FUNCTIONAL INVESTIGATION OF ARABIDOPSIS CALLOSE SYNTHASES AND THE SIGNAL TRANSDUCTION PATHWAY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Xiaoyun Dong, M.S. (Plant Biology)
The Ohio State University
2005

Dissertation Committee:

Dr. Desh Pal S. Verma, Adviser

Dr. Biao Ding

Dr. Terry L. Graham

Dr. Guo-liang Wang

Approved by

______________________________________________

Adviser

Graduate Program in Plant Pathology
ABSTRACT

Callose synthesis occurs at specific stages of cell wall development in all cell types, and in response to pathogen attack, wounding and physiological stresses. We isolated promoters of 12 Arabidopsis callose synthase (CalS1-12) genes and demonstrated that different callose synthases are expressed specifically in different tissues during plant development. That multiple CalS genes are expressed in the same cell type suggests the possibility that CalS complex may be constituted by heteromeric subunits. Five CalS genes were induced by pathogen (Peronospora parasitica, a causal agent of downy mildew) or salicylic acid (SA) treatments, while seven CalS genes were not affected by these treatments. Among the genes that are induced, CalS1 and CalS12, showed the highest responses. When expressed in npr1, a mutant impaired in the response of pathogen related (PR) genes to SA, the induction of CalS1 and CalS12 genes by the SA or pathogen treatments was significantly reduced. The patterns of expression of the other three CalS genes were not changed significantly in the npr1 mutant. These results suggest that the high induction observed of CalS1 and CalS12 is NPR1-dependent while the weak induction of all five CalS genes is NPR1-independent. In a T-DNA knockout mutant of CalS12, callose encasement around the haustoria on the infected leaves was reduced and the mutant was found to be more resistant to downy mildew as compared to the wild type plants.
Arabidopsis contains 12 callose synthase (CalS) genes that have evolved in order to catalyze callose synthesis in different locations and in response to biotic and abiotic cues. We demonstrate that one of these genes, CalS5 is responsible for the synthesis of callose deposited to the primary callose wall of meiocytes, tetrads and microspores, and is essential for the exine formation and pollen viability. CalS5 encodes a transmembrane protein of 1923 amino acid residues with a molecular mass of 220 kD. Knockout of the CalS5 gene by T-DNA insertion resulted in a severe reduction in fertility. The reduced fertility in cals5 mutants was attributed to the degeneration of microspores. However, megagametogenesis is not affected and the female gametes are completely fertile in cals5 mutants. CalS5 gene is expressed in several organs with the highest expression in meiocytes, tetrads, microspores and mature pollens. Callose deposition in these tissues in cals5 mutants was nearly completely depleted, suggesting that this gene is essential for the synthesis of callose in these tissues. The pollen exine wall was not formed properly in the mutant and tryphine appeared to be transported from the pollen outer wall into the central vacuole presumably via endocytosis. These data suggest that callose synthesis has a vital function in building the exine sculpture, integrity of which is essential for pollen viability.

Using the cell plate specific CalS1 as a bait to screen an Arabidopsis cDNA library constructed in the yeast two-hybrid vector, we obtained two positive clones. One of these interacting clones, RLK1, encodes protein kinase and may play a role in the regulation of CalS1 activity during cell plate formation. Another clone, UGP1, encodes an UDP-glucose pyrophosphorylase and may act to provide alternative source of UDP-
glucose for the synthesis of callose.

In summary, induction of callose synthase genes by pathogen infection or SA treatment involves both \textit{npr1}-dependent and \textit{npr1}-independent signaling pathways. \textit{CalS5} is required for exine formation during microgametogenesis and pollen viability in \textit{Arabidopsis}. UDP-Glc Pyrophosphorylase could be a component of callose synthase complex.
Dedicated to my mother
ACKNOWLEDGMENTS

During my Ph.D. program at The Ohio State University, Dr. Desh Pal S. Verma has been continually supportive and patient. I admire and appreciate his intellectual guidance. His sincere efforts and encouragement made this dissertation possible.

I would also like to thank my committee members, Dr. Terry L. Graham, Dr. Biao Ding, and Dr. Guo-liang Wang, Dr. Erich Grotewold for their valuable suggestions and advices.

My sincere gratitude to Dr. Zonglie Hong, Dr. Sunghan Kim, Dr. Magdy Mofouz, Dr Sunyong Jeong, Dr Asuka Itaya, Niloufer Irani, Shalaka Patel, Jennifer Truong, and Sonia Malaoui. I also appreciate their friendship throughout the years of my program. Their encouragement was essential for the completion of this work.

vi
VITA

1987 .............................................................  B.S. Nanjing University, China

2000 .............................................................  M.S. The Ohio State University, Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Plant Pathology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>vi</td>
</tr>
<tr>
<td>Vita</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xiv</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xv</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Callose and cellulose in plants</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Wounding Callose</td>
<td>6</td>
</tr>
<tr>
<td>1.3 Haustoria Callose</td>
<td>7</td>
</tr>
<tr>
<td>1.4 Cell Plate Callose</td>
<td>10</td>
</tr>
<tr>
<td>1.5 Callose in pollen development</td>
<td>11</td>
</tr>
</tbody>
</table>
2. Tissue-Specific Expression of Different Callose Synthases and Involvement of both \textit{NPR1}-Dependent and \textit{NPR1}-Independent Signaling in Pathogen Induction of Affected Genes ................................................................. 14

2.1 Introduction .............................................................................................................. 14

2.2 Results ...................................................................................................................... 17

2.2.1 Tissue-Specific Expression of \textit{CalS} Genes ................................................ 17

2.2.2 Induction of \textit{CalS} Genes by Salicylic Acid ............................................ 22

2.2.3 \textit{NPR1} Requirement for the Induction of \textit{CalS} Genes by SA Treatment ................................................................. 24

2.2.4 Induction of \textit{CalS} Genes by Pathogen Infection .................................... 28

2.2.5 Haustoria Callose Deposition and the Infection of Downy Mildew in cals1 and cals12 Mutant ................................................................. 2

2.3 Discussion .................................................................................................................. 34

2.3.1 Expression of Different \textit{CalS} Genes is Regulated in a Tissue-Specific Manner ................................................................. 34

2.3.2 Multiple Roles of Callose in Plant Development and in Response to Pathogen Attack ................................................................. 35

2.3.3 Requirement of \textit{NPR1} in the Induction of \textit{CalS1} and \textit{CalS12} by SA and Pathogen ................................................................. 36

2.3.4 The Weak Induction of \textit{CalS} Genes by Pathogen Infection is \textit{NPR1}-Independent ................................................................. 37

2.4 Materials and methods ......................................................................................... 39

2.4.1 Promoter-Reporter Constructs and Plant Transformations ................. 40
Callose synthase (cals5) is required for exine formation during microgametogenesis and pollen viability in Arabidopsis

3

3.1 Introduction .................................................................46
3.2 Results and discussion....................................................52
3.2.1 Isolation of cals5 T-DNA knockout mutants.................52
3.2.2 CalS5 is a transmembrane protein and contains multiple functional motifs .......................................................53
3.2.3 Sterility in cals5 mutant.................................................53
3.2.4 Arrest in tetrad and microspore development during male gametogenesis ..........................................................54
3.2.5 Megasporogenesis and megagametogenesis in cals5 plants are not affected .................................................................54
3.2.6 Absence of viable pollen grains in cals5 mutants .........57
3.2.7 Tissue-specific expression of CalS5 gene ......................58
3.2.8 Meiosis male gametophyte is normal in the cals5 mutant..........................................................61
3.2.9 Defective exine formation.............................................62
3.3 Discussion ........................................................................63
3.3.1 CalS5 gene encodes a callose synthase required for microgametogenesis .................................................................63
3.3.2  *CalS5* is expressed highly in anthers and its role in microgametogenesis cannot be replaced by other *CalS* genes ................................................................. 64

3.3.3  Callose is required for exine wall formation of pollen grains ................................................................................................................................. 67

3.4  Materials and Methods ..................................................................... 70

3.4.1  Promoter-reporter constructs and plant transformation ......... 70

3.4.2  GUS staining and GFP detection .............................................. 71

3.4.3  RNA extraction and RT-PCR ................................................. 71

3.4.4  Genomic DNA extraction and isolation of a T-DNA insertion line .................................................................................................................. 71

3.4.5  Callose staining and pollen viability assay ......................... 72

3.4.6  Light microscopy ..................................................................... 72

3.4.7  Electron microscopy .................................................................. 73

4  Callose Synthase Complexes in *Arabidopsis* .................................... 74

4.1  Introduction .................................................................................. 74

4.2  Results .......................................................................................... 78

4.2.1  CalS1 interacts with RLK1 and UGP1 ............................. 78

4.2.2  Localization of RLK1, UGP1 and SuSys ......................... 78

4.3...Discussion .................................................................................. 79

4.3.1  The role of *RLK1* ............................................................... 79

4.3.2  The role of *UGP1* .............................................................. 80

4.3.3  Multiple components in the callose synthase complexes ... 80

4.3.4  Sucrose synthases .............................................................. 83

4.4  Methods ......................................................................................... 85

4.4.1  Bacterial and yeast strains and tobacco cell culture .......... 85

4.4.2  Cloning of *SuSy* cDNA .................................................. 85

4.4.3  Yeast two-hybrid assay ......................................................... 86
4.4.4 Expression of the GFP fusion protein and fluorescence detection ................................................................. 86

Bibliography ......................................................................................................................................................... 87
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Different roles of callose in plant</td>
<td>4</td>
</tr>
<tr>
<td>2.1</td>
<td><em>CalS</em>:GUS constructs and the presence of <em>cis</em>-elements in various <em>CalS</em> promoter</td>
<td>18</td>
</tr>
<tr>
<td>2.2</td>
<td><em>CalS</em>:GUS expression in vegetative tissues of transgenic plants</td>
<td>19</td>
</tr>
<tr>
<td>2.3</td>
<td><em>CalS</em>:GUS expression in different reproductive tissues of transgenic plants</td>
<td>20</td>
</tr>
<tr>
<td>2.4</td>
<td>Induction of <em>CalS</em> genes by SA and MeJA</td>
<td>25</td>
</tr>
<tr>
<td>2.5</td>
<td>Induction of <em>CalS</em> genes in <em>npr1</em> Mutant by SA treatment</td>
<td>26</td>
</tr>
<tr>
<td>2.6</td>
<td>Induction of <em>CalS</em> genes by <em>P. parasitica</em> infection</td>
<td>27</td>
</tr>
<tr>
<td>2.7</td>
<td>Characterization of T-DNA insertional knockout lines of the <em>CalS1</em> and <em>CalS1</em> genes</td>
<td>30</td>
</tr>
<tr>
<td>2.8</td>
<td>Conidiophore production and callose deposition in <em>cals1</em> and <em>cals12</em> plants</td>
<td>31</td>
</tr>
<tr>
<td>2.9</td>
<td>A model for the regulation of <em>CalS1</em> and <em>CalS12</em> gene expression</td>
<td>38</td>
</tr>
<tr>
<td>3.1</td>
<td>Identification of T-DNA insertional knockout lines of the <em>CalS5</em> gene</td>
<td>39</td>
</tr>
<tr>
<td>3.2</td>
<td>Reduced fertility in <em>cals5</em> mutants</td>
<td>40</td>
</tr>
<tr>
<td>3.3</td>
<td>Male and female gametophyte development in <em>cals5</em> mutant lines</td>
<td>41</td>
</tr>
<tr>
<td>3.4</td>
<td>Arrest in microspore development in <em>cals5</em> mutants</td>
<td>55</td>
</tr>
<tr>
<td>3.5</td>
<td>Tissue-specific expression of <em>Cal5S</em>:GUS and <em>Cal5S</em>:GFP in transgenic plant</td>
<td>56</td>
</tr>
<tr>
<td>3.6</td>
<td>Callose deposition during microsporogenesis</td>
<td>59</td>
</tr>
<tr>
<td>3.7</td>
<td>Ultrastructure of pollen grains</td>
<td>60</td>
</tr>
<tr>
<td>3.8</td>
<td>Role of the callose wall in exine formation during microgametogenesis</td>
<td>68</td>
</tr>
<tr>
<td>4.1</td>
<td>Localization of RLK1, UGP1 and SuSys</td>
<td>74</td>
</tr>
<tr>
<td>4.2</td>
<td>Production of UGP-Glc</td>
<td>81</td>
</tr>
<tr>
<td>4.3</td>
<td>Plant callose complex</td>
<td>82</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Expression of <em>CalS:GUS</em> in different tissues of transgenic plants</td>
</tr>
<tr>
<td>2.2</td>
<td>Primers used for amplification of <em>CalS</em> promoters by PCR</td>
</tr>
<tr>
<td>2.3</td>
<td>Primers used for amplification of <em>CalS</em> transcripts by RT-PCR</td>
</tr>
<tr>
<td>4.1</td>
<td>SuSy interacts with CalS1 or UGT1</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

as-1 activating sequence 1
BTH benzothiadiazole
bp base pairs
CalS callsoe synthase
cDNA complementary DNA
kb kilo base
kDa kilo Dalton
mRNA messenger RNA
GSL glucan synthase-like
GUS b-glucuronidase
JA jasmonic acid
MeJA methyjasmonic acid
NahG salicylate hydroxylase
npr1 nonexpresser of PR genes

SA salicylic acid

SAR systemic acquired resistance

W-box the sequence motif TTGAC
CHAPTER 1

INTRODUCTION

1.1 Callose and cellulose in plants

Callose is a polymer of β-1, 3-glucan linkages with some β-1, 6-branches, and it is different from cellulose, a polymer of β–1, 4-glucan crystallized to form cellulose microfibrils (36 glucan chains are in each elementary fibril), which is the major component of the plant cell wall. In plants, callose is also found in seeds, leaf and stem hairs, secondary walls and thickenings of developing xylem, sieve plates and plasmadesmata canals, transient walls of microsporogenic and megasporogenic tissues, pollen and pollen tube (Fink et al., 1987; Gregory et al., 2002; Verma and Hong, 2001; Østergaard et al., 2002). Callose was detected to exist on the cell plate by aniline blue staining and immunocytochemical studies (Northcote et al., 1989, Verma and Hong, 2001). A silencing mutant of β-1, 3-glucanase decrease the plant sensitivity to viruses, after the invasion of the virus, the plasmadesmata are found to be smaller than in wild-type plants, because of the accumulation of callose in the plasmadesmata, which is degraded in the wild-type plants (Iglesias et al., 2000). When not needed, callose is degraded by β-1, 3-glucanase. This suggests that callose synthesis and the degradation is regulated precisely during the plant development. Callose also plays important roles such
as providing the impermeability of the coat, cause of the dormancy of the seeds (Bevilacqua et al., 1989; Leubner-Metzger et al., 2001), temporary components of the cell wall, and resistance to pathogen and wounding (Figure 1.1).

In higher plants, cellulose is synthesized on the plasma membrane by a membrane associated cellulose synthesizing complex, a six-member hexagonal structure as detected by freeze-fracture that extrude cellulose microfibrils (Brown et al., 1996). In addition to cellulose synthase (the catalytic unit), other protein components are likely to make up the rosettes as well. Other proteins, which have been implicated to be the components of the rosettes, are annexin (Andrawis and Solomon, 1993), and a membrane integral protein (MIP). These proteins have been found to be associated with the cellulose synthase fraction in the product entrapment experiments. Recently, it has been found that sucrose synthase (SuSy) is associated with the plasma membrane and may supply UDP-Glc directly to cellulose synthase. The pattern of its localization, which is parallels to the deposition of cellulose, suggests that SuSy may be part of the rosette complex (Amor et al., 1995).

Callose is similar to cellulose in structure, and both polymers are synthesized with UDP-Glc as substrate. Since CalS activity is often found in the cellulose synthase fraction in the plasma membrane in plats, it has been suggested that the same enzyme(s) synthesize both β-1, 3-glucan and β-1, 4-glucan (Jacob et al., 1985). Membrane protein from coleoptiles and first leaves of young barley synthesizes β- (1, 3)(1, 4) and β-1, 3-glucans (Becker et al., 1995). Similar results were reported from maize coleoptiles (Gibeaut et al., 1993). Genes essential for the production of a linear, bacterial β-1, 3-
glucan, curdlan, is cloned from Agrobacterium sp. ATCC31749 (Stasinopoulos et al., 1999). The predicted CrdS protein (540 amino acids) has high similarities with glycosyl transferases with repetitive action pattern. These homologous glycosyl transferases include bacterial cellulose synthases, which form β-1, 4-glucans. But no similarity was found with putative β-1, 3-glucan synthases from yeast and filamentous fungi. These results suggested that cellulose synthase might produce cellulose as well as callose (Dhugga 2001).

However evidences also support that there are different enzymes for callose and cellulose synthesis. Mung bean β-1, 3-glucan synthase activity can be physically separated by non-denaturing gel electrophoresis (Kudlicka et al., 1997). After electrophoresis on non-denaturing gel, the in situ glucan synthesis shows two product bands identified as callose and cellulose, respectively. Interestingly both of the enzyme bands for the 2 glucans synthesis are composed of multiple peptides (Kudlicka et al., 1997). The presence of both callose synthase and cellulose synthase homologues in Arabidopsis genome suggests that the synthesis of callose and cellulose may be achieved by separated proteins (Henrissat et al., 2001). Another evidence supporting this is that in a cyr1 mutant, the cells can accumulate excessive callose but not synthesize cellulose (Nickle et al., 1998, Lukowitz et al., 2001). The debate is finally resolved by the cloning of CalS1 and UGT1 which are the callose synthase complex for callose synthesis on the cell plate (Hong et al., 2001 a, b; Verma, 2001 a, b). CalS1 is localized at the growing
Figure 1.1 Different roles of the same polymers β-1, 3-glucan (callose) in plant development and callose synthesis corresponding to biotic and abiotic signals
cell plate, the over-expression of CalS1 in transgenic tobacco cells enhances callose synthesis on the forming cell plate, and these cell lines show higher levels of CalS activity. These data demonstrate that there are separate synthase complexes for the callose and cellulose, and their properties are different.

In Arabidopsis, there are 12 CalS genes which fall into two groups, one (CalS1-10) contain 50 exons and the other (CalS11-12) with 2-3 exons. Callose synthase (CalS) genes are also referred as glucan synthase-like (GSL) genes (Richmond and Somerville, 2001) and two of them have been demonstrated to encode the catalytic subunit of callose synthases (Hong et al., 2001a; Østergaard et al., 2002; Jacob et al., 2003; Nishimura et al., 2003). Overexpression of Arabidopsis CalS1 in tobacco BY-2 cells results in the enhanced callose synthesis at the forming cell plate (Hong et al., 2001a), while knockout of CalS12 leads to the callose-less encasements of papillae in Arabidopsis upon pathogen infection (Jacobs et al., 2003; Nishimura et al., 2003). Multiple CalS isoenzymes have evolved in higher plants to catalyze callose synthesis in different locations and in response to mechanical, physiological stresses, and pathogen attack (Verma and Hong, 2001).

1.2 Wounding Callose

After wounding, plant cells rapidly synthesize callose. The mechanisms behind this phenomenon are not well understood, and there may be several possible wounding-caused changes that may trigger callose synthesis. Callose synthase may be activated by perturbed conditions, which lead to some loss of membrane permeability (Kohle et al.,
Membrane perturbations cause the activation of callose synthase, possibly because the perturbation results in membrane leakage, and the apoplastic Ca$^{2+}$ leaks into the cytosol to increase the local concentration of Ca$^{2+}$, which activates callose synthase. It is reported that several annexin-type molecules that are known to respond to Ca$^{2+}$ levels interact with CalS (Andrawis et al., 1993); CalS may be activated by annexin interaction. After wounding, the membrane lipids may change and affect the activity of callose synthase (Klaus et al., 1996). Wounding may affect membrane fraction that may activate callose synthase (Klaus et al., 1991). After wounding the protease may acquire access to the callose synthase complex that activate the otherwise latent enzyme. It is also suggested that after wounding callose synthase activator may redistribute from where they are isolated from the callose synthase to trigger callose synthesis (Ohana et al., 1992, 1993).

The signal for wounding callose may be mediated by Rho-like proteins, which is shown to be an integral part of the CalS complex (Hong et al., 2001b). The regulation of CalS activity could be regulated by interaction with G-proteins since a G-protein binding signature exists in the AtCalS1 sequence (Hong et al., 2001b). Wound callose is greatly reduced in GSL5 ds RNAi transgenic Arabidopsis lines compared with that in GSL6, GSL11 ds RNAi lines, and control lines. It shows that GSL5 is required for wound callose (Jacobs et al., 2003).

Wounding or pathogen invasion can induce callose synthesis in plants. This may ameliorate the consequences of wounding or help stop the pathogen from spreading to other places (Kauss, 1987). Synthesis of callose within the sieve pores can help to seal off damage or prepare the cells for developmental changes. Callose deposition is
reversible.

1.3 Haustoria Callose

During the fungal infections, callose is deposited in cell wall appositions (papillae) that form beneath infection sites and are thought to provide a physical barrier to penetration. However we know little about the signaling pathway leading to callose synthesis during plant-microbe interaction. Callose is induced in carrot (*Daucus carota L.*) cell suspensions treated with a spirostanol saponin from Yucca. The induction mechanism is not known, but differs from those mediated by Ca$^{2+}$ (Messianen et al., 1995). Over-expression of the tomato disease resistance gene PTO induces callose deposition (Tang et al., 1999). It is not known whether the induction of callose deposition is caused by expression level of callose synthases or by activation of the latent enzymes, although *pto* over-expression increases expression of pathogenesis-related genes. Arabidopsis plants treated with the synthetic acquired resistance (SAR) inducer benzo thiadiazole (BTH) also increase callose deposition, as well as the expression of resistance genes (Kohler et al., 2002). One of the callose synthases of Arabidopsis, CalS12, is suggested to be induced by salicylic acid (SA) at transcription level (Østergaard et al., 2002).

One of Arabidopsis mutants resistant to the powdery mildew *Erysiphe cichoracearum, pmr4*, is also resistant to other biotrophic pathogens (*E. orontii* and *Peronospora parasitica*), and resistance appears to act after the pathogen has penetrated the plant cell wall (Jacob *et al.*, 2003; Nishimura *et al.*, 2003). PMR4 gene was mapped to the top arm of chromosome 4, a region that contains a glucan synthase like gene
(GSL5=CalS12, At4g03550), it is further confirmed by genetical complementation experiment (Nishimura et al., 2003). Also pmr4 produces dramatically less callose in response to powdery mildew infection or wounding. It indicates that CalS12 is required for the callose synthesis during powdery mildew infection or wounding. A CalS12 cDNA can partially complement a callose synthase-deficient yeast mutant. Double-mutant analysis indicated that blocking the salicylic acid defense signaling pathway was sufficient to restore susceptibility to pmr4 mutants. Callose or callose synthase negatively regulated the SA pathway. These data suggest that callose synthesized by CalS12 might protect the fungus growth during pathogenesis from plant recognition and callose deposition may impede plant defenses against pathogen attacks.

After penetrated by the powdery mildew *Erysiphe cichoracearum*, callose is deposited along the entire cell margin detected by an intense aniline blue fluorochrome staining in wild-type plants, whereas cells in *cals12* mutants showed only a punctuate callose staining pattern at the cell periphery. The punctuate staining pattern in *cals12* plants may be plasmadesmata callose, callose is typically deposited in the cell wall area immediately surrounding the orifice of a plasmadesmata, especially when a tissue is wounded or during aldehyde fixation (Hughes and Gunning, 1980; Northcote et al., 1989; Vaughn et al., 1996; Jacob et al., 2003). This result suggests that massive callose accumulation normally occurs along the cell margin during pathogen-triggered cell death and that CalS12 is required for the cell margin callose synthesis and the plasmadesmata callose are synthesized by different callose synthase isoforms of the same cell.
To investigate the signaling transduction pathway leading to callose synthesis during plant microbe interaction, we isolated promoters of 12 Arabidopsis callose synthase (CalS1-12) genes and demonstrated that different callose synthases are expressed specifically in different tissues during plant development. Our data suggests that CalS complex may be constituted by heteromeric subunits since multiple CalS genes are found to be expressed in the same cell type. Five CalS genes were induced by pathogen (Peronospora parasitica, a causal agent of downy mildew) or salicylic acid (SA) treatments, while seven CalS genes were not affected by these treatments. Among the genes that are induced, CalS1 and CalS12, showed the highest responses. When expressed in npr1, a mutant impaired in the response of pathogen related (PR) genes to SA, the induction of CalS1 and CalS12 genes by the SA or pathogen treatments was significantly reduced. The patterns of expression of the other three CalS genes were not changed significantly in the npr1 mutant. These results suggest that the high induction observed of CalS1 and CalS12 is NPR1-dependent while the weak induction of all five CalS genes is NPR1-independent. In a T-DNA knockout mutant of CalS12, callose encasement around the haustoria on the infected leaves was reduced and the mutant was found to be more resistant to downy mildew as compared to the wild type plants.

1.4 Cell Plate Callose

During cytokinesis, the Golgi-derived vesicles fuse in the center of the phragmoplast to start the process of cell plate formation. Callose is synthesized shortly after the initial fusion of the cell plate vesicles. And the maximum level of callose deposition is detected in the network consolidation phase (Samuels et al., 1995). Callose
fills the tubulo-vesicular network, and it is suggested that the callose provide spreading force on the membranes to help widen the tubules and converts the network into fenestrated sheet (Samuels et al., 1995). This spreading effect of callose may be increased from the phragmoplastin polymerization and squeezing of phragmoplastin polymers (Verma 2001b), since one of subunits of callose synthase complex interact with phragmoplastin (Hong et al., 2001b). After the cell plate has maturated to a cell wall by cellulose deposition, the cell plate callose may be degraded by cell plate specific β-1, 3-glucanase. The callose cannot be detected in the wall except at the plasmodesmata (Samuels et al., 1995; Hong et al., 2001). It shows that callose synthesis and degradation is highly regulated during cell plate formation and cell division.

In Arabidopsis, the cell plate callose synthase complex on cell plate consists of at least 2 proteins, CalS1 and UGT1 (Hong et al., 2001a, b). It has been shown that CalS1 shares homology to yeast callose synthase and UGT1 shares homology to other UGT. CalS1 and UGT1 both are localized on the cell plate and interact with phragmoplastin. Both proteins are present in the complex purified from product entrapment procedures. CalS1 over-expressing BY2 cells show higher callose synthase activity in the microsomal fractions and accumulate more callose the cell plate than the wild type cells. Other components for the cell plate callose complex include Rop1, SuSy and phragmoplastin (Hong et al., 2001a, b; Verma, 2001a, b).

Using the cell plate specific CalS1 as a bait to screen an Arabidopsis cDNA library constructed in the yeast two-hybrid vector, we obtained two positive clones. One of these interacting clones, RLK1 encodes a protein kinase and may play a role in the regulation of CalS1 activity during cell plate formation. Another clone, UGP1, encodes
an UDP-glucose pyrophosphorylase and may act to provide alternative source of UDP-glucose for the synthesis of callose.

1.5 Callose in pollen development

In microsporocyte of developing anthers, callose is synthesized as a temporary cell wall between the primary cell wall and plasma membrane. After two consecutive divisions of meiosis, the microspore tetrad is encased in a callose wall. Along with the initiation of microspore exine synthesis, a β-1, 3-glucanase (callase) is secreted by the tapetum cells and released into the locular space. During the first meiotic division the callase activity is low in the anthers but increases rapidly at the end of the second meiotic division. As the temporary callose wall is degraded by callase and microspores are released in the locular space (Frankel et al., 1969; Steiglitz and Stern, 1973; Steiglitz, 1977). The process of callose synthesis and degradation is highly regulated during the pollen development. Several mutants in the callose wall formation and degradation have been characterized in petunia, and it has been suggested that the timing of callose wall formation and degradation is important for microsporegenesis and pollen development (Izhar and Frankel, 1971; Warmke and Overman, 1972). In transgenic tobacco expressing a β-1, 3-glucanase in tapetum cells, the callose wall of microspore is dissolved prematurely, causing male sterility (Worrall et al., 1992).

This temporary callose wall plays many important roles in the pollen. The callose wall may be built temporarily to prevent cell cohesion and fusion and, upon its degradation, and at late stage to facilitate the release of free microspores to the locular space (Waterkeyn, 1962). It is suggested that the callose wall may also function as a
molecular filter protecting the developing microspores from the influence of the surrounding diploid tissues (Heslop-Harrison and Mackenzie, 1967). It can also provide a flexible wall that may help prevent premature swelling and bursting of the microspores. Finally, the callose wall can act as a mold where the primexine is generated soon after the completion of meiosis during microsporogenesis and then the primexine provides a blueprint for the formation of exine patterns on mature pollen grains (Waterkeyn and Beinfait, 1970; Stanley and Linskens, 1974).

Several *Arabidopsis* mutants defective in exine formation have also been isolated and characterized. These include *defective in exine formation 1* (*dex1*), *male sterility 2* (*ms2*), *faceless pollen-1* (*flp1*), and *no exine formation* (*nef1*). Sporopollenin is deposited irregularly on the plasma membrane of *dex1* microspores leading to the degeneration of pollen grains (Paxson-Sowders et al., 2001). In the *ms2* mutant, the exine wall is thin and sensitive to acetolysis treatment (Aarts et al. 1997). In the *flp1* mutant, the microspores and their exine were visually normal as wild type, but the exine pattern is sensitive to acetolysis (Ariizumi et al., 2003). In *nef1* mutant, sporopollenin is synthesized but is deposited on the locular wall (Ariizumi et al., 2004). However, there is no abnormality in callose synthesis during microsporogenesis in these four exine-defective mutants.

To study the role of callose in microsporogenesis, we have isolated and characterized T-DNA insertional mutants in *Arabidopsis CalS* genes. Two independent insertions in CalS5 were analyzed, and they show a similar phenotype. The *cals5* mutant is male sterile and lacks the normal callose wall and exine pattern in microspores. Tryphine was synthesized but formed aggregates irregularly distributed on the surface of microspores. After the release from the tetrad, microspores did not survive and become
deformed and degenerated. These results demonstrate that the CalS5 gene is responsible for the synthesis of callose in the temporary callose wall of microspores and is required for the normal exine formation during microgametogenesis in Arabidopsis. The pollen exine wall was not formed properly in the mutant and tryphine appeared to be transported from the pollen outer wall into the central vacuole presumably via endocytosis. These data indicate that callose synthesis plays an important role in building the exine sculpture, integrity of which is essential for pollen viability.
CHAPTER 2

TISSUE-SPECIFIC EXPRESSION OF DIFFERENT CALLOSE SYNTHASES AND INVOLVEMENT OF BOTH NPR1-DEPENDENT AND NPR1-INDEPENDENT SIGNALING IN PATHOGEN INDUCTION OF AFFECTED GENES

2.1 INTRODUCTION

During normal development of a plant, callose is found at many locations, e.g. the forming cell plate, pollen, pollen tube, seeds, leaf and stem hairs, sieve plates, plasmadesmata, transient walls of the microsporogenic and megasporogenic tissues, secondary walls and thickenings of the developing xylem. Other biotic (pathogen infection and wounding) and abiotic (desiccation, treatment with heavy metals and environmental stresses) factors also induce the synthesis of callose (Gregory et al., 2002; Stone and Clarke, 1992; Verma and Hong, 2001). The Arabidopsis genome contains 12 callose synthase (CalS) genes, also referred as glucan synthase-like (GSL) genes (Richmond and Somerville, 2001), which have been demonstrated to encode the catalytic subunit of callose synthases (Hong et al., 2001a; Jacob et al., 2003; Nishimura et al., 2003; Østergaard et al., 2002). Overexpression of Arabidopsis CalS1 in tobacco BY-2
cells enhanced callose synthesis at the forming cell plate (Hong et al., 2001a), while disruption of the CalS12 rendered callose-less encasements of papillae in Arabidopsis upon pathogen infection (Jacobs et al., 2003; Nishimura et al., 2003). Apparently multiple CalS genes have evolved in plants to meet the need of callose synthesis in different locations and in response to different physiological and developmental signals (Verma and Hong, 2001). Moreover, the composition of the callose synthase complex is not known (Verma, 2001) and it is possible that this complex is composed of multiple homologous or heterologous subunits.

Nondevelopmental callose production is associated with defenses against fungal and oomycete pathogens, the hypersensitive response elicited by diverse pathogens on nonhost species (Stone and Clark, 1992). P. parasitica is a naturally occurring oomycete parasite of Arabidopsis. It spreads via the production of sexual oospores and vegetative conidiospores. Upon contact with young leaf surface, conidiospores germinate within few hours, develop hyphal networks within leaf tissues and produce haustoria which invade the host cells. In response, the host cells synthesize and deposit callose around the haustoria (Donofrio and Delaney, 2001).

Plants have adopted a variety of induced defense systems to protect themselves against the invading pathogens and insects. These defense responses are regulated by cross communicating signal transduction pathways in which salicylic acid (SA), jasmonic acid (JA) and ethylene play key roles (Thomma et al., 1998; van Wees et al., 2000). Upon pathogen infection, plants increase SA levels, which induce systemic acquired
resistance (SAR). Treatment with the synthetic compound benzothiadiazole (BTH) enhances the expression of several resistance genes, as well as increases callose deposition (Kohler et al., 2002).

Little is known about the signal transduction pathway leading to callose synthesis during plant-pathogen interactions. *NPR1* gene functions in a signaling pathway leading to the induction of pathogen-related (*PR*) genes and the onset of SAR (Ryals et al., 1996; Cao et al., 1997). NPR1 protein does not bind DNA directly, but interacts with the transcription factors TGA2, TGA3, TGA5, and TGA6 (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Fan and Dong, 2002), which bind to the *as-1 cis*-element (TGACG motifs). It has been demonstrated that NPR1 plays an important regulatory role in plant defense (Cao et al., 1994, 1997; Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Fan and Dong, 2002). However, it is not known if *NPR1* is required for the induction of callose synthesis.

We report here that different CalS isoforms are expressed in a tissue-specific manner, and five *CalS* genes are induced by the treatment with SA or *Peronospora parasitica*, a naturally occurring oomycete biotrophic pathogen of *Arabidopsis*. Furthermore, expression analysis of the *CalS* promoter in the WT and *npr1* backgrounds revealed an *NPR1*-dependent *CalS1* and *CalS12* induction and an *NPR1*-independent induction of the five *CalS* genes. These results suggest that there are two SA signaling pathways involved in the induction of different *CalS* genes during pathogen infection.
2.1 Results

2.2.1 Tissue-Specific Expression of CalS Genes

To localize the expression of different CalS genes at the cellular level, we cloned promoter fragments of all 12 CalS genes in Arabidopsis and fused them with the uidA gene (GUS, Figure 1.1). Arabidopsis plants expressing different CalS:GUS constructs were produced and used for histochemical analysis of GUS activity in various plant tissues. The expression of CalS:GUS in different tissues is summarized in Table 1.1. All CalS genes except CalS4 were found to be expressed in the root (Table 1.1, Figure 1.2C). CalS1, 2, 3, 5, 9, 10 and 11 were expressed in the cells of the entire root tip including root meristematic zone, vasculature, pericycle, and cortex. In contrast, CalS6, 7 and 8 were expressed only in the vasculature of the elongation zone. CalS12 was expressed strongly at the root tip and weakly in the vasculature of elongation zone. CalS1, 3 and 9 were also expressed in the root hairs (see Table 1.1).

Expression of all CalS genes except CalS4 and 12 were detected in the cotyledons and true leaves of the seedling (Figure 1.2A-B). CalS1 and 2 were expressed both in the mesophyll cells and the vascular tissue, whereas CalS3, 5, 6, 7, 8, 9, 10 and 11 were detected mainly in the vascular tissue (Table 1.1). The expression of CalS12 was undetectable in leaves under control conditions, but became intensive around the infection sites when the leaves were challenged with the pathogen P. parasitica (Figure 1.2B, CS12), indicating that the expression of this gene is induced by pathogen infection.
Figure 2.1. *CalS*:GUS constructs and the presence of *cis*-elements in various *CalS* promoters. *CalS* promoters amplified from Arabidopsis genomic DNA were ligated in frame with the *uid* gene (GUS coding region) and used for plant transformation. For the *CalS3*, *4* and *6* genes, the promoters were defined to start from the end of the immediate upstream gene. For the rest of the genes, a 2 kb fragment was used, except for the *CalS1* promoter (2.5 kb). The presence of TGACG motif and *W-box* are marked by solid triangle and star, respectively. Alternative name of each *CalS* gene is included in parenthesis.
**Figure 2.2** *CalS:GUS* expression in vegetative tissues of transgenic plants. A, Seedlings. Arrows indicate shoot apical meristems. *CalS4:GUS* (CS4) expression was observed only at the lateral bud (LB) but not in the stem (ST) or other parts of the plant. CS, *CalS:GUS*. Bar = 2 mm except in CS4 where bar = 0.03 mm. B, Leaves. *CalS12:GUS* (CS12) was not expressed in the leaf, but its expression was induced by pathogen infection (arrows indicate infection sites). Bar = 1 mm. C, Roots. Bar = 0.1 mm.
Figure 2.3. CalS:GUS expression in different reproductive tissues of transgenic plants. A, Flowers. Bar = 0.2 mm. B, Pollen grains. Bar = 0.05 mm. C, Siliques. Bar = 0.2 mm. D, Developing seeds. Bar = 0.03 mm.
Table 2.1. Expression of *CalS*:GUS in different tissues of transgenic plants.

Transgenic plants expressing *CalS*:GUS were incubated in GUS staining solution overnight and GUS expression was scored as detected (+) and not detected (-).
The expression of CalS4 was constrained specifically within the axillary meristem (Figure 1.2A, CS4), whereas CalS1, 2, 3, and 9 were expressed in the entire shoot apical meristem.

In flowers, the expression of CalS1 and 4 was undetectable, while CalS2, 3, 6, 7, 8 and 11 were found to be co-expressed in the same cells of anther connective tissue (Figure 1.3A). Co-expression of multiple CalS genes in the same cell suggests that these genes may be functionally redundant or their gene products may form a heteromeric complex in the cell. Similarly, CalS5, 9, 10, and 12 were co-expressed in the pollen grains and embryos (Table 1.1). The expression of CalS2, 9 and 10 was found in both carpels and embryos, whereas CalS1 was expressed only weakly in the carpels but not in the embryos. CalS6, 7 and 8 were expressed in the funicular and vascular tissue in the carpel, while a weak expression of the CalS6 gene was observed in these tissues. CalS11 was expressed only in the carpel, while CalS12 expressed in the embryo. No activity of CalS4:GUS was detected in the pollen grains, carpel and embryo, and this gene was used as a negative control (Figure 1.3B-D).

2.2.2 Induction of CalS Genes by Salicylic Acid

To understand the signal transduction pathway leading to the induction of CalS genes in response to pathogen attacks and physical wounding, four week-old transgenic plants containing different CalS:GUS constructs were treated with various chemicals (SA, JA or H2O2) and GUS activities of the leaf extracts were measured after 24 h of
treatment. Three different lines for each promoter were used for GUS activity assay, they showed similar expression patterns, and data of a representative line were described. As shown in Figure 1.4, five CalS genes were found to be induced by the SA treatment as compared to the mock (water) control. The most profound induction was observed in CalS1 and CalS12 genes, with 2 to 5-fold increase over the control (Student’s t test, P < 0.05). A moderate induction (ranging from 50% to 200% increase) was found in CalS5, 9 and 10 genes. SA treatment did not have any effect on the rest of the CalS genes (CalS2, 3, 4, 6, 7, 8 and 11). Treatment with methyljasmonic acid (MeJA), a JA derivative, did not have significant effect on the expression of CalS genes. SA- and JA-dependent defense pathways have been shown to cross-communicate, providing the plant with a regulatory potential to fine tune the defense reaction depending on the type of attackers encountered (Felton and Korth, 2000; Feys and Parker, 2000; Pieterse et al., 2001). Our data suggests that JA has no antagonistic effect on the SA signaling leading to the expression of CalS genes. Treatment with H2O2 had no significant effect on the induction of any CalS genes (data not shown).

The GUS induction results were verified independently using RT-PCR. Total RNA isolated from four week-old wild type plants treated with water (mock control) or SA for 6 hours was used in RT-PCR with CalS gene-specific primers (Table 1.2). CalS1 and CalS12 transcripts were up-regulated 6.0 h after SA treatment (Figure 1.5A left panel). These transcripts continued to increase 24 h after the treatment (data not shown). No significant induction upon SA treatment was detected by RT-PCR for the rest of the
CalS genes, including CalS5, 9 and 10, which showed 50% to 200% increases in the GUS activity. This suggests that the RT-PCR approach may not be as sensitive as the GUS activity assay, which may be due to the stability of GUS. The RT-PCR results on the induction of CalS1 and 12 by SA treatment are consistent with those observed using the GUS activity assay (Figure 4), because these induction levels are high.

### 2.2.3 NPR1 Requirement for the Induction of CalS Genes by SA Treatment

We searched the CalS promoters for the presence of the TGACG motif and W-box (TTGAC), the two most important regulatory motifs implicated in the mediation of pathogen-induced gene expression in plants (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). One or multiple copies of TGACG motif and W-box were found in all CalS promoters, except CalS12, which contains two W-boxes but no TGACG motif (Figure 1.1). The TGACG motif of the Arabidopsis PR-1 gene has been shown to serve as a binding site for TGA-bZIP transcription factor, which interacts with the NPR1 protein and mediates PR-1 gene expression upon SA treatment (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Fan and Dong, 2002). Because the TGACG motif is absent in the CalS12 promoter, we investigated if the induction of CalS genes by SA treatment is dependent on the NPR1-pathway.

CalS:GUS constructs were used to transform Arabidopsis npr1 mutant plants. GUS activity measured in transgenic npr1 plants expressing CalS1, 5, 9, 10 and 12 were
**Figure 2.4.** Induction of *CalS* genes by SA and MeJA. GUS activity in plants expressing *CalS:GUS* constructs in response to treatments with 2.0 mM SA or 50 µM MeJA for 24 h. Mock treatment with water served as controls. Each value represents the mean of four replicates. The error bars correspond to the SD of four replicate measurements for each line.
Figure 2.5. Induction of CalS genes in npr1 Mutant by SA treatment. A, RT-PCR of CalS genes in wild type (WT) and npr1 plants treated with water (-) or 2 mM SA (+) for 6 h. RNA transcript levels of CalS genes were measured by RT-PCR using total RNA from leaves. Actin transcript was used as an internal control. B, GUS activity in npr1 plants expressing CalS:GUS constructs (CS1, 5, 9, 10, 12). GUS activity was measured in protein extracts prepared from leaves 24 h after treatment with 2.0 mM SA or water (control). Each value represents the mean of four replicates. The error bars correspond to the SD of four replicate measurements for each line.
Figure 2.6. Induction of CalS genes by *P. parasitica* infection. A, GUS activity in transgenic plants expressing CalS:GUS constructs (CS1–CS12) 4 days post inoculation with water (control) or *P. parasitica* (pathogen). Each value represents the mean of four replicates. The error bars correspond to the SD of four replicate measurements for each line. B, Expression levels of CalS transcripts measured by RT-PCR using total RNA from leaves 4 days post inoculation with water (-) or *P. parasitica* (pathogen +). Actin transcript was used as an internal control. C, GUS activity in *npr1* plants expressing CalS:GUS (CalS1 and CalS12) 4 days post inoculation with water (control) or *P. parasitica* (pathogen). Each value represents the mean of four replicates. The error bars correspond to the SD of four replicate measurements for each line.
found to be increased (less than two fold; Figure 1.5B) by the SA treatment as compared to that in plants treated with water (Student’s $t$ test, $P < 0.05$), suggesting that the weak induction of these genes by SA is $NPR1$-independent. Interestingly, the high induction of $CalS1$ and $CalS12$ by the SA treatment was not observed in the $npr1$ mutant background (Figure 1.5B), suggesting that the SA signaling pathway leading to the high induction of the $CalS1$ and $CalS12$ genes is $NPR1$-dependent. We also verified these results using the RT-PCR approach. The RT-PCR products of $CalS1$ and $CalS12$ were increased after SA treatment as compared to the mock control (Figure 1.5A right panel). No significant differences in the amount of RT-PCR products before and after SA treatment were found for the rest of the $CalS$ genes. These results were consistent with that obtained using GUS activity assay, except that the latter is more sensitive and could detect the low levels of induction of $CalS$ gene expression. Taken together, these data indicate that the high induction of $CalS1$ and $CalS12$ requires $NPR1$, whereas the weak induction of $CalS$ genes ($CalS1, 5, 9, 10$ and $12$) is $NPR1$-independent in the SAR signaling pathway.

2.2.4 Induction of $CalS$ Genes by Pathogen Infection

The Emco5 of $P. parasitica$ is compatible with $Arabidopsis$ accession Columbia-0 (Holub and Beynon, 1997; McDowell et al., 1998). One week-old $Arabidopsis$ seedlings expressing $CalS:GUS$ constructs were challenged with $P. parasitica$ isolate Emco5. GUS activity was measured 4 days after inoculation. Five $CalS$ genes, $CalS1, 5, 9, 10, and 12$, were found to be induced by the pathogen treatment (Figure 1.6A). These are the same five genes that were induced by the SA treatment (Student’s $t$ test, $P < 0.05$, Figure 1.4).
The most significant induction was observed in CalS1 and I2, which is consistent with the results obtained with the SA treatment (Figure 1.4).

We also used the RT-PCR approach to verify whether the CalS1 and I2 transcripts were increased in wild type plants after pathogen infection. Primers specific to each of the 12 CalS genes were used to amplify PCR products using RNA samples isolated before or after pathogen infection. As shown in Figure 1.6B, CalS1, and I2 transcripts were up-regulated significantly 4 days after P. parasitica inoculation. No significant differences were detected by RT-PCR for the mRNA levels of the rest of the CalS genes, which is consistent with that obtained after SA treatment (Figure 1.5A, left panel). To test if NPR1 plays a role in pathogen signaling leading to the CalS gene expression, P. parasitica was inoculated onto npr1 plants containing CalS1:GUS and CalS12:GUS transgenes. As shown in Figure 1.6C, the increase in GUS activity in npr1 expressing CalS1:GUS and CalS12:GUS was low after pathogen infection, suggesting that NPR1 is required for the high level induction of CalS1 and CalS12 genes by pathogen infection. This data is consistent with that obtained for the SA treatment (Figure 1.5B).

2.2.5 Haustoria Callose Deposition and the Infection of Downy Mildew in calsl and calsl2 Mutants

One of the significant host responses to P. parasitica invasion is the production of callose around the haustoria invaginating the host plasma membrane. To investigate possible roles of the SA signaling and the CalS1 and CalS12 genes in the formation of callose encasements around the haustoria, we identified CalS1 and CalS12 T-DNA
Figure 2.7. Characterization of T-DNA insertional knockout lines of the CalS1 and CalS12 genes. A and D, CalS1 and CalS12 genes (thin line) contains 42 and 5 exons (thick lines), respectively. Salk_142792 and Salk_002911 lines have a T-DNA inserted in the CalS1 and CalS12 genes. Primers used to identify the homozygous line were marked by arrows. PCR products (937 and 450 bp for CalS1, and 908 and 690 bp for CalS12) of genomic DNA from wild type (WT) and homozygous T-DNA insertional lines, respectively, were indicated. B and E, PCR products of genomic DNA from WT and homozygous T-DNA lines. C and F, RT-PCR of total RNA from WT and T-DNA lines prepared 6 h after treatment with water (-) or 2 mM SA (SA+). No RT-PCR products were detected in the T-DNA lines.
Figure 2.8. Conidiophore production and callose deposition in *cals1* and *cals12* plants. A, Number of conidiophores per leaf in WT plants, *cals1 (cs1)*, *cals12 (cs12)* and plants of two other genotypes defective in SA response (*npr1*) or SA accumulation (*nahG*). The value represents the mean of conidiophores counted on five leaves, each from an individual plant of the same genotype. The error bars correspond to the SD of five replicate measurements for each line. B-D, Leaf phenotypes (B) and haustoria (C-D) developed on leaves of WT, *cals1, cals12, npr1*, and *nahG* plants 6 days post inoculation with *P. parasitica*. Leaves were stained with aniline blue and photographs were taken in bright field (C) or with UV light (D). Bar = 1 mm in B and 0.025 mm in C, D.
knockout mutant lines from the Salk *Arabidopsis* T-DNA lines collection. To identify a homozygous T-DNA line, genomic DNA was isolated from leaves of individual plants of Salk_142792 for *CalS1* and Salk_002911 for *CalS12* genes. Using gene specific primers to these two *CalS*-genes and a T-DNA primer (Figure 1.7A), PCR of genomic DNA from wild type plants produced a band of 900 bp (Figure 1.7B and E), while the genomic DNA of a homozygous T-DNA insertional plant generated a PCR product of about 650 bp for *CalS1* and 690 bp for *CalS12*. Homozygous T-DNA insertional lines, designated as *cals1* and *cals12*, were verified for the absence of CalS1 or CalS12 RNA transcripts using RT-PCR. For this purpose, total RNA was isolated from wild type and the T-DNA insertional plants before and after SA treatment, which induces *CalS1* and *CalS12* gene expression (see Figure 1.4). RT-PCR of RNA from wild type plants produced a band of 900 bp, whereas the RNA from the knockout plants failed to produce a PCR product (Figure 1.7C and F). *cals1* and *cals12* mutant lines showed no observable phenotypes as compared with the wild type plants.

The callose encasements and number of conidiophores in *cals1* and *cals12* mutant lines were compared with that formed in the wild type, *npr1* and *nahG* (transgenic, salicylate hydroxylase-expressing) plants. The *npr1* mutant is known to be defective in the response to SA treatment (Cao et al., 1994; Dong, 1998), whereas *nahG* plants are unable to accumulate SA (Ryals et al., 1996). Conidiophores were produced on all host genotype as early as 6 days post-infection. The number of conidiophores per leaf in *cals12* plants was lower than in wild type plants (Student’s *t* test, *P* < 0.05, Figure 1.8A,
B-C), suggesting that the knockout mutant is more resistant to downy mildew and mutation of CalS12 plays an important role in disease resistance by depositing callose around the haustoria. There was no significant difference in the number of conidiophores formed per leaf between the cals1 mutant and the wild type plants (Student’s t test, P < 0.05, Figure 1.8A). The npr1 and nahG plants, which are compromised in SA signaling or SA accumulation, were more prolific, having 51 ± 7.4 and 81 ± 6.5 conidiophores per leaf, respectively (Figure 1.8A, D-E).

We stained leaves of wild type, cals1, cals12, npr1 and nahG plants with aniline blue for detecting the presence of callose 6 days post-infection. Three patterns of callose distribution around the haustoria were distinguished, a) full encasement by callose; b) callose deposited in a collar-like form around the base or neck of the haustorium; c) minimal callose deposition (see also Donofrio and Delaney, 2001). All three types of callose distribution were detected in the leaves of the five genotypes, but the percentage of each type differed among the genotypes. The frequency of collar-like callose distribution around the haustoria in cals1 and cals12 mutants (25%) was not different from that in wild type (25%), but was much lower than that in npr1 mutant (30%) or nahG plants (50%). Callose deposition is much lower around the haustoria in cals12 than in wild type, cals1, npr1 and nahG plants, typical distribution of callose around the haustoria in these genotypes is shown in Figure 1.8 F-N. It is possible that this different distribution pattern is due to the expression of different CalS isoforms.
2.3 DISCUSSION

2.3.1 Expression of Different CalS Genes is regulated in a Tissue-Specific Manner

There are 12 CalS genes in Arabidopsis distributed over the five chromosomes (Hong et al., 2001a). All CalS genes fall into two groups, one (CalS11 and CalS12) containing 2-3 exons and the other (CalS1-10) up to 50 exons. It has been suggested that multiple CalS genes may have evolved in higher plants for the callose synthesis in different locations and in response to different physiological and developmental signals (Verma and Hong, 2001).

Our data on CalS promoter:GUS analyses have shown that all CalS genes (except CalS4) are expressed in the growing root. This may be a combination several phenomenon such as cell division, cell elongation, root hair emergence, vascularization, or constitutive expression to encounter soil pathogens. CalS4 expression is constrained to the primordia of branching axial buds (Figure 1.2A). CalS1, 2, 3, 5, 6, 7, 8, 9, 10, and 11 were expressed in the leaf. Interestingly CalS1, 3 and 9 were also found to be expressed in the root hairs. These data show that multiple CalS genes are expressed in the same cell-type. CalS5, 9, 10 and 12 exhibited a very similar pattern of gene expression in pollen grains and developing embryos. These four genes may be responsible for the deposition of callose during reproductive events in Arabidopsis. Callose accumulation is important during megasporogenesis, microsporogenesis and pollen tube growth (Tucker et al., 2001; Worrall et al., 1992). CalS2, 3, 6, 7 and 8 were
expressed at the junction of filaments and anthers. Four CalS genes are expressed in pollen at a low level while CalS5 is expressed at a very high level. Mutation in this gene causes partial male sterility (our unpublished data). These results indicate that several CalS isoforms may be required for callose production in a specific tissue. It remains to be tested if different CalS isoforms interact with each other to form heteromeric complexes (Verma and Hong, 2001), or they form homomeric complexes that are co-localized. Such an analysis will await dissection of the CalS complexes.

2.3.2 Multiple Roles of Callose in Plant Development and in Response to Pathogen Attack

Callose encasements have been implicated in the host defense against pathogens by providing a physical barrier that impedes nutrient transfer from the host to the pathogen or possibly delay of pathogen growth long enough for the other host defenses to become active (Allen and Friend, 1983). It has also been suggested that callose may reinforce the plant cell wall at attempted site of parasite penetration or may provide a medium for blocking of toxic compounds or toxins secreted by the pathogen (Kovats et al., 1991; Skou et al., 1984; Stone and Clarke, 1992). However, Arabidopsis pmr4 mutant, a cals12 mutant (gsl5, Jacobs et al., 2003; Nishimura et al., 2003) failed to produce pathogen-induced callose, but was surprisingly more resistant to powdery mildew and downy mildew, rather than being more susceptible as observed by Vogel and Somerville (2000) using Erysiphe cichoracearum.
In this study, we isolated a T-DNA insertional mutant of *CalS12* (Figure 7) and demonstrated that callose deposition around haustoria was reduced and the plants were more resistant to *P. parasitica* (Figure 1.8). This result is consistent with the two other recent reports (Jacobs et al., 2003; Nishimura et al., 2003) and suggests a negative role of callose in plant defense against pathogen infection. Callose in papillae may in fact act as a protection layer for the fungus during pathogenesis, and lack of callose may expose the pathogen to the defense machinery of the host plant (Jacobs et al., 2003). It is also possible that pathogen-induced callose may negatively regulate the SA signaling pathway of the plant, and lack of callose in *cals12* mutant may thus enhance the SA signaling leading to the increased resistance to pathogen infection (Nishimura et al., 2003). Therefore, callose may play multiple roles in the interaction between the pathogen and host.

2.3.3 Requirement of NPR1 in the Induction of *CalS1* and *CalS12* by SA and Pathogen

We have demonstrated that five *CalS* genes could be induced by either SA or pathogen treatments. Among them, *CalS1* and 12 were increased up to 5 fold by these treatments. Weak induction of *CalS5*, 9 and 10 by SA and pathogen treatments was detected in transgenic plants expressing *CalS:GUS* constructs. Østergard et al. also provide the evidence that CalS12 (GSL5) is under the control of the SA pathway using Northern blot analysis (Østergard et al., 2002). Our results suggest that the high induction of *CalS1* and *CalS12* is NPR1-dependent whereas the weak induction of *CalS1*, 5, 9, 10 and 12 genes is NPR1-independent. The *Arabidopsis* NPR1 plays an important
role in inducible plant disease resistance (Johnson et al., 2003; Kinkema et al., 2000; Li et al., 1999; Ryals et al., 1996). The expression of NPR1 is also induced by SA and pathogen treatments (Cao et al., 1997). CalS1 promoter contains six TGACG motif and eight W-box cis-elements, and CalS12 promoter has only two W-box elements (Figure 1.1). The TGACG motif is recognized by TGA-bZIP transcription factors, and the Arabidopsis genome contains 10 TGA-bZIP genes. W-box elements are recognized by WRKY transcription factors and the Arabidopsis genome has 74 WRKY genes. A group of TGA-bZIP and WRKY genes are known to be induced by SA and pathogen in a NPR1-dependent manner (Yu et al., 2001; Kalde et al., 2003). Therefore, it is likely that NPR1 and distinct members of the TGA-bZIP and WRKY transcription factor families may function together and mediate the induction of CalS1 and CalS12 genes (Figure 1.9). A WRKY binding site in the PR-1 promoter has been shown to be a negative regulatory element in response to SA (Eulgem et al., 2000), whereas the parsley WRKY1 acts as a positive regulator of defense gene expression (Rushton et al., 1996).

2.3.4 The Weak Induction of CalS Genes by Pathogen Infection is NPR1-Independent

Because a subset of TGA-bZIP and WRKY genes are known to be induced by pathogen infection independently of the NPR1 gene function (Yu et al., 2001; Dong et al., 2003; Kalde et al., 2003), the weak induction of CalS genes (CalS1, 5, 9, 10 and 12) by SA/pathogen may be mediated via the interaction with TGA-bZIP and WRKY transcription factors (Figure 1.9). Although a direct interaction between NPR1 and members of the TGA-bZIP family is required for the SA-mediated activation of PR genes
Figure 2.9. A model for the regulation of CalS1 and CalS12 gene expression. The high induction of CalS1 and CalS12 genes is regulated in an NPR1-dependent manner, whereas the weak induction of CalS1, 2, 5, 6, 9, 10, 12 genes may involve unidentified factors (?) and is independent of NPR1.
such an interaction may not be needed for the weak induction of the five CalS genes. It is interesting to note that the expression of NPR1 is induced by SA and pathogen, but such an induction is not abolished in the npr1 mutant (Cao et al., 1997). This suggests that the induction of NPR1 itself is NPR1-independent and a separate pathway other than the NPR1-dependent one, must be involved in this process. It is possible that the pathway that controls NPR1 induction is also involved in the weak induction of the CalS genes. Other promoter elements besides TGACG motif and W-box, and other transcription factors besides TGA-bZIP and WRKY may also be required to achieve precise regulation of different CalS genes expressed during SAR.

Transgenic plants expressing inducible CalS promoters may be used for the dissection of the signal transduction pathways leading to the induction of CalS gene expression in response to pathogen attacks, especially the NPR1-independent pathway in the SA signaling. It could become a unique model system to study plant-pathogen interaction, since extrahaustorial callose could be visualized under UV after aniline staining which should facilitate screening of the mutants defective in the induction/activation of callose synthases following fungal infection. This system would also allow the identification of other cis-regulatory element(s) that are present in the CalS12 gene that makes it NPR1-independent.
2.4 Materials and Methods

2.4.1 Promoter-Reporter Constructs and Plant Transformations

Promoter regions of the 12 CalS genes were amplified by PCR using genomic DNA from Arabidopsis thaliana, ecotype Columbia. The forward primer for each CalS gene (see Supplemental Table I) was designed to start from the end of the immediate upstream gene, or from a position that would generate a promoter of about 2 kb in length, except the CalS1 promoter for which a 2.5 kb fragment was used. The reverse primers were designed to amplify part of the coding regions of each CalS gene (20 amino acid residues from the N-termini). PCR-amplified fragments were cloned in pCR2.1 (Invitrogen, Carlsbad, CA), verified by DNA sequencing, and subcloned into pBI101.2 vector (DB Biosciences, Palo Alto, CA) as a translational fusion in frame with the β-glucuronidase (GUS) coding region. The constructs were electroporated into Agrobacterium tumefaciens strain GV3101 and used to transform Arabidopsis plants Col-0 and npr1-3 mutants by vacuum infiltration method (Bechtold et al., 1993). Kanamycin (50 µg/mL)-resistant seedlings were selected, transferred to soil and grown at 22 °C in a growth chamber with a 12-h-light/-dark cycle. T3 homozygous plants were used for further experiments.

2.4.2 Oomycete Infection

The oomycete Peronospora parasitica isolate Emco5 was maintained on its susceptible host Arabidopsis Ws-0 through weekly culture onto two week-old plants
(Donofrio and Delaney, 2001). One week-old seedlings of wild type (Col-0), npr1-3, nahG, cals1, cals12 and transgenic plants containing CalS:GUS constructs were spray-inoculated with a conidiospore suspension of approximately 5 to 8 x 10^4 conidiospores per ml of water. Plants were harvested 4 days post inoculation for GUS activity assay, RNA extraction and microscope analysis.

2.4.3 Histochemical Staining and GUS Activity Assay

Histochemical staining of GUS expression was performed as described (Jefferson et al., 1987). After staining, chlorophyll was extracted from photosynthetic tissues with 70% ethanol. For each CalS:GUS construct, 15 transgenic lines were analyzed and one representative line was selected for further experiments. For activity assay, four week-old plants were sprayed with 2 mM SA, 50 mM MeJA (in 0.1% ethanol), or 20 mM H_2O_2. Salicylic acid was dissolved in water as 2 mM solution and adjusted to pH 7.0 with KOH. Leaves were collected 24 hr after the treatment and homogenized in extraction buffer (50 mM NaH_2PO_4-pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM b-mercaptoethanol). The GUS activity in the supernatant was measured (Jefferson et al., 1987) and expressed as nanomoles of 4-methylumbelliferone produced per minute per mg total protein.

2.4.4 RNA extraction and RT-PCR

Rosette leaves were harvested 6 h after treatment with 2 mM SA or 4 days after conidiospore inoculation. Total RNA was isolated using the Trizol kit (Invitrogen).
Reverse transcription of RNA (50 ng) was carried out using SuperScript II (Invitrogen) and PCR was performed for 25 cycles (94 °C-1 min, 55 °C-1 min, 72 °C-1 min). Primers (see Supplemental Table II) specific to each CalS gene were designed to amplify PCR products of approximately 800-1000 bp. RT-PCR of actin-2 served as an internal control.

2.4.5 Genomic DNA extraction and isolation of a T-DNA insertion line

Seedlings of T-DNA insertional lines Salk_142792 for CalS1 and Salk_002911 for CalS12 were grown individually. One fully expanded leaf of a 4 week-old plant was used to extract genomic DNA. Two CalS-gene specific primers (CS1-LP 5’-AGAAGATCGCAGGTCAAACCAAT-3’ and CS1-RP 5’-AGGAAAGTCAAGACATTCTGTGTGG-3’ for CalS1 or CS12-LP 5’-CGCTTGACTTGA-CTGTACAAGCTG-3’ and CS12-RP 5’-AAGAAAGCAATCCGCGCTCCTC-3’) and one T-DNA primer (LBb1 5’-GCGTGGACCGCTTGCTGCAACT-3’) were used in PCR using genomic DNA as template (Siebert et al., 1995). Genomic DNA from wild type plants produced a PCR product of approximate 900 bp, whereas homozygous plants produced a 650 bp PCR band for CalS1 and 690 bp for CalS12. Both PCR bands were present using genomic DNA from heterozygous plants. Seeds from a homozygous plant, designated as cals1 and cals12, were collected and used for further experiments.
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CalS1F</td>
<td>SaII 5'-ACGTCGACCAAATTTTGGTCAAGTTGACAATG-3'</td>
</tr>
<tr>
<td>CalS1R</td>
<td>SmallI 5'-TTCCCGGGCTGAGTCCGCAGAATCCGCCTCT-3'</td>
</tr>
<tr>
<td>CalS2F</td>
<td>HinDIII 5'-AAAAAAGCTTTGTGCACATGGTTTAC-3'</td>
</tr>
<tr>
<td>CalS2R</td>
<td>BamHI 5'-CGGGATCTTGAGTCCGCAGAATCCGCCTCT-3'</td>
</tr>
<tr>
<td>CalS3F</td>
<td>SaII 5'-TACTCAGTGGAGATTTGAGCTC-3'</td>
</tr>
<tr>
<td>CalS3R</td>
<td>BamHI 5'-CGGGATCCAAATCCCGCGCTGCTGAGCTGAGA-3'</td>
</tr>
<tr>
<td>CalS4F</td>
<td>HinDIII 5'-CAAACAAAGCTTTACATACTCTGATAC-3'</td>
</tr>
<tr>
<td>CalS4R</td>
<td>BamHI 5'-CGGGATCCAGTTTGGAGAATCTGCGCTCTG-3'</td>
</tr>
<tr>
<td>CalS5F</td>
<td>HinDIII 5'-CAAC CAA AGC TTA CAT ATG CAC CA-3'</td>
</tr>
<tr>
<td>CalS5R</td>
<td>BamHI 5'-CGGGATCCAGATGGCGCTCATAGACGCCCCCT-3'</td>
</tr>
<tr>
<td>CalS6F</td>
<td>SaII 5'-AGGTCTGACCATTACAGCGACGACCTGGCT-3'</td>
</tr>
<tr>
<td>CalSR</td>
<td>BamHI 5'-CGGG ATC CCG GAG CTC TCC GAG AAA GAG ACC-3'</td>
</tr>
<tr>
<td>CalS7F</td>
<td>SaII 5'-CGAAGCTTGGGATCTATGCGAAAAAATCCTTAATTATG-3'</td>
</tr>
<tr>
<td>CalS7R</td>
<td>BamHI 5'-AGGTTCGACCATTACGAGGAGCAGCTG-3'</td>
</tr>
<tr>
<td>CalS8F</td>
<td>SaII 5'-TAATCGACCAAGCTGATACGACGACGAC-3'</td>
</tr>
<tr>
<td>CalS8R</td>
<td>BamHI 5'-CGGGATCCGGGCTGGTATTCTGCTGAGTAACCTGAGGAA-3'</td>
</tr>
<tr>
<td>CalS9F</td>
<td>XbaI 5'-GACGGAAGAGAAGAGCTCTAGAAGAC-3'</td>
</tr>
<tr>
<td>CalS9R</td>
<td>SmaI 5'-TTCCCGGGGCTAAACCTCTCTGAAGACGACT-3'</td>
</tr>
<tr>
<td>CalS10F</td>
<td>SaII 5'-ATGCGACCAATATCCGCGCGAGCTGAGA-3'</td>
</tr>
<tr>
<td>CalS10R</td>
<td>BamHI 5'-CGGGATCCAGGAGACGAGGAGACGAG-3'</td>
</tr>
<tr>
<td>CalS11F</td>
<td>SaII 5'-CAGTGGACCAAGAATGGAATCTCTCTGAGAATG-3'</td>
</tr>
<tr>
<td>CalS11R</td>
<td>BamHI 5'-CGGGATCCGGGCTGAGGAGCAGCGCATCTGAGAATG-3'</td>
</tr>
<tr>
<td>CalS12F</td>
<td>SaII 5'-AAGTCGACCGGATGTTGGAGAAGAGACGAG-3'</td>
</tr>
<tr>
<td>CalS12R</td>
<td>SmaI 5'-TTCCCGGGGAAGCCTTCTGCGCGACTGAGA-3'</td>
</tr>
</tbody>
</table>

Table 2.2. Primers used for amplification of CalS promoters by PCR
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CalS1F</td>
<td>5'-GGAAAAAATCAGAGCTTTTGGG-3'</td>
</tr>
<tr>
<td>CalS1R</td>
<td>5'-AGAGATTTGTAGACCTCTACTAAAG-3'</td>
</tr>
<tr>
<td>CalS2F</td>
<td>5'-AGCAACCAGAAGGCTTTTGCATCAACAT-3'</td>
</tr>
<tr>
<td>CalS2R</td>
<td>5'-CCGGAATACAAACTCTAAAGAACCTATA-3'</td>
</tr>
<tr>
<td>CalS3F</td>
<td>5'-AAGTACGCAAAAGGACATCCGAGACATAA-3'</td>
</tr>
<tr>
<td>CalS3R</td>
<td>5'-GGGATAAAATTTGATCTCCCTCTGTGCT-3'</td>
</tr>
<tr>
<td>CalS4F</td>
<td>5'-AATTGGGAAACAGCTATGATGATGGA-3'</td>
</tr>
<tr>
<td>CalS4R</td>
<td>5'-TCAGTCTTTATCTCTGGAC-3'</td>
</tr>
<tr>
<td>CalS5F</td>
<td>5'-TTGCAGCAGAAAGGAGATAGTTTCTCT-3'</td>
</tr>
<tr>
<td>CalS5R</td>
<td>5'-TTATTTCTGCTTCTTTACCAC-3'</td>
</tr>
<tr>
<td>CalS6F</td>
<td>5'-TTTTGAGACTCGCAAGCCTGAGCCTTTGCAA-3'</td>
</tr>
<tr>
<td>CalS6R</td>
<td>5'-GACAGAAGAAACCCCGAAACACTGAGTA-3'</td>
</tr>
<tr>
<td>CalS7F</td>
<td>5'-CTGCAAAAGTGCAAGTGATGATGATGACCAA-3'</td>
</tr>
<tr>
<td>CalS7R</td>
<td>5'-GCAAAGAGAGAGCAGCTTTTGAGCCTCTACA-3'</td>
</tr>
<tr>
<td>CalS8F</td>
<td>5'-AAGACGCTATTCTCTTGAGGCGAGAAGGAA-3'</td>
</tr>
<tr>
<td>CalS8R</td>
<td>5'-TCTATGATTTCTTTCTCTCC-3'</td>
</tr>
<tr>
<td>CalS9F</td>
<td>5'-TTTCGAGAAGAGCTATCTTTCTTGAGATGAT-3'</td>
</tr>
<tr>
<td>CalS9R</td>
<td>5'-CCCATCATTTACTACTGTACACTGCTCGTA-3'</td>
</tr>
<tr>
<td>CalS10F</td>
<td>5'-GCTGATCGTGCAATATCAAGTAGCTCAA-3'</td>
</tr>
<tr>
<td>CalS10R</td>
<td>5'-CACACTAATCTCTCTCTCTCTCTCTCAT-3'</td>
</tr>
<tr>
<td>CalS11F</td>
<td>5'-AGTAGCAGCAATGAAGCTCTTGGACGAA-3'</td>
</tr>
<tr>
<td>CalS11R</td>
<td>5'-CCCAAACCTCTCTGAGCACCACACTTAA-3'</td>
</tr>
<tr>
<td>CalS12F</td>
<td>5'-TCCGCTCTGACAGAGCATGCAGAGACACCAA-3'</td>
</tr>
<tr>
<td>CalS12R</td>
<td>5'-GGCCATTATCCATGAAATCACAGA-3'</td>
</tr>
</tbody>
</table>

Table 2.3. Primers used for amplification of *CalS* transcripts by RT-PCR
2.4.6 Callose Staining

Rosette leaves from wild type, *cals1*, *cals12*, *npr1*, *nahG* plants were fixed in ethanol/acetic acid (3:1, v/v/) and stained with 0.01% aniline-blue (Hong et al., 2001a). The tissue was viewed in a fluorescent microscope using a UV filter.
3.1 Introduction

In developing anthers in angiosperms, microsporocytes synthesize a specialized temporary cell wall consisting of callose (a β-1,3-linked glucan) between the primary cell wall and plasma membrane. Callose continues to be deposited through meiosis and eventually the microspore tetrad is encased in a callose wall. Multiple roles of this temporary wall have been proposed over the last 40 years. It is believed that this callose layer is formed temporarily to prevent cell cohesion and fusion and, upon its degradation, facilitates the release of free microspores to the locular space (Waterkeyn, 1962). The callose wall may also function as a molecular filter protecting the developing microspores from the influence of the surrounding diploid tissues (Heslop-Harrison and Mackenzie, 1967). It can also provide a physical barrier that may help prevent premature swelling and bursting of the microspores. Finally, the callose wall appears to act as a mold where the primexine that provides a blueprint for the formation of exine patterns on mature pollen grains is formed soon after the completion of meiosis during microsporogenesis (Waterkeyn and Beinfait, 1970; Stanley and Linskens, 1974).
After the completion of meiosis and the initiation of microspore exine synthesis, a β-1,3-glucanase (callase) is secreted by the tapetum cells and released into the locular space. The callase activity is low in the anthers during the first meiotic division but increases rapidly at the end of the second meiotic division. The temporary callose wall is degraded and microspores are released in the locular space (Frankel et al., 1969; Steiglitz and Stern, 1973; Steiglitz, 1977).

Several mutants in the callose wall formation and dissolution have been characterized in petunia, and it has been suggested that the timing of callose wall formation and degradation is pivotal for normal pollen development (Izhar and Frankel, 1971; Warmke and Overman, 1972). In transgenic tobacco expressing a β-1,3-glucanase in tapetum cells, the callose wall is dissolved prematurely, causing male sterility (Worrall et al., 1992).

Several Arabidopsis mutants defective in exine formation have also been isolated and characterized. These include defective in exine formation 1 (dex1), male sterility 2 (ms2), faceless pollen-1 (flp1), and no exine formation (nef1). Sporopollenin is deposited randomly on the plasma membrane of dex1 microspores leading to the degeneration of pollen grains. DEX1 protein could be a component of either the primexine matrix or endoplasmic reticulum (ER) and may participate in the assembly of primexine precursors (Paxson-Sowders et al., 2001). In the ms2 mutant, the exine wall is thin and sensitive to acetolysis treatment. The MS2 protein is a fatty acyl reductase that reduces long-chain fatty acids to fatty alcohol, one of the reactions required for the formation of sporopollenin (Aarts et al. 1997). In the flp1 mutant, the microspores and their exine
were visually normal, but the exine pattern is sensitive to acetolysis. FLP1 protein appears to be a transporter or a catalytic enzyme involved in fatty acid biosynthesis, which is a required step for the synthesis of sporopollenin and wax crystals (Ariizumi et al., 2003). In nef1 mutant, sporopollenin is synthesized but is deposited on the locular wall. NEF1 is an integral membrane protein in the plastids of the tapetum cells where lipid synthesis takes place (Ariizumi et al., 2004). However, there is no abnormality in callose synthesis during microsporogenesis in these four exine-defective mutants.

Twelve callose synthase (CalS) genes, also referred as glucan synthase-like (GSL) genes (Richmond and Somerville, 2001), are present in Arabidopsis and two of them have been demonstrated to encode the catalytic subunit of callose synthases (Hong et al., 2001a; Jacob et al., 2003; Nishimura et al., 2003; Østergaard et al., 2002). Callose is synthesized at many locations during the normal plant development (Gregory et al., 2002; Stone and Clarke, 1992; Verma and Hong, 2001). The synthesis of callose is also induced by other biotic (pathogen infection and wounding) and abiotic (desiccation, treatment with heavy metals and environmental stresses) factors. Overexpression of Arabidopsis CalS1 in tobacco BY-2 cells results in the enhanced callose synthesis at the forming cell plate (Hong et al., 2001a), while knockout of CalS12 leads to the callose-less encasements of papillae in Arabidopsis upon pathogen infection (Jacobs et al., 2003; Nishimura et al., 2003). Thus different CalS genes meet the demand of callose synthesis in different locations and in response to different physiological and developmental signals (Verma and Hong, 2001).
Figure 3.1. Identification of T-DNA insertional knockout lines of the CalS5 gene.

(A) CalS5 gene (thin line) contains 39 exons (thick lines) in the coding region. Salk_009234 and Salk_026354 lines have a T-DNA inserted in the CalS5 gene. Primers used to identify the homozygous line were marked by arrows. PCR products (907 and 600 bp for CalS5-1, and 971 and 650 bp for CalS5-2) of genomic DNA from wild type (WT) and homozygous T-DNA insertional lines, respectively, were indicated.

(B-C) PCR products of genomic DNA from wild type (WT) and homozygous T-DNA lines of cals5-1 (cs5-1) and cals5-2 (cs5-2).

(D) RT-PCR of total RNA from WT and T-DNA lines. A PCR product of 1200 bp was produced when total RNA from wild type plants was used, whereas no RT-PCR product was detected in the T-DNA lines. The expression of Actin-2 gene served as control.

(E) Transmembrane (TM) probability of CalS5 peptide predicted by the transmembrane hidden Markov model (TMHMM) program.

(F) Topology of CalS5 in membrane. The long rectangle indicates the membrane, and the vertical black bars represent the transmembrane helices of CalS5. The length of the peptide chain in each non-membrane-spanning segment is indicated by the number of amino acid residues.
**Figure 3.2.** Reduced fertility in *cals5* mutants.

(A) No apparent morphological defects except siliques were found in *cals5-1* (5-1) and *cals5-2* (5-2) mutants as compared to wild type plants (WT).

(B-C) Comparison of flowers and mature siliques between the wild-type and *cals5* mutant lines. Note that the length of flower stalks is similar but that of the siliques different between the wild type and mutants.

(D-E) Comparison of flowers. Note that anthers of *cals5-1* mutant line are shrunken. Bar = 50 µm.

(F-G) Comparison of anthers. Note that anthers of *cals5-1* mutant line contain few pollen grains. Bar = 50 µm.
Figure 3.3. Male and female gametophyte development in *cals5* mutant lines. (A-B) Developing pollen mother cells shows uniform meiocytes in the wild type (A) and mutant plants (B). Bar = 10 µm for all pictures. (C-D) Tetrad of microspores that are products of meiosis. The microspores of wild type plants are encased in callose walls and are separated by clear boundaries. In the mutants, the four microspores form a clamp and the boundaries are barely recognizable. (E-F) Comparison of microspores. After the release from the tetrad, microspores continue to develop into round and mature pollen grains in the wild type plants, whereas they start to deform and degenerate in the mutants. (G-H) Enlarged views of the developed microspores in wild type plants and the degenerated microspores in the mutants. (I-L) Megaspore mother cell (arrow, MMC) of the wild type (I) and mutant (K), and embryo sack (arrowhead, ES) of the wild type (J) and mutant (L). Note that there are no apparent differences in embryo sac development between the wild type and mutant plants.
To study the role of callose in microsporogenesis, we have isolated and characterized T-DNA insertional mutants in *CalS5* gene (*cals5*). The *cals5* mutant exhibited male sterility and lacked the normal callose wall and exine pattern in microspores. Tryphine was synthesized but formed aggregates randomly distributed on the surface of microspores. Upon the release from the tetrad, microspores did not survive and collapse of the pollen wall occurred. These results demonstrate that the *CalS5* gene is responsible for the synthesis of callose in the temporary callose wall of microspores and is essential for exine formation during microgametogenesis in *Arabidopsis*.

3.2 Results

3.2.1 Isolation of *cals5* T-DNA knockout mutants

To investigate possible roles of *CalS* genes in plant development, we screened a collection of the Salk *Arabidopsis* T-DNA lines that contain an insertion in one of the 12 *CalS* genes. We identified two independent lines that exhibited severely reduced silique length and seed yield in otherwise apparently “normal” plants. The two lines, Salk_009234 and Salk_026354, contain a T-DNA insertion in intron 15 of the *CalS5* gene. Their homozygous lines are referred as *cals5-1* and *cals5-2*. To identify a homozygous T-DNA plant, genomic DNA was isolated from leaves of individual plants of each line. Using two *CalS5*-gene specific primers and a T-DNA primer (Figure 2.1A), PCR of genomic DNA from wild type plants produced a band of 907 and 971 bp, while the genomic DNA of a homozygous T-DNA insertional plant generated a PCR product of about 600 bp for *cals5-1* and 650 bp for *cals5-2* (Figure 2.1B-C). Complete knockout of
CalS5 gene expression in these homozygous lines was verified for the absence of CalS5 RNA transcripts using RT-PCR. For this purpose, total RNA was isolated from inflorescences of wild type and mutant plants. RNA from wild type plants produced a PCR band of approximately 1200 bp, whereas RNA from the knockout plants failed to produce a PCR product (Figure 2.1D).

3.2.2 CalS5 is a transmembrane protein and contains multiple functional motifs

The predicted coding region of CalS5 gene is composed of 39 exons (Figure 2.1A) and encodes a peptide of 1923 amino acid residues with a calculated molecular mass of 220 kD. Topology analysis of CalS5 revealed that similar to CalS1 (Hong et al., 2001a), it is a transmembrane protein with 16 transmembrane helices that are clustered in two regions separated by a large hydrophilic domain (727 residues). It also has a hydrophilic N terminus (485 residues) that contains a GTP-binding motif (Prosite PDOC00185) and an ABC transporter motif (Prosite PDOC00185; Figure 2.1F). The central hydrophilic loop contains a calcium-binding domain (Prosite PDOC00018) and a β-1,3-glucan synthase component that is conserved in the glycosyltransferase 48 family that includes β-1,3-glucan synthases from yeast and plants (Verma and Hong, 2001). 

3.2.3 Sterility in cals5 mutants

There were no observable differences in vegetative parts of the plant between the mutant and wild type. They were similar in plant size, and had comparable growth rates and leaf numbers throughout the vegetative growth (Figure 2.2A). However, the mutant plants developed abnormal flowers with shrunken anthers (Figure 2E, G) and were
partially or completely sterile. Siliques formed on homozygous *cals5* mutants were very short and contained no seeds or only few seeds, as compared with those on wild type plants (Figures 2.2C). Flowers of the mutant plants were open for considerably longer periods than those of wild-type plants, and many remained unfertilized. The flowers were completely fertile and developed normal-sized siliques, when cross-pollinated with wild type pollen grains (see below), suggesting that the female sex organs of the mutant are normal.

3.2.4 Arrest in tetrad and microspore development during male gametogenesis

The defects in pollen formation and fertility led us to analyze in more detail the development of male and female gametophytes. Both wild-type and *cals5* mutant developed uniformly sized meiocytes in their anthers (Figures 2.3A-B). However, the tetrads, the products of meiosis, were abnormal in *cals5* mutant plants. Under light microscope, the border between the four cells of a tetrad became undistinguishable (Figure 2.3C-D), suggesting that the cell wall may start to collapse. Microspores of the mutant plants exhibited severe morphological defects (Figure 2.3E-F). They were shrunken, broken, and degenerated eventually (Figure 2.3G-H). As a consequence, no viable pollen was developed in most of the mature anthers in the mutant plants. A few anthers were able to develop 1-15 mature pollens that were viable and fertile, whereas a normal anther of the wild type plant may contain about 800 mature pollen grains.

3.2.5 Megasporogenesis and megagametogenesis in *cals5* plants are not affected
Figure 3.4. Arrest in microspore development in *cals5* mutants.

(A-C) Viability of pollen grains as assayed with Alexander staining. Anther of the wild type plants is filled with viable, purple-stained pollen grains (purple; A), while that the *cals5* mutants contains only nonviable pollen grains (green; B) or only a few viable pollen grains (arrow; C). Bar = 25 µm.

(D-F) Transverse sections of anthers. Wild-type anthers are dehiscent and contain round, well-developed pollen grains (D). Anthers of the mutants contain no pollen grains (E), or rarely a few grains (F). They are dehiscent at the mature stage and do not show an altered structure compared with that of the wild type. Note that degenerated remnants of microspores (arrowheads) are visible in the mutant anthers (E-F) but not in the wild type (D). Bars = 200 µm.
Figure 3.5. Tissue-specific expression of Cal5S:GUS and Cal5S:GFP in transgenic plants. (A-F) GUS staining of plants expressing Cal5S:GUS. Expression of Cal5S was detected in the vascular tissues of cotyledons (A), leaf (C) and petals (D), in the shoot meristem (A), root tip (B), pollen grains (G), young silique (F) and developing seed (F, inset). E, pollen grains of wild type plants. (H-S) GFP fluorescence in living plants expressing Cal5S:GFP. Expression of Cal5S was detected in the tetrad (K), pollen grain (O) and growing pollen tube (S). Shown are bright field and fluorescence images of tetrad from wild type (H-I) and the mutant (J-K); pollen grain of wild type (L-M) and the mutant (N-O); and growing pollen tube of wild type (P-Q) and the mutant (R-S).
Megasporocytes in *cals5* mutants undergo normal cell divisions during early ovule development as observed in the wild type plants (Figure 2.3I, L). After the first two mitotic divisions of the functional megaspore during megagametogenesis, the developing embryo sac contained four nuclei that follow a final mitotic division and led to the eight nuclei contained in the mature gametophyte. One visible central cell in the embryo sac was eventually developed at the late stage during megagametogenesis (Figure 2.3J, L). No apparent differences in megasporogenesis and megagametogenesis between *cals5* mutant and wild type plants were observed, suggesting ovule development in *cals5* mutants are not affected. When cross-pollination with the wild-type *Arabidopsis* pollens was performed, the mutant flowers developed normal size siliques that contained an average of approximately 40 seeds per silique, which was comparable with 42 seeds per silique recovered on the wild-type plants under the same growth conditions. The F1 plants of the backcross were also normal, and F2 generation exhibited a 3:1 segregation of fertile versus sterile individuals. This result shows that both ovule development and female fertility in *cals5* mutants are normal and the sterility is due to defective male gametes.

3.2.6 Absence of viable pollen grains in *cals5* mutants

Mutants with a severe sterility phenotype had small recessed anthers that were brown and appeared to lack pollen grains. Under differential interference contrast (DIC) microscope, anthers of *cals5* mutants were partially empty and contained shrunken and malformed pollen grains (Figure 2.4B-C; E-F), as compared with the control anthers that
were filled with round and well-formed pollen grains (Figure 2.4A, D). To determine whether the malformed pollen grains were viable, we stained with Alexander solution. Anthers of the wild type plants contained full of purple-stained (viable) pollen grains (Figure 2.4A), whereas those of cals5 mutants were empty and pollen grains that remained were nonviable (green grains) (Figure 2.4B-C). Only a few purple pollen grains were occasionally observed in the mutant anthers (arrow in Figure 2.4C). Although anthers of the cals5 mutants were smaller than that of the wild type at an equivalent stage of development, they did not appear altered in their morphology. Transverse sections of the flower confirmed that these empty and smaller anthers still continued to develop and became partially dehiscent at the mature stage (Figure 2.4E-F). Remnants of degenerated pollen grains were visible in the anthers of the mutant plants (arrowhead, Figure 2.4E-F).

3.2.7 Tissue-specific expression of CalS5 gene

To localize the expression of CalS5 gene at the cellular level, we cloned the promoter of the CalS5 gene and used it to drive the expression of GUS and GFP as reporters (Figure 2.5). Arabidopsis plants expressing CalS5:GUS and CalS5:GFP constructs were produced and used for histochemical analysis of GUS activity and fluorescence detection of GFP in different plant tissues. As shown in Figure 2.5, CalS5 was found to be expressed in the cells of the entire root tip including root meristem, vasculature, pericycle, and cortex. The expression level in the maturation zone of the root was relatively weak (data not shown), which is in contrast to the expression patterns
Figure 3.6. Callose deposition during microsporogenesis. Reproductive tissues were fixed and stained with aniline blue for callose. Bright field and UV fluorescence images were taken under an epifluorescence microscope. Note that callose deposition was nearly absent in meiocytes, tetrads, microspores and pollen grains in the mutant plants. 

(A-D) Meiocytes of wild type (A-B) and mutant (C-D). Bar = 10 µm for A-L. 

(E-H) Tetrads of wild type (E-F) and mutant (G-H). 

(I-L) Microspores of wild type (I-J) and mutant (K-L). 

(M-R) Pollen grains of wild type (M-N), round-shaped mutant pollen (O-P) and shrunken mutant pollen (Q-R). Bar = 10 µm.
Figure 3.7. Ultrastructures of pollen grains.

(A-B) TEM of pollen grains of wild type (A) and mutant (B). Note that a large central vacuole is present only in the mutant pollens. Bar = 0.1 μm for A, B and E.

(C-D) SEM of pollen grains of wild type (C) and mutant (D). Bar = 5 μm.

(E) The central vacuole in the mutant pollen contained numerous multivesicular bodies (MVB) and endocytosed trypsin granules (SP) that were encapsulated by a membrane.

(F-G) Extine structure of wild type (F) and mutant (G). Note the disappearance of the bacula (Ba) and tectum (Tc) in the mutant pollen. Bar = 0.5 μm.
of other CalS genes including CalS6, 7 and 8, which are highly expressed in the vasculature of the elongation zone (Dong et al., 2004). In hypocotyls and stems, CalS5:GUS expression was primarily restricted to the vascular bundles, while little expression is found in the parenchyma and epidermal tissues. In cotyledons, leaves and sepals, CalS5:GUS was expressed in the vascular bundles (Figure 5A-D). In flowers, CalS5 was expressed at high levels in the anthers, particularly in pollens and embryos (Figure 2.5E-F). In contrast, four other CalS genes (CalS9, 10, 11, and 12) that are also expressed in pollen grains (Dong et al., 2004) had much lower activity than CalS5, and apparently they cannot compensate the loss of the CalS5 gene. Transgenic plants expressing CalS5:GFP were also analyzed in order to follow the expression of CalS5 gene in live tissues. Expression of CalS5:GFP was detected throughout microsporogenesis, pollen development and pollen tube growth (Figure 2.5H-S).

3.2.8 Meiosis male gametophyte is normal in the cals5 mutants

We followed the progression of meiosis in cals5 mutants in order to understand if callose is involved in the process of meiosis in higher plants. The contents of anthers at various developmental stages were analyzed by staining for callose with aniline blue. Microsporocytes of both mutant and wild type anthers appeared normal before the initiation of meiosis. Sporocytes of cals5 mutants produced much less callose, however, as compared to that of the wild type seen as bright blue fluorescence, but underwent apparently normal meiosis (Figure 2.6A, D). The four products of meiosis that were held together within the tetrads could be recognized in cals5 mutants (Figure 2.6E-H)
were filled with round and well-formed pollen grains (Figure 2.4A, D). To determine whether the malformed pollen grains were viable, we stained with Alexander solution. Anthers of the wild type plants contained full of purple-stained (viable) pollen grains (Figure 2.4A), whereas those of *cals5* mutants were empty and pollen grains that remained were nonviable (green grains) (Figure 2.4B-C). Only a few purple pollen grains were occasionally observed in the mutant anthers (arrow in Figure 2.4C). Although anthers of the *cals5* mutants were smaller than that of the wild type at an equivalent stage of development, they did not appear altered in their morphology. Transverse sections of the flower confirmed that these empty and smaller anthers still continued to develop and became partially dehiscent at the mature stage (Figure 2.4E-F). Remnants of degenerated pollen grains were visible in the anthers of the mutant plants (arrowhead, Figure 2.4E-F).

3.2.9 Defective exine formation

To understand the mechanism for the pollen degeneration in *cals5* mutant, we compared the ultra-structures of pollen cell wall of the mutant and wild type using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Pollen grains of *cals5* mutant had a spotted surface pattern that was in contrast to the reticulate pattern observed on the wild type pollens (Figure 2.7C-D). Further observations using TEM suggested that *cals5* mutant completely lacks the exine (baculum and tectum) structure (Figure 2.7F-G). Tryphine, derived mainly from the remnants of the tapetum, formed a pollen coat that filled the interstices of the exine of the
wild type pollens (Figure 2.7F), whereas it aggregated into numerous large agglutinations on the pollen surface in cals5 mutant (Figure 7G). In addition, numerous large agglutinations of sporopollenin-like material were found inside the pollen that appeared to be endocytosed into the central vacuole that was not present in the wild type pollens (Figure 2.7A-B). There was also a significant difference in the plasma membrane between the mutant and the wild type pollen. The plasma membrane of the wild type pollen was flat and the intine maintained the same thickness surrounding the plasma membrane (Figure 2.7A, F). In contrast, the plasma membrane of the cals5 pollen was irregular waved (Figure 2.7B, G), suggesting an intensive activity of endocytosis at the plasma membrane. This was consistent with other two observations that tryphine was transferred from the pollen surface to the central large vacuole and that the central vacuole contained numerous multivesicular bodies (Figure 2.7E). Taken together, these data suggest that the callose wall of microspores may provide a structural basis for the formation of exine. Removal of the callose wall in cals5 may have two consequences: the exine cannot be formed and the intine becomes “leaky” having extremely active endocytosis on the plasma membrane. As a result, these microspores appear to undergo apoptosis, stop development and eventually become deformed and degenerate.

3.3 Discussion

3.3.1 CalS5 gene encodes a callose synthase required for microgametogenesis

Two of the twelve CalS genes, CalS1 and CalS12, have been characterized previously (Hong et al., 2001a; Østergaard et al., 2002; Nishimura et al., 2003; Jacobs et
In this report, we describe the isolation and characterization of two insertional mutants of CalS5 and demonstrate that callose synthesized by CalS5 is essential for the male fertility in Arabidopsis.

CalS5 encode a peptide of 1923 amino acid residues. Like other callose synthases in the CalS protein family, it contains multiple transmembrane domains that are clustered in two segments. The N-terminus (480 residues) and a large central hydrophilic loop (750 residues) of this protein are predicted to face the cytoplasm and may play a role in the interaction with other components of the callose synthase complex (Verma and Hong, 2001). The central loop contains the putative catalytic site which has been divided into two, the UDP-glucose binding domain (domain 1) and the glycosyltransferase domain (domain 2; Kelly et al., 1996). The domain 2 in CalS5 and other CalS proteins is characterized by the absence of the QXXRW motif that is highly conserved in the CesA superfamily of proteins (Saxena et al., 1995; Verma and Hong, 2001). It appears that plant CalS may not directly bind UDP-glucose; instead, this function may be carried out by UDP-glucose transferase (UGT1) that contains a UDP-glucose binding signature and has been shown to tightly interact with the catalytic subunit of CalS1 (Hong et al., 2001a). Results presented here clearly show that CalS5 is responsible for the synthesis of callose that is required for pollen development and male fertility in Arabidopsis. In the absence of this enzyme in the knockout cal5 mutants, callose is depleted on the cell wall of meiocytes, tetrads, microspores and pollen grains (Figure 2.6D, H, L, P, R).
3.3.2 *CalS5* is expressed highly in anthers and its role in microgametogenesis cannot be replaced by other *CalS* genes

The expression of each of the 12 *CalS* genes in *Arabidopsis* is regulated differently in order to meet the demands for the callose synthesis in different locations and in response to different physiological and developmental signals (Verma and Hong, 2001; Dong *et al*., 2004). *CalS1* and *CalS12* have localized to the forming cell plate and to the site of pathogen infection, respectively (Hong *et al*., 2001a; Nishimura *et al*., 2003; Jacobs *et al*., 2003). Consistent with their functions in plants, *CalS1* gene is expressed at high levels in the meristems where cell division and cytokinesis are active (Dong *et al*., 2004). *CalS12* gene is induced during pathogen infection and knockout or knockdown of this gene causes the depletion of callose around the infection site and renders resistance to pathogens (Nishimura *et al*., 2003; Jacobs *et al*., 2003; Dong *et al*., 2004). *CalS1* gene is also induced during pathogen infection (Dong *et al*., 2004), however, its role in disease resistance is not known. *CalS4* expression is confined to the primordia of branching axial buds (Dong *et al*., 2004) and the exact role of this gene in bud initiation and formation also remains to be determined.

It has been shown that multiple *CalS* genes are expressed in the same cell-type, *e.g.* *CalS1, 2, 3, 5, 6, 7, 8, 9, 10, and 11* are expressed in the leaf (Dong *et al*., 2004). Because of this redundancy in gene expression, it is not surprised that no apparent defects were observed in the leaves and other tissues except the anther in the *cals5* mutant plants. It is likely that the role of *CalS5* protein in these tissues in the *cals5* mutants is overlapping partially or completely by that of other *CalS* genes that are expressed in the
same tissues. In anthers where CalS5 is highly expressed, its role in microgametogenesis cannot be replaced apparently by other CalS genes, such as CalS9, 10, 11, and 12 that are also co-expressed in the anthers (Dong et al., 2004). This cell type-specific high level expression appears to be coupled with the formation of specialized cell wall in pollens.

3.3.3 Callose is required for exine wall formation of pollen grains

In most plant species, pollen grain wall has similar architecture with the inner layer, intine and the outer layer, exine. The exine consists of a simple inner layer and an outer sculptured layer, called exine and sexine, respectively (Figure 2.8A). The sexine is built by rod-like structures called bacula, which are subsequently covered by a roof in different form, called tectum. In the final stage of wall development, additional proteins, phenolics, and fatty acid derivatives are deposited within the exine, forming a layer called tryphtine. The exine pattern of pollen grains in cals5 mutants was altered dramatically. The sexine, consisting of baculae and tectums, was missing, whereas the tryphtine that fills the interstices of sexine on wild type pollen grains formed aggregates on the outer layer of the mutant pollens (Figure 2.7C-D, 2.8B). This exine-defective phenotype resembles that of transgenic plants expressing a callase (β-1,3-glucanase) in the anther locule (Worrall et al., 1992), but is different from that of the ms2, dex1, flp1, and nef1 mutants that have previously been characterized to be defective in exine formation. The sporopollenin failed to be deposited onto the microspore wall in nef1, whereas it was randomly aggregated along the microspore wall the in the dex1 mutant (Paxson-Sowders et al., 1997; 2001; Ariizumi et al., 2004). The occasional pollen grains produced by the
ms2 mutant have a very thin exine, whereas the exine of flp1 mutant appears to be normal but is acetolysis sensitive (Aarts et al., 1997; Ariizumi et al., 2003).

It has been proposed that the callose wall of tetrads acts as a mold that is filled by the primexine, on which sporopollenin is deposited, leading to the formation of the final patterned exine wall (Waterkeyn and Beinfait, 1970; Stanley and Linskens, 1974). In the cals5 mutant, callose was depleted from the cell wall in meiocytes, tetrads, and microspores during microsporogenesis (Figure 6). Thus a proper exine wall fails to develop in mature pollen grains of the cals5 mutant (Figure 7-8). Without an exine pattern, the sticky trypheine, which consists of various proteins, phenolics and fatty acid derivatives, could not form a thin layer covering the pollen grains. Instead, it aggregated into electron-dense granules randomly distributed on the pollen wall in cals5 mutant (Figure 2.7). In the dex1 mutant, in which callose deposition is normal, abnormal deposition of sporopollenin onto the plasma membrane occurred (Paxson- Sowders et al., 1997). In the nef1 mutant that develops normal callose wall, sporopollenin is not deposited onto the plasma membrane of microspores because of the lack of normal primexine, and finally, aggregates and accumulates on the inner surface of the locule wall (Ariizumi et al., 2004). In cals5 mutant, trypheine was only found on the pollen surface and in the remnant of the degenerated pollen wreckage, but not on the inner surface of the locule wall (Figure 2.4).

It appears that timing of microsporogenesis is not affected by the change in the callose wall because the four microspores are produced and held together in a tetrad-like
Figure 3.8. Role of the callose wall in exine formation during microgametogenesis. 
(A) Structure of a mature pollen grain wall. Pm, plasma membrane. (B) Pollen exine formation in wild type and calls5 mutant plants. Meiocytes and microspores of tetrads are encapsulated in a thick callose wall. Upon the completion of meiosis, the primexine is formed between the plasma membrane and the callose wall. The probaculum becomes evident at the locations where the plasma membrane protrudes into the expanded primexine layer. Protosporopollenin is synthesized in the microspores and secreted only to the probaculae. Protosporopollenin is a partially polymerized precursor for sporopollenin and is resistant to the acetolysis treatment. As the primexine dissipates, the nexine is formed as a foot-layer of the exine. The callose wall is degraded by callase, a β-1,3-glucanase secreted by the tapetum cells. Following microspore release from the tetrads, protosporopollenin is rapidly converted to sporopollenin via cross-linking. Sporopollenin secreted from the tapetum cells plays an important role in the formation of the acetolysis-resistant exine pattern (baculae and tectum). Finally, the intine is formed and tapetally-derived tryphine fills the interstices of the exine pattern. In calls5 mutant, the callose wall is not formed. The exine pattern cannot be formed in the calls5 mutant. Coating with tryphine and the formation of nexine and intine layers are not affected by the lack of the callose wall. Tc, tactum. Ba, baculum. Tp, tryphine. Ne, nexine. In, intine. Pm, plasma membrane.
structure in the anthers of the \textit{cals5} mutant (Figure 2.3, 2.6). This suggests that material other than callose is capable of holding the microspores in a tetrad. Alternatively, small amount of callose could also be synthesized in the \textit{cals5} mutant because of four other \textit{CalS} genes (\textit{CalS9}, 10, 11, and 12) that are known to be expressed in the anther tissues as well (Dong \textit{et al.}, 2004). Release of the microspores from the tetrad is also observed in the mutant anthers, suggesting that factors other than callase may also be required in this process (Steiglitz, 1977; Sexton \textit{et al.}, 1990). Because cellulose is also part of the tetrad cell wall, cellulase may be required for the release of the microspores. Callose, however, appears to play a crucial role in the formation of a normal microspore cell wall. In its absence, the exine wall lacks regular sculpturing and is composed of an unusual structure overlaid by apparently random deposits of a material that is probably trypthine. Further work is required to determine whether the primexine is actually formed in \textit{cals5} mutants. Callose synthesis is important during megasporogenesis, microsporogenesis and pollen tube growth (Tucker \textit{et al.}, 2001; Worrall \textit{et al.}, 1992). \textit{CalS2, 3, 6, 7} and \textit{8} are co-expressed at the junction of filaments and anthers (Dong \textit{et al.}, 2004), suggesting that several CalS isoforms may be required for callose production in specific tissues.

It remains to be tested if different CalS isoforms interact with each other to form heteromeric complexes, or they form homomeric complexes that are co-localized (Verma and Hong, 2001). The fact that callose deposition affects exine formation suggests that this homopolymer interacts with other proteins creating a bridge with the plasma membrane where baculae are formed. The full protein composition of pollen wall
remains to be elucidated.

3.4 Experimental procedures

3.4.1 Promoter-reporter constructs and plant transformation

Promoter region of the *CalS5* gene was amplified by PCR using genomic DNA from *Arabidopsis thaliana*, ecotype Columbia. The forward primer for *CalS5* gene was designed to start from the end of the immediate upstream gene. The reverse primers were designed to amplify the coding regions of *CalS5* gene for the first 20 amino acid residues from the N-terminus. PCR-amplified fragments were cloned in pCR2.1 (Invitrogen, Carlsbad, CA), verified by DNA sequencing, and subcloned into pBI101.2 vector (DB Biosciences, Palo Alto, CA) as a translational fusion in frame with the £]-glucuronidase (GUS) coding region. The construct was electroporated into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis* plants *via* flower infiltration. Kanamycin (50 µg/mL)-resistant seedlings were selected, transferred to soil and grown in a growth chamber. Promoter fragment was cloned in pENTR1A and then subcloned into pBGWFS7 as a translational fusion in frame with GFP by LR recombination reaction (Invitrogen). The construct was electroporated into *Agrobacterium tumefaciens* strain ABI and used to transform *Arabidopsis* plants *via* flower infiltration. 50 µg/ml of BASTA (Sigma-Aldrich, St. Louis, MO) in water was sprayed on 6-day old seedlings after germination and was applied three times every other three days. Basta-resistant seedlings were selected, transferred to pots and grown in a growth chamber.
3.4.2 GUS staining and GFP detection

Histochemical staining of GUS expression was performed as described (Jefferson et al., 1987). After staining, chlorophyll was extracted from photosynthetic tissues with 70% ethanol. Fifteen transgenic lines expressing either CalS5-GUS or CalS5:GFP construct were analyzed and one representative was selected for further experiments. Localization of GFP was performed using an epifluorescence microscope.

3.4.3 RNA extraction and RT-PCR

Total RNA was isolated from flowers using the Trizol kit (Invitrogen). Reverse transcription of RNA (50 ng) was carried out using SuperScript II (Invitrogen) and PCR was performed for 25 cycles (94°C-1 min, 55°C-1 min, 72°C-1 min). Primers CalS5F, 5’-TTGCAGCGAAAGGAGATAGTTCTT-3’, CalS5R, 5’-TTATTTCTGCTTCTTACCACC-3’ were designed to amplify PCR products of approximately 1200 bp. RT-PCR of actin-2 served as an internal control.

3.4.4 Genomic DNA extraction and isolation of a T-DNA insertion line

Seedlings of T-DNA insertional lines Salk_009234 for CalS5-1 and Salk_026354 for CalS5-2 were grown individually. One fully expanded leaf of a 4 week-old plant was used to extract genomic DNA. Two CalS-gene specific primers (CS5-1-LP 5’-TGCAACTCAGGATCCATTTTCTT-3’ and CS5-1-RP 5’-CCAGAAAAACTACCTACCTCTCCAAACG-3’ for CalS5-1 or CS5-2-LP 5’-
and C S S 2 - R P 5’-G C A T A C C A A A A T T G A G T G T C C A T - 3’) and one T-DNA primer (L B b 1 5’-G C G T G A C C G C T T G C T G C A A C T - 3’) were used in PCR using genomic DNA as template (Siebert et al., 1995). Genomic DNA from wild type plants produced a PCR product of approximate 907 and 971 bp, whereas homozygous plants produced a 600 bp PCR band for CalS5-1 and 650 bp for CalS5-2. Both PCR bands were present using genomic DNA from heterozygous plants. Seeds from a homozygous plant, designated as cals5-1 and cals5-2, were collected and used for further experiments.

3.4.5 Callose staining and pollen viability assay

For callose staining, flowers were fixed in ethanol/acetic acid (3:1, v/v) and stained with 0.01% aniline-blue (Hong et al., 2001a). The tissue was viewed in a fluorescent microscope using a UV filter. For pollen viability assay, anthers were stained in Alexander solution and observed by light microscopy (Alexander, 1969). Differential interference contrast microscopy was used to observe anthers or ovules that had been cleared using Herr’s solution (phenol:chloral hydrate:85% lactic acid:xylene:oil of clove [1:1:1:0.5:1]).

3.4.6 Light microscopy

Anthers were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0 and were post fixed in 2% osmium in the same buffer. An ethanol dehydration series was performed and the samples were embedded in Spurr resin. Transversal sections of 1.5

72
μm were cut using an LKB Ultratome III ultramicrotome, colored with 0.5% toluidine blue, and observed with a light microscope.

3.4.7 Electron microscopy

For scanning electron microscopy, fresh pollen grains were coated with 8 nm gold, and observed on a Jeol JSM-8404 microscope. For transmission electron microscopy, anthers were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0 and were post fixed in 2% osmium in the same buffer. An ethanol dehydration series was performed and the samples were embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate. Micrographs were taken using a Phillips CM12 transmission electron microscope (Philips Electronic Instruments Co., Mahwah, NJ) at 60 kV.

Acknowledgements

We thank ABRC (Ohio State University) for seeds of Arabidopsis T-DNA insertional lines.
CHAPTER 4
CALLOSE SYNTHASE COMPLEXES IN *ARABIDOPSIS*

4.1 Introduction

Callose is synthesized at many places in plant and at different stages of plant growth and development. Callose synthesis is induced by biotic and abiotic stress. One model of CalS complex is built based on the studies of the complex on the cell plate (Hong et al., 2001 a and b; Verma, 2001 a and b). It is believed that there are different callose synthase complexes for the synthesis of callose at different places and under different regulation.

It is unknown whether different CalS isoforms interact with each other to form heteromeric complexes for callose synthesis. For cellulose synthesis, it is suggested that at least 2 cellulose synthase genes are required to express in the same cells for the cells to have cellulose synthesis activity. For the primary cell wall synthesis during cell elongation, *CesA6* and *CesA1* are both required, and mutation of either of these genes resulted in a similar phenotype (Fagard *et al*., 2000). Multiple CalS genes are expressed in the same cell-type. It indicates that several CalS isoforms may be required for callose production in a specific tissue (Dong *et al*., 2004).
Interestingly, some reports suggested that a single peptide might have callose synthase activity. A 48 Kda protein from peanut cotyledon was suggested to have callose synthase activity (Kamat et al., 1992). Kamat et al., (1992) purified callose synthase to one peptide band examined in SDS-PAGE. A 65 Kda protein from French bean was thought to be the catalytic subunit of callose synthase activity (McCormack et al., 1997). In pollen tube membrane, a 190 kDa protein was shown to have callose synthase activity (Turner et al., 1998). But none of these observations excluded the possibility of the contribution of other proteins present at lower level. One *Arabidopsis* callose synthase (AtCalS12) was show to have callose synthase activity in yeast cells (Østergaard et al., 2002). Expression of this protein in yeast mutant fks1 (β-1, 3-glucan synthase) partially complement the mutation, suggesting that AtCalS12 may have callose synthase activity in yeast cells. But it is also possible that some yeast proteins may assist AtCalS12 in synthesizing callose. Purification of the protein and tests of callose synthesis activity in vitro is needed to confirm its callose synthesis activity by itself alone.

However, other reports suggested that plant callose synthesis enzyme may be composed of multiple polypeptides for the callose synthesis. Both 55 kDa and 70 kDa proteins were shown to have callose synthase activity in pea (Dhugga and Ray, 1994). Wasserman at al.(1992)postulated that callose synthase might be composed of 6 to 9 components. Based on the studies of the CalS complex on the cell plate, it seems that callose synthase complex contains at least 3 components: the catalytic subunit, UDP-glucose transferase (UGT) and sucrose synthase (Verma, 2001 a; Hong et al., 2001 a, b). Using the cell plate specific *CalS1* as a bait to screen an *Arabidopsis* cDNA library
Table 4.1 Results of yeast two-hybrid and GFP localization

<table>
<thead>
<tr>
<th>Interaction with Callose Synthase Or UDP-glucosyl Transferase</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>SuSy1</td>
<td>no</td>
</tr>
<tr>
<td>SuSy2</td>
<td>?</td>
</tr>
<tr>
<td>SuSy3</td>
<td>no</td>
</tr>
<tr>
<td>SuSy4</td>
<td>no</td>
</tr>
<tr>
<td>SuSy5</td>
<td>?</td>
</tr>
<tr>
<td>SuSy6</td>
<td>no</td>
</tr>
</tbody>
</table>

Table 4.1 Results of yeast two-hybrid and GFP localization
Figure 4.1 Subcellular Localization of RLK1, UGP1, SuSy1 and SuSy6. 
(A) and (B) BY-2 cell expressing the GFP alone as a bright-field image (A) and a fluorescent image (B). 
(C) and (D) BY-2 cell expressing the RLK1-GFP fusion protein shown as a bright-field image (C) and a fluorescent image (D) 
(E) and (F) BY-2 cell expressing the RLK1-GFP fusion protein shown as a bright-field image (E) and a fluorescent image (F) 
(G) and (H) BY-2 cell expressing the UGP1-GFP fusion protein shown as a bright-field image (G) and a fluorescent image (H) 
(I) and (J) BY-2 cell expressing the SuSy1-GFP fusion protein shown as a bright-field image (I) and a fluorescent image (J) 
(K) BY-2 cell expressing the SuSy6N-GFP fusion protein shown as a fluorescent image
constructed in the yeast two-hybrid vector, we obtained two positive clones. One of these interacting clones, **RLK1**, encodes protein kinase and may play a role in the regulation of CalS1 activity during cell plate formation. The other clone, **UGP1**, encodes an UDP-glucose pyrophosphorylase and may act to provide alternative source of UDP-glucose for the synthesis of callose.

### 4.2 Result

#### 4.2.1 CalS1 Interacts with RLK1 and UGP1

Using the cell plate specific CalS1 as a bait to screen an *Arabidopsis* cDNA library constructed in the yeast two-hybrid vector, we obtained two positive clones. One of these interacting clones, **RLK1**, encodes protein kinase and may play a role in the regulation of CalS1 activity during cell plate formation. The clone, **UGP1**, encodes a UDP-glucose pyrophosphorylase and may act to provide alternative source of UDP-glucose for the synthesis of callose. In the same way, **SuSy1, 2, 3, 4, 5, 6** were cloned into PAS2 vector, **CalS1** N terminal 1500bp, **CalS1** center loop (2000bp) and **UGT1** full length were cloned into PACT2 vector, there were no interaction detected between **SuSy** and **CalS1 or UGT1** (table 3.1).

#### 4.2.2 Localization of RLK1, UGP1 and SuSys

To determine the subcellular location of **RLK1, UGP1 and SuSys**, we expressed a GFP– RLK1, UGP1 and SuSys fusion protein under the control of the cauliflower mosaic virus (CaMV) 35S promoter in transgenic tobacco BY-2 cells. We examined 15
transgenic lines for each construct and found that all lines expressed the GFP fusion protein. Green fluorescence was checked in cells undergoing cytokinesis (Figures 3.1). As a control, GFP alone, expressed under the control of the CaMV 35S promoter, was found to be distributed throughout the nuclei and cytoplasm, and the fluorescence was not confined to the cell plate (Figure 3.1). For RLK1-GFP, green fluorescence was detected at plasma membrane and it needs to be further confirmed by other experiments. For UGP1-GFP, green fluorescence was detected at cytoplasm with some very weak expression at the cell plate. For SuSys-GFP, green fluorescence was detected at cytoplasm except SuSy6 with very weak expression at the cell plate.

4.3 DISCUSSION

4.3.1 The role of \textit{RLK1}

\textit{RLK1} is a novel lectin-containing Receptor-Like Kinase (\textit{lecRLK1}). It contains a lectin-like fragment in the N-terminus, followed by a transmembrane domain and a C-terminal protein kinase domain. The \textit{Arabidopsis} genome contains a large number (620) of receptor-like kinases (\textit{RLK}) implicated in the regulation of diverse metabolic and developmental processes including self-incompatibility, pathogen interaction, hormone perception and organ biogenesis. In the \textit{lecRLK} subfamily of receptor-like kinases, there are 38 homolog proteins in \textit{Arabidopsis} and little is known about their ligands and biological functions. Because different lectins are known to bind to specific polysaccharides, the N-terminal lectin-like region of RLK1 may be able to recognize with
specific polysaccharides such as callose and cellulose. Callose and cellulose are synthesized and deposited in the extracellular space of the cell, where the N-terminal lectin-like domain of RLK1 is also localized according to the protein topology prediction. The middle part of RLK1 is a transmembrane domain, which may anchor the protein to the plasma membrane. It is possible that RLK1 may receive a signal via binding to callose or cellulose, and regulate CalS1 activity by phosphorylation on the N-terminus of CalS1.

4.3.2 The role of $UGP1$

$UGP1$, encodes an UDP-glucose pyrophosphorylase, is also referred as UTP:glucose-1-phosphate pyrophosphorylase. $UGP1$ catalyzes the conversion of glucose-1-phosphate to UDP-glucose in the presence of UTP. Glucose-1-phosphate can be generated from fructose by the activities of three other cytosolic enzymes (Fig.3.2). Fructose is a byproduct of sucrose breakdown by SuSy, which provides the direct source of UDP-glucose. Therefore, it appears that both SuSy and $UGP1$ may provide CalS1 with UDP-glucose from the degradation of sucrose (Figure 3.2).

4.3.3 Multiple components in the callose synthase complexes

Altogether, there are 12 callose synthase family members in Arabidopsis genome with homology to yeast callose synthase. According to our promoter analysis we know that different callose synthase may be expressed in different organs, or function at different cell sites, or under different regulation. CalS1 is considered as the catalytic
Fig. 4.2. Production of UDP-Glc from Sucrose by SuSy and UGP1.
Sucrose is converted by SuSy into UDP-Glc and fructose. UDP-Glc is used directly as a substrate for the synthesis of callose and cellulose. Fructose can be converted into Glucose-1-P by the activities of hexokinase (HK), Phosphoglucose isomerase (PGI) and phosphoglucose mutase (PFM). PGI and PGM. UGP1 catalyzes the synthesis of UDP-Glc from glucose-1-P in the presence of UTP.
Figure 4.3 Hypothetical model of the callose synthase complex. It shows that callose synthase N-terminal region and hydrophilic loop interact with annexin, Rop1, UGT1, RLK1, UGP1 or SuSys.
component of callose synthase complex in the cell plate, and it interacts with UGT1 which encodes an UDP-glucose transferase. In *Arabidopsis* genome there are more than 120 members of UDP-glycosyltransferase. UGT1 is localized on the cell plate (Hong et al., 2001 b). UGT1 interacts with CalS1, and both proteins are in the callose synthesis complex purified by product entrapment, suggesting the synthesis complex require two proteins (Hong et al., 2001 a, b).

From cotton fiber, several polypeptides can be photolabeled with $\alpha^{32}$P UDP-glucose (Delmer et al., 1990), one of them with 52 kDa associated with callose synthesis activity. This labeling with $^{32}$P UDP-glucose is activated by Ca$^{2+}$, and this polypeptide is taken as part of the callose synthase complex. The 52 kDa peptide may be an UDP-glucose transferase, as it has similar size as *Arabidopsis* UGT1. In pea a peptide of 55 kDa is required for callose synthesis, and it can be labeled by ultraviolet irradiation of the plasma membranes in the presence of $\alpha^{32}$P UDP-glucose under callose synthesis conditions (Dhugga et al., 1994). These results suggested that the 55 kDa peptide might be a UGT.

4.3.4 Sucrose synthase (*SuSy*)

Sucrose synthase (*SuSy*, EC 2.4.1.13; sucrose +UDP reversible UDP-glucose + fructose) may function to provide the callose synthase complex with UDP glucose from sucrose (Figure 3.3). Since the patterns of immunolocalization of *SuSy* in cotton fibers are similar to those of callose deposition. More than half of the total *SuSy* of the developing cotton fibers is tightly associated with plasma membrane. When detached
and permeabilized cotton fibers were used for glucan synthesis, carbon from sucrose was converted at high rates to cellulose and callose. This form of SuSy might serve to channel carbon directly from sucrose to cellulose and/or callose synthases in the plasma membrane (Amor et al., 1995).

SuSy can be phosphorylated and the phosphorylation sites and peptide sequence around it is conserved (Angunot et al., 1999; Huber et al., 1996; Winter et al., 1997). Treatment of maize seedlings with anoxia increase the portion of dephosphorylated SuSy, and the SuSy becomes membrane associated. This change occurred before callose deposition started. In sh1 mutants, which have sustained SuSy phosphorylation, this anoxia-induced relocalization of SuSy does not exist. In the roots of this mutant, anoxia treatment causes less callose deposition (Subbaiah et al., 2001). It is suggested that phosphorylation of SuSy is involved in the release of the membrane-bound enzyme in part as a result of decreased surface hydrophobicity. After dissociation from the membrane, SuSy separates from the Callose synthase complex, which limits the activity of the complex in plants.

Our data on CalS promoter:GUS analyses show that multiple CalS genes are expressed in the same cell-type. These results indicate that several CalS isoforms may be required for callose production in a specific tissue. It remains to be tested if different CalS isoforms interact with each other to form heteromeric complexes (Verma and Hong,
2001), or they form homomeric complexes that are co-localized. Such an analysis will await dissection of the CalS complexes.

4.4 METHODS

4.4.1 Bacterial and Yeast Strains and Tobacco Cell Culture

*Escherichia coli* strain Top10F’ (Invitrogen, Carlsbad, CA) was used for plasmid manipulation. *Saccharomyces cerevisiae* strain J69-4A (MATa trp1 his3 ura3 leu2::6 LexAop-LEU2) containing pSH18-34 (beta-galactosidase reporter plasmid; Gyuris et al., 1993) was grown in yeast nitrogen base (YNB) medium (0.67 g/L YNB [Sigma], 0.6 g/L amino acid dropout mix [Bio101, La Jolla, CA], and 20 g/L glucose or galactose). Tobacco BY-2 cells overexpressing GFP fusion proteins were maintained in Murashige and Skoog (1962) medium.

4.4.2 Cloning of *SuSy* cDNA

One pair of oligonucleotides corresponding to the sequences around the N-terminal and C-terminal of *SuSy* was synthesized. Total RNA was obtained from shoot apical meristems of 3-week-old *Arabidopsis* (ecotype Columbia) seedlings. Reverse transcription (RT) of the RNA was performed using oligo (dT) as a primer and Moloney murine leukemia virus reverse transcriptase (Gibco BRL). cDNA fragments were amplified by polymerase chain reaction (PCR) using Platinum Taq DNA polymerase (Gibco BRL) and cloned in the pT-Adv vector (Clontech, Palo Alto, CA). The DNA sequence was determined using an Applied Biosystems (Foster City, CA) model 373A
DNA sequencer. To eliminate possible PCR errors, any inconsistency between cDNA and genomic sequences was verified by sequencing independent clones.

4.4.3 Yeast Two-Hybrid Assay

The *RLK1*, *UGP1* and *SuSys* cDNAs were cloned in the EcoRI-XhoI sites of PAS2 (Estojak et al., 1995; Golemis et al., 1996) with a human influenza (hemagglutinin [HA]) tag. Plasmid PACT-CalS1, and PACT-UGT1 were constructed by *CalS1* N-terminal 1500bp or *UGT1* full length in the BamHI-XhoI sites of PACT2. Cloning vectors PAS2 and PACT2 were used as controls. Interaction assays were performed on YNB medium (YNB-His-Trp-Ura) containing 75 mg/L 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in the presence of galactose (for induction) or glucose (as a control).

4.4.4 Expression of the GFP Fusion Protein and Fluorescence Detection

*RLK1*, *UGP1* and *SuSys* full-length cDNAs were fused with green fluorescent protein (GFP) under the control of the cauliflower mosaic virus (CaMV) 35S promoter and expressed in tobacco BY-2 cells (Gu and Verma, 1997). Localization of the GFP fusion protein was performed using an epifluorescence microscope.
BIBLIOGRAPHY


Dong, X., Hong, Z., Kim, S and Verma, D.P.S. 2004 Induction of Callose Synthase Genes by Pathogen Infection Involves both NPR1-Dependent and NPR1-Independent Signaling Pathways *Plant MOI. Biol.*


Gibeaut DM, Carpita NC. (1993) Synthesis f (1, 3), (1, 4)-b-glucan in the Golgi apparatus of maize coleoptiles. Proc Natl Acad Sci USA 90 (9):3850-4


Kudlicka K and RM Brown (19970 Cellulose and callose biosynthesis in higher plants. Plant Physiology 115:643-656


Lukowitz w, Nickle tc, Meinke DW, Last RL, Conklin PL, Somerville CR (2001) Arabidopsis cyt1 mutants are deficient in a mannose-1-phosphate guanylytransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis Proc Natl Acad Sci USA 98(5):2262-7


anther cellulose (P(1,4)-glucan hydrolase) from Lathyrus ororatus L. and its relationship to the similar enzyme found in abscission zones. Plant Sci. 67, 169-176.


