component, HRGPs are predominantly proteinaceous. Most common among these are extensins, proteins that are likely to comprise a large proportion (~15%) of total cell wall biomass. They possess arabinose side-chains attached to hydroxyproline (Hyp)-rich regions (reviewed in Lamport \textit{et al.}, 2011). However, it must be acknowledged that both AGP
and extensin features may occur on a wide variety of proteoglycans, so they cannot be considered exclusive from one another (reviewed in Lamport et al., 2011). It has been proposed that extensin self-assembles into large, positively-charged scaffolds, and that the alternating hydrophilic and hydrophobic regions of extensins promote interactions with a diversity of cell wall constituents (Rapaport, 2006). Scaffolding appears to be facilitated by extensin peroxidase, as this enzyme yielded an insoluble, tyrosine-cross-linked extensin substrate, AtEXT3/ROOT- SHOOT- HYPOCOTYL-DEFECTIVE (RSH) (Cannon et al., 2008). The authors of this study propose that the positively-charged RSH scaffold joins with pectin (net negative charge) to form extensin pectate within cell walls, thus providing a template for subsequent cell wall formation processes. Classical extensins are not GPI-anchored and likely behave more exclusively as structural proteins that cross-link with other glycan-containing cell wall constituents in cell wall stiffening; extensin is thus a mis-nomer.

1.3.5 Lignin
Lignins are complex polymers that form through enzymatic oxidation and non-enzymatic coupling of monolignol subunits (p-coumaryl, coniferyl, and sinapyl alcohols) within various regions of the apoplast (Buchanan et al., 2000). This polymerization is irreversible and lignin is known to covalently cross-link with many adjacent polysaccharide and proteinaceous cell wall constituents (Caffall & Mohnen, 2009). Thus, lignification presents a major mechanical impediment to introduction of cell wall modifications thought to be required for cell expansion, as well as forming an impermeable barrier that isolates cells that are committed to programmed cell death (PCD).

Lignification appears specific to regions of secondary cell wall thickening where expansion has finished. Massive lignification is coincident with secondary cell wall
thickening of the interfascicular fibre region at a point along Arabidopsis stems where expansion is likely to have ceased (Ehlting et al., 2005). In the elongating hypocotyl, lignification appears limited to annular and helical regions of secondary cell wall deposition in the protoxylem (Dharmawardhana et al., 1995). Study of fescue leaf blades revealed an apparent relationship between the accumulation of p-coumarylated lignins and cessation of leaf expansion (MacAdam & Grabber, 2002). It has been proposed that oxidative coupling of ferulates of lignin to xylan has a role in cessation of elongation (Grabber, 2005).

However, it is unclear if lignification is a cause or consequence of cessation of elongation. There is little empirical evidence for a causal link between lignification and cell wall expansion. While an ectopic lignification mutant (ell1) exhibited altered cell expansion (Cano-Delgado et al., 2000), overall growth of this plant was severely affected and the gene remains uncharacterized. Interestingly, localized lignification of the root stele has been observed to result in turgor drop and reduced maize root elongation (Fan et al., 2006), and it remains to be seen if this response is widespread in the normal course of cessation of diffuse elongation, outside of pathogen-induced responses where such localized lignification is well-known.

One approach to understanding the conditions that facilitate lignification is to study the mode of lignin deposition. The mode of localized deposition of monolignols also remains a mystery (Vanholme et al., 2010). Deposition of lignols via TGN/vesicle trafficking was eliminated as a possibility (M. Kaneda, 2009), while a mechanism to deliver monolignols via a cytosolic pathway does not appear to directly involve ABC transporters (M. Kaneda et al., 2011). Closer examination of the roles of regulators of lignin-related biosynthesis pathways, such as R2R3-MYB, LIM, MADS, and KNOX transcription factors (reviewed in Rogers & Campbell, 2004) may reveal effects on
other cellular processes associated with wall expansion and/or secondary wall deposition that could yield insight into the monolignol delivery mechanism.

1.4 Regulation of differentiation
Anisotropic expansion and the associated processes of primary and secondary cell wall formation are embedded within a program of organ differentiation; the process by which homogeneous cohorts of meristematic cells diverge in morphology and function to form complex and mature tissues. Aside from localized expansion phenomena such as tip-growing epidermal layers, anisotropic cell expansion in growing tissues is coincident with other differentiation processes such as cell fate determination. The senescence program is also relevant to many expanding organs such as the primary inflorescence stem of annuals, including Arabidopsis (an over-wintering annual). Coordination of these processes is facilitated by a complex network of systemic, diffusible signals (morphogens) such as hormones, and more localized effecters such as transcription factors.

1.4.1 Regulation of vascular cell fate
A significant proportion of organ differentiation in primary growth has been characterized as ‘vascular differentiation’, and may more precisely be characterized as vascular cell fate determination. Central to this patterning in Arabidopsis is the establishment of the spatial arrangement of vascular bundles within the surrounding parenchyma. Cytokinin signalling appears to mediate procambial formation, as suggested by mutation of CYTOKININ RESPONSE 1 (CRE1)/ WOODEN LEG which impairs procambial formation in the nascent vascular bundle (De Leon et al., 2004). Both empirical evidence and modelling have pointed to the coordinate action of both auxin and brassinosteroid (BR) signalling pathways in configuring the alternating arrangement of vascular bundles and interfascicular regions (Ibañes et al., 2009).
Brassinosteroids are thought to induce procambial divisions at periodic points of auxin maxima established around the circumference of the primary stem. The role of auxin (indole-3-acetic acid, ‘IAA’) in this model appears to be through auxin response factor (ARF) transcription factors such as MONOPTEROS (MP/ARF12) (Hardtke & Berleth, 1998) and BODENLOS/IAA PROTEIN 12 by its association as an ARF inhibiting MP/ARF12 (Hamann et al., 2002). This BR-mediated asymmetric patterning of xylem and phloem within regions of vascularization appears largely dependent upon tissue-specific control of class III homeodomain-leucine zippers (HD-ZIP) and KANADI transcription factor genes along periclinal gradients (reviewed in Carlsbecker & Helariutta, 2005).

Interestingly, the action of AGPs is not limited to [aforementioned] cell wall formation processes since these proteins seem to play a role in vascular cell fate determination. Xylogen, a *Zinnia elegans* lipid transfer protein (LTP) that contains the AGP structure, triggers differentiation in *Zinnia elegans* (Motose et al., 2004), while epitopes recognized by AGP-specific antibodies have been closely associated with the initiation of proto- and metaxylem (reviewed in Seifert & Roberts, 2007).

Furthermore, auxin appears to be required for the proliferation of the cambium into the interfascicular region at the base of the Arabidopsis stem, thus promoting the initiation of interfascicular xylem and establishing secondary growth (Little et al., 2002). Close histological, molecular and genetic examination of this region of the stem collectively indicates that jasmonic acid (JA) signaling pathway components also play important roles in this interfascicular cambial proliferation (Sehr et al., 2010). It thus appears that vascular cell fate determination is biphasic, including a phase of vascular bundle establishment at the root or shoot apex, followed by interfascicular cambial formation in
the hypocotyl region of the primary stem where procambial activity is retained long after primary growth has stopped occurring.

**1.4.2 Regulation of cell polarity, expansion, and secondary cell wall formation**

Aside from this program of cell fate determination, a subset of these regulators has been found to directly influence various aspects of anisotropic cell expansion, primary cell wall formation and secondary cell wall formation. The first step of anisotropic cell expansion is to establish cell polarity, and this appears to be due in part to the role of BR in mediating auxin flux through alteration of auxin efflux protein (PIN2) localization at the boundary between root tip and elongation zone (Li et al., 2005). Gibberellic acid (GA) signalling, in addition to influencing seed germination and flowering, has long been recognized as a key hormonal pathway in mediating the extent/rate of cell wall expansion in plant organs (Hedden, 2003). Many mutants with cell expansion defects have been found to possess low levels of active GA (Hanzawa et al., 2000). Also, many mutants exhibiting hyper-elongation phenotypes have led to discovery of genes which negatively regulate GA signalling. As one example, gain-of-function mutation of the **OVATE FAMILY PROTEIN 1** (**OFP1**), a repressor of GA biosynthesis enzyme AtGA20ox1, resulted in impaired elongation in many Arabidopsis organs (Wang et al., 2007). Furthermore, repressor screens of dwarf GA-biosynthetic mutants, suggest that GA functions largely through targeted ubiquitination of DELLA growth repressors (reviewed in Schwechheimer, 2008). Mutation of the spermine synthase gene **ACAULIS 5** produces a mutant (**acl5**) with deficient cell expansion yet with normal GA levels (Hanzawa et al., 1997), indicating a role for spermine signalling in cell expansion, operating downstream of GA.

Many regulators appear to predominantly affect cell wall thickening, perhaps largely independent of cell wall expansion. In Arabidopsis, auxin appears to be required for cell
wall thickening and lignification of both xylary and interfascicular fibres, which are associated with the aforementioned proliferation of interfascicular cambium (Little et al., 2002). Interestingly, α-naphthalene acetic acid (α-NAA), the synthetic analogue to IAA, suppresses secondary cell wall synthesis and enhances elongation, emphasizing the importance of subtle spatiotemporal effects of IAA in mediating secondary cell wall formation (Singh et al., 2009). Ethylene also appears to influence the helical cell wall thickenings of pea epicotyl internodes and mung-bean hypocotyls (Roberts et al., 1985).

The inositide-signalling pathway has also been implicated since mutation of FRAGILE FIBER 3 (a myo-inositol polyphosphate 5-phosphatase) affects secondary cell wall synthesis and actin organization in fibres (Zhong et al., 2004). Roles in xylem or fibre development have been ascribed to several NAC (NAM, ATAF1/2 and CUC2) -domain transcription factors. In particular, VASCULAR-RELATED NAC-DOMAIN6 (VND7) and VND6 can induce secondary cell wall formation in the protoxylem and metaxylem, respectively, while NST1 and NST3/SND1 appear to act specifically in the interfascicular fibres (reviewed in Demura & Fukuda, 2007). It must be acknowledged that mutant characterizations have been limited and so the reported association of genes/proteins may not be exclusive to specific processes. Also, significant gaps remain in our current understanding of the regulation of cell wall expansion and secondary cell wall formation, so our perception of the roles of regulatory players could be significantly revised.

1.5 Systems biology for cell wall expansion
Extension of our knowledge of cell wall dynamics, particularly those of primary cell expansion and secondary cell wall formation, is made challenging by the complexity of such phenomena. The sheer number of molecular agents that may affect cell wall dynamics forces us to apply reductionist approaches to inferring their function. There
are so far approximately 1000 genes that have been associated at some level with cell wall processes (Somerville et al., 2004), and likely even more metabolic players are also contributing indirectly. Furthermore, myriad possible interactions appear to exist among these components, particularly within the complex and varied environment of the developing cell wall. At the other end of the organizational spectrum, cell wall expansion phenomena appear to be governed by organ-wide, systemic signals, and the pathways of such signalling are complex and largely uncharacterized.

Thus, reductionist approaches to dissecting these pathways on a gene-by-gene basis are greatly limited by human resource as well as by being confounded by in planta complexity. ‘Oomics’ research approaches are capable of capturing global, in planta views of an increasing range of biochemical and molecular processes. Acknowledging that the whole is greater than the sum of its parts, ‘systems biology’ integrates and analyzes such multidimensional biological information to network and model these complex processes thereby providing an alternate route to deducing the roles of individual players (genes, proteins, metabolites, etc…)(reviewed in Yuan et al., 2008).

1.5.1 Model systems for study of directional cell expansion
To tackle the complexity of cell wall dynamics, researchers have employed a large arsenal of biochemical, molecular, genetic, and computational tools. It has been acknowledged that ‘complete understanding’ of root [or stem] biology would require an integrated, ‘systems biology’ approach (Iyer-Pascuzzi et al., 2009; Yuan et al., 2008). Much attention has been focused on developing new ‘omics’ technologies, data integration methodologies, and data analysis methodologies (Yuan et al., 2008). However, there is considerable heterogeneity among studies with regard to the biological material being examined due to poorly defined developmental categories in the tissues sampled.
Inherent in complex systems such as developing plant organs are numerous developmental processes, including cell division and whole-plant processes such as senescence. It is therefore necessary to study a developmental continuum that is sufficiently narrowly defined to allow us to infer functional associations between specific cell wall expansion processes operating at different levels, ranging from molecular signals to cell morphology.

Stems, like roots, are cylindrical organs with a relatively uniform anisotropic (basipetal, bi-directional) expansion. Much of the inflorescence architecture is formed prior to bolting during the period initiated by flowering transition in the rosette (Hempel & Feldman, 1994; Pouteau & Albertini, 2009). This differs from the situation in roots, where lateral root emergence occurs after elongation and confounds the cell expansion gradient with lateral root emergence patterning (Moreno-Risueno et al., 2010).

1.5.2 Defining the gradient of primary stem anisotropic expansion

Given the radical changes in transcriptome profile that occur over narrow spatiotemporal regions of the expanding inflorescence stem (Ehlting et al., 2005; Imoto et al., 2005; Ko & Han, 2004; Minic et al., 2009; Oh et al., 2003), imprecision in describing various growth phases among experiments significantly confounds efforts to integrate, or find corroboration among, datasets. A developmental framework has been proposed for studying Arabidopsis in which its growth is divided into six major stages (with sub-division) including leaf development, rosette growth, inflorescence emergence, flower production, silique ripening, and senescence (Boyes et al., 2001). However, this approach has been used by only one of the transcriptomic studies cited above (Minic et al., 2009), and offers very limited resolution in terms of describing specific developmental regions within the inflorescence stem. Importantly, there is currently no standardized means to identify the point of cessation, at which the transition from
primary to secondary cell wall biosynthesis occurs in the lateral cell walls. Publicly available databases do not yet offer standardized fields of entry on such a fine developmental scale for inflorescence primary stems (ex Genevestigator) or any other expanding organ. As a result, ‘systems biology’ approaches aimed at ‘complete understanding’ of primary and secondary cell wall formation processes have yet to develop a common framework upon which to build that understanding. The lack of such a developmental platform also affects the value that can be derived from the phenotyping of cell wall mutants, since the timing of the emergence or the maximum impact of cell wall irregularities needs to be placed within the context of cell wall expansion.

1.6 Kinematic analysis for characterization of the expansive growth field in root and shoot
Measures of the distribution of elongation rates along the stem effectively describe the distribution of anisotropic cell wall expansion events in the underlying cells. Kinematics, the study of the motion of bodies, has traditionally been used to describe the movement of various plant regions relative to a spatially fixed position such as a root tip (Gandar, 1983), and thus shows great potential for characterizing anisotropic cell wall expansion. Because of their simple, macroscopic morphology, roots have been the primary focus of efforts to characterize anisotropic cell expansion. Erickson and Sax (1956) were the first to quantitatively characterize plant root growth by monitoring corn root expansion on a 2D surface through time-lapse imagery and manual measurement (Erickson & Sax, 1956). Such root tip displacement rates collectively form what is referred to as the ‘velocity field’ (Gandar, 1983).

Modern applications of this approach have utilized a high density of natural features along a continuously visible, expanding surface. In one study, leaf expansion in Ricinus
(castor) plants was monitored using time-lapse imagery to generate complex velocity fields describing the expanding leaf surface (Schmundt et al., 1998; Walter et al., 2002). However, this approach can produce unreliable estimates when the expanding regions are highly visually uniform, since they lack sufficiently high density of optical features to define the expanding surface (Schmundt et al., 1998). Additional noise can be introduced into the measurements when identification of those features is not precisely determined (manually or automatically). One strategy to circumvent the challenge of generating velocity fields when natural features are limiting has been to add synthetic markers, such as ink marks, to expanding surfaces (Suh et al., 2005).

Ultimately, however, velocity field data provide a cumulative view of organ expansion, which does not provide a convenient means to identify points of maximum growth rate. To circumvent this limitation, it is possible to present growth rate data in terms of ‘relative elemental growth rate’ (REGR) for discrete regions along a specific transect of an organ, such as the midline of a root. Erickson & Silk (1956) (Ralph O. Erickson & Sax, 1956) first demonstrated that acceleration rates for extension of specific root regions relative to the root tip could simply be derived through mathematical differentiation of velocity field data. Since the authors considered these region-specific acceleration derivations to represent local expansion rates, they described such output as ‘relative elemental rate[s] of elongation’. This same approach has since been used to convert velocity field data determined through manual (Gandar, 1983) or automated (Basu et al., 2007) means to the more intuitive REGR curves (Gandar, 1983). However, ‘relative elemental’ growth rates may be obtained more directly from direct measurement of distances between appropriately spaced natural or synthetic features along the surface of the plant organ, thereby providing REGRs without requiring velocity field data acquisition. For example, Sugimoto et al. (2000) measured the change in the
distance between adjacent graphite particles scattered along Arabidopsis root tips to determine REGR. An analogous approach to measuring REGRs, using inflorescence stem segments tagged with synthetic optical markers, could potentially provide the cell expansion data needed to guide harvesting of tissue samples that would consistently represent specific cell expansion states.

1.7 Problem statement

Primary and secondary cell wall formation processes are conveniently arranged in sequential spatial and temporal manner within the Arabidopsis inflorescence stem, which makes this an ideal system for studying these processes, as well as the transition between them. However, there is no established methodology for precisely determining the developmental status of cell wall expansion within the context of primary stem extension. Growth kinematic analysis of stem extension, and generation of REGR data, has the potential to identify specific stages of cell wall development on the basis of cell wall expansion performance. Such an approach would seem to be possible in growing Arabidopsis inflorescence stems, since REGR data were computed earlier for corn coleoptiles (Collings et al., 1998). It is also obvious that growth kinematic analysis conducted on individual plants could provide a guide to harvesting stem segments that consistently represent specific stages of cell wall development, thus allowing pooling of developmentally-matched samples for various molecular analyses. If successful, this approach could therefore provide a common developmental scaffold upon which to assemble a systems biology approach to understanding cell wall formation.

1.8 Research/thesis objectives

The objective of this work is to identify the changes in molecular patterns (gene transcription and cell wall structural epitopes) occurring along the cell wall differentiation
continuum of *Arabidopsis thaliana* primary stems. In order to be able to identify and pool developmentally-matched tissue samples from multiple individual plants for these analyses, I also wished to establish a common developmental framework of cell wall expansion based on growth kinematic profiling.
Chapter 2. Developmentally equivalent tissue sampling based on growth kinematic profiling of Arabidopsis primary stems

2.1 Introduction

The *Arabidopsis thaliana* inflorescence stem undergoes a radical change in form and composition as the compressed internodes of newly formed primordial axillary stem elongate rapidly to their fully expanded state in the mature inflorescence. As in roots, this primary stem growth is accomplished through an iterative process of division, elongation, and maturation of successive cohorts of cells occurring within a developmental cassette at the plant apex. It has long been established that the anisotropic tissue expansion accompanying stem elongation requires a massive increase in the length of axial cell walls (reviewed in Erickson & Sax, 1956). Unlike roots, lateral branching of stems is established prior to elongation and, under normal long-day growing conditions, much of the aerial architecture of the primary stem, including transition from cauline leaf zone to true inflorescence, is already formed prior to primary stem bolting (Hempel & Feldman, 1994; Pouteau & Albertini, 2009). The dramatic bolting of the Arabidopsis inflorescence presents a simple and powerful system to study cell wall expansion phenomena. Furthermore, since extensive secondary cell wall formation and rigidification occurs within a subset of cell types below the point of cessation of general cell wall expansion (Ye *et al.*, 2002), this system also shows great potential for discovery of key processes and regulatory mechanisms associated with secondary cell wall formation (Ehlting *et al.*, 2005; Zhong *et al.*, 2008).

Examination of developmental processes through an integrated systems biology approach is primarily challenged by 1) accurate identification of tissues at distinct
developmental stages (Boyes et al., 2001) and 2) the requirement for adequate amounts of those tissues for assays such as proteomics, metabolomics and transcriptomics (Pu & Brady, 2009). The challenge of pooling developmentally-matched tissues across sufficient replicate plants is particularly acute in study of cell wall expansion processes, given the narrow spatiotemporal window at either root or shoot apices. A high degree of spatiotemporal resolution has been demonstrated in expression profiling studies of Arabidopsis root development that use fluorescence-based sorting of protoplasted Arabidopsis root cells from fluorescent marker-bearing lines (Birnbaum et al., 2003; Birnbaum et al., 2005). However, uniform protoplasting of often-lignified primary stem tissues remains an intractable problem.

It would be desirable to be able to physically isolate tissues of a known developmental state en masse from macroscopically-discernable regions of the plant. Some studies of stem elongation have attempted to accomplish this by harvesting the base of the inflorescence stem at standardized principal growth stages as defined by flower status (Boyes et al., 2001; Minic et al., 2009) or inflorescence maturity in a subset of plants (Ko & Han, 2004). Others have compared different developmental stages co-occurring along the primary stem (Ehlting et al., 2005; Imoto et al., 2005), using microscopic examination of a subset of plants to establish guidelines for harvesting and pooling samples from a larger set of plants. Methods that group tissue samples selected on morphometric measures along the stem rely upon the assumption that plants that appear morphologically similar also share similar developmental proportioning. However, genetic and environmental variables, as well as the innately stochastic nature of developmental programming, make it unclear that such an assumption is valid.
Inflorescence elongation is a dynamic process that lends itself to kinematic analysis (study of the motion of bodies) as a means of establishing how the process of cell elongation is distributed throughout the inflorescence stem. Manual measurement of the rates of separation of ink marks positioned 5mm apart along the Arabidopsis inflorescence stem, relative to the base of the stem, did establish that no elongative growth occurred at distances >7cm below the apex of the stem in Columbia plants over a 24 hour period, which made it possible to contrast pre- versus post-cessation states (Suh et al., 2005). These samples were presumed to broadly represent growth stages involving predominantly primary and secondary cell wall formation, respectively, but this approach of tracking ink mark movement cannot isolate regions of maximum tissue expansion rate.

Kinematic analysis of root growth was first realized through the development of time-lapse imagery of corn roots marked by carbon particles (Erickson & Sax, 1956). Measurement of the rates of displacement of carbon particles from the root tip allowed derivation of the ‘relative elemental growth rate’ (REGR) for points along the root, essentially providing a growth kinematic profile. More modern approaches have utilized root tip displacement rates, also referred to as the velocity field (Gandar, 1983), as the basis for direct calculation of REGR as a percentage change in length for elements (segments) over time (Basu et al., 2007; Schmundt et al., 1998). REGR data have also been generated indirectly for a number of species (not including Arabidopsis) through a modelling approach that uses a logistic function conditioned by estimated parameters in order to substitute for the missing velocity field data (Morris & Silk, 1992). This logistic function was used to model strain rates (cell wall expansion), essentially equivalent to
REGR, from cell length data (destructive sampling) and overall root growth rate (Baskin, 2005).

Unlike the apex-based growth analyses of roots, derivation of REGRs for inflorescence stems requires a much different approach, because 1) the inflorescence stem apices are obscured by floral clusters, and 2) lateral branching and flowers in the region of stem expansion prevent direct observation of all regions of the elongating primary stem. These impediments also preclude use of the recently developed 'structure tensor' methods for automated determination of velocity fields (optical flow) (French et al., 2009; Palaniappan et al., 2004; Schmundt et al., 1998; Walter et al., 2002), which depend upon a high density of natural features along a continuously visible, expanding surface.

In Zea mays L., manual measurement of internode lengths enabled REGR (mm/day) values to be derived for individual, numbered internodes between 30 and 65 days post-germination (Collings et al., 1998) but the internode density of Arabidopsis is too low to allow detailed growth kinematic profiling. I reasoned that regularly spaced synthetic optical markers (plant tags) that extend beyond visual obstructions (branching) would provide a finer scale of measurement with which to compute localized REGR, largely irrespective of the degree of primary stem concealment. Physical tagging of plant stems would also permit post-observation isolation of specific, numbered segments that correspond to regions of interest along the cell wall expansion continuum. The finer spatial and temporal resolution provided by this strategy should, when applied to the Arabidopsis inflorescence stem, result in a powerful platform on which to build analyses of the developmental program of cell wall expansion in this organ.
Here, I outline a rapid, non-invasive means of establishing fine-scale growth kinematic profiles of individual elongating Arabidopsis primary stems. This system facilitates both growth-rate characterization and isolation of tissue from specific stages of elongative development. To validate my approach, I compared cell length between growth kinematic profile-identified developmental states for each of two diffusely growing cell types. I also use the technique to examine the position of specific developmental transitions relative to the shoot apex, and to the overall height of the plant, as a test of the common assumption that individual plants of similar stature have similar growth kinematic profiles.

2.2 Results

2.2.1 Synthetic optical markers (tags) allow precise tracking of inflorescence elongation

To record the progress of cohorts of cells relative to other cohorts, I fixed a series of synthetic optical markers (paper tags of fixed dimensions) along the stem surface at a density that allowed precise tracking of those epidermal cell cohorts (Figure 2.1). While manual placement of either paper tags or polyvinylidene fluoride (PVDF) discs (Figure A.1) at distinct intervals requires care, their use offers distinct advantages over other marking systems. They allow for fine-scale characterization of primary stem segment REGRs, whereas natural features on the stem surface are typically not present in sufficiently high density, and do not present precise proxy to cohorts of cells. Similarly, the arbitrary placement of synthetic marks such as graphite particles on roots (Erickson & Sax, 1956; Mullen et al., 1998; Sugimoto et al., 2000) or silicone beads on trichomes (Schwab et al., 2003) substantially complicates the tracking and harvesting of the marked segments.
Figure 2.1 Images of a plant within growth chamber, just after tagging (start of the observation period) and then just prior to harvesting (end of the observation period). Primary stem growth was constrained within front and back plates of vertical nylon lines. The pair of images selected from a 2h:50m time-series are shown here to demonstrate a clear change in stem height. Normally, images were captured every minute and final calculation of the growth kinematic profile was made on the final 10 minute interval before harvest.

Although the optical marker cannot always be positioned exactly horizontally, it is possible to routinely identify within the images the point at which the top edge of each
paper tag intersects the left and right edge of the plant stem, and to use this information to precisely quantify the length of individual between-tag stem segments as they change over time (Figure 2.2). Assuming that a tag is attached at a single point on the stem (tangential point of attachment), the location of the point of contact can be inferred by averaging the XY coordinates of the left and right tag/plant intersection points (Figure 2.2, inset). Restriction of the primary stem to a two-dimensional plane allowed us to digitize the spatial arrangement of left and right tag/plant intersection points on an XY plane (Figure 2.3), and to calculate the segment length as the Euclidean distance between the means of tag/plant intersection points for adjacent tags with a high degree of precision. I could also calculate the width of the stem at the tagged point, which I found not to measurably increase over the duration of the observation period. Further precision in establishing these intersection points was achieved by doubling the number of pixels in the image.
Figure 2.2 Illustration of segment length determination in tagged plants. Tag position is defined as the intersection point between the midline of the primary stem and the top edge of the tag (large arrows). Segment length change is computed as the change in the start (*) and end (**) distance between two tags over the time interval. Inset: Calculation of tag position was formally computed as the mean Euclidean distance in XY space between the left and right tag/plant intersection points (arrowheads), where either the left or right edge of the primary stem was considered. An alternate tracking strategy for round disks is similarly portrayed in Supporting Information Figure A.1.

While the top of the plant was also tracked in this time-lapse imagery, flowers typically obscured the shoot apex. The flowers themselves did not provide a reliable indicator of apex movement due to floral organ movements obscuring the tip of the primary stem. The top segment (#1 in Figure 2.3, inset) was therefore not incorporated into the kinematic analysis of primary stem elongation.
Figure 2.3 XY coordinates of tag/plant intersection points derived from feature tracking dataset. Two marks for the left and right side of the stem enable calculation of stem width and mean tag placement on plant. The XY coordinates have been normalized such that the base of the stem (asterix) is the origin, while the top of the plant is indicated as a triangle. Arrows labeled R1 and R2 depict calibration reference marks that remain a fixed distance apart (5cm) throughout the time course to correct for the effect of digital focusing on scale. Blow-up: dotted vertical lines bisect the span between the left and right stem-tag intersection points for the segments numbered from the top downwards.
2.2.2 Calculation of relative growth rates from tag movements

To calculate the elongation rates of tag-defined segments over time, I chose an approach that closely parallels previous methods of deriving REGR from dimensional change in composite segments of roots over time (Sugimoto et al., 2000; Wenzel et al., 2000).

Accounting for differences in absolute lengths of segments, and specifying the unit of time in hours, I established the following formula:

\[
\text{REGR} = \left( \frac{[x_{n,f} - x_{n,i}]}{x_{n,i}} \right) / t_{\text{int}} \times 100 \quad (1)
\]

where:

- \( n \)=segment number
- \( x_f \)= final length of segment (cm)
- \( x_i \)= initial length of segment (cm)
- \( t_{\text{int}} \)=time interval (hours)

REGR values, expressed as the rate of percentage change in length per hour, were calculated for all segments spanned by tags using equation (1). These REGR values were then plotted as a function of distance from the base of the stem to generate growth kinematic profiles that reflect the positioning of elongative growth along the bolting inflorescences through the course of observation (surface plots, Figure 2.4). Variations in segment length over time due to measurement error were addressed with a smoothing function (linear regression) to provide predicted segment lengths for all time intervals (Supporting Information Figure A.2), and thus more robust estimation of the REGRs during the final interval prior to harvest. Since the REGR values generally fell within 65% confidence intervals for the best-fit ‘locally-weighted linear regression’ (LOWESS) curve, I chose to use the LOWESS curves to provide a standardized approach to best identify specific stages of elongative development.
In this manuscript, I present growth kinematic profiles for all plants used for multi-plant analyses of growth stage transitions and cell length measurements (Figures 2.5 and 2.6). I identified the points of maximal growth rate and cessation in 24 individual Columbia plants using either paper tag (Fig A.3; A1-E5) or PVDF disk (Fig A.3; A5,B5) optical markers. Initial time-lapse imagery of unrestrained plants indicated that circumnutation was pronounced in some individual plants (video not shown) and may contribute to apparent increases or decreases in the size of marker-spanned segments as a result of stem movement toward or away from the camera. The resulting inaccuracy in REGR determination may have led to increased noise in growth kinematic profiles observed for plants grown under those conditions (Figure A.3; A1-F1).

It is worth noting that accurate recording of dimensional change along the primary stem requires restriction of this expansion to a single dimension for high-resolution imagery, and precise tracking of the changes over short time intervals. Primarily for this reason, assessment of REGRs from 2D observation of growing roots (single-point perspective) has largely been conducted on restrictive 2D surfaces such as glass plates (Basu et al., 2007; Erickson & Sax, 1956) or petri dishes (Mullen et al., 1998; Nagel et al., 2006; Hummel et al., 2007). This data is recognized as being informative despite ongoing debate of the effect of such confinement on actual growth rate (reviewed in Baskin, 2005). In the system I present, stem growth was essentially constrained to a single linear trajectory through use of fine vertical nylon guide lines that permit uninterrupted branching and floral development while constraining circumnutatory movement, without impeding vertical extension (Figure A.1). Maximum REGRs for growth kinematic profiles of these plants (Figure A.3, columns 2-6) do not appear negatively impacted (3-4% change in length per hour in the nylon-restrained environment compared to 2-3%
change in length per hour for the unrestrained condition). Such confinement appears unlikely to impact expansive growth, in comparison to more invasive means of confining growth to 2D, such as were employed in a study of leaf expansion where tensile force was being exerted by leaf tethers (Hsiao et al., 1970; Schmundt et al., 1998). The latter process has documented effects on normal tensile forces involved in leaf expansion (Walter et al., 2002).

Growth kinematic profiles derived in this fashion generally presented identifiable zones where the elongation rate was increasing, where elongation was maximal, where the elongation rate was declining, and where elongation had effectively ceased (cessation zone) (Figure 2.4). Thus, while it is clear that no sharp developmental boundaries exist along the inflorescence stem, these profiles allow me to locate points of maximal cell expansion and of growth cessation along individual stems, and to then harvest, pool and compare tissues with contrasting elongative growth behaviours.

While the point of cessation of elongation was clearly evident in most of the kinematic profiles, a precise point of maximal elongation rate was often less clear due to extended regions of similar elongation rate and/or growth rate anomalies. In 11 out of 24 plants, enhanced elongation at the apex generated two maximal growth rate peaks. In seven of these cases, an abrupt spike in the relative growth rates of near-apical segments was observed at various times during the observation period. In other cases, specific segments within the zone of greatest elongation rate displayed distinctly suppressed growth relative to their neighbours (Figure 2.4c), greater than the measurement variability observed in the growth rates for regions below the point of cessation, which likely reflected measurement error and/or length-smoothing artifacts. Since one goal for the present study was to enable highly actively elongating tissues to be compared with
those that had ceased elongating, I selected as the *de facto* representative of the region of maximal elongation rate, the segment within that region that was located nearest to the defined point of growth cessation.
2.2.3 The position of growth stage transitions is widely variable among individual plants

My growth kinematic profiling system allows formal testing of the assumption that plants of equal height have similar developmental proportioning. For this purpose, I compared the distances from the shoot apex of the experimentally-determined points of maximum growth rate and cessation for 24 individual Columbia plants. These plants ranged in height from 10 to 15 cm (Figure 2.5, Figure A.3), but while the distribution in plant heights (Figure 2.5a) is shown to be normal through both a Shapiro-Wilks test and quantile-quantile (Q-Q) plot for normality (Figure 2.5b), I found considerable spread in the distributions of the values for maximum growth-rate (mean=2.57, st.dev. = 0.954, n=24) and cessation (mean=5.75, st.dev. = 1.16 n=24) distance from the apex. This points to a high degree of variability in the position of these growth phases within individual plants (Figures 2.5c and 2.5e, respectively). To test the significance of the differences in these distributions, I performed a Welch 2-sample t-test to reveal that t=-10.1532 (deg.fr.=42.46) for a p-value of 6.3 x 10^{-13}. The maximum growth rate distribution had a mean of 2.58 and a 95% confidence interval (CI) of +/-3.8, while the
cessation distribution had a mean of 5.75 and a 95% CI of +/-2.54. While these means are significantly different from each other, the populations do overlap. Scatterplot graphing also demonstrates that the relationship between overall plant height and the distances of cessation ($r = 0.24$) was even weaker than that of maximum growth rate ($r = 0.33$) from the apex (Figures 2.5d and 2.5f, respectively). I also found that the distance required to transition from maximum growth-rate to cessation along a given stem was quite variable, with a wide distribution in this metric (mean = 3.174, st.dev. = 0.67, n=24) and a poor correlation with overall plant height ($r=-0.06$) (Figure 2.5g and 5h, respectively). This indicates that identification of either the point of maximum growth rate or the point of growth cessation does not allow reliable prediction of the position of the other.
2.2.4 Growth kinematic profiles facilitate isolation of distinct stages of cell length

To assess the precision with which developmentally-matched cell cohorts could be isolated from stem segments of different plants based on positional information gleaned from their growth kinematic profiles, I examined longitudinal sections of the relevant segments and measured the cell lengths within two stem tissues – the photosynthetic cortical cells located immediately below the epidermal layer, and a layer of more elongated cells that forms a barrier between the cortical cells and developing fibres in the interfascicular region. Cell length data were collected from young (sub-apical) tissue just beginning to elongate, from maximally elongating tissues, from tissue identified as having just ceased elongating, and from tissue located >7cm below the apex, a zone in which cells are no longer expanding.

This morphometric analysis of two cell types across a sample of six plants (average plant height = 12.3cm, st.dev. = 1.6) revealed that, for both cell types, significant differences in cell length existed between young tissue (0.5-1.0mm below apex), maximum growth rate tissue (mean = 2.6cm from apex, st.dev = 0.58) and growth cessation tissues (mean = 5.7cm from apex, st.dev. = 0.71). Importantly, there was no
significant difference in lengths of either cell type between the tissues identified as growth cessation tissues and tissues from below the 7cm point (mature, non-expanding stem). The uniformity within each cell type population, and the significant differences observed between the tissue populations, are both consistent with my expectation that cells and tissues harvested as guided by GKP analysis represent developmentally discrete populations.

**Figure 2.6** Summary of mean cell lengths (um) for cortex and endodermal cell types at young, maximum growth-rate, cessation, and a post-cessation sampling points (7cm), where 20 cortical and 10 endodermal cells were measured. Error bars denote standard deviations (n=6, α=0.05).
2.3 Discussion

The measure of success of any tissue sampling methodology is whether pooled and compared tissues consistently represent the biological context of interest, so that the results of molecular/cytometric/biochemical analyses can be legitimately correlated with differences in developmental state. In order to study molecular events that regulate directional tissue expansion in the bolting Arabidopsis inflorescence stem I wished to isolate and pool tissue samples representing developmentally distinct growth stages across biological replicate tissues. Many recent studies have inferred the developmental status of tissues based upon the assumption that tissues harvested from a particular stem region, or at a specific time in the plant growth cycle, will exhibit similar developmental proportioning (Ehlting et al., 2005; Imoto et al., 2005; Ko & Han, 2004). However, the validity of this assumption depends upon a high degree of uniformity in the developmental process, both along the developmental continuum that the stem represents, as well as from plant to plant.

In contrast to this assumption, I demonstrate here that developmental proportioning of the bolting inflorescence stem is highly irregular among plants of the widely-studied Columbia ecotype (Figure 2.5, Figure A.3). It has not been established whether this level of variability is also observed for other Arabidopsis genotypes that have been used in stem elongation studies, such as Landsberg erecta (Ehlting et al., 2005).

The resulting weak correlation between plant height and location of the regions of maximal growth rate and growth cessation can be expected to interfere with accurate analysis of cellular and molecular events accompanying this major developmental transition because of the averaging effect of pooling developmentally mismatched tissues.
My optical marker tracking system, developed for examination of primary inflorescence stems, reports the REGR status of individual stem segments, each of which can then be processed for cytological and/or molecular assays. This relatively high resolution view of stem expansion reveals surprisingly frequent irregularities associated with the more apical region of the growth kinematic profiles. Pronounced dips in REGR were recorded for specific segments on individual plants (e.g. A1:seg8, A2:seg7, C2:seg9, A5:seg8, B5:seg11). In addition, many of the 24 plants profiled showed elevated growth rate at the apex. While it is possible that elongation in these plants is actually greatest at the apex, I cannot exclude the possibility that tags bounding these upper segments were inadvertently attached to silique pedicels appressed to the primary stem. This can introduce a technical error as the rapid movement of these tags would reflect the combined elongation of primary stem and silique pedicel. Despite these anomalous patterns in the growth of the upper part of the stem, I am able to routinely identify and harvest segments representing points of maximal growth rate and cessation, and morphometric analysis of the resulting sample pools yielded morphologically distinct cell populations (Figure 2.6).

Fifty years after the establishment of REGR calculation of root systems (Erickson & Sax, 1956), my approach finally provides a practical means to overcome the challenges of time-lapse imaging of the Arabidopsis inflorescence stem architecture, and comprehensively and accurately characterizes the distribution of expansive growth across this important model organ. This methodology is amenable to a broader range of biological questions through modest technical refinements.

Rather than present the full range of inflorescence development, the data presented here focus on a narrow developmental window that is of greatest relevance to those
studying both cell wall expansion and the transition from primary to secondary cell wall formation (Ehlting et al., 2005; Imoto et al., 2005; Ko & Han, 2004). The current image chamber design (Figure A.5) was optimized to observe the top 10cm of the primary stem of 10-15cm plants, although modifications to growth chamber dimensions, focal distance and camera resolution could conceivably address the entire inflorescence over a broader developmental window.

While my approach to deriving growth kinematic profiles has proven useful in isolating developmentally matched segments from different plants, the REGR data also represent one aspect of the plant phenotype, and as such they reflect both genetic and environmental influences. In *Ricinus communis* (castor oil plant), averaged growth kinematic profiles of multiple plants for leaf mid-rib expansion have been shown to be remarkably different among experimental groups (genotype, treatment) through a diurnal cycle (Walter et al., 2002), and a similar pattern was observed for root expansion in Arabidopsis under differing light levels (Nagel et al., 2006). I anticipate that quantifiable differences would also be evident among growth kinematic profiles of inflorescence primary stems for experimental groups (ex. genotypes) with divergent aerial architectures.

My system may also meet the demands of systems biology, quantitative genetics, and mutant screening if the rate of image analysis can be increased. In addition to the 24 plants presented in this study, I have analyzed an additional 51 plants for immunohistochemical and transcriptome analysis (data to be published separately). Manual analysis of an image set for a single plant currently takes approximately two hours, but exciting new advances are being made in the establishment of velocity fields, based upon optical flow (Basu et al., 2007; Nagel et al., 2006; Schmundt et al., 1998).
This could potentially facilitate observation of hundreds of plants within a single experiment if such automated feature tracking approaches that rely on natural features of the expanding surface can be adapted to deal with the challenges of imaging primary stems.

Finally, the use of relatively large-scale synthetic optical markers for tagging stem segments provides several advantages over other synthetic markings such as small, randomly-placed particles (ex. graphite) or ink marks. Use of larger tags with outer dimensions that extend beyond visual barriers such as cauline leaves not only allows segments to be individually monitored without interference, but avoids any changes in tag dimensions over time, in contrast to ink marks. These larger tags also allow convenient cataloging of segments for future use in downstream analyses (Video A.3). In addition, since structure tensor methods are not restricted to tracking small particles (ex. graphite) or secondary leaf veins, my tagging system is, in principle, also amenable to automated generation of velocity fields, and hence, REGRs. Larger tags may also support the future development of 3D tracking of nastic movements from a single camera perspective, by taking advantage of the apparent changes in imaged tag dimensions associated with movement of the plant toward or away from the camera.
Figure 2.7 Workflow for growth kinematic profile-guided harvesting of inflorescences. A. Gantt chart depicting a typical process timeline for GKP-guided harvesting of plant tissue for downstream analysis. B. Flow chart showing how the growth phenotyping can guide and inform a range of downstream analyses of preserved tissue. C, Gantt chart depicting the GKP-guided harvesting process when equipped with real-time, automated feature tracking.
2.4 Materials and methods

2.4.1 Plant material and growth conditions

Cold-treated Columbia seeds were sown in 32-plug tray inserts with soil-less potting mix (Sunshine Mix #5, Sun Gro Horticulture Canada Ltd., Seba Beach, Alberta) supplemented with liquid fertilizer 20N-20P-20K (Plant-prod soluble fertilizer, Plant Products Co. Ltd, Brampton, Ontario), then grown on short-day conditions (Day 8 hours, 21°C; Night 16 hours, 19°C) for 6 weeks. To induce bolting, plants were transferred to long-day conditions (Day 16 hours, 21°C; Night 8 hours, 21°C) until the inflorescence reached a height of 10-15cm. Plants were then removed from the growth chamber for image analysis and harvest.

As detailed in Supporting Information Video A.1, 11mm x 0.5mm tags cut from 28lb bright inkjet paper were glued to inflorescence stems with the application of a small drop of white glue to the center of the tag, bringing the tag in contact with the stem using forceps to allow the glue to bind tag to stem. Tags were placed ~5mm apart, a suitable separation for downstream analysis, and positioned so as to be perpendicular to the stem, for ease of tracking. While every attempt was taken to avoid fastening tags to developing siliques at the apex, this occurred ~20% of the time for the uppermost tag.

2.4.2 Growth imaging and image processing

Tagged plants were immediately loaded into single-plant sub-chambers/restraints (Supporting Information Figure A.4). For plants grown without nylon strings constraint, front- and backplates were constructed of plexiglass, spaced 3cm apart to allow the full range of lateral movement (circumnutation) of primary stems during elongation (growth profiles in Figure A.4, column 1). Otherwise, plants were placed within sub-chambers
such that axillary branches and siliques protruded through the vertical nylon strings of the front-plate. Nylon-strung backplates were placed behind the plants to constrain the primary stems, with rearward branches and siliques similarly protruding. To ensure uniform soil moisture during the imaging period, plants were watered prior to loading into the imaging chamber (Supporting Information Figure A.5).

Once all plants were tagged and loaded, the growth chamber (Supporting Information Figure A.5) was closed and a time-lapse series (1 minute interval) of all six plants was captured using a 10 megapixel compact digital camera (CoolPix p5000, Nikon) fitted with a polarizing lens (UR-E20, Nikon) to reduce Plexiglas reflectance. A neutral density gradient filter was added to the reflected light path in order to normalize image contrast from top to bottom. Exhaust fans ensured that ambient room temperature (21°C) was maintained within the imaging chamber.

Plants were subsequently removed at thirty minute intervals, between image capture events, for harvesting and preservation. The overall harvesting period for the six plants was centered around 2pm, the midpoint of the day on a long-day cycle beginning at 6am.

Upon completion of the harvesting of segments, six-plant image sets (3648pix × 2736pix) were subdivided into six separate, single-plant image sets using an action script in ImageReady (Creative Suite 1, Adobe). This script also increased the size of the images to provide additional precision in pinpointing intersection points of tag and plant within ImageJ. Final single-plant image dimensions were 1412pix × 5472pix (JPEG), restricted by RAM memory limits within ImageJ (~300MB for the time series)
2.4.3 Time-lapse series analysis

As detailed in Supporting Information Video A.2, the single-plant JPEG image series was imported into ImageJ as an 8-bit RGB stack, and the ‘point tool’ used to record XY coordinates for single points of interest that could then be tracked through the series sequentially. Reference marks 5cm apart were first recorded to allow for calibration of image scale fluctuations resulting from digital camera autofocus. A proxy for the stem base (not visible to the camera) was recorded and the offset distance to the stem was later measured after removal of the plant from the chamber (~2cm). From the bottom upwards, left- then right-side tag/plant intersection points (Figure 2.2, inset; Figure 2.3, inset) were recorded throughout the image sequence. Finally, the position of the apex of the stem was recorded. Feature tracking data (image set ID, X coor, Y coor, image #) were exported in tab-delimited form for import into the R programming environment (http://www.R-project.org).

2.4.4 Growth kinematic profiling

All calculations and plotting of growth kinematic data were carried out in a script provided in its general form as Supplemental Script A.1, and here outlined in the order in which these processes are carried out in R. XY coordinates (pixels) were first normalized relative to reference marks prior to conversion to a centimeter scale based upon the 5cm span between left and right reference marks. Plots such as Figure 3 (excluding inset) were generated for each time point for quality assurance, while calculation of stem width, mean tag locations, and segment length were scripted to perform as described in the results section. Segment lengths were treated with a locally weighted linear regression (LOWESS) smoothing function ('loess' function, base package, R) to dampen the effect of spurious measures on estimates of length in the
final intervals before harvest. Calculation of relative elongation rate was made using formula '1' (results), and LOWESS regressions curves generated via the 'loess' function for co-plotting with the relative elongation rates along with maxima and minima for the growth kinematic profiles as in Figure 4. Identification of developmental zones is addressed in the results section. Segments corresponding to maximum growth rate and cessation stages were identified and distances from the apex derived from the y-axis of the growth kinematic profile plots. Histograms, scatterplots, Shapiro-Wilks test for normality, Q-Q plotting, and Pearson correlations were carried out using R base package functions.

2.4.5 Tissue harvesting for microscopic analysis

Stem segments bounded by paper tags were harvested from individual plants in sequence from top to bottom over the course of approximately 10 minutes (Supporting Information Video A.3). Upon excision, segments were immersed in 150ul fixation buffer (stock 2X PME; 15.12g 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid (PIVES), 120mg MgSO4, 380mg EGTA), within 0.2ml dome-cap thermal cycler tubes. Segments were then subjected to three consecutive cycles of 5-minute vacuum infiltration at 20 inches Hg and washed three times in 1X PME prior to long-term storage at 4°C in 1X PME.

2.4.6 Morphometric analysis

Segments were trimmed to a uniform 3mm length and longitudinally sectioned to bisect the interfascicular region along the length of the segment. To demarcate cell boundaries, each segment was stained with the xylan/cellulosic stain Congo Red (Cat#:60910, Fluka, Buchs, Switzerland) and imaged with a Zeiss Meta Inverted confocal microscope. Z-stacks (225µm x 225µm x ~35µm) were collected through an
LD C-Apochromatic 40x/1.1 W M27 objective (pinhole 88µm) using a 488nm laser, excluding intrinsic fluorescence with a BP 575-615 IR filter. Confocal Z-stacks were stitched together using the '3D stitching' function of the Fiji version of ImageJ (http://pacific.mpi-cbg.de/) in order to provide a wider field of view for morphometric analysis. For each segment, 20 cortical and 10 endodermal cells from each segment were traced with the freehand line tool of ImageJ and entered as regions of interest (ROIs) into the ROI editor (Supporting Information Video A.4). Cell length was extracted from 'major axis' metric (maximum dimension in microns) of the ROI summaries and used to compute mean cell lengths for cell types of a specific segment. These means were then used in statistical tests of consistency among cell lengths of the six replicate plants for each cell type, using base package statistical tools in R (Figure 2.6).
Chapter 3. Stage-specific transcriptome profiling across the cell wall expansion continuum in the Arabidopsis primary stem

3.1 Introduction

Directional cell wall expansion is an integral part of most, if not all, plant developmental processes, facilitating morphological changes necessary for proper cell and organ morphogenesis. Such expansion is coupled with cell wall fortification and, in many cell types, thickening processes necessary to confer structural integrity of the organ. Together, these processes imply that a high degree of context-specific coordination of cell wall building/remodelling is required to harness the underlying driving force of turgor pressure in a spatially-defined manner (reviewed in Cosgrove, 1997; Szymanski & Cosgrove, 2009 and discussed further in Chapter 1. As a measure of the associated complexity, a thousand or more genes of the approximately twenty seven thousand present in the Arabidopsis genome have been shown to have some association with cell wall synthesis and remodelling (Somerville et al., 2004). This number grows substantially when considering all the genes that may contribute indirectly to cell expansion and cell size determination.

Reductionist examination of genetic regulation of cell wall expansion in Arabidopsis has relied heavily upon classical [forward] genetics, reverse genetics, and quantitative genetic approaches. Mutant screens, coupled with efficient biochemical screening approaches have identified many genes essential or important for cell wall expansion and/or secondary cell wall formation. These approaches become problematic when considering genes that are essential for individual plant survival, since embryo lethality
and extreme phenotypes (such as dwarfism) can prevent inference of primary gene
function due to pleiotropic effects, while functional redundancy among genes can
require construction of double-, triple- or quadruple-mutant lines to expose a detectable
impact. On the opposite end of the spectrum, many traits, such as morphometric
properties (e.g., cell length, cell wall thickness) are often polygenic in
nature. Quantitative genetics approaches are useful for isolating large effect loci and
ultimately isolating associated candidate genes in Arabidopsis through high resolution
mapping of large effect loci with respect to, for example, growth rate (Beemster et al.,
2002), cell wall expansion (Mouille et al., 2006), and light response (Wolyn et al.,
2004). However, such efforts are labor intensive and time consuming.

The transcriptome is a genome-wide inventory of gene transcripts (mRNAs) that are
present within cells (Velculescu et al., 1995), and thus provides a convenient and
informative report of gene activities relating to all molecular functions and biological
processes taking place within cellular compartments. Changes in the transcriptome are
predicted to reflect developmental trajectories to the extent that 1) transcription occurs
rapidly in response to external cues, and 2) mRNA pools are tightly regulated through
processes such as RNA synthesis, turnover, alternative splicing and down-regulation by
small silencing RNAs in response to developmental cues (Ghildiyal & Zamore,
2009). Since the Arabidopsis genome is fully sequenced (Arabidopsis Genome
Initiative, 2000), quantitative assessment of the transcript abundance for all coding
sequences is now routine, and has resulted in the generation of global gene expression
datasets which will have increasing utility as the functional characterization of the
encoded genes is gradually extended. Within studies that use suitable tissue samples,
the availability of relevant sample pools for parallel study allows transcriptome profiling
to be integrated with other global analyses, such as immunohistochemical evidence of cell wall composition (Chapter 3). Such integration of complementary approaches makes it possible to adopt a systems biology strategy, a step that is thought to be critical to understanding complex biological processes such as cell wall building (Somerville et al., 2004).

The transcriptome has been studied in several Arabidopsis organs in an effort to define gene co-expression networks associated with cell wall expansion and secondary cell wall formation. For example, seedling hypocotyls undergo rapid cell elongation without significant cell division during the later phase of post-germination growth (Gendreau et al., 1997; Arabidopsis Genome Initiative, 2000), a situation that lends itself to transcriptomic (and proteomic) studies of the biosynthetic and regulatory players in cell wall development (Jamet et al., 2009). Roots have also been a popular system for studying organ development, although directional cell expansion has not been the main focus of transcriptomic studies in this organ (Birnbaum et al., 2003). Lateral root emergence in this organ presents a significant complication to its use as a model system for cell wall expansion study, given recent evidence that the initiation of this emergence is dictated by oscillatory hormonal regulation mechanisms within the elongation region (Moreno-Risueno et al., 2010), a process that is presumably superimposed upon the process of primary root elongation.

By contrast, inflorescence stem architecture is largely established prior to bolting, and thus provides a simpler context within which to view transcriptome changes through cell wall expansion. In addition, inflorescence stems grown under secondary growth-promoting conditions can also be used for study of secondary cell wall formation (Ko & Han, 2004; Zhao et al., 2005).
Only a subset of the transcriptomic studies conducted in the inflorescence stem have directly investigated the changes occurring between specific developmental stages (Brown et al., 2005; Ehlting et al., 2005; Imoto et al., 2005; Minic et al., 2009). However, these studies have commonly compared tissues that were physically isolated from macroscopically discernable regions of sample plants, operating under the assumption that these plants all have similar developmental proportioning. In addition, the partitioning guidelines for harvested plants were established from destructive inspection of an entirely different set of plants, identifying certain morphological or histological features associated with stage-specific events such as the lignification of interfascicular fibres of the primary stem (Ehlting et al., 2005). However, the work I present in Chapter 2 provides evidence that the spatial distribution of cell expansion activity along the inflorescence stem can vary markedly between plants of similar height, thus undermining the assumption that such indirect proportioning approaches will allow reliable pooling of developmentally matched tissues for analysis. Furthermore, the correlation of these indirectly selected and harvested regions to actual cell wall extension or modification stages is typically not well established, which prevents the biological interpretation of transcriptome profile data in the context of the gradient of cell wall expansion.

In this Chapter, I have taken advantage of the availability of developmentally-defined stem samples harvested through use of growth kinematic profiling of individual plants (Chapter 2), and used these to compare transcriptomes across the cell wall expansion developmental continuum. The result is the first transcriptomic study that has been placed accurately within a developmental context of cell wall expansion performance (relative elemental growth rates) in an Arabidopsis organ. The data are presented as
shortlists of differential gene expression between stages, accompanied by an assessment of functional commonality among genes belonging to co-expression clusters. To assess the degree to which my results agree with other studies of inflorescence development, and to place those studies in the context of this growth kinematic profile guided study, I have also directly compared my dataset to those generated in similar Arabidopsis inflorescence primary stem transcriptomic studies (Ehlting et al., 2005; Imoto et al., 2005) that, however, employed different sampling approach.

3.2 Results
3.2.1 Sampling approach to stage-specific transcriptomic analysis of cell wall expansion

In order to ensure that the transcriptome profiles I generated are derived from accurately defined points along the cell wall expansion continuum, I have employed growth kinematic profiling, which establishes relative elemental growth rates (REGRs) for segments of individual plants (described in Chapter 2). The growth kinematic profiles facilitated identification of three developmental stages; an apical region where directional cell growth is initializing (termed 'young', or YNG), a region where directional growth is most rapid (termed 'maximum growth-rate', or MGR), and a region where elongation is finishing (termed 'cessation', or CSS). These stages are represented in this array study by multiple pooled segments, each of which is centered upon specific growth kinematic profile-identified phases (ex. MGR or CSS). The tissue selection protocol is outlined in Figure 3.1A-C, while the growth kinematic profiles for all plants examined in this study are provided in Figure B.1. To facilitate comparison of my data with similar transcriptome studies of inflorescence stems (Ehlting et al., 2005; Imoto et al., 2005; Ko & Han, 2004; Minic et al., 2009; Oh et al., 2003), the base of the stem
(termed 'mature', or OLD), was included as a fourth sample, although the age of this tissue relative to the GKP-defined time of growth cessation was not standardized.

**Figure 3.1 Representative growth profiling and harvesting.** A) Representative surface plot of relative elongation growth rates (% change per hour, vertical axis) plotted against the number of segments (defined by optical marker tags) from the apex downwards, over the duration of the imaging period in 10 minutes intervals. The red-shaded, nearest profile denotes the last 10-minute interval before harvest, depicted in the greater detail in the right-hand scatterplot. B) Corresponding scatter plot of growth rates (% change in length per hour) against distance from the stem base for specific segments. Segments are numbered from the top of the plant downwards in the right-hand margin. The LOWESS regression curve follows the best fit through the growth rate data for this plant over a given 10' interval. Green dotted lines represented 65% confidence intervals for the LOWESS regression curve. Closed-box/arrow indicates the stem position that matches the maximum growth rate of the regression curve (segment 5), plotted as the right-most vertical dotted line, while the open-box/arrow indicates the first position below the top of the stem where the growth rate falls to zero (segment 10). C) Harvesting zones for young (YNG), maximum growth-rate (MGR), cessation (CSS), and stem base (OLD) zones based upon LOWESS curve. See methods for description of zone establishment. See Figure B.1 for complete set of 34 growth kinematic profiles.
To assay the entire Arabidopsis genome, I employed custom two-channel, microarrays spotted with 26,929 70-mer oligonucleotides originally synthesized on the basis of ‘The Arabidopsis Information Resource’ (TAIR) ‘5’ release of the Arabidopsis genome (www.arabidopsis.org), with gene annotations subsequently updated to the current genome release (TAIR10). To maximize both statistical power and flexibility in analysis, I used a complete factorial experimental design coupled with a ‘mixed effects model’ analysis (Tempelman, 2005) to compare four developmental stages on the basis of six biological replicates, each pooled from a common set of thirty-four randomly-assigned plants (Figure 3.2). Such an approach permits computation of conventional differential expression values between any two stages and statistical measures of significance (p- and q-values). Use of the mixed effect modelling also facilitates computation of ‘estimates’ for gene expression level at a particular stage relative to the overall mean signal intensity; an approach that is useful for clustering of genes on the basis of expression trajectories across all stages. Gene annotations, raw data, statistical analysis, mean differentials, mean estimates, and gene categorization for the full genome are provided in Table B.1, as well as through ArrayExpress following the conventions established by the 'Minimum Information About Microarray Experiments' (MIAME) initiative (Brazma et al., 2001), as accession E-MEXP-3525.
3.2.2 Examination of differential expression between stages

The primary aim of this study was to identify genes whose expression differs most strongly between different developmental stages, since these genes should represent the loci most actively involved in the transcriptional reprogramming accompanying stem developmental transitions. The mixed effects model-based analysis of this experiment generates six possible pair-wise comparisons between developmental stages. In Table B.1, I present the complete statistical analysis for all the pair-wise comparisons, but I have chosen to focus on three comparisons in detail within this 'Results' section, and these are based on the arithmetic differences between the mean (log₂) signal intensities of the compared stages: YNG-MGR, CSS-MGR, and CSS-OLD.
Forty of the biggest gene expression differences within each comparison are presented as conventional fold-change ratios ($\log_2(YNG/MGR)$, etc...), along with measures of false-discovery-corrected statistical significance of two-sample t-test scores (q-values), for YNG/MGR (Table 3.1), MGR/CSS (Table 3.2), and CSS/OLD (Table 3.3) comparisons. Since I have applied stringent filtering criteria, these “short lists” should be predominantly populated by genes whose transcript abundances are being modulated in a radical fashion during each associated developmental transition.

By definition, the genes featured in these short-lists are being co-expressed, which suggests that many of them are responding to common recruitment and/or suppression mechanisms that are invoked during specific developmental transitions. These shortlists can thus be considered to represent at least a portion of the associated regulatory networks. To examine potential functional relationships among these short-listed genes, annotations and accessions for up- and down-regulated genes were separately examined for gene ontology (GO) and promoter motif enrichment. Specifically, GO term enrichment was determined relative to whole-genome averages using the ‘AtCoeCis’ web-tool (Vandepoele et al., 2009), reporting enrichment (fold-change), statistical significance (p-value), and proportion of short-listed genes containing that specific GO term (‘score’)(Tables B.2-B.4).

To establish the status of the stem transcriptome prior to the period at which the maximal elongation rate has been achieved, gene expression profiling was conducted on the top 1cm of the plant (without flowers) and this YNG profile was then compared to the growth kinematic profile-identified maximum growth rate (MGR) phase. Genes whose expression is biased to one stage relative to the other stage are presumed to be associated with processes that are not commonly active at both stages. Table 3.1
presents forty of the genes exhibiting high transcript copy number in the YNG stage sample relative to the MGR stage (positive fold-change values), or MGR stage relative to the YNG stage (negative fold-change values). Two of the four most up-regulated genes in the YNG-dominant set are identified as *ELONGATION FACTOR 1-ALPHA* (EF-1-α; At1g07930, At1g07940), and AtCoeCis assessment of GO term enrichment of this set (Table B.2) indicates that molecular function terms containing ‘translation’ are enriched up to 85-fold over the full-genome representation. Also within this list are genes related to signalling (*RLK902; CLE16; LOX2*; At1g62950, a LRR protein kinase) as well as transcription factors (*ZF-HD class AtHB33; NAC063, At3g55210*). While cell wall-associated GO terms are not over-represented within the YNG-dominated list, a putative glucan endo-1,3-β-glucosidase (At4G14080) (Dobritsa et al., 2011) is up-regulated 15.4-fold over the MGR stage.

Although cell wall expansion is likely to be taking place in both the YNG and the MGR stage, several genes related to cell wall biogenesis are up-regulated in the MGR stage, relative to the YNG stage. The most strongly up-regulated (40-fold) is a gene encoding a peroxidase (*PER64*) that appears to be up-regulated in stems due to mechanical load (Koizumi et al., 2009), and more specifically (GUS expression) in the protoxylem (Wenzel et al., 2008), where lignification in the annular and helical cell wall thickenings is known to occur during elongation. This list contains several genes more directly related to primary cell walls, including the xyloglucan endotransglycosylase/hydrolase *MERISTEM-5 (MERI5B/XTH24)*; a putative pectinase (At1g80170); and two arabinogalactan proteins (*AGP12, AGP13*). Also in this list is a MYB transcription factor (*MYB61*) with broad ranging function (see ‘Discussion’) that has previously been shown to contribute to a suite of different biological functions (Liang et al., 2005; Newman et al.,
2004; Penfield et al., 2001) (see ‘Discussion’). Relative to the YNG stage, the MGR stage is enriched in hormone-associated GO terms, particularly those associated with gibberellic acid (100-fold enrichment), as reflected in the presence of genes with GA-related annotations; GA-responsive MINI ZINC FINGER 1 (MIF1) (Hu & Ma, 2006) and a putative GA-responsive protein (At1g74670). The GO term for the biological process ‘gibberellic acid-mediated signalling’ is 100-fold up-regulated over the full-genome largely because of the occurrence in the list of these two genes. The sum of enrichment scores of ‘Higher in MGR’ (2.95) is marginally elevated over that of the ‘Higher in YNG’ list (2.49) due to enrichment of terms associated with stress and hormone signaling, including auxin and ethylene in addition to gibberellic acid-associated genes.
Table 3.1 Top 40 genes with the greatest differential expression between YNG and MGR stages (q-value<4.7e-02), ranked according to fold-change values derived from log2 ratios of MGR (numerator) and CSS (denominator). Gene descriptions are abbreviated from TAIR10 genome release.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession</th>
<th>Gene descriptions</th>
<th>YNG/MGR Fold-change</th>
<th>YNG/MGR q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G07930</td>
<td>elongation factor 1-alpha / EF-1-alpha</td>
<td>22.5</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>AT5G25754</td>
<td>unknown protein</td>
<td>19.2</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>AT1G11520</td>
<td>spliceosome associated protein-related</td>
<td>18.9</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>AT1G07940</td>
<td>elongation factor 1-alpha / EF-1-alpha</td>
<td>18.9</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>AT5G22430</td>
<td>unknown protein</td>
<td>17.8</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 33 (AtHB33)</td>
<td>AT1G75240</td>
<td>transcription factor with homeobox domain, ZF-HD class (InterPro:IPR006455)</td>
<td>16.2</td>
<td>0.01</td>
</tr>
<tr>
<td>MATERNAL EFFECT EMBRYO ARREST 48 (MEE48)</td>
<td>AT4G14080</td>
<td>O-glycosyl hydrolase (family17) located in the endomembrane system</td>
<td>15.4</td>
<td>0.02</td>
</tr>
<tr>
<td>AT1G01300</td>
<td>aspartyl protease family protein located in membrane, plant-type cell wall</td>
<td>14.6</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>AT2G07739</td>
<td>unknown protein</td>
<td>14.4</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>AT3G13470</td>
<td>chaperonin, putative with domain Cpn60/TCP-1 (InterPro:IPR002423)</td>
<td>13.1</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>AT4G34850</td>
<td>chalcone and stilbene synthase family protein involved in phenylpropanoid biosynthetic process</td>
<td>13.0</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>RECEPTOR-LIKE KINASE 902 (RLK902)</td>
<td>AT3G17840</td>
<td>Encodes a receptor-like kinase found at the cell surface of various tissues. Its function remains unknown.</td>
<td>12.6</td>
<td>0.03</td>
</tr>
<tr>
<td>NA</td>
<td>AT1G52030</td>
<td>myrosinase binding protein, putative</td>
<td>12.2</td>
<td>0.02</td>
</tr>
<tr>
<td>GDSL-MOTIF LIPASE 7 (GLIP7)</td>
<td>AT5G15720</td>
<td>Contains lipase signature motif and GDSL domain.</td>
<td>11.6</td>
<td>0.04</td>
</tr>
<tr>
<td>EMBRYO DEFECTIVE 2296 (EMB2296)</td>
<td>AT1G70830</td>
<td>structural constituent of ribosome involved in embryonic development ending in seed dormancy</td>
<td>11.5</td>
<td>0.04</td>
</tr>
<tr>
<td>AT5G62080</td>
<td>protease inhibitor/seed storage/lipid transfer protein (LTP) family protein</td>
<td>11.4</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>CLAVATA3/ESR-RELATED 16 (CLE16)</td>
<td>AT2G01505</td>
<td>Member of a large family of putative ligands homologous to the Clavata3 gene</td>
<td>10.8</td>
<td>0.04</td>
</tr>
<tr>
<td>LIPOXYGENASE 2 (LOX2)</td>
<td>AT3G45140</td>
<td>Chloroplast lipoxygenase required for wound-induced jasmonic acid accumulation in Arabidopsis</td>
<td>10.6</td>
<td>0.02</td>
</tr>
<tr>
<td>AT5G62950</td>
<td>leucine-rich repeat transmembrane protein kinase</td>
<td>10.4</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 63 (anac063)</td>
<td>AT3G55210</td>
<td>Arabidopsis NAC domain containing protein 63 (anac063)</td>
<td>10.0</td>
<td>0.03</td>
</tr>
<tr>
<td>(CPK21)</td>
<td>AT4G04720</td>
<td>member of Calcium Dependent Protein Kinase</td>
<td>9.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Gene name</td>
<td>Accession</td>
<td>Gene descriptions</td>
<td>YNG/MGR Fold-change</td>
<td>YNG/MGR q-value</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>-------------------</td>
<td>---------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>ARABINOGLACTAN PROTEIN 12 (AGP12)</td>
<td>AT3G13520</td>
<td>Encodes a GPI-anchored arabinogalactan (AG) peptide</td>
<td>-6.8</td>
<td>0.03</td>
</tr>
<tr>
<td>MYB DOMAIN PROTEIN 61 (MYB61)</td>
<td>AT1G80170</td>
<td>putative polygalacturonase, putative</td>
<td>-7.3</td>
<td>0.01</td>
</tr>
<tr>
<td>RARE-COLD-INDUCIBLE 2A (RCI2A)</td>
<td>AT3G05880</td>
<td>unknown, highly hydrophobic protein with two potential transmembrane domains.</td>
<td>-7.5</td>
<td>0.01</td>
</tr>
<tr>
<td>MYB DOMAIN PROTEIN 61 (MYB61)</td>
<td>AT1G09540</td>
<td>putative transcription factor involved in mucilage extrusion</td>
<td>-7.4</td>
<td>0.03</td>
</tr>
<tr>
<td>RARE-COLD-INDUCIBLE 2A (RCI2A)</td>
<td>AT3G05880</td>
<td>similar to 1-aminocyclopropane-1-carboxylate oxidase Gl:3386565 from (Sorghum bicolor)</td>
<td>-7.5</td>
<td>0.02</td>
</tr>
<tr>
<td>SPIRAL1-LIKE5 (SP1L5)</td>
<td>AT1G73730</td>
<td>auxin-responsive protein-related with domain Auxin responsive SAUR protein (InterPro:IPR003676) cortical microtubule organization, anisotropic cell expansion.</td>
<td>-8.0</td>
<td>0.03</td>
</tr>
<tr>
<td>SPIRAL1-LIKE5 (SP1L5)</td>
<td>AT1G72430</td>
<td>similar to 1-aminocyclopropane-1-carboxylate oxidase Gl:3386565 from (Sorghum bicolor)</td>
<td>-7.5</td>
<td>0.02</td>
</tr>
<tr>
<td>SPIRAL1-LIKE5 (SP1L5)</td>
<td>AT4G23496</td>
<td>unknown, highly hydrophobic protein with two potential transmembrane domains.</td>
<td>-7.5</td>
<td>0.02</td>
</tr>
<tr>
<td>SPIRAL1-LIKE5 (SP1L5)</td>
<td>AT4G03205</td>
<td>SOUL heme-binding family protein with domain SOUL haem-binding protein (InterPro:IPR006917)</td>
<td>-8.1</td>
<td>0.02</td>
</tr>
<tr>
<td>SPIRAL1-LIKE5 (SP1L5)</td>
<td>AT1G77885</td>
<td>unknown protein</td>
<td>-8.2</td>
<td>0.03</td>
</tr>
<tr>
<td>ARABINOGLACTAN PROTEIN 13 (AGP13)</td>
<td>AT4G26320</td>
<td>arabinogalactan protein 13 (AGP13)</td>
<td>-8.8</td>
<td>0.02</td>
</tr>
<tr>
<td>BRANCHED-CHAIN AMINOTRANSFERASE4 (BCAT4)</td>
<td>AT3G19710</td>
<td>methionine-oxo-acid transaminase</td>
<td>-9.6</td>
<td>0.02</td>
</tr>
<tr>
<td>BRANCHED-CHAIN AMINOTRANSFERASE4 (BCAT4)</td>
<td>AT5G48560</td>
<td>basic helix-loop-helix (bHLH) family protein (InterPro:IPR001092)</td>
<td>-9.9</td>
<td>0.04</td>
</tr>
<tr>
<td>BRANCHED-CHAIN AMINOTRANSFERASE4 (BCAT4)</td>
<td>AT3G55240</td>
<td>Overexpression leads to PEL (Pseudo-Etiolation in Light) phenotype.</td>
<td>-10.0</td>
<td>0.02</td>
</tr>
<tr>
<td>BRANCHED-CHAIN AMINOTRANSFERASE4 (BCAT4)</td>
<td>AT1G74670</td>
<td>gibberellin-responsive protein, putative (InterPro:IPR003854)</td>
<td>-10.2</td>
<td>0.03</td>
</tr>
<tr>
<td>MERISTEM-5 (MERIS5)</td>
<td>AT4G30270</td>
<td>like endo xyloglucan transferase in sequence</td>
<td>-11.5</td>
<td>0.02</td>
</tr>
<tr>
<td>MINI ZINC FINGER 1 (MIF1)</td>
<td>AT1G74660</td>
<td>gibberellin (GA) response for cell elongation</td>
<td>-12.0</td>
<td>0.00</td>
</tr>
<tr>
<td>MINI ZINC FINGER 1 (MIF1)</td>
<td>AT4G29905</td>
<td>unknown protein</td>
<td>-12.9</td>
<td>0.04</td>
</tr>
<tr>
<td>MERISTEM-5 (MERIS5)</td>
<td>AT3G45160</td>
<td>unknown protein</td>
<td>-15.3</td>
<td>0.01</td>
</tr>
<tr>
<td>MINI ZINC FINGER 1 (MIF1)</td>
<td>AT5G05960</td>
<td>protease inhibitor/seed storage/lipid transfer protein (LTP) family protein</td>
<td>-24.2</td>
<td>0.02</td>
</tr>
<tr>
<td>MINI ZINC FINGER 1 (MIF1)</td>
<td>AT5G42180</td>
<td>peroxidase 64 (PER64) (P64) (PRXR4) located in plant-type cell wall</td>
<td>-40.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Comparison of the MGR stage to the CSS stage provides another view of those genes that are most relevant to cell wall expansion. The twenty genes listed in each set of ‘Higher in MGR relative to CSS’ and ‘Higher in CSS relative to MGR’, are representative of genes that are highly active in the MGR and CSS stages, respectively (Table 3.2). AtCoeCis assessment of GO term enrichment shows moderate over-representation of ‘response to…’ biotic/abiotic stimuli, where the most significantly over-represented term (‘response to biotic stimulus’,) exhibits an 8-fold enrichment and is associated with five of the twenty genes (Table B.3). Interestingly, this differs from the dominance in hormone response terms seen in the ‘Higher in MGR relative to YNG’ list (Table B.2) and, in fact, the gene lists for ‘Higher in MGR relative to YNG’ and ‘Higher in MGR relative to CSS’ do not share any common genes (Tables 3.1 and 3.2, respectively). Most highly up-regulated among this set of MGR-biased genes is MAJOR LATEX PROTEIN 423 (MLP423) which belongs to the BET V1 class of allergens, and exhibits sequence homology to ABA- and stress-responsive proteins from various plant species (EMBL-EBI database information).

By contrast, the ‘Higher in CSS relative to MGR’ set exhibits a higher tally of all term scores present in the last column of Tables B.2-B.4 (3.75) than is seen in the MGR-biased list (2.46), indicating a higher degree of functional similarity/alignment within this short list of genes (Table B.3). In this case, ‘cell wall’ associated terms appear enriched up to 140-fold (‘cell wall biosynthesis (sensu Magnoliophyta)’). Examination of the content of the ‘Higher in CSS relative to MGR’ list reveals that all three of the cellulose synthase genes previously associated with secondary cell wall biosynthesis (CESA4/IRX5, CESA7/IRX3, and CESA8/IRX1) (Brown et al., 2005; Gardiner et al., 2003; Hamann et al., 2004; Holland et al., 2000) are co-associated in this list of twenty genes. Also included in this list are genes previously associated with secondary cell
wall building, including the glycosyl transferases involved in xylan synthesis, *IRREGULAR XYLEM 9 (IRX9)* (Wu et al., 2010) and *FRAGILE FIBER 8 (FRA8)* (Lee et al., 2009). In addition, genes associated with cell wall remodelling are up-regulated, including two encoding putative pectinesterases (At2g43050, At2g45220) and a xyloglucan-specific endotransglycosylase/hydrolase 19 (*XTH19*) (Maris et al., 2011), as well as genes that have been associated with lignification; *CHITINASE-LIKE PROTEIN 2 (CTL2)* (Hossain et al., 2010), and a laccase, *IRREGULAR XYLEM 12 (IRX12/LAC4)* (Berthet et al., 2011). Expression of *LAC4* was previously reported to be biased toward the interfascicular fibre (IFF) region and vascular bundles in the inflorescence stem and implicated in lignification (Berthet et al., 2011). Only seven of the twenty genes in this shortlist (At5g25110, At5g59290, At1g03740, At1g22480, At2g28315, At2g03200, At1g63910) do not have any apparent formal association with cell wall biology in their TAIR10 annotations, although their involvement in secondary cell wall formation cannot be excluded. In all, therefore, the genes found in the ‘Higher in CSS relative to MGR’ list appear to be functionally biased toward secondary cell wall biosynthesis.
Table 3.2 Top 40 genes with the greatest differential expression between MGR and CSS stages (p-value<6.72E-02), ranked according to fold-change values derived from log2 ratios of MGR (numerator) and CSS (denominator). Gene descriptions are abbreviated from TAIR10 genome release.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession</th>
<th>Gene descriptions</th>
<th>MGR/CSS Fold-change</th>
<th>MGR/CSS p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLP-LIKE PROTEIN 423 (MLP423)</td>
<td>AT1G24020</td>
<td>Similar to major latex protein gene family, Bet v 1 superfamily</td>
<td>16.7</td>
<td>2.9E-03</td>
</tr>
<tr>
<td>PHLOEM PROTEIN 2-B7 (AtPP2-B7)</td>
<td>AT2G02320</td>
<td>Phloem protein 2-B7 (AtPP2-B7)</td>
<td>5.2</td>
<td>6.6E-03</td>
</tr>
<tr>
<td>LIPID TRANSFER PROTEIN 1 (LP1)</td>
<td>AT2G38540</td>
<td>Non-specific calmodulin-binding lipid transfer protein</td>
<td>5.2</td>
<td>5.0E-05</td>
</tr>
<tr>
<td>VEGETATIVE STORAGE PROTEIN 1 (VSP1)</td>
<td>AT5G24780</td>
<td>Acid phosphatase</td>
<td>4.8</td>
<td>3.6E-03</td>
</tr>
<tr>
<td>PLANTACYANIN (ARPN)</td>
<td>AT2G02850</td>
<td>Encodes plantacyanin one of blue copper proteins.</td>
<td>3.9</td>
<td>2.4E-03</td>
</tr>
<tr>
<td>SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3)</td>
<td>AT2G33810</td>
<td>Squamosa-promoter binding protein-like gene family</td>
<td>3.8</td>
<td>4.2E-02</td>
</tr>
<tr>
<td>LI-TOLERANT LIPASE 1 (LTL1)</td>
<td>AT3G04290</td>
<td>LI-TOLERANT LIPASE 1 (LTL1)</td>
<td>3.7</td>
<td>2.4E-03</td>
</tr>
<tr>
<td>GERMIN 3 (GER3)</td>
<td>AT5G20630</td>
<td>Encodes a germin-like protein</td>
<td>3.5</td>
<td>3.8E-04</td>
</tr>
<tr>
<td>SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3)</td>
<td>AT2G33810</td>
<td>Squamosa-promoter binding protein-like gene family</td>
<td>3.8</td>
<td>4.2E-02</td>
</tr>
<tr>
<td>CHAPERONIN 20 (CPN20)</td>
<td>AT5G20720</td>
<td>Encodes a chloroplast co-chaperonin</td>
<td>3.2</td>
<td>7.6E-03</td>
</tr>
<tr>
<td>GAST1 PROTEIN HOMOLOG 4 (GASA4)</td>
<td>AT5G15230</td>
<td>Gibberellin-regulated (GASA4)</td>
<td>3.4</td>
<td>3.1E-04</td>
</tr>
<tr>
<td>NA</td>
<td>AT1G55490</td>
<td>Rubisco subunit binding-protein beta subunit/60 kDa chaperonin beta subunit</td>
<td>3.6</td>
<td>9.1E-04</td>
</tr>
<tr>
<td>GERMIN 3 (GER3)</td>
<td>AT5G20630</td>
<td>Encodes a germin-like protein</td>
<td>3.5</td>
<td>3.8E-04</td>
</tr>
<tr>
<td>GAST1 PROTEIN HOMOLOG 4 (GASA4)</td>
<td>AT5G15230</td>
<td>Gibberellin-regulated (GASA4)</td>
<td>3.4</td>
<td>3.1E-04</td>
</tr>
<tr>
<td>CHAPERONIN 20 (CPN20)</td>
<td>AT5G20720</td>
<td>Encodes a chloroplast co-chaperonin</td>
<td>3.2</td>
<td>7.6E-03</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT3G47340</td>
<td>Glutamine-dependent asparagine synthetase</td>
<td>3.2</td>
<td>1.3E-02</td>
</tr>
</tbody>
</table>
Table 3.2 (continued).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession</th>
<th>Gene descriptions</th>
<th>MGR/CSS Fold-change</th>
<th>MGR/CSS p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBL-INTERACTING PROTEIN KINASE 25 (CIPK25)</td>
<td>AT5G25110</td>
<td>member of AtCIPKs</td>
<td>-5.1</td>
<td>1.8E-02</td>
</tr>
<tr>
<td>(ATPMEPCRD)</td>
<td>AT2G43050</td>
<td>enzyme inhibitor activity, pectinesterase activity</td>
<td>-5.5</td>
<td>2.8E-03</td>
</tr>
<tr>
<td>XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 19 (XTH19)</td>
<td>AT4G30290</td>
<td>putative xyloglucan endotransglycosylase/hydrolase</td>
<td>-5.5</td>
<td>2.8E-04</td>
</tr>
<tr>
<td>NA</td>
<td>AT5G59290</td>
<td>U-dependent epimerase/dehydratase family</td>
<td>-5.6</td>
<td>5.8E-04</td>
</tr>
<tr>
<td>IRREGULAR XYLEM 12 (IRX12)</td>
<td>AT2G38080</td>
<td>putative member of laccase family (17 members in Arabidopsis).</td>
<td>-6.0</td>
<td>4.1E-04</td>
</tr>
<tr>
<td>IRREGULAR XYLEM 9 (IRX9)</td>
<td>AT1G70830</td>
<td>The IRX9 gene encodes a putative family 43 glycosyl transferase</td>
<td>-6.1</td>
<td>4.3E-04</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT5G46340</td>
<td>O-acetyltransferase-related</td>
<td>-6.1</td>
<td>3.0E-03</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT1G03740</td>
<td>Protein kinase superfamily protein</td>
<td>-6.1</td>
<td>7.8E-06</td>
</tr>
<tr>
<td>TRICHOME BIREFRINGENCE-LIKE 3 (TBL3)</td>
<td>AT5G01360</td>
<td>TBL (TRICOME BIREFRINGENCE-LIKE) gene family</td>
<td>-6.9</td>
<td>2.3E-03</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT2G28315</td>
<td>Nucleotide/sugar transporter family protein</td>
<td>-7.3</td>
<td>4.0E-03</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT1G22480</td>
<td>plastocyanin-like domain-containing protein</td>
<td>-7.8</td>
<td>3.6E-04</td>
</tr>
<tr>
<td>CELLULOSE SYNTHASE A7 (CESA7/IRX7)</td>
<td>AT5G17420</td>
<td>xylem-specific cellulose synthase</td>
<td>-8.8</td>
<td>1.6E-04</td>
</tr>
<tr>
<td>NA</td>
<td>AT3G18660</td>
<td>glycogenin glucosyltransferase (glycogenin)</td>
<td>-9.0</td>
<td>5.7E-04</td>
</tr>
<tr>
<td>CELLULOSE SYNTHASE A8 (CESA8/IRX1)</td>
<td>AT4G18780</td>
<td>cellulose synthase family involved in secondary cell wall biosynthesis</td>
<td>-9.5</td>
<td>9.4E-04</td>
</tr>
<tr>
<td>CHITINASE-LIKE PROTEIN 2 (CTL2)</td>
<td>AT3G16920</td>
<td>chitinase-like protein</td>
<td>-10.1</td>
<td>1.3E-05</td>
</tr>
<tr>
<td>FRAGILE FIBER 8 (FRAB8)</td>
<td>AT2G28110</td>
<td>glycosyltransferase family 47</td>
<td>-10.3</td>
<td>4.8E-04</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT2G03200</td>
<td>aspartyl protease family protein</td>
<td>-10.4</td>
<td>9.6E-05</td>
</tr>
<tr>
<td>MYB DOMAIN PROTEIN 103 (AIMYB103)</td>
<td>AT1G63910</td>
<td>member of MYB3R- and R2R3-type MYB- encoding genes</td>
<td>-11.5</td>
<td>2.3E-03</td>
</tr>
<tr>
<td>CELLULOSE SYNTHASE A4 (CESA4/IRX5)</td>
<td>AT5G44030</td>
<td>cellulose synthase involved in secondary cell wall biosynthesis</td>
<td>-11.5</td>
<td>5.6E-05</td>
</tr>
<tr>
<td>no gene name</td>
<td>AT2G45220</td>
<td>pectin methylesterase inhibitor</td>
<td>-38.4</td>
<td>1.8E-05</td>
</tr>
</tbody>
</table>
While growth kinematic data cannot precisely position the base of the stem along the developmental continuum (growth kinematic profiling can only distinguish stem regions on the basis of their rates of expansion), it is clear that the OLD stage captures an advanced stage of cell wall maturation in the 10-15cm tall plants examined in this study. Table 3.3 presents the twenty genes whose expression is ‘Higher in CSS relative to OLD’ and twenty genes whose expression is ‘Higher in OLD relative to CSS’. Based on our present understanding of the stem maturation process, the CSS and OLD samples are expected to contain tissues actively engaged in early and late stages of secondary cell wall formation, respectively. AtCoeCis examination of GO term over-representation (Table B.4) shows enrichment of terms likely related to cell wall building/remodelling and turgor pressure. Among this list of genes highest in the CSS stage relative to the OLD stage are several hydrolases (At3g15720, At1g80280, At2g05720, At1g68560/ALPHA-XYLOSIDASE 1) and lyases (At1g64660, At3g07010); both classes may be related to pre-processing or restructuring of the cell wall constituents. GERMIN3 (At5g20630) and GERMIN-LIKE PROTEIN 1 (GER1/GLP1) (At1g72610) exhibit second- and fourth-highest fold-changes (respectively). A germin-like protein (GLP10, At3G62020) was found to be highly co-expressed with secondary cell wall-associated CESAs (4, 7, and 8) in regression analysis of public datasets (Persson et al., 2005). There is a dearth of published data for this family of proteins, though germins (‘germination-related’) have been defined as homopentameric glycoproteins with oxalate oxidase function that reside in the apoplast and participate in embryo hydration (Lane et al., 1993). As with the relationship of the MGR lists to the ‘before’ (YNG) and ‘after’ (CSS) stages, the contents of this “Higher in CSS relative to OLD” list have little in common with the ‘Higher in CSS relative to MGR’ gene list of Table 3.2, which may indicate that the genes most active in both the CSS and OLD stages, such as
secondary cell wall-related cellulose synthases, are distinct from those most active in the MGR stage.

The ‘Higher in OLD relative to CSS’ list exhibits a greater degree of GO term enrichment than the ‘Higher in CSS relative to OLD’ list, measured as sums of enrichment scores of 3.15 and 2.58, respectively (Table B.6). Fifteen of the eighteen terms in this list relate to cell wall biology (aka ‘external encapsulating structure’) or appear to be defense-related (‘response to…’, ‘toxin metabolism’ or ‘toxin catabolism’). The most significant (GO:0005618) and most enriched (GO:0005199) terms are both cell wall-associated, suggesting that cell wall processes still demand the highest rates of transcription, even at this late stage in development. However, six of the eight most highly up-regulated genes in this list are PLANT DEFENSIN (PDF) genes, that encode small cysteine-rich peptides (Thomma et al., 2002). Since both CSS and OLD tissues were harvested only seconds apart, an artifactual pattern of gene induction through mechanical wounding is not likely. Instead, it appears that accumulation of the products of such classical “defense” genes forms an integral part of the normal maturation of the inflorescence stem.
Table 3.3 Top 40 genes with greatest differential expressions between cessation and old tissues (q-value<4.9e-02), ranked according to fold-change values derived from log2 ratios of CSS (numerator) and OLD (denominator). Gene descriptions are abbreviated from TAIR10 genome release.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession</th>
<th>Gene descriptions</th>
<th>CSS/OLD Fold-change</th>
<th>CSS/OLD p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G12845</td>
<td>unknown protein</td>
<td>10.1</td>
<td>4.1E-02</td>
<td></td>
</tr>
<tr>
<td>AT5G20630</td>
<td>Encodes a germin-like protein</td>
<td>6.5</td>
<td>1.6E-02</td>
<td></td>
</tr>
<tr>
<td>AT3G07010</td>
<td>pectate lyase family protein</td>
<td>6.3</td>
<td>7.8E-03</td>
<td></td>
</tr>
<tr>
<td>AT1G72610</td>
<td>germin-like protein (GLP1)</td>
<td>6.2</td>
<td>4.0E-02</td>
<td></td>
</tr>
<tr>
<td>AT1G64660</td>
<td>functional methionine gamma-lyase</td>
<td>5.7</td>
<td>9.6E-03</td>
<td></td>
</tr>
<tr>
<td>AT3G15720</td>
<td>glycoside hydrolase family 2B protein / polygalacturonase (pectinase) family protein</td>
<td>5.3</td>
<td>3.5E-02</td>
<td></td>
</tr>
<tr>
<td>AT1G80280</td>
<td>hydrolase, alpha/beta fold family protein</td>
<td>5.3</td>
<td>3.4E-02</td>
<td></td>
</tr>
<tr>
<td>AT1G88600</td>
<td>unknown protein</td>
<td>5.0</td>
<td>4.3E-02</td>
<td></td>
</tr>
<tr>
<td>AT5G38430</td>
<td>ribulose bisphosphate carboxylase small chain 1B / RuBisCO small subunit 1B (RBCS-1B) (ATS1B)</td>
<td>4.8</td>
<td>4.3E-02</td>
<td></td>
</tr>
<tr>
<td>AT2G39010</td>
<td>PLASMA MEMBRANE INTRINSIC PROTEIN 2E (PIP2E)</td>
<td>4.8</td>
<td>2.2E-02</td>
<td></td>
</tr>
<tr>
<td>AT2G38540</td>
<td>LIPID TRANSFER PROTEIN 1 (LP1)</td>
<td>4.7</td>
<td>2.8E-02</td>
<td></td>
</tr>
<tr>
<td>AT3G16240</td>
<td>DELTA TONOPLAST INTEGRAL PROTEIN (DELTA-TIP)</td>
<td>4.7</td>
<td>2.2E-02</td>
<td></td>
</tr>
<tr>
<td>AT1G403205</td>
<td>(hemf2)</td>
<td>4.4</td>
<td>3.9E-02</td>
<td></td>
</tr>
<tr>
<td>AT3G48970</td>
<td>copper-binding family protein in metal ion transport</td>
<td>4.3</td>
<td>4.9E-02</td>
<td></td>
</tr>
<tr>
<td>AT1G75900</td>
<td>family II extracellular lipase 3 (EXL3), carboxylesterase activity, acyltransferase activity</td>
<td>4.3</td>
<td>1.5E-02</td>
<td></td>
</tr>
<tr>
<td>AT2G05790</td>
<td>glycosyl hydrolase family 17 protein</td>
<td>4.2</td>
<td>7.8E-03</td>
<td></td>
</tr>
<tr>
<td>AT5G38420</td>
<td>ribulose bisphosphate carboxylase small chain 2B / RuBisCO small subunit 2B (RBCS-2B) (ATS2B)</td>
<td>4.2</td>
<td>4.1E-02</td>
<td></td>
</tr>
<tr>
<td>AT1G68560</td>
<td>bifunctional alpha-L-arabinofuranosidase/beta-D-xylanase (glycoside hydrolase family 3)</td>
<td>4.2</td>
<td>4.1E-02</td>
<td></td>
</tr>
<tr>
<td>AT5G22580</td>
<td>unknown protein</td>
<td>4.2</td>
<td>2.8E-02</td>
<td></td>
</tr>
<tr>
<td>AT3G12610</td>
<td>DNA-DAMAGE REPAIR/TOLERATION 100 (DRT100)</td>
<td>4.1</td>
<td>2.2E-02</td>
<td></td>
</tr>
<tr>
<td>AT3G30180</td>
<td>BRASSINOSTEROID-6-OXIDASE 2 (BR6OX2)</td>
<td>4.1</td>
<td>4.3E-02</td>
<td></td>
</tr>
<tr>
<td>Gene name</td>
<td>Accession</td>
<td>Gene descriptions</td>
<td>CSS/OLD Fold-change</td>
<td>CSS/OLD p-value</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>-----------</td>
<td>------------------------------------------------------------------------------------</td>
<td>---------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>EXTENSIN 3 (ATEXT3)</td>
<td>AT1G21310</td>
<td>Encodes extensin 3.</td>
<td>-3.9</td>
<td>4.8E-02</td>
</tr>
<tr>
<td></td>
<td>AT5G09840</td>
<td>unknown protein</td>
<td>-4.1</td>
<td>3.7E-02</td>
</tr>
<tr>
<td></td>
<td>AT3G54580</td>
<td>proline-rich extensin-like family protein, structural constituent of cell wall</td>
<td>-5.0</td>
<td>4.8E-02</td>
</tr>
<tr>
<td>MYB DOMAIN PROTEIN 49 (MYB49)</td>
<td>AT5G54230</td>
<td>Encodes a putative transcription factor (MYB49).</td>
<td>-5.0</td>
<td>4.1E-02</td>
</tr>
<tr>
<td></td>
<td>AT2G28780</td>
<td>unknown protein</td>
<td>-5.0</td>
<td>2.9E-02</td>
</tr>
<tr>
<td></td>
<td>AT1G70830</td>
<td>Bet v I allergen family</td>
<td>-5.2</td>
<td>3.9E-02</td>
</tr>
<tr>
<td></td>
<td>AT2G02930</td>
<td>Encodes glutathione transferase belonging to the phi class of GSTs.</td>
<td>-5.4</td>
<td>3.0E-02</td>
</tr>
<tr>
<td></td>
<td>AT4G15390</td>
<td>acyl-transferase family protein</td>
<td>-6.1</td>
<td>1.6E-02</td>
</tr>
<tr>
<td></td>
<td>AT4G08780</td>
<td>peroxidase, putative</td>
<td>-8.2</td>
<td>4.5E-02</td>
</tr>
<tr>
<td></td>
<td>AT1G02930</td>
<td>glutathione transferase belonging to the phi class of GSTs.</td>
<td>-8.6</td>
<td>4.3E-02</td>
</tr>
<tr>
<td></td>
<td>AT2G36120</td>
<td>glycine rich protein that is involved in leaf vascular patterning</td>
<td>-8.8</td>
<td>3.5E-02</td>
</tr>
<tr>
<td></td>
<td>AT1G19530</td>
<td>unknown protein</td>
<td>-9.7</td>
<td>3.1E-02</td>
</tr>
<tr>
<td></td>
<td>AT1G75830</td>
<td>plant defensin (PDF) family</td>
<td>-9.9</td>
<td>1.5E-02</td>
</tr>
<tr>
<td></td>
<td>AT2G26020</td>
<td>plant defensin (PDF) family</td>
<td>-10.7</td>
<td>4.3E-02</td>
</tr>
<tr>
<td></td>
<td>AT5G22490</td>
<td>condensation domain-containing protein</td>
<td>-11.5</td>
<td>4.5E-02</td>
</tr>
<tr>
<td></td>
<td>AT5G44420</td>
<td>ethylene- and jasmonate-responsive plant defensin</td>
<td>-13.1</td>
<td>3.9E-02</td>
</tr>
<tr>
<td></td>
<td>AT2G26010</td>
<td>plant defensin (PDF) family</td>
<td>-16.2</td>
<td>4.4E-02</td>
</tr>
<tr>
<td></td>
<td>AT4G16260</td>
<td>O-glycosyl hydrolase family</td>
<td>-17.9</td>
<td>4.9E-02</td>
</tr>
<tr>
<td></td>
<td>AT5G44430</td>
<td>plant defensin (PDF) family</td>
<td>-19.9</td>
<td>3.6E-02</td>
</tr>
<tr>
<td></td>
<td>AT3G56700</td>
<td>FATTY ACID REDUCTASE 6 (FAR6)</td>
<td>-55.4</td>
<td>7.8E-03</td>
</tr>
</tbody>
</table>

Higher in OLD relative to CSS
3.2.3 Stage-specific, whole-genome co-expression analysis

While they can provide initial insights into the biology underlying individual developmental transitions, differential gene expression datasets such as the MGR/YNG, CSS/MGR, and OLD/CSS lists still provide only a limited view of regulatory networks (or modular gene programs). This is especially true in cases where the change between sequential developmental stages is gradual but consistent, apparent only when viewed across the broader developmental range. Functionally related co-expression patterns may therefore be better extracted by considering transcript abundances across all the sampled developmental stages. The underlying rationale is that genes co-expressed at one stage would be predicted to retain a similar pattern of co-expression throughout development since they share common cis-regulatory motifs.

Hierarchical clustering has previously been used to isolate co-expression sets across developmental stages of the inflorescence primary stem (Ehlting et al., 2005; Ko & Han, 2004), and between tissue types at the base of the mature primary stem (hypocotyl secondary growth) (Oh et al., 2003). While Ehlting et al. 2005 clustered expression ratios generated from conventional analysis of two-channel microarrays, Ko et al. 2005 clustered expression values obtained from Affymetrix arrays that approximated the values of each stage relative to the overall experiment, a procedure that provides a more intuitive view of gene trajectory.

The mixed effects model approach used in my study also facilitated the generation of developmental stage 'estimates' from two-channel arrays; these can be expressed as mean fold-change values (biological replicates=6) of transcript abundance at one stage relative to a hypothetical mean value across the entire experiment of zero.
noted that these 'estimates' may be computed with the same statistical power as applies to the log₂ differential expression ratios. Such a view provides a more intuitive means of visualizing gene expression trajectories, and provides the basis for co-expression analysis. Gene associations with all co-expression sets (clusters) presented in this section are provided in a filterable column within the full-genome dataset (Table B.1).

Hierarchical divisive clustering was performed on the 4635 genes whose means were most significantly different from the gene-wise mean of all stages (q-value<0.05) (Figure 3.3), thereby filtering out the great majority (22 294) of genes whose expression displayed little perceptible change during the course of elongative development. All 4635 genes fall within eight major co-expression clusters that exhibit distinct developmental trajectories. With the exception of cluster 2 (Figure 3.3, 3.4), which exhibits elevated expression at only the YNG and OLD stages, genes within the major clusters exhibit a single peak in transcriptional activity associated with a discrete developmental stage, accompanied by lower expression in all the sets before and/or after this peak. Each co-expression set (cluster) thus appears to have a uniquely defined developmental window in which the associated genes act, and the clusters are engaged sequentially during elongation and maturation of the inflorescence stem. It is interesting that most clusters (1-7) contain at least 443 genes, while Cluster 8 (out-group of Clusters 5-7), which contains genes that are up-regulated only at the base of the stem (OLD), has only 16 genes.
Figure 3.3 Hierarchical clustering of 4635 differentially expressed genes (q-value<0.05) on the basis of relative expression between cell wall expansion stages as outlined in Figure 3.1; top 1cm of plant (YNG), maximum growth-rate (MGR), cessation of elongation (CSS) and base of primary stem at rosette (OLD). Inset; 11-level colourimetric fold-change scale. Clusters (1-8) and stage-specific sub-clusters (1.1,2.1,5.1,5.2) are numbered for subsequent examination. The positions of representative genes associated with cell wall processes have been indicated along the right margin (described in ‘Results’ and/or ‘Discussion’). PMEI=pectin methyl-esterase inhibitor, At2g45220.
The primary stem undergoes radical change through the examined developmental continuum, from cell division and differentiation processes at the YNG stage, through to cell wall fortification processes in the CSS and OLD stages. Since these eight co-expression clusters exhibit such distinct development-correlated activity patterns, it was of interest to see whether the genes co-associated with different primary stem developmental phases would also exhibit any clear functional relatedness when examined for GO term enrichments for the major gene ontology categories of 'cellular compartment', 'molecular function', and 'biological process'. However, GO term enrichment analysis revealed significant over-representation only for high-level GO terms (data not shown).

The Gene Ontology Consortium also provides top-level parent terms, called 'GO SLIMs', that assign GO terms of the broadest (parent) categories of 'cellular compartment', 'molecular function', and 'biological process' to the submitted gene lists. When I used the TAIR bulk tool 'Functional Categorization' to summarize the GO SLIM enrichments across my clusters, (Figure B.2), the most dramatic over-representation included the cellular compartment 'ribosome', the molecular function 'structural molecule', and the biological process terms 'energy pathways' and 'DNA or RNA metabolism'. Collectively, these enrichment patterns suggest that the transcriptional programming changes may be dominated by modifications to the transcriptional and translation machinery itself. This implies the degree to which translational machinery must be modified to accommodate the changes in protein profile, a pattern reflected in other transcriptional studies (Hall et al., 2007).

While these trends are pronounced, such GO SLIM term enrichment remains difficult to interpret because of the broad generality of the functional categorization. I therefore
considered whether narrower definition of the co-expression sets might provide a clearer view of functional relationships. While the majority of the major clusters exhibit up-regulation across more than one developmental stage, several sub-clusters exhibit stage-specific up- or down-regulation (Figure 3.3; sub-clusters 1.1, 2.1, 5.1, 5.2, and Cluster 8), suggesting a high degree of temporal co-regulation of their respective genes during inflorescence stem development. [Box-] plotting of the mean ‘estimates’ for these sub-clusters emphasizes the extent to which these co-expression sets are up- or down-regulated with respect to the other stages (Figure B.3).

AtCoeCis analysis of each sub-cluster reveals only modest GO term enrichment among these small subsets of co-expressed genes (Table B.5). In Cluster 1.1 (‘High only in YNG’), 10 of 29 genes are tagged with the ‘chloroplast’ GO terms, a ~4-fold enrichment. However, the exhibited 86-fold enrichment of ‘translation elongation factor activity’ reported for the ‘Higher in YNG relative to MGR’ list (Table 3.1) is not evident in this sub-cluster. In Clusters 2.1 (‘Low only in MGR and CSS’) and 5.1 (‘High only in MGR’), little enrichment exists for GO terms, while cis-regulatory elements show no more than 4.5-fold and 6.9-fold enrichment, respectively. In cluster 5.1, six of the 23 genes are associated with protein synthesis, whereas GO term enrichment analysis for ‘Higher in MGR relative to YNG’ (Table B.2) and ‘Higher in MGR relative to CSS’ (Tables 3.3) suggested dominance in the processes of hormonal response. Interestingly, it is Cluster 5.2 (‘High only in CSS’) that shows enrichment of GO terms associated with hormonal response, with ‘response to endogenous stimulus’ appearing on nine genes (14% of the cluster). Also prevalent in this list are terms associated with ‘channel or pore class transporter activity’. This is consistent with a 21-fold over-representation of ‘GO: 0015267’ for the ‘Higher in CSS relative to OLD’ (Table B.4). In Cluster 8 (‘High
only in OLD’), ‘cell wall’ appears in 31% of genes together with ‘defense response to pathogen’ and associated child (subsidary) terms. This is consistent with the enrichment of stress response terms for the original differential list ‘Higher in OLD relative to CSS’ (Table B.4). Collectively, these sub-cluster AtCoeCis reports suggest that considerable heterogeneity persists among highly co-expressed subsets of genes.

3.2.4 Stage-specific co-expression analysis of cell wall-related genes

Many previously published gene expression-profiling studies focused on investigating plant cell wall processes have limited the features on their microarrays to those genes specific to cell wall building/remodelling. For example, Roach et al. (2007) produced a flax cDNA array whose probes were derived specifically from phloem scrapings, in order to investigate flax fibre development, while cotton fibre elongation has been examined using cDNAs derived from cotton ovule libraries (Wu et al., 2007). In rice, 735 cell wall-related genes were used to generate a microarray specifically for investigation of cell wall processes (Guillaumie et al., 2007). In Arabidopsis, Imoto et al. (2005) generated a microarray from 765 cell wall-related genes gleaned from full genome annotations. The datasets produced from such ‘simplified’ array platforms provide useful information concerning the co-expression of the cell wall-related genes. Similarly, data generated from full genome 70-mer oligonucleotide array experiments, such as that described here, can be filtered for cell wall-related genes already known to be involved in various cell wall formation processes (ex. cellulose, phenylpropanoid metabolism) (Ehlting et al., 2005; Ko & Han, 2004), thereby facilitating a more direct view of the co-expression of these known cell wall players.

To assemble a list of genes whose function is potentially related to cell walls, I first acquired all Arabidopsis accessions (AGI codes) which were designated by TAIR as
encoding carbohydrate active enzymes (CAZy) (Cantarel et al., 2009), including 83 carbohydrate esterases, 353 glycoside hydrolases, 321 glycosyltransferases, and 28 polysaccharide lyases. Additional accessions were derived from keyword searches in the TAIR database, which yielded 34 expansins, 60 arabinogalactan proteins (AGPs), 203 hydroxyproline-rich glycoproteins (HRGPs), 304 pectin-related genes, 177 cellulose- and hemicellulose-related genes, and 46 xyloglucan-related genes. A list of lignin-related accessions was derived from both TAIR searches and accessions listed in supplemental tables in Ehlting et al. (2005). In all, I considered 742 accessions to be directly related to cell wall building and remodelling, and these designations are included in Table B.1.

Hierarchical clustering of expression values was performed on the basis of mean 'estimates' for all 742 genes across four stages of development (Figure 3.6) to generate a view of their co-expression patterns. This yielded six co-expression groups that exhibited expression in either one stage or in two consecutive stages. For stage-specific clusters, I then tested for over-representations of functional categories (GO term enrichment) using the AtCoeCis tool (Table B.6). Approximately thirty percent of the genes found in Cluster 3 ('High only in MGR') are tagged as having 'pectinesterase inhibitor activity' (GO:0046910), coinciding with the region where cell wall plasticity should be at its greatest. By contrast, Cluster 5 ('High only in CSS') exhibits enrichment of 'pectinesterase activity' but not 'pectinesterase inhibitor activity', consistent with the prediction that the pectin de-esterification process would dominate at a stem developmental stage where cell wall rigidification dominates, i.e. at cessation. These observations are consistent with the finding in Arabidopsis seedling hypocotyls that the degree of pectin methyl esterification (where pectinesterase activity is inhibited) is
highest where tissue expansion rate is greatest (Derbyshire et al., 2007). Interestingly, both ‘pectinesterase activity’ (34-fold) and ‘pectinesterase inhibitor activity’ (25-fold) are the most dramatically enriched terms in Cluster 7 (‘High only in OLD’). This dichotomy implies that a homeostatic balance between these two opposing forces is being actively maintained, or that both are simultaneously required in different tissue/cellular regions to enact proper cell wall maturation in this late stage of inflorescence development. Indeed, clustering of 253 pectin-related genes (Figure B.5) reveals co-expression of pectin methylesterases (PMEs) and pectin methylesterase inhibitors (PMEIs) for a number of distinct gene trajectories, suggesting that PMEIs may be highly specific to PMEs that are presumably required for stage-specific production of methylesterified pectins. Such apparent relationships were discussed earlier, in the context of pectin-specific immunofluorescence patterns, in the ‘Discussion’ section of Chapter 3.

Cell wall-specific hierarchical clustering thus reveals pronounced co-expression sets, while gene ontology analysis reports clear functional differences between those groupings, consistent with the dramatic changes in cell wall composition detected earlier in my immunohistochemical analysis (Chapter 3). While ‘cell wall’ associations have been assigned to these 742 selected genes, based on the current annotations of their predicted biochemical function, many of the genes in question are still essentially uncharacterized. This co-expression data may ultimately assist us in understanding the functional relationships between members of this group of putative cell wall-related genes.
3.2.5 Assessment of commonality between the published transcriptome studies of primary stem development

Several microarray-based gene expression profiling experiments have now been carried out on bolting Arabidopsis inflorescence stems (Ehlting et al., 2005; Imoto et al., 2005; Ko & Han, 2004; Minic et al., 2009; Oh et al., 2003). Collectively, these datasets would be predicted to cross-validate the performance of specific genes within a common developmental context, and perhaps also to provide a more complete and integrated view of this developmental process, to the extent that somewhat different aspects of inflorescence growth may have been captured in different experiments. However, meaningful cross-platform 'meta' analysis of multiple experiments within a unified data processing pipeline is challenging because of the experimental noise and bias inherent to each array platform (Clarke & Zhu, 2006). Effective analysis also requires that we have the ability to access the original raw data. Finally, as noted in Chapter 2, the sampling methodologies employed in previous primary stem transcriptomic studies have relied upon the untested assumption that plants of similar height have very similar growth kinematic profiles. These factors make it very difficult to determine the appropriate experimental groupings for such a unified meta-analysis.

However, I found that two of these previous studies that have investigated primary stem development, Imoto et al. (2005) (designated ‘Im’) and Ehlting et al. (2005) (designated ‘Eh’), could still potentially be compared to my own dataset (designated ‘Ha’) (Figure 3.4). While the ‘Eh’ study was carried out on the Landsberg erecta (Ler) accession, as opposed to the ‘Im’ and ‘Ha’ studies conducted on Columbia (Col-0), it is likely that the underlying cellular processes are highly conserved across this narrow phylogenetic gap,
and there is also sufficient similarity in experimental approach to allow direct comparison.

Both of these studies performed comparative analysis of different portions of bolting inflorescence primary stems at a similar stage of stem maturation (~10-15cm in height). These studies are also similar to my study insofar as they each conducted analyses that identified gene co-expression sets having different developmental trajectories. Importantly, each study also has provided complete accession lists for each co-expression set, information that allowed me to use computational methodology for assessment of commonality between experiments, provided that common categories of developmental trajectory could also be identified.

In order to facilitate such comparison, co-expression sets identified in these three studies were grouped, where necessary, into super-categories ('Developmental Trajectory') designated as 'high early' (HE), 'high at middle' (HM), and 'high at late' (HL) (Table 3.4). Such broad categorization partially circumvents the problem of determining developmental equivalence when comparing the results of studies in which clear distinction between developmental stages represented by the tissues sampled is not available. A fourth category for both the ‘Eh’ study and my study, includes genes with low activity during the 'middle' phase. This category was designated 'low middle' (LM). It should be noted that the 'middle' classification used for this meta analysis includes the CSS stage from my own study.
Table 3.4 Inter-study grouping co-expression sets, identified in Ehlting et al. (2005), Imoto et al. (2005) and this study. Studies are referred to in the Venn diagram (Figure 3.5) according the alphabetic designator, A-C. Data sources for accessions lists (and associated figures) are provided for each study. 'Categories' column indicates the grouping of accession categories into similar developmental trajectories for comparison with other data sets. "High Early" (HE) genes are highest in the young tissue of the primary stem apex, "High MGR" (HM) have peak expression below the apex and above the point where cessation is assumed to occur, and "High Late" (HL) have highest expression at or after the point of cessation. "Low MGR" (LM) indicates a group of genes lower in regions spanned by the shoot apex and the presumed point of cessation. The number of accessions for each category available for analysis is indicated in the left-most column. See Fig 3.4 for visualization of the experimental designs/categories and Table B.1 (column 68) for a complete list of the accessions.

<table>
<thead>
<tr>
<th>Alphabetic Designator</th>
<th>Data source Reference</th>
<th>Figure Referenc e</th>
<th>Categories</th>
<th>Developmental Trajectory</th>
<th>Number of accessions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Ehlting ('Eh')</td>
<td>TPJ_2403_sm_SuppTable1.xls , Ehlting et al. (2005)</td>
<td>Figure 2, Ehlting et al.(2005)</td>
<td>Categories 2,3,6</td>
<td>High Early (HE)</td>
<td>2197</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Category 8</td>
<td>High MGR (HM)</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Categories 1,4 and 5</td>
<td>High Late (HL)</td>
<td>2323</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Category 7</td>
<td>Low MGR (LM)</td>
<td>128</td>
</tr>
<tr>
<td>B Hall ('Ha')</td>
<td>Table B.1 (column 66)</td>
<td>Figure 4.3, Figure 4.4</td>
<td>Clusters 1,3 and 4</td>
<td>High Early (HE)</td>
<td>2091</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cluster 5</td>
<td>High MGR (HM)</td>
<td>864</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Clusters 6 and 7</td>
<td>High Late (HL)</td>
<td>1156</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cluster 2</td>
<td>Low MGR (LM)</td>
<td>513</td>
</tr>
<tr>
<td>C Imoto ('Im')</td>
<td>11103_2005_Article5344_M OESM1_ESM.doc, Imoto et al.(2005)</td>
<td>Figure 4, Imoto et al. (2005)</td>
<td>Type 'A'</td>
<td>High Early (HE)</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Type 'B'</td>
<td>High MGR (HM)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Type 'C'</td>
<td>High Late (HL)</td>
<td>45</td>
</tr>
</tbody>
</table>

84
Commonality between the results of these experiments was first investigated by comparing the accessions identified in the ‘Eh’ and ‘Ha’ studies, both of which were conducted on the same full-genome scale (22,891 common probes) oligonucleotide microarray platform. This comparison has been expressed in the form of Venn diagrams where comparisons are made between the same expression trajectory (HE, HM, or LM) for each experiment (‘intra-stage’), or between different expression trajectories of experiments (‘inter-stage’). Insets in the diagrams show the statistical values expressing the significance of the observed commonality (Figure 3.5). To generate the Venn diagrams, I determined the number of accessions found in both datasets (intersection), as well as the proportion of the smaller list in the intersecting set (pair-wise commonality score, expressed as a percentage), for each of HE, HM, and HL co-expression categories (‘intra-stage’ comparisons). The ‘Fisher’s exact’ test was used to compute the probability of each intersection value occurring by chance. HE and HL showed 28% and 30% agreement, respectively, between experiments, despite the use of different genotypes (Landsberg erecta and Columbia), and different experimental conditions and sampling methodologies for the ‘Eh’ and ‘Ha’ studies, respectively. Fisher’s exact test values show a high degree of significance in this data overlap (p-values of 1.4E-136 and 6.9E-76 for HE and HL sets, respectively). In contrast, the consensus between ‘Eh’ and ‘Ha’ sets for the HM category is considerably lower, with a commonality of only 5.5% and a Fisher’s exact p-value of 0.45.
Figure 3.4 Schematic of comparison of co-expression datasets for four transcriptome studies of Arabidopsis primary stem development. Each figure depicts a separate experiment and is composed of two parts; left, a schematic of the harvesting methodology and right, re-grouping of co-expression sets identified in each study into similar categories of 'high-early' (HE), 'high-middle' (HM), 'high-late' (HL), and low-middle' (LM). A) Ehlting et al. (2005) study of Landsberg erecta primary stems. B) Current study of Columbia primary stems. C) Imoto et al. (2005) study of Columbia primary stems. Boxplots depict mean signal intensity distributions of clusters 1-8 featured in Figure 3.3.
Figure 3.5 Pair-wise comparison of commonality among co-expression sets from transcriptome studies of primary stem development. A & B) Venn diagrams showing commonality of matched (intra-stage comparisons, center) and mismatched (inter-stage comparisons) co-expression sets at or between stages of 'high-early' (HE), 'high-middle' (HM) and 'high-late' (HL). A) Comparison between Ehlting et al. (2005) study (Eh) and this study (Ha), considering 22,891 genes (n) common to both microarray platforms. B) Intra-stage comparison between Eh, Ha, and Imoto et al. (2005) study (Im), considering 585 genes (n) common to all three array platforms. Values within Venn circles indicate values that are unique to each set, or common to all considered sets. White text in black boxes indicate the percentage of the smaller co-expression set (bracketed values below outlined text; 'Eh', 'Ha', or 'Im') that falls within the intersection set, while values in white boxes indicate Fisher's exact p-values of the likelihood that the intersection set occurs by chance alone. No probabilities were calculated for the intersection of the three sets in 'B', or where the intersection set is empty. See Figure 3.6 for a complete list of pairwise comparison of gene commonality.

To assess the possible functional basis of the observed commonality, GO term enrichment was assessed for all intra-stage intersection sets (Table B.7). Common amongst the HE lists for the full-genome sets is significant enrichment in translational machinery (‘HE:Eh’ to ‘HE:Ha’). When considering only cell wall-specific genes with the
HE trajectory across all experiments, I observe significant enrichment in glycosylase and transferase activity. Glycosylases also comprise approximately sixty percent of the genes common to all cell wall-specific HL lists. There is no significant functional commonality evident between ‘Eh’ and ‘Ha’ HM sets, and there was a surprisingly low significance of enrichment of GO terms between the ‘Eh’ and ‘Ha’ sets for the HL trajectory, despite the 30% overlap in list commonality.

Despite the low level of agreement among experiments when compared at the HM stage, it seemed possible that these experiments would exhibit more commonality when examined at the level of different co-expression categories. In fact, pair-wise comparisons between categories (‘inter-stage’) reveal greater similarity than ‘intra-stage’ comparison of ‘Eh’ and ‘Ha’ for ‘HE:Eh’ to ‘HM:Ha’ (21%), ‘HM:Ha’ to ‘HL:Eh (9%)’, and highest overall similarity between ‘HM:Eh’ to ‘HL:Ha’ (31%) (p-value = 2.1E-18). The lowest level of agreement between ‘Eh’ and ‘Ha’ sets exists between ‘HE:Ha’ and ‘HM:Eh’ (0.8%, p-value=4.6E-04).

When comparing these two studies to the Imoto et al. (2005) study, which was limited to 765 cell wall-related genes, a common set of 585 genes can be examined (Figure 3.5B). Interestingly, the Imoto et al. 2005 study shows a greater level of agreement with the data from the ‘Ha’ study than with that of the ‘Eh’ study for all developmental stages, especially the stage corresponding to maximum growth rate, ‘HM’ (50%, p-value = 1.5E-05). To examine the relationship between HE, HM, and HL sets across these three experiments, commonality scores were computed for all possible pairs, which resulted in a similarity matrix (Figure 3.6A). When a dendrogram was generated from this similarity matrix (Figure 3.6B), reveals that the ‘HE:Eh’ set aligns most closely with the HM clade of both the ‘Im’ and the ‘Ha’ experiments. Furthermore, the ‘HM:Eh’ set is
seen to be the outlier in a clade composed of HL sets from all three experiments. Interestingly, the HE sets from the ‘Im’ and ‘Ha’ studies align more closely with HL than with HM sets, which raises the possibility that the HM co-expression set includes genes that diverge from a transcriptional program that occurs both early and late in the cell wall formation process.
Figure 3.6 Summary of likeness of co-expression sets based upon co-occurrence of genes (accessions) in co-expression sets outlined in Table 3.4. A) Matrix of pair-wise commonality, expressed as the percentage of genes of the smaller co-expression set (see Table 3.4; Figure B.4) that fall within the intersection set in each pair-wise comparison of sets; four shading categories are based upon the percentage values. B) Dendrogram derived from values in 'A', showing divisive clustering of sets on the basis of dissimilarity. Co-expression sets are annotated by stage (prefix) and study (suffix) where; HE='high-early', HM='high-middle', HL='high-late', Eh=Ehlting et al. (2005) study, Ha=this study, and Im =Imoto et al (2005). 'NA'=not applicable.
3.3 Discussion

Cell wall building/remodelling necessarily undergoes radical change within the narrow developmental range of primary stem elongation, implying radical transcriptional reprogramming between growth kinematic profile-identified stages. Full-genome mRNA inventories captured at various stages of cell wall expansion thus have the potential to provide important insight into the regulatory mechanisms underlying cell wall development. While cell expansion will not be the only biological process undergoing change during stem maturation, the universal occurrence of cell walls and the scale of metabolic commitment to this particular carbon sink mean that the demands of cell wall re-modelling can be expected to be strongly reflected in transcriptomes sampled across the developmental gradient.

Since the biological processes being sampled in these data are not restricted to, or necessarily associated directly with cell wall formation/modification, I have attempted to facilitate independent analyses of this broader landscape of transcriptional changes, while still highlighting hypothesis-generating insights on stage-specific cell wall building and remodelling processes.

3.3.1 ‘Young’ stage

The YNG stage sampled in this study captures the top 1cm of the Arabidopsis stem and so encompasses a developmentally complex region containing the shoot apical meristem, and up to twenty internodes which bore siliques prior to harvest. Dominant in this pool of nascent tissue are two EF-1-α accessions (Table 3.1), proteins that are associated with protein translation. Interestingly, occurrence of these two tandemly duplicated genes within the list of twenty short-listed genes (Tables B.2) represents an 85-fold enrichment over the full-genome occurrence of this functional process, and these genes are expressed at the YNG stage 22.5- (At1g07930) and 19-fold
(At1g07940) higher than in the MGR stage. It seems likely that the bulk of protein translational machinery required for subsequent developmental stages is being established at this early stage.

The absence of genes clearly associated with cell cycle processes from this shortlist may at first seem surprising. However, the most actively dividing shoot apical meristem resides within a very small region ~0.4mm from the shoot tip; which represents ~4% of the length of the harvested region, and even less so by volume, given the conical shape of this section (maximum 0.5mm diameter at the base of the segment). The overall YNG mRNA pool is therefore presumably dominated by the transcriptional activity associated with the bulk of the tissue, where cell wall formation/remodelling processes, among others, likely prevail.

However, cell wall-forming processes do not seem predominant in the YNG stage in comparison with the MGR stage, with the exception of a putative glucan endo-(1→3)-ß-glucosidase (MEE48) whose high relative expression appears limited to the YNG stage (Figure 3.3). Interestingly, MEE48 has previously been characterized as anther-specific in its expression, and has been proposed to be involved in callose degradation during pollen exine formation (Dobritsa et al., 2011). However, in my study all floral tissue had been deliberately removed from the YNG tissue at the time of sampling. If callose hydrolysis is an accurate description of the biochemical function of the MEE48 protein, it is not obvious why it should be such a dominant feature of the YNG transcriptome.

Perhaps high MEE48 expression is associated with removal or modification of callose in the plasmodesmata that occur abundantly within the phloem (Zavaliev et al., 2011). Another alternative would be a role in modulating the callose buildup often associated with wound response (Thomas & Hall, 1979). However, there is no evidence of other wound-induced transcriptional responses in the YNG stage transcriptome. GLUCAN
SYNTHASE-LIKE 5 (GPL5), which is thought to be required for callose synthesis in Arabidopsis (Jacobs et al., 2003), is not significantly differentially expressed among any stages of my study. It is conceivable that, even though stem extension growth was guided by nylon strings in my single-plant growth chambers, lateral forces, including gravity could still impact cell wall mechanics and trigger MEE48 expression at this early stage of development. Another 1-3-ß-glucosidase (BGL2) has been reported to be down-regulated when mechanical stress is relieved from Arabidopsis stems (Koizumi et al., 2009), which might point to a role for callose degradation in response to mechanical stress in the developing Arabidopsis primary stems, but no BGL2 homologues were differentially expressed at any developmental stage in my study.

As reviewed in Chapter 1 (‘General Introduction’), auxin plays important roles in tissue differentiation as well as acid-induced growth. AUXIN RESPONSE FACTORS (ARFs) ARF8, ARF18, and ARF12 are found significantly up-regulated in the YNG stage (Figure 34.3, cluster 1). Although ARF12 and ARF18 have not been the specific focus of research, negative regulation of ARF8 expression (arf8-1) resulted in hyper-elongation of Arabidopsis hypocotyls (Tian et al., 2004). ARF8 is highly conserved among spermatophytes, and study in rice demonstrates that ARF8 is a negative regulator of IAA conjugating enzyme OsGH3-2 (Yang et al., 2005). Thus, up-regulation of ARF8 likely reflects reduction in IAA-conjugation and consequently increased levels of free auxin. Transcription factors previously associated with vascular/non-vascular differentiation (reviewed in Demura & Fukuda, 2007), as well as genes classified in TAIR9 annotations as auxin response factors, were co-clustered (Figure B.6) revealing several other ARFs (2, 5/MONOPTEROS, 23) that up-regulated in the YNG stages relative to MGR and CSS stages.
3.3.2 ‘Maximum growth-rate’ stage
Both the YNG/MGR comparison and MGR/CSS comparison lists provide a perspective on the genes up-regulated in tissue undergoing maximum rate of growth (MGR). Most up-regulated in MGR relative to YNG was the PEROXIDASE 64 (PER64) gene, whose expression appears to be specific to the MGR stage, based upon hierarchical clustering (Figure 3.3). Peroxidases are thought to be at least partially responsible for cross-linking of lignin monomers, and PER64 has been previously associated with maturing interfascicular fibres (Koizumi et al., 2009), but the MGR stage is not characterized by any extensive accumulation of lignin. Although PER64 expression is high, it is not known whether the PER64 protein is necessarily active at this stage. Interestingly, PER64, like BGL2, was shown to be down-regulated upon relief of mechanical load on Arabidopsis stems (Koizumi et al., 2009).

Genes associated with gibberellic acid (GA)-mediated elongation are predominant at the MGR stage, consistent with long-standing knowledge of gibberellic acid as a key modifier of directional cell growth (Schwechheimer, 2008). In particular, GO terms associated with GA are enriched as much as 100-fold over their full-genome occurrence (Table B.2). The gene encoding the putative [cysteine-rich] signal peptide, GA-STIMULATED ARABIDOPSIS 4 (GASA4), is 3.4-fold up-regulated in MGR relative to CSS (Table 3.2), which is consistent with the notion that GA-responsive genes would be more highly expressed where GA is most active.

Consistent with GA involvement in rapid, directional cell expansion, the transcription factor MINI ZINC FINGER 1 (MIF1), previously characterized as GA-responsive (Hu & Ma, 2006), is up-regulated 12-fold in the MGR stage relative to the YNG stage. Mutation of MIF1 results in unresponsiveness to GA and inflorescence stem dwarfism (Hu & Ma, 2006). It is therefore likely that up-regulation of MIF1 is central to proper GA-mediation.
of inflorescence stem elongation. Closely associated with MIF1 is MERISTEM-5 (MERI5B, At4g30270), which is up-regulated in MGR relative to YNG, and remains clustering with MIF1 across all the developmental stages studied (Figure 3.3). MERI5B/XTH24 is a group 2 xyloglucan endotransglycosylase/hydrolase (XTH) (Rose et al., 2002), a group of proteins that facilitate the remodelling of hemicellulose known to be required for cellulose microfibril separation and ‘creep’ during anisotropic cell wall expansion (Cosgrove, 1997). Interestingly, MIF1 and MERI5B co-cluster with the arabinogalactan protein, AGP13, in a set that exhibits peak expression at the onset of cessation (Figure 3.3, cluster 7).

In contrast, expression of another XTH, ENDOXYLOGUCAN TRANSFERASE A1 (EXGT-A1), required for normal cell wall expansion (Akamatsu et al., 1999), is restricted to the MGR stage (Figure 3.3, cluster 5) along with AGP12, which exhibits 6.8-fold up-regulation in the MGR stage over the YNG stage. XTHs target xyloglucan chains, and not arabinogalactan chains, so the co-expression of the AGP12-with-EXGT-A1 and AGP13-with-MERI5B pairs is intriguing. An additional five AGPs (AGP14, 21, 22, 24 and FLA13) appear significantly up-regulated (q-value<0.05) in the MGR stage relative to YNG stage (Table B.1), suggesting that a suite of AGPs may be contributing to the unique structural dynamics of rapidly expanding cells at the MGR stage.

While no AGPs appear to be significantly differentially expressed between the MGR and CSS stages, AGP18 is down-regulated at the OLD stage relative to the MGR stage (Tables B.1). AGP18 is a member of a lysine-rich, GPI-anchored sub-family that includes AGP17 and AGP19, and the loss-of-function mutant possesses shortened inflorescence stems (Yang et al., 2007). High ovule abortion rates resulting from RNAi-silencing of AGP18 has been attributed to failure of the megagametophyte to
expand (Acosta-Garcia & Vielle-Calzada, 2004), implying that AGP18 may play a role in a broad spectrum of cell expansion contexts.

Other AGPs also exhibit significant increases in expression when comparing the MGR stage to the OLD stage (Table B.1). *FASCICLIN-LIKE 8 (FLA8)* is up-regulated at the OLD stage relative to the MGR stage (Table B.1). *FLA8/AGP8* belongs to sub-family of AGPs that contain a fasciclin domain that often possesses a glycosyl phosphatidyl inositol (GPI) anchor (MacMillan et al., 2010). *FLA8* was observed to be up-regulated significantly in tension wood, but not opposite wood, in poplar when compared to its expression in differentiating xylem (Lafarguette et al., 2004). Interfascicular fibres in the Arabidopsis stem develop a thickening, pliable inner wall during the MGR stage of extension growth, a layer which resembles the G-layer of tension wood. It is tempting to speculate that my earlier (Chapter 4) observation that the arabinogalactan-specific antibodies JIM8 and PN16-4B4 exhibit their most intense fluorescence when applied to stem tissues at the CSS stage (Figures 4.5-4.8) might be due to binding of these antibodies to the arabinogalactan side-chains of FLA8.

*AGP21* also appears significantly up-regulated in the OLD stage relative to the MGR stage (Table B.1). Interestingly *AGP21*, which is homologous to *AGP12* and *AGP14* (Shinsaku et al., 2005), is down-regulated ~4-fold upon silencing of the transcription factor *PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1/MYB75)* (Nossen background), coincident with increased cell wall thickness in xylary and interfascicular fibres (Bhargava et al., 2010). Also of interest is *FLA11*, which was associated with *IRX3/CESA7* in a regression analysis of publicly available transcript profiling datasets (Persson et al., 2005). Its expression was also up-regulated in the lower half of Columbia stems with 2-3 expanded siliques where secondary cell wall formation is most likely dominant (Brown et al., 2005). While *FLA11* exhibits no significant difference
among sequential developmental stages in my study, its expression intensity does increase markedly throughout the maturation process, most notably from the onset of cessation onwards (Figure 3.3). This is consistent with in situ evidence of localization of FLA11 protein to secondary cell walls in mature stems (Shinsaku et al., 2005). Interestingly, the in situ data suggest that the localization is specific to sclerenchyma cells of the interfascicular fibre (IFF) region. Recent biomechanical assessment of Arabidopsis stem strength showed a significant reduction in stress yield in fla11 mutants, and more so in fla11 fla12 mutants, compared to WT plants (MacMillan et al., 2010). As no significant change in lignin accumulation was observed in these mutant lines, authors posit that structural integrity may be lowered by reduction in cellulose deposition. My expression data, as well as this published evidence, suggest members of the large AGP family have functionally diverged, and that this divergence plays out in part through differences in spatiotemporal regulation of their expression (Rose et al., 2002).

Another gene that has been previously linked to the process of cell expansion is SPIRAL1-LIKE 5 (SP1L5). Mutation of SP1L5, like that of SPIRAL1, results in strong, right-handed axial twisting of etiolated hypocotyls and roots (Nakajima et al., 2006). This response is likely due to the aberrant cortical microtubule arrangement observed in SP1 mutants (Sedbrook et al., 2004). The predicted protein structure of SP1 suggests that SP1 may function as an intermolecular linker, and within the cell the protein appears to be associated with the growing (plus) ends of microtubules (Sedbrook et al., 2004). This behavior has led to the designation of SP1 and SP1-like proteins (including SP1L5) as +TIP proteins (Young & Bisgrove, 2011). The effect of mutation of SP1L5 on microtubule dynamics has yet to be examined.
Also co-expressed with *MIF1*, *MERI5B* and *AGP13* is *MYB61*, which showed dramatic rise in expression from YNG to MGR stages (Table 3.1), yet appears most highly up-regulated in CSS and OLD stages. *MYB61* has been associated with seed coat mucilage production (Penfield *et al.*, 2001), but also a wide-ranging of other processes including stomatal closure (Liang *et al.*, 2005) and ectopic lignification (Newman *et al.*, 2004).

### 3.3.3 ‘Cessation’ stage

From the perspective of cell wall biology, the composition of the shortlist of twenty genes most up-regulated at the CSS stage relative to the MGR stage (Table 3.2) is particularly striking. Thirteen of the 20 genes in this list appear to be functionally related to secondary cell wall biosynthesis. It is also notable that the population of this gene list is distinct from that of the list of genes dominant in CSS relative to OLD, suggesting that these are likely genes whose expression remains elevated through both developmental stages. Central among these are the three cellulose synthases that are essential for secondary cell wall formation, which have been shown to co-localized (Gardiner *et al.*, 2003) and exhibit similar co-expression patterns (Persson *et al.*, 2005). *CESA8* is thought to belong to the same multi-protein biosynthetic complex as *CESA4* and *CESA7*, and the model of the cellulose synthase complex also predicts that the three component CESA proteins co-occur in a 1:1:1 ratio. However, in my co-expression analysis, *CESA8* clusters differently from *CESA4* and *CESA7*, primarily due to increasingly elevated expression in the OLD tissue sample (Figure 3.3, cluster 8), in contrast to *CESA4* and *CESA7* (Figure 3.3, cluster 6), which do not change significantly in expression from the CSS to OLD stages. Since the relative stability and turnover rates for the three CESA proteins are unknown, these differences in gene expression do not necessarily conflict with the predicted abundance of the proteins in the plasma
membrane. It is also possible that the relative proportion of secondary cell wall-associated CESAs is not fixed throughout secondary cell wall formation.

By definition, only primary cell walls are capable of expanding (McNeil et al., 1984), and the majority of this expansion is certainly occurring above the point of cessation. We therefore expect that cellulose synthase genes associated with primary cell wall formation (CESA1, 3, & 6) will be up-regulated in the YNG and MGR stages relative to their expression in the CSS and OLD stages. Surprisingly, in my dataset, CESA3 is more highly expressed in the CSS and OLD stages than in the YNG and MGR stages (Figure 3.3). In an earlier study that examined gene expression in the base of immature, intermediate, and mature stems (5cm, 10-15cm, and >25cm in height, respectively), elevated expression of AtCESA3 was observed at all developmental stages (Ko & Han, 2004). Further, expression of CESA3 does not appear correlated with primary cell wall formation in primary stems of Landsberg erecta (Ler) plants, appearing more highly expressed at the base of the stem than the region five to seven centimeters below the apex coinciding to expansion or cessation regions (Ehlting et al., 2005). Another study found that AtCESA4, 7, & 8 were up-regulated in mature Columbia stems, but none of the canonical primary cell wall CESAs (CESA1, 3, 5) were found in the list of CESAs expressed early in development (Imoto et al., 2005). Instead, a series of CESA-LIKE genes was found (At5g16190 (CSLA11), At5g22740 (CSLA2), At1g02730 (CSLD5)).

Other canonical primary and secondary cell wall-associated cellulose synthases also do not conform strictly to the pre- (YNG and MGR) and post- (CSS and OLD) cessation division. CESA6, which is generally considered to be specific to primary cell walls, is most highly up-regulated in the MGR and CSS stages (Figure 3.3, Cluster 5), where it is co-expressed with CESA2 (radial wall reinforcement) and CESA10.
The profiles of CESAs discussed here collectively suggest that employment of particular cellulose synthases may not be exclusive to either diffusely elongating tissues or post-elongation tissue, canonically associated with primary or secondary cell wall formation processes, respectively. Alternatively, a more diverse, heterogeneous mixture of cell wall forming processes may persist in the sampled tissues after cessation of elongation to account for late expressions such as CESA3. If the latter case is true, one explanation may be that intrusive growth of interfascicular fibres or cambial activity (radial cell proliferation) persists at or below the point of cessation.

While it could be argued that cessation of elongation does not accurately capture the transition from primary to secondary cell wall formation, accumulation of glucuronylarabinoxylans (GAXs, or just ‘xylans’) may be taken as an indicator that secondary cell wall formation is taking place. My immunoprofiling data revealed the appearance of xylan hemicellulose (LM10-related fluorescence) at the point of cessation within the interfascicular fibre region (Figures 4.4-4.5). Furthermore, the expression of genes previously associated with xylan synthesis (IRX7/8/9 & GATL-1) (Brown et al., 2007), appear to be highly correlated with the CSS and OLD stages where secondary cell wall synthesis is occurring (Figure 3.3, cluster).

3.3.4 ‘Old’ stage
The base of the stem of 10-15cm Columbia plants contains live, photosynthetically active cells located adjacent to thick-walled, highly lignified fibres of the interfascicular region that are presumably in the advanced stages of programmed cell death. From the expression data from this study, and from other studies examining such mature tissues (Ehlting et al., 2005; Imoto et al., 2005; Ko & Han, 2004), it is clear that genes related to secondary cell wall synthesis are active in this region of the lower stem. Figure 3.3 presents several co-expression sets (clusters 2, 6, 7 and 8) whose expression in the
OLD stage is higher than in one or more of the earlier developmental stages. Interestingly, *CESA8*, which appears in cluster 8, exhibits its highest level of expression at this stage, as does *XYLOGLUCAN ENDOTRANSGLYCOZYSLASE HYDROLASE 18 (XTH18)*. Both this expression data, and my observation of strong immunosignals from the xyloglucan-specific CCRC-M1 antibody in the CSS stage tissues (Figure 4.7) are consistent with other results linking xyloglucan deposition with late stages of secondary cell wall synthesis. For instance, xyloglucan deposition has been observed to occur in cotton fibres after cessation (Tokumoto *et al.*, 2002), and PttXET16 activity has been observed in secondary vasculature of poplar (Bourquin *et al.*, 2002).

Interestingly, the chloroplast-localized *FATTY ACID REDUCTASE 6 (FAR6)* gene is the most up-regulated of all the genes at the OLD stage relative to the CSS stage. A similar phenomenon has been observed in other studies: increased expression of FAR6 was observed through microarray analysis in epidermal peels from the base of the stem of Columbia (Suh *et al.*, 2005) as well in stem tissue harvested from the base of 35cm (mature) Columbia plants (Ko & Han, 2004). FAR6 is presumably contributing primary alcohols for the cuticle in mature stems, and not for suberin since FAR1, 4 and 5 were investigated as more likely candidates of suberin synthesis (Domergue *et al.*, 2010).

Several genes whose products are associated with the shikimic acid pathway and lignification (reviewed in Humphreys & Chapple, 2002) exhibit an expected pattern of behaviour within this dataset, though differences in clustering suggest subtle distinctions in the timing of their expression (Figure 3.3). *PHENYLALANINE AMMONIA- LYASE 1 (PAL1)*, whose activity is required for phenylalanine allocation to phenylpropanoid metabolism from the shikimate acid biosynthesis pathway, appears up-regulated in both the CSS and OLD stages, coincident with up-regulation of *3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE (DAHP) SYNTHASE*. 3
(DHS3), which regulates the intake of carbon into the shikimate pathway. Interestingly, maximum expression of PAL4 is restricted to the OLD stage, although PAL1 and PAL4 function have so far not been distinguished. CINNAMYL ALCOHOL DEHYDROGENASE 4/5 (CAD4/5) and CINNAMIC ACID 4-HYDROXYLASE (C4H) genes which have been referred to as metabolic gatekeepers for lignin biosynthesis (Sibout et al., 2005), are not present in the short list of clustered genes (Figure 3.3). CINNAMOYL CoA REDUCTASE (COMT) is significantly up-regulated in CSS and OLD stages relative to elongating tissues (Figure B.3). COMT produces sinapic acid from 5-hydroxyferulic acid and thus is involved in the production of syringyl (S-) lignin over guaiacyl (G-) and p-hydroxyphenyl (H-) lignin monomers. Arabidopsis fibres are enriched in S- versus G-lignin (Chapple et al., 1992), so up-regulation of COMT may account for the extensive deposition of S-lignin in the fibers in lower stem regions where elongation is presumed to have ceased and fibre-specific cell wall formation processes likely dominate (Ehlting et al., 2005), independent of CAFFEYOYL COA O-METHYLTRANSFERASE (CCoAOMT) which is not significantly differentially expressed across stages. This suggests a strong contribution of S-lignin deposition in the interfascicular fibre region in older stems. However, F5H, which is the rate-limiting step of sinapic acid production (Humphreys & Chapple, 2002) is not noticeably up-regulated, so it is difficult to speculate on the impact of up-regulation of COMT on S-lignin deposition.

IAA12/BODENLOS and ARF4 transcription factors also show highest up-regulation in the OLD stage. Interestingly, ARF4 is known to act redundantly with ETT in establishing organ polarity (abaxial fate in leaves, for instance) (Pekker et al., 2005), thus ARF4 expression in my dataset presents a novel context in which ARF4 expression is associated. IAA12/BODENLOS, an Aux/IAA repressor of the ARF
MONOPTEROS/IAA24 (Hamann et al., 2002) has been associated with cambial formation. Since interfascicular cambial formation is known to be an auxin-dependent mechanism, up-regulation of IAA12 provides a possible mechanism by which wood formation is arrested in the senescing inflorescence (Little et al., 2002).

One of the most pronounced patterns within this expression set is the occurrence of five genes annotated as ‘defense-related’ in the list of twenty most significantly up-regulated in the OLD stage relative to the CSS stage (Table 3.3). Four plant defensins (PDF1.2, 1.2b, 1.2c and 1.3) possess different accession codes, though sequence similarity among them is high (data not shown), and non-specific binding to the associated probes is likely. Complementing this set is an RDF-family defensin, LOW MOLECULAR WEIGHT CYSTEINE-RICH 67 (LCR67), which also exhibits a high degree of sequence similarity with the PDF-family members (Eigen-value = 4e-33). It is therefore not possible to resolve whether mRNA abundance for one or all of these accessions is elevated in this dataset.

Plant defensins are small, basic peptides that have been so named for their putative roles in pathogen defense (Thomma et al., 2002). To date, however, transcriptional characterization of these genes has been limited to pathogen studies and they have not been associated with cell wall development. Defense response to pathogens often involves the process of local cell wall fortification, termed cell wall apposition, and lignification has been observed to play a significant role in resistance (Bhuiyan et al., 2009). As noted earlier, the process of callose induction is not reflected in the expression data, and tissue from the OLD stage was harvested (snap-frozen) less than one minute after the CSS tissue. It is conceivable that one or more of these defensins might play an important signalling role in the advanced lignification of primary stems, possibly independent of an external, biotic stimulus.
3.3.5 Comparison to other transcriptomic studies of inflorescence stems

This expression profiling study potentially complements several extensively cited analyses that have also addressed transcriptional changes in Arabidopsis inflorescence stem maturation (Ehlting et al., 2005; Imoto et al., 2005; Jamet et al., 2009; Ko & Han, 2004; Persson et al., 2005). My transcriptional examination potentially integrates with these datasets to provide a more complete picture of transcriptional reprogramming in primary stem maturation; one that is placed with a more precisely defined context of directional cell expansion.

Comparison of this study to that of Ehlting et al. (2005) showed as high as 31% agreement between lists, yet it is not clear if this represents a significant consensus. Other studies that have tried to assess the level of reproducibility of expression profiling experiments between labs that are using the same genetic accessions have demonstrated higher correlations ($r^2 > 0.9$) (Massonnet et al., 2010). Since the microarray hybridization methodologies used in the Ehlting et al. (2005) study and in my study (direct labelling of microarrays printed at the same facility), are very similar, the lack of a higher level of agreement between the datasets from these two experiments is likely to have a biological basis.

One possible reason that the gene trajectories derived from the Ehlting and other microarray studies represent different sets of genes is that they reflect fundamental differences in sampling methodology. The inflorescence stem developmental stages selected for microarray analysis in the Imoto study were most analogous to my YNG and MGR stages (MGR zone ~2.6cm from apex coincides with Imoto zone III). On the other hand, the Ler ecotype used in the Ehlting study has a somewhat dwarfed growth habit relative to Col-0, reaching a final height of ~20cm under our growth conditions (SD→LD). It is therefore clear that the growth kinematic profiles of these two ecotypes
will be substantially different, in addition to the compounding effects of developmental signalling differences that may exist between Col-0 and Ler accessions. If the Ler GKP is compressed, then the HM category of genes showing elevated expression from 3-9cm in the Ehlting ('Eh') study likely corresponds to a later stage in development in my ('Ha') study, as the greater similarity between ‘HM:Ha’-to-‘HL:Eh’ over other comparisons would suggest (Figure 3.4).

The significance values (Fisher’s exact tests) indicate that the commonalities in gene expression that do exist among many intersection sets are highly significant. In silico functional genomic analysis of the genes that populate the intersection sets obtained when the results of the Ehlting et al. (2005) and Imoto et al. (2005) studies are mapped onto this study (Table B.7) already provides some hints of a functional basis for such shared expression, but these would need to be explored more directly by pursuing reverse genetics on a gene-by-gene basis. Genes shared within expression categories of these different experiments are listed in the supplemental Table B.1 for those who may be interested in narrowing their list of candidate genes by selecting those that have been identified as common players in these different experimental settings.

Overall, however, the large plant-to-plant variation in growth kinematic profiles that I have been able to identify through growth kinematic profiling makes it clear that earlier studies in which multiple plants have been pooled to create biological replicate samples of inflorescence stems are inevitably compromised in their ability to accurately place cellular changes in an elongative development context (Chapter 2). As a result, although some degree of overlap between the datasets generated in those earlier studies and my own dataset can be expected, the development-linked accuracy of the gene expression profiling reported here has established a new standard for such analyses.
3.4 Experimental procedures

3.4.1 Plant growth, imaging, preservation, and growth kinematic profiling

Plant growth and imaging was conducted as described in Chapter 2, where paper tags (and not PVDF disks) were utilized as synthetic optical markers. Plants were harvested sequentially between 1 and 3pm (mid-day where daylight cycle occurs between 6am and 10pm on a 16L:8D regime) at 20 minute intervals. Segments were immediately snap-frozen in liquid nitrogen and deposited into 0.2mL PCR tubes for -80°C storage. Growth kinematic profiling was conducted as described in Chapter 2 for paper tag feature tracking. Segments were pooled on the basis of growth kinematic profiling and design objectives as outlined in Figure 3.1 on the basis of growth kinematic profiles outlined in Figure B.1.

3.4.2 RNA processing

Whole-segments (pooled according to growth kinematic profile equivalence) were homogenized in liquid nitrogen with mortar and pestle then transferred to 1.5mL eppindorf tubes, weighed and combined with 1mL TRItzo™ (cat#15596-026, Invitrogen) per 100mg of tissue, vortexed and incubated at room temperature for 5 minutes. 0.2mL chloroform was added for each 1mL of TRItzo™, vortexed for 15 seconds, incubated for 1 minute at room temperature, then centrifuged at 15000g for 10 minutes at 4°C. Aqueous phase was transferred to fresh RNAse-free tubes and then combined with an equal volume of isopropanol and incubated 20 minutes on ice. RNA_{total} was pelleted by centrifugation at 15000g for 10 minutes, and pellets were washed with 1mL 75% ethanol in RNAse-free water. Following a 5-minute pellet drying phase, pellets were resuspended in 25µL RNAse-free water and incubated on ice for 1 hour. Each resuspension was treated with 1/10th volume (~2µL) 10X DNase I buffer and 1µL 10X
DNAse I (from RNAqueous® Micro kit; cat#AM1931, Ambion) for 20 minutes at 37°C followed by addition of 2µL DNAse inactivation reagent (also from RNAqueous® Micro kit) and incubated at room temperature for 2 minutes. Samples were centrifuged at 13000g for 1.5 minutes and supernatant transferred to RNase-free tubes and stored at -80°C.

3.4.3 Reverse transcription and labelling

For each biological replicate, approximately 20µg RNA_{total} was incubated with 2ug of 0.5µg/µL oligo(dT) primer (cat#18418-012, 0.5ug/ul, Invitrogen) in a 22.5µL volume of RNA-primer mix and denatured at 70°C for 10 minutes. Reaction mix was prepared such that each sample contained 9µL 5X First Strand buffer (supplied with Superscript II, Invitrogen, cat#18064-014), 0.23µL each of 0.1mM dATP (cat#10216-108, Invitrogen), dCTP (cat#10217-016, Invitrogen), and dGTP (cat#10218-014,Invitrogen), as well as 0.045µL dTTP (cat#10219-012, Invitrogen) for a total reaction mix volume of 18.5µL. This reaction mix was combined with 18.5µL of RNA-primer mix along with 1.5µL (1.5moles/µL) of the appropriate Cyanine dye; Cy5-dUTP (cat#45-000-740, Fisher) or Cy3-dUTP (cat#45-000-738, Fisher). After incubation at 42°C for 2 minutes, 1uL 40U/ul RNAase Inhibitor (cat#10777-019, 40U/ul, Invitrogen) and 1.2ul 200 U/µL Superscript II (cat#18064-014,200U/ul, Invitrogen) were added for a final volume of 45µL which was incubated at 42°C for 2.5 hours, then deactivated with 0.5M NaH/50mM EDTA at 65°C for 15 minutes. The reaction was neutralized with 7.1uL 1M Tris-HCl (pH7.5). Samples were cleaned of unlabeled probe via centrifugal filtration using Amicon 0.5-Ultra30kDa filters (cat#UFC503096, Millipore) prior to array hybridization.
3.4.4 Array hybridization

Microarray slides were first pre-conditioned by incubating slides in Coplin jars with 50°C 2X SSC for 20 minutes followed by room temperature washes with 0.2X SSC and ddH2O for 5 and 3 minutes, respectively using the Advawash AV400 machine (Advalytix/Beckmen-Coulter). Prehybridization solution of 1X formamide-based hybridization buffer (pre-warmed to 80°C) from Vial 7 of the 3DNA Array 350 kit (cat#W300130, Genisphere) was then added to gap between each slide and a pre-placed m-Series lifterslip (cat#48382-251, VWR) within the Slidebooster (Advalytix/Beckmen-Coulter) hybridization chamber and subsequently incubated for 1-1.5 hours at 50°C with sonication (power=15, pulse=1 second 'on', 9 seconds 'off'). Slides were then washed in 2X SSC, 0.2% SDS for 15 minutes at 65°C followed by room temperature washes in 2X SSC and 0.2X SSC for 10 minutes each, and centrifuged at 700rpm till dry in Advatubes (cat# OAX05216, Advalytix/Beckmen-Coulter). 25μL solutions of labeled (Cy3 and Cy5) mixes were combined with 25μL 2X formamide buffer (Vial 7, 3DNA Array350 kit) and the 50μL hybridization mix added to the gap between the 42°C pre-warmed slides and the pre-placed m-Series lifterslips. Slides were then incubated at 42°C for 16-18 hours with sonication (power=15, pulse=1 second 'on', 9 seconds 'off'). Post-hybridization washing was carried out in reduced lighting with 42°C 2X SSC, 0.2% SDS for 15 minutes followed by room temperature washes at of 2X SSC and 0.2X SSC for 15 minutes each, then centrifuged until dry at 700rpm. Slides were stored in a light-proof desiccating chamber until fluorescence scanning.
3.4.5 Microarray scanning and spot quantification

Arrays were scanned with using the ScanArray Express HT (Perkin-Elmer) scanner and associated software using 543nm laser for Cy3 and 633nm laser for Cy5 fluorescence, with laser power adjusted for each slide individually within the range of 95-100% such that ~1-2% of spots (presumed positive controls) were saturated in signal. PMT gain ranged from 60-95%, set for each slide such that fluorescence intensity of sub-grid regions surrounding spots did not exceed 400 (16-bit grayscale). TIFF images of array scans were imported into Imagene (Biodiscovery Software) and grid templates roughly placed then 'auto-adjust' function applied to best fit subgrid on a per-spot basis, allowing spot size variation from 15-20µm. Median pixel intensities computed from spot regions are used to represent spot intensity in subsequent analyses.

3.4.6 Microarray data analysis

Data analysis was carried out in the statistical programming environment R (cran.r-project.org/) using custom scripts and contributed packages. To remove local, background noise, the mean signal intensity of the dimmest five percent of spots within each of 48 subgrids was subtracted from each array element using a custom script, then variance stabilization normalization (VSN) was applied to each channel to normalize for non-linearity in variance across spot intensities (Huber et al., 2002) using the function ‘vsn’ (‘vsn’ package, Bioconductor). Normalized intensities were then fit to the mixed effects model (Tempelman, 2005) using the ‘lme’ function (‘nlme’ package), and all pairwise differential expressions for array elements were computed as the log2 intensity difference values between treatment class intensities. Associated measures of significance (p-values relative to null hypothesis, log2 difference equals zero) were corrected for false-discovery rate using a custom script according to Story (2002), and
‘estimates’ were computed as the log₂ intensity difference of each treatment class to the mean of all treatment classes (normalized to zero). Associated measures of significance (p-values relative to null hypothesis of log₂ difference = 0) were also corrected for false-discovery rate as described above. Raw and output data were exported along with TAIR10 annotations in the supplemental data (Table B.1). For hierarchical clustering, dissimilarity matrices were computed from filtered datasets using the ‘diana’ function (‘cluster’ package) (Maechler et al., 2005) and rendered as dendrograms using the ‘dendro’ function (‘cluster’ package) (Maechler et al., 2005). Heatmaps were generated using the 'heatmap.2' function (‘gplots’ package) (Warnes, 2010).
Chapter 4. Epitope profiling across the cell wall expansion continuum in the Arabidopsis primary stem

4.1 Introduction

Anisotropic cell wall expansion is a process of fundamental importance to plant growth and development, and is integral to establishing plant organ shape. The properties of cellulose microfibrils, particularly their length and crystallinity, are central to the ability of the cell to generate the differences in tensile strength among its walls necessary for controlled directional cell expansion (Fujita et al., 2011). However, many other cell wall carbohydrate constituents, such as cross-linking glycans and glycoproteins, interact with the cellulose microfibrils to condition the degree of cell wall extensibility and to affect the direction of cell growth. Non-enzymatic and enzymatic modifications to these components dynamically alter the way in which they interact with cellulose (reviewed in Cosgrove, 1997).

The developing Arabidopsis inflorescence stem provides an experimentally accessible model for studying the coordinated interaction of diverse tissue types during a period of dramatic, directional elongation as the plant generates a rigid seed-dispersal structure. During this process, the parenchymous ground tissue differentiates into an outer, multi-layered band of photosynthetic cortical cells encased in the protective epidermis. The inner tissues consist of a radial series of vascular bundles (phloem and xylem) nested within the periphery of the central pith. Together, these tissues provide both additional photosynthetic capacity and the transport conduits required to support morphological transformation of this organ as it develops. At the interface of these outer and inner tissue bands is a layer of endodermal cells which are analogous to
endodermis/pericycle of the root, although lacking any suberized cell walls. Although such endodermal layers, also referred to as the starch sheath (Esau, 1965; Fleurat-Lessard, 1981), are found in aerial organs of many embryophytes (Lersten, 1997), this cell layer is not yet well characterized in Arabidopsis.

As stem elongation completes its apically-driven growth, the interfascicular fibre (IFF) region, located between vascular bundles and immediately interior to the endodermis (Lev-Yadun et al., 2005), undergoes extensive secondary cell wall thickening along with the neighbouring xylem cells (fibre, tracheid and vessel element cells) to form a 'wavy band' (Lev-Yadun et al., 2005), that rigidifies the inflorescence stem in a turgor-independent manner, and supports the stem through its senescent, drying phase.

While there are different functions and diverse structural end-points for cell walls across these different tissue types, the organ itself must elongate in a unified, diffuse fashion. One model for such coordinated growth would be that the lateral (expanding) walls of the various cell types all share a common, homogeneous composition until elongation ceases, at which point the differentiation program that defines tissues such as the IFF region and xylem cells would switch from the common program to more specialized processes such as secondary cell wall formation. Alternatively, specialized cell types could be envisioned to diverge in wall structure much earlier, during their elongation phase, but still maintain matched, diffuse cell wall expansion despite these different wall properties.

One way to gain some insight into the apparent paradox between similarity in diffuse growth characteristics and diversity of structural endpoints is through examination of the composition of the cell walls of these diverse cell types at distinct stages in their
elongative development. Histochemical staining of plant tissues is a traditional, but
imprecise, method for identifying the presence of a limited set of cell wall biomolecules
such as cellulose (Calcofluor White, Toluidine Blue, Pontamine Fast Scarlet Red 4B)
and lignin (phloroglucinol, Maüle) (reviewed in Ruzin, 1999) (Ruzin, 1999). Microspectrophotometric techniques, such as Fourier-Transform Infra-Red (FT-IR) microscopy, offer more precise means to quantify the spectral properties of tissue samples (Gorzsás et al., 2011; G. Mouille et al., 2003), but are still limited in their ability to distinguish amongst the hundreds of spectroscopically-similar chemicals present. Bulk chemical analyses, such as analysis of sugar composition for specific tissues, provide detailed knowledge of the relative abundance of component sugars (Pattathil et al., 2010), but do not inform us of their linkages or spatial arrangement in different cell types.

By contrast, various types of molecular probes now provide great precision in identifying specific cell wall constituents. Forty-two families of carbohydrate binding modules (CBMs), binding domains of microbial glycosidic hydrolase enzymes, are now cataloged within the “carbohydrate active enzyme” (CAZy) database (http://afmb.cnrs-mrs.fr/CAZY/). In Arabidopsis, a subset of these CBMs has been shown to target cellulose, as well as a number of cross-linking glycans, including mannans and glucomannans. Initial examination of a subset of CBMs, employed as fluorescence-recombinant proteins, suggests that CBMs exhibit a high degree of target specificity in planta despite the broad range of targets bound by CBMs in vitro (Knox & McCartney, 2005). However, the library of available CBMs is not yet comprehensive and individual CBMs are not necessarily specific to unique glycosidic linkages. By contrast, monoclonal antibodies (mAb) raised against cell wall components are typically
epitope targets *in planta* in a 1:1 fashion. These mAbs can, through indirect fluorescent-labelling approaches, provide quantitative, cell type-specific and subcellular localization information for epitopes in plant cells, wherever the epitopes are accessible to the mAb. An extensive array of cell wall-directed mAbs is now publicly available which target a diverse set of polysaccharide-containing cell wall constituents (Pattathil et al., 2010). While it has been observed *in vitro* that particular epitopes may not necessarily be unique to single glycan polymers (Clausen et al., 2003; McCartney et al., 2005; Moller et al., 2008; Pattathil et al., 2010; Verhertbruggen et al., 2009), indirect immunolabelling nonetheless provides valuable semi-quantitative information about changes in the structural/compositional status of the cell wall. The large number and diversity of available mAb molecular probes, as well as the robust technology that enables rapid assessment of mAb binding, provide an opportunity for simultaneous analysis of many cell wall constituents across cell types of multiple samples. The resulting data can provide a global view of cell wall heterogeneity with regard to all major cell wall classes in different cell types. This general strategy has been employed in a limited number of recent studies, including use of 27 different antibodies to examine seven sub-regions of G-fibres in tension wood of sweetgum (*Liquidambar styraciflua*) (Bowling & Vaughn, 2008). Twenty-four antibodies were also used to explore cell wall composition within the phloem fibres of hemp (*Cannabis sativa* L.) plants at three separate regions along the primary stem (Blake et al., 2008). In principle, antibodies can therefore be used in this manner to interrogate cell wall composition across a broader set of cell types at specific stages of elongative development.

Here, I present a more comprehensive view of epitope abundance for 55 carbohydrate-directed antibodies in the developing inflorescence stem of *Arabidopsis thaliana*, using
Here, I present a more comprehensive view of epitope abundance for 55 carbohydrate-directed antibodies in the developing inflorescence stem of *Arabidopsis thaliana*, using tissues from three experimentally-defined growth stages along a continuum of elongative development identified by growth kinematic profiling (see 'Chapter 2'). To enable concurrent examination of a large set of antibodies with cell type-specific resolution, I employed a 96-well format, thick-section (non-embedded) indirect immunolabelling approach. The resulting growth stage-specific immunolabelling micrographs were used to conduct an analysis of quantified cell type-specific fluorescence intensities which allowed me to 1) highlight major shifts in epitope abundance/accessibility across developmentally-distinct tissues, 2) identify similarly behaving antibodies that displayed similar reporting patterns, and 3) detect novel cell type-specific patterns of immunolabelling. The complete set of micrograph images have also been made available as a public resource (http://www.plantometrics.com/antibody_summary), for the plant biology research community.

4.2 Results

4.2.1 High throughput immunological survey for stage-specific epitope profiling

Regions representing distinct elongative developmental states were identified by growth kinematic profiling of individual Arabidopsis plants, which establishes relative growth rates for segments of individual plants and thereby permits identification and isolation of tissues at specific stages of development (see Chapter 2). This sampling approach enabled me to perform comparative immunohistochemical analysis of three stages of elongative development; 1) an apical region where directional cell growth is initializing (termed 'young', or 'YNG'), 2) a region where this directional growth is most rapid
(termed 'maximum growth-rate, or 'MGR'), and 3) a region where this elongation is finishing (termed 'cessation', or 'CSS') (growth kinematic analyses detailed in Figure C.1A-C).

Efficient examination of 55 antibodies and negative controls across seven tissues in three biological replicate plants of Columbia (Col-0) demanded several high-throughput procedural modifications to immunolabelling, confocal microscopy imaging, image processing, and image analysis. I chose an immunofluorescence approach whereby formaldehyde-fixed segments were cut into 40µm-thick transverse sections by use of a vibratory microtome. For high-throughput treatment, an immunofluorescence and counterstaining approach was used on free-floating 40µm sections in six separate 96-well plates, which allowed me to address ~27 antibodies (per 96-well array) and controls under standardized conditions. Confocal imaging represents a workflow bottleneck in the mounting and imaging of large numbers of sections. For high-throughput confocal imaging of 96 cross-sections, I employed a custom confocal imaging dish with 96 2mm wells (Figure C.1D). Finally, image acquisition was restricted to the central 20µm optical sections of the 40µm confocal viewing field to accelerate acquisition and reduce file sizes.

Use of a fluorescence counterstain, Congo Red, which binds to both xyloglucan and cellulose (Wood, 1980) allowed me to uniformly demarcate cell walls of all cell types throughout development, and provided sufficient morphological context from which to establish tissue boundaries. To establish the density and distribution of primary antibody labelling of accessible epitopes, I utilized an Alexa-Fluor™ pre-labelled secondary antibody whose chromophore emits in a spectral range distinct from that of Congo Red. This allowed two distinct signal channels to be generated for each
image. This two-channel approach enabled me to generate confocal stacks that portrayed the morphological context necessary for accurate tissue identification (using the 561nm emission channel), while permitting observation of secondary antibody immunofluorescence intensity and distribution (the 488nm channel). The resulting images were assembled into antibody-specific views for browsing (Figures C.2.1-C.2.58), as well as 96-well, two-channel montages (Figures C.4.1-C.4.6), which supported subsequent analysis of ‘regions of interest’ (ROI). For each antibody, the immunofluorescence intensity was quantified in seven readily distinguished cell types (Figure 1) in ROIs selected from within tissue boundaries. Ultimately, profiles were generated for 55 antibodies across seven tissue types (plus three negative controls) in the inflorescence stems of three biological replicate plants sampled at three developmental stages.
**Figure 4.1 Representative developmental series for fluorescence intensity scoring** A developmental series for the JIM11 antibody (antigen=extensin, potato lectin) in a single primary flowering stem at YNG (A & B), MGR (C & D) and CSS (E & F) stages. Tissues were identified from maximum projection overlays of Congo Red counterstain (blue) channel with Alexa-Fluor™ antibody fluorescence (green) channel (panels A,C,& E) as indicated above. Ep=epidermis, Co=cortex, En=endodermis, IFF=interfascicular fibre region, Pi=pith, Ph=phloem, and Xy=xylem. The antibody-only channel (panels B,D, & F) was examined separately to quantify fluorescence signals of seven cell types and background (denoted Bk) for each 40µm section. See Supplemental dataset A.1-A.57 for the complete set of confocal panels for 55 antibodies and negative controls, presenting maximum projection images for three biological replicates at each stage in two-channel and 488-channel-only view.
Two analytical approaches were used in this study to examine commonality among the tissue-specific fluorescence intensity responses. First, changes in fluorescence intensities associated with each antibody across development were assessed; these reflect the most prominent changes in epitope abundance for each cell type between stages. Second, relative intensities ($I_{rel}$) among tissue types were derived, thus establishing antibody-specific epitope patterning from which groups of antibodies can be identified that share similarity in their tissue-specific localization. Since epitope specificity remains largely uncharacterized for the majority of available cell wall-directed antibodies, including most of those used in the current study, I have first presented my antibody-specific results in the context of the ‘binding specificity’ clades (bracketed items after the introduction of each antibody). These clades were defined in a recent extensive study of cell wall-directed antibodies, in which they were classified on the basis of polysaccharide recognition patterns (Pattathil et al., 2010). In this section, I discuss the relation of my antibody-specific results to this and other published evidence for antibody targeting, while I examine more global system observations and issues in the ‘4.3 Discussion’ section.

### 4.2.2. Assessment of developmental equivalence among biological replicates

Prior to examining the binding of individual antibodies in tissues at YNG, MGR, and CSS stages, I first sought to confirm that the quantitative data generated for the biological replicate segments accurately reflected the distinct development stages that I expected to be captured in segments from YNG, MGR and CSS regions of the inflorescence stem. Since there are 399 unique antibody:ROI pairings for each segment (biological replicate), those labelling patterns provide an overall immunoprofile
for each segment. To test the hypothesis that the overall profile for biological replicate segments would be more similar within a stage than between stages, I carried out a hierarchical clustering (HCL) analysis. The results can be visualized as a dendrogram showing the relationship between biological replicates on the basis of dissimilarity in fluorescence intensities for their antibody:ROI profiles (Figure 2). The HCL analysis revealed that biological replicates separated into distinct clades of YNG, MGR, and CSS stages, and also that MGR and CSS stages are more similar to each other than either are to the YNG stage. Furthermore, the association of biological replicates within each developmental stage/clade changes between stages, indicating that no systemic difference between replicate plants is maintained throughout development. This analysis demonstrates that antibody-associated fluorescence intensities are correlated with the process of developmental change across these three harvested regions, and thus have the potential to report structural differences that may be associated with the fundamental underlying processes.
Figure 4.2 Dendrogram of immunofluorescence similarity of biological replicates at three stages

Fluorescence intensities for 55 antibodies were assayed in seven tissue types across three developmental stages. Dissimilarity scale values are proportional to the root sum-of-squares of intensity differences (Euclidean distances) among clade members. 'BR' denotes biological replicate (number 1-3) for each developmental stage.

4.2.3 Epitope abundance change across development

Those primary antibodies associated with the most dramatic changes in tissue-specific fluorescence intensity from one developmental stage to the next were most directly assessed as arithmetic means (change in mean intensities, or ‘Δ-I’) between fluorescence intensities of YNG and MGR (i.e. MGR-YNG), and between MGR and CSS (i.e. CSS-MGR). I first identified the most consistent (p-value<0.05) and pronounced (Δ-I>=10 absolute intensity units) responses for each comparison to yield
short lists for MGR-YNG (Table C.2) and CSS-MGR (Table C.3) comparisons. Micrographs showing results for the antibodies associated with the most striking trends are presented as 2-channel image panels (Figures C.5-C.9), along with a review of published information of antibody specificity. It should be noted that the 488nm-channel images should be viewed independent of counter-stain channel for the most accurate rendering of epitope distribution. Such views are accessible in either the antibody-specific panels (Figures C.2.1-C.2.58) or the 96-well montages used for ROI analysis (low resolution-renderings provided as Figures C.4.1-C.4.6).

For changes between YNG and MGR, 36 of 55 antibodies were associated with marked changes (p-value<\(\leq\)-0.05, \(\Delta-I\)=10) in fluorescence intensities, including 62 increases and 31 decreases in tissue-specific fluorescence intensity (Table C.2). Most pronounced of these changes involve modified fluorescence intensities associated with three antibodies in at least six of the seven tissue types (Figure C.5); CCRC-M7 ('RG-I/AG' clade, increasing), CCRC-M13 ('RG-I/AG' clade, increasing), and JIM1 ('RG-I/AG' clade, decreasing). An additional five antibodies exhibited marked fluorescence intensity increases in all tissues except the cortex and pith (Figure C.6); CCRC-M22 ('RG-I/AG' clade), CCRC-M32 ('RG-I/AG' clade), CCRC-M12 ('RG-I/AG' clade), CCRC-M31 ('AG-3' clade) and JIM14 ('AG-2' clade). Also emerging from this short list of fluorescence intensity changes were two antibodies, CCRC-M34 ('pectic backbone' clade) and MAC204 ('AG-1' clade), associated with dramatic and opposite \(\Delta-I\) values for two adjacent tissues (Figure C.7, Figures C.2.16, C.2.51).

When comparing changes between the MGR and CSS stages, 31 of the 55 antibodies were associated with marked changes in fluorescence intensities, 26 of which were increases while 36 were decreases (Table C.3). As with the YNG-to-MGR transition,
only a small subset (four) of those 31 antibodies was associated with widespread and radical changes in fluorescence between the MGR and CSS tissue samples. Most dominant of these universal changes were decreases in fluorescence intensities in sections probed with four antibodies thought to be specific to arabinogalactan proteins; MAC265 (‘AG-undefined’ clade), JIM12 (‘AG-2’ clade), JIM14 (‘AG-2’ clade), and MAC266 (‘AG-3’ clade) (Figure C.8).

4.2.4 Similarity of epitope patterning among antibodies at three stages of development

Examination of the 55 antibody-specific panels (Figures C.2.1-C.2.55) reveals some striking patterns of label distribution and intensity for individual antibodies across different stages of elongative development. Similarity in patterning also exists among some of these antibodies, and such similarity provides important insight into the degree of co-occurrence of different epitopes in tissues at specific stages of elongative development. To explore this phenomenon more closely, I used the background-normalized, mean fluorescence gray value intensity ($\bar{I}_{bn}$) for seven tissue-specific regions of interest for a given antibody across biological replicate sections to effectively establish a pattern, or signature, that reflects that antibody’s performance (tissue response) at a given stage of development. This pattern can be described in terms of relative fluorescence intensity scores ($\bar{I}_{rel}$) for each of the seven tissues, thus representing the distribution of signal across the tissue. This quantitative measure of fluorescence distribution can be used as the basis for grouping antibodies that behave similarly at a particular developmental stage, which presumably reflects epitope co-occurrence. In addition, the 55 mean $\bar{I}_{rel}$ scores associated with each antibody may be correlated with tissue type, thereby providing a measure of the bias of each antibody (or technically, of the 55 associated epitopes) for each tissue. In this second analytical
approach that addresses the three stages (YNG, MGR, and CSS) individually, I used hierarchical clustering methods to assess the degree of similarity among 1) antibodies, based on their associated \( I_{rel} \) scores for the seven tissue types and 2) tissue types, based on their associated relative abundance scores for all antibodies, thus grouping 'like-antibodies' and 'like-tissues', respectively.

For this purpose, antibodies were grouped according to similarity via HCL and then organized in dendrogram form (vertical-axis), while a heat map rendered their \( I_{rel} \) scores on a nine-level colourimetric scale for the seven cell types (Figure 4.3A, 3.4A, and 3.5A for the YNG, MGR, and CSS stages, respectively). Tissue types were also grouped according to their similarity in \( I_{rel} \) scores to yield a two-axis clustering view that provides the clearest presentation of biologically relevant patterns. Mean gray value scores (\( \bar{I}_{bnr} \), see Methods) have been added to the plots as a metric for overall antibody signal abundance. Since many of these antibodies had already been assigned to particular groupings according to a published report of the similarity in their binding specificity to cell wall glycans \textit{in vitro} (Pattathil \textit{et al.}, 2010), I was interested in comparing the distribution of those clade assignments to the results of my own HCL \textit{in planta} analysis (Figures 4.3B, 4.4B, and 4.5C). To simplify this added layer of complexity, clades from Pattathil \textit{et al.} (2010) that shared common roots were combined into super-clades that were then grouped column-wise, thus providing a higher-order view of epitope class. Sub-clade information may still be viewed as text within the cells of these super-clade columns. There is additional published information on their target specificity of many of these antibodies, as detailed in Table C.1 and also provided at WallBioNet (glycomics.ccrc.uga.edu/wall2/index.html). I will first briefly discuss the clustering results
for each developmental stage, followed by a discussion of the important overall co-abundance groupings.

4.2.5 ‘Young’ stage clustering

Two-axis clustering on the basis of similarity of relative abundance scores for 55 antibodies over seven tissue types yielded six clearly distinguished clusters bifurcated within the dendrogram. With minor exceptions, these may be generalized as a cluster of antibodies whose signal is dominant in the vascular tissue (Figure 4.3, cluster #1), those dominant in the peripheral tissue of cortex and epidermis (Figure 4.3, cluster #2), those where the endodermis is dominant to the phloem and IFF region (Figure 4.3, cluster #3), those that are xylem-dominant but present in other tissues (Figure 4.3, cluster #4), and those with either widespread and/or low-level intensity scores across all tissues (Figure 4.3, cluster #5). In addition, two antibodies thought to recognize partially methyl-esterified homogalacturonan (HG) appear highly specific to separate, single tissues (i.e. clustering out-groups); JIM5 (‘pectic backbone’ clade, Figure C.2.26) to the protoxylem and CCRC-M34 (‘pectic backbone’ clade, Figure C.2.16, Figure C.7) to the cortex. Differences in the $I_{rel}$ distribution patterns of JIM5 (Clausen et al., 2003; Verhertbruggen et al., 2009) and CCRC-M34 (information from WallBioNet) may therefore reflect regional differences in distribution of partially methyl-esterified forms of HG.

In terms of identifying tissues whose antibody binding profile is most similar in YNG sections, tissue type-specific clustering identified the phloem and xylem as being most similar, while the epidermis is the out-group. On the basis of epitope profile, parenchymal tissues are less clearly distinguished from each other at this stage. At this stage, strong patterning is not evident in glycan-specific clade assignments (Figure
4.3B), although some homogeneous groups (double and triple) exist, perhaps indicative of epitope co-occurrence or cross-reactivity.
Figure 4.3 Cluster analysis in YNG segments of relative fluorescence intensities associated with 55 antibodies. A) Hierarchical clustering of both antibodies (vertical axis) and tissue types (horizontal axis) based upon relative, mean antibody fluorescence. Numerical values within heat map cells indicate mean intensities used to derive relative intensity clustering values. Horizontal dotted lines indicate major clade divisions that are numbered in the left margin. Cell colours representing relative intensities are restricted to nine levels. Histogram (inset, top-left) indicates frequency of relative intensity values in nine levels. B) Higher-order (super) clades in which the corresponding antibodies belong (vertical columns), as described in Pattathil et al. (2010). Cells shaded black or grey without text are not featured in the Pattathil et al. (2010) glycan survey but placed according to their likely grouping based on the current literature. LIMu = linseed mucilage.
4.2.6 ‘Maximum growth-rate’ stage clustering

For the MGR stage, cell types are expanding most rapidly in unison, presumably with similar, primary cell wall composition. Two-axis HCL presents antibodies grouped as dominant in the IFF region (Figure 4.4, cluster #1; outgroup), as dominant in the cortex (Figure 4.4, cluster #2), as largely absent in the IFF region (Figure 4.4, cluster #3), as centred on the endodermis but present in adjacent interior tissues of phloem and IFF region (Figure 4.4, cluster #4), as dominant in the pith but absent in the endodermis (Figure 4.4, cluster #5), as present in the epidermis and either of cortex or pith (Figure 4.4, cluster #6), and as widespread and/or low-to-absent in all tissues (Figure 4.4, cluster #7). JIM4 (‘AG-3’ clade) signal abundance in the pith places it as an out-group for the common branch to clusters #3-7 (Figure 4.4). In terms of tissue similarity, the IFF region is a prominent out-group, while phloem and xylem no longer appear most similar to each other, and the parenchymal grouping is lost. Glycan clade distribution for clusterings at the MGR stage (Figure 4.4B) presents a remarkably different picture than that of YNG tissue, where glycan clade-specific antibodies form homogeneous groups of up to seven members (Figure 4.3, cluster #4) and groups of 3-4 members of the same super-clade are common (Figure 4.3, clusters #3,4, & 6). While it is conceivable that such antibodies co-cluster on the basis of common antigen (cross-reactive), the pairings (or triplets) of like-clade antibodies are not conserved with high frequency between YNG and MGR suggesting that co-clustering likely reflects co-occurrence of distinct epitopes on single or multiple antigenic polysaccharides. There appears to be overall bias in the distributions of 'RG-I/AG' clade members in the pith (nine members in clusters #5-7, Figure 3.6), while 'AG-1' and 'AG-2' cluster together, separately from 'AG-3' in most but not all cases (i.e. Figure 4.4, cluster #5).
Figure 4.4 Cluster analysis in MGR segments of relative fluorescence intensities associated with 55 antibodies. A) Hierarchical clustering of both antibodies (vertical axis) and tissue types (horizontal axis) based upon relative, mean antibody fluorescence. Numerical values within heatmap cells indicate mean intensities used to derive relative intensity clustering values. Horizontal dotted lines indicate major clade divisions that are numbered in the left margin. Cell colours representing relative intensities are restricted to nine levels. Histogram (inset, top-left) indicates frequency of relative intensity values in nine levels. B) Higher-order (super) clades in which the corresponding antibodies belong (vertical columns), as described in Pattathil et al., 2010. Cells shaded black or grey without text are not featured in the Pattathil et al., 2010 glycan survey but placed according to their likely grouping based on the current literature.
4.2.7 ‘Cessation’ stage clustering

At the point of cessation, primary walls have discontinued anisotropic expansion and secondary cell wall formation (thickening and rigidification) can proceed on lateral (periclinal and anticlinal) cell walls. Antibodies appear to group in six distinct clusters at this stage, including those dominant in the IFF region and xylem (Figure 4.5, cluster #1; out-group), those dominant in the peripheral tissue of the cortex and/or the epidermis (Figure 4.5, cluster #2), those present in the phloem but also the adjacent IFF region and endodermis (Figure 4.5, cluster #3), those dominant in the pith and/or highly abundant in all tissues (Figure 4.5, cluster #4), those dominant in the fibre-possessing tissue of the IFF region and the xylem (Figure 4.5, cluster #5), and those with ubiquitous and/or low abundance across tissues (Figure 4.5, cluster #6). Notable out-groups to clusters #2-6 are the co-clustered antibodies JIM93 ('AG-1' clade) and JIM94 ('AG-1' clade). JIM93 and JIM94 also appear co-clustered at the MGR stage, along with other 'AG-1' and 'AG-2' clade members (Figure 4.4, cluster #4), but only JIM94 showed appreciable abundance in YNG tissue (Figure 4.3, cluster #1). Since both antibodies bind to unknown epitopes, it is quite possible that their epitopes occur independently on common or different antigens.

As at the MGR stage, the IFF region is again the pronounced out-group among tissue types, with the cortex being separated from other tissues to a lesser degree. While the MGR stage exhibited the largest homogeneous grouping of clade members, the distribution of 'RG-I/AG' members remains focussed on a few clusters (Figure 4.4, clusters #4 & 6), as do certain core groupings of 'AG-3'/4 clade members (Figure 4.4, cluster #1).
Figure 4.5 Cluster analysis in CSS segments of relative fluorescence intensities associated with 55 antibodies. A) Hierarchical clustering of both antibodies (vertical axis) and tissue types (horizontal axis) based upon relative, mean antibody fluorescence. Numerical values within heatmap cells indicate mean intensities used to derive relative intensity clustering values. Horizontal dotted lines indicate major clade divisions which are numbered in the left margin. Cell colours representing relative intensities are restricted to nine levels. Histogram (inset, top-left) indicates frequency of relative intensity values in nine levels. B) Higher-order (super) clades in which the corresponding antibodies belong (vertical columns), as described in Pattathil et al. 2010. Cells shaded black or grey without text are not featured in the Pattathil et al., 2010 glycan survey but placed according to their likely grouping based on the current literature.
4.2.8 Clusters of particular interest

One of the most pronounced clades appearing in the hierarchical clustering for the three stages was that comprised of 'AG-3' glycan clade members JIM8, JIM15, PN 16.4B4, and 'AG-4' clade member JIM13 (Figures 4.3-4.5, cluster #1). In YNG tissues, the fluorescence intensity signals for these four antibodies are limited to the vessel elements of the protoxylem and a small group of cells in the protophloem, but these signals then appear within the interfascicular fibre region during the most rapid elongation phase of the inflorescence stem growth (MGR), and persist through to the CSS stage in all tissues. Interestingly, fluorescence associated with these antibodies remains limited to the interiors/inner-walls of vessel elements at the CSS stage, but also appears in the inner walls of the live fibres during the MGR stage. A high degree of similarity in the fluorescence pattern is observed in tissues exposed to JIM8, JIM13, JIM15, and PN16-4B4, most strikingly as concentrated signal associated with the interior of IFF region (fibre) cells (Figure 4.6). It is interesting that, while these JIM antibodies were reported to display a high degree of cross-reactivity in vitro (Pattathil et al., 2010) (PN16-4B4 was not addressed in that study), JIM13 does group in a distinct glycan-specific clade ('AG-4'), implying that it recognizes a specific class of arabinogalactan side-chain other than that of the 'AG-3' clade. Cross-reactivity evidence (Pattathil et al., 2010) and the HCL analysis presented here (Figure 4.4-4.5, cluster #1; Figure 4.6) together raise the possibility that the presence of epitopes for JIM13 and the 'AG-3' clade members could be tightly linked to early stages of secondary cell wall formation during this most rapid phase of anisotropic cell expansion.
At the CSS stage, two additional antibodies appear tightly clustered with this group (Figure 4.5, cluster #1); JIM84 (‘AG-3’ clade), and the aforementioned LM10 (‘xylan’ clade) which is known to target low-methyl-substituted xylans in secondary walls. JIM84 was raised against a carrot ‘coated vesicle’ preparation, and has been observed to bind Golgi bodies as well as plasma membranes of many cell types; biochemical analysis implicates the protein portion of a glycoprotein as the likely epitope for this antibody (Horsley et al., 1993). Thus, common localization of both JIM84 and LM10 to the IFF region at MGR and CSS stages may indicate co-association of a glycoprotein with un-substituted xylan at the CSS stage though there is currently no evidence that these two epitopes are linked. Since LM10 identifies un-substituted xylans, it is also possible that two separate polysaccharide molecules are physically associated, as opposed to two different epitopes co-occurring on the same antigen.
Figure 4.6  Immunofluorescent labelling of YNG, MGR, and CSS tissue with antibodies identified in clustering as specific to inner walls of rapidly expanding fibres. Maximum projection, two-channel views of arabinogalactan-specific JIM8 (A-C), JIM13 (D-F), JIM15 (G-I), and PN 16-4B4 (J-L). Antibodies that bind later in development (CSS) are added to panel for comparison and include JIM84 (M-O), also arabinogalactan-specific, and xylan-specific LM10 (P-R) at three stages of development; YNG, MGR, and CSS. Congo Red counterstain channel is represented as blue, and antibody fluorescence as green. All images for biological replicate #1 (BR1). See supplemental image series of these antibodies to view all biological replicate images as well as view the antibody-specific channel independently.
One distinct cluster appearing at both the MGR stage (Figure 4.4, cluster #4) and the CSS stage (Figure 4.5, cluster #3) exhibits pronounced localization to the endodermis, a layer of cells that is morphologically distinct from both the cortical cells (endodermal cells have a longer anticlinal axis than cortical cells) and IFF region (endodermis is more polyhedral than fibre cells). While the endodermis is found in aerial plant organs of many species (Lersten, 1997) current knowledge of the biological role of the endodermis in Arabidopsis is limited to its participation in regulation of the shoot gravitropic response (Fukaki et al., 1998), possibly involving starch granules which have been observed within this cell type in *Mimosa pudica* L. (Fleurat-Lessard, 1981). This endodermis-specific cluster includes JIM11 ('AG-1' clade), JIM19 ('AG-2' clade), JIM20 ('AG-1' clade), and MAC204 ('AG-1' clade), while JIM12 ('AG-2' clade) appears within this cluster only during the MGR stage (Figure 4.4, cluster #4). As with other AG clade members in our study, the labelling appears specific to the inner wall of particular cells, which may be taken as an indication that the antibody fails to penetrate intact walls, and can only label those cortical cells cut open by the sectioning process.
Figure 4.7  Immunofluorescent labelling of YNG, MGR, and CSS tissue with antibodies identified in clustering as abundant within endodermis at MGR and CSS. Maximum projection, two-channel views of arabinogalactan-specific JIM11 (A-C), JIM19 (D-F), JIM20 (G-I), and MAC204 (J-L) at 3 stages of development; three stages of development. Congo Red counterstain channel is represented as blue, and antibody fluorescence as green. All images for biological replicate #1 (BR1), chosen arbitrarily. Antibodies co-clustered at both MGR (cluster#4) and CSS (cluster #3). JIM12 (M-O) co-clusters with this group at MGR so has been added. See supplemental image series of these antibodies to view all biological replicate images as well as view the antibody-specific channel independently.
Also prominent in this set of stage-specific hierarchical clusterings is a group of 'pectic backbone' clade members that are associated with ubiquitous fluorescence at the MGR stage (Figure 4.4, cluster#3), but then become localized to the IFF region and xylem at the CSS stage (Figure 4.5, cluster#5). This group includes LM19 (recognizes un-esterified HG), LM18 (recognizes partially methyl-esterified HG), and LM20 (recognizes methyl-esterified HG), depicted in Figure 4.8. Binding specificities for these antibodies were determined via differential response of antibody binding to treatment with CAPS buffer (pH 9.5) and sodium carbonate (pH 11.4) generating low and high degrees of de-esterification, respectively (Verhertbruggen et al., 2009).

Two additional antibodies of the 'pectic backbone' clade that are shown in Figure 4.8 behave similarly to either LM19 or LM20 in both this study and in that of Verhertbruggen et al. (2009). JIM5 signal patterns are similar to those of LM19, while JIM7 is similar to LM20. This similarity suggest that the respective pairs of antibodies may have similar epitope targets. My clustering data suggests that “un-esterified HG-specific” JIM5 and LM19 actually behave differently from each other at the MGR stage (Figure 4.4, cluster#2 and #4, respectively), largely on the basis of signal differences in the cortex, but they co-cluster at the CSS stage (Figure 4.5, cluster #5), suggesting convergence in localization of two forms of un-esterified HG epitope at the developmental point of growth cessation. One striking pattern common to both JIM5 and LM19 (and also LM18) is a peripheral zone of absence of signal in the phloem, which creates a ‘halo effect’ at the CSS stage (Figure 4.8D-I). LM19 and JIM5 signals are also localized to the middle lamella in three-cell junctions within the pith at both the YNG and MGR stages (Figure 4.8D,G). Gaps between these three-cell junctions (i.e. two-cell boundaries) subsequently display fluorescence at the CSS stage, potentially indicative of a spatiotemporal process of de-esterification associated with tighter cell-cell
associations as cessation is established within the pith. The three-cell junction labelling pattern has been observed in other studies using JIM5 (VandenBosch et al., 1989; Verhertbruggen et al., 2009) and LM19 (Verhertbruggen et al., 2009). In contrast to the similarity in epitope signal patterns for JIM7 and LM20 reported in tobacco (Verhertbruggen et al., 2009), these two antibodies exhibit markedly different behaviours in Arabidopsis stems, where they cluster differently at both the MGR (Figure 4.4, cluster#3 and #5, respectively) and CSS (Figure 4.5, clusters#4 and #3, respectively) stages. While both signals are found ubiquitously at the MGR and CSS stages, LM20 exhibits a strong bias toward certain tissue types (cortex and IFF region), while JIM7 does not (Figure 4.8J-L and M-O, respectively). Thus, while the tissue type-specific distribution of exposed methyl- and de-methyl-esterified HG epitopes appears similarly concentrated within a subset of tissues in the elongating Arabidopsis stem, subtle differences do exist between the un-esterified and methyl-esterified epitope patterns, perhaps indicating that different tissue types have distinct strategies for synchronizing their elongative growth with diffuse growth in neighbouring tissues.
<table>
<thead>
<tr>
<th></th>
<th>YNG</th>
<th>MGR</th>
<th>CSS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LM18</strong></td>
<td><img src="LM18_YNG.png" alt="Image" /></td>
<td><img src="LM18_MGR.png" alt="Image" /></td>
<td><img src="LM18_CSS.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>LM19</strong></td>
<td><img src="LM19_YNG.png" alt="Image" /></td>
<td><img src="LM19_MGR.png" alt="Image" /></td>
<td><img src="LM19_CSS.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>JIM 5</strong></td>
<td><img src="JIM5_YNG.png" alt="Image" /></td>
<td><img src="JIM5_MGR.png" alt="Image" /></td>
<td><img src="JIM5_CSS.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>LM20</strong></td>
<td><img src="LM20_YNG.png" alt="Image" /></td>
<td><img src="LM20_MGR.png" alt="Image" /></td>
<td><img src="LM20_CSS.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>JIM7</strong></td>
<td><img src="JIM7_YNG.png" alt="Image" /></td>
<td><img src="JIM7_MGR.png" alt="Image" /></td>
<td><img src="JIM7_CSS.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 4.8 Immunofluorescent labelling of YNG, MGR, and CSS tissue with antibodies identified in clustering as specific to xylem and IFF region at CSS. Maximum projection, two-channel views of arabinogalactan-specific LM18 (A-C), LM19 (D-F), JIM5 (G-I), and LM20 (J-L), and JIM7 (M-O) at 3 stages of development; YNG, MGR, and CSS. Congo Red counterstain channel is represented as blue, and antibody fluorescence as green. All images for biological replicate #1 (BR1), except JIM5 and JIM7 where BR3s were chosen to best represent the outlying attributes leading to distinct clustering.
4.2.9. Persistent co-association of epitopes on enduring glycans

Of the 55 cell wall-associated antibodies studied, signal patterns from five pairs appear to co-cluster within our HCL analysis through all three stages of development, suggesting persistent co-association of certain epitopes, presumably on an enduring glycan cell wall component. Common to all five pairings is the potential for the associated epitopes to occur on the same antigen; either exclusively polysaccharide (first two to be discussed) or proteoglycan (last three to be discussed). Two ‘RG-I/AG’ clade members, CCRC-M7 and CCRC-M12, exhibit a similar pattern throughout the HCL series and likely share a common, conserved antigen substrate. CCRC-M7 is believed to bind specifically to a trimer (or larger oligomer) of $\beta-(1,6)$-Gal typical of an RG-I side-chain, while CCRC-M12, has no defined epitope, although both of these antibodies demonstrate similar high affinity for sycamore and maple RG-I, relative to their affinity for gum tragacanth and larchwood arabinogalactan (Puhlmann et al., 1994). The JIM84 and LM10 antibodies also behave similarly across the HCL series. While LM10 (McCartney et al., 2005) binding is well characterized as showing specificity for un-substituted xylan, the JIM84 epitope structure remains uncharacterized. It is not likely that JIM84 is specific to xylan in light of the detection of JIM84 signals in both the Golgi and plasma membrane of carrot (Horsley et al., 1993), possibly related to secondary growth, and Arabidopsis protoplast (Zabotina et al., 2008b), not so clearly linked to secondary cell wall formation processes.

Another pair of similarly behaving antibodies detected in the HCL analysis, PN16-4B4 and JIM8, both belong to the ‘AG-3’ clade and may share a common glycoprotein antigen. While JIM8 specificity is unknown, the PN16.4B4 epitope has been narrowed to the carbohydrate component of a glycoprotein (Kjellbom et al., 1997). Although
JIM11 (‘AG-1’ clade) and JIM19 (‘AG-2’ clade), belong to separate AG clades, both appear to share extensin as a common target (Moller et al., 2008; Paul Knox et al., 1995; Smallwood et al., 1994). Likewise, JIM13 (‘AG-4’ clade) and JIM15 (‘AG-3’ clade) associate consistently throughout the HCL developmental series, suggesting that their antigens, AGP2 and AGP1, respectively, also co-occur. Interestingly, JIM14 (‘AG-2’ clade), like JIM13, was also against AGP2 but shows strikingly different patterning throughout the HCL series. A precise epitope for JIM14 is currently unknown but it may be a more widely distributed carbohydrate moiety, since JIM14 signals are associated with multi-tissue targets across the developmental gradient of cell wall expansion (Figures 4.3-4.5). While other co-associations of antibodies may also exist through the three developmental series, the most obvious are limited to this ten-member subset of all the antibodies tested (55). Overall, the evidence suggests a high degree of separation of epitopes on different antigenic substrates and low degree of antigen commonality among this set of antibodies in the context of the elongating Arabidopsis inflorescence stem.
4.3 Discussion

The currently available library of cell wall-directed monoclonal antibodies provides a powerful tool to survey the location and relative abundance of many important classes of cell wall-associated glycans including pectins, glycoproteins, and cross-linking xylans (hemicellulose). My analysis of the binding patterns of a large set of these antibodies in the Arabidopsis inflorescence stem sampled at experimentally defined developmental stages has yielded an array of unique changes in epitope presence and patterning. These dynamic changes seem very likely to be related directly to phases of directional cell wall expansion.

Despite the inability to directly infer absolute polysaccharide abundance using this approach, the trends between stages (YNG, MGR, and CSS), and the patterning amongst tissue types, together provide specific knowledge of how cell wall structure differs between stages of cell wall differentiation in the inflorescence stem.

One of the most pronounced clusters included several ‘AG-3’ glycan clade members (JIM13, JIM8, and PN16-4B4), which appear to recognize a unique class of epitopes specific to the interfascicular fibres MGR and CSS stages coincident with inner cell wall accumulation (Figures 4.4-4.5). While JIM8 and PN16-4B4 exhibit elevated fluorescence intensity at the MGR and CSS stages, strong onset of JIM13 at the YNG stage suggests that JIM13 targets a distinct arabinogalactan-bearing cell wall component. Indeed, JIM13 was raised against the protein of *ARABINOGALACTAN PROTEIN 2* (AGP2) and is known to bind its glycan component, ($\beta$)GlcA1-$\rightarrow$3($\alpha$)GalA1-$\rightarrow$2Rha though it is unknown to what extent this glycan is found on other AGPs. The JIM8 fluorescence pattern correlates with global gene expression data for AGP2 (Figure 3.3, Table B.1), which indicates that AGP2 expression is early and sustained through to
cessation of elongation. If JIM13 is indeed AGP2-specific, such coincidence of AGP transcript and epitope at the YNG stage implies that little lag time exists in the localization and epitope maturation of AGP2.

By contrast, JIM8 and PN16-4B4, were raised against different constituents of suspension cultured cells; whole-protoplasts and cell membranes, respectively. It is likely that JIM8 and PN16-4B4 are targeting one or more of the 40 known Arabidopsis AGPs (Knox & McCartney, 2005) that may have been present in the glycan preparations, since 'AG-3' clade members have previously been described as AGP-specific (Moller et al., 2008; Pattathil et al., 2010). Seven AGPs (AGP12,13,14,21,22 & 24) exhibit significant up-regulation in the MGR stage relative to the YNG stage (see Chapter 3 ‘Discussion’). While these AGPs are closely related among the full suite of AGPs and fasciclin-containing AGPs (FLAs)(Shinsaku et al., 2005), differences in structure and expression timing, particularly of AGP12 (GPI-anchor, peak expression at MGR) and AGP13 (no GPI-anchor, peak at CSS), imply functional differences exist among these glycoproteins. Such differences may therefore account for the distinct, IFF-specific binding of JIM8 and PN16-4B4 at MGR and CSS stages compared to the seven other ‘AG-3’ clade members examined in this study, which do not exhibit this specificity. In the absence of specific knowledge of JIM13 and PN16-4B4 epitopes, these data nonetheless implicate a unique class of epitopes, possibly of AGP12 and/or AGP13, specific to early stages of the inner cell wall thickening, prior to the formal cessation of elongation where secondary cell wall formation is generally thought to be active.

Another of the most striking clusters includes antibodies with varying degrees of concentration around the endodermis; JIM11 ('AG-1' clade), JIM19 ('AG-2' clade),
JIM20 ('AG-1' clade), MAC204 ('AG-1' clade), and JIM12 ('AG-2' clade) (Figure 4.7). It is intriguing that, whereas JIM11, JIM12, JIM19 and JIM20 all recognize putative extensin antigens (Paul Knox et al., 1995; Smallwood et al., 1994) and also bind very strongly to the endodermis, JIM11 is unique within this group in not finding targets in the IFF region at the CSS growth stage. Indeed, the specificity exhibited by JIM11, whose signal appears almost exclusively within the endodermis across three developmental stages (Figure 4.7A-C), is remarkable. JIM11 has also been reported to label regions of the carrot (*Daucus carota*) root pericycle, the analogous tissue layer to the aerial endodermis, adjacent to the phloem (Smallwood et al., 1994). Experimental evidence suggests that JIM11 is specific to the oligosaccharide portion of either extensin or lectins (Smallwood et al., 1994).

Lectin receptor-like kinases (LecRKs) bear lectins in their extracellular regions that putatively act as receptors to monitor cell wall integrity (Humphrey et al., 2007). Of six annotated LecRKs assessed in the microarray experiment (Chapter 3), only *ARABIDOPSIS THALIANA* LECTIN-RECEPTOR KINASE (ATHLECRK) showed significant change between stages, being elevated at the OLD stage relative to the MGR stage (Table C.1). Extensin is one of the better-studied hydroxylproline-rich glycoproteins with demonstrated impact on plant growth (Humphrey et al., 2007), yet functional roles for extensins in maturing cell walls of inflorescence stems are still largely unexplored. They may be involved in cell wall assembly, possibly through their propensity to cross-link with pectins and thereby to act as cell wall stabilizers (Lamport et al., 2011a). That EXTENSIN 3 (ATEXT3, At1g21310) is up-regulated significantly in the OLD stage relative to the MGR and CSS stages (Table 3.3, Figure 3.3) implies that ATEXT3 plays an increasingly dominant role in stabilizing cell walls after elongation has
possibly within cells that do not undergo appreciable fortification by lignification. Interestingly, ATEXT3 but not ATHLECRK exhibited elevated expression in YNG stage relative to the MGR and CSS stages, and this may best account for presence of the JIM11 epitope in the xylem and phloem of the YNG tissue.

Another conspicuous cluster exhibiting ubiquitous fluorescence at the MGR stage, and thus coincident with rapid, diffuse cell expansion, is a group of ‘pectic-backbone’ clade members, LM19 (recognizes un-esterified HG), LM18 (recognizes partially methyl-esterified HG), and LM20 (recognizes methyl-esterified HG). Co-occurrence of epitopes of various degrees of methyl-esterification at both MGR and CSS stages is somewhat surprising, since there is some evidence that the level of pectin methyl-esterification in cell walls is proportional to the degree of their extensibility (Pio M. J. Szyjanowicz et al., 2004). For example, reduced levels of pectin methyl-esterification have been correlated with reduced cell wall expansion in Arabidopsis hypocotyls (Derbyshire et al., 2007), and elevated pectin methylesterase activity was thought to impede symplastic (diffuse) and intrusive growth in poplar secondary growth (Siedlecka et al., 2008). However, LM19 does exhibit a tissue type-specific binding pattern that is distinct from that of both LM18 and LM20, most notably through the general absence of LM19 signal from the cortex at both the MGR (Figure 4.4, cluster #3) and CSS (Figure 4.5, cluster #5) growth stages. This implies that cell wall extensibility may be established outside of the cortex by means other than the level of methyl-esterification.

Interestingly, the LM20 signal is predominantly detected in the epidermis, cortex, and xylem at the YNG and MGR stages (Figure 4.3, cluster#3; Figure 4.4, cluster#2), but becomes more ubiquitous at the CSS stage. While LM20 is not among those whose signal decreases most generally from the MGR to CSS stage (Table C.3), LM19 is
associated with a marked increase of fluorescence intensity over this period in the endodermis, epidermis, and IFF region, implying that the ratio of methyl- to un-methyl-esterified forms of HG is decreasing in these tissues as the cell walls cease expanding. Given that 60% pectin methyl-esterification has been shown to be sufficient to support elongative growth in Arabidopsis hypocotyls (Derbyshire et al., 2007), it seems likely that subtle differences exist in the extent of esterification between stages, rather than a purely dichotomous condition. Such a hypothesis is supported by experimental evidence for a linear decline in methyl-esterified forms of cell wall pectin as corn coleoptiles progress through all phases of elongative growth, as opposed to a spike in uronic acid esters in regions of the stem undergoing rapid cell wall expansion (Kim & Carpita, 1992).

Consistent with the notion that pectin methylesterase activity should be low in labile, expanding cell walls, my examination of co-expression of cell wall-related genes revealed that the ‘MGR’ stage was enriched in genes associated with pectinesterase inhibitor activity, while the CSS stage was enriched in genes associated with pectinesterase activity (Table C.6). Interestingly, the OLD stage exhibited enrichment of both terms, implying that tight regulation of pectin methylesterase (PME) activity at this stage was likely. Furthermore, a PME and a PMEI are among the twenty most up-regulated genes in the CSS stage relative to the MGR stage; ATPMEPCRD and AT2G45220 (no gene name), respectively (Table 3.2). Co-expression analysis of the 253 pectin-related genes further supports this notion in demonstrating that PMEs and PMEIs are largely co-expressed in distinct clades which exhibit unique trajectories through development, altogether creating a complex transcriptional landscape of pectin synthesis and modification. In light of the immunohistochemical evidence presented in
this Chapter which reveals tissue-specific methyl-esterification patterns at the YNG, MGR and CSS stages (Figure 4.8), it seems likely that methylesterification is tightly regulated in spatial manner, possibly accounting for the large numbers of co-expressed PMEs and PMEIs.

Despite the wealth of novel information produced by this study, it must be acknowledged that our ability to draw firm conclusions about the absolute abundance of specific polysaccharides in a particular tissue sample still faces technical challenges, most notably the general lack of verified knowledge of the binding specificities of most of the antibodies. Epitopes may appear on different classes of carbohydrate-bearing cell wall polymers, as suggested by the general lack of specificity in vitro of 180 antibodies to 54 plant polysaccharides (Pattathil et al., 2010). Indeed, cross-reactivity has been observed for many monoclonal antibodies (Clausen et al., 2003; McCartney et al., 2005; Moller et al., 2008; Pattathil et al., 2010; Verhertbruggen et al., 2009), implying that structurally similar epitopes certainly do occur on multiple antigens. Studies involving global assessment of cross-reactivity among many antibodies have so far relied on plant extracts from multiple species (Moller et al., 2008; Pattathil et al., 2010), and associated data do not allow clear deduction of the potential for antibodies to bind to multiple antigens within a specific system such as Arabidopsis thaliana inflorescence stems. Certainly, epitopes for ten antibodies in this study appear to co-occur on antigens which persist at all developmental stages. However, that 45 of 55 antibodies are unique from all other antibodies at one or more stages of development provides evidence for the degree to which epitopes are uncoupled from common, persistent antigens within this experimental context.
In addition, the issue of the impact of changes in the accessibility of the antigenic site(s) to the antibody (i.e. epitope masking) remains difficult to resolve. While the primary aim of this study was not to attempt to address such issues, our findings nonetheless provide some clues as to degree to which these factors might be complicating the analysis.

The accessibility of the epitope to the primary antibody-containing solution is potentially affected by physical barriers to diffusion of the antibody solution. Such a barrier is evident where the antibody is localized to the inner cell wall or plasma membrane, and fluorescence only appears where the cells have been physically opened by the sectioning process as with CCRC-M44 (RG-I/AG), JIM16 (RG-I/AG), JIM20 (AG-1), JIM101 (RG-1c), JIM131 (RG-I/AG), and MAC207 (AG-2). The phenomenon of 'epitope masking' is a more likely factor affecting inference of the abundance of epitope. This phenomenon involves localized interference with antibody binding through such processes as H-bonding of the epitope with other cell wall constituents. Xyloglucan epitope accessibility has been shown to be affected by pectin (Marcus et al., 2008), yet CCRC-M1, known to bind fucosylated xyloglucan (Puhlmann et al., 1994), shows steady increase in total fluorescence intensity for all tissues through the YNG, MGR and CSS stages (404, 512, and 677 grayscale units, respectively). Further, xylan-specific LM10 fluorescence intensity increases through the YNG, MGR and CSS stages consistent with previous reports of LM10 binding specifically to secondary cell walls (Bowling & Vaughn, 2008; M. Kaneda, 2009; Persson et al., 2007). HCL data sets for the YNG, MGR and CSS stages collectively indicate that masking is not a global issue; the mean overall intensities for each stage are 16.9, 19.6, and 21.3 grayscale units (respectively), consistent with a gradual increase in overall epitope abundance.
Despite the caveats described above, this dataset provides strong evidence that anisotropic cell wall expansion may be enacted through very different means for cell types with diverse structural endpoints. Furthermore, the data provide *in planta* evidence of the behaviours of 55 antibodies within seven major cell types of Arabidopsis tissues, a platform that will support future systems biology investigations in this important model organism.
4.4 Experimental procedures

4.4.1 Plant material

Cold-treated Columbia (Col-0) seeds were sown in 32-plug tray inserts with soil-less potting mix (Sunshine Mix #5, Sun Gro Horticulture Canada Ltd., Seba Beach, Alberta) supplemented with liquid fertilizer 20N-20P-20K (Plant-prod soluble fertilizer, Plant Products Co. Ltd, Brampton, Ontario), then grown on short-day conditions (Day 8 hours, 21°C; Night 16 hours, 19°C) for 6 weeks. To induce bolting, plants were transferred to long-day conditions (Day 16 hours, 21°C; Night 8 hours, 21°C) until the inflorescence reached a height of 10-15cm. Plants were then removed from the growth chamber for image analysis and harvest.

4.4.2 Growth kinematic profiling

Developmentally-specific tissues for young (YNG), maximum growth-rate (MGR) and cessation (CSS) tissues were isolated essentially as described in Chapter 2 'Methods', utilizing as optical markers 1mm diameter disks cut from 45µM polyvinylidene fluoride (PVDF) membrane (Cat# IPVH00010, IPVH 00010, Millipore Co., Bedford, MA) using a CTR 6500 Laser microdissection system (Leica, Switzerland) that were applied to the stem at 5mm intervals via electrostatic charge when held in close proximity to the stem with forceps. Feature tracking (ImageJ) was carried out as described in Chapter 2 'Methods'. Specifically, an oval of fixed dimension (red) is 'best-fit' by eye for each disk through the time series. The pixelated nature of the shape in ImageJ allows for the tracking of left- and right-edges (blue marks) of the oval through the series (refer to Chapter 2, Figure A.1 'Electrostatically-attached disks; an alternative to glued paper tags as synthetic optical markers'). Growth kinematic profiles of relative growth rates for tag-defined segments were used to identify points of maximum growth-rate and
cessation for three biological replicate plants are provided in supplemental Figure C.1 of this chapter.

**4.4.3 Tissue harvesting for microscopic analysis**

Stem segments bounded by paper tags were harvested from individual plants in sequence from top to bottom. Upon excision, segments were immersed in 150µL of fixation buffer (stock 2X PME; 50mM 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid (PIPES), 2mM MgSO₄, 2mM EGTA), within 0.2mL dome-cap thermal cycler tubes. Segments were then subjected to three consecutive cycles of five minute vacuum infiltration at 20 inches Hg and washed 3 times in 1X PME prior to long-term storage at 4°C in 1X PME. Segments were individually encased in 1cm³ blocks of 5% agar at 65°C, and stored at 4°C to set. 40µm-thick transverse sections were cut from segments using a vibrating microtome (model VT100S, Leica), separated from agar encasement with a sable-hair (‘00’) brush, then blocked for at least one hour in 5% bovine serum albumin (BSA) in 1X TBST (10mM TRIS, 0.25M NaCl, 0.1% TWEEN). Sections were mixed to randomize developmental difference, randomly allocated from each biological replicate pool, along with 100µL of fresh blocking solution, to wells of 96-well plates (ex. Cat# 353912, BD Falcon) according to layouts (Figure C.3), grouping three biological replicates row-wise according to antibody (Figures C.2.1-55) or negative control (Figures C.2.56-58). Antibodies included in this experimental are listed in Table C.1 providing source animal, supplier, immunogen, epitope structure, and antigen; information derived from CCRC database, WallBioNet (glycomics.crc.uga.edu/wall2/index.html).
4.4.4 Immunolabelling and counterstaining

Antibodies were randomly selected from the SKCMA-AR1/AM1 kits (CCRC, Athens, GA) and examined along with those obtained from Dr. Lacey Samuels (originally generously provided by Dr. Paul Knox; JIM5, JIM7, and LM10). Blocking solutions were swapped with 15µL 1:36 dilutions of supplied antibody solutions (see Table C.1) using gel-loading tips, then sections were incubated at 4°C for 16 hours. Sections were washed two times in 100µL 1X TBST, then incubated one hour at 21°C in the dark in secondary antibody; either 15µL of 2µg/µL Alexa Fluor™ 488 donkey anti-rat IgG (H+L) (Cat#:A-21208, Invitrogen) or 2µg/µL Alex Flour™ 488 goat anti-mouse IgG (H+L) (Cat#:A-21131, Invitrogen) based upon the requirements of the primary antibodies (see Table C.1). Sections were again washed 2 times in 100µL 1X TBST, prior to counterstaining with 0.015% Congo Red (Cat#:60910, Fluka, Buchs, Switzerland). Sections were again washed 2 times in 100µL 1X TBST to remove excess counterstain and unbound secondary antibody.

4.4.5 Confocal imaging

Sections were mounted on a glass bottom confocal imaging dish (series GWSt-5030, WillCo Wells, Amsterdam) custom-fitted with a 40µm PVDF membrane containing 96, 2mm-diameter wells, and individually covered with a 1mm-thick, 2mm diameter, 2% agar film to prevent evaporative loss during loading and imaging, further managed by hydration with 1X TBST as needed. Sections were imaged row-wise through the 96-well array on a spinning disk confocal (Ultraview VoX, Perkin-Elmer) fitted with an electron multiplier CCD camera (9100-02, Hamamatsu, place) using an inverted microscope (DMI6000, Leica). Congo Red was excited with a 561nm laser, and emitted light was then filtered with an emission wheel allowing 525nm (width=50) and 640nm
(width=120) wavelengths. Alexa Flour™ 488 secondary antibodies were excited with 488nm laser, and emitted light filtered with an emission wheel allowing 527nm (width=55). Laser powers were adjusted section-wise to minimize background fluorescence and maximize contrast. Z-stacks were captured from the center 20µm of each 40µm section using Volocity software (Improvision, Perkin-Elmer).

### 4.4.6 Image processing and analysis

Maximum projection (2D) renderings of both 561nm (Congo Red) and 488nm (secondary antibody) stacks were exported together as an RGB JPEG image (blue channel blank). A 488nm 8-bit grayscale image was produced from the 488nm channel for display alongside the two-channel (red/green) images in panels for each of the 55 antibodies and negative controls (Figures C.2.1-C.2.58), where all three biological replicates at the three developmental stages are shown. Figures depicted in the main body of the text were converted from red/green to blue/green for color-blind view.

For region-of-interest (ROI) quantification, two-channel images were re-assembled into the 96-well layouts in 8000px X 5610px RGB images (Figure C.4.1-C.4.6) and imported into ImageJ for region of interest (ROI) selection using the 'ROI' tool, identifying seven tissue types (and background) discernable on the basis of morphology in the 561nm channel (see Figure 4.1 for a list of these tissue types). Gray value histograms, recording the frequency of pixels having values in 256 grayvalue bins, were computed on the 488nm channel for each of the 4608 ROIs depicted in Figures C.4.1-C.4.6. These histograms were exported via macro from ImageJ then imported into the statistical programming environment R (http://cran.r-project.org) for computation of the 75th quartile measures (i.e. grayvalue for 75th brightest pixel) chosen to provide the best estimate of cell wall signal above background fluorescence. Background signals
for each image (single well in 96-well layout) were subtracted from grayvalue measures for each of the seven cell types. To correct for persistent differences in the total fluorescence between cell types, stemming from differences in the proportion of cell wall to vacuolar region, grayvalues were normalized such that the sum of all grayvalues for each cell type (58 treatments x 3 stages = 174 obs.), across the entire experiment, were equal (all biological replicates included). Clustering of biological replicates (Figure 4.2) was performed on these background-corrected, normalized grayvalue scores ($I_{bn}$) in R using the 'diana' method of the 'cluster' package, where dissimilarity matrices were computed on sum of squares 'Euclidean' distance, converted to the class 'dendrogram', and plotted using the standard plotting function in R.

For assessment of epitope abundance change across development, background-corrected, normalized grayvalue scores ($I_{bn}$) for three biological replicates of a given stage:antibody combination were used to calculate means of $I_{bn}$ ($\bar{I}_{bn}$), the arithmetic difference between $\bar{I}_{bn}$ of two stages ($\Delta \bar{I}_{bn}$ values), t-test p-values (two-sample, two-tailed). These are collectively featured in Tables C.2 and C.3. For assessment of relative intensity among tissue types, $\bar{I}_{bn}$ values for each tissue type were computed as the proportion of the sum of all $\bar{I}_{bn}$ values (7 tissue types) for each antibody:stage combination. Relative intensity scores ($\bar{I}_{rel}$) were computed as the proportion of each $\bar{I}_{bn}$ value to the sum of all seven intensities for that antibody ($\sum I_{bn}$). $I_{rel}$ scores were adjusted using a custom logistic threshold function in order to reduce the contribution of background noise and residual fluorescence anomalies in cases where all seven values were below 20 units of the 256-level grayscale (see Figure C.10 for schematic and details). Hierarchical clustering of $\bar{I}_{rel}$ values (Figures 4.3-4.5) employed methods described above to compute dissimilarity matrices for antibodies across $\bar{I}_{rel}$ scores for
seven tissue types, and for each of the seven tissue types across $\tilde{T}_{rel}$ scores of each antibody surveyed in that tissue. Dendrograms were co-plotted with a heatmap of $\tilde{T}_{rel}$ scores and corresponding $\tilde{T}_{bn}$ scores using the 'heatmap.2' function of the 'gplots' package in R.
Chapter 5. Conclusions and future directions

5.1 The ‘molecular phenotype’
Cell wall metamorphosis is a key aspect of the anisotropic growth of every plant organ, and the associated processes, which include expansion, modification and fortification, present a bewildering level of complexity. The overarching aim of this doctoral research was to exploit several broad-spectrum, molecular profiling assays that would enable me to identify key features of specific stages of cell wall development, or changes marking the transition between these stages. The choice of assays allowed me to explore both changes in the levels of gene expression (relative mRNA abundance), and changes in cell wall structure (relative epitope abundance).

It is helpful to view cell wall ontogenesis as a continuum of states, each displaying a pattern of traits conditioned by genetic and environmental factors. The term “phenotype” may arguably be extended beyond its common usage in describing macroscopic traits of genetically distinct populations, to include the complex and dynamic features of the molecular landscape, such as gene expression (Chapter 3) and cell wall structure (Chapter 4), as they vary through development. Characterization of such context-specific patterns, which involve both known and uncharacterized regulators and components of cell walls, provides a powerful platform upon which to construct testable hypotheses for exploration of the origins and functions of specific wall traits.

The utility of these ‘molecular phenotypes’ is largely dependent on the accuracy with which they reflect their developmental context, since this linkage is a crucial element of our ability to generate informative interpretations, either of the phenotypic impact of gene mis-expression studies or, in the case of the work presented in this thesis, of cellular changes occurring during the passage through ontogenic phases.
Plant cell wall expansion has been long known to occur across a steep spatiotemporal developmental gradient. However, previous studies of the plant cell wall expansion process have failed to include a rigorous method for routinely isolating developmentally equivalent tissues from multiple plants for the sample pooling and comparison required by currently “biomaterial-intensive” molecular assays. I felt that this gap between the reporting of molecular evidence and the accurate definition of the developmental processes with which those molecular events are associated represented a significant challenge to our ability to reliably interpret the results of these assays. To address this problem, I set about to develop a standardized method for the isolation of specific developmental stages of tissue for use in molecular assays, focusing my efforts on the Arabidopsis inflorescence stem.

5.2 Establishing developmental context for the molecular phenotype

In Chapter 2, I demonstrated that the positions of growth stage transitions along the Arabidopsis inflorescence stem were widely variable among individual plants within the Columbia ecotype. This finding validated my concerns about the reliability of the common assumption inherent in many sampling methods reported in the literature, that plants of similar height will have similar developmental proportioning. Growth kinematic profiling made it possible to isolate distinct stages of cell length development, and thereby created the opportunity to explore the associated molecular changes. This approach was validated as a means by which developmental equivalence could be established on a plant-by-plant basis for experiments requiring unperturbed molecular snapshots of tissues. However, it was also clear that growth kinematic profiling conducted in the manner I had devised is a time-consuming process, due to the manual nature of plant tagging and feature tracking. The systematic tagging
of plants does provide a useful form of synthetic markers for growth kinematic profiling of individual plants, and the use of large tags, in particular, did facilitate precise characterization of stem movement through time, where natural surface features are likely to be too dispersed or imprecisely positioned. At this time, I cannot conceive of an alternative method that includes these strengths while simultaneously allowing me to identify specific stem regions for later harvest and analysis. Introduction of some form of automated feature tracking is perhaps the most obvious point of future improvement. Automation of feature tracking could potentially facilitate the observation of hundreds of plants, enabling large-scale phenotyping of growth kinematic profiles in natural or mutant populations. My preliminary development of an automated feature tracking system (not presented in this dissertation) indicates that large tags such as I employed here would provide a suitable means by which stem movements can be monitored in both two and three dimensions from a single camera perspective, using modern, sophisticated feature tracking approaches.

Since such automation would make it possible to accurately capture the growth proportioning of primary stems in other accessions, it would be of interest, for example, to directly test the developmental precision of “stage-specific” sampling methods employed in other studies such as the gene expression profiles of expanding stems in the Ler ecotype, reported in Ehlting et al., 2005.

Growth kinematic profiles themselves represent a potential phenotype in elongating organs, so broader application of this methodology in forward and reverse genetics studies would make it possible to assess the functional implications of genotypic differences, which would obviously be of particular interest in the case of mis-regulation of cell wall-related genes. While root growth performance has been examined through kinetic study of root length rate change by hormone or stress treatments (Binder et al.,
2004; Folta & Spalding, 2001), the possibility of alterations to growth kinematic profiles by such treatments has not been studied in either roots or shoots.

5.3 Emergent biology from the phenotypic landscape

Once I had developed the means to isolate developmentally equivalent and specific stages along the cell wall expansion continuum, this made it possible for me to survey the transcriptome at several, distinguishable stages along this continuum. Furthermore, examination of the binding of 55 randomly chosen monoclonal antibodies to seven discernible cell types (Chapter 4) at three developmental stages across the cell wall expansion continuum (YNG, MGR, and CSS) provided a wealth of information, and novel insights into the distribution of structural differences within diffusely elongating stem tissue. Together, these assays provide a synergistic view of the dominant processes accompanying cell wall expansion as Arabidopsis primary stems grow.

5.3.1 Pre-cessation curiosities

One very interesting observation arising from parallel study of both the transcriptome and epitope profiles was the up-regulation of the callose-specific endo(1→3)-ß-glucosidase MEE48 at the YNG stage relative to the MGR stage (Table 3.1). This represents a previously uncharacterized response of this gene outside the context of anther development. Expression of another (1→3)-ß-glucosidase, BGL2, is induced in Arabidopsis stems by gravitropic manipulation (Koizumi et al., 2009), yet no BGL2 homologues were significantly differentially regulated in my study. It would be interesting to investigate the cell type-specific localization of callose, that likely provides the substrate for this ß-glucosidase, and to monitor the expression of other callose-related genes using qRT-PCR in the context of stem elongation. Unfortunately, the epitope survey (Chapter 4) did not include examination of the distribution of callose
epitopes. Such a study could employ the callose-specific dye Aniline Blue, or the polyclonal antibody specific to callose (Hasegawa et al., 1996).

At the stage coincident with maximum growth-rate (MGR), the high level of expression of PER64 was unexpected, since the role of cell wall-related peroxidase is in rigidifying, not loosening cell walls. As with BGL2, PER64 was previously found to be up-regulated in the apical stem region in response to mechanical stress in the elongating Arabidopsis stem (Koizumi et al., 2009). In the absence of evidence that PER64 is involved specifically in lignification, I can at least speculate that the cell wall-specific PER64 participates in modifications to cell wall mechanical properties.

It is interesting that the endodermis of stems (a.k.a. starch sheath) is thought to participate in gravitropism responses (Fukaki et al., 1998), and my epitope-profiling data (Chapter 4) revealed a remarkable specificity of extensin-specific JIM11 for endodermis-associated epitopes. Cross-linking of extensins via peroxidases is a key mechanism in controlling cell wall rigidity (Lamport et al., 2011), and the potential capacity of PER64 to affect gravitropic responses through modification to the extensin network has yet to be tested. Perhaps effort should first be focused on identifying the epitope targets of JIM11 and other ‘AG-1’ and ‘AG-2’ clade members featured in Figure 3.7 (JIM19, JIM20, MAC204, & JIM12). This information, along with biochemical characterization of the relevant extensin structures, would help clarify the role of extensin in modifying the structural properties of the cell wall during anisotropic expansion.

Another dominant feature of the MGR stage transcriptome was the relatively high expression of many arabinogalactan proteins (AGP12,13,14,21,22,24 & FLA13) a pattern consistent with an active role for AGPs in mediating cell wall expansion. This notion is corroborated by pronounced, cell type-specific AGP-related epitope patterning
in the MGR stage relative to YNG and CSS stages, particularly the abundance of JIM8, JIM13 and PN16-4B4 (‘AG-3’ clade) epitopes within the rapidly elongating interfascicular fibres (Figures 3.4 & 3.6). The diversity of patterning for these epitopes among different tissues suggests that functional diversity in arabinogalactan side-chains is of high spatiotemporal significance in this developmental context. The structural implications of this antibody-related fluorescence cannot be fully evaluated since the exact epitope specificities are still unknown for many of the available monoclonal antibodies. That observation that cell wall thickenings can be seen within fibres during this phase of rapid expansion (Figure 3.6), challenges the notion that divergence in primary cell wall structures occurs after cessation, and highlights the potential need for different cell types to meet the requirement of diffuse stem elongation by different means.

Future analysis of the functional diversification of AGPs must also include characterization of the expression of AGPs with a higher spatial resolution. Cell type-specific expression profiling of AGPs already observed to be up-regulated in the MGR stage in this study (Chapter 3) could be carried out by using laser-microdissected samples and qRT-PCR of a limited set of AGPs, or transcriptome profiling, by using microarrays or ‘Next Gen’ sequencing. Either of these approaches would likely reveal differences in transcript abundance of AGPs between tissues that I found to exhibit differences in epitope labelling. Combining mis-regulation (knock-out/over-expression) of specific AGPs and growth kinematic profiling of Arabidopsis primary stems would potentially provide novel insights into the role of AGPs in cell expansion processes. Much work still needs to be done to characterize the function and the cell type-specific localization of the enzymes that modify AGP side-chains, as these appear to be the ultimate determinants of AGP structure and function. This quest is especially
challenging given the broad distribution of the AGP motif across the genome, likely beyond the 30 genes so far placed in each of the AGP and FLA categories (Seifert & Roberts, 2007).

5.3.1 Post-cessation curiosities
Transcriptome profiling of cessation (CSS) and most-matured (OLD) stages of the primary stem revealed patterns that largely corroborated published transcriptome descriptions of secondary cell wall formation. Interestingly, CESA3 expression appears oddly placed in the context of cell wall expansion, where it was found to exhibit its greatest expression after the point of cessation. This finding is consistent with other studies that show continued expression of CESA3 in tissues associated with secondary cell wall formation (Ehlting et al., 2005; Ko & Han, 2004). Since CESAs appear to belong to exclusive primary and secondary cell wall-specific CESA complexes (Brown et al., 2005; Gardiner et al., 2003; Hamann et al., 2004; Holland et al., 2000), further cell type- and stage-specific assessment of transcript abundance for CESAs and CESA-likes (CSLs) would likely provide important spatiotemporal evidence of the distribution of these processes within the context of the developing inflorescence stem. It still remains a mystery why such functional divergence exists, and it is unclear if these functional associations translate into structural differences between the cellulose of primary and secondary walls. An expanded epitope profiling study could include examination of the abundance of (1→4)-ß-D linkages of cellulose and cross-linking glycans using carbohydrate-binding modules (CBMs) specific to those linkages (Knox & McCartney, 2005). Unfortunately, it was not feasible to include the OLD stage (base of stem) in the epitope profiling examination, so the full extent of post-cessation modification to epitope abundance was not explored. Future study of this late stage of cell wall development may reveal important insights into the function of CESAs and other
cellulose/hemicellulose-modifying enzymes that appear active through to this stage (Chapter 3).

The transcriptome profiling study presented in this thesis provides corroborating evidence for the participation of previously identified primary and secondary cell wall-associated genes, with unparalleled precision in linking that participation to specific stages of development. Beyond this corroboration of previous predictions of gene function, many other ‘unknown’ genes also appear to be involved in these growth transitions, and these make likely candidates for regulators/participants of cell wall expansion processes. The ‘Working On Walls’ (WoW) initiative (http://wow.msl.ubc.ca/) is currently examining knock-out and/or over-expression mutants of many of these genes (+60 lines) for possible growth/developmental phenotypes.

5.5 Wider application of growth kinematic profile-guided sampling

Growth kinematic profiling provides the means to isolate developmentally equivalent tissues en masse for any conceivable study of molecular assays. Application of growth kinematic profiling is only limited by the availability of technologies that may capture and catalogue the sophisticated mechanisms of anisotropic cell wall expansion. Next-Gen sequencing would also be useful in corroborating this mRNA-specific evidence, and would also provide quantitative data for miRNA and siRNA abundances, thereby revealing patterns reflective of processes of storage/decay in p-bodies and cytosolic destruction, respectively. It is also possible to conduct cell type-specific global gene expression profiling by utilizing the latest advances in laser-microdissection and RNA amplification technologies. My effort to adapt these technologies for transcriptomic study of individual cell types in expanding stems is documented in Appendix ‘C’. While
the results were disappointing, it is clear that cell type specific sampling and RNA amplification are both possible. However, only a few examples of cell type-specific gene expression profiling have been published, and none of those studies have clearly demonstrated that the results successfully portray true mRNA abundances in the laser-microdissected tissue.

Clearly then, the discovery-based transcriptional and epitope profiling presented in this thesis provide only an initial foray into the biological application of growth kinematic profile-guided sampling. Nevertheless, the cell expansion-anchored ‘molecular phenotypes’ presented in this study provide a valuable scaffold upon which to assemble new knowledge of inflorescence stem development, cell differentiation, anisotropic cell expansion, and cell wall formation/remodelling processes; all the means that make multicellular terrestrial plants so very un-blob-like.
Bibliography


Warnes GR. 2010. Gplots: Various R programming tools for plotting data (*cran.r-project.org/package=gplots*)


Appendices

The data is available through the Faculty of Graduate Studies on-line publishing resource, cIRcle. Supplemental resources are listed in the order in which they appear within the document.

**Appendix A – Chapter 2 supplementals**

<table>
<thead>
<tr>
<th>Supplemental</th>
<th>Filename (FTP)</th>
<th>Size (MB)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure A.1</strong> Electrostatically-attached PVDF disks as an alternative optical marker system.</td>
<td>FigureA.1.pdf</td>
<td>5.6</td>
</tr>
<tr>
<td><strong>Figure A.2</strong> Effect of smoothing length measurements on growth kinematic profiling.</td>
<td>FigureA.2.pdf</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>Figure A.3</strong> Surface plots of REGRs and LOWESS-predicted growth kinematic profiles for 24 plants.</td>
<td>FigureA.3.pdf</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Figure A.4</strong> Tagged plants shown placed within plant-specific growth restraints and chamber.</td>
<td>FigureA.4.pdf</td>
<td>5</td>
</tr>
<tr>
<td><strong>Figure A.5</strong> Illustrations of growth imaging chamber.</td>
<td>FigureA.5.pdf</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Script A.1</strong> Growth kinematic profiling analysis from ImageJ feature tracking data</td>
<td>ScriptA.1.txt</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Video A.1</strong> Tagging of 1 complete plant with ~15 paper rectangles for growth analysis within the imaging chamber</td>
<td>VideoA.1.mov</td>
<td>28.4</td>
</tr>
<tr>
<td><strong>Video A.2</strong> Video depicting the process of feature tracking plants in ImageJ.</td>
<td>VideoA.2.mov</td>
<td>26.2</td>
</tr>
<tr>
<td><strong>Video A.3</strong> Video depicting the harvesting of segments bounded by tags.</td>
<td>VideoA.3.mov</td>
<td>34.1</td>
</tr>
<tr>
<td><strong>Video A.4</strong> Video rendering of an image series of cell outlines used to calculate cell length.</td>
<td>VideoA.4.mov</td>
<td>11.2</td>
</tr>
</tbody>
</table>
## Appendix B – Chapter 3 supplementals

<table>
<thead>
<tr>
<th>Supplemental</th>
<th>Filename (FTP)</th>
<th>Size (MB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure B.1 Surface plots of relative elongation growth rates and LOWESS-predicted growth kinematic profiles</td>
<td>FigureB.1.pdf</td>
<td>2</td>
</tr>
<tr>
<td>Figure B.2 Gene ontology (GO) SLIM term enrichment analysis for clusters depicted in Figure 3.3.</td>
<td>FigureB.2.pdf</td>
<td>0.25</td>
</tr>
<tr>
<td>Figure B.3 Boxplots depicting distribution of estimates of relative gene expression (fold-change) of each developmental stage</td>
<td>FigureB.3.pdf</td>
<td>0.17</td>
</tr>
<tr>
<td>Figure B.4 Hierarchical clustering of 742 cell wall-related genes</td>
<td>FigureB.4.pdf</td>
<td>1.5</td>
</tr>
<tr>
<td>Figure B.5 Hierarchical clustering of pectin-related genes</td>
<td>FigureB.5.pdf</td>
<td>0.9</td>
</tr>
<tr>
<td>Figure B.6 Hierarchical clustering of cell wall-associated transcription factors</td>
<td>FigureB.6.pdf</td>
<td>0.28</td>
</tr>
<tr>
<td>Table B.1 Raw and processed data for stage-specific (whole-segment) microarray experiment</td>
<td>TableB.1_(master-dataset).xlsx</td>
<td>34.6</td>
</tr>
<tr>
<td>Table B.2 AtCoeCis reports for enrichment of gene ontology (GO) terms for top 40 genes most different between YNG and MGR stages.</td>
<td>TableB.2.xlsx</td>
<td>0.05</td>
</tr>
<tr>
<td>Table B.3 AtCoeCis reports for enrichment of gene ontology (GO) terms for top 40 genes most different between MGR and CSS stages.</td>
<td>TableB.3.xlsx</td>
<td>0.05</td>
</tr>
<tr>
<td>Table B.4 AtCoeCis reports for enrichment of gene ontology (GO) terms for top 40 genes most different between CSS and OLD stages.</td>
<td>TableB.4.xlsx</td>
<td>0.05</td>
</tr>
<tr>
<td>Table B.5 AtCoeCis reports for enrichment of gene ontology (GO) terms for [sub] clusters of genes with stage-specific expression.</td>
<td>TableB.5.xlsx</td>
<td>0.05</td>
</tr>
<tr>
<td>Table B.6 AtCoeCis reports for enrichment of gene ontology (GO) terms for stage-specific clusters of co-expressed cell wall-related genes</td>
<td>TableB.6.xlsx</td>
<td>0.05</td>
</tr>
<tr>
<td>Table B.7 AtCoeCis reports for enrichment of gene ontology (GO) terms</td>
<td>TableB.7.xlsx</td>
<td>0.05</td>
</tr>
<tr>
<td>Supplemental</td>
<td>Filename (FTP)</td>
<td>Size (MB)</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Figure C.1A-C Growth profiles of plants used in this study (A-C) and 96-well confocal imaging (D)</td>
<td>FigureC.1.pdf</td>
<td>14.5</td>
</tr>
<tr>
<td>Figures C.2.1- C.2.58. Antibody-specific confocal panels for biological replicates (folder with 58 images)</td>
<td>FigureC.2_antibody-panels</td>
<td>~25 x 58 (1280)</td>
</tr>
<tr>
<td>Figure C.3. Layout for 96-well imaging runs (Sets1-6) for all developmental stages.</td>
<td>FigureC.3.pdf</td>
<td>0.5</td>
</tr>
<tr>
<td>Figure C.4.1-3.4.6 96-well montages depicting region of interest (ROI) selection (folder with six images)</td>
<td>FigureC.4_ROI-panels</td>
<td>~130 x 6 (807)</td>
</tr>
<tr>
<td>Figure C.5 Immunofluorescent labelling of YNG and MGR tissue for antibodies associated with most dramatic intensity changes in six or more tissues from YNG to MGR (i.e. ubiquitous).</td>
<td>FigureC.5.pdf</td>
<td>34.8</td>
</tr>
<tr>
<td>Figure C.6. Immunofluorescent labelling of YNG and MGR stages for antibodies associated with marked intensity increases in 5 tissues from YNG to MGR.</td>
<td>FigureC.6.pdf</td>
<td>13.9</td>
</tr>
<tr>
<td>Figure C.7. Immunofluorescent labelling of YNG and MGR stages for antibodies associated with concurrent and opposing intensity among tissues from YNG to MGR.</td>
<td>FigureC.7.pdf</td>
<td>20.7</td>
</tr>
<tr>
<td>Figure C.8 Immunofluorescent labelling of MGR and CSS tissue for antibodies associated with ubiquitous decline from MGR to CSS.</td>
<td>FigureC.8.pdf</td>
<td>24.8</td>
</tr>
<tr>
<td>Figure C.9 Immunofluorescent labelling of YNG, MGR and CSS tissue for antibodies associated with intensities localized to the epidermis and parenchyma at CSS.</td>
<td>FigureC.9.pdf</td>
<td>20.7</td>
</tr>
<tr>
<td>Figure C.10 Schematic representation of calculation of $I_{rel}$ scores for hierarchical clustering.</td>
<td>FigureC.10.pdf</td>
<td></td>
</tr>
<tr>
<td>Table C.1 Summary of information for antibodies used in this study</td>
<td>TableC.1(Antibody_summary).xlsx</td>
<td>0.45</td>
</tr>
<tr>
<td>Table C.2. Antibodies with greatest change in abundance from young to maximum growth-rate stages</td>
<td>TableC.2.xlsx</td>
<td>0.1</td>
</tr>
<tr>
<td>Table C.3 Antibodies with greatest change in abundance from Young to Maximum growth-rate stages</td>
<td>TableC.3.xlsx</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Appendix D – Stage- and cell type-specific transcriptomics

This section was prepared as a chapter for the main body, but omitted since the material provided in this section was not considered essential to the main body of the thesis. However, I wish to document the techniques and challenges of this microgenomics approach. Experimentation and analyses are accredited as for Chapter 4, with the exception of assistance with laser microdissection from volunteers Zhixia (Stella) Cao, Qing ling (Crystal) Cheung, and Florence Leung. References for this appendix are listed separately from the rest of main body of the thesis. Terms exclusive to this appendix are provided in a ‘list of terms’ within this appendix. Otherwise, abbreviations are defined in the ‘list of terms’ for the main body of the thesis. The following table presents the items found within the ‘Appendix_D’ folder. These items are not directly referenced in the body of thesis outside of this table.

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AppendixD.pdf</td>
<td>Chapter-style presentation of cell type-specific expression profiling</td>
</tr>
<tr>
<td>FigureD.1.pdf</td>
<td>Growth kinematic profiles for all plants utilized in this study</td>
</tr>
<tr>
<td>FigureD.2.pdf</td>
<td>Comparison of whole-segment and LMD microarray transcriptional change between stages</td>
</tr>
<tr>
<td>FigureD.3.pdf</td>
<td>senseRNA quality assessment for each sample utilized in the experiment</td>
</tr>
<tr>
<td>FigureD.4.pdf</td>
<td>Microarray slide quality assessment</td>
</tr>
<tr>
<td>TableD.1(master-data).xlsx</td>
<td>Master microarray data set including all raw data from scanning and statistical analysis.</td>
</tr>
<tr>
<td>TableD.2.xlsx</td>
<td>Top 100 genes most significantly up-regulated in the IFF cells relative to the cortical cells at the CSS stage.</td>
</tr>
<tr>
<td>TableD.3.xlsx</td>
<td>Top 100 genes most significantly down-regulated in the IFF cells relative to the cortical cells at the CSS stage.</td>
</tr>
<tr>
<td>TableD.4.xlsx</td>
<td>AtCoeCis reports for enrichment of GO terms of the [sub] clusters of genes with cell type- AND stage-specific expression</td>
</tr>
<tr>
<td>VideoD.1.mov</td>
<td>Demonstration video of cryosectioning and laser microdissection</td>
</tr>
</tbody>
</table>