ROLES OF THE CLAVATA1 AND ERECTA CLADE MEMBERS IN ARABIDOPSIS ANther DEVELOPMENT

A Thesis in

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by

Carey LeeAnna Hendrix Hord

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The thesis of Carey LeeAnna Hendrix Hord was reviewed and approved* by the following:

Hong Ma  
Professor of Biology  
Thesis Advisor  
Chair of Committee

Teh-hui Kao  
Professor of Biochemistry and Molecular Biology  
Chairman, Intercollege Graduate Degree Program in Plant Biology

Timothy McNellis  
Associate Professor of Plant Pathology

Paula McSteen  
Assistant Professor of Biology

*Signatures are on file in the Graduate School
ABSTRACT

Anther development, and therefore male fertility, relies on the coordinated differentiation of several adjacent cell types. Establishment of these cell layers likely involves cell-to-cell communication pathways as plants rely more on positional cues than strict cell lineage for their development. Currently, only a few genes are known to be involved in Arabidopsis early anther development, particularly in the establishment of these different cell layers. The SPOROCYTELESS/NOZZLE (SPL/NZZ) gene functions in early anther development to promote formation of the sporogenous cell type. Subsequently the EXCESS MICROSPOROCYES1/EXTRA SPOROGENOUS CELLS (EMS1/EXS) gene specifies the formation of the tapetum. SPL/NZZ encodes a putative transcription factor, while EMS1/EXS encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) and is thought to function with other known LRR-RLKs to mediate tapetum differentiation. CLAVATA1 (CLV1) belongs to a four member clade of LRR-RLKs including BAM1 (for BARELY ANY MERISTEM1), BAM2 and BAM3. The bam1 bam2 and bam1 bam2 bam3 mutants are male sterile, suggesting that BAM1, BAM2 and BAM3 are important for normal anther development. In addition, recent work has shown that loss of the three ERECTA (ER) genes, ER, ERL1 and ERL2, results in underdeveloped and presumably sterile anthers, indicating that this gene family may also play a role in anther development. The objective of this research was to understand the function of the BAM and ER genes in Arabidopsis anther development. Also, the relationship between SPL/NZZ, EMS1/EXS and the BAM genes in specifying anther cell differentiation was examined. Analyses using molecular markers and cytological
techniques shows that bam1 bam2 anthers lack the normal anther somatic cell layers and produce only pollen mother-like cells (PMLs), indicating a very early defect in cell fate specification. Furthermore the bam1 bam2 bam3 triple mutant anther can produce the somatic cell types and BAM3 expression is drastically increased in the bam1 bam2 mutant flowers. This indicates that BAM1/2 mediate a signal to negatively regulate BAM3 expression in order to properly specify the parietal cell type. In addition, the meiotic cells of the bam1 bam2 double and bam1 bam2 bam3 triple mutants degrade, suggesting these cells are defective and BAM1, BAM2 and BAM3 may be important for normal meiosis. Mutant analysis also demonstrates that SPL/NZZ is epistatic to BAM1/2, and BAM1/2 are in turn epistatic to EMS1/EXS. Finally, phenotypic analysis of the ER-family mutants indicates that these genes act redundantly to promote cell proliferation during anther development and to direct normal growth and differentiation of anther cell layers, particularly the tapetum.
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Chapter 1

Literature Review
GENETIC CONTROL OF EARLY ANther DEVELOPMENT

The Cytology of Anther Stages

Flowering plants, the angiosperms, rely on microsporogenesis and microgametogenesis for male reproduction, which is necessary for the propagation of the species (Li and Ma, 2002; Ma, 2005). Microsporogenesis is the formation of the microspores that precedes the development of the male gametophytes (pollen). Microgametogenesis occurs within the developing pollen grain and leads to the generation of sperm cells. These complex processes require coordinated development of sporophytic and gametophytic cell types within the stamen, the male floral organ. In Arabidopsis, stamen specification occurs in the third whorl of floral organs and is directed by the homeotic genes APETELA3 (AP3), PISTILATA (PI) and AGAMOUS (AG) (Bowman et al., 1991). Arabidopsis flowers have four long stamens and two short ones, which begin developing slightly later than the four long ones (Scott et al., 2004). Each stamen consists of an anther, where pollen development takes place, and a filament, which provides structural support and nutrients to the anther (Goldberg et al., 1993). The cross section of an Arabidopsis anther is butterfly-shaped, with two adaxial and two abaxial lobes. During the early stages of anther development, these lobes develop somewhat asynchronously and are at slightly different stages at a given time during development.

Anther development in Arabidopsis has been divided into fourteen stages on the basis of the anther morphology visible in a cross section under light microscopy (Table 1-
1) (Sanders et al., 1999). In addition, specific cellular events at some anther stages are only visible using transmission electron microscopy (TEM) (Owen and Makaroff 1999). Anther histogenesis, establishment of the cell layers, occurs within the first five stages Figure 1-1. The emergence of anther primordia takes place at flower stage 5 and is called anther stage 1 (Smyth et al., 1990; Sanders et al., 1999). The cross section of these round primordia shows three layers, designated from outer to inner as L1, L2 and L3, as they are defined in a shoot apical meristem (Satina and Blakeslee, 1941). L1 gives rise to the epidermis. The L2 cells differentiate into several parietal cell layers and the sporogenous cell type. The L3 layer develops into the vascular and connective tissues. The further characterization of stages is based primarily on the morphology of the L2-derived cells.

Figure 1-1: Anther Histogenesis. Branch points mark periclinal cell division events; dashed line indicates exact cell lineage is not known.
<table>
<thead>
<tr>
<th>Anther stage⁶</th>
<th>Major events and morphological markers</th>
<th>Tissues present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stamen is round with three major cell layers</td>
<td>L1, L2, L3</td>
</tr>
<tr>
<td>2</td>
<td>Stamen becomes oval shaped; archesporial cells are in the four corners of the L2 layer.</td>
<td>Ep, Ar, L3</td>
</tr>
<tr>
<td>3</td>
<td>Archesporial cells have divided parallel to the epidermis to form the primary parietal and primary sporogenous cells in the lobes</td>
<td>Ep, PP, PSp, L3</td>
</tr>
<tr>
<td>4</td>
<td>Primary parietal cells have divided parallel to the epidermis to form the inner secondary parietal and outer secondary parietal cell layers; they begin to form concentric rings around the sporogenous cells. The Vascular and connective become distinguishable</td>
<td>Ep, ISP, OSP, Sp, V</td>
</tr>
<tr>
<td>5</td>
<td>One of the secondary parietal layers has divided periclinally at the outer surface; three L2-derived cell layers surround the pollen mother cells. At late stage five, callose begins to form around PMCs</td>
<td>Ep, En, ML, T, PMC, C, V</td>
</tr>
<tr>
<td>6</td>
<td>A thick callose layer is apparent around the pollen mother cells, which enter and go through meiosis. The middle layer becomes compressed and appears as a thin line. Tapetum becomes vacuolated.</td>
<td>Ep, En, ML, T, Mc, PMC, V</td>
</tr>
<tr>
<td>7</td>
<td>Meiosis has completed with cytokinesis. Tetrads of microspores appear in the locules.</td>
<td>Ep, En, ML, T, Td, C, V</td>
</tr>
<tr>
<td>8</td>
<td>Microspores are free within in the locules after the callose wall is degenerated. Middle layer is no longer visible.</td>
<td>Ep, En, T, Msp, C, V</td>
</tr>
<tr>
<td>9</td>
<td>Microspores generate an exine wall and become vacuolated. Septum is visible using TEM.</td>
<td>Ep, En, T, Msp, C, V, Sm</td>
</tr>
<tr>
<td>10</td>
<td>Tapetum degeneration is initiated</td>
<td>Ep, En, T, Msp, C, V, Sm</td>
</tr>
<tr>
<td>11</td>
<td>Microspores undergo three rounds of mitotic divisions. Tapetum continues to degenerate. Endothecium and epidermis appear highly vacuolated. Septum cells begin to degenerate</td>
<td>Ep, En, T, MMsp, C, V, Sm, St</td>
</tr>
<tr>
<td>12</td>
<td>Tricellular pollen grains are visible. Septum degeneration and breakage cause the anthers to become bilocular. Differentiated stonium visible using TEM.</td>
<td>Ep, En, PG, C, V, St</td>
</tr>
<tr>
<td>13</td>
<td>Breakage along stonium allows for pollen release-dehiscence.</td>
<td>Ep, En, PG, C, V, St</td>
</tr>
<tr>
<td>14</td>
<td>The stamen senesces as the cells shrink.</td>
<td>Ep, En, C, V</td>
</tr>
</tbody>
</table>

⁶Anther stages were taken from Sanders et al. (1999). Ar, archesporial cells; Ep, epidermis; PP, primary parietal cells; PSp, primary sporogenous cells; ISP, inner secondary parietal cells; OSP, outer secondary parietal cells; Sp, sporogenous cells; V, vascular tissue; En, endothecium; ML, middle layer; T, tapetum; PMC, pollen mother cells; C, connective tissue; Mc, meiocyte; Td, tetrads; Msp, microspores; Sm, septum; St, stonium; PG, pollen grain.
At stage two, the anther has enlarged somewhat and become more oval. The L2 cells at the four corners are called archesporial cells (Sanders et al., 1999). These cells divide in a periclinal orientation, parallel to the surface of the epidermis. The two resulting daughter cells are called the primary parietal and primary sporogenous cells, with the parietal cell being adjacent to the epidermis and the sporogenous cell being further interior. The appearance of the primary parietal and sporogenous cells designates stage three. The primary parietal cells then undergo a second round of periclinal division thus forming the inner and outer secondary parietal cells at stage four. At this stage it becomes clear that the parietal cell types are actually forming sequential rings around the sporogenous cells within each of the four anther lobes. From stage two to four, the primary sporogenous cells continue to divide, forming a single central core of pollen mother cells (PMCs) in each lobe.

From stage four to five, further periclinal division of secondary parietal cells results in three cell layers between the epidermis and PMCs. From outer to inner they are the endothecium, middle layer, and tapetum. In *Arabidopsis*, it is not clear which of the two secondary parietal cell layers then divides periclinal to establish two of these three cell layers (Scott et al., 2004). It is possible that the precise lineage of cell types may vary from anther to anther, lobe to lobe, and even from one part of the lobe to another. Furthermore, the final identity of a cell may depend more on the relative position of each cell than on their direct lineage (Jorgenson and Crane, 1927; Szymkowiak and Sussex, 1996; Berger et al., 1998; Scheres and Benfey, 1999). Although the relative roles that position and cell lineage play in anther cell differentiation have yet to be elucidated (Goldberg et al., 1993; Scott et al., 2004) recent studies strongly support a role of cell-cell
communication in determining positional cues (see below). It is important to determine precisely the developmental histology of the anther from stage four to five as it affects the interpretation of several mutant phenotypes and our understanding of anther development. For example, one proposed mechanism for the differentiation of the sporophytic cells is that the centrally located sporogenous cells send a signal to their adjacent cells, directing them to divide and differentiate (Yang et al., 1999; Zhao et al., 2002; Scott et al., 2004; Albrecht et al., 2005) (see below for more details).

Regardless of the mechanism, by early anther stage five, the cell layers are fully established with the endothecium, middle layer and tapetum forming rings around the developing PMCs. At this stage, the PMCs and tapetal cells are connected to each other and to their own cell type by plasmodesmata (Owen and Makaroff, 1995). Post-histogenesis development also requires the coordinated development and function of sporophytic and gametophytic cells. Of particular importance is the development of the tapetum (Mariani et al., 1990; Pacini, 1990; Mariani et al., 1991; Denis et al., 1993). During stage five, the tapetum appears to undergo further expansion and specialization. In addition, at late stage 5, a callose ($\beta$-1, 3-glucan) layer is formed that separates the PMCs from each other and from the tapetum layer, severing their plasmodesmatal connections (Owen and Makaroff, 1995). At the same time, the middle layer becomes very thin, while the tapetal cells expand. During anther stage six, the PMCs enter and complete meiosis.

Meiosis produces four haploid daughter cells from one diploid parent cell via two phases of nuclear division: meiosis I and meiosis II. Both of these phases are further divided into stages based on chromosome behavior observable under light microscopy.
After DNA replication, chromosomes condense and undergo homologous recombination during prophase I. Prophase I is further divided into five sub-stages: leptotene, zygotene, pachytene, diplotene and diakinesis, each of which has distinctive chromosomal behavior. Metaphase I is marked by the alignment of conjoined sister chromatids (called bivalents) at the equatorial plane of the cell. Unique to meiosis I, homologous chromosomes, instead of sister chromatids, separate during anaphase I. After segregation, the chromosomes decondense during telophase I. The chromosomes recondense during prophase II and the two sets of chromosomes align at their respective division planes in metaphase II. During anaphase II, sister chromatids separate forming four groups of chromatids, which decondense during telophase II to form four haploid nuclei. In most eudicots, cytokinesis occurs between the four haploid nuclei simultaneously at the end of meiosis II, resulting in the formation of four microspores (Owen and Makaroff, 1995; Brown and Lemmon, 2001).

While the PMCs undergo meiosis, tapetal cells become binucleate, slightly vacuolated, and begin to separate from each other and the middle layer. By stage seven, the tapetal cell walls have completely degenerated and extensive layers of endoplasmic reticulum (ER) surround the nuclei. In addition, the epidermis and endothecium become progressively more vacuolate beginning at stage six. Throughout anther development, the anther continues to grow and expand in size. As a result, from stage seven onward, each anther locule becomes a somewhat open tube, allowing for the growth of the developing pollen. Stage seven is marked by the appearance of tetrads. During this stage, the secretory tapetum releases a mixture of enzymes (called callase), which includes endoglucanases, exoglucanases and cellulases (Pacini, 1990; Murgia et al., 1991;
Owen and Makaroff, 1995; Scott et al., 2004). Callase releases the microspores from the tetrads by dissolving the external and intersporal walls. The release of microspores from the tetrads marks stage eight. The tapetal cells continue to release materials into the locule and clusters of small vesicles are seen throughout their cytoplasm. At stage nine, an exine wall is formed on the microspores and numerous vacuoles in the microspore have fused into a single large vacuole. In addition, the stacks of ER, vesicles and ribosomes become more prominent in the tapetal cells.

The sequence of tapetum degeneration and pollen mitotic divisions is unclear, but stage ten is designated as the initiation of tapetum degeneration (Sanders et al., 1999). Stage eleven comprises the two rounds of haploid pollen mitosis, resulting in tri-cellular pollen grains at stage twelve. The majority of tapetal cell degeneration and release of their cellular contents occurs after pollen mitosis II, during stage eleven. Tapetum degeneration and its timing are very important for proper microspore development (Wu and Cheung, 2000). By stage twelve, the tapetum and middle layer have disappeared. The anther becomes bilocular as the septum separating the locules on each side of the anther breaks. It is during stages eleven and twelve that the stamen filament elongates dramatically, placing the anther in position to pollinate the stigma (Smyth et al., 1990). Anther dehiscence occurs during stages thirteen and fourteen; the stamen begins to senescence as anther cells shrink during stage fourteen. The morphological description of Arabidopsis anther development provides a basis for the characterization of mutants defective in anther development.
Control of Early Anther Development by \textit{SPL/NZZ}

Relatively few genes have been identified that are important for the very early stages of anther development. The earliest known acting gene is \textit{SPOROCYTELESS/NOZZLE (SPL/NZZ)}, which is required for the formation of the PMC\(\text{s}\) and also has a role in ovule development (Schiefthaler et al., 1999; Yang et al., 1999; Balasubramanian and Schneitz, 2000). Yang et al. (1999) reported that in \textit{spl} anthers, the archesporial cells underwent a normal periclinal cell division to form the primary parietal cells (PPCs) and primary sporogenous cells (PSC\(\text{s}\)) at stage 3. The PPC\(\text{s}\) then underwent a further round of cell division to form secondary parietal cells (SPC\(\text{s}-\)stage 4), but cell division was arrested at stage 4, when the PSC\(\text{s}\) became abnormally vacuolated (Yang et al., 1999). On the other hand, Schiefthaler et al. (1999) reported that the \textit{nzz} mutant anther failed to show differentiation of the archesporial cells. It is possible that the difference in phenotypic descriptions reflect allelic variations and/or environmental effects. Alternatively, because there are no biochemical or molecular markers for early anther cell types, the identification of these cells is difficult and therefore open to interpretation. Nevertheless, it is clear that \textit{SPL/NZZ} is required for normal early anther development.

\textit{SPL/NZZ} encodes a putative transcription factor (Schiefthaler et al., 1999; Yang et al., 1999). It was recently shown that the floral homeotic protein AGAMOUS (AG) can directly bind to the 3’ end of \textit{SPL/NZZ} and induce its transcription (Ito et al., 2004). While AG is necessary to activate \textit{SPL/NZZ} expression, it may not be needed to maintain it (Ito et al., 2004). Using a fusion of \textit{SPL} to the rat glucocorticoid receptor gene
(35S::SPL-GR) in a strong ag mutant background, it was shown that SPL/NZZ is sufficient to induce microsporogenesis. This places SPL/NZZ as a central mediator for the promotion of microsporogenesis by AG (Ito et al., 2004). The induced 35S::SPL-GR flowers did not form stamens, but produced whorl 3 petals with locules near their edges containing morphologically normal pollen grains. Thus, the SPL/NZZ function alone was not sufficient to produce the normal stamen structure. Also, the fact that SPL/NZZ could only induce microsporogenesis in whorl 3, where stamens normally form, implies the existence of other whorl-specific factors involved in this process (Ito et al., 2004).

The spatial and temporal domain of SPL/NZZ expression and function remains somewhat unclear, as the different groups reported slightly different results. Using in situ hybridization and SPL-GUS transgenic plants, Yang et al. (1999) detected SPL/NZZ expression in sporogenous cells of late stage 3, stage 4 and stage 5 anthers, but not in the developing parietal tissues (Yang et al., 1999). This expression pattern is in line with their observed mutant phenotype seen between stages 3 and 4.

In contrast, Schiefthaler et al. (1999) reported the detection of NZZ transcript by in situ hybridization throughout the anther in stage 7 flowers (anther stage 2); after stage 2, the signal in the epidermis became less obvious (Schiefthaler et al., 1999). By early meiosis (anther stage 6), the NZZ transcript was restricted to the tapetum and PMCs, and continued in these two cell types until microspores are released from the tetrad (Schiefthaler et al., 1999). Early SPL/NZZ expression was also reported by Ito et al. (2004). Using a fusion construct of SPL-GUS they saw GUS staining at the lateral edges of stamen primordia in floral stage 6 (late anther stages 1 and/or 2); the GUS activity then expanded to cover the developing stamen during floral stages 6 to 8 (anther stages 2 to 4).
The early expression of $SPL/NZZ$ supports an early function as reported by Schiefthaler et al. (1999).

The temporal and spatial domain of the $SPL/NZZ$ function is important to our understanding of anther development. The expression of $SPL/NZZ$ at stages 1 and/or 2 and the possible failure of the archesporial cells to properly differentiate implies that the $SPL/NZZ$ function is necessary for promoting cellular division and differentiation of this cell type (Schiefthaler et al., 1999). It is possible that PPCs and PSCs not only differ in position, but also in molecular characteristics, and that $SPL/NZZ$ promotes the differentiation if these two cell types. Alternatively, $SPL/NZZ$ might not be necessary for the division of the archesporial cells into PPCs and PSCs, but is required for promoting the differentiation of the sporogenous cells beginning at late stage 3. Because of the lack of specific molecular markers for these early anther cell types, it is difficult to know whether PPCs and PSCs are correctly formed at stage 3 and these hypotheses cannot yet be distinguished.

Furthermore, it is not known whether $SPL/NZZ$ is directly or indirectly necessary for the development of the parietal cell types. A current theory in anther development is that the sporogenous cells promote the differentiation and development of the parietal cell layers possibly through cell-cell communication (Yang et al., 1999; Yang et al., 2003; Scott et al., 2004; Albrecht et al., 2005). This model would support an indirect role for $SPL/NZZ$ in sporophytic development, by regulating a number of genes necessary for the formation of sporogenous cells, which then promote the differentiation of adjacent cells into the sporangium. Alternatively, it is possible that the $spl/nzz$ mutants are not able to properly specify the archesporial cell type between stages 1 and 2. Thus, the reported
result that the anther was filled with vacuolated parenchyma cells (Yang et al., 1999) may be the result of a defect in this specification. In this case, SPL/NZZ would play an earlier and more direct role in specifying the parietal cells.

Tapetum Specification Requires Cell-to-Cell Signaling

As stated earlier, the proper development of the parietal cell types, particularly the tapetum, is essential for the sporogenous cells to develop normally. The tapetum cell layer supports pollen development by producing and releasing essential proteins (Izhar and Frankel, 1971; Stieglitz, 1977). When the tapetum cells are selectively destroyed the plant fails to produce pollen (Koltunow et al., 1990; Mariani et al., 1990; Mariani et al., 1991). Recent discoveries have revealed several genes that are critical for the formation of the tapetum layer and that may function in the same signaling pathway. Two groups using independent genetic approaches isolated mutant alleles of the same gene called *EXCESS MICROSPOROCYTES1 (EMS1)/EXTRA SPOROGENOUS CELLS (EXS)* (Canales et al., 2002; Zhao et al., 2002). According to morphological analysis, both the *ems1* and *exs* mutants lack the tapetal cell layer and produce a greater than normal number of PMCs, or microsporocytes. The *ems1* mutant phenotype was further supported by in situ hybridization experiments with meiosis-specific and tapetum-specific probes (Zhao et al., 2002). Specifically, the *ems1* mutant showed no signal for the tapetum-specific probe *ATA7*, whereas the expression domain of the meiosis-specific probe *SDS* was expanded to the extra PMCs.
In the wild type at anther stage 6, PMCs are detached from the tapetum and each other; in contrast, the \textit{ems1} mutant PMCs were abnormally enlarged and adhered to each other and the adjacent cells (Zhao et al., 2002). This may be due to abnormal callose accumulation/deposition on the mutant meiocytes (Canales et al., 2002). This supports the hypothesis that tapetum is necessary for normal callose deposition and the physical separation of the PMCs. Despite the abnormal morphology of \textit{ems1}/\textit{exs} mutant meiocytes, both mutants undergo meiotic nuclear events from prophase I to telophase II (Canales et al. 2002; Zhao et al. 2002). However, in the \textit{ems1} mutant, meiocytes fail to undergo cytokinesis and degenerate without forming microspores (Zhao et al., 2002). In addition, the \textit{ems1} anther has a persistent middle layer, whereas the \textit{exs} anther has a variable number of cell layers in the anther cell wall. In the \textit{exs} anther, the inner secondary parietal cells either degenerate, are crushed, or remain as indeterminate cells on the epidermal side of the locule (Canales et al., 2002). These differences may be due to allelic strength, ecotype variation, or technical differences, such as the thickness of the sections. In any case, the germ line in both \textit{ems1} and \textit{exs} mutants degenerates near the time of meiotic cytokinesis, resulting in the complete failure to produce any normal microspores.

Using in situ hybridization, Zhao et al. (2002) detected the \textit{EMS1}/\textit{EXS} transcript initially in archesporial cells at stage 2 and subsequently in the L2-derived cells at stages 3 and 4. At stage 5, the \textit{EMS1}/\textit{EXS} transcript was detected in PMCs and more strongly in the tapetum (Zhao et al., 2002; Yang et al., 2005). The \textit{EMS1}/\textit{EXS} expression is greatly reduced at stage 6 and gradually decreases to an undetectable level at stage 9. Canales et al. (2002) reported detection of \textit{EMS1}/\textit{EXS} expression using situ hybridization and
reporter genes in the floral meristem, developing anther, and ovule primordia. The
expression in the anther is very similar to that described by Zhao et al. (2002), whereas
the early expression in the floral meristem and the ovule expression suggest possible
additional roles for EMS1/EXS in floral development.

The EMS1/EXS gene encodes a leucine-rich repeat receptor-like kinase (LRR-RLKs) (Canales et al., 2002; Zhao et al., 2002). LRR-RLKs are the largest family of
RLKs in plants and have been shown to regulate several developmental processes,
including maintenance of meristem activity (see “Functions of the CLAVATA1 clade”
below), cell proliferation, guard cell patterning (see “Functions of the ERECTA clade”
below), disease resistance, floral organ abscission, and brassinosteroid signaling (Song et
al., 1995; Clark et al., 1996; Torii et al., 1996; Wang et al., 1996; Li and Chory, 1997;
Wang et al., 1998; He et al., 2000; Jinn et al., 2000; Hecht et al., 2001; Shpak et al., 2001;
Wang et al., 2001; Li et al., 2002; Nam and Li, 2002; Rojo et al., 2002; Dievart et al.,
2003; Lenhard and Laux, 2003; Shpak et al., 2004; Masle et al., 2005; Shpak et al.,
2005). Transient expression in onion epidermal cells of an EMS1-GFP fusion suggests
that EMS1 is localized to the cell surface; furthermore, an in vitro assay showed that
EMS1/EXS has autophosphorylation activity (Zhao et al. 2002). These results support
the hypothesis that it functions as a receptor-like protein kinase, which mediates an
important developmental signal.

Different scenarios have been proposed to explain the failure of the ems1/exs
mutant anthers to produce the tapetum and the concomitant overproduction of PMCs.
One possibility is that cell fate specification is altered between stages 4 and 5 when the
tapetum differentiates. Zhao et al. (2002) suggested that cell fate is switched from
tapetum to PMC, implying a default pathway leading to PMC formation. However, it is difficult to identify histologically a tapetum-positioned cell layer that later differentiates into excess PMCs. Another explanation is that the inner secondary parietal cells fail to divide and differentiate into the middle layer and tapetum; at the same time, extra PMCs arise from additional cell divisions of the sporogenous cells (Albrecht et al., 2005). This suggests that the tapetum negatively regulates PMC proliferation (Albrecht et al., 2005), and requires that the middle layer and tapetum form from a periclinal cell division of the inner secondary parietal cells. As discussed in the introduction, morphological studies have not provided a definitive lineage for the formation of the middle layer and tapetum in *Arabidopsis* (Scott et al., 2004).

In addition to *EMS1/EXS*, two other similar receptor-like protein kinases, *SOMATIC EMBRYOGENESIS1* (*SERK1*) and *SERK2*, play critical roles in tapetum formation. Although neither the *serk1* nor the *serk2* single mutants showed any obvious defects, the *serk1 serk2* double mutant has the same male fertility and anther development phenotypes as those of *ems1* mutant (Albrecht et al., 2005; Colcombet et al., 2005). Therefore, *SERK1* and *SERK2* act redundantly in a way similar to *EMS1/EXS*.

The expression patterns of *SERK1* and *SERK2* in the anthers largely coincide with the expression pattern of *EMS1/EXS*, with some minor differences. Using Fluorescence Resonance Energy Transfer (FRET), it was shown that SERK1 and SERK2 can form homo- or hetero-dimers in the plasma membrane, suggesting that SERK1 and SERK2 act interchangeably in the same complex (Albrecht et al., 2005). *SERK1*, but not *SERK2*, transcript and protein YFP-fusion were observed in stage 2 anthers throughout the anther primordia. At stage 3 both SERK1-YFP and SERK2-YFP were visible in all L2-derived
cell layers. Interestingly, while SERK1 and SERK2 transcript were detected in the L2-derived cells at stages 4 and early 5, including the PMCs, only very faint SERK1/2-YFP signal was seen in the PMCs at these stages, suggesting that the SERK1 and SERK2 may be regulated at the protein level. Strong RNA and GFP signals were seen in the tapetum at late stage 5 for SERK1 and SERK2. Similar to EMS1/EXS, SERK1/2 transcript signal faded after stage 8 or 9. The apparent lack of SERK2 at stage 2 and the evidently normal phenotype of the serk1 mutant together suggest that the function of SERK1/2 at this stage may not be necessary for subsequent tapetum specification. The serk1 serk2 mutant phenotypes and expression patterns of SERK1 and SERK2 are consistent with their being regulators of anther cell differentiation between stages 3 and 5.

The similarity of ems1 and serk1 serk2 mutant anther phenotypes suggests that these genes might mediate the same signaling pathway. It is possible that EMS1/EXS forms a receptor complex with SERK1 and SERK2 that binds to one or more ligands (Albrecht et al., 2005; Colcombet et al., 2005). Although the ligand(s) for EMS1/EXS, SERK1 and/or SERK2 has(have) not yet been identified, currently the most likely candidate is TAPETUM DETERMINANT1 (TPD1), a small 176 amino acid protein (Yang et al. 2003). The tpd1 mutant exhibits an identical phenotype (Yang et al., 2003) to those of the ems1/exs single mutant and serk1 serk2 double mutant, with no tapetum and extra PMCs. In addition, expression of the PMC marker SDS is expanded in the tpd1 mutant and that of the tapetum marker ATA7 is absent. A double mutant between tpd1 and ems1/exs1-2 has nearly identical anther phenotypes to those of the ems1 and tpd1 single mutants (Yang et al., 2003), suggesting that these genes act in the same signaling pathway.
In situ hybridization of TPD1 on WT tissue showed that it is expressed strongly in the first two to three layers of the inflorescence meristem, the floral meristem, and in anther and ovule primordia (Yang et al., 2003). In the anther it appears to be expressed in all of the L2-derived cells at stages 2-4, and at stage 5 it is expressed predominantly in the PMCs and tapetum. At early stage 5, TPD1 expression is strongest in the PMCs. The expression is slightly reduced at stage 6 and gradually declines until it is no longer detectable at stage 10, similar to the EMS1/EXS, SERK1 and SERK2 expression. Yang et al. (2003) also compared the expression patterns of TPD1 and EMS1/EXS; they saw that at late stage 4 and early stage 5, TPD1 and EMS1/EXS are expressed in the PMCs and in what they referred to as “pre-tapetal” (PT) cells, with TPD1 being strongest in the PMCs and EMS1/EXS being strongest in the PT cells. At mid/late stage 5, TPD1 is expressed predominantly in both PMCs and tapetum cells and EMS1/EXS is still predominantly in tapetum. Thus TPD1 and EMS1/EXS have largely overlapping expression patterns, but show predominant expression at early stage 5 in the PMCs and tapetal cells, respectively. The expression of TPD1 was not reduced in the ems1/extra1-2 mutant background, nor was EMS1/EXS expression reduced in the tpd1 mutant background (Yang et al., 2005). Therefore, while TPD1 and EMS1/EXS may function in the same pathway, they do not regulate one another at the mRNA level (Yang et al., 2005).

Expression of TPD1 under the 35S promoter in transgenic lines caused a range of abnormal phenotypes (Yang et al., 2005). Overexpression of TPD1 caused wider siliques to develop by increasing cell number, and the most severe lines were male sterile. Cytological analysis showed that in these plants the tapetal cells were enlarged and did not degenerate at anther maturity. In addition, the microspores did not separate from
each other or the tapetum. Weaker transgenic lines were fertile, but the tapetal cells still appeared to be slightly enlarged and tapetum degeneration was significantly delayed compared to the wild type. Pollen grains from these plants were released from the tetrad walls and were fertile but were stuck together at anther maturity. In addition, overexpression of TPD1 in the ems1/exs1-2 mutant background did not cause the wide carpel phenotype, while introducing a wild type copy of EMS1/EXS recovered this phenotype. Together these results indicate that TPD1 requires EMS1/EXS in order to regulate reproductive development (Yang et al., 2005). In addition, the expression patterns of EMS1/EXS, TPD1, SERK1 and SERK2 suggests that these genes represent signaling pathway(s) that respond to signal(s) from the central region of the PMCs and direct differentiation of the tapetum, which then support meiosis and pollen development (Ma 2005).

FUNCTIONS OF THE CLAVATA1 CLADE

CLAVATA1

In higher plants, the generation of aerial organs, such as leaves and flowers, depends on the continuous production of pluripotent stem cells at their apical meristems. The apical meristem consists of a central core of slowly dividing stem cells, the central zone, which overlies a larger mass of differentiating cells, the rib zone, and both of which are flanked by more rapidly dividing and differentiating tissue, the peripheral zone (Bowman and Eshed, 2000). Normal organ development relies on a strict balance
between stem cell proliferation in the central zone and differentiation in the peripheral zone, which differentiation leads to organ primordia initiation. Leaf primordia form at the flanks of the shoot apical meristem while flowers are produced by inflorescence meristems.

*Arabidopsis thaliana* mutations causing the clavata or ‘club-like’ phenotype of the apical meristem were first described in the early 1960’s (McKelvie, 1962). One of these mutants was in the *CLAVATA1 (CLV1)* gene (Clark et al., 1993; Clark et al., 1997b). To date, thirteen *clv1* mutants have been characterized in order to better understand the role *CLV1* plays in regulating the size of the apical meristem (Koornneef et al., 1983; Leyser and Furner, 1992; Clark et al., 1993; Pogany et al., 1998; Dievart et al., 2003). The *clv1* mutants all have enlarged shoot and inflorescence meristems due to an enlarged central zone (Clark et al., 1993). The *clv1* mutants have a range of phenotypes, categorized as weak (*clv1*-6, *clv1*-7, *clv1*-11, *clv1*-12 and *clv1*-13), intermediate (*clv1*-1, *clv1*-2 and *clv1*-5) and strong (*clv1*-3, *clv1*-4, *clv1*-8 and *clv1*-10) (Clark et al., 1993; Dievart et al., 2003). The strong mutant alleles produce fasciated inflorescence meristems, which may be caused by the extension of the central zone into the peripheral zone, in addition to the enlargement of the central zone (Clark et al., 1993; Clark et al., 1997b).

Incorrect maintenance of the central zone and peripheral zone at the inflorescence meristem leads to abnormal flower development. *Arabidopsis* wild-type flowers have four concentric rings of floral organs, called whorls. From outer to inner they consist of four sepals, four petals, six stamens, and two fused carpels (Smyth et al., 1990). The classical ‘ABC’ model of flower development suggests that floral organ identity is directed by three sets of homeotic genes (Bowman et al., 1991). The ‘A’ function genes
**APETALAI (API)** and **AP2** direct sepal formation. ‘A’ function combined with ‘B’ function genes, **AP3** and **PI**, specify petal development. Together ‘B’ and ‘C’ function genes (**AG**) direct stamen development; and ‘C’ function alone specifies the carpels.

While normal floral organ identity is maintained in the **clv1** mutants, floral organ number is increased. Although the weak **clv1** alleles did not show a significant increase in sepal or petal number, the number of stamens and carpels were greater than the wild-type (Clark et al., 1993; Dievart et al., 2003). In addition, the intermediate and strong alleles had a higher number of all four floral organ types, with the most dramatic increase seen in the number of stamens and carpels. Interestingly, 4-6% of the stamens produced by the intermediate and strong alleles were antherless (Clark et al., 1993; Dievart et al., 2003).

In addition to producing supernumerary floral organs, the **clv1** mutants also produce additional whorls of organs. In the wild-type, the floral meristem terminates with the formation of the gynoecium, which consists of two fused carpels. However, in the **clv1** mutants each of the gynoecium produced one or more addition gynoecium inside, which in the strongest mutant allele (**clv1-4**) sometimes resembled meristematic tissue. Molecular studies showed that at least in the strong and intermediate mutants, the formation of the additional whorl corresponds to an abnormal lack of **AG** expression and persistence of **API** expression in the center of the floral primordia. Abnormal **API** expression is probably due to the lack **AG** expression, which normally represses **API** (Gustafson-Brown et al., 1994). Thus, **CLV1** expression is important for the determinate growth of floral meristems, similar to **AG** (Bowman et al., 1989, 1991; Mizukami and
Ma, 1995), and may help maintain floral meristem identity in the center of the flowers, possibly in part through \textit{AG} (Clark et al., 1993).

It was recently revealed that all of the intermediate and strong \textit{clv1} alleles are dominant negative and that \textit{clv1} null alleles display the weak phenotype (Dievart et al., 2003). This indicates that the intermediate and strong \textit{clv1} phenotypes are likely caused by interference with other unknown factors that share functional overlap with CLV1. Based on the observed differences between the weak and intermediate/strong alleles, these factors may contribute to maintaining appropriate apical and floral meristem size, promoting floral meristem determinacy and down regulating floral organ number in all four whorls. Interestingly, crossing the \textit{clv1} mutants into various ecotypes of \textit{Arabidopsis} revealed that genetic background significantly affects the mutant phenotype (Dievart et al., 2003). The presence of the \textit{er} mutation was one factor that significantly enhanced the mutant phenotype of both weak and strong \textit{clv1} alleles. In addition, an even greater enhancement was seen in the \textit{Ler} background, indicating that there are other factors, in addition to \textit{ER}, that contribute to meristem maintenance (Fletcher, 2001; Carles and Fletcher, 2003; Carles et al., 2005).

Although many of the players are still unknown, the pathway controlling meristem size and maintenance is one of the best understood in \textit{Arabidopsis}. \textit{CLV1} encodes an LRR-RLK that is a central mediator of this pathway (Clark et al., 1997b). In situ hybridization showed that \textit{CLV1} is expressed in the L3 portion of the central zone and appears to extend into the rib zone (Clark et al., 1997b; Bowman and Eshed, 2000). \textit{CLV1} functions with \textit{CLV2}, an LRR receptor-like protein, and \textit{CLV3}, a small extracellular protein, to negatively regulate stem cell proliferation (Clark et al., 1993,
WUS is a homeodomain transcription factor that promotes stem cell proliferation and formation, thus acting antagonistically to the *CLV1/CLV2/CLV3* pathway (Laux et al., 1996; Mayer et al., 1998). In addition, the *SHOOTMERISTEMLESS* (*STM*) gene also codes for a putative transcription factor that is essential for the establishment and maintenance of the undifferentiated central zone cells (Barton and Poethig, 1993; Clark et al., 1996; Long et al., 1996). Together, *WUS* and *STM* can revert differentiation and promote ectopic meristem development and cell division (Gallois et al., 2002; Lenhard et al., 2002). They also act via a non-cell autonomous signal to promote *CLV1* expression and organogenesis, thus forming a feedback loop (see below). Significantly, *CLV3* was shown to prevent reversion of differentiated cells in the peripheral zone to central zone cells, thereby acting antagonistically to *WUS* and *STM* (Reddy and Meyerowitz, 2005). This is likely mediated through the *CLV1/CLV2/CLV3* signal transduction pathway.

The *CLV* genes also negatively regulate the central zone by repressing *WUS* expression, restricting it to a small population of cells in the L3 portion of the central zone (Brand et al., 2000; Schoof et al., 2000). The *CLV1* expression domain overlaps with and surrounds the area of *WUS* expression (Clark et al., 1997b). In addition, *CLV3* expression is mainly limited to L1 and L2 layers of the central zone and only minimally overlaps with *CLV1* expression, but not with *WUS* expression (Fletcher et al., 1999; Bowman and Eshed, 2000). However, the CLV3 protein is exported to the extracellular space and is thereby able to interact with CLV1/CLV2 in a non-cell autonomous manner (Fletcher et al., 1999; Brand et al., 2000; Rojo et al., 2002). Furthermore, it was shown
that CLV3 is necessary for CLV1 to be incorporated into a functional signaling complex and may be a ligand for CLV1 (Trotochaud et al., 1999). The CLV1 signaling complex also contains a kinase-associated protein phosphatase (KAPP), which is a negative regulator of CLV1 activity, and a Rho GTPase-related protein, which may act to transduce the signal mediated by CLV1/CLV3 (Williams et al., 1997; Trotochaud et al., 1999).

**BAM1, BAM2 and BAM3**

In addition to CLV1 itself, the CLV1 clade contains three other members, BAM1 (for BARELY ANY MERISTEM 1), BAM2 and BAM3 (Shiu and Bleecker, 2001; DeYoung et al., 2006). BAM1 and BAM2 share over 85% sequence identity and are most closely related to each other. Currently, the relative placement of CLV1 and BAM3 within the clade is not clear. Two mutant alleles have been examined for both BAM1 and BAM2, namely *bam1-1, bam1-3, bam2-1* and *bam2-3*, and one mutant allele of BAM3 has been studied, *bam3-2* (DeYoung et al., 2006). All of the *bam1, bam2* and *bam3* single mutant plants appear similar to the wild-type, while *bam1 bam2* double mutants display pleiotropic defects, indicating that *BAM1* and *BAM2* act redundantly. In addition, while the *bam1 bam3* and *bam2 bam3* double mutant appear normal, the *bam1-1 bam2-1 bam3-2* triple mutant exhibits pleiotropic defects similar to the *bam1 bam2* double mutants, but stronger. This suggests that *BAM3* function is additive to the role of *BAM1/BAM2*, but it is not essential when functional *BAM1* or *BAM2* are present.
BAM1, BAM2 and BAM3 are important for shoot and flower meristem development (DeYoung et al., 2006). Unlike the clv1 mutants, which have enlarged apical meristems, the bam1 bam2 double mutants have shoot meristems that are significantly reduced in size compared to the wild-type. In addition, most of the bam1-1 bam2-1 bam3-2 triple mutant plants have terminated shoot meristems. Furthermore, while the bam1 bam2 mutants specify the normal number of carpels, the bam1-1 bam2-1 bam3-2 mutant has significantly reduced numbers of stamens and carpels, reminiscent of the wus and stm mutants (Barton and Poethig, 1993; Clark et al., 1996; Laux et al., 1996; DeYoung et al., 2006). Unlike the wus mutant, however, the bam1 bam2 and bam1-1 bam2-1 bam3-2 mutants produce no viable pollen and are therefore completely male sterile (Deyhle et al., 2006; DeYoung et al., 2006). Moreover, while the bam1 bam2 mutants are fertile, they produce about half the number of ovules seen in the wild-type (DeYoung et al., 2006). In addition, both the bam1 bam2 and bam1-1 bam2-1 bam3-2 mutants develop gynoecia that are bent and twisted in shape, and which emerge precociously, indicating additional roles for BAM1, BAM2 and BAM3 in flower development.

The pleiotropic defects exhibited by the bam1 bam2 double and bam1-1 bam2-1 bam3-2 triple mutants also include altered leaf morphology, reduced rosette size, and moderately delayed bolting and flowering (DeYoung et al., 2006). Altered leaf morphology was due in part to reduced vascular branching and abnormally large veins along the margins of the leaves. As with the other defects described, the bam1-1 bam2-1 bam3-2 triple mutant is more severely affected than the bam1 bam2 double mutants.
RT-PCR analysis showed that $BAM1$, $BAM2$ and $BAM3$ are weakly to moderately expressed in most tissues, including seedling, root, rosette leaf, stem, inflorescence, flower and silique, with the highest expression seen in the inflorescence (DeYoung et al., 2006). Using in situ hybridization, the expression patterns of $BAM1$ and $BAM2$ in the floral meristem were examined. Both genes were expressed at the flanks of the apical meristem, and did not appear to overlap with the known $CLV1$ expression domain (Clark et al., 1997b). In addition, $BAM1$ and $BAM2$ expression were observed in all four whorls of floral organs (DeYoung et al., 2006). In particular, expression was seen throughout developing tissues of the anther locules and gynoecium, including at later stages in the ovules. Together with the observed mutant defects, the expression patterns of $BAM1$, $BAM2$ and $BAM3$ support that these genes play a role in regulating meristem size, promoting leaf vasculature, and controlling floral organ growth, particularly in pollen and ovule development.

Interestingly, expression of $CLV1$ under the $ERECTA$ ($ER$) promoter ($pER:CLV1$) is capable of completely rescuing the $bam1-1$ $bam2-1$ double mutant phenotypes. This is reminiscent of the observation that the $pER:CLV1$ construct can partially rescue the short pedicel phenotype seen in Ler plants (Dievart et al., 2003). In addition, $pER:BAM1$ and $pER:BAM2$ constructs, which fully compliment the $bam1$ $bam2$ mutant, can partially rescue the $clv1-11$ null mutant phenotype (DeYoung et al., 2006). This suggests that $CLV1$, $BAM1$ and $BAM2$ can function in multiple developmental processes. Furthermore, while the $BAM$ genes appear to function antagonistically to $CLV1$, this appears to be based in part on the restricted expression domains of each. The exact role of the $BAM$ genes in meristem development is unclear, but they may function in a manner
analogous to WUS or STM in promoting stem cell maintenance (Barton and Poethig, 1993; Clark et al., 1996; Laux et al., 1996; DeYoung et al., 2006). In addition, the BAM genes appear to play a critical role male and female gametophyte development, which is not yet understood.

FUNCTIONS OF THE ERECTA CLADE

ERECTA

The importance of the ERECTA (ER) gene in controlling plant height has been known for decades, as the Landsberg erecta ecotype has a much more compact inflorescence than other Arabidopsis ecotypes (Rédei, 1992; Bowman, 1993). Over twenty er mutant alleles have been examined to date; generally these alleles display only subtle differences in the severity of the mutant phenotype (Torii et al., 1996; Lease et al., 2001). Most of the alleles examined are considered ‘strong’ alleles and do not vary significantly from a null mutant, er-105. As previously mentioned, the er mutants have a compact inflorescence, caused in part by reduced internode elongation, and are shorter than their wild-type counterparts on average by about 50% (Torii et al., 1996; Shpak et al., 2004). In addition, er mutants have very short pedicels and cluster their flower buds at the apex of the inflorescence. Overall silique length and silique valve height are also reduced in the er mutants, while silique, pedicel and inflorescence stem width are significantly increased (Torii et al., 1996; Shpak et al., 2004). Longitudinal sections of er-105 and wild-type pedicels were examined and revealed that endodermal and cortical
file cells in the mutant are greatly enlarged compared to the wild-type, and longitudinal cell number is decreased (Shpak et al., 2003). In addition, the cortex cells expand radially inward, contributing to the increase in pedicel width. Interestingly, in both the strong er-105 and intermediate er-103 mutants, the increase in cortex cell size was correlated with an increase in endoploidy relative to the wild-type. These observations support a role for ER in promoting cell proliferation and cell division in several different tissues.

RNA gel blot analysis and in situ hybridization experiments have been used to examine the expression pattern of the ER gene (Torii et al., 1996; Yokoyama et al., 1998). As would be expected from the er mutant phenotypes, ER is expressed in many different tissues. Moderate to weak expression was seen in stem and leaf tissue. Silique and young rosette tissues showed moderate levels of ER expression, and flower and inflorescence tissue had very strong expression. In particular, ER was expressed broadly throughout the apical meristem and floral primordia. Weak expression was observed at the shoot apical meristem early in plant development and became progressively stronger during the transition from vegetative to reproductive growth. In addition, expression was strongest in organ primordia and immature organs, and weak in mature organs. Similarly, ER expression was strong in floral primordia and became progressively weaker and concentrated in the inner whorls. Expression was seen longest in the carpel tissue. Thus, ER is expressed in rapidly dividing tissue, consistent with a role in promoting cell division.
Use of the Landsberg *erecta* ecotype has revealed interactions between *ER* and several other genes and pathways (Kanyuka et al., 2003; Xu et al., 2003; Qi et al., 2004). For example, the *er* mutation increases stomatal density and *ER* was shown to contribute to regulating transpiration efficiency (Masle et al., 2005; Shpak et al., 2005). Also, the *er* mutation enhances the internode elongation defect of both auxin and gibberellin response mutants (Peng et al., 1997; Fridborg et al., 2001; Kanyuka et al., 2003; Woodward et al., 2005). In addition, activation tagging of *YUCCA5*, which caused increased levels of free auxin (IAA) and enhanced auxin response, could suppress the weak *er-103* mutant phenotype (Woodward et al., 2005). Further study showed that presence of the *er* mutation does not significantly affect auxin response, indicating that while the auxin and *ER* pathways overlap in promoting cell proliferation, they are largely independent. In addition, a functional *ER* gene has been shown to attenuate the *brevipedicellus* (*bp*) mutant phenotype, which corresponds to a null mutation in the *KNAT1* homeobox gene (Douglas et al., 2002). The *bp* mutants have reduced pedicel length and plant height, similar to the *er* mutants. These characteristics are much stronger in *bp er* double mutants, indicating an additive role for *ER* and *KNAT1*. Additional investigation revealed that *ER* and *KNAT1* promote chlorenchyma development, which is necessary to maintain radialized or symmetric tissue differentiation important for normal internode growth and elongation.

In addition to affecting internode development, the *ER* gene is important for establishing proper leaf polarity (Xu et al., 2003). Double mutant analyses indicate that *ER* functions with the *ASYMMETRIC LEAVESI* (*ASI*) and (*A S2*) pathway to promote the adaxial cell fate in leaves. The exact role of *ER* in this process is unclear. The presence
of a functional *ER* gene is not sufficient to completely rescue *as2* abaxial-adaxial defects, and *er* single mutants are not defective in this process (Torii et al., 1996; Xu et al., 2002a; Xu et al., 2003). This indicates that additional factors are involved and that *AS1* and *AS2* may function upstream of *ER* (Xu et al., 2003). Interestingly, *as1 er* and *as2 er* seedlings grown at high temperatures, or temporarily heat shocked, had a much higher frequency of abaxial-adaxial leaf polarity defects than the *as1* or *as2* single mutants, respectively (Qi et al., 2004). This suggests that *ER* also functions in the AS1-AS2 pathway to reduce seedling sensitivity to heat stress during abaxial-adaxial leaf polarity establishment.

Similar to the work done with *CLV1*, use of a dominant-negative version of the *ER* receptor, which lacked the kinase domain, revealed overlap in the *ER* signaling pathways (Dievart et al., 2003; Shpak et al., 2003). The presence of the dominant-negative receptor construct in the wild-type background caused growth defects similar to the strong *er* mutants, due to interference with the *ER* pathway (Shpak et al., 2003). In the *er-105* mutant background, the dominant-negative receptor greatly enhanced the mutant phenotype, including reduced plant height, pedicel length, silique length and loss of cortex cell organization. This strongly suggests that additional factors are involved in the *ER* signaling pathway.

**ERECTA, ERL1 and ERL2**

In an effort to identify additional components of the *ER* signaling pathway, proteins with high sequence similarity were identified and studied (Shiu and Bleecker, 2003; Shpak et al., 2004). ERECTA-LIKE1 (ERL1) and ERL2 are about 60% identical
to ER and share 78% identity with each other (Shpak et al., 2004). *ERL1* and *ERL2* are likely the result of a recent duplication event and form a sister clade to ER. The *erl1-2* and *erl2-1* single mutants are null alleles, but show no phenotypic differences with wild-type plants. Furthermore, the *erl1-2 erl2-1* double mutant does not exhibit any detectable defect in plant growth. However, mutations in *ERL1* and *ERL2* are able to uniquely enhance subsets of the *er* mutant defects. The *er-105 erl1-2* double mutant was not affected in plant height compared to the *er-105* single mutant, but has significantly shorter siliques and pedicels. In addition, the *er-105 erl1-2* double mutant had enlarged cortex cells, similar to the *er-105* mutant, but they were less organized. In contrast, the *er-105 erl2-1* double mutant has reduced internode elongation and is much shorter than *er-105* single mutant. This double mutant had tightly clustered flowers, likely due to the defect in internode elongation. In addition, pedicel length did not appear affected and siliques length was only mildly reduced. Also, the *erl2-1* did not appear to affect the organization of the cortex cells as the *erl1-2* did. Interestingly, in both the *er-105 erl1-2* and *er-105 erl2-1* double mutants, the siliques valve width is increased and siliques style height is decreased. Thus, *ERL1* and *ERL2* are functionally redundant with *ER* in distinct and overlapping aspects of plant development. Furthermore, both *ERL1* and *ERL2* are capable of rescuing the *er* mutant phenotypes when expressed under the *ER* promoter. This indicated that ERL1 and ERL2 can replace ER function, and their different roles may be due to their different expression patterns.

Strikingly, the *er-105 erl1-2 erl2-1* triple mutant is severely affected in several aspects of plant development (Shpak et al., 2004). Triple mutant plants are drastically shorter than the wild-type, by over 90%, and are also markedly smaller than the other
single and double mutants. Cotyledon, leaf and pedicel growth are also strongly reduced. In addition, floral organ development is disrupted to various degrees. Some flowers lack floral organs, while others occasionally produce fused organs. Even in flowers that produce all four types of floral organs, their development is abnormal. In general, the triple mutant floral organs are much smaller than in the wild-type and they do not appear to develop to maturity, particularly the stamen and carpels. Stamens produce small, apparently undifferentiated anther and the ovules do not develop inside the triple mutant carpels. In addition, cortex cells are enlarged, disorganized and severely reduced in number. Interestingly, the affected organs rely on cell division and differentiation to develop properly. In contrast, hypocotyl elongation, which relies solely on cell elongation, was normal in the er-105 erl1-2 erl2-1 triple mutant. This indicates that the ER-family genes function redundantly and synergistically to promote cell proliferation during growth of all above ground organs.

As previously mentioned, the er mutation confers increased stomatal density (Masle et al., 2005; Shpak et al., 2005). In order to investigate the role of the ER-family genes in guard cell development, the single, double and triple mutant combinations were examined (Shpak et al., 2005). Guard cell development is normally tightly controlled by pre-existing stomata, such that stomata develop at least one cell away from each other (Nadeau and Sack, 2002; Shpak et al., 2005). In addition, stomata differentiation involves highly oriented asymmetric cell divisions. Meristemoid mother cells differentiate meristemoids, which behave like stem cells but undergo at most three asymmetric cell division events. Each cell division gives rise to a large stomatal-lineage ground cell (SLGC) and a small daughter cell, which retains the stem cell characteristics.
of the meristemoid until it differentiates into a guard cell (Bergmann, 2004; Shpak et al., 2005). The erl1, erl2 and erl1 erl2 mutants have reduced numbers of SLGCs, while the er mutant was shown to have an increased number of SLGCs, which failed to differentiate into guard cells (Shpak et al., 2005). In addition, the er mutant also undergoes a higher number of asymmetric cell divisions than normal, suggesting that ER functions to repress guard cell differentiation. Furthermore, in the er erl1 double mutant guard mother cells are able to differentiate from the supernumerary SLGCs, while in the er erl2 double mutant, guard mother cells differentiation is not restored, but greater numbers of SLGCs are produced. Finally, the er erl1 erl2 triple mutant produces dramatically high numbers of stomata, which fail to differentiate at least one cell apart and form dense clusters. This further supports that the ER-family genes function together to negatively regulate stomata differentiation.

The expression domains of the ER-family genes were examined using RT-PCR and promoter GUS fusion constructs (Shpak et al., 2004). ER expression was seen in all above ground tissues, in agreement with previous results (Torii et al., 1996; Shpak et al., 2004). Expression of ERL1 and ERL2 was also observed in the above ground tissues, except that ERL1 appeared largely absent in rosette leaves, and both ERL1 and ERL2 were weaker in the leaf, stem and petiole tissues than ER. GUS expression analyses revealed that the ER-family genes are expressed in actively growing tissues, such as the shoot and inflorescence meristems and young leaves. Expression was also seen in the floral primordia and subsequent unique, yet overlapping expression was observed in the carpels of late stage flowers. ER is expressed strongly in the mesocarp and weakly in the ovules, ERL1 is strongly expressed in the ovules, and ERL2 is expressed in the ovules.
and style. Overlapping expression in the ovules is compatible with observed triple mutant defect in ovule development. In addition, while ER expression was not observed in the epidermal cells, ERL1 and ERL2 both showed strong expression in the meristemoids, guard mother cells and immature guard cells, and very weak expression in the SLGCs and mature guard cells, supporting a role in guard cell differentiation (Shpak et al., 2005).

Mutant and expression analysis indicate that the ER-family genes interact synergistically in regulating stomata differentiation and to promote cell proliferation during above ground organ development (Shpak et al., 2004; Shpak et al., 2005). The roles of ER, ERL1 and ERL2 in guard cell formation have been better elucidated using combined mutant and expression analysis with a known regulator of this pathway, TOO MANY MOUTHS (TMM) (Yang and Sack, 1995; Shpak et al., 2005). The model for the combined action of these genes is as follows: ER, and to a lesser extent ERL1 and ERL2, negatively regulate the initial asymmetric cell division of meristemoid mother cells, and promote their symmetric cell division. TMM appears to positively regulate stomatal differentiation by inhibiting the ER-family genes at this stage. After the meristemoid mother cell has undergone the first asymmetric cell division, forming a meristemoid daughter cell, ERL1 strongly and ERL2 weakly inhibit the subsequent asymmetric divisions leading to guard mother cell formation. In addition, TMM likely acts to block ERL1 and ERL2 inhibition of guard mother cell formation, thereby promoting stomatal differentiation.

The ER-family genes clearly play a role in promoting cell proliferation in a synergistic and overlapping manner. Loss of one or two of these genes causes defects in
growth and loss of all three results in dramatically stunted development of all above
ground tissues. In the er-105 erl1-2 erl2-1 triple mutant, WUS expression is somewhat
reduced, suggesting a decrease in the stem cell population. However, the expression of
STM and other genes important for meristem function are not affected. In addition, the
phenotype of the apical meristems has not yet been examined and may reveal a role for
the ER-family genes in meristem function. Furthermore, the er-105 erl1-2 erl2-1 triple
mutant is apparently male and female sterile and further investigation may yield insights
into the nature of these defects.

The goal of this dissertation is to understand the function of the CLV1 clade
members, BAM1, BAM2 and BAM3 and the ER-family genes in Arabidopsis anther
development. In addition, the research will attempt to elucidate the relationship between
certain of these genes and other genes previously known to be important for anther
development in Arabidopsis.
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Chapter 2

The BAM1/BAM2 Receptor-Like Kinases are Essential for Cell Fate Specification in the Early Arabidopsis Anther

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ABSTRACT

Cell differentiation is critical for the development of multicellular organisms. In flowering plants, anther development involves the formation of several adjacent cell types required for normal male fertility. Currently, only a few genes are known to be involved in early anther development, particularly in the establishment of these different cell layers. *BAM1* and *BAM2* encode CLAVATA1 (CLV1)-related leucine-rich repeat receptor-like kinases that appear to have redundant or overlapping functions. We characterized anther development in the *bam1 bam2* flowers and found that *bam1 bam2* anthers appear to be abnormal at a very early stage and lack the endothecium, middle, and tapetum layers. Analyses using molecular markers and cytological techniques of *bam1 bam2* anthers revealed that cells interior to the epidermis acquire characteristics of pollen mother cells (PMCs), indicating defects in cell fate specification. The pollen mother-like cells (PMLs) degenerate prior to completion of meiosis, suggesting these cells are defective. In addition, the *BAM1* and *BAM2* expression pattern supports both an early role in promoting somatic cell fates and a subsequent function in the PMCs. Therefore, analysis of *BAM1* and *BAM2* uncovered a cell-cell communication process important for early anther development, including aspects of cell division and differentiation. This may have implications for the evolution of multiple signaling pathways in specifying cell types for microsporogenesis.
INTRODUCTION

Understanding the mechanisms that regulate cell division and differentiation is an essential goal for developmental biologists. In plants, cellular differentiation occurs as an interplay between a cell’s lineage and its relative position within a structure (Szymkowiak and Sussex, 1996; Berger et al., 1998; Scheres and Benfey, 1999). The relative importance of these determining factors appears to vary depending on the specific developmental process. For example, analysis using chimeras between *Solanum luteum* and tomato (*Solanum lycopersicum*) leaf cell layers indicated that trichome development was determined by the genotype of the differentiating L1 cells (autonomous development), whereas leaf shape was controlled by the genotype of the neighboring cell layer (non-autonomous development) (Jorgenson and Crane, 1927; Szymkowiak and Sussex, 1996).

For several aspects of plant development, cell fate specification has been shown to rely more on positional information rather than on cell lineage (Szymkowiak and Sussex, 1996). For instance, it was shown in both leaf and root that cells are able to respond to a new position and differentiate accordingly when they are displaced from one layer into an adjacent layer, irrespective of their lineages (Stewart and Dermen, 1975; van den Berg et al., 1995). Positional signals can be mediated by either apoplastic, via receptors, or symplastic, through plasmodesmata, mechanisms (Hake and Freeling, 1986; Fletcher et al., 1999; Rojo et al., 2002; Kwak et al., 2005). Apoplastic cell-cell communication is often achieved via receptor-ligand interactions (Bergmann, 2004).
example, the leucine-rich repeat receptor-like kinase (LRR-RLK) SCRAMBLED (SCM) is required for normal epidermal cell-type patterning in the *Arabidopsis* root (Kwak et al., 2005). In addition, the *ERECTA* (*ER*) and *ERECTA-LIKE* (*ERL1* and *ERL2*) genes encode LRR-RLKs that control position-dependent guard cell differentiation (Shpak et al., 2005). Hence, positional cues via receptor-mediated intercellular signaling from adjacent cells help control cell division and differentiation (Hake and Freeling, 1986; Berger et al., 1998; Kwak et al., 2005).

In the male reproductive organ of flowering plants, the anther, the differentiation of sporogenous and parietal cell types is essential for the propagation of the species. In *Arabidopsis thaliana*, development of the male gametophytes, the pollen grains, occurs within the four lobes of the anthers (Goldberg et al., 1993; Sanders et al., 1999). Within each lobe, cells divide and differentiate to form distinct somatic cell layers surrounding the developing reproductive cells, or the pollen mother cells (PMCs). The proper formation and development of these somatic cell layers is critical for the development and eventual release of pollen grains (Mariani et al., 1990; Mariani et al., 1991; Denis et al., 1993; Ross et al., 1996; Zhao et al., 2002; Zhang et al., 2006; Hord and Ma, 2007).

Anther development in *Arabidopsis* has been divided into specific stages according to morphological characteristics (Sanders et al., 1999). In the emergent anther primordium (stage 1), there are three distinct cell layers derived from the floral meristem: from outer to inner they are L1, L2, and L3 (Sanders et al., 1999). The four corners of the anther primordia develop into the four lobes during further anther development, as described previously (Goldberg et al., 1993; Sanders et al., 1999). The L1 layer develops
into the epidermis, and the L2-derived cell layers develop via a series of periclinal (parallel to the adjacent outer surface and creating additional cell layers) and anticlinal (perpendicular to the outer surface and increasing cell number in a layer) cell divisions. At stage 2, L2 cells called archesporial cells divide periclinally to form primary parietal (outer) and primary sporogenous (inner) cells. Then at stage 3, periclinal division of the primary parietal cells forms the inner and outer secondary parietal cells. Subsequently, in each of the four anther lobes, cells of one of the secondary parietal layers divide again at stage 4, resulting in three somatic layers at stage 5: endothecium, middle layer and tapetum, which immediately surrounds the L2-derived PMCs. The tapetum is important for providing the developing pollen with nutrients and materials (Mariani et al., 1990; Mariani et al., 1991; Denis et al., 1993). The L3 layer gives rise to the vascular and connective tissues of the anther.

To date, only a few genes are known to be involved in the early anther cell division and cell differentiation events, including SPOROCYTELESS/NOZZLE (SPL/NZZ), EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS (EMS1/EXS), SOMATIC EMBRYOGENESIS1 (SERK1), SERK2, and TAPETUM DETERMINANT1 (TPD1) (Schiefthaler et al., 1999; Yang et al., 1999; Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Ito et al., 2004; Albrecht et al., 2005; Colcombet et al., 2005; Ma, 2005; Yang et al., 2005; Hord and Ma, 2007). SPL/NZZ was shown to promote microsporogenesis under the control of AGAMOUS (AG) in whorl three floral organs (Ito et al., 2004). In the spl and nzz mutants, the L2-derived cells do not develop properly and they are unable to form PMCs (Schiefthaler et al., 1999; Yang
The detailed descriptions of the spl and nzz mutants differ somewhat. The spl mutant was reported as having primary sporogenous and secondary parietal cells (Yang et al., 1999), while the nzz mutant forms an undifferentiated mass of archesporial cells (Schieffthaler et al., 1999) suggesting that SPL/NZZ may act at the stage when the archesporial cells divide to form the primary sporogenous cells and primary parietal cells. SPL/NZZ has been cloned and encodes a putative transcription factor (Schieffthaler et al., 1999; Yang et al., 1999).

The ems1/exs, serk1 serk2 and tpd1 mutations affect cellular differentiation by altering cell fate at a later stage than spl/nzz (Schieffthaler et al., 1999; Yang et al., 1999; Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005). Instead of forming the four normal L2-derived cell types, these mutants form endothecium, middle layer cells, and a greater than normal number of PMCs. The mutant anther lobes completely lack the nutritive tapetum layer. Although the excess PMCs enter meiosis and undergo nuclear division, they fail to complete cytokinesis and degrade completely by anther stage 7 (Ma, 2005; Hord and Ma, 2007). EMS1/EXS, SERK1 and SERK2 encode LRR-RLKs, indicating an essential role for a position-dependent intercellular signaling event (Canales et al., 2002; Zhao et al., 2002; Albrecht et al., 2005; Colcombet et al., 2005). TPD1 encodes a small putatively secreted protein that may act in the same pathway as that of EMS1/EXS and SERK1/2 (Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005; Yang et al., 2005). In addition to its role in anther development, SERK1 was initially shown to promote somatic embryogenesis (Schmidt et al., 1997; Hecht et al., 2001).
LRR-RLKs compose one of the largest gene families in *Arabidopsis thaliana*; however, relatively few have known functions (Shiu and Bleecker, 2001, 2003). For instance, *CLAVATA1 (CLV1)* is a LRR-RLK that acts with *CLV3* to limit the size of the shoot apical and floral meristems and may promote the transition of central zone cells to peripheral zone cells (Clark et al., 1995; Clark et al., 1996; Fletcher et al., 1999; Trotochaud et al., 1999; Brand et al., 2000; Gallois et al., 2002; Rojo et al., 2002; Lenhard and Laux, 2003; Reddy and Meyerowitz, 2005). In addition, *BRASSINOLIDE INSENSITIVE1 (BRI1)* and *BRI1-ASSOCIATED RECEPTOR KINASE (BAK1/SERK3)* are involved in the brassinosteroid-signaling pathway (He et al., 2000; Wang et al., 2001; Li et al., 2002; Nam and Li, 2002).

**BAM1** (for BARELY ANY MERISTEM) and **BAM2** encode LRR-RLKs (DeYoung et al., 2006) that share high levels of amino acid sequence identity and form a four-gene monophyletic clade along with *CLV1* and **BAM3** (Shiu and Bleecker, 2001; DeYoung et al., 2006). Single mutants in **BAM1** and **BAM2** do not exhibit any obvious morphological defects, indicating that they have redundant functions (DeYoung et al., 2006). In contrast, *bam1 bam2* double mutants display multiple developmental defects, including a reduction of meristem size, altered leaf shape, size and venation, male sterility and reduced female fertility (DeYoung et al., 2006). Herein we show that the *bam1 bam2* mutations affect normal cell division and differentiation during early anther development. The *bam1 bam2* double mutant does not produce the somatic cell layers that are derived from the archesporial cells; instead they only form cells that are characteristic of PMCs, which then degrade before completing meiosis. The **BAM1** and
BAM2 genes are expressed in the area of archesporial cells as early as stage 2 and also preferentially in the sporogenous cells and PMCs at later stages. The very early BAM1/2 expression pattern and the early morphological defects suggest that these genes promote cell division and differentiation, including the specification of the parietal cells that give rise to the endothecium, middle layer and tapetum. BAM1/2 expression in the sporogenous cells and PMCs and the degeneration of PMCs in the bam1 bam2 double mutant suggest that these gene also play a role in the development and/or function of PMCs.

RESULTS

Anther Development in the bam1 bam2 Mutant is Abnormal

The bam1 bam2 mutant plants are male sterile and the mutant anthers fail to produce pollen (DeYoung et al., 2006). In order to better understand the overall morphological differences between the mutant and wild-type anthers, scanning electron microscope (SEM) images of dissected buds were examined (Figure 2-1). At approximately flower stage 8 (Smyth et al., 1990), the size and shape of wild-type and mutant anthers appeared similar (Figures 2-1 A and D). Subsequently, mutant anthers had lobes that appeared less full (Figures 2-1 B and E) at approximately late stage 9, although the stage of the mutant flowers was difficult to determine at this resolution due to the abnormal size and morphology of other floral organs. Close to stage flower stage 10, the mutant anthers had a shriveled appearance, suggesting that the locules had
collapsed (Figures 2-1 C and F). Anther development appeared to be defective at or before flower stage 9, during which several key developmental processes occurred, including the establishment of the five cell layers and meiosis. The anther morphology of the bam1-1 bam2-1 (not shown) is similar to that of bam1-3 bam2-3.

Figure 2-1: SEM Images of Dissected Buds. Several floral organs have been removed to allow for better visualization of the anther defects. (A) to (C) Wild-type (Ler) floral buds. (D) to (F) bam 1-3 bam 2-3 double mutant (Ler) floral buds. (A) A floral bud at stage 8 (anther stage 4). (B) A floral bud at stage 9 (anther stage is between 5 and 7). (C) A floral bud at stage 10 (anther stage is between 7 and 8). (D) A floral bud near stage 8. The anthers appear similar to those of the wild type (A). (E) A mutant floral bud near stage 9 showing anthers that are less full than the wild type (B). (F) A floral near stage 10, with anthers that have apparently collapsed locules.

The bam1 bam2 Mutant is Defective in Formation of Anther Cell Layers

In order to better understand the defect in anther development, we prepared and analyzed transverse sections of wild-type and the bam1-3 bam2-3 mutant anthers.
At stage 1 of anther development cells from all three cell layers, L1, L2 and L3, appeared slightly larger in the bam1 bam2 anthers than in the wild type (Figures 2-2 A and D). At the same time, the average number (± standard deviation) of sub-epidermal cells (L2 and L3) in a cross section of the stage 1 bam1 bam2 anther (27.9 ± 3.5, n=11) was slightly smaller than the number in the wild type (Ler= 31.9 ± 5.6, n=44). This reduction is much less dramatic than the size reduction of the bam1 bam2 inflorescence meristem (DeYoung et al., 2006). At stage 2, both wild-type and mutant anthers appeared to have a similar structure and overall cell patterning, but the cells in the mutant remained slightly larger than those of the wild type (Figures 2-2 B and E). At anther stage 3, the wild-type anther has well defined lobes and the archesporial cells have undergone a periclinal cell division, forming the primary sporogenous and primary parietal cells, which lie roughly parallel to the epidermis at the outermost part of each lobe (Figure 2-2 C). Mutant anthers at stage 3 appeared to have fewer and larger cells in each lobe (Figure 2-2 F). Although evidence of a periclinal cell division was occasionally observed, in general there were no clearly defined primary parietal and primary sporogenous cells.

At stage 4, the wild-type sporogenous cells are visible at the center of each lobe (Figure 2-2 G). At this stage, the primary parietal cells have divided periclinally to form the outer secondary parietal and the inner secondary parietal cells. At stage 4 in the mutant, the width and thickness of the anther began to appear substantially greater than the wild-type dimensions (Figure 2-2 I). Although at times there appeared to have been cell divisions with orientations close to periclinal and anticlinal, the mutant anthers still
did not form the cell layers that are characteristic of normal anthers and the cellular pattern appeared to be disorganized.

Figure 2-2: Semi-thin Sections of Anthers. (A) to (C), (G), (H) and (K) Wild type (Ler). (D) to (F), (I), (J), and (L) bam 1-3 bam 2-3 (Ler). (A) Wild-type anther at stage 1. (B) Wild-type anther at stage 2. (C) Wild-type anther at stage 3. (D) Mutant anther at stage 1. (E) Mutant anther at stage 2. (F) Mutant anther at stage 3 does not have normally organized L2-derived cell layers. (G) Wild-type anther at stage 4. (H) Wild-type anther at stage 5 showing five distinct cell layers in each lobe. (I) A mutant anther at stage 4 has the epidermis, but the distinct developing cell layers are missing. (J) A mutant anther at stage 5, which appears to completely lack the normal sub-epidermal cell layers. The cells in each lobe appear enlarged. (K) Wild-type anther at stage 6 showing thick callose surrounding the meiotic sporogenous cells. (L) A mutant anther at stage 6; the L2-derived cells begin to degrade. ep, epidermis; ar, archesporial cells; sl, sporogenous-like cells; pp, primary parietal cells; ps, primary sporogenous cells; osp, outer secondary parietal cells; isp, inner secondary parietal cells; pmc, pollen mother cells; pml, pollen mother-like cells; en, endothecium; mi, middle layer; t, tapetal cells; c, callose. Bar = 20µm; all panels are at the same magnification.
In the wild-type anther at stage 5, the PMCs are formed at the center of each lobe and are surrounded sequentially, from inner to outer, by the tapetum, middle layer, and endothecium, with the epidermis encasing the entire anther (Figure 2 H). With the exception of the epidermis, the mutant anthers never formed these organized sporophytic cell layers (Figure 2 J). Instead, their enlarged cells appeared slightly fewer in number (see below) and less organized. In addition, the nuclei of these cells appeared unusually large, a characteristic of meiotic cells. Meiosis in the wild type commences during anther stage 6 and the PMCs become isolated from each other and from the tapetum as a thick callose wall is formed around them (Figure 2 K). Some cells within the bam1 bam2 anther lobes had callose around them, while some other cells exhibit signs of degradation (Figure 2 L). Eventually most or all the L2-derived cells in the mutant degraded, causing the lobes to collapse and the anther to appear shriveled (Figure 1 F). In addition, occasionally a bam1 bam2 anther was observed that had a small, apparently undifferentiated lobe (not shown).

The cells in the bam1 bam2 mutant anthers appeared larger in size and fewer in number than their wild-type counterparts. To obtain more quantitative information, the number (Figure 2-3 A) and dimensions of the L2-derived cells in each lobe were analyzed using cross-sections. At stage 5 in the wild-type the average number ± standard deviation of PMCs per lobe was 3.8 ± 0.9 and the total number of L2-derived cells per lobe was 36.9 ± 3.8 (Figure 2-3A). In contrast, the number of L2-derived cells in the double mutant was 15.8 ± 1.8. Although this number is obviously smaller than the total number of L2-derived cells in the wild-type, it is also much larger than the average number of PMCs seen per lobe in the wild-type, by more than four fold. The average
length (16.0 ± 5.4 µm; parallel to the epidermis) and height (14.1 ± 5.0 µm; perpendicular to the epidermis) of the mutant cells was significantly greater than the wild-type PMCs (5.8 ± 2.5 µm long, 4.7 ± 2.1 µm high). Interestingly, although the length of the mutant juxta-epidermal cells at stage 5 did not differ significantly from the corresponding wild-type cells (16.0 ± 5.4 µm mutant vs. 16.1 ± 4.2 µm wild-type), the height of the cells was significantly greater in the mutant (14.1 ± 5.0 µm mutant vs. 6.9 ± 1.6 µm wild-type). Together these observations indicate that cell division and/or cell expansion were altered in the *bam1 bam2* mutant. In particular, it seems that cells in the double mutant expanded without some of the cell divisions that normally produced the secondary parietal cells or subsequent cell layers in the wild type.

The cell layers in the anther are normally formed by periclinal cell divisions of the sub-epidermal cells. In order to better quantify the cell division defect, we counted the number of cell walls per lobe, interior to the epidermis and exterior to the PMCs, that appeared to have arisen via a periclinal cell division for stages 4 to 5 (Figure 2-3 B). The wild type at stage 4 had an average of 3.5 ± 0.8 periclinal cell division events per lobe, which was not statistically different from the lobes transitioning from stage 4 to 5 that had 5.9 ± 2.2. However, at stage 5 the wild-type had an average of 10.8 ± 1.9 periclinal cell division events per lobe, which was significantly higher than both stage 4 and stage 4 to 5. In the *bam1 bam2* mutant, the numbers of periclinal cell divisions were similarly small at stages 4 (1.6 ± 0.9) and 5 (1.3 ± 0.7), and severely reduced compared with those seen at stages 4 and 5 in the wild type. Sections of *bam1-1 bam2-1* at stages 5-6 (not shown) appeared to be very similar to those of *bam1-3 bam2-3*. Similar anther sections
of bam1-3 bam2-3 plants carrying a ProER-BAM1-FLAG construct (DeYoung et al., 2006) were normal (not shown).

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Figure 2-3: A Comparison of the Number of Cells and Cell Division Events Between Wild Type and bam1 bam2. (A) Average numbers of cells per cross section in each lobe. (B) Average numbers of cell division events per cross section in each lobe that were parallel to the epidermis, between the epidermis and the PMCs. Bars indicate standard deviation. SE, sub-epidermal L2-derived cells; M, PMCs.

In summary, the bam1 bam2 mutant anthers did not form the normal somatic cells layers in the L2-derived position. The cells that formed in their place were larger, fewer in number, and appeared randomly organized; these cells degraded near anther stage 6.
Periclinal cell division events in the mutant were reduced compared to the wild type. Although the cells formed by the bam1 bam2 anthers were significantly larger than the normal sporophytic cells, they appeared similar to the PMCs formed in wild-type anthers. We concluded that the bam1 bam2 anther formed pollen mother-like cells (PMLs) in place of the three normal somatic cell types. These PMLs were clearly abnormal because they degenerate before producing microspores (see below for more characterization).

The L2-derived Cells in the bam1 bam2 Anthers Express PMC Markers

The L2-derived cells in the bam1 bam2 mutant anthers have size, shape and organization that are similar to the characteristics of the PMCs, which are normally found interior to the tapetum. To test whether the L2-derived mutant cells have additional properties of PMCs, we examined the expression of known cell-identity markers. In wild-type anthers, the meiotic genes ATRAD51 and SDS are strongly expressed in PMCs at stage 6 in the center of each lobe (Figures 2-4 A and B, respectively) (Azumi et al., 2002; Li et al., 2004). In stage 6 bam1 bam2 mutant anthers, ATRAD51 and SDS appeared to be expressed (Figures 2-4 D and E, respectively) in the L2-derived cells that had not yet degraded, including the cells that occupied a position immediately interior of the epidermis. This suggests that the L2-derived PMLs in the bam1 bam2 anther share molecular properties with normal PMCs. To further verify the lack of tapetal cells in the mutant anther, we examined the expression of DYT1, which is very strongly expressed in the wild-type tapetal cells at late anther stage 5 and stage 6 (Figure 2-4 C) (Zhang et al.,
2006). No *DYT1* expression was detected in the *bam1 bam2* mutant anthers at stages 5 or 6 (Figure 2-4 F and data not shown), indicating the absence of tapetal cells.

**PMLs in the *bam1 bam2* Anthers Can Enter but Fail to Complete Meiosis**

The *ems1* mutant lacks the tapetum layer and produces excess PMCs, which proceed to meiosis II (Zhao et al., 2002). The expression of meiotic genes in the *bam1 bam2* PMLs suggests they may also undergo meiosis. To test this hypothesis, we analyzed DAPI-stained chromosome spreads of wild-type and *bam1-3 bam2-3* meiocytes. Over 200 cells from mutant anthers were examined. Based on chromosome morphology, most cells appeared to be entering or undergoing meiosis. Among these, at least a quarter of them were at the leptotene or zygotene stages of prophase I. In addition, about ten percent of the cells had pachytene-like chromosomes, at mid-prophase I. Therefore, a substantial fraction of the PMLs had entered meiosis. Amazingly, two of the examined cells were at metaphase I and one was at anaphase I (not shown). The chromosomes in the metaphase I cells were highly condensed and aligned, but appeared to be partially degraded (data not shown). In addition, in the anaphase I cell the chromosomes segregated unequally between the two poles of the meiocyte. Furthermore, chromosomal fragments were frequently observed at different stages of meiosis (Figure 2-4 J and data not shown).
Figure 2-4: Molecular and Cytological Analyses of Wild-type and bam1 bam2 Anthers at Stage 6. (A) to (C), (G) and (I) Wild type. (D) to (F), (H) and (J) bam1 bam2 mutant. (A) and (D) ATRAD51 was expressed in wild-type PMCs (A) at a high level and in the bam1 bam2 mutant (D) interior to the epidermis (arrows). (B) and (E) SDS was expressed in the wild-type (B) at the center of the lobe at the position of the PMCs (arrow) and in bam1 bam2 mutant (E) in the cells interior and juxtaposed to the epidermis (arrow). (C) and (F) DYT1 expression. In the wild type (C), DYT1 signal could be detected in the tapetal layer, but no detectable DYT1 signal could be seen in bam1 bam2 (F). (G) and (H) Sections of wild-type and bam1 bam2 mutant anthers stained with DAPI. A wild-type lobe (G) with PMCs undergoing meiosis in the center surrounded by the tapetal layer, middle layer, endothecium and epidermis. A lobe of bam1 bam2 (H) showing a greater number of PMCs only surrounded by the epidermis and connective tissue. (I) and (J) DAPI-stained chromosome spreads of wild-type and bam1 bam2 mutant meiotic cells showing pachytene-like chromosomes. Fragments of chromosome can be seen in the bam1 bam2 meiotic cells (J) (arrow) but not in the wild type (I). ep, epidermis; en, endothecium; mi, middle layer; t, tapetum; pmc, pollen mother cell; pml, pollen mother-like cell. Bar = 20 μm in (A) and (G); bar = 10 μm in (I). (A) to (F) are of the same magnification; (G) and (H) are at the same magnification; (I) and (J) are at the same magnification.
To verify that the cells able to enter meiosis were not confined to the center of the lobes, we performed DAPI staining of sectioned anthers. Whereas in the wild type, the four sporophytic cell layers surrounding the meiotic cells were clearly observable (Figure 2-4 G), in the *bam1 bam2* mutant anthers only the epidermis was seen encasing and juxtaposed to a mass of randomly organized PMLs, many of which were evidently undergoing meiosis (Figure 2-4 H).

In summary, the L2-derived cells in *bam1 bam2* anthers possessed attributes of PMCs and were partially able to enter meiosis, indicating that the L2-derived cell fates are altered in the mutant anthers. Furthermore, the PMLs exhibit defects at meiotic stages much earlier than the PMCs in the *ems1/exs, serk1 serk2*, and *tpd1* mutants, with most cells unable to complete prophase I prior to degeneration. Therefore, *BAM1* and *BAM2* are also important for normal PMC development.

**Expression of the *BAM1* and *BAM2* Genes Supports Their Function in Anther Development**

To obtain clues about the mechanisms of *BAM1* and *BAM2* action, we analyzed their expression during early anther development (Figure 2-5). Overall, *BAM1* and *BAM2* had very similar expression patterns. Both were expressed in the archesporial cells at anther stage 2 (Figures 2-5 A and H), and in the primary parietal and primary sporogenous cells near the lateral edges of stage 3 anthers (Figures 2-5 B and I). Interestingly, at stage 4, both genes appeared to be predominantly expressed in the sporogenous cells and might have a low level of expression in the L2-derived secondary parietal cells (Figures 2-5 C and J). At stage 5, *BAM1* and *BAM2* were highly expressed
in the PMCs, with a very low level of expression in the tapetum (Figures 2-5 D and K). During anther stage 6, both BAM1 and BAM2 were very strongly expressed in the PMCs and tapetum, and might be weakly in the middle layer (Figures 2-5 E and L). Similarly, at stage 7, strong BAM1 expression was seen in the tapetum and a lower level was seen in the tetrads (Figure 2-5 F). By anther stage 9, there was no detectable BAM1 or BAM2 expression (not shown). Also, there was no detectable expression in the bam1 bam2 double mutant tissues (not shown).

In summary, BAM1 and BAM2 appear to be expressed at stage 2 in the archesporial cells and at stage 3 in the primary sporogenous and primary parietal cells; subsequently they were preferentially expressed in the sporogenous cells at anther stage 4, after which their expression becomes restricted to the tapetum and PMCs. These expression patterns support an early function that promotes the formation of the primary parietal cells, which are the progenitors of the L2-derived somatic cell layers, and a later function in support of PMC development.
Figure 2-5: BAM1 and BAM2 in Situ Hybridization of Wild-type Ler Anthers. (A) to (F) BAM1 expression. (G) Sense control probe. (H) to (L) BAM2 expression. (A) and (H) Stage 2 anthers, showing BAM1 and BAM2 are expressed in lateral L2-derived archesporial cells (arrows). (B) and (I) Stage 3 anthers; BAM1 and BAM2 are expressed predominantly in the primary sporogenous cells. (C) and (J) Stage 4 anthers, BAM1 and BAM2 expression is predominantly seen in the sporogenous cells. (D) and (K) Stage 5 anthers, both genes were highly expressed in PMCs and may have a very low level of expression in the tapetal layer. (E) and (L) Stage 6 anthers showing that BAM1 and BAM2 were very strongly expressed in the tapetum, PMCs, and faintly in the middle layer. (F) Stage 7 anther showing strong BAM1 expression in the tapetum and somewhat weaker expression in the tetrads. (G) Sense probe showing no cross-hybridization. pmc, pollen mother cells; t, tapetal cells; ms, microspores in tetrads. All panels are at the same magnification.
DISCUSSION

_BAM1 and BAM2 are Important for Normal Cell Division and Differentiation in Early Anther Development_

We have demonstrated here that _BAM1_ and _BAM2_ together are important for normal early anther development. Our phenotypic studies indicate that the _bam1 bam2_ double mutant anthers have only a slightly reduced number of cells at very early stages, in contrast to the dramatic size reduction of the _bam1 bam2_ meristems (DeYoung et al., 2006). Therefore, the _bam1 bam2_ anther defects do not seem to be a direct consequence of a greatly reduced anther primordium that resulted from the small mutant meristem. Nevertheless, analysis of subsequent anther stages clearly indicates a dramatic reduction of L2-derived cell numbers, with the _bam1 bam2_ anther producing fewer than half of the wild-type number of sub-epidermal cells per lobe, suggesting that cell division is significantly decreased in the mutant. In addition, our analyses showed that the _bam1 bam2_ mutant failed to specify the normal identity of L2-derived somatic cell layers, completely lacking the endothecium, middle layer, and tapetum. Moreover, morphological, cytological and molecular studies indicate that the L2-derived cells in the mutant had properties of PMCs. Furthermore, _BAM1_ and _BAM2_ were expressed in archesporial cells at stage 2 and in the _bam1 bam2_ double mutant abnormal cell patterning was visible by stage 3. As _BAM1_ and _BAM2_ encode LRR-RLKs, we propose that they mediate a developmental signal that promotes the differentiation of archesporial cells at stage 2 (Figure 2-6 A). Our results strongly support the idea that _BAM1_ and _BAM2_ contribute to the differentiation of the parietal and perhaps sporogenous
cell types at stage 3. It is possible that $BAM1$ and $BAM2$ regulate the asymmetric cell division of the archesporial cells into the primary sporogenous and primary parietal cells.

In the wild-type anther, the number of somatic cells increases considerably during stages 4 and 5, whereas the sporogenous cells expand in size but do not change dramatically in number during this period. In the $bam1$ $bam2$ anther, the decrease in L2-derived cell number is accompanied by the change of somatic cells to PMLs. In the wild type, PMCs are larger than the L2-derived somatic cells; therefore, it is possible that the reduction in number and expansion in size of the mutant L2-derived cells are a reflection of these cells being similar to PMCs. A similar phenomenon was seen in the $ems1/exs$, $tpd1$ and $serk1$ $serk2$ mutants, which possess a total number of PMCs that is less than the combined number of tapetum and PMCs seen in the wild type, but is greater than the number of PMCs seen in the wild type (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005). It is possible that when progenitor cells are directed to form PMCs (or PMLs), they divide less frequently and develop into larger cells. An alternative explanation is that in these mutants a negative regulation of PMC proliferation is lacking due to the absence of the adjacent somatic cells, allowing the sporogenous cells to proliferate abnormally. Therefore, the function of $BAM1$ and $BAM2$ in regulating cell division and differentiation may be coupled, and the normal function of these genes is important for the formation of correct cell types in the anther.
**BAM1 and BAM2 May Play a Role in Sporogenous Cells**

In addition to the BAM1/BAM2 function in very early anther development, strong BAM1 and BAM2 expression was seen from anther stages 4 to 6, preferentially in the sporogenous cells. This suggests possible additional roles for these genes later in anther development. In addition, although the L2-derived cells in the mutant resemble PMCs, they are not normal. Some of the PMLs in the bam1 bam2 mutant were able to initiate meiosis, but none were able to complete meiosis I, and all mutant PMLs eventually degenerated. Sporogenesis requires the coordinated development of multiple adjacent cell types that probably involves cell-cell communication; therefore, it is plausible that BAM1/2 might be needed to receive signaling directed toward the PMCs in order to promote their normal differentiation. In addition, BAM1 and BAM2 may be required in the meiocytes for the completion of meiosis. Another possibility is that one or more of the somatic cell layers may be required for meiosis. For example, the tapetum may provide materials and nutrients needed for normal meiosis, or BAM1 and BAM2 may mediate a response to a developmental signal(s) normally released from neighboring cells that promotes the meiotic process.

LRR-RLKs compose a very large gene family, but only a small number of them have been characterized functionally. If BAM1 and BAM2 indeed promote PMC differentiation and/or function, this would be a new function for LRR-RLKs. It is possible that other LRR-RLKs are involved in this complex process, perhaps by interacting with BAM1 and BAM2. Further genetic and molecular studies are needed to uncover and better elucidate LRR-RLK functions in anther development.
Figure 2-6: A Model for the Control of Early Anther Cell Differentiation by BAM1 and BAM2. (A) BAM1/2 function in early anther development. In the wild-type anther by stage 3, archesporial cells have divided to form primary parietal (pp) and primary sporogenous (ps) cells. In the absence of BAM1 and BAM2 function, the asymmetric division of the archesporial cells might be abnormal, resulting in the lack of primary parietal cells and the formation of primary sporogenous-like cells. At anther stage 5, the wild-type anther has three clearly defined concentric cell layers surrounding the pollen mother cells; in the bam1 bam2 mutant, however, only pollen mother-like cells are observed in the area interior of the epidermis. Therefore, BAM1 and BAM2 define a cell-cell signaling pathway critical for the differentiation of the primary parietal and primary sporogenous cells. The abbreviations for cell types are the same as in Figure 2. (B) Interaction of CLV1, CLV3, and WUS in controlling the stem cell pool in meristems (left); a proposed interaction of BAM1/2, SPL, and a CLE-type gene for a putative ligand for BAM1/2 (right), regulating cell fates in the anther. Arrows indicate positive genetic interactions; a line with a bar at the end denotes negative interactions; open arrows represent positive effect on the cellular process.

A Model for BAM1/2 Function in Anther Development

BAM1 and BAM2 regulate several aspects of development, including meristem size and leaf development (DeYoung et al., 2006). We show here that they are also
important for the formation of somatic cell types in early anther development. The
detailed analysis of the bam1 bam2 anthers suggests that the mutant defect in somatic cell
formation is not likely to be a direct consequence of the reduced meristem. Although the
occasional loss of an anther locule may be analogous to reduced cell proliferation at the
meristem, the change in cell fate does not appear to be. Therefore, we believe the
BAM1/2 function in anther development is largely different from that in regulating
meristem size. BAM1 and BAM2 are closely related in sequence to CLV1 (DeYoung et
al., 2006) (Figure 2-7), which acts in the meristem (Clark et al., 1996). CLV1 may
achieve the correct balance of the central zone and periphery zone in the meristem by
limiting cell proliferation in the central zone cells and/or promoting the transition of
central zone cells to peripheral zone cells (a form of differentiation) (Clark et al., 1996;
Clark et al., 1997a; Gallois et al., 2002; Reddy and Meyerowitz, 2005) (Figure 2-6 B).
We found that BAM1 and BAM2 negatively regulate the number of sporogenous cells at
the center of the anther lobes, seemingly by promoting the differentiation of the
peripheral somatic cells, and/or possibly by reducing the division of sporogenous cells.
Therefore, the BAM1/BAM2 function in the early anther may be to promote
differentiation (and limit proliferation) in a manner analogous to the role of CLV1 in the
meristem (Figure 2-6 B). Just as CLV1 acts to restrict proliferation of the cells in which it
is expressed and may promote the differentiation of adjacent cells, BAM1/2 expression
and the bam1 bam2 mutant phenotype suggest that BAM1 and BAM2 may restrict
sporogenous cell proliferation while promoting differentiation of the adjacent parietal
cells.
Further support for the functional similarity between BAM1/2 and CLV1 came from transgenic experiments. It was shown that high levels of BAM1 or BAM2 were able to partially rescue the *clv1-11* mutant phenotype and expression of *CLV1* using the *ER* promoter, which is active in the anther, could completely rescue the *bam1 bam2* mutant phenotype (DeYoung et al., 2006). These results suggest that these highly similar proteins have retained some conserved biochemical activities and, when expressed outside their normal expression domains, are able to interact with components of related signaling pathways (DeYoung et al., 2006). Thus, while CLV1 normally functions to limit meristem stem cell population size and/or to promote the transition of central zone cells to peripheral zone cells, when expressed in the developing anther it can function in the place of BAM1/2 to promote the differentiation of parietal cells and to limit the sporogenous cells. *CLV3* is thought to encode the ligand for CLV1 and is a member of a conserved gene family in flowering plants, called the *CLEs* (Cock and McCormick, 2001; Carles and Fletcher, 2003); however, *clv3* mutants are fertile (Clark et al., 1996), suggesting that either CLV3 is not a ligand for BAM1/2 or that the *CLV3* gene is functionally redundant with another gene. Nevertheless, the biochemical similarity of BAM1/2 and CLV1 suggests that the ligand for BAM1/2 may be a member of the CLE family (Cock and McCormick, 2001).

Our results and previous studies strongly support the hypothesis that both *SPL/NZZ* and *BAM1/BAM2* act very early in anther development to promote the formation of several cell types. The *spl/nzz* mutants do not produce any sporogenous cells, whereas the *bam1 bam2* mutant generates an abnormally large number of PMLs,
which have several properties of PMCs. Therefore, SPL/NZZ and BAM1/2 seem to act in opposing ways in regulating the number of sporogenous (-like) cells. BAM1/2 and SPL/NZZ, as well as the ligand for BAM1/2 may form a regulatory loop (Figure 2-6 B), in a way somewhat similar to the interactions between CLV1, WUS, and CLV3 (Figure 2-6 B) (Carles and Fletcher, 2003).

The BAM1 BAM2 Function and the Evolution of Sporophytic Cell Types

In the ems1/exs, serk1 serk2, tpd1 and bam1 bam2 mutants, cell differentiation is altered to the end that one or more normal cell layers do not form, but PMCs or PMLs form in their place. These results support the idea that a default pathway leads to the formation of PMCs and that the development of the other cell layers requires differentiation mediated by additional signaling pathways. It is known that non-flowering plants have relatively simple structure in the microsporangium. For example, in leptosporangiate ferns a series of precise cell divisions results in the formation of the sporangium that has an outer wall (one cell layer thick) and a two-cell-layered tapetum that surrounds and provides nutrients to the developing spore mother cells (Raven et al., 1999). When the sporophytic sac opens spores are released that lack the highly decorated wall found on pollen grains. It appears that as vascular plants evolved, particularly angiosperms, they acquired additional developmental pathways that allowed for greater complexity in the structure of the male sporangium, which in angiosperms is the anther lobe. Our results support the hypothesis that through evolution, cell divisions that would have resulted in the formation of sporogenous cells in an ancestor were restricted or
otherwise altered in order to produce other cell types, among which the earliest and most important appears to be the tapetum.

The specification of the primary parietal cell fate by the BAM1 and BAM2 LRR-RLKs represents a novel-signaling pathway that has implications for the evolution of the sporophytic cell types involved in sporogenesis. The development of PMLs seems to be a default pathway and the development of the somatic cell types is achieved through the specification of a progenitor cell type (parietal cells), as a result of the acquisition of new signaling pathways, perhaps like that defined by the BAM1 and BAM2 receptors.

Distinct homologs of each member of the BAM/CLV1 clade have been identified in poplar (Populus trichocarpa) and rice (Oryza sativa) (Figure 2-7), suggesting that the function of BAM1 and BAM2 might be conserved in other flowering plants, and different from the roles of CLV1 and BAM3. It is known that the homolog of EMS1 in rice, MSP1, has a very similar function to that of EMS1; the rice msp1 mutant also forms excess PMCs in the anther and concomitantly lacks the tapetum (Nonomura et al., 2003).

Further investigation of the rice homologs of BAM1 and BAM2 is needed to test whether they also have conserved functions.
Figure 2-7: A Neighbor Joining Tree of Selected *Arabidopsis*, Rice, and Poplar LRR-RLK Amino Acid Sequences. Gene ID numbers starting with “At” indicate genes from *Arabidopsis thaliana*; names of genes with functional information are given after the gene ID numbers. Gene ID numbers starting with “Os” indicates genes from rice (*Oryza sativa*); *MSP1* is also shown as *Os MSP1*. “Pt” indicates genes from Poplar (*Populus trichocarpa*), with temporary names given according to sequence similarity to the closest *Arabidopsis* genes. Bootstrap values are show near the relevant nodes.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Two mutant alleles were found for each of the *BAM1* (At5g65700) and *BAM2* (At3g49670) genes and were described in detail previously (DeYoung et al., 2006).
Briefly, the bam1-1 and bam1-3 alleles were generated in the Columbia (Col-0) ecotype and backcrossed into the Landsberg erecta (Ler) ecotype. The bam1-1 allele contains a dSpm insertion and is from the SLAT collection. bam1-3 is a SALK T-DNA line. The bam2-1 and bam2-3 alleles are in the Ler ecotype and each contains a Ds insertion. bam2-1 is from a launching pad line and bam2-3 is from the Cold Spring Harbor TRAPPER collection. The two pairs of double mutants used in this study were bam1-1 bam2-1 and bam1-3 bam2-3. Ler plants were used as the wild-type control. Arabidopsis thaliana seeds were planted directly, or transplanted after germinating on MS plates, on potting mixture and were grown with a 16 hr light/8 hr dark cycle at 18 to 23°C.

**Characterization of the Mutant Phenotype**

SEM analysis was performed on flowers from various stages as previously described (Dievart et al., 2003). Some organs were removed to expose the inner floral organs. Flower buds and inflorescences were prepared for sectioning using a method described previously (Owen and Makaroff, 1995) with some minor modifications (Zhao et al., 2002). Semi-thin (0.5 µm) sections were made using either a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems, Nussloch, Germany) or an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and were stained with 0.1% of Toluidine Blue in 0.1% Na₂B₄O₇ for 30 seconds. Images were photographed using an Olympus BX51 microscope (Tokyo, Japan) and a SPOT II RT Slider digital camera with SPOT software version 3.5.8 for Windows (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Images were edited using PHOTOSHOP 7.0 (Adobe system
Inc., San Jose, CA, USA). Measurement of cell length and height were done using GIMP version 2.2.4 software (http://www.gimp.org). For both the wild-type and the mutant, only the cells juxtaposed to the epidermis of stage 5 anther transverse sections were measured. Average cell number and standard deviations were calculated using Microsoft Excel (Seattle, WA, USA). DAPI staining and chromosome spreads were performed as previously described (Ross et al., 1996). DAPI staining of tissue sections was done with young inflorescences that were fixed by methanol:acetone (4:1) fixative for 45 minutes on ice. They were then embedded in wax and sectioned at 10 μm thick. Dewaxed slides were directly stained with DAPI. Images were taken using a Nikon E800 microscope (Tokyo, Japan) and a Hamamatsu C4742 digital camera (Hamamatsu City, Japan) with Image Pro Plus software version 4.5.1.27 for Windows (Media Cybernetics Inc., Silver Spring, MD, USA).

**In Situ Hybridization Experiments**

Non-radioactive RNA in situ hybridization was performed essentially as described (Xu et al., 2002b). Young inflorescences of wild type and bam1 bam2 double mutants were fixed in FAA fixative for at least 2 hours at room temperature. The tissue was then dehydrated and embedded in Fisher (Hampton, NH, USA) paraffin. 10 μm thick sections were made using a Shandon Finesse paraffin microtome (Thermo Electron Co., Pittsburg, PA, USA) and were mounted onto slides so that each slide had a similar number of wild-type and bam1 bam2 sections. All slides were de-waxed with Histoclear, treated with protein kinase for 30 minutes, and then dehydrated and baked at 42 °C.
for at least 2 hours. The dried slides were used immediately or stored at –80 °C for up to 6 months. RNA probes labeled with digoxigenin (DIG) were used for the hybridization. After hybridization, Anti-DIG-AP and NBCI were used in order to detect any hybridization signal. Images were taken using a BX51 Olympus microscope (Tokyo, Japan) with a SPOT II RT camera or using a Nikon E800 microscope (Tokyo, Japan) coupled with a Nikon D50 SLR digital camera. All images were then edited using PHOTOSHOP 7.0 (Adobe system Inc., San Jose, CA, USA).

A fragment of the *BAM1* cDNA from 821 to 2250 bp after the ATG initiation codon was amplified using the primers (5’-

ATCTAGATCTTCTCCGGTCCATTAAACTTGG-3’) and (5’-

ACTCGAGTCTGTGTCTTATCCTTCCTAAG-3’) and cloned into the T/A site of the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA) (DeYoung et al, 2006). The resulting construct (pMC 3021) was linearized with NotI or SpeI and transcribed using SP6 or T7 to generate the antisense and sense probes, respectively. The *BAM2* probes were similarly synthesized using in vitro transcription from a linearized fragment of the *BAM2* cDNA, which was amplified with gene-specific primers (5’-

ATCTAGACATTACAGGGACAATAACTCAA-3’) and (5’-

ACTCGAGCTGTGTCTTACCTACTAACCTAGC-3’) and cloned into pCRII-TOPO, resulting in pMC 3022. The plasmid was linearized with BglIII, and transcribed using SP6 for the antisense probe. The *SDS* and *ATRAD51* probes were synthesized as previously described (Azumi et al., 2002; Li et al., 2004). The *DYT1* probe was synthesized using a cDNA clone (Zhang et al., 2006).
Construction of Phylogenetic Tree

To generate the phylogenetic tree shown in Figure 8, BAM1, BAM2, BAM3, CLV1, ER, EMS1, and BRI1 protein sequences from *Arabidopsis thaliana* were used to perform a Basic Local Alignment Search Tool (BLASTp) search of the TIGR Rice pseudomolecules:protein sequences database with no filter. The 4 members of the BAM/CLV1 clade were also used to search the *Populus trichocarpa* genomic sequences at the PlantTribes website (http://www.floralgenome.org/cgi-bin/tribedb/tribe.cgi). Redundant sequences were removed. Full length sequences were imported into ClustalX (Plate-Forme de Bio-Informatique, Illkirch Cedex, France) and a multiple sequence alignment was performed (Supplemental Figure 1). Phylogenetic analysis was conducted using MEGA version 3.0 (Kumar et al., 2004) (http://www.megasoftware.net/index.html) and the neighbor-joining algorithm and a bootstrap analysis with 1000 replicates to test the significance of the nodes. Default parameters were used, including random seed initiation, the amino: Poisson correction model, uniform rates among sites, and gaps were deleted. Gene ID: BAM1, At5g65700; BAM2, At3g49670.


Scheres, B., and Benfey, P.N. (1999). Asymmetric cell division in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 505-537.


Chapter 3

Abnormally High Expression of BAM3 Contributes to the Developmental Defect Observed in bam1 bam2 Arabidopsis Anthers

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ABSTRACT

In flowering plants, normal pollen development requires the formation of several different anther cell types. Only a few genes important for the development of these cell types have been uncovered. The four member CLAVATA1 (CLV1)-clade of Leucine-rich repeat receptor-like kinases (LRR-RLKs) includes CLV1, BAM1 (for BARELY ANY MERistem), BAM2 and BAM3. Recently, BAM1 and BAM2 were shown to function redundantly in promoting the parietal cell type during early anther development (Chapter 2). In addition, the SPOROCYTELESS/NOZZLE (SPL/NZZ) gene functions in early anther development, but promotes the formation of the sporogenous cells. Subsequent to SPL/NZZ and BAM1/2 function, the LRR-RLK EXCESS MICROSPOROCYES1/EXTRA SPOROGENOUS CELLS (EMS1/EXS) specifies the formation of the tapetum. Here we demonstrate that SPL/NZZ is epistatic to BAM1/2, and BAM1/2 are in turn epistatic to EMS1/EXS. Because BAM3 was previously reported to function additively to BAM1/2 in other aspects of plant development, we sought to understand its role in anther development. Here we report that while the bam3 single mutant anther appears normal, the bam1 bam2 bam3 triple mutant anther can produce the somatic cell types. Furthermore, BAM3 expression is drastically increased in the bam1 bam2 mutant flowers. Together these results suggest that BAM1/2 mediate a signal to negatively regulate BAM3 expression, and this regulation is important for the normal specification of the parietal cell type.
INTRODUCTION

Cell differentiation is critical for the development of multicellular organisms. In flowering plants, male fertility is dependent on the coordinated differentiation of different anther cell types. The male floral organ, the stamen, consists of an anther connected to a filament; the anther is the site of pollen production and the filament serves as a structural support and provides the anther with nutrients through the vascular tissues (Goldberg et al., 1993; Sanders et al., 1999; Scott et al., 2004; Ma, 2005). Typically, each anther has four locules. Within each locule, concentric rings of somatic cell layers form around a central core of sporogenous cells. At anther stage 5, the epidermis surrounds the entire anther and within each locule the other cell layers are, from outer to inner: the endothecium, middle layer, tapetum and pollen mother cells (PMCs) (Goldberg et al., 1993; Owen and Makaroff, 1995; Sanders et al., 1999). The formation of these cell types requires coordinated cell proliferation, oriented cell divisions and cell type specification (Chapter 2) (Laux et al., 1996; Sanders et al., 1999; Lohmann et al., 2001; Reddy and Meyerowitz, 2005).

In recent years, some of the genes important for the development of the different anther cell types have been uncovered. The *SPOROCYTELESS/NOZZLE (SPL/NZZ)* gene is believed to function very early in anther development and promotes the formation of the sporogenous cells in *Arabidopsis* (Schiefthaler et al., 1999; Yang et al., 1999; Ito et al., 2004). The *spl/nzz* mutant does not form the endothecium, middle layer, tapetum or PMCs (Schiefthaler et al., 1999; Yang et al., 1999). Also, it was recently shown that
SPL/NZZ transcription is under the control of \textit{AGAMOUS (AG)} (Ito et al., 2004). In addition, the CLAVAT1-related Leucine-rich repeat receptor-like kinases (LRR-RLKs) BAM1 (for BARELY ANY MERISTEM) and BAM2 also function very early in anther development, potentially at the same time and in the same cells as SPL/NZZ (Chapter 2). \textit{BAM1} and \textit{BAM2} function redundantly in the early anther to promote the parietal cell type, a role opposite that of \textit{SPL/NZZ} (Chapter 2) (DeYoung et al., 2006).

The \textit{EXCESS MICROSPOROCYES1/EXTRA SPOROGENOUS CELLS (EMS1/EXS)} gene encodes an LRR-RLK that appears to function later than \textit{SPL/NZZ} and \textit{BAM1/2} and specifies the tapetum cell layer (Canales et al., 2002; Zhao et al., 2002). The \textit{EMS1/EXS} signaling pathway likely involves two other LRR-RLKs, \textit{SOMATIC EMBRYOGENESIS1 (SERK1)} and \textit{SERK2}, and a small protein, \textit{TAPETUM DETERMINANT1 (TPD1)} (Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005; Yang et al., 2005). To date, the relationship between \textit{SPL/NZZ}, \textit{BAM1/2} and \textit{EMS1/EXS} in anther development has not been elucidated.

\textit{BAM1} and \textit{BAM2} were first identified and investigated due to their sequence similarity to CLAVATA1 (CLV1) (DeYoung et al., 2006). CLV1 is believed to function with CLV2 and CLV3 to negatively regulate the expression of the transcription factor \textit{WUSCHEL (WUS)} in order to properly maintain the stem cell population size at the shoot and floral meristems (Clark et al., 1993, 1995; Laux et al., 1996; Clark et al., 1997; Kayes and Clark, 1998; Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000; Gallois et al., 2002; Lenhard and Laux, 2003; Reddy and Meyerowitz, 2005). Interestingly, due to their different expression patterns, the function of \textit{BAM1/2} in the
meristem is opposite that of CLV1, which can rescue the bam1 bam2 mutant phenotypes when expressed under a broader promoter (DeYoung et al., 2006). Additionally, the bam1 bam2 mutant revealed that BAM1/2 function is also important for vascular strand formation in the leaves and for male and female fertility (Chapter 2) (DeYoung et al., 2006).

In addition to BAM1/2, the CLV1-clade contains a highly related protein, BAM3 (Shiu and Bleecker, 2001; DeYoung et al., 2006). The phylogenetic analysis performed by DeYoung et al. (2006), which was based on a portion of the kinase domain, was unable to resolve the position of BAM3 relative to BAM1/2 and CLV1. However, our analysis using the full-length protein showed that BAM3 is more closely related to BAM1/2 than is CLV1, and BAM1, BAM2 and BAM3 form a sister clade to CLV1 (Figure 2-7). This suggests that the role of BAM3 in development may be more like BAM1/2 than CLV1. Indeed, while the bam3-2 single mutant appeared normal, the bam1-1 bam2-1 bam3-2 mutant was reported to have a more severe or enhanced mutant phenotype compared to the bam1 bam2 mutant (DeYoung et al., 2006). This included a reduced and sometimes terminated shoot meristem, reduced leaf venation, reduced female fertility and reduced carpel and stamen number, which the bam1-1 bam2-1 mutant did not have (DeYoung et al., 2006). These plants were also male sterile; however, the defect causing this sterility was not characterized (DeYoung et al., 2006). We therefore sought to understand the role of BAM3 in anther development.

Here we report that while the bam3 single mutant anther is similar to the wild-type, the bam1 bam2 bam3 triple mutant displays a range of defects in anther
development. The majority of the triple mutant anthers have one or more locules that do not differentiate. In addition, the bam1 bam2 bam3 triple mutant sometimes resembles the bam1 bam2 double mutant, but is often able to produce the somatic cell types, namely endothecium, middle layer and tapetum. However, the cells are enlarged and their cell layers appear less organized, indicating that cell type specification and cell patterning are abnormal. Eventually, most or all of the cells within the triple mutant locule degrade. Significantly, BAM3 expression is drastically increased in the bam1 bam2 mutant flowers. Taken together these results suggest that BAM1/2 mediate a signal to negatively regulate BAM3 expression, and this regulation is important for the normal specification of the parietal cell types. In addition, our analyses indicate that SPL/NZZ is epistatic to BAM1/2, and they may form a regulatory loop similar to that of CLV1 and WUS (Schoof et al., 2000; Gallois et al., 2002). Furthermore, our results support that BAM1/2 are epistatic to EMS1/EXS.

RESULTS

The bam3-2 Single Mutant Anther Produces the Somatic and Sporogenous Cell Layers Similar to the Wild-type

In agreement with previous results, the bam3-2 single mutant vegetative and reproductive development appeared similar to the wild-type (not shown) (DeYoung et al., 2006). In order to verify that anther development was normal, semi-thin cross sections of wild-type and bam3-2 single mutant anthers were generated and analyzed (Figure 3-1). At stage 5, wild-type anthers have four well defined locules: two abaxial and two adaxial
(Figure 3-1 A). Each locule has five cell layers, from outer to inner they are: epidermis, endothecium, middle, tapetum and pollen mother cells (PMCs) (Figure 3-1 A). Similar to the wild-type, the **bam3-2** single mutant formed four well-defined locules with the normal somatic and sporogenous cell layers and was fertile (Figure 3-1 B). These cells were similar in size and shape to those seen in the wild-type. In addition, the **bam2-1 bam3-2** double mutant and **bam1-1 (+/−) bam2-1 bam3-2** mutant were fertile and appeared to develop normally, consistent with previous results (not shown) (DeYoung et al., 2006).

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**Figure 3-1**: Semi-thin Sections of Anthers at Stage 5. (A) Wild-type (Col) (B) **bam3-2** single mutant. Anther stage defined as in (Sanders et al., 1999). ep, epidermis; en, endothecium; mi, middle layer; t, tapetum cells; pmc, pollen mother cells. Bar = 20µm; all panels are of the same magnification.

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**The bam1 bam2 bam3 Triple Mutant Anther Can Produce Somatic Cell Types**

Similar to the **bam1 bam2** double mutant, the **bam1 bam2 bam3** triple mutant is male sterile (DeYoung et al., 2006). In order to gain a better understanding of the defect causing this sterility, semi-thin transverse sections of wild-type and **bam1-1 bam2-1 bam3-2** anthers were prepared and analyzed (Figure 3-2). The **bam1-1 bam2-1 bam3-2**
mutant anthers showed a range of phenotypes. In the *bam1 bam2 bam3* triple mutant the majority of the anthers observed had at least one undifferentiated locule (Figure 3-2 A), and a few anthers appeared to not differentiate any locules (Figure 3-2 D). Interestingly, in the *bam1 bam2* mutant, occasionally an anther was observed that had an undifferentiated locule (not shown). Thus a mutation in *BAM3* appears to enhance this aspect of the *bam1 bam2* double mutant phenotype. In both mutant genotypes, these locules generally contained large, highly vacuolated parenchyma-like cells (Figure 3-2 D to G and I). In addition, some *bam1-1 bam2-1 bam3-2* anthers were observed that had large, disorganized, pollen mother-like cells throughout most or all of the locules (Figure 3-2 B, E and H), similar to the *bam1 bam2* mutant anthers (Figure 3-2 B), but with cells that appeared larger. Often a small portion of these locules would display what appeared to be organized cell layers, while the remainder of the cells resembled enlarged PMCs (Figure 3-2 B and E).

Unexpectedly, many of the anthers had locules that appeared to have somatic cell layers (Figure 3-2 C, F to I, K and L). Although these cells were generally enlarged and less organized than the wild-type (compare tapetum in Figure 3-1 A to Figure 3-2 H and I), the layers were morphologically distinct and specific cell types could be identified (i.e. endothecium, middle layer, tapetum and PMCs). At late stage 5/early stage 6, the endothecium cells were relatively small and vacuolated, while the middle layer appeared as a thin line of cells; both cell types were identified based on their position and appearance (Figure 3-2 L). Tapetum cells at this stage were larger, not highly vacuolated and sometimes binucleate (Figure 3-2 L), similar to the wild-type (not shown).
Figure 3-2: Semi-thin Sections of bam1-1 bam2-1 bam3-2 Anthers. (A) to (C) are at anther stage 4. (D) to (F) and (K) are at anther stage 5. (G) to (I) and (L) are late stage 5 to early stage 6. (J) is at late stage 6. Arrows in (B) and (F) point to portions that appear to contain somewhat organized cell layers. (L) is a larger version of the upper right lobe in (I). u, undeveloped lobe; ep, epidermis; en, endothecium; mi, middle layer; t, tapetal cells; pmc, pollen mother cells; pml, pollen mother-like cells; d, degrading cells; c, callose; e, empty locule; i, incomplete cell layer. Bar = 20µm; (A) to (F) are at the same magnification; (G) to (J) are at the same magnification.
The tapetum cells and PMCs were also identified based on their position as well as their overall appearance. In some locules all four of the somatic cell types could be identified, while in others one or more of these cell layers was partially or completely missing (Figure 3-2 F and K). As the L2-driven somatic cell types were never observed in the bam1 bam2 double mutant, this indicates that a mutation in BAM3 can attenuate the change in cell fate seen in the bam1 bam2 double mutant anthers. Finally, similar to the bam1 bam2, ems1/exs, serk1 serk2 and tpd1 mutants, at or during anther stage 6, the cells inside the anther locules begin to degrade, leaving the locule largely empty (Figure 3-2 G, I and J). Together with the abnormal cell size and patterning observed in the bam1-1 bam2-1 bam3-2 mutant anthers, this suggests that these cells do not develop normally, and cell fate specification may be irregular.

To support that the bam1 bam2 bam3 triple mutant anthers sometimes have restored somatic cell types, we examined the expression of known molecular markers compared to bam1 bam2 and the wild-type (Figure 3-3). SDS is a meiosis specific gene that has been used as a marker for meiotic cells in mutants with abnormal anther development as well as in the wild-type (Azumi et al., 2002; Zhao et al., 2002; Yang et al., 2003; Zhang et al., 2006). DYT1 is expressed preferentially in the tapetum and is currently the most reliable molecular marker for the tapetal cell type at early stages (Zhang et al., 2006). Previously, in situ hybridization experiments showed bam1 bam2 anthers have expanded SDS expression and no observable DYT1 expression, consistent with changes in cell fate (Figure 2-4). Real-time PCR results were consistent with the in situ observations. Compared to the wild-type, bam1 bam2 inflorescences had very high SDS expression and almost no DYT1 expression ($p < 0.05$) (Figure 3-3). Conversely, the
bam1 bam2 bam3 inflorescences showed slightly elevated levels of both SDS and DYT1 expression, but these were not significantly different from the wild-type (p > 0.2). This supports the presence of tapetal-like cells in the triple mutant, unlike the bam1 bam2 double mutant, and a loss of PMCs in the triple mutant relative to the double mutant.

Figure 3-3: Real-time PCR of DYT1 and SDS Expression. There was no significant difference in DYT1 or SDS expression in the bam1-1 bam2-1 bam3-2 sample compared to the wild-type. For the DYT1 experiment, Ler and bam1-1 bam2-1 n=2, bam1-1 bam2-1 bam3-2 n=3. For the SDS experiment, Ler n=3, bam1-1 bam2-1 and bam1-1 bam2-1 bam3-2 n=2. * indicates significant difference between the mutant and wild-type (Ler) sample (p< 0.05).

BAM3 Expression is Relatively Low in Wild-type Anthers

The bam3-2 single mutant anther is phenotypically similar to wild-type, indicating that BAM3 expression is not essential to normal anther development and may therefore be low in wild-type anthers. In order to gain a better understanding of BAM3 expression, data from a previously performed microarray study was examined and a preliminary in situ hybridization was performed. From the microarray study, the relative expression levels of the CLV1-clade members could be estimated and compared to other
known anther genes (W. Zhang, Y. Sun, A. Wijeratne, W. Liu, and H.M., unpublished data). In stage 4 to 6 anthers, the microarray analysis suggests that expression levels of *BAM1, BAM2, EMS1* and *AG* are not significantly different from each other. In addition, *CLV1* and *BAM3* were expressed at around the same level, but their expression was much lower than that of *BAM1, BAM2, EMS1* and *AG*. Therefore, *BAM3* expression appears relatively low in the early anther. Similarly, the preliminary in situ hybridization experiment showed that *BAM3* may be only very weakly expressed during early stages of anther development, if at all, but expression can be seen at later stages in the center of the lobes (Figure 3-4 A and B). Thus, the *BAM3* expression pattern may be similar to that of *BAM1* and *BAM2* during anther development, but at a much lower level.

**BAM3 Expression is Dramatically Increased in the bam1 bam2 Mutant**

The *bam1-1 bam2-1 bam3-2* anthers often have restored somatic cell types compared to the *bam1-1 bam2-1* anthers, suggesting that the *bam3-2* mutation attenuates some aspects of the *bam1 bam2* mutant phenotype. Furthermore, the low level of *BAM3* expression in the wild-type and normal phenotype of the *bam3-2* single mutant indicates that in the *bam1 bam2* mutant background, *BAM3* expression is abnormal. In order to test this, we performed RT-PCR and real time PCR of *BAM3* on wild-type, *bam1-1 bam2-1* and *bam1-1 bam2-1 bam3-2* young flower buds (Figure 3-4 D and E). Both experiments showed a drastically high increase of *BAM3* expression in the *bam1 bam2* mutant compared to the wild-type and little or no expression in the *bam1-1 bam2-1*
bam3-2 mutant. This suggests that abnormally high expression of BAM3 contributes to the bam1 bam2 mutant anther phenotype.

Figure 3-4: Expression of BAM3 in the Wild-type and bam1-1 bam2-1 mutant. (A) and (B) in situ hybridization of BAM3. (C) Sense probe. (D) RT-PCR of BAM3 in stage 1-10 flowers. (E) Real-time PCR experiment for BAM3 expression in stage 1-10 flowers. Expression was mainly seen in the center of stage 5 and older anther locules (arrows) (A) and (B). The sense probe showed very little background signal (C). RT-PCR of BAM3 showed increased expression in the bam1-1 bam2-1 mutant stage 1-10 flowers compared to the wild-type and no observable difference in the Actin2 control (D). Real-time PCR confirmed that BAM3 expression in the bam1-1 bam2-1 mutant compared to wild-type and decreased in the bam1-1 bam2-1 bam3-2 mutant (E). Bar in (A) to (C) = 100µm. Cycle number in (D) = 35. In (E) the numbers in parentheses indicates experimental replicate. Experiment (1) n=2. Experiment (2) Ler n=2, bam1-1 bam2-1 n=3. * indicates significant difference between the mutant and wild-type (Ler) sample (p< 0.05).
Possible Relationships of *BAM1/2* with *SPL*

*BAM1/2* appear to have a role in promoting the differentiation of the parietal cell type at the same time *SPL* may function to promote the differentiation of the sporogenous cells. In order to better understand the relationship between *BAM1/2* and *SPL/NZZ*, we generated *bam1 bam2 spl* triple mutants and analyzed anther development compared to wild-type, *bam1 bam2* and *spl* (Figure 3-5 A-C and F). The overall plant morphology of *bam1 bam2 spl* mutants was similar to the *bam1 bam2* mutant (not shown) (DeYoung et al., 2006). In the wild-type at anther stage 5, each of the four locules forms concentric rings of somatic cells surrounding the PMCs (Figure 3-5 A). As previously described, the *bam1 bam2* mutant anthers are larger than the wild-type and form pollen mother-like cells (PMLs) in the place of the somatic cell types (Figure 3-5 B and Figure 2-2). Conversely, the *spl* mutant produces anthers that are thin in the abaxial/adaxial plane and are filled with cells similar in morphology to the connective-tissue (Figure 3-5 C) (Schiefthaler et al., 1999). Analysis of the *bam1 bam2 spl* triple mutant anther showed that it was clearly similar to the *spl* mutant anther, indicating that *SPL/NZZ* is epistatic to *BAM1/2* (Figure 3-5 F).
Figure 3-5: Semi-thin Sections of Wild-type and Mutant Anthers at Stage 5. (A) Wild-type (Ler) (B) bam1-1 bam2-1 (C) spl (D) ems1 (E) bam1-1 bam2-1 ems1 (F) bam1-1 bam2-1 spl. The bam1-1 bam2-1 ems1 triple mutant anther in (E) is similar to the bam1-1 bam2-1 double mutant in (B). The bam1-1 bam2-1 spl triple mutant anther in (F) is similar to the spl single mutant anther in (C). ep, epidermis; en, endothecium; mi, middle layer; t, tapetal cells; pmc, pollen mother cells; pml, pollen mother-like cells; pl, parenchyma-like cells. Bar = 50µm; all panels are of the same magnification.

In order to gain further insights into the relationship between BAM1/2 and SPL/NZZ, we performed RT-PCR, real-time PCR and in situ hybridization experiments using mutant and wild-type tissue. Real-time PCR showed no significant difference between SPL expression in the wild-type and bam1-1 bam2-1 double mutant young flower buds and no significant difference between BAM1 or BAM2 expression in the spl mutant young flower buds (Figure 3-6 A). Interestingly, in situ hybridization experiments did reveal some differences in SPL and BAM1 expression patterns between the wild-type and bam1 bam2 and spl mutants, respectively (Figure 3-7 A to I). At anther stages 2 and 4, there was no obvious difference in SPL/NZZ expression between the wild type (Figure 3-7 A and B) and bam1 bam2 (Figure 3-7 D and E). At stage 5, however,
when SPL/NZZ expression was restricted to the PMCs in the wild type (Figure 3-7 C), SPL/NZZ expression in the bam1 bam2 anther had expanded to all or most of the L2-derived cells (Figure 3-7 F), consistent with these cells being similar to PMCs.

BAM1 expression in the spl mutant appeared normal at stage 2 (Figure 3-7 G and Figure 2-5), but in the L2-derived cells it continued to be restricted to the juxta-epidermal cells at later stages (Figure 3-7 H and I). Also, BAM1 expression in the spl mutant appeared to extend into the epidermal cells. The restricted expression of BAM1 is consistent with the failure of the spl mutant to form sporogenous cells and PMCs and suggests that the L2-derived cells may remain undifferentiated or archesporial-like. Therefore, while the BAM1/2 and SPL/NZZ expression at anther stage 2 seems independent of each other, SPL/NZZ may subsequently promote BAM1 expression in the sporogenous cells.

Possible Relationships of BAM1/2 with EMS1

Based on mutant analyses, BAM1/2 are thought to function prior to EMS1/EXS in promoting differentiation of parietal cells (Chapter 2). To test this hypothesis, we generated bam1 bam2 ems1 triple mutants and analyzed their morphology. The ems1 mutant does not form the tapetal cell layer and produces extra PMCs in its place (Figure 3-5 D) (Zhao et al., 2002). However, the bam1 bam2 ems1 triple mutant anther produces only enlarged, disorganized, PMLs, similar to the bam1 bam2 anthers (Figure 3-5 B and E). This supports that BAM1/2 function is epistatic to EMS1 function in specifying the parietal cell type.
Figure 3-6: Expression Analyses of SPL, BAM1, BAM2 and BAM3. (A) to (C) Real-time PCR experiments of stage 1-10 flower buds. (D) RT-PCR of BAM3 and Actin2 control in stage 1-10 flower buds; cycle number = 35. There was no significant difference in BAM1 or BAM2 expression in the spl mutant and no significant difference in SPL expression in the bam1-1 bam2-1 or bam1-1 bam2-1 bam3-2 mutants (A). BAM1 and BAM2 were both increased in the ems1 mutant (B) and EMS1 expression was higher in the bam1-1 bam2-1 mutant (C). In addition to bam1-1 bam2-1, the spl mutant also showed an increase in BAM3 expression.
Figure 3-7: In Situ Hybridizations of BAM1/2, SPL/NZZ and EMS1/EXS. (A) to (C) SPL expression in wild-type anthers. (D) to (F) SPL expression in bam1 bam2 mutant anthers. (G) to (I) BAM1 expression in spl mutant anthers. (J) to (L) EMS1 expression in wild-type anthers. (M) to (O) EMS1 expression in bam1 bam2 mutant anthers. (P) to (R) BAM1 expression in ems1 mutant anthers. (A) and (D) SPL expression in wild-type and bam1 bam2 mutant anthers, respectively, was seen in most of the L2-derived cells at stage 2 (arrows). (B) and (E) SPL expression in wild-type and bam1 bam2 mutant anthers, respectively, was strongest at the center of each lobe and somewhat fainter in the remaining L2-derived cells at stage 4. (C) In the wild type at stage 5 strong SPL expression was seen in the PMCs. (F) In the bam1 bam2 mutant at stage 5 strong SPL expression was seen throughout the lobe in the pollen mother-like cells. (G) BAM1 expression in the spl mutant anther at stage 2 was seen in the archesporial cells (arrows). (H) BAM1 expression in the spl mutant anther at stage 4 was mainly confined to the cells immediately adjacent to the epidermis. (I) BAM1 expression in the spl mutant anther at stage 5 was seen in the epidermis and juxta-epidermal cells at the lateral edges of the lobes (arrow). (J) EMS1 expression in wild-type at stage 3 was seen in the L2-derived cells and in parts of the epidermis (arrows); this may be broader than what was seen in the bam1 bam2 mutant anther at this stage (M). (K) EMS1 expression in the wild type at early stage 5 was predominantly in the PMCs and weakly in the tapetum. (L) EMS1 expression in the wild type at late stage 5/early stage 6 was almost exclusively in the tapetum. (M) EMS1 expression in the bam1 bam2 mutant anther at stage 3 was in the L2-derived cells (arrows). (N) and (O) EMS1 expression in the bam1 bam2 mutant anther at early stage 5 and early stage 6, respectively, was seen throughout the anther lobes in pollen mother-like cells. (P) BAM1 expression in the ems1 mutant anther at stage 2 was seen in the archesporial cells (arrows). (Q) BAM1 expression in the ems1 mutant anther at stage 4 was seen predominantly in the center of the lobe, and weakly in the other L2-derived cells. (R) Strong BAM1 expression was seen in the ems1 mutant anther at stage 5 in the PMCs. t, tapetum; pmc, pollen mother cell; pml, pollen mother-like cell. Bar = 20µm; all panels are of the same magnification.

In order to gain a greater understanding of the relationship between BAM1/2 and EMS1/EXS, we performed real-time PCR and in situ hybridization experiments (Figure 3-6 and Figure 3-7). Interestingly, EMS1/EXS expression was significantly higher in the bam1-1 bam2-1 mutant flower buds than in the wild-type (Figure 3-6 C). As EMS1/EXS is expressed in the PMCs, this may reflect the change in cell fate to PMLs in the bam1 bam2 mutant. Furthermore, BAM1 and BAM2 expression were both slightly higher in the
ems1 mutant than in the wild-type flower buds) (Figure 3-6 B). This is also likely due to the increase in PMC number in the mutant.

In situ hybridization experiments for EMS1/EXS expression showed that at early stages there was no clear difference in the expression pattern between the wild type and bam1 bam2 (Figure 3-7 J and M and data not shown). At early wild-type stage 5, EMS1/EXS expression was strong in the PMCs and moderate in the tapetum (Figure 3-7 K). At late stage 5/early stage 6, EMS1/EXS expression was almost exclusively in the tapetum and very weakly expressed, if at all, in the PMCs (Figure 3-7 L). The bam1 bam2 stage 5 anthers had strong and expanded EMS1/EXS expression in the PMLs (Figure 3-7 N), filling the lobes. Furthermore, strong EMS1/EXS expression continued in the L2-derived cells at stage 6 (Figure 3-7 O), suggesting that the PMLs were different from normal PMCs. BAM1 expression in the ems1 mutant was similar to the wild type through stage 4 (Figure 3-7 P). During stages 4 and 5 BAM1 expression was seen in the PMCs of ems1 (Figure 3-7 O and Figure 3-6 R), consistent with the normal expression of BAM1 in PMCs at these stages. Similarly, BAM1 and BAM2 expression were slightly higher in the ems1 mutant young flower buds than in the wild-type (Figure 3-6 B). Therefore, BAM1/2 may be involved in regulating the reduction of EMS1 expression in the PMCs, but EMS1 does not seem to affect the BAM1 expression pattern.

Possible Relationships of BAM3 with SPL and EMS1

Previous results from a microarray study showed that BAM3 expression in stage 4-6 spl anthers was significantly increased compared to the wild-type, but no significant
difference was observed in *ems1* anthers (A. Wijeratne, W. Zhang, Y. Sun, W. Liu, D. Zhao and H.M., unpublished data). In order to gain further insight into the relationship between *BAM3* with *SPL/NZZ* and *EMS1/EXS*, RT-PCR and real-time PCR experiments were performed (Figure 3-6 D). Real-time PCR experiment for *BAM3* showed 

\[\text{Ler} = 1.0 \pm 0.8 \ (n=2) \text{ vs. } \text{spl} = 237 \pm 87 \ (n=3) \text{ with } p < 0.05.\]

RT-PCR and real-time PCR experiments confirmed that *BAM3* expression was increased in the *spl* mutant, though not as strongly as in the *bam1-1 bam2-1* mutant (Figure 3-6 D and compare Real-time expression above to Figure 3-4 E). This suggests that *SPL* function down-regulates *BAM3* expression. In addition, real-time PCR showed no significant difference between *SPL* expression in the wild-type and *bam1-1 bam2-1 bam3-2* mutant young flower buds (Figure 3-6 A), indicating that *SPL* expression is normal in the triple mutant, and *BAM3* function does not regulate *SPL* expression. Furthermore, RT-PCR did not detect a significant difference in *BAM3* expression between the wild-type and *ems1* mutant (Figure 3-6 D), in agreement with the microarray results. Thus, *EMS1* function does not appear to affect *BAM3* expression.

**DISCUSSION**

**Increased Expression of BAM3 Contributes to the Change in Cell Fate in bam1 bam2 Anthers**

We have characterized the anther developmental defects in the *bam1-1 bam2-1 bam3-2* triple mutant and compared it to the *bam1-1 bam2-1* double mutant, the *bam3-2* single mutant and the wild-type. Morphological and molecular studies showed that while
the bam1 bam2 mutant fails to specify the identity of L2-derived somatic cell layers, the bam1-1 bam2-1 bam3-2 does sometimes produce endothecium, middle layer, and tapetum, although they appear abnormal (Figure 3-2 and Figure 3-3). Furthermore, expression studies showed that BAM3 expression was dramatically higher in young bam1-1 bam2-1 flowers compared to the wild-type (Figure 3-4). In addition, the bam3-2 single mutant did not have any obvious defect in specifying the somatic and sporogenous cell types (Figure 3-1) (DeYoung et al., 2006). Taken together these results suggest that increased expression of BAM3 in the bam1 bam2 double mutant anthers is partially responsible for the change in cell fate observed in the double mutant and that the BAM1/2 signaling pathway normally inhibits BAM3 expression (Figure 3-8). The observation that some bam1-1 bam2-1 bam3-2 locules resemble the bam1 bam2 mutant indicates that there are additional unknown factors downstream of BAM1/2 that affect parietal cell differentiation (Figure 3-8). Furthermore, the ability of some bam1-1 bam2-1 bam3-2 locules to produce the somatic cell types suggests that some other pathway(s) is able to promote the parietal cell type independent of BAM1/2.

Similar to the bam1-1 bam2-1 mutant, the bam1-1 bam2-1 bam3-2 PMCs degrade during or prior to anther stage 6. In addition, when the tapetal and middle layer cells are present, they also degrade. Furthermore, the L2-derived cell types in the triple mutant are abnormally enlarged and their patterning is irregular. This indicates that development of the PMCs, tapetum and middle layer cells in the triple mutant is abnormal and that the defect seen in bam1 bam2 meiocytes may not be due to increased BAM3 expression. This supports the idea that BAM1/2 may play a role in the progression of sporogenous
cells (Figure 3-8). Alternatively, abnormal tapetum development may contribute to the failure of the sporogenous cells to progress beyond stage 6.

**BAM3 Function is Additive to BAM1/2 Function in Some Aspects of Anther Development**

The *bam3-2* mutation was reported to enhance the other Bam\(^\ast\) phenotypes, including a reduced and sometimes terminated shoot meristem, and reduced leaf venation (DeYoung et al., 2006). In addition, the *bam1-1 bam2-1 bam3-2* mutant had reduced carpel and stamen number, which the *bam1-1 bam2-1* mutant did not have (DeYoung et al., 2006). Interestingly, some of the phenotypes observed in the *bam1-1 bam2-1 bam3-2* anthers also appear to be enhanced, including loss of differentiated locules and increased cell size. These results suggest that BAM3 function is additive to BAM1/2 function in young floral and anther primordia, similar to its role at the meristem. The reduced number of stamen and undifferentiated anther locules in the *bam1-1 bam2-1 bam3-2* mutant may be due to a lack of stamen primordia progenitor cells, suggesting that *BAM1*, *BAM2* and *BAM3* promote proliferation as well as regulate differentiation in very early anther development (Figure 3-8).
Figure 3-8: Schematics of Anther Development in the *bam1 bam2* and *bam1 bam2 bam3* Mutants and in the Wild-type. (A) In the *bam1 bam2* double mutant, the parietal cell types are lost and anthers produce only Pollen Mother Cells. Also, *BAM3* expression is increased and meiosis fails to complete. (B) In the *bam1 bam2 bam3* triple mutant, the parietal cell types are sometimes present, but meiosis fails to complete. (C) A model for the function of *BAM1, BAM2* and *BAM3* in wild-type anther development. Open arrows and bracket indicate positive genetic interactions; a line with a bar at the end denotes negative interactions; branched lines signify a periclinal cell division events; dashed line indicates exact cell lineage is not known.
In addition, increased cell size is often related to defects in cell cycle progression (Urbani et al., 1995; Wang et al., 2000; De Veylder et al., 2001). Thus the enlarged anther cells observed in the bam1-1 bam2-1 and bam1-1 bam2-1 bam3-2 mutants may be caused by disruption of the cell cycle. As BAM1, BAM2 and BAM3 encode LRR-RLKs, it is likely that they mediate a developmental signal that promotes cell division and differentiation. Furthermore, BAM3 function appears to be additive to BAM1/2 function, but is not essential in the presence of either BAM1 or BAM2 (DeYoung et al., 2006).

While BAM3 appears to function synergistically with BAM1/2 in some developmental processes, it is not able to compensate for the loss of BAM1/2 in early anther cell differentiation; in fact increased expression of BAM3 appears to significantly contribute to the observed defect in differentiation. Thus, while increased BAM3 expression can partially compensate for loss of BAM1/2 in leaf and meristem development, increased BAM3 expression in the anther results in a dominant negative effect, analogous to the effect that some CLV1 mutants and a truncated ER receptor have in the CLV1 and ER signaling pathways, respectively (Dievart et al., 2003; Shpak et al., 2003). The ability of some bam1-1 bam2-1 bam3-2 locules to produce the somatic cell types suggests the presence of another pathway that is capable of promoting parietal cell differentiation independent of BAM1/2. Furthermore, this pathway appears distinct from the pathways involving BAM1, BAM2 and BAM3 in other aspects of plant development. In the anther, BAM3 may disrupt the function of the BAM1/2-independant pathway by sequestering its ligand or co-receptor without activating it, thereby blocking that signaling pathway.
Establishment of cell polarity has been shown to be important for asymmetric cell division and differentiation in both plants and animals [for review see (Scheres and Benfey, 1999)]. In addition, positional information transmitted via cell-cell communication has been shown to influence differentiation more than cell lineage (Berger et al., 1998). Therefore, drastically increased $BAM3$ expression in early anther development may disrupt cell polarity or the signaling pathways important for transferring positional information, as described above. Thus, archesporial cells at anther stage 2 are not able to asymmetrically divide into primary parietal and primary sporogenous cells. Moreover, subsequent positional information essential for cell type specification could likewise be disrupted.

**SPL/NZZ is Epistatic to $BAM1/2$, which is Epistatic to $EMS1/EXS$**

$SPL/NZZ$ and $BAM1/2$ appear to function very early in anther development in an opposing manner; $SPL/NZZ$ promotes the formation of the sporogenous cell type, while $BAM1/2$ promote the parietal cell type (Figure 3-9 and Figure 2-6) (Schiefthaler et al., 1999; Ito et al., 2004). Our triple mutant analysis of $bam1-1$ $bam2-1$ $spl$ indicates that $SPL/NZZ$ is epistatic to $BAM1/2$ during early anther differentiation. In addition, $BAM1$ expression in the $spl$ mutant remains fairly restricted to the juxta-epidermal cells at later stages, consistent with a failure in archesporial cell differentiation. Similarly, $SPL$ expression was spatially expanded in the $bam1$ $bam2$ mutant.
Likewise, changes in SPL/NZZ expression may be the results of altered cell fate specification in the bam1 bam2 mutant. Alternatively, from stages 2 through 5, SPL/NZZ may indirectly promote expanded BAM1 expression as a part of its role in promoting differentiation of the sporogenous and somatic cell types. This suggests that while the expression patterns of these genes at stage 2 are not affected by each other, subsequently, BAM1/2 may limit the domain of SPL/NZZ expression and SPL/NZZ promotes BAM1 expression in the central sporogenous cells. It is likely that in the wild-type BAM1/2 negatively regulate SPL/NZZ expression indirectly, possibly via the specification of somatic L2-derived cell types, which have reduced SPL/NZZ expression. Given the increased expression of BAM3 in the spl young flowers and stage 4 to 6 anthers, SPL/NZZ appears to negatively regulate BAM3 expression (Figure 3-10). Understanding the relationship between SPL/NZZ and BAM3 may yield further insight into the role of SPL/NZZ in promoting the sporogenous cell type.
Our results from the analysis of *bam1-1 bam2-1 ems1* triple mutants also indicate that *BAM1/2* are epistatic to *EMS1/EXS*. The expanded *EMS1/EXS* expression in the stage 5 *bam1 bam2* anther most likely reflects a change in cell type because *EMS1/EXS* is expressed in the early PMCs. However, normal *EMS1/EXS* expression is reduced in meiocytes and strong in the tapetum; therefore, the strong *EMS1/EXS* expression in the *bam1 bam2* PMLS at the time of meiosis suggests that *BAM1/2* may negatively regulate *EMS1/EXS* expression at that time (Figure 3-9). Furthermore, expanded *EMS1/EXS* expression might be an indication of a defect in meiocyte development in *bam1 bam2*, supporting a role of the *BAM1/2* genes in meiocytes.

**A Model for *SPL/NZZ*, *BAM1/2*, and *BAM3* Function in Anther Development**

Previous studies have strongly suggested that *BAM1/2* and *SPL/NZZ* function in an opposing manner early in anther development to promote the parietal and sporogenous cell types, respectively (Figure 3-9 and Figure 2-6) (Schiefthaler et al., 1999; Yang et al., 1999; Ito et al., 2004). In addition, our analyses indicate that *SPL/NZZ* is epistatic to *BAM1/2* and that while their expression appears independent at early stages, subsequently they may form a regulatory feedback loop similar to that of *CLV1*, *CLV3* and *WUS* (Figure 3-10 and Figure 2-6) (Brand et al., 2000; Fletcher and Meyerowitz, 2000; Schoof et al., 2000; Gallois et al., 2002). *CLV1* and *CLV3* promote the differentiation of the peripheral zone cells and negatively regulate the transformation of peripheral zone cells into central zone cells (Clark et al., 1996; Clark et al., 1997; Reddy and Meyerowitz, 2005). The mechanism for this regulation is not known, although it may be through...
inhibiting the expression of \textit{WUS}, which promotes the central zone (Laux et al., 1996; Gallois et al., 2002). Interestingly, the negative regulation of \textit{BAM3} expression by \textit{BAM1/2} appears essential for the formation of the somatic cells and may prevent the transformation of somatic cells into sporogenous cells, analogous to \textit{CLV1/CLV3} function (Reddy and Meyerowitz, 2005).

Figure 3-10: A Model for the Control of Early Anther Cell Differentiation by \textit{BAM1/2}, \textit{BAM3} and \textit{SPL}. Interaction of \textit{CLV1}, \textit{CLV3}, and \textit{WUS} in controlling the stem cell pool in meristems (left); a proposed interaction of \textit{BAM1/2}, \textit{BAM3}, \textit{SPL}, and a \textit{CLE}-type gene as a putative ligand for \textit{BAM1/2} (right), regulating cell fates in the anther. Arrows indicate positive genetic interactions; a line with a bar at the end denotes negative interactions; open arrows represent positive effect on the cellular process; dashed lines indicate an inferred effect.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

The mutant alleles of the \textit{BAM1}, \textit{BAM2} and \textit{BAM3} genes that were used were described previously (DeYoung et al., 2006). Briefly, \textit{bam3-2} was a SALK T-DNA line
generated in the Col-0 ecotype (Alonso and Stepanova, 2003). Plants homozygous for
bam1-1 bam2-1 bam3-2 were generated by crossing bam3-2 pollen into bam1-1 bam2-1 plants, which had been backcrossed into Ler (DeYoung et al., 2006). The double and
triple mutants were backcrossed into the Ler to ensure they were mutant for ER also (B.
DeYoung personal communication). Ler plants were used as the wild-type control.

Arabidopsis thaliana seeds were planted directly, or transplanted after germinating on
MS plates, on potting mixture and were grown with a 16 hr light/8 hr dark cycle at
approximately 18 to 23°C.

Characterization of the Mutant Phenotype

Flower buds and inflorescences were prepared for sectioning using a method
described previously (Owen and Makaroff, 1995) with some minor modifications (Zhao
et al., 2002). Semi-thin (0.5 µm) sections were made using an Ultracut UCT
ultramicrotome (Leica Microsystems, Wetzlar, Germany) and were stained with 0.1% of
Toluidine Blue in 0.1% Na₂B₄O₇ for 10-30 seconds. Images were photographed using an
Olympus BX51 microscope (Tokyo, Japan) and a SPOT II RT Slider digital camera with
SPOT software version 3.5.8 for Windows (Diagnostic Instruments, Inc., Sterling
Heights, MI, USA). Images were edited using PHOTOSHOP 7.0 (Adobe system Inc.,
San Jose, CA, USA).
**RT and Real-time PCR Experiments**

The primers for RT and real-time PCR are as follows: *SPL* (oMC 2362 & 2363), *DYTI* (oMC 1834 & 1872), *EMS1* (oMC 2364 & 2365), *SDS* (oMC 2281 & 2282), *BAM1* (oMC 2386 & 2387), *BAM2* (oMC 1977 & 2388), *BAM3* (oMC 2389 & 2390) and *Actin2* (oMC 1533 & 1534) (Table 3-1) (Zhang et al., 2006). Stage 1 to 9/10 flower buds were collected and quickly frozen in liquid nitrogen (Smyth et al., 1990). Total RNA was extracted using the RNeasy Plant Kit (Qiagen, Valencia, CA) and reverse transcription was preformed according to the manufacturer’s instruction to synthesize cDNA (Invitrogen, Carlsbad, CA). The generated cDNA was used directly as PCR templates. The PCR and data treatment were carried out as described previously for the comparative C$_T$ method for relative quantification, with minor modifications, where if $x=$(sample-control value) ‘$x$’ is subjected to the calculation $2^{-x}$, then these values were averaged for each replicated sample instead of first taking the average of ‘$x$’ for each replicate and then calculating $2^{-\text{Average of replicates } x}$ (AB, 2003; Ni et al., 2004). Averages and standard deviations were calculated using Microsoft Excel (Seattle, WA, USA). Student T-test was performed using [http://home.clara.net/sisa/t-test.htm](http://home.clara.net/sisa/t-test.htm).

**In Situ Hybridization Experiments**

For *SPL/NZZ, EMS1/EXS, BAM1* and *BAM2*, non-radioactive RNA in situ hybridization was performed as previously described (Xu et al., 2002). The *SPL/NZZ, EMS1*, and *BAM1* probes were synthesized as previously described (Chapter 2) (Zhao et al., 2002; Sieber et al., 2004). Images were taken using a BX51 Olympus microscope.
(Tokyo, Japan) with a SPOT II RT camera or using a Nikon E800 microscope (Tokyo, Japan) coupled with a Nikon D50 SLR digital camera. RNA in situ hybridization was essentially carried out as described previously (Larkin et al., 1993). For BAM3, in situ hybridization was performed essentially as previously described (Larkin et al., 1993). All images were then edited using PHOTOSHOP 7.0 (Adobe system Inc., San Jose, CA, USA).

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Chapter 4

The ERECTA-Family Receptor-like Kinases are Important for Arabidopsis Anther Development at Multiple Stages

Acknowledgments

The er-105, erl1-2, erl2-1, er-105 erl1-2, er-105 erl2-1, erl1-2 erl2-1, er-105 erl1-2 (+/-) erl2-1 mutant lines and pERECTA::GUS, pERL1::GUS and pERL2::GUS expression lines were obtained from Dr. Keiko Torii’s lab at the University of Washington. GUS expression images were taken by Yi Hu. I conducted all other experiments.
ABSTRACT

Floral organ formation and therefore fertility rely on coordinated cell proliferation and differentiation. The ERECTA-family genes, consisting of ERECTA (ER), ERECTA-like1 (ERL1) and ERL2, are Leucine-rich repeat receptor-like kinases that function together to direct stomatal cell fate specification and promote cell proliferation during organ growth. It was also shown that the er-105 erl1-2 erl2-1 triple mutant is defective in anther development. We have characterized anther development in the ER-family single, double and triple mutants and found that while the single and double mutants are similar to wild-type, the triple mutant displays varying degrees of aberrant phenotypic severity. The er-105 erl1-2 erl2-1 triple mutant has a reduced number of stamens, the majority of which possess completely undifferentiated or underdifferentiated anthers. A few of the anthers produce one or two lobes that differentiate somatic and sporogenous cell layers, but the cells are larger and more disorganized than the wild-type. Furthermore, the tapetum and middle layer cells appear delayed in their development, and the er-105 erl1-2 erl2-1 mutant anthers do not dehisce. Taken together, these results suggest that the ER-family genes act redundantly to promote cell proliferation and proper differentiation throughout anther development.
INTRODUCTION

The coordination of cell proliferation and differentiation are essential for normal plant growth. Correct floral organ number, for example, requires the appropriate maintenance of stem cell population size at the meristem. This is known to involve a feedback loop between CLAVATA1 (CLV1), CLV3 and WUSCHEL (WUS). Floral organ identity is subsequently controlled by homeotic genes of the well known ABC model (Coen and Meyerowitz, 1991).

Stamens, the male floral organ, are composed of a filament, which elongates prior to and during dehiscence (pollen release) and an anther, which has four locules where pollen is produced. Anther development in Arabidopsis has been divided into stages based on anther morphology (Sanders et al., 1999). An epidermis encases the entire anther, and within the locules adjacent rings of cells are formed surrounding the sporogenous cells. Stage 5 is marked by the establishment of the four cell layers present within each locule. From outer to inner, they are: endothecium, middle layer, tapetum, and pollen mother cells (PMCs). During stage 6 the PMCs enter and undergo meiosis, forming tetrads at stage 7. Microspores are then released from tetrads during stage 8 and form an exine layer at stage 9. Tapetum degeneration commences during stage 10 and ends by stage 12. During stage 11, the two pollen mitotic divisions occur and the septum, which separates the abaxial and adaxial anther locules, begins to degenerate. Stage 12 is marked by the absence of the tapetum and the breakdown of the septum, causing the
anthers to become bilocular. Finally, dehiscence occurs during anther stage 13 when the stonium cells break apart allowing the release of the mature pollen grains.

Stamen identity is conferred by the combined action of \textit{APETELA3 (AP3)}, \textit{PISTILATA (PI)} and \textit{AGAMOUS (AG)}, which regulate a subset of genes that promote anther development (Bowman et al., 1989, 1991; Ito et al., 2004). To date, relatively few of the genes involved in this process have been identified. The \textit{SPOROCYTELESS/NOZZLE (SPL/NZZ)} gene encodes a putative transcription factor that acts very early in anther development and promotes microsporogenesis in whorl three floral organs under the control of \textit{AG} (Schiefthaler et al., 1999; Yang et al., 1999; Ito et al., 2004). In addition, two \textit{CLV1}-related genes, \textit{BAM1} and \textit{BAM2}, act redundantly in anther development to promote the parietal cell type (DeYoung et al., 2006; Hord et al., 2006). Subsequently, the \textit{EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS (EMS1/EXS)}, \textit{SOMATIC EMBRYOGENESIS1 (SERK1)}, \textit{SERK2}, and \textit{TAPETUM DETERMINANT1 (TPD1)} genes promote the differentiation of the tapetal cell type (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005; Ma, 2005; Yang et al., 2005).

Normal tapetum development and degeneration are essential for proper pollen development (Mariani et al., 1990; Mariani et al., 1991; Denis et al., 1993; Owen and Makaroff, 1995; Wu et al., 1997; Bih et al., 1999; Kapoor et al., 2002; Zheng et al., 2003). Some of the genes known to be important for tapetum development and function in \textit{Arabidopsis} include \textit{MYB33} and \textit{MYB65}, \textit{DYSFUNCTIONAL TAPETUM1 (DYTI)}, \textit{ABORTED MIRCROSPORES (AMS)}, \textit{Arabidopsis thaliana} glycerol-3-phosphate
acyltransferase (AtGPAT1), and MALE STERILE1 (MS1). The myb33 myb65 double mutant and single mutants of the other genes are aberrant in tapetum development at different stages, but all result in partial or complete male sterility, underscoring the importance of the tapetum in pollen development.

Several of the genes found to be involved in anther development encode leucine-rich repeat receptor-like kinases (LRR-RLKs), including BAM1, BAM2, EMS1/EXS, SERK1, and SERK2. The LRR-RLK gene family is one of the largest gene families in Arabidopsis, although very few of its members have known functions (Shiu and Bleecker, 2001, 2003). Interestingly, some of the LRR-RLKs have been shown to play roles in multiple processes. SERK1, for example, was originally isolated for its ability to promote somatic embryogenesis (Schmidt et al., 1997; Hecht et al., 2001). In addition to a role in anther development, BAM1 and BAM2 work with BAM3 to maintain meristem size and to promote proper development of leaf vasculature (DeYoung et al., 2006). In addition, the ERECTA gene has long been known to be important for plant height, as the Landsberg erecta ecotype has a much more compact inflorescence than other ecotypes, and was recently shown to function with ERECTA-like1 (ERL1) and ERL2 to promote cell proliferation and control differentiation (Rédei, 1992; Torii et al., 1996; Shpak et al., 2004; Shpak et al., 2005).

The ER-family genes have overlapping functions, but with distinct roles in development based on their expression patterns (Shpak et al., 2004; Shpak et al., 2005). Together with TOO MANY MOUTHS (TMM), ER, ERL1 and ERL2 regulate stomatal cell fate specification (Shpak et al., 2005). In addition, the er-105 erl1-2 erl2-1 triple
mutant was shown to be disrupted in ovule and anther development (Shpak et al., 2004). Here we show that the \textit{ER}-family genes are important for anther cell differentiation throughout anther development. The majority of \textit{er-105 erl1-2 erl2-1} triple mutant stamens have anthers that do not differentiate the normal cell types. In addition, while mutant anthers do differentiate the somatic and sporogenous cell types, the cells are larger and more disorganized than the wild-type. Furthermore, the tapetum and middle layer cells may be delayed in their development, and the \textit{er-105 erl1-2 erl2-1} mutant anthers do not dehisce. This suggests that the \textit{ER}-family genes are important for coordinating cell proliferation and differentiation during anther development.

\section*{RESULTS}

\textbf{The \textit{ER}, \textit{ERL1} and \textit{ERL2} Genes Have Overlapping Expression Patterns in Anther Development}

\textit{ER}, \textit{ERL1} and \textit{ERL2} were previously shown to have unique or overlapping roles depending on the overlap of their expression domains. In order to gain an understanding of \textit{ER}-family gene expression patterns during anther development, promoter \textit{GUS} transcriptional fusion lines were examined (Figure 4-1) (Shpak et al., 2004). Expression of \textit{ERECTA::GUS}, \textit{ERL1::GUS} and \textit{ERL2::GUS} in anthers from stage 10 to 14 flowers largely overlapped (Smyth et al., 1990). In all three lines, \textit{GUS} expression could be seen quite strong throughout the anther and filament in anthers from stage 10 flowers (anther stages 8 and 9) (Sanders et al., 1999). \textit{ERECTA::GUS} expression becomes progressively weaker, similar to previous observations (Yokoyama et al., 1998), but remains
Figure 4-1: GUS Expression of ER-family Genes. (A) and (B) Flowers and anthers expressing pER::GUS, respectively. (C) and (D) Flowers and anthers expressing pERL1::GUS, respectively. (E) and (F) Flowers and anthers expressing pERL2::GUS, respectively. Bar in (A) and (B) = 1.0 mm. (A), (C) and (E) are at the same magnification. (B), (D) and (F) are at the same magnification. Flowers in (A), (C) and (E) are, from left to right, approximately flower stage 10, 12, 12-13, 13 and 14 (Smyth et al., 1990). Anthers in (B), (D) and (F) were taken from corresponding buds in (A), (C) and (E), respectively.

throughout the anther until flower stage 13 when it becomes restricted to the very center of the anther (Figure 4-1 B). Similarly, ERL1::GUS and ERL2::GUS were expressed throughout the anther with strong expression seen in the center of the anther at flower
stage 13 and restricted expression at flower stage 14 (Figure 4-1 B and C), comparable to
ERECTA::GUS. The largely overlapping expression pattern of the ER-family genes
indicates they may function redundantly in anther development.

The er-105 erl1-2 erl2-1 Triple Mutant Displays a Range of Anther Defects and a
Reduced Number of Stamen

As previously described, the er-105 erl1-2 erl2-1 triple mutant forms stamen with
anthers that do not appear to differentiate normally (Shpak et al., 2004). In order to better
understand the defect in anther development, Alexander’s stain was used to test for viable
pollen in the er and erl mutant backgrounds (Figure 4-2). Wild-type anthers had round,
roughly uniform pollen grains that stained bright magenta, indicating their viability
(Figure 4-2A). Anthers from control plants known to produce dead pollen had non-
uniform, shriveled pollen grains that stained aqua green, indicating that they were not
viable (not shown). The er-105, erl1-2, erl2-1 single mutants, er-105 erl1-2, er-105 erl2-
1, erl1-2 erl2-1 double mutants and er-105 erl1-2 (+/-) erl2-1 mutant had anthers that
were comparable in size to the wild-type and produced round, uniform pollen grains that
stained bright magenta, similar to the wild-type (Figure 4-2 B to H).

Many of the er-105 erl1-2 erl2-1 flowers did not produce stamens (not shown)
(Shpak et al., 2004). In er-105 erl1-2 erl2-1 flowers that did produce stamens, the anther
developmental defect varied in severity. The most severe defect observed was filament-
like stamens that appeared to completely lack an anther (Figure 4-2 I). Stamens were
also observed that had apparently undifferentiated anthers that varied in size (Figure 4-2
J).
Figure 4-2: Alexander’s Staining for Viable Pollen. (A) Wild-type (Col) (B) er-105 (C) erl1-2 (D) erl2-1 (E) er-105 erl1-2 (F) er-105 erl2-1 (G) erl1-2 erl2-1 (H) er-105 erl1-2 (+/-) erl2-1 (I) er-105 erl1-2 erl2-1 filament-like stamen with no anther (J) er-105 erl1-2 erl2-1 stamens with underdeveloped anthers (K) er-105 erl1-2 erl2-1 anther showing one locule with viable pollen (L) er-105 erl1-2 erl2-1 anther possessing two locules with viable pollen. Images were taken at the same magnification.

Finally, some stamens were seen that produced magenta-stained pollen in one or two of their anther locules (Figure 4-2 K and L). These anthers were larger than those that did not differentiate, but were still drastically reduced in size compared to the wild-type. Furthermore, the average number of anthers in the er-105 erl1-2 erl2-1 triple mutant was reduced and varied more compared to the wild-type (3.06 ± 1.18 vs. 5.77 ±
0.56, respectively) (Figure 4-3 A). Almost three-fourths of the stamens observed (72.63%) had undifferentiated anthers, 10.53% were filament-like and less than one fifth (16.84%) produced pollen (Figure 4-3 B). No aqua green (non-viable) pollen was ever observed in er-105 erl1-2 erl2-1 triple mutant anthers. Interestingly, the pollen grains in the triple mutant appeared slightly smaller than the wild-type and the locule of pollen grains was sometimes surrounded by a sheath not seen in the wild-type (not shown). In addition, although they produced viable pollen, these anthers did not dehisce and pollen grains seemed sticky when anthers were manually broken, indicating additional later defects.
The average number of stamens ± standard deviation in wild-type (Col) (n=78), erl1-2 erl2-1 (n=26), er-105 erl1-2 (+/-) erl2-1 (n=54) and er-105 erl1-2 erl2-1 mutants (n=95). (B) The percent anther type seen in the er-105 erl1-2 erl2-1 mutant.

**The er-105 erl1-2 erl2-1 Triple Mutant is Defective in Anther Formation and Differentiation**

To gain further insight into the development of the anther cell layers, semi-thin transverse sections of wild-type and the ER-family single, double and triple mutants were analyzed (Figures 4-4 and 4-5). At anther stage five, each wild-type locule has five cell layers (Figure 4-4 A). From outer to inner they are: epidermis, endothecium, middle layer, tapetum and pollen mother cells (PMCs). Similar to the wild-type, the er-105,
erl1-2, erl2-1 single mutants, er-105 erl1-2, er-105 erl2-1, erl1-2 erl2-1 double mutants and er-105 erl1-2 (+/-) erl2-1 mutant produced the five anther cell layers (Figure 4-4 A to H). In general, these cell layers were similar in appearance to the wild-type cell layers. Occasionally, the er-105 mutant was observed to undergo an additional periclinal cell division in the middle layer (Figure 4-4 B), but this did not appear to affect pollen development.

Figure 4-4: Semi-thin Cross Sections of Anther Lobes. (A) Wild-type (Col) (B) er-105 (C) erl1-2 (D) erl2-1 (E) er-105 erl1-2 (F) er-105 erl2-1 (G) erl1-2 erl2-1 (H) er-105 erl1-2 (+/-) erl2-1. Bar = 20µm; all panels are at the same magnification.

Analogous to the er-105 erl1-2 erl2-1 stamens observed using the Alexander’s stain, cross-sections of the triple mutant flowers showed stamens that were defective in anther development to varying degrees (Figure 4-5 C to F). Filament-like stamens appeared fairly round with a central vascular bundle (Figure 4-5 E and F). Stamens that appeared to produce undifferentiated anthers varied in size and shape, but were overall oval or kidney-shaped (Figure 4-5C through F). These anthers generally had a vascular
bundle near the center surrounded by large, disorganized cells that were similar in appearance to connective tissue. Anthers that did differentiate the somatic and sporophytic cell types were seen at different stages of development within the same flower (Figure 4-5C) and even between locules of the same anther (not shown). Only the abaxial lobes were ever observed to differentiate, and therefore no more than two of the four locules were ever observed to differentiate.
Figure 4-5: Semi-thin Cross Sections of Flowers and Anthers. (A) A wild-type (Col) bud at anther stage 5 (B) A wild-type (Col) bud at anther stage 6 (C) An er-105 erl1-2 erl2-1 bud with an anther at stage 5 (D) An er-105 erl1-2 erl2-1 bud with anthers at stage 6 (E) A wild-type anther at stage 6 (F) An er-105 erl1-2 erl2-1 anther at stage 6 with the two abaxial locules that have differentiated. The tapetum cells are highly vacuolated, enlarged and disorganized compared to the wild-type in (E), and some of the tapetal cells may have been degraded. The other cell layers are larger than the wild-type in (E). (G) An er-105 erl1-2 erl2-1 anther at stage 6 with one differentiated abaxial locule. The tapetum cell layer is similar to (F), and the middle layer appears somewhat discontinuous. Arrow in (E) to (G) point to tapetal cells. Panels (A) and (B), (C) and (D) and (E) through (G) are at the same magnification. Bar in (A) and (C) = 50µm Bar in (E) = 20µm.

In general, when a locule of an er-105 erl1-2 erl2-1 anther differentiated, the five cell layers could be seen. Cell types were identified based on positional and overall appearance. The cells of the epidermis and endothecium appeared slightly larger in the triple mutant than in the wild-type, but were otherwise fairly normal. Where in the wild-type the middle layer cells appear adjacent to each other (Figure 4-5 A and B), in the triple mutant they appear to overlap with one another (Figure 4-5 C and D). In addition, sometimes two periclinally adjacent middle layer cells were seen, indicating an additional cell division similar to the er-105 mutant; whereas other times the middle layer cells did not appear to completely surround the tapetum (Figure 4-5 D). Furthermore, these cells were also larger at stage 6 than in the wild-type, giving the impression that they persisted longer than normal (Figure 4-5 F and G). The most striking cellular defect was seen in the tapetum cells. In the wild-type, the tapetum forms a single layer of rectangular cells surrounding the PMCs at stage 5 (Figure 4-5 A), which become separated from each other and from the meiocytes by a callose layer during stage 6 (Figure 4-5 B and E). In the er-105 erl1-2 erl2-1 mutant, the tapetal cells did not form a single layer, but appeared as a somewhat disorganized mass of cells that were greater in number compared to the
wild-type (Figure 4-5 D, F, and G). In addition, the tapetum did not always completely surround the meiocytes, possibly due to degradation of some of the tapetal cells. Furthermore, the tapetal cells were greatly enlarged and abnormally shaped with large vacuoles, and they did not appear to separate from each other during stage 6. When the PMCs had developed into microspores (stage 8), the tapetal cells remained predominately attached to each other and to the somatic cells surrounding them and their degeneration appeared delayed (not shown).

In summary, the ER-family single, double and er-105 erl1-2 (+/-) erl2-1 mutants produced viable pollen and the five normal cell layers similar to the wild-type. The er-105 erl1-2 erl2-1 triple mutant produced fewer stamens and the anther developmental defect varied from antherless stamens to anthers with no more than the two abaxial lobes producing the somatic and sporogenous cell layers. In addition, the cells of the triple mutant anthers appeared larger and more disorganized than the wild-type. Tapetum development appeared particularly affected, and tapetum and middle layer cells may have been delayed in their development. Finally, although they sometimes produce viable pollen, the er-105 erl1-2 erl2-1 mutant anthers do not dehisce.

**DISCUSSION**

*ER, ERL1 and ERL2 Act in Anther Formation and Development*

Previous studies have shown that the ERECTA-family genes promote cell proliferation, synergistically regulate guard cell differentiation and are important for
normal anther development (Torii et al., 1996; Shpak et al., 2003; Shpak et al., 2004; Shpak et al., 2005). Here we show that together, the ER-family genes are important for early and late anther differentiation. Expression of ER, ERL1 and ERL2 appears to overlap during anther development (Figure 1). Furthermore, anther development in the ER-family single, double and er-105 erl1-2 (+/-) erl2-1 mutants is similar to wild-type (Figures 2 and 4). Together these results indicate that these genes act redundantly in this process and that a single functional copy of ERL1 is sufficient for anther development to proceed normally.

The reduced number of stamens in the er-105 erl1-2 erl2-1 triple mutant may be due to reduced cell proliferation in the floral primordia, resulting in fewer stamen primordia being initiated (Figure 3A). Alternatively, the correct number of stamen primordia may initiate, but some fail to progress. In addition, the lack of anther development or tissue differentiation in many of the er-105 erl1-2 erl2-1 stamens may be due to a lack of stamen primordia progenitor cells and/or because the ER, ERL1 and ERL2 genes promote proliferation and regulate differentiation in anther primordia (Shpak et al., 2004).

While the er-105 erl1-2 erl2-1 anther developmental defects vary, they do sometimes produce lobes with the five anther cell layers, indicating that the ER, ERL1 and ERL2 genes are not absolutely essential for the formation of the epidermis, endothecium, middle layer, tapetum or PMCs. There may be weaker parallel pathways that promote this differentiation, or that are normally activated by ER/ERL1/ERL2 and are weakly activated by an independent source, allowing for differentiation to occur in one or two lobes. In addition, the variability in the anther, and even flower, phenotype
suggests the need for the ER-family genes in order to maintain the normal homeostasis of floral organ regulatory genes, such as AG, AP3 and PI, and the factors downstream of them. A single copy of one of the ER-family genes appears sufficient to maintain homeostasis of the pathways needed for normal flower development. The observed range in flower morphology also hints that other factors, such as environmental conditions, overall plant health and inflorescence branch age, may also affect regulatory pathways in the absence of the ER-family genes.

The ER-Family Genes are Essential for Normal Tapetum Development and Anther Dehiscence

Although the five cell types can be established in the er-105 erl1-2 erl2-1 mutant, tapetum development is consistently abnormal (Figure 5C, D, F and G). The tapetum cells are enlarged, highly vacuolated and their cell patterning is disrupted. In addition, tapetum development appears delayed. This indicates that ER, ERL1 and ERL2 redundantly control the normal development and differentiation of the tapetum cells.

In recent years, some of the genes involved in tapetum specification and maturation have been uncovered. The ems1/exs, serk1 serk2 and tpd1 mutants fail to produce the tapetum layer and form excess PMCs instead, indicating that these genes likely function in the same pathway to specify tapetum formation (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005). As the er-105 erl1-2 erl2-1 triple mutant can form tapetum cells, the ER-family genes may act in a separate pathway than the EMS1/EXS, SERK1, SERK2 and TPD1 genes. However, the delay in tapetum degeneration in the er-105 erl1-2 erl2-1 mutant is
reminiscent of \textit{TPD1} weak overexpression lines (Yang et al., 2005). An analysis of \textit{TPD1} expression in the \textit{er-105 erl1-2 erl2-1} mutant could yield evidence for a relationship between \textit{TPD1} expression and the \textit{er-105 erl1-2 erl2-1} mutant phenotype. In the \textit{myb33 myb 65} and \textit{dyt1} mutants, the tapetum becomes vacuolated and hypertrophies around anther stage 5 (Millar and Gubler, 2005; Zhang et al., 2006). Subsequently the tapetal cells enlarge inward leaving little space inside the locule and apparently crushing the PMCs, neither of which was observed in the \textit{er-105 erl1-2 erl2-1} mutant. This indicates that the ER-family genes are not likely to function upstream of either the MYB33/MYB65 or DYT1 pathways. Furthermore, none of the known loss of function \textit{Arabidopsis} tapetum mutants resemble the \textit{er-105 erl1-2 erl2-1} mutant in abnormal tapetum patterning, indicating a novel pathway (Wilson et al., 2001; Canales et al., 2002; Ito and Shinozaki, 2002; Sorensen et al., 2002; Zhao et al., 2002; Steiner-Lange et al., 2003; Yang et al., 2003; Zheng et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005; Millar and Gubler, 2005).

The failure of the \textit{er-105 erl1-2 erl2-1} mutant anthers to dehisce is likely due to defects in anther development caused by the lack of the \textit{ER}-family gene function rather than a direct result of their function in this process, as no \textit{ER}-family gene expression was observed in septum or stonium cells. Anther dehiscence requires proper stonium and septum cell differentiation and degeneration, and it was recently shown that \textit{WUS} is essential to this process (Deyhle et al., 2006). Interestingly, Shpak et al. showed that \textit{WUS} expression is decreased in \textit{er-105 erl1-2 erl2-1} mutant inflorescences (Shpak et al., 2004). Therefore, it is possible that the failure of \textit{er-105 erl1-2 erl2-1} mutant anthers to dehisce is due to decreased \textit{WUS} expression during anther development. The \textit{ER}-family
genes may positively regulate *WUS* expression in anther development. An alternative explanation is that the triple mutant anthers may not fully develop to maturity.

The ER-Family Functions in Anther Development are Analogous to Their Roles in Other Aspects of Plant Growth

The *ER*-family genes promote cell proliferation and control differentiation in various aspects of plant development (Torii et al., 1996; Shpak et al., 2003; Shpak et al., 2004; Shpak et al., 2005). The *er-105* mutation confers a reduced number of enlarged cortical cells in the pedicel (Shpak et al., 2003; Shpak et al., 2004). Strikingly, the cortex of the *er-105 erl1-2 erl2-1* mutant pedicel had somewhat larger, irregularly shaped cells that appeared disorganized and had gaps between them (Shpak et al., 2004). In addition, the triple mutant was severely reduced in longitudinal cell number, but did not appear to be affected in the number of cell files (Shpak et al., 2004). As a seeming paradox, the number of guard cells seen in the *er-105 erl1-2 erl2-1* mutant epidermis was dramatically increased (Shpak et al., 2005). This was proposed to be due to the synergistic function of the *ER*-family genes in negatively regulating asymmetric cell division and guard mother cell differentiation (Shpak et al., 2005). Thus the *ER*-family genes promote cell proliferation and regulate differentiation, linking the two to cell patterning and organ growth (Shpak et al., 2003; Shpak et al., 2004; Shpak et al., 2005).

Analogous roles can be seen in the *ER*-family’s control of anther development. At very early stages of anther formation and development, *ER, ERL1* and *ERL2* appear to redundantly promote the propagation of cells necessary for normal anther formation. The
enlarged and disorganized cells observed in some of the *er-105 erl1-2 erl2-1* triple mutant anthers is similar to the phenotype observed in the pedicel cortex. While disruption of cell cycle can lead to increased cell size, this does not account for the aberrant cell patterning or increased number of the tapetum cells (Urbani et al., 1995; Wang et al., 2000; De Veylder et al., 2001). It appears that the *ER*-family genes communicate signals necessary for normal tapetum cell division and differentiation, perhaps analogous to their role in stomatal cell specification. In anther locule formation, asymmetric cell divisions occur periclinally, while symmetric cell divisions occur longitudinally and anticlinally. Hence anther cell differentiation is distinct from epidermal cell patterning in that symmetric cell divisions do not typically occur in the same plane as the asymmetric cell division events (Sanders et al., 1999; Scheres and Benfey, 1999; Geisler et al., 2000; Scott et al., 2004). Therefore, although all anther cell types are sometimes present in the *er-105 erl1-2 erl2-1* mutant, the lack of ER-family function leads to aberrant cell patterning and increased numbers in the tapetum and sometimes the middle layer cells. Thus the *ER*-family genes may function in repressing periclinal cell division events and promoting longitudinal cell divisions in anther development, as well as contributing to normal cell patterning.
MATERIALS AND METHODS

Plant Material and Growth Conditions

The mutant alleles used for *ERECTA* (At2g26330), *ERECTA-LIKE1* (*ERL1*) (At5g62230) and *ERL2* (At5g07180) were described in detail previously (Torii et al., 1996; Shpak et al., 2004). Briefly, the *er-105* allele was generated in the Columbia (Col) ecotype in the *glabrous1* (*gl1*) mutant background by fast-neutron-irradiated seeds and contains a T-DNA insert from an unknown source in the *ERECTA* promoter region (Lehle Seeds, Round Rock, TX, USA) (Torii et al., 1996). The *erl1-2* and *erl2-1* mutants carry T-DNA insertions in the LRR domain and were generated by the Arabidopsis Biological Resource Center (Figure 6) (Shpak et al., 2004). Col plants were used as the wild-type control and *gl1* mutant as a background control for the *er-105* mutant. *Arabidopsis thaliana* seeds were planted directly, or transplanted after germinating on MS plates, on potting mixture and were grown with a 16 hr light/8 hr dark cycle at 18-23°C.

Expression of *ERECTA, ERL1* and *ERL2* in *Arabidopsis* Anthers

The GUS expressing lines used were described previously (Shpak et al., 2004). GUS staining of floral tissue was performed essentially as described by Meyerowitz and Sieburth (http://iprotocol.mit.edu/protocol/119.htm) with the placing the tissue directly into 100% ethanol after staining instead of going through a graded ethanol series. Anthers and other floral organs were dissected and images were taken using a Nikon
dissecting microscope (Nikon Corp., Tokyo, Japan) and an Optronics Digital camera (Optronics Inc., Goleta, CA, USA).

**Characterization of the Mutant Phenotype**

Pollen viability was determined by fixing flowers in Carnoy’s fixative (100% ethanol:Chloroform:Acetic Acid= 6:3:1) for at least 1 hour and staining with Alexander’s stain for at least 7 hours at 55-73°C (Alexander, 1969). Flowers were then briefly washed with 10% glycerol and anthers were dissected from them. Images were taken using either a Nikon Eclipse E400 or E800 microscope (Nikon Corp., Tokyo, Japan) with an Optronics Digital camera (Optronics Inc., Goleta, CA, USA). Average anther number, standard deviations and percent anther type were calculated using Microsoft Excel (Seattle, WA, USA). Flower buds and inflorescences were prepared for sectioning by embedding in Spurr’s resin as previously described (Owen and Makaroff, 1995; Zhao et al., 2002). Semi-thin (0.5 µm) sections were made using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and were stained with 0.1% of Toluidine Blue in 0.1% Na₂B₄O₇ for 5-30 seconds. Images were photographed using an Olympus BX51 microscope (Tokyo, Japan) and a SPOT II RT Slider digital camera with SPOT software version 3.5.8 for Windows (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Images were edited using PHOTOSHOP 7.0 (Adobe system Inc., San Jose, CA, USA).
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Chapter 5

Conclusions and Future Work
CONCLUSIONS AND SPECULATIONS

The primary objective of this thesis was to gain a greater understanding of the role the CLV1 clade members, BAM1, BAM2 and BAM3 and the ER-family genes play in Arabidopsis anther development. In addition, this work sought to uncover relationships between the BAM genes and SPL/NZZ and EMS1/EXS, which were previously known to be important for anther development in Arabidopsis. To this end, two main experimental approaches were used: i) phenotypic analysis of single, double and triple mutant anthers ii) gene expression analysis. Additionally, phylogenetic analysis was used to explore the relationship of the CLV1 clade with potential homologs from other species.

The role of the BAM and ER-family genes in anther development had not been elucidated when this research was started and several key findings have come into view. First, BAM1 and BAM2 act redundantly to direct differentiation of the parietal cell type and appear to function during the transition from anther stage 2 to stage 3 (Chapter 2). In addition, the differentiation of parietal cells requires, in part, inhibition of BAM3 expression, which is mediated by the BAM1/BAM2 pathway (Chapter 3). However, BAM3 function is additive to BAM1/BAM2 in promoting cell proliferation during anther development, but it is not essential in the presence of BAM1/BAM2 function (Chapter 3).

Second, SPL/NZZ is epistatic to BAM1 and BAM2 in directing early anther cell differentiation. SPL/NZZ function is necessary for normal BAM1/BAM2 expression, and it appears to act indirectly as a positive regulator of BAM1/BAM2 (Chapter 3). In addition, BAM1 and BAM2 are epistatic to EMS1/EXS in promoting the parietal cell fate and may act as a negative regulator of EMS1/EXS expression in pre-meiotic cells during
late stage 5 (Chapter 3). Third, *BAM1* and *BAM2* are directly or indirectly important for normal meiotic progression (Chapters 2 and 3). Finally, the *ER*-family genes act redundantly in promoting cell proliferation and proper differentiation throughout anther development. This includes a role in normal tapetum development. In addition, the *ER*-family genes are not essential for the production of viable pollen, but are required for anther dehiscence (Chapter 4).

Several implications about development in general can be drawn from our observations. First, the variable phenotypes observed support that regulatory networks include multiple interplaying factors and that normal development requires maintaining their homeostasis. These factors may be influenced by regional and/or general environmental conditions. For example, altered central zone size leads to a variable number of floral organs (Laux et al., 1996; DeYoung et al., 2006). Second, there is a connection between regulation of cell division and appropriate differentiation. Cell division, differentiation and patterning are interconnected processes. For example, in cancer cells, changes in cell fate lead to changes in cell division and cell patterning. In plants, cell fate specification relies on positional cues via cell-to-cell communication. Therefore, cell patterning as well as altered cell cycle can influence differentiation.

Most interestingly, our observations support some specific and universal ideas about evolution and raise further questions. First, establishment of floral structures appears to use several factors involved in meristem maintenance, which likely existed before the evolution of angiosperms (Laux et al., 1996). Second, we see several examples of gene redundancy, where genes that have arisen via duplication share functionality (i.e. *BAM1/BAM2* and the *ER*-family genes). However, there is also
evidence for divergence in function. This specification appears to be a result of changes in transcriptional regulation and/or changes in the protein coding sequence. For example, in several processes, the ER-family genes are able function redundantly, but under normal conditions they do not because their expression does not overlap (Shpak et al., 2004). Furthermore, apparently loosely related genes (CLV1 and ER) that have similar roles (promote cell proliferation) can partially replace each other when expressed outside their normal domain (Dievart et al., 2003; DeYoung et al., 2006). Thus, evolution of regulatory sequences contributes highly to functional divergence.

In addition, there is also evidence for functional diversification beyond transcriptional regulation and overall sequence homology. For example, BAM3 shares higher homology with BAM1/BAM2 than does CLV1 across the LRR domain, kinase domain and the entire protein (data not shown). Nevertheless, CLV1 is capable of completely replacing BAM1/BAM2 function, while BAM3 may be able to substitute for only a subset of the BAM1/BAM2 roles and apparently disrupts others (Chapter 3) (DeYoung et al., 2006). Furthermore, while many of the roles of the ER-family genes overlap, they synergistically interact in other processes (Chapter 4) (Shpak et al., 2004; Shpak et al., 2005). This creates a paradox; while there is evidence for the idea that highly similar proteins share common functions, there is also evidence for functional divergence between highly related genes. One possible explanation is that changes in specific amino acid residues contribute to changes in function, such as changing a proteins ability to interact with ligands, substrates and/or other proteins.
FUTURE RESEARCH

From the results obtained in this research, several new questions have emerged. First, how much of the bam1 bam2 anther defect is due to loss of BAM1/BAM2 function vs. increased BAM3 expression? One way to test this would be to drive BAM3 expression using the AP3 or PI promoter in the wild-type background, thereby increasing BAM3 expression throughout anther development (Bowman et al., 1991). In addition, the question “How does increased expression of BAM3 contribute to the bam1 bam2 anther phenotype?” has yet to be addressed. More generally, the roles of BAM1/BAM2 and BAM3 in ovule development need to be explored.

Second, what is the relationship between SPL/NZZ and BAM3? BAM3 expression is increased in the spl mutant anther (Chapter 3; W. Zhang, Y. Sun, A. Wijeratne, W. Liu, and H.M., unpublished data), but it is unclear how this contributes to the mutant phenotype, if at all. Furthermore, while SPL/NZZ and BAM1/BAM2 appear to play opposing roles in anther development, BAM3 expression is elevated in both of their mutant backgrounds and this appears to contribute significantly to the bam1 bam2 anther defect. This creates a paradox that further experimentation may resolve. This could include analysis of an spl bam3 double mutant and of BAM3 expression in the bam1 bam2 spl triple mutant.

Third, the bam1 bam2 and bam1 bam2 bam3 mutants are in the Landsberg erecta background; does the presence of the er mutation affect their development? Our analysis of the ER-family genes showed that the er mutant occasionally displayed increased cell number in one of the anther cell layers. In addition, the er mutation has been shown
previously to enhance other mutant phenotypes (Douglas et al., 2002; Xu et al., 2003; Qi et al., 2004). Therefore, in order to better understand the roles of BAM1, BAM2 and BAM3, the bam1 bam2 and bam1 bam2 bam3 mutants should be examined in the Landsberg ERECTA background.

Fourth, does CLV1 play a yet undiscovered role in anther development? Although the clv1 mutants are fertile, CLV1 is expressed in the anther, albeit at a low level (W. Zhang, Y. Sun, A. Wijeratne, W. Liu, and H.M., unpublished data), indicating that it may function in the anther (Clark et al., 1993; Dievart et al., 2003). In addition, CLV1 and BAM1/BAM2 appear to function antagonistically in meristem development, but are able to compensate for the loss of each other when expressed outside their normal domain (DeYoung et al., 2006). Therefore, examination of CLV1 expression in the bam1 bam2 and bam1 bam2 bam3 mutant anthers in addition to analyzing bam1 bam2 clv1 and bam1 bam2 bam3 clv1 mutants may yield new insights into the role of CLV1 in anther development.

Fifth, what are the down-stream targets of the BAM and ER signaling pathways? Furthermore, what are the ligands and interacting partners of the BAM and ER-family proteins in anther development? A mitogen-activated protein kinase kinase kinase (MAPKK kinase) mutant called yoda, bears striking similarity to the er-105 erl1-2 erl2-1 triple mutant in both overall plant growth and stomatal patterning (Bergmann et al., 2004; Wang et al., 2007). Therefore, one possible mechanism for ER, or perhaps even BAM, signaling is through a MAP kinase signaling cascade.

Finally, what is the relationship between the BAM and ER signaling pathways? The bam1-1 bam2-1 bam3-2 and er-105 erl1-2 erl2-1 triple mutants share some
remarkable similarities in their anther developmental defects. Both had anthers with undeveloped or underdeveloped lobes, disrupted cell patterning and greatly enlarged cells, especially the tapetum. In addition, expression of \textit{CLV1} in pedicels was capable of rescuing the \textit{er} mutant phenotype, supporting that their functions are interchangeable to a degree (Dievart et al., 2003). Together, this suggests that the \textit{BAM} and \textit{ER} function may overlap somewhat in anther development. However, one important difference between the \textit{bam1-1 bam2-1 bam3-2} and \textit{er-105 erl1-2 erl2-1} triple mutant anthers is that at around anther stage 6 (meiosis), cells inside the \textit{bam1-1 bam2-1 bam3-2} locules degrade, while the \textit{er-105 erl1-2 erl2-1} anthers go on to make viable pollen. This indicates a key difference in the \textit{BAM} and \textit{ER}-family function, elucidation of which will require further experimentation.
REFERENCES


VITA

Carey LeeAnna Hendrix Hord

Education

May, 2007  **Ph.D. in Plant Biology**, The Pennsylvania State University
Dissertation: Roles of the CLAVTA1 and ERECTA Clade Members in *Arabidopsis* Anther Development
Thesis Advisor: Hong Ma, Ph.D.

May, 2000  **B.S. in Plant Biology**, The Arizona State University

Experience

Teaching Assistant in the Department of Biology, The Pennsylvania State University

Publications


Professional Presentations

August 2006  The BAM1/BAM2 LRR-Receptor-Like Kinases are Essential for Normal Cell Fate Specification in the Early *Arabidopsis* Anther. Presentation at the American Society of Plant Biologists annual meeting, Boston, MA.

Memberships

Feb., 2006  American Society of Plant Biologists

Nov., 2005  American Association for the Advancement of Science