FUNCTIONAL STUDY OF POLLEN-EXPRESSED EXPANSINS IN

ARABIDOPSIS THALIANA

A Dissertation in

Plant Physiology

by

Lei Zhao

© 2008 Lei Zhao

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2008
The dissertation of Lei Zhao was reviewed and approved* by the following:

Daniel J. Cosgrove  
Eberly Professor of Biology  
Dissertation Advisor  
Chair of Committee

Teh-hui Kao  
Professor of Biochemistry and Molecular Biology  
Chair, Plant Biology Graduate Program

Hong Ma  
Distinguished Professor of Biology

Mark Guiltinan  
Professor of Plant Molecular Biology

*Signatures are on file in the Graduate School
ABSTRACT

Expansins are wall proteins that are capable of promoting plant cell wall extension. They are encoded by a gene superfamily, which consists of four distinct families, EXPA, EXPB, EXLA, and EXLB. EXPA and EXPB were discovered as they reconstitute wall extension of the heat-inactivated cucumber hypocotyls whereas EXLA and EXLB were identified based on the sequence similarity to the first two families. The mechanism of expansin function is proposed as weakening the hydrogen bonds between cellulose microfibrils to facilitate wall loosening. Large numbers of expansins have been identified in plants from mosses to angiosperms, and in non-plants as well. However, \textit{in vivo} functional study of expansins is very limited.

In this dissertation, a group of pollen-expressed \textit{Arabidopsis} expansins were selected for functional study in both vegetative and reproductive growth. \textit{EXPA13} expression was observed in microsporogenesis and in the differentiating vessel elements. Reverse genetic studies were performed in three \textit{expa13} mutants. \textit{Atexpa13-1}, a \textit{qrt1} background mutant with increased gene expression, produces half-collapsed tetrads in the heterozygous mutant, in which wild-type pollen tends to break. It is proposed that imbalanced microspore expansion in heterozygous tetrad leads to the phenotype. No significant change in pollen size was observed in two other knockout mutants, \textit{atexpa13-2} and \textit{atexpa13-3}. The attempts to obtain \textit{expa13 qrt1} double mutant by crossing \textit{atexpa13-2} and \textit{qrt1-1} plants have failed.

Deficient vegetative growth has been observed in \textit{EXPA13} knockout mutant with differential severities on different ecotypes. Transcript analysis and mutant observation
suggest that EXPA13 controls cell elongation as well as wall thickening during xylem
element development. Combining the phenotypic changes in microsporogenesis and
stem development, I conclude that EXPA13 facilitates microspore expansion and xylem
element elongation.

EXPA4, EXPA24, and EXPB5 are expressed in Arabidopsis mature pollen grains
and elongating pollen tubes. This indicates that both α- and β-expansins are functioning
in the reproductive development of Arabidopsis. Their expression profiles were
constructed, and knockout mutants have been identified for EXPA4 and EXPB5, named
atexpa4-1 and atexpb5-1 respectively. No phenotypic changes were noticed in either
line. No EXPA24 mutant has been isolated from public stocks. The biological function
of Arabidopsis pollen-expressed expansins is hypothesized as facilitating wall assembly
of elongating pollen tube and wall separation of the female tissues as the pollen tubes
pass through.
TABLE OF CONTENTS

LIST OF FIGURES ......................................................................................................................... ix

LIST OF TABLES .............................................................................................................................. xi

ACKNOWLEDGEMENTS .................................................................................................................. xii

Chapter 1  Introduction and Literature Review .............................................................................. 1
  1.1 Motivation .............................................................................................................................. 1
  1.2 Plant Cell Wall Components and Architectural Models .................................................. 3
    1.2.1 Plant Cell Wall Polysaccharides ......................................................................................... 3
    1.2.2 Cell Wall Structural Proteins ............................................................................................. 4
    1.2.3 Cell Wall Enzymes ............................................................................................................. 5
    1.2.4 Replace with Subject Heading ............................................................................................ 6
  1.3 Cell Growth and Wall Extensibility ....................................................................................... 7
    1.3.1 Cell Expansion ................................................................................................................... 7
    1.3.2 Wall Extensibility ................................................................................................................. 8
  1.4 Wall Loosening and the Candidate Agents ............................................................................ 9
    1.4.1 Endoglucanase ................................................................................................................... 9
    1.4.2 Xyloglucan Endotransglucosylase/Hydrolase (XTH) ..................................................... 10
    1.4.3 Hydroxyl Radical .............................................................................................................. 10
  1.5 Expansins ............................................................................................................................. 11
    1.5.1 The Super Gene Family of Expansins ................................................................................. 11
    1.5.2 High Specificity of Expansin Expression .......................................................................... 12
    1.5.3 Techniques for the Study of Expansin Structure and Function ....................................... 15
      1.5.3.1 In Vitro Techniques ...................................................................................................... 15
      1.5.3.2 In Vivo Techniques ...................................................................................................... 19
    1.5.4 Model Plants for Expansin Study ...................................................................................... 20
  1.6 Organization of This Dissertation ......................................................................................... 22

References ......................................................................................................................................... 23

Chapter 2  EXPA13 and the Early Pollen Development in Arabidopsis ................................................. 31

  2.1 Introduction ............................................................................................................................. 31
    2.1.1 Male Gametophyte Development in Arabidopsis ............................................................... 31
    2.1.2 Correlation between Pollen Development and the Stages of Flower Development ................... 32
    2.1.3 Wall Alteration during Gametogenesis ............................................................................. 34
  2.2 Materials and Methods .......................................................................................................... 36
    2.2.1 Plant Material and Growth Conditions ............................................................................. 36
    2.2.2 Collection of Mature Pollen Grain and Developing Anther .............................................. 36
    2.2.3 Histochemical GUS Staining and Observation ................................................................. 37
2.2.4 Crossing of *Arabidopsis* Plants ............................................................38
2.2.5 PCR-Based Mutant Screening ..............................................................38
2.2.6 RNA Extraction and RT-PCR ..............................................................41
2.2.7 Microscopy, Photography and Image Measurement ............................42

2.3 Results.............................................................................................................43

2.3.1 GUS Expression in Developing Pollen ................................................43
2.3.2 Transcript Analysis by RT-PCR ...........................................................46
2.3.3 Phenotypic Observation in *Expa13-1* ................................................47
  2.3.3.1 Genotyping and Transcript Analysis of *expa13-1* ......................47
  2.3.3.2 Half-Collapsed Tetrads in *Expa13-1* Heterozygous Mutant ......49
    2.3.3.2.1 Phenotypic Observation ...................................................49
    2.3.3.2.2 Linkage between the Phenotype and Genotype ...............51
    2.3.3.2.3 Observation of Premature Pollen .....................................52
    2.3.3.2.4 Genotyping of Collapsed Pollen ......................................52
  2.3.4 Observation of *Expa13-2* and *Expa13-3* ........................................53
    2.3.4.1 Genotyping and Transcript Analysis .........................................53
    2.3.4.2 Pollen Size in *Expa13-2* and *Expa13-3* ..............................54
    2.3.4.3 Pollen Fertility in *Expa13-2* .......................................................55
    2.3.4.4 Analysis of *Expa13 Qrt1* Double Mutant ................................56

2.4 Discussion .......................................................................................................58

2.4.1 *EXPA13* Expression Profiles in Microsporogenesis .............................58
2.4.2 Pollen Collapse in *Expa13-1* ..............................................................58
2.4.3 Pectin and Expansin Function ..............................................................60

2.5 Summary.........................................................................................................63

References.............................................................................................................64

Chapter 3  *EXPA13* and the Vegetative Growth of *Arabidopsis* .................68

3.1 Introduction .....................................................................................................68

3.1.1 Leaf Development in *Arabidopsis* .......................................................68
3.1.2 Inflorescence Stem Elongation in *Arabidopsis* .................................69
3.1.3 Vascular Development in *Arabidopsis* .................................................70
3.1.4 Xylem Development in *Arabidopsis* ..................................................71

3.2 Materials and Methods ...................................................................................73

3.2.1 Plant Materials and Growth Conditions ...............................................73
3.2.2 RNA Extraction and Semi-quantitative PCR .......................................73
3.2.3 Histochemical GUS Staining and Observation .....................................75
3.2.4 Mutant Screening .................................................................................75
3.2.5 Growth Measurement ............................................................................77
3.2.6 Complementation Test for *Expa13-2* .................................................78
3.2.7 Light Microscopy ..................................................................................79
3.2.8 Wall Extension Assay of *Expa13-2* WT and Mutant ...........................79

3.3 Results.............................................................................................................80

3.3.1 *EXPA13* Expression in Rosette Leaves .............................................80
Chapter 5  Summary and Future Directions ................................................................. 138

5.1 Expansins in Developing Microspores ............................................................... 138
5.2 Expansins in Mature Pollen Grains ................................................................. 140
5.3 Prospective Directions and General Discussions ............................................ 141
   5.3.1 Application of qrt1 in Expansin Study .................................................... 141
   5.3.2 Synergistic Function of Expansin and Pectin-Degrading Enzymes ....... 141
   5.3.3 Expansins in Vascular Development ..................................................... 143
   5.3.4 Mature Pollen-Expressed Expansins ...................................................... 144
References ............................................................................................................... 145
LIST OF FIGURES

Figure 2.1: Locations of the T-DNA/transposon insertion sites in \textit{EXPA13} ............41
Figure 2.2: Histochemical staining of \textit{EXPA13} promoter::GUS lines...................45
Figure 2.3: Transcript analysis of \textit{EXPA13} in microspores and mature pollen grains.................................................................46
Figure 2.4: RT-PCR analyses for \textit{EXPA13} expression in the whole flower of \textit{expa13-1} .........................................................................................48
Figure 2.5: Light microscopic images of pollen grains in \textit{expa13-1} mutant in the \textit{qrt1-2} background ...............................................................50
Figure 2.6: Comparison of pollen grain diameter in \textit{expa13-1} .........................51
Figure 2.7: RT-PCR analyses for \textit{EXPA13} expression in the whole flower of \textit{expa13-2} and \textit{expa13-3} ........................................................54
Figure 2.8: Comparison of pollen size in \textit{expa13-2} and \textit{expa13-3} ..............55
Figure 2.9: Light microscopic images of pollen grains in \textit{EXPA13/expa13 qrt1/qrt1} ...........................................................................................................57

Figure 3.1: Transcript level of \textit{EXPA13} in the rosette leaves of 21-day-old \textit{Arabidopsis} ..............................................................................................81
Figure 3.2: Transcript levels of \textit{EXPA13} gene in the elongating inflorescence stem and siliques of \textit{Arabidopsis} ..................................................................................82
Figure 3.3: The elongation rate of inflorescence stem in \textit{Arabidopsis Ler-1} .............84
Figure 3.4: Phenotype of \textit{expa13-2} in rosette leaves ............................................85
Figure 3.5: Phenotype of inflorescence stem length in \textit{expa13-2} .........................86
Figure 3.6: Seed production reduced in \textit{expa13-2} mutant .....................................88
Figure 3.7: Phenotype of \textit{expa13-3} ........................................................................90
Figure 3.8: Light microscopic images of \textit{EXPA13} promoter::GUS transgenic plants.................................................................92
Figure 3.9: Cross section of *Arabidopsis* leaf, showing vascular bundle of wild type sibling and homozygous mutant of *expa13-2* ........................................ 94

Figure 3.10: Longitudinal view and curvature quantification of the tertiary vein in *expa13-2* ........................................................................................................ 96

Figure 3.11: Acid-induced extension of *Arabidopsis* stem tip in *expa13-2* wild type and mutant ..................................................................................................... 98

Figure 4.1: Histochemical staining of *EXPA4* promoter::GUS lines ...................... 119

Figure 4.2: Histochemical staining of *EXPA24* promoter::GUS lines ..................... 121

Figure 4.3: Histochemical staining of *EXPB5* promoter::GUS lines ....................... 123

Figure 4.4: Transcript analyses of *EXPA4*, *EXPA24* and *EXPB5* in mature pollen grains of *Arabidopsis* ................................................................. 125

Figure 4.5: Location of the T-DNA insertion in *EXPA4* and gene transcripts levels in *expa4-1* ............................................................................................................. 127

Figure 4.6: Location of the T-DNA insertion in *EXPB5* and gene transcript analysis ............................................................................................................. 129
LIST OF TABLES

Table 2.1: The landmark events of flower development and pollen development in *Arabidopsis* ................................................................. 33
Table 2.2: Three primers for PCR-based screening of *expa13-1* ......................... 39
Table 2.3: Three primers for PCR-based screening of *expa13-2* .......................... 39
Table 2.4: Three primers for PCR-based screening of *expa13-3* ......................... 40
Table 2.5: Primers for RT-PCR of *EXPA13* transcript .................................. 42

Table 3.1: Primers for semi-quantitative RT-PCR of *EXPA13* mRNA ............... 74
Table 3.2: Three primers for PCR-based screening of *expa13-2* ......................... 76
Table 3.3: Three primers for PCR-based screening of *expa13-3* ......................... 76
Table 3.4: Primers used for the complementation test of *expa13-2* .................. 78

Table 4.1: Primers for semi-quantitative RT-PCR of *EXPA4*, *EXPA24*, and *EXPB5* mRNA ................................................................. 114
Table 4.2: Summary of the promoter::GUS staining patterns and RT-PCR results of the mature pollen expressed expansins in *Arabidopsis* ......................... 125
Table 4.3: Three primers for PCR-based screening of *expa4-1* ......................... 128
Table 4.4: Three primers for PCR-based screening of *expb5-1* ........................ 129
ACKNOWLEDGEMENTS

I wish to express sincere gratitude to my advisor, Professor Daniel J. Cosgrove, for invaluable guidance, advice, support, and encouragement during the course of this project. Sincere thanks also go to Professor Teh-hui Kao, Professor Hong Ma, and Professor Mark Guiltinan for serving as committee members and providing their expert guidance.

I thank my colleagues, Dr. Nikolas Nikolaidis, Dr. Javier Sampedro, Dr. Akira Tabuchi, Dr. Takumi Takada, Dr. Lian-Chao Li, Dr. Elene Valdivia, Dr. Robert Carey, Dr. Nuwan Umantha Sella Kapu, and Young Bum Park, for their great help and providing a stimulating, enjoyable and friendly working environment. I would also like to thank Dan Durochko for providing everything I need, Ed Wagner for always being ready to help.

I would like to thank my classmates, Jori Sharda, Sarah Nilson, Amelia Henry, Jennelle Heyer, Solmaz Barazesh, and Tim Gookin for their friendship and encouragement. A special thanks to my friends in China and US, including but not limited to Shanwu Wang, Aiyu Zhang, Yanxing Wang, Fuhua Ma, Chunxia Chen, Danning You, Nan Yu, Tao Liu, Yinghui Shan, Ying Huang, Xuelei Huang, Irmgard Seidl-Adams, Sarah Melissa, and Katalin Boroczky for their friendship and encouragement.

To my family, I convey my sincere appreciation for their understanding, encouragement, and moral support at all times. Last but not least, my deepest appreciation and love go to my parents, Jingguo Zhao and Peishan Jin, and my husband,
Nan Zong, who are always there to support me and provide me with endless care and encouragement. I dedicate this thesis to my family.
Chapter 1
Introduction and Literature Review

1.1 Motivation

About fifty years ago, protons were proposed as the wall-loosening factor as they cause rapid cell elongation in raw and isolated coleoptile tissues. This idea subsequently developed into the acid growth theory (Rayle & Cleland 1970; Hager 1971). The acid growth theory states that plant cells enlarge rapidly at low pH (4.5 – 6.0). Many biotic and abiotic agents, including auxin, regulate cell growth at least partially by changing wall pH (Cosgrove 2005). As the major protein showing acidic preference similar to acid growth, expansin was originally discovered as a cell wall protein that could reconstitute wall extension of the heat-inactivated cucumber hypocotyls (McQueen-Mason et al. 1992). This reconstituted extension shares many common characteristics of the native acid growth. For example, they both display maximum extension activity between pH 3.5 and 4.5. They react similarly to enhancing agent (i.e., thiol-reducing DTT) and inhibiting agent (i.e., copper and aluminum ions). They can both be eliminated after boiling in water but survive methanol boiling.

Although expansins, as suggested by many recent research findings, are involved in wall loosening processes, including cell enlargement (Choi et al. 2003), fruit softening (Brummell et al. 1999), abscission (Belfield et al. 2005) and pollination (Cosgrove et al. 1997), the mechanism of expansin action is not well understood. In contrast to the initial
assumption that they might be hydrolytic enzymes, expansin enhanced the stress relaxation of isolated walls without physical weakening (McQueen-Mason et al. 1992 and 1995, Li et al. 1993, McQueen-Mason & Cosgrove 1994, Wu et al. 1996, Li et al. 2003). Based on in vitro experiments, it was proposed recently that expansin breaks the non-covalent bands between wall polysaccharides (Cosgrove 2005). Very limited work, so far, has been conducted regarding the in vivo function of expansin.

Expansin is not only essential for plant growth but also important to humans in the sense of public health and commerce. For example, grass group-1 pollen allergens constitute a group of β-expansins. They cause hay fever and asthma in about one quarter of the world’s population. Expansin participates in parasitism (nematode and potato, Qin et al. 2004; fungus and tomato, Cantu et al. 2008), which directly correlates with food yield. Expansin also facilitates enzymatic disassembly of cellulose, which is of potential application in biofuel production (Hurley 2007). Owing to its wide involvement in our daily life, curiosity has been inspired in the functional study of expansins which will be potentially beneficial to future applications in commercial and public health applications.

The main objective of this dissertation is to improve the understanding of expansin function in plants. A group of pollen-expressed expansins in *Arabidopsis*, including *AtEXPA13, A4, A24, and B5*, were investigated. Their expression profiles were established and phenotypes of mutant lines were characterized at vegetative growth and reproductive growth stages. This work provides insight into the mechanism of expansin function and the possible cooperation between expansin and pectic methylesterase in cell separation.
1.2 Plant Cell Wall Components and Architectural Models

The plant cell wall was first observed under a primitive microscope by English physicist Robert Hooke in 1665. This event deepened our view of organisms to the cellular level and is regarded as a major milestone in the history of science. However, the impression of cell wall as a static, dead structure was held until 1980s. It was only during the past 20 years that the cell wall began to be recognized as a dynamic and living structure for plants and many other organisms.

The cell wall is the outermost thin layer of a cell. Except animals and most protists, cell walls are widely found in plants, bacteria, archaea, fungi, and algae. The diameter of plant cells ranges from 5 to >100 μm, and the plant cell wall is about 0.2 – 2 μm thick (Raven et al. 1999). In terms of volume, the wall accounts for only 5% of the growing tissue (Cosgrove & Cleland 1983). Although trivial in size, plant cell walls play a vital role in mechanical support and protection. Cell wall material is also of great importance for human health and animal nutrition, as well as for the paper, textile and lumber industries.

1.2.1 Plant Cell Wall Polysaccharides

The primary cell wall is deposited during cell growth. It consists mainly of polysaccharides classified as cellulose, hemicellulose, and pectin. Cellulose is composed of linear polysaccharide chains of β(1, 4) linked D-glucan. These long glucan molecules are packed into microfibrils of about 2 to 4 nanometers in diameter. They are embedded in a polysaccharide matrix containing hemicellulose and pectin. Xyloglucans and
arabinoxylans are the most common hemicelluloses in flowering plants. Unlike cellulose, hemicelluloses often have short side chains which can prevent them from assembling into crystalline microfibrils. It has been generally accepted that hemicelluloses tightly bind to the surface of cellulose microfibrils by hydrogen bonds and thus tether adjacent microfibrils and play a significant role in regulating cell enlargement.

Pectin is also a heterogeneous group of polysaccharides. They are characterized as containing a high proportion of galacturonic acid residue (York et al. 1985). Three pectins are present in all plant primary cell walls: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II). They are diverse in molecular complexity and abundance in plants. HG is the most abundant pectic polysaccharide accounting for ~57% - 69% of extractable pectin (O’Neill et al. 1990).

The secondary cell wall is formed after the cell ceases growth. It is located between the primary cell wall and the plasma membrane. Compared to the compositions of primary cell wall, it has no pectin, different hemicelluloses, and additional substances like lignin, cutin, and suberin. The secondary cell wall of vessel elements is deposited in special patterns, corresponding to their structural function as water transporting conduits.

1.2.2 Cell Wall Structural Proteins

In addition to polysaccharides, the plant cell wall contains many proteins. The annotation of Arabidopsis shows that ~17% of its genome (~5,000 genes) encodes proteins with predicted signal peptides, which target proteins to the secretory pathway (Jamet et al. 2006). Considering the biochemical functions of wall proteins and the
potential alternative splicing and post-translational modifications, a reasonable estimate will be 1,000 to 2,000 wall proteins in *Arabidopsis* (Jamet *et al.* 2006). They may be involved in wall component modifications, wall structure alterations, signaling, and/or interactions with plasma membrane proteins. The structural proteins of the cell wall are unusually rich in one or two amino acids and contain domains of highly repetitive sequence. Many of them are highly glycosylated. Wall structural proteins are named according to their predominant amino acids, such as, hydroxyproline-rich glycoproteins (HRGPs), arabinogalactan proteins (AGPs), glycine-rich proteins (GRPs), and proline-rich proteins (PRPs). This classification, however, became blurred as proteins with multiple predominant amino acids and modular/hybrid proteins were found (Cosgrove 1997).

1.2.3 Cell Wall Enzymes

Wall enzymes are defined as enzymes located in the cell wall. According to their substrates, cell wall enzymes may participate in (1) modification of structural carbohydrates (e.g., endoglucanases, xylosidases, pectin methyl esterases, xyloglucan endotransglycosylases), (2) pathogen defense (e.g., chitinases and β-1,3-glucanases), or (3) alteration of other substrates (e.g., peroxidases and phosphatases).
1.2.4 Architectural Models of the Plant Cell Wall

The structure and interactions of wall components is not clearly defined and considerable debate exists. As for the wall structure, there are four models in general, the Albersheim model, the sticky network model, the multi-coat model, and the stratified hybrid model (Cosgrove 2000). Keegstra et al. (1973) first proposed the “Albersheim model”, in which hemicelluloses and structural proteins are covalently linked to each other and cellulose is bonded to xyloglucans by hydrogen bonds. In the “Sticky network” model (Hayashi 1989, Fry 1989), xyloglucan directly cross-links celluloses by adhesion to its surface or by entrapment within the cellulose microfibrils. This cellulose-xyloglucan network is embedded in a pectic network. According to this model, xyloglucan plays a key role in regulating wall expansion. However, endoglucanases, which can hydrolyze xyloglucan, failed to induce significant wall extension in cucumber hypocotyl walls (Cosgrove & Durachko, 1994). The multi-coat model was proposed by Talbott and Ray (1992). It assumes that cellulose is enwrapped in layers of xyloglucan and other hemicellulose. In addition, pectin fills the interstices. This is consistent with the results of the hydrolytic enzyme assay mentioned above. The stratified layer model (Ha et al 1997) proposed that celluloses are cross-linked by xyloglucan through hydrogen bonds. They form layers and are separated by strata of pectin. The major difference of those four models lies in the spatial location and types of associations between the major wall components.

Multiple ideas exist regarding the interactions between cellulose and hemicellulose. Hemicelluloses could bind to the surface of cellulose and tether them
together. Xyloglucan may be entrapped during microfibril formation and the free end of xyloglucan may help to anchor the cellulose microfibrils to neighboring matrix polymers. Covalent bonding between xyloglucan and pectin has been proposed based on evidence from suspension-cultured rose cells (Thompson & Fry 2000) and pea (Cumming et al. 2005). Furthermore, these linkages may each contribute to part of the overall wall architecture (Cosgrove 2005).

1.3 Cell Growth and Wall Extensibility

Animals build up organs through cell migration and motility. However, plant cells are formed at meristems and are constrained by cell walls from moving around. The meristem cells are uniform in appearance: small and cuboidal in shape, thin and flexible cell wall, high nucleus to cytoplasm ratio, and dense cytoplasm. At maturity, cells of 30 to 40 distinct types exist, which are all derived from these identical meristem cells and play diverse roles in plants. The major construction process used to shape plant cells is the selective expansion of cells.

1.3.1 Cell Expansion

Plant cells typically enlarge 10 to 100 times in volume as they approach maturity, and water uptake accounts for most of this increase in volume (Cosgrove 1993). As the driving force of cell expansion, turgor pressure is built up due to the semipermeability of
the plasma membrane and the differential solute concentrations on its two sides. The cell expansion rate can be described by the following equation:

\[
\frac{\Delta V}{\Delta t} = A \times Lp \left( \Delta \Psi_w \right)
\]  

(1)

where \(\Delta V/\Delta t\) is the rate of water uptake, \(A\) the surface area of the cell, \(Lp\) the permeability of the plasma membrane to water, and \(\Delta \Psi_w\) the water potential difference. This equation indicates that the rate of water uptake relies on cell area, membrane permeability, and the water potential difference.

1.3.2 Wall Extensibility

As water moves into vacuoles along concentration gradients, the water pressure will push the plasma membrane tightly against cell walls. In growing cells, the cell wall will be loosened when the turgor pressure exceeds the yield threshold (the minimum turgor value beyond which the cell begins to enlarge). As a result, the wall will be stretched irreversibly. This process is described by below equation

\[
GR = m \left( \Psi_p - Y \right)
\]  

(2)

where \(GR\) is the cell grow rate, \(m\) the wall extensibility, \(\Psi_p\) the turgor pressure, and \(Y\) the yield threshold. Both the wall extensibility and the yield threshold are internal factors which can affect wall loosening (Cosgrove 2003).
1.4 Wall Loosening and the Candidate Agents

“Wall loosening” is defined as the relaxation of wall stress through the modification of the wall network (Cosgrove 2005). It may result from scissoring or sliding of a wall stress-bearing crosslink without influencing the dimension of wall. Recently, a lot of attention has been given to four candidate wall loosening agents, including expansin, endoglucanase, xyloglucan endotransglycolase/hydrolase, and hydroxyl radical. Expansins will be discussed in details in Section 1.5 and the others are briefly reviewed below.

1.4.1 Endoglucanase

A fungal endo-\(\beta (1 \rightarrow 4)\) D-glucanase was identified to induce wall extension of heat-inactivated hypocotyls (Yuan et al. 2001). This similarity to expansin implies that endoglucanase could be an endogenous wall loosening agent. Endoglucanase is a glycoside hydrolase with cellulose and xyloglucan as the potential substrates. In higher plants, endoglucanases are encoded by a multi-gene family of which most are of unknown function. Ohmiya et al. reported the overexpression and antisense suppression of endoglucanase leaded to enhanced and reduced growth, respectively (Ohmiya et al. 1995, 2000).
1.4.2 Xyloglucan Endotransglucosylase/Hydrolase (XTH)

Xyloglucan endotransglucosylase/hydrolase (XTH) was also proposed to have wall loosening function (Fry et al. 1992, Nishitani & Tominaga 1992). XTH belongs to a multi-gene family. Some XTHs take part in the cleavage and rejoining of xyloglucan chains (known as xyloglucan endotransglucosylase, XET) and a few of them cut xyloglucan and use water as acceptor substrate (known as xyloglucan hydrolase, XEH). XTH gene expression and enzymatic action correlates well with plant growth (Vissenberg et al. 2003, Hyodo et al. 2003). Down-regulated expression of AtXTH18 and AtXTH27 results in phenotypic changes of *Arabidopsis* (Matsui et al. 2005, Osato et al 2006). However, direct proof of XTHs inducing wall stress relaxation and extension is still lacking (reviewed in Cosgrove 2005).

1.4.3 Hydroxyl Radical

Reactive oxygen species (ROS) are highly active molecules taking part in stress signaling and cell death (Cosgrove 2005). As one form of ROS, hydroxyl radical is impressive for the highly reactive property and the extremely short half-life of several nanoseconds. Hydroxyl radical can attack almost all biomolecules, especially DNA, proteins and lipids, by breaking the intramolecular bonds at its diffusion rate. Although generally regarded toxic to organisms, hydroxyl radical was proposed as a wall loosening agent (Fry 1998). Schopfer (2001) reported hydroxyl radical induced wall extension. However, compared to the 40 – 100% wall extension derived from acid growth, this extension is very limited (~1% extension) before leading to wall breakage (unpublished...
data). More questions arise concerning the control of hydroxyl radical production and the side effect to living cells (Cosgrove 2005). The assessment of hydroxyl radical as a wall loosening agent will largely depend on how these questions are answered.

1.5 Expansins

Expansin was first identified from cucumber hypocotyls in 1992 and then cloned in 1995 (McQueen-Mason et al. 1992, Shcherban et al. 1995). Since then, expansins have been found in plants from mosses to flowering plants and non-plants (e.g., slime mold, fungus, nematode, and mollusk) (review in Sampedro & Cosgrove 2005, Li et al. 2002).

1.5.1 The Super Gene Family of Expansins

The genes encoding expansin proteins constitute a gene superfamily. For example, 36 expansins were discovered in Arabidopsis, 58 expansins in rice and 35 expansins in poplar (review in Sampedro & Cosgrove 2005). This gene superfamily consists of four distinct families designated as EXPA (α-expansins), EXPB (β-expansins), EXLA (expansin-like A), and EXLB (expansin-like B). EXPAs are phylogenetically related to the first-identified cucumber expansins, whereas EXPBs are closely related to the group-1 grass pollen allergens. Both EXPA and EXPB were discovered by their wall loosening activity on isolated cell walls (McQueen-Mason et al. 1992).
1992, Cosgrove et al. 1997). However, EXLA and EXLB were identified merely based on sequence similarity.

By microsynteny analysis, the expansin superfamily was subdivided into 17 clades. There are 12 clades of EXPa, 2 clades of EXPB, 1 clade of EXLA, and 2 clades of EXLB. Each clade came from one gene in the last common ancestor of Arabidopsis and poplar (Sampedro et al. 2005 & 2006). In the Arabidopsis lineage, Sampedro et al. (2005) also proposed there were 67 gene birth and 48 gene deaths since monocots and eudicots diverged. This gave rise to the current number of expansin genes in Arabidopsis. Phylogenetic analyses of the moss (Physcomitrella patens) genome (Carey & Cosgrove 2007) suggested a minimum of 2 EXPAs and 1 EXPB in the most recent ancestor of angiosperms and Physcomitrella. The large number of expansin in angiosperms suggests redundant and/or highly specialized gene functions.

1.5.2 High Specificity of Expansin Expression

Gene expression at the “right” time and in the “correct” place is essential for plant growth and development. The expression pattern of a gene can shed light on its function in vivo. The majority of the existing expansin studies are focused on gene identification and expression profiles in various plant tissues or organs. Four representative examples are given below.

① Arabidopsis thaliana has 26 EXPAs, 6 EXPBs, 3 EXLAs, and 1 EXLB identified. The promoter-GUS lines (Cosgrove et al., 2000) indicated that expansins vary in their expression profiles temporally and spatially. It is rare for two expansin genes to show
identical expression patterns. For example, although both are expressed in root, 
*AtEXPA14* expression is restricted to root tips, but *AtEXPA11* expression is observed in 
the root buds and cortex. *AtEXPA4* and *AtEXPA16* are closely related expansins 
belonging to Clade IV. *AtEXPA4* transcript was detected in guard cells, pollen, and 
pollen tubes whereas *AtEXPA16* was transcribed exclusively in guard cells. Exceptions 
include the two root hair expansins observed in *Arabidopsis* (Cho & Cosgrove 2002).

② Valdivia *et al.* (2007a) identified 15 *EXPBs* from maize pollen, of which most are 
pollen specific. They are the dominant allergenic components of grass pollen, known as 
group 1 allergens in immunology. Considering their pollen specific expression, the 
biological function of group 1 allergens is proposed as wall loosening in pollen-stigma 
recognition, stigma penetration and/or pollen tube elongation. Supporting this idea, 
maize pollen carrying a *ZmEXPB1* mutation was less competitive than those carrying 
wild type *ZmEXPB1* when large loads of mixed pollen were deposited on the stigma 
(Valdivia *et al.* 2007b).

③ Tracheary elements (TE) are the conducting cells of the xylem. The differentiation of 
TE involves a series of complicated cell morphology and wall modifications: 
Procambium cells enlarge radially and longitudinally first, and then lay down the 
secondary cell wall in a spiral, annular, reticulate, or pitted pattern (Aloni 1987). To 
decipher this complicated process, *Zinnia elegans* mesophyll cells have been used to 
study vascular differentiation *in vitro*. *Zinnia* mesophyll cells are capable of 
synchronized redifferentiation into TE upon auxin and cytokinin treatment (Fukuda & 
Komamine 1980). A large number of biochemical characterizations have been done
using this system and at least some of the genes expressed in this system turned out to be expressed *in vivo* (Demura & Fukuda 1994). Three α-expansins, ZeExp1, ZeExp2, and ZeExp3, were identified during the cell elongation of developing TE in the *Zinnia* mesophyll system. In situ localization showed that ZeExp1 and ZeExp3 mRNA are localized at the apical tip, whereas ZeExp2 mRNA is located in the basal tip. This polar distribution suggests that expansins play a role in localized wall loosening during xylem differentiation (Im et al. 2000).

Fruit ripening is a process involving substantial depolymerization and solubilization of pectic and hemicellulosic polymers (Brummell et al. 1999). Hemicellulose disassembly is the primary determinant in this process. Cloning of expansins from expanding and ripening tomato fruit suggests that expansins act not only on wall extension but also on wall break-down. The cloned tomato fruit expansins include *LeExp1* (Rose et al. 1997, 2000, Brummell et al. 1999), *LeExp2* (Catala et al. 2000), and *LeExp3 - LeExp7* (Brummell 1999). They exhibit differential expression patterns. *LeExp1* expression is specific to ripening fruit. *LeExp3* is expressed throughout the fruit growth and ripening stage, while all the others appear either in expanding fruit or in ripening fruit with mRNA peak appears at different times. This high specificity of expansin expression is common in most studied expansins. Large gene numbers and high expression specificity are two characteristics prevalent in cell wall proteins. This may result from the varying wall properties of diverse cell types over different growth stages.
1.5.3 Techniques for the Study of Expansin Structure and Function

Early study of expansin structure was only based on sequence analysis and comparison. Some features were revealed as conserved traits among expansins of cucumber, rice, *Arabidopsis* and pea (Cosgrove 1996), such as the signal peptide, the domain 1 resembling family-45 glycoside hydrolase (GH45), the cysteine-rich region, and the domain 2 similar to the cellulose-binding domain (CBD) of bacterial cellulases. By x-ray crystallography, expansin (*ZmEXPB1*) was revealed as a two-domain protein with a long and shallow groove for potential polysaccharide binding (Yennawar *et al.* 2006). Some details of expansin structure were also revealed. Domain 1 has a β-barrel flanking by short loops and α-helices and domain 2 is composed of mainly two antiparallel β-sheets. Post translational modification consists of disulfide bonds between the conserved cysteines, H-bonds and salt bridges between domains which may account for their compact alignment.

Various experimental methods have been used to investigate the biochemical and biological functions of expansins. This section briefly summarizes these methods using the broad categories of *in vitro* and *in vivo* techniques.

1.5.3.1 *In Vitro* Techniques

These techniques are classified as “*in vitro*” since they are conducted using tissue or cells outside of a living plant. With simplified conditions and reduced variables, *in vitro* techniques provide information about effects of a single variable on a simplified system.
Creep Measurement

Wall “creep” is interpreted as the alteration of relative positions between wall polysaccharides. As described in the plant cell growth equation (Equation 2), turgor pressure provides the mechanical force to drive wall creep. Following this idea, the wall specimen is clamped under constant load. When submerged in specific buffer, the specimen changes in length (extension) which is recorded by a displacement transducer. This “extension-meter” is known as an extensometer and is widely used in monitoring long-term wall extension (Cleland et al. 1987, Cosgrove 1989). When neutral buffer is exchanged for acid buffer, wall extension (acid growth) is promoted, induced in native cell wall samples (Cleland et al. 1987). Heat-inactivated and mature tissue displayed no acid growth, as assessed by creep measurement (Cosgrove 1989, Cosgrove & Li 1993). Expansins were discovered as they restored the acid growth of inactivated samples (McQueen-Mason et al. 1992). Zhao et al. (2008) reported that the susceptibility of mature cucumber hypocotyls to expansins was reconstituted after fungal pectinases and calcium chelator EGTA treatment. Their data suggested that pectin and its calcium bridges are important mediators of wall sensitivity to expansin.

Significant progress in the research of expansin mechanism has also been made through creep measurement. Expansins induced creep of pure cellulose paper without detectable cellulase activity (McQueen-Mason & Cosgrove 1994), which suggests that expansins induce wall extension through weakening the hydrogen bonds between cellulose microfibrils. As a simplified and homogenous “wall”, crystalline cellulose synthesized by Acetobacter xylinus was used for creep measurement (Whitney et al. 2000). Sensitivity to expansins appeared when the cellulosic pellicles were stretched in
the presence of xyloglucan. The observation implies the cellulose-xyloglucan interface as the target of expansin action.

A programmable extensometer was developed for studying wall-yielding properties in cucumber hypocotyls (Takahashi et al. 2006). By increasing the load in a stepwise or linear manner, the method provides the estimates of wall extensibility (m) and yield threshold (Y) (see Equation 2). Takahashi et al. (2006) reported that expansins caused changes in both parameters.

**Stress-Relaxation Measurement**

The method of stress-relaxation involves extending wall specimens to a constant length and then recording the reduction in wall stress (holding force) over a period of time (Cosgrove 1989). When a wall sample is extended, wall stress increases. Molecular rearrangements of wall polymers subsequently lead to a decrease in wall stress. This change is recorded by a force transducer. The change in force (dF) against the change in log time (dlog) is plotted to generate a stress relaxation spectrum. One hallmark of expansins is that they restore the stress relaxation spectrum of heat-inactivated wall samples to that of native cell walls. Therefore, stress-relaxation measurement is usually performed for testing expansin functions, in addition to creep measurements.

**Instron (Stress/Strain) Measurement**

Under pressure, wall extends both reversibly and irreversibly (Cleland 1984, Cosgrove 1989). The reversible deformation is known as elasticity and the irreversible part is referred as plasticity. When a wall specimen is stretched over a load, the slope of
the extension curve represents its total extensibility, which contains both elasticity and plasticity. A subsequent round of stretch over the same load will be due only to elastic deformation as plastic deformation can not be recovered during the relaxation between two extensions. Subtracting elasticity from the total extensibility gives rise to the plasticity of the cell wall. This method is widely used to measure the elasticity and plasticity of wall samples.

**Other Techniques for Single Cell Measurement**

Multiple *in vitro* techniques have been developed for studying the mechanical properties in single plant cell. Micromanipulation is used to measure the force required to burst single tomato cells from suspension cultures (Blewett *et al.* 2000). Single tomato cells are compressed between a probe and a glass surface, and the output force is recorded by a force transducer connected to the probe. Wang *et al.* (2008) detected highest elasticity at pH 4.5, which is the pH optimum for expansin activity. The addition of exogenous expansins enhanced the wall elasticity at all pH values.

Other techniques developed for measurement of wall properties include the pressure probe (Tomos and Leigh 1999), micropenetration (Hiller *et al.* 1996), and ball tonometry (Lintilhac *et al.* 2000). They are generally applied to individual cells within tissues, which means that cell-cell interactions and intercellular water flows may affect the results.
1.5.3.2 *In Vivo* Techniques

Analysis of transgenic plants with increased, decreased, or absent expression of a specific gene provides a powerful tool for *in vivo* functional study. However, this technique has been of limited usage in expansin investigation up to the present time.

In general, transgenic plants with down-regulated expression of expansin showed reduced plant growth and up-regulated expression resulted in increased or abnormal growth. Transgenic *Arabidopsis* with sense or antisense constructs of *AtEXPA10* exhibited increased or decreased growth of petioles, respectively (Cho and Cosgrove 2000). Pedicel abscission was also enhanced or retarded in the transgenic plants. Choi *et al.* (2003) generated transgenic rice with sense and antisense constructs of *OsEXP4*. Antisense plants were shorter and flowered earlier than control plants whereas overexpression lines were taller. In tobacco, local expression of expansins within the tobacco meristem initiated the precocious leaf development and altered leaf shape (Pien *et al.* 2001). Lee *et al.* (2003) reported that the ecotopic expression of soybean *GmEXP1* accelerated the root growth of tobacco seedlings. A similar phenomenon was observed in trees. In transgenic lines with overexpressed *PttEXPA1*, aspen showed increased stem internode elongation and leaf expansion. Transgenic methods were also used for studying the role of the ripening-specific expansin *LeEXP1* in tomato. Suppression of *LeEXP1* resulted in firmer fruits and overexpression softened the fruits (Brummell *et al.* 1999). This investigation supports the proposal that expansins contribute to fruit softening during ripening.
Compared with the progress of gene identification, *in vivo* functional study of expansins is lagging behind. For example, out of 36 *Arabidopsis* expansins, only three (*AtEXPA10, 7, and 18*) have been investigated in detail. A detailed investigation of this area will provide useful information about protein function and working mechanism.

### 1.5.4 Model Plants for Expansin Study

A variety of plants have been used for expansin study, including both dicots and monocots. A number of representative plants are reviewed here.

Cucumber is the plant originally used for expansin extraction and the reconstitution assay (McQueen-Mason *et al* 1992). Many advantages of cucumber hypocotyls make it the preferred source of α-expansin protein and wall samples for *in vitro* measurement. Cucumber seedlings grow fast and easily. In dark and humid environment, it takes four days from seeds to seedlings that are ready for harvest. The ease of obtaining large amounts of plant tissue compensates for the difficulty and losses during expansin extraction. The size of cucumber hypocotyl also makes for easy handling for *in-vitro* techniques. One piece of interesting work about expansin identification was performed in cucumber fruit. *CsEXP10* was discovered from young cucumber fruit and the gene expression level was highly correlated to the fruit expansion process (Sun *et al*. 2005). The obvious disadvantage of cucumber as a model plant is the limited genomic data that are available.

Another popular model plant with limited genomic resources is maize. Maize pollen is a rich source of β-expansins. Upon soaking pollen in sodium acetate solution
and subsequent chromatography, multiple β-expansins isoforms are readily purified (Li et al. 2003). Because of the abundance of maize pollen β-expansins and the severity of their immunological influence, maize pollen β-expansins have been the preferred subject for numerous biochemical, biological, and immunological studies. Expansins were also discovered in some other grasses, including rice (Cho & Kende 1997, Lasanthi-Kudahettige et al. 2007), oat (Cosgrove & Li 1993), wheat (Liu et al. 2007), barley (Kwasniewski & Szarejko 2006), and sorghum (Buchanan 2005), etc.

Tomato is the plant in which expansin was found to be involved in fruit ripening (Brummell et al. 1999). Kalamaki et al. (2003) reported that the simultaneous suppression of expansin and polygalacturonase affected tomato juice viscosity. This interesting strategy is novel for studying wall protein function. Limited genomic information is also a constraining factor for wide applications of tomato as a model plant. Fruit ripening expansins were observed in a variety of other plants including strawberry (Dotto et al. 2006), pear (Hiwasa et al. 2003), peach (Hayama et al. 2006), grape (Schlosser et al. 2008), and melon (Nishiyama et al. 2007).

*Arabidopsis thaliana* is the most favored model organism in plant biology (Meinke et al. 1998). The *Arabidopsis* genome has been sequenced and it is the most thoroughly studied plant species. *Arabidopsis* is small in size and easy to grow. It has a short generation time of about six weeks and produces over 5,000 seeds in one plant. Transformation of *Arabidopsis* is highly efficient and many T-DNA mutants of *Arabidopsis* are easy to obtain from public sources as well. Despite these advantages of *Arabidopsis* as a model plant, functional study has only been performed for three *Arabidopsis* expansin genes. There are three reasons for this. First of all, 36 expansins
exist in *Arabidopsis* and their expression patterns are very complicated. Tissue specific expression in different organs and coexpressions of multiple expansins in single organs are widely observed in promoter::GUS lines (Cosgrove *et al*. 2000). Both situations can fail the attempt of accurate localization of expression and thus mislead functional study. Second, some mutant phenotypes may be manifested as subtle changes, which may need a sensitive methodology for their detection. Third, functional redundancy is a potential challenge to decipher single gene function. To overcome these difficulties, a group of pollen-expressed *Arabidopsis* expansins were selected for functional study. Their differential expression profiles were studied carefully. Phenotypic analysis of mutants implies both high specificity and function redundancy of expansins.

### 1.6 Organization of This Dissertation

In Chapter 1, an introduction and literature review about cell wall and expansin is presented. The functional study of *AtEXPA13* in early pollen development and in vegetative growth is detailed in Chapter 2 and Chapter 3, respectively. Chapter 4 contains the expression and functional analysis of three additional pollen-expressed expansins. Chapter 5 ties the research data together and presents an in-depth discussion and suggestions for further investigation of expansins.
References


conserved, multigene family of proteins that mediate cell wall extension in plants. *Proc Natl Acad Sci USA* 92: 9245-9249.


Chapter 2

*EXPA13* and the Early Pollen Development in *Arabidopsis*

2.1 Introduction

*Arabidopsis thaliana* is a small plant belonging to the mustard family (*Brassicaceae, Cruciferae*) (Meinke *et al.* 1998). It is a predominantly self-fertilizing and annual species. Although native to Eurasia and Northern Africa, *Arabidopsis* is now naturalized widely in the world. It is also extensively used as a model plant in all aspects of biological research, especially in flower development study. As the first plant chosen for genome sequencing (The Arabidopsis Genome Initiative 2000), *Arabidopsis* has about 28,000 genes which encode approximately 35,000 proteins. The availability of this sequenced genome greatly enhances our knowledge of the genes expressed by a typical flowering plant and paves the way for a thorough analysis of gene function. The map-based cloning of genes, T-DNA and transposon tagging, and gene silencing are thus very convenient approaches for functional studies in *Arabidopsis*.

2.1.1 Male Gametophyte Development in *Arabidopsis*

*Arabidopsis* growth can be divided into vegetative and reproductive growth stages. After germination, *Arabidopsis* plants initially grow as a rosette and then bolt to produce an inflorescence. All the aboveground organs derive from the shoot apical meristem (SAM). After a transition stage, the SAM changes to an inflorescence
meristem. Flowers arise from the floral meristem which is further developed from the inflorescence meristem. A typical eudicot flower is composed of four whorls of organs. From the outermost to the innermost, they are the sepal, petal, stamen, and pistil. Pollen grains, the male gametophytes, are produced within the four pollen sacs of the stamen.

In *Arabidopsis*, the division of diploid sporophytic cells (“archesporial cells”) represents the initiation of male gametogenesis. This division gives rise to the tapetal initial (“primary parietal layers”) and the sporogenous initial (“pollen mother cell”, PMC). The primary parietal layer experiences two rounds of mitosis and forms four nonreproductive layers: the epidermis, endothecium, middle layer, and the tapetum, all of which surround the reproductive cells. The PMCs undergo meiosis to yield tetrads of haploid cells called microspores. As the callose-impregnated tetrad walls are degraded by callase, microspores are freed into the pollen sac. Subsequently, microspores undergo an asymmetric mitosis which leads to bicellular pollen grains, a smaller generative cell fully enclosed within a larger vegetative cell. As in other *Cruciferae* species, the generative cell divides to form two sperm cells before the pollen is shed from the anthers of *Arabidopsis*. After mitosis, the pollen grain undergoes dehydration and is ready for pollination upon anther dehiscence (Ma 2005, Boavida *et al.* 2005).

### 2.1.2 Correlation between Pollen Development and the Stages of Flower Development

In early 1990s, flower development of *Arabidopsis* was studied at the organ level and pollen development was investigated mainly at the cellular level (Smyth *et al.* 1990,
Bowman et al. 1994). The stages of flower development are easy to identify as the landmark events are macroscopical whereas the stages of pollen development cannot be determined without tissue sectioning and light microscopy. To conveniently determine pollen development status, the correlation between pollen and flower development stages is summarized (Table 2.1) and used in this dissertation. It is primarily based on the work of Smyth et al. (1990) and Bowman et al. (1994).

Table 2.1: The landmark events of flower development and pollen development in Arabidopsis (based on Smyth et al. 1990, Bowman 1994). Modified from Table 1 of Smyth et al. 1990.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Landmark event at beginning of stage</th>
<th>Pollen Development (Bowman 1994)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flower buttress arises</td>
<td>7 Archesporial cells divide to give rise to primary parietal and sporogenous cells</td>
</tr>
<tr>
<td>2</td>
<td>Flower primordium forms</td>
<td>8 Microsporocytes are conspicuous</td>
</tr>
<tr>
<td>3</td>
<td>Sepal primordia arise</td>
<td>9 PMC's separate from each other &amp; from tapetum; PMC's undergo meiosis &amp; form tetrads</td>
</tr>
<tr>
<td>4</td>
<td>Sepals overlie flower meristem</td>
<td>10 Microspores separate &amp; lie freely in pollen sac; Microspores round up, wall thickens due to exine formation</td>
</tr>
<tr>
<td>5</td>
<td>Petal and stamen primordia arise</td>
<td>11 Tapetum degenerate; 1st mitosis of microspores follows resorption of prominent vascular; 2nd mitosis</td>
</tr>
<tr>
<td>6</td>
<td>Sepals enclose bud</td>
<td>12 Dehiscence.</td>
</tr>
<tr>
<td>7</td>
<td>Long stamen primordia stalked at base</td>
<td>13 Fertilization.</td>
</tr>
<tr>
<td>8</td>
<td>Locules appear in long stamens</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Petal primordia stalked at base</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Petals level with short stamens</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Stigmatic papillae appear</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Petals level with long stamens</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Bud opens, petals visible, anthesis</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Long anthers extend above stigma, 0 HAF</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Stigma extends above long anthers</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Petals and sepals withering</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>All organs fall from green siliques</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Siliques turn yellow</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Valves separate from dry siliques</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Seeds fall</td>
<td></td>
</tr>
</tbody>
</table>
2.1.3 Wall Alteration during Gametogenesis

Complex cell wall changes occur during pollen development. The cytological aspects of these changes have been well documented (Blackmore and Barnes 1990, Owen and Makaroff 1995). Before meiosis, the PMC wall is similar to the ordinary primary wall with plasmodesmatal connections between adjacent PMCs. At the onset of meiosis, the PMC begins to secrete callose ($\beta$-1,3-glucan) between the plasma membrane and the primary cell wall. As more and more callose is deposited, the plasmodesmata are enlarged and then blocked, which may be meaningful for synchronizing meiosis at first and then facilitating the development of genetically distinct pollen grains (Mascarenhas 1975). During the two-cell and four-cell stages of meiosis, each cell (dyad and tetrad) is surrounded by a callose wall. At the tetrad stage, the microspore starts to lay down its own wall (“primexine”). Callose degradation, pectin degradation, and microspore release happen simultaneously. In the _quartet_ mutant, pectins are persistent in the microspore wall (Rhee & Somerville 1998), which suggests pectin degradation is essential for microspore separation. At about the same time, the tapetal cell walls degrade (Owen and Makaroff 1995). The outer layer of the microspore wall (the “exine”) is deposited on the surface, apparently mediated by secretions from the tapetum. The inner layer of pollen wall, the intine, is derived from the microspore and is deposited between the exine and the plasma membrane during expansion. The distinction between the exine and intine lies in their deposition time, structure, and chemistry. The exine is laid down earlier, which starts even when the grain is enclosed in the callose wall. It is often highly sculptured and composed mainly of cellulose, sporopollenin, and glycoprotein. The
intine forms during the free microspore stage (Knox and Heslop-Harrison 1970). In structure and composition, the intine is similar to a primary wall of somatic cells, but quite thick, containing cellulosic microfibrils, a matrix of hemicellulose, pectin and protein. The intine is exposed to the exterior at the aperture of the pollen grain. Both the exine and the intine contain diffusible substances, which are important in pollen-stigma interactions.

Genes expressed during male gametophyte development can be generally classified into early and late genes (McCormick 1993). “Early gene” transcripts are detected soon after meiosis but not detected in mature pollen. “Late gene” transcripts are detected after microspore mitosis and still detectable as pollen matures. Genes with continuous expression throughout pollen development have also been identified (Twell et al. 1993). Most of the research has been done on “late genes” (Honys & Twell 2003) owing to difficulty in isolating “early genes” of gametogenesis.

A pioneering study of expansin function in the early pollen development of Arabidopsis was performed and reported herein. The expression of the EXPA13 gene was detected in developing pollen. Three mutant lines were examined for gene functional study. The phenotype observed in the qrt1-background mutant, EXPA13-1, is very interesting and suggests gene function in microspore expansion. The failure of constructing an expa13 qrt1 double mutant implied synergistic action between expansin and pectin methylesterase. The function of EXPA13 in Arabidopsis vegetative growth will be discussed in Chapter 3.
2.2 Materials and Methods

2.2.1 Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh, Columbia-0, were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). Sources of other seeds will be described in section 2.2.4. *Arabidopsis* seeds were surface-sterilized by soaking in 60% ethanol for 2 min, followed by 50% bleach plus 0.02% Triton X-100 for 8 min. Seeds were then washed four times in sterile water and were sown on plates of MS medium (Murashige & Skoog 1962) supplemented with 1% sucrose and 0.08% phytagar. Vernalization was performed at 4°C for two days, after which seeds were transferred to a growth chamber with the temperature range of 22 °C /16 °C (16 h light/8 h dark) and fluorescent lighting with a photon flux density of 70 μmol m⁻² s⁻¹. After about 10 days, seedlings were transplanted from the plates to pots containing a 6:1 mix of Miracle-Gro potting mix and vermiculate (Miracle-Gro Lawn Products, Inc., Marysville, OH) and then grown in the same conditions.

2.2.2 Collection of Mature Pollen Grain and Developing Anther

Mature pollen grains were collected in bulk from flowering inflorescences following the procedure of Honys and Twell (2003). Inflorescences were collected in a large flask with 300 mL of ice-cold 0.3 M mannitol. After being vigorously shaken for 1 min, the pollen suspension was then sequentially filtered through 100 and 70 μm nylon
mesh. Pollen grains were concentrated by repeated centrifugation steps (50 mL Falcon tubes, 450 g, 5 min, 4°C). The final compact pollen pellet was stored at -80°C.

Anthers from the flowers of Stage 10 to 12 were collected from unopened buds. The characteristic of flowers at these stages is that the petals are longer than the short stamens but shorter than the long ones. Partial filaments attached to the anthers were contained in the anther samples. Samples were stored at -80°C.

### 2.2.3 Histochemical GUS Staining and Observation

The construction of the *EXPA13* promoter::GUS fusion was reported by Cosgrove *et al.* (2000). A DNA fragment of 1490 bp upstream from the 5’ flanking region of *EXPA13* was amplified from *Arabidopsis* genomic DNA by polymerase chain reaction (PCR). The primers used in the PCR reactions were 5’-CTAGTCTAGAGCTTACCCTGTTTACGGTTG -3’ and 5’ – TCCCCCCCAGGGAGAGAGAAATGCG -3’, which introduced *XbaI* and *XmaI* sites into the product ends. The PCR fragment was cloned into Bluescript and then verified by restriction digestion and sequencing. The *XbaI/XmaI* fragment was inserted into a binary vector pGPTV-HPT (Becker *et al.* 1992) for a recombinant transcription unit *EXPA13 promoter::GUS*. The construct was introduced into *Arabidopsis* through *Agrobacterium* (GV3101) – mediated transformation by the floral infiltration method (Tague 2001). The independent transformants were screened on MS media containing 0.8% phytagar and 20 μg ml⁻¹ hygromycin. The homozygous T2 plants were used for GUS histochemical staining.
Histochemical staining of GUS was performed following the modified protocol of Jefferson *et al.* (1987). Briefly, plants were incubated in staining buffer (pH 7.0) containing 0.5 mg/mL X-Gluc at 37°C in dark for 24 h. The staining solution was then replaced by 70% ethanol to remove chlorophyll from tissues. Stained tissues were then mounted with pure lactic acid for microscopy. Lactic acid helps turn tissue transparent and simplifies the observation of delicate structures. Flowers were dissected when certain structures, such as developing microspores, were observed.

### 2.2.4 Crossing of *Arabidopsis* Plants

Unopened flowers were selected from the female parent right before the white petals were visible. All other flowers were eliminated. Under the dissecting microscope, all flower organs except the pistil were removed. The female parent was put back into the growth chamber overnight before crossing. Flowering inflorescences were chosen from the male parent. Anthers were removed by nipping off filaments with forceps and tapping the open anthers gently against the exposed stigma. A total of 5 – 6 flowers were pollinated and labeled. Seeds were harvested after dried.

### 2.2.5 PCR-Based Mutant Screening

*Expa13-1* (SAIL_190_E07, *Col-0 qrt1-2* background) is a T-DNA mutagenized line from the Syngenta Arabidopsis Insertion Library (SAIL) collection (Sessions *et al.* 2002). For genotyping, the genomic DNA was extracted from leaf tissues using a rapid
DNA extraction protocol (Kasajima et al. 2004). DNA from individual plants was analyzed by PCR using combinations of three primers, including the gene specific primers (MA13CLP and MA13CRP) and the insertion specific primer LBSYN1 (Table 2.2). All the gene specific primers mentioned in this chapter were designed using T-DNA Primer Design (http://signal.salk.edu/tdnaprimers.2.html) of SIGnAL (Salk Institute Genomic Analysis Laboratory).

Table 2.2: Three primers for PCR-based screening of expa13-1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA13CLP</td>
<td>TCTAGGCACAATGCGGTAAAC</td>
</tr>
<tr>
<td>MA13CRP</td>
<td>TTTCCCTTTTATTTTGGTTTGGGG</td>
</tr>
<tr>
<td>LBSYN1</td>
<td>CATCTGAATTTCATAACAAATCTCGA</td>
</tr>
</tbody>
</table>

Expa13-2 (GT_5_8306, Ler-1 background) is a Ds gene trap Line (Sundaresan et al. 1995) produced at the John Innes Centre (Norwich, UK). It is generated by the mobilization of a gene-trap Ds transposable element which can be screened using kanamycin. For genotyping purpose, the gene specific primers (MA13ELP and MA13ERP) and the insertion specific primer Ds3-1 JIC-GT were used for PCR (Table 2.3).

Table 2.3: Three primers for PCR-based screening of expa13-2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA13ELP</td>
<td>AGCACACGGATATCGAAGAAG</td>
</tr>
<tr>
<td>MA13ERP</td>
<td>CAAATGGTCAAATTGTCCAAAATG</td>
</tr>
<tr>
<td>Ds3-1 JIC-GT</td>
<td>ACCCGACC GGATCGTATCGGT</td>
</tr>
</tbody>
</table>
Expal3-3 (GABI_290B03, Col-0 background) is a GABI-Kat T-DNA insertion line from the Max Planck Institute for Plant Breeding Research (Cologne, Germany) (Rosso et al. 2003). The antibiotic sulfadiazine was applied for mutant screening. PCR-based screening was performed with gene specific primers (MA13FLP and MA13FRP) and the T-DNA specific primer MA13FBP (Table 2.4).

Table 2.4: Three primers for PCR-based screening of expal3-3.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA13FLP</td>
<td>AATTACGGATTTGATCCCGAC</td>
</tr>
<tr>
<td>MA13FRP</td>
<td>TCAATGTGTCCGTCTCTGTCTC</td>
</tr>
<tr>
<td>MA13FBP</td>
<td>CCCATTTGGACGTGAATGTAGAC</td>
</tr>
</tbody>
</table>

As for PCR, the primer annealing temperature was predicted using Gene Runner 3.03 software (Hastings Software, Inc., Hasting, NY) with the settings of 100,000 pM probe concentration and 50 mM salt concentration. Taq DNA polymerase (GeneChoice, Inc., Frederick, MD) was used for PCR (Figure 2.1). All PCR reactions were conducted in a PTC-100 Programmable Thermal Controller or PTC-200 Peltier Thermal Cycler (MJ Research, Inc., South San Francisco, CA). The following programming was used for PCR reactions: 2 min at 94°C for denaturation, followed by 35 cycles of 94°C for 25 sec, 58°C for 1 min, and 72°C for 1 min, and finally one cycle of 5 min extension step at 72°C.
2.2.6 RNA Extraction and RT-PCR

Total RNA was extracted from plant tissues using an RNeasy plant kit (Qiagen, Valencia, CA) following the manufacturer’s handbook. An on-column DNase I treatment was performed using RNase-free DNase set (Qiagen, Inc., Valencia, CA).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was employed to detect gene transcript levels. RT-PCR was performed via a Titan One-Tube RT PCR kit (Roche Applied Science, Indianapolis, IN). The starting amount of RNA template (10 ng per reaction) was determined using 260 nm absorption (GeneQuant RNA/DNA Calculator, Pharmacia Biotech, Cambridge, England) and then finely normalized using actin or 18S rRNA band as reference. The gene specific fragment was then amplified using primers AE13SpSn and AE13SpAs (Table 2.5). The primer 13CEXON1Rvs and 13exonsF, AE13SpSn and MA13AF were also employed to detect

Figure 2.1: Locations of the T-DNA/transposon insertion sites in EXPA13. The filled boxes represent coding regions and the empty box indicates untranslated region (UTR). Triangle and arrow head show the insertion site and direction. Horizontal arrows show the direction and localization of primers. Bar = 200 bp.
the partial segment of *EXPA13* transcripts. *ACT2* or *18S rRNA* served as internal loading controls. The intensity of ethidium bromide-stained band was photographed with a Polaroid camera (Gelcam, UK) and then quantified using ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA).

**Table 2.5: Primers for RT-PCR of *EXPA13* transcript.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Band size</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE13SpSn</td>
<td>CGACCTCTTCACCTTCTTCGTCTCCTC</td>
<td>986 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>AE13SpAs</td>
<td>GCTTTCGCTCCTTAGTAATGTAACAAATAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13CEXON1Rvs</td>
<td>TGGATACGGAGATCTCGTCAAATC</td>
<td>705 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>13exonsF</td>
<td>ACCAAGATCGATACTTTGCGCATCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AE13SpSn</td>
<td>CGACCTTTACCTTTCTCTCGTCCTC</td>
<td>391 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>MA13AF</td>
<td>GCTTTCCAATAAGCGATCTTCTCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18rRNAAF</td>
<td>TTGTGTGGGGCTCGGGATCGGAGTAAT</td>
<td>446 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>18rRNAR</td>
<td>TGCACCCACCACCATAGAATCAAGAAAGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**2.2.7 Microscopy, Photography and Image Measurement**

Microscopic observation was performed with a Zeiss Axioplan (Opto-systems, Inc., Jenkintown, PA). Digital images were captured through a ProgRes C14 camera and software (JENOPTIK Laser System GmbH, Munich, Germany). Image measurement was acquired using software SPOT 3.5.2 (Diagnostic Instruments, Inc., Sterling Heights, MI).
2.3 Results

2.3.1 GUS Expression in Developing Pollen

The inflorescences of *EXPA13* promoter::GUS T2 plants were picked from shoot tips and immediately submerged in staining solution (containing 50 mM sodium phosphate buffer, pH 7.0, 0.2% Triton X-100, 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, and 1 mM X-gluc) for 24 hours. As a well-defined boundary of staining was clearly distinguishable in the stained tissue, the diffusion of GUS product to nearby non-expressing tissue was minimal after 24 hour staining. The highly specific expression pattern was consistently observed in at least three independent lines.

Anthers from GUS lines of multiple developmental stages were examined by light microscopy (Figure 2.2). Figure 2.2A indicated that the earliest *EXPA13* promoter::GUS expression was in the anther of stage 10 flowers. The diagnostic characteristic of the stage 10 flower is that petals begin to overgrow the shorter stamens but stigmatic papillae have not appeared (Smyth *et al.* 1990). During this stage, microspores are freed from tetrads and enlarged in size and the exine is deposited (Bowman 1994). As the petals elongated during stage 11, the GUS signal in anthers was found to accumulate. Until stage 12, the GUS staining was clearly constrained to the entire innermost layer surrounding the pollen sac (mostly likely the tapetum layer) (Figure 2.2B). Immediately before anther dehiscence at stage 13, GUS staining was present in individual microspores but not in the tapetum any more (Figure 2.2C). The high specificity of GUS expression in microspore was seen clearly in an anther opened manually with forceps (Figure 2.2D).
At maturity, pollen grains showed no staining whether they were inside the anther or on the stigma (Figure 2.2E and F).
Figure 2.2: Histochemical staining of *EXPA13* promoter::GUS lines. **A.** An anther from a stage 10 flower of a GUS plant. **B.** At stage 12, the GUS staining was apparently limited to the tapetum. Microspores had no staining. **C.** At early stage 13, the GUS expression was observed within individual microspores. **D.** Microspores from C were manually released from anther using forceps. **E.** An anther at the dehiscence stage with a few pollen grains remained. **F.** Stigma covered by mature pollen grains. Bar = 80 μm (A, D, E, F), 50 μm (B, C).
2.3.2 Transcript Analysis by RT-PCR

To verify the expression of *EXPA13* during pollen development, plant tissues were collected for RNA extraction and RT-PCR (Figure 2.3). Due to the difficulty in separating microspores from somatic tissues in *Arabidopsis*, *EXPA13* transcript analysis in developing pollen was performed using stamens from Stages 10 – 12 flowers. Mature pollen grains were obtained from inflorescences following the protocol described in Section 2.2.2. RT-PCR results showed that *EXPA13* transcripts were present in RNA from the whole flower of Stages 10 – 12, as well as in the developing anthers. No transcript, however, was detectible in RNA from the mature pollen grains. The result is consistent with the *EXPA13* promoter::GUS line observations discussed in Section 2.3.1 (Figure 2.2).

Figure 2.3: Transcript analysis of *EXPA13* in microspores and mature pollen grains. A. *EXPA13* transcript analysis in *Arabidopsis* flowers at stage 10-12. Lane 1 & 2, pool of stamens (anther plus partial filament) from stages 10-12 (Smyth *et al.* 1990) flowers. Lane 3, whole flowers at stages 10-12. B. *EXPA13* shows no expression in mature pollen grains. *18S rRNA* and *ACT2* served as internal control.
2.3.3 Phenotypic Observation in *Exp*13-1

2.3.3.1 Genotyping and Transcript Analysis of *expa*13-1

In order to identify the role of *EXPA13* in regulating pollen development, three independent lines, *expa*13-1, *expa*13-2, and *expa*13-3, were obtained from the public T-DNA / transposon insertion mutant collections. *Exp*13-1 is a SAIL T-DNA insertion mutant in the Col-0 *qrt1-2* background. T-DNA stands for transferred-DNA from *Agrobacterium tumefaciens*, which is the most well-established insertional mutagen in *Arabidopsis* (Krysan *et al*. 1999). The T-DNA insertion site of *expa*13-1 is 161 bp downstream of the stop codon but within the 3’-UTR of *EXPA13*. Genomic PCR was used to distinguish wild type, heterozygous, and homozygous mutants. Using the primers spanning the T-DNA insertion site, no wild-type transcript was detected in the homozygous mutants by RT-PCR. However, *EXPA13* transcripts were detected using primers that amplified the upstream sequence of the insert (Figure 2.4A). This observation suggests that *EXPA13* is transcribed in *expa*13-1. As the T-DNA insertion is outside the coding sequence, functional proteins may still be produced in the homozygous mutant.
Figure 2.4: RT-PCR analyses for *EXPA13* expression in the whole flower of *expa13-1*.  
**A.** RT-PCR indicates *EXPA13* transcription is not disrupted in *expa13-1*. Lane 1 & 2 are wild type siblings and lane 3 & 4 are homozygous mutants. 35-cycle PCR with the template of 10 ng total RNA was performed as described in the Materials and Methods. The *EXPA13* band of 986 bp was from primer AE13SpSn and AE13SpAs whereas the 705 bp was from primer 13CEXON1Rvs and 13exonsF. The former two primers span the T-DNA insertion site but the latter two only cover exon 1. *ACT2* served as internal control.  

**B.** Semi-quantitative RT-PCR to access the gene transcription level in *expa13-1*. PCR with primer 13CEXON1Rvs and 13exonsF were terminated after 23 (Lanes 1 & 4), 24 (Lanes 2 & 5), and 25 cycles (Lanes 3 & 6) before saturation was reached. The mRNA templates were obtained from WT sibling (Lanes 1, 2, & 3) and homozygous mutant (Lane 4, 5, & 6). *18S rRNA* served as internal control. Two replicates were performed. Data shown are means ± SE.
The 3’-UTR is part of the mature mRNA sequence but is not translated. It contains information for translation regulation and mRNA stability. A question arising here is whether the T-DNA insertion in the 3’-UTR affects the transcript level of *EXPA13*. To explore gene expression levels, semi-quantitative RT-PCR was performed to determine mRNA levels using *18S rRNA* as loading control (Figure 2.4B). A 36% increase of *EXPA13* transcripts was revealed in the homozygous mutant compared to that of the wild type sibling. This study suggested a possible correlation between the increased *EXPA13* transcript and the T-DNA insert in 3’-UTR.

2.3.3.2 Half-Collapsed Tetrads in *Expa13-1* Heterozygous Mutant

2.3.3.2.1 Phenotypic Observation

*Expa13-1* is in the *qrt1-2* background. *QRT1* is essential for microspore separation, and thus the mature pollen grains of *qrt1-2* are released as tetrads. It has no influence on pollen viability or fertility. The wild-type siblings and homozygous mutants of *expa13-1* produce the *quartet* pollen as well. However, the heterozygous mutants of *expa13-1* form tetrads with two collapsed pollen grains (Figure 2.5). Comparison of pollen grain diameters between wild type sibling and homozygous mutant indicated no significant difference (Figure 2.6). Thus, the collapse of the pollen grains occurred only in the heterozygous lines in the *qrt1-2* background.
Figure 2.5: Light microscopic images of pollen grains of the expa13-1 mutant in the qrt1-2 background.  

A. Mature pollen grains from the wild type sibling and homozygous mutant of expa13-1.  
B. Mature pollen grains of the heterozygous mutant of expa13-1.  
C. Developing pollen grains from the heterozygous plant.  
Tetrads from stage 11 (C1), early stage 12 (C2 & C3), late stage 12 (C4), and stage 13 (C5).  
C2 and C3 are the same tetrad in different focal planes.  
Bar = 30 μm.
2.3.3.2 Linkage between the Phenotype and Genotype

To examine the linkage between the phenotype and the genotype, two experiments were performed. 57 plants from two generations of expa13-1 were subjected to PCR-based genotyping and pollen examination. The half-collapsed tetrads and the heterozygous insertion co-segregated. In another experiment, the cross between homozygous mutant and wild type plant gave rise to 32 progeny plants, all of which produced half-collapsed pollen. These experiments confirmed the linkage between the phenotype and the heterozygous T-DNA insertion in expa13-1. ExpA13-1 was also crossed with Col-0, which produced healthy pollen grains and indicated that the half-collapsed pollen phenotype is dependent on the qrt1-2 background.

Figure 2.6: Comparison of pollen grain diameters in expa13-1. n = 24. p = 0.96 by two-tail T-test. Data shown are means ± SE.
2.3.3.2.3 Observation of Premature Pollen

In order to trace the formation of the half-collapsed tetrad, unopened anthers from stage 10-11 and 12 flowers were dissected. At stage 10-11, the four microspores in a tetrad were of similar size but two of them already showed signs of breakage. At stage 12, the two intact microspores were enlarged greatly in size whereas the other two had ceased expansion. As the pollen grains approached maturity, the dead microspores collapsed further and stayed attached to the other two healthy ones (Figure 2.5C). These observations indicate that microspore breakage happens after tetrad formation but before pollen expansion. The affected gene function may lead to the microspore collapse directly or indirectly by influencing the interaction between microspores.

2.3.3.2.4 Genotyping of Collapsed Pollen

A question arising with this phenotype is whether the collapse segregates with the wild type or mutant allele of EXPA13. To answer this question, pollen grains from EXPA13/expa13 in qrt1-2 were used to pollinate stigmas of Col-0. Seeds produced from the cross were genotyped. In principle, if the pollen collapse happens in a random manner, the heterozygous mutant will produce equal amounts of microspores carrying wild type or mutated allele of EXPA13. Since all eggs formed in the Col-0 ovary bear only the wild type allele, the offspring will have equal opportunity to be heterozygous or homozygous for the EXPA13 gene. In this experiment, 56 plants from the cross were genotyped. Among them, six plants were homozygous for the wild type EXPA13 allele and 50 plants were heterozygous. In other words, 10.7% of the successful pollen grains
carried the wild type \textit{EXPA13} allele whereas 89.3\% carried the mutant gene. By Chi-
square Goodness-of-fit test, the chi-square value is 34.571 with one degree of freedom.
The two-tailed P value is less than 0.0001, which indicates the observed ratio of homozygous wild type to heterozygous mutant is statistically different from the predicted ratio. From this result I conclude that, in the heterozygous mutant of \textit{expa13-1}, wild type pollen grains are inclined to collapse.

\subsection*{2.3.4 Observation of \textit{Expa13-2} and \textit{Expa13-3}}

\subsubsection*{2.3.4.1 Genotyping and Transcript Analysis}

Two independent lines of \textit{EXPA13} knock-out mutants were obtained for functional study. \textit{Expa13-2} is a transposon mutagenized line in the \textit{Ler-1} background. Transposons insert themselves into genes and cause mutation. The phenomenon was first discovered in maize by McClintock (1950) and now has been identified in almost all organisms. One of the plant transposon systems is named Ac/Ds, where the Ac element is a transposase and Ds is a stable element lacking transposase activity. Sundaresan \textit{et al.} (1995) integrated the maize Ac/Ds elements into a gene trap construct, which was used as an insertional mutagen in \textit{Arabidopsis}. In \textit{expa13-2}, the insertion is located 7 bp downstream of the start codon but in the opposite direction to the gene. There is also a 30 bp deletion immediately after the insertion site (Figure 2.1). Gene specific primers not spanning the insertion region were used for RT-PCR. No transcript was detected, indicating the \textit{EXPA13} gene was effectively disrupted in \textit{expa13-2} (Figure 2.7A).
Expa13-3 is a T-DNA mutant line with the insertion at 144 bp after the beginning of exon 2, a 225 bp deletion flanks the T-DNA on the downstream side of the insertion. In the homozygous mutant, the \textit{EXPA13} transcript was not detected by gene specific primers (AE13SpSn and AE13SpAS) (Figure 2.7B). Primers specific to Exon 1 detected transcription (data not shown). However, as the T-DNA insertion occurs within the exon, the mRNA cannot be translated into functional protein.

\textbf{2.3.4.2 Pollen Size in Expa13-2 and Expa13-3}

One hypothesis about \textit{EXPA13} function is that it promotes pollen expansion during microsporogenesis. To test this hypothesis, pollen from open flowers (Stage 14, the long
anthers extend above stigma according to Smyth et al. 1990) was dispersed on a glass slide and then observed immediately under a microscope. Pictures were taken and then used for a blind measurement of pollen grain diameter using SPOT 3.5.2. The data indicate that the pollen grains from homozygous mutants are of similar size to pollen from wild type siblings in both knockout mutants (Figure 2.8). My conclusion is that *EXPA13* single mutants are not significantly different in pollen size compared to wild type siblings. *EXPA13* gene function may promote microspore expansion in a subtle way.

![Figure 2.8: Comparison of pollen size in expa13-2 (A) and expa13-3 (B).](image)

Figure 2.8: Comparison of pollen size in *expa13*-2 (A) and *expa13*-3 (B). For each independent line, pollen grains were randomly collected from four wild type and four homozygous mutant plants. Blind tests were performed. n = 26 - 40. A. P = 0.450, two-tailed T-test. B. P = 0.674, two-tailed T-test. Data shown are means ± SE.

### 2.3.4.3 Pollen Fertility in *Expa13*-2

Segregation analysis was performed with heterozygous *expa13*-2 to check the effect of *EXPA13* mutation on pollen fertility. Sixty progeny from a selfed heterozygous
mutant gave rise to 21 wild type siblings, 30 heterozygous mutants, and 9 homozygous mutants. The “Goodness of fit” test yielded a chi-square value of 4.8, with which the p value is 0.0907 (df = 2). These data suggest that EXPA13 knock-out mutant was not significantly reduced in fertility. However, homozygous expa13-2 mutants exhibited defects in vegetative growth and the seed yield was consequently reduced, which will be discussed in Chapter 3.

2.3.4.4 Analysis of Expa13 Qrt1 Double Mutant

The potential interaction of EXPA13 and QRT1 was implied by my observations of the expa13-1 line. In order to explore this idea further, attempts were made to generate double mutant of expa13-2 and qrt1 by crossing the single mutants. Pollen grains from expa13-2 were used to pollinate exposed stigmas of qrt1-1 (Landsberg background) to produce a F1 generation heterozygous for both mutations. F1 generation plants were subsequently self-pollinated to produce F2 plants with segregating phenotypes. Ten out of 62 F2 plants produced quartet pollen grains, indicating that they were homozygous for qrt1. By PCR-based genotyping of these ten plants, four individuals were formed to be homozygous for wild-type EXPA13 and the other six were heterozygous EXPA13 (EXPA13/expa13). It is interesting that no expa13 qrt1 double mutant was identified. The “Goodness-of-fit test” yielded the Chi-square value of 3.6, which gave rise to the two-tailed P value of 0.1651 (df = 2). To further confirm whether this happened just by chance or not, F3 seeds from one EXPA13/expa13 qrt1/qrt1 plant were grown for genotyping. In 27 F3 plants, there were 11 individuals carrying only wild type EXPA13,
15 heterozygous mutants, and one homozygous mutant in EXPA13. The chi-square value of this experiment was 7.741 (df = 2) with the two-tailed P value of 0.0209. These data confirmed that expa13 qrt1 double mutant is lethal for Arabidopsis. I hypothesize that pollen grains carrying the mutated alleles of QRT1 and EXPA13 are lethal as the pollen tube development is blocked. To test this idea, the data mentioned above were subjected to the “Goodness-of-fit” test, which gave rise to the chi-square value of 0.615 (df = 1). Therefore, the two-tailed P value was equal to 0.433, which indicated that the hypothesis is supported statistically.

Another interesting observation in the experiment was that abnormal pollen growth appeared in EXPA13/expa13 qrt1/qrt1 plants (Figure 2.10). In the qrt1 single mutant, the four pollen grains in one tetrad are of even size and similar morphology. However, when one allele of EXPA13 gene was disrupted, pollen development was affected. Two of the pollen grains were of smaller size, and sometimes one or both of them were collapsed as shown in the most right panel (Figure 2.9). This defect in pollen development may account for the lack of both EXPA13 and QRT1 genes.

Figure 2.10: Light microscopic images of pollen grains in EXPA13/expa13 qrt1/qrt1 (B & C) and qrt/qrt (either EXPA13/EXPA13 or expa13/expa13, A). Bar = 30 μm.
2.4 Discussion

2.4.1 EXPA13 Expression Profiles in Microsporogenesis

By RT-PCR analysis and EXPA13 promoter::GUS analysis, EXPA13 expression was observed in microsporogenesis during stage 10 to 12 of flower development. The cell wall of microspores is actively modified during these stages since multiple wall proteins are reported with tapetum specific expression. The EXPA13 expression is both sporophytic and gametophytic based on the GUS staining. Transcriptome analysis by microarray also suggests that EXPA13 is expressed at the uninucleate stage of microspore development (Honys & Twell 2004). Based on the developmental events during these stages, EXPA13 may function in microspore separation or expansion.

2.4.2 Pollen Collapse in Expa13-1

Expa13-1 is in the qrt1 background. Quartet (qrt) is an Arabidopsis mutant identified by Preuss et al. (1994). The four pollen grains within a tetrad are fused together. They share exine which extends between the pollen grains. However, there is no evidence of fused intine or common cytoplasm. The qrt pollen grains are normal in all other aspects, including viability and fertility. The qrt gene has been previously applied in two fields. First, it has been widely used for genetic analysis (“tetrad analysis”) as the allele segregation through meiosis can be traced by analysis of the attached pollen grains. Examples include: mapping of Arabidopsis centromeres, distinguishing between sporophytic and gametophytic genes, and studies of crossover
interference (Copenhaver et al. 2000). Second, qrt1 has been extensively used for studying microsporogenesis and pollen cell wall development (Rhee et al. 2003). Three genes (QRT1, QRT2, and QRT3) could lead to the quartet pollen phenotype when mutated. QRT1 has been identified as a pectin methylesterase (PME) (Francis et al. 2006) and QRT3 as a polygalacturonase (PG) (Rhee et al. 2003). The enzyme activities were demonstrated by expression in Escherichia coli (PME) and yeast (PG). The site of expression of both genes has been localized to the tapetal layer during early pollen development, stages 9 through 11 for QRT1 and stage 10 for QRT3. It has been proposed that they demethylesterify and cleave pectin polymers, which leads to pectin degradation and microspore separation in consequence.

The phenotype of expa13-1 is special in that the microspore carrying the wild type copy of EXP13 tends to break in heterozygous mutants in the qrt1 background. This suggests the pollen failure is not directly due to the T-DNA insertion. To explain the phenotype in heterozygous mutant, we propose that the pollen collapse is due to the imbalanced expansion of the four microspores in the locked tetrad. As revealed by semi-quantitative RT-PCR, the mutant shows enhanced transcript level of EXP13, which may be because the insertion in 3′-UTR leads to increased mRNA stability (see review of mRNA stability in higher plants by Green 1993 and Schwartz et al. 2006). The increased mRNA probably corresponds to more expansin produced and consequently induced more expansion of the mutated microspores. As microspores are fused together in qrt1, the cell wall load-bearing polymers, which bridge microspores of different genotypes, will undergo differential stretching in the four cells. This unbalanced stretching tends to pull the wall apart in the less expanded microspore and results in breakage of pollen bearing
the *EXPA13* allele. However, in wild type siblings and *expa13-1* homozygous mutants, the four microspores expand synchronously, bringing equal pressure to bear their cell walls. Therefore, wild type siblings and mutant produce normal *quartet* pollen grains. The observation in *expa13-1* supports the idea that *EXPA13* functions in microspore expansion.

### 2.4.3 Pectin and Expansin Function

There are three major components of pectin in the primary cell wall, namely homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (reviewed in Cosgrove 2005). The dominant sugar residue of all three types is galacturonic acid (GalA). During pectin synthesis, the GalA is highly methylesterified (Schols & Voragen 2002). Much more unesterified GalA is detected in the older, non-elongating tissue (Fujino & Toh 1998, Liberman *et al.* 1999). It has been proposed that pectin cleavage is involved in cell expansion (e.g., guard cell expansion, Nadeau & Sack 2002) and wall separation (e.g., fruit ripening, Giovannoni 2001, Kalamaki 2003). Pectin cleavage may be accomplished through GalA demethylesterification by PME (Pelloux *et al.* 2007) and subsequent backbone scission by PGs (Tucker & Seymour, 2002). PME is encoded by a large multigene family showing high tissue specificity and functions in both vegetative and reproductive development. In addition to the function in microspore separation indicated in the *qrt1* mutant, PME is also involved in xylem cell differentiation (Micheli *et al.* 2000) and fibre development (Pelloux *et al.* 2007). Ectopic expression of PME generally led to dwarf plants and inhibited growth (Hasunuma *et al.* 2004, Bosch &
PG is also encoded by a large gene family with over 50 genes in Arabidopsis. Study of PG has predominantly focused on fruit ripening, where PGs were implicated in polyuronide depolymerization and solubilization during ripening. However, fruit softening is not affected in RNA silencing and mutant investigations (see review of Brummell & Harpster, 2001).

The synergistic action between expansin and pectinase has long been proposed. Cosgrove & Durachko (1994) observed that the expansin-induced reconstituted wall extension was enhanced by fungal pectinase pretreatment. Since no direct interaction was observed between expansin and pectin, a synergistic action between expansin and pectinase was proposed for the first time. Zhao et al. (2008) found that fungal pectinase or EGTA treatment could restore the sensitivity of mature tissues to α-expansin. Their results suggest the cooperative action between pectinase and expansin in wall relaxation and also imply that calcium bridges function as a crosslinking factors in wall stiffening.

The interaction of expansin and polygalacturonase was investigated in fruit softening studies. Brummell et al. (1999) reported that tomato fruit ripening was delayed or accelerated by suppression or over-expression of LeExp1, respectively. Polyuronide depolymerization was prevented as LeExp1 transcript was down-regulated but was not affected in the overexpression line. Simultaneous suppression of LePG and LeExp1 resulted in increased viscosity of tomato juice compared with WT and suppression of single genes (Kalamaki et al. 2003). Not only juice rheology but also pathogen susceptibility was affected in the LePG and LeExp1 simultaneous suppression line (Cantu et al. 2008). Simultaneous suppression of both genes reduced the fruit susceptibility to Botrytis cinerea whereas no change was observed in neither LePG nor LeExp1 single
suppression line. The double-suppression tomato fruit was notable in greatly reduced pectin depolymerization but no alteration in hemicellulose. This change of wall components also served as an evidence of synergistic relation between expansin and polygalacturonase.

The synergistic interaction between expansin and pectin-degrading enzyme is also suggested by the failed attempts to obtain EXPA13 qrt1 double mutant. In the qrt1 mutant, pollen fusion happens without influencing pollen viability or fertility. For the EXPA13 single mutant, no visible influence on pollen activity was observed in two independent knockout lines. However, the attempt to obtain expa13 qrt1 double mutants failed. This may be due to the pollen abnormality observed in EXPA13/expa13 qrt1/qrt1 mutant. It implies that EXPA13 and QRT1 gene function interact synergistically. A hypothesis about the mechanism of their interaction is proposed and described below. In the tetrads of EXPA13/expa13 qrt1/qrt1, two microspores carry wild type EXPA13 and the other two carry the mutant allele. QRT1 is a sporophytic gene expressed in the tapetal layer. For the two microspores carrying wt allele of EXPA13, normal protein function allows other tapetum-produced, expansion-related pectinases access to pectin polymers. The normal expansion of microspores is then enabled. In the two double-mutated microspores, the lack of EXPA13 protein not only affects sliding of cellulose microfibrils and hemicellulose chains but also prevents tapetal pectinases from gaining access to pectins in the pollen cell wall. The stiffened pollen wall retards pollen expansion and sometimes leads to tearing when high turgor pressure built inside. As more polysaccharides are integrated into the pollen wall, the survived, double-mutated microspores end up with a thick and tough wall, which prohibit them from germination
on stigma. Analysis of pollen viability and pollen tube in-vitro growth of 
EXPA13/expa13 qrt1/qrt1 pollen will be an excellent supplement to test this hypothesis.

2.5 Summary

EXPA13 is expressed in microsporogenesis during the stages 10 to 12 of flower 
development and therefore is proposed to function in microspore separation and 
expansion. The expression was supported by RT-PCR, promoter::GUS observations, and 
microarray data from the literature. The GUS staining pattern suggests both sporophytic 
and gametophytic expression of EXPA13. Expa13-1, a mutant with enhanced EXPA13 
transcript on qrt1 background, produces half-collapsed tetrads in heterozygous mutant. 
Wild type microspores tend to collapse, which is explained by the imbalanced expansion 
of microspores in heterozygous tetrads. As for the two knockout mutants, expa13-2 and 
expa13-3, the size of pollen grains was not significantly different from wt. The attempt 
to obtain expa13 qrt1 double mutant failed. I proposed that this is due to the synergistic 
function between expansin and pectin-degrading enzymes. In this work, QRT1 mutant 
served as a nice background for exploring expansin function. It enabled me to disclose a 
phenotype due to the altered EXPA13 expression. It also provides additional evidence for 
expansin-PME interaction, thus widening the proposed role of expansin in cell wall 
functions.
References


Chapter 3

EXPA13 and the Vegetative Growth of Arabidopsis

3.1 Introduction

Leaf expansion and stem elongation are two major events in the vegetative growth of Arabidopsis. The leaf is the organ where photosynthesis occurs whereas the stem functions to lay out leaves and inflorescences for high efficiency of light interception and reproduction. The details of their development, still under investigation, are complicated by multiple cell types and complex patterns of growth that involve both cell division and expansion.

3.1.1 Leaf Development in Arabidopsis

All the aerial parts of a plant come from a group of small dividing cells forming the shoot apical meristem (SAM), which is located at the shoot tip. The number of stem cells in the SAM is maintained by a balance between cell division and cell differentiation, which is controlled by genes such as WUS, CLV1-3, KAPP, and STM. In dicots, the SAM is composed of three layers, L1, L2 and L3, from the outermost to the innermost. Cell lineage studies showed that the leaf epidermis is derived from the L1 layer, whereas the mesophyll and vascular tissues are from the L2 and L3 layers, respectively (review in Steeves and Sussex 1989).
In *Arabidopsis*, the earliest leaf primordia are initiated as small spherical bulges at the periphery of the embryo SAM. High expression of the *PIN1* gene, which encodes an auxin-efflux-protein, was detected on the sites of leaf primordia (Reinhardt et al. 2003, Wisniewska et al 2006). At the same location, high auxin distribution was detected using the *DR5rev::GFP* construct (Smith et al. 2006). Both lines of evidence suggest auxin accumulates and functions in the leaf primordia. Shortly afterwards, two bands of cells form on the sides of the primordium. The bands will develop into the leaf blade whereas the central region will differentiate into the midrib. Leaf growth is dependent on both cell division and cell expansion. As cell division ceases, cells continue to expand, attaining sizes that are 20 to 50 times the size of meristem cells (Maksymowych 1973). Leaf shape and size are largely controlled by genes and also regulated by environmental factors, such as light and water (Tsukaya 2005).

Like most plants, *Arabidopsis* produces leaves of varied morphology at different stages. Rosette leaves are formed in early development and have long petioles and broad blades. Cauline leaves are produced on the nodes of the inflorescence stem and have narrower blades without petioles. Rosette leaves were investigated in this study of *EXPA13* function.

### 3.1.2 Inflorescence Stem Elongation in *Arabidopsis*

The plant stem is generally divided into node and internode regions. The node is where leaves or branches are located whereas the internode is located between nodes. During *Arabidopsis* rosette formation, very little internode growth takes place. As the
plant is transiting to reproductive development, internodes elongate rapidly between the last two to three leaf nodes. Therefore, the inflorescence stem of *Arabidopsis* is an elongated shoot above the rosette in ontogeny. Many mutants with defects in internode elongation were identified as gibberellic acid (GA)-biosynthetic or GA-insensitive mutants. This suggests that GA functions in internode elongation.

3.1.3 Vascular Development in *Arabidopsis*

In *Arabidopsis*, procambium precursor cells are distinguishable as the embryo transits from the globular to the heart stage (West and Harada 1993). In the mature embryo, procambial cells form a continuous network extending through the cotyledons, hypocotyl, and root without differentiation (Busse and Evert, 1999). The procambium differentiates into xylem and phloem after seed germination. Both xylem and phloem are complex tissues consisting of multiple cell types. In addition to ground tissues like fibers and parenchyma cells, xylem has tracheary elements (TE) and phloem contains sieve elements and companion cells. TE differentiation in *Arabidopsis* is a genetically controlled process, which will be discussed in Section 3.1.4. At maturity, xylem acts as the conduit of water and soluble minerals whereas phloem functions as the photosynthate-transporting tissues of the plant.

Leaf vasculature is arranged into a net-like network known as vein. In *Arabidopsis*, the primary vein extends from leaf base to apex (acropetally), with 6 to 10 secondary veins branching out. The adjacent secondary veins connect and form closed loops. The tertiary and quaternary veins may subdivide the closed area or form free ends.
Veins other than the primary vein develop basipetally (from leaf apex to base). In the Arabidopsis stem, five to eight vascular bundles (xylem inside and phloem outside) are arranged in a circle, formed during primary vascular tissue development. Xylem differentiation progresses from stem center to edge (Turner & Somerville 1997). Phloem differentiation is hard to visualize but appears to precede xylem development.

Leaf vascular patterning involves auxin. Jacobs (1950) demonstrated that increasing concentration of auxin quantitatively induced vasculature regeneration. Thompson and Jacobs (1966) observed that regeneration could be reduced by auxin polar transport inhibitors. By means of PIN1::GFP and DR5rev::GFP observation and recent technique of time-lapse imaging, auxin accumulations were detected at the incipient leaf base and then in the leaf margin before vascular differentiation in Arabidopsis (Scarpella et al. 2006, Sawchuk et al. 2007). The best supported mechanism of leaf vascular patterning is the “canalization hypothesis”. It states that, as auxin is transported through leaf cells, it promotes cell differentiation into vasculature. This will in return improve the cell capability of auxin transport (Sachs 1989).

3.1.4 Xylem Development in Arabidopsis

As the primary conducting cell of the xylem, tracheary elements (TE) have two cell types, the tracheids and the vessel elements (VE). Seedless vascular plants and most gymnosperms (except Gnetophyta) possess only tracheids as water conducting cells whereas most angiosperms and Gnetophyta have both. Wall pores form in the cell walls of adjacent VE (known as perforations) and distinguish VE from tracheids. The
corresponding structure in tracheids is the pit pair through which water flows between tracheids.

TE development involves complicated modifications of the cell wall. The differentiating TE first undergoes significant elongation. In zinnia stems, expansin mRNA is preferentially localized to the ends of differentiating TE, which suggests expansin involvement in the polar extension of cell walls. A secondary wall is then laid down in annular, spiral, reticulate or pitted patterns. The deposition pattern is precisely defined by the function of cortical microtubules (MTs), which control the deposition of cellulose microfibrils. The majority of mature TEs construct a secondary cell wall. In addition to cellulose, it is also composed of hemicellulose (mainly xylan in dicots wood) and lignin. Lignins are polymers of monolignols, which strengthen the plant vasculature together with the deposited cellulose. After the secondary wall thickens, differentiating TEs undergo the programmed cell death and perforation plate formation. A mature vessel element is subsequently ready for water transport (Busse & Evert 1999).

The aim of this work is to study the function of expansin gene, *EXPA13*, in vegetative growth of *Arabidopsis*. The correlation between *EXPA13* transcript and the growth pattern of leaf expansion and stem elongation has been investigated through semi-quantitative RT-PCR and GUS staining analysis. A substantial defect in vegetative growth and consequent reduction of seed yield has been observed in two independent *EXPA13* knockout mutants. Because of the different genetic backgrounds of the different ecotypes, the mutant phenotypes are not of the same extent in the two lines. *Expa13-2*, which shows a more severe phenotype, has been employed for a complementation test.
Comparison of vascular morphology in wild type siblings and homozygous mutant gave hints to the function of *EXPA13* in vasculature development.

### 3.2 Materials and Methods

#### 3.2.1 Plant Materials and Growth Conditions

All studies were carried out using the *Arabidopsis thaliana* Columbia (Col) or *Landsberg erecta* (Ler) plants. For all experiments, seeds were surface-sterilized for 2 min in 70% ethanol and then for 8 min in 50% bleach plus 0.02% Triton X-100. Seeds were then rinsed in water and poured onto MS plates as described in Section 2.2.1 of Chapter 2. After two days of cold treatment at 4 °C, plates were placed into growth chamber under long-day conditions (16 h light at 22 °C and 8 h dark at 16 °C). Photon fluence rate was 70 µmol m⁻² s⁻¹.

For transcript analysis, rosette leaves from 21-day-old *Arabidopsis* were collected and stored at -80 °C. Stem tissue for transcript analysis was collected from the inflorescence stem of 34-day-old *Arabidopsis* and stored at -80 °C.

#### 3.2.2 RNA Extraction and Semi-quantitative PCR

Total RNA was extracted from plant tissues using an RNeasy plant kit (Qiagen, Valencia, CA) following the manufacturer’s handbook. An on-column DNase I treatment was performed using RNase-free DNase set (Qiagen, Inc., Valencia, CA).
Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was employed to assay gene transcript level. RT-PCR was performed with a Titan One-Tube RT PCR kit (Roche Applied Science, Indianapolis, IN). The starting amount of RNA template (10 ng per reaction) was determined using absorbance at 260 nm (GeneQuant RNA/DNA Calculator, Pharmacia Biotech, Cambridge, England) and then finely normalized using actin or 18S rRNA band as reference. The gene specific fragment was then amplified using primers AE13SpSn and AE13SpAs. The primer 13cexon1R and 13exonsF were also used to detect the partial segment of EXPA13 transcript (Table 3.1). The intensity of ethidium bromide-stained band was photographed with a Polaroid camera (Gelcam, UK) and quantified using ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA).

Table 3.1: Primers for semi-quantitative RT-PCR of EXPA13 mRNA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Band size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE13SpSn</td>
<td>CGACCTCTTCACCTTCTTCGTTCCTC</td>
<td>986 bp</td>
</tr>
<tr>
<td>AE13SpAs</td>
<td>GCTTTCGCTCCTTAGTAATGTAAACAAATAG</td>
<td></td>
</tr>
<tr>
<td>13cexon1R</td>
<td>TGGATACGGAGATCTCGTCAAATC</td>
<td>705 bp</td>
</tr>
<tr>
<td>13exonsF</td>
<td>ACCAAGATCGATATACTTGCCATCA</td>
<td></td>
</tr>
<tr>
<td>18rRNA_F</td>
<td>TTGTGTGCTGCTCGGGATCGGAGTAAT</td>
<td>446 bp</td>
</tr>
<tr>
<td>18rRNA_R</td>
<td>TGCACCACCACCATAGAATCAAGAAAGA</td>
<td></td>
</tr>
</tbody>
</table>
3.2.3 Histochemical GUS Staining and Observation

A transcriptional fusion of the *EXPA13* gene promoter with the GUS gene was made by Cosgrove *et al.* (2000). The detailed procedure was described in Section 2.2.3 of Chapter 2. For histochemical GUS staining, rosette leaves from the homozygous T2 plants were incubated in staining buffer (Jefferson *et al.* 1987) containing 0.5 mg/mL X-Gluc at 37 °C in dark for 24 h. The tissues were then cleared in 70% ethanol and examined under a microscope.

3.2.4 Mutant Screening

*Expa13-2 (GT_5_8306, Ler-1 background)* is a Ds gene trap Line (Sundaresan *et al.* 1995) produced at the John Innes Centre (Norwich, UK). It was generated by the mobilization of a gene-trap Ds transposable element which could be screened using kanamycin. For genotyping purpose, the gene specific primers (MA13ELP and MA13ERP) and the insertion specific primer Ds3-1 JIC-GT were used for PCR (Table 3.2). The gene specific primers used in this work were designed using T-DNA Primer Design ([http://signal.salk.edu/tdnaprimers.2.html](http://signal.salk.edu/tdnaprimers.2.html)) of SIGnAL (Salk Institute Genomic Analysis Laboratory).
Expa13-3 (GABI_290B03, Col-0 background) is a GABI-Kat T-DNA insertion line from the Max Planck Institute for Plant Breeding Research (Cologne, Germany) (Rosso et al. 2003). The antibiotic sulfadiazine was applied for mutant screening. PCR-based screening was performed with gene specific primers (MA13FLP and MA13FRP) and the T-DNA specific primer MA13FBP (Table 3.3).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA13ELP</td>
<td>AGCACACGGATATCGAAGAAG</td>
</tr>
<tr>
<td>MA13ERP</td>
<td>CAAATGTCAAATTGATCCAAATG</td>
</tr>
<tr>
<td>Ds3-1 JIC-GT</td>
<td>ACCCGACCGGATCGTATCGGT</td>
</tr>
</tbody>
</table>

As for PCR-based genotyping, genomic DNA was extracted from young rosette leaf following a rapid DNA extraction protocol (Kasajima et al. 2004). The primer annealing temperature was predicted using Gene Runner 3.03 software (Hastings Software, Inc., Hasting, NY) with the settings of 100,000 pM probe concentration and 50 mM salt concentration. Taq DNA polymerase (GeneChoice, Inc., Frederick, MD) were used for PCR. All PCR reactions were conducted in a PTC-100 Programmable Thermal Controller or PTC-200 Peltier Thermal Cycler (MJ Research, Inc., South San Francisco,
CA). The following programming was used for PCR reactions: 2 min at 94°C for denaturation, followed by 35 cycles of 94°C for 25 sec, 58°C for 1 min, and 72°C for 1 min, and finally one cycle of 5 min extension step at 72°C.

3.2.5 Growth Measurement

For leaf measurement, *Arabidopsis* rosettes were photographed (Nikon, Coolpix 990, Japan) at 12 pm of day 27, 29, and 31 after seed germination. The total leaf area, whole leaf length, and blade length were then measured using SPOT 3.5.2 (Diagnostic Instruments, Inc., Sterling Heights, MI) software. The stem elongation profile was obtained when the inflorescence stem reached 7 cm long, measured from the rosette leaf to the base of the terminal flower. Fifteen marks were made which evenly demarcated the whole stem into fourteen segments, 5 mm each. After 48 hours, the length between adjacent marks was measured and used to calculate the stem elongation rate. The equation used for calculation is indicated below:

\[
\text{Stem Elongation Rate} = \frac{(\text{Segment length after 48h} - \text{Initial segment length})}{\text{Initial segment length} \times 48 \text{ h}} \times \%
\]

( % h\(^{-1}\) )

To trace the day-to-day elongation of the inflorescence stem in wild types and mutants of *expa13-2*, the stem length was measured at 12 pm daily starting with the appearance of white petal in the first flower and ending when flowering stopped. The silique measurement was performed on siliques of representative length by ignoring the first few siliques formed in the stems. For the measurement of seed yield, the plants were
bagged when the first few siliques began to turn yellow. After the whole plants dried out, the seeds were harvested and weighed.

3.2.6 Complementation Test for *Expa13-2*

The complementation of *expa13-2* was conducted by introducing the wild type genomic DNA of *EXPA13* into the mutant plant. DNA was extracted from wild type siblings with phenol/chloroform and used for cloning a 3.5 kb genomic DNA, which harbored the *EXPA13* gene and the 1.5 kb sequence upstream of the start codon. Primers 13CPMF and 13CPMR (Table 3.4) were used for cloning and introducing Bam H1/Pst I restriction sites into the cloned sequence. The Bam H1/Pst I fragments was then inserted into a binary vector, pCambia1300 (CAMBIA, Canberra, Australia). Transformation was performed to transfer the binary vector into competent *Agrobacterium tumefaciens* (GV3101) cells. *Arabidopsis* transformation was carried out using the floral infiltration method of Tague (2001). The homozygous T2 generation was screened and used for further observation. Six complementation lines were carefully examined for phenotype analysis. One representative line was subjected to detailed growth measurement.

Table 3.4: Primers used for the complementation test of *expa13-2*. The underlined sequence introduced two restriction sites, Bam H1 and Pst I, into the cloned sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>13CPMF</td>
<td>GGATCCAAATTGCAAGTCGGTCCAAAG</td>
</tr>
<tr>
<td>13CPMR</td>
<td>CTGCAGGTCCATGTCATGCTCAATGC</td>
</tr>
</tbody>
</table>
3.2.7 Light Microscopy

Whole leaves of *expa13-2* wild type siblings and homozygous mutants were collected from fully expanded rosettes. They were vacuum-fixed in a fixative containing 4% paraformaldehyde, 0.1% gluteraldehyde, and 50 mM Na phosphate buffer (pH 7) for 24 h at 4°C. Tissues were trimmed to proper size and then embedded in 5% low melt agarose to help with positioning in resin. Dehydration was then carried out stepwise through 10, 25, 50, 70, 80% ethanol. Later on, the tissue blocks were infiltrated with LR white resin/ethanol (3:7, 1:1, and then 3:1) for 2 h at each step before being embedded in 100% LR white resin (London Resin Co., Ltd., Berkshire England). Sections of 2 μm thick were cut and stained with 1.5% safranin-o (Fisher Scientific Company, Pittsburgh PA, USA) for microscopy. Microscopic observation was performed with a Zeiss Axioplan (Opto-systems, Inc., Jenkintown, PA) and a UV filter set. Digital images were captured through a ProgRes C14 camera and software (JENOPTIK Laser System GmbH, Munich, Germany). Image measurement was acquired using SPOT 3.5.2 (Diagnostic Instruments, Inc., Sterling Heights, MI) software.

3.2.8 Wall Extension Assay of *Expa13-2* WT and Mutant

Creep extension assays were performed following Cosgrove (1989). The tip region of the inflorescence stem was collected as the whole stem reached 7 cm in length and stored at -80°C. Frozen samples were then abraded using carborundum, rinsed and pressed under 200 g weight. Tip regions of 1 cm were then clamped in an extensometer with a constant tension of 20 g. Samples were first incubated in 50 mM Na Acetate (pH
6.8) for 30 min and then changed to the same buffer at pH 4.5 for another 120 min. The wall extension was recorded by a position transducer connected to a computer for data acquisition and exported later for data analysis.

3.3 Results

3.3.1 EXP413 Expression in Rosette Leaves

Figure 3.1 shows the transcript levels of EXP413 in Arabidopsis rosette leaves. Rosette leaves from a 21-day-old plant was collected for RNA extraction and semi-quantitative RT-PCR. To normalize mRNA level, 18S rRNA primers were used for RT-PCR with 18 cycles of PCR. EXP413 gene specific primers, 13cexonsR and 13exonsF (Table 3.1) were used in RT-PCR with 30 cycles of PCR. Quantification of RT-PCR bands indicated that EXP413 is transcribed in Arabidopsis rosette leaves (Figure 3.1A) and the expression level is modulated according to the stage of leaf development. Younger leaves showed higher EXP413 transcript levels compared to expanded leaves (Figure 3.1B).
EXPA13 transcript level in elongating inflorescence stem was detected by semi-quantitative RT-PCR using primers shown in Table 3.1. The stem segments (20 mm) were harvested from different positions of the inflorescence stem (basal, intermediate, and tip region, Figure 3.2 sample #1 - #3). To avoid the potential effect from gene expression in the node region, an internode-only sample (Figure 3.2 sample #4) was also obtained for transcript detection. Figure 3.2 showed that EXPA13 is expressed in the
elongating inflorescence stem. The expression level in the tip region is more than four times higher than that of the basal region. The RT-PCR of internode-only sample showed that \textit{EXPA13} expression was not constrained to the node region of elongating stem. This suggests \textit{EXPA13} functions along the whole developing stem. \textit{EXPA13} expression was also evident in elongating siliques.

Figure 3.2: Transcript levels of \textit{EXPA13} gene in the elongating inflorescence stem and siliques of \textit{Arabidopsis}. A. mRNA levels were detected by semi-quantitative RT-PCR using endogenous control \textit{18S rRNA}. B. Quantification data obtained using ImageQuant 5.2. Percentage was calculated with reference to sample #3. Samples #1 - #4 were collected from the inflorescence stem of 34-day-old \textit{Ler}, which include: sample #1, 20 mm basal region; sample #2, 20 mm intermediate region; sample #3, 20 mm tip region not containing any flower stalk or bud; and sample #4, 20 mm tip region, internode only. Sample #5 was pool of five elongating siliques.
3.3.3 Stem Elongation Kinetics

To exam whether the *EXPA13* expression profile is correlated with the growth distribution in the developing stem, stem elongation kinetics was studied. Figure 3.3 displays the elongation distribution of the inflorescence stem in *Arabidopsis Ler-1*. This measurement was performed on 7-cm-long stems as they were undergoing the most rapid elongation throughout the whole process (with reference to Figure 3.11 A). The length change of each 5 mm segment was recorded and calculated for the elongation rate (% h\(^{-1}\)) using the equation mentioned in Section 3.2.5. Most plants were over 14 cm after 48 h. The results indicate that the tip region has highest elongation rate whereas the basal region is not growing. The growth rate at the tip region was over twenty times that of the intermediate region. The trend of decreasing growth rate from the tip to the basal region is consistent with the decreasing *EXPA13* transcript level along the same regions.
3.3.4 Phenotypic Observation in Expa13-2

3.3.4.1 Rosette Leaf Phenotype

In the knockout mutant *expa13-2*, a substantially reduced growth of rosette leaves was observed in the homozygous mutant (Figure 3.4). The mutant leaves were shorter in both the blade and the petiole compared to the wild type siblings. The reduction in leaf width was less than that in the length but still showing significant difference. In addition to the size change, mutant leaves were also different in shape. The mutant leaf lamina lost curvature and seemed flat on the side view. The leaf phenotype is largely complemented in the complementation line as indicated in Figure 3.4.

Figure 3.3: The elongation rate of inflorescence stem in *Arabidopsis Ler-1*. Plants with 7 cm long stem were used for elongation measurement (around 34 days after germination). The stem was marked as 14 segments of 5 mm each. After 48 hr, measurement was performed again to determine the elongation rate (% h⁻¹). n = 11. Data shown are means ± SE.
Figure 3.4: Phenotype of *expa13-2* in rosette leaves. A. Semi-quantitative RT-PCR showing *EXPA13* transcript in WT (lane 1), homozygous mutant (lane 2), and complementation line (lane 3). B. Rosettes of 27-day-old WT, homozygous mutant, and complementation line. Embedded pictures show side view of rosette leaves. Bar = 10 mm. C. Overall rosette leaf area was measured on pictures of 27, 29, and 31-day-old plants using SPOT3.5.2. n=8. The overall leaf size of *expa13-2* homozygous mutants are significantly different from the WT siblings (Two-tail T-test, p<0.05). D. Whole leaf length, leaf blade length and width were measured in 31-day-old WT and *expa13-2*. n=8. Significant differences were detected in whole leaf length (Two-tail T-test, p<0.05). Data shown are means ± SE.
### 3.3.4.2 Inflorescence Stem Phenotype

The flowering time of *Arabidopsis* is defined to begin as floral buds become clearly visible, which is followed by rapid stem elongation. In *expa13-2*, no difference of flowering time was observed between the wild type sibling and mutant. However, the rate of stem elongation was significantly reduced in the homozygous mutant. This resulted in significant difference in the length of inflorescence stem after growth ceased. This phenotype was fully complemented in the complementation line (Figure 3.5).

![Figure 3.5: Phenotype of inflorescence stem length in *expa13-2*. A. Inflorescence stem of wild type siblings, homozygous mutant, and complementation line of *expa13-2*. Bar = 5 cm. B. Inflorescence stem length after most flowers ceased flowering. n = 12. Significant difference was detected between wild type siblings and the homozygous mutant (Two-tail T-test, p<0.05). Data shown are means ± SE.](image)

### 3.3.4.3 Phenotype of Seed Production

Seed production in *expa13-2* homozygous mutant was greatly reduced compared to wild type siblings and the complementation line (Figure 3.6F). As no ovary defect was observed in the homozygous mutant, it was proposed that the seed production reduction was due to a decreased growth of the stem. As shown in Figure 3.6C, silique length of
homozygous mutant was about 20% shorter than that of wild type. Comparable to this, approximately 20% less seeds were produced in individual siliques of the mutant compared with the wild type siliques (Figure 3.6D). Silique number in the mutant was almost half that of the wild type and complemented plants. In wild type siblings (45-day-old, Figure 3.6A) and in the complementation line (55-day-old, Figure 3.6B), the fully expanded siliques were straight and slim in shape and the elongating siliques were of similar appearance but shorter. However, siliques of the expa13-2 mutant were stunted and even curly, which might constrain seeds from normal development. When harvested, a large number of aborted seeds were observed in the mutant. Flowers of expa13-2 were also examined which showed no visible defect in flower development and pollination. In summary, it is suggested that the reduced yield of EXPA13 mutant is a secondary effect derived from decreased stem and silique elongation.
Figure 3.6: Expa13-2 mutant is greatly reduced in seed production compared to wild type and complementation line. A. Tip region of inflorescence stem in 45-day-old WT and homozygous mutant. B. Tip region of inflorescence stem in 55-day old complementation line. C. Expa13-2 mutant siliques are significantly shorter than wild type. Bar = 10 mm. D. Expa13-2 mutant has less seeds produced in individual silique compared to wild type. E. Less normal siliques formed in expa13-2 mutant than wild type. F. Seed yield in expa13-2 mutant is lower than wild type. n = 8, p<0.05, two-tail T-test. Data shown are means ± SE.
3.3.5 Phenotypic Observation in *Expa13-3*

To investigate whether the phenotypes observed in *expa13-2* were found in another independent mutant line, I screened for another knockout mutant *expa13-3*, which is a *Col-0* background T-DNA mutant as indicated in Chapter 2 (Section 2.3.4.1). Data of leaf size, inflorescence stem length, and siliques length were obtained from wild type siblings and homozygous mutants and are presented in Figure 3.7. It is demonstrated that similar phenotypes, including smaller leaf, shorter stem and siliques, were observed in *expa13-3*. The growth reduction due to *EXPA13* mutation is less severe in *expa13-3* than in *expa13-2*. This suggests that *EXPA13* contributes unequally to *Arabidopsis* growth in different ecotypes.
Histochemical analysis of **EXPA13** promoter::GUS plants was used to examine the localization of promoter activity. Consistent with the semi-quantitative RT-PCR results, GUS staining was most intense in expanding leaves and gradually disappeared with age. (Figure 3.8). The GUS staining was limited to vascular bundle areas as shown in Figure 3.8A. After transition to the phase of reproductive growth, GUS staining was
found in the vasculature of the stem tip. Figure 3.8C shows that intense GUS staining was observed in the vessel elements which were undergoing secondary wall thickening. However, the GUS staining was faint either in differentiating xylem as they just became apparent as narrow cells without secondary wall thickening (Figure 3.8B) or in formed vessel elements (Figure 3.8D). GUS staining was visible not only around the vein termini but also along the connected veins which jointed to pre-existing vasculature at both ends. This expression pattern suggested that *EXPA13* facilitates vessel element development, during cell expansion and/or secondary wall deposition stage. *EXPA13* expression in the vascular bundle is consistent with the overall deficit in the vegetative growth of the knockout mutant.
3.3.7 Vasculature Observation

As the GUS staining pattern implicated the vasculature, especially developing vessel elements, as the location of EXPA13 protein function, a morphology observation of EXPA13 mutant vasculature may provide more information about its function. To reduce developmental variation, fully expanded leaves from wild type siblings and

Figure 3.8: Light microscopic images of EXPA13 promoter::GUS transgenic plants. Leaf #8 from 20-day-old rosette was used for GUS staining and observation. Bar = 400 μm (A), 50 μm (B & D), 25 μm (C). Arrow (C) points to an emerging vessel element with a few bands of secondary cell wall thickening visible.
homozygous mutants of *expa13-2* were used for resin embedding and sectioning. Cross sections were made 1/3-leaf-length to the base of petiole and perpendicular to the leaf main vein. Since the side veins were generally less than 50 μm in diameter, only sections of main vein were shown here (Figure 3.9).

In wild type vasculature, mature vessel elements were conspicuous due to their evenly thickened cell wall. The secondary cell wall was of slightly purple color with Safaonin-O staining and gave high fluorescence intensity with UV excitation. Different from the “ring” shape in wild type, vessel elements of the mutant seemed not homogeneously thickened in their secondary cell wall. Therefore, only curves of bright blue were observed under UV light (Figure 3.9F). Two other remarkable phenotypes were found in the mutant vasculature. First, cell walls were missing in mutant vascular bundles, giving rise to multicellular cavities as indicated in Figure 3.9B and F. Multiple cavities were evident in mutant vasculature but not in wild type siblings. This was likely due to cell breakage during vessel element development but before the secondary cell wall was formed. Second, excessive cell wall thickening was observed in cells on the periphery of vascular bundle. This might be attributed to the reduced xylem elongation, which largely limited the space of nearby cells and constrained their assembly in a compact way.
Figure 3.9: Cross section of *Arabidopsis* leaf, showing vascular bundle of wild type sibling (A, C, E) and homozygous mutant (B, D, F) of *expa13-2*. A and B are light microscopic images after Safranin-O staining. C - F were fluorescence microscopic images. The white rectangle areas of C and D were magnified and showed in E and F, respectively. Arrows indicate vascular regions which were absent of cellular structure. Bar = 50 μm (A – D), 100 μm (E & F).
Vascular morphology was also investigated on the plane of the leaf surface. Fully expanded rosette leaves were collected from wild type and expa13-2 mutants. Leaves were decolored in 70% ethanol and then mounted in acetic acid to clarify the tissue. Microscopic observation was performed on tertiary veins considering their structural simplicity. As shown in Figure 3.10A and B, mutant veins were more curly compared to wild type veins. This difference was quantified by measuring the angle of curvature using the measurement tool of software SPOT3.5.2. The angle value between 0 - 180° was recorded for quantification. Wild type vasculature was of significantly larger angle value compared to mutant, which corresponds to less curvature in appearance (Figure 3.10C).
3.3.8 Acid-induced Extension of Arabidopsis Stem Tip

Acid-induced extension is one of the characteristic features of expansin (Cosgrove 2005). As the most efficient and reproducible technique for measuring this extension, the wall extension assay (Cosgrove 1989) is conventionally used in biophysical studies of expansins. However, Arabidopsis is not a preferred species for this creep assay. In this work, stem elongation kinetics was traced after flowering (Figure 3.11A). Tip regions

Figure 3.10: Longitudinal view (A & B) and curvature quantification (C) of the tertiary vein in expa13-2. A. Wild type sibling. B. Homozygous mutant. C. Quantification of vein curvature using SPOT3.5.2. n = 38 – 42. Two-tail T-test, p = 8.5E-10. Data shown are means ± SE. Bar = 50 μm.
from the most quickly elongating part of the stem (7 cm-long stem) were used for the measurement of acid-induced extension. Stem tips were collected from wild type plants 3 days after flowering. However, at the same time, the mutant stems were just half of the wild type but larger in diameter (~30% higher in dry weight). To avoid difference arisen from sample dimension, mutant stem tips were collected 6 days after flowering, at which point they were also 7 cm in stem length.

As depicted in Figure 3.11B and C, the differences of acid-induced elongation between wild type and mutant stem tips were not statistically significant. As for the change in length, \( p = 0.155 \) at 60 min and \( p = 0.080 \) at 80 min. As for the creep rate, \( p = 0.068 \) at 60 min and \( p = 0.056 \) at 80 min. This may be because that \( EXPA13 \) is only expressed in vessel element which only accounts for a small proportion of the whole stem cells. However, more samples may be helpful to give more affirmative answers to this study. This experiment also suggests that the stem tip of \( Arabidopsis \) is an applicable sample for extensometer measurement.
Figure 3.11: Acid-induced extension of *Arabidopsis* stem tip in expa13-2 wild type and mutant. **A.** Length and growth rate of inflorescence stem in WT and expa13-2. Stem length was measured daily after flower appeared. n = 16. **B.** Length and rate profiles (averages of samples) of acid-induced extension for wild type and mutant stem tip. **C.** Sample length and creep rate at 60 min and 80 min. n = 4 ~ 6. Data shown are means ± SE.
3.4 Discussion

This study describes the function of expansins in xylem vessel development of Arabidopsis thaliana, which has been barely investigated to date.

3.4.1 EXPA13 Expression Pattern in Rosette Leaf and Inflorescence Stem

Gene expression analysis by RT-PCR and GUS observation revealed that the EXPA13 transcript is broadly distributed in expanding rosette leaves and elongating inflorescence stems of Arabidopsis. The EXPA13 expression pattern is consistent with the growth pattern in Arabidopsis vegetative growth.

Arabidopsis leaves are “determinate structures”, which means they expand during a given period of time until they reach certain size and form. The lamina area of the Arabidopsis leaf increases exponentially during the initial stages of leaf expansion and subsequently at a decreasing expansion rate (Granier et al. 2002). Therefore, younger leaves have a higher expansion rate compared with older leaves in the rosette. Semi-quantitative RT-PCR results indicated that EXPA13 had a higher expression level in expanding rosette leaves than in older leaves. As EXPA13 transcript was detected throughout the leaf expanding stage, it is suggested to affect leaf expansion through regulating cell expansion.

EXPA13 transcript displayed a trend of increasing levels from the basal to the tip region of the elongating stem. To exam the correlation between this trend and the stem elongation pattern, a growth profile was obtained from fast elongating inflorescence stems. Over the 48 hours between two measurements, inflorescence stems double their
length and the elongation rates ranged from over 4% h\(^{-1}\) at the tip region down to zero at the base. Therefore, the highest \(EXPA13\) transcription happens in the most rapidly elongating region of stem and the lowest transcript level is detected in the non-elongating region.

Consistent with the RT-PCR results, analysis of GUS activity showed this preferential expression in young leaves and stem tips. Affymetrix data also implied increasing \(EXPA13\) transcript level from older to younger leaves (Schmid \textit{et al.} 2005), from the bottom to the top of the stem (Suh \textit{et al.} 2005), and from established to elongating siliques (Schmid \textit{et al.} 2005). Based on these investigations, I conclude that \(EXPA13\) is widely expressed in expanding rosette leaves, elongating stems and siliques, with higher expression level in younger tissues.

\subsection*{3.4.2 \textit{EXPA13} Function in Vessel Element Development}

\subsubsection*{3.4.2.1 Expansins in Vascular Development}

Expansin function in vasculature development has been proposed in some studies. Im \textit{et al.} (2000) reported the apical localization of three \(\alpha\)-expansins (\(ZeEXP1\), 2, and 3) in differentiating tracheary elements (TEs) from zinnia. The work suggested that expansin might be involved in the elongation of xylem cells. Pesquet \textit{et al.} (2005) identified another two expansins, \(ZeEXP4\) and 5, from differentiating zinnia cells and localized \(ZeEXP5\) in TEs. Gray-Mitsumune \textit{et al.} (2004, 2008) identified multiple xylem-formation-associated expansin genes (\(PttEXPA1\), 5, 7, 15, and 16) in poplar. \(PttEXPA1\) over-expression in aspen resulted in increased stem internode elongation and
leaf expansion, and larger leaf epidermis cells. Geisler-Lee et al. (2006) proposed some poplar expansins are involved in wood cell expansion whereas some function in secondary wall formation of xylem based on their expression profile. In *Arabidopsis*, *EXPA10* gene suppression and over-expression resulted in smaller and larger leaf blade size, respectively (Cho & Cosgrove 2000). *EXPA10* expression was observed only in midrib and petiole. Coordination between midrib growth and blade expansion was proposed to explain this phenotype. These investigations proposed expansin function in vasculature development and potentially regulate plant size.

### 3.4.2.2 Vegetative Growth Defect in *EXPA13* Mutants

In this work, two *EXPA13* knockout mutants were found to show reduced vegetative growth, including smaller leaf size, reduced stem elongation, and suppressed silique growth. These two mutants are in different genetic backgrounds and the severity of the phenotype is different. About 30% reduction of vegetative growth was observed in *expa13-2* (Landsberg background) whereas around 10% inhibition was found in *expa13-3* (Columbia background). Phenotypes were almost completely recovered in complementation test, confirming the *EXPA13* function in *Arabidopsis* vegetative growth.

### 3.4.2.3 Localization of *EXPA13* Expression

Based on GUS staining analysis, *EXPA13* expression is localized to differentiating vasculature. As intense staining is present around the thickening vessel elements, I propose that *EXPA13* functions in expansion of vessel elements and/or in
secondary wall deposition. Microarray data from Poplar eFP Browser (bar.utoronto.ca) indicated that the ortholog of *EXPA13* in poplar is also expressed in xylem.

Similar phenotypes, including smaller rosette, lower inflorescence stem, and shorter siliques, were observed in *irregular xylem* (*irx*) mutants of *Arabidopsis* (Turner & Somerville 1997, Brown *et al.* 2005). The *irx* mutants were characterized by their collapsed xylem vessels. Due to cellulose deficient in their secondary cell wall, xylem vessels are too weak to withstand the negative pressure associated with water transport. The *irx* loci have been identified as cellulose synthase or other cellulose and lignin deposition related proteins (Persson *et al.* 2007). Studies on *irx* mutants imply that xylem development defect could result in overall decreased vegetative growth. Therefore, xylem vessels are suggested as a control factor of plant size. Phenotype similarities also support the hypothesized function of *EXPA13* in xylem development.

### 3.4.2.4 Hypothesis of *EXPA13* Function in Vessel Element Development

Multiple differences were observed in the vasculature of the *expa13-2*, including abnormal wall thickness, missing cell wall, and altered secondary wall thickening. Other cells in the periphery of the vascular bundle showed thickened cell walls in the mutant. Considering the heterogeneous origins of different cell types, it is logical to propose that inhibited vessel element elongation constrains other vascular cells from normal expansion in the long axis of the leaf. As a result, wall materials deposit into limited intracellular space and thus produce thick-wall cells. Mesophyll cells are arranged less compactly compared to vascular cells. Therefore, very limited wall thickening is observed in the bundle sheath cells and no visible wall change in distant cells. The xylem in the mutants
was observed much curlier. My proposed explanation is that xylem appears curly on the overlapping region of adjacent vessel elements. Since mutants produce shorter vessel elements, they have a higher density of junction regions in certain leaf areas compared to wild type siblings which form long vessel elements. Therefore, the leaf vein is curlier in the mutant than in wild type.

Unlike wild type, the expa13-2 mutant has unevenly thickened secondary cell wall in the xylem vessels. This suggests that normal EXPA13 function is essential for secondary wall deposition in xylem cells. Cavities were observed in vasculature. Based on their morphology, they are most likely derived from the fusion of multiple dead cells. Their localization and size implicate them as failed vessel elements. My hypothesis is, due to the loss of EXPA13 function and consequent defect in secondary wall formation, some defective xylem vessels easily break when under negative pressure as water conduit. The broken vessel elements fuse as one pipeline and conduct water transport.

3.4.3 The “Epidermal-Growth-Control” and “Tensile Skin” theory

It has long been debated whether the epidermis or inner tissue of plants control the organ growth. In 1867, Kraus first postulated that the inner tissue provides the driving force for organ elongation whereas the epidermis determine the growth rate by imposing a mechanical constraint. Sachs (1887) showed a substantial length increase of inner tissue when the epidermis was removed. This suggests that the epidermis constrains tissue enlargement, but does not drive it. However, auxin studies indicated that the epidermis but not inner tissue is auxin responsive. Kutschera et al. (1987)
proposed the cooperation of epidermis and inner tissues during plant growth. Savaldi-Goldstein et al. (2007) argued the epidermis both promotes and constricts Arabidopsis stem growth. In their experiment, a brassinosteroid (BR)-biosynthetic enzyme or receptor was expressed in the epidermis of BR mutant. Transgenic plants overcame their dwarf phenotype. Studies of EXPA13 mutants and irx mutants support the conclusion that vasculature (especially xylem) development is not completely passive in Arabidopsis vegetative growth. Inhibited xylem elongation leads to reduced plant vegetative growth.

3.4.4 EXPA13 and Acid-Induced Wall Extension

The inflorescence stem tip of Arabidopsis has proven to be an excellent plant tissue for extensometer assays. Advantages include the ease of gene manipulation and convenient sample size. As indicated in the growth profile, the tip region harvested three days after flowering is good for fundamental study of EXPA13. Extensometer assay of acid-induced cell wall extension displayed a reduction in mutant samples but not statistically significant. I hypothesize that this is because the vessel element, where EXPA13 is proposed to function, accounts for only a limited proportion of stem tissues.

3.5 Summary

In this work, the expression profiles of the EXPA13 gene in the expanding leaf and elongating stem of Arabidopsis has been characterized. Semi-quantitative RT-PCR and promoter::GUS investigation demonstrated that EXPA13 was transcribed in the
vasculature of the expanding leaf and elongating inflorescence stem. Based on mutant analysis and complementation test, EXPA13 protein was predicted to regulate cell elongation as well as cell wall thickening during vessel element development. Extension assay were performed using the tip region of *Arabidopsis* stem but no significant reduction of acid-induced extension was observed in the mutant line. This experiment suggested that *Arabidopsis* stem tip is an excellent tissue sample for wall biophysical study.
References


Chapter 4

Expansins in Mature Pollen Grains of *Arabidopsis*

4.1 Introduction

At maturity, grass pollen grains contain large quantities of group-I pollen allergens. The group-I allergens are major elicitors of hay fever and asthma in human and comprise a subfamily of the β-expansin family (Cosgrove et al. 1997). Their biological function was largely unknown until presently when they were implicated in pollen development, pollen-stigma recognition, and pollen tube penetration of the stigma and style (Cosgrove *et al.* 1997, Wu *et al.* 2001). In this chapter, their homologues in mature pollen grain of *Arabidopsis* were studied.

4.1.1 Cell Wall of *Arabidopsis* Pollen Grains

The cell wall of mature pollen grains can be divided into three layers: exine, intine, and pollen coat. The rudimentary structure of exine, namely primexine, is laid down at the tetrad stage (soon after the completion of meiosis) during microsporegenesis. Using the primexine as a template, exine is deposited on the surface of microspores. The exine may be highly sculptured (with “apertures” formed) and is composed mainly of cellulose, sporopollenin, and glycoprotein. The intine forms between exine and plasma membrane during the free microspore stage. In structure and composition, the intine is similar to a primary wall of somatic cells, but is quite thick, containing cellulose
microfibrils, hemicellulose, pectin and protein. The intine is exposed to the exterior at the aperture of the exine. The pollen coat fills the sculptures of exine and is composed of lipids, proteins, pigments, and aromatic compounds (Brett & Waldron 1996).

4.1.2 Pollen Tube Growth in *Arabidopsis*

The pollen grain is highly desiccated when released from the anthers. When the pollen grain lands on the stigma surface, the pollen coat accumulates to the site of contact and forms a “foot” of lipid-rich material. After a compatible interaction between pollen grain and the stigma surface of *Arabidopsis*, the pollen grain hydrates, germinates, and forms a pollen tube. The pollen tube initially penetrates between the wall of stigmatic papilla cells, and then passes through the transmitting tract of the style and septum. Eventually, the pollen tube emerges onto the septum epidermis and travels subsequently toward ovule. Finally, the two sperm cells are delivered through the pollen tube to the embryo sac for fertilization of the egg and the central cell, respectively (review in Edlund *et al.* 2004).

The pollen tube wall is chemically distinct from somatic cell walls, since it is made up principally of callose (β-1,3-glucan) and pectin. Pollen tube growth is also unusual in that it elongates exclusively at the extreme apex (reviewed in Franklin-Tong, 1999; Lennon and Lord, 2000; Hepler *et al.*, 2001; Holdaway-Clarke and Hepler, 2003). Callose plugs are laid down at regular intervals behind the growing tip and the region behind the plug becomes vacuolated. The formation of the plug serves to maintain a region of concentrated cytoplasm, containing organelles, and the sperm nuclei, near the
growing tip. There is a highly dynamic clear zone at the extreme end of the growing tip, which contains vesicles and cell wall precursors. The activities in this zone include continual biosynthesis of cell wall and plasma membrane and turnover of the cytoskeletal components during tube elongation. Interactions with the surrounding tissues are said to guide the pollen tube growth toward the ovule (reviewed in Feijo et al. 2004). The key features of growing pollen tubes include a steep tip-focused Ca\textsuperscript{2+} gradient, oscillatory Ca\textsuperscript{2+} influx at the growing tip, complex interactions among Ca\textsuperscript{2+} influx, ROP GTPases and the actin cytoskeleton (Li et al., 1999; Gu et al., 2003). The growth of the pollen tube also exhibits an oscillatory pattern, which most likely can be attributed to oscillatory changes in extracellular pH and intracellular Ca\textsuperscript{2+}. The high concentration of protons in the tip region is correlated with the most rapid growth rates (Feijo et al., 1999).

4.1.3 Expansins in Maize Pollen Grains

Expansins are wall loosening proteins proposed to disrupt the non-covalent bonding between cellulose and hemicellulose, or between cellulose microfibrils (review in Cosgrove 2005). The sequence similarity between expansin and group-I allergens were first revealed by Shcherban et al. (1995). Cosgrove et al. (1997) reported the activity of maize group-I allergens, collectively called “Zea m 1”, in promoting cell wall extension and stress relaxation in vitro, which is characteristic of expansins. Four β-expansins (Zea m 1 a-d isoforms) were then purified from maize pollen extracts (Li et al. 2003) and each of them enhanced wall extension and wall stress relaxation in grass samples. As the most abundant isoform of Zea m 1, ZeEXPB1 was used for studying
expansin crystal structure because of its efficient purification (Yennawar et al. 2006). Valdivia et al. (2007) observed that, when large pollen loads were deposited onto a stigma, the pollen grains carrying mutated ZeEXPB1 gene were less competitive than the ones bringing the wild type copy. By immunogold electron microscopy, Zea m 1 was located in the pollen coat as well as in the cytoplasm (Wang et al. 2006). It was proposed that Zea m 1 is involved in pollen germination on the stigma.

In this chapter, mature-pollen-grain expressed expansins, including EXPA4, EXPA24, and EXPB5, were studied in Arabidopsis. Their expression profiles were constructed based on GUS activity analysis and RT-PCR. Knockout mutants were also used for the functional study of EXPA4 and EXPB5.

4.2 Materials and Methods

4.2.1 Plant Materials and Growth Conditions

All studies were carried out using Arabidopsis thaliana Columbia (Col) or Landsberg erecta (Ler) plants. For all experiments, seeds were surface-sterilized for 2 min in 70% ethanol and then for 8 min in 50% bleach plus 0.02% Triton X-100. Seeds were then rinsed in water and poured onto MS plates as described in Chapter 2 Section 2.2.1. After two days of cold treatment at 4°C, seeds were placed in a growth chamber under long-day conditions (16 h light at 22 °C and 8 h dark at 16 °C). Photon fluence rate was 70 μmol m⁻² s⁻¹. Ten-day seedlings were transplanted into soil and grown in the same chamber.
Mature pollen grains were collected in bulk from flowering inflorescences following Honys and Twell (2003). Inflorescences were collected in a large flask with 300 mL of ice-cold 0.3 M mannitol. After vigorous agitation for 1 min, the pollen suspension was filtered through 100 and 70 μm nylon mesh. Pollen grains were concentrated by repeated centrifugation steps (50 mL Falcon tubes, 450 g, 5 min, 4°C). The final compact pollen pellet was stored at -80°C.

4.2.2 RNA Extraction and Semi-Quantitative PCR

Total RNA was extracted from plant tissues using an RNeasy plant kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. An on-column DNase I treatment was performed using RNase-free DNase set (Qiagen, Inc., Valencia, CA).

RT-PCR was performed via a Titan One-Tube RT PCR kit (Roche Applied Science, Indianapolis, IN). Ten ng of total RNA, estimated by 260 nm absorbance (GeneQuant RNA/DNA Calculator, Pharmacia Biotech, Cambridge, England), was used as template for RT-PCR. 18S rRNA served as loading control. Gene specific primers, including AE4SpSn, AE4SpAs, AE24SpSn, AE24SpAs, B5SpSn, and B5SpAs, were used for gene specific amplification (Table 4.1). The intensity of ethidium bromide-stained bands was photographed with a Polaroid camera (Gelcam, UK) and then quantified using ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA).
4.2.3 Histochemical GUS Staining and Observation

Translational fusions of the expansin promoters with the GUS gene were made by Cosgrove et al. (2000). For EXP4, the XbaI to HaeII promoter fragment from BAC 17A14 was cloned into a binary vector pGPTV-HPT after blunting HaeII. For EXP24, the promoter sequence was cloned from BAC K3K3 using the following primers, 5’-TTTCTCTATTTACCACATTTGTTC-3’ and 5’-TTTCGAACACTCGCCCTTAAAC-3’. The amplified fragment was then ligated into the XbaI and SalI sites of the vector. The EXPB5 promoter was cloned from BAC T8B10 using primers, 5’-CTCTTCCATTATTTCCAGTCCG-3’ and 5’-GCTTCCTCATCTCTCAAATG-3’. The promoter sequence was then introduced into the SalI and SmaI sites of the vector. Agrobacterium tumefaciens (GV3101) was used for Arabidopsis transformation by the floral infiltration.

Table 4.1: Primers for semi-quantitative RT-PCR of EXP4, EXP24, and EXPB5 mRNA and their annealing temperature.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Band size</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE4SpSn</td>
<td>GCAATTCTATTTACCACATTTGTTC</td>
<td>310 bp</td>
<td>58°C</td>
</tr>
<tr>
<td>AE4SpAs</td>
<td>TTTCGAACACTCGCCCTTAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AE24SpSn</td>
<td>TATGGATTGCACCTATGACTAACGGGC</td>
<td>860 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>AE24SpAs</td>
<td>CTTTCGATTACATTTCCAGTCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5SpSn</td>
<td>GCTTCCTCATCTCTCAAATG</td>
<td>828 bp</td>
<td>55°C</td>
</tr>
<tr>
<td>B5SpAs</td>
<td>CTTTCGATTACATTTCCAGTCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18rRNA_F</td>
<td>TTGTGTTGGCTTCGGGATCGGAGTAAT</td>
<td>446 bp</td>
<td>58°C</td>
</tr>
<tr>
<td>18rRNA_R</td>
<td>TGCACCACCCACCAATAGAATCAAGAAAGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
method (Tague 2001). For histochemical GUS staining, rosette leaves from the homozygous T2 plants were incubated in staining buffer (Jefferson et al. 1987) containing 0.5 mg/mL X-Gluc at 37°C in dark for 24 h. The tissues were then cleared in 70% ethanol and examined under microscope. Dissection was performed when delicate structures, such as the elongating pollen tube, was observed.

4.2.4 Microscopy, Photography and Image Measurement

Microscopic observation was performed with a Zeiss Axioplan (Opto-systems, Inc., Jenkintown, PA). Digital images were captured through a ProgRes C14 camera and software (JENOPTIK Laser System GmbH, Munich, Germany). Image measurement was acquired using software SPOT 3.5.2 (Diagnostic Instruments, Inc., Sterling Heights, MI).

4.2.5 PCR-Based Mutant Screening

For genotyping, the genomic DNA was extracted from leaves using a rapid DNA extraction protocol (Kasajima et al. 2004). DNA from individual plants was analyzed by PCR using combinations of gene-specific and insertion-specific primers. All the gene-specific primers mentioned in this chapter were designed using T-DNA Primer Design (http://signal.salk.edu/tdnaprimers.2.html) of SIGnAL (Salk Institute Genomic Analysis Laboratory). The annealing temperatures of primers were estimated using the software Gene Runner 3.03 software (Hastings Software, Inc., Hasting, NY) with the settings of
100,000 pM probe concentration and 50 mM salt concentration. Taq DNA polymerase (GeneChoice, Inc., Frederick, MD) was used for PCR. All PCR reactions were conducted in a PTC-100 Programmable Thermal Controller or PTC-200 Peltier Thermal Cycler (MJ Research, Inc., South San Francisco, CA). The following programming was used for PCR reactions: 2 min at 94°C for denaturation, followed by 35 cycles of 94°C for 25 sec, 58°C for 1 min, and 72°C for 1 min, and finally one cycle of 5 min extension step at 72°C.

4.3 Results

4.3.1 GUS Analysis of Pollen Expressed Expansins

Based on histochemical analysis of GUS activity, four expansin genes showed pollen expression in Arabidopsis. As discussed in Chapter 2, EXPA13 was found to be transcribed in developing microspores. The other three expansins, including EXPA4, EXPA24, and EXPB5, showed GUS expression in mature pollen grains.

4.3.1.1 EXPA4 Promoter::GUS Expression

The GUS staining results indicated that the mature pollen expansins are expressed differentially in space and time. EXPA4 was expressed from the stages 13 to 17 of Arabidopsis flower development. The earliest expression was limited to individual pollen grains and was observed within the pollen sac. As the GUS expression was not synchronous in pollen grains of the same anther, EXPA4 was evidently transcribed by
gametophyte. Later on, all pollen grains showed GUS staining. *EXPA4* was highly expressed in mature pollen grains since the GUS staining was intense after only a brief period of staining (Figure 4.1A-D). The latest *EXPA4* staining in pollen grains was observed in the flower of Stage 17, at which point the collapsed stigmatic papillae were stained. GUS staining was also observed in pollen tubes (Figure 4.1E), root tips (Figure 4.1F) and in the vasculature (Figure 4.1G). Although the GUS staining in the root tip region was highly diffuse, darkest color was focused in the root cap cells. *EXPA4* was expressed in the whole vasculature, which is different from the xylem-specific expression of *EXPA13* (Figure 4.1H).
4.3.1.2 *EXPA24* Promoter::GUS Expression is Restricted to Pollen

*EXPA24* expression was observed exclusively in mature pollen grains and pollen tubes. As shown in Figure 4.2A, the earliest expression of *EXPA24* was detected in stage 13 flowers. GUS staining appeared first within longer stamens and then in all stamens. The staining was limited to individual pollen grains and was not synchronized in each anther. *EXPA24* expression was also observed along the pollen tube (especially the tip region) as it penetrated through the stigma wall (Figure 4.2C), grew through the ovary (Figure 4.2D), and passed through the micropyle (Figure 4.2E) for fertilization. The latest staining in the ovary was observed in the embryo sac after pollination (Figure 4.2F). The staining on stigmatic papillae lasted until stage 18 (Figure 4.2H).
The expression pattern of EXPB5 was similar to EXPA24 except that the earliest staining was in the stage 12 flower (Figure 4.3A). The distinguishing characteristic of stage 12 was that the bud was still closed but petals were leveled with the long stamens. EXPB5 was also exclusively expressed in mature pollen grains and elongating pollen tubes. The GUS staining of EXPB5 was not synchronized in each pollen sac which suggested the gene expression is controlled gametophytically (Figure 4.3B). In mature pollen grains, the GUS staining was densely located in the anther (Figure 4.3C). As pollen grains germinated on the surface of stigmatic papillae, GUS staining was observed on both pollen and stigma (Figure 4.3D). Since unpollinated stigma showed no staining, mature pollen grains are the origin of staining. EXPB5::GUS staining was also observed in the tip of the pollen tube as the pollen tube elongated in the ovary and penetrated the ovule micropyle (Figure 4.3E & F). The latest staining of EXPB5 was observed in the stigma at stage 18. Since the hydrolysis product of GUS could diffuse away from where it is produced, diffuse staining patterns need careful interpretation. Additional elements outside of the promoter region may influence gene expression, which will not be reflected
by the promoter::GUS fusion. The protein stability of GUS makes promoter::GUS analysis not a feasible strategy to observe brief gene transcription.
Figure 4.3: Histochemical staining of *EXPB5* promoter::GUS lines.  A. The earliest expression of *EXPB5* GUS is observed in stage 12 flower.  B. Stamen from a stage 12 flower.  C. Intense GUS staining in a stage 12 flower.  D. GUS staining is highly diffused around the stigmatic papillae.  E. GUS stained pollen tube in a dissected developing silique.  F. A pollen tube passes through ovule micropyle.  Bar = 500 µm (A, C), 100 µm (B, D, F), 20 µm (E).
4.3.2 Gene Transcript Analysis

Semi-quantitative RT-PCR was performed to investigate the transcription of \textit{EXPA4}, \textit{EXPA24}, and \textit{EXPB5} in mature pollen grains (Figure 4.4). Mature pollen grains were collected in bulk from inflorescences following the procedure of Honys and Twell (2003). RNA extraction and RT-PCR were carried out as described in Section 4.2.2. \textit{EXPA13} transcript was not detected in mature pollen grains although expression was confirmed in microspore development (Figure 4.4, Lane 2) as detailed in Chapter 2. \textit{EXPA21} showed no expression in mature pollen grains although GUS staining was observed in mature pollen grains (Figure 4.4, Lane 3). RT-PCR results confirmed that \textit{EXPA4}, \textit{EXPA24} and \textit{EXPB5} were transcribed in mature pollen grains of \textit{Arabidopsis}. They also suggested that \textit{EXPA4} and \textit{EXPB5} may be transcribed at higher levels than \textit{EXPA24} assuming no difference in PCR conditions. GUS observation and RT-PCR results are summarized in Table 4.2.
Honys & Twell (2004) detected *EXPA4* expression in mature pollen grains using Affymetrix ATH1 genome arrays. The transcription level of *EXPA4* increased greatly.

**Figure 4.4:** Transcript analyses of *EXPA4*, *EXPA24* and *EXPB5* in mature pollen grains of *Arabidopsis*. 10 ng RNA was used as template for semi-quantitative RT-PCR. Lane 1: *EXPA4*. Lane 2: *EXPA13*. Lane 3: *EXPA21*. Lane 4: *EXPA24*. Lane 5: *EXPB5*. 18S rRNA served as internal control.

**Table 4.2:** Summary of the promoter::GUS staining patterns and RT-PCR results of the mature pollen expressed expansins in *Arabidopsis*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Staining in Promoter::GUS Plants</th>
<th>Gene Transcript in Mature Pollen (by RT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EXPA24</em></td>
<td>Mature pollen grains. Elongating pollen tubes.</td>
<td>Yes</td>
</tr>
<tr>
<td><em>EXPB5</em></td>
<td>Mature pollen grains. Elongating pollen tubes.</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**4.3.3 Microarray Data of Pollen Expressed Expansins**

Honys & Twell (2004) detected *EXPA4* expression in mature pollen grains using Affymetrix ATH1 genome arrays. The transcription level of *EXPA4* increased greatly.
(over 800 times) as pollen developed from the uninucleate microspore stage to mature pollen. These data are consistent with high \textit{EXPA4} transcript levels observed by GUS and RT-PCR. As shown by GUS analysis, a root-cap-focused expression of \textit{EXPA4} was also reported in the ATH1 GeneChips data by Birnbaum \textit{et al}. (2003). The highest expression level in root tip was about 1/10 that of the mature pollen and approximately 4 times that of the rosette leaf. All rosette leaves displayed similar \textit{EXPA4} expression, which is different from the preferred expression of \textit{EXPA13} in younger leaves (Chapter 3).

The peak of \textit{EXPA24} expression appeared in tricellular pollen and subsequently declined to half this level in the mature pollen grain, according to the Affymetrix data (Honys & Twell 2004). In mature pollen, the \textit{EXPA24} expression level was about 1/10 that of \textit{EXPA4}, which is consistent with the semi-quantitative results. \textit{EXPA24} expression in \textit{Arabidopsis} embryo was also revealed by the microarray data from the Lindsey lab (Spencer \textit{et al}. 2006).

The microarray data indicate that \textit{EXPB5} is expressed exclusively in mature pollen grains with the similar magnitude to \textit{EXPA4} (Honys & Twell 2004). Almost 200-fold increase of \textit{EXPB5} transcript level was detected as the uninucleate microspore develops into the mature pollen grain.

\textbf{4.3.4 Mutant Analysis of Mature-Pollen-Grain Expressed Expansins}

\textit{Expa4-1} (GABI\textunderscore 061D02, \textit{Col-0} background) is a GABI-Kat T-DNA insertion line from the Max Planck Institute for Plant Breeding Research (Cologne, Germany)
(Rosso et al. 2003). The antibiotic sulfadiazine was used for mutant screening. PCR-based screening was performed with gene specific primers (MA4BLP and MA4BRP) and the T-DNA specific primer pAC161LB (Figure 4.5, Table 4.3). RNA transcript was detected using primer AE4SpSn and AE4SpAs (Figure 4.5, Table 4.1).

Figure 4.5: Location of the T-DNA insertion in EXPA4 and the transcript levels in expa4-1. A. Locations of T-DNA insertion and primers used for PCR-based screening and RT-PCR. The filled boxes represent the coding regions and the empty boxes indicate untranslated regions (UTR). The insertion site and direction is indicated by a triangle and an arrow head. Arrows show the direction and location of the primers. Bar = 200 bp. B. RT-PCR analysis of EXPA4 in wild type siblings (Lanes 1 & 2) and homozygous mutants (Lanes 3 & 4). 18S rRNA served as internal control.
Expb5-1 (CSHL_ET8666, *Landsberg erecta* background) is a Ds-based enhancer trap line (Sundaresan *et al*. 1995) generated at Cold Spring Harbor Lab. For genotyping purpose, the gene specific primers (MB5CLP and MB5CRP) and the insertion specific primer Ds3_2 were used for PCR (Figure 4.6, Table 4.4). RNA transcript was detected using primer B5SpSn and B5SpAs (Figure 4.6, Table 4.4).

**Table 4.3**: Three primers for PCR-based screening of *expa4-1*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA4BLP</td>
<td>CCCGTTGATCTAACAACGTACGG</td>
</tr>
<tr>
<td>MA4BRP</td>
<td>GGTCTTTGGCAATATTTGAAG</td>
</tr>
<tr>
<td>pAC161LB</td>
<td>ATATTGACCACCTACTCATTGC</td>
</tr>
</tbody>
</table>
UP to now, mutants of *EXPA24* are not available from public sources of seed stocks, including the Versailles T-DNA lines collection from INRA, Syngenta *Arabidopsis* Insertion Library (SAIL) collection, John Innes Centre (JIC, Norwich, UK) Ds gene trap insertion lines, GABI-Kat T-DNA insertion line from the Max Planck
Institute for Plant Breeding Research (Cologne, Germany), as well as the seed stocks of the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA) and the Nottingham Arabidopsis Stock Center (NASC, Loughborough, UK).

In expa4-1, the mature pollen morphology and size, germination rate, in vitro pollen tube elongation, segregation of heterozygous mutant, and seed yield were investigated, but no defect was detected in homozygous mutants. Because of EXPA4::GUS staining in the vasculature, I compared leaf and shoot size between the mutant and wild type. The results indicated that the EXPA4 knockout mutant is not reduced in vegetative growth. In the expb5-1 mutant, no difference in mature pollen size, pollen tube elongation, and segregation analysis was detected between wild type siblings and the mutant. The lack of phenotype suggests that functional homologues of these two genes exist. It is possible that phenotypes may be found in lines with stacked mutations.

4.4 Discussion

4.4.1 Expression Profiles of Mature-Pollen-Grain Expressed Expansins

4.4.1.1 Expression Analysis

The time and location of gene expression provides clues to gene function. In this study, three Arabidopsis expansins were found to be expressed in mature pollen grains by examining expansin promoter::GUS lines and subsequent confirmation by RT-PCR. The mature-pollen-grain expressed expansins include two α-expansins (EXPA4 and EXPA24)
and one β-expansins (EXPB5). All three genes display their highest expression level in pollen grains. However, their expression profiles are differentiated from one another. In addition to its expression in pollen grains, EXPA4 is widely expressed in Arabidopsis, notably the root tips and vasculature. Based on GUS analysis, EXPA4 and EXPA13 are different in the localization and timing of their vascular expression. EXPA4 promoter::GUS is distributed evenly within the vascular bundle whereas EXPA13 promoter::GUS is limited to differentiating xylem elements. In siliques harboring globular embryos, EXPA4 transcript is detected with a similar level to that in the root cap, based on the microarray data. The transcript level is then halved at the heart embryo stage. This may suggest a transcription peak in early embryogenesis or in the vasculature of developing siliques.

Both EXPA24 and EXPB5 transcripts were detected in mature pollen grains. However, the peaks of their transcript levels are not overlapping based on the Affymetrix ATH1 array data. The highest expression of EXPA24 is in tricellular pollen and the most abundant level of EXPB5 transcript takes place in mature pollen grains. Outside pollen grains, EXPA24 is only suggested to be transcribed in developing embryo, based on Affymetrix data (Spencer et al. 2006). However, it is necessary to confirm this embryo expression since very high variation was reported in their data. EXPB5, as the only β-expansin identified in mature pollen grain of Arabidopsis so far, displayed exclusive transcription in pollen grains and reached the highest transcript level in mature pollen.

My expression analysis indicates a highly specific expression pattern for Arabidopsis expansins. Although all three genes are expressed in the mature pollen grain, their expression levels are different and the transcript peaks appear at different
stages of pollen development. The co-expressions in mature pollen grain and pollen tube suggest a possible functional redundancy.

4.4.1.2 Mutant Analysis

No phenotype was observed with regard to pollen development or reproduction in the knockout mutants of \textit{EXPA4} and \textit{EXPB5}. Although \textit{EXPA4} is also expressed in the vasculature, no phenotypic change was observed in the vegetative growth of \textit{expa4-1}. This lack of phenotype upon disruption of a single expansin gene suggests the presence of functional homologues in mature pollen grains. Identification of these two single mutant lines, \textit{expa4-1} and \textit{expb5-1}, provides the initial step towards screening of stacked expansin mutants.

Many attempts were made to search the public collections for \textit{EXPA24} mutants but without success to date. Instead of insertion mutant analysis, RNA silencing may be an efficient approach for defining gene function. An inverted-repeat construct driven by the gene specific promoter can be used to induce post transcription gene silencing in plants (Curtin \textit{et al.} 2007).

4.4.1.3 Function Prediction

Since gene transcripts of \textit{EXPA4}, \textit{EXPA24}, and \textit{EXPB5} exist in both the mature pollen grain and elongating pollen tube, I propose that they may have a function in pollen germination and pollen tube elongation through the stigma and style. The pollen grain
navigates a long way through sporophytic tissue to reach an ovule. In *Arabidopsis*, the pollen tube first grows within the cell wall of the stigmatic papillae towards the base. After emergence, it continues to grow intercellularly through the style and transmitting tissue until it arrives at ovule. Pollen-produced enzymes have been suggested to take part in this process, including pectin-degrading enzymes such as pectin esterase (Kim *et al.* 1996), pectate lyase (Wu *et al.* 1996), and polygalacturonase (Kim *et al.* 2006), and cellulose- and hemicellulose-degrading enzymes like glucanases (Kotake *et al.* 2000) and xylanases (Suen & Huang 2007).

Recently, pectin methylesterases (PMEs) were shown to be essential in *Arabidopsis* pollen tube growth both *in vitro* and *in vivo* (Jiang *et al.* 2005, Tian *et al.* 2006). *Vanguard1* (Jiang *et al.* 2005) was identified as an *Arabidopsis* mutant with reduced male fertility. The *vgd1* pollen tube growth was retarded in the style and transmitting tract and seeds were only produced in the upper part of the silique. Molecular cloning showed that the *VGD1* gene encodes a pectin methylesterase (PME)-homologous protein. As the VGD1-GFP fusion protein appeared in the pollen tube wall, it was proposed that the loss of *VGD1* function in the extracellular space could reduce the efficiency of the degradation of the female tissue cell wall and lead to retarded pollen tube elongation. The reverse genetic study of another PME gene, *AtPPME1*, showed that gene silencing affected pollen tube elongation and morphology *in vitro* but no influence on seed production was reported (Tian *et al.* 2006).

The synergistic function between expansin and pectin-degrading enzymes has been proposed as reviewed in Chapter 2. The co-localization of expansins and PME (*VGD1* and *AtPPME1*) in the elongating pollen tube may again suggest their cooperation
in cell wall separation as the pollen tube migrates intercellularly. Taking the double mutant phenotype of expansin and pectin-degrading enzyme (reviewed in Chapter 2) into account, I propose that crossing expa4-1 or expb5-1 with vgd1 or atppme1 would result in highly reduced pollen tube elongation, even male sterility. This experiment will be very intriguing for testing the synergistic function of expansin and PME in pollen tube growth.

4.5 Summary

The experiments discussed in this chapter show that three expansins are expressed in mature pollen grains, as well as in the elongating pollen tubes. They are EXPA4, EXPA24, and EXPB5. Their expression patterns have been characterized through promoter::GUS analysis, RT-PCR, and public microarray data. These data also reveal their differential expression profiles. In addition to expression in reproductive growth, EXPA4 is also expressed in vasculature and root cap cells. EXPA24 and EXPB5 are exclusively expressed in mature pollen grains, with the highest transcript levels appearing in tricellular pollen and mature pollen respectively. For reverse genetic study, two knockout mutants, expa4-1 and expb5-1, have been screened although no phenotypic changes were found in either of them. This suggests the functional redundancy of mature-pollen expressed expansins. No mutant of EXPA24 has been identified from the public stock centers. The mature-pollen expression expansins are proposed to function in cell wall separation of female tissues during pollen tube elongation.
References


conserved, multigene family of proteins that mediate cell wall extension in plants. *Proc Natl Acad Sci USA* **92**: 9245-9249.


Chapter 5

Summary and Future Directions

The broad objective of this thesis is to examine the biological function of a group of pollen-expressed expansins in Arabidopsis. Although widely identified in plants and non-plants, expansins have been reported very limited in their biological functions. As for pollen-expressed expansins, a large number of β-expansins, known as group-I allergens, have been identified in maize pollen and proposed to loosen the silk walls, allowing pollen tube growth (Valdivia et al. 2007a, b). In the work presented in this thesis, pollen-expressed expansins were studied in Arabidopsis for the first time. One expansin has been identified to function in developing pollen and three other expansins in mature pollen grains. Employing reverse genetics, I propose their biological functions in both vegetative and reproductive growth. Summaries and future directions are discussed in this chapter.

5.1 Expansins in Developing Microspores

In this work, EXPA13 was found to be expressed during microsporogenesis in the Arabidopsis flower of stages 10 to 12. Evidence includes promoter::GUS observations, RT-PCR, and microarray data from public sources (Winter et al. 2007). Based upon its expression pattern, I propose that EXPA13 functions in microspore separation and/or expansion. Three mutant lines were identified for reverse genetic study. Expa13-1, a qrt1
background mutant with increased expression of EXPA13, produces half-collapsed tetrads when expa13-1 is present in the heterozygous state. As wild type microspores were found to collapse when linked to expa13-1-bearing pollen in the tetrad (quartet) state, I propose that the imbalanced expansion of microspores in heterozygous tetrads leads to pollen collapse. In the other two knockout mutants, expa13-2 and expa13-3, no significant difference of pollen size was observed. The attempts to obtain EXPA13 QRT1 double mutant by crossing expa13-2 and qrt1-1 plants failed to produce double mutants. This failure most likely means that expansin and pectin-modifying enzyme act synergistically in microspore wall expansion.

One unexpected observation in the EXPA13 mutant is defective leaf expansion and stem elongation. There is approximately 30% reduction in growth in expa13-2 plants in Landsberg erecta (Ler-1)-background and about 10% reduction in the expa13-3, which is Columbia (Col-0)-background. Semi-quantitative RT-PCR and promoter::GUS analysis suggests that EXPA13 is transcribed in the vasculature, most likely in differentiating xylem elements, of expanding leaves and the elongating inflorescence stem. Mutant analysis and microscopic observation suggest that EXPA13 is necessary for normal cell elongation as well as cell wall thickening during vessel element development.

Combining the phenotypic changes in microsporogenesis and stem elongation observed in EXPA13 mutants, I conclude that EXPA13 functions in the process of microspore expansion and xylem element elongation. The gene function is subtle in single pollen and individual xylem element but evident in qrt1 pollen and in the whole xylem. The mutant analysis also suggests that microspore and xylem development share common mechanisms in cell expansion.
5.2 Expansins in Mature Pollen Grains

As detailed in Chapter 4, three expansins, \textit{EXPA4}, \textit{EXPA24}, and \textit{EXPB5}, are expressed in \textit{Arabidopsis} mature pollen grains and elongating pollen tubes. This observation indicates that both α- and β-expansins function in the reproductive growth of \textit{Arabidopsis}. The expression profiles of pollen-expressed expansins have been constructed through promoter::GUS analysis, RT-PCR, and public microarray data. Gene expression patterns are not the same in these three genes. Based on the microarray data, the transcript peaks for \textit{EXPA4} and \textit{EXPB5} appear simultaneously in mature pollen grains whereas the highest \textit{EXPA24} transcript levels appear in tri-cellular pollen. In contrast to the exclusive expression of \textit{EXPA24} and \textit{EXPB5} in pollen grains, \textit{EXPA4} is also expressed in vasculature and root cap cells. Knockout mutants have been identified for \textit{EXPA4} and \textit{EXPB5}, named \textit{expa4-1} and \textit{expb5-1} respectively. No phenotypic change has been noticed in either line, possibly indicating the functional redundancy of pollen-expressed expansins. Attempts to identify \textit{EXPA24} mutants from public stocks failed. RNA silencing of \textit{EXPA24} will be a promising future direction to decipher this gene function in wild type plants as well as in \textit{expa4-1} and \textit{expb5-1} plants. Because all pollen-expressed expansins are expressed in mature pollen grains and elongating pollen tubes, I hypothesize that these expansins function in wall assembly during pollen tube elongation and/or in wall separation of female tissues as pollen tubes pass through.
5.3 Prospective Directions and General Discussions

5.3.1 Application of qrt1 in Expansin Study

Analysis of expa13-1 has shown qrt1 plants to be an informative background for the functional study of EXPA13. As proposed in Chapter 2, the fused pollen grains may provide a platform to detect subtle changes of microspore expansion. According to my interpretation, when microspore enlargement is not synchronous in the qrt1 tetrads, cell collapse occurs in two of the pollen grains, possibly because of cell wall tearing in the joined cell walls. Although further evaluation of this hypothesis is necessary, the work in this dissertation suggests qrt1 as a novel system for expansin function study.

5.3.2 Synergistic Function of Expansin and Pectin-Degrading Enzymes

Studies of expa13-1 and expa13-2 support a synergistic function between expansin and pectin-modifying enzymes, which was proposed by Cosgrove & Durachko (1994). In mature pollen grains and elongating pollen tubes, expansins and pectin methylesterases, such as Vanguard1 (Jiang et al. 2005) and AtPPME1 (Tian et al. 2006), are co-expressed. As reported in the literature, reduced fertility was observed in vanguard1, but no effect in atppme1. It will be of great interest to examine the phenotype of mutants defective in both pollen-expressed expansin and PME. As single mutants of pollen-expressed expansins have been identified (expa4-1 and expb5-1) and PME mutants reported by Jiang et al. (2005) and Tian et al. (2006), this experiment
should be easy to do. The prediction is that pollen tube formed in the pollen with stacked
gene mutations will be difficult to elongate and leads to male infertility.

Many functional studies of expansins by reverse genetics have yielded subtle
changes or no change in mutant plants. Some of these cases are likely because of
functional redundancy whereas some others are due to lack of delicate techniques for
capturing the wall alterations. Similar situations were facing in the study of many other
cell wall proteins, such as the “non-functional” polygalacturonase (as reviewed in
Chapter 2). Efficient tools will bring breakthrough to decipher the functions of wall
protein gene families. In this dissertation, I propose using qrt1 (and/or other pectin-
modifying enzyme mutants) as background for expansin functional study. This proposal
is based on a literature survey of expansin-pectin enzyme interaction and also based on
my investigation on the EXPA13 mutant of qrt1 background. This mutant study based on
another mutant may not only shed light on expansins-pectin enzymes interaction but also
uncover the potential underestimated function of expansins in pectin modification. The
potential challenge of this proposed technique is wall proteins show highly specific
expression patterns, which suggest the interactions are between highly specific genes.
Identification and finely localization of these gene expressions are essential for further
functional studies.
5.3.3 Expansins in Vascular Development

Two pollen-expressed expansins, *EXPA4* and *EXPA13*, are observed expressed within the vasculature but in different cell types. It will be intriguing to study their function in double mutants by crossing the single mutants. By investigations on the *Arabidopsis* expansin promoter::GUS plants, several other genes were found as vasculature-expressed (data not shown). Considering the significant defect of vegetative growth in *EXPA13* mutant, it will be of interest to characterize other expansin genes expressed in the differentiating xylem cells.

Because of the xylem cell defects noted in the *expa13* mutant, further study of *EXPA13* protein localization may shed more light on the function of this protein in wall formation or breakdown. A technique to fluorescently label wall protein in living cells by 4′,5′-bio(1,3,2-dithioarsolan-2-yl) fluorescein has been reported by Griffin et al. (1998). As a small fluorescein receptor with only six amino acids incorporated into the protein of interest, this technique is extremely useful to label small proteins like expansins, which may help to deepen our understanding of the EXPA13 localization to subcellular level.

*EXPA13* is one of the few expansins showing clear phenotype in knockout mutants so far. My explanation is that although subtle change in individual vessel element of the *EXPA13* mutant, the end-to-end arrangement of vessel elements enables the changes to be added up in the whole xylem. This makes a significantly visible change in the whole stem length.
5.3.4 Mature Pollen-Expressed Expansins

Single mutants have been identified for two of the mature pollen-expressed expansins; however, no phenotype was observed. As a prospective further study on mature pollen-expressed expansins, an analysis of double mutants and lines with RNA silencing may be informative. My hypothesis is that as all the redundant expansins are mutated simultaneously, the pollen tube will be obstructed from normal wall assembly and/or normal penetration of the pistil tissues and thus will exhibit severe male infertility. The novelty in this work is that both α- and β-expansins are identified in mature pollen grains and they are of similar transcript levels. This is different from what has been observed in maize pollen grains, in which only abundant β-expansins were identified (reviewed in Sampedro & Cosgrove 2005). This implies that although α- and β-expansins are strictly separated in the phylogenetic tree, they are simultaneously expressed in the mature pollen grains and may work together to loosen cell walls. The relatively low transcript level of EXPA24 suggests it a fine tuning apparatus facilitating wall assembly in the tip region of the elongating pollen tube.
References


VITA - Lei Zhao

204 Life Science Building, The Pennsylvania State University, University Park, PA 16802
814-883-2215, LZZ107@psu.edu

Education:

08/03~12/08 Ph.D. in Plant Physiology, The Pennsylvania State University, University Park, PA
08/97~06/01 B.S in Biology, East China Normal University, Shanghai, P.R.China

Research Experience:

12/03~12/08 Graduate student. The Pennsylvania State University, University Park. Thesis title:
Functional Study of Pollen-Expressed Expansins in Arabidopsis. Advisor: Dr. Daniel J. Cosgrove
07/01~07/03 Research assistant. Yunnan Agricultural University, Kunming Yunnan. Research
title: Survey and Evaluation of Seed Plant Resources in Yunlong Reservoir Area,
Yunnan. Supervisor: Prof. Rongchun Li
01/01~06/01 Undergraduate student. East China Normal University, Shanghai. Thesis title:
Studies of the Digestive Enzymes Activities in the Embryogenesis of Eriocheir
sinensis. Advisor: Dr. Yunlong Zhao

Teaching Experience:

2005~2008 (Fall) Teaching assistant – Biology 110: Basic Concepts and Biodiversity
The Pennsylvania State University, University Park
07/01~07/03 Teaching fellow – Lectures and laboratory in Botany.
Yunnan Agricultural University, Kunming Yunnan, P.R.China
09/00~03/01 Teacher (intern) – Biology
Shixi Middle School, Shanghai.

Publications:

Li S, Zhao C, Zhang L, Li R, Zhao L, Li X. 2006. Investigation and Analysis on the wild resources of
toxic seed plants from Yunlong Reservoir area in Yunnan Province. Journal of Yunnan Agricultural


Honors and Scholarships:

06/01 The ECNU Excellent Graduate Award
06/01 The ECNU Excellent Bachelor Thesis Award
06/01 Huawei Outstanding Student Scholarship
1997~2001 Normal University Undergraduate Registration Scholarship
1997~2000 The ECNU Scholarship