ROLES OF AUXIN RESPONSE FACTORS IN *ARABIDOPSIS* FLOWER DEVELOPMENT

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ABSTRACT

MIIN-FENG WU: Roles of Auxin Response Factors in *Arabidopsis* Flower Development

(Under the direction of Jason Reed)

The plant hormone auxin regulates organ initiation, growth, and development. The Auxin Response transcription Factors (ARFs) mediate transcriptional responses to auxin. Under low auxin concentration, the ARF proteins bind Aux/IAA proteins, which inhibit transcription. As auxin concentration is elevated, Aux/IAA proteins are rapidly degraded, thus allowing ARFs to activate target genes. Two closely related *ARF* genes, *ARF6* and *ARF8*, regulate flower maturation by promoting stamen elongation and gynoecium development. *ARF6* and *ARF8* are cleavage targets of plant microRNA, *miR167*. Phenotypes and transcript expression patterns of *miR167*-insensitive *mARF6* and *mARF8* transgenic plants showed that *miR167* patterns *ARF6* and *ARF8* transcript distribution in the ovule and in the anther, and this patterning activity is important for development of these two organs. Silencing *ARF6* and *ARF8* in the style and in the ovule funiculus by expressing a *miR167* precursor gene, *MIR167a*, further showed that *ARF6* and *ARF8* promote stigmatic papillae elongation and pollen tube growth in these two different floral tissues. To further reveal functions of other *ARF* genes in flowers, especially during ovule formation, we

repressed activity of ARF proteins by expressing a gain-of-function *aux/iaa* gene in the ovule outer integument and in the funiculus. We found that auxin response in the ovule is important for the asymmetric growth of the outer integument and for differentiation of the entire ovule. These results showed that auxin and the ARF-mediated auxin responses regulate multiple aspects of flower development.

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AG	AGAMOUS
ANT	AINTEGUMENTA
ARF	Auxin Response Factor
AuxRE	Auxin Response Element
ATS	Aberrant testa shape
AXR3	Auxin-Resistant 3
BDL	BODENLOS
ETT	ETTIN
GUS	β-glucuronidase
INO	INNER-NO-OUTER
IAA	Indole-3-Acetic Acid
KAN	KANADI
MP	MONOPTEROS
NPH4	Nonphototropic hypocotyl
SAUR	Small Auxin Up RNA
SHY2	Suppressor of *hy2* mutation 2
SLR	Solitary root
STK	SEEDSTICK
SUP	SUPERMAN
UAS	Upstream Activation Sequence
X-gluc	5-bromo-4-chloro-3-indolyl-D-glucuronic acid

CHAPTER I

Reproductive Organ Development in *Arabidopsis thaliana* Flower

Arabidopsis flowers are initiated continuously at the floral meristem in a spiral phyllotaxy, and thus a single inflorescence has flowers of all developmental stages in sequence according to the order in which they emerged (Smyth et al., 1990). Arabidopsis flower development stages have been described by Smyth et al. (1990). During stages 1-12, Arabidopsis flowers develop protective structures for male and female gametophytes which support pollen and egg cell development. The flower opens at stage 13, and anthesis and pollination also occur at this stage (Smyth et al., 1990).

Formation of the ovule

The ovule is the diploid structure where female gametophyte development and embryogenesis occur (Robinson-Beers et al., 1992). After fertilization, ovules develop into seeds, the propagation unit of plants and an important food source of many animal species. The ovule is initiated as a radially symmetric structure in stage 9 flowers (Schneitz, 1995). The ovule primordia can be divided into three different parts along the proximal-distal axis: thefuniculus, the chalaza and the nucellus (Fig. I-1). The nucellus harbors the megaspore mother cell which undergoes meiosis to form

the megagaspore. The megagaspore undergoes nuclear divisions to produce the eight-nuclear embryo sac, which then cellularizes. After fertilization, the embryo and endosperm develop from the egg and central cell at the megagametophyte (Bowman et al., 1991; Robinson-Beers et al., 1992; Yang and Sundaresan, 2000).

The ovule switches to bilateral symmetry after the inner integument initiates at the flanks of the chalazal region, and the outer integument initiates right after the inner integument. The integuments form the protective seed coat after fertilization. The outer integument grows more extensively at the gynobasal (toward the base of the gynoecium) side of the ovule whereas the growth at the gynoapical (toward the style) side is limited (Skinner et al., 2004). The asymmetric growth of the outer integument makes the ovule curves toward the transmitting tract as it matures. This curvature places the micropylar opening close to the funiculus, which facilitates the pollen tube entry into the ovule at fertilization.

The funiculus connects the ovule to the placenta which latter develops into septum and transmitting tract. Vascular tissues form inside the funiculus and transport nutrients into the ovule to support growth of the ovule and later, the embryo.

Ovule development takes place within carpels. In the Arabidopsis flower, two carpels fuse to form the gynoecium, and ovules are initiated on the flanks of the meristematic placental tissues of carpel margins (Skinner et al., 2004; Smyth et al., 1990). Developing and maintaining the meristematic carpel placenta requires tight regulation of several genes. *SHOOT MERISTEMLESS* (*STM*) is an important regulator during shoot meristem formation and maintenance (Long and Barton, 1998b; Long et al., 1996). *STM* is expressed in the carpel medial region to maintain

the meristem in this area (Skinner et al., 2004). *stm* loss-of-function mutants have phenotypes in the carpels ranging from reduced formation of placental tissues, inhibited carpel fusion to complete loss of carpel development, whereas overexpressing *STM* causes ectopic carpel formation in the ovule (Scofield et al., 2007). *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2* are expressed at the boundaries of incipient cotyledon and leaf primordia and are required for organ separation (Aida et al., 1997; Aida et al., 1999). Expression domains of *CUC* genes overlap with those of *STM* in the carpel medial region (Takada et al., 2001). *CUC1* and *CUC2* are both required for the expression of *STM* at the shoot apical meristem, and proper *STM* expression is also required for correct expression pattern of *CUC2* gene (Aida et al., 1999). The interdependency between *CUC* and *STM* genes can also be true in the carpels. Proper expression of *CUC* and *STM* might be important for specifying the meristematic placental tissues. In addition, development of carpel margins requires *SPATULA* (*SPT*) (Alvarez and Smyth, 1999; Bowman et al., 1999). *SPT* functions in supporting carpel margin growth. Loss-of-function *spt* mutant carpels do not form transmitting tract and have fewer ovules (Alvarez and Smyth, 1999).

Carpel identity requires the class C MADS-box gene *AGAMOUS* (*AG*) (Bowman et al., 1991; Yanofsky et al., 1990), and the presence of *AG* transcripts in the placental tissues and ovule integument implies that *AG* regulates ovule development (Ferrandiz et al., 1999). However, ovules formed on the carpelloid sepals of *ap2 ag* mutants indicates that *AG* alone is not absolutely required for ovule formation (Alvarez and Smyth, 1999). *AG* functions redundantly with three other MADS-box genes,

SHATTERPROOF1 (*SHP1*), *SHP2* and *SEEDSTICK* (*STK*), to specify ovule identity, and *stk shp1 shp2* triple mutant plants form ovules with leaf- or carpel-like structures (Pinyopich et al., 2003). Another class of MADS-box genes, *SEPALLATA* (*SEP*), interacts with class B and C MADS-box genes to specify floral organ identity also regulate the formation of ovule identities (Favaro et al., 2003; Honma and Goto, 2001). *SEP1/sep1 sep2 sep3* mutants have similar ovule phenotypes to those of *stk shp1 shp2* triple mutants, and SEP3 can bridge protein interactions among STK, AG, and SHP proteins, suggesting that multimeric MADS-box protein complex might participate in defining ovule identity (Favaro et al., 2003).

BELL1 (BEL1), a homeodomain transcription factor, maintains ovule identity by repressing *AG* expression in the ovule around anthesis (Modrusan et al., 1994; Ray et al., 1994; Reiser et al., 1995). Loss-of-function *bel1* mutant ovules form collar-like, amorphous structures in the chalazal region. Some of the mutant ovules continue to grow and transform into carpel-like structures (Ray et al., 1994; Reiser et al., 1995; Robinson-Beers et al., 1992). *BEL1* does not inhibit *AG* expression before anthesis. The formation of carpel-like ovules in *bel1* mutant flowers might suggest that loss of *BEL1* might cause *AG* to predominate at anthesis and cause the amorphous integument to turn into carpel in a self-reinforcing manner (Skinner et al., 2004).

The proper development of ovule integuments requires regulation of several genes that also participate in other lateral organ formation. AINTEGUMENTA (ANT), an AP2-domain transcription factor that regulates cell growth and organ size by maintaining the meristemic nature of cells during organ development is important for integument initiation in the ovule (Krizek, 1999; Mizukami and Fischer, 2000).

ANT is only expressed in the chalazal area of the ovule, and loss-of-function *ant* mutants form naked ovules that lack integuments and only have nucellus and funiculus (Elliott et al., 1996). Similarly to *ant* mutant ovules, loss-of-function *wuschel* (*wus*) mutant ovules also lack integument formation (Gross-Hardt et al., 2002). *WUS* is a homeodomain protein that is important for meristem maintenance in both shoot and floral meristems (Mayer et al., 1998). In the ovule, *WUS* is expressed only in the nucellus and thus regulates integument initiation non-cell-autonomously (Gross-Hardt et al., 2002). In the floral meristem, initiation of *AG* expression requires *WUS*, and *AG* can later inhibit *WUS* expression to terminate the floral meristem (Lenhard et al., 2001; Lohmann et al., 2001). However, loss of *WUS* activity does not affect *AG* expression in ovules (Gross-Hardt et al., 2002). In addition, *ANT* expression is also not affected in the *wus* mutant ovules and vice versa, suggesting integument initiation might require independent inputs from both the nucellus and the chalaza (Gross-Hardt et al., 2002).

Multiple gene families have been identified to participate in integument growth. Arabidopsis class III homeodomain-leucine zipper family (HD-Zip III) proteins promote the formation of adaxial fate in lateral organs (Long and Barton, 1998a; McConnell and Barton, 1998; McConnell et al., 2001; Otsuga et al., 2001; Prigge et al., 2005). Loss-of-function mutants of multiple HD-Zip III genes cause loss of bilateral symmetry in embryos and abaxialization of leaves (Emery et al., 2003; Prigge et al., 2005). Two genes of the HD-Zip III family, *PHABULOSA* (*PHB*) and *REVOLUTA* (*REV*), are expressed in the ovule (Sieber et al., 2004a; Sieber et al., 2004b). *PHB* is expressed in the placental region before ovule emergence and in the

inner integument later. Ovules of the heterozygous gain-of-function *phb-1d* mutant have growth arrested outer integuments, suggesting that in the ovule, as in other lateral organs in *phb-1d* mutant, the abaxial fate is lost (Sieber et al., 2004a). *REV* is expressed in the entire ovule primordium and in both integuments (Sieber et al., 2004b). However, the function of *REV* in the ovule is still not clear.

Members of *KANADI* (*KAN*) and *YABBY* gene families specify abaxial fate (Eshed et al., 2001; Eshed et al., 2004; Kerstetter et al., 2001; Sawa et al., 1999; Siegfried et al., 1999). Ectopically expressing *KAN* genes causes lateral organs to be abaxialized, whereas loss-of-function *kan1 kan2* mutant results in adaxialized lateral organs (Eshed et al., 2001; Eshed et al., 2004; Kerstetter et al., 2001). Similar results have been obtained from both the gain-of-function and the loss-of-function *yabby* mutants (Sawa et al., 1999; Siegfried et al., 1999). It is proposed that the initial primordium polarity establishment requires antagonistic juxtaposition between domains expressing *KAN* and *HD-Zip III* genes, and the following lamina expansion requires abaxial expression of *YABBY* genes (Eshed et al., 2004; Hudson and Waites, 1998; Waites, 1995).

Similarly to *phb-1d* mutants, outer integument growth of *kan1-2 kan2-1* mutant ovules is also arrested (Eshed et al., 2001). One member of the *KAN* gene family, *ABERRANT TESTA SHAPE* (*ATS*)/*KAN4*, is expressed in the outer cell layer of the inner integument, and loss-of-function *ats/kan4* causes congenital fusion of the inner and the outer integuments (McAbee et al., 2006). The *kan1 kan2 ats/kan4* triple mutant has complete loss of laminar expansion of both integuments, revealing that

KAN1 and *KAN2* define the outer integument abaxial fate whereas *ATS/KAN4* defines the inner integument abaxial fate (McAbee et al., 2006).

INNER-NO-OUTER (*INO*) is the only *YABBY* gene expressed in the ovule. *INO* is only expressed in the abaxial (outer) cell layer of the gynobasal outer integument (Villanueva et al., 1999). In strong loss-of-function *ino-1* mutant ovules, the initiation and growth of the outer integument is completely absent (Schneitz et al., 1997; Villanueva et al., 1999). In contrast, ectopic *INO* expression in the gynoapical side of the ovule causes symmetric growth of the outer integument (Meister et al., 2002). *INO* maintains its own expression through a feedback regulation on its own promoter (Meister et al., 2002). *SUPERMAN* (*SUP*), a C2H2-type zinc finger transcription factor, controls cell proliferation in stamen and carpel primodia and in ovule outer integument (Bowman et al., 1992; Gaiser et al., 1995; Sakai et al., 1995; Schultz et al., 1991). Loss-of-function *sup* ovule outer integuments grow symmetrically on both gynobasal and gynoapical sides (Gaiser et al., 1995), and this symmetrically growth is attributed to ectopic *INO* expression in gynoapical outer integument (Villanueva et al., 1999). In wild-type ovules, SUP inhibits *INO* activity by disrupting the feedback activation of *INO* on its own promoter in gynoapical outer integument, and expressing *SUP* in the *INO* expression domains causes arrested outer integument growth (Meister et al., 2002).

In addition to the abaxial-adaxial and gynobasal-gynoapical axes of ovules, the proximal-distal axis is also patterned to achieve proper ovule formation. *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*), a putative transcription factor, participates in the proximal-distal axis patterning (Schiefthaler et al., 1999; Yang et al., 1999).

Loss-of-function *spl/nzz* ovules have shifted proximal-distal boundary. The *spl/nzz* mutant ovules develop smaller nucellus and longer funiculus, and the smaller nucellus lacks a megaspore mother cell in the developing ovule nucellus (Balasubramanian and Schneitz, 2000). *AG* activates *SPL/NZZ* expression directly by binding to the 3' untranslated region on *SPL/NZZ* gene (Ito et al., 2004). This indicates that *AG* participates in female gametophyte formation by regulating *SPL/NZZ*, and the development of megasporophyte in the nucellus might affect the funiculus growth.

Anther development

Anther development and pollen formation occur in stamens. Stamen primordia are initiated at floral stage 6 and differentiate into anther and filament later in the development (Smyth et al., 1990). There are two major phases of anther development (Goldberg et al., 1993). In phase I, the basic form of the anther is established, and the sporophytic cells in the anthers undergo meiosis to form microspores. During phase II, the microspores undergo further mitosis and differentiate into male gametophytes or the pollen grains. At the same time, the filament undergoes extensive growth and pushes the anther to reach the top of the stigma. When the flower opens, pollen grains are released from the anther to the stigmatic papillae (Goldberg et al., 1993).

The pollen grains are encased by sporophytic tissues of the anther. These tissues are the endothecium, the stomium, the connective, the septum, and the tapetum. They play important roles during pollen development and the following dispersal of mature pollen grains (Sanders et al., 1999). Several genes have been identified to be

important regulators during anther and pollen development (Sanders et al., 1999; Scott et al., 2004).

In addition to promoting nucellus development in the ovule, *SPL/NZZ* also regulates anther cell division and differentiation (Yang et al., 1999). In wild-type anthers, hypodermal cells in the four anther lobes develop into archesporial cells, and the archesporial cells further differentiate into primary sporogenous cells and primary parietal cells (Sanders et al., 1999). The latter develops into sterile tissues surrounding the anther locules, including the tapetum and the endothecium. Meanwhile, the primary sporogenous cells differentiate into pollen mother cells and undergo meiosis to generate the microspores (Sanders et al., 1999). In *spl/nzz* mutant anthers, development of both the primary sporogenous cells and primary parietal cells are affected. The primary sporogenous cells do not form pollen mother cells, and the primary parietal cells fail to differentiate (Yang et al., 1999). Both female and male gametophyte development in *spl/nzz* mutant flowers are arrested at a similar time point, suggesting that *SPL/NZZ* is a general regulator of female and male gametophyte formation, both of which are activated by *AG* (Ito et al., 2004).

A putative leucine-rich repeat receptor protein kinase gene (LRR-RPK), *EXCESS MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS* (*EMS1/EXS*), regulates late steps of pollen development (Canales et al., 2002; Zhao et al., 2002). *ems1/exs* mutant anthers lack the tapetal cells and have excess microsporocytes due to lack of cytokinesis during meiosis (Zhao et al., 2002), showing that LRR-RPK-mediated cell to cell signaling might be important for tapetum differentiation and pollen formation (Ma, 2005). The discovery of *TAPETUM DETERMINANT 1* (*TPD1*) further supports

this hypothesis. *TPD1* encodes a small peptide molecule and its loss-of-function mutant has almost identical phenotypes to those of *ems1/exs* mutants, suggesting that *TPD1* could be the ligand of *EMS1/EXS* (Yang et al., 2003). One possible model for anther development is that TPD1 binds to EMS1/EXS and triggers a series of phosphorylation events of downstream targets, which allows the following developmental events to occur (Ma, 2005). *DYSFUNCTIONAL TAPETUM 1* (*DYT1*), a bHLH transcription factor, could be one of the downstream targets of *EMS1/EXS* (Zhang et al., 2006). *dyt1* mutant anthers have abnormal tapetal cells and arrested meiotic cytokinesis. Moreover, *DYT1* expression levels are reduced in *spl/nzz* and *ems1/exs* mutants (Zhang et al., 2006). This genetic and molecular evidence suggest that the interactions between tapetum cells and microsporocytes are essential for pollen grain formation. The tapetum could provide the enzymes and signals needed for pollen grain maturation.

After pollen grains mature, the anther has to break and release pollen. This process is called anther dehiscence or anthesis. The sterile tissues in anthers not only support the development of pollen but they also facilitate pollen release. During anther development, these sterile tissues undergo a series of changes called the pre-dehiscence program (Sanders et al., 1999). The pre-dehiscence program includes: tapetum degeneration, expansion of the endothecial layer, and deposition of fibrous bands in endothecium and connective tissues. This program involves a series of cell death and desiccation processes that initiate anther breakage and pollen release; disruption in any of these events may lead to anther indehiscence (Goldberg et al., 1993; Sanders et al., 1999). At flower stage 13, anthesis occurs when the longer

stamens have reached the height of the gynoecium. Anther stomium cells break to release pollen grains onto the receptive stigmatic papillae, and fertilization ensues. Pollens rehydrate, germinate, and form pollen tubes when they reach stigma.

Studies have found that jasmonic acid (JA) plays important roles during anther dehiscence in Arabidopsis. Known JA-deficient mutants often share similar phenotypes, such as shorter stamen filaments, delayed anther dehiscence and unviable pollen (Scott et al., 2004). The *DELAYED DEHISCENCE 1* (*DDE1*) gene encodes 12-oxophytodienoate reductase, an enzyme in the JA biosynthetic pathway, and its loss-of-function mutant anther indehiscent phenotypes are caused by delayed stomium cell degeneration (Sanders et al., 2000). Another gene called *DEFFECTIVE IN ANTHER DEHISCENCE 1* (*DAD1*) encodes a chloroplast phospolipase A1 which catalyze the first step of JA biosynthesis, and its loss-of-function mutant has similar delayed dehiscence phenotypes (Ishiguro et al., 2001). In addition to JA biosynthetic mutants, JA-insensitive mutant *coronatine-insensitive 1* (*coi1*) which is defective in an F-box protein in JA signaling pathway also has indehiscent anthers (Xie et al., 1998). These studies show that JA is a critical signal for anther dehiscence to occur. Both *DDE1* and *DAD1* are expressed in the stamen filament, not in the anther, suggesting that these genes regulate JA levels in the stamen filament (Ishiguro et al., 2001; Sanders et al., 2000). The force that breaks the stomium cells at anther dehiscence comes from dehydration and shrinkage of the endothecium and connective cells. It is therefore proposed that JA in the stamen filament might affect the anther desiccation by regulating water transport (Ishiguro et al., 2001).

Pollen tube growth and guidance

For land plants, successful fertilization requires precise growth and guidance of the pollen tubes to the female gametophyte. In crucifers, such as Arabidopsis thaliana, pollen tube growth is initiated when the pollen grain adheres to the dry stigma. The pollen grains monitor the interactions between the stigma and itself, and the pollen tube will only start growing on a compatible stigma (Johnson and Preuss, 2002). The stigma is coated with cuticle and a superficial proteinaceous layer with several unidentified molecules, which is required for interacting with pollen grains (Gaude and Dumas, 1986). The outer pollen wall, the exine, is important for the initial species-specific recognition step (Zinkl et al., 1999). Molecules important for stigma-pollen interactions have been uncovered by studying natural variants of the self-incompatible *Brassica* (Dickinson et al., 1998; Stein et al., 1996; Stein et al., 1991). The self-incompatibility locus (S locus) encodes two stigma proteins, a transmembrane receptor kinase (SRK) and an extracellular glycoprotein (SLG), and a pollen-specific protein, a cysteine-rich protein (SCR) (Dickinson, 2000; Schopfer et al., 1999). The SRK and SCR interaction is the determinant factor for specificity, and this interaction is enhanced by the SLG molecule (Johnson and Preuss, 2002; Takayama and Isogai, 2003). In self-incompatible species, the interaction between SRK and SCR blocks water uptake of the pollen grains and inhibit pollen tube initiation. In Arabidopsis thaliana genome, the S locus is not completely lost. Instead, subtle mutations occurred in the SRK and SCR loci to inactivate the genes and make Arabidopsis thaliana self-fertile (Kusaba et al., 2001).

In Arabidopsis, pollination requires rehydration of the desiccated pollen grains, and this process is mediated by the lipid-rich pollen coat which facilitates water uptake (Mayfield and Preuss, 2000). GRP17 is an abundant pollen coat protein that contains an oil-binding oleosin domain, and mutation in *GRP17* gene impairs pollen rehydration (Mayfield and Preuss, 2000). *GRP17* belongs to the *GRP* gene family and the arrangement of the *GRP* gene clusters might facilitate speciation (Mayfield et al., 2001).

After pollen hydration and germination, the pollen tube enters the stigma and grows between the stigma cell wall through the extracellular matrix. Since bacterial or fungal infection through the stigma is rare, specific recognition signals must exist on both the pollen tube and the stigma (Johnson and Preuss, 2002). However, the nature of these signals remains unclear.

After pollen tubes exit the style, they enter the transmitting tract. The transmitting tract cells are surrounded by extracellular matrix, and signals required for pollen tube growth might be embedded there (Lord, 2000). Arabinogalactan proteins (AGPs) are one of the prominent transmitting tract extracellular components and function in cell to cell interactions (Showalter, 2001). Tobacco AGPs, transmitting tissue-specific proteins (TTSs), can support and direct pollen tube growth. In transgenic plants with reduced TTS levels, the pollen tube growth rate is slower (Cheung et al., 1995). However, TTS alone might not be sufficient for providing signal gradient to support pollen tube elongation, and multiple signals may be required (Johnson and Preuss, 2002). In addition, calcium ions and pH in the

transmitting tract can also be important for pollen tube growth (Cheung et al., 1995; Holdaway-Clarke et al., 2003).

To reach the female gametophyte, the pollen tube must exit the transmitting tract and the placental tissue, grow to reach the funiculus and enter the micropyle. Studies have shown that the ovule and the female gametophyte might send out signals to attract pollen tube growth (Hulskamp et al., 1995; Ray et al., 1997). It is believed that there are two signals guiding pollen tubes toward the female gametophyte: the funicular guidance and the micropylar guidance (Higashiyama et al., 2003). The funiculus guidance signals attract the pollen tube to exit the transmitting tract and guide the pollen tube from the placenta to the funiculus. The micropylar guidance signals attract the pollen tube to enter the female gametophyte. Pollen tubes in the female gametophyte mutant *magatama* (*maa*) fail to enter the micropyle, but the funiculus guidance is not affected in the *maa* mutant flowers (Shimizu and Okada, 2000), suggesting the funiculus guidance signals might come from tissues other than the female gametophyte . Mutants affecting ovule morphology can have defects in pollen tube guidance. In *ino* mutant gynoecia, the pollen tube grows randomly after it exits the septum, whereas the wild-type pollen tube grows along the funiculus and enter the microyle (Baker et al., 1997), suggesting the outer integument can be important for funiculus guidance. The genetic analysis of *pop2* mutant might reinforce the idea that ovule can regulate pollen tube guidance. *POP2* encodes a transaminase of γ-amino butyric acid (GABA) (Palanivelu et al., 2003). In *pop2* mutant flowers, the GABA concentration in the gynoecium is abnormally high. However, the pollen tube growth in the transmitting tract is not affected despite the

high GABA concentration, and the pollen tube guidance is only affected after the pollen tubes exit the septum. *POP2* is expressed in the outer integument where the GABA concentration is low in the wild-type gynoecium (Palanivelu et al., 2003), suggesting that *POP2* and similar genes affecting GABA concentration in the ovule outer integument is important for funiculus guidance.

Laser ablation of *Torenia fournieri* synergid cells showed that synergid cells secrete signals guiding pollen tubes to the micropyle (Higashiyama et al., 2001). Small peptide EGG APPARATUS 1 (EA1) secreted by the maize synergid cells has been shown to be a signal guiding pollen tube to the female gametophyte (Marton et al., 2005). However, the absence of EA1 in dicotyledons suggests that EA1 might not be a general signal. It has been suggested that synergid cells participate in multiple fertilization processes, such as the arrest of pollen tube growth, the release of the pollen contents and the migration of the sperm cells to the egg cell and the central cell (Weterings and Russell, 2004). A structure called filiform apparatus at the micropylar side of the synergid cell plays important roles during the fertilization process. One of the suggested functions of the filiform apparatus is to secrete the pollen tube guidance signals (Higashiyama et al., 2003; Weterings and Russell, 2004). Mutation in transcription factor *MYB98* arrests filiform apparatus development, and also affects the mycroplar phase of pollen tube guidance, supporting this model (Kasahara et al., 2005). In addition to small peptides, calcium secreted by the synergid cells might also serve as pollen tube attractant, but calcium might need to work together with other signals to achieve species specificity of pollen tube guidance (Higashiyama et al., 2003).

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Figure

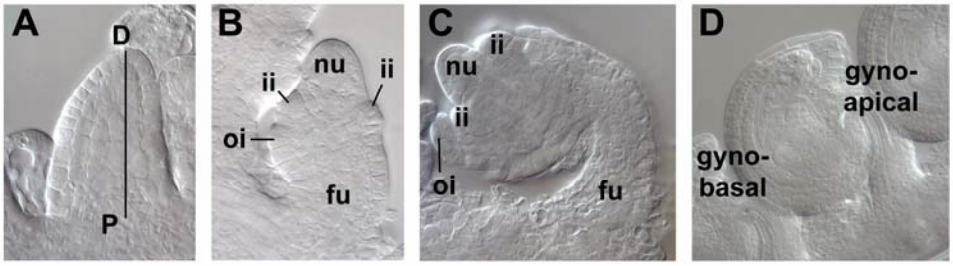

Figure I-1. Arabidopsis ovule development.

(A) A stage 1-II ovule. (B) A stage 2-III ovule. (C) A stage 3-I ovule. (D) A mature stage 4-I ovule at anthesis. D: distal; P: proximal; fu: funiculus; ii: inner integument; oi: outer integument; nu: nucellus. In each panel, the gynobasal side of the ovule is at left.

CHAPTER II

Molecular Mechanisms of Auxin Transport and Signaling

The physiological roles of the plant hormone auxin were first described in the nineteenth century. Theophili Ciesielski studied the geotropic response of plants (Ciesielski, 1872). Charles and Francis Darwin observed a substance that induced shoots to bend toward light (Darwin, 1880). These studies led to later discovery of the diffusible growth stimulating factor eventually named auxin.

Auxin regulates multiple aspects of development. At the cellular level, auxin promotes cell expansion and cell division. Auxin also regulates cell differentiation, such as vascular differentiation. Coordinated cell division, cell growth and differentiation eventually lead to developmental phenomena, such as tropisms, apical dominance, organ initiation and embryo pattern formation.

Polar auxin transport

The developmental fate of a cell is often determined by auxin. Homeostasis of auxin is also required to maintain the initiated developmental status of the plant. In vascular tissue, auxin is transported by phloem from the source leaves to the sink organs. Since auxin is transported passively in the phloem sap and can be affected by

a lot of factors, auxin transport by the vascular tissues is thus considered as non-polar auxin transport (Davies, 2004).

Intercelluar auxin transport that is carrier-mediated and requires energy is considered as polar auxin transport. Polar auxin transport is subject to multiple regulatory influences and is important for fine-tuning of developmental events. The classical concept of polar auxin transport is that auxin, such as IAA (indole-3-acetic acid), is protonated in the more acidic cell wall space (pH 5.5) and enters the cell either by passive diffusion or by influx carriers (Rubery and Sheldrake, 1973). Once in the cell, the more basic environment (pH 7.0) dissociates IAA and retains IAA anions in the cell. An efflux carrier is thus necessary to export IAA to the extracellular space. Polar auxin transport is the net result of differences of auxin efflux between two ends of the cells (Davies, 2004). Auxin diffuses passively through the intercellular space and is taken up into the next cell by diffusion or by influx carrier when rapid uptake is required (Leyser, 2005).

PIN: the auxin efflux carrier

The first identified auxin efflux carrier was PIN-FORMED 1 (PIN1) protein. Loss-of-function *pin1-1* mutant plants have inflorescences devoid of any floral organ formation, resembling plants treated with the polar auxin transport inhibitor N-(1-naphthyl)phthalamic acid (NPA) (Galweiler et al., 1998; Okada et al., 1991). *PIN1* encodes a transmembrane protein similar to a family of bacterial amino acid transporters (Gälweiler et al., 1998). Even though how PIN transport auxin has not been shown, PIN proteins can mediate auxin efflux in yeast or mammalian cells

without the assistance of other plant proteins (Luschnig et al., 1998; Petrasek et al., 2006). Moreover, inducible PIN protein is the rate-limiting factor of auxin efflux and its activity is specific to auxin (Petrasek et al., 2006). Another line of evidence supporting the role of PIN proteins as auxin efflux carriers is that polarities of PIN proteins cellular distribution always point toward the direction of auxin flux, and loss of the polar localization causes changes in auxin accumulation (Benkova et al., 2003; Friml et al., 2002a; Luschnig et al., 1998).

There are eight PIN genes in the Arabidopsis genome (PIN1-PIN8) (Paponov et al., 2005). Five of the PIN proteins (PIN1-4, 7) have been shown to be involved in multiple developmental processes, such as organogenesis, vascular tissue formation, root gravitropism, root meristem patterning, and early embryo development (Benkova et al., 2003; Blilou et al., 2005; Friml et al., 2002a; Friml et al., 2002b; Luschnig et al., 1998; Reinhardt et al., 2003). Some PIN proteins act redundantly. A p*in1 pin3 pin4 pin7* quadruple mutant is embryonic lethal whereas single mutants have subtler effects (Friml et al., 2003). *PIN* genes can be ectopically induced in loss-of-function of other *PIN* genes. For example, *PIN4* is ectopically expressed in the *PIN7* expression domain in *pin7* embryos, and *PIN1* can also be ectopically induced in the *PIN2* expression domains in *pin2* roots (Blilou et al., 2005; Vieten et al., 2005). Similarly, *PIN1* and *PIN2* are ectopically expressed in *PIN3*, *PIN4*, and *PIN7* expression domains in *pin3 pin4 pin7* mutant roots (Blilou et al., 2005). Cross-regulation among *PIN* genes and compensation effects in *pin* mutants explains the functional redundancy and synergistic effects among *PIN* gene family members.

PIN proteins are multispan transmembrane proteins that continuously recycle through endomembrane compartments, suggesting that a vesicle trafficking system is involved in regulating this process (Geldner et al., 2001). The identification of *gnom* (*gn*) mutant revealed that PIN1 protein is regulated by intracellular vesicle trafficking. PIN1 localization in *gn* embryos is disoriented and shows no coordinated polar localization (Steinmann et al., 1999). *GN* encodes a membrane-associated guanine-nucleotide exchange factor on ADP-ribosylation factor G protein (ARF-GEF), and is sensitive to brefeldin A (BFA) inhibition (Steinmann et al., 1999). BFA inhibits vesicle trafficking and ARF-GEFs are the primary targets. PIN1 polar localization is disrupted by BFA treatment, but the polar localization is restored when a BFA-resistant GN is present, meaning that GN is responsible for correct localization of PIN1 on the membrane (Geldner et al., 2003). Further analysis also showed that GN regulates PIN1 recycling between endosomal compartment and plasma membrane (Geldner et al., 2003). Phenotypes of various *gn* mutant alleles include various aspects of defective auxin transport, and they also suggest that auxin induced organ patterning requires active vesicle transport (Geldner et al., 2004).

Another input to PIN regulation is phosphorylation and dephosphorylation. *PINOID* (*PID*) encodes a serine/threonine kinase and loss-of-function *pid* mutants closely resemble *pin1* mutants (Bennett et al., 1995). Transgenic plants with ectopically expressed *PID* show various phenotypes similar to auxin-insensitive mutants as well as decreased auxin accumulation in the roots, suggesting that PID is a negative regulator in auxin signaling (Christensen et al., 2000; Friml et al., 2004). Loss-of-function *pid* causes PIN distribution to shift basally, and excess *PID*

expression causes PIN proteins to shift apically, both of which result in unidirectional auxin flux (Friml et al., 2004). Another factor affecting *PIN* expression could be the ARF-Aux/IAA pathway. *PIN1* expression is reduced in Aux/IAA protein gain-of-function mutants (Sauer et al., 2006; Vieten et al., 2005), suggesting that downstream auxin signaling can feed back on auxin transport.

Auxin transport and organogenesis

PIN1 protein was up-regulated at sites of incipient floral primordia formation, and the distribution of PIN1 protein predicted the pattern of primordium initiation (Heisler et al., 2005; Reinhardt et al., 2003). Furthermore, the PIN1 protein localization was also directed by exogenously applied auxin (Reinhardt et al., 2003). It thereby indicated that auxin plays an instructive role during flower primordium initiation, and that the distribution of auxin efflux carriers creates local peaks of auxin concentration at the newly forming primordia. The current model of organogenesis is that polarized PIN1 protein concentrates auxin at sites of incipient primordia, and auxin is depleted from the surrounding region. Once a primordium is specified, PIN1 orientation is reversed in the older primordium, allowing auxin to move toward new primordium anlagen. The upregulation of PIN1 in new primordia results in accumulating auxin and further enhances PIN1 expression, which causes a sharp increase in auxin concentration that defines the next primordium (Heisler et al., 2005; Reinhardt, 2005).

AUX1: the auxin influx carrier

The *AUX1* gene encodes a transmembrane plant amino acid permease protein and is a proton-driven symporter involved in transporting the tryptophan-like molecule, IAA (Bennett et al., 1996). *aux1* mutant roots are insensitive to IAA and 2,4-D, but are selectively inhibited by the membrane permeable synthetic auxin, 1-naphthaleneacetic acid (NAA) (Marchant et al., 1999; Yamamoto and Yamamoto, 1998). Moreover, the *aux1* mutant agravitropic root phenotypes can be rescued by applying NAA (Marchant et al., 1999). Expressing AUX1 protein in *Xenopus* oocytes shows that AUX1 is the factor responsible for auxin uptake (Yang et al., 2006).

AUX1 protein is localized at the upper plasma membrane of root protophloem cells, opposite to the basal localization of PIN1 proteins in these cells, suggesting that directional auxin flow is achieved by asymmetric localization of the influx and the efflux carriers (Swarup et al., 2001). In the shoot apical meristem, AUX1 is expressed in the abaxial epidermis and L1 layer, and its presence in L1 layer can prevent auxin diffusion to the inner layer when PIN1 promotes directional auxin transport and primordia formation (Reinhardt et al., 2003)

Molecular mechanism of auxin signaling

Aux/IAA proteins

Auxin affects developmental processes by directly altering gene expression (Woodward and Bartel, 2005). Genes rapidly induced by auxin independent of de novo protein synthesis are called primary auxin response genes (Abel and Theologis,

1996). Known primary auxin response genes fall into three major classes: *SAUR*, *GH3*, and *Aux/IAA* (Hagen and Guilfoyle, 2002). *GH3* genes encode auxin conjugating enzymes, and are involved in light signaling, stress responses and root growth (Khan and Stone, 2007; Park et al., 2007; Takase et al., 2003). To date, functions of *SAUR* genes are still unknown. Among the three classes of primary auxin response genes, the *Aux/IAA* genes are the best studied.

There are 29 members in the *Aux/IAA* gene family in the Arabidopsis genome (Remington et al., 2004). The Aux/IAA proteins are short-lived nuclear proteins that are 25 to 35 kD in size (Abel et al., 1995; Abel et al., 1994). Most Aux/IAA proteins have four conserved motifs (Liscum and Reed, 2002; Tiwari et al., 2001). Motif I at the N terminus contains a sequence similar to a motif found in ethylene response factor (ERF)-associated amphiphilic repressors (EAR) and in SUPERMAN (Hiratsu et al., 2003; Hiratsu et al., 2004; Ohta et al., 2001). In vitro transient expression assays showed that motif I of various Aux/IAA proteins had transcription repression activity (Tiwari et al., 2004). The conserved leucine residues LxLxL in EAR motifs are important for the transcriptional repression activity, and mutations in any one of them completely abolished the repression activity (Tiwari et al., 2004).

Motif II of Aux/IAA proteins contains a destabilizing signal. This signal lies in a 13-amino acid consensus in motif II. Among these 13 amino acids, GWPPV (positions 4-8) are required for low stability of the protein (Ramos et al., 2001). Fusing Pea Aux/IAA protein PS-IAA6 motif II to firefly luciferase protein (LUC) was sufficient to shorten the fusion protein life span (Worley et al., 2000), and auxin induced rapid degradation of the reporter fusion protein (Zenser et al., 2001).

Moreover, point mutations in the 13-amino acid sequence of corresponding *iaa17/axr3-1* mutant proteins increased protein stability several fold over that of the corresponding wild-type proteins (Gray et al., 2001; Ouellet et al., 2001).

Motif III and possibly motif IV of Aux/IAA are the dimerization motif. These two motifs can mediate homodimerization between Aux/IAA proteins or heterodimerization between Aux/IAA and auxin response factor (ARF) proteins, which also have these two motifs at their C termini (Kim et al., 1997; Ouellet et al., 2001). Mutation in *axr3/iaa17* motif III eliminates both homo- and hetero-dimerization among different Aux/IAA proteins (Ouellet et al., 2001), suggesting that motif III could be the key contact point.

Gain-of-function mutants with pleiotropic auxin-related phenotypes, such as *axr2-1/iaa7*, *axr3-1/iaa17*, *shy2-2/iaa3*, *bdl-1/iaa12*, and *iaa18-1* all have point mutations in the highly conserved motif II, suggesting that protein stability plays a key role in Aux/IAA protein function (Hamann et al., 2002; Nagpal et al., 2000; Rouse et al., 1998; Tian and Reed, 1999). This also suggests that a robust protein degradation system is indispensable for proper Aux/IAA-related signaling pathways. Indeed, Aux/IAA proteins can be stabilized by proteasome inhibitors (Gray et al., 2001; Ramos et al., 2001; Tian et al., 2003). A protein called Transport Inhibitor Response 1 (TIR1), identified in a genetic screen of mutants with auxin-resistant phenotypes, is an F-box protein in an E3 ubiquitin-ligase complex, known as SCF^{TIR1} (Ruegger et al., 1998). The SCF^{TIR1} complex includes Cullin homolog 1 (CUL1), Arabidopsis SKP1-like (ASK1), RING-box protein 1 (RBX1) and TIR1 proteins (Cardozo and Pagano, 2004; Gray and Estelle, 2000; Gray et al., 2002). Stability of

Aux/IAA proteins increases in *tir1-1* or in *axr1* mutants, which lack proteins required for SCF^{TIR1} function (Gray et al., 2001; Tian et al., 2003). TIR1, as well as its closely related F box proteins AFB1, AFB2 and AFB3, interact directly with auxin in the presence of Aux/IAA motif II peptide (Dharmasiri et al., 2005a; Dharmasiri et al., 2003; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005; Tian et al., 2003). TIR1 and the SCF^{TIR1} adaptor protein ASK1 form a mushroom-like structure with TIR1 leucine-rich repeat (LRR) domain as the cap and the rest as the stem (Tan et al., 2007). Auxin binds directly to the LRR domain cavity and the Aux/IAA peptide sits directly on top of auxin, suggesting that auxin serves as a molecular glue between TIR1 and Aux/IAA proteins (Tan et al., 2007).

Auxin Response Factors

Promoters of primary auxin response genes often contain consensus 5'-TGTCTC-3' auxin response elements, and these sequences were able to mediate auxin induced transcriptional activation (Guilfoyle, 1999; Ulmasov et al., 1995). The auxin response element (AuxRE) led to the discovery of a family of transcription factors called Auxin Response Factors (ARFs) (Ulmasov et al., 1997a). There are 22 ARF proteins in the Arabidopsis genome. The ARF proteins have a conserved N-terminal DNA binding motif that binds to the AuxRE element (Hagen and Guilfoyle, 2002; Ulmasov et al., 1997a). Most of the ARF proteins, except for ARF3 and ARF17, contain C-terminal motifs III and IV homologous to the corresponding motifs in Aux/IAA proteins. ARF and Aux/IAA proteins are able to form heterodimers through these motifs (Guilfoyle et al., 1998; Ouellet et al., 2001; Ulmasov et al.,

1997b). In addition, the ARF proteins contain variable middle regions. Some of the ARF proteins (ARF5, 6, 7, 8, 19) have glutamine (Q)-rich middle regions and act as transcriptional activators in protoplast transient assays, whereas other ARF proteins with proline/serine/threonine (P/S/T)-rich middle regions (ARF1, ARF2, ARF3, ARF4) repress transcription (Tiwari et al., 2003; Ulmasov et al., 1999a). However, data discussed below suggest that whether ARFs activate or repress gene expression may depend on the regulatory context.

A working model of auxin-regulated gene expression

ARF proteins dimerize with other proteins, possibly with Aux/IAAs or with other ARFs, to stabilize their association with the AuxRE containing promoters (Ulmasov et al., 1999b). Although Aux/IAA motif III is homologous to a prokaryotic ββα class transcriptional repressor and dimerization of such repressors results in DNA binding (Morgan et al., 1999), there is still no evidence that homodimer Aux/IAA proteins can act as transcription factors independently of their interactions with ARFs. Currently, the most widely accepted model is that Aux/IAA inhibits transcriptional activities of ARF proteins by dimerizing with ARFs and by recruiting additional proteins that can inhibit gene expression. When auxin concentration elevates in the cell, Aux/IAA proteins are degraded by the ubiquitin-proteasome pathway. The ARF protein is thus available to homodimerize with itself or heterodimerize with another ARF protein to regulate transcription (Fig. II-1) (Leyser, 2006).

Outstanding Questions

The auxin response pathway appears very short and simple, having just one regulated step. However, given the fact that every component of the pathway is encoded by multiple genes, one can predict some diversity in the makeup of the signaling pathway in different tissues, in the Aux/IAA, ARF, and AFB components and in possible interacting proteins. The various distinct contexts could result in distinct developmental outputs, and these considerations thus raise several questions about how *ARF* and *Aux/IAA* genes regulate development.

(I) Redundancy and Specificity

Loss-of-function mutants in many Arabidopsis ARF genes have been characterized to reveal their developmental functions (Table II-1). *ARF* gene functions are often masked by redundancy with their most closely related genes, and can only be revealed by multiple mutants. However, distinctive functions still exist among closely-related *ARF* genes. Based on phylogeny, ARFs have been grouped into four different classes, and functions of most of the class I, II, and III ARFs have been studied (Table II-1) (Remington et al., 2004; Hagen and Guilfoyle, 2002).

Besides the divergent activities among ARF proteins, specificity of auxin responses can also be achieved by unique ARF-Aux/IAA protein pairs. ARF proteins can dimerize with multiple Aux/IAA proteins in yeast two-hybrid experiments with little apparent specificity (Tatematsu et al., 2004). Specificity of ARF and Aux/IAA function might thus be achieved in part by transcriptional control. In specific tissues or organs, ARF proteins might be inhibited by Aux/IAA proteins specifically expressed in that particular place. For example, *mp/arf5* and gain-of-function

bdl/iaa12 have similar phenotypes and overlapping expression domains in the embryo (Hamann et al., 2002; Hamann et al., 1999). MP/ARF5 and BDL/IAA12 proteins can physically interact in the embryo to specify embryonic root (Weijers et al., 2006). Similarly, gain-of-function mutations in *SOLITARY-ROOT (SLR)/IAA14* and *MASSUGU2(MSG2)/IAA19* cause loss-of-function *nph4/arf7* mutants phenotypes, each decreases lateral root formation, suggesting that SLR/IAA14 and MSG/IAA19 may regulate NPH4/ARF7 (Fukaki et al., 2005; Tatematsu et al., 2004).

In addition to specificity provided by tissue-specific expression, different Aux/IAA proteins can also have divergent biochemical activities. In promoter swapping experiments among *shy2/iaa3*, *bdl/iaa12*, and *iaa13*, it was found that *shy2/iaa3* has weaker effects on inhibiting *MP/ARF5* activities and the embryonic defects of misexpressing *shy2/iaa3* in the *bdl/iaa12* expression domain are not as strong as those in *bdl/iaa12* mutants (Weijers et al., 2005). On the other hand, *bdl/iaa12* has weaker effects on inhibiting *NPH4/ARF7* and *ARF19*-regulated gravitropism and auxin induced gene expression in roots than *shy2/iaa3* does, suggesting that propensities to interact might still vary among different ARF-Aux/IAA pairs (Weijers et al., 2005). *AXR3/IAA17* and *SHY2/IAA3* also have divergent activities in root development. Studies on gain-of-function *axr3/iaa17* and *shy2/iaa3* mutants show that *axr3/iaa17* mutation inhibits root hair initiation and elongation whereas similar *shy2/iaa3* mutation promotes and prolongs root hair elongation, and this difference persisted even when a common promoter was used (Knox et al., 2003).

Differential stabilities among Aux/IAA proteins can add another layer of differential regulation onto auxin response signaling specificities. For example, AXR2/IAA7 has a half-life of around 10 minutes, whereas AXR3/IAA17 has a much longer half-life of about 80 minutes (Gray et al., 2001; Ouellet et al., 2001). In addition, sequences outside the Aux/IAA motif II can also affect the protein stability (Dreher et al., 2006). Differential affinities with the TIR1 protein or other members of the AFB proteins might also affect Aux/IAA protein stability and hence the developmental outcome of auxin signaling (Leyser, 2006).

(II) How do Q-rich ARFs work?

Q-rich ARF proteins activate transcription of reporter proteins driven by synthetic auxin response elements in in vitro protoplast transient assays, and the activation function resides in the Q-rich middle region in these assays (Tiwari et al., 2003; Ulmasov et al., 1999a). In vivo data also show that Q-rich ARF proteins can activate transcription of auxin responsive genes. However, some evidence also suggests that Q-rich ARF proteins can also be inhibitors under some circumstances.

Expression profile analyses of *nph4/arf7*, *arf19* and *nph4/arf7 arf19* mutants showed that auxin inducible gene expression is reduced in these mutants (Okushima et al., 2005b). Similarly, auxin inducible expression of some early auxin response genes, such as *IAA1*, *IAA2*, and *IAA4*, are reduced in *mp/arf5* and *arf6-2 arf8-3* mutant plants, indicating that these Q-rich ARFs can be activators for transcription of these primary auxin response genes (Hardtke et al., 2004; Nagpal et al., 2005). *nph4/arf7* leaf mesophyll protoplasts cells have reduced *DR5::GUS* auxin response

reporter expression, and this reduction can be restored when these cells are transfected with the *NPH4/ARF7* full-length gene (Wang et al., 2005b). *DR5::GUS* expression was affected less in mesophyll protoplast of other loss-of-function *arf* mutants, including *arf6*, *arf8* and *arf19*. Moreover, transfecting other Q-rich *ARF*s (*ARF5*, *ARF6*, *ARF8*, and *ARF19*) into *nph4/arf7* mesophyll protoplasts only restored *DR5::GUS* activity partially. This suggests that the Q-rich ARFs might have different strengths to activate gene transcription, and in leaf mesophyll cells, NPH4/ARF7 is apparently a stronger activator than other Q-rich ARFs (Wang et al., 2005b).

mp/arf5 mutants have defects in provascular cell development and do not form embryonic roots (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998). Sometimes adventitious roots can be induced in *mp/arf5* mutants, which allows analysis of *mp/arf5* adult phenotypes (Przemeck et al., 1996). Adult *mp/arf5* mutant plants often form pin-like inflorescences without any flowers, and have distorted vascular strands (Przemeck et al., 1996). The most closely related ARF gene to *MP/ARF5* is *NPH4*/*ARF7* (Remington et al., 2004). *nph4/arf7* mutants have altered phototropic and gravitropic responses in hypocotyls and roots and reduced leaf cell expansion (Stowe-Evans et al., 1998; Watahiki and Yamamoto, 1997). *mp/arf5 nph4/arf7* double mutant plants have more severe embryonic and post-embryonic phenotypes than *mp/arf5* plants do, suggesting that *MP/ARF5* and *NPH4/ARF7* have common functions (Hardtke et al., 2004). However, *MP/ARF5* has activities that *NPH4/ARF7* lacks. Overexpressing *MP/ARF5* can rescue *nph4/arf7* mutant hypocotyl and leaf phenotypes, while overexpressing *NPH4/ARF7* fails to rectify the vasculature defects

of *mp/arf5* mutants (Hardtke et al., 2004). Taken together, these results suggest that some degree of specificity of function among Q-rich ARFs, which may reside in the most diverse middle region.

ARF6 and *ARF8* are another two closely related members in the *ARF* gene family. Single loss-of-function mutations of either one of these two genes only cause slightly reduced stamen length and fecundity, but the *arf6 arf8* double mutant arrests flower development right before flowers open, suggesting that *ARF6* and *ARF8* both regulate flower maturation (Nagpal et al., 2005). In addition to promoting flower maturation, *ARF8* can inhibit fruit development before fertilization occurs, and *arf8* mutants develop fruit in the absence of fertilization (parthenocarpic fruit) (Goetz et al., 2006; Vivian-Smith et al., 2001). Auxin promotes fruit development, suggesting that ARF8 inhibits this auxin response. ARF8 may inhibits fruit development gene expression by recruiting Aux/IAA proteins before fertilization, and the inhibitory effect may be lifted when Aux/IAA proteins are degraded by elevated auxin concentration upon fertilization (Goetz et al., 2006). In *arf8* mutant plants, the ARF8 binding sites in ARF8 target genes could be occupied by other ARF proteins, which might activate transcription of fruit development genes that would otherwise be repressed in wild-type plants. Another model could be that ARF8 induces transcription of *GH3* genes and of some *Aux/IAA* genes. In *arf8* mutant plants, the decreased *GH3* expression could result in increased bioactive auxin pool, and the decreased *Aux/IAA* expression might increase ARF protein transcription activities. These might cause other ARF proteins to promote transcription of fruit development genes. This model can also explain how ARF8 regulates hypocotyl development.

Light-grown *arf8-1* mutant seedlings have long hypocotyls, and this phenotype may also be caused by decreased *GH3* expression, leading to higher free auxin level and promoting hypocotyl elongation (Tian et al., 2004). These examples show that ARF8, and possibly other Q-rich ARF proteins, might inhibit auxin responses by recruiting Aux/IAA proteins or by inducing transcription of *GH3* and *Aux/IAA* genes.

(III) How do P/S/T-rich ARFs work?

P/S/T-rich ARF proteins are considered as repressor ARFs since they can repress transcription of reporter genes in protoplast transient expression assays when co-expressed with Q-rich ARF proteins (Tiwari et al., 2003; Ulmasov et al., 1999a). The inhibitory effects of these ARF proteins depend on the synthetic auxin response element used in the experiment (Tiwari et al., 2003), and it is still uncertain how the P/S/T-rich ARF proteins work in vivo.

ARF2 protein can act as inhibitors on a few developmental processes based on the phenotypes of the loss-of-function mutants. Loss-of-function *arf2* mutants delay senescence and floral organ abscission, and these defects are enhanced by *arf1* mutations which do not cause these phenotypes on their own (Ellis et al., 2005; Li et al., 2004). In addition, *ARF2* can inhibit cell growth and division in the ovule, which is not observed in *arf1* mutants (Schruff et al., 2005). Despite the overlapping functions between *ARF1* and *ARF2*, only *ARF1* can inhibit auxin-inducible gene expression whereas *ARF2* does not have any effect on these genes (Ellis et al., 2005; Okushima et al., 2005a). ARF2 protein could either target different sets of early auxin response genes or does not repress transcription in vivo. NPH4/ARF7 and

ARF19 are Q-rich ARFs, and their mutant plants do not have any senescence phenotypes. However, mutations in *NPH4/ARF7* and *ARF19* enhance *arf2* mutant plant senescence phenotypes (Ellis et al., 2005). This suggests that Q-rich ARF proteins can act together with P/S/T-rich ARF proteins to regulate certain developmental pathways.

ettin (ett)/arf3 mutant plants have reduced gynoecia with complete loss of valve tissue in plants with strong alleles. Flowers of *ett* mutants sometimes have increased number of sepals and petals and decreased number of stamens (Sessions et al., 1997; Sessions and Zambryski, 1995). *ARF4* acts redundantly with *ETT/ARF3*. *arf3 arf4* double mutants have a gynoecium that is reduced to a bump without any ovule formation or sometimes with very few exposed ovules (Christine Ellis, unpublished data). In addition, *arf3 arf4* double mutant rosette leaves form abaxial outgrowths indicative of adaxialization (Pekker et al., 2005).

ETT/ARF3 lacks the two C-terminal motifs III and IV, and the Aux/IAA inhibition model therefore does not apply for ETT/ARF3. However, ETT/ARF3 still participates in auxin related developmental processes. For example, applying the auxin polar transport inhibitor NPA to developing gynoecia phenocopied *ett* mutant (Nemhauser et al., 2000). *ETT/ARF3* together with other genes in the auxin signaling pathway, including *MP/ARF5*, *PIN*, and *PID*, participate in floral organ formation and patterning. Roles of *ETT/ARF3* in floral organ development is reinforced by the finding that *seuss* (*seu*) is a modifier of *ett/arf3* mutant and can interact physically with ETT/ARF3 protein (Pfluger and Zambryski, 2004). *ett seu* double mutants have filamentous petals, mispositioned sepals and reduced number of stamens (Pfluger and

Zambryski, 2004). SEUSS is an inhibitor of the floral homeotic gene *AGAMOUS* (*AG*), and loss-of-function *seu* causes ectopic *AG* expression (Franks et al., 2002). However, ectopic *AG* does not cause the *ett* and *seu* phenotypes since loss of *AG* does not rectify the filamentous petals (Pfluger and Zambryski, 2004). *seu* mutant seedlings show various auxin resistant defects and reduced *DR5::GUS* expression, confirming that *SEU* participates in auxin-related developmental regulation (Pfluger and Zambryski, 2004). *SEU* encodes a Q-rich protein with a putative dimerization domain (Franks et al., 2002). SEU interacts with LEUNIG (LUG) which is also an inhibitor of *AG* (Conner and Liu, 2000; Sridhar et al., 2004). The inhibitory activity of LUG requires functional histone deacetylases (Sridhar et al., 2004), suggesting that ETT/ARF3 might repress transcription by associating with chromatin remodeling complexes by interacting with SEU.

ETT/ARF3 also acts together with KANADI (KAN) proteins, transcription factors defining abaxial identify of lateral organs. *ett/arf3* suppresses ectopically expressed *KAN1* (Pekker et al., 2005). However, *ETT/ARF3* and *ARF4* expression is not affected in *kan1 kan2* mutant and ectopic *ETT/ARF3* or *ARF4* does not rescue *kan1 kan2* phenotypes, meaning that *ETT/ARF3* and *ARF4* are not direct targets of KAN proteins (Pekker et al., 2005). Still, these data are the first evidence suggesting that ARF proteins could be regulators in establishing organ polarity and could also be components in complex transcription machineries.

(IV) What are the downstream targets of ARFs?

Many of the primary auxin response genes, including *Aux/IAA*, *GH3* and *SAUR* genes, have AuxRE elements in their promoters and are presumed to be direct targets of ARF genes. However, developmental functions of several of these genes remain elusive. Therefore, to understand the in vivo activities of different ARF proteins in different tissues, several recent efforts have attempted to reveal other direct target genes of various *ARF* genes.

As previously discussed, NPH4/ARF7 and ARF19, two Q-rich ARF proteins, can activate downstream target genes directly by binding to their promoters. LATERAL ORGAN BOUNDARIES DOMAIN 16 (LBD16) and LBD29, two LOB domain containing proteins, are among these direct targets that are expressed in wild-type seedling but not in *nph4/arf7 arf19* mutant seedlings (Okushima et al., 2005b). The LOB proteins are plant-specific and in some cases function in boundary establishment in lateral organs (Shuai et al., 2002). Overexpressing *LBD16* and *LBD29* can restore lateral root formation in the absence of *NPH4/ARF7* and *ARF19*, and expression of *LBD16* and *LBD19* require the presence of *NPH4/ARF7* and *ARF19* (Okushima et al., 2007). Moreover, overexpressing *LBD16* fused to a transcription repression domain inhibited lateral root formation, indicating that the lateral root-inducing activity of NPH4/ARF7 and ARF19 might be mediated by LBD16 (Okushima et al., 2007). Both *LBD16* and *LBD29* have AuxRE elements in their promoters and the ARF7 DNA binding domain can bind directly to these elements. In addition, *LBD16* and *LBD29* are rapidly induced by auxin and activated by cyclohexamide, which qualifies these two genes as primary auxin response genes (Okushima et al., 2007). Similar lateral root induction activity of LOB domain proteins has been described in maize.

The maize *rootless concerning crown and seminal roots* (*rtcs*) mutant lacks a LOB domain protein and is deficient in embryonic and post-embryonic root development (Taramino et al., 2007). Similarly, rice *ADVENTITIOUS ROOTLESS 1*(*ARL1*), a LOB domain protein, promotes adventitious root primordia initiation and is responsive to auxin (Liu et al., 2005). These findings suggest a general role of LOB domain in auxin-regulated root development.

MYB21 and *MYB24*, two closely related genes, are not expressed in *arf6-2 arf8-3* double mutant flowers. In wild-type stage 12 flowers, *MYB21* and *MYB24* are expressed in overlapping domains with *ARF6* and *ARF8* (Paul Reeves, unpublished data). Loss-of-function *myb21* or *myb21 myb24* mutant flowers have short stamen filaments and delayed flower opening (Mandaokar et al., 2006). Even though *MYB21* and *MYB24* are important downstream effectors of *ARF6* and *ARF8* in promoting flower maturation, there is still no evidence whether ARF6 or ARF8 binds to *MYB21* or *MYB24* promoters.

(V) Mechanisms of Aux/IAA activities

Aux/IAA proteins inhibit ARF protein transcription activities not just simply by binding to ARF proteins or by blocking dimerization between ARF proteins. Aux/IAA proteins could also repress transcription directly through the transcription repression domain of motif I. Mutation of *PICKLE*, a CHD3 chromatin remodeling factor involving in transcriptional repression, suppressed the gain-of-function *slr/iaa14* mutation (Eshed et al., 1999; Fukaki et al., 2006; Ogas et al., 1999). These data suggest that recruiting chromatin remodeling complex protein, possibly through

motif I, to silence transcription might be one mechanism by which Aux/IAA proteins inhibit transcription.

(VI) What are the functions of feedback regulatory loops in auxin response?

Auxin induces expression of multiple components in its signaling pathway. For example, auxin activates expression of *ARF19* and *ARF4*, which can amplify the auxin signal. Auxin also induces *PIN* expression, and this regulation may be important component of intercellular patterning formation. On the other hand, auxin induces *Aux/IAA* and *GH3* gene expression to repress the signaling. We still do not know how the homeostasis of the positive and negative feedback are reached, and how these regulations fit into particular developmental contexts. Biochemical and kinetic properties of the feedback regulatory loops might determine the developmental outcomes. To answer this, functions of primary auxin response genes need to be revealed. In addition, modeling approaches may also help in reaching broad understanding of the response system.

(VII) Inputs of other signals

Small RNAs, including microRNAs (miRNAs) and trans-acting siRNAs (ta-siRNAs), regulate some of the *ARF* genes. Both miRNAs and ta-siRNAs are non-coding RNAs that interact with their target transcripts and guide the transcripts to cleavage (Bartel, 2004). *ARF6* and *ARF8* are targets of *miR167*, and overexpressing *miR167* causes transgenic plants having identical phenotypes to *arf6 arf8* double mutants (Wu et al., 2006). *miR167* excludes *ARF6* and *ARF8* transcripts from the

ovule and the anther to ensure proper pattering and development of these reproductive organs. *miR160* targets *ARF10*, *ARF16*, and *ARF17* (Mallory et al., 2005; Wang et al., 2005a). Overexpressing *miR160* recapitulates *arf10 arf16* mutant root phenotypes (Wang et al., 2005a). *miR160*-resistant *ARF17* causes various developmental defects, including leaf development, floral organ patterning/formation and embryo patterning, and *miR160*-resistant *ARF16* has few root hairs, curled leaves and reduced fertility (Mallory et al., 2005; Wang et al., 2005a).

The third class of *ARF* genes regulated by small RNAs are *ARF2*, *ARF3* and *ARF4*. These genes are regulated by ta-siRNA encoded by the *TAS3* gene (Allen et al., 2005). ta-siRNAs differ from miRNAs in that they are arise from double-stranded RNA precursors instead of stem-loops, and they are processed by DICER-LIKE 4 (DCL4) instead of DCL1 as miRNAs are (Allen et al., 2005; Jones-Rhoades et al., 2006). Loss of *TAS3* regulation of *ETT/ARF3* causes defects in leaf development phase change and in floral organ patterning (Fahlgren et al., 2006).

Other plant hormones might interact with auxin to regulate plant development. One such hormone is brassinosteroid (BR) which regulates some overlapping developmental pathways with auxin. Expression profile analyses have shown that some of BR-regulated genes are auxin responsive genes, and the AuxRE element is enriched in genes induced both by auxin and BR (Goda et al., 2004). Moreover, BR activates primary auxin response DR5::GUS reporter expression, indicating that BR can activate auxin responses through the AuxRE element (Nakamura et al., 2003). Gain-of-function *aux/iaa* mutants, *axr3-1/iaa17* and *axr2-1/iaa7*, have abnormal BR-responses, and microarray analysis of *axr2-1/iaa7* showed some altered BR-inducible

gene expression, suggesting that the Aux/IAA proteins might regulate BR-responses in certain organs (Nakamura et al., 2006). It is therefore suggested that auxin and BR regulations might converge at the promoters of the shared target genes (Nemhauser et al., 2006; Nemhauser et al., 2004). Mutation in *BREVIS RADIX* (*BRX*), a rate-limiting enzyme in BR biosynthesis, causes reduced auxin responses in roots, and *BRX* expression is induced strongly by auxin and slightly repressed by brassinolide (the most potent form of BR), suggesting that auxin might also participate in the feedback loop of BR biosynthesis (Mouchel et al., 2006). However, auxin and BR activate auxin response gene expression with different kinetics, and the detailed mechanisms will require further studies to clarify dynamics of interaction between these two hormones.

Conclusions

The current information we have on ARF and Aux/IAA proteins only explains how they function in specific cells or under unique context. There are still several unanswered questions regarding the general mechanism.

The transcription activating activities of some Q-rich ARFs (ARF7 and ARF19) have been described in lateral root formation and in leaf mesophyll cells. However, this information still does not explain how these and other Q-rich ARFs activate transcription in other developmental pathways. In addition, the direct targets of these ARF proteins are largely unknown, and the transcriptional activities of other non-Q-rich ARFs remain elusive. Developmental roles of some of these ARF proteins have been described, but we still do not know how they regulate transcription.

Currently, limited loss-of-function phenotype has been reported probably due to the extensive redundancy among Aux/IAA genes. Gain-of-function *aux/iaa* mutants often have overlapping phenotypes, and these phenotypes all have some aspects of auxin insensitivity. Nevertheless, these gain-of-function mutants could be neomorphic and have little to do with their in vivo functions. Some data have suggested that Aux/IAA could inhibit transcription by recruiting histone acetylase associated chromatin remodeling complex. To clarify this point, more in vivo and in vitro physical interaction data will be needed.

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Figure

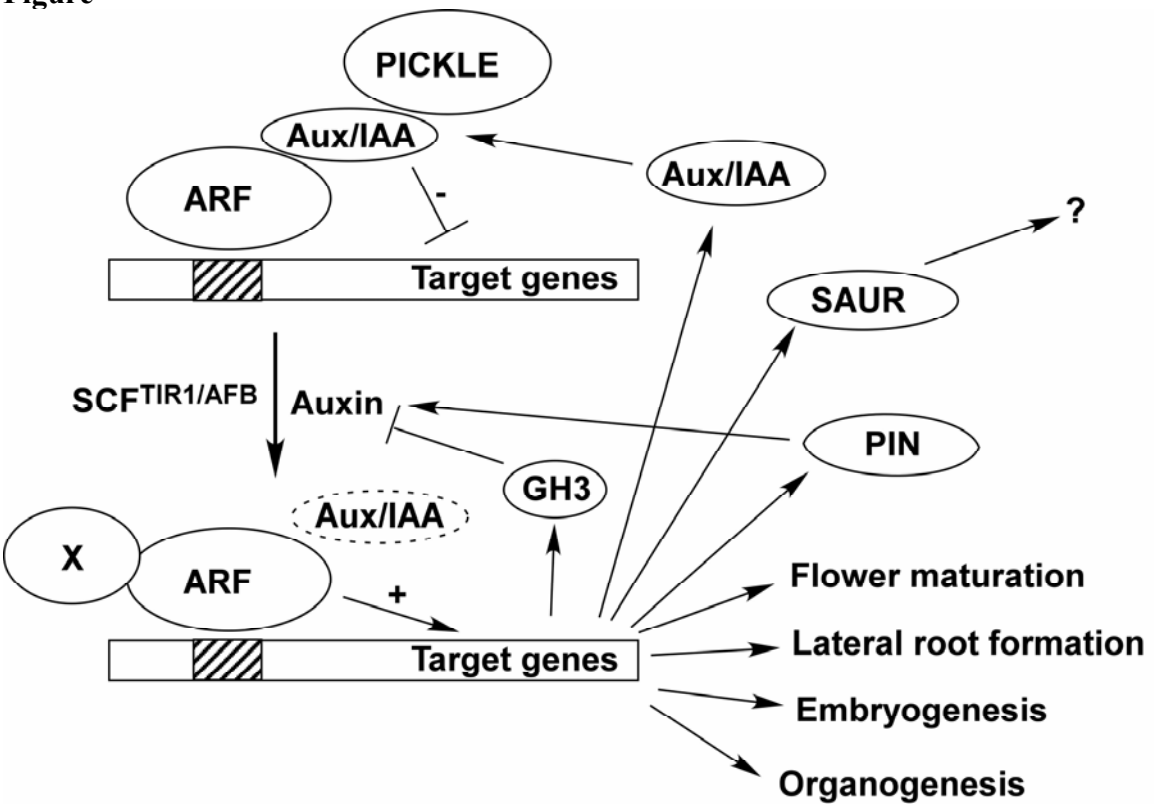

Figure II-1. Diagram of auxin response.

In the absence of auxin, ARF proteins bind to Aux/IAA proteins which recruit PICKLE to inhibit transcription by chromatin remodeling. Auxin induces Aux/IAA turnover, freeing ARF proteins to interact with unidentified protein X to activate transcription. Negative feedback loops of GH3 and Aux/IAA proteins, positive feedback loop of PIN proteins and developmental pathways regulated by ARF proteins are shown.

Table II-1 Loss-of-function mutations in *ARF* genes.

Mutant	Most notable phenotypes
Class I (P/S/T-rich)	
arf1	Single mutant has no phenotype; increased *IAA* expression
arf2	Delayed leaf senescence and floral organ abscission; large ovules
arf1 arf2	Exaggerated dark-grown seedling hypocotyl hook; enhanced *arf2* phenotypes
Ett/arf3	Abnormal apical-basal gynoecium development
arf4	Single mutant has no phenotype
ett/arf3 arf4	Adaxial leaf outgrowths; reduced gynoecium
arf9, arf11, arf18	Unknown
Class II (Q-rich)	
mp/arf5	Severely reduced leaf vasculature; rootless embryo
nph4/arf7	Impaired differential growth responses in aerial tissues; reduced leaf size
mp/arf5 nph4/arf7	Enhanced *mp/arf5* embryonic and vasculature phenotypes
arf19	Single mutant has no phenotype
nph4/arf7 arf19	Lacks lateral roots, reduced leaf expansion
arf6	Slightly reduced stamen length and fecundity
arf8	Slightly reduced stamen length and fecundity; parthenocarpic fruit
arf6 arf8	Curled rosette leaves, short inflorescence, arrested flower maturation
Class III	
arf10 arf16	Root cap defects, agravitropic root growth
arf17	Unknown
Class I'	
Arf12, arf13, arf14, arf15, arf20, arf21, arf22	Unknown

Remintong et al., 2004. ARF1, ARF2: Li et al., 2004; Ellis et al., 2005, Schruff et al., 2005; Okushima et al., 2005. ARF3, ARF4: Sessions et al., 1995; Pekker et al., 2005. MP/ARF5: Berleth and Jürgens, 1993; Hardtke et al., 1998; Hardtke et al., 2004. NPH4/ARF7, ARF19: Liscum et al., 1995; Harper et al., 2000; Wilmoth et al., 2005; Okushima et al., 2005. ARF6, ARF8: Nagpal et al., 2005; Goetz et al., 2006. ARF10, ARF16, ARF17: Wang et al., 2005

CHAPTER III

Arabidopsis microRNA167 controls patterns of *ARF6* and *ARF8* expression and regulates both female and male reproduction

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Summary

In flowering plants, diploid sporophytic tissues in ovules and anthers support meiosis and subsequent haploid gametophyte development. These analogous reproductive functions suggest that common mechanisms may regulate ovule and anther development. Two Arabidopsis Auxin Response Factors, ARF6 and ARF8, regulate gynoecium and stamen development in immature flowers. Wild-type pollen grew poorly in *arf6 arf8* gynoecia, correlating with *ARF6* and *ARF8* expression in style and transmitting tract. *ARF6* and *ARF8* transcripts are cleavage targets of the microRNA *miR167*, and overexpressing *miR167* mimicked *arf6 arf8* phenotypes. Mutations in the *miR167* target sites of *ARF6* or *ARF8* caused ectopic expression of these genes in domains of both ovules and anthers where *miR167* was normally present. As a result, ovule integuments had arrested growth, and anthers grew abnormally and failed to release pollen. Thus, *miR167* is essential for correct patterning of gene expression and fertility of both ovules and anthers. The essential patterning function of *miR167* contrasts with cases from animals in which miRNAs reinforce or maintain transcriptionally established gene expression patterns.

Introduction

Plant life cycles alternate between diploid sporophyte and haploid gametophyte phases. In flowering plants, the more prominent sporophyte supports meiosis and subsequent gametophyte development in specialized female and male organs within flowers. Ovules, the female sporophyte organs, support development of the embryo sac and growth of embryos and seeds after fertilization. Anthers, the male sporophyte organs, support formation, development and subsequent release of pollen. Gametophyte development and successful reproduction thus require correct pattern formation of ovules and anthers.

Arabidopsis ovules initiate as finger-like structures on the flanks of carpel margin meristems at around floral stage 8 (Smyth et al., 1990). Megaspore mother cells which later give rise to the female gametophyte reside in the distal nucellus end of ovules. Proximal to the nucellus is the chalaza where both inner and outer integuments initiate. Inner and outer integuments grow out to enclose the entire ovule as the ovule matures, and asymmetric growth of the outer integument causes the developing ovule to curve. After fertilization and embryo development, integuments form the seed coat. Ovules are connected to the placental tissues by funiculi which supply nutrients to support ovule and seed growth (Schneitz et al., 1997; Skinner et al., 2004).

Stamen primordia initiate at floral stage 6 and form a filament which holds an anther at its distal end. Several distinct cell types in anthers are important for male gametogenesis and anther dehiscence (Goldberg et al., 1993; Smyth et al., 1990). Some of these undergo cell death or desiccation to allow dispersal of pollen grains at anthesis. Prior to anthesis, tapetum cells that coat the anther locule wall and septum cells between two anther locules are degraded. Stomium cells then break to allow pollen dispersal (Sanders et al., 1999).

Endogenous small non-coding RNAs called microRNAs (miRNAs) regulate several developmental events in Arabidopsis (Baker et al., 2005; Bao et al., 2004; Chen, 2004; Emery et al., 2003; Laufs et al., 2004; Mallory et al., 2004a; Williams et al., 2005). miRNA precursor genes (*MIR*s) are transcribed by RNA polymerase II in both animals and plants (Kurihara and Watanabe, 2004; Lee et al., 2004; Xie et al., 2005). DICER-LIKE 1 (DCL1), an Arabidopsis DICER RNase III family homolog, cleaves the pri-miRNA and pre-miRNA hairpin precursors to produce a miRNA:miRNA* duplex in the nucleus (Jones-Rhoades et al., 2006). The duplex is transported to the cytoplasm where the mature miRNA is incorporated into the RNA induced silencing complex (RISC). The RISC complex then identifies target mRNAs with specificity provided by base pairing between the miRNA and the target site (Bartel, 2004).

Most plant miRNAs have high sequence complementarity to their target binding sites,

allowing straightforward prediction of the genes they regulate (Rhoades et al., 2002). In most cases, plant miRNAs shut down their target gene activities by transcript cleavage (Axtell and Bartel, 2005; Schwab et al., 2005). Over-expressing *MIR* precursor transcripts in transgenic plants decreased the corresponding target gene transcript levels (Schwab et al., 2005). In addition, cleavage products of computationally predicted miRNA targets have been detected in wild-type plants (Allen et al., 2005; Kasschau et al., 2003; Mallory et al., 2005; Xie et al., 2005). Nevertheless, miRNAs can act by other regulatory mechanisms, including translational inhibition and methylation induced gene silencing (Bao et al., 2004; Bartel, 2004; Chen, 2004; Kurihara and Watanabe, 2004).

More than half of the known Arabidopsis miRNA target genes encode transcription factors, suggesting that miRNAs regulate various developmental processes (Jones-Rhoades et al., 2006). The importance of plant miRNAs is further supported by the finding that most Arabidopsis miRNA families are conserved among other species of land plants, both vascular and in some cases, lower plants (Axtell and Bartel, 2005; Floyd and Bowman, 2004; Reinhart et al., 2002; Rhoades et al., 2002; Sunkar et al., 2005).

Among miRNA targets are several *ARF* genes encoding Auxin Response Factors. *ARF6* and *ARF8* are targeted by *miR167*, whereas *ARF10*, *16*, and *17* are targeted by *miR160* (Mallory et al., 2005; Rhoades et al., 2002; Wang et al., 2005). ARF proteins bind to Auxin

Response promoter elements and mediate gene expression responses to the plant hormone auxin (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002; Mallory et al., 2005; Tiwari et al., 2003). Different ARF proteins regulate embryogenesis, root development and floral organ formation (Hardtke and Berleth, 1998; Hardtke et al., 2004; Mallory et al., 2005; Sessions et al., 1997; Wang et al., 2005).

We previously found that *ARF6* and *ARF8* regulate flower maturation (Nagpal et al., 2005). Flowers of *arf6 arf8* double loss-of-function mutant plants were arrested at stage 12, just before wild-type flower buds normally open. Stamens of *arf6 arf8* flowers were short, and anthers did not dehisce to release pollen. The double mutant anther indehiscence was due to a lack of jasmonic acid (JA) production, and pollen release could be restored by spraying the flower buds with JA or its precursors. *arf6 arf8* double mutant flowers were also female sterile and their stigmatic papillae did not elongate as did those of wild-type flowers. Single loss-of-function *arf6* or *arf8* mutants had only subtly reduced fecundity resulting from shorter stamen filaments and delayed anther dehiscence, indicating that ARF6 and ARF8 act largely redundantly.

To determine the developmental functions of *miR167*, we have over-expressed *MIR167* coding sequences, mutated *ARF6* and *ARF8* to make them immune to *miR167*-mediated effects, and studied expression of *MIR167*, *ARF6* and *ARF8* genes. Our

results indicate that *miR167* regulates the pattern of *ARF6* and *ARF8* expression, which is vital for both ovule and anther development.

Materials and Methods

Plant materials and Constructs

Most plants used in this work were in the Columbia (Col-0) ecotype. *arf6-2*, *arf8-3*, and *arf6-2 arf8-3* mutants were isolated and described previously (Nagpal et al., 2005). The *ino-1* mutant (Villanueva et al., 1999) was in the Landsberg *erecta* ecotype.

MIR167a (At3g22886; stem-loop sequence accession number: MI0000208), *MIR167b* (At3g63375; stem-loop accession number: MI0000209), *MIR167c* (stem-loop accession number: MI0001088), and *MIR167d* (stem-loop accession number: MI0000975) were PCR amplified from wild-type genomic DNA using the following primers: *MIR167a*, 5'-cacccactttcgacccttaaactctcca-3' and 5'-tgaagctaggaaagaggagctttg-3'; *MIR167b*, 5'-cacctcaggcttctttaattcgtggtg-3' and 5'-aacttagactgtgcaaagccaaa-3'; *MIR167c*, 5'-caccccatgggtgagaaagtgaaaa-3' and 5'- tcatgattgtcacactagcacaa-3'; *MIR167d*, 5'-cacctgaatgaaactgtccaaacaca-3' and 5'-cgtcgctagctaccaacaaa-3'. PCR products were cloned into pENTR/D-TOPO (Invitrogen) and then subcloned into binary vector pB7WG2 (Karimi et al., 2002) by LR clonase (Invitrogen).

A genomic *ARF6* (*gARF6*) fragment including the 5' and 3' regulatory sequences (chromosome 1 positions 10693520-10680841) was cut out from BAC clone T4K22 with *BamHI* and subcloned into pBS SK$^-$ (Stratagene) (Nagpal et al., 2005). The *miR167* target site on *ARF6* was mutated by PCR using primers: 5'-gaccctgtgcgtagtggatggcagctggtatttg-3' and 5'-caaataccagctgccatccactacgcacagggtc-3'. Both *gARF6* and *mARF6* were cloned into binary vector pBAR (Holt et al., 2002). Genomic *ARF8* (*gARF8*) was obtained from BAC clone K15O15 by PCR (chromosome 5 position 14645242-14652007) in three fragments using the following primer pairs: 5'-ctcgagtgagaactgaggctggcttt-3' and 5'-gtctaattcaacttcaagaa-3'; 5'-tcttccttctctccactgtatcg-3' and 5'-gaccctcttcagagctctactca-3'; 5'-caccatcgatcatgctggcacatcatcttt-3' and 5'-ctcgagctaggcactgtttatg-3', and then ligated together. *mARF8* was obtained by mutating the *miR167* target site by the same method as for *mARF6*. Both *gARF8* and *mARF8* were first cloned into pENTR/D-TOPO (Invitrogen) and then into binary vector pKWG (Karimi et al., 2002) by LR clonase (Invitrogen).

gARF6, *mARF6*, *gARF8*, and *mARF8* fragments excluding their stop codons and 3' untranslated regions were cloned into pENTR/D-TOPO (Invitrogen) and then introduced into pGWB3 (a kind gift from Dr. Tsuyoshi Nakagawa, Shimane University, Japan) by LR clonase (Invitrogen) to obtain the protein GUS fusions.

$P_{MIR167a}$, $P_{MIR167b}$, $P_{MIR167c}$, and $P_{MIR167d}$ were PCR amplified from wild-type genomic DNA using the following primers: $P_{MIR167a}$, 5'-caccaagtttcgagtagaccgtga-3' and 5'-tcagatgccggtgcaccata-3'; $P_{MIR167b}$, 5'-caccagggtagagggtttctcaag-3' and 5'-ttgtggacttgtcttcaaaa-3'; $P_{MIR167c}$, 5'-cacccgttgtgtggtgtttccaac-3' and 5'-tacatggtatacatacagacatga-3'; $P_{MIR167d}$, 5'-cacctcacgtttctatggacccaat-3' and 5'-tagataattgaaaaagaatgagaag-3'. These promoters were cloned into pENTR/D-TOPO (Invitrogen) and subcloned into binary vector pBGWFS7 (Karimi et al., 2002) to produce P_{MIR167}*:GFP-GUS* constructs. Only GUS activity was assayed in plants carrying these constructs.

Northern blots and in situ hybridization

Total cellular RNA was isolated from flower clusters of long-day-grown plants by Trizol reagent (Invitrogen). RNA gel blot analysis was performed as previously described (Tian et al., 2003). *ARF6* (coding region position 1346-2211) and *ARF8* (coding region position 1151-2106) probes were amplified from cDNA with the following primers: *ARF6*, 5'-cggaattcaggcattgatcctgcaaaag-3' and 5'-cgggatccaaggtttgacattccgttcg-3'; *ARF8*, 5'-cgggatccgaaggggtgatttgggaagt-3' and 5'-ctcgaggttggacgagttaatctgtcc-3'. A probe recognizing Arabidopsis β-tubulin-4 (At5g44340) was used as a loading control in RNA gel blot hybridizations.

For low molecular weight RNA, 30 μg of total cellular RNA was suspended in 20 μl loading buffer (95% formamide, 5mM EDTA, 0.025% SDS, 0.025% bromophenol blue, and 0.025% xylene cyanol FF) and separated in 15% denaturing polyacrylamide gel containing 8 M urea. Antisense *miR167* (5'-tagatcatgctggcagcttca-3') and U6 snRNA probes (5'-ctcgatttatgcgtgtcatccttgc-3') were end-labeled by T4 polynucleotide kinase (New England Biolabs) in the presence of γ^{32}P-ATP.

In situ hybridization was performed as previously described (Long and Barton, 1998). *ARF6* and *ARF8* fragments used in Northern blots were cloned into plasmid pGEM-T (Promega). Probes were labeled by in vitro transcription with SP6 polymerase using a DIG RNA labeling kit (Roche). Wild-type and *mARF6* hybridizations were done together so as to increase comparability of results. *INNER NO OUTER* probe was amplified from wild-type flower cDNA using primers described in Sieber et al. (Sieber et al., 2004) and cloned into pGEM-T (Promega).

Histology and microscopy

Flower X-gluc staining was performed as described by Sessions et al. (Sessions et al., 1999) and the concentration of potassium ferro- and ferricyanide used depended the constructs. For *MIR167* promoter:GFP-GUS lines, the concentration used was 5 mM each. For *ARF6* and *ARF8* protein:GUS fusions, it was 0.5 mM each for ovules and 0.2 mM each

for flowers.

For tracking pollen tube growth, stigmas were dusted with pollen from *LAT52:GUS* plants (Johnson et al., 2004). 24 hours after pollination, carpel walls were removed and gynoecia were stained with X-gluc overnight at 37^{o}C.

Ovules for DIC microscopy were fixed in 3:1 ethanol:acetic acid for 15 min, incubated in 70% ethanol for another 15 min, cleared in chlorohydate solution (chlorohydrate:water=8:2), and observed under DIC microscopy. Scanning electron microscopy was performed as previously described (Nagpal et al., 2005). Anthers were fixed and sectioned based on methods in Ellis et al. (Ellis et al., 2005).

Results

***ARF6* and *ARF8* are required to support pollen tube growth**

Our previous analyses of promoter:GUS plants suggested that both *ARF6* and *ARF8* were expressed in multiple flower organs, but would not have revealed effects of *miR167* or other regulatory elements missing from the promoter:GUS constructs. We therefore analyzed expression patterns of *ARF6* and *ARF8* in wild-type flowers by in situ hybridization (Fig. III-1). We also analyzed X-gluc staining patterns in plants carrying genomic translational fusions to the *GUS* reporter gene (Fig. III-2). These *gARF6:GUS* and

gARF8:GUS constructs were able to increase fecundity of an *arf8* null mutant (data not shown), suggesting that they were partially functional (although as discussed below *miR167*-resistant versions conferred weaker phenotypes than unfused genes). In most tissues, staining patterns of the GUS fusions were very similar to the distribution of transcripts shown by in situ hybridization.

In wild-type flowers, *ARF6* transcript was present in the carpel medial ridge (which later forms the transmitting tract for pollen tube growth), in placental tissues and in young ovule primordia as they emerged (Fig. III-1B and D). As integuments initiated on the flanks of ovules (ovule stage 2-II), *ARF6* transcript became restricted to the ovule funiculus and the placental tissues and was excluded from the integuments and the nucellus (Fig. III-1E and F). These expression patterns persisted at least through flower stage 12, just before fertilization would normally occur. *ARF6* transcript was also detected at a low level in the vasculature of flower stems and stamen filaments, in petals, and in nectaries (Fig. III-1A and C). Consistent with the in situ hybridization data, *gARF6:GUS* stained in transmitting tract, ovule funiculi and nectaries, and faintly in stamen filaments (Fig. III-2A-E).

ARF8 was expressed in a similar pattern to *ARF6*, with strong expression in the funiculus and placenta (Fig. III-1J). *ARF8* was also detected in stigmatic papillae in flowers approaching anthesis (data not shown). Similarly, g*ARF8:GUS* was expressed in

transmitting tract, placenta, funiculi, and stamen filaments (Fig. III-2K and M). Stigmatic papillae expression was also detected in some strongly expressing *gARF8:GUS* lines (data not shown).

In addition, we detected weak X-Gluc staining in the style and in the valves of both *gARF6:GUS* and *gARF8:GUS* plants, but we did not detect *ARF6* or *ARF8* transcript in these tissues by in situ hybridization.

Expression of *ARF6* and *ARF8* in style, transmitting tract, and funiculus suggests that ARF6 and ARF8 may regulate fertilization rather than gametophyte development. To explore why *arf6 arf8* flowers were female sterile, we pollinated wild-type and *arf6 arf8* stigmas with pollen from the *LAT52:GUS* reporter line (Johnson et al., 2004). Whereas pollen grew efficiently in wild-type transmitting tracts and fertilized the majority of ovules, pollen tubes elongated very little in *arf6 arf8* transmitting tracts (Fig. III-4M). These results indicate that ARF6 and ARF8 may act within the stigma, style, or transmitting tract to regulate production of some component necessary for pollen tube germination or growth.

MIR167 genes can decrease *ARF6* and *ARF8* transcript levels

ARF6 and *ARF8* mRNA cleavage products ending within the *miR167* target site have been detected in wild-type plants (Allen et al., 2005; Jones-Rhoades and Bartel, 2004; Rhoades et al., 2002). To test whether *miR167* targets only these two genes, we made

transgenic plants expressing the stem-loop regions of each of the four predicted Arabidopsis *MIR167* precursor genes behind the strong *Cauliflower Mosaic Virus 35S* promoter (P_{35S}*::MIR167a*, *b*, *c*, and *d*). Only P_{35S}*::MIR167a* caused twisted leaves, short inflorescences, and arrested flower development, thereby fully recapitulating *arf6 arf8* mature plant phenotypes (Fig. III-3B,C and Table III-1). We did not examine seedling or root phenotypes in these sterile plants. P_{35S}*::MIR167b* and P_{35S}*::MIR167c* caused weaker mutant phenotypes, whereas P_{35S}*::MIR167d* plants all appeared identical to wild-type plants (Fig. III-3B,C and Table III-1). The phenotypic strengths of plants expressing different *MIR167* precursor genes correlated with the amount of mature *miR167* produced and with the degree of reduction of *ARF6* and *ARF8* transcript levels (Fig. III-3D). These results confirm that *miR167* can remove or destabilize *ARF6* and *ARF8* transcripts in vivo. No additional leaf or flower phenotype was observed in transgenic plants carrying any of the four constructs, suggesting that *miR167* targets only *ARF6* and *ARF8* in adult leaves and flowers.

***miR167*-immune *mARF6* and *mARF8* flowers are sterile**

To elucidate the developmental function of *miR167*, we introduced eight translationally silent mutations into *miR167* target sites in both *ARF6* and *ARF8* coding sequences in the context of their normal 5' and 3' flanking sequences (Fig. III-3A, *mARF6* and *mARF8*), and

transformed these constructs into wild-type plants. These mutations disrupted base-pairing between *miR167* and its target site, and should therefore render *mARF6* and *mARF8* transcripts immune to *miR167*-mediated turnover. Corresponding wild-type genomic constructs (*gARF6* and *gARF8*) increased fecundity of the loss-of-function mutants (Nagpal et al., 2005); data not shown), indicating that these genomic constructs were functional. *mARF6* and *mARF8* T1 plants had the same spectrum of phenotypes (Figs. III-4, S1), supporting our previous conclusion that *ARF6* and *ARF8* have similar activities (Nagpal et al., 2005). We focus here on our phenotypic studies of *mARF6* plants.

The severity of phenotypes of *mARF6* plants correlated with the level of *mARF6* transcript being expressed (Fig. III-4A). *mARF6-I* transgenic plants with the highest *ARF6* levels (12 of 63 T1 plants) had small leaves and sterile flowers (Figs. III-4A,B and S2). *mARF6-II* plants, with *ARF6* levels higher than wild-type plants but lower than *mARF-I* plants (36/63), had slightly smaller leaves than wild-type plants and sterile flowers (Figs. III-4A,B and S2). *mARF6-III* plants (15/63), with similar *ARF6* levels as wild-type plants, had leaves similar in size to those of *mARF6-II* or wild-type plants, but did produce seeds (Figs. III-4A,B and S2). However, *mARF6-III* seeds were small and could not germinate. As described below, embryos in these seeds were arrested.

Wild-type plants transformed with genomic *ARF6* or *ARF8* constructs or expressing

the wild-type *ARF6* coding sequence behind the CaMV 35S promoter (*P_{35S}::ARF6*) had fertile flowers despite having *ARF6* or *ARF8* transcript levels similar to or higher than those of *mARF6-II* or *mARF6-III* plants (Fig. III-4A). A small proportion (less than 5%) of *P_{35S}::ARF6*, *gARF6*, and *gARF8* plants also had small leaves. Thus, whereas elevated *ARF6* expression level inhibited leaf growth, only loss of *miR167* regulation caused flowers to be sterile.

***miR167* regulates ovule development**

Female sterility in *mARF6* plants arose from defects in ovule development. Early stage 2-IV ovules from *mARF6-II* plants had indistinguishable morphology from wild-type ovules, with inner and outer integuments initiated properly on ovule flanks (Fig. III-4C and G). However, whereas wild-type outer integuments grew to encase the entire nucellus (Fig III-4D and E), *mARF6-II* outer integuments only grew slightly (Fig. III-4H and I). In *mARF6-I* ovules, both inner and outer integuments and the nucellus were developmentally arrested (Fig. III-4K). In *mARF6-III* ovules, outer integuments extended farther than in *mARF6-II* ovules, but they nevertheless failed to envelop the nucellus completely (Fig. III-4L). In contrast to these effects on integument growth, cell morphology and arrangement in funiculi of *mARF6* ovules appeared normal (Fig. III-4E and I).

These ovule integument defects affected both pollen tube guidance to the ovule and

embryo development. Wild-type pollen tubes grew normally in transmitting tracts of *mARF6-II* gynoecia (Fig. III-4M). However, only a small proportion of *mARF6* ovules (12%, n=195) were fertilized by wild-type pollen (Fig. 4F and J), whereas 84% (n=70) of *gARF6* ovules were fertilized. Moreover, fertilized *mARF6* ovules still failed to support embryo development. Seven days after pollination, *gARF6* embryos had developed to mid-torpedo stage (Fig. III-4N), whereas embryos on *mARF6* plants were developmentally arrested at the 4-cell stage (Fig. III-4O). Embryos formed in self-fertilized *mARF6-III* flowers also developed only to the 4-cell stage. Similarly, absence of the outer integument in the *inner no outer-1* (*ino-1*) mutant, deficient in a member of the *YABBY* gene family (Villanueva et al., 1999), also caused reduced fertilization efficiency and arrested embryo development (data not shown). Thus, a primary defect in integument growth accounts for female sterility.

To determine whether altered distribution of *ARF6* and *ARF8* transcripts could account for these phenotypes, we examined *ARF6* and *ARF8* expression patterns in flowers of *mARF6-II* and *mARF8-II* plants by in situ hybridization (Fig. III-1). As a second method, we also compared X-gluc staining patterns in plants carrying *miR167*-insensitive (*mARF6:GUS*, *mARF8:GUS*) translational GUS fusions to the staining patterns of the *gARF6:GUS* and *gARF8:GUS* plants described above (Fig. III-2). In some strongly staining

mARF8:GUS lines, a subset of ovules had reduced outer integument growth similar to *mARF6-III* ovules (Fig. III-2N), suggesting that these constructs were partially functional. However, most *mARF6:GUS* and *mARF8:GUS* plants had fertile flowers, and these reporter constructs thereby revealed expression patterns largely independently of effects of the *mARF6* or *mARF8* mutations on ovule or anther development.

Consistant with Northern blot results, *ARF6* expression in *mARF6-II* ovules appeared stronger in tissues where *ARF6* was expressed in wild-type ovules (Fig. III-1E-F 1H-I, 2C-E and 2H-G). Moreover, *mARF6* (Fig. 1H and I) and *mARF6:GUS* (Fig. III-2H-J) expression also appeared in the integuments and nucellus. In stage 4-I ovules, staining of *mARF6:GUS* persisted most strongly in the chalazal domain of the mature ovule but decreased in the tips of the integuments (Fig. III-2J).

In *mARF8* ovules, the expression of *ARF8* only expanded into the integuments but not the nucellus (Fig. III-2K), suggesting that the expanded expression of *ARF8* into the integument region might be sufficient to arrest outer integument growth. Similarly, *mARF8:GUS* was expressed in both funiculi and ovules (Fig. III-2L and N).

INO was expressed in outer integuments of ovules, and *ino* mutations also caused arrested outer integument growth (Villanueva et al., 1999). However, *mARF6* ovules had a normal *INO* expression pattern, and *ino-1* ovules had a normal *ARF6* expression pattern (Fig.

III-S3), suggesting that *mARF6* affects integument growth independently of the INO pathway.

MIR167a is expressed in ovules and anthers

The *mARF6* and *mARF8* expression data indicated that *miR167* limits *ARF6* and *ARF8* transcript expression domains in ovules. To determine *MIR167* expression domains, we made transgenic plants carrying approximately 2 kb promoter fragments upstream of the stem-loop sequences of *MIR167a*, *b*, *c*, and *d* fused to a GFP-GUS reporter gene ($P_{MIR167a,\ b,\ c,\ d}$*:: GUS*), and analyzed promoter activities by X-gluc staining. In ovules, $P_{MIR167a}$*::GUS* expression (Fig. III-2P-R), and to a lesser degree $P_{MIR167b}$*::GUS* and $P_{MIR167c}$*::GUS* expression (Fig. III-2V and X), correlated with *miR167* functions revealed by mutating target sites. $P_{MIR167a}$*::GUS* expression first appeared at late ovule stage 1, in the cells from which both the inner and outer integuments would later be initiated (Fig. III-2P). As both integuments enveloped the nucellus and the ovule began to grow asymmetrically, staining expanded into the entire nucellus and integuments, but was always absent from the funiculus (Fig. III-2Q and R). $P_{MIR167a}$*::GUS* also stained in anthers and in sepal vasculature (Fig. III-2O). $P_{MIR167b}$*::GUS* was expressed in the ovules and nectaries but was not detected in other floral organs in the open flower (Fig. III-2U), and staining in mature $P_{MIR167b}$*::GUS* ovules was restricted mostly to the tips of inner and outer integuments (Fig. III-2V).

$P_{MIR167c}$*::GUS* stained mainly in the stamen filaments with trace amount of staining in the ovules (Fig. III-2W and X), and $P_{MIR167d}$*::GUS* only stained in sepals and petals, but not in the internal floral organs (Fig. III-2Y). In situ hybridization results have also shown that in both *Nicotiana benthamiana* and Arabidopsis, *miR167* is present in ovules but not in funiculi, and in anther vasculature (Valoczi et al., 2006).

miR167 regulates anther development

Male sterility of *mARF6* and *mARF8* flowers was due to indehiscent anthers (Fig. III-4B). Anthers of *mARF6* and *mARF8* flowers appeared normal before stage 10. However, *mARF6-II* anthers grew to be 20% larger than wild-type anthers due to enlarged connective cells without any significant increase in cell number (Fig. III-5A and D). In constrast, the vascular bundles of *mARF6-II* anthers were smaller than those of wild-type anthers (Fig. III-5B and E). In the oldest closed wild-type flower bud, anther tapetum and septum had entirely degraded, and as flowers opened stomium cells broke apart to allow release of pollen grains (Fig. III-5A). In *mARF6-II* anthers, traces of tapetum were present within the anther locules of the oldest closed flower bud, and the septum did not degrade so that the two anther locules did not fuse. Septum cell breakage occurred in *mARF6-II* anthers after flower opening, but the stomium still remained intact, resulting in lack of anther dehiscence (Fig. III-5D). Unlike the *arf6 arf8* double mutant, spraying with JA did not

restore *mARF6* anther dehiscence.

Whereas wild-type *ARF6* and *ARF8* were expressed in stamen filaments but not anthers (Fig. III-2A, B and K), *mARF6* and *mARF8* transcripts were also present in anther vasculature after floral stage 9 (Fig. III-1G, data not shown). $P_{MIR167a}$*::GUS* was expressed in anther primordia as they differentiated, and throughout young anthers (Fig. III-2S). As anthers matured, $P_{MIR167a}$*::GUS* expression became restricted to anther connective cells (Fig. III-2T). We also transformed the *mARF6* construct into plants with the synthetic auxin responsive reporter construct *DR5::GUS* (Ulmasov et al., 1997). In T1 plants showing *mARF6-I* phenotypes, we detected ectopic *DR5::GUS* expression in stage 13 flower anther locules but not in vascular or connective cells (Fig. III-5C and F).

Discussion

miR167 regulates both female and male floral organ development. Loss of *miR167* regulation in *mARF6* and *mARF8* flowers expanded the domains of *ARF6* and *ARF8* expression, and caused arrested ovule development and anther indehiscence. Plants that overexpressed *ARF6* or *ARF8* but had normal *miR167* regulation were fertile, indicating that loss of *miR167*-regulated patterning of *ARF6* and *ARF8* gene expression, rather than higher expression level, caused these phenotypes. *miR167* directs *ARF6* and *ARF8* transcript

cleavage, but might also affect *ARF6* and *ARF8* transcription, since it has been shown that *miR165/166* decreases *PHB* and *PHV* transcription by promoting DNA methylation in the coding regions downstream of the miRNA target sites (Bao et al., 2004).

Of the four predicted *MIR167* genes, when overexpressed only *MIR167a* caused high *miR167* production and arrested flower development to the same extent as in *arf6 arf8* plants. DCL1 might recognize or process the stem-loop structure of *MIR167a* more efficiently than the others. In addition, *miR167b* and *miR167c* might have weaker activities toward *ARF6* and *ARF8* transcripts, and *MIR167d* may be a pseudogene that does not have activity. *MIR167a* is therefore most likely to be the main functional *miR167* precursor gene in vivo. Consistent with this idea, $P_{MIR167a}$*::GUS* expression in ovules correlated precisely with *miR167* functions revealed in *mARF6* and *mARF8* plants.

In ovules, the complementary *ARF6*, *ARF8* and *miR167* expression patterns and the arrested development of *mARF6* and *mARF8* integuments indicate that *miR167* functions to clear *ARF6* and *ARF8* transcripts from cells that will become integuments, thereby allowing integument growth. Persistence of the expression patterns at later ovule stages suggests that *miR167* both establishes and maintains the correct pattern. *ARF2*, encoding another ARF protein, is normally expressed in the integuments and nucellus and inhibits integument growth (Schruff et al., 2006). The ectopic ARF6 and ARF8 activity caused by blocking

miR167 function may therefore activate pathways that ARF2 normally activates to restrict integument growth. Future studies may reveal the extent to which different ARF proteins have different activities, and why different *ARF* genes are expressed in mutually exclusive domains.

In anthers, *miR167* was present in vascular cells where *mARF6* and *mARF8* accumulated (Valoczi et al., 2006), indicating that *miR167* patterns gene expression in anthers as it does in ovules. However, although anther vasculature was altered in *mARF6* and *mARF8* plants, the strongest anther phenotypes were in connective cells, which grew abnormally large, and in locules, which failed to break open to release pollen and in some cases ectopically expressed the auxin-responsive marker *DR5::GUS*. *mARF6* and *mARF8* therefore have non-cell-autonomous effects in anthers. Anther dehiscence requires a series of desiccation events (Ishiguro et al., 2001), and excess *ARF6* and *ARF8* transcripts in the vasculature might increase water uptake, leading to excess connective cell expansion and preventing dehiscence.

Although *miR167* accumulated in anther vasculature (Valoczi et al., 2006), we detected $P_{MIR167a}$*::GUS* expression in connective cells but not in vasculature. This difference suggests that *miR167* processing or stability may differ in different cell types, or that *miR167* may move between cells.

Just as ectopic *mARF6* and *mARF8* appear to act cell-autonomously in ovules but non-cell-autonomously in anthers, wild-type *ARF6* and *ARF8* also appear to act autonomously in gynoecium transmitting tracts, but non-automonously on anthers by affecting JA production from other tissues (Nagpal et al., 2005). Moreover, *mARF6* and *mARF8* restrict growth in ovules, but cause extra growth in anthers. These observations suggest that ARF6 and ARF8 may activate distinct target genes in ovules and anthers.

In Drosophila, microRNAs have been suggested to function to reinforce transcriptional repression patterns (Stark et al., 2005). In contrast, the function of *miR167* to restrict distribution of its target transcripts is an essential patterning function that is not conferred by transcriptional controls of *ARF6* and *ARF8* alone. *miR165/166* also affects development by excluding expression of its target transcripts from the abaxial domain of lateral organs (Juarez et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2004b). In fact, the *miR165/166*-insensitive *phb-1d/+* mutant also has arrested outer integuments (Sieber et al., 2004), suggesting that both *miR165/166* and *miR167* might regulate common pathways during ovule formation.

miR167 is present in angiosperms and gymnosperms but not in mosses, lycopods or ferns (Axtell and Bartel, 2005). Angiosperms and gymnosperms are seed plants, and form integuments around the female gametophyte that later form the seed coat. Gymnosperm

male gametophytes are also surrounded by sterile cells similar to angiosperm anther connective cells (Gifford and Foster, 1988). Appearance of *miR167* in seed plants but not in lower plants therefore suggests that regulation by *miR167* could have arisen as plants evolved formation of sporophytic structures that protect gametophytes.

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Figures

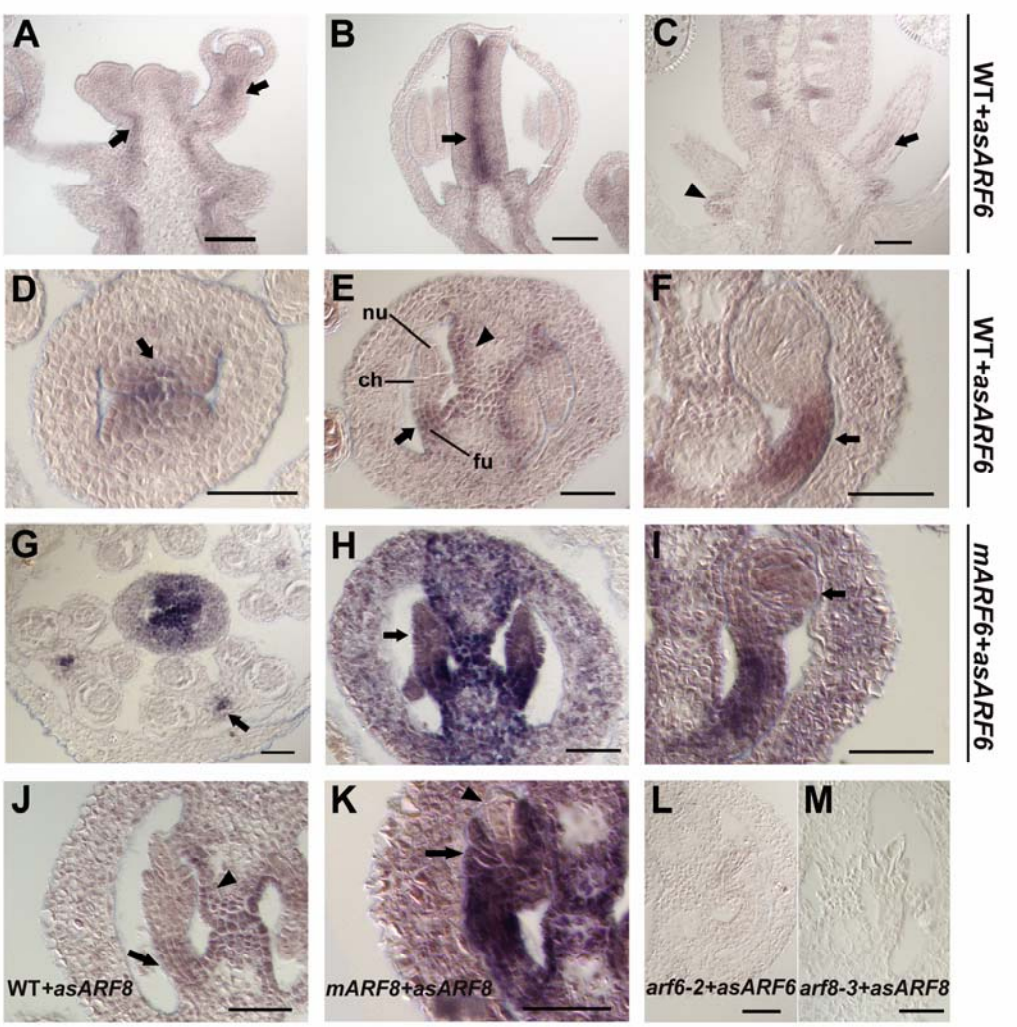

Figure III-1. *ARF6* and *ARF8* mRNA expression patterns.

(A-I, L) Sections of wild-type (A-F), *mARF6* (G-I) and *arf6-2* (L) flowers hybridized with an antisense *ARF6* probe. (J, K, M) Sections of wild-type (J), *mARF8* (K), and *arf8-3* (M) flowers hybridized with an antisense *ARF8* probe.

(A) Longitudinal section of inflorescence. Arrows indicate vasculature. (B) Longitudinal

section of stage 9 flower. Arrow indicates *ARF6* expression in medial ridge of carpels. (C) Longitudinal section of a stage 12 flower. Arrow indicates stamen filament vasculature and arrowhead indicates nectary. (D) Cross section of a stage 9 flower gynoecium. Arrow indicates medial ridge of carpels. (E) Stage 2-II ovule. Arrow indicates funiculus and arrowhead indicates the placental region. (F) Stage 3-I ovule. Arrow indicates funiculus. (G) Cross section of a stage 9 flower bud. Arrow indicates anther vasculature. (H, I) Stage 2-III (H) and 3 (I) ovules. Arrows indicate the integument and nucellus regions. (J) Stage 2-II ovule. Arrow indicates funiculus and arrowhead indicates the placental region. (K) Stage 3-I ovule. Arrow indicates integuments and arrowhead indicates nucellus. ch: chalaza; fu: funiculus; nu: nucellus. Scale bars: (A-C, G) 60 μm; (D-F, H-M) 30 μm.

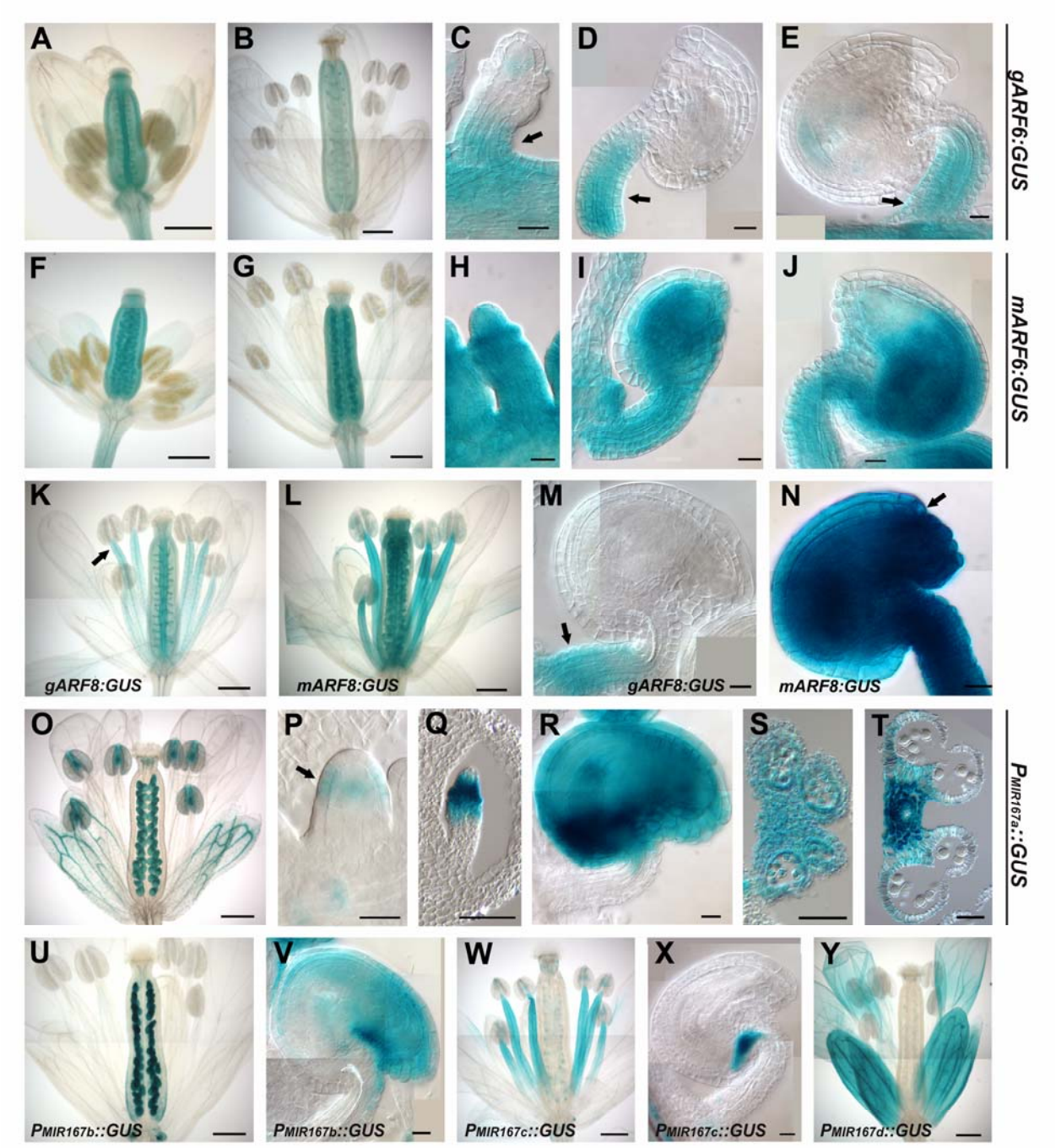

Figure III-2. Expression patterns of *gARF6*, *mARF6*, *gARF8*, and *mARF8* protein:GUS fusions, and *MIR167* promoter::GUS fusions.

(A-E) *gARF6:GUS*. (F-J) *mARF6:GUS*. (K, M) *gARF8:GUS*. (L, N) *mARF8:GUS*.

(O-T) $P_{MIR167a}$::*GUS*. (U, V) $P_{MIR167b}$::*GUS*. (W, X) $P_{MIR167c}$::*GUS*. (Y) $P_{MIR167d}$::*GUS*. (A, B) *gARF6:GUS* staining patterns in stage 11 (A) and stage 13 (B) flowers. (D-E) *gARF6:GUS* expression in stage 2-III (C), stage 3-I (D), and stage 4-I (E) ovules. Arrows indicate funiculus. (F, G) *mARF6:GUS* expression in stage 11 (F) and stage 13 (G) flowers. (H-J) *mARF6:GUS* expression in ovules. Ovule development stages in H, I, and J are equivalent to those in C, D, and E, respectively. (K) *gARF8:GUS* staining in stage 13 flower. Arrow indicates stamen filament expression. (L) *mARF8:GUS* staining in stage 13 flower. (M) *gARF8:GUS* staining in stage 4-I ovule funiculus (arrow). (N) *mARF8:GUS* expression in stage 4-I ovule. Arrow indicates reduced outer integument growth. (O) $P_{MIR167a}$::*GUS* expression in stage 13 flower. (P-R) $P_{MIR167a}$::*GUS* in stage 1-II (P), stage 2-III (Q) and stage 3-IV (R) ovules. (S, T) $P_{MIR167a}$::*GUS* expression in floral stage 10 (S) and stage 13 (T) anthers. (U, V) $P_{MIR167b}$::*GUS* expression in stage 13 flower (U) and stage 4-I ovule (V). (W, X) $P_{MIR167c}$::*GUS* expression in stage 13 flower (W) and stage 4-I ovule (X). (Y) $P_{MIR167d}$::*GUS* expression in stage 13 flower. Scale bars: (A, B, F, G, K, L, O, U, W) 3 mm; (C-E, H-J, M, N, P, R, V, X) 12 μm; (Q, S, T) 30 μm.

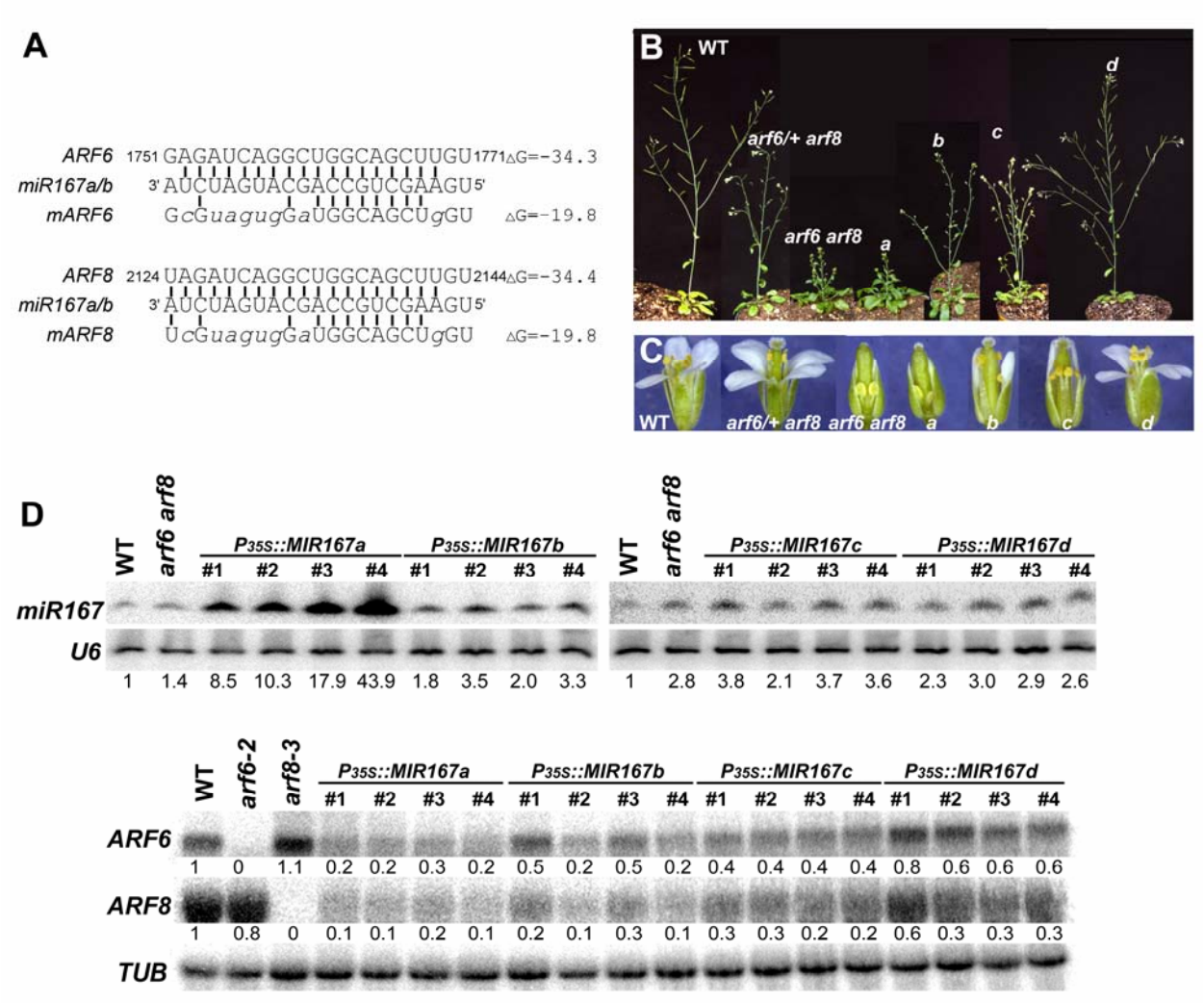

Figure III-3. Effects of over-expressing *MIR167* genes.

(A) Sequences of *miR167* target sites on *ARF6* and *ARF8* mRNA, and the mutated target sites of *mARF6* and *mARF8*. The Watson-Crick base pairings to the miR167 sequence are shown. ΔG (kcal/mol) was calculated by Mfold (Zuker, 2003). Mutated nucleotides are in lower case. (B) Phenotypes of plants over-expressing four different *MIR167* genes. From left to right: wild type (WT), a*rf6/+ arf8*, *arf6 arf8*, P_{35S}*::MIR167a* (a), P_{35S}*::MIR167b* (b), P_{35S}*::MIR167c* (c), and P_{35S}*::MIR167d* (d). (C) Stage 13 flowers of P_{35S}*::MIR167* plants.

Genotypes are the same as in (B). (D) Northern blot analyses of *MIR167*-overexpressing transgenic plant flowers. U6 snRNA and β-tubulin are included as loading controls. Numbers beneath lanes indicate relative transcript levels normalized to loading controls.

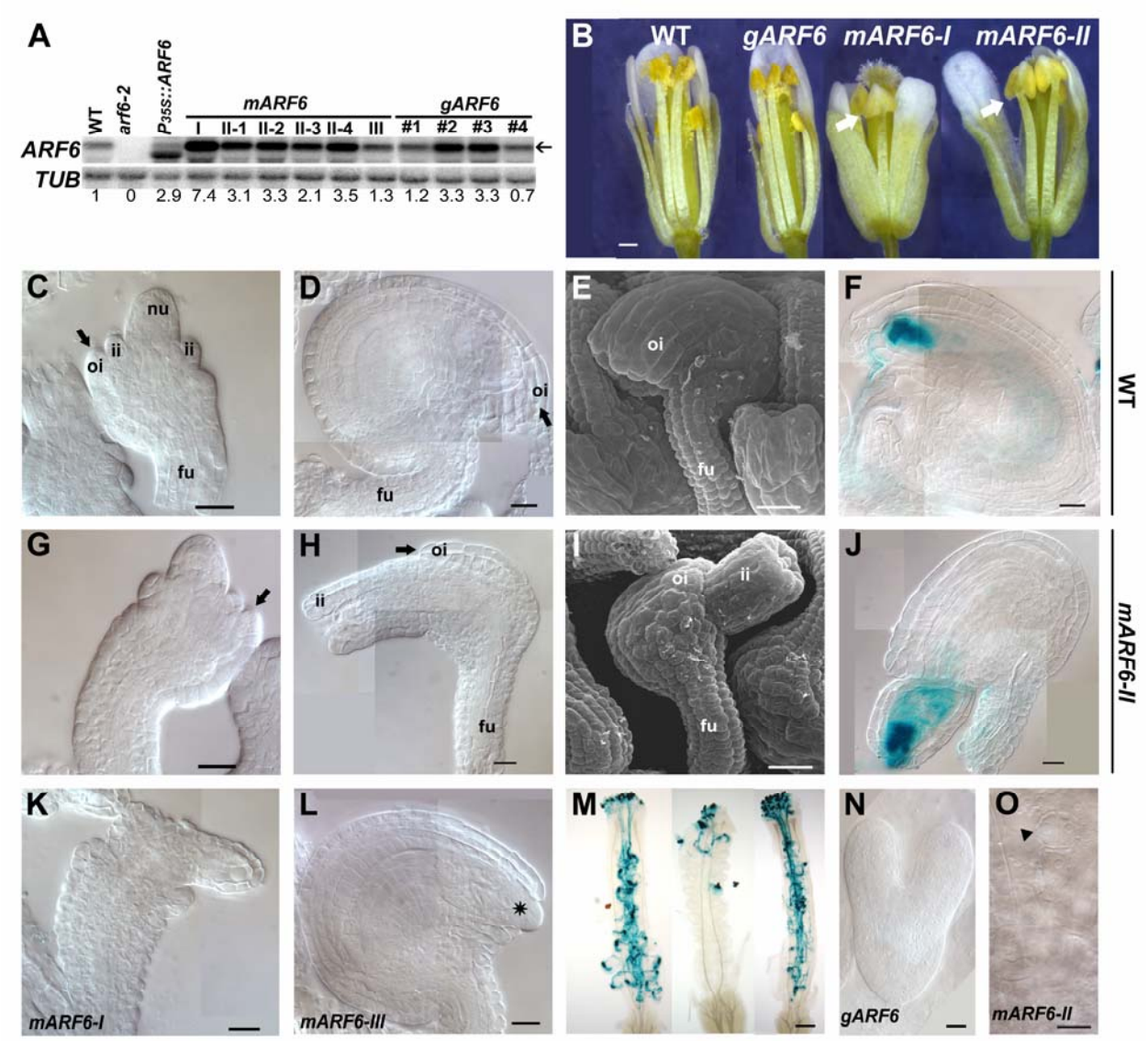

Figure III-4. *ARF6* expression and flower and ovule phenotypes of *mARF6* plants.

(A) Northern blot analysis of *ARF6* transcript levels in wild-type, *arf6-2*, *P*$_{35S}$*::ARF6*, and individual *mARF6* and *gARF6* transgenic plant flowers. Transcript of *P*$_{35S}$*::ARF6* is shorter because it lacks the 5' and 3' UTRs. Arrow indicates the *ARF6* transcript. Numbers beneath lanes indicate relative *ARF6* transcript levels normalized to the β-tubulin loading control. (B) Wild-type, *gARF6*, *mARF6-I* and *mARF6-II* flowers. Arrows indicate

indehiscent anthers. (C-F) Wild-type ovules. (G-J) *mARF6-II* ovules. (C, G) Stage 2-IV ovules. (D, E, H, and I) Stage 4-I ovules. Arrows in (C), (D), (G) and (H) indicate outer integuments. (K) Stage 4-I *mARF6-I* ovule. (L) Stage 4-I *mARF6-III* ovule; asterisk indicates exposed embryo sac. (F,J,M) Wild-type (F, M left), *arf6 arf8* (M middle) and *mARF6-II* (J, M right) gynoecia (M) and ovules (F, J) after pollination with the pollen-specific reporter *LAT52:GUS* pollen (Johnson et al., 2004). (N, O) Embryos of *gARF6* (N) and *mARF6-II* (O) plants 7 days after pollination with wild-type pollen. Arrow in (O) indicates arrested embryo. fu: funiculus; ii: inner integument; oi: outer integument; nu: nucellus. Scale bars: (B,M) 0.3 mm. (C, D, F, G, H, J-L, N, O) 12 µm. (E, I) 20 µm.

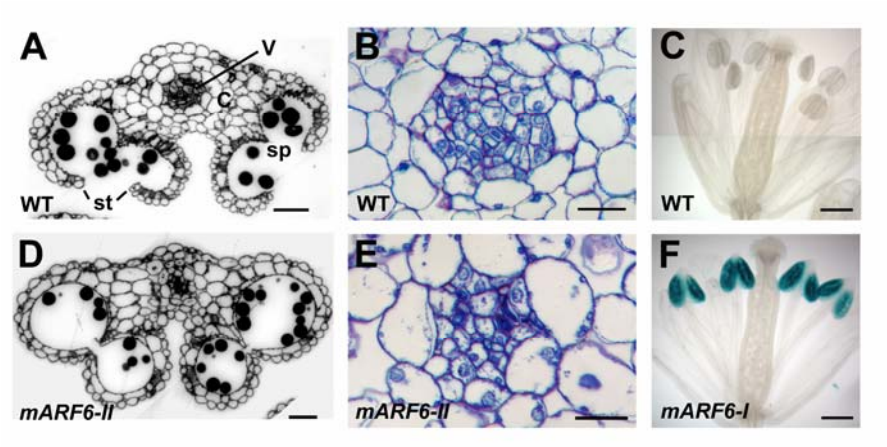

Figure III-5. Anther phenotypes of *mARF6* plants.

(A) Anther from stage 13 wild-type flower. c: connective cells; v: vascular bundle; sp: septum; st: stomium. (D) Anther from stage 13 *mARF6-II* flower. (B, E) Enlarged views of anther vascular bundles and surrounding connective cells from (A) and (D). (C, F) *DR5::GUS* staining patterns in wild-type (C) and *mARF6-I* (F) stage 13 flowers. Scale bars: (A, D) 30 µm. (B, E) 12 µm. (C, F) 0.3 mm.

Table III-1. Summary of *P_{35S}::MIR167* T1 plant phenotypes.

Gene	Predicted Sequence [A]	Strong (*arf6 arf8*-like)	Medium	Weak	No phenotype
MIR167a	5'-ugaagcugccagcaugaucua-3'	100	12	0	0
MIR167b	5'-ugaagcugccagcaugaucua-3'	0	3	101	8
MIR167c	5'-uuaagcugccagcaugaucuu-3'	0	0	91	7
MIR167d	5'-ugaagcugccagcaugaucugg-3'	0	0	0	109

A: (Jones-Rhoades and Bartel, 2004; Rhoades et al., 2002)

Supplementary Figures

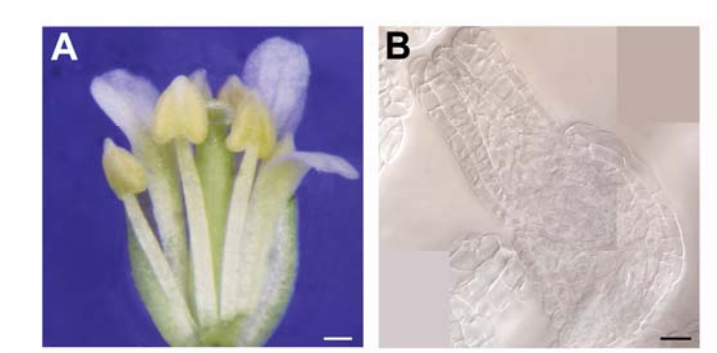

Figure III-S1. Flower and ovule of *mARF8* plants.

(A) Stage 13 *mARF8-II* flower. (B) Stage 4-I *mARF8-II* ovule. Scale bars: (A) 0.3 mm. (B) 12 μm.

Figure III-S2. Rosettes of 4-week-old wild-type, *gARF6*, *mARF6-I*, *mARF6-II* and *mARF6-III* plants. Scale bar: 2 cm.

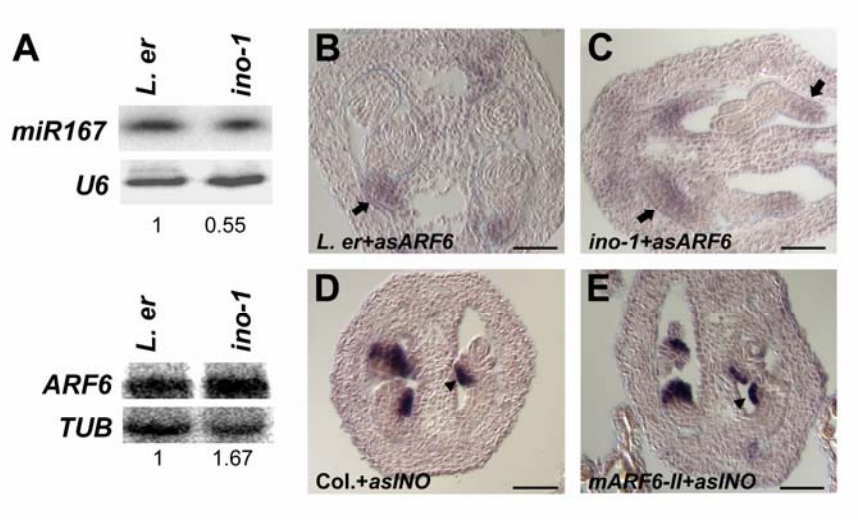

Figure III-S3. *miR167* and *ARF6* expression patterns in *ino-1* mutant flowers, and *INO* expression patterns in *mARF6-II* flowers.

(A) Northern blot analyses of wild type (L. *er*) and *ino-1* flowers. U6 snRNA and β-tubulin are included as loading controls. Numbers beneath lanes indicate relative transcript levels normalized to loading controls. (B, C) Cross sections of wild-type (L. *er*) (B) and *ino-1* (C) ovules hybridized with antisense *ARF6* probe. Arrows indicate ovule funiculus. (D, E) Cross sections of wild-type (Col.) and *mARF6-II* flowers hybridized with antisense *INO* probe. Arrowheads indicate outer integument. Scale bars: 30 μm.

CHAPTER IV

ARF6 and *ARF8* Promote Stigma Growth and Pollen Tube Elongation

Summary

Coordinated development between the female and the male reproductive organs is important for self-fertilization to occur in Arabidopsis flowers. *Auxin Response Factors 6* (*ARF6*) and *ARF8* regulate flower maturation, and *arf6 arf8* double mutant flowers arrest development right before flower opening. To understand how *ARF6* and *ARF8* promote flower maturation and fertilization in different parts of the flower, we expressed the negative regulator of the *ARF6* and *ARF8*, *MIR167a*, in the style or in the ovule funiculus. These experiments revealed that *ARF6* and *ARF8* in the style and the stigma are important for stigmatic papillae elongation. In addition, *ARF6* and *ARF8* in the ovule funiculus and in the transmitting tract regulate pollen tube growth. These results showed that *ARF6* and *ARF8* in different floral tissues can regulate different aspects of flower maturation and fertilization.

Introduction

For self-fertilizing plants, such as the model organism Arabidopsis thaliana, development of the gynoecium and stamens has to be coordinated to allow successful fertilization to occur. In Arabidopsis flowers, stamens assume rapid growth shortly before flower opening and reach the length of the gynoecium when the flower opens. Anther dehiscence occurs as the flower opens and pollen grains are released onto stigmatic papillae at the apex of the gynoecium.

The delivery of sperm to female gametophyte of angiosperms requires a pollen tube. Pollen grains hydrate and germinate shortly after they land on the stigma, and the pollen tubes start to elongate. To reach the female gametophyte, the pollen tube has to penetrate the style, grow intercellularly through the transmitting tract, leave the septum, grow along the funiculus and enter the ovule through the micropyle. During this process, pollen tubes must navigate successfully through these female tissues (Krichevsky et al., 2007). It has been shown that both the diploid ovule and haploid female gametophyte send out signals to direct pollen tube growth (Hulskamp et al., 1995; Marton et al., 2005; Ray et al., 1997). Synergid cells at the micropylar pole are required for directed pollen tube growth (Higashiyama et al., 2001). Several candidate signals involved in pollen tube guidance have been identified, including small peptides secreted by synergid cells and γ-amino butyric acid (GABA) (Marton et al., 2005; Palanivelu et al., 2003). Glycoproteins, as well as pH and calcium ions in the extracellular matrix of the transmitting tract can also determine the direction of pollen tube growth (Cheung et al., 1995; Holdaway-Clarke et al., 2003).

Immature gynoecia do not support pollen germination and pollen tube growth, indicating that developmental changes must occur in the gynoecium to allow pollination. Mutations that affect gynoecium to support pollen tube growth and/or guidance have been isolated (Crawford et al., 2007; Johnson et al., 2004). *ARF6* and *ARF8*, two closely related *ARF* genes, are important regulators during flower maturation (Nagpal et al., 2005). *arf6-2 arf8-3* double mutant flowers arrest development right before flower opening. The *arf6-2 arf8-3* double mutant flowers never open and have short stamens whose anthers do not dehisce. In addition, development of the double mutant gynoecia is also arrested. Stigmatic papillae on the top of the *arf6-2 arf8-3* gynoecia do not elongate, and the gynoecium does not support pollen tube growth (Nagpal et al., 2005; Wu et al., 2006).

In addition to promoting flower maturation, *ARF8* also participates in regulating fruit development. Loss-of-function *arf8* mutant plants form partially developed fruits in the absence of fertilization (Goetz et al., 2006; Vivian-Smith et al., 2001). In the absence of fertilization, ARF8 may inhibit expression of genes that promote fruit development by recruiting Aux/IAA proteins (Goetz et al., 2006) or by activating expression of *GH3* and *Aux/IAA* genes which thereby inhibit other fruit-promoting ARF protein activities. In tomato, fruit development can be initiated in the absence of fertilization be treating the ovary with exogenous auxin or by expressing an auxin synthesis gene in the ovary (Abad and Monteiro, 1989; Rotino et al., 1997). The inhibitory effect of *ARF8* on fruit development suggests that *ARF8* can be an inhibitor of auxin signaling, despite the fact the *ARF8* encodes a Q-rich ARF protein.

ARF6 and *ARF8* transcript levels are negatively regulated by *microRNA167* (*miR167*) (Jones-Rhoades and Bartel, 2004; Jones-Rhoades et al., 2006; Reinhart et al., 2002; Rhoades et al., 2002). miRNAs are small non-coding RNAs that can decrease gene expression by promoting transcript cleavage, translational inhibition or DNA methylation (Bao et al., 2004; Bartel, 2004). Arabidopsis miRNA target genes are involved in various pathways, such as hormone signaling transduction, pathogen-induced defense responses, organ polarity formation, environmental stress responses and feedback regulation on miRNA processing (Achard et al., 2004; Juarez et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2005; Mallory et al., 2004; Navarro et al., 2006). *miR167* negatively regulates *ARF6* and *ARF8* transcripts by transcript cleavage (Axtell and Bartel, 2005; Schwab et al., 2006). Overexpressing the *miR167* precursor gene *MIR167a* in plants recapitulates *arf6 arf8* double mutant phenotypes (Wu et al., 2006). *miR167*-resistant *mARF6* and *mARF8* transgenic plants have anthers that do not dehisce, and the ovule outer integument of *mARF6* and *mARF8* plants is arrested, showing that *miR167* regulation of *ARF6* and *ARF8* is important for proper reproductive organ development (Wu et al., 2006).

In flowers, *ARF6* and *ARF8* are expressed in vasculature of stamen filaments, in necteries, in valve tissues, in placental tissues of young flowers and in funiculi of developing ovules (Wu et al., 2006). The expression patterns suggest that *ARF6* and *ARF8* control multiple aspects during flower maturation. To reveal roles of *ARF6* and *ARF8* in two different parts of gynoecium, we used GAL4/UAS two component system and a funiculus-specific promoter to drive expression of *MIR167a*.

Expressing *miR167* precursor gene should remove *ARF6* and *ARF8* transcripts and reveal their functions in these areas of flowers.

Materials and Methods

Plant materials and constructs

Columbia (Col-0) ecotype was used as the wild-type strain. *arf6-2* and *arf8-3* were isolated and described previously (Nagpal et al., 2005).

GAL4 enhancer trap driver line *E254* (ABRC Number: CS70021) was generated by the Scott Poethig lab (http://enhancertraps.bio.upenn.edu/). *MIR167a* was PCR amplified (Wu et al., 2006) and cloned into pSDM7023 (Weijers et al., 2005) behind a *GAL4-UAS* fragment. The *UAS-MIR167a* fragment was then subcloned into binary vector pBAR (Holt et al., 2002). *UAS-GUS* plants were introduced into *E254* plants by crosses.

The *STK* promoter was amplified from wild-type genomic DNA using the following primiers: 5'-cacctgatggcgcatgtagcttag-3' and 5'-ccttcattttaaacatcaaacaac-3'. PCR products were cloned into pENTR/D-TOPO vector (Invitrogen) and subcloned into pBGWFS7 (Karimi et al., 2002). *MIR167a* was amplified from genomic DNA as described previously (Wu et al., 2006) and cloned into pKGW-Yc9 (Karimi et al., 2002) to obtain pKGW-*MIR167a*. The *STK* promoter was cloned into pKGW-*MIR167a* by LR clonase (Invitrogen).

Constructs were introduced into Agrobacterium strain GV3101 by electroporation and transformed into plants by the floral dip method (Clough and Bent, 1998).

In situ hybridization

Anti-sense *ARF6* and *ARF8* probes were synthesized as described previously (Wu et al., 2006). In situ hybridizations were performed as previously described (Long and Barton, 1998).

Histology and Microscopy

Pollen from *LAT52:GUS* plants (Johnson et al., 2004) was used for tracking pollen tube growth. X-gluc staining and microscopy were performed as described previously (Wu et al., 2006).

Results and Discussion

***ARF6* and *ARF8* in the style and stigma promote stigmatic papillae growth**

To clarify the expression patterns of GAL4 enhancer trap driver *E254*, we introduced a *UAS-GUS* reporter construct into *E254* plants to obtain *E254>>GUS*, and studied the X-gluc staining patterns. Prior to flower opening, *E254>>GUS* was expressed mainly in the style, the stigma, and the valves, and was also detected in the anthers (Fig. IV-1A). After the flower opened, *E254>>GUS* expression in the gynoecium was restricted only to the style and was still detected at the tips of anthers (Fig. IV-1B). Based on the X-gluc staining patterns of *E254>>GUS*, *E254* is a good driver for misexpressing genes in the style and stigma.

Gynoecia of the *arf6-2 arf8-3* double mutant plants have short stigmatic papillae (Nagpal et al., 2005). To study how *ARF6* and *ARF8* affect stigmatic papillae and

style development, we therefore used *E254* to misexpress *MIR167a* (*E254>>MIR167a*), the precursor gene of the *ARF6* and *ARF8* negative regulator *miR167*. Unlike *arf6-2 arf8-3* mutant flowers which did not open (Nagpal et al., 2005), *E254>>MIR167a* transgenic plant flowers opened as wild-type flowers did (Fig. IV-1E and F). Petals of *E254>>MIR167a* transgenic plant flowers grew longer than the sepals as in wild-type flowers, but the stamens were still slightly shorter than the gynoecium (Fig. IV-1F). Stigmatic papillae of *E254>>MIR167a* transgenic plant flowers were shorter than those of wild-type plants (Fig. IV-1E and F). Scanning EM of the apical part of the *E254>>MIR167a* gynoecium confirmed that the stigma was short, and also revealed that the boundaries between style and valves did not differentiate as obviously as in wild-type plants, whereas the cell size and number in the style did not deviate much from the wild-type (Fig. IV-1G and H). The *E254>>MIR167a* gynoecia thus appeared similar to those of the *arf6-2 arf8-3* double mutant flowers (Nagpal et al., 2005).

Stigmatic papillae are required for the adhesion of the pollen grains. The shorter stigmatic papillae of *E254>>MIR167a* transgenic plants may therefore interfere with pollination. To answer this, we tracked pollen tube growth using the pollen-specific reporter line *LAT52:GUS* (Johnson et al., 2004). Unlike *arf6-2 arf8-3* mutant plants which only had very limited pollen germination and growth, pollen tube growth appeared normal in *E254>>MIR167a* transgenic plants (Fig. IV-2A-C), suggesting that the shorter stigmatic papillae did not interfere with pollen grain germination and subsequent fertilization.

miR167 down-regulates *ARF6* and *ARF8* activities by inducing transcript cleavage (Axtell and Bartel, 2005; Schwab et al., 2006). To correlate *E254>>MIR167a* transgenic plant phenotypes to the levels of *ARF6* and *ARF8* transcripts, *in situ* hybridizations were performed on *E254>>MIR167a* plants using antisense *ARF6* and *ARF8* probes. In stage 12 wild-type flowers, we found that *ARF6* was not expressed in the style whereas prominent *ARF8* expression was detected in the area right beneath the stigmatic papillae (Fig. IV-3A and C). This expression was absent in *E254>>MIR167a* flowers (Fig. IV-3D). These results suggest that *ARF8* might be responsible for the short stigmatic papillae phenotypes in *E254>>MIR167a* flowers. However, *arf8* mutant flowers have normal stigmatic papillae (Nagpal et al., 2005), suggesting that *ARF6* might also contribute to stigma development, perhaps at earlier stages .

ARF6 and *ARF8* in the funiculus is required for pollen tube growth

ARF6 and *ARF8* are expressed in the ovule funiculus and in the transmitting tract, but their functions in these areas remain unclear (Wu et al., 2006). To understand roles of *ARF6* and *ARF8* might play in the ovule funiculus, we used the promoter of a funiculus-specific gene *SEEDSTICK* (*STK*) to misexpress *MIR167a*. *STK* expression was initiated in stage 9 flowers in the placenta tissues and in the funiculi in developing ovules (Fig. IV-1C) (Pinyopich et al., 2003; Rounsley et al., 1995). In ovules of stage 11 flowers, some P_{STK}*::GUS* expression could be detected in the ovule chalaza (Fig. IV-1D). The expression rapidly declined after floral stage 12, and disappeared after the flower opened (Rounsley et al., 1995).

Flowers of the P_{STK}::*MIR167a* transgenic plants did not have obvious morphological phenotypes, but some of the transgenic plants (23 out of 50 T1s) were less fecund than the wild-type plants. Ovules in these transgenic plants also did not have obvious morphological phenotypes (data not shown). However, only the ovules in the apical half of the transgenic plant siliques were fertilized.

To understand the cause of reduced fecundity, we studied the pollen tube growth patterns in P_{STK}::*MIR167a* transgenic plant flowers. We found that pollen tube growth was arrested in the transmitting tract of P_{STK}::*MIR167a* transgenic plants (Fig. IV-2D). Among pollen tubes that did elongate, guidance toward the ovule micropyle was not disrupted, and successful fertilization usually occurred. The arrested pollen tube growth in these plants suggests that *ARF6* and *ARF8* in the transmitting tract and/or the funiculus might regulate accumulation of biochemical cues important for substantial pollen tube elongation. Direct test of *ARF6* and *ARF8* expression patterns in these plants will provide an additional test for this idea.

After pollen tubes exit the style tissues, they enter the transmitting tract and assume rapid growth (Johnson and Preuss, 2002). The transmitting tract cells are coated with extracellular matrix, and signals required for pollen tube growth might be embedded there (Lord, 2000). Arabinogalactan proteins (AGPs) are one of the prominent transmitting tract extracellular components and function in cell to cell interactions (Showalter, 2001). Tobacco AGPs, transmitting tissue-specific proteins (TTSs), can support and direct pollen tube growth. In transgenic plants with reduced TTS levels, the pollen tube growth rate is slower (Cheung et al., 1995). However, TTS alone might not be sufficient for providing signal gradient to support pollen tube

elongation, and multiple signals may be required (Johnson and Preuss, 2002). The arrested pollen tube growth in P_{STK}::*MIR167a* transgenic plant gynoecia suggests that auxin signaling pathway might regulate expression of multiple signaling molecules required for pollen tube growth in the transmitting tract. One recently identified transmitting tract-specific transcription factor, *NO TRANSMITTING TRACT* (*NTT*), is expressed in overlapping domains with *ARF6* and *ARF8* (Crawford et al., 2007). In addition, *ntt* mutant phenotypes are similar to those of P_{STK}::*MIR167a* plants, suggesting that *NTT* might be one of the downstream targets of *ARF6* and *ARF8* in the transmitting tract.

Previously we have reported that ectopic expression of *miR167*-resistant *mARF6* or *mARF8* into ovules caused reduced fertilization rate (Wu et al., 2006). Most of the pollen tubes in *mARF6* or *mARF8* gynoecia wandered around the ovule without entering the micropyle (M.-F. Wu, unpublished data). These phenotypes might indicate that *ARF6* and *ARF8* might inhibit expression of pollen tube guidance signals in the ovule. One candidate of such molecules could be γ-amino butyric acid (GABA). Gradients of GABA in the Arabidopsis transmitting tract can provide positional cues to pollen tubes. Mutation in a GABA transaminase gene, *POP2*, disrupts the gradient and causes pollen tubes to grow around the ovule aimlessly (Palanivelu et al., 2003). *POP2* is mainly expressed in the outer integument where the relative GABA levels are low (Palanivelu et al., 2003). It is possible that ectopic *ARF6* and *ARF8* may affect expression of *POP2* or other genes affecting GABA gradient in the outer integument, which might then have caused the pollen tube phenotypes we observed in *mARF6* or *mARF8* transgenic plants. Taken together,

ARF6 and *ARF8* might promote pollen tube growth in the transmitting tract and funiculus, and their absence in the ovule might ensure proper expression of the ovule guiding signals.

Parthenocarpic fruit development

arf8-3 mutant plants, as well as loss-of-function *arf6-2* mutant plants, formed partially elongated fruit in the absence of fertilization (Table IV-1). To reveal where *ARF6* and *ARF8* regulate fruit development, we studied fruit development of *E254>>MIR167a* and P_{STK}*::MIR167a* in the absence of fertilization. We found that neither transgenic plant had elongated fruit in the absence of fertilization (Table IV-1), suggesting that *ARF6* and *ARF8* might regulate fruit initiation process in tissues other than the style and the funiculus. However, *arf6-2 arf8-3* double mutant did not have elongated fruit development. On the contrary, *arf6-2 arf8-3* double fruits were shorter than wild-type fruits in the absence of fertilization. These results suggest that a more informative experiment would be to silence either *ARF6* or *ARF8*, but not both, in selected tissues. In addition, we need to assess whether valve margins differentiate in the fruits, another indication of parthenocarpy.

In developing flowers, *arf6* and *arf8* mutations enhance each other, so the mutual suppression of fruit elongation in *arf6 arf8* double mutant suggests that an intermediate dose of ARF6 and ARF8 may promote fruit elongation. *arf8* single mutant light-grown seedlings have longer hypocotyls than wild-type hypocotyls and reduced *GH3* expression levels (Tian et al., 2004). Similarly, *arf6*, *nph4/arf7 arf6*, *nph4/arf7 arf8*, and *arf6 arf8* mutant seedlings also have elongated hypocotyls, and

only *arf6 nph4/arf7 arf8* mutants that lack all three of the relevant ARF proteins, have shorter hypocotyls (Jason Reed, unpublished data). The single or double mutant hypocotyl phenotypes suggest that decreasing *ARF* gene expression might actually increase outputs of auxin signals, probably by upregulating other *ARF* gene activities. Only when all ARF proteins participating in the same pathway are removed, can we see the phenotypes of reduced auxin response. A similar dynamic could be true during fruit development. *ARF6* and *ARF8* might act together to regulate fruit development, and loss of just one of them might increase the output of the other, which could result in increased fruit growth in the single mutants even in the absence of fertilization.

Previously, it was suggested that *ARF8* inhibits fruit development before fertilization by recruiting Aux/IAA proteins to the promoters of fruit inducing genes. An auxin increase upon fertilization could then cause Aux/IAA proteins to turn over, releasing ARF8 to activate transcription of these genes (Goetz et al., 2006). ARF6 and ARF8 also activate expression of auxin signaling inhibitors Aux/IAA and GH3 genes (Nagpal et al., 2005; Tian et al., 2004). These pathways could all integrate together to inhibit fruit development before fertilization.

The auxin responsive *DR5::GUS* reporter is expressed in the funiculus and the ovule chalaza in fertilized ovules (Aloni et al., 2006), suggesting that fertilization can trigger auxin responsive signals in these areas of ovules to promote fruit growth. It was reported that a transient *ARF8* expression in the ovule chalaza right before fertilization might be important for fruit growth induction (Goetz et al., 2006). We also observed that the *ARF8* promoter was downregulated after fertilization and

remained highly expressed in unfertilized ovules (Nagpal et al., 2005). However, we did not observe any *ARF8* expression in the ovule using a *gARF8-GUS* reporter construct or by in situ hybridization with an antisense *ARF8* probe (M.-F. Wu, unpublished data). Tissue-specific silencing of *ARF6* and/or *ARF8* may help to resolve which cells that express *ARF6* and *ARF8* are important for fruit initiation and growth.

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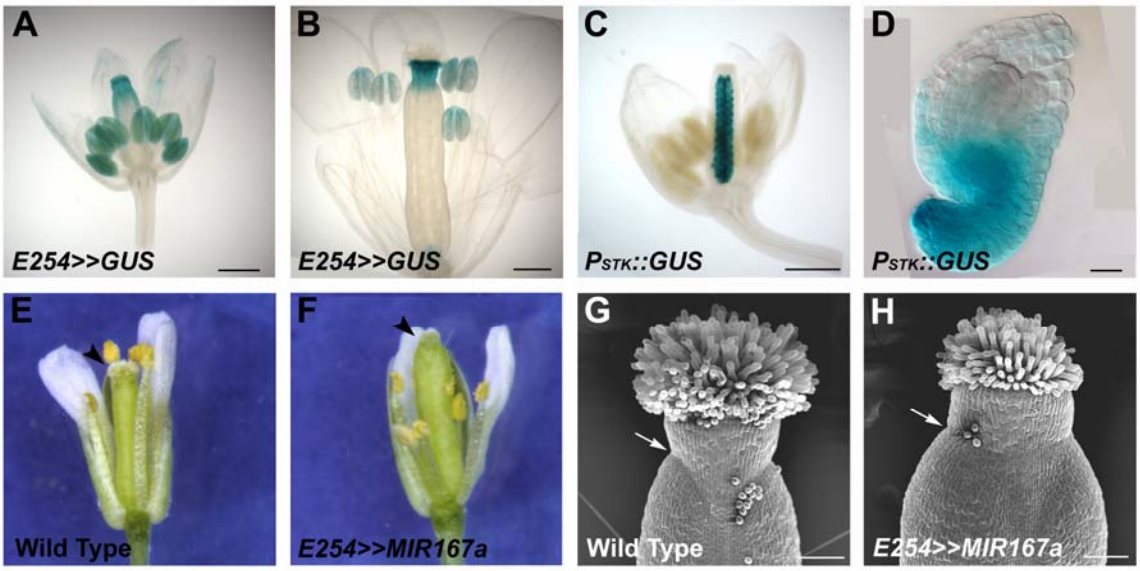

Figure IV-1. Expression patterns of *E254>>GUS* and P_{STK}*::GUS* and phenotypes of *E254>>MIR167a* flowers.

(A, B) X-gluc staining patterns of *E254>>GUS* in stage 10 (A) and stage 13 (B) flowers. (C, D) X-gluc staining patterns of P_{STK}*::axr3-1* in stage 10 flower (C) and in stage 3-I ovule (D). (E, F) Wild-type (E) and *E254>>MIR167a* (F) stage 13 flowers. Arrowheads indicate the arrested stigma. (G, H) SEM of wild-type (G) and *E254>>MIR167a* (H) style and stigma areas. Arrows indicate the boundaries between the style and the valve. Scale bars: (A-C, E, F) 0.3 mm; (D) 12 μm. (G, H) 100 μm.

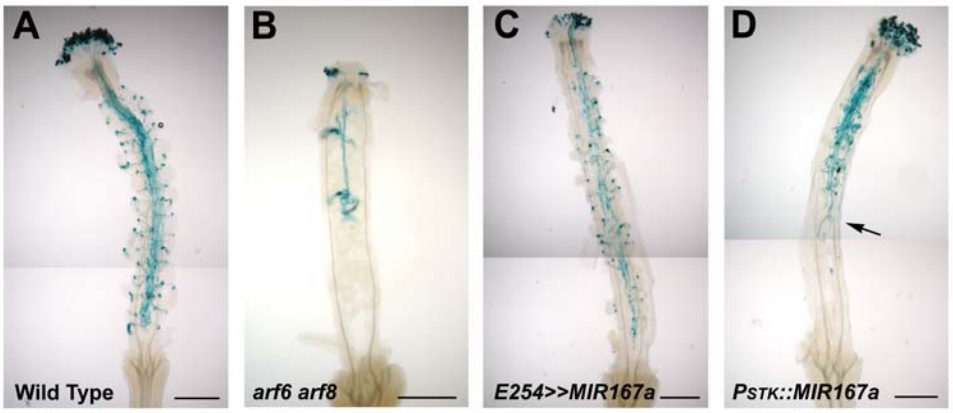

Figure IV-2. Pollen tube growth patterns.

Wild-type (A), *arf6 arf8* mutant (B), *E254>>MIR167a* (C) and P_{STK}*::MIR167a* (D) gynoecia were pollinated with *LAT52:GUS* pollens (Johnson et al., 2005). Arrow in (D) indicates arrested pollen tube growth. Scale bars: 0.3 mm.

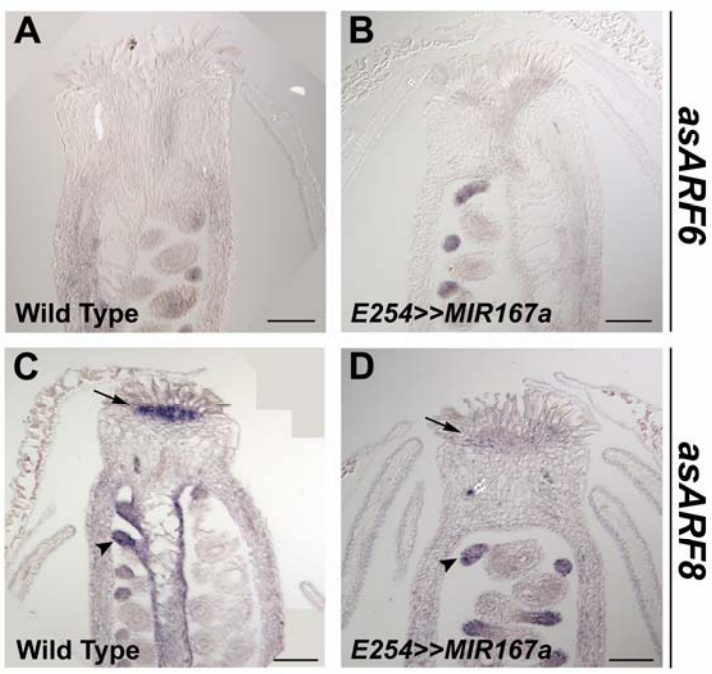

Figure IV-3. Expression patterns of *ARF6* and *ARF8* in *E254>>MIR167a* flowers. (A, B) Longitudinal sections of wild-type (A) and *E254>>MIR167a* (B) stage 12 flowers hybridized with antisense *ARF6* probes. (C, D) Longitudinal sections of wild-type (C) and *E254>>MIR167a* (D) stage 12 flowers hybridized with antisense *ARF8* probes. Arrowheads indicate expression in the funiculus. Arrowheads in (C, D) indicate *ARF8* expression beneath the stigmatic papillae. Scale bars: 60 μm.

Table IV-1. Gynoecium lengths one week after emasculation.

Genotype	**Length, mm ± SD**
Wild type	4.5 ± 0.5
arf6-2	5.5 ± 0.9
arf8-3	7.7 ± 0.9
arf6-2 arf8-3	2.8 ± 0.2
E254>>MIR167a	4.4 ± 0.9
P_{STK}*::MIR167a*	4.5 ± 0.4

CHAPTER V

ROLES OF AUXIN RESPONSE FACTORS DURING OVULE DEVELOPMENT

Summary

Angiosperm ovules are the predecessor of seeds, the propagation units. Therefore proper formation and development of the ovule is important for successful angiosperm reproduction. Auxin regulates various steps during ovule formation. To understand how auxin signaling regulates ovule development, we studied functions of multiple Auxin Response Factors (ARFs) in ovules. *ETT/ARF3* was expressed prominently in the inner integument, and promoted ovule growth and maturation. *MP/ARF5* was expressed in the inner integument and funiculus and might function with other *ARF* genes redundantly to regulate ovule growth. Silencing ARF activity in the outer integument by misexpressing the gain-of-function *axr3-1* mutant gene caused symmetric growth of the outer integument, whereas silencing ARF activity in the funiculus affected ovule differentiation and patterning. These results showed that the ARF-Aux/IAA signaling pathways regulate both ovule growth and pattern formation.

Introduction

Seeds, the propagation unit of plant and a major food source of many species, develop from the ovule after fertilization. Ovules have three major parts along the proximal-distal axis: funiculus, chalaza and nucellus. The nucellus is at the distal end of the ovule, and is also the place where female gametophyte development, fertilization and embryogenesis occur. The chalaza region is just proximal to the nucellus. The inner and outer integuments initiate from the chalazal region and develop into the seed coat after fertilization. The funiculus connects the ovule to the placenta. Vascular tissue forms in the funiculus and transports nutrients to support growth of the female gametophyte and later, the embryo and seed (Schneitz, 1995; Sieber et al., 2004a).

The unique developmental process and reproductive function of the ovule makes it an excellent model to study organ formation. In Arabidopsis, ovules arise from the placental tissues in carpels of stage 9 flowers as radially symmetric finger-like structures (Skinner et al., 2004). At ovule development stage 2, inner (adaxial) then outer (abaxial) integuments grow out at the flanks of the ovule (Schneitz, 1995). During the course of ovule development, the ovule shifts from a radially symmetric structure to a bilaterally symmetric structure after the initiation of the inner integument (Sieber et al., 2004a). In addition, the asymmetric growth of the ovule later during its development can further specify the ovule into gynobasal and gynoapical domains. A mature ovule, due to the more extensive outer integument

growth at the gynobasal side, curves toward the style of the gynoecium on the gynoapical side (Skinner et al., 2004).

Proper formation of ovules requires multiple tightly regulated patterning events. Multiple gene families have been identified to participate in pattern formation of the ovule. Arabidopsis class III homeodomain-leucine zipper family (HD-Zip III) proteins promote the formation of adaxial fate in lateral organs (Long and Barton, 1998; McConnell and Barton, 1998; McConnell et al., 2001; Otsuga et al., 2001; Prigge et al., 2005). Triple mutants lacking three HD-Zip III genes cause loss of bilateral symmetry in embryos and have abaxialized leaves (Emery et al., 2003; Prigge et al., 2005). Two genes of the HD-Zip III family, *PHABULOSA* (*PHB*) and *REVOLUTA* (*REV*), are expressed in the ovule (Sieber et al., 2004a; Sieber et al., 2004b). *PHB* is expressed in the placental region before ovule emergence and in the inner integument later. The heterozygous gain-of-function *phb-1d* mutant has *phb-1d* ectopically expressed in the outer integument, and growth of the mutant ovule outer integument is arrested, suggesting that in the ovule, as other lateral organs in *phb-1d* mutant, the abaxial fate is lost (Sieber et al., 2004a). *REV* is expressed in the entire ovule primordium and in both integuments (Sieber et al., 2004b). However, the function of *REV* in the ovule is still not clear.

Members of *KANADI* (*KAN*) and *YABBY* gene families specify abaxial fate (Eshed et al., 2001; Eshed et al., 2004; Kerstetter et al., 2001; Sawa et al., 1999; Siegfried et al., 1999). Ectopically expressing *KAN* genes causes lateral organs to be abaxialized, whereas loss-of-function *kan1 kan2* mutant results in adaxialized lateral organs (Eshed et al., 2001; Eshed et al., 2004; Kerstetter et al., 2001). Analogous

results have obtained from both the gain-of-function and the loss-of-function *yabby* mutants (Sawa et al., 1999; Siegfried et al., 1999). It was proposed that initial establishment of primordium polarity requires juxtaposition between abaxial and adaxial tissues that express *KAN* and HD-Zip III gene families, and the following lamina expansion requires abaxial expression of *YABBY* genes (Eshed et al., 2004; Hudson and Waites, 1998; Waites, 1995). *INNER-NO-OUTER* (*INO*) is the only *YABBY* gene expressed in the ovule. *INO* is only expressed in the abaxial (outer) cell layer of the gynobasal outer integument (Villanueva et al., 1999). In strong loss-of-function *ino-1* mutant ovules, the initiation and growth of the outer integument is completely absent (Schneitz et al., 1997; Villanueva et al., 1999). In contrast, ectopic *INO* expression on the gynoapical side of the ovule causes symmetric growth of the outer integument (Meister et al., 2002). Similarly to *ino-1* and *phb-1d* mutants, outer integument growth of *kan1-2 kan2-1* mutant ovules is also arrested (Eshed et al., 2001). One member of the *KAN* gene family, *ABERRANT TESTA SHAPE* (*ATS*)/*KAN4*, is expressed in the outer cell layer of the inner integument, and loss-of-function *ats/kan4* causes congenital fusion of the inner and the outer integuments (McAbee et al., 2006). *kan1 kan2 ats/kan4* triple mutants completely lack of laminar expansion of both integuments, revealing that *KAN1* and *KAN2* define the outer integument abaxial fate whereas *ATS/KAN4* defines the inner integument abaxial fate (McAbee et al., 2006).

Besides genes regulating organ polarity formation, other genes involved in different pathways also regulate ovule development. *WUSCHEL* (*WUS*) encodes a homeodomain protein that acts in stem cell maintance in shoot and floral meristems

(Mayer et al., 1998). In the ovule, *WUS* is expressed only in the nucellus, and loss-of-function *wus* ovules do not have integuments, implying that *WUS* regulates integument formation non-cell-autonomously (Gross-Hardt et al., 2002). AINTEGUMENTA (ANT), an AP2 domain containing protein that promotes initiation and growth of lateral organ primordia, also promotes integument initiation and is expressed mainly in the chalazal area of the ovule (Elliott et al., 1996; Krizek, 1999). Loss-of-function *ant* ovules do not form integuments, and the female gametophyte development is arrested (Elliott et al., 1996). The *SUPERMAN* (*SUP*) gene encodes a C2H2-zinc finger protein that defines the boundary of floral organ identity B class genes (Goto and Meyerowitz, 1994; Lohmann and Weigel, 2002). Outer integuments of loss-of-function *sup* ovules grow symmetrically around the nucellus, resulting in an elongated tubular-shaped ovule (Gaiser et al., 1995). *SUP* negatively regulates *INO* expression in the gynoapical outer integument, and loss of *SUP* activity causes ectopic *INO* expression and integument growth in this area (Meister et al., 2002).

The formation of organs requires initiation of primordia, cell division and growth, and finally differentiation. The developmental process and the regulatory genes during ovule formation share striking similarities to those of other lateral organs. It has long been known that the plant hormone auxin regulates these basic steps of organogenesis (Reinhardt, 2005; Reinhardt et al., 2003). Auxin is required for organ primordium initiation at the meristems, and the route of auxin flux deduced from localization of the auxin efflux carrier, PIN1, suggests how auxin movement affects organ initiation (Benkova et al., 2003; Heisler et al., 2005; Reinhardt et al.,

2000; Reinhardt et al., 2003). In developing lateral organ primordia, the amount of auxin peaks at the tip of incipient primordia and drains through the central vascular tissue (Benkova et al., 2003). The peak of auxin leads to formation of the organ primordium, and then auxin flows away from primordia toward sites of initiation of other organs (Heisler et al., 2005).

Auxin activates a family of transcription factors called AUXIN RESPONSE FACTORS (ARFs). ARF proteins have a conserved N-terminal DNA binding motif and in most classes, C-terminal dimerization motifs III and IV (Liscum and Reed, 2002). In the absence of auxin, another group of small nuclear proteins called Aux/IAA proteins bind to ARF proteins through the shared domains III and IV and inhibit their activity. When auxin enters the cell, Aux/IAA proteins are rapidly degraded, thus freeing ARF proteins to control gene transcription. Aux/IAA proteins have a consensus motif responsible for the protein stability called motif II. Point mutations in this motif in any of several different *Aux/IAA* genes cause Aux/IAA proteins to be insensitive to auxin induced degradation (Leyser, 2006). Gain-of-function mutations cause pleiotropic auxin resistant phenotypes (Fukaki et al., 2005; Hamann et al., 1999; Leyser et al., 1996; Nagpal et al., 2000; Tian and Reed, 1999).

There are 22 *ARF* genes in Arabidopsis, many of which regulate various developmental processes (Liscum and Reed, 2002; Remington et al., 2004). *ARF2* regulates senescence, floral organ abscission, hypocotyl apical hook formation, and ovule growth (Ellis et al., 2005; Li et al., 2004; Okushima et al., 2005; Schruff et al., 2005). *ETTIN* (*ETT*)/*ARF3* is important for gynoecium patterning and floral organ number (Nemhauser et al., 2000; Sessions and Zambryski, 1995). *MONOPTEROS*

(*MP*)/*ARF5* regulates basal domain development during embryogenesis, vasculature differentiation, and floral organ initiation (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998). *NONPHOTOTROPIC HYPOCOTYL* (*NPH4*)/*ARF7* acts redundantly with *MP*/*ARF5* in vasculature differentiation and embryo development (Hardtke et al., 2004). In addition, *NPH4*/*ARF7* also acts together with *ARF19* in promoting lateral root growth (Okushima et al., 2007; Okushima et al., 2005; Wilmoth et al., 2005). *ARF6* and *ARF8* promote flower maturation and are expressed in ovule funiculi (Nagpal et al., 2005; Wu et al., 2006). When *miRNA167* regulatory sites in *ARF6* and *ARF8* were mutated, their mRNA expanded into the integuments and inhibited integument growth, indicating that auxin signaling might regulate ovule patterning (Wu et al., 2006). Similarly, *ARF2* inhibits integument growth (Schruff et al., 2005). Microarray data showed that *ARF3*, *ARF5*, *ARF11*, and *ARF18* are also expressed in the ovule (C. Gasser, personal communication).

We hypothesize that one or more of these genes might promote ovule growth or differentiation. To reveal roles of *ARF* genes in the ovule, we studied the expression patterns of some of these *ARF* genes. We also looked at the phenotypes of some loss-of-function *arf* mutant ovules. In addition, to inhibit multiple ARF protein transcriptional activities in the ovule, we utilized two ovule-specific promoters to drive the expression of a gain-of-function mutant *IAA* gene, *axr3-1*. Our results showed the functional *ARF* genes are important for growth of the outer integument and for ovule differentiation. Once the ARF-mediated auxin signaling responses were disturbed, the auxin flux in the ovule can be reduced, which can result in altered ovule organ polarity.

Materials and Methods

Plant Materials and constructs

Columbia (Col-0) ecotype was used as the wild-type strain. *arf2-8*, *arf6-2*, and *arf8-3* were isolated and described previously (Ellis et al., 2005; Nagpal et al., 2005). The *arf3* (SALK031544) and *arf4* (SALK070506) alleles were isolated and identified by Christine Ellis (unpublished data).

The *axr3-1* (At1g04250) coding region was amplified from *axr3-1* 10-day-old seedling first strand cDNA using the following primers: 5'-caccactagtatgatgggcagtgtcgagct-3' and 5'-ccgagctctcaagctctgctcttgcact-3'. Wild-type *AXR3* was amplified from wild-type 10-day-old seedling first strand cDNA using the same primer pair. The PCR products were cloned into pENTR/D-TOPO vector (Invitrogen) and subcloned into pKGW-Yc9 (Karimi et al., 2002) using *Spe*I and *Sac*I sites to obtain pKGW-axr3-1 and pKGW-AXR3. *INO* and *STK* promoters were amplified from wild-type genomic DNA using the following primers: INO, 5'-cacctggaacaattctttgcgaca-3' and 5'-agagagtgtgtgtgtacgatgaatg-3'; STK, 5'-cacctgatggcgcatgtagcttag-3' and 5'-ccttcattttaaacatcaaacaac-3'. PCR products were cloned into pENTR/D-TOPO vector (Invitrogrn) and subcloned into pKGW-axr3-1 or into pKGW-AXR3 by LR clonase (Invitrogen) to obtain P_{INO}*::axr3-1*, P_{INO}*::AXR3*, P_{STK}*::axr3-1*, and P_{STK}*::AXR3*. Constructs were introduced into Agrobacterium strain GV3101 by electroporation and transformed into plants by the floral dip method (Clough and Bent, 1998).

PIN1:GFP seeds were a gift from Dr. Jiří Friml (Benkova et al., 2003). P_{INO}*::axr3-1* and P_{STK}*::axr3-1* were introduced into *PIN1:GFP* by crosses.

In situ Hybridization

ARF2, *ARF3* and *ARF5* probes were amplified using the following primers: *ARF2*, 5'-cggaattcccggctttgggactaacata-3' and 5'-ccaagcttaaggatcgtcaccaacaagc-3'; *ARF3*, 5'-ccctcgagtgcttccctcctctggacta-3' and 5'-gagagcaatgtctagcaaca-3'; *ARF5*, 5'-cggaattcaaacgtcagatccatccatc-3' and 5'-ccaagcttcagaagggtggttctggaaa-3'. The amplified fragments were cloned into pGEM-T vector (Promega). *INO* probe was cloned and synthesized as previously described (Wu et al., 2006). Full-length *axr3-1* was also cloned into pGEM-T vector (Promega) for in situ hybridization probes. Antisense probes were synthesized by in vitro transcription with SP6 RNA polymerase using a DIG RNA labeling kit (Roche). In situ hybridizations were performed as previously described (Long and Barton, 1998).

Microscopy

Ovules were fixed as previously described (Wu et al., 2006). Ovule photographs were taken with a Nikon E800 photomicroscope using differential interference contrast (DIC) optics.

For *PIN1:GFP* images, ovules were dissected out from the carpels and fixed in 4% paraformaldehyde in 1X PBS, pH 7.4 for 1 hour at room temperature. Ovules were rinsed twice with 1X PBS, pH 7.4 and mounted in 10% glycerol in 1X PBS, pH

7.4. GFP images were taken with a Zeiss LSM 510 laser scanning confocal microscope.

Results

ARF2 and *ETT/ARF3* regulate ovule growth

As described earlier (Schruff et al., 2005), *arf2-8* ovules were larger than wild-type ovules due to excess cell division and cell growth (Fig. V-1A and B). To correlate observed ovule phenotypes to the distribution of the *ARF* gene transcripts, we used in situ hybridization to locate the transcripts of *ARF2*. *ARF2* expression was present in the inflorescence meristem (Fig. V-2A) and in anther primordia (Fig. V-2B). After the ovule formed, *ARF2* transcript was detected all over the entire ovule (Fig. V-2C), which corresponded to what has been described earlier using *ARF2:GFP* protein-reporter construct (Schruff et al., 2005).

ett/arf3 mutant flowers often had narrow gynoecia and exposed ovules (Sessions et al., 1997; Sessions and Zambryski, 1995). In contrast to *arf2* ovules, *ett/arf3* ovules were smaller than wild-type ovules (Fig. V-1C). Whereas the micropylar side of the wild-type ovule bent toward the base of the funiculus, the *ett/arf3* ovules did not bend as much as wild-type. Moreover, the outer integument of *ett/arf3* ovules did not fully encase the inner integument and nucellus (Fig. V-1C). In some cases, the outer integument was slightly shorter than the inner integument.

Expression of *ETT/ARF3* is first initiated in stage 1 flower primordia and then present in the abaxial sides of stamen and gynoecium (Sessions et al., 1997). In

ovules, *ETT/ARF3* was first detected in the chalazal area which later gave rise to the integuments (Fig. V-2D). After the inner integument initiated, *ETT/ARF3* became restricted to the inner integument (Fig. V-2E), and persisted into later developmental stages (Fig. V-2F). These results suggest that *ETT/ARF3* might promote integument growth and may therefore act antagonistically to *ARF2* in the ovule.

Gynoecia of *ett/arf3 arf4* flowers reduced to a small mound and lacked several gynoecial tissues, including valve, style and stigmatic papillae (Pekker et al., 2005). Most of the *ett/arf3 arf4* flowers did not have ovules, but sometimes exposed ovules were formed at the apex of the gynoecial mound (Fig. V-1D). A radially symmetric ovule often formed at the very apex (Fig. V-1D), whereas ovules on the flanks of the gynoecial mound were similar to those of the *ett/arf3* single mutant. These results suggest that loss of *arf4* does not aggravate the developmental defects of *arf3* ovules. The radially symmetric ovule at the top of the gynoecium could be due its position at the apex, where normal asymmetric cues may be absent.

Consistent with our observation that *arf4* did not enhance *ett/arf3* ovule phenotypes, *ARF4* was expressed in the floral meristem and in the gynoecium and anther vascular bundles but not in the ovule (Fig. V-S1).

Manipulating auxin response signaling in the funiculus epidermis and outer integument affects integument growth

The distinct ovule expression patterns of *ARF* genes, including *ARF2*, *ARF3*, *ARF5*, *ARF6* and *ARF8*, suggest that they might regulate different aspects of ovule growth. However, loss-of-function mutants in ARF genes may not reveal functions in

ovules because they are often redundant, and because loss-of-function *ett/arf3* and *mp/arf5* mutants either form very few ovules or arrest development long before flowers develop. To inhibit activities of multiple *ARF* proteins, except for *ETT/ARF3* (which lacks C-terminal dimerization domains), and to obtain an ovule-specific inhibition, we used ovule specific promoters to drive expression of gain-of-function *aux/iaa* genes, *axr3-1/iaa17*, *shy2-2/iaa3*, and *iaa18-1* (Leyser et al., 1996; Rouse et al., 1998; Tian and Reed, 1999). These genes have point mutations in the consensus destabilizing signal within motif II that render the proteins insensitive to auxin-induced degradation. This generally leads to constitutive inhibition of ARF proteins although axr3-1 mutants have some phenotypes suggesting increased auxin responses (Leyser et al., 1996). We found that most transgenic plants with misexpressed *shy2-2* did not have any phenotype whereas transgenic plants with misexpressed *axr3-1* or with *iaa18-1* had similar ovule phenotypes. *shy2-2* may be less potent than *axr3-1* and *iaa18-1* because *shy2-2* protein may be less stable, or it may interact weakly with relevant ARF proteins in the ovule. Because the endogenous *IAA18* transcript was also expressed in the ovule, we decided to focus our studies on transgenic plants with misexpressed *axr3-1* to avoid confusion between the introduced transgenes and the endogenous wild-type copy.

Expanded expression of *miR167*-resistant mutant *ARF6* and *ARF8* transcripts into the ovule caused arrested outer integument growth (Wu et al., 2006), suggesting that ectopic auxin signaling in the ovule outer integument might interfere with outer integument formation. To understand whether silencing auxin signaling can also affect outer integument growth, we used the promoter of *INNER-NO-OUTER* (*INO*)

which drives expression in the outer integument. *INO* expression is initiated in the epidermal cell layer of stage I ovules and is then restricted to the abaxial cell layer of the outer integument on the gynobasal side (Villanueva et al., 1999). We constructed P_{INO}*::axr3-1* to misexpress *axr3-1* in the outer integument. As controls, we cloned wild-type *AXR3* behind the same promoter. In addition, we also constructed P_{INO}*::GUS* as mean to identify expression patterns of the promoter. We found that expression of P_{INO}*::GUS* was detected in the gynobasal outer integument, and expanded into the funiculus (Fig. V-3L).

In wild-type ovules, the outer integument grew more on the gynobasal side than on the gynoapical side of the ovule, which caused the micropylar end to orient toward the funiculus (Fig. V-3A-C). P_{INO}*::axr3-1* ovule integuments initiated properly at stage 2 ovule, as in wild-type ovules (Fig. V-3A and D). However, P_{INO}*::axr3-1* outer integuments did not envelop the nucellus, possibly due to less cell expansion than in wild type (Fig. V-3B and E). Meanwhile, the P_{INO}*::axr3-1* gynoapical outer integument also started to grow, in contrast to the limited growth of the wild-type gynoapical outer integument (Fig. V-3B and E). The abnormal growth patterns of the P_{INO}*::axr3-1* outer integuments caused the mature ovule to stand up straight instead of bending toward the transmitting tract (Fig. V-3C and F). We refer to these ovules as straightened ovules. Surprisingly, fertilization and embryo development was not affected in P_{INO}*::axr3-1* ovules. Although seeds of P_{INO}*::axr3-1* transgenic plants were more crescent-shaped than wild-type seeds, which were oval (Fig. V-3G and H), they germinated properly.

To assess whether *axr3-1* was misexpressed in these ovules, we perform in situ hybridization with an antisense *axr3-1* probe. The endogenous *AXR3* was not expressed in the wild-type ovule (Fig. V-3K). In P_{INO}*::axr3-1* plants, *axr3-1* was first expressed in the gynobasal outer integument at ovule stage 1-II as was the *INO* transcript (Fig. V-3M, Fig. V-4A). However, unlike *INO* transcript which was restricted to the abaxial outer integument throughout ovule development, *axr3-1* transcript was expanded to the epidermis of the outer integument and the funiculus on the gynoapical side of the ovule (Fig. V-3N).

Manipulating auxin response signaling in the funiculus affects both ovule symmetry and differentiation

To reveal possible roles of *ARF*s in the ovule funiculus, we expressed *axr3-1* in the funiculus using *SEEDSTICK* (*STK*) promoter. *STK* is expressed in the placental tissue just before ovule formation and in the chalaza and funiculus at later stages (Pinyopich et al., 2003; Rounsley et al., 1995).

P_{STK}*::axr3-1* transgenic plants had much more severe ovule defects than those of P_{INO}*::axr3-1* transgenic plants. Most P_{STK}*::axr3-1* ovules did not develop female gametophyte or vascular tissue, and those plants that had an ovule phenotype (18 out of 39 T1s) could be roughly divided into two classes. Class I (n=8) transgenic plants also had excess integument growth similar to P_{INO}*::axr3-1* straightened ovules; however, their funiculi were exceptionally long compared to both wild-type and P_{INO}*::axr3-1* ovules (Fig. V-3I). Class II (n=10) transgenic plants had completely radially symmetric ovules (Fig. V-3J). Most class II transgenic plants had a mixture

of straightened and radialized ovules. In addition, some class II P_{STK}*::axr3-1* plants had fewer ovules than wild-type plants.

In P_{STK}*::axr3-1* flowers, *axr3-1* was expressed only in the funiculus (Fig. V-3O and P), which mirrored those of *STK* transcript in the ovule. In contrast to P_{INO}*::axr3-1* in which *axr3-1* was expressed only in the epidermis of the funiculus, *axr3-1* was expressed in all cell layers in P_{STK}*::axr3-1* funiculus.

P_{INO}*::axr3-1* and P_{STK}*::axr3-1* cause ectopic *INO* expression

The straightened P_{INO}*::axr3-1* ovules had defects in growth of the outer integuments, and the radially symmetric P_{STK}*::axr3-1* ovules had both growth and patterning defects. To understand the basis for these phenotypes, we examined expression patterns of several genes that regulate ovule pattern formation in these transgenic plants using in situ hybridization. We also included P_{INO}*::iaa18-1* and P_{STK}*::iaa18-1* since both transgenic plants had identical phenotypes to those with misexpressed axr3-1. The symmetric ovules in both P_{INO}*::axr3-1/*P_{INO}*::iaa18-1* and P_{STK}*::axr3-1/*P_{STK}*::iaa18-1* were similar to *sup* ovules, whose phenotype was caused by expanded *INO* expression (Meister et al., 2002; Villanueva et al., 1999). We therefore checked *INO* expression in these two transgenic plants. In wild-type ovules, *INO* was restricted to the abaxial cell layer of the gynobasal outer integument (Fig. V-4A and B). As in *sup* mutant ovules, *INO* transcript in P_{INO}*::axr3-1/*P_{INO}*::iaa18-1* was expanded to the gynoapical outer integument (Fig. V-4C and D). Sometimes, *INO* transcript expanded into the upper funiculus, which mirrors the expression patterns of *axr3-1* in these transgenic plants. *INO* transcript was also expanded to the

gynoapical outer integument in P_{STK}*::axr3-1/*P_{STK}*::iaa18-1* ovules (Fig. V-4E and F). Furthermore, in multiple experiments *INO* transcript expanded further into the epidermis of most of the funiculus. These results suggested that the misexpressed *axr3-1* and *iaa18-1* in the funiculus induced *INO*. We also examined expression patterns of *ANT* and *PHB*, two other regulators of ovule pattern formation, but we did not see any difference in expression patterns between wild-type and transgenic plants (Fig. V-S2).

PIN1:GFP localization in wild-type and in P_{INO}*::axr3-1* and P_{STK}*::axr3-1* ovules

Previous reports have shown that the ARF-Aux/IAA pathway regulates PIN protein localization, which contributes to patterning (Sauer et al., 2006). In gain-of-function *aux/iaa* mutants, *PIN1* expression is often greatly reduced (Vieten et al., 2005; Weijers et al., 2006), suggesting that *PIN1* expression might be upregulated by ARF proteins.

To understand how auxin transport correlates to ovule formation, we examined PIN1 protein localization utilizing the *PIN1:GFP* reporter construct (Benkova et al., 2003). In wild-type ovules, *PIN1:GFP* expression was detected as early as ovule primordium emergence (Fig. V-5A). In stage 1 ovules, *PIN1:GFP* was present throughout the epidermis of the ovule primordium, and the orientation of PIN1 localization in epidermal cells pointed toward the tip of the primordium (Fig. V-5A). As in lateral organ primordia, *PIN1:GFP* was also present in the center of the ovule primordium, which marked the position of future vasculature (Benkova et al., 2003) (Fig. V-5A). *PIN1:GFP* in provascular cells was oriented toward the base of the

primordia. As the young ovule further differentiated into nucellus, chalaza and funiculus, *PIN1:GFP* became restricted to the epidermis of the nucellus and the central vasculature of the funiculus (Fig. V-5B). In early stage 2 ovules when the initiation and rapid growth of integuments occurred, *PIN1:GFP* was detected at the tip of inner integuments (Fig. V-5C) but disappeared in stage 3 ovules when the outer integument started to encase the ovule (Fig. V-5I). In stage 3 ovules, *PIN1:GFP* gradually diminished in the nucellus area but still remained prominent in the vasculature (Fig. V-5I).

In all P_{STK}*::axr3-1* ovules, *PIN1:GFP* expression and localization appeared similar to those in wild-type ovules as ovule emerged (Fig. V-5D). However, as the ovules grew further, the *PIN1:GFP* expression in the central vasculature disappeared (Fig. V-5E) compared to the bright expression in the same area in wild-type ovules (Fig. V-5B). Transient induction of *PIN1:GFP* in the inner integument was still observed in those straightened P_{STK}*::axr3-1* that developed integuments (Fig. V-5F), and sometimes weak *PIN1:GFP* signal could be seen in the position where vasculature should form in older ovules. *PIN1:GFP* was not detectable in those radially symmetric P_{STK}*::axr3-1* ovules in either the epidermis of the nucellus ot the vasculature of the funiculus (Fig. V-5G).

PIN1:GFP expression and localization in P_{INO}*::axr3-1* ovules did not differ from wild-type ovules during early ovule development. However, persistent *PIN1:GFP* expression was detected in the inner integument of stage 3 P_{INO}*::axr3-1* ovules whereas *PIN1:GFP* was not detectable in wild-type ovule inner integument at this stage (Fig. V-5H and I).

ARF6, *NPH4/ARF7*, *ARF8* and *ARF19* do not regulate ovule growth or morphology

Q-rich ARFs regulate growth of stem and flower organs, and might also contribute to ovule growth. To test this idea and to identify ARF proteins that might be affected in the P_{INO}*::axr3-1* and in the P_{STK}*::axr3-1* transgenic plants, we examined ovules of mutants deficient in Q-rich ARFs.

Previously we found that *ARF6* and *ARF8* were expressed in the funuculus (Wu et al., 2006), but *arf6-2 arf8-3* double mutant ovules were morphologically normal. To reveal if *ARF3* and *ARF4* could act in the same pathway as *ARF6* and *ARF8*, we examined ovules of an *ett/arf3 arf4 arf6 arf8* quadruple mutant. We found that loss of *arf6* and *arf8* did not enhance the *ett/arf3* ovule phenotypes (Fig. V-S3), suggesting that *ARF3* does not interact with *ARF6* and *ARF8* in regulating ovule development.

Another possible reason why *arf6 arf8* double mutant plants did not have an ovule phenotype might be that *ARF6* and *ARF8* act redundantly with other *ARF* genes to regulate ovule development. To address this, we constructed an *arf6 nph4/arf7 arf8 arf19* quadruple mutant lacking four Q-rich ARF genes. Despite severely affected vegetative growth and arrested floral buds at stage 12, the quadruple mutant ovules appeared normal (Fig. V-S3). These results showed that these *ARF* genes do not regulate growth of the ovules. Rather, *ARF6* and *ARF8* regulate pollen tube guidance (see chapter IV).

MP/ARF5 regulates organ formation and patterning at several stages, and was also expressed in ovules. *MP/ARF5* expression was detected in the placental tissues before ovule primordia initiated (Fig. V-2G). In young ovules, *MP/ARF5* was expressed in the center of the funiculus where the vascular tissue would form (Fig. V-2H). After the integuments initiated, *MP/ARF5* transcript was detected in the inner integument and in the center of the funiculus (Fig. V-2I). As the ovule matured, *MP/ARF5* transcript accumulated in the chalazal part of the ovule, and was still present in the inner integument and in the developing vascular tissue (Fig. V-2J).

Discussion

Phenotypic studies of *arf* mutant ovules suggest that multiple *ARF* genes have various or even opposite functions during the formation of the ovule. *arf2* mutant has larger ovules due to extra cell division in both inner and outer integuments (Schruff et al., 2005). Similarly, expanded *ARF6* and *ARF8* expression into the integuments caused by loss of *miR167* regulation inhibits outer integument growth (Wu et al., 2006). These results suggest that *ARF2* restricts ovule integument growth and that *ARF6* and *ARF8* might have similar potential as *ARF2*. In contrast, *ett/arf3* mutant ovules are growth arrested and smaller than wild-type ovules. This observation suggests that in inner integument, where *ETT/ARF3* is expressed, can limit growth of the outer integument. Failure of *arf6 arf8* mutations to rectify *ett/arf3* ovule phenotypes suggests that *ETT/ARF3* does not act in restricting *ARF6* and *ARF8* activities in the ovules. Previous studies have suggested that *ETT/ARF3* interacts

with *KAN1* and *KAN2* to specify lateral organ abaxial fate, and *ett/arf3 arf4* and *kan1 kan2* share similar rosette phenotypes (Pekker et al., 2005). However, unlike *kan1 kan2* mutant ovules which lack lamina expansion in outer integument, *ett/arf3 arf4* ovules do not have such phenotypes. This suggests that *ETT/ARF3* and *KAN* genes might act in different pathways in the ovule.

Currently, only *arf2* and *ett/arf3* mutants have ovule phenotypes. However, the distinct ovules expression patterns of *MP/ARF5*, *ARF6*, *NPH4/ARF7* and *ARF8* suggest that these proteins might also regulate ovule development (Hardtke et al., 2004; Wu et al., 2006). MP/ARF5, ARF6, NPH4/ARF7, ARF8, and ARF19 all have a Q-rich middle region and form a clade on the phylogenetic tree (Remington et al., 2004). Activities of these Q-rich ARF proteins could therefore be masked by their functional redundancy. However, the normal ovules of *arf6-2 nph4-1/arf7 arf8-3 arf19-2* show that we might need to add the *mp/arf5* mutation to see any defect. *MP/ARF5* participates in multiple developmental pathways, and strong *mp/arf5* mutants do not reach adulthood (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Hardtke et al., 2004; Przemeck et al., 1996). Some *mp/arf5* mutants with weaker alleles do develop flowers, but the gynoecia are often valve-less and empty, suggesting that the early placenta expression of *MP/ARF5* might be important for ovule initiation. However, roles of *MP/ARF5* in the inner integument and in the funiculus remain to be identified. Since *mp/arf5* mutants often lack lateral organs, *MP/ARF5* may be required for vascular differentiation and integument outgrowth.

The straightened ovule phenotype of P_{INO}*::axr3-1* suggests that active ARF proteins in the outer integument are required for the asymmetric integument growth.

The extra outer integument growth is opposite to *miR167*-resistant *mARF6* or *mARF8* transgenic plant ovules, which have arrested outer integuments (Wu et al., 2006). These results suggest that excessive auxin signaling in the outer integument can inhibit growth, whereas reduced/silenced auxin signaling can promote ectopic gynoapical outer integument growth. However, we have not identified *ARF* genes that are expressed in the outer integument; therefore it is also possible that *axr3-1* can act independently of the ARF proteins. Motif I of Aux/IAA proteins has transcription repression activity (Tiwari et al., 2004), and Aux/IAA proteins recruit chromatin remodeling complexes (Fukaki et al., 2006). These observations suggest that gain-of-function *aux/iaa* protein might be capable of repressing gene transcription without the presence of ARF proteins.

Unlike P_{INO}*::GUS* plants in which GUS expression is only detected in the gynobasal side of the ovule, in P_{INO}*::axr3-1* ovules ectopic *axr3-1* expression was detected in the gynoapical side of the ovule. Increased *INO* expression in the gynoapical side of P_{INO}*::axr3-1* ovules suggests that *axr3-1* might activate *INO*, and this activation can bypass the inhibitory effects of *SUP* on *INO* (Meister et al., 2002). The transgene would thus create a positive feedback of *INO* on its own transcription, which then results in ectopic *INO* expression and gynoapical outer integument growth. Previous reports have shown that *INO* transcript is required for upregulating of the *INO* promoter (Meister et al., 2002; Villanueva et al., 1999). The natural positive feedback of *INO* expression might also include auxin response components.

P_{INO}*::axr3-1* starts to deviate from the wild-type development around the onset of stage 3 when the outer integument starts to envelop the ovule. This phenotype might

be caused by persistent PIN1 expression in the inner integument in stage 3 ovule when PIN1 expression is no longer detectable in the wild-type ovule. Since PIN1 is never expressed in the outer integument, *axr3-1* in the outer integument might therefore affect PIN1 in the inner integument non-cell-autonomously. The persistent PIN1 expression in the inner integument could then send back signals affecting growth of the outer integument. This also indicates that diminishing PIN1 signal and hence auxin accumulation in the inner integument in stage 3 ovules regulates growth of the outer integument. A similar phenomenon has been observed in the embryo. Functional *MP/ARF5-BDL/IAA12* pathway in the proembryo is required to produce a signal in apical domain cells, which then activate hypophysis differentiation in the basal cell lineage (Weijers et al., 2006).

Misexpressing *axr3-1* in the funiculus causes the ovule to grow symmetrically. In more severely affected cases, the ovule was completely radialized and lacked famale gametophyte and vasculature. This suggests that disrupting the ARF-Aux/IAA pathway in the funiculus can affect differentiation in the distal parts of the ovule. Similarly, *axr3-1* in the funiculus could affect the funiculus-specific ARF protein transcriptional activity, or it may act in an ARF-independent manner. The known *ARF* genes that are expressed specifically in the funiculus are *ARF2*, *MP/ARF5*, *ARF6* and *ARF8*. The morphologically normal ovules in *arf6 arf8* mutant flowers suggest that other *ARF* genes might act redundantly with *ARF6* and *ARF8* in the funiculus.

MP/ARF5 transcript distribution patterns overlap with those of *PIN1:GFP* localization in the funiculus and in the inner integument, and also overlap in the

embryo (Hardtke and Berleth, 1998; Steinmann et al., 1999), suggesting that *MP/ARF5* could be a general regulator of *PIN1* expression. However, the fact that *mp/arf5* embryo has wild-type PIN1 localization (Steinmann et al., 1999) and PIN1 protein is still polarly localized in *mp/arf5* inflorescences (Reinhardt et al., 2003) implies that *MP/ARF5* is not the sole factor controlling PIN1 expression. Still, proper PIN1 function is dependent on the ARF-Aux/IAA pathway. In gain-of-function *axr3-1/iaa17* and *slr/iaa14* roots, PIN1 expression is compromised (Vieten et al., 2005). PIN1 relocalization at the site of lateral root formation also does not occur in *shy2-2/iaa3* roots (Sauer et al., 2006). Furthermore, PIN1 expression is absent in *bdl/iaa12* embryos, and is absent in the area where *bdl/iaa12* is misexpressed (Weijers et al., 2006). In P_{STK}*::axr3-1* ovule, *PIN1:GFP* expression is either absent or diminished in the funiculus where the *axr3-1* gene is misexpressed. In wild-type ovules, PIN1 is localized at the basal end of the provascular cells in the funiculus and therefore probably drains auxin away from the developing nucellus. Absence of PIN1 expression in the funiculus might cause auxin to accumulate in the apical part of developing ovule, which could then result in the symmetric integument growth of some of the P_{STK}*::axr3-1* ovules. *PIN1:GFP* expression is not detectable in radialized P_{STK}*::axr3-1* ovules. These ovules might have higher *axr3-1* expression, and such high level of *axr3-1* could inhibit PIN1 expression in young ovule primordia.

INO expression in both P_{INO}*::axr3-1* and P_{STK}*::axr3-1* ovules is expanded to both sides of the ovule. In addition, the expression is further expanded into the funiculus in P_{INO}*::axr3-1* and P_{STK}*::axr3-1* ovules. In P_{INO}*::axr3-1* ovules, *axr3-1* might directly induce *INO* transcription in the gynoapical outer integument. In contrast, the

effects might be indirect in P_{STK}*::axr3-1* ovules since the *axr3-1* transcript is only present in the funiculus. During flower primordium development in the inflorescence meristem, *PIN1* expression marks the boundaries of the adaxial and abaxial domain (Heisler et al., 2005). The same could be true in the ovule. In P_{STK}*::axr3-1* ovules, PIN1 expression is absent in the funiculus area, which might disrupt the boundaries among domains of various parts of the ovule. However, expression of *PHB*, the adaxial marker, remains the same as in wild-type ovules. It is possible that the ARF-Aux/IAA pathway regulates organ polarity only by defining the expression of the abaxial marker genes, but studies in other organs are needed to confirm this point.

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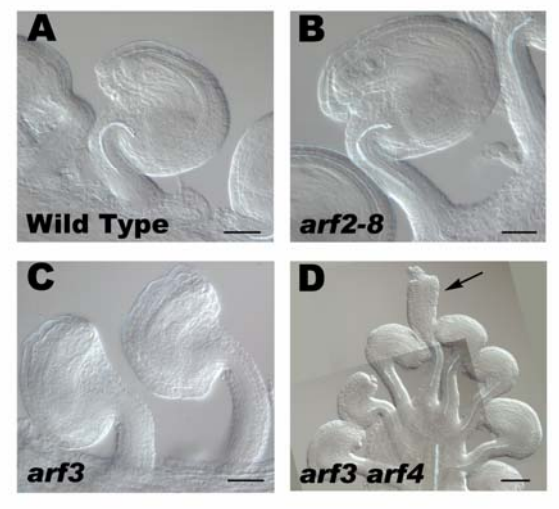

Figure V-1. Wild-type and *arf* mutant ovules at floral stage 13.

(A) Wild-type. (B) *arf2-8*. (C) *arf3*. (D) *arf3 arf4*. Arrow in (D) indicates the radially symmetric ovule at the top of the gynoecium. Scale bars: (A-C) 30 μm; (D) 60 μm.

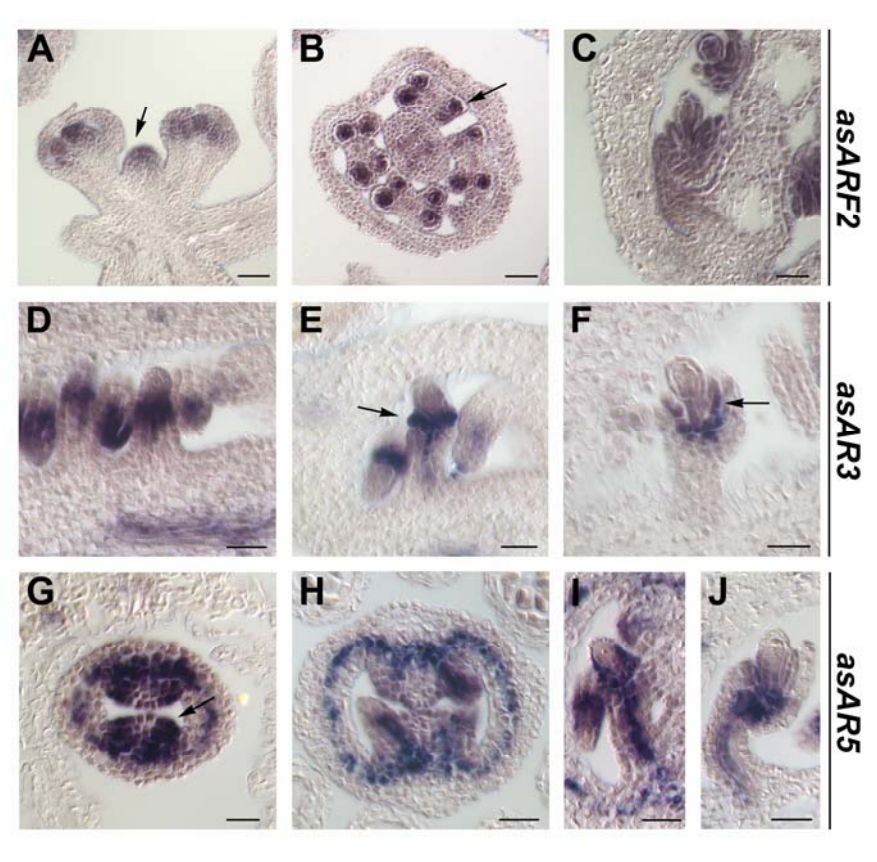

Figure V-2. Expression patterns of *ARF2*, *ARF3* and *ARF5* in different stages of flowers and ovules.

Sections of wild-type flowers were hybridized with antisense *ARF2* (A-C), *ARF3* (D-E) and *ARF5* (G-J) probes.

(A) Longitudinal section of inflorescence. Arrow indicates the inflorescence meristem. (B) Cross section of a stage 8 flower. Arrow indicates the anther primordium. (C) Stage 3-I ovule. (D) Stage 2-I ovule. (E) Stage 2-III ovule. (F) Stage 3-I ovule. Arrows in (E) and (F) indicate the inner integument. (G) Cross section of a stage 9 gynoecium. Arrow indicates the placenta. (H) Stage 1-II ovule. (I) Stage 2-III ovule. (J) Stage 3-I ovule. Scale bars: (A) 30 μm; (B-J) 15 μm.

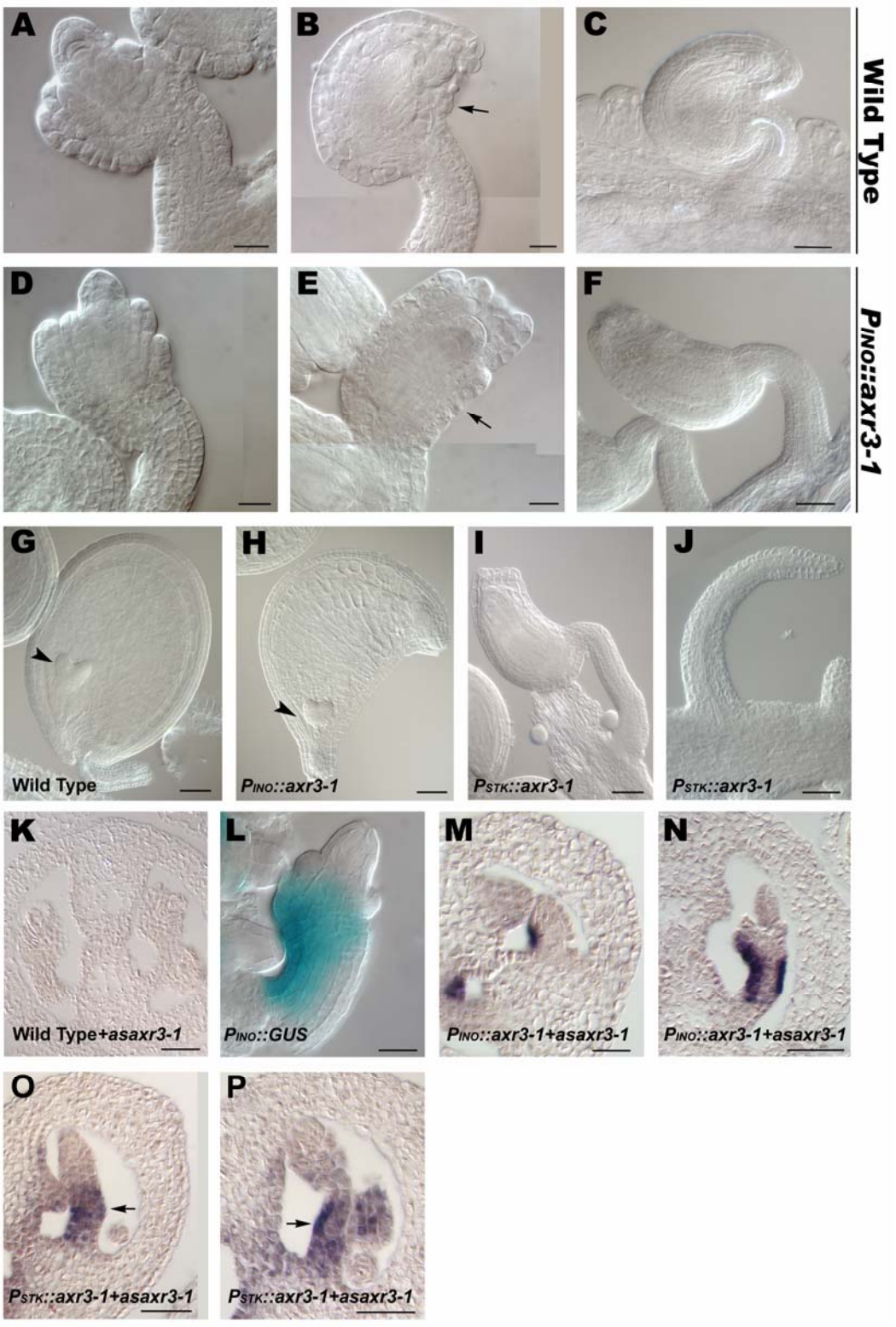

Figure V-3. Ovules of transgenic plants misexpressing *axr3-1*, and *axr3-1* expression patterns in these ovules.

(A-C, G, K) Wild-type ovules. (D-F, H, M) *P_{INO}::axr3-1* ovules. (I, J, N) *P_{STK}::axr3-1* ovules. In each panel, the gynobasal side of the ovule is at left. (A, D) Stage 3-I ovules. (B, E) Stage 3-II ovules. Arrows indicate the gynoapical outer integument. (C, F) Stage 4-I ovules. (G, H) Fertilized ovules with heart stage embryos. Arrowheads indicate the developing embryos. (I, J) Stage 4-I ovules. Class I (I) and class II (J) *P_{STK}::axr3-1* ovules.

(K) Cross section of a wild-type gynoecium hybridized with an antisense axr3-1 probe. (L) X-gluc staining pattering of *P_{INO}::GUS* in a stage 2-III ovule. (M, N, O, P) *P_{INO}::axr3-1* (M, N) and *P_{STK}::axr3-1* (O, P) ovules hybridized with antisense *axr3-1* probes. Arrows in (O, P) indicate the funiculus. Scale bars: (A, B, D, E, L) 12 µm. (C, F, G-K, N-P) 30 µm. M 15 µm.

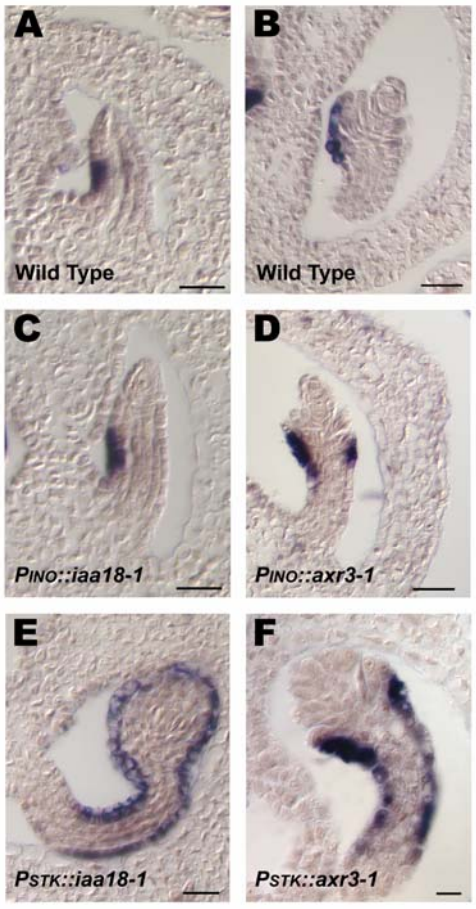

Figure V-4. *INO* expression patterns in wild-type, P_{INO}*::axr3-1*, P_{INO}*::iaa18-1*, P_{STK}*::axr3-1*, and P_{STK}*::iaa18-1* ovules.

(A, B) Wild-type stage 1-II (A) and 3-I (B) ovules. (C, D) P_{INO}*::iaa18-1* stage 1-II (C) and P_{INO}*::axr3-1* stage 2-V (D) ovules. (E, F) P_{STK}*::iaa18-1* stage 3-I (F) and P_{STK}*::axr3-1* stage 2-V (F) ovules. The gynobasal side of the ovule is at left. Scale bars: 12 μm.

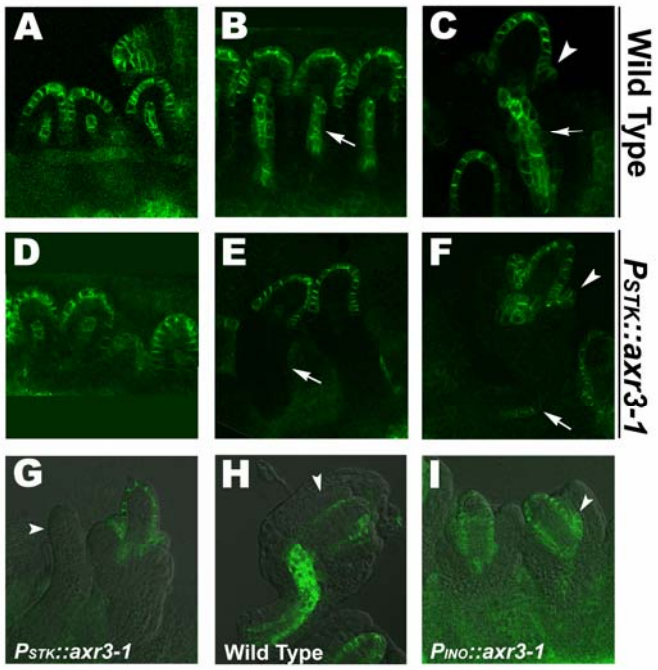

Figure V-5. *PIN1-GFP* patterns in wild-type, P_{INO}*::axr3-1*, and P_{STK}*::axr3-1* ovules. (A, D) Stage 1-II wild-type (A) and P_{STK}*::axr3-1* (D) ovules. (B, E) Stage 2-II wild-type (B) and P_{STK}*::axr3-1* (E) ovules. (C, F) Stage 3-I wild-type (C) and P_{STK}*::axr3-1* (F) ovules. Arrows in (B, C, E, F) indicate the vasculature in the funiculus. Arrowheads in (C, F) indicate the inner integument. (G) Stage 3-I P_{STK}*::axr3-1* ovules. Arrowhead in (G) indicates a radially symmetric ovule. (H, I) Wild-type (H) and P_{INO}*::axr3-1* (I) stage 3-II ovules. Arrowheads in (H, I) indicate the inner integument.

Supplementary Figures

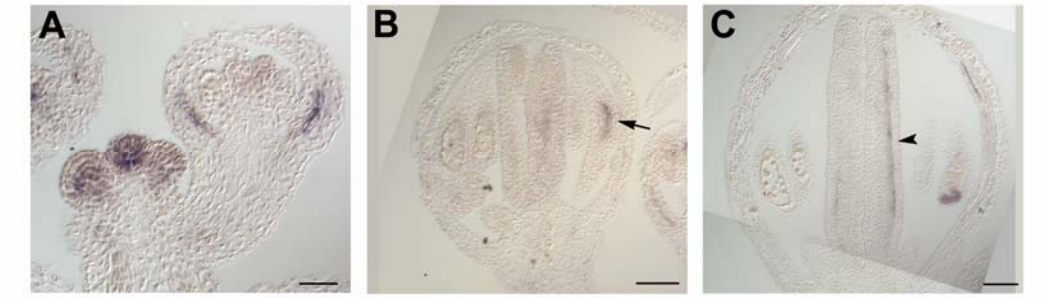

Figure V-S1. Expression patterns of *ARF4* in flowers.

(A) A longitudinal section of the inflorescence meristem and a stage 6 flower. (B) A longitudinal section of a stage 8 flower. Arrow indicates the anther vasculature. (C) A longitudinal section of a stage 9 flower. Arrowhead indicates the gynoecial vasculature. Scale bars: 60 μm.

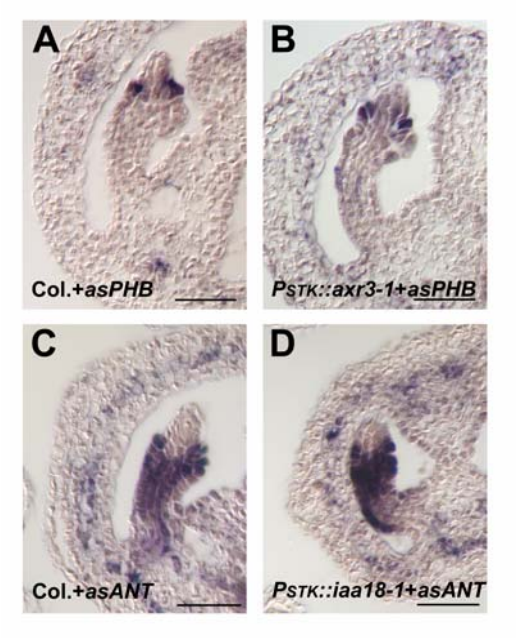

Figure V-S2. Expression patterns of *ANT* and *PHB* in wild-type and P_{STK}*::axr3-1* ovules.

(A, B) Wild-type (A) and P_{STK}*::axr3-1* (B) stage 2-V ovules hybridized with antisense PHB probes. (C, D) Wild-type (C) and P_{STK}*::iaa18-1* (D) stage 2-V ovules hybridized with antisense ANT probes. Scale bars: 30 μm.

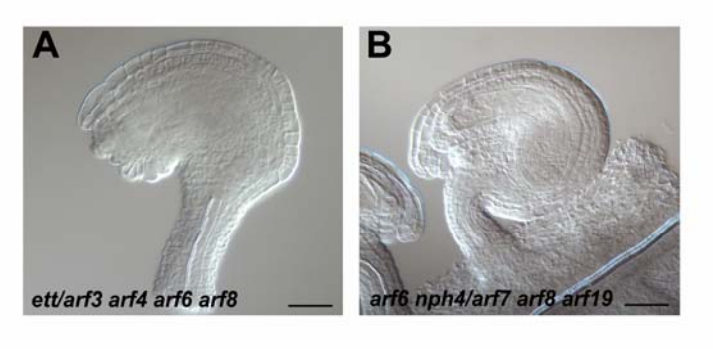

Figure V-S3. *ett/arf3 arf4 arf6 arf8* (A) and *arf6 nph4/arf7 arf8 arf19* (B) mutant ovules. Scale bars: 30 μm.

CHAPTER VI

Conclusions

Roles of *microRNA167*

When I started working in the Reed lab in the summer of 2003, we only knew that *ARF6* and *ARF8* could be regulated by one of the plant miRNAs, *miR167*, based on sequence complementarity. Back then, the plant microRNAs and their targets were just predicted by computational methods (Jones, 2002; Rhoades et al., 2002). We had limited information on how miRNAs worked in vivo and how miRNAs regulated development. Over the years, work from other researchers has gradually revealed how miRNAs regulate their targets by promoting transcript turnover and how plant miRNAs can actually participate in various developmental pathways. Two papers published in 2004 showed that *miR165/166* excluded adaxial cell fate determining genes, *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), from the abaxial domains in leaf primordia (Juarez et al., 2004; Kidner and Martienssen, 2004). It was also shown that silent mutations in miRNA target sites in *PHB* or in *REVOLUTA* (*REV*), another adaxial cell fate determinant gene, caused their transcripts to expand into the abaxial domains in leaf primordia (Emery et al., 2003; Sieber et al., 2004). We used similar approaches, i.e. introducing silent mutations into the *miR167* target sites in *ARF6* and *ARF8* coding sequences, and studied the phenotypic and molecular

outcomes. We first validated that *miR167* can target *ARF6* and *ARF8* in vivo by overexpressing *MIR167* precursor genes, and we found that one of the four *MIR167* precursors, *MIR167a*, had the strongest effect on *ARF6* and *ARF8* transcript levels. Later, the in vivo *ARF6* and *ARF8* transcript cleavage by *miR167* was shown by other labs (Axtell and Bartel, 2005; Schwab et al., 2005), which further confirmed the regulatory roles of *miR167* on *ARF6* and *ARF8*.

Our studies on *miR167* and its regulation on *ARF6* and *ARF8* revealed roles of *miR167* during reproductive organ formation. Expanded expression of *miR167*-insensitive *mARF6* or *mARF8* into the ovule and the anther caused arrested growth of the outer integument and aberrant anther development which led to anther indehiscence. We used promoter reporter fusions to study *miR167* expression patterns, and these revealed that *miR167* precursor was expressed in cells where *miR167* regulates *ARF6* and *ARF8*. The complementary expression between *miR167* and *ARF6/ARF8* showed that *miR167* is essential for the correct transcript distribution of *ARF6* and *ARF8* in ovules and anthers. Similarly to other plant miRNA, such as *miR165/166* and *miR164*, *miR167* patterns *ARF6* and *ARF8* transcript distribution and this patterning function is important for ovule and anther development.

Even though we have shown how *miR167* defines *ARF6* and *ARF8* expression patterns, we know very little about how *miR167* expression is regulated. The arrested outer integument of *mARF6* and *mARF8* ovules were similar to those of *phb-1d/+* and of *kan1 kan2* mutant ovules (Eshed et al., 2001; Sieber et al., 2004), which suggested that *mARF6* and *mARF8* ovules might have acquired ectopic adaxial cell fate. We therefore asked whether *mARF6* and *mARF8* flower phenotypes were caused by

ectopic *PHB* expression. We found that *PHB* expression patterns were the same as those in wild-type in *mARF6* or in *mARF8* flowers. In addition, transforming mARF6 into *phb-6 phv-5* mutant plants still caused those phenotypes we observed in the wild-type background. The arrested outer integument was not caused by reduced *INO* expression, either. *INO* expression patterns were not altered in *mARF6* or *mARF8* ovules, and *ARF6* was not expanded in *ino-1* mutant ovules. We next went on to ask whether other abaxial determining genes, including *KAN1* and *KAN2*, could regulate *miR167* expression. We introduced $P_{MIR167a}$*::GUS* into *kan1 kan2* mutant background and failed to see any change in the X-gluc staining patterns of the reporter. These results suggested that *miR167* and *ARF6/ARF8* might not interact directly with the known abaxial and adaxial determining genes, but the phenotypes of *mARF6* and *mARF8* flowers indicate that *miR167* regulates organ patterning. Further efforts, such as studying the expression profiles of *mARF6* or *mARF8* flowers, are needed to reveal what genes might be responsible for the phenotypes we observed.

Recent unpublished data suggest that *miR167* might regulate quantitative responses to external signals in addition to its role in patterning. Nitrogen induces *miR167* in root pericycle, and this appears to affect lateral root growth (M. Gifford and G. Cornzzi, personal communication). In addition, pathogen induces *miR167* expression, suggesting that limiting auxin responses is part of plant immunity. To understand how *miR167* acts in these pathways, we could examine expression patterns of the *miR167*, *ARF6* and *ARF8* reporter constructs, or analyze expression profiles of *mARF6* and *mARF8* plants under different environmental/growth conditions.

Auxin Response Factors and Ovule Development

The roles of auxin in plant organ initiation have been studied in detail in recent years. One of the earliest indications that auxin promotes organ initiation came from loss-of-function *pin* mutants. *pin* mutant inflorescences did not form any organ, resembling the phenotypes of polar auxin transport inhibitor NPA-treated inflorescences (Gälweiler et al., 1998). Later works showed that the localization of PIN1 proteins predicted the patterns of organ primordia initiation (Heisler et al., 2005; Reinhardt et al., 2003). Studies have shown that *PIN* gene expression is regulated by *ARF*-mediated auxin signaling (Blilou et al., 2005; Sauer et al., 2006; Vieten et al., 2005). *ARF* genes therefore could be involved in auxin-induced organ initiation and development. Many studies have reported that *ARF* genes can regulate organogenesis. For example, *MP/ARF5* regulates the formation of the embryonic root and vascular tissue development (Hardtke and Berleth, 1998; Weijers et al., 2006). Furthermore, adding other *arf* mutations to *mp/arf5* mutants aggravated the *mp/arf5* embryonic phenotypes (Hardtke et al., 2004; Sara Ploense, unpublished data). Similarly, *ARF* genes and hence auxin signaling might affect ovule formation, possibly using the same mechanisms as those during embryogenesis.

The distinct expression patterns of *ARF2*, *ETT/ARF3* and *MP/ARF5* in the ovule showed that these genes could be such candidates. *ARF2* inhibits cell growth and division of the outer integument (Schruff et al., 2005), and *ETT/ARF3* seems to promote growth and maturation of the ovule. Nevertheless, we still know very little about how other *ARF* genes, including *MP/ARF5*, regulate ovule formation. To

inhibit multiple ARF protein activity in the ovule, we misexpressed a gain-of-function *aux/iaa* gene, *axr3-1*, in the outer integument and in the funiculus of the ovule. The ovule phenotypes of these transgenic plants suggested that multiple *ARF* genes might regulate the outer integument growth in the outer integument and ovule differentiation in the funiculus. To have a clearer picture on this scenario, we will need to identify the ARF proteins that were inhibited by the misexpressed *axr3-1*. Cell sorting techniques using fluorescent reporter lines and the expression profile analyses on the sorted cells have been conducted successfully in the root (Birnbaum et al., 2005). We could utilize outer integument-specific or funiculus-specific fluorescent reporters to isolate the outer integument or the funiculus cells and study their expression profiles. Similar methods could also apply to the transgenic plants with misexpressed *axr3-1* to find out the genes that caused the ovule phenotypes. Currently, we have only identified that *INO* was expanded in the transgenic plant ovules, but we are still unclear on what caused *INO* to be ectopically expressed. Thus, identifying the expression profiles of these transgenic plants may help us answering this question.

Another interesting thing we found out in these set of experiments is how altered auxin signaling affected ovule outer integument symmetry. In *mAR6* or *mARF8* ovules, the ectopic *mARF6* and *mARF8* expression into the outer integument might cause excessive auxin signaling, and this excessive auxin signaling arrested outer integument growth. On the contrary, expression of *axr3-1* in the outer integument should cause reduced auxin signaling, and this reduction of auxin signaling caused excessive and symmetric outer integument growth. In other reports, it has been

suggested that arrested outer integument growth indicates loss of abaxial cell fate, whereas symmetric outer integument growth reflects expanded abaxial cell fate (Villanueva et al., 1999). Our results might suggest that auxin signaling could be a determining factor of organ polarity. In fact, *ETT/ARF3* and *ARF4* interact with *KAN* genes to determine abaxial cell fate in the leaf (Pekker et al., 2005), and similar mechanisms could also be true in the ovule. Auxin might promote organ primordia imitation as well as organ patterning, and elements in the auxin signaling pathway may even interact with genes determining organ identity. Sorting out these potential interactions is another interesting direction for future studies.

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