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FUNCTIONAL ANALYSIS OF *PLUMBAGO ZEYLANICA* SPERM-SPECIFIC ISOPENTENYLTRANSFERASE (IPT) GENE IN HETEROLOGOUS SYSTEMS

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FUNCTIONAL ANALYSIS OF *PLUMBAGO ZEYLANICA* SPERM-SPECIFIC ISOPENTENYLTRANSFERASE (IPT) GENE IN HETEROLOGOUS SYSTEMS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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ABSTRACT

Double fertilization is a defining characteristic of flowering plants (angiosperms), which requires a pair of sperm cells to be transported to the embryo sac and then fuse with egg cell and central cell to form a zygote and endosperm, respectively. Understanding this process is vital to the world ecology and agriculture. Since its discovery over a century ago, the process has been cytologically well characterized. In recent years, tremendous efforts have been made to dissect the molecular mechanisms involved in the process. Pollen consists of one vegetative cell and one generative cell or two sperm cells and is considered as a key player for successful double fertilization. This review focuses on male germline initiation and male gametophyte development. The review begins with an introduction of double fertilization process in flowering plant, followed by a brief overview of morphology and cytology of male gametophyte development. This chapter will focus on the recent advances in molecular characterization of male gametophyte and male germline development. The review concludes with the current understanding of male gametophyte and germline development and future perspectives on male reproductive research.

Key words: double fertilization, male gametophyte development, pollen, sperm cell, germline, gene expression, transcriptomic profile, *Arabidopsis*, *Plumbago*, lily, maize, cell cycle, cell fate.

Abbreviations: EST – expressed sequence tag; FACS - fluorescence-activated cell sorting; FGU - female germ unit; MGU - male germ unit; PMI – pollen mitosis I; PMII – pollen mitosis II.

I. Introduction to double fertilization

In flowering plants, the life cycle alternates between two distinct forms, diploid and haploid, or sporophyte and gametophyte. The change from haploid to diploid requires double fertilization to form a seed to begin the next generation. Double fertilization is one of defining characteristics of flowering plant (angiosperm), in which two fertilization events take place—one sperm cell from the mature male gametophyte fusing with the egg in the embryo sac to give rise to the embryo, and another sperm cell being incorporated into the central cells to give rise to the nutritive endosperm (Russell, 1992b; Southworth, 1996; Lord and Russell, 2002; Raghavan, 2003; Weterings and Russell, 2004; Berger, 2008). Since double fertilization was discovered more than a century ago by Guignard (Guignard, 1899) and Nawaschin (Nawaschin, 1898), this unique reproductive biological process has attracted enormous interests from molecular biologists to crop breeders. Double fertilization contributes to the success of flowering plants which dominate plant life on the earth and thus is a vital biological process to the world ecology. Seeds or grains, which are the products of double fertilization, make up most of food consumed by humans. The manipulation of double fertilization process may help create more productive crops.

Three components of the plant are especially important in double fertilization – pollen (containing the male germ unit, MGU), embryo sac (containing the female germ unit, FMU), and the sporophytic tissue (mainly tapetum in anther and female

reproductive tract) (Huang and Russell, 1992; Mogensen, 1992). Successful double fertilization requires that all three parts develop normally, and the cell cycles of the male and female gametes are synchronized (Christensen et al., 1998; Friedman, 1999; Weterings and Russell, 2004; Borges et al., 2008).

Male gametogenesis starts with cell division of diploid sporophytic cells which results in a sporogenous initial – pollen mother cell (PMC). The PMC undergoes meiosis to form a haploid tetrad. The tetrad separates and releases free microspores. These uninucleate microspores undergo a subsequent asymmetric mitotic division to produce a binucleate pollen grain, with a small generative cell housed within a large vegetative cell. The generative cells then divide to form two sperm cells before pollen maturity in plants with trinucleate pollens (e.g. *Arabidopsis* and maize), or divide after pollen germination to form two sperm cells in the pollen tube for plants with binucleate pollens (e.g. tobacco and petunia) (Cresti et al., 1992; McCormick, 1993; Southworth, 1996; McCormick, 2004; Weterings and Russell, 2004; Ma, 2005).

Female gametogenesis also starts from a diploid tissue called the nucellus. One cell of the nucellus develops into a megaspore mother cell, which undergoes meiosis to give rise to four haploid megaspores. The three micropylar megaspores undergo programmed cell death. The chalazal end megaspore becomes functional and undergoes three rounds of mitosis to form an eight-nucleate embryo sac within an ovule. The two polar nuclei fuse to form a central cell during late megagametogenesis. The typical unfertilized mature embryo sac consists of seven cells: three antipodal cells, one large central cell, one egg cell and two synergids (Cresti et al., 1992; Robinson-Beers et al.,

1992; Christensen et al., 1997; Christensen et al., 1998; Weterings and Russell, 2004; Sundaresan and Alandete-Saez, 2010; Yang et al., 2010).

When pollination starts, pollen grains germinate after they land on the stigma, grow through the style and are guided towards FGU to meet their female partners. The pollen tube penetrates the micropyle and releases its two sperm cells into one of two synerdids. Then, the two sperm cells are delivered to the egg and central cell finishing double fertilization. The flowering plant life cycle, gametogenesis, and fertilization process based on *Arabidopsis* are illustrated in Figure I-1 according to Cresti 1992, Russell 1992 and Weterings 2004 (Cresti et al., 1992; Russell, 1992a; Weterings and Russell, 2004).

Several major steps are critical to the double fertilization process: (1) male and female gamete development, (2) tapetum development and anther dehiscence, (3) pollen tube growth and traveling within the female tract, (4) female guidance of pollen tube growth and pollen tube arrest, (5) release of sperm cells into the synergids, (6) gamete recognition and fusion, and (7) reprogramming parental genes for zygote and endosperm development (Cresti et al., 1992; Russell, 1992a; Christensen et al., 1998; Faure, 2002; Raghavan, 2003; Ma, 2005; Berger, 2008). The morphologic and cytological aspects of these steps have been well studied over the past century. In the last two decades, with the completion of *Arabidopsis* and other plant genome sequencing projects, availability of T-DNA insertion knockdown mutants and development of new technology (Microarray, high-throughput sequencing, FACS cell sorting, etc.), our understanding of gene expression regulation in plant reproduction has expanded greatly.

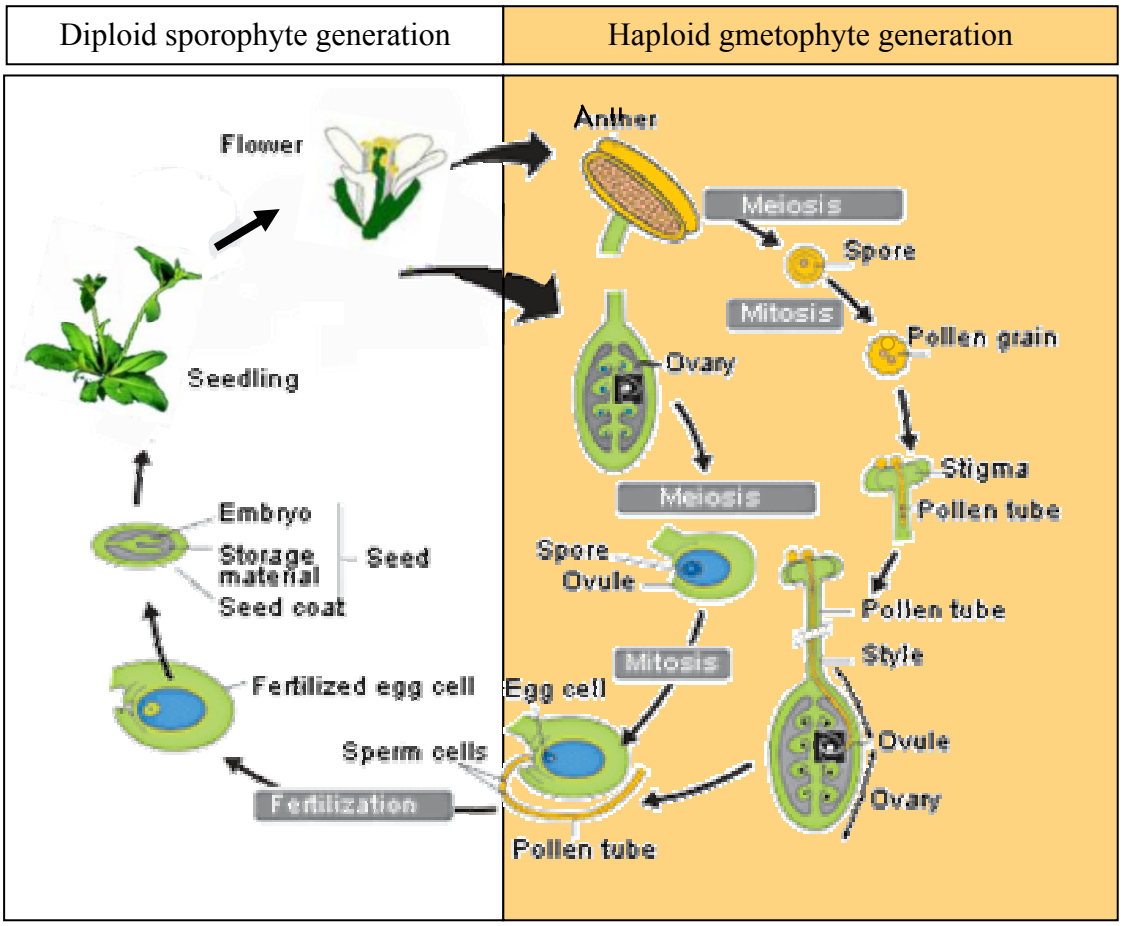

Figure I-1. Angiosperm life circle. Modified from Lidwien van der Horst and John Slippens (www.vcbio.science.ru.nl/images/pollen).

II. The morphologic and cytological aspects of male gametophyte development: an overview

The formation of the male gametophyte occurs in anthers of very young flower buds. The meristematic tissue in young flower bud primordium grows and differentiates to form vegetative tissues and organs, eventually developing into reproductive organs containing diploid sporogenous cells. The sporophytic cell divides to produce a tapetal initial cell and a sporogenous initial cell (also called pollen mother cell, PMC). Each PMC

undergoes meiosis to form a tetrad of haploid cells, which are held together by a wall of callose. The tetrad separates and releases as free microspores by the action of callase, an enzyme produced by the tapetum layer of the anther. The uninucleate microspore enlarges. Consequently, a single large vacuole is formed in the center of the cell and the microspore nucleus migrates to a peripheral position against the cell wall (Owen and Makaroff, 1995; Yamamoto et al., 2003). The microspore then undergoes an asymmetric cell division known as Pollen Mitosis I (PMI) to produce a larger vegetative cell and a smaller generative cell. The asymmetric division at PMI is essential for the correct cellular patterning of the male gametophyte, since the resulting two daughter cells each harbors a distinct cytoplasm and possesses unique gene expression profiles that confer their distinct structures and cell fates (Twell et al., 1998). The vegetative cell receives most of the microspore cytoplasm. The entire generative cell is surrounded by the unique "cell-within-a cell" structure (Russell et al., 1996). After PMI, the large vegetative cell has dispersed nuclear chromatin and exits the cell cycle in G1. The vegetative cell nurtures the developing germ cell and grows into the pollen tube following successful pollination. This pollen tube grows through the stylar tissues of the gynoecium to deliver twin sperm cells to the embryo sac. On the other hand, the smaller generative cell undergoes morphogenesis and migration, entering the interior of the vegetative cell and forming a highly polarized spindle-shaped cell. This cell has condensed nuclear chromatin and continues through a further round of mitosis, called Pollen Mitosis II (PMII), to produce two small sperm cells (Mogensen, 1992; Russell and Strout, 2005). The male gametic lineage is immersed in and thus dependent on the pollen vegetative cell for nutrition and transportation. This second cell division can take place during pollen

maturation in the anther for plants with trinucleate pollen (e.g., *Arabidopsis, plumbago zeylanica* and maize), or can divide after pollination to form two sperm cells in the pollen tube for plants with binucleate pollen (e.g., lily, tobacco and petunia). The diagram illustrated in Figure I-2 describes these steps. Additional descriptions can be found in several reviews (Russell, 1986; Mogensen, 1992; McCormick, 1993; Southworth, 1996; McCormick, 2004; Singh and Bhalla, 2007; Singh et al., 2008; Borg et al., 2009).

Extensive light and electron microscopic studies on *Plumbago zeylnica* have revealed that the sperm cells are elongate, spindle-shaped, intact cells with a plasma membrane enclosing the usual organelles such as a nucleus, mitochondia, endoplasmic reticulum (ER), ribosomes, golgi bodies, vesicles, microtubules, and in some sperm cells, plastids. In the mature pollen of *Plumbago zeylanica*, the two sperm cells are enclosed together within the inner vegetative cell plasma membrane, and linked by a common cell wall traversed by plasmodesmata. One sperm cell is physically associated with the vegetative nucleus (Svn, or Sperm associated with vegetative nucleus) and possesses more mitochondria than the other sperm cell (Sua, or Sperm unassociated with vegetative nucleus) which, in contrast, is unassociated with the vegetative nucleus and is rich in plastids (Figure I-3) (Russell, 1980; Russell and Cass, 1981; Russell, 1984; Russell, 1986; Russell et al., 1996). The cytological evidence also supports that sperm cytoplasmic organelles can be transmitted to the egg cell and central cell along with sperm nuclei (Russell, 1980; Russell, 1983; Russell, 1986).

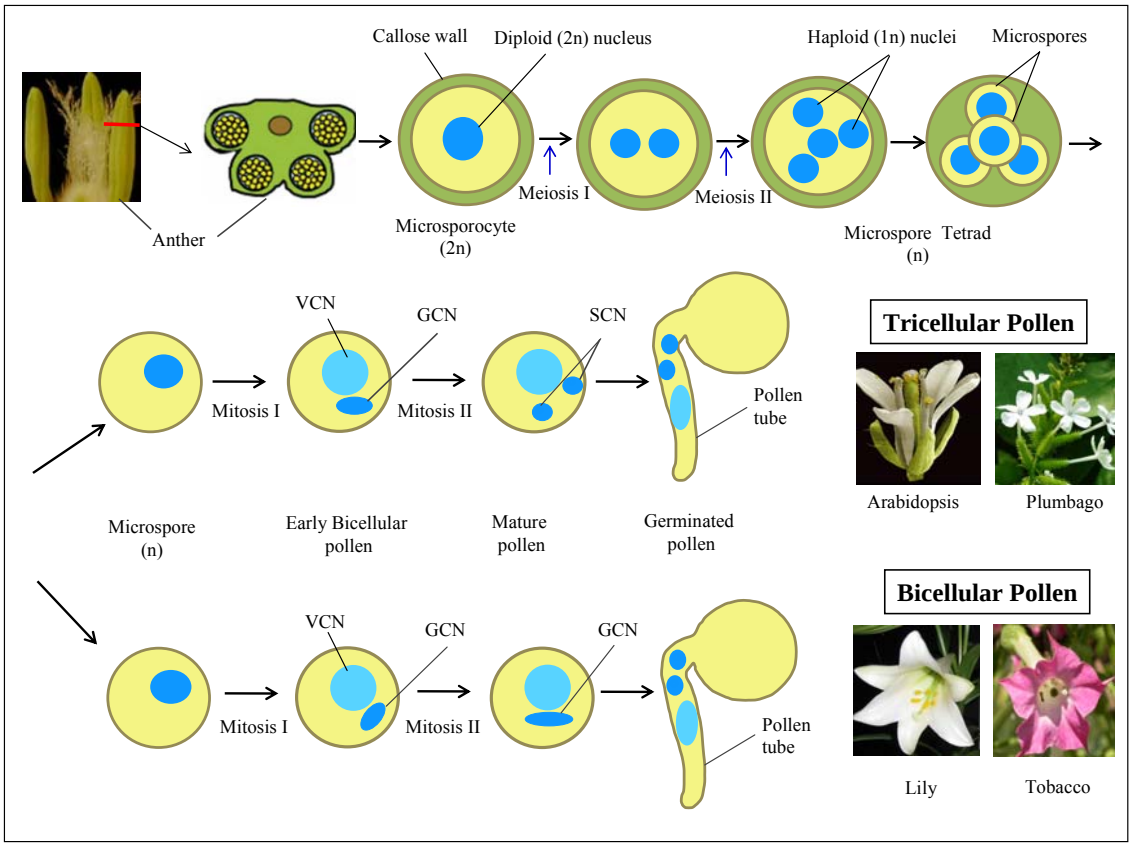

Figure I-2. Schematic description of male gametophyte development. GCN, generative cell nucleus; SCN, sperm cell nucleus; VCN, vegetative cell nucleus. Modified from Sing, 2007; Twell, 2002; Southworth, 1996 (Southworth, 1996; Twell, 2002; Singh and Bhalla, 2007).

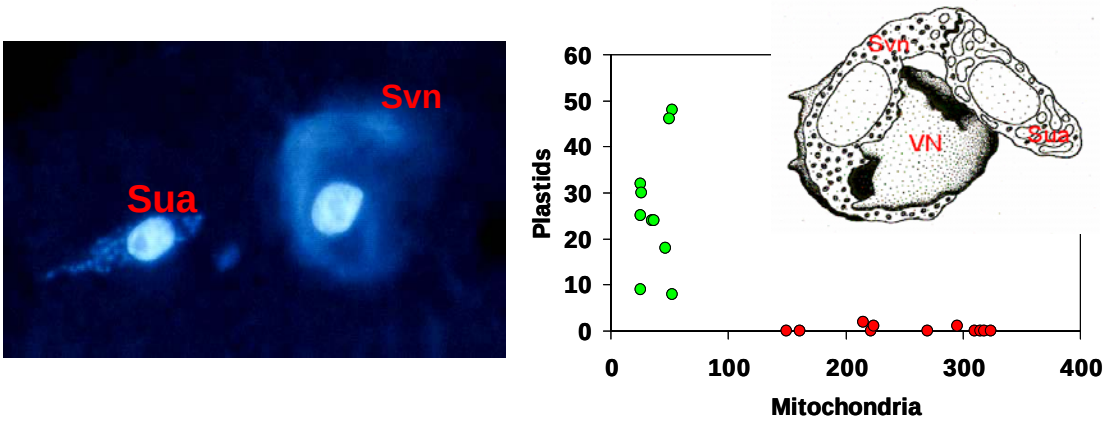

Figure I-3. Two dimorphic sperm cells in *Plumbago zeylanica* mature pollen. VN: vegetative nucleus. S_{vn}: the sperm cell associated with the vegetative nucleus, numerous mitochondria and a few plastids. $S_{ua:}$ the sperm cell unassociated with the vegetative nucleus, numerous plastids and a few mitochondria (Russell, 1984).

III. Genetic control of anther and tapetum development

The meristematic tissue in young flower buds differentiates to produce sporogenous cells. The sporophytic cell divides to give rise to a sporogenous initial cell as well as a tapetal initial cell. The tapetum is derived from subsequent division of tapetal initial cells. The tapetum and other layers of nonreproductive cells make up the wall of the anther lobes and surround the microsporocytes. The tapetum plays critical functions for microspores releasing from tetrad. Several genes have been identified that are critical to early anther development (Scott et al., 2004; Ma, 2005). The *Arabidopsis SPOROCYTELESS* (*SPL*)/*NOZZLE* (*NZZ*) gene is important for early anther cell division and differentiation (Schiefthaler et al., 1999; Yang et al., 1999b). Mutations in *SPOROCYTELESS* (*SPL*) block sporocyte formation in *Arabidopsis.* Sporogenous cells and nonreproductive tissues, including the tapetum, are absent in *spl* mutants (Schiefthaler et al., 1999; Yang et al., 1999b). Consequently, the *spl*/*nzz* mutants fail to produce pollen and are male sterile. *SPL*/*NZZ* is strongly expressed in the microsporocytes and tapetum. The *SPL* gene encodes a novel nuclear protein related to MADS box transcription factors and is expressed during microsporogenesis and megasporogenesis. This data suggests that the *SPL* gene product is a transcriptional regulator of sporocyte development in *Arabidopsis* (Yang et al., 1999b). *Arabidopsis* homeotic gene *AGAMOUS* (*AG*) is well-known for the specification of reproductive organs (stamens and carpels) during the early steps of

flower development (Bowman et al., 1989; Yanofsky et al., 1990; Bowman et al., 1991). *AG* encodes a MADS-box transcription factor in that controls microsporogenesis by activation of the *SPL/NZZ* (Yanofsky et al., 1990; Ito et al., 2004). Using an inducible system for AG activity, *SPL* is one of the genes transcriptionally activated after AG is induced, suggesting that the AG protein may be a direct regulator of *SPL* expression (Ito et al., 2004).

The leucine-rich repeat receptor like kinase family (LRR-RLK) has over 200 members in Arabidopsis. They are involved in cell-cell communication and signal transduction (Shiu and Bleecker, 2001; Fritz-Laylin et al., 2005). The EXCESS MICROSPOROCYTES1/ EXTRA SPOROGENOUS CELLS (EMS1/EXS) belongs to the same subfamily X of the LRR-RLKs as BRI1 (BRASSINOSTEROID INSENSITIVE1) does (Shiu and Bleecker, 2001). Mutations in the *EMS1/EXS* gene cause the formation of additional male sporocytes (MMCs) along with a lack of tapetal cells (Canales et al., 2002; Zhao et al., 2002). As a result, these mutants cannot produce any viable pollen grains and are male sterile. The *ems1*/*exs* mutant anthers lack the tapetum, but meiocytes can complete the meiotic nuclear division. Although the *ems1* male sporocytes can complete meiotic nuclear divisions, they fail to carry out cytokinesis. Instead, these cells undergo cell degeneration and thus fail to produce microspores. The action of EMS1/EXS is hypothesized to be a trigger for a signaling pathway essential for tapetal cell differentiation (Zhao et al., 2002). Moreover, the same developmental phenotypes are observed for mutations in the *TAPETUM DETERMINENT1 (TPD1)* gene, which encodes a predicted small secreted protein (Yang et al., 2003). *TPD1* could regulate cell fate in coordination with *EMS1/EXS* (Yang et al., 2003; Yang et al., 2005).

Another two LRR-RLK genes in subfamily II of the LRR-RLKs are also discovered to have the same function (Albrecht et al., 2005; Colcombet et al., 2005). The *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)* genes encode LRR-RLKs belonging to the 14-member subfamily II (Shiu and Bleecker, 2001). *Arabidopsis SERK3*, also named *BAK1* (*BRI1-Associated Kinase1*), is a component in brassinosteroid perception/signaling pathways (Li et al., 2002; Nam and Li, 2002). *SERK2* is the closest homolog to *SERK1* and is also related to *SERK3/BAK1*. Phenotypic analyses of s*erk1 serk2* double mutants show that *SERK1* and *SERK2* have a crucial and redundant function in anther development and male gametophyte maturation. Single knockout mutants of *SERK1* and *SERK2* show no obvious phenotypes. But the *serk1 serk2* double mutant produced more sporogenous cells that were unable to develop beyond meiosis. Furthermore, *serk1 serk2* double mutant anthers lack tapetal cell layer deveopment, which accounts for the microspore abortion and male sterility. Fertility can be restored by a single copy of either *SERK1* or *SERK2* gene. The SERK1 and SERK2 proteins can form homodimers or heterodimers in vivo, suggesting they are interchangeable in the SERK1/SERK2 signaling complex. Taken together, these findings demonstrate that the SERK1 and SERK2 receptor kinases function redundantly as an important control point for sporophytic development which is key to male gametophyte production (Albrecht et al., 2005; Colcombet et al., 2005).

Additionally, several MYB transcription factor genes are also involved in tapetum differentiation and anther development. *AtMYB103* is expressed specifically in the tapetum and is required for normal tapetum morphology and pollen development (Higginson et al., 2003). *AtMYB33* and *AtMYB65* are microRNA-regulated genes that

function redundantly in tapetum formation and anther development. In the *myb33 myb65* double mutant, the tapetum undergoes hypertrophy at the pollen mother cell stage, resulting in premeiotic abortion of pollen development. However, *myb33 myb65* sterility is conditional, where fertility increases under both higher light or lower temperature conditions. Thus, *MYB33*/*MYB65* facilitate, but are not essential for, anther development (Millar and Gubler, 2005).

Other tapetum related genes include *ABORTED MICROSPORES* (*AMS*) (Sorensen et al., 2003), and *MALE STERILE1* (*MS1*) (Wilson et al., 2001). The *ams* mutant's anthers have both the tapetum and male sporocytes and can complete meiosis, but the tapetum and microspores degenerate soon after meiosis. The *ms1* mutant's anthers exhibit microspore degeneration following their release from the tetrad, and tapetum becomes abnormal at this time. A particularly interesting *Arabidopsis* mutant is called *fat tapetum*, which appears normal in early anther development, but the mutant tapetum becomes enlarged at meiosis and the middle layer persists and also enlarges in a way similar to the tapetum (Sanders et al., 1999). More *Arabidopsis* mutant analyses (Sanders et al., 1999; Boavida et al., 2009) indicate that additional genes likely participate in the differentiation of anther cell layers.

IV. Plant male meiosis and its genetic control

In the anthers of flowering plants, pollen mother cells undergo meiosis to produce haploid microspores. Meiosis is essential for sexual reproduction because it facilitates stable sexual reproduction as well as recombination and independent assortment of homologous chromosomes, allowing for a greater diversity of genotypes in the population. During meiosis, the nuclear DNA of a diploid germ cell undergoes DNA

replication followed by two rounds of division, meiosis I and meiosis II, resulting in four haploid daughter cells (Dawe, 1998; Yang and Sundaresan, 2000; Ma, 2005). Meiosis I is unique and involves the segregation of homologous chromosomes (homologs), whereas meiosis II is similar to mitosis and results in the segregation of sister chromatids. Following meiosis II, the cell undergoes cytokinesis to produce four haploid cells. The process can be divided to Prophase I (including leptotene, zygotene, pachytene, diplotene, and diakinesis), Metaphase I, Anaphase I, Telophase I, prophase II, Metaphase II, Anaphase II, and Telophase II.

Plant meiosis has been traditionally studied using cytological and genetic analysis. Over the past two decades, new experimental approaches such as forward and reverse genetics have been used to identify genes involved in meiosis in model plants such as *Arabidopsis.* Because meiotic defects could result in sterile or semi-sterile male gametes, many novel meiotic genes have been successfully uncovered using mutants with defects in meiosis.

During meiosis, in the stage before anaphase I, sister chromatid cohesion requires the cohesion complex consisting of several subunits. Mutations in the *Arabidopsis SYN1/DIF1* gene (*Arabidopsis* homolog of the yeast *REC8* gene, which encodes a meiosis-specific cohesin subunit) (StoopMyer and Amon, 1999; Watanabe and Nurse, 1999), result in defects in chromosome condensation and pairing (Bai et al., 1999; Bhatt AM, 1999), suggesting that the cohesion complex also plays a role in condensation, which may indirectly affect pairing. The localization pattern of this protein indicates that it plays an important role in maintaining both chromosome arm and centromere cohesion during late stages of meiosis I (Cai et al., 2003). In addition to *SYN1*, the *Arabidopsis*

SWI1/DYAD gene is also required for sister chromatid cohesion and centromere organization during meiosis (Siddiqi et al., 2000; Mercier et al., 2001; Agashe et al., 2002; Ravi et al., 2008). In *swi1/dyad* mutant male meiosis, sister chromatid cohesion is lost and 20 separated chromatids are seen at late prophase I to metaphase I instead of the normal 5 bivalents. Female meiosis in the *swi1* mutant undergoes a mitosis-like division, presumably because the defect in cohesion causes the centromeres to behave like mitotic centromeres. These defects lead to the production of two diploid cells in place of four haploid megaspores, and failure to form a female gametophyte. The *dyad* allele of *SWI1* causes female specific sterility without affecting pollen development. The *dyad* plants are partial sterile, and a few seeds (1 to 10) per plant can be produced. The most fertile ovules in *dyad* plants form seeds that are triploid, arising from the fertilization of an unreduced female gamete by a haploid male gamete. This result suggests that a single gene of *SWI1* is responsible for the successful induction of functional apomeiosis, which is a major component of apomixis (Ravi et al., 2008).

Chromosome pairing, synapsis, and recombination ensure the appropriate recognition and association of homologs and the proper segregation of genetic information into haploid meiotic products. The wheat *Pairing homoeologous* locus *Ph1* is required for correct homologs pairing (pairing of chromosomes from the same subgenome) but suppressing homeologous pairing (pairing of chromosomes from different subgenomes), since in *ph1* mutant wheat meiotic cells, homeologous chromosomes may also pair (Gill and Gill, 1996; Martinez-Perez et al., 2001). The *Arabidopsis asy1* mutant is defective in synapsis in male and female meiosis, resulting in the formation of only one to three bivalents per cell, instead of the normal five (Ross et

al., 1997; Caryl et al., 2000). The rice *pair2* mutation affects a rice homolog of the *Arabidopsis ASY1* gene, and the *pair2* mutant is defective in pairing at pachytene and has univalents at diakinesis (Nonomura et al., 2004). Another *Arabidopsis* gene, *AHP2*, is also involved in pairing and bivalent formation (Schommer et al., 2003). *Arabidopsis* homologs for yeast pairing and recombination genes, such as *At*SPO11-1 (Grelon et al., 2001), *AtRAD50* (Gallego et al., 2001), *AtMRE11* (Puizina et al., 2004), *AtDMC1* (Couteau et al., 1999), and *AtXRCC3* (Bleuyard and White, 2004), show important functions in chromosome pairing and recombination during meiosis in *Arabidopsis*.

Several Arabidopsis genes seem to be involved in the process for homologous chromosome separation and segregation, such as *ASK1*, *MMD1*/*DUET*, and a homolog to yeast *CDC45*. The *Arabidopsis ask1* mutant is abnormal in chromosome separation and segregation at anaphase I (Yang et al., 1999a). An *Arabidopsis* mutant (*atk1*) with reduced male fertility was isolated from a population of transposon insertional lines. The *atk1* and formed abnormal meiotic spindles and had uneven chromosome segregation (Chen et al., 2002). *Arabidopsis MMD1*/*DUET* gene that encodes a protein with a C-terminal PHD-finger domain may be a critical regulator of meiosis (Reddy et al., 2003; Yang et al., 2003). Mutations in this gene cause meiotic defects, a failure to produce normal pollen, and male sterility. During early meiosis, the *mmd1* (*male meiocyte death1*) mutant appears normal up to diakinesis (Yang et al., 2003). From diakinesis to telophase II, meiotic cells show signs of programmed cell death, including chromosome fragmentation and cytoplasmic shrinkage. All meiotic cells die before cytokinesis. The *duet* mutant shows a delayed meiotic cytokinesis and formation of mostly dyads instead

of tetrads (Reddy et al., 2003). The abnormal microspores produced from the mutant meiocytes then undergo one or two mitotic divisions before degeneration.

An *Arabidopsis* homolog to the yeast cell-cycle gene *CDC45* is expressed in early floral buds that include meiotic cells. RNAi transgenic plants where *CDC45* is silenced have fertility defects (Stevens et al., 2004). Further analysis indicates that the RNAi lines produce polyads instead of tetrad from meiosis and subsequent nonviable pollen grains.

Several genes are genetically important for meiotic cytokinesis. Among these mutants are the *Arabidopsis stud* (*std*) and *tetraspore* (*tes*) mutants. In the mutant meiocytes, the nuclear meiotic events are normal, but cell plate formation is incomplete during cytokinesis, resulting in a giant microspore with four nuclei. During the subsequent pollen development, the four nuclei undergo mitotic divisions separately, producing four vegetative nuclei and up to eight sperm cells within a large abnormal "pollen grain." This pollen grain behaves as a single male gametophyte and produces only one pollen tube, resulting in significantly reduced male fertility (Hulskamp et al., 1997; Spielman et al., 1997).

V. Male gametophyte development and gene control

After the tetrad separates and releases as free microspores, each uninucleate microspore undergoes an asymmetric mitotic division to produce a small generative cell and a larger vegetative cell. The generative cell then divides to form two sperm cells (Cresti et al., 1992; Mogensen, 1992; McCormick, 1993; Southworth, 1996; McCormick, 2004; Weterings and Russell, 2004; Ma, 2005). Although the unique "cell-within-a cell" structure means that the male gametic lineage -- sperm cells and their precursor generative cell depend on the pollen vegetative cell for nutrition and transportation, male

germ cells are known to have their own enriched and unique molecular repertoire and gene regulation network (Twell, 2002; McCormick, 2004; Singh and Bhalla, 2007; Singh et al., 2008; Borg et al., 2009; Gou et al., 2009). Over last two decades, significant progresses have been made on male gametophyte specific gene expression, regulatory elements for male lineage identity, molecular mechanisms controlling male gametophyte development, and male gene involved in fertilization and early embryogenesis.

5.1. Genome-wide transcriptome studies of pollen

In recent years, genomics approaches such as high-throughput transcriptome sequencing and microarray assays have enabled the analysis of male gametophyte gene expression on a genome-wide scale.

The first *Arabidopsis* pollen microarray assays were performed by two independent group using first generation of Affymetrix Arabidopsis Genome Gene Chip microarray (Becker et al., 2003; Honys and Twell, 2003). Since this 8K chip only covers approximately one-third of the *Arabidopsis* genome, even with FACS sorted pollen, a limited number of genes were identified as pollen specific (Becker et al., 2003; Honys and Twell, 2003; Borg et al., 2009). The development of the Affymetrix 23K *Arabidopsis* ATH1 array, which covers approximately 80% of *Arabidopsis* genes, enables transcriptome profiling of the male gametophyte on a much larger scale. The first large scale *Arabidopsis* pollen microarray dataset was generated from four stages of male gametophyte development (uninucleate microspores, bicellular pollen, tricellular pollen, and mature pollen) from ecotype Landsberg *erecta* (Honys and Twell, 2004). A total of 13,977 genes are expressed in the male gametophyte, and 9.7% of the expressed genes seem to be male-gametophyte-specific. The different stages of pollen have overlapping,

yet different expression profiles. The transition from bicellular to tricellular pollen is accompanied by a decline in the number of diverse mRNA species and an increase in the proportion of male gametophyte-specific transcripts. The majority of male gametophyte-expressed genes (52%) can be grouped into four clusters comprising early expressed genes repressed after PMII. Several large gene clusters collectively containing 1,899 genes (13.6%) are associated with pollen maturation, and they are activated or upregulated between the bicellular pollen (BCP) and tricellular pollen (TCP) stages, In contrast, a discrete cluster set of 298 genes is upregulated only after the TCP stage. The core cell-cycle genes, transcription factors and core translation factors that could correspond to components of gametophytic regulatory networks were identified from the assay.

Another dataset is derived from mature pollen grains from *Arabidopsis* ecotype Columbia by using Affymetrix Arabidopsis ATH1 arrays (Pina et al., 2005). The comparison of gene family and Gene Ontology (The Gene Ontology Consortium, 2000) representation in the transcriptome of pollen and vegetative tissues indicates a smaller and overall unique transcriptome (6,587 genes expressed) with greater proportions of selectively expressed (11%) and enriched (26%) genes in pollen grains than in any vegetative tissue. Relative Gene Ontology category representations in pollen and vegetative tissues reveal functions of the pollen transcriptome toward signaling, vesicle transport, and the cytoskeleton, suggesting that these pollen expressed genes may be involved in pollen germination and pollen tube growth. In addition, factors associated with G2/M in the cell cycle are strongly expressed revealing that may play a role in the first mitotic division of the zygote.

An increasing number of publicly available sporophytic datasets have allowed comparative analyses to be performed with the male gametophyte transcriptome (Zimmermann et al., 2004). Using bioinformatic tools to compare a number of sporophytic datasets , Twell et al. (Twell et al., 2006) estimated that ~5% of genes expressed in mature pollen are pollen-specific, a number about half of the initial estimates of 10% from individual datasets (Honys and Twell, 2004; Pina et al., 2005). The genes with specific or enhanced expression in the male gametophyte-specific genes are often characterized by having very high expression levels. Many of these genes encode proteins with predicted functions related to pollen germination or tube growth (Honys and Twell, 2004; Pina et al., 2005; Twell et al., 2006; Borg et al., 2009).

Some pollen-specific genes likely code for allergens. Russell et al. recently profiled rice pollen gene expressions using the Affymetrix 57K rice GeneChip microarray, and found that some most abundant pollen-specific genes to be putative allergens (Russell et al., 2008).

5.2. Gametophytic mutants affecting pollen development and pollen specific gene expression

A number of interesting mutants showing defective pollen phenotypes have been described in *Arabidopsis* based on morphology or DAPI (4',6-diamidino-2-phenylindole) staining characteristics of pollen (Chen and McCormick, 1996; Johnson and McCormick, 2001; Lalanne and Twell, 2002). Among them, *sidecar pollen* (Chen and McCormick, 1996) and *gemini pollen* (Park et al., 1998) are two representative (or best characterized) mutants. At the mature pollen stage, 7% of heterozygotes *sidecar pollen* show the sidecar phenotype, namely an extra cell within the pollen exine. The *sidecar pollen* (*scp*)

mutation causes some of the developing pollen grains to undergo a premature and symmetric cell division. This extra mitosis produces two vegetative cells prior to the asymmetric division, and thus, 43% of the mutant pollen grains are aborted. As a result, these two cells are then unable to undergo the normal mitotic divisions to form the generative cell and two sperm cells. The *gemini pollen1* (*gem1*) mutant also exhibits defects in the divisional pattern. Among the mutant pollen grains, equal, unequal, and partial divisions can all be observed at the first mitotic division (Park et al., 1998), suggesting that the mutation may affect the positioning of the nucleus. GEM1 is identical to MOR1 (Whittington et al., 2001). MOR1/GEM1 belongs to the MAP215 family of microtubule-associated proteins and plays a vital role in microspore polarity and cytokinesis by stimulating growth of the interphase spindle and phragmoplast microtubule arrays at pollen mitosis I (Twell et al., 2002). In another mutant, *limpet pollen*, the generative cell remains outside the cytoplasm of the vegetative cell and against the pollen wall, suggesting that the migration of the generative cell is defective (Howden et al., 1998).

In mature pollen grain, the vegetative nucleus and the two sperm cells are components of the MGU. Screening of pollen grains from mutagenized plants has identified mutants with abnormal organization or positioning of the MGU (Lalanne and Twell, 2002). The *gum* (*germ unit malformed*) and *mud* (*MGU displaced*) mutants are defective in the organization of the MGU such that the vegetative nucleus stays near the pollen wall and away from the sperm cells. In *mud* mutant pollen grains, the entire MGU is mislocalized near the pollen wall on one side of the cytoplasm. Further analysis of

these mutants and the corresponding genes should provide new insights into the control of the integrity and positioning of the MGU.

The analysis on two *Arabidopsis* plastidic glucose 6-phosphate/phosphate translocator GPTs indicate that GPT1 is essential to pollen maturation and embryo sac development (Niewiadomski et al., 2005). The homozygous T-DNA insertion mutants *gpt1-1* and *gpt1-2* are lethal, and the heterozygous line shows distorted segregation and reduced male and female transmission rates. TEM observations show that the *gpt1* mutant pollen development is associated with reduced formation of lipid bodies and small vesicles and the disappearance of dispersed vacuoles, resulting in disintegration of the pollen structure and thus abortion of the pollen. Meanwhile, the embryo sac development in the mutant is also defective. Although the embryo sac cell division is normal, the polar nuclei fail to fuse. The loss of GPT1 function probably results in disruption of the oxidative pentose phosphate cycle, which in turn affects fatty acid biosynthesis (Niewiadomski et al., 2005).

Notably, the tomato *LAT52* gene encoding a cystein-rich protein is specifically expressed in the vegetative cell during pollen maturation (Twell et al., 1990; Bate and Twell, 1998). The *LAT52* promoter, isolated and analyzed by several groups (Twell et al., 1991; Eyal et al., 1995; Bate et al., 1996; Bate and Twell, 1998) by testing gain-of-function and loss-of-function gene constructs in transient assays in pollen and in somatic cells as well as in stably transformed plants, seems to contain a 30-bp element that defines pollen specificity. Gene expression driven by the promoter can be detected in uninucleate microspores in *Arabidopsis* (Eady et al., 1994) and this promoter shows strong gene expression at all developmental stages after the bicellular pollen stage, even

after delivery into mature pollen via particle bombardment (Twell et al., 1991; Eyal et al., 1995).

Genes regulating pollen tube growth and navigation inside in female tract are also important for successful fertilization. Recently, Arabidopsis *VANGUARD1 (VGD1)* was identified to play an important role in pollen tube growth. *VGD1* encodes a pectin methylesterase homologous protein that may modify the cell wall and thus enhances the interaction of pollen tube with the female style and transmitting tract. The *vdg1* mutant showed retarded pollen tube growth and resulted in a significant reduction of male fertility (Jiang et al., 2005).

A recent report on a collection of Ds transposon insertion mutants associated with defects in pollen development, pollen germination, pollen tube growth and pollen tube-embryo sac interaction in *Arabidopsis* could help decipher a more complete understanding of the genetic basis of pollen development and functions (Boavida et al., 2009).

5.3. Male germ cells (generative cell and sperm cells) transcriptional profile

Although sperm cells and their precursor generative cells are small and dependent on the vegetative cell, male germ cells are known to have their own enriched and unique molecular repertoire with both separate and overlapping systems of genetic control (Singh et al., 2008). Male germline cells transcribe their own separate pools of stable, translatable mRNA (Zhang et al., 1993; Blomstedt et al., 1996), and synthesize proteins unique to the germline (Ueda and Tanaka, 1995b, 1995a). EST sequencing and microarray assay are powerful tools for the analysis of gene expression in generative cells and sperm cells. Data generated using these tools have been available for several model

plant species. The cDNA libraries have been constructed from the generative cells of *Lilium longi-florum* (lily) (Xu et al., 1998; Okada et al., 2006a), sperm cells of *Plumbago zeylanica* (Gou et al., 2009), sperm cells of *Oryza sativa* (rice) (Gou et al., 2001), sperm cells of *Zea mays* (maize) (Engel et al., 2003), and generative cells of *Nicotiana tobacco* (tobacco) (Xu et al., 2002). cDNA microarray and Affymetrix GeneChip arrays have been employed for comparative transcriptome studies of *Arabidopsis*, lily, rice and *Plumbago* (Okada et al., 2007; Borges et al., 2008; Gou et al., 2009; Russell et al., 2010b). Genes identified as male germline-expressed from the above studies can be classified into three categories: (1) housekeeping genes expressed constitutively in male germline, pollen vegetative cell and other plant cells; (2) genes found in several cell types, but up-regulated in the male germ cells; and (3) germ cell specific genes (Singh et al., 2008). Some germ cell-specific genes share functions with somatic counterparts, whereas others are unique in sequence and function exclusively in germline cells. The latter is the most attractive to plant reproduction community as this may recover molecular mechanisms underlying male gamete development and for controlling gamete interactions. Much of current transcriptomic information is from four plants: lily, *Plumbago*, maize and *Arabidopsis.*

5.3.1. Lily generative cell transcriptional profile

As model for bicellular pollen biology, lily is a classical experimental material for the study of pollen development and male gamete gene regulation because it has abundant pollens and there is a well-established protocol for isolation of sufficient intact generative cells (Tanaka, 1988; Blomstedt et al., 1996; Xu et al., 1998; Okada et al., 2006a). Xu et al. (Xu et al., 1999b) constructed the first generative cells cDNA library using purified lily

generative cells by a discontinuous Percoll gradient centrifugation. Initial analysis identified several germline-specific genes including *LGC1* which encodes a membrane protein (Xu et al., 1999b), male germline histone isoforms gcH2A and gcH3 (Xu et al., 1999a; Ueda et al., 2000), a polyubiquitin gene *LG52* (Singh et al., 2002), and a strongly upregulated gene which is a highly conserved DNA repair gene and homologous to human *ERCC1* (Xu et al., 1998).

Another study by Okada et al. (Okada et al., 2006a) is on male gamete cell gene expression through EST analysis of cDNAs derived from isolated lily generative cells. In order to gain new insight on expression profile of male gamete genes, the group also compared lily generative cell expressed genes with genes in other plants. A total of 886 ESTs derived from lily generative cell cDNA library are assembled into 637 unique contigs, 39% of which show sequence similarity to *Arabidopsis* genes with known functions. Interestingly, genes related to the ubiquitin pathway are over-represented, suggesting the key role of ubiquitin-dependent proteolysis in gametogenesis. A total of 168 and 129 lily generative cell contigs are highly similar to maize sperm cell ESTs and *Arabidopsis* male gametophyte-specific transcripts, respectively, and 55 male gametophyte-specific genes appear to be conserved in lily, *Arabidopsis* and maize, suggesting functional conservation of the male gamete-expressed genes across different plant genera.

Microarray experiments of lily generative cells found that only 17% of the generative cell-expressed genes can hybridize to sporophytic sources of mRNA. Thus, 83% of lily generative cell transcripts seem to be male germ cell-specific. No other plant

cell type analyzed to date produces such a high ratio of cell-specific transcripts (Okada et al., 2007).

5.3.2. Plumbago dimorphic sperm cells transcriptional profile

plumbago zeylanica is a model plant for the study of sperm cell dimorphism and preferential fertilization. Sperm cell dimorphism and preferential fertilization may be common in flowering plants. A survey of 19 plant species found that 17 display dimorphism (Saito et al., 2002; Weterings and Russell, 2004). Preferential fertilization has been reported in other flowering plant species too, including maize and tobacco (Roman, 1948; Carlson, 1969, 1986; Rusche et al., 1997; Tian et al., 2001; Faure et al., 2003).

In *plumbago zeylanica*, mature pollen is tricellular and contains a vegetative cell and two strongly heteromorphic sperm cells (Russell and Cass, 1981; Russell, 1984). The sperm cell associated with the vegetative nucleus (Svn) is the larger cell and contains many mitochondria (mean 256.18) and very few plastids (mean 0.45). The smaller sperm cell (Sua) is unassociated with the vegetative nucleus and contains abundant plastids (mean 24.3) and smaller, less numerous mitochondria (mean 39.8) (Russell, 1984; Russell, 1986). Because the embryo sac of the plant lacks synergids, a property that greatly simplifies the observation of events occurred inside the embryo sac, *Plumbago zeylanica* is frequently used to trace the fate of sperm cells during double fertilization (Russell, 1980; Russell, 1982; Russell, 1983). Using this model plant, preferential fertilization in plant reproduction was first documented about two decades ago. Sua selectively fuses with the egg in >95% of cases examined, whereas Svn usually fuses with central cells (Russell, 1985; Russell, 1986). The differences between the two sperm

cells may correlate with differences in gene expression that affects post-fertilization development, since the male cytoplasm in this plant is known to be transmitted into female gametes during double fertilization (Russell, 1980; Russell, 1983; Weterings and Russell, 2004).

To elucidate possible expressional differences between the sperm cells, a method to isolate and collect two sperm cell types based on micropipetting individual sperm cell types has been developed (Zhang and Russell, 1998). Two cDNA libraries have been prepared from each sperm type, and sequencing of clones from each has highlighted some differences in the repertoire of genes expressed in each sperm type. EST expression patterns and functional classification of the transcripts using Gene Ontology (The Gene Ontology Consortium, 2000) suggest strong transcriptome divergence in the two *Plumbago* sperm cell types. Over 1500 representative ESTs from these two sperm cell types have been submitted to GenBank, including 893 sequences isolated from Sua and 629 sequences from Svn. The Sua ESTs are assembled into 426 distinct sequences, comparing to 419 distinct sequences for Svn ESTs. Of the ~ 1500 genes, 13.3% represent products with unknown function and 60.8% of the sequences do not have any homology in other plants. Only 25.9% could be classified into functional categories, where the largest groups were genes involved in metabolism, protein modification, transcription and biosynthesis. Of those sequences with no known homology in the GenBank protein database, the Svn has slightly more unique sequences, with 62.8% having no hits, versus 58.9% in the Sua. The high percentage of no hits and unclassified sequences indicates that current databases provide inadequate coverage of genes involved in angiosperm male gamete biology. Similar to lily generative cells, there is a conspicuous upregulation of

ubiquitin, as well as metabolic, transcriptional, and biogenetic activity. Interestingly, ubiquitin expression in terms of EST count is much higher in the Svn (Gou et al., 2009). Genes that are highly upregulated in only one of the two sperm cells illustrates the likelihood of independent regulatory elements controlling expression in each of the two sperm cells of *Plumbago* (Singh et al., 2002).

The differential expression between Svn and Sua was validated through suppression subtractive hybridization, cDNA microarrays, real-time RT-PCR and in situ hybridization. Some male germline genes were differentially expressed in the Sua, while others specifically expressed in the Svn. The Sua, which targets the egg cell, has a greater abundance of transcripts relating to transcription, translation and protein modification, and thus appears to reflect a profile similar to anticipated patterns of expression in the embryo. The Svn, which targets the central cell and forms the endosperm, displays greater abundance of transcripts relating to metabolism and phytohormone biosynthesis. Notably, multiple copies of *IPT (isopentenyl transferase*) transcripts were detected in Svn cDNA library. IPT is an enzyme governing the limited-rate step for cytokinin biosynthesis (Mok and Mok, 2001; Miyawaki et al., 2004; Miyawaki et al., 2006). Although cytokinin causes negligible responses in pollen (Miyawaki et al., 2004), cytokinin levels are strongly elevated during endosperm proliferation and appear to control endosperm growth and development (Day et al., 2008). The Svn appears to have a more strongly divergent program than the Sua, since many upregulated genes from the SSH cDNA library of the Svn lack homology with other sequences in public database (Gou et al., 2009; Russell et al., 2010a).

5.3.3. Maize sperm cells transcriptional profile

There are 5,093 ESTs representing maize sperm-expressed transcripts in GenBank. They were sequenced from a maize cDNA library constructed using sperm cells isolated by FACS, followed by EST sequencing (Engel et al., 2003). A characteristic feature of the grass genome has been a relatively high representation of transposable elements, for which the sperm lineage is no exception. Annotated retrotransposons represent 9.46% of the maize sperm ESTs, but there are fewer annotated transposons (0.06%). In contrast, female gametophytes showed less than one-fifth the number of retrotransposons (1.69%) and more annotated transposons (1.44%) (Yang et al., 2006), reflecting differential expression of such elements in the male germlineage. Sperm-specific EST sequences from maize have been successfully used to identify *Arabidopsis* sperm-specific gene candidates, many of which have been validated to be expressed specifically in sperm cells of *Arabidopsis* (Engel et al., 2005). Several such genes expressed in male germ cells in *Arabidopsis* (*Gamete Expressed 1*, 2, *3, GEX1, 2, 3*) were identified by comparative analysis of the maize sperm cell EST sequences with the *Arabidopsis* genome (Engel et al., 2005; Alandete-Saez et al., 2008).

5.3.4. Arabidopsis sperm transcriptional profile

Arabidopsis is widely used as a model plant for functional genomics. However, its sperm transcriptomic studies are hard to perform because the sperm cells are not easily accessible. The first genome-wide Arabidopsis sperm cell transcritome study was reported by Borges et al. (Borges et al., 2008) who used FACS to isolate sperm cells from *Arabidopsis* for assay. To use FACS, they first transformed *Arabidopsis* plant with GFP driven by the *AtGEX2* promoter. The promoter is known to express strongly in sperm

cells. The direct comparison of their transcriptome with those of pollen and seedlings, as well as with additional ATH1 data sets from a variety of vegetative tissues shows that sperm cells have fewer expressed genes (5829 genes, or 27% of genes represented in the chip) than those detected from pollen (7177 genes, or 33%) and seedling (14464 genes, or 64%). However, the sperm cell seems to have a distinct and diverse transcriptional profile. Functional classifications of genes up-regulated in sperm cells shows that genes involved in DNA repair, ubiquitin-mediated proteolysis, and cell cycle progression are overrepresented. The candidate genes involving in sperm cell development and fertilization identified in this study can be directly tested in Arabidopsis. These results provide useful information to decipher the role of sperm-expressed proteins (Borges et al., 2008).

To summarize, plant sperm cell transcriptome profiling from several model plants indicates that a number of sperm-specific genes are conserved across species, and sperm expressed genes seem to be involved in general metabolism, cellular organization, DNA synthesis, chromatin structure, and protein degradation.

5.4. Male germline specific gene expression

The previous session reviewed global patterns of sperm transcriptome profile. Here I will review individual genes specifically expressed in sperm cells and their regulation. There are numerous interests in identifying and characterizing germ-specific genes since they may play vital roles in germline development and fertilization. Many germline specific genes have been identified from either genome-wide transcriptomic study or gametophytic mutant screening. These genes have various molecular functions and are involved in different biological processes.

5.4.1. Male germline specific genes involved in ubiquitin pathway

Ubiquitin is a highly-conserved regulatory protein that is ubiquitously expressed in all eukaryotic cells. Ubiquitin is mainly involved in the ubiquitination pathway which was initially characterized as an ATP-dependent proteasomal degradation system. The ubiquitin system provides a critical regulatory mechanism in many cellular processes, including protein degradation, transcription, DNA repair, chromatin structure, signal transduction, autophagy, and cell-cycle control (Kerscher et al., 2006). Mammalian systems show that ubiquitin has specialized functions in mammalian gametogenesis (Baarends et al., 1999). Mouse gene knockout studies have shown that inactivation of components of the ubiquitin system leads to impaired gametogenesis (Roest et al., 1996).

In flowering plants, genes encoding ubiquitin-pathway-related proteins such as polyubiquitin, proteasome subunit, ubiquitin-conjugating enzyme, Skp1 and Ring box protein are highly upregulated in the generative cells of lily (Okada et al., 2007), sperm cells of *Arabidopsis* (Borges et al., 2008), sperm cells of *Plumbago* (Singh et al., 2002; Gou et al., 2009), and sperm cells of maize (Engel et al., 2003). In Arabidopsis, ubiquitination related genes are the second most abundant in sperm-enriched genes (Borges et al., 2008). While in *Plumbago*, polyubiquitin, ubiquitin ligase, and ubiquitin-conjugating enzyme are the most abundant in both Svn and Sua sperm cell cDNA libraries (Gou et al., 2009)..

In lily generative cell cDNA library, the clone *LG52* contains two ubiquitin units. To determine whether the ubiquitin gene was generative cell-specific, its expression was measured in various tissues including the generative cells, mature pollen, leaf, stem and petal. A strong hybridization signal was detected in generative cells, but no signal was

detected in other floral and vegetative organs except in the cytoplasm of mature pollen where only a weak hybridization signal was detected, indicating that the gene corresponding to *LG52* cDNA is transcribed in the generative cell nucleus (Singh et al., 2002). Using the lily generative cell mRNA to cross-hybridize *Plumbago* sperm-expressed genes, a *Plumbago* homolog was identified which encodes a polyubiquitin with three ubiquitin repeats. Both RT-PCR analysis and in situ hybridization experiments demonstrated that the *Plumbago* polyubiquitin was expressed in both sperm cells, but expression in mitochondria-rich sperm (Svn) cells was much higher than in the plastid-rich sperm (Sua) cells (Singh et al., 2002; Singh et al., 2008). The high level of expression of ubiquitin-pathway-related genes in generative cells and one of the *Plumbago* sperm cell types suggests that the ubiquitin proteolysis system plays a critical role in the male gametogenesis of higher plants (Singh et al., 2002).

5.4.2. Male germline specific histone variants and epigenetic remodeling in male germlines

Microarray expression profiling in *Arabidopsis* has revealed that among several tissue types, sperm cells and pollen have the fewest genes expressed (Becker, 2007; Borges et al., 2008). Comparing to the vegetative nucleus, the generative nucleus and sperm have very condensed chromatin and DNA is heavily methylated (Slotkin et al., 2009). In lily, 2D gel electrophoresis identified at least five nuclear basic proteins which are either specific to or enriched in generative cell nuclei (Ueda and Tanaka, 1994). Two of these generative cell specific proteins gH3 and gH2B are variants of histones H3 and H2B. Immunocytochemical staining of these histone variants demonstrated that they were not only present in generative cells but also in the two sperm cells produced from the division

of the generative nucleus in pollen tube (Ueda and Tanaka, 1995b). In a lily generative cell cDNA library, transcripts encoding histone variants are generally abundant, and ESTs for histone H3 are particularly over-represented (Xu et al., 1999a; Ueda et al., 2000; Okada et al., 2005a; Okada et al., 2005b; Okada et al., 2006b). Generative cell specific expression of histone variants, especially histone H3, may be a common characteristic of the male germline in flowering plants (Xu et al., 1999a; Okada et al., 2005b; Singh et al., 2008). Immunocytochemical studies have further demonstrated that histone variants gH3 and gcH3 are incorporated in male germline chromatin in a replication independent manner (Singh et al., 2008).

In a maize sperm cell cDNA library, 20 out of 1,100 ESTs are for histone H3, while < 5 ESTs are found for other histones (Engel et al., 2003). In *Plumbago* sperm cDNA libraries, male gamete-expressed chromatin related genes included histones *H2A*, *H2B*, *H3* and *H4* that are up-regulated in both Svn and Sua sperm cells (Gou et al., 2009). In *Arabidopsis*, at least three *H3*-variant genes are expressed in male germline cells, including the male gamete-specific variant gene *AtMGH3* and variant *H3.3* (Okada et al., 2005b). Male germline histone variants possibly play an important role in epigenetic regulation of gene expression (Grewal and Moazed, 2003). The lily variant *gH3* histone, for instance, can only be localized in generative nucleus chromatin. In addition to variant histones, generative cells also show evidence of chromatin modification, as indicated by the localization of a strong methylation signal at lysine residue position 4 (H3K4) and position 9 (H3K9) of the generative nucleus H3 histone (Okada et al., 2006b).

Sequence similarity searches using lily germline specific histone H3 as the query identified *MGH3*, an *Arabidopsis* male germline-specific gene (Okada et al., 2005a). *In*

situ hybridization and monitoring of promoter activity with the β-glucuronidase (GUS) reporter demonstrates that *MGH3* is specifically expressed in both the germ cell and sperm cells (Okada et al., 2005a). However, an *MGH3* insertion mutant did not show aberrant phenotypes. This may arise from functional redundancy among histone H3 genes (Okada et al., 2005a). Recent studies on the dynamics of the male germlineage marker MGH3-mRFP1 and the centromeric histone H3 marker HTR12-GFP in the zygote and endosperm nuclei indicated active replication-independent replacement of paternal histone *H3.3* in the zygote and replication-coupled removal in the endosperm. This study also revealed a spatial segregation of paternal chromatin (marked by HTR12-GFP) from maternal chromatin in the endosperm, but not in the zygote. Thus, the differential paternal chromatin remodelling involving histone *H3* variants, which may also be coupled to parental imprinting of the endosperm, distinguishes the two products of fertilization (Ingouff et al., 2007).

In *Arabidopsis*, the chromatin remodeling *ATPase DECREASE IN DNA METHYLATION 1 (DDM1)* is down-regulated in the vegetative nucleus but accumulates in sperm cells. DDM1 is a master regulator of transposable element (TE) activity. In the vegetative nucleus most TE lost DNA methylation and active TE transcription is observed (Slotkin et al., 2009). Generally, TE transcripts are sources for 24nt siRNAs which negatively regulate gene expression at transcriptional level by guiding DNA methylation. Interestingly, siRNAs of 21nt from Athila retrotransposons are generated in the vegetative nucleus. The 21mer siRNAs seem to be mobile and can accumulate in the sperm cells to specifically silence TE.

5.4.3. Genes associated with male germline specification

The genome-wide germline transcriptomic assay is valuable to identify germline up-regulated or specific genes (Xu et al., 1998; Xu et al., 1999b; Engel et al., 2003; Engel et al., 2005; Okada et al., 2006a; Borges et al., 2008; Gou et al., 2009). Genes specifically or highly expressed in germline cells may play an important role in the specification of functional sperm cells.

LGC1 (Lily Generative Cell-specific 1) is the most impressive such gene that is exclusively expressed in the male gametic cells of lily. Identified from a RNA gel blot with cDNA clones from generative cell cDNA library (Xu et al., 1999b), *LGC1* showed strong hybridization signal to RNA of generative cells, but weak hybridization signal to RNA of pollen, and no detectable hybridization to RNAs of other tissues when cDNA of *LGC1* was used as probe for Northern blot hybridization. RT-PCR also confirmed the results of Northern blot hybridization. *In situ* hybridization further showed that *LGC1* mRNA is restricted to the generative cell in mature pollen. The temporal expression analysis of *LGC1* showed *LGC1* mRNA was not detected in the microsporocyte, tetrad, or unicellular microspores; but *LGC1* mRNA started to transcribe only in the generative cell at the later bicellular stage of pollen. Very high level of *LGC1* mRNA was detected in mature pollen and sperm cells after generative cell division (Xu et al., 1999b). *LGC1* encodes a small protein of 128 amino acids with a hydrophobic domain having characteristics of a GPI anchor, suggesting membrane localization of this protein. The male gametic cell specific *LGC1* and membrane surface characteristic suggested a putative function in sperm-egg recognition and fusion (Xu et al., 1999b).

LGC1 is abundantly expressed in the lily germline, as 11 of 886 sequenced lily generative cell ESTs were found in generative cell cDNA library (Okada et al., 2006a). *LGC1* homologs are also present in *Arabidopsis* and rice genomes. The *LGC1* ortholog in *Arabidopsis* is also exclusively expressed in generative and sperm cells (Singh et al., 2008).

GCS1 (Generative Cell-Specific 1) is another lily generative cell specific gene (Mori et al., 2006). *GCS1* cDNA clone was detected in the generative cell and the expression specificity was validated by RT–PCR. The immunolocalization of GCS1 protein in lily pollen grains showed GCS1 was distributed in the region surrounding the generative nucleus in pollen grains. The knockout mutation in *Arabidopsis GCS1* specifically blocked male transmission and fertilization. During double fertilization, sperm cells were observed within the degenerated synergid but failed to fuse with the female gametes. Mori and colleagues thus presented two lines of evidence that support an important role for *GCS1* in angiosperm fertilization. First, expression of *GCS1* is specific to male gametes and location of expression is at the cell surface; second, disruption of *GCS1* in *Arabidopsis* prevents gamete interaction, indicating that GCS1 is essential for successful gamete attachment, fusion, or both. However, the failure of gamete fusion in *gcs1* mutants could also arise from defective transport of sperm cells to their respective fusion sites on the egg and central cells. *GCS1* encodes a novel protein without known domains. All homologues have a common structure with an N-terminal putative transmembrane peptide and a prominent hydrophobic putative transmembrane domain located in the C-terminal region, followed by a unidefined histidine-rich region. GCS1 may be anchored by its C-terminal transmembrane domain, which localize to the sperm

surface and serve as facilitators of gamete fusion (Mori et al., 2006). Single-copy *GCS1* homologues with a conserved domain structure are present in *Arabidopsis,* rice, green and red algae, slime mould and protozoan parasites. All these evidences point to a fundamental role of *GCS1* in membrane fusion during fertilization. *Arabidopsis* plants with a gene knockout for a *GCS1* homologue gene, *HAP2*, also fail in fertilization. Sperm cells of *hap2* mutant plants are incapable of fertilizing the egg or central cell, leading to the degeneration of the egg cell (von Besser et al., 2006; Frank and Johnson, 2009). HAP2/GCS1 protein is the first identified flowering plant sperm surface protein that is essential for fertilization (Mori et al., 2006; von Besser et al., 2006; Frank and Johnson, 2009).

Comparative study of maize sperm cell specific transcripts with *Arabidopsis* sequences led to the identification of germline specific genes *GEX1, GEX2* and *GEX3* and their promoters in *Arabidopsis* (Engel et al., 2005; Alandete-Saez et al., 2008). These genes belong to membrane-associated proteins which are expressed in male germ cells in *Arabidopsis*. GEX1 and GEX2 are predicted to have three and six transmembrane domains, respectively, while GEX3 is predicted to have only one. Fluorescent protein GFP fusions indicate that all three proteins are plasma membrane-associated. Promoter::GFP analysis indicated that *GEX1* is expressed in sperm cells, ovules, root, and guard cells, but not in generative cells and vegetative cells. *GEX2* is expressed in both the generative cells and sperm cells, but not in vegetative cell or other tissues (Engel et al., 2005). *GEX3* is expressed in the vegetative cell and sperm cells, and a low level of expression in the egg cell of the female gametophyte (Alandete-Saez et al., 2008). The function of *GEX1* and *GEX2* has not yet been elucidated, while analysis of transgenic

GEX3 knockdown and over-expression lines revealed reduced seed set caused by a female defect (Alandete-Saez et al., 2008). The discovery of these different gamete surface proteins with potential roles in signaling during pollen tube guidance and fertilization suggest complex communications between the different cells of the male and female gametophyte (Borg et al., 2009).

Several other interesting germline-specific genes are identified by screening of *Arabidopsis* pollen defective mutants. These genes include *DUO1*, *DUO2*, *DOU3*, *MGH3/HTR10*, *HAP2/GCS1*, *HAP5*, and *HAP12*.

Arabidopsis duo1 and *duo2* mutants appear to have normal pollen morphology but generative cell division is blocked due to the formation of bicellular pollen at anthesis (Durbarry et al., 2005). Both *duo* mutants progress normally through the first haploid division at pollen mitosis I (PMI) but fail at distinct stages of generative cell cycle. The generative cells in *duo1* pollen complete S-phase but fail to enter mitosis II at G2-M transition, whereas the generative cells in *duo2* pollen enter PMII but arrest at pro-metaphase suggesting a specific role for *DUO2* in mitotic progression (Durbarry et al., 2005). *DUO1* is expressed exclusively in the male germline, with expression first detected in the germ cell soon after asymmetric division at PMI (Rotman et al., 2005). A map-based cloning experiment revealed *Arabidopsis* gene At3g60460 corresponds to mutations in *duo1* mutants. *DUO1* encodes a novel R2R3-MYB transcription factor that is expressed specifically in the male germline and the DUO1 protein reportedly accumulated in the nucleus of the generative and sperm cells, with a proposed function of promoting the generative cell by activating specific targets such as cyclin genes.

DUO3 has overlapping roles with *DUO1* in male germ cell division and sperm cell specification (Brownfield et al., 2009a; Brownfield et al., 2009b). *DUO3* is conserved throughout the land plants and contains motifs conserved in the GONADLESS-4 (GON-4) protein, a cell lineage regulator of gonadogenesis in *Caenorhabditis elegans* (Friedman et al., 2000). The generative cells in mutant *duo3-1* either fail to divide or show a delay in division, unlike *DUO1*, *DUO3* which promotes entry into mitosis independent of the G2/M regulator *CYCB1;1*. Further studies indicate that *DUO3* is a positive regulator of germ cell fate, and like *DUO1*, *DUO3* is required for the normal expression of the germline markers *GCS1* and *GEX2*. However, *DUO3* is not required for *MGH3* expression, distinguishing the role of *DUO3* in sperm cell specification from that of *DUO1*. Furthermore, *DUO3* has an essential sporophytic role in cell division and embryo patterning. Together, *DUO3* plays essential developmental roles in cell cycle progression and cell specification in both gametophytic and sporophytic tissues (Brownfield et al., 2009a; Brownfield et al., 2009b).

Pollen tube guidance has been known controlled by female gametophyte (FG) factors. Several female gametophytic genes, such as *AtFER* (*FERONIA*) (Escobar-Restrepo et al., 2007), *ZmEA1* (*Zea mays EGG APPARATUS1*) (Marton et al., 2005), *TfLUREs (Torenia fournieri Cysteine-Rich Polypeptides) (Okuda et al., 2009), AtMYB98* (Kasahara et al., 2005), and *AtCCG* (*CENTRAL CELL GUIDANCE*) (Chen et al., 2007) have been reported for their important roles in pollen tube guidance and reception. However, the recent report of *Arabidopsis* sperm-specific gene *HAP2* shows its unique function involved in pollen tube guidance and fertilization (von Besser et al., 2006; Frank and Johnson, 2009). *Arabidopsis* mutant *hap2* was identified through screening for

distorted inheritance (Johnson et al., 2004). *HAP2* is characterized as a sperm-specific gene. In defective mutant, generative cell divides normally but sperm cells fail to fuse with either the egg cell or the central cell (Johnson et al., 2004; von Besser et al., 2006). The absence of fertilization in *hap2* mutants is not due to defects in sperm development and migration of sperms within the pollen tube or pollen tube growth, but due to misdirecting pollen tube to its ovule targeting and failure of initiation of fertlization. *HAP2* was found to be allelic to *GCS1* (Mori et al., 2006) as described in previous paragraph. The data generated from different groups suggest that *HAP2* has unique function involved in pollen tube guidance and fertilization (Mori et al., 2006; von Besser et al., 2006; Frank and Johnson, 2009).

5.4.4. Genes involved in cell division during Pollen Mitosis I (PMI) and Pollen Mitosis II (PMII)

After the tetrad separates and releases from anther as free microspores, each microspore undergoes an asymmetric mitotic division (PMI) to produce a small generative cell and a larger vegetative cell. The vegetative cell exits the cell cycle in G_1 while the generative cell continues through a further round of mitosis at PMII to produce two sperm cells. Both PMI and PMII are critical for male gamete development.

The germline cells transcriptomic studies provide genome-wide snapshot for genes controlling cell cycle during male gametogenesis. Affymetrix ATH1 arrays of *Arabidopsis* four stages of male gametophyte development (Honys and Twell, 2004) and mature pollen (Pina et al., 2005) revealed that a significant number of cell cycle genes are expressed in pollen.

Cell divisions in plants are controlled by universally conserved molecular machinery across higher eukaryotes, and the core key components are Ser/Thr kinases called cyclin-dependent kinases (CDKs). Cell cycle progression depends on correct localization of CDKs that are regulated by phosphorylation, cyclin proteolysis, and protein-protein interaction (Boruc et al., 2010). Among 61 core cell-cycle genes predicted in a genome-wide analysis in Arabidopsis (Vandepoele et al., 2002), fifty-five genes were present on the ATH1 GeneChip. ATH1 array data generated from four stages of male gametophyte development (uninucleate microspores, bicellular pollen, tricellular pollen, and mature pollen) showed 45 CDKs (82% of 55) were expressed in the male gametophyte (Honys and Twell, 2004), and all CDK families and subfamilies were representated. The majority of gametophytic core cell-cycle genes showed similar expression profiles, with a decline in mRNA abundance after UNM stage to zero (or low levels) at TCP and MPG stages. This pattern is consistent with the termination of proliferation of the microspore and generative cell before pollen maturation. The dynamic expression of core cell cycle is presented in Figure I-4 based on Honys and Twell's additional data (Honys and Twell, 2004).

ATH1 GeneChip array analysis revealed that 23 of the 55 genes analyzed are expressed in mature pollen grains (Pina et al., 2005). The comparison of pollen core cell cycle genes expression profile with vegetative tissues is shown in Figure I-5. The chart is generated from data list in Pina, 2005 (Pina et al., 2005).

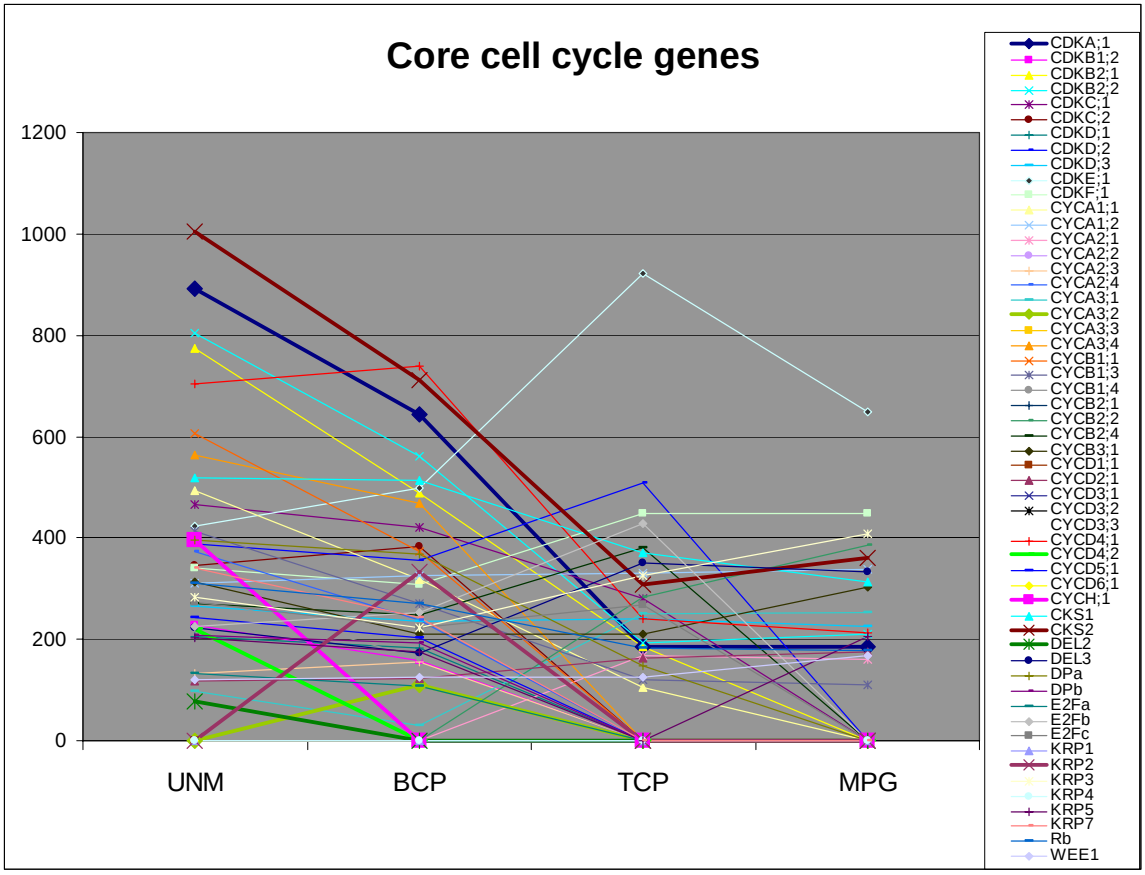

Figure I-4. *Arabidopsis* male gametophyte transcriptomic expression profiles of core cell cycle genes in haploid spores of different developmental stages. The chart is generated from additional data presented by Honys and Twell (Honys and Twell, 2004). UMN, uninucleate microspores; BCP, bicellular pollens; TCP, immature tricellular pollens; MPG, mature pollen grains.

Sperm cells in Arabidopsis stay in the S-phase at anthesis and continue through the cell cycle during pollen tube growth to reach G2 just prior to fertilization (Friedman, 1999). The vegetative nucleus is thought to be arrested in G1. These data together with a recent schematic overview of the mechanistic regulation of the G1/S transition in plants (De Veylder et al., 2003), suggest that the block could be achieved by a combination of

absence of essential components needed for the G1/S transition and an up-regulation of potential repressors and of potential factors of increased cell cycle duration (Pina et al., 2005).

Both ATH1 array analyses showed overlapping gene expression profiles (Honys and Twell, 2004; Pina et al., 2005). Interestingly, the D3-type cyclin *CYCD3;1*, *CYCD3;2*, and *CYCD3;3* were not expressed in pollen in both datasets. D3-type cyclin has been characterized as promoting S-phase entry (Schnittger et al., 2002; Dewitte et al., 2003). It can form complexes with A-type cyclin-dependent kinases (CDK). The CDK interacting proteins CKS1 and CKS2 are expressed in pollen, and CKS2 is highly enriched. Overexpression of *CKS1* leads to growth inhibition in roots through increased cell cycle duration, associated with an equal extension of both the G1 and G2 phases (De Veylder et al., 2001). *CKS2a* assumably has similar function with *CKS1*. Its over-expression in pollen might be an additional factor inhibiting cell cycle progression. The detailed analysis of the complement of mRNAs in pollen encoding CDKs and cyclins reveals a rather unexpected picture. Following a schematic overview of the mechanistic regulation of the G2/M transition (De Veylder et al., 2003), pollen seems to feature most of the transcripts needed for the G2/M transition, although they seem to be kept in their inactive state.

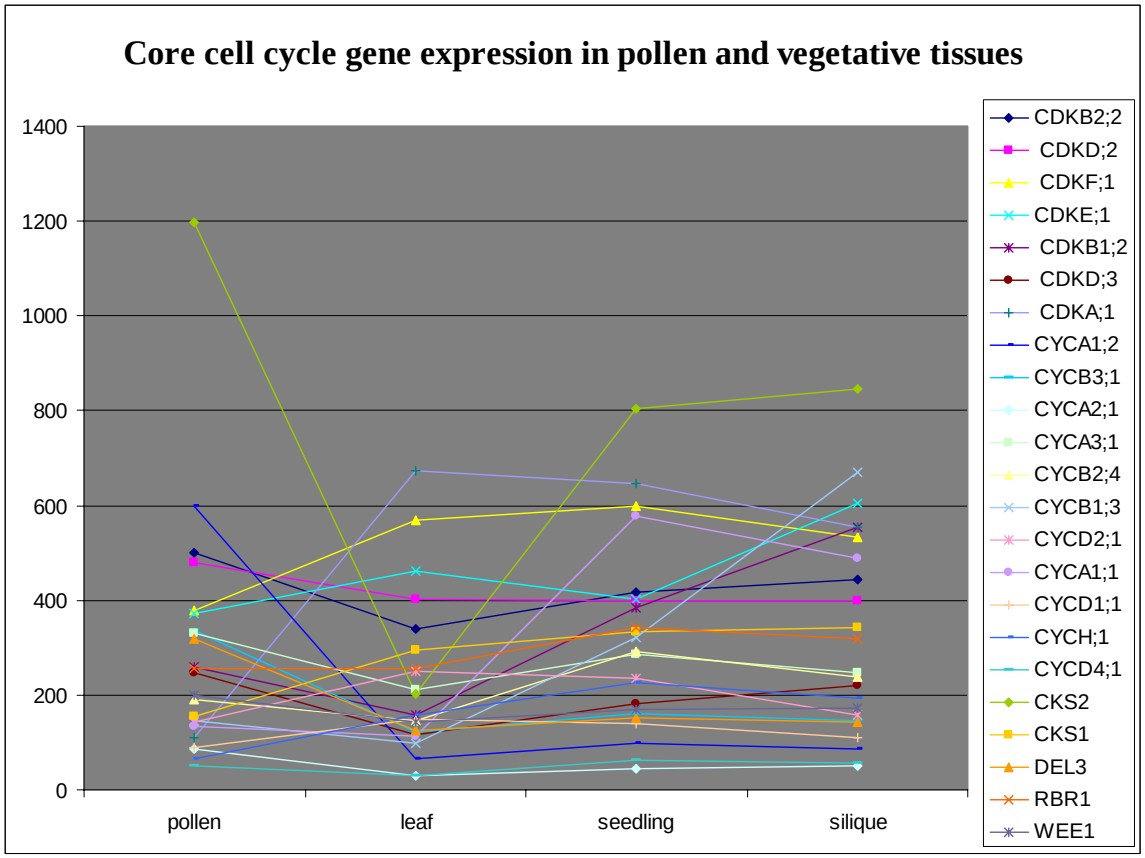

Figure I-5. Core cell cycle gene expression profile in pollen, leaf, seedling and silique. The chart is generated from Pina 2005 (Pina et al., 2005).

The *Arabidopsis* sperm Genechip ATH1 array showed that the cell cycle genes are also highly enriched in sperm cells (Borges et al., 2008).

The genetic studies have also provided insights into the genes important during cell cycle progression. The analysis of Chromatin Assembly Factor-1 (CAF-1) pathway mutants (*fas1, fas2, msi1*), indicates that chromatin integrity is also important for germ cell division (Chen et al., 2008). The loss-of-function mutants in CAF-1 pathway display a delay and arrest of cell cycle during pollen development. The *fas1, fas2* and *msi1* mutants showed range of phenotypes with some pollens arresting before PMI, some arresting before PMII, and some successfully dividing to produce tricellular pollen. This

indicates that the CAF-1 pathway has a wide role in male gametophyte cell division that could involve direct or epigenetic deregulation involving nucleosome and chromatin reassembly following replication (Chen et al., 2008). CAF-1 deficient pollens are able to fertilize and the bicellular pollen correctly expresses germ cell-fate markers (Chen et al., 2008).

A number of mutants have been identified in *Arabidopsis* in which bicellular pollen (a single germ cell within the vegetative cell) is produced due to a failure of PMII. Analysis of T-DNA insertion mutants in the single A-type cyclin-dependent kinase *CDC2A* (homolog of *CDC2,* also called *CDKA;1*) in *Arabidopsis* revealed an essential role in germ cell division (Iwakawa et al., 2006; Nowack et al., 2006). In *cdka;1* mutants, PMII (generative cell division) fails and DNA synthesis (S) phase of the cell cycle is delayed. However, this single germ cell is able to fertilize exclusively with the egg cell.

A similar phenotype of the *cdka;1* mutant was observed in the *fbl17* (the *F-box-Like 17*) mutant (Kim et al., 2008). F-box proteins associate with Skp1 and CUL1 to form SKP1-CUL1-F-box protein (SCF) E3 ubiquitin protein ligase complexes. These SCF complexes are involved in the ubiquitination of proteins targeted for proteasome-dependent degradation (Petroski and Deshaies, 2005). Substrate specific F-box proteins play a critical role in controlling cell cycle and diverse developmental processes through targeted degradation of various proteins (Cardozo and Pagano, 2004). *FBL17* is transiently expressed in the male germline after PMI and targets the CDK inhibitors *KRP6* and *KRP7* for proteasome-dependent degradation, enabling the germ cell to progress through S-phase (Kim et al., 2008). Conversely, vegetative cell cycle progression is inhibited since *FBL17* is not expressed in the vegetative cell and persistent

levels of *KRP6/7* continue to inhibit *CDKA;1*. The *fbl17* mutant pollen phenotype and its similarity to *cdka;1* mutant pollen can be explained by stabilization of *KRP6/7* in the germ cell in the absence of *FBL17*, resulting in continued inhibition of *CDKA;1*. Germline-specific expression of *FBL17* thus enables differential control of the cell cycle in the germ and vegetative cells, and allows the progression of germ cells through S-phase (Kim et al., 2008; Borg et al., 2009).

A single germ cell phenotype is also present in *duo pollen* (*duo*) mutants as reviewed in 5.4.3. In these mutants, asymmetric microspore division at PMI is completed, but the germ cell fails to undergo cell division at PMII. Unlike *fbl17*, *cdka;1* and CAF-1 pathway-deficient mutant pollen, *duo1* pollen cannot fertilize. This suggests that, in addition to cell cycle defects, key features of gamete differentiation and function are incomplete in *duo1*. *DUO1* may therefore act as a germ cell fate determinant linking cell division and gamete specification. *DUO1* orthologues are present throughout the angiosperms (Rotman et al., 2005; Borg et al., 2009).

5.4.5. Transcriptional regulation of male germ-line-specific gene expression

As previously mentioned, transcriptome analyses and mutant screening have identified a significant number of male germline-specific genes which are expressed exclusively in flowering plants. Naturally we want to know the transcriptional regulation control of the cell specificity of male germ-cell-specific genes. Germline Restrictive Silencing Factor (GRSF) identified in lily (Haerizadeh et al., 2006) has illustrated one such mechanism.

Singh et al. (Singh et al., 2003) demonstrates that a 0.8 kb promoter sequence of *LGC1* is sufficient to regulate the expression of reporter genes in a cell-specific manner. The transient expression of GFP driven by the promoter in lily pollen was only observed

in the generative cell, while stable transgenic tobacco with *P-LGC1::GUS* also showed the generative cell-specific expression, indicating that the transcriptional factors required to control the specificity of expression of *LGC1* promoter are conserved in male germ-line cells. Deletion analysis of the *LGC1*promoter showed the presence of a 43 bp nucleotide regulatory silencer element whose absence from the promoter led to a constitutive pattern of expression of the truncated promoter in all the plant tissues tested. Gel retardation assays showed that nuclear extracts of lily petal cells contain a protein that specifically interacts with the *LGC1* silencer sequence (Singh et al., 2003). The gene encoding this repressor protein was recently cloned by southwestern blotting of a lily petal cDNA expression library (Haerizadeh et al., 2006). The protein named GRSF is a DNA-binding repressor protein encoded by a gene expressed ubiquitously in plant tissues with the exception of generative cells. Immunolocalization showed that GRSF is present in the nuclei of uninucleate microspores and pollen vegetative cells but is absent in the generative cell nucleus. Chromatin immunoprecipitation assays showed that GRSF interacts with *LGC1* promoter and the male germline specific histone *gcH3*. Promoter mutagenesis experiments led to the identification of a conserved 8-bp motif in the *LGC1* and *gH3* promoters. This sequence motif is likely to be core-binding site for GRSF. These evidences support the hypothesis that the expression of male germ-cell-specific genes is controlled by GRSF through repressing their expression in non-germline cells (Haerizadeh et al., 2006; Singh and Bhalla, 2007).

Interestingly, the putative GRSF binding sites have been found in the promoter regions of three *Arabidopsis* germline expressed genes, *DUO1*, *MGH3*, and *GEX2*. These *Arabidopsis* genes may be direct targets of GRSF or a similar functionally conserved

repressor that represses their expression in non-male germline cells (Haerizadeh et al., 2006). However, the core GRSF-binding domain is not conserved in the promoter regions of *AtGEX1* and *GCS1/HAP2* which are only expressed in sperm cells, suggesting that they could be regulated by a different, GRSF-independent mechanism. A model for male germline development control proposed by Borg et al based on the data from lily and *Arabidopsis* is presented in Figure I-6 (Borg et al., 2009).

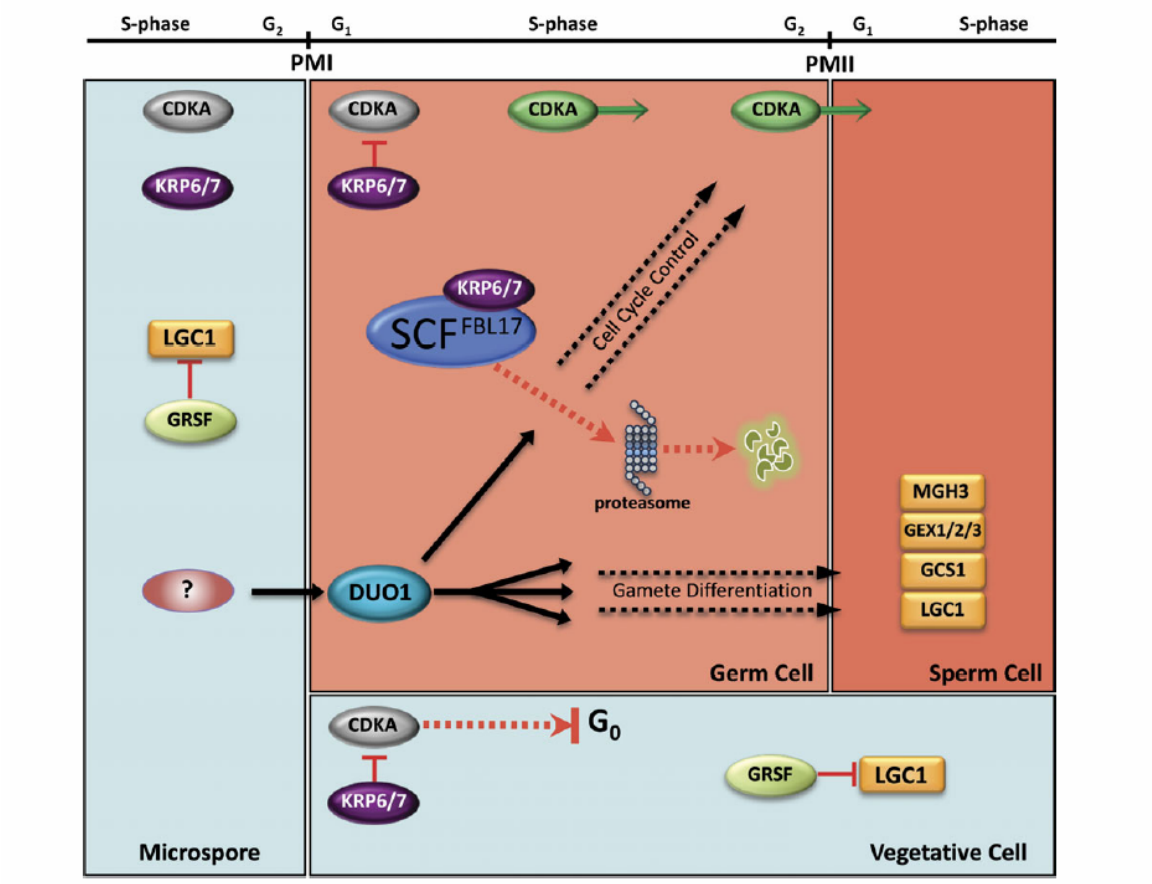

Figure I- 6. A schematic model for male germline specification and maintenance with data incorporated from lily and Arabidopsis proposed by Borg and Twell (Borg et al., 2009).

VI. Summary and Perspectives

Double fertilization in flowering plants requires that pollen and pollen tube precisely deliver a pair of sperm cells to the embryo sac to fuse with their female partners – egg cell and central cell to form the zygote and endosperm, respectively. Highly specialized pollen grains consisting of one vegetative cell and one generative cell or two sperm cells are considered as a key player for successful double fertilization. Recent advances in the molecular mechanisms underlying male germline initiation and male gametophyte development reviewed in this chapter help us better understand double fertilization from a new angle beyond morphology and cytology.

Pollen development starts from diploid sporogenous cells and undergoes meiosis to produce microspores. Genes controlling anther development and meiosis are mainly identified from male sterile mutant screening in *Arabidopsis*. The comprehensive transcriptomic studies for male gametophytes from several different plant species have extended our knowledge about the complexity and dynamics of haploid gene expression in the developing gametophyte and germline cells at a genome level. In coupling with both forward and reverse genetic approaches, a number of genes have been identified that are expressed exclusively in germlines. These genes regulate germline specification and gametophyte formation (Twell et al., 2006; Singh and Bhalla, 2007; Singh et al., 2008; Borg et al., 2009).

However, progresses in molecular mechanisms in male germline development are mainly from a few model plants. For other unique plant systems for reproduction study such as *Plumbago* and *Torenia*, lacking of genomic sequence and genetic tools limits functional characterization for interesting genes. The next generation sequencing

technology appears to be a very powerful tool for reproduction studies in plants which have not been tractable with traditional genomics approaches. Using the new sequencing technology, we can quickly and cheaply profile expressions of coding genes, non-coding genes and small RNAs, and monitor epigenetic status of the genome. Comprehensive gene regulatory networks can be obtained by analyzing these different types of data using systems biology approach.

Despite recent findings on GRSF transcriptional repression to differentiate somatic from germ cell lineages in lily (Haerizadeh et al., 2006), other regulatory mechanisms for male germ-cell differentiation are likely to exist and should be uncovered. Studies to define consensus cis-element motifs and/or shared transcriptional factors that control the male germ-line specificity of different gene clusters will likely yield valuable data (Singh and Bhalla, 2007).

miRNAs and siRNAs are small regulatory RNAs which regulate gene expression at transcriptional and post-transcriptional levels. Many biological processes seem to be regulated by these small RNAs. The latest finding on sperm-specific siRNA regulation in facilitating gametophyte formation and double fertilization (Ron et al., 2010) opens a new avenue for study miRNA and siRNA pathway in gamete development.

The application of proteomic technologies in this field will further define the developmental synthesis and functional roles of proteins involved in germline development, pollen tube growth and fertilization (Becker, 2007; Chen et al., 2007).

In conclusion, although much of knowledge on male gametophyte development has been gained through transcriptome expression profiling and genetic approaches in last two decades, much remains to be discovered and uncovered.

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Chapter II

Functional Analysis of *Plumbago zeylanica* Sperm-Specific Isopentenyltransferase (IPT) Gene in Heterologous Systems Reveals Its Role in Gametophytic Cell Division

ABSTRACT

Plumbago zeylanica is a unique model plant for the study of flowering plant heterospermy and preferential fertilization. During double fertilization, one sperm cell, which is physically associated with the **v**egetative **n**ucleus (S_{vn}) and possesses more mitochondria, will fuse with central cell to form endosperm. Whereas, the other sperm cell (S_{ua}), which is **u**n**a**ssociated with the vegetative nucleus and is rich in plastids, will fuse with the egg to form zygote. In order to determine whether gene expression differences in the two sperm cells of *Plumbago zeylanica* relate to their fertilization fate, populations of S_{ua} and S_{vn} sperm cells were isolated from mature pollen and representative cDNA libraries were constructed. We also employed the suppression subtractive hybridization (SSH) and cDNA microarray to identify cell type-specific genes in each sperm type. Among the differentiatially expressed transcripts, one group of ESTs which are highly up-regulated in S_{vn} seems to encode isopentenyltransferase (IPT). We obtained the full length cDNA and promoter sequence of the *Plumbago zeylanica IPT* gene, named *PzIPT1*. Expression analysis of *PzIPT1* using pollen whole mount *In situ* hybridization, semi-quantitative and quantitative RT-PCR showed that *PzIPT1* was highly expressed in S_{vn}, but much lowly expressed in S_{ua} and pollen. We further characterized the gene's function in *Arabidopsis* and tobacco. To examine the expression specificity of the gene in cell/tissue types, we transformed *Arabidopsis* and tobacco with reporter genes of GFP and GUS driven by the *PzIPT1* promoter. The

pPzIPT1::GFP/GUS in *Arabidopsis* are predominantly expressed in the sperm cell and synergids. In tobacco, however, the reporter genes are expressed in the vegetative cells of pollen and ovule. In transgenic *Arabidopsis,* the reporter genes are also detected in vegetative tissues including maturation zone of root, leaf of young seedling, tip of mature rosette leaf and cauline leaf. The expression pattern of *pPzIPT1::GFP/GUS* is different from those of nine *Arabidopsis IPTs promoter::GUS*, suggesting that *PzIPT1* is functionally different from known vegetative expressed *IPTs.* Overexpression of *PzIPT1* driven by *AtIPT3* promoter can restore Arabidopsis *AtIPT3, 5, 7* knockout triple mutant *atipt357* phenotype. Cytokinin oxidases (CKX) are enzymes that catalyze the irreversible degradation of *N6*-substituted purine cytokinins and can be used for cytokinin-deficient phenotype analysis. We used *PzIPT1* promoter to drive *AtCKX1* expression in *Arabidopsis*, and the transgenic plants showed reduced seed setting but otherwise were normal. The detailed cytological study showed that cytokinin deficiency is responsible for defects in both male and female gametogenesis. The plant with insufficient cytokinin has the first gametophytic mitosis blocked. The defective microspore and megaspore were arrested in uninucleate stage. These results suggest that *PzIPT1* is a gametophyte-specific *IPT* and plays an important role in gametophytic cell division. We also showed that *PzIPT1* paternal transcripts and proteins can be transmitted to the embryo sac and may initiate early cell division in endosperm and embryo development.

Key words: isopentenyltransferases, cytokinin, cytokinin oxidases, gametophyte development, sperm cell, pollen, ovule, cell division, paternal transmission, *Plumbago zeylanica*, *Arabidopsis*, tobacco.

Abbreviations: IPT – isopentenyltransferases; CK – cytokinin; CKX – cytokinin oxidase; EST – expressed sequence tag; HAP – hours after pollination; S_{vn} – sperm cell associated with the **v**egetative **n**ucleus; S_{ua} – sperm cell **u**n**a**ssociated with the vegetative nucleus; PMI – pollen mitosis I; PMII – pollen mitosis II; WT – wild type; FACS - fluorescence-activated cell sorting; VN – vegetative nucleus; DAPI – 4',6-diamidino-2-phenylindole.

INTRODUCTION

Double fertilization is a sexual reproduction system unique to flowering plants. In the system, three parts of plant are involved: pollen (or the Male Germ Unit, MGU) (Mogensen, 1992), embryo sac (or the Female Germ Unit, FMU) (Huang and Russell, 1992), and the sporophytic tissue (mainly tapetum in anther and female reproductive tract) (Ma, 2005). During the double fertilization process, twin sperm cells are transported to embryo sac via a pollen tube and fuse with their female partners – the egg cell and central cell to form zygote and endosperm, respectively (Russell, 1992; Southworth, 1996; Lord and Russell, 2002; Raghavan, 2003; Weterings and Russell, 2004; Berger, 2008). The twin sperm cells are not always identical. They may differ in size, shape, organelle types and numbers, and nuclear DNA content (Carlson, 1969; Russell and Cass, 1981; Russell, 1984; Carlson, 1986; Mogensen, 1992; Saito et al., 2002; Weterings and Russell, 2004). The dimorphic sperms are called heterospermy cells. Sperm cell dimorphism may be common in flowering plants, as a survey of 19 plant species found that 17 displayed dimorphism (Saito et al., 2002; Weterings and Russell, 2004). In plants with sperm cell dimorphism, the sperm cells do not randomly fertilize the egg or the central cell. Rather, the sperm with a certain morphology selectively fuses with the egg while the other sperm

with a diffrernt morphology tends to fuse with the central cell. This phenomenon is called "preferential fertilization" (Russell, 1983; Russell, 1985).

Plumbago zeylanica is a well-documented model plant for both cytoplasmic heterospermy and preferential fertilization. Its mature pollen contains a vegetative nucleus and two strongly heteromorphic sperm cells (Russell and Cass, 1981; Russell, 1984). The sperm cell that physically associated with the **v**egetative **n**ucleus (S_{vn}) is the larger cell and contains many mitochondria (mean = 256.18) and few plastids (mean = 0.45). The other sperm cell (S_{ua}) **u**n**a**ssociated with the vegetative nucleus is smaller and contains abundant plastids (mean = 24.3), less numerous mitochondria (mean = 39.8) (Russell and Cass, 1981; Russell, 1984; Russell, 1986). During double fertilization, the dimorphic sperm cells, S_{ua} and S_{vn}, selectively fuse with the egg to form the zygote and central cells to form the endosperm, respectively (Russell, 1985; Russell, 1986). Because the embryo sac of *Plumbago* lacks synergids, a property that greatly simplifies the observation of events occurring inside the embryo sac, the plant is an ideal model to trace the fate of sperm cells during double fertilization (Russell, 1980; Russell, 1982; Russell, 1983). The differences between the two sperm cells may have different gene expression profiles that may determine the fate of the two sperm cells and affect post-fertilization development, since the male cytoplasm in this plant is known to be transmitted into female gametes during double fertilization (Russell, 1980; Russell, 1983; Weterings and Russell, 2004). Maize (*Zea mays*) is another example of heterospermy and preferential fertilization. Some lines of maize carry supernumerary B chromosomes which are frequently distributed unevenly at the second pollen mitosis. As a result, one sperm cell contains B chromosomes and the other has none (Roman, 1948). The fate of the two

maize dimorphic sperm cells is different: the sperm cell carrying B chromosomes is selectively targeted to the egg cell (Roman, 1948; Carlson, 1969, , 1986).

Although the cytology of double fertilization has been well established, the molecular mechanism of this process remained largely unknown until the last decade. As many genetic and genomic tools become available, such as genome sequences from a number of plants, high throughput gene expression profiling and FACS cell sorting; significant progresses have been made in understanding the molecular mechanisms controlling gametophyte development, double fertilization and early embryogenesis.

Sperm cells and their progenitor – the generative cell - are small in size, and therefore, it is generally believed that the sperms may be dependent on cytoplasm of vegetative cell for nutrition and transport. But studies in recent years have showed that male germ cells have their own enriched and unique molecular repertoire and gene regulatory networks (Twell, 2002; McCormick, 2004; Singh and Bhalla, 2007; Singh et al., 2008; Borg et al., 2009; Gou et al., 2009). Comprehensive transcriptomic studies for male gametophytes from lily, maize, *Arabidopsis,* rice and tobacco have extended our knowledge of the complexity and dynamics of haploid gene expression in the developing gametophyte and germline cells. In coupling with both forward and reverse genetic approaches, a significant number of genes have been identified expressed exclusively in the germline that regulate gamete specification and gametophyte formation (Twell et al., 2006; Singh and Bhalla, 2007; Singh et al., 2008; Borg et al., 2009). Over the last decade, several cDNA libraries have been constructed for generative cells of lily (*Lilium longi-florum*) (Xu et al., 1998; Okada et al., 2006a), sperm cells of rice (*Oryza sativa*) (Gou et al., 2001; Russell et al., 2010b), sperm cells of maize (*Zea mays*) (Engel et al., 2003), and

generative cells of tobacco (*Nicotiana tobacco*) (Xu et al., 2002). cDNA microarray and Affymetrix GeneChip array have been employed for comparative transcriptome studies of germ cells in *Arabidopsis*, lily, and rice (Okada et al., 2007; Borges et al., 2008; Russell, 2010). Data generated from the above studies have revealed a genome wide picture of the transcriptional profile of male germline cells. Functional characterization of these ESTs and microarray datasets shows that similar classes of genes are expressed in the germ cells of different species and many of the genes are conserved across species. These genes are involved in general metabolism, cellular organization, DNA synthesis, chromatin structure, and protein degradation. Interestingly, proteins involved in the ubiquitin-mediated proteolysis pathway are upregulated in plant germline cells. Ubiquitin-pathway-related proteins such as polyubiquitin, proteasome subunit, ubiquitin-conjugating enzyme, Skp1 and Ring box protein are highly upregulated in the generative cells of lily (Okada et al., 2007), sperm cells of *Arabidopsis* (Borges et al., 2008), sperm cells of *Plumbago* (Singh et al., 2002), and sperm cells of maize (Engel et al., 2003). Singh et al. (Singh et al., 2002) used cross hybridization with lily generative cell mRNA to screen sperm-expressed genes in *Plumbago*, and identified a clone which encodes a polyubiquitin gene with three ubiquitin repeats. Polyubiquitin genes from both species are specifically expressed in their germ cells (Singh et al., 2002). Moreover, the *Plumbago* polyubiquitin gene is expressed at a much higher level in mitochondria-rich sperm (S_{vn}) cells than in plastid-rich sperm (S_{ua}) cells (Singh et al., 2002; Singh et al., 2008). The recent finding and functional characterization of the F-box protein FBL17 provides another line of evidence supporting the importance of the ubiquitination pathway in male gametophyte development (Kim et al., 2008). Another conserved class

of genes encodes male germline-specific histone variants possibly involved in chromatin remodeling. Generative cell-specific expression of histone variants, particularly histone H3, is likely a common characteristic of the male germline in flowering plants (Xu et al., 1999a; Okada et al., 2005b; Singh et al., 2008). Histone H3 proteins are upregulated in germ cells of lily, maize, *Plumbago* and *Arabidopsis* (Engel et al., 2003; Okada et al., 2005a; Okada et al., 2005b; Okada et al., 2006b; Gou et al., 2009).

Since germline-expressed genes may play vital roles in germline development and fertilization, tremendous efforts have been made to identify such genes. The identified germline-expressed genes are involved in different biological processes. *LGC1* (*Lily Generative Cell-specific 1*) is the most impressive such gene that is exclusively expressed in the male gametic cells of lily. It was identified from a RNA gel blot with cDNA clones from generative cell cDNA libray (Xu et al., 1999b). The male gamete-specific expression and membrane surface characteristic suggested a putative function for *LGC1* in sperm-egg recognition and fusion (Xu et al., 1999b). *LGC1* homologs are also present in *Arabidopsis* and rice genomes. The *Arabidopsis* ortholog seems to be expressed only in generative and sperm cells (Singh et al., 2008). Deletion analysis of the *LGC1* promoter identified a 43 bp regulatory silencer element. GRSF (Germline Restrictive Silencer Factor) is a protein specifically interacting with the *LGC1* silencer sequence. GRSF gene seems to be expressed ubiquitously in all plant tissues but absent in generative cells (Haerizadeh et al., 2006). GRSF represses the expression of *LGC1* in non-germline cells (Haerizadeh et al., 2006; Singh and Bhalla, 2007).

GCS1 (Generative Cell-Specific 1) is another lily generative cell-specific gene isolated from lily male germ cells by differential display (Mori et al., 2006). The

knockout mutant in *Arabidopsis GCS1* ortholog *HAP2* specifically blocks male transmission and fertilization (Mori et al., 2006) (von Besser et al., 2006; Frank and Johnson, 2009). In the mutant, sperm cells are observed within the degenerated synergid but fail to fuse with the female gametes (Mori et al., 2006). GCS1 protein is not similar to any genes of known functions and has no obvious functional motifs. Functional analysis from both groups show HAP2/GCS1 protein is associated with sperm surface and essential for successful gamete attachment, fusion, or both (Mori et al., 2006; von Besser et al., 2006; Frank and Johnson, 2009). *GCS1* orthologs from *Arabidopsis*, rice, green and red algae, and in slime mould and protozoan parasites share a conserved domain structure, suggesting a fundamental role in membrane fusion during fertilization.

AtGEX1, *AtGEX2* and *AtGEX3* (*Gamete Expressed 1*, 2, *3, GEX1, 2, 3*) are *Arabidopsis* genes expressed in sperm cells identified by comparative analysis of maize sperm cell specific ESTs with *Arabidopsis* sequences (Engel et al., 2005; Alandete-Saez et al., 2008). These three genes belong to a membrane-associated protein family expressed in male germ cells in *Arabidopsis*. A number of *Arabidopsis* germline-specific genes such as *DUO1, DUO2, DUO3, MGH3/HTR10, HAP1, HAP5, and HAP12* were identified by screening of *Arabidopsis* pollen defective mutants. *DUO1 and DUO2* are germline specific and can block generative cell division, resulting in the formation of bicellular pollen at anthesis (Durbarry et al., 2005). *DUO1* encodes a novel R2R3-MYB transcription factor which is expressed specifically in the male germline and the protein accumulates in the nucleus of generative and sperm cells. *DUO1* may function to promote generative cell by activating specific targets such as cyclin genes (Rotman et al., 2005). *DUO3* has overlapping roles with *DUO1* in male germ cell division and sperm

cell specification (Brownfield et al., 2009a; Brownfield et al., 2009b). The generative cells in mutant *duo3-1* either fail to divide or show a delay in division. *DUO3* plays essential developmental roles in cell cycle progression and cell specification in both gametophytic and sporophytic tissues (Friedman et al., 2000).

As above described, most mutant screenings for and transcriptomic studies on the sperm cell-expressed and specific genes are in *Arabidopsis* and lily, whose sperms are not dimorphic. Therefore, findings from these studies are not directly applicable to the understanding of the double fertilization process for plants with dimorphic sperms. Although maize is a good genetic system for molecular characterization of fertilization (Dresselhaus et al., 1994; Engel et al., 2003; McCormick, 2004; Marton et al., 2005), the sperm cell dimorphism and preferential fertilization is characterized by the differences in the number of B-chromosomes in the sperm (Rusche et al., 1997) and may be different from cytoplasmic heterospermy represented by *Plumbago zeylanica*. To determine whether gene expression differences relate to fertilization fate in the two sperm cells of *Plumbago*, we have employed a series of tools to characterize the molecular mechanisms controlling the sperm dimorphism and preferential fertilization. The tools include the isolation of two populations of S_{ua} and S_{vn} sperm cells from the mature pollen (Zhang and Russell, 1998), representative cDNA library construction (Gou, Yuan, Wei & Russell, 2009), expression profiling by EST sequencing, subtractive cDNA library construction, and microarray analysis. We obtained 826 ESTs from S_{ua} and 606 ESTs from S_{vn} representative cDNA libraries. Gene Ontology (The Gene Ontology Consortium, 2000) annotation of these ESTs suggests a strong transcriptome divergence in the two *Plumbago* sperm cell types S_{ua} and S_{vn} (Gou, Yuan, Wei & Russell, 2009). The most

abundant ESTs are involved in protein turnover and cell maintenance, suggesting that ubiquitin-dependent proteolysis may play a critical role in gametogenesis, zygote initiation and early embryo development. We randomly selected a total of 4,608 SSH clones for microarray analysis. Clones with 4-fold or higher expression difference between the S_{ua} and S_{vn} clones were sequenced. A total of 106 up-regulated clones from the S_{ua} and 149 up-regulated clones from the S_{vn} were obtained with high quality EST sequences. Interestingly, one group of transcripts is S_{vn} sperm-specific and also highly expressed. 11 clones have been found among the 149 up-regulated clones of the S_{vn}. Sequence comparison suggest that this group of transcripts is homologous to isopentenyltransferase (IPT) gene (Gou, Yuan, Wei & Russell, 2009). The IPT enzyme catalyzes the first step of cytokinin biosynthesis and governs the rate limiting steps in cytokinin biosynthesis (Kakimoto, 2003). It is already known that, in *Arabidopsis*, the expression of *IPTs* is highly correlated with endosperm growth (Miyawaki et al., 2004). Because *Plumbago* S_{vn} sperm specifically targets central cell during double fertilization to produce endosperm, this S_{vn} up-regulated *IPT* may play an important role in endosperm development. As the sperm cytoplasm is known to be transmitted during fertilization in *Plumbago* (Russell, 1980), sperm-contributed *IPT* mRNA and protein are probably transmitted during fertilization and could affect early endosperm development. We therefore propose that S_{vn} sperm IPT is transmitted to the endosperm during its fusion with the central cell, and the transmitted IPT initiates endosperm cell division and development.

To test our hypothesis, we cloned the full-length cDNA sequence and the promoter sequence of this gene for further expression and function analysis. Full-length

cDNA of the *Plumbago IPT* was recovered from the S_{vn} cDNA library via plaque hybridization screening. The screening yielded several positive clones with very similar sequences. The sequence, designated as *PzIPT1*, was used for further functional analysis (Yuan, 2007). The deduced polypeptide sequence (shown in Figure II-1) indicates the presence of an ATP/GTP binding signature motif GxxxGK(S/T) of IPTs (Saraste and Wittinghofer, 1990) in PzIPT1 at the N-terminal (22-33 position), and the whole protein sequence matches the common pattern of IPTs: GxTxxGK[ST]xxxx[VLI]xxxxxxx[VLI][VLI]xxDxxQx(57,60)[VLI][VLI]xGG[ST], where x represents any amino acid residue, and x(m,n) denotes amino acid residues of m to n in number (Kakimoto, 2001; Kakimoto, 2003). The structure of the predicted amino acid sequence of the full-length cDNA of *PzIPT1* indicates that *PzIPT1* belongs to the family of *IPTs* (Yuan, 2007).

Our preliminary results from semi-quantitative RT-PCR, quantitative RT-PCR, and *in situ* hybridization revealed that *PzIPT1* is highly expressed in the S_{vn} (Yuan, 2007). We used RT-PCR and real-time RT-PCR approaches to get *PzIPT1* tissue expression profiles on a panel of *Plumbago* tissue types (roots, stems, leaves, sepals, petals, unpollinated mature ovaries, pollinated ovaries, unicellular pollen, bicellular pollen, mature pollen, sperm cell S_{vn} and S_{ua}) (Figure II-2A, II-2B). Of all the examined tissues, the *PzIPT1* transcripts can only be detected in the male gametophyte and male gamete (mature pollen and sperm cells) but not any other tissues. Since pollen contains sperm cells, the weak signal presumably reflects sperm cell transcripts. The results also show higher *PzIPT1* expression levels in S_{vn} compared to S_{ua}. The whole-mount *in situ* hybridization in mature pollen of *Plumbago* using an antisense *PzIPT1* RNA probe

detected a strong signal only in S_{vn}, but not in S_{ua} or the vegetative cell (Figure II-2C). These results strongly support that *PzIPT1* is preferentially expressed in S_{vn}.

We cloned the promoter of *PzIPT1* from a *Plumbago* genomic DNA library using a nested PCR technique. A large PCR product (1089 bp) was obtained from the upstream region of *PzIPT1* and the sequence analysis identified a typical TATA box (TATAAA) located at -29 from the transcription start site and a number of putative transcriptional regulatory elements (Yuan, 2007). The *PzIPT1* promoter does not possess the putative *GRSF* binding site which is found in the promoter regions of three germline expressed genes, *DUO1*, *MGH3*, and *GEX2* in *Arabidopsis* (Haerizadeh et al., 2006).

We have established a highly efficient tissue culture system in *Plumbago* (Wei et al., 2006). However, a stable transformation system for this species is not yet available. Thus, it is difficult to analyze functions of *PzIPT1* in *Plumbago*. As a model plant widely used for gene characterization, *Arabidopsis* was chosen for our experiments for *PzIPT1* promoter specificity and other general functional analysis. we also examined *PzIPT1* promoter activity in transgenic tobacco which has been reported for sperm cell dimorphism (Tian et al., 2001),

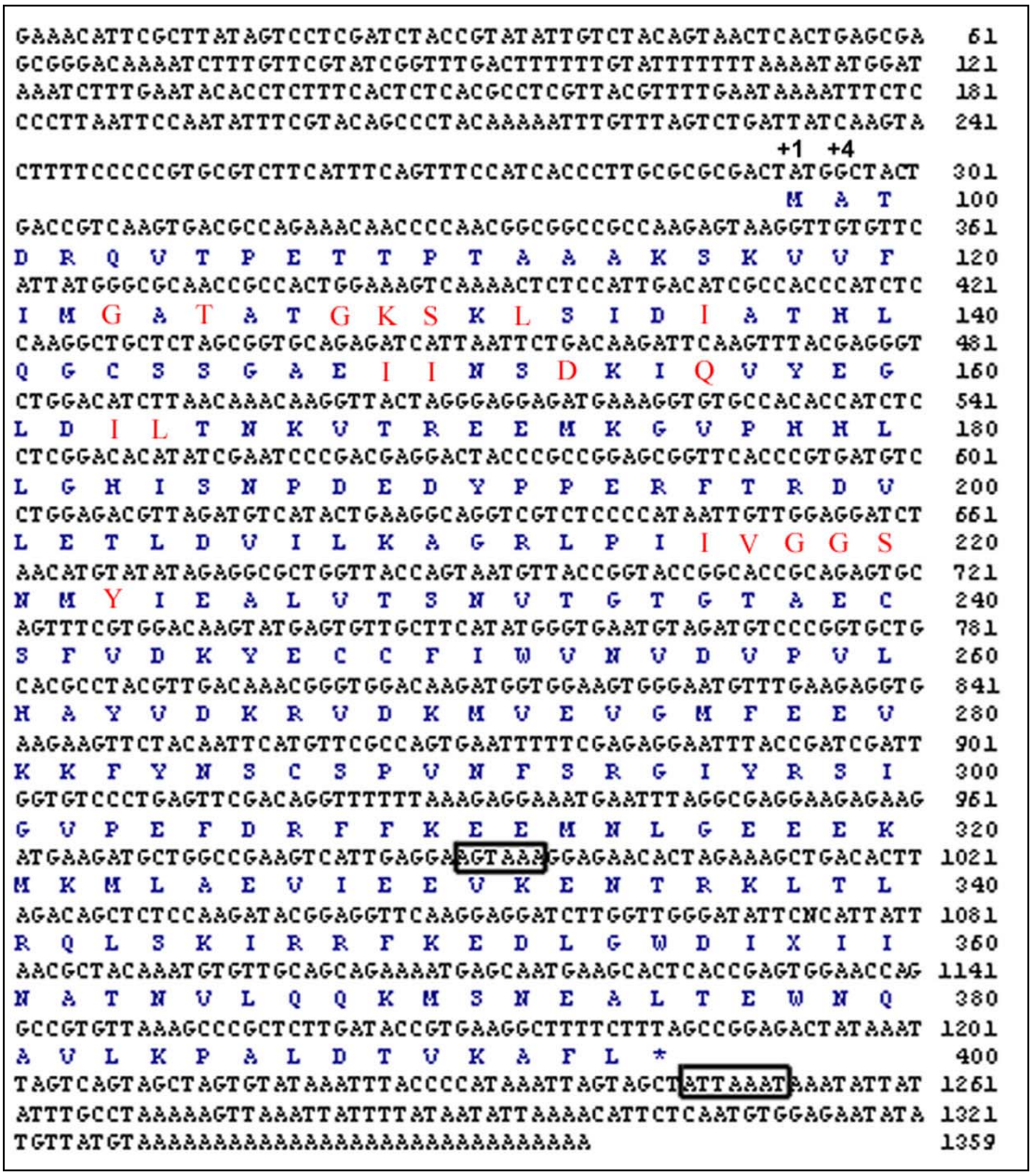


Figure II-1. Nucleotide and derived amino acid sequences of *PzIPT1* cDNA. Box indicates the putative polyadenylation consensus signals AGTAAA and (A)TTAAA(T). The consensus pattern of IPTs (GxTxxGK[ST]xxxx[VLI]xxxxxxx[VLI][VLI] xxDxxQx(57,60)[VLI][VLI]xGG[ST]) is shown in red letters. * = stop codon (Yuan, 2007).

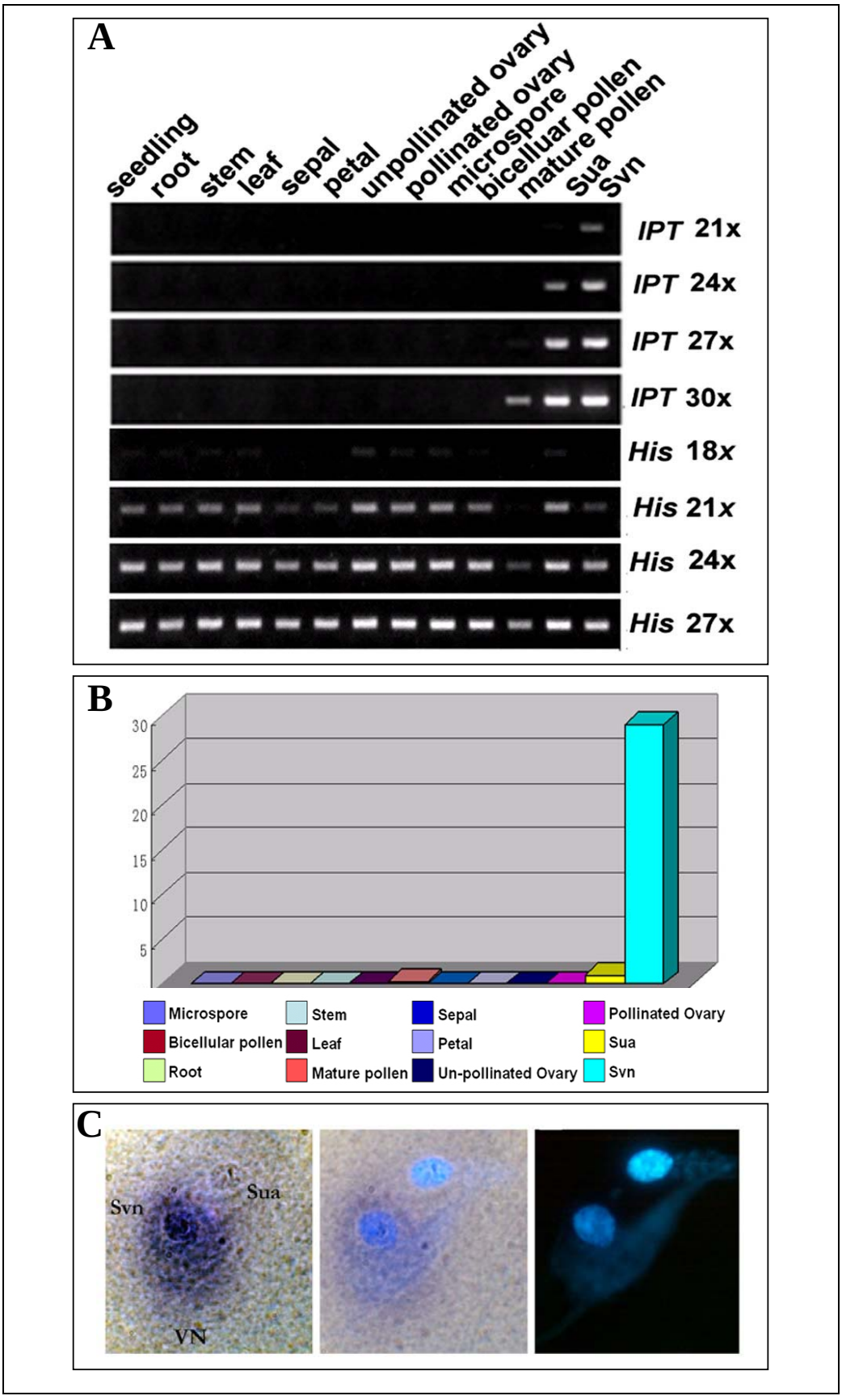

A
seedling
root
stem
leaf
sepal
petal
unpollinated ovary
pollinated ovary
microspore
bicellular pollen
mature pollen
Sua
Svn
IPT 21x
IPT 24x
IPT 27x
IPT 30x
His 18x
His 21x
His 24x
His 27x
B
30
25
20
15
10
5
Microspore
Stem
Sepal
Pollinated Ovary
Bicellular pollen
Leaf
Petal
Sua
Root
Mature pollen
Un-pollinated Ovary
Svn
C
Svn
Sua
VN

Figure II-2. Semi-quantitative RT-PCR, quantitative RT-PCR, and *in situ* hybridization verified that *PzIPT1* is highly expressed in S_{vn}. **A.** Semi-quantitative RT-PCR analyses of the expression patterns of *PzIPT1* in multi-tissues. The number in the right indicated PCR cycles. *PzIPT1* transcript is highly expressed in S_{vn}, with low expression in S_{ua}, and very low expression in other tissues. *Histone 3.3* was used as a loading control. **B.** Real-time PCR analysis confirms the results obtained from RT-PCR using 10ng cDNA from different tissues and cells. Different colors represent different samples; the expression level in S_{ua} was normalized to 1. **C.** *In situ* hybridization of mature pollen of *Plumbago zeylanica* with *PzIPT1* antisense probe shows that *PzIPT1* is exclusively expressed in S_{vn}. Left: brightfield microscopy; center: overlayed DAPI epifluorescence/brightfield microscopy; Right: DAPI epifluorescence microscopy. VN = vegetative nucleus (Yuan, 2007).

RESULTS

I. *PzIPT1* is a paralog of *Arabidopsis AtIPT3*

Cytokinins (CKs) are a group of phytohormones that play an important role in plant growth and development by regulating cell division and cell differentiation, leaf senescence, apical dominance, shoot meristem formation, root initiation, seed germination, flower development, photosynthesis, chloroplast differentiation and pathogen responses (Mok and Mok, 2001). The cytokinin content in plant cell is mainly determined by two opposite biological processes -- biosynthesis and degradation. Isopentenyltransferase (IPT), first identified from slime mold (Taya et al., 1978) and subsequently from *A. tumefaciens* (Barry et al., 1984), is a cytokinin biosynthesis enzyme

catalyzing the first and rate-limiting step of cytokinin biosynthesis. *IPTs* are found in different organisms, including bacteria, fungi, plants, insects and mammals. Figure II-3A shows a phylogenetic tree for deduced *PzIPT1* amino acid sequence and previously reported *IPTs*, generated by CLUSTALW program (http://www.ddbj.nig.ac.jp/). The phylogenetic tree has three branches: DMAPP:ATP/ADP isopentenyltransferases, DMAPP:AMP isopentenyltransferases, and DMAPP:tRNA isopentenyltransferases (Figure II-3A). *PzIPT1* is in the DMAPP: ATP/ADP isopentenyltransferase group. This group contains only plant *IPTs*, suggesting that DMAPP:ATP/ADP isopentenyltransferases play a major role in plant cytokinin biosynthesis (Kakimoto, 2001; Kakimoto, 2003).

In *Arabidopsis,* there are 9 *AtIPTs* identified computationally (Takei et al., 2001; Miyawaki et al., 2004). Among them, *AtIPT1, 3–8* shows DMAPP:AMP isopentenyltransferase activity (Takei et al., 2001); and *AtIPT2* and *AtIPT9* code for DMAPP : tRNA isopentenyltransferases (Takei et al., 2001). A phylogenetic tree built using *PzIPT1* and nine *Arabidopsis AtIPTs* is shown in Figure II-3B. *PzIPT1* is closely related to *AtIPT3* with 49% sequence similarity. pAtIPT3::GUS/GFP is highly expressed in the vegetative phase with strong GUS/GFP activities in the phloem of vascular tissue and weak activities in the pericycle of roots (Miyawaki et al., 2004; Takei et al., 2004). Quantitative RT-PCR showed the expression of *AtIPT3* in all organs, but at very low level in the reproductive tissues and siliques (Takei et al., 2004). Comparing to *Plumbago PzIPT1* expression pattern in quantitative RT-PCR and *in situ* hybridization experiments shown in Figure II-2, it appears that *PzIPT1* is a DMAPP: ATP/ADP isopentenyltransferase and may have similar function with *AtIPT3* but has distinctly

different tissue-specificity. Therefore, *PzIPT1* and *AtIPT3* seem to be paralogous rather than orthologous. The *Arabidopsis AtIPT3* is also a key determinant of CK biosynthesis in response to nitrate (Miyawaki et al., 2004; Takei et al., 2004). The loss-of-function mutant *atipt3* has no visible phenotype, but *atipt357* triple mutant and *atipt1357* quadruple mutant have short and thin plants, fewer rosette leaves, reduced shoot apical meristem size and thin inflorescence (Miyawaki et al., 2006). The *atipt1357* quadruple mutant also shows aborted seeds (Miyawaki et al., 2006).

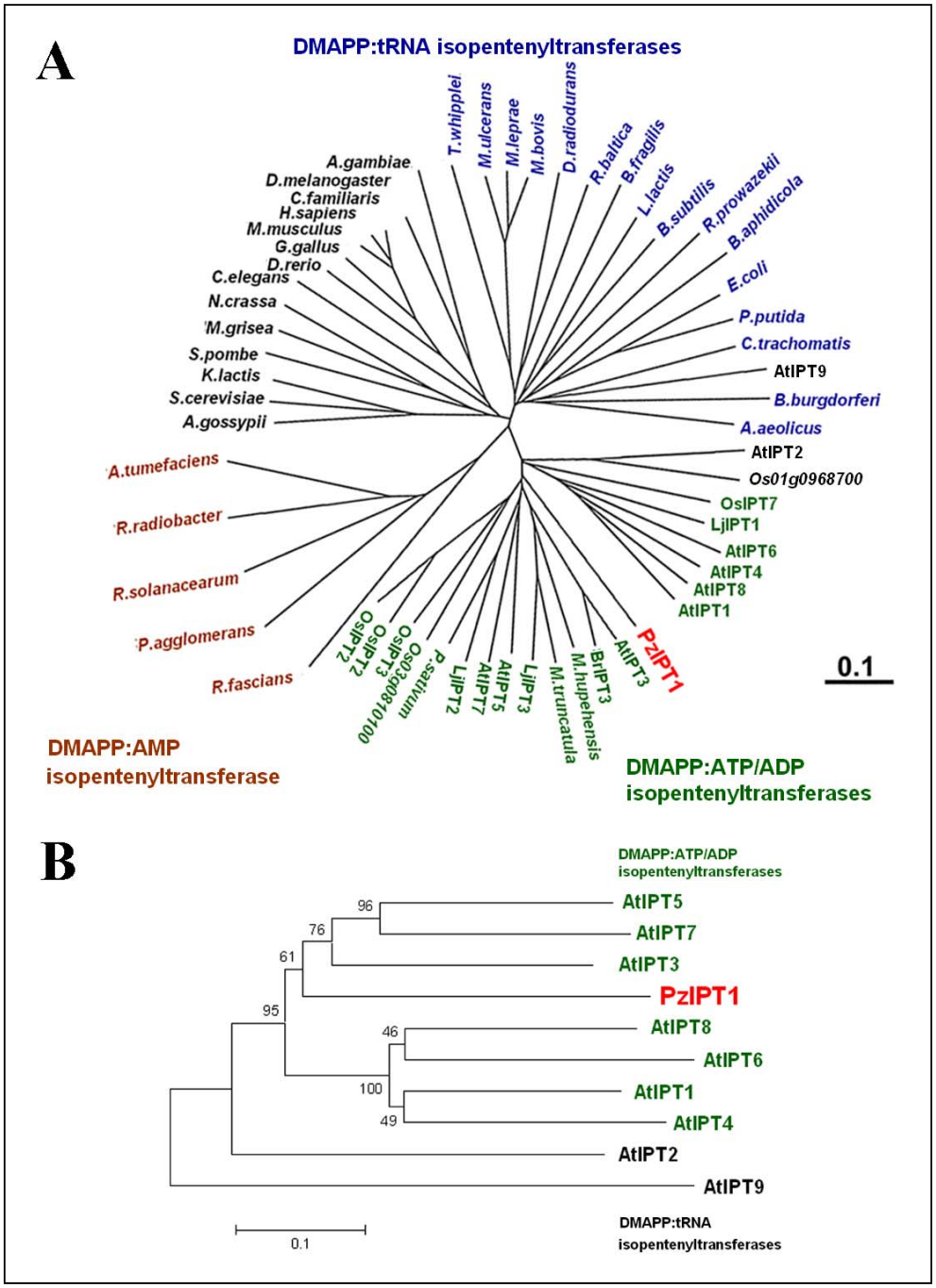


Figure II-3. Comparison of *PzIPT1* and previously reported *IPTs*

A. The phylogenetic tree of representative isopentenyltransferases. There are three groups of IPTs:DMAPP:AMP isopentenyltransferases, DMAPP:tRNA isopentenyltransferases, and DMAPP:ATP/ADP isopentenyltransferases. *PzIPT1* belongs to DMAPP:ATP/ADP

isopentenyltransferase. CLUSTALW program (http://www.ddbj.nig.ac.jp/) was used to generate the tree. Relative branch lengths are approximately proportional to phylogenetic distance. **B.** An unrooted phylogenetic tree generated from the amino acid sequences of *PzIPT1* proteins and its homologs in *Arabidopsis*. The *PzIPT1* shares high similarity with *AtIPT3*.

II. *PzIPT1* promoter::GUS/GFP expression in heterologous system

2.1. *pPzIPT1::GUS/GFP* expression pattern in transgenic Arabidopsis

Since the transformation system in *Plumbago zeylanica* has not been established, we examined the expression specificity of *PzIPT1* in *Arabidopsis* by observing the expression of *beta-glucuronidase* (GUS) (Jefferson et al., 1987) and green fluorescent protein (GFP) (Siemering et al., 1996; Tsien and Miyawaki, 1998) reporter genes driven by the *PzIPT1* promoter. The constructs of p*PzIPT1::GUS* and p*PzIPT1::GFP* (Figure II-4A) were introduced into *Arabidopsis* wild type (WT) Col-0 by floral dipping (Clough and Bent, 1998). We screened for homozygous lines in T3 generation based on the segregation of Basta resistance. Basically, we chose 3:1 (resistant : susceptible) segregation patterns in T2 generation and then selected all resistant line in T3 as homozygous line. For each construct, at least 5 independent transgenic homozygous lines were observed for reporter gene expression. The expression patterns described here are from the majority of the lines. The GUS histochemical expression assays were carried out following Jefferson's protocol with minor modifications (Jefferson et al., 1987), and GFP expression patterns were observed by epifluorescence or confocal microscopy. The expression of the reporter genes was examined in male and female gametophytes as well as vegetative tissues throughout the plant life circle. As expected, GUS and GFP are

expressed in both sperm cells, but not in vegetative cells of the pollen (Figure II-4B, C, D, and F). Because *Arabidopsis* sperm cells are not dimorphic, it is not surprising that the reporter genes are expressed in both sperm cells. Interestingly, reporter genes are also expressed in unpollinated and pollinated ovules (Figure II-4E, G, H and J), specifically in synergids of unpollinated ovule (Figure II-4E and J). Since *Plumbago* does not have synergids, the information about whether *PzIPT1* is expressed in synergids in *Plumbago* can not be determined.

To examine the dynamic expression of *PzIPT1*, we performed a time course of *pPzIPT1*::GUS/GFP expression in *Arabidopsis* ovule and pollen. The young flowers of *pPzIPT1::GUS* homozygous line were hand emasculated at floral mid stage 12 according to Smyth et al. and Schneitz et al. (Smyth et al., 1990; Schneitz et al., 1995). In this experiment, the time of 30 hours after emasculation is set as 0 HAP (hour after pollination). This is because that natural pollination occurs at stage 13, which is 30 hours after the mid stage 12. We sampled at different time points from -30 HAP to 96 HAP. For each time point, 30 - 50 siliques were collected and analyzed by GUS staining. Flower and ovule development stages are determined based on Christensen et al. (Christensen et al., 1997) and Smyth et al. (Smyth et al., 1990).

The GUS expression was observed in developing ovules at -30 HAP with even distribution within embryo sac (Figure II-5B). Anthers were manually removed at this time point which corresponds to flower development stage mid 12 and the ovule development early two-nucleate stage (stage FG2) (Figure II-5A). At -15 HAP when flower development stage is between mid - late 12 and the ovule is in late two-nucleate stage (stage FG3) or early four-nucleate stage (stage FG4) (Figure II-5A), GUS

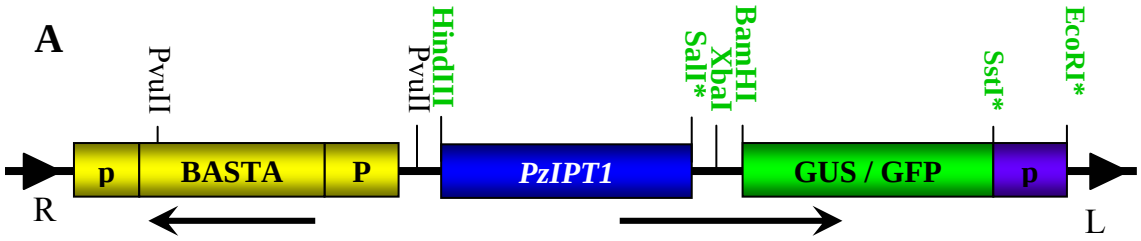
expression in the embryo sac increases in the micropylar end (Figure II-5C). At 0 HAP, when natural pollination occurs if anthers have not been removed, the flower development stages is between late 12 and 13, and the ovule is in early four-nucleate stage (stage FG4) or eight-nucleate stage (FG5) (Figure II-5A). GUS expression in the embryo sac becomes even stronger in the micropylar end (Figure II-5D). At +12 HAP, GUS expression is mainly distributed in the embryo sac near the micropylar end (Figure II-5E). Flower development stage at this time is between late 12 and 14 and the ovule reaches seven-celled stage (stage FG6) (Figure II-5A). The GUS expression pattern remains the same at +18 HAP (Figure II-5F) as that at +12 HAP. Flower development stage at +18 HAP is between 13 and 14 and the ovule is in four-cell stage (stage FG7) (Figure II-5A). Six hours later at +24 HAP, the ovule is mature and in four-celled stage (stage FG7) (Figure II-5A). The GUS expression is specifically restricted to the synergids in the embryo sac (Figure II-5G). The unpollinated ovule at +36 HAP is still at a four-cell stage (stage FG7) (Figure II-5A), and the GUS expression pattern does not change much from the previous time point (Figure II-5H). At +48 HAP, flower organs begin to fall off, and the unpollinated ovule is about to degenerate. A very weak GUS signal is detected in the micropylar end at this time point (Figure II-5I). At +96 HAP, no GUS signal is detected in the developing ovule and the unpollinated ovule has collapsed (Figure II-5J).

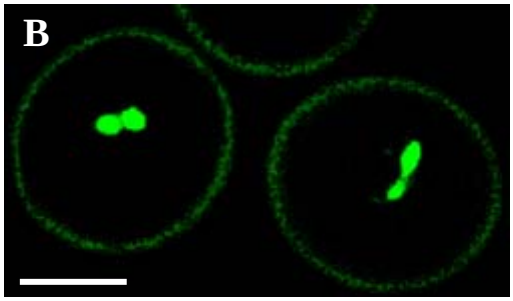
Transgenic *Arabidopsis* with the *pPzIPT1::GFP* construct was also used to follow the time course of GFP expression during the ovule and pollen development. GFP expression showed similar trend to GUS expression (data not shown). At +24 HAP, GFP signal was restricted in synergids (Figure II-5K). During pollen development, the GFP signal was detected early in the generative cell of bicellular pollen (Figure II-5L and 5M).

The time course study of reporter gene expression in male and female gametophytes suggests that the *PzIPT1* promoter is active during both male and female gametogenesis of *Arabidopsis*. The reporter genes driven by the *PzIPT1* promoter are expressed as early as the first gametophytic mitosis – GFP and GUS expression were detected in the generative cell of bicellular pollen (Figure II-5L and 5M) and ovule at two-nucleate stage of (Figure II-5B).

The systematic examination of *pPZIPT1::GUS* expression in transgenic *Arabidopsis* lines showed that GUS was also expressed in vegetative tissues too. In seeds imbibed for two days, no GUS expression was detected (Figure II-6A and B). But strong GUS signal was observed in radicles of 2-day old germinated seedlings (Figure II-6C). In 5-day old seedlings, GUS was expressed in the tip of cotyledon and also at the junction of hypocotyl and radicle (Figure II-6D). In 7-day-germinated seedlings, GUS expression was observed in the tip of true leaf, cotyledon and root maturation zone (Figure II-6E). In older seedlings (10-day and 2-week old seedlings), GUS was expressed in the tip of young true leaf, cotyledon and root maturation zone, but not in the older leaves (Figure II-6F and G). At the reproductive phase (flowering stage), GUS was expressed in the tip of cauline leaf, but not in rosette leaf (Figure II-6L and M). GUS expression was detected in stigma and the abscission zone of pollinated silique, but not in the unpollinated young flower (Figure II-6N). Notably, GUS expression was not detected in shoot and root meristems (Figure II-6C, 6D, 6E, 6F, 6G, 6H, 6I, and 6N). In the radicle and secondary roots, GUS expression was only in the maturation zone (zone of differentiation), but not in elongation zone or root meristematic zone (Figure II-6H, J and K). It has been well-accepted that the root tip is the major site of cytokinin synthesis. The

cambium, the shoot apex, and immature seeds are also thought to synthesize cytokinins (Letham, 1994 ; Emery et al., 2000; Kakimoto, 2003). Cytokinin content analysis also showed that cytokinins are most abundant in young, rapidly dividing cells of shoot and root meristems (Kakimoto, 2003). However, since p*PzIPT1*::GUS was not expressed in root tip, shoot and root meristems, this novel expression patterns indicates that *PzIPT1* is distinctly different from all other known *IPTs,* whose function is associated with vegetative tissues. Therefore, we propose that *PzIPT1* is probably a germline (sperm and synergid) specific *IPT* involved in gametogenesis and embryogenesis.


A
PvuII
HindIII
PvuII
BamHI
XbaI
SalI*
SstI*
EcoRI*
p
BASTA
P
PzIPT1
GUS / GFP
p
R
L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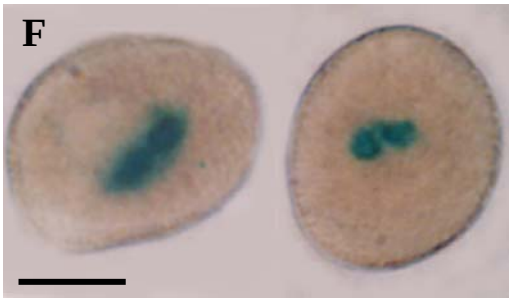
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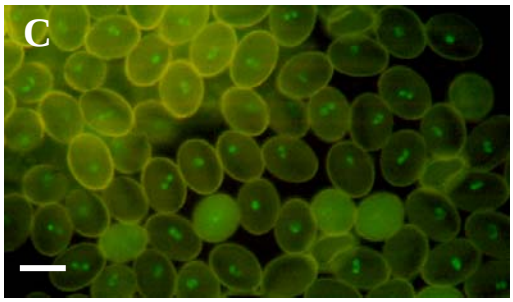
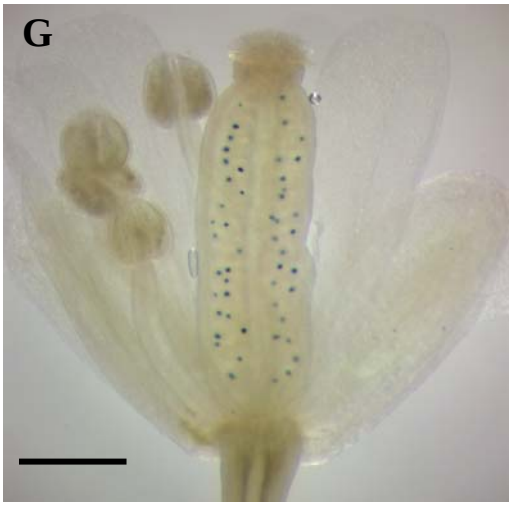

C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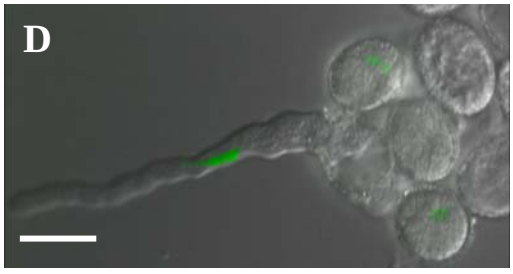
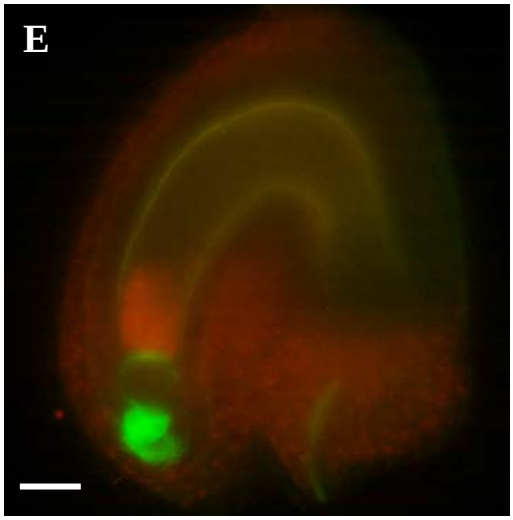

D


H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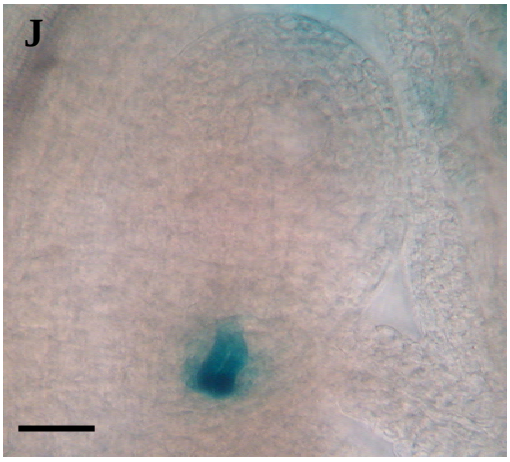

J

Figure II-4. GUS and GFP expression in sperm cells, unpollinated and pollinated ovule of transgenic *Arabidopsis* harboring *pPzIPT1*::GUS/GFP. **A**. Schematic illustration of *pPzIPT1*::GUS/GFP construct for *Arabidopsis* transformation. **B.** GFP expression in sperm cells (Confocal microscopy). Bar = 10 μm. **C**. GFP expression in sperm cells in homozygous line (epifluorescence microscopy). Bar = 20 μm. **D**. Pair of green (GFP) sperm cells in germinated pollen tube (Confocal microscopy image is courteously provided by Lili Ge). Bar = 20 μm. **E**. GFP expression in synergid cells of unpollinated ovule. Bar = 20 μm. **F**. GUS expression in sperm cells. Bar = 10 μm. **G**. GUS expression in unpollinated ovule. Bar = 500 μm. **H**. GUS expression in pollinated ovule. Bar = 1 mm. **I.** GUS expression in synergid cells of unpollinated ovule. Bar = 30 μm.

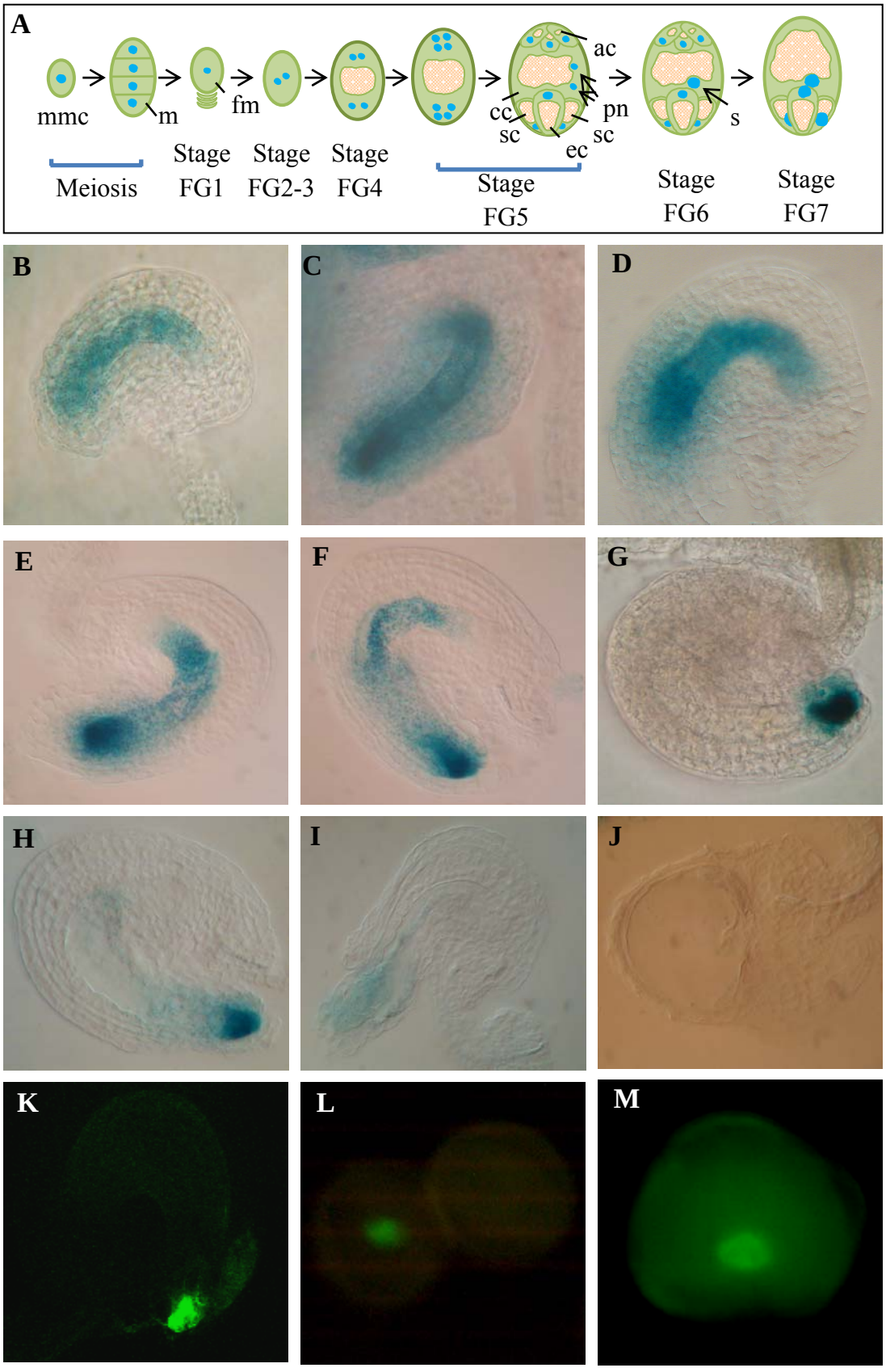

A
mmc
m
fm
ac
cc
pn
sc
sc
ec
s
Meiosis
Stage FG1
Stage FG2-3
Stage FG4
Stage FG5
Stage FG6
Stage FG7
B
C
D
E
F
G
H
I
J
K
L
M

Figure II-5. GUS and GFP expression pattern in developing ovule (hand emasculated) and pollen in transgenic Arabidopsis plants harboring *pPzIPT1::GUS/GFP*. **A.** Schematic illustration of megagametogenesis stage modified from Christersen (Christensen et al., 1997) **B.** GUS expressed in developing ovule at -30 HAP. At this stage, anthers were manually removed. Flower development stage is in mid 12 and ovule is in early two-nucleate stage (stage FG2). **C.** GUS expressed in developing ovule at -15 HAP. At this time point, flower development stage is between mid and late 12 and the ovule is in late two-nucleate stage (stage FG3) or early four-nucleate stage (stage FG4). **D.** GUS expressed in developing ovule at 0 HAP. At this stage, natural pollination occurs if anthers have not been removed. Flower development stage is between late 12 and 13 and the ovule is in early four-nucleate stage (stage FG4) or eight-nucleate stage (FG5). **E.** GUS expressed in developing ovule at +12 HAP. At this time point, flower development is between late stage 12 and stage 14 and ovule is in seven-celled stage (stage FG6). **F.** GUS expressed in developing ovule at +18 HAP. At this time, flower development stage is between 13 and 14 and ovule is in four-celled stage (stage FG7). **G.** GUS expressed in developing ovule at +24 HAP. At this time point, flower development stage is between 13 and late 14 and ovule is in four-celled stage (stage FG7). **H.** GUS expressed in developing ovule at +36 HAP. At this time point, flower development stage is at late 14 and ovule is still in four-celled stage (stage FG7). **I.** GUS expressed in developing ovule at +48 HAP. At this time point, flower organs begin to fall off and unpollinated ovule is about to degenerate. **J.** GUS expressed in developing ovule at +96 HAP. At this stage, unpollinated ovules have collapsed. **K.** At +24 HAP, GFP signal was restricted in

synergids. **L** and **M.** GFP signal was detected early in the generative cell of bicellular pollen.

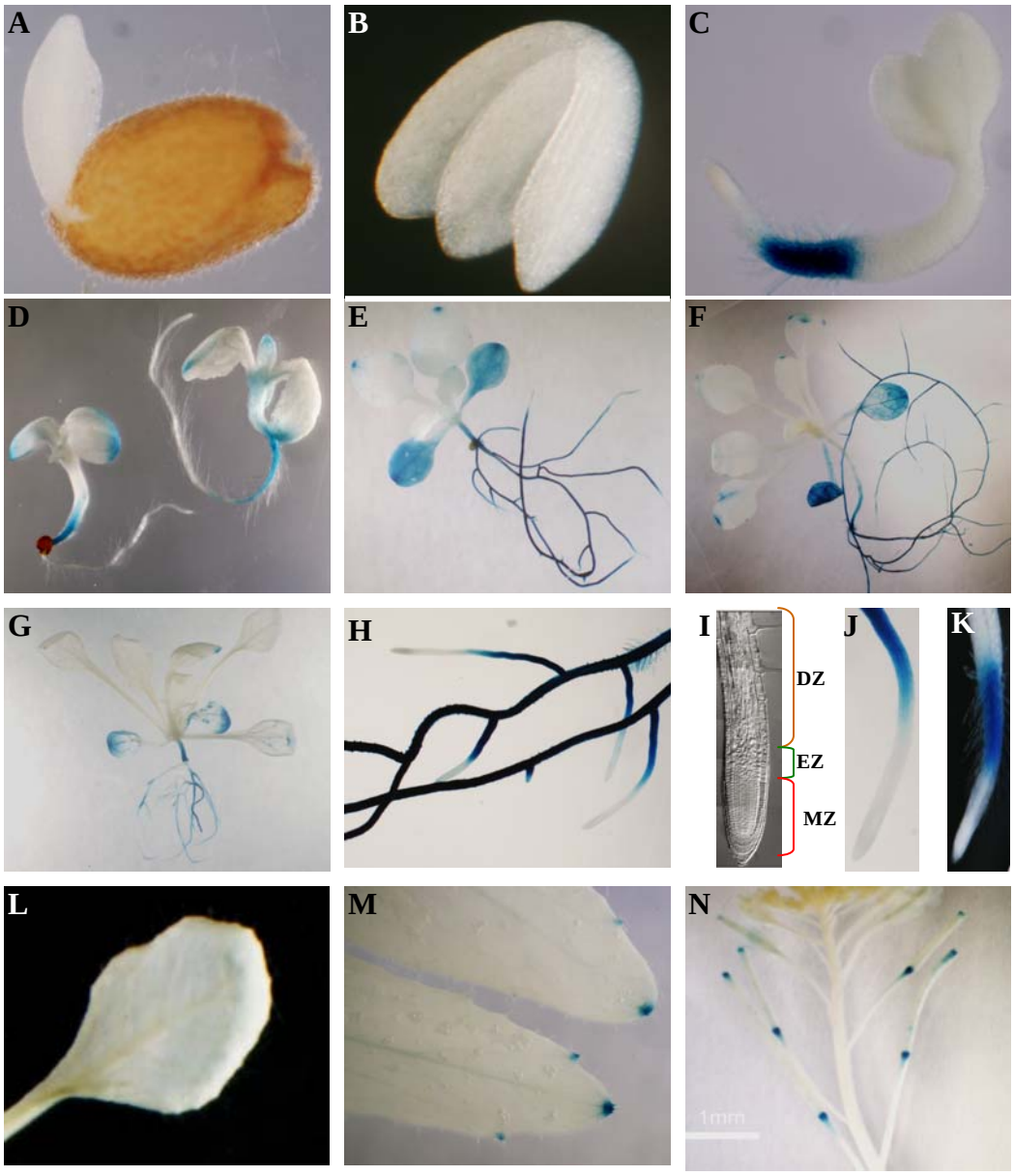


Figure II-6. GUS expression in vegetative tissues of transgenic *Arabidopsis* harboring *pPzIPT1*::GUS/GFP. **A**. No GUS expression in imbibed seeds (with seed coat). **B**. No GUS expression in imbibed seeds (without seed coat). **C**. GUS expression in junction of hypocotyl and radicle in 2-day old germinated seedlings. **D**. GUS expression in the tip of

cotyledon and at the junction of hypocotyl and radicle in 5-day old seedlings, but not in the shoot meristem or root meristem. **E**. GUS expression in the tip of true leaf, cotyledon and root in 7-day old seedlings, but not in the shoot meristem and root meristem. **F**. GUS expression in the tip of true leaf, cotyledon and root in 10-day old seedlings, but not in the shoot meristem and root meristem. **G**. GUS expression in the tip of young true leaf, cotyledon and root in 2-week old seedlings, but not in the older leaf, shoot meristem and root meristem. **H**. GUS expression in zone of cell maturation (zone of differentiation) of root, but not in elongation zone and meristematic zone of root. **I**. DZ, differentiation zone; EZ, elongation zone; MZ, meristematic zone of the *Arabidopsis* root (Grieneisen et al., 2007). **J**. Close look of root from 7-day-germinated seeling. GUS expression in zone of cell maturation (zone of differentiation) of root, but not in elongation zone and meristematic zone of root. **K**. Close look of radicle and hypocotly from 2-day-germinated seeling. GUS expression in zone of differentiation of root and junction of hypocotyl and radicle, but not in elongation zone and meristematic zone of root. **L**. No GUS expression in rosette leaf of mature plant (flowering stage). **M.** GUS expression in tip of cauline leaf. **N.** GUS expression in stigma and abscission zone of pollinated silique, but not in stigma and abscission zone of unpollinated young flower.

2.2. pPzIPT1::GUS/GFP expression in transgenic tobacco

Since *Arabidopsis* sperm cells are not dimorphic and the *PzIPT1* promoter driving reporter genes in transgenic *Arabidopsis* are expressed in both sperm cells, we also assayed the expression of *pPzIPT1*::reporter in tobacco whose sperm cells appear to be dimorphic when they enter the pollen tube (Tian et al., 2001). The *pPzIPT1:GFP/GUS* constructs (Figure II-7A). were introduced into tobacco via *A. tumefacien*-mediated

transformation following Horsch's protocol with modifications (Horsch et al., 1985). Briefly, the *in vitro* cultured leaf explants were inoculated with *Agrobacterium* strain LBA4401 containing the binary vector plasmid of *pPzIPT1*::GUS/GFP. Infected leaf stripes were then cultured on MS shoot-inducing medium with kanamycin selection (Figure II-7B). The survived shoots were transferred to root-inducing medium (Figure II-7C). Transgenic plantlets were transferred to the greenhouse and allowed to grow to flowering stage for GUS and GFP analysis (Figure II-7D). Tobacco mature pollen is bicellular and contains a generatetive cell and a vegetative cell (see Figure I-2). The generative cell undergoes PMII in the pollen tube to produce sperm cells. We obtained pollen tubes by a *semi-vivo* culture protocol (Cao et al., 1996; Tian et al., 2001). Pollen, pollen tube, ovule and other vegetative tissues were subjected to GUS histochemical staining for GUS expression assessment or direct epifluorescence microscopy for GFP expression pattern analysis. Surprisingly, *PzIPT1* promoter in transgenic tobacco lost its sperm cell specificity, and GUS was expressed in the vegetative cell of pollen (Figure II-7E and 7F), newly germinated pollen tube (Figure II-7H), ovule (Figure II-7G), petals and young seedling (data not shown), but not in the generative cell and sperm cells (Figure II-7H, 7I, 7J and 7K). GFP also showed the same expression pattern as GUS (data not shown). The differences of *PzIPT1* promoter activities in *Plumbago, Arabidopsis* and tobacco suggest that different plant species may have different transcriptional machinery.

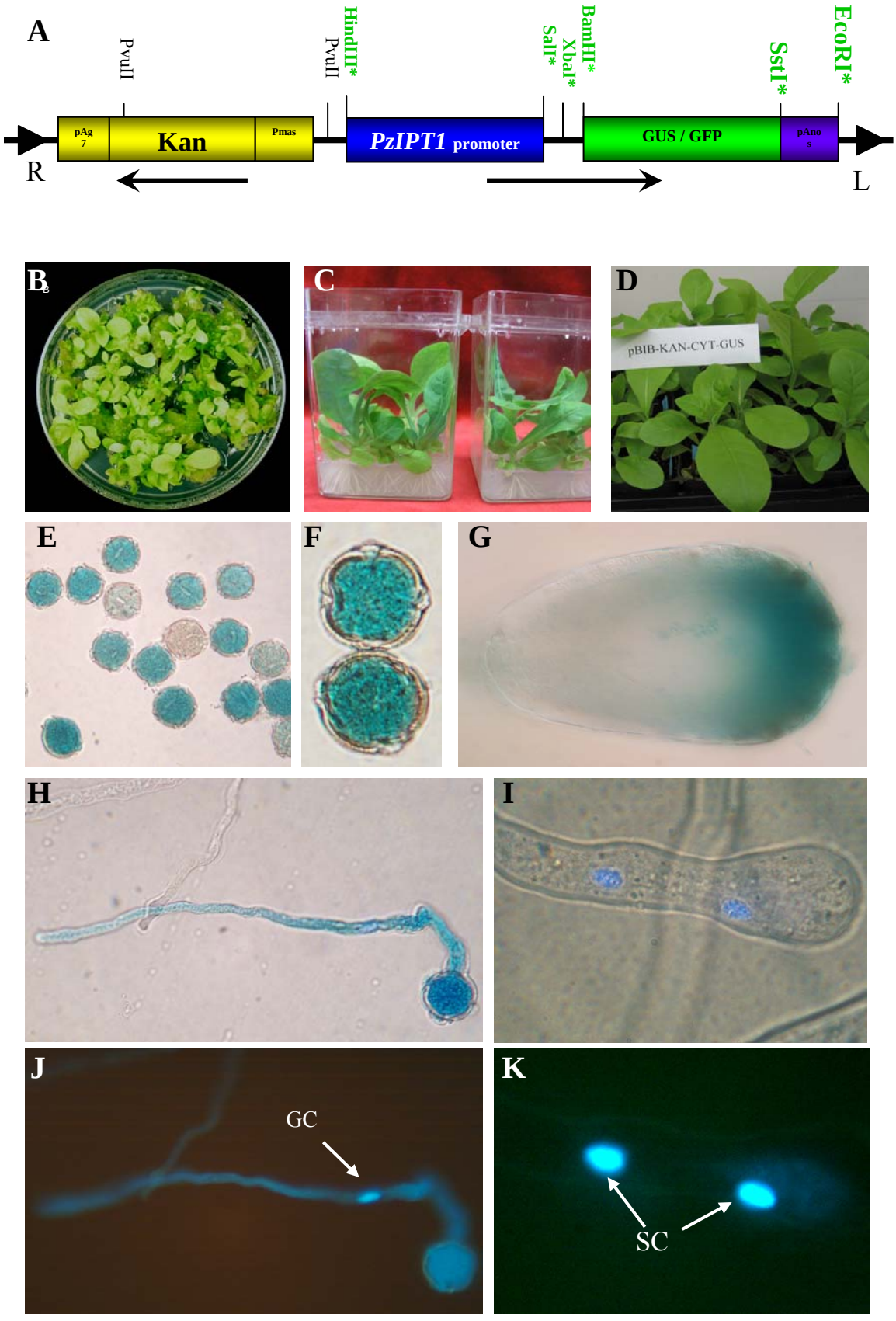

A
PvuII
HindIII*
PvuII
BamHI*
XbaI*
SalI*
SstI*
EcoRI*
pAg 7
Kan
Pmas
PzIPT1 promoter
GUS / GFP
pAno s
R
L
B
C
D
pBIB-KAN-CYT-GUS
E
F
G
H
I
J
GC
K
SC

Figure II-7. GUS expression pattern in transgenic tobacco driven by *PzIPT1* promoter. **A**. Schematic illustration of *pPzIPT1*::GUS/GFP constructs for *tobacco* transformation. **B**. Transgenic tobacco shoots arise on the selective medium. **C**. transgenic shoots are rooted in the root inducing medium**. D.** Transgenic tobacco plants are transferred to greenhouse**. E.** GUS expression in the pollen of R1 transgenic plants harboring *pPzIPT1*::GUS. **F.** GUS proteins are evenly distributed in the vegetative cell of the pollen. **G.** GUS is expressed in the chalazal end of the ovule. **H** and **J.** GUS is expressed in the newly formed pollen tube, but not in the generative cell. **H** shows light microscopy image and **J** shows epifluorescence microscopy image of DAPI stained generative cell nucleus. **I** and **K**. GUS expression is not detected in the tip of germinated/elongated pollen tube nor in sperm cells. **I** shows light microscopy image and **K** shows epifluorescence microscopy image of DAPI stained sperm cell nucleus.

2.3. PzIPT1 is localized in the cytoplasm

Because the p*PzIPT1::GFP* construct used for *Arabidopsis* transformation does not contain *PzIPT1,* the localization of GFP which is evenly distributed in the sperm cell (Figure II- 4B, 4C, and 4D) may not reflect the subcellular localization of PzIPT1. So we built another construct where GFP was fused with C-terminal of PzIPT1 protein driven by the *PzIPT1* promoter (*pPzIPT1::PzIPT1-GFP*) (Figure II-8A). The construct was stably transformed into *Arabidopsis* to determine the localization of the expressed PzIPT1 protein in the cell. The epifluorescence microscopy images and confocal microscopy optical sections in transgenic *Arabidopsis* pollen show that the fusion protein is distribute in the cytoplasm of sperm cell, with a weak signal detected in the sperm nucleus (Figure II-8B and 8C). The construct was also transiently expressed in tobacco

leaves by *Agrobacterium* infiltration. The PzIPT1-GFP fusion protein was also detected in the cytoplasm of epidermal cells of tobacco leaf (Figure 8D). The localization of PzIPT1 in the cytoplasm is consistent with AtIPT3's subcellular localization (Miyawaki et al., 2006), and also supports the proposed function of PzIPT1 as an ATP/ADP dependent isopentenyltransferase catalyzing the first step of cytokinin biosynthesis in cytoplasm.

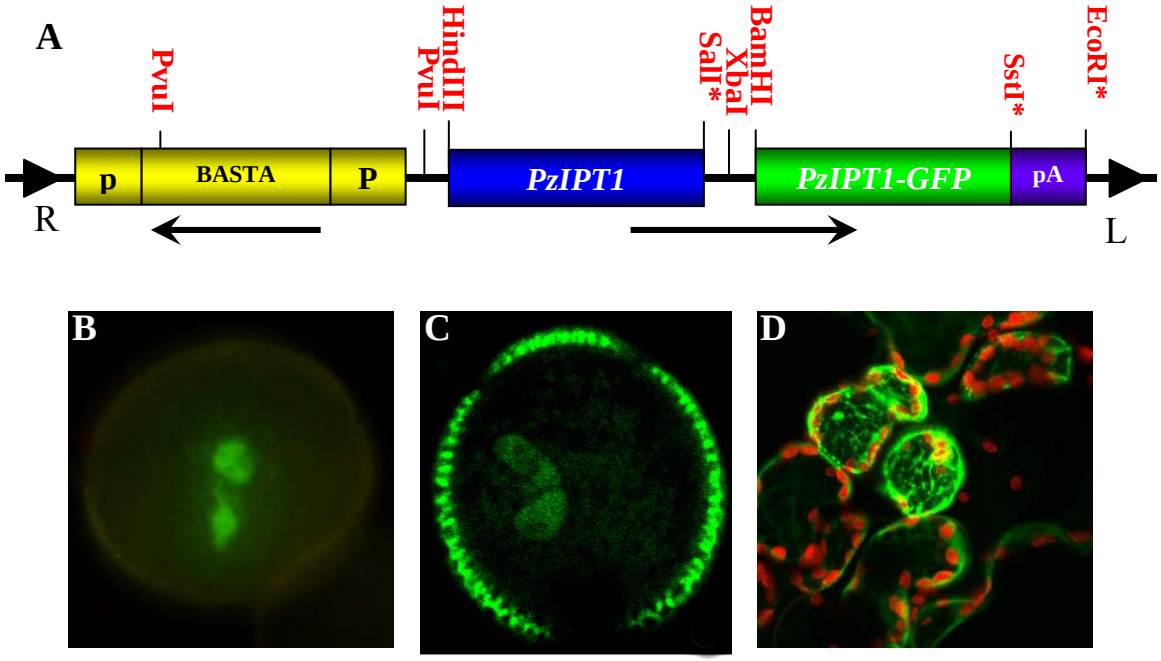


Figure II-8. Subcellular localization of PzIPT1. **A.** Schematic illustration of *pPzIPT1*::*PzIPT1-GFP* construct. **B.** The epifluorescence microscopy image showing PzIPT1-GFP fusion protein expressed in the cytoplasm of sperm cells in transgenic *Arabidopsis* pollen. **C.** The confocal microscopy optical sections in transgenic *Arabidopsis* pollen showing PzIPT1-GFP fusion protein is distribute in the cytoplasm of sperm cells, but weak signal in nuclei of sperm cells. **D.** The PzIPT1-GFP fusion protein is expressed transiently in the cytoplasm of epidermal cells of tobacco leaf.

III. The complementation of *PzIPT1* in Arabidopsis *atipt* 357 triple mutant indicates that *PzIPT1* is a functional *IPT*

As mentioned in previous paragraphs, sequence similarity search identified nine *IPT* homologs in *Arabidopsis* genome (Kakimoto 2001; Takei, et al. 2001). Seven of them encode ATP/ADP isopentenyltransferases (*AtIPT1, 3-8*) and play a major role in cytokinin biosynthesis. We are particular interested in *AtIPT3* because *PzIPT1* shares high sequence similarity with *AtIPT3*. *PzIPT1* and *AtIPT3* are phylogenetically classified into a subfamily of which another two *Arabidopsis IPT*s, *AtIPT5* and *AtIPT7*, are also members (Figure II-3B). T-DNA insertion knockout mutants analysis did not detect visible phenotypes in single or double mutants of any possible mutant combination for *AtIPT1*, *3*, *5*, and *7*. However, the *atipt357* triple mutant and the *atipt1357* quadruple mutant are short, with thin aerial parts, fewer rosette leaves, reduced shoot apical meristem size and thin inflorescence. The quadruple mutant has a more severe defective phenotype including seed development (Miyawaki et al., 2006). The *atipt1357* quadruple mutant phenotype can be partially rescued by trans-zeatin (tZ) which is an active cytokinin (Miyawaki et al., 2006), suggesting that the growth defect in *atipt1357* is caused by cytokinin deficiency. The overexpression of *AtIPT3* under native promoter *pATIPT3* in *atipt3567* mutant background was able to completely recover the phenotype of *atipt3567* mutant. This strongly suggests that the mutation in *AtIPT3* is mainly responsible for the cytokinin deficient phenotype (Miyawaki et al., 2006). We compared *atipt357* triple and *atipt1357* quadruple mutants for their reproductive development and did not detect any defects in pollen and ovule development in both mutants (Figure II-9 and Table 1). We also examined the male and female gametophyte development of

atipt357 triple mutant and *atipt1357* quadruple mutant. The flower of *atipt357* is normal as in WT (Figure II-9A and 9B), but the flower of *atipt1357* has smaller petals and shorter anther filaments which barely reaches stigma in anthesis (Figure II-9C). The pollen viability by Alexander's staining method (Alexander, 1969) showed all pollen in *atipt357* triple mutant and the *atipt1357* quadruple mutant are viable (Figure II-9D, 9E, and 9F). However, *atipt1357* quadruple mutants have mild anther dehiscence defects. Not all pollen is freely released from the anther. Pollen has a rough pollen wall and stick together (Figure II-9F). We used DAPI DNA stain to study male gametophyte development. Pollen development from meiosis to 3-nucleate pollen stage seems to be normal (Figure II-9M, 9N, and 9O). We also observed ovule development using cleared whole-mount ovule (Liu and Meinke, 1998) and did not find any defects in *atipt357* triple mutants and *atipt1357* quadruple mutants (data not shown). Seed development in *atipt357* triple mutants seems also normal compared to WT (Figure II-9G, 9H, 9K, and 9J, Table II-1), while seed abortion in *atipt1357* quadruple mutants was observed (Figure II-9I and 9L, Table II-1). Because there are no major defects in pollen and ovule in *atipt1357* quadruple mutants, seed abortion is most likely from abnormal development of the pollinated ovule or caused by short anther filaments. Our observations, together with results from others (Miyawaki et al., 2006), point to a role of *AtIPT3* in plant vegetative growth but not in male and female development.

Although *pPzIPT1::GUS/GFP* is mainly expressed in sperms and synergids in *Arabidopsis*, it is also expressed in vegetative tissues, indicating that *PzIPT1*'s function is not restricted to the gametophyte development. To test whether *PzIPT1* can complement the phenotype of *atipt357* triple mutant and *atipt1357* quadruple mutant, we expressed

PzIPT1 under *AtIPT3* promoter in these mutants (Figure II-10A). The T1 transgenic plants of *atipt357* triple mutant showed a partially rescued mutant phenotype (Figure II-10B and 10C). This result confirms that *PzIPT1* is a functional *IPT*.

Table II-1. Seed setting comparison for *atipt357*, *atipt1357* and Col-0

Lines	Siliques analyzed	Seed setting (%)	Standard Error
Col-0	22	98.5	8.03
atipt357	19	98.6	8.12
atipt1357	17	90.9	13.69

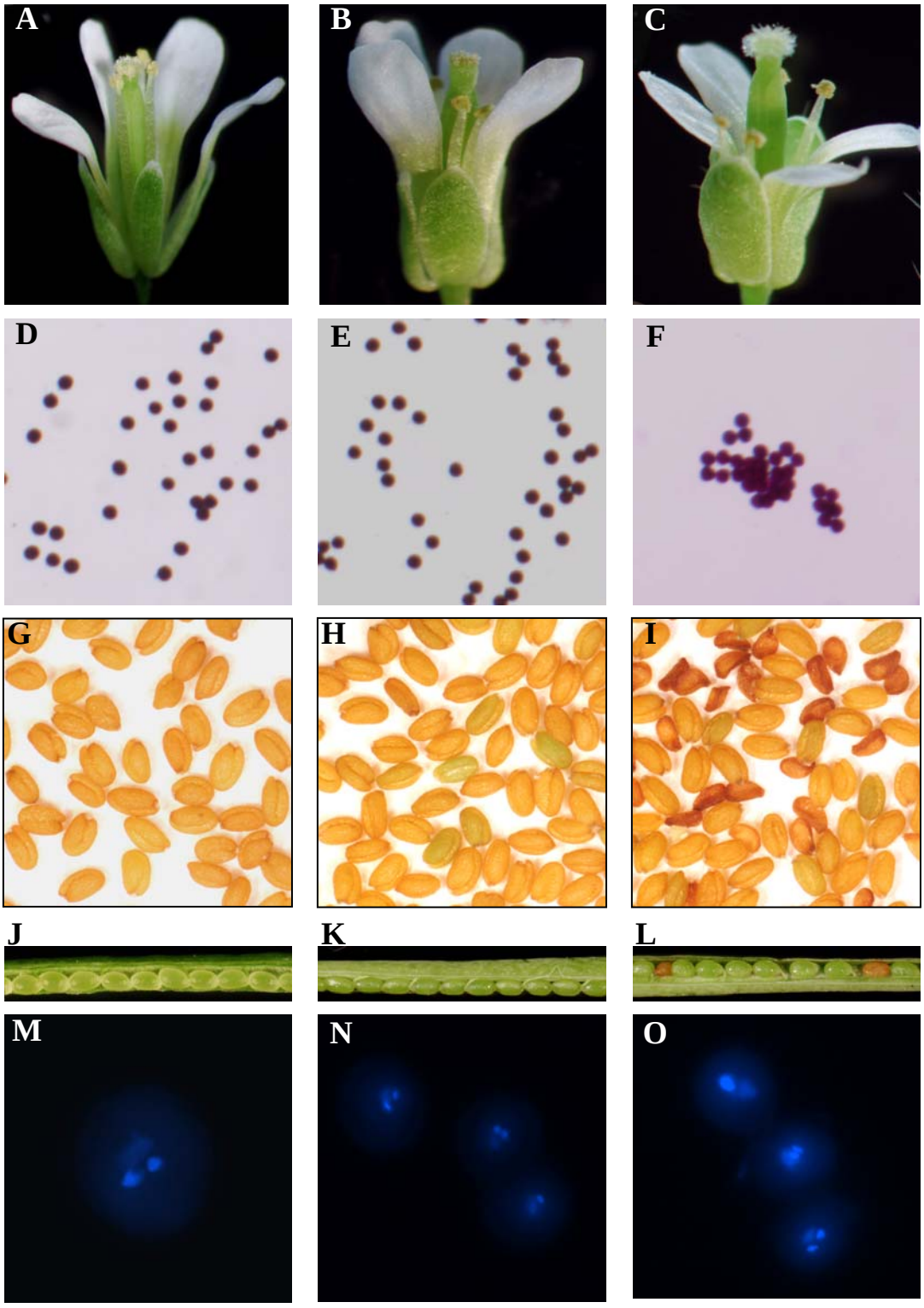


Figure II-9. Phenotype of *atipt* 357 and *atipt1357* mutant flower, pollen and seed. Left panel: Flower and seed phenotype of WT Col-0. **A.** Flower; **D.** Alexander's staining of

pollen. **G.** Mature seeds. **J.** developing seeds in silique. **M**. DAPI staining of mature pollen. Middle panel: Flower and seed phenotype of *atipt357* triple mutant. **B**. Flower; **E.** Alexander's staining of pollen. **H**. Mature seeds. **K**. Developing seeds in silique. **N**. DAPI staining of mature pollen. Right panel: Flower and seed phenotype *atipt1357* quadruple mutant. **C**. Flower phenotype; **F**. Alexander's staining of pollen. **I.** Mature seeds. **L.** Developing seeds in silique. **O**. DAPI staining of mature pollen.

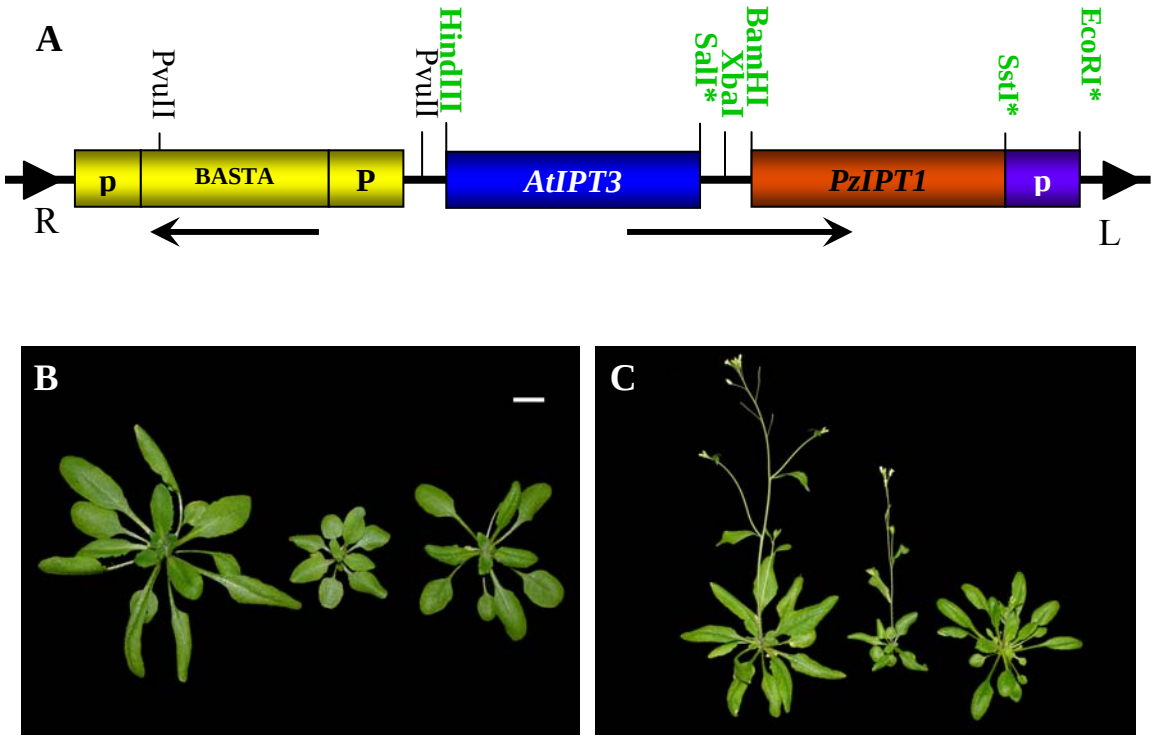


Figure II-10. Complementation of *PzIPT1* in *atipt357* triple mutant. **A.** schematic diagram for *pAtIPT3::PzIPT1* construct. **B.** *pAtIPT3::PzIPT1* recovering the phenotype of *atipt357* mutant at early stage. Left, Col-0; middle, *atipt357* mutant; right, T1 transgenic plant of *pAtIPT3::PzIPT1* in *atipt357* mutant. **C.** *pAtIPT3::PzIPT1*

complementation of *atipt357* at late stage. Left, Col-0; middle, *atipt357* mutant; right, T1 transgenic plant of *pAtIPT3::PzIPT1* in *atipt357* mutant.

IV. Overexpressing of Arabidopsis cytokinin oxidase1 (*CKX1*) in male and female gametophytes driven by *PzIPT1* promoter reveals *PzIPT1* function in gametophytic cell division

Cytokinin homeostasis in cells is determined by rates of biosynthesis, import, export and degradation (Mok and Mok, 2001). Cytokinin biosynthesis and degradation are two opposite biological processes which govern cytokinin concentration in tissues. In contrast to *IPT* which is essential for cytokinin biosynthesis, cytokinin oxidases/dehydrogenases (CKXs) are responsible for cytokinin catabolism and degradation (Schmülling et al., 2003a). CKXs catalyze the irreversible degradation of the cytokinins of isopentenyladenine and zeatin, and their ribosides in a single enzymatic step by oxidative side-chain cleavage (Mok and Mok, 2001; Schmülling et al., 2003a). CKXs are important in controlling local cytokinin levels and contribute to the regulation of cytokinin-dependent processes. In *Arabidopsis,* there are seven CKXs, which have different expression patterns. Overexpressing *AtCKXs in Arabidopsis and* tobacco showed enhanced CKX activities, reduced cytokinin concentration and a typical cytokinin-deficiency phenotype (Werner et al., 2001; Werner et al., 2003). Constitutive overexpression of individual *AtCKX* genes driven by 35S promoter led to an approximately 30%–45% reduction in the endogenous content of different cytokinin metabolites. The phenotypical analysis of these cytokinin-deficient plants identified developmental and physiological processes that are under cytokinin control (Werner et al., 2001; Schmülling et al., 2003b; Werner et al., 2003).

Since knockdown or knockout mutants for *PzIPT1* are lacking and chemical inhibitors that can specifically target the gene are also not available, we overexpressed *AtCKX1* driven by the gamete-specific *PzIPT1* promoter, in the hope that the strategy might deplete cytokinins in male and female gametophytes and thus result in a phenotype mimicking *PzIPT1*'s 'loss of function' phenotype. This alternative approach may help decipher the function of cytokinins during male and female gametophyte development.

Figure II-11A shows a construct that *AtCKX1* (At2g41510) is under the control of *PzIPT1* promoter. The *pPzIPT1::AtCKX1* transgenic plants exhibit varied fertility by the term of seed production. Seed setting reduction ranges from 0 – 80%. But for most transgenic lines, fertility is reduced by 40 – 50% (Table II-2). Except for the defects in seed setting, the transgenic plants seem to be as normal as the WT (Figure II-11). Further analysis focused on the lines with ~ 40-50% fertility reductions. Based on Basta resistance segregation patterns in T2 generation, we observed a distorted segregation which does not follow the 3:1 resistant:susceptible pattern in all independent transgenic lines. Generally, T2 lines have more susceptible plants than expected. To screen transgenic homozygous lines based on Basta resistance, we screened multiple generations in several independent lines but failed to obtain any homozygous lines (Table II-3). We suspect that the homozygous seeds are either aborted during embryo development or embryo-lethal during germination. Thus, heterozygous lines were used for further analysis.

Table II-2. Seed setting percentage of different *Arabidopsis* transgenic lines and Col-0

Lines (T5)	Siliques analyzed	Seed setting average (%)	Standard Error
CKX-1-6	49	54.4	20.00
CKX1-3-3	41	53.2	19.32
CKX1-4-9	42	53.6	19.71
CKX1-6-11(fertile)	14	99.0	13.71
Col-0	22	98.5	8.03

Table II-3. Basta resistant plants percentage in different generations of *pPzIPT1::AtCKX1* (%)

Lines	T3	T4	T5	T6	Average	Standard Error
CKX1-1-6	58.8	55.1	61.7	51.9	56.9	18.32
CKX1-3-3	50.0	48.9	56.4	53.1	52.1	11.41
CKX1-4-9	57.1	62.8	63.3	57.5	60.2	10.96
Expected	75	75	75	75	75	0

The transgenic plants seem to have a normal vegetative growth from young seedling to anthesis (Figure II-11B). In late fruit maturation stages, transgenic plants are taller than the WT (data not shown) due to longer inter-nodes of the transgenic plant (Figure II-11C). Compared to the WT, transgenic plants have taller stigma (Figure II-11D) and shorter siliques (Figure II-11C, 11H, and 11I). In young siliques, most aborted ovules are due to a fertilization failure (Figure II-11F and II-12). Occassioally, fertilized ovules

aborted during later stages of seed development (Figure II-11F arrow), resulting in abnormally developed seeds from transgenic plants (Figure II-11E).

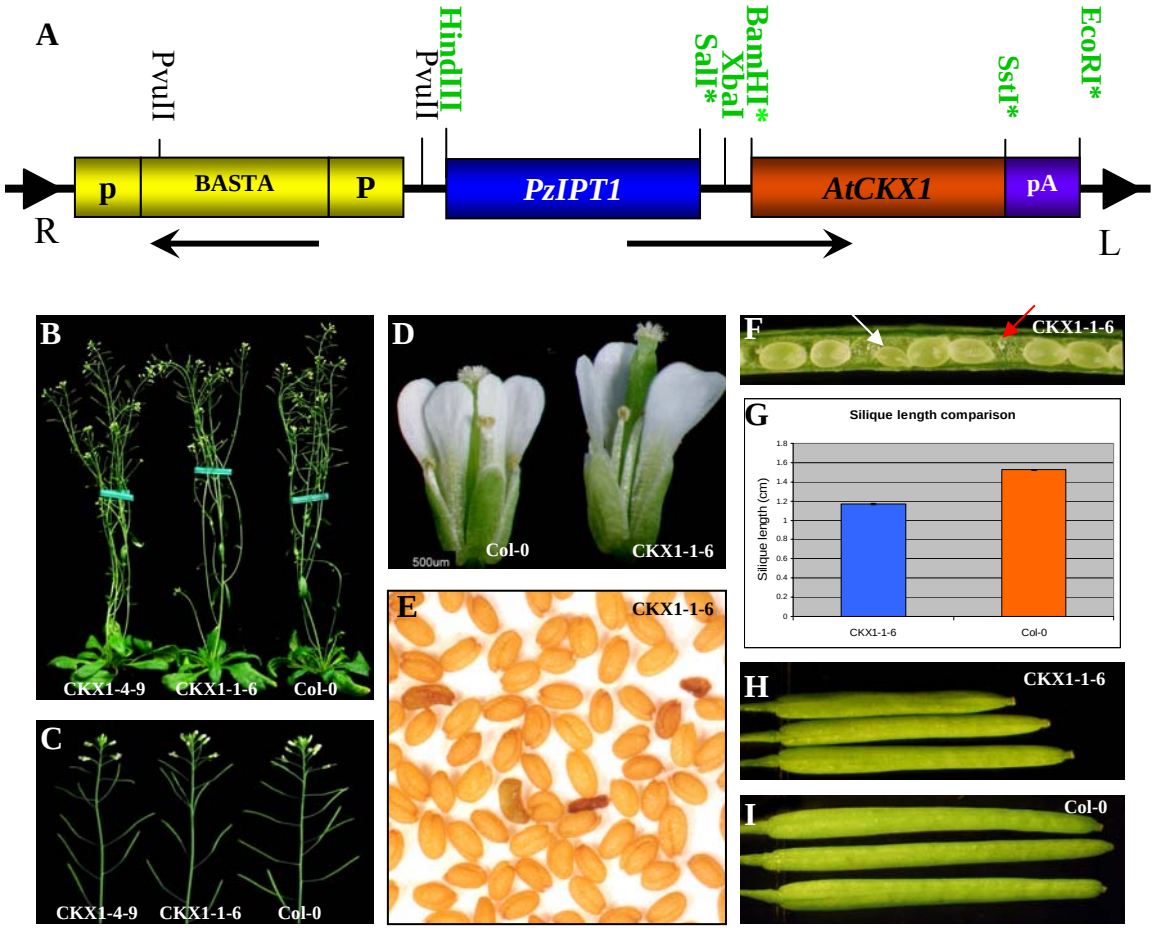


Figure II-11. Phenotype of transgenic plants harboring *pPzIPT1::AtCKX1*. **A**. Schematic diagram for *pPzIPT1::AtCKX1* construct; **B**. Transgenic plants (left and middle) and the WT (right); **C**. Internodes of transgenic plants (left and middle) are longer than those of the WT (right); **D**. Flowers of the WT (left) and the transgenic plant (right. Note taller stigma in the transgenic plant flower); **E.** Mature seeds from transgenic plants; **F**. Developing seeds in a silique. Arrows indicating aborted seeds; **G**. Average silique length. Blue: transgenic plant, orange: WT plant; **H**. Siliques of the transgenic plant; **I**. Siliques of the WT.

As reported by Werner et al. (Werner et al., 2003), constitutive overexpression of *AtCKX1* under 35S promoter resulted in strong phenotypic changes during all growth phases. Shoot and leaf development was retarded and the size of the meristematic cells was significantly decreased. The transgenic plants show severely reduced vegetative growth and delayed flowering (Werner et al., 2003). However, p*PzIPT1::AtCKX1* plants had no vegetative growth defects, indicating that *PzIPT1* only plays a minor role in vegetative growth. Although *pPzIPT1::GUS* was expressed in vegetative tissues as described in section 3, it was never expressed in shoot and root meristems. This may explain why *PzIPT1* does not affect plant vegetative growth.

We made reciprocal crosses of the transgenic plant with WT Col-0 to evaluate whether male, female or both affects fertility. The seed settings for F0 were counted and the result is in Table II-4. Assuming near perfect fertility of the WT as either male or female parent, any reduction in fertility should be due to the transgene. Then the result of the reciprocal crosses between CKX1-1-6 and Col-0 suggets that the female parent is responsible for sterility and the male parent does not seem to matter much, since CKX1-1-6 (♀) × Col-0 (♂) gives 71.7% seed settings, while the seed setting for the cross Col-0 (♀) × CKX1-1-6 (♂) is up to 94.7% (Table II-4). However, the pollen Alexander's staining showed about 30-50% of transgenic pollen are abnormal and non-viable (Figure II-12), suggesting that the male parent should be responsible for reduced seed settings. This inconsistency may be due to repetitive manual pollination of the same flower. The process may have increased the population of normal pollen on the stigma and thus shadowed the effects of non-viable pollen on seed setting. To evaluate the artifact, we compared manual pollination of CKX1-1-6 heterozygous line with natural pollination (no

human interruption). Manual pollination increased seed setting by ~20% (Table II-4). Thus, both parents seem to cause sterility.

Table II-4. Reciprocal cross of *pPzIPT1::AtCKX1* with WT Col-0 seed setting comparison

Lines	Siliques analyzed	Seed setting average (%)	Standard Error
Col-0(♀) × CKX1-1-6 (♂)	37	94.7	14.50
CKX1-1-6 (♀) × Col-0 (♂)	49	71.7	18.57
CKX1-1-6 natural	49	54.4	10.00
CKX1-1-6 manual	29	74.2	4.74
Col-0 natural	22	98.5	8.03
Col-0 manual	17	98.8	5.01

The Basta resistance test for F1 plants of reciprocal crosses showed less than 40% percent of plants were carrying the transgene, regardless of whether the transgenic plant was male or female parent (Table II-5). This result indicates that the transgene can be transmitted equally through male and female parents.

Table II-5. The Basta resistant plant percentage for F1 of reciprocal crosses

Cross	F1 Basta ^{r} (%)
♀Col-0 × ♂CKX1-1-6	30.5
♀CKX1-1-6 × ♂Col-0	39.8
Expected	50

We conducted a RT-PCR experiment to evaluate *AtCKX1* expression in different independent lines, including three lines used for phenotype analysis (CKX1-1-6, CKX1-3-3 and CKX1-4-9) and one line without fertility reduction (CKX1-6-11). The rosette leaves, cauline leaves and flowers were sampled from plants at anthesis stage for RNA extraction. The primers were designed spanning the intron for AtCKX1 (At2g41510) to yield an ~500 bp PCR product. *Arabidopsis* tubulin8 was used as internal control. In lines with reduced fertility (CKX1-1-6, CKX1-3-3 and CKX1-4-9), the expression of AtCKX1 was higher in the flower compared to the rosette leaf, cauline leaf and WT flower (Figure II-12) as we expected. Ovule abortion and reduced pollen viability were observed in these lines (Figure II-12). For the fertile line CKX1-6-11, the expression level in the flower was about the same as in the WT flower, but lower than in other transgenic lines (Figure II-12). This is consistent with the observed phenotype of viable pollen and fertile ovules (Figure II-12).

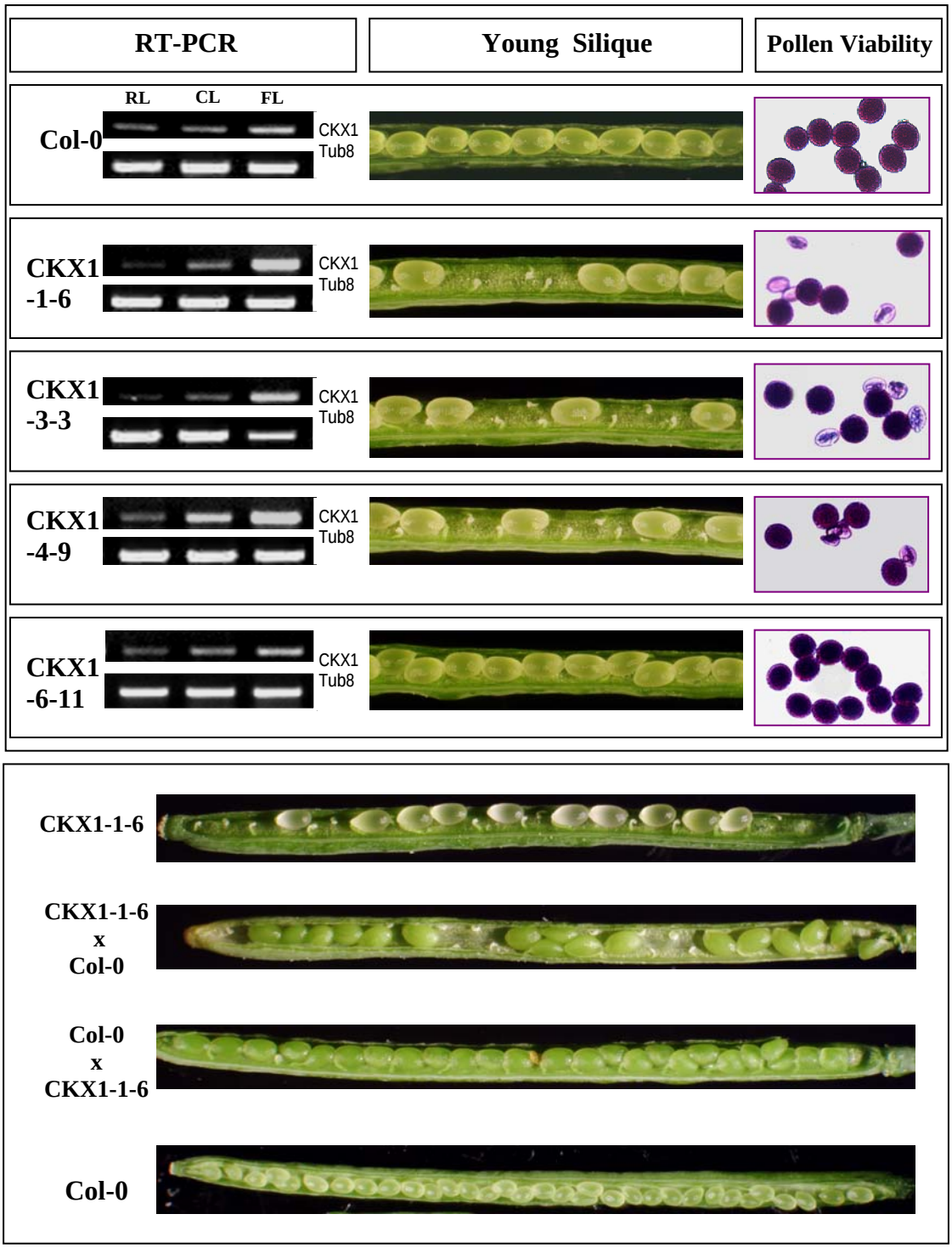


Figure II-12. *AtCKX1* expression driven by *pPzIPT1* was elevated in the flower tissue of transgenic plants and causes unviable pollen and aborted seeds. Top panel, Left: RT-PCR showing *AtCKX1* is highly expressed in flower of different transgenic lines. RL- rosette

leaf; CL- cauline leaf; FL- flower; Tub8- tubulin8, served as internal control. Middle: young silique showing aborted ovules and normal WT ovule. Right: pollen viability showed by Alexander's staining. The bottom panel shows young siliques of reciprocal crosses of CKX1-1-6 and Col-0 and parent lines.

Since *AtCKX1* catalyzes the degradation of cytokinins, driven by *PzIPT1* promoter, the level of cytokinins in male and female gametophyte can be dropped below the normal level. We examined whether cytokinin-deficiency reduced fertility and caused defects in male gametogenesis. As mentioned before, we could not obtain homozygous *pPzIPT1::AtCKX1* transgenic plants, so we used *pPzIPT1::AtCKX1* heterozygous transgenic lines which produce both WT and transgenic pollens. Actually, the heterozygous plant is advantageous because it provides a uniform developmental environment for the WT and transgenic pollen development. Since the development of all pollen within an individual flower is synchronized, we can track in which pollen development stage the defect for transgenic spores occur in the same flower. We examined male gametophyte development from early development stages -- pollen mother cell meiosis I, meiosis II, tetrad, to uninucleate microspores, bicellular, and mature tricellular pollen using DAPI nuclear staining. Before the uninucleate microspore stage, there was no obvious difference between transgenic and WT plants (Figure II-13B-I). However, about 36% of spores were arrested at the uninucleate microspore asymmetric mitosis I stage (772 arrested uninucleate microspores out of 2130 total spores examined) (Figure II-13J-K). DAPI nuclear stain showed the arrested uninucleate pollen with loose and specked nuclei when the WT pollen advanced to bicellular and early tricellular stages (Figure II-13J-M). Light microscopy showed shrunken cytoplasm and

nucleus in the arrested uninucleate pollen (Figure II-13K, M, O, P). As the WT pollen progressed to mature tricellular stage, the nucleus of the arrested uninucleate pollen became smaller in size or totally disappeared (Figure II-13P). We could occasionally observe the symmetric pollen mitosis I (Figure II-13N) and the retarded pollen development (Figure II-13O). This suggested that *PzIPT1* played an important role in pollen development, particularly for the advancement from mitosis I to later stages.

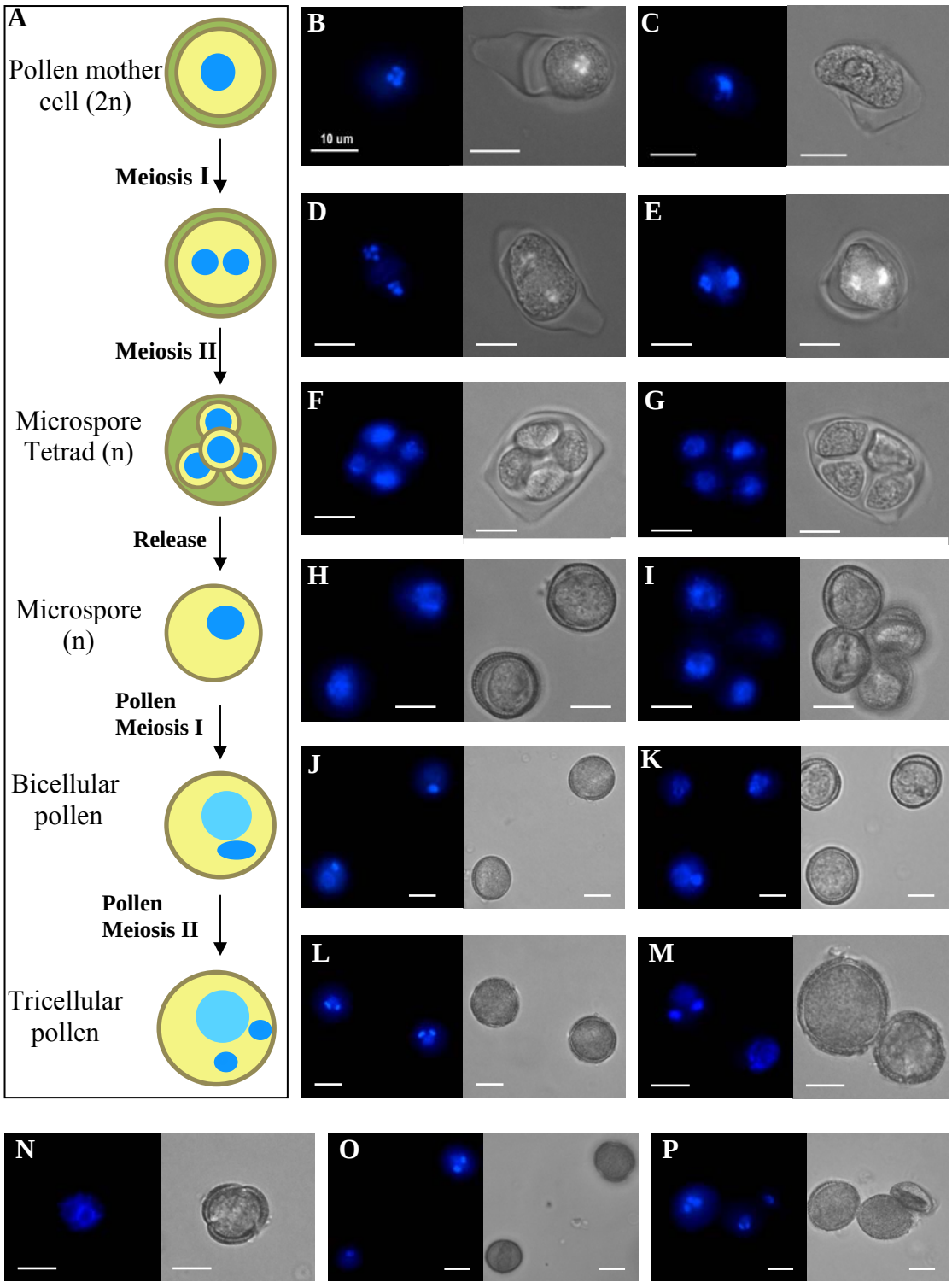

Pollen development scheme
WT
P-PzIPT::AtCKX1
A
Pollen mother cell (2n)
Meiosis I
Meiosis II
Microspore Tetrad (n)
Release
Microspore (n)
Pollen Meiosis I
Bicellular pollen
Pollen Meiosis II
Tricellular pollen
B
10 um
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure II-13. Pollen development in WT and *pPzIPT1::AtCKX1* transgenic plants. **A.** schematic depiction showing microgametogenesis in *Arabidopsis* WT plants. **B** and **C** showing pollen mother cells in the WT **(B)** and *pPzIPT1::AtCKX1* **(C). D** and **E** showing pollen mother cells undergoing meiosis I in the WT **(D)** and *pPzIPT1::AtCKX1* **(E)**. **F** and **G** showing tetrads in the WT **(F)** and *pPzIPT1::AtCKX1* **(G)**. **H** and **I** showing uninucleate microspores in the WT **(H)** and *pPzIPT1::AtCKX1* **(I)**. **J** and **K** showing bicellular pollen in the WT **(J)** and *pPzIPT1::AtCKX1* **(K).** Some pollen were arrested at uninucleate stage **(K). L** and **M** showing tricellular pollens in the WT **(L)** and *pPzIPT1::AtCKX1* **(M).** Some pollen were still arrested at uninucleate stage. **N** showing symmetric unclear division during PMI in *pPzIPT1::AtCKX1* transgenic lines. **O** showing occasionally, pollen development can be retarded. The defective pollen is in bicellular stage (left) while WT progressed to tricellular stage (right). **P** showing arrested uninucleate pollen was shrunken and unviable. Scale bar = 10 um.

To examine whether *pPzIPT1::AtCKX1* affects female gametogenesis, we also compared female gametophyte development in *pPzIPT1::AtCKX1* heterozygous transgenic plants. Similar to synchronization of pollen development in a flower, ovule and embryo sac development are synchronous within a silique in *Arabidopsis*. We compared each development stage of transgenic and WT female gametophyte from the same individual siliques. In the early stages of a WT ovule, a single sporophytic cell - megaspore mother cell undergoes meiosis to produce four haploid megaspores during megasporogensis. While three of the megaspores near the micropylar end undergo programmed cell death, the chalazal end megaspore becomes functional and undergoes three rounds of mitosis to form an eight-nucleate embryo sac within an ovule. Subsequent

fusion of two polar nuclei (form a central cell) to form a seven-cell-mature embryo sac consisting: three antipodal cells, one large central cell, one egg cell and two synergids. The three antipodal cells then degenerated to form mature four-nucleate embryo sac (Christensen et al., 1997; Drews and Yadegari, 2002) (Figure II-14A).

The whole mount clearing method (Liu and Meinke, 1998) in combination with differential interference contrast (DIC) microscopy was used to investigate ovule development in *pPzIPT1::AtCKX1* transgenic plants. Aniline blue staining was performed to visualize callose accumulation in the newly formed cell wall during megasporogenesis (Rodkiewicz, 1970). In both WT and *pPzIPT1::AtCKX1* ovules, meiosis was normal (Figure II-14B and C) because we could observe the accumulation of three bands of callose, which marks the position of newly formed cell walls that divide the four meiotic nuclei into the tetrad. In the functional megaspore stage FG1 (FG1 and all other developmental stages are defined by Christensen et al., 1997), all ovules in *pPzIPT1::AtCKX1* seemed as normal as the WT (Figure II-14D, E, and F). In stage FG2-3, about 30-40% ovules were arrested at the uninucleate stage (Figure II-14H and I) whereas the WT ovules in the same silique developed normally to the two-nucleate stage (Figure II-14G and H). The arrested uninucleate embryo sac did not undergo any cell division and retained uninucleate stage afterwards, while their corresponding WT siblings underwent sequential two mitotic divisions and cellularization to form four-nucleate, eight-nucleate, seven-nucleate and mature late-four-nucleate embryo sac (Figure II-14J to 14X). The arrested embryo sac had no central vacuole and did not enlarge (Figure II-14J to 14X), while the embryo sac in their WT siblings enlarged and formed a big central vacuole and several small vacuoles. The arrested embryo sac did not enlarge, the inner

and out integuments in arrested ovule still underwent active cell division and elongated like the WT. In the WT, the elongated inner and outer integuments enclose the embryo sac, but in the arrested ovule, the inner and out integuments remain open.

Both male and female gametophytogenesis were arrested at the first round of mitosis in *pPzIPT1::AtCKX1* plants, strongly suggesting that *PzIPT1* is involved in both male and female gametophyte development, particularly in gametophyte mitosis I.

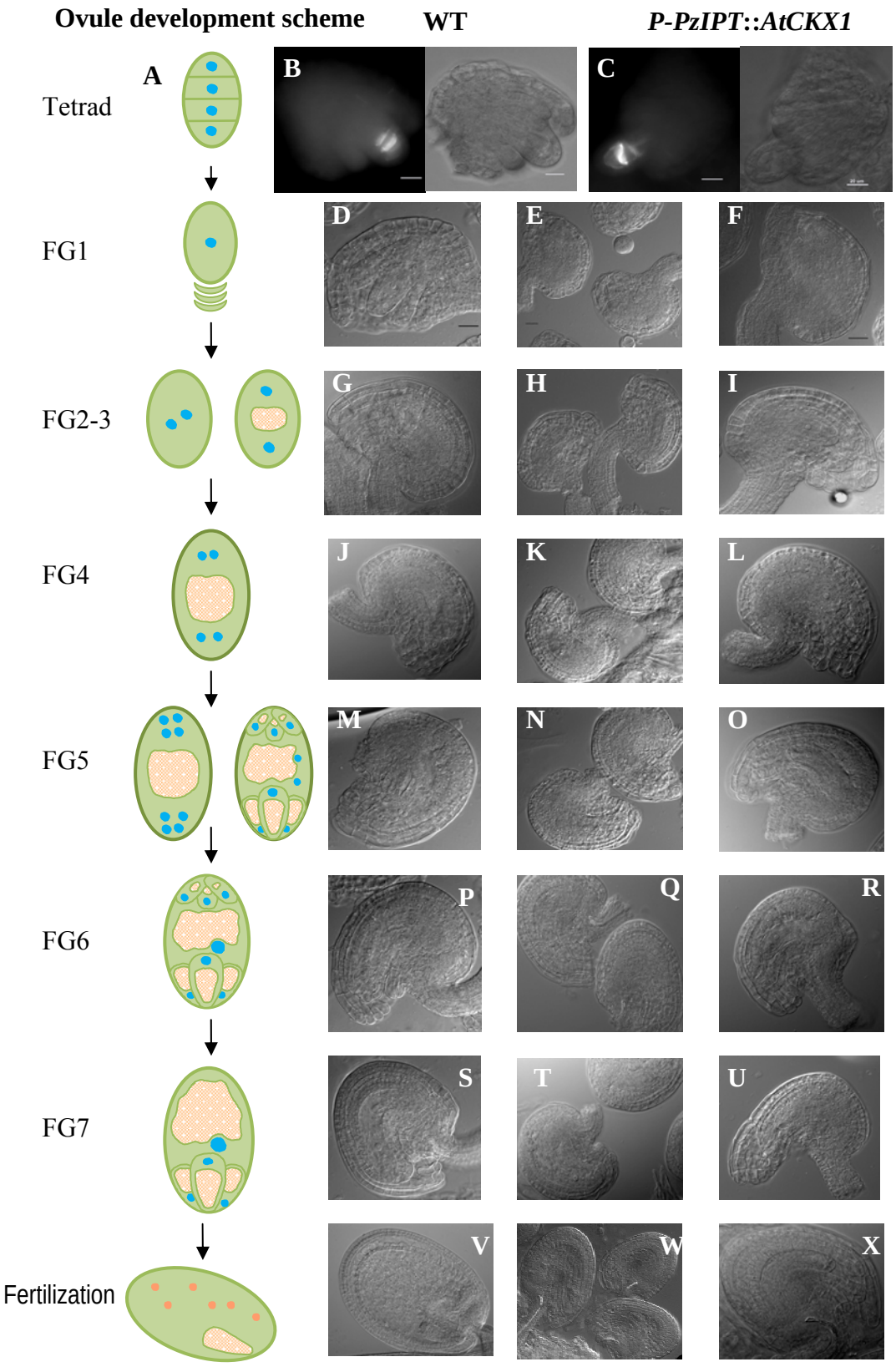

Ovule development scheme
WT
P-PzIPT::AtCKX1
Tetrad
FG1
FG2-3
FG4
FG5
FG6
FG7
Fertilization
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X

Figure II-14. Megagametogenesis in WT and *pPzIPT1::AtCKX1* transgenic plants. **A.** schematic depiction showing megagametogenesis in *Arabidopsis* WT plants. **B** and **C** showing megaspore mother cell undergoes meiosis to produce four haploid megaspores in WT and *pPzIPT1::AtCKX1* transgenic plants, respectively. In both B and C, left: UV micrographs showing three bands of callose accumulation, which represents the position of newly formed cell walls that divide the four meiotic nuclei into the tetrad; right: light micrographs corresponding to UV micrographs. **D, E,** and **F** showing at FG1 stage, all ovules in *pPzIPT1::AtCKX1* were found normal as that in WT. **H, K, N, Q, T,** and **W** showing two ovules in the same silique, one WT ovule developed normally whereas the other was arrested at the uninucleate stage at FG2-3, FG4, FG5, FG6, FG7 and post pollination stages, respectively. **G, J, M, P, S,** and **V** showing higher magnification micrograph of WT ovules developed normally at FG2-3, FG4, FG5, FG6, FG7 and post pollination stages, respectively. **I, L, O, R, U, and X** showing higher magnification micrograph of arrested ovules in the same silique with their WT siblings at FG2-3, FG4, FG5, FG6, FG7 and post pollination stages, respectively.

V. *Plumbago* S_{vn}-specific *PzIPT1* can be transmitted to the ovule and may play an important role in early embryogenesis

It has been long assumed that sperms only pass over the haploid male genome to embryo. But the cytological evidence in *Plumbago zeylanica* indicated that the male cytoplasm can be transmitted into female gametes during double fertilization (Russell, 1980). Recent studies from mammals also suggested that sperm cells deliver transcripts and proteomes to the egg that are crucial to zygote and embryo early development (Ostermeier et al., 2004). Since *PzIPT1* is specifically expressed in S_{vn} in *Plumbago* and

expressed in both sperm cells in *Arabidopsis*, we investigated whether or not *PzIPT1* transcripts and proteomes are transmitted to female gametes during double fertilization.

The RT-PCR experiment was performed in *Plumbago* pollinated and unpollinated ovules at different HAP (Hours After Pollination). In *Plumbago zeylanic*, fertilization usually occurs around 7-8 HAP. Our RT-PCR result showed *Plumbago* S_{vn}-specific *PzIPT1* transcripts were detected in fertilized ovules (9HAP) shortly after fertilization (Figure II-15), but not detectable in later stages. The experiment was repeated, and the same results were obtained.

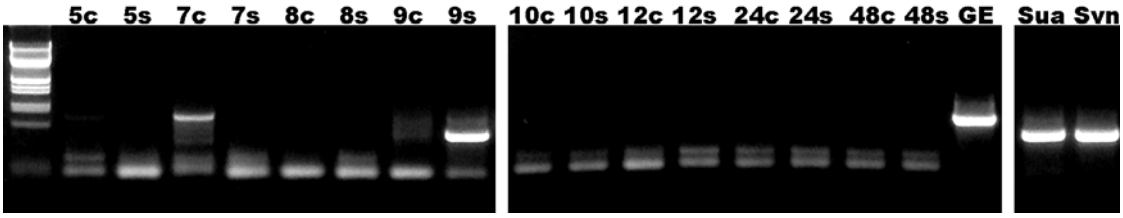
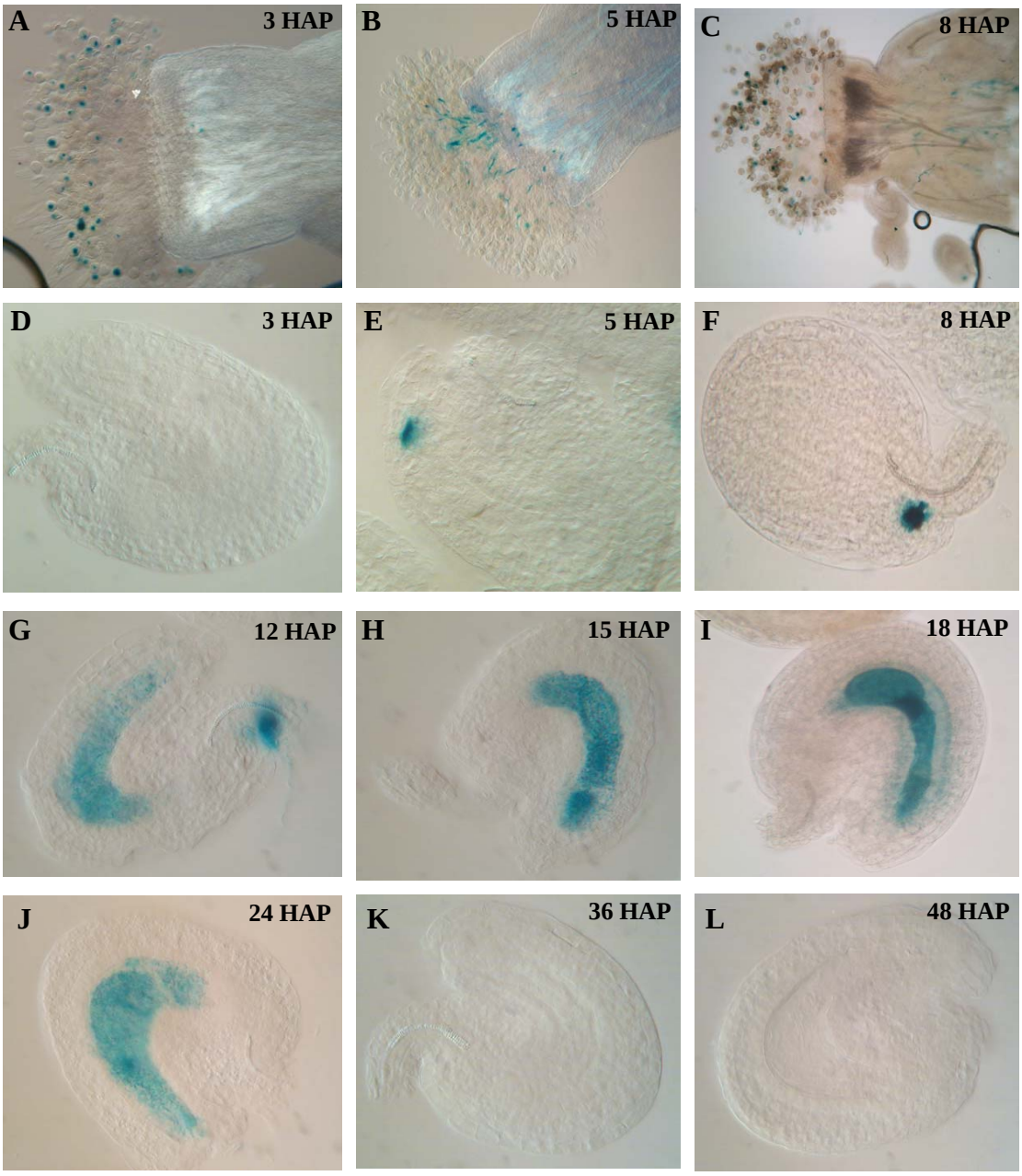


Figure 15. RT-PCR result for *PzIPT1* expression in different stage of pollinated ovules of *Plumbago***.** Numbers for HAP (Hour After Pollination); C – Control, hand emasculated and unpollinated ovules; S – Sample, hand emasculated and pollinated ovules. GE – genomic DNA as PCR template.

To analyze paternal transmission of *PzIPT1* proteomes in *Arabidopsis*, the homozygous line of *pPzIPT1:GUS* transgenic *Arabidopsis* was used as the male parent to cross with the WT Col-0. Then, the pollinated ovules were sampled from 0 - 80 HAP for GUS staining and light microscope examination. GUS expression in early zygotic and embryonic developmental stages (5 to 36 HAP) was observed. GUS expression started in pollinated ovules when the sperm cell just arrived within the synergids, accumulating over a 12-hour period to reach the highest expression level (18 HAP), then the GUS

signal started to decrease. At 36 HAP, we could not detect any GUS signal. Is the strong GUS expression at 18 HAP from paternal transmission or from activation of delivered sperm genome after fertilization? The result from Dr. Ueli Grossniklaus's lab (Vielle-Calzada JP, 2000) showed that most of the paternal genome is transcriptionally inactive during early embryogenesis. Transcription of paternal genes is initiated after fertilization, most likely in the 32-64 cell stage of embryo formation (>68 HAP) in *Arabidopsis* (Vielle-Calzada JP, 2000). Although their conclusion may not be true for every individual gene (Vielle-Calzada et al., 2001; Weijers et al., 2001), it is reasonable to believe that at least some, if not all, *pPzIPT1:GUS* transcripts are delivered to the zygote and endosperm by sperm cells, along with some *pPzIPT1::GUS* protein products. To further test this hypothesis, we used DsRed1-E5 fluorescent protein in pTimer vector (http://www.clontech.com) driven by *pPzIPT1* to discriminate newly translated and aged fluorescent proteins.

GUS expression in *Arabidopsis* Wild type (female) X *P-PzIPT1:GUS* (male)



HAP: Hours After Pollination

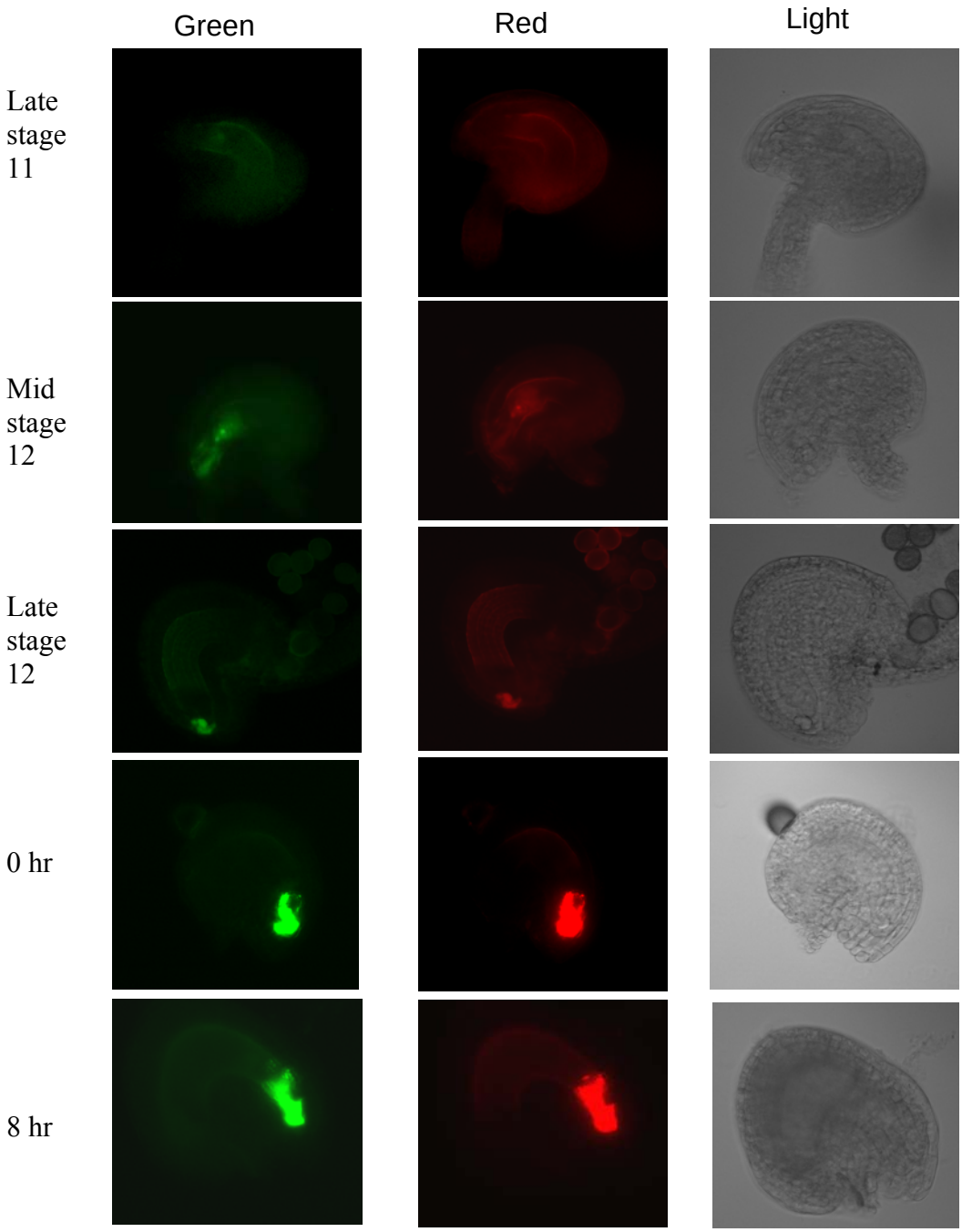
Figure II-16. GUS expression in ovules of WT (♀) x *pPzIPT1::GUS* (♂) (HAP – hours after pollination)

The DsRed1-E5 protein is a mutant form of the DsRed fluorescent reporter, containing two amino acid substitutions which increase its fluorescence intensity and endow it with a distinct spectral property: as the fluorescent protein matures, it changes color in a matter of hours, depending on the expression system used. When first synthesized, DsRed1-E5 is green. As time passes, the green fluorophore undergoes additional changes that cause its fluorescence to shift to longer wavelengths—when fully matured, the protein is bright red (Matz MV, 1999; Terskikh A, 2000). DsRed1-E5's invariable green-to-red shift can be used as a timer to track the on-off phases of gene expression during embryogenesis and cell differentiation (Terskikh A, 2000). These properties indicate that the ratio of green to red fluorescence can be used to determine the age of DsRED-E5 protein, which could facilitate the visualization of dynamics of promoter activity and reflect promoter corresponding gene expression (Terskikh et al., 2000). Analysis of the fluorescence ratios clearly provided more accurate insight into the timing of promoter activity (Terskikh A, 2000; Mirabella et al., 2004).

We placed DsRed1-E5 under the control of *PzIPT1* promoter, and transformed it into *Arabidopsis*. We obtained five independent transgenic homozygous lines by Basta resistance screening. All lines showed strong expression of DsRed1-E5 in sperm cells and synergids. This was consistent with our previous GUS and GFP expression patterns. From the time course study of unpollinated ovule, the earliest expression of DsRed1-E5 in the ovule can be detected at the flower developmental stage late 11 (Figure II-17A). At this stage, only green fluorescent protein can be detected, indicating the beginning of DsRed1-E5 protein synthesis. After mid-stage 12, both green and red fluorescence of DsRed1-E5 were detectable in the ovule with strong expressions from Stage 13 (referred

as 0 hr at this time point, when anthers were manually removed to prevent male interference with female expression pattern) to 48 hours after stage 13 (Figure II-17B, C, D, E, F, and G). Then, DsRed1-E5 protein started to turn over (Figure II-17H, I, and J). In the ovule, the green fluorescent signal was coupled with the red fluorescent signal. This indicated that in the ovule, DsRed1-E5 protein kept synthesizing while the mature protein also accumulated.

In male gametophyte, pollens were sampled from the anther at anthesis stage (pollen just start to shed), 12 hours after shedding and 24 hours after shedding to examine DsRed1-E5 protein expression. When pollen just shed, both green and red fluorescence were detected in sperm cells (Figure II-18A). This indicated that DsRed1-E5 protein was synthesized before the pollen developed to mature stage. Then, at 12 hours after shedding, most of DsRed1-E5 protein became red (mature) and only weak green fluorescence could be detected (Figure II-18B). At 24 hours after shedding, all DsRed1-E5 protein was red (mature) and no green fluorescence could be detected (Figure II-18C), indicating no active translation of DsRed1-E5 protein. To analyze the DsRed1-E5 protein translation and maturation in sperm cells during pollination, *in vitro* germination of pollen tube was performed based on the protocol Boavida et al. developed (Boavida and McCormick, 2007). The observation also showed no newly translated protein in pollen tube after 24-hour germination (Figure II-19A to 19E).


Green
Red
Light
Late stage 11
Mid stage 12
Late stage 12
0 hr
8 hr

(Continued…)

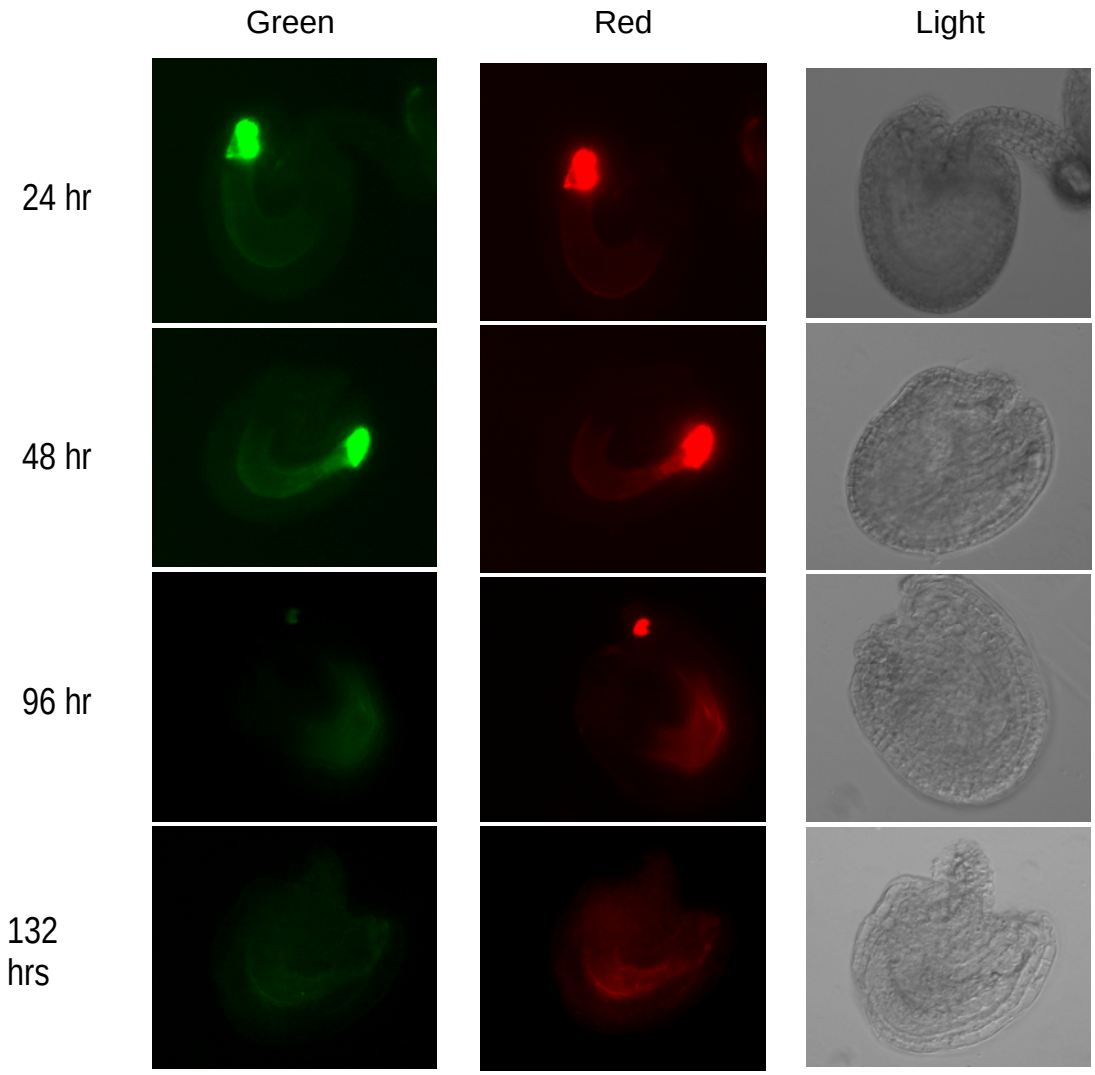


Figure II-17. DsRed1-E5 expression in the unpollinated ovule.

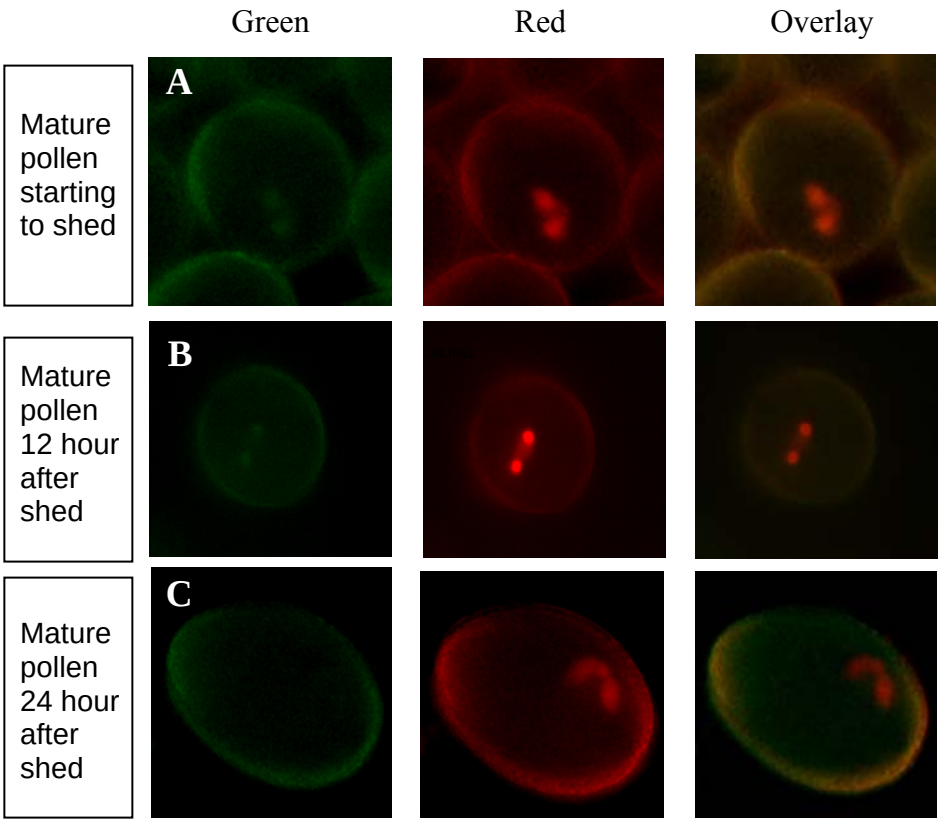


Figure II-18. DsRed1-E5 expression in mature pollen.

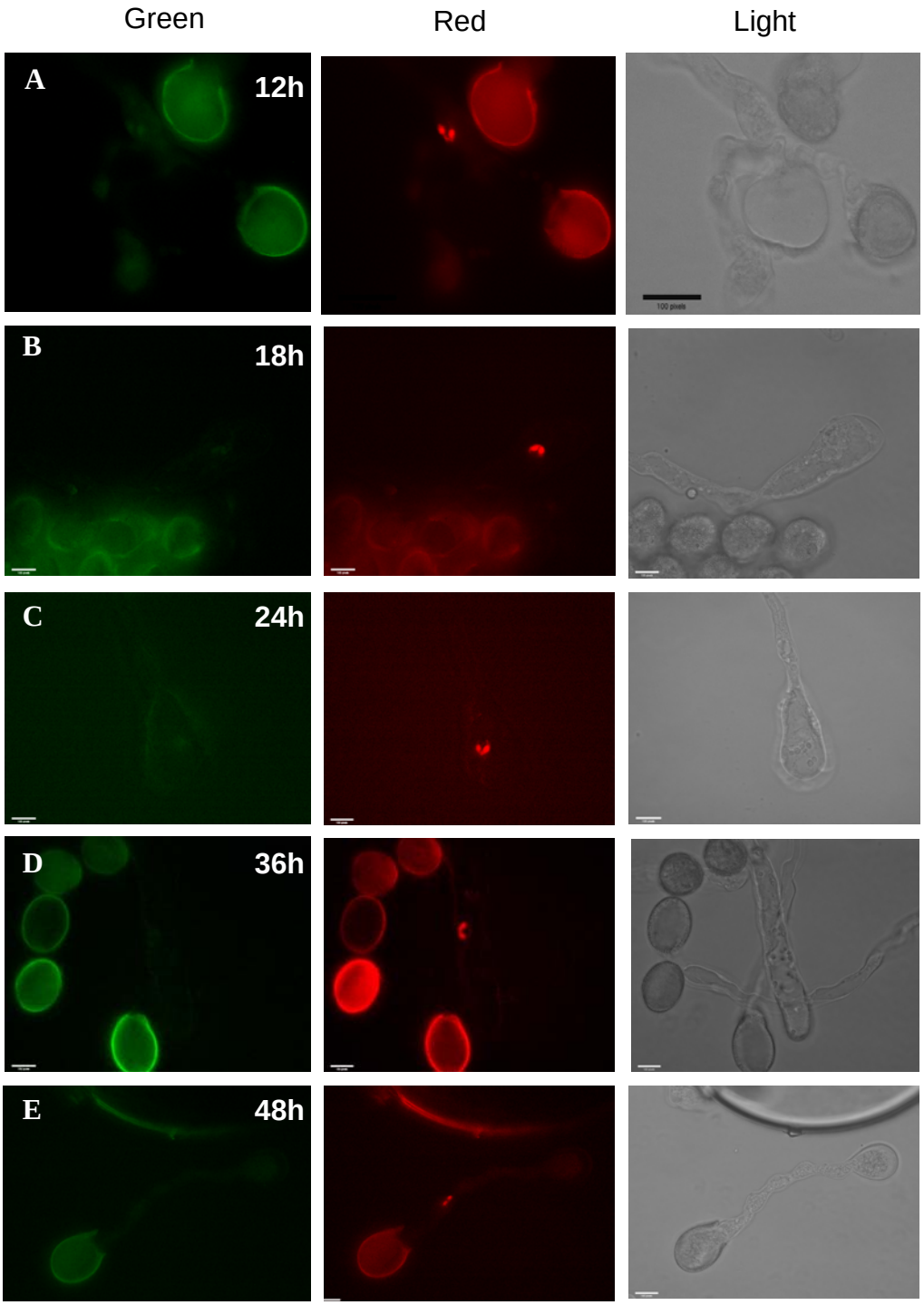
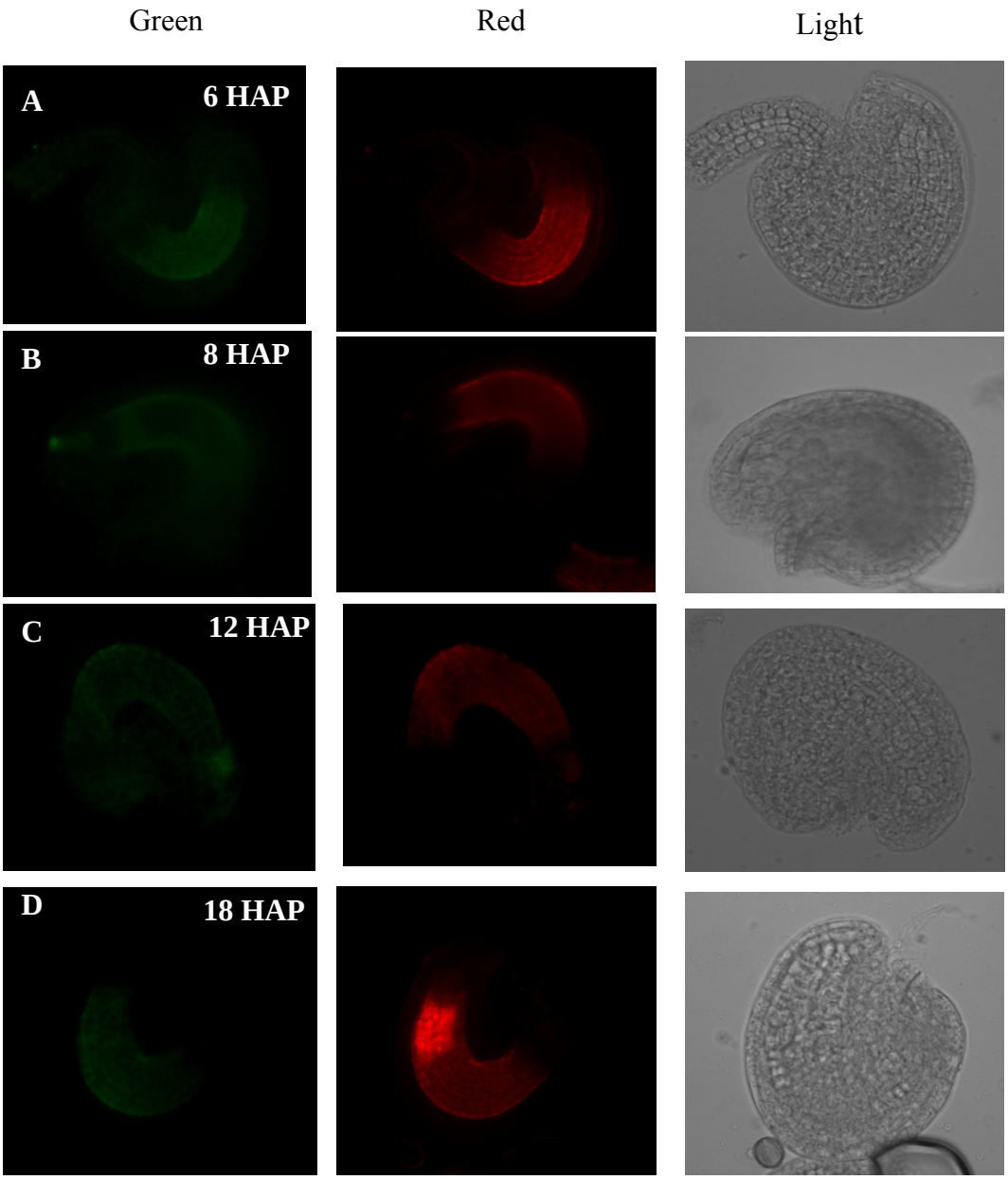


Figure II-19. DsRed1-E5 expression in the germinated pollen tube.

To discriminate sperm transcripts and proteomes transmission from genome DNA activation in the pollinated ovule, we made the cross between the WT (♀) and homozygous lines of *pPzIPT1::DsRed1-E5* (♂). The pollinated ovules were sampled from 0 HAP to 72 HAP for epifluorescent microscope observation. Both green and red fluorescence was detected in the ovule at 8 HAP and 12 HAP (Figure II-20 B and C). At 18 HAP, a strong red fluorescence signal and a weak green fluorescence signal were detected in the ovule. The mature DsRed1-E5 protein was present in the ovule until 48 HAP, then DsRed1-E5 protein was turned over. This observation indicated that no active DsRed1-E5 gene transcription and translation occurred after the fertilization (around 8 HAP). The observations from both Col-0 x *pPzIPT1::GUS* and Col-0 x *pPzIPT1::*DsRed1-E5 strongly suggest that transcripts and proteomes sperm *PzIPT1* can be transmitted to the pollinated ovule and may play an important role in early embryogenesis.


Green
Red
Light
A
6 HAP
B
8 HAP
C
12 HAP
D
18 HAP

(Continued…)

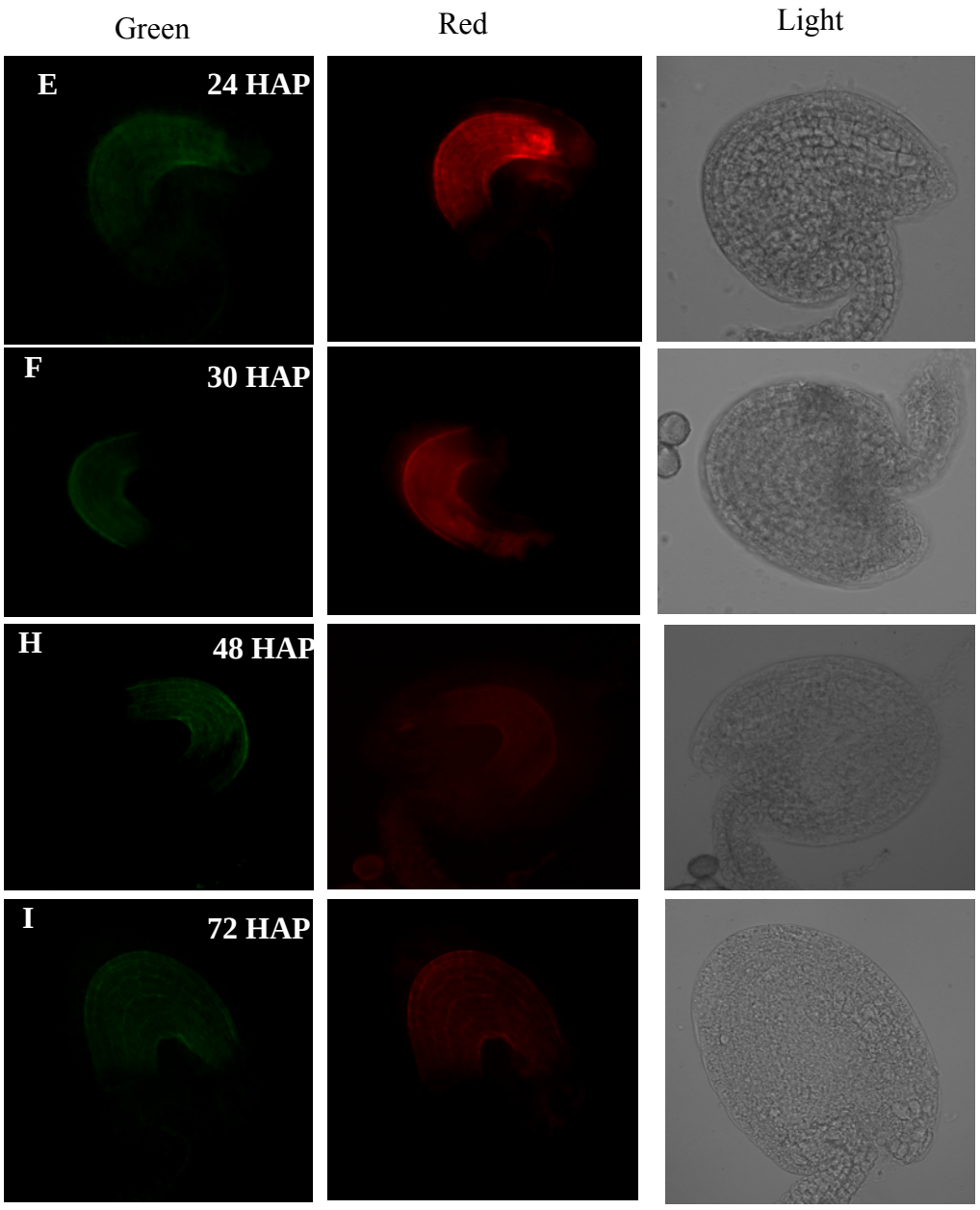


Figure II-20. DsRed1-E5 expression in the pollinated ovule of WT (♀) x *pPzIPT1::*DsRed1-E5 (♂)

DISCUSSION

PzIPT1 is a gametophyte-specific isopentenyltransferase and catalyzes cytokinin biosynthesis locally for germline cell division

It is generally assumed that the root is the main site for cytokinin synthesis. The other organs, such as cambium, the shoot apex, and immature seeds are also thought to synthesize cytokinins (Letham, 1994 ; Emery et al., 2000; Kakimoto, 2003). Although *AtIPTs* promoter::GUS expressed in a wide range of organs and cell types (Miyawaki et al., 2004; Miyawaki et al., 2006), to our knowledge, there have been no reports showing expression patterns of known plant *IPTs* linked to male and female germline. *Arabidopsis IPTs* are most abundant in young, rapidly dividing cells of shoot, root meristems and other vegetative tissue (Kakimoto, 2003; Miyawaki et al., 2004). Our results from *pPzIPT1::GUS/GFP/DsRed1-E5* showed no reporter gene expressed in root tip, shoot and root meristems (Figure II-6), but strong expression in male and female gametophyte. This indicated that *PzIPT1* is distinctly different from the known *IPTs* and is a gametophyte-specific isopentenyltransferase. It has been also accepted that cytokinins can be transported easily through vascular tissues to the place where plant needs for cell division and other biological functions (Oka, 2003; Hirose et al., 2008). The microspore and megaspore are not diploid sporophytic tissues anymore and may lost vascular connection to their maternal origins for efficient cytokinins translocation. Thus, locally synthesized cytokinins by *PzIPT1* may play an important role for germline cell division. Recent report from Sundaresan's lab about auxin-dependent patterning and female gamete specification also support our hypothesis (Pagnussat et al., 2009). They showed that localized auxin synthesis, along with auxin degradation/conjugation have

more important influence in establishing the female gametophyte patterning, rather than auxin efflux that regulates patterning in the diploid sporophytic tissues. Is locally synthesized cytokinin by *PzIPT1* sufficient for germline cell division? Our results from overexpression of AtCKX1, a major enzyme for cytokinin degradation in *Arabidopsis,* under *PzIPT1* promoter showed the microspore and megaspore mitosis I were impaired. Together, we believe that PzIPT1 is a gametophyte-specific isopentenyltransferase and catalyzes cytokinin biosynthesis locally for germline cell division.

Overexpression of *AtCKX1* under *PzIPT1*promoter partially blocks germline mitosis I, but not totally arrest mitosis I

The data we generated from reciprocal crosses of *pPzIPT1::AtCKX1* and WT (Table II-4 and II-5) indicated that not all microspores and megaspores carrying transgene (*CKX1*) were arrested at mitosis I, but some can "escape" or "bypass" the defective stage and undergo normal mitosis. Thus, the transgenic progenies can be obtained by self cross and reciprocal crosses with WT. This is true because we got heterozygous transgenic progenies from self cross of heterozygous lines. In table II-5, transgene can be transmitted to their progenies at 30% for male gametes and 39% for female gametes (expecting 50% if no impairment for gametes). As mentioned before, WT ovules were pollinated multiple times with heterozygous *pPzIPT1::AtCKX1* pollen, which increased chances for WT pollen to successful pollination. This is why we get higher percentage of female transmission rate than male although the male gametes were expected to be transmitted at the same rate as female.

Phenotype difference between *35S::AtCKXI* and *PzIPT1::AtCKX1* supports that *PzIPT1* is a gametophyte-specific *IPT*

It is interesting that the phenotype of overexpressing *AtCKXI* under constitutive 35S promoter in *Arabidopsis* is very different from expressing *AtCKX1* gene under *PzIPT1* promoter. *35S::AtCKX1* had severe defective vegetative growth and delayed flowering (Werner et al., 2003); whereas *PzIPT1::AtCKX1* vegetative growth was normal as WT except the reduced fertility. Noteworthy, the dwarf and stunted *35S::AtCKX1* also showed reduced seed setting (Werner et al., 2003). The authors also they conducted experiments to discriminate potential defects in male and female gametophyte development (personal communication). They did reciprocal crosses for heterozygous *35S:CKX1* and WT and result showed that the transgene in female gametophyte was perfectly transmitted (almost 1:1 of sensitive vs. hygromycin-resistant F1 progenies); whereas the transmission of transgene in male gametophyte was slightly affected (approximately 1:1.3 of resistant vs. sensitive progenies). They believed this male impairment was caused by extremely severe plant that could only produce very small amounts of pollen grains and aborted ovules were due to lacking of pollen grains and consequently abortion of non-fertilized ovules. This may be similar to our observation for quadruple mutant of *atipt1357,* in which, male and female gametophytes developed normally and aborted seeds were due to insufficient pollen grains or fertilized ovules stopped developing. The phenotypic difference between *35S::AtCKXI* and *PzIPT1::AtCKX1* also supports that *PzIPT1* is a gametophyte-specific *IPT.*

Cyclin genes and gametophyte cell division

Cytokinin is well-known for its function in promoting cell division. The CYCD3 D-type cyclins were shown the essential role for cytokinin-mediated cell division in vegetative tissues (Dewitte et al., 2007). The Affymetrix ATH1 arrays of *Arabidopsis* four stages of

male gametophyte development (Honys and Twell, 2004) and mature pollen (Pina et al., 2005) revealed that a significant number of cell cycle genes are expressed differently in four stages of male gametophyte development (see chapter I for detail). Among them, several genes seems linked to pollen mitosis I. CYCH;1, CYCD4;2, KRP2, CDKA;1, CYCA3;2, DEL2 and CKS2 showing big fold changes between uninucleate microspores and bicellular pollen could be involved in pollen mitosis I. The further pursuing of gametophyte cell division related cyclin genes linked with *PzIPT1* will be the most interesting.

Is *PzIPT1* regulated by environmental stress?

The *PzIPT1*'s close parolog *AtIPT3* was reported to be regulated by nitrogen availability and is a key determinant of cytokinin biosynthesis in response to rapid changes in the availability of nitrogen (Miyawaki et al., 2004; Takei et al., 2004). *AtIPTs* may also be regulated by other environmental changes (Takei et al., 2001; Miyawaki et al., 2004; Takei et al., 2004). In our hand, it seems *PzIPT1* is also regulated by some environmental stresses. In the crosses of WT (♀) x *pPzIPT1:GUS/DsRed1-E5* (♂), even the strong sperm-expressed GUS/DsRed1-5E homozygous lines were used to pollinate WT ovules, we were not able to get every pollinated ovule with expression of paternal transmitted GUS or DsRed1-E5. This may be due to manually disrupted pollination process and therefore creating stress for small and sensitive male and female gametes. Another coincidence also leads to this consideration. The *pPzIPT1::AtCKX1* plants were usually grown in long day (16 hr light / 8 hr dark). For one time, due to mechanical problem with the growth chamber, the plants were temporally moved to short day growth chamber (12 hr light / 12 hr dark) when plants were about to flower. The fertility

increased significantly after light/dark period changed (60% - 70% seed setting comparing to 50% in long day). The well-designed experiment should be performed in the future to uncover the mystery here.

CONCLUSION

Plumbago zeylanica is a representative for both sperm dimorphism and preferential fertilization. Isolation and characterization of genes differentially expressed in two dimorphic sperm cells that related to their fertilization fate is vital to understand the molecular control of sperm dimorphism and preferential fertilization. *Plumbago* isopentenyltransferase1 (*PzIPT1*) was identified as such a gene which is highly expressed in S_{vn} sperm cell. The *PzIPT1* expression specificity was confirmed by pollen whole mount *In situ* hybridization, semi-quantitative and quantitative RT-PCR in *Plumbago*. Here, we showed the further functional analysis of *PzIPT1* in heterologous systems (*Arabidopsis* and tobacco). The *pPzIPT1::GFP/GUS* in transgenic *Arabidopsis* were predominantly expressed in the sperm cells and synergids; and were also expressed in vegetative tissues but not in root and shoot meristems. The expression pattern of *pPzIPT1::GFP/GUS* is different from those of nine *Arabidopsis IPTs* promoter::*GUS*, suggesting that *PzIPT1* is functionally different from known vegetative expressed *IPTs* and is probably a germline-specific IPT. Overexpression of *PzIPT1* driven by *AtIPT3* promoter can restore the phenotype of *Arabidopsis atipt357* triple mutant suggests that *PzIPT1* is a functional *IPT*. The overexpression of *Arabidopsis* cytokinin oxidase 1 (*AtCKX1*) gene under *PzIPT1* promoter which mimicks 'loss of function' reveals *PzIPT1* is involved male and female gametophyte mitosis I. Together, we believe that PzIPT1 is a gametophyte-specific isopentenyltransferase and catalyzes cytokinin biosynthesis locally

for gametophytic cell division. We also showed that *PzIPT1* paternal transcripts and proteomes can be transmitted to the embryo sac and may initiate early cell division in endosperm and embryo development.

MATERIALS AND METHODS

Plant material

Plants of *Plumbago zeylanica* L. were grown in the greenhouse at University of Oklahoma under a light cycle of 14 h light/10 h dark at 25-28°C. The anthers were emasculated before anthesis for hand pollination. The pollinated and unpollinated ovules were sampled at different time point for RT-PCR experiment to examine the *PzIPT1* expression.

All *Arabidopsis thaliana* plants used in this study were in the Col-0 background. The seeds were sowed in moist soil and kept at 4°C for 2 days to one week. Then move to growth chamber with a light cycle of 16 h light/8 h dark at 22°C. For systematic analysis of *pPzIPT1::GUS* expression pattern, homozygous transgenic seeds were surface sterilized three times with 70% ethanol, followed by one time with 100% ethanol. Then seeds were dried in clean hood. The sterilized seeds were sprinkled on ½ MS medium and germinated in growth chamber with same condition as soil-grown plant.

Tobacco seeds were geminated on ½ MS medium and the leaves were used for Agrobacterium-mediated transformation. The transgenic plants were grown in the same condition as *Plumbago*.

Plant transformation

Agrobacterium tumefaciens-mediated *Arabidopsis* transformation was performed mainly based on a simplified floral dipping method (Clough and Bent, 1998). For selection of

transformants, seeds were selected based on the herbicide Basta resistance with the Basta concentration at 200 mg L^{-1}.

Tobacco transgenic plants were produced by *Agrobacterium*-mediated transformation of leaf discs and screened on semi-solid one-half Murashige and Skoog (Murashige and Skoog, 1962) agar plate plus 50 μg/ml kanamycin and 1% sucrose. Transgenic tobacco plants were transferred into soil to grow till flowering.

Molecular cloning

All the constructs were created in the backbone of pBIB vector (Becker, 1990). For herbicide screening, the Pnos promoter and the kanamycin resistance gene in the T-DNA region of pBIB-HYG-35S and pBIB-HYG were replaced by the Pmas promoter and BASTA coding region PCR-amplified from vector pSKI015 and flanked by *Hind*III and *Bgl*II restriction sites to make binary plant transformation vectors pBASTA-35S and pBASTA, respectively. The Gateway cassette and GFP coding sequence were PCR amplified from pEarleyGate103 and inserted into pBASTA-35S and pBASTA at the *Kpn*I and *Sac*I sites to create pBASTA-35S-GWR, pBASTA-35S-GWR-GFP and pBASTA-GWR-GFP. The S_{vn}-specific promoter *pPzIPT1* was PCR amplified with primers CYTPromHindIII (5'-AGTAAGCTTGCTGCAGAAAAATTAACCAAAT-3') and CYTProm3Kpn (5'-TAAGGTACCCCGCTCGCTCAGTGAGTTACTGT-3') and cloned into pBASTA-35S-GWR-GFP and pBASTA-35S-GWR at the *Hind*III and *Sal*I sites to replace the 35S promoter, resulting in pBASTA-pPzIPT1-GWR-GFP and pBASTA-pPzIPT1-GWR, respectively.

To investigate the subcellularlocalization of PzIPT1 protein, the coding region of *PzIPT1* was amplified by primers IPTBPB1 (5'-AAAAAGCAGGCTATGGCTAC

TGACCGTCAAGTGAC-3') and PzIPT1PB2 (5'-AGAAAGCTGGGTCAAGAA AAGCCTTCACGGTATCA-3') and cloned into pBASTA-pPzIPT1-GWR-GFP to obtain expression construct pBASTA-pPzIPT1-PzIPT1-GFP by *in vitro* DNA recombination with the help of Invitrogen Gateway technology.

To rescue the mutant phenotype of *atipt357*, promoter of *Arabidopsis AtIPT3* and the coding region of *PzIPT1* were fused by PCR with primers Pipt3PB1g (5'-AAAAAGCAGGCTTTTGCCAAGAGTTCAAGCGAAC-3'), PzIPT1Pipt3-R (5'-TCACTTGACGGTCAGTAGCCATGATGAAACGCTTTGCAATATAAA-3'), Pipt3PzIPT1-F (5'-TTTATATTGCAAAGCGTTTCATCATGGCTACTG ACCGTCAA GTGA-) and PzIPT1PB2, and the resulting fusion product *pAtIPT3::PzIPT1* was cloned into pBASTA-GWR-GFP with the help of Gateway technology to obtain expression construct pBASTA-pAtIPT3::PzIPT1-GFP.

RT-PCR was performed to amplify Arabidopsis *AtCKX1* coding sequence using primers ATCKX1PB1 (5'-AAAAAGCAGGCTATGGGATTGACCTCATCCTTACG-3') and ATCKXPB2 (5'-AGAAAGCTGGGTTTATACAGTTCTAGGTTTCGGCAGT-3'). The amplified *AtCKX1* coding sequence was cloned into pBASTA-pPzIPT1-GWR to obtain expression construct pBASTA-pPzIPT1-AtCKX1.

The reporter gene GFP was PCR amplified from pBIN-m-gfp5-ER and inserted into pBASTA at the *Xba*I and *Sac*I sites, resulting in vector pBASTA-GFP. To clone the promoter of *PzIPT1*, an 1129 bp PCR fragment containing 63 bp 5' UTR cDNA sequence of *PzIPT1* was inserted into pBASTA-GFP at the *Hind*III and *Sal*I sites to make pBASTA-pPzIPT1-GFP. The coding region of Timer (pTimer, Clontech) was cloned into sites *Xba*I and *Not*I of pBluescript II SK(+), resulting in pSK-Timer. Then pSK-Timer

was digested with *Xba*I and *Sac*I to transfer the Timer sequence into pBASTA-pPzIPT1-GFP to replace the GFP reporter gene. The resulting construct was named pBASTA-pPzIPT1::Timer.

RNA isolation

Ten pollinated and unpollinated ovules of *Plumbago* were sampled each time point at 5HAP, 7HAP, 8HAP, 9HAP, 10HAP, 12HAP, 24HAP, and 48HAP for RNA extraction. Pooled samples from 10-15 of *pPzIPT1::AtCKX1* transgenic *Arabidopsis* plants were collected for rosette leaf, cauline leaf, and flower samples. All tissues were frozen immediately in liquid nitrogen and stored at -80ºC until used. Total RNA was isolated from 200 mg tissue powder using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

RT-PCR

Semi-quantitative reverse transcription PCR (RT-PCR) analysis was carried out using the SuperScript ^{TM} One-Step RT-PCR with Platinum Taq Kit (Invitrogen). Prior to RT-PCR reaction, RNA samples were treated using the TURBO DNA-free Kit (Ambion) to remove contaminating DNA. For each RNA sample, 0.5µg RNA template was used in each reaction (25 µl). The primers used in *pPzIPT1::AtCKX1* transgenic plants are: At2g41510_For (5'-TTGACCTCATCCTTACGGTTCC-3') and At2g41510_Rev (5'-CACCACCTGAGACATCAACA-3'). *Arabidopsis* β-Tubulin8 used as internal control and primer pair of β-Tubulin8_F (5'-CGTGGATCACAGCAATACAGAGCC-3') and β-Tubulin8_R (5'-CCTCCTGCACTTCCACTTCGTCTTC-3') were used. The following program was used for all RT-PCR reactions: 50ºC for 15 min (for cDNA synthesis), 94ºC for 5 min followed by 25 or 28 cycles of 15 seconds at 94ºC, 30 seconds at 55ºC, and 45

seconds at 72ºC. The RT-PCR products were visualized in 1% agarose gels under UV light.

Preparation of whole-mount cleared ovules

Cleared pistils of *pPzIPT1::AtCKX1* transgenic *Arabidopsis* plants at different developmental stages were prepared as described by Liu 1998 (Liu and Meinke, 1998) with slight modification. Briefly, the inflorescence was fixed in ethanol-acetic acid (3:1) overnight at 4°C and dehydrated by two subsequent 1 h steps in 90% and 70% ethanol. The pistil was then cleared in Hoyers solution (100 g chloral hydrate, 5 ml glycerol in 30 ml water) overnight at 4°C. Pistils were dissected and observed using Nikon Eclipse E 800 widefield microscope equipped with DIC optics. Images were recorded with Retiga1300 image camera.

Alexander's staining of pollen

Dissected anthers from fresh flower were incubated with Alexander's staining solution (Alexander, 1969) under the coverslip for 15 min at room temperature. Observation was performed with a Nikon Eclipse E 800 widefield microscope. Images were recorded with Retiga1300 image camera.

GUS staining

Transgenic *Arabidopsis* plants harboring *pPzIPT1-GUS* were used for histochemical detection of GUS activity (Jefferson et al., 1987). Plant tissues were vacuum-infiltrated in GUS staining solution containing 100 mM NaPO4, 2 mM $K_3Fe(CN)_6$, 0.1% Triton-X-100 and 0.5 mg ml^{-1} X-Gluc, incubated at 37°C overnight and de-colored in 70% ethanol.

***Semi-vivo* culture of tobacco pollen tube**

In order to examine GUS/GFP expression in sperm cells of transgenic tobacco plants harboring *pPzIPT1-GUS,* a semi-vivo culture of tobacco pollen tube method was used to get viable sperm cells (Cao et al., 1996). Briefly, unopened flowers just before the anthesis were hand pollinated. After7-9 h of pollen tube growth *in vivo*, styles were excised and floated on the surface of the modified Brewbaker's medium (15% sucrose + 0.01% (W/V) boric acid + 0.03% (W/V) $Ca(NO_3)_2$, at pH 4.60) with end immersed in the medium. The cultured styles were kept at 27°C in growth chamber until pollen tube emerged. After sperm cells were formed, the styles were stained with GUS solution or directly observed for GFP expression using an epifluorescence microscope (Leitz Dialux 20) equipped with GFP filters set.

***Arabidopsis* pollen tube *in vitro* germination**

Freshly anther-dehisced flowers were used for pollen tube germination according to Boavida et al. (Boavida and McCormick, 2007). Anthers were dabbed onto surface of semi-solid germination medium on microscope slides. The medium contains 0.01% boric acid, 5mM CaCl2, 5mM KCl, 1mMMgSO4, 10% sucrose pH7.5, 1.5% low-melting agrose. Then, the slides were incubated in moisture chamber (square plastic plate with wet paper towel) at 25°C.

DAPI staining of pollen

DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) from Molecular Probes (Invitrogen) was dissolved in de-ionized water at a concentration of 10 mg/ml to make 1000X stock. The working solution can be diluted in de-ionized water or PBS buffer. Apply a few drops working solution on fixed or fresh pollen and stain for 10-30 minutes.

The excitation wavelengths around 370 nm and emission peaks around 450 nm were used to set filter.

Aniline Blue staining

0.01% in 50 mM K_2HPO_4 decolored aniline blue solution was used to stain callose accumulation in the newly formed cell wall during megasporogenesis (Rodkiewicz, 1970). The stained cell wall was visualized with the same wavelengths setting as for DAPI staining.

Microscopy analysis of fluorescent protein

GFP specimens were observed under epifluorescence microscopes (Leitz Dialux 20 and Nikon Eclipse E 800 widefield microscope) equipped with GFP filter setting of the excitation wavelengths around 488 nm and emission peaks around 515 nm. Reproductive organs with GFP reporter were also observed by an Olympus FluoView 500 laser scanning microscope with argon laser excitation at 488 nm and 505–550 emission filter set.

DsRed1-E5 specimens were observed under Nikon Eclipse E 800 epifluorescence microscopes equipped with both GFP filter setting and DS-Red filter setting of the excitation wavelengths around 560 nm and emission peaks around 610 nm. The DsRed1-E5 specimens were also observed by a Zeiss LSM 510 Meta laser scanning microscope with argon laser settings for GFP (excitation 488 nm / emission 500-530 nm), and DS-Red (excitation 545 nm / emission 565-615 nm).

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