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REGULATORY NETWORK CONTROLLING OVULE DEVELOPMENT IN *Arabidopsis thaliana*

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It is hard to tell
that the world we live in
is either a reality or a dream

3-Iron
Kim Ki-duk

Just have patience, persistence
and good luck

adapted from Richard Amasino

Part I

ABSTRACT

In flowering plants ovules develop as lateral organ from the placenta and, once fertilized, give rise to seeds. My Ph.D. was mainly focused on the identification of the mechanisms controlling ovule number and development in *Arabidopsis thaliana*.

AINTEGUMENTA (ANT), CUP-SHAPED COTYLEDON 1 (*CUC1*) and *CUC2* are involved in ovule primordia initiation, since *ant* single and *cuc1 cuc2* double mutant produce fewer ovule compared to wild type. Through the contemporary silencing of *ANT*, *CUC1* and *CUC2* we showed that these genes have additive roles in the determination of ovule number.

Among all the developmental processes in which hormones are involved, their role in ovule primordia formation have been proposed for auxin and cytokinins.

We show that *CUC1* and *CUC2* are required for correct auxin transport and that they, together with *ANT* are direct target of MP, a member of AUXIN RESPONSIVE FACTOR (ARF) family. Interestingly we saw that cytokinin treatments restore ovule number defects in our mutants increasing the expression of *PINFORMED1* (*PIN1),* an auxin efflux carrier.

Once the primordium is formed, the three MADS box transcription factors SEEDSTICK (STK), SHATTERPROOF1 (SHP1), and SHP2 redundantly regulate ovule development and, together with the SEPALLATA proteins determine ovule identity. I have contributed to the characterization of VERDANDI (VDD), the first direct target of the Arabidopsis ovule identity complex.

STATE OF THE ART

Ovule development in *Arabidopsis thaliana*

The evolutionary success that Spermatophyte had during the second half of the Cretaceous Period, when they began to dominate temperate lands, is linked to their capability in developing different reproductive strategies. Undoubtedly, the most successful one is the production of seeds that allowed plants to colonize rapidly the Earth. Seeds develop from the ovules that are the place where fertilization process takes place.

In *Arabidopsis thaliana*, the gynoecium is composed of two carpels fused along their margin forming the pistil and ovules develop as small finger like primordia that emerge from the sub-epidermal meristematic tissue of the carpel, named placenta (J. Bowman, 1993).

Ovule development consists mainly of three different stages: primordia formation, megasporogenesis and megagametogenesis (Schneitz et al., 1995). Ovule primordia develop as result of periclinal divisions of the placenta ‘s cells. The primordium differentiates in three zones: the funiculus, that connects the ovule to the placenta, the chalaza, from which the integuments are formed and the nucellus, the tissue in which the Megaspore Mather Cell (MMC) differentiates forming the embryo sac (Figure. 1) (Schneitz et al, 1995).

The plant life cycle is characterized by the alternation of the aploid (gametofitic) and diploid (sporofitic) generations. In ovules, the two generations coexist. In fact, integuments and funiculus, which are sporofitic tissues are formed synchronously with the embryo sac, that represents the gametophyte.

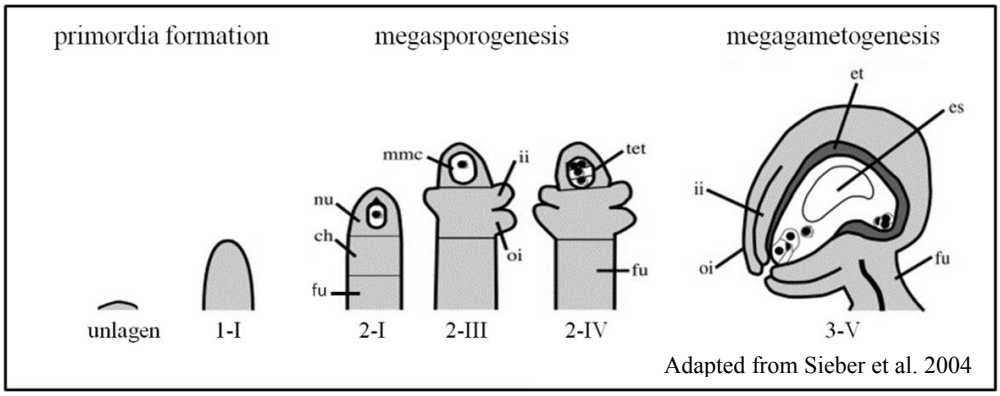

Figure. 1 Schematic representation of *Arabidopsis thaliana* ovule development.
Ovule developmental stages according to Schneitz et al., 1995. Gametophytic tissue is represented in white, sporophytic tissue in gray. Abbreviations: ad, adaxial; ab, abaxial; nu, nucellus; ch, chalaza; fu, funiculus; mmc, megaspore mother cell; ii, inner integument; oi, outer integument; tet, tetrad; et, endothelium; es, embryo sac.

At stage 2-I of ovule development, the sub-epidermal cell layer of the nucellus differentiates into the MMC, containing a large nucleus and a big vacuole, that make it distinguished from the other nucellus cells. At the same stage, the chalazal cells undergo periclinal divisions, starting integuments formation that develop asynchronously. In particular, the inner integument starts to be formed at stage 2-II, whereas the outer integument initiates at stage 2-III. The outer integument grow faster covering the inner integument and the nucellus.

During stage 2 of ovule development, megasporogenesis takes place. The MMC undergoes meiosis forming a tetrad of haploid megaspores. The three megaspores at the micropylar end of the nucellus degenerate and only the most distal one becomes the Functional Megaspore (FM), also known as FG1 embryo sac, and proceeds to megagametogenesis (Schneitz et al., 1995, Drews and Yadegari 2002).

Stage 3 of ovule development starts with the megagametogenesis, consisting of three rounds of mitosis of the FM that consequently produces an eight-cells embryo-sac (Figures 1, 2). In particular, the first mitosis produces a two-nucleate (FG2) embryo sac with a big vacuole that separates the two nuclei. The second mitosis leads to the formation of a four-nucleate female gametophyte (FG4). During this stage, the endothelium is formed from the inner layer of the inner integument. (Christensen et al. 1997; Schneitz et al., 1995). The third mitosis produces a FG5 eight-nucleated female gametophyte with four nuclei at each pole and it is characterized by two events: the migration of one nucleus from each pole (polar nuclei) to the central region of the embryo sac and the beginning of the embryo sac cellularization (Christensen et al. 1997). At FG6 stage polar nuclei fuse together and cellularization is completed giving rise to a mature female gametophyte composed of seven cells: one egg cell, two synergids, a bi-nucleated central cell and three antipodals (Figure 2, 3).

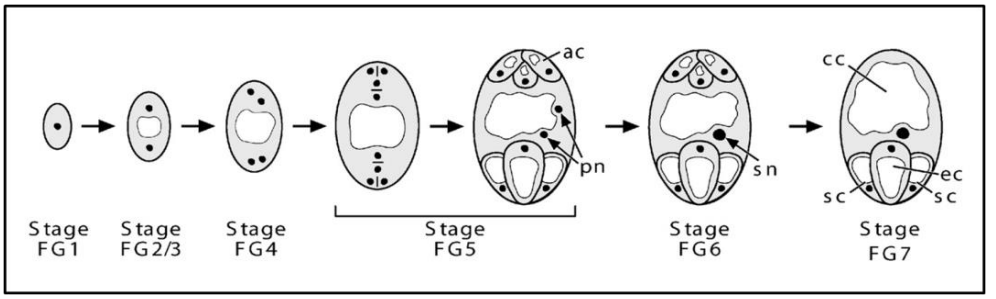

Figure 2 Description of megagametogenesis in Arabidopsis.

Ovule developmental stages as described in Drews and Yadegari, 2002. Citoplasmatic areas are represented in gray, vacuoles in white and nuclei in black. In FG5, black lines between the nuclei represent partial cell walls. Abbreviations: ac, antipodal cells; cc, central cell; ec, egg cell; pn, polar nuclei; sc, synergid cell; sn, secondary nucleus (fused polar nuclei).

Female gametophyte cell specification

The mature female gametophyte is composed of two synergids and one egg cell, located close to the micropyle, three antipodal cells located at the opposite chalazal pole, and a central cell in the middle of the gametophyte (Figure 3). After both the female and male gametophytes are matured, fertilization process takes place and a new sporophytic generation develops. In flowering plants, the reproductive process has been implicated in their evolutionary success (Friedman 2006) and it is considered a double fertilization because one of the two sperm cell fertilizes the egg cell giving rise to the zygote (first fertilization), while the other fuses with the central cell originating the first triploid cell of the endosperm (second fertilization). The mechanisms of differentiation of the female gametophyte cells are under tight control and cell specification is fundamental for reproductive success (Sprunck and Groß-Hardt, 2011).

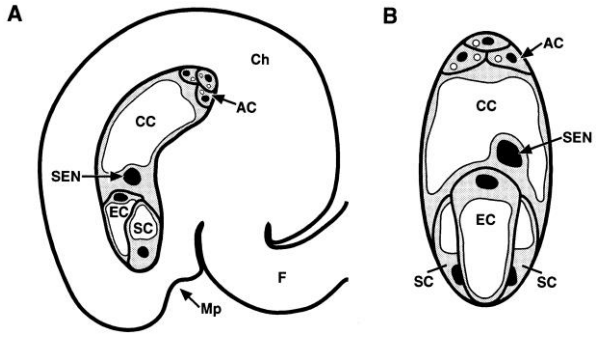

Figure 3 Female Gametophyte of Arabidopsis (Drews et al., 1998)

Female gametophyte, longitudinal (A) and perpendicular section. Cytoplasm is indicated in gray, vacuoles in white and nuclei in black. AC, antipodal cells; CC, central cell; Ch, chalazal region; EC, egg cell; F, funiculus; Mp, micropyle; SC, synergid cell; SEN, secondary endosperm nucleus.

The correct nuclei position of the female gametophyte cells, and in particular the opposite nuclei polarization of the central and egg cells are important for the male nuclei fusion and the following fertilization process (Berger et al. 2008; Sprunck, 2010).

The analysis of some mutants revealed a correlation between cell fate and the position of the single cell nuclei inside the female gametophyte (GrossHardt et al. 2007; Moll et al. 2008; Pagnussat et al. 2007). Among the nuclei present in the micropylar zone, the one that is most chalazally positioned always becomes the egg cell nucleus after cellularization (Russell 1993). This have been also confirmed in maize through the analysis of the *retinoblastoma-related 1* (*rbr1*) mutant. The lack of *RBR1* leads to the nuclei overproduction, but they always differentiate according to their position inside the female gametophyte (Ebel et al. 2004; Evans, 2007). This phenomenon is particularly evident in *eostre*, *lachesis* (*lis*), *clotho* (*clo*), and *atropos* (*ato*) mutants in which the number of FG nuclei is normal, but mis-specified (GrossHardt et al. 2007; Moll et al. 2008; Pagnussat et al. 2007). In particular, the *oestre* mutant shows nuclear migration abnormalities and two functional egg cells due to the fact that one of the synergids loses its identity (Pagnussat et al. 2007). In *lis*, *clo*, and *ato* mutants, synergids and central cell can differentiate into the egg cell, and antipodals can adopt central cell fate, indicating that all the FG cells can differentiate into gametes, thus a developmental plasticity of the female gametophyte itself (Groß-Hardt et al. 2007; Moll et al. 2008; Sprunck and Groß-Hardt, 2011).

Additionally, it has been discovered that LIS, CLO and ATO are implicated in spliceosome formation, suggesting a connection between female gametophyte cell specification and pre-mRNA splicing (Moll et al. 2008).

Genes involved in ovule number determination

In the last decades, the study of the mutants with defects in ovule development processes have been used as a successful approach to understand ovule development in *Arabidopsis thaliana*.

Aintegumenta (*ant*) mutant has been isolated as a female sterile mutant of *Arabidopsis thaliana* (Elliott et al., 1996). *ANT* encodes a AP2-like transcription factor mainly involved in growth control of lateral organs through the regulation of cell proliferation (Mizukami et al., 2000). *ANT* is expressed in almost all organ primordia, in the newly arising vasculature and, in some cases, its expression appears before the primordial arise, confirming its role in controlling organ initiation (Elliott et al., 1996). In *ant* mutants are produced half of the ovules respect to wild type and they are completely sterile (Baker et al., 1997). This sterility is due to the fact that ovules do not develop correctly the two integuments and the embryo sac arrests at FG1 stage, suggesting that *ANT* has multiple functions during all stages of ovule development (Elliott et al., 1996; Klucher et al., 1996; Schneitz et al., 1997; Losa et al., 2010) (Figure 4).

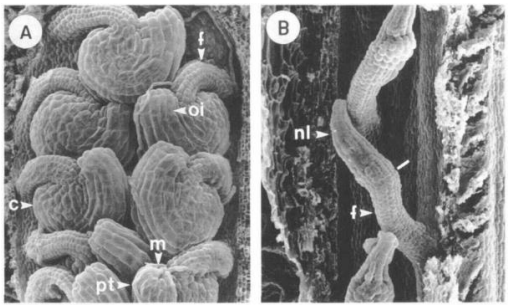

Figure 4 Mature ovules of wild-type and *ant* mutant (Elliott et al., 1996)
Scanning electron microscopy of wild type (A) and *ant* (B) mutant ovules
c, chalaza; f, funiculus; m, micropyle; nl, nucellus-like structure; oi. outer integument

Furthermore, ANT plays an important role in the placenta determination, since in the *ant lug* and *ant seuss* double mutant, as well as in *ant shp1 shp2 crc* quadruple mutant, placenta is not formed and ovules cannot develop (Liu *et al*., 2000; Azhakanandam *et al.*, 2008; Wynn *et al*., 2011; Colombo *et al*., 2008).

CUP-SHAPED-COTYLEDON (CUC) genes are involved in ovule primordia number determination, since a severe reduction in ovule number has been observed in *cuc1 cuc2* double mutant background (Ishida et al., 2000). CUCs have been described as transcription factors involved in boundaries establishment in organ primordia. *cuc1* and *cuc2* single mutants hardly show severe phenotype, while the *cuc1 cuc2* double mutant completely lacks the shoot apical meristem and the two cotyledons are fused along their margin forming a cup-shaped structure (Aida et al.,1997) (Figure 5B). These observations indicate that *CUC1* and *CUC2* are functionally redundant.

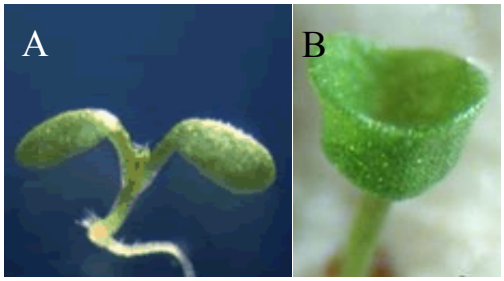

Figure 5: wild-type and *cuc1cuc2* double mutant seedlings
Cotyledons of a wild-type (A) and *cuc1cuc2* double mutant (B) plant

The young cup-shaped seedling dies few days after germination, thus studying *CUC1* and *CUC2* role during ovule formation was only possible on adventitious shoots that occasionally developed from *cuc1 cuc2* mutant calli

in tissue culture (Ishida et al., 2000). The regenerated plant produces flowers with stamen and sepals fusion. Pollen is fertile, even if flowers are sterile (Ishida et al., 2000). Inside *cuc1 cuc2* double mutants carpel, ovule number is drastically reduced indicating that the initiation of ovule primordia from the placenta is inhibited. Furthermore, *cuc1 cuc2* double mutant ovules show several defects. In particular, very few ovules are fertile, whereas the the majority of them have integument or gametophytic defects. Ishida et al., 2000) (Figure 6).

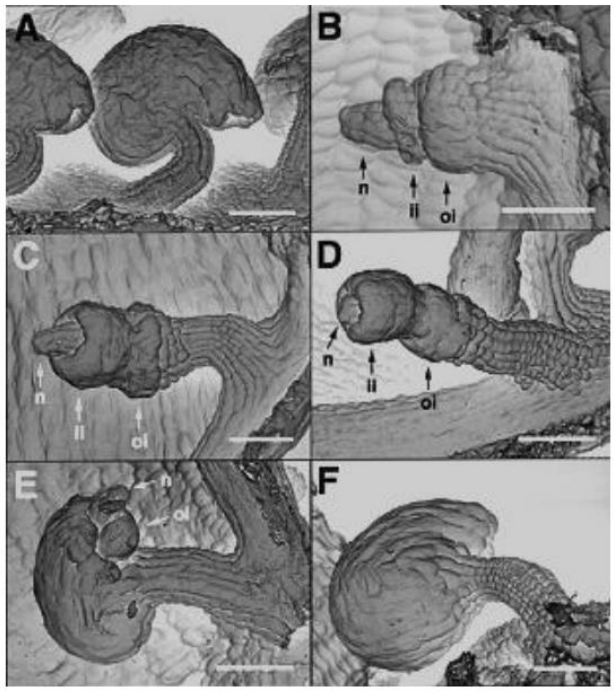

Figure 6 Ovules of the cuc1cuc2 double mutant (Ishida et al., 2000)

A) Wild-type ovule at anthesis;

B - F) Ovules of the *cuc1 cuc2* double mutant.

n, nucellus; ii, inner integument; oi, outer integument.

Genetic network controlling ovule identity

Through the analysis of homeotic mutants it has been possible to study the mechanisms controlling floral organ identity. In *Arabidopsis thaliana*, ovule identity is determined by three redundant MADS-box transcription factors: SEEDSTICK (STK), SHATTERPROOF1 (SHP1) and SHP2, (Brambilla et al., 2007).

STK is expressed in the placenta and in developing ovules. In particular, this gene is important for the funiculus development required for seed dispersal. In *stk* mutant, ovules have a longer and larger funiculus respect to wild-type and seeds are not released when mature (Pinyopich et al., 2003).

SHP1 and *SHP2* have a similar expression profile. They are expressed in the carpel valve margin, in the replum, in ovule primordia and in the funiculus (Liljegren et al., 2000). The *shp1* and *shp2* single mutant do not have any defects, whereas in *shp1 shp2* double mutant the differentiation of the dehiscent zone is not formed, thus seeds are not released (Liljegren et al., 2000). The ectopic expression of *STK*, as well as of *SHP1* and *SHP2* resulted to the homeotic conversion of sepals in carpelloid structures containing ovules (Favaro et al., 2003).

The characterization of the *stk shp1 shp2* triple mutant brought to the conclusion that these three genes act redundantly in the determination of ovule identity. In fact, in *stk shp1 shp2* triple mutant plants, integuments were homeotically converted in carpelloid structures and the female gametophyte development is arrested (Pinyopich et al., 2003) (Figure 7C).

The SEPALLATA (SEP) are MADS-box transcription factors playing an important role during ovule identity determination and in *Arabidopsis thaliana* genome the *SEP* genes are named *SEP1, SEP2, SEP3* (Pelaz et al.,

2000) and *SEP4* (Ditta et al., 2004). Single *sep* mutants do not have strong defects, whereas *sep1sep2sep3* triple mutant showed indeterminate flower composed only of sepals. Interestingly in the *SEP1/sep1sep2sep3* mutant ovules phenotype is similar to the one of *stk shp1shp2* triple mutant, displaying a homeotic conversion of ovules in carpelloid structures (Favaro et al., 2003) (Figure 7).

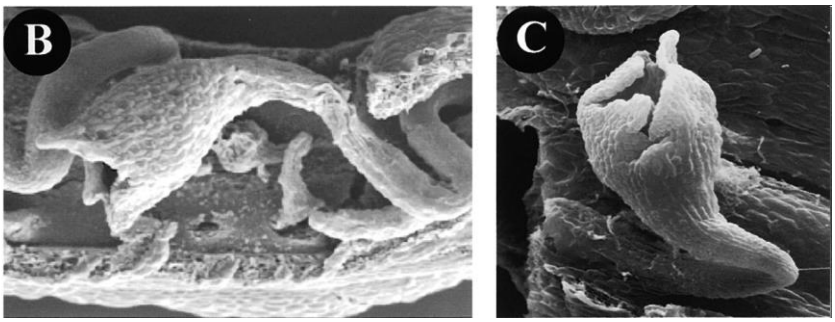

Figure 7 *SEP1/sep1sep2sep3* and *stk shp1* shp2 ovule (Favaro et al., 2003)
Homeotic conversion of the *SEP1/sep1sep2sep3*(B) *and stk shp1 shp2*(C) triple mutant ovules in carpelloid structures.

Yeast protein-protein interaction studies revealed that STK, SHP1 and SHP2 ovule identity factors can interact only in presence SEP proteins (Favaro et al., 2003). Yeast experiment, together with the genetic approach showed that the multimeric complex formation between STK, SHP1, SHP2 with SEP proteins is fundamental for the ovule identity determination in Arabidopsis.

Thanks to experiment conducted in other model species it was possible to understand that this identity complex is evolutionary conserved in other species such as *Oryza sativa* and *Petunia hybrida* (Colombo et al., 2008).

The role of the phyto-hormones

Phyto-hormones are plant specific factors able to coordinate several cellular functions triggering specific responses. While animal hormones are produced in a specific tissue and, through blood, transported in the target cells, most if not all, plant organs are able to produce hormones, which can act in their origin cells or be transported in distant cells through different mechanisms.

In the early half of the 20th century the five classical phyto-hormones have been identified. They are ethylene (Neljubov, 1901), auxin (Went, 1926), gibberellins (Kurosawa, 1926), cytokinins (Skoog, 1950s) and abscisic acid (ABA) (Bennett-Clark and Kefford, 1950s). More recently it has been almost universally accepted that other compound are included in the list of plant hormones like brassinosteroids (BRs), jasmonates, salicylates, and strigolactones.

Phyto-hormones can have different functions, but all of them have a pivotal role in plant growth and development, affecting all stages of plant lifecycle and in plant response to both biotic and abiotic stresses. In particular, for auxin and cytokinins it was suggested a role during ovule primordia formation (Galweiler et al., 1998; Benková et al., 2003; Cheng et al., 2006; Stepanova et al., 2008; Higuchi et al. 2004; Bartrina et al., 2011).

The auxin biosynthesis pathways

Auxins are probably the most studied class of phyto-hormones, composed of related compounds. The most abundant form of auxin present in

nature is Indole-3-Acetic Acid (IAA), thus, auxin is often used as synonymous of IAA.

Auxin is universally present in all plants species including green, red and brown algae, although in these organisms it is not so well characterized. It influences all the aspect of plant development mainly promoting growth and cell elongation, but also specifying vascular tissues, root and leaf initiation, maintaining stem cell populations and forming developmental patterns (Laskowski et al., 1995; Reinhardt et al., 2000; Benkovà et al., 2003).

IAA is synthetized through two different pathways: one is tryptophan (Trp) dependent and the other one is Trp-independent (Woodward and Bartel, 2005; Chandler, 2009; Normanly, 2010). The Trp-independent via uses indole-3-glycerol phosphate or indole as precursor, but this pathway is poorly characterized (Ouyang et al., 2000; Zhang et al., 2008). On the other hand several parallel or interacting pathways control the synthesis of the IAA starting from Trp: the indole-3-acetamide (IAM) pathway, the indole-3-pyruvic acid (IPA) pathway, the YUCCA (YUC) pathway, and the indole-3-acetaldoxime (IAOX) pathway (Woodward and Bartel, 2005; Pollmann et al., 2006a; Chandler, 2009; Mano et al., 2010; Normanly, 2010; Zhao, 2010). Although several pathways have been discovered, little is known about the genetic control of the key enzymes involved in auxin biosynthesis. For instance, the conversion of the IAM to IAA by AMIDASE 1 (AMI1) has been demonstrated, but it is not clear how *IAM* expression is regulated. This pathway has been identified in several species (Pollmann et al., 3003; Lehmann et al., 2010). On the contrary it seems that the IAOx pathway, that convert Trp to IAOx through CYP79B family, is present in few plant species (Bak et l., 1998; Hull et al., 2000; Mikkelsen et al., 2000; Zhao et al., 2002;

Sugawara et al. 2009). Previous works demonstrated that both TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC) flavin monooxygenase-like proteins are necessary for IAA biosynthesis. Recently it has been confirmed the important role for these proteins and it has been proposed that they act in the same pathway. TAA controls the synthesis of IPA that is converted into IAA buy YUC proteins (Mashiguchi et al., 2011).

The importance of auxin transport

Once auxin is synthetized, it moves throughout the plant where it is required for several developmental processes. Auxin distribution depends on two different ways of transport, one through the phloem, fast and non-polar and the other one is the cell-to-cell polar transport (PAT). The majority of long-distance auxin transport occurs through the non-polar transport via phloem. For this reason the importance of this transport should not be underestimated, even if genetic and physiological data showed the pivotal role of PAT in regulating plant developmental processes.

The PAT allows auxin to be transported in and out of cells by families of auxin influx and efflux carriers, creating a spatial auxin maxima and minima concentration. This gradient provides the required directional and positional information for the correct plant growth, controlling many developmental processes like embryogenesis, organogenesis, root meristem maintenance, vascular tissue differentiation, growth responses to environmental stimuli, fruit development, apical hook formation and many others that have been reviewed in the last years (Davies, 2004; Tanaka et al.,

2006; Vieten et al., 2007; Zazimalova et al., 2007; Petrasek and Friml, 2009; Vanneste and Friml, 2009; Zazimalova et al., 2010; Peer et al., 2011).

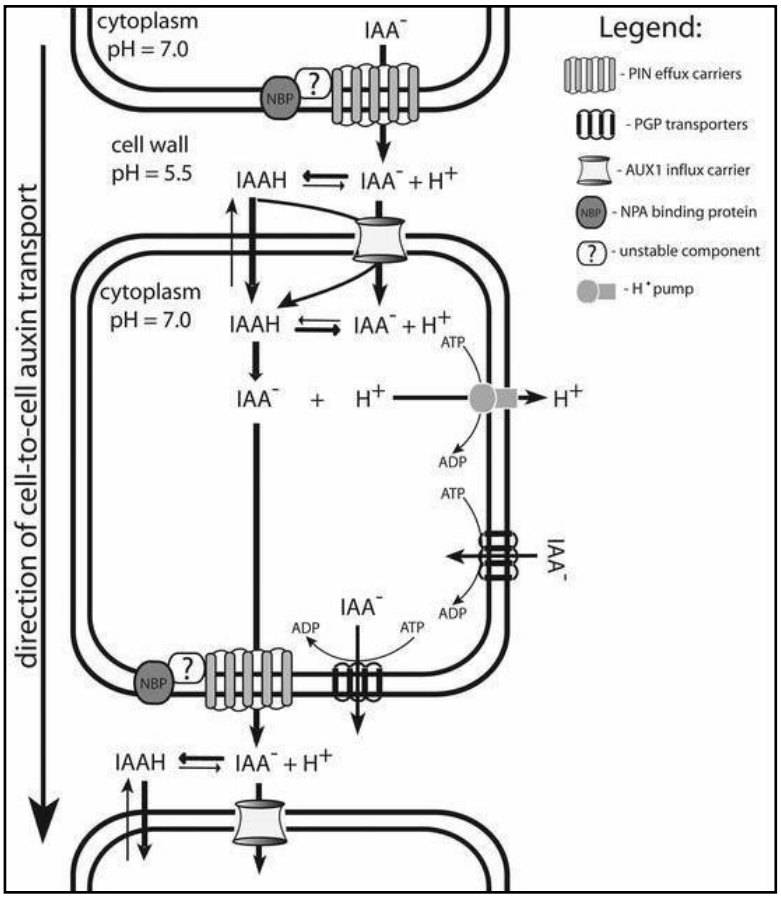

Figure 8 Model for polar auxin transport (Michniewicz et al., 2007)

Almost 40 years ago, a chemiosmotic model was proposed, which postulated the presence of auxin-specific carriers (Rubery and Sheldrake, 1973; Raven, 1975). The difference of pH between the cell wall (pH 5,5) and the cytoplasm (pH 7,0) promotes the simple diffusion of the IAA inside the cell, were the pH lead to the de-protonation of the IAA giving rise to polar

IAA anions. At this point, deprotonated IAA can only leave the cell via active efflux mediated by specific efflux carriers. If the last ones are predominantly localized to one side of the cell, the unidirectional polar auxin flux is generated (Figure 8). In addition, it was also demonstrated the presence of auxin influx carrier (AUX1) that facilitate auxin uptake into the cell (Bennett et al., 1996). This model was strengthened by the identification of the auxin efflux carrier PIN proteins (Gälweiler et al., 1998; Luschnig et al., 1998).

The first auxin efflux carrier, that belongs to the PIN1 family, was identified through the analysis of the Arabidopsis *pin-formed1* (*pin1*) mutant, which was linked to defects in PAT (Okada et al., 1991; Gälweiler et al., 1998). *pin1* phenotype is very severe, with needle-like inflorescences that mimicked the one of wild type plants treated with auxin efflux inhibitors (Okada et al., 1991; Müller et al., 1998; Friml et al., 2002a, b; Friml et al., 2003b; Benková et al., 2003; Blilou et al., 2005). Subsequently, other seven *PIN* genes have been identified in *Arabidopsis thaliana*. *PIN2*, also known as *ETHYLENE INSENSITIVE ROOT 1* (*EIR1*) and *AGRAVITROPIC 1* (*AGR1*) affects root gravitropism (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Utsuno et al., 1998). *PIN3, PIN4* and *PIN7* are required for tropism, root meristem patterning and early embryo development, respectively (Friml et al., 2002a, b; Friml et al., 2003b). PIN5 and PIN8 transporters have opposite roles in the regulation of intracellular IAA homeostasis, respect to the previous described PIN proteins (Mravec et al., 2009). It has recently been published that *PIN6*, plays a role in nectary and stamen development (Bender et al., 2013).

The PIN polar localization at different sides of the cell determines the auxin flux direction, thus, the polarity of all the tissue (Wiśniewska et al.,

2006). Endo and exocytosis between the plasma and the endosomal membranes create continuous movement of the PIN proteins that is necessary for PIN polarization (Dhonukshe et al., 2007; Dhonukshe et al., 2008).

It is known that the fungal toxin brefeldin A (BFA) interfere with vesicle trafficking processes in cells and target the GDP-to-GTP exchange factors (GEFs) that activates the ADP-ribosylation factors (ARFs), small guanine-nucleotide-binding proteins that regulate membrane traffic in eukaryotic cells. In particular, once the inactive GDP-bound form of ARF moves to the active GTP-bound form, it tightly binds to the membrane where leads to changes in protein and lipid composition of the membrane, resulting in membrane modulation of both structure and function (Donaldson and Jackson, 2000).

In Arabidopsis, the BFA-sensitive endosomal ARF-GEF GNOM is required for the polar localization and recycling of PIN1 (Steinmann et al., 1999; Geldner et al., 2001). The inhibitory effect of BFA on PIN1 cycling in the root cells is due to its effect specifically on GNOM activity (Geldner et al., 2003). Hence, GNOM is clearly involved in the vesicle transport, required for the recycling of the PIN1 protein to the basal (root apex-facing) side of the cell. Moreover, GNOM activity is also involved in dynamic transcytosis of PIN proteins from one side of the cell to the other, regulating PIN-dependent cell repolarization (Kleine-Vehn et al., 2008a).

PIN proteins localizes preferentially to different sides of the cell. For example, PIN1 localizes to the basal side, while PIN2 to the apical one. It suggests that the protein sequence itself regulates the polarity (Wiśniewska et al., 2006). It has been proposed that the polarity determinants are related with the phosphorylation state of the PINs (F. Huang, M. Kemel-Zago, A.

van Marion, C.G. Ampudia, and R. Offringa, unpublished data; Zhang et al., 2009). The protein serine/threonine (Ser/Thr) kinase PINOID (PID) and the PROTEIN PHOSPHATASE 2A (PP2A) mediate the phosphorylation status of PIN proteins (Bennett et al. 1995; Benjamins et al. 2001; Friml et al. 2004; Michniewicz et al. 2007). In particular, PP2A is responsible of the de-phosphorylation of PIN that in this form is targeted to the basal plasma membrane, while phosphorylated PIN proteins are targeted to the apical plasma membrane (Friml et al., 2004; Michniewicz et al., 2007).

Auxin Response Factors (ARFs) mediate auxin response

Auxin signaling plays a crucial role during plant correct growth since many developmental processes are mediated through auxin-regulated gene expression. Auxin response is mediated by two groups of transcription factors. One is the Auxin/Indole 3-Acetic Acid (AUX/IAA) genes family of 29 members and the second one is the Auxin Response Factors (ARFs) gene family consisting of 23 members (Guilfoyle and Hagen, 2001; Liscum and Reed, 2002).

ARFs bind to AuxREs element (TGTCTC) in the promoter of auxin responsive genes mediating their transcription (Ulmasov et al., 1997). Most ARFs present an amino-terminal DNA binding domain (DBD), a middle region that can act as activator (AD) or repressor (RD) domain and a carboxy-terminal dimerization domain (CTD) carboxy-terminal dimerization domain (CTD) (Guilfoyle and Hagen, 2001; Tiwari et al., 2003). Among 23 ARFs, just ARF5, 6 and 19 have been identified as activators, while the other ARFs are considered repressors even if it is possible that an ARF classified as an activator can function as a repressor and vice versa in

particular cell types or environmental conditions (Guilfoyle and Hagen, 2001).

Aux/IAAs encode 18 to 35 kD short-lived nuclear proteins that are composed of four conserved domains. Domains II, III and IV are responsible for homo and hetero-dimerization with other Aux/IAA, while the first domain is necessary for the transcriptional repression (Reed et al., 2001; reviewed in Hagen and Guilfoyle, 2002; Kepinski and Leyser, 2002).

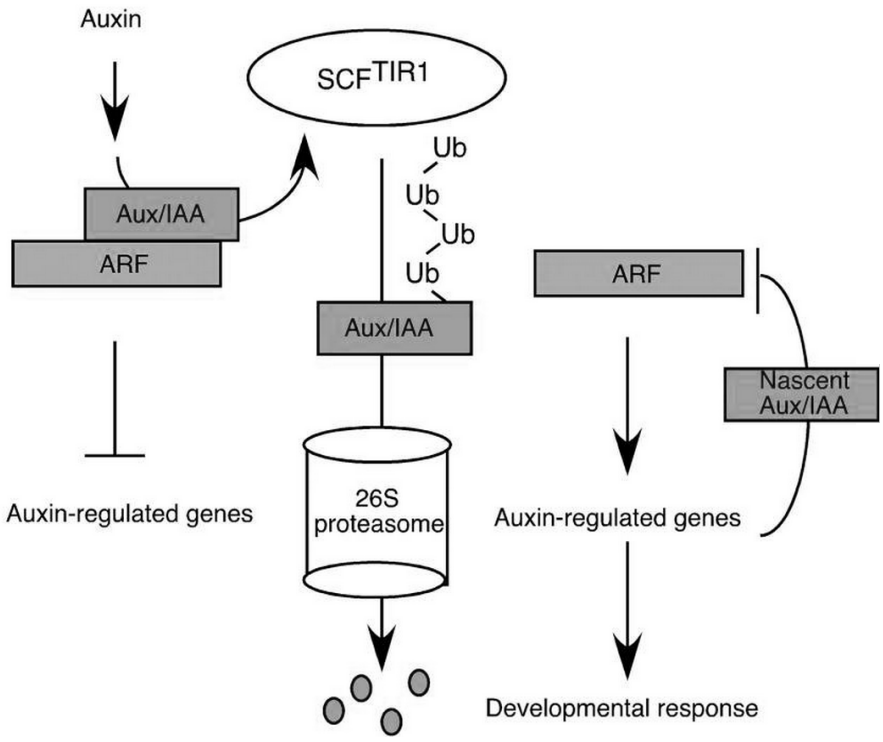

Figure 9 Auxin response model (Gray., 2004)

The F-box protein TRANSPORT INHIBITOR RESPONSE1 (TIR1) has been identified as an auxin receptor and a component of a SCF ubiquitin

ligase complex. The ubiquitinated proteins are transfer to the 26S proteasome, which degrades proteins. When bound to auxin, TIR1 also specifically binds to Aux/IAA repressors, targeting them for proteolysis (Gray et al., 2001; Kepinski et al., 2005; Dharmasiri et al., 2005; Tan et al., 2007). The ARFs and Aux/IAA can interact via their C-terminal domain to form homo and heterodimers.

When auxin concentration is low, Aux/IAA proteins form dimers with ARFs, repressing auxin responses. When auxin level increases, the SCF TIR1 complex binds to auxin and to Aux/IAA proteins leading them to ubiquitination and to the following degradation. In such a way ARFs repression is released and auxin response genes can be transcriptionally regulated (Figure 9) (Rogg and Bartel, 2001; reviewed by Kepinski and Leyser, 2002; Gray, 2004).

MONOPTEROS (*MP*), also known as *ARF5,* encodes a member of the AUXIN RESPONSE FACTOR (ARF) family. It has been observed that *mp* loss of function mutant results in an embryo lethal phenotype, while *mp* partial loss of function mutants have normal embryo development however reproductive development is compromised (Hardtke and Berleth, 1998). It was reported that *MP* loss of function mutants result in a rootless phenotype (Barleth and Jürgens 1993; Weijers et al. 2006). *MP* is also involved in vascular tissue development since loss of function *MP* alleles present reduced vain formation, while the semi-dominant gain of function mp^{abn} allele results in vain proliferation of leaves (Wenzel et al., 2007; Garrett et al., 2012). It have been suggested that MP play a role in lateral organ formation (Przemeck et al., 1996). The partial lost of function mutant have the phenotype similar to the mutants of auxin transport inhibitors (Okada et al., 1991).

The cytokinins biosynthesis pathway

Cytokinins (CKs) are a family of N6-substituted adenine related compounds first isolated, crystallized, characterized and synthesized almost 60 years ago (Miller et al., 1955a, 1955b, 1956). The name refers to their role in promoting cell division, but in the last decades it has been discovered several role in which they are involved. CKs can promote cell differentiation, seed germination, chloroplast differentiation, apical dominance, flower and fruit development and senescence (Sakakibara, 2006; Werner and Schmülling, 2009). Recently it has been also demonstrated that high doses of CKs are able to induce programmed cell death (PCD) (Ishii et al.,2002; Mlejnek and Prochazka, 2002; Carimi et al., 2003). Furthermore cytokinins have been reported to play a role during ovule primordia formation (Higuchi et al. 2004; Bartrina et al., 2011).

Several compounds with CK activity have been identified including several natural CKs with aromatic side chains (Horgan, 1973; Strnad, 1997) and isoprenoid N6 -(2 -isopentenyl) -adenine (iP), tZ, cis-zeatin (cZ), and dihy-drozeatin (DZ) (Letham, 1963). Among them, the major derivatives depend on plant species, but tZ- and iP-type CKs are the major forms in Arabidopsis (Sakakibara et al., 2006).

In Arabidopsis seven *ATP/ADP-ISOPENTENYLTRANSFERASE* (*IPT*) genes have been identify to catalyze the CKs biosynthesis limiting step (Takei et al., 2001; Miyawaki et al., 2006; Kamada-Nobusada and Sakakibara, 2009). In particular, *IPTs* overexpression determine cytokinin overproduction (Kakimoto et al., 2001;Sun et al., 2003; Miyawaki et al., 2004).

iP nucleotide is produced as a CK precursor by IPT using dimethylallyl diphosphate and adenosine phosphate as substrates (Kakimoto, 2001; Takei et al., 2001a; Sakamoto et al., 2006), while tZ nucleotide is formed by cytokinin hydroxylase, which hydroxylates the *trans*-end of the prenyl side chain of iP nucleotide (Takei et al., 2004a). After that, both nucleotides are converted in the corresponding active nucleo-bases, but the mechanisms of action for this process are still unclear. It has been proposed that LONELY GUY (LOG) could mediate this conversion (Kurakawa et al., 2007). *LOG* encodes a cytokinin riboside 5′-monophosphate phosphoribohydrolase that releases cytokinin nucleobase and ribose 5′-monophosphate. It was discovered through the analysis of *Oryza sativa* mutants affecting the maintenance of shoot meristems (Kurakawa et al., 2007). Even if it has been suggested that LOG is specifically involved in cytokinin activation, it is unknown if it plays a role just in rice shoot meristem.

The regulation of cytokinins catabolism

A controlled balance between cytokinin synthesis and catabolism regulates cytokinins homeostasis (Sakakibara et al., 2006; Werner et al., 2003). In Arabidopsis seven *CYTOKININ OXIDASE/DEHYDROGENASEs* (*CKX1–7*) are involved in CKs catabolism, catalyzing the irreversible degradation of CKs (Werner et al., 2003). They are differentially expressed during plant development, having a particular intense activity in growing tissues like shoots, root meristem and leaves primordia (Werner et al., 2003). *CKXs* are induced by CKs indicating the important role of feedback loop regulation in cytokinins homeostasis.

It has been observed that plants overexpressing CKSs have the same phenotype observed in *ipt* mutants, with enhanced root growth and reduced shoot and leaves growth (Werner et al., 2003). Analysis of *ipt* or *ckx* mutants underlined the important role of cytokinins during plant development (Werner et al., 2003, Miyawaki et al., 2006)

Cytokinins do not have a specific transport system

Unlike the highly elaborated polar transport system existing for auxin, CKs seems not to have a specific transporter system. Spatio-temporal expression analysis demonstrated that the distribution of cytokinin precursor do not always coincide with those of active cytokinin form (Miyawaki et al., 2004; Takei et al., 2004b). It indicates that CKs are mobile hormones that can act as long distance signal. Xylem and phloem mainly contain CK precursors and it can be that from there, the precursors moves to other tissues where they are activated (Corbesier et al., 2003; Hirose et al., 2008).

It seems that the major CK traslocator forms are nucleosides. This selective translocation is mediated by members of the equilibrative nucleoside transporter (ENT) family. In rice there are four different *ENT* genes (*OsENT1- OsENT4*) and it has been suggested that *OsENT2* may have a role in the selective transport of CK nucleosides in the vascular tissues (Hirose etal., 2005). The *ENT* gene, *SOI33/AtENT8* in *Arabidopsis thaliana* was proposed to function in CK nucleoside transport (Sun et al., 2005). It remains to prove if CKs translocation contribute to global growth coordination.

Cytokinins perception and signaling network

CK signaling is mediated by a His kinase (HK) multistep phosphorelay pathway that resembles bacterial two-component systems for sensing environmental stimuli (Kakimoto, 2003; To and Kieber, 2008).

The Arabidopsis genome encodes three transmembrane histidine kinases receptors: the ARABIDOPSIS HIS KINASE 2 (AHK2), AHK3 and AHK4/WOL1 (WOODENLEG 1)/CRE1 (CYTOKININ RESPONSE 1) (Riefler et al., 2006; To and Kieber, 2008; Inoue et al., 2001; Hwang and Sheen, 2001). Once cytokinins bind to the receptors at the plasma membrane, they auto-phosphorylate and then transfer the signal through phosphorylation of the ARABIDOPSIS HIS PHOS-PHOTRANSFER PROTEINS (AHPs) (Choi and Hwang, 2007; Hellmann et al., 2010) (Figure 10). This proteins move to the nucleus where phosphorylate members of ARABIDOPSIS RESPONSE REGULATORS (ARRs type A and B) protein family. The five known AHPs (AHP1-5) in Arabidopsis are highly redundant regulators of the cytokinins signaling and the multiple knockout of these genes causes CK insensitivity, while the AHP6 is a pseudo-AHP known to negatively regulate cytokinins signaling interfering with the CK receptors and response regulators (Mähönen et al., 2006).

Based on their C terminal sequence it is possible to divide the ARRs in three different classes. In Arabidopsis there are 11 type-B ARRs acting as transcriptional activators, 10 type-A and two type-C ARRs acting as negative regulators of CK signaling (To et al., 2007; Perilli et al., 2010). Even if it is not clear how this mechanism of action works, it is very probable that the type-A ARRs compete with type-B ARRs for the phosphorylation. Once phosphorylated, type-B ARRs are able to

transcriptional activate the CYTOKININ RESPONSE FACTORS (CRFs) (Rashotte et al., 2006).

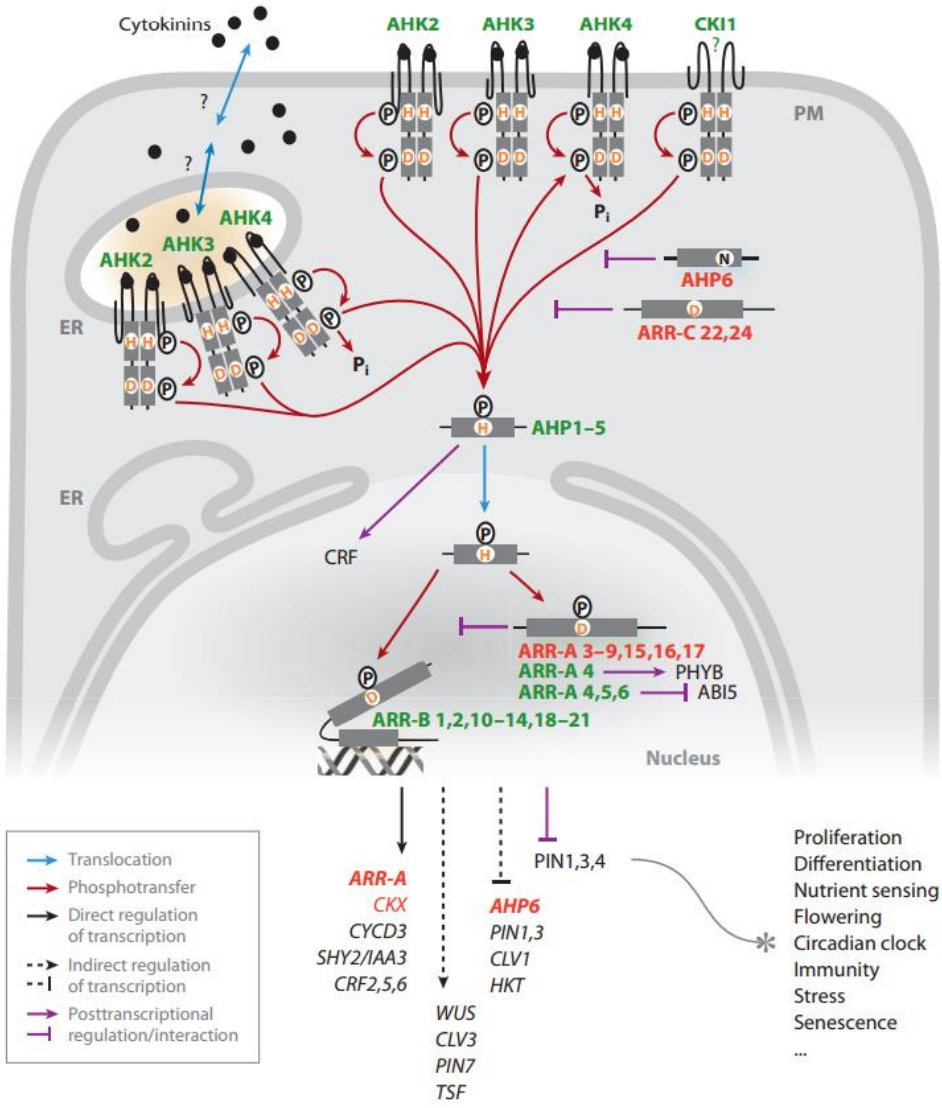

Figure 10 Cytokinin signaling mechanism (Hwang et al. 2012)

The role of hormones during ovule development

Although it has been proposed that auxin and cytokinin are both paying important roles during ovule development (Galweiler et al., 1998; Benková et al., 2003; Cheng et al., 2006; Stepanova et al., 2008; Higuchi et al. 2004; Bartrina et al., 2011; Bencivenga et al., 2012) their involvement in ovule patterning is still not well described.

In 2003 it was proposed that lateral organ formation initiate in correspondence of an auxin accumulation (maximum) (Figure 11) surrounded by auxin depletion and that auxin synthesis and transport play a crucial role in ovule formation (Benková et al., 2003).

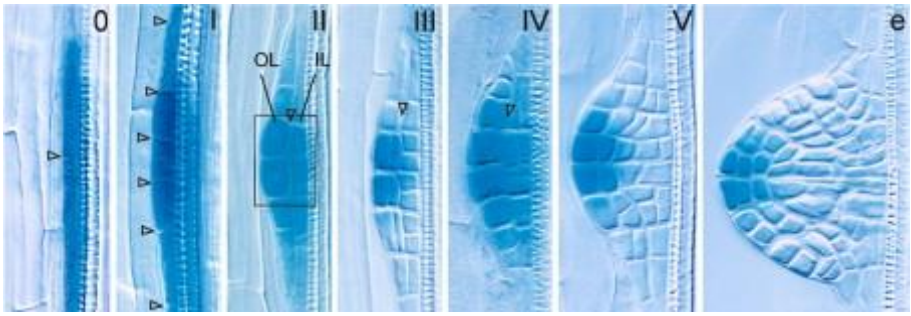

Figure 11 Auxin distribution in developing lateral roots (Benková etal., 2003)

Indeed, plants treated with auxin efflux inhibitors, which develop a placenta without ovules (Nemhauser et al., 2000). Mutants affecting local auxin synthesis like *yucca1 yucca4* (*yuc1 yuc4*) and *weak ethylene insensitive8 tryptophan aminotransferase related2* (*wei8 tar2*) double mutants presented carpels without ovules (Cheng et al., 2006, Stepanova et al., 2008) and a similar phenotype was observed for *pin1-5*, a partial loss of

function mutant that develops pistils with very few ovules (Bencivenga et al., 2012). Furthermore, a role of auxin gradient was also described for the correct cell identity of the female gametophyte (Pagnussat et al., 2009).

Not just auxin, but also cytokinins have been described as important molecules for the correct ovule development, since plants affected in CKs synthesis or perception present a drastic reduction in ovule number and female fertility (Werner et al., 2003; Hutchison et al., 2006; Miyawaki et al., 2006; Riefler et al., 2006; Kinoshita-Tsujimura and Kakimoto, 2011; Bencivenga et al., 2012)

What is more, in cytokinin *response1-12 histidine kinase2-2 histidine kinase3-3* (*cre1-12 ahk2-2 ahk3-3*) triple mutant, ovule number is severely affected, due to a high reduction in CKs responses (Higuchi et al. 2004). On the contrary, when CKs are not degraded, as in *ckx3 ckx5* double mutants, the number of ovule primordia increases (Bartrina et al., 2011). All these evidences indicate a correlation between cytokinins and ovules.

It was published that the overexpression of a gibberellin inactivation gene is able to alter seed development in Arabidopsis (Singh et al., 2010) and that ethylene is involved in ovule senescence process (Carbonell-Bejerano et al., 2011), but until now it was not reported any evident correlation between these or other hormones and ovule development. Very recently it has been reported that *brassinazole-resistant1-1D* (*brz1-1D*) mutants display more ovules compared with the wild-type, indicating a correlation between brassinosteroid signal and ovule number (Huang et al., 2013). It indicate that we cannot exclude that other hormones are involved in ovule formation and development, thus, it would be of high interest investigate in this direction.

AIM OF THE PROJECT

The aim of my project was to elucidate the network controlling ovule primordium formation and development in *Arabidopsis thaliana*.

By genetic analysis of the mutants already characterized, I have identified a new possible network that integrates the auxin signaling pathway with the transcription factors controlling ovule primordium formation already described in literature.

Furthermore I analyzed the role of the ovule identity genes in other aspect of ovule development such as ovule primordia formation and ovule fertility.

MAIN RESULTS

It was published that *CUC1, CUC2* and *ANT* are involved in ovule number in *Arabidopsis thaliana* (Baker et al., 1997; Mizukami et al., 2000; Aida et al., 1997). We demonstrate that these genes have additive effects on ovule primordia formation and that they are direct target of MP, an auxin response factor. Furthermore, we were able to show that the CUCs control PIN1 expression and localization, which is necessary for the correct ovule primordia formation.

We saw that during ovule development, MP is also able to regulate *STK*, which together with *SHP1, SHP2* and *SEP* determine ovule identity (Pinyopich et al., 2003; Favaro et al., 2003; Brambilla et al., 2007).

To better understand the molecular network controlling ovule development we have identified STK targets among which *VDD* was fully characterized (Matias-Hernandez et al., 2010).

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Part II

Integrative model to control ovule primordia formation

Submitted to *the* plant journal

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# equal contribution

Running title: ovule primordia development.

Key words: ovule development, *AINTEGUMENTA*, Arabidopsis, *CUP-SHAPED COTYLEDON*, PIN1.

Summary

Upon hormonal signaling, ovules develop as lateral organs from the placenta. Ovule numbers ultimately determine the number of seeds that can develop and thereby control the final seed yield in crop plants. We demonstrate here that *CUP-SHAPED COTYLEDON 1 (CUC1), CUC2* and *AINTEGUMENTA* (*ANT*) have additive effects on ovule primordia formation. We show that expression of the *CUC1* and *CUC2* genes is required to redundantly regulate *PINFORMED1* (*PIN1)* expression, which in turn is needed for ovule primordia formation. Furthermore, our results suggest that *ANT, CUC1* and *CUC2* expression might be directly regulated by the auxin response factor *MONOPTEROS (MP/ARF5)*. Based on our findings we propose a model in which MP plays a crucial role in the molecular network controlling the early stages of ovule development.

Introduction

In *Arabidopsis*, ovules arise from the placenta as lateral organs, and both auxin synthesis and transport play important roles in their formation (Benková *et al.*, 2003). In the *yucca1 yucca4* (*yuc1 yuc4*) and *weak ethylene insensitive8 tryptophan aminotransferase related2* (*wei8 tar2*) double mutants, the local auxin response is compromised and the pistil develops lacking ovules (Cheng *et al*., 2006; Stepanova *et al*., 2008). A similar phenotype has been described for *mp S319*, a MP partial loss-of-function mutation (Cole *et al*., 2009; Lohmann *et al*., 2010). *MP* encodes a member of the AUXIN RESPONSE FACTOR (ARF) family (Hardtke and Berleth, 1998) that can bind, as a monomer or a dimer, to the promoters of target

genes to control their transcription (Ulmasov *et al*., 1997). Although total loss of function *mp* mutants are unable to correctly form embryos, embryo development is unaffected in *mp S319* plants suggesting that the MP dimerization domain is required for activities mainly occurring in the post-embryonic phase of plant development (Lau *et al*., 2011).

Further indications that auxin could influence ovule primordium number is given by the characterization of the partial loss-of-function mutant *pin1-5* that develops pistils having fewer ovule primordia compared to wild-type (Bencivenga *et al*., 2012).

Cytokinins have also been reported to play a role in ovule primordium formation. The *cytokinin response1-12(cre1-12) histidine kinase (ahk)2-2 ahk3-3* triple mutant shows a reduction in the cytokinin response, and the number of ovule primordia is drastically reduced (Higuchi et al. 2004). By contrast, the *cytokinin oxidase/dehydrogenase3* (*ckx3*) *ckx5* double mutant, which is affected in cytokinin degradation, develops twice as many ovules as the wild-type (Bartrina et al., 2011). It has recently been shown that cytokinin modulates auxin fluxes during ovule development by regulating *PIN1* expression (Bencivenga *et al*., 2012).

Besides the auxin and cytokinin signaling pathways, transcription factors such as ANT, (Elliott *et al*., 1996) CUC1 and CUC2 (Ishida et *al*., 2000) have been demonstrated to be important for ovule primordia formation.

In the *ant* single mutant, a reduced number of ovule primordia are formed. Furthermore *ant* ovules lack of the two integuments and the embryo sac arrests at the one-cell stage, suggesting that *ANT* has multiple functions during ovule development (Klucher *et al*., 1996; Elliott *et al*., 1996; Schneitz *et al.,* 1997; Losa *et al.,* 2010).

Although *cuc1* and *cuc2* single mutants are very similar to wild-type plants (Aida *et al*., 1997), *cuc1 cuc2* double mutants completely lack the shoot apical meristem, and the two cotyledons are fused to form a cup-shaped structure (Aida *et al*., 1997). Studying the roles of *CUC1* and *CUC2* in ovule formation was therefore only possible on adventitious shoots that occasionally developed from *cuc1 cuc2* mutant calli in tissue culture (Ishida *et al*., 2000). In *cuc1 cuc2* double mutants the number of ovules was reduced compared to wild-type and many of them were found to be sterile (Ishida *et al*., 2000).

In the work reported here we study CUC1 and CUC2 function during ovule development using RNAi-based silencing of *CUC1* under the control of an ovule specific promoter in the *cuc2* mutant background. By crossing these plants with the *ant* mutant we show that *ANT*, *CUC1* and *CUC2* have additive roles in the determination of ovule number. Furthermore, we show a role for CUC1 and CUC2 in the localization and expression of *PIN1*.

Recently it has been shown that MP plays a key role in flower primordium initiation, controlling the expression of *LEAFY* (*LFY*), *ANT* and *AINTEGUMENTA-LIKE6/PLETORA3* (*AIL6/PLT3*) (Yamaguchi *et al.*, 2013). In this manuscript, we show that *ANT* is not expressed in the pistil of the *mp S319* mutant, thus confirming that MP controls *ANT* expression during the reproductive developmental phase. Moreover we reveal that *CUC1* and *CUC2* are direct targets of MP and, based on our findings, we propose a model in which MP integrates the auxin signaling required for ovule primordium formation to regulate the expression of *ANT*, *CUC1* and *CUC2* transcription factors.

Results

ANT, CUC1 and CUC2 are required for ovule initiation.

In the *cuc1 cuc2* double mutant and in the *ant* single mutant the number of ovules is reduced (Ishida *et al*., 2000; Elliott *et al.*, 1996). To study the interaction among these three genes, *CUC1* was silenced, in the *ant-4 cuc2-1* double mutant background, using a *CUC1* specific RNAi construct under the control of the ovule-specific S*EEDSTICK* promoter *(pSTK)* (Kooiker *et al*., 2005), which is already active in the placenta before ovule primordia arise (Figure 1a, 1b).

All mutant combinations were morphologically analyzed by differential interference contrast microscopy (DIC). For each genotype we analyzed 10 pistils of 6 plants. The down regulation of *CUC1* due to the specific RNAi was verified using Real time PCR (Figure 1c). The *ant-4* (Figure 1e) and *cuc2-1 pSTK::CUC1_RNAi* mutant plants (Figure 1f) showed reduced ovule number compared to wild type (Figure 1d) in agreement with previously published data (Ishida *et al*., 2000; Elliott *et al*., 1996). The ovules of the *ant-4 cuc2-1* double mutant resembled those of the *ant-4* single mutant (data not shown). In the *ant-4 cuc2-1 pSTK::CUC1_RNAi* transgenic plants we observed a drastic further reduction in the number of developing ovule primordia (Figure 1g). The *cuc2-1 pSTK::CUC1_RNAi* mutants developed an average of 30 ovules per pistil, while *ant-4* developed an average of 20 ovules per pistil. Finally the *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants developed an average of 7 ovule primordia per gynoecia (Figure 1h), suggesting that *ANT*, *CUC1* and *CUC2* act additively in controlling the number of ovule primordia that develop.
Despite the reduction in ovule number in the mutant background the size of the pistils were not reduced (Figure S1).

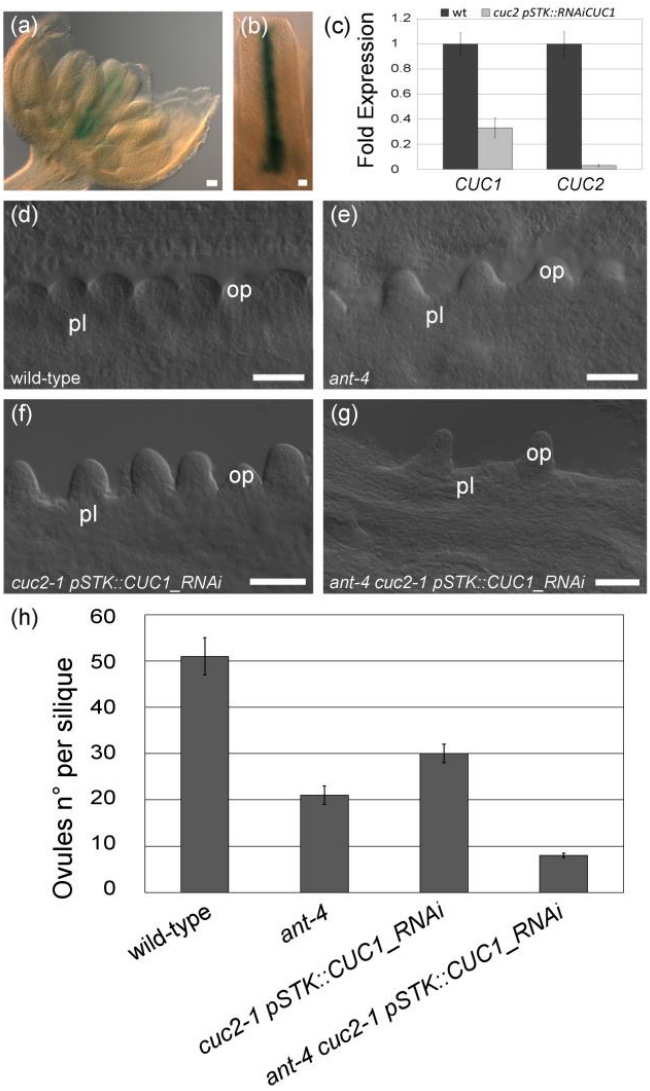

Figure 1. *Arabidopsis thaliana* ovule primordia in wild-type and mutant plants.

(a-b) pSTK::GUS is expressed in the placenta at stage 8 (a) and stage 9 (b) of flower development. (c) Expression level of *CUC1* and *CUC2* in wild-type and in *cuc2-1 pSTK::CUC1_RNAi* plants.

(d-g) *Arabidopsis thaliana* ovule primordia at stage 1-I in wild-type (d), *ant-4* (e), *cuc2-1 pSTK::CUC1_RNAi* (f), *ant-4 cuc2-1 pSTK::CUC1_RNAi* (g) plants.

(h) ovule primordia number in wild-type, *ant-4*, *cuc2-1 pSTK::CUC1_RNAi*, *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants. pl, placenta; op, ovule primordia. Scale bars: 20 µm.

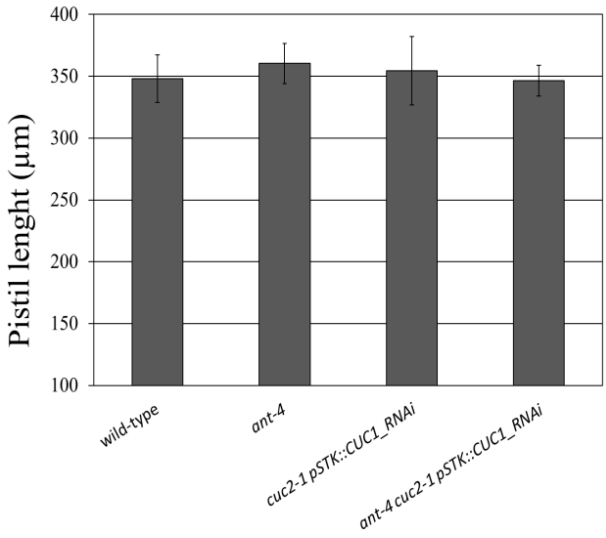

Figure S1. *ant-4, cuc2-1 pSTK::CUC1_RNAi* and *ant-4 cuc2-1 pSTK::CUC1_RNAi* pistil sizes.

Pistil length of wild-type, *ant-4*, *cuc2-1 pSTK::CUC1_RNAi* and *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants. The length was measured at stage 8-9 of flower development. Error bars indicate the standard error.

CUCs control *PIN1* expression and PIN1 localization required for correct ovule primordia formation.

A reduction in ovule primordia was also observed in the *pin1-5* mutant (Bencivenga *et al*., 2012) and in the *cre1-12 ahk2-2 ahk3-3* triple mutant (Higuchi *et al.*, 2004). Recently it has been proposed that cytokinin regulates *PIN1* expression in the placenta (Bencivenga *et al.*, 2012) and that PIN1 is required to form ovule primordia as later organs (Benkova *et al.*, 2008; Bencivenga *et al*., 2012).

To analyze whether the reduction in ovule number observed in the *ant-4 cuc2-1 pSTK::CUC1_RNAi* mutant was due to *PIN1* down regulation, we crossed the *ant-4/ANT cuc2-1 pSTK::CUC1_RNAi* plants with those containing the *pPIN1::PIN1-GFP* reporter construct (Friml *et al*., 2003).

Analyses of these reporter line plants showed that during early stages of pistil development, *PIN1-GFP* is expressed in the ovule primordia before stage 1-I (Figure 2a) and at stage 1-I (Figure 2b) in the plasma membrane of

the epidermal cell layer of the ovule primordia. The expression and localization of PIN1-GFP in the plasma membrane in the *ant-4* and *cuc2-1* ovule primordium was similar to what we observed in wild-type plants (Figure S2).

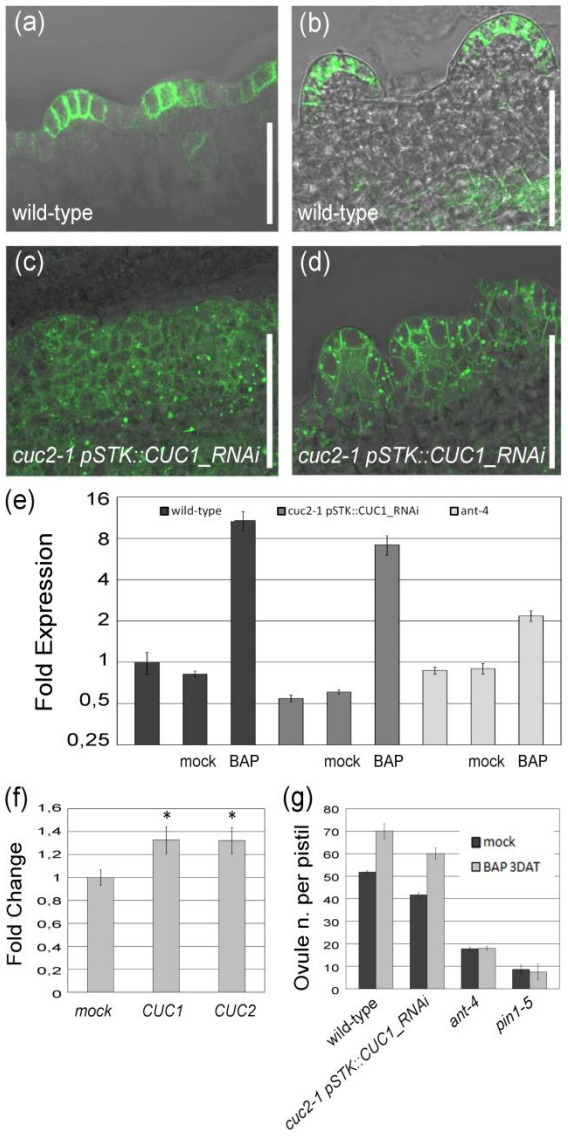

Figure 2. *PIN1* expression in developing *Arabidopsis* wild-type and mutant ovules.

(a-b) *pPIN1::PIN1-GFP* in wild-type ovule, before stage 1-I (a) and at stage 1-I (b). (c-d) *pPIN1::PIN1-GFP* in *cuc2 pSTK::RNAi_CUC1* ovules, before stage 1-I (c) and at stage 1-I (d). (e) *PIN1* expression levels in Bap or mock-treated pistils. (f) Transient expression of *PIN1::LUC* in tobacco BY-2 protoplasts. *PIN1* expression is regulated by *CUC1*(2) and *CUC2* (3). Transactivation is expressed relative to the normalized luciferase (LUC) activity. Error bars indicate the standard error (n = 8 separate transfection events and measurements). Statistical significance was determined by Student's t-test. (g) Number of ovules in mock- and BAP-treated pistils of wild-type, *cuc2-1 pSTK::CUC1_RNAi*, *ant-4*, *pin1-5* plants. Scale bars: 20 µm.

In contrast, the *cuc2-1 pSTK::CUC1*_RNAi plants showed that the PIN1-GFP recombinant protein was hardly visible, and partially included in vesicles (Figure 2c, d). Furthermore, in *cuc2-1 pSTK::CUC1*_RNAi plants, *PIN1* seems to be expressed in all the cells of the primordium as well as in the boundary between ovules (Figure 2d). Real time PCR analysis showed that *PIN1* expression was down-regulated in *cuc2-1 pSTK::CUC1*_RNAi plants when compared to *cuc2*, *ant* single mutants and wild-type plants (Figure 2e). This result suggests that CUC1 and CUC2 might redundantly regulate *PIN1* expression during early stage of ovule development.

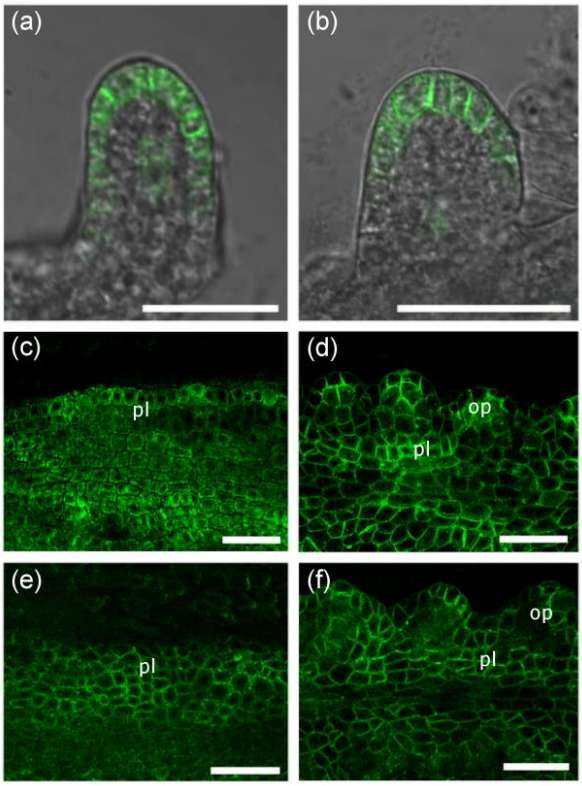

Figure S2. *PIN1* expression in wild-type, *ant-4* mutant and in BAP treated wild-type and *cuc2 pSTK::RNAi_CUC1* ovules.

(a-b) *pPIN1::PIN1-GFP* ovules at stage 1-I /1-II of ovule development in wild-type (a) and *ant-4* (b) mutant. (c-d) wild-type *pPIN1::PIN1-GFP* pistils before ovule primordia are formed (c) and ovules at stage 1-I (d) treated with BAP.

(e-f) *cuc2 pSTK::RNAi_CUC1 pPIN1::PIN1-GFP* pistils before ovule primordia are formed (e) and ovules at stage 1-I (f) treated with BAP. Scale bars: 20 µm

To verify whether CUC1 and CUC2 are able to induce *PIN1* expression *in vivo*, we performed transient expression assays in BY-2

tobacco protoplasts. A significant increase in *pPIN1::LUCIFERASE* (*pPIN1::LUC*) reporter expression, when co-transformed with CUC1 or CUC2, further confirmed that these transcription factors can act as positive regulators of *PIN1* (Figure 2f). These results confirm that CUC1 and CUC2 might directly regulate *PIN1* expression at early stages of ovule development.

To understand whether the reduction in ovule number observed in the *cuc2-1 pSTK::CUC1_RNAi* plants, might be caused by the down-regulation of *PIN1* expression we decided to up-regulate *PIN1* expression in the *cuc2-1 pSTK::CUC1_RNAi* pistil. It was shown that cytokinin 6-Benzylaminopurine **(**BAP) treatment is able to increase *PIN1* expression in pistils (Bencivenga *et al.,* 2012).

We therefore, counted the number of ovules in wild-type flowers at stage 8-9 of (L*er*) treated with mock or with 1mM BAP, at 3 days after the treatment (3DAT). We observed an increase in ovule number in the BAP treated pistil compared to the mock treated ones (Figure 2g). Similar treatments were performed using stage 8-9 *pin1-5* flowers. In this case we did not observed new ovules primordia in both the control and in the BAP treated plants confirming that PIN1 is required for the increase of ovule primordia in BAP treated plant (Figure 2g). These results are coherent with those reported by Bencivenga *et al.,* (2012). Interestingly, we also observed an increase in ovule number in *cuc2-1 pSTK::CUC1_RNAi* carpels of BAP treated inflorescences (Figure 2g). The increase in ovule numbers in the *cuc2-1 pSTK::CUC1_RNAi* background (46.04%) was comparable with the one obtained in the treated wild-type plants (46.3%) (Figure 2g). To verify whether the phenotypic complementation observed in 3DAT BAP treated plants was link to the restoration of *PIN1* expression levels, we analyzed

pPIN::PIN1-GFP (Figure S2) and performed qRT-PCR (Figure 2h), in BAP and mock wild type and *cuc2-1 pSTK::CUC1_RNAi* treated plants. This showed that the expression of *PIN1* is clearly increased in BAP treated plants (Figure 2e).

We also tested the effect of cytokinin on ovule numbers in the *ant*-4 mutant background. BAP treatment could not complement the ovule number reduction (Figure 2g) even if PIN1 expression is increased after the treatment (Figure 2e), suggesting that ANT might act in an independent pathway respect to CUC1 and CUC2 as suggested also by our genetic data.

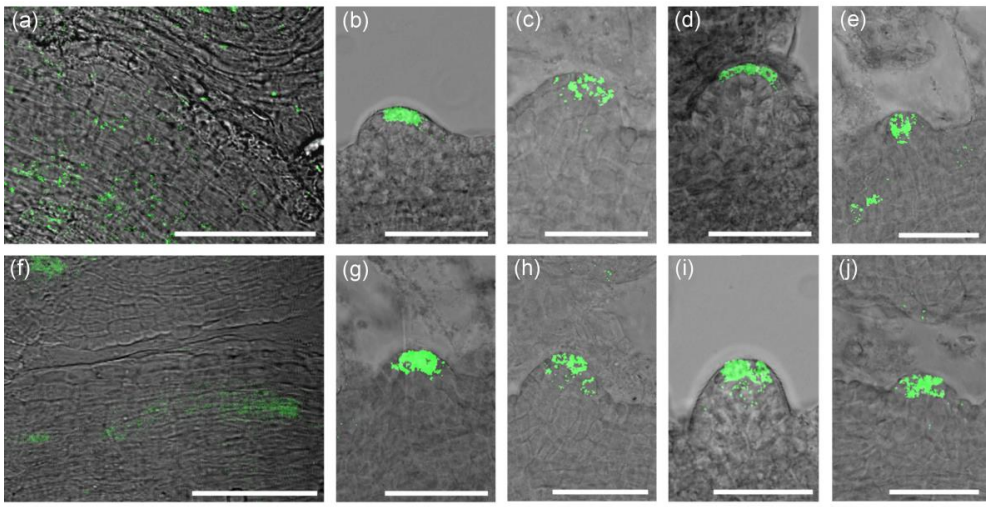

Figure S3. Auxin accumulation in developing *Arabidopsis* wild-type ovule.

(a-e) *pDR5rev::GFP* localization in wild-type and mutant pistils: wild-type stage 1-I (a), wild-type stage 1-II (b), *ant-4* stage 1-II (c), *cuc2-1 pSTK::CUC1_RNAi* stage 1-II (d), *ant-4 cuc2-1 pSTK::CUC1_RNAi* stage 1-II (e).

(f-j) *pDRN::GFP* localization in wild-type and mutant pistils: wild-type stage 1-I (f), wild-type stage 1-II (g), *ant-4* stage 1-II (h), *cuc2-1 pSTK::CUC1_RNAi* stage 1-II (i), *ant-4 cuc2-1 pSTK::CUC1_RNAi* stage 1-II (j). Scale bars: 20 µm

Auxin maxima are not affected in *cuc2-1 pSTK::CUC1_RNAi* ovule primordia

To analyze whether auxin accumulation is compromised in *cuc2-1 pSTK::CUC1_RNAi* flowers, we crossed *cuc2-1 pSTK::CUC1_RNAi* plants with the auxin-responsive reporter line *pDR5rev::GFP* (Benková *et al.*, 2003). The GFP signal was not visible using *pDR5rev::GFP* in the placenta and in ovule primordia at stage 1-I (Figure S3). Moreover the GFP signal, reflecting auxin accumulation, was visible in *cuc2-1 pSTK::CUC1_RNAi* mutant ovule primordia starting from stage 1-II (Figure S3). The expression as observed in this mutant was identical to the one of wild-type plants, suggesting that although *PIN1* expression is affected in these plants, the auxin maximum is still established in ovule primordia.

It was previously shown that *DORNRÖSCHEN* (*DRN)*, which encodes an AP2-domain transcription factor, is transcriptionally regulated by auxin signaling, and that its expression anticipates the auxin accumulation visible using the *pDR5* promoter (Chandler *et al.*, 2011). Therefore, to investigate if we could observe auxin induced signals earlier during ovule development, we crossed *cuc2-1 pSTK::CUC1_RNAi* with *pDRN::GFP* plants. In wild-type plants like in *cuc2-1 pSTK::CUC1_RNAi* plants, the *DRN* promoter was already active from stage 1-I, however we were not able to detect GFP before the ovule primordia formed (Figure S3). These experiments do not allow us to verify if in *cuc2-1 pSTK::CUC1_RNAi* plants *PIN1* down-regulation and auxin accumulation in the , however, we can conclude that once the primordia are formed, the auxin gradient along the ovule axis is not affected.

***ANT, CUC1,* and *CUC2* are targets of MONOPTEROS.**

The analysis of the *yuc1 yuc4* double mutant and the *mp* mutant suggests that auxin is also required for ovule development (Cheng *et al*., 2006; Cole *et al*., 2009; Lohmann *et al.,* 2010). The *mp S319* mutant produces few flowers all of which are defective and develop a reduced organ number and, importantly, the pistils do not develop ovules (Compare Figure 3a,b with 3c,d) (Cole *et al.,* 2009; Lohmann *et al.,* 2010). To explore whether MP could directly regulate *CUC1, CUC2* and *ANT* expression in the pistil we focused our analysis on MP expression during early stages of ovule primordium development. We analyzed *pMP::MP-GFP* plants using Confocal Laser Scanning Microscopy (CLSM). In these plants, GFP is visible in the placenta before ovule primordia are formed (Figure 3e). Once the primordia arise (stage 1-I), GFP expression is observed in the epidermal cell layer of the primordia (Figure 3f). Starting from stage 1-II the MP-GFP is mainly localized in the ovule boundaries (Figure 3g).

ANT (Figure 3h, i), *CUC1* (Figure 3j,k), and *CUC2* (Figure 3l,m) are, like MP, expressed in the placenta before ovule primordia arise and in ovules at stage 2-II.

To study if MP regulates *ANT, CUC1*and *CUC2* expression, we have performed qRT-PCR using pistils (5) of the *mp S319* mutant and of the wild type. This analysis revealed that *ANT, CUC1* and *CUC2* were all down-regulated in this mutant (Figure 4a).

Interestingly, the rosette leaf margins of the *mp S319* mutant also show less serration in respect to wild-type leaves (Figure 4b,c). This phenotype was previously described for the *cuc2* mutant (Bilsborough *et al*., 2011). Consequently, we have performed a RT-PCR to analyze whether MP controls *CUC2* expression also in leaves. As shown in Figure 4d, *CUC2* is

down regulated in *mp S319* leaves suggesting that MP controls *CUC2* expression also in these organs.

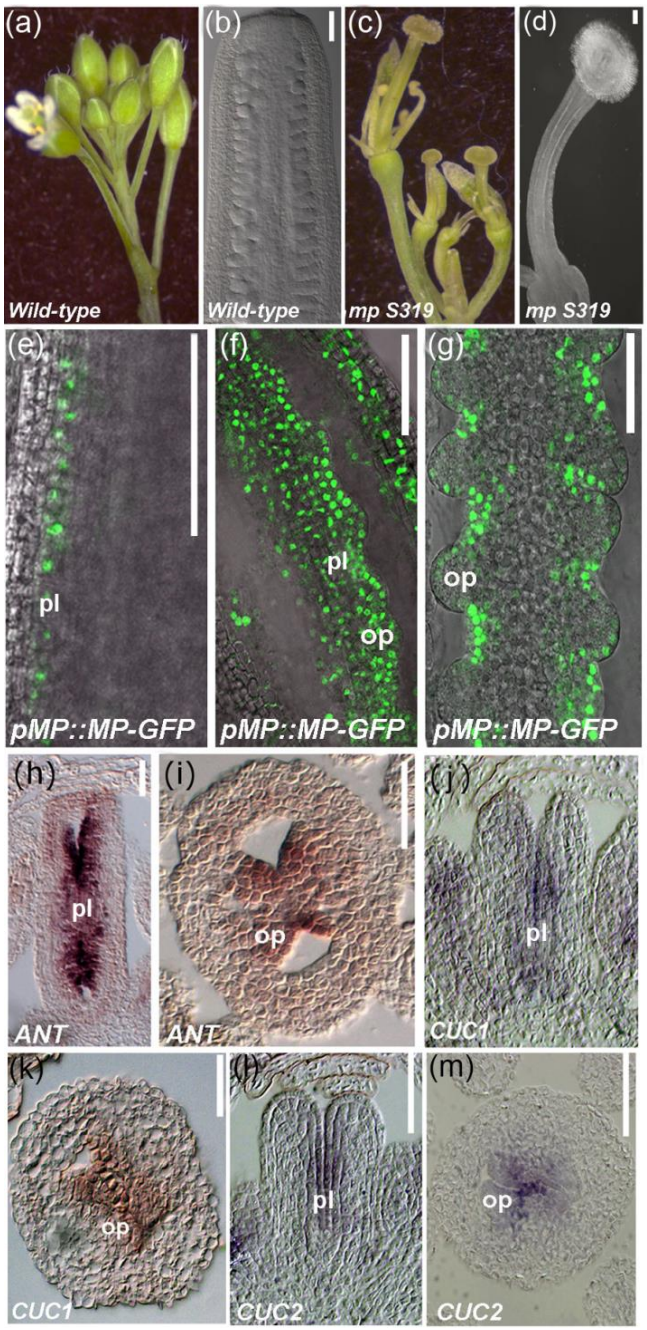

Figure 3. *MP, ANT, CUC1* and *CUC2* expression patterns.

Wild-type inflorescence (a) wild-type pistil (b) *mp S319* inflorescence (c) *mp S319* pistil (d). (e-g) the *MP* expression profile was deduced analyzing *pMP::MP-GFP* plants: a signal is observed in the placenta before ovule primordia are formed (e), in ovule primordium before stage 1-I (f) and in developing ovules at stage 1-I (g). *In situ* hybridizations were performed in a wild-type background using specific *ANT* (h-i), *CUC1* (j-k) and *CUC2* (l-m) antisense probes. (h, j, l): the stage before ovule primordia are formed; (i, k, m): stage 1-I of ovule development. pl: placenta; op: ovule primordium. Scale bars: 50 µm.

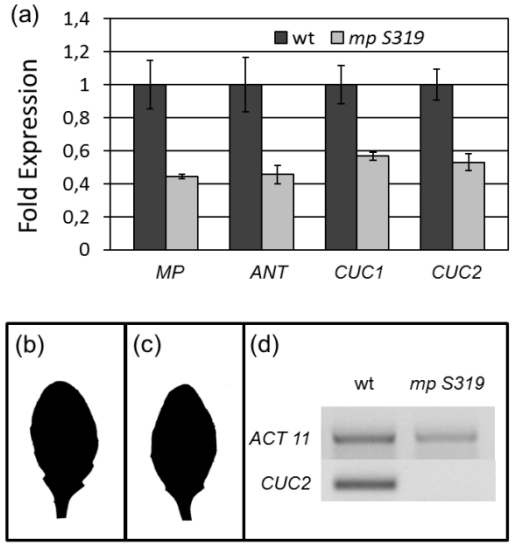

Figure 4. MP regulates the expression of *ANT*, *CUC1* and *CUC2*.

(a) *MP*, *ANT*, *CUC1* and *CUC2* expression in *mp S319* mutant pre-fertilized pistils is reduced compared to wild-type. (b-c) Silhouette of rosette leaf in wild-type (b) and *mp S319* (c) mutant background. (d) *CUC2* expression analysis by RT-PCR on wild-type and *mp S319* rosette leaf cDNAs. *ACT11* was used as a control.

Recently it has been published that ANT is a direct target of MP during (Yamaguchi et. al., 2013). To understand whether MP could directly regulate *CUC1* and *CUC2* we performed Chromatin Immuno-Precipitation (ChIP) experiments using chromatin extracted from flower tissues of *pMP::MP-GFP mp/mp* plants before fertilization and anti-GFP antibodies. The MP-GFP fusion protein fully complements the *mp* phenotype, indicating that the MP-GFP protein is biologically functional (Schlereth *et al*., 2010). Pre-fertilized wild-type inflorescences were used as negative control whereas *ARABIDOPSIS RESPONSE REGULATOR15* (*ARR15)* and *ANT*, direct target of MP, were used as positive control (Zhao *et al*., 2010; Yamaguchi *et al*., 2013).

Within the genomic regions of *CUC1* and *CUC2* starting from 3 kb upstream of the ATG start codon until 0.5 kb downstream of the STOP codon, some putative ARF binding sites were identified (Figure 5a). Three independent quantitative real-time PCRs for each of the three independent immuno-precipitated chromatin samples were performed. This analysis

showed that MP binds to the ARF binding site present on the *CUC1* genomic region (Figure 5b), to the ARF binding site present in the *CUC2* genomic region at 34 bp after the start codon (Figure 5a,b), while no evident enrichment was observed in correspondence to the ARF binding site present at 1734 bp before the *CUC2* start site (Figure 5b). These data obtained support that *CUC1,* and *CUC2* might be direct targets of MP during pistil development.

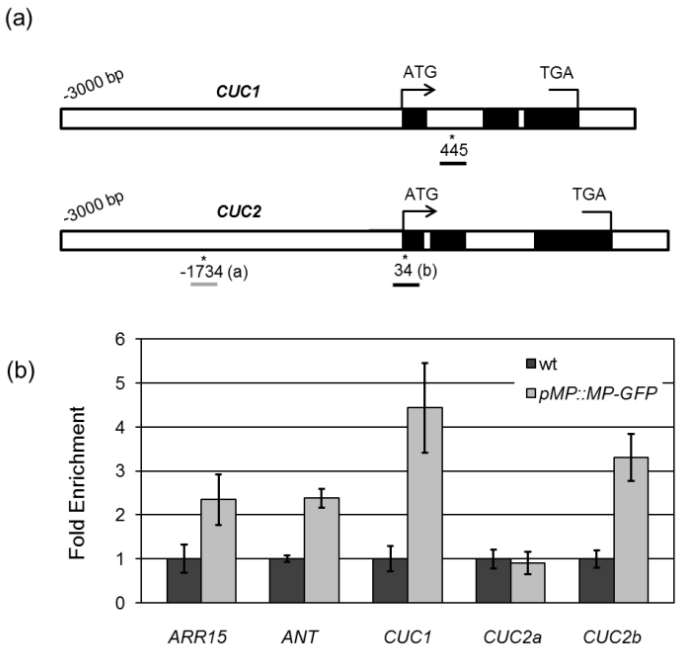

Figure 5. MP directly binds *ANT, CUC1* and *CUC2* genomic regions.

(a) Schematic representation of *CUC1* and *CUC2* genomic loci with the putative ARF binding sites (asterisks). Numbers indicate nucleotide positions from the site of initiation of translation. Black underlined asterisks indicate the enriched loci. MP binding was verified by ChIP experiments. Black boxes represent exons.

(b) ChIP experiments. Chromatin was extracted from pre-fertilized flower tissues of *pMP::MP-GFP mp/mp* plants, while wild-type pre-fertilization flowers were used as a negative control. ChIP experiments were performed using anti-GFP antibodies. Error bars indicate the propagated error value using three replicates (see Material and Methods).

Discussion

Ovule primordia formation: an integrative model.

Lateral organ formation requires the integrated action of hormones such as auxin and cytokinin which have been shown to play a pivotal role in the control of organ development (Ruzicka *et al.,* 2009). Although the local auxin response and auxin transport are needed to form the Carpel Margin Meristem (CMM) (Nemhauser *et al.,* 2000), it seems that the cytokinin pathway-related genes also play an important function in establishing ovule primordia formation (Kinoshita-Tsujimura and Kakimoto, 2011; Werner *et al.,* 2003; Bencivenga *et al*., 2012). Once the primordium is formed, PIN1 is involved in re-establishing the auxin gradient along the axis of the newly developing organ, with a maximum occurring at its tip (Benkovà *et al*., 2003). We have previously shown that *PIN1* expression and localization in ovules is controlled by cytokinin through the action of BELL1 (BEL1) and SPOROCYTELESS/NOZZLE (SPL/NZZ) (Bencivenga *et al.,* 2012). However, these two transcription factors seem to be important for establishing ovule pattern without influencing ovule number (Bencivenga *et al.,* 2012) since they are expressed from stage1-I when the primordium is already formed (Schiefthaler *et al*., 1999; Balasubramanian and Schneitz, 2000). In roots, *PIN* gene expression and PIN protein localization are also controlled by cytokinin which modulates cell-to-cell auxin transport and consequently auxin levels (Ruzicka *et al*., 2009).

That *PIN1* is involved in the determination of ovule number was inferred from the characterization of the *pin1-5* mutant since it develops fewer ovules compared to wild-type plants (Bencivenga *et al.,* 2012). According to our data, CUC1 and CUC2 are required for both correct *PIN1* expression and PIN1 localization (Figure 2c,d). The involvement of CUC2 in

PIN1 expression has already been proposed for the formation of leaf serrations (Bilsborough *et al*., 2011). Although we have shown that CUC1 and CUC2 are able to induce *PIN1* expression *in vivo* using protoplast assays, we cannot exclude the possibility that *PIN1* transcriptional activation is indirect.

Our study shows that treatments with BAP result in restored ovule numbers in *cuc2-1 pSTK::CUC1_RNAi* plants. BAP treatment however does not have any effect if PIN1 is mutated as is the case in *pin1-5* plants. It seems that cytokinin acts downstream of the CUC1 and CUC2 transcription factors to induce *PIN* expression. It will be of interest to study if this regulatory mechanism is also conserved in other organs such as leaves where it has been shown that a CUC2–dependent regulatory pathway controls PIN1-mediated auxin efflux (Bilsborough *et al*., 2011).

Finally, the observation that BAP treatment cannot complement the *ant* phenotype suggests that ANT functions in a pathway independent to that of CUC1 and CUC2. This is supported by the additive effects seen on the reduction in ovule numbers in *ant cuc2-1 pSTK::CUC1_RNAi* plants.

MP is a key player in ovule primordia formation

MP is broadly expressed and is involved in the transcriptional regulation of several auxin responsive genes (Cole *et al*., 2009; Donner *et al.,* 2009; Schlereth *et al*., 2010). Our data suggest that MP directly regulates the expression of *ANT*, *CUC1* and *CUC2* during the early stages of ovule primordia formation. Accordingly, the expression of these genes is reduced in a partial loss-of-function *mp* mutant (*mp S319)*. The role of ANT in pistil development has already been described. For instance in *ant lug* and *ant seuss* double mutants as well as in the *ant shp1 shp2 crc* quadruple mutant,

placenta formation is seriously compromised and ovules are not formed (Liu *et al*., 2000; Azhakanandam *et al.*, 2008; Wynn *et al*., 2011; Colombo *et al*., 2008). We suggest that in the pistil, MP responds to auxin by activating the expression of *ANT* which is required for correct pistil and ovule primordia formation. In this regard, it has recently been suggested that MP also regulates *ANT* expression in the floral meristem (Yamaguchi *et al*., 2013). Furthermore, we suggest that MP might regulate the expression of *CUC1* and *CUC2* in the pistil which are required for *PIN1* expression.

Based on our results we propose a model in which MP regulates *ANT*, *CUC1* and *CUC2* expression during the early stages of ovule development (Figure 6). The expression of *CUC1* and *CUC2* is necessary for correct *PIN1* expression which in turn is a prerequisite for ovule primordia formation. Once *PIN1* is expressed, an auxin maximum is formed in the apex of the nucellus (Figure. 6). This model is very similar to that proposed for the development of leaf serration (Bilsborough *et al*., 2011).

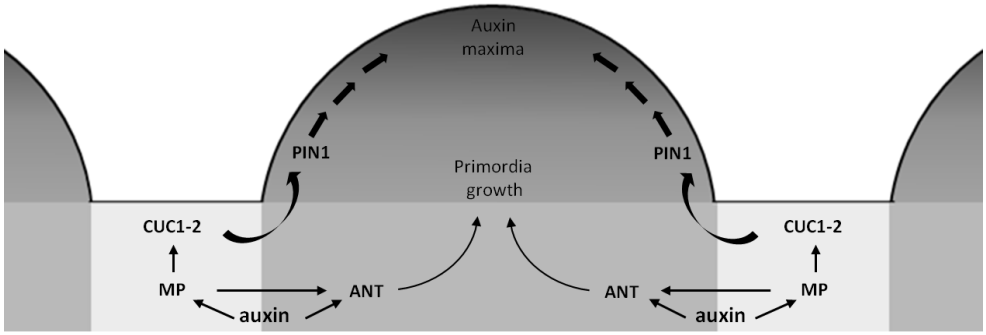

Figure 6. Regulatory network controlling ovule primordia formation.

MP is required for *ANT*, *CUC1* and *CUC2* expression during ovule primordia formation. In particular, ANT controls cell proliferation, whereas CUC1 and CUC2 control *PIN1* expression and PIN1 localization which is required for correct ovule primordia formation. Once the primordia are formed auxin is accumulated at the edge of the developing ovule.

Once ovules are formed, the interaction between auxin and cytokinin provides a signaling system for the correct growth and development of ovules (Bencivenga *et al*., 2012; Marsch-Martínezet *et al.,* 2012). Interestingly, our results seem to confirm the importance of cytokinin in controlling ovule numbers (Werner *et al*., 2003; Kinoshita-Tsujimura and Kakimoto, 2011; Bencivenga *et al.,* 2012), suggesting that the interaction between the auxin and cytokinin pathways is needed for the formation of the ovule primordia.

Experimental procedures

Plant materials

Arabidopsis thaliana wild-type and mutant plants were grown at 22°C under short-day (8 hours light/16 hours dark) or long-day (16 hours light/8 hours dark) conditions. *ant-4* (Baker *et al.*,1997), *cuc1-1* (Takada *et al*., 2001) and *cuc2-1* (Aida *et al*., 1997) mutant seeds were obtained from the Nottingham Arabidopsis Stock Center (http.//nasc.life.nott.ac.uk/). *pDRN::GFP* was obtained by Wolfgang Werr (University of Cologne, Cole *et al.,* 2009). *pMP::SV40-3xGFP* (Rademacher *et al.,* 2011), *pMP::MP:GFP mp-5/mp-5* (Schlereth *et al*., 2010) and *mp S319* (Cole *et al.,* 2009) have been described previously.

Plant treatments

BAP treatments were performed on flowers at stage 8-9 of development as previously reported by Bencivenga et al. 2012. We collected treated inflorescences 3 DAT and counted the numbers of ovules in pistils.

Optical and confocal microscopy

GUS staining was performed overnight as described previously (Liljegren *et al.*, 2000).

Siliques and carpels were collected and cleared as reported by Yadegari *et al*., (1994). Pistils were observed using a Zeiss Axiophot D1 microscope (http://www.zeiss.com) equipped with differential interface contrast (DIC) optics. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

For Confocal Laser Scanning Microscopy (CLSM) fresh material was collected, mounted in water and immediately analyzed. CLSM analysis was performed using a LEICA TCS SPE with a 488-nm argon laser line for excitation of GFP fluorescence. Emissions were detected between 505 and 580 nm. Confocal scans were performed with the pinhole at 1 Airy unit. Images were collected in the multi-channel mode and the overlay images were generated using the Leica analysis software LAS AF 2.2.0.

Plasmid Construction and *Arabidopsis* Transformation:

To construct *pSTK::CUC1_RNAi*, a specific *CUC1* fragment (nucleotides 730-925) was amplified using the primers AtP_2916 and AtP_2917 and recombined into the RNAi vector pFGC5941 (Karimi *et al*., 2002) through a LR reaction (Gateway system, Invitrogen). The promoter CaMV35S of the pFGC5941 vector was removed and substituted by the *STK* promoter (amplified with AtP_590 and AtP_591) (Kooiker *et al.*, 2005). This construct was used to transform *ant-4 cuc2* plants using the floral dip method (Clough and Bent, 1998). Detailed information about the pBGW0 and pFGC5942 vectors is available at http://www.psb.ugent.be/gateway and at

http://www.chromdb.org/rnai/vector respectively. The list of the primers used is available in Table S1.

Reverse Transcription-PCR and quantitative Real-Time (qRT-PCR) analysis

Total RNA was extracted from pistil at stage 8-10 of flower development (Roeder and Yanofsky, 2006) using the LiCl method (Verwoerd *et al.,* 1989). Total RNA was treated with the Ambion TURBO DNA-free DNase kit and then reverse transcribed using the ImProm-II™ Reverse Transcription System (Promega). The cDNAs were standardized relative to *UBIQUITIN10* (*UBI10*) and *ACTIN 2-8* (*ACT 2-8*) transcripts and gene expression analyses was performed using the iQ5 Multi Colour Real-Time PCR detection system (Bio-Rad) with a SYBR Green PCR Master Mix (Biorad). Baseline and threshold levels were set according to the manufacturer's instructions. For RT-PCR and qRT-PCR primers, see Table S1.

Protoplast transfection

Protoplast preparation and transient expression experiments were carried out as described in De Sutter et al. (2005). Protoplasts were prepared from a Bright Yellow-2 tobacco cell culture and co-transfected with a reporter plasmid containing the *fLUCIFERASE* reporter gene driven by the *PIN1* promoter, along with a normalization construct expressing *RENILLA LUCIFERASE* (*rLUC*) and effector constructs expressing *CUC1* and *CUC2*, respectively, under the control of the *35S* promoter. The reporter construct was generated as follows: *pEN-L4-PIN1-R1* vector (*PIN1* promoter 2098 bp

upstream of the coding sequence) was recombined together with *pEN-L1-fLUC-L2* by multisite Gateway LR cloning with *pm42GW7* (Karimi *et al.*, 2007). For the effector constructs, *pEN-L1-ORF-R2*, the ORFs were either CUC1 (1565 bp) or CUC2 (1128 bp) introduced ì by Gateway LR cloning into *p2GW7* for overexpression. 2 µg of each construct was added, and total effector amount was equalized in each experiment with *p2GW7-GUS* mock effector plasmid. After transfection, protoplasts were incubated overnight and then lysed; fLUC and rLUC activities were determined with the Dual-Luciferase reporter assay system (Promega). Variations in transfection efficiency and technical error were corrected by normalization of fLUC by rLUC activities. All transactivation assays were conducted in an automated experimental set-up that involved eight separate transfection experiments and were performed at least twice.

Chromatin immunoprecipitation (ChIP) assays

ChIP experiments were performed as a modified version of a previously reported protocol (Gregis *et al*., 2008) using the commercial antibody GFP:Living Colors full-length A.v. polyclonal antibody (Clontech). Chromatin was extracted from stage 8-10 flowers of p*MP::MP-GFP* plants and from wild-type plants (Col-0) as a control. The DNA fragments obtained from the immune-precipitated chromatin were amplified by qRT-PCR using specific primers (see table S1). Three real-time PCR amplifications on three independent chromatin extractions were performed. For the complete primer sets see Table S1. Enrichment of the target region was determined using the iQ5 Multi Colour Real-Time PCR detection system (Bio-Rad) with a SYBR Green PCR Master Mix (Biorad). The qRT-

PCR assays and the fold enrichment calculation were performed as previously described (Matias-Hernandez *et al*., 2010).

In situ hybridization

In situ hybridization experiments were performed as previously described (Dreni *et al*., 2011). The *ANT*, *CUC1* and *CUC2* probes were amplified as described in Elliott *et al.*, (1996) for *ANT* and Aida et al. (2002) for *CUC1* and *CUC2*, and subsequently cloned in the pGEMT-Easy Vector (Table S1).

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Table S1. List of primers.

Primers used for *in situ* hybridization

CUC1 fw	AtP_3733	5'-TGAATGGGTTCTCTGTAAAG-3'
CUC1 rev	AtP_3734	5'-TAATACGACTCACTATAGGGCAGAGAGTAAACGGCCACAC-3'
CUC2 fw	AtP_3731	5'-ATGAATGGGTGATCTCTAGG-3'
CUC2 rev	AtP_3732	5'-TAATACGACTCACTATAGGGCAAATACAGTCAAGTCCAGC-3'

Primers used for reverse transcription PCR and quantitative Real Time PCR

CUC1 fw	RT 332	5'-TGCATGAGTATCGCCTTGAC-3'
CUC1 rev	RT 333	5'-GGTGACGGCAGAAGAAGAAG-3'
NAC1 fw	RT 328	5'-TCAGATTTCACCCGAAGGAC-3'
NAC1 rev	RT 329	5'-CCGTTGCTCGGTTAGTTCTC-3'
At5g07680 fw	RT 356	5'-ATTGGGTGATGCATGAGTATAGGC-3'
At5g07680 rev	RT 357	5'-GGTTTTGTCGTTGTATGGTGAAGAAT-3'
At5g61430 for	RT 358	5'-TCCTGGGTTCAGGTTTCATC-3'
At5g61430 rev	RT 359	5'-GGCTTCAGTTGCTCGGTTAG-3'
MP fw	RT 513	5'-GTTCATCAGGGATGAGAAGTCAC-3'
MP rev	RT 514	5'-CAAGAACACCGATGTGCATACTA-3'
ANT fw	AtP_3398	5'-CACTCAGATCTGATGGTTCTC-3'
ANT rev	AtP_3399	5'-GGGAAGCTAAAGAACTCTTG-3'
CUC2 fw	RT 360	5'-AAAGGAAGAGCTCCGAAAGG-3'
CUC2 rev	RT 361	5'-TCACAGTTGCTCCTCCTCCT-3'
CUC2 rev	RT 923	5'-TTACGCTCACAGTTGCTCCTC-3'

Ubiquitin 10 fw	RT 147	5'-CTGTTCACGGAACCCAATTC-3'
Ubiquitin 10 rev	RT 148	5'-GGAAAAAGGTCTGACCGACA-3'
PP2A fw	RT 670	5'-CAGCAACGAATTGTGTTTGG-3'
PP2A rev	RT 671	5'-AAATACGCCCAACGAACAAA-3'
Retrotranscription fw	VeP_0021	5'-GCCAAAGCAGTGATCTCTTTGCTC-3'
Retrotranscription rev	VeP_0033	5'-CACTCCTGCCATGTATGTCGCTAT-3'

Primers used for Plasmid Construction

CUC1_RNAi fw	AtP_2916	5'-CACCAGCCACGTCACGTCGGTGATG-3'
CUC1_RNAi rev	AtP_2917	5'-TAAACGGCCACACACTCACGG -3'
miR164 fw	AtP_2248	5'-CACCTCACGTTTTCAAATATCAAACC-3'
miR164 rev	AtP_2249	5'-TCTCCTGTCTAATACTCGCTAACC-3'
STK promoter 1 fw	AtP_590	5'-CTCAGAATTCGTTGGGTATGTTCTCACTTTC-3'
STK promoter 1 rev	AtP_591	5'-GTCACTCGAGTCCCATCCTTCATTTTAAACAT-3'
35S fw	AtP_1663	5'-CGAGCTCGCGGCCATGCTAGAGTCCGC-3'
35S rev	AtP_1664	5'-CGAGCTCGAGGTCACTGGATTTTGGTTTTAGG-3'
STK promoter 2 fw	AtP_1507	5'-TCTGACGTCAGGCGTTTTTGTTGGGTATGTTCTCAC-3'
STK promoter 2 rev	AtP_1508	5'-TCTGACGTCAGGCATCCTTCATTTTAAACATC-3'
AP1 promoter fw	AtP_3082	5'- CCGAGCTCTCAAAACTCAGGACGTACAT-3'
AP1 promoter rev	AtP_3083	5'- CCACTAGTAGCTCAGACTTTGGTATGAA-3'

Table S2. Number of ovules in BAP treated pistils.

	mock*	+ 1 mM BAP		
		2 DAT	3 DAT	4 DAT
wild-type	51,8 +/-0,6 (37)	62 +/- 1,6 (9)	70,1 +/- 3,2 (7)	75,2 +/- 2,9 (15)
cuc2-1 pSTK::RNAi_CUC 1	41,7 +/- 0,9 (31)	54,7 +/- 2,8 (25)	60,0 +/-2,6 (18)	60,9 +/- 2,0 (13)
ant-4	17,8 +/- 0,7 (5)	17,7 +/- 0,9 (7)	17,9 +/- 0,8 (15)	17,9 +/- 1,2 (6)
pin1-5	8,6 +/- 2 (15)	7,3 +/- 2,6 (3)	7,5 +/- 3,5 (12)	8,4 +/- 1,8 (4)

Number of ovules counted for mock- and BAP-treated wild-type, *cuc2-1 pSTK::CUC1_RNAi ant-4* and *pin1-5* pistils at 2, 3 and 4 DAT. Asterisks indicate the mean of the number of ovules in the mock-treated pistils at 2, 3 and 4 DAT. The total number of carpels analyzed is given in parentheses.

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VERDANDI Is a Direct Target of the MADS Domain Ovule Identity Complex and Affects Embryo Sac Differentiation in *Arabidopsis* [W]

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In *Arabidopsis thaliana*, the three MADS box genes *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*), and *SHP2* redundantly regulate ovule development. Protein interaction studies have shown that a multimeric complex composed of the ovule identity proteins together with the SEPALLATA MADS domain proteins is necessary to determine ovule identity. Despite the extensive knowledge that has become available about these MADS domain transcription factors, little is known regarding the genes that they regulate. Here, we show that *STK*, *SHP1*, and *SHP2* redundantly regulate *VERDANDI* (*VDD*), a putative transcription factor that belongs to the plant-specific B3 superfamily. The *vdd* mutant shows defects during the fertilization process resulting in semisterility. Analysis of the *vdd* mutant female gametophytes indicates that antipodal and synergid cell identity and/or differentiation are affected. Our results provide insights into the pathways regulated by the ovule identity factors and the role of the downstream target gene *VDD* in female gametophyte development.

INTRODUCTION

In *Arabidopsis thaliana*, ovule primordia appear from the placental tissue at stage 8 of flower development (Smyth et al., 1990). Ovule differentiation is complete at stage 13, when the mature embryo sac awaits fertilization (Schneitz et al., 1995). It has been shown that the MADS box genes *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*), and *SHP2* redundantly regulate the identity of the ovule integuments that develop from the chalazal region, since in the *stk shp1 shp2* triple mutant, the integuments are transformed into carpelloid structures leading to complete sterility (Pinyopich et al., 2003; Brambilla et al., 2007). Furthermore, genetic and protein interaction studies have shown that these ovule identity factors interact with SEPALLATA (SEP) MADS domain factors and that these interactions are essential for their function in ovule development (Favaro et al., 2003). It is clear that these MADS domain transcription factors are key regulators of ovule development. However, there is very limited information about the genes that are regulated by them. It could well be that they are involved in the regulation of both sporophyte and gametophyte development since not only integument development but also embryo sac development was arrested in the *stk shp1 shp2* triple mutant (Brambilla et al., 2007; Battaglia et al., 2008).

Differentiation of the embryo sac occurs contemporarily and in coordination with the development of the diploid sporophytic tissues of the ovule. Megasporogenesis takes place in the nucellus when integument primordia elongate from the chalazal region. Shortly after, the functional megaspore undergoes three rounds of mitosis without cellularization to form the syncytial female gametophyte, or embryo sac, with eight haploid nuclei. Subsequently, nuclear migration and cellularization take place, and the mature female gametophyte consists of seven cells: three antipodal cells, two synergid cells, one egg cell, and one central cell containing two polar nuclei that fuse prior or during fertilization (Schneitz et al., 1995). The formation of the next sporophytic generation depends on long- and short-range interactions between male and female gametophytes. The male gametophyte, or pollen tube, follows chemotactic signals produced by the female gametophyte and is guided into the micropylar opening of the ovule (Hülskamp et al., 1995; Ray et al., 1997). As the pollen tube approaches the micropyle, one of the synergid cells initiates degeneration and is penetrated by the pollen tube, which arrests its growth, bursts, and releases the two sperm cells to ensure double fertilization. These processes are referred to as pollen tube guidance and reception (reviewed in Weterings and Russell, 2004). Both male and female gametophytes play fundamental roles in the control of male gamete delivery (Johnson and Lord, 2006).

Recently, some of the mechanisms underlying double fertilization in angiosperms have been dissected at the molecular level. The identification and characterization of female gametophytic mutants showing defects in embryo sac cell differentiation

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allowed the determination of the contributions of specific embryo sac cells to pollen tube guidance and reception (Higashiyama et al., 2001; Huck et al., 2003; Kasahara et al., 2005; Portereiko et al., 2006; Chen et al., 2007; Pagnussat et al., 2007; Punwani et al., 2007; Bemer et al., 2008; Shimizu et al., 2008; Steffen et al., 2008; Okuda et al., 2009; Srilunchang et al., 2010). A key role during the fertilization process is played by the synergid cells. These haploid cells are not only responsible for the production and secretion of a signal that guides the pollen tubes toward the embryo sac (Higashiyama et al., 2001; Kasahara et al., 2005; Okuda et al., 2009, Tsukamoto et al., 2010), but they also mediate pollen tube reception (Huck et al., 2003; Rotman et al., 2003; Escobar-Restrepo et al., 2007). Once pollen tubes correctly reach the micropyle, synergid-specific expression of the FERONIA (FER) receptor-like kinase is required for pollen tube growth arrest, rupture, and sperm cell discharge (Huck et al., 2003; Escobar-Restrepo et al., 2007). In *fer* mutants, the pollen tubes fail to arrest and keep growing within the embryo sac, leading to pollen tube overgrowth (Huck et al., 2003; Rotman et al., 2003). Such pollen tube overgrowth has also been observed in the absence of the *LORELEI* function (Capron et al., 2008), in self-fertilized *absence of mutual consent* mutants (Boisson-Dernier et al., 2008), and in *scylla* (*syl*) mutant embryo sacs (Rotman et al., 2008). Interestingly, at low frequency, *syl*/*SYL* heterozygous plants show proliferation of the central cell nucleus in the absence of fertilization, indicating that the pollen tube overgrowth phenotype may also depend on some central cell functions (Rotman et al., 2008).

Here, we report the functional characterization of *VERDANDI* (*VDD*), a direct target gene of the ovule identity factors STK, SHP1, and SHP2. *VDD* plays a role in female gametophyte development and fertilization. Since in the *vdd* mutant both synergids and antipodal cells lose their cellular identity, we named this mutant after one of the three Norns, the goddesses of fate in Norse mythology (Brodeur, 1916). Although pollen tubes get attracted, the transmission efficiency of the *vdd* mutant allele is drastically reduced through the female gametophyte due to a defect in the subsequent fertilization process.

RESULTS

Identification of Genes Expressed in Ovule Primordia

In the model plant *Arabidopsis*, ovule identity is redundantly regulated by the activity of the MADS box genes *STK*, *SHP1*, and *SHP2* (Pinyopich et al., 2003). Genetic and biochemical evidence showed that ovule identity proteins interact with SEP factors in order to function (Favaro et al., 2003). These interactions might result in higher-order MADS domain complexes as has been suggested previously (Egea-Cortines et al., 1999). To identify target gene(s) of the ovule identity complex, we isolated cells from ovule primordia (stage 8-9 of flower development) using laser microdissection (see Supplemental Figure 1 online). RNA was extracted, amplified, and used for RNA profiling studies based on the Affymetrix ATH1 GeneChip. Using the GeneSpring 7.2 program, these expression data were analyzed, revealing that more than 14,000 genes are expressed in ovule primordia (see Supplemental Data Set 1 online). For our further analyses of these candidates, we considered only genes that were annotated as putative transcription factors. The reason for doing this is that we would like to study the network that is regulated by these MADS domain factors and because until now the majority of the direct target genes that have been identified for MADS domain proteins encode transcription factors (Sablowski and Meyerowitz, 1998; Wagner et al., 1999; Hepworth et al., 2002; Lamb et al., 2002; Ito et al., 2004; William et al., 2004; Gómez-Mena et al., 2005; Sundström et al., 2006). This reduced our set of genes to 1024 putative transcription factor–encoding genes (see Supplemental Data Set 2 online).

Since MADS domain proteins recognize and bind CArG boxes [$CC(A/T)_6GG$] (Nurrish and Treisman, 1995), we further restricted our sample through the identification of ovule primordial-expressed transcription factors that contain CArG box consensus sequences in their genomic region (see Methods). For each gene, we considered a region comprising 3 kb upstream of its putative transcription start site, its complete coding sequence, including introns, and 1 kb downstream of the termination codon. Since the ovule identity proteins probably form a higher-order MADS domain complex that binds to multiple CArG boxes (Egea-Cortines et al., 1999; Favaro et al., 2003), we considered only those genes that had at least two MADS domain binding sites in their genomic region, with a distance between them of <300 bp. These selection criteria allowed us to identify a subset of 15 transcription factor–encoding genes as putative targets of the ovule identity complex (see Supplemental Table 1 online).

VDD Is a Direct Target of the Ovule Identity Complex

To investigate which of these 15 genes were indeed targets of the ovule identity MADS domain factors, chromatin immunoprecipitation (ChIP) experiments were performed using an STK antibody. Since STK is redundant for its ovule identity function with SHP1 and SHP2, we used flower tissue isolated from the *shp1 shp2* double mutant for these experiments. This approach likely increased the amount of STK protein that binds to the target sites and optimized the ChIP analysis.

These experiments demonstrated the binding of STK to CArG box-containing regions in three of the 15 putative targets (see Supplemental Table 1 online). Here, we focus on the *VDD* gene encoding a transcription factor belonging to a poorly characterized gene family, namely, the reproductive meristem (REM) family (Franco-Zorrilla et al., 2002; Swaminathan et al., 2008; Romanel et al., 2009). Sequence analysis of the *VDD* genomic region revealed the presence of three putative CArG boxes (Figure 1A). Quantitative real-time PCR performed on chromatin immunoprecipitated using the anti-STK antibody showed an enrichment of the genomic regions containing CArG boxes 1 and 3, while the putative CArG box 2 sequence was not bound by STK (Figure 1B). Chromatin immunoprecipitated from the *stk* single mutant was used as negative control. These data strongly indicate that the STK protein directly interacts with the promoter region of the *VDD* gene.

Since SEP proteins are necessary for the formation of ovule identity protein complexes (Favaro et al., 2003), we also tested the binding of SEP3 to the CArG box–containing region of *VDD*.

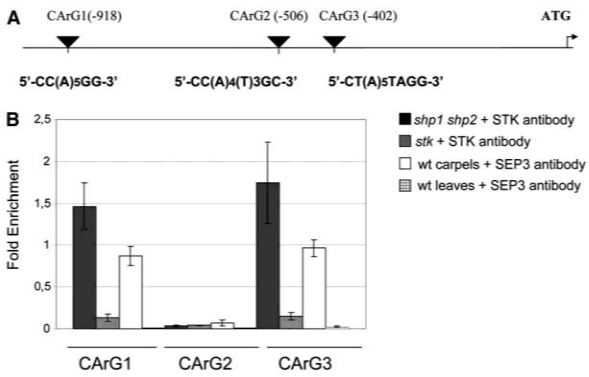

Figure 1. Quantitative Real-Time PCR on Chromatin Immunoprecipitated with STK and SEP3 Antibodies.

(A) Schematic representation of the position of the CArG boxes in the promoter region of the *VDD* gene.
(B) ChIP enrichment tests by quantitative real-time PCR show STK- and SEP3-specific binding to the CArG boxes 1 and 3. The *stk* single mutant was used as a negative control in the STK-ChIP and wild-type leaves as negative control for the SEP3-ChIP assays. Fold enrichment was calculated over the negative controls. Error bars represent the propagated error value using three replicates (see Methods).

Our results showed an enrichment of fragments containing the same CArG boxes (1 and 3) that are bound by the STK protein (Figure 1B). These results demonstrate that the ovule identity protein complex composed of SEP3 and STK proteins directly interacts with the promoter region of the *VDD* gene.

VDD Is Not Expressed during Ovule Development in the *stk shp1 shp2* Triple Mutant

We studied the spatial and temporal expression pattern of *VDD* through quantitative real-time PCR (Figure 2A). This experiment showed that *VDD* is highly expressed in the reproductive tissues and during early stages of seed development. The amount of *VDD* transcript strongly decreased during late stages of seed formation (Figure 2A). To analyze better the *VDD* expression profile at the cellular level, in situ hybridization experiments were performed (Figures 2B to 2E). This analysis revealed that *VDD* is expressed in the inflorescence and floral meristems (Figure 2B). During ovule formation, *VDD* is expressed during all the stages of ovule development, including the developing embryo sac (Figures 2C to 2E).

The *VDD* expression profile in the developing ovule and megagametophyte was confirmed by analyzing reporter gene expression of a construct consisting of 1221 bp upstream of the *VDD* translation start site, the complete genomic *VDD* coding region fused to the *uidA* reporter gene that encodes β-glucuronidase (GUS), and 389 bp corresponding to the *VDD* 3′ untranslated region. Transgenic plants transformed with this construct showed GUS activity in the same tissues where we observed *VDD* expression in our in situ hybridization experiments before fertilization (Figures 3A to 3D). However, we could not detect GUS expression in the developing seeds, indicating that this construct did not include the regulatory regions that are necessary for *VDD* transcription following fertilization (Figure 3E). We did not test longer constructs because the neighboring genes immediately flank the sequences we used: *At5g18005* maps 1222 bp upstream of the *VDD* start site, while *At5g17990* is located immediately downstream of the 3′ untranslated region.

Since *VDD* is a direct target of STK, we were interested in investigating whether the expression of *VDD* is regulated by the ovule identity factors. Therefore, we analyzed *VDD* expression in the *stk*, *shp1 shp2*, and *stk shp1 shp2* mutants (Figures 2F to 2N). Compared with the expression in wild-type ovules, a reduction in expression of *VDD* was observed in the *stk* single mutant (Figures 2G and 2H). Interestingly, no hybridization signal could be detected in *stk shp1 shp2* triple mutant ovules, indicating that *VDD* expression within the ovule is strictly dependent on the activity of the ovule identity factors STK, SHP1, and SHP2 (Figures 2M and 2N). *VDD* was normally expressed in other floral tissues of the triple mutant, suggesting that other factors may regulate *VDD* expression in these organs (Figure 2L).

The *vdd-1* Mutation Causes a Female Gametophytic Defect

To understand the function of *VDD*, we characterized the *vdd-1* mutant allele that carries a T-DNA insertion in the first intron (Figure 4A). Segregation analysis of the progeny of plants heterozygous for the *vdd-1* allele revealed a distorted segregation from the expected 1:2:1 ratio (wild type:*vdd-1*/*VDD*:*vdd-1*). We obtained wild-type and heterozygous plants in a segregation ratio 1.0:1.3 but did not recover any plant homozygous for the *vdd-1* mutation (Table 1).

To test whether female, male, or both gametophytes were affected due to the lack of *VDD* activity, we performed reciprocal crosses of *vdd-1*/*VDD* and wild-type plants. Crossing wild-type female with heterozygous pollen showed that transmission

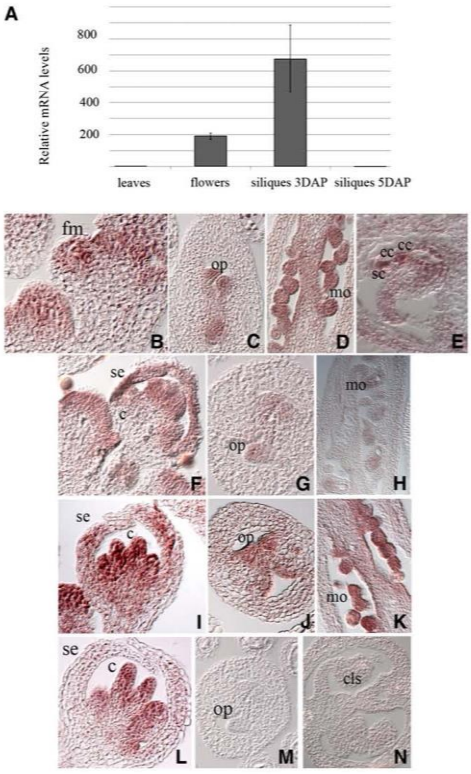

Figure 2. Spatial and Temporal Expression Pattern of *VDD* in Wild-Type, *stk*, *shp1shp2* Double, and *stk shp1 shp2* Triple Mutant Backgrounds.

(A) Quantitative real-time RT-PCR performed on cDNA obtained from leaves, flower, and siliques at 3 d after pollination (DAP) and siliques at 5 DAP. The relative mRNA levels indicate that *VDD* is strongly expressed in the reproductive tissues before fertilization and in the early stages of seed development. Error bars represent the propagated error value using three replicates.

(B) to **(E)** In situ hybridization experiment performed in wild-type plants.

(B) *VDD* is expressed in the floral meristem and in developing carpels and stamens.

(C) During ovule formation *VDD* mRNA is present in ovule primordia.

(D) *VDD* expression is detectable during later stages of ovule formation.

(E) In the mature embryo sac, *VDD* transcripts are found in the synergids, egg, and central cells.

(F) to **(H)** In situ hybridization experiment performed in the *stk* single mutant background.

(F) *VDD* is expressed in young flowers.

(G) Within ovule primordia, the signal is reduced compared with wild-type plants.

(H) Mature ovules show a decreased signal compared with wild-type plants.

efficiency (TE; Howden et al., 1998) via the male gametophyte was only slightly reduced (TE_{male}= 84.8%) and not significantly different from the expected 1:1 segregation (χ^2 test, P value = 0.17). By contrast, crossing a heterozygous female with wild-type pollen showed a strongly reduced TE through the female gametophyte (TE_{female}= 27.5%), significantly deviating from the expectation (P value = 2.7 e-20) (Table 1). Assuming a normal TE through the male gametophyte, we expected to obtain 6.9% *vdd-1/vdd-1* homozygous seedlings in the progeny of a selfed plant heterozygous for the *vdd-1* mutation. However, such homozygous plants were not identified (n = 212), indicating that homozygous *vdd-1* is also zygotically lethal.

Analysis of the siliques of plants heterozygous for the *VDD* T-DNA insertion showed that two different ovule abortion phenotypes could be distinguished (Figures 4B and 4C): abortions of ovules that were not fertilized (35%), which are expected to be due to the female gametophyte defect, and seed abortion postfertilization (10%, n = 5451). The latter phenotype probably explains why we did not observe homozygous *vdd-1* plants. In siliques of wild-type sibling plants grown under the same conditions, ovule abortion was around 2%, which is in agreement with previously reported observations (Acosta-García and Vielle-Calzada, 2004). In this article, we will focus our attention on the role of *VDD* during female gametophyte formation. Concerning the seed phenotype, differential interference contrast (DIC) microscopy analysis showed that 8% of the developing seeds in the *vdd-1* heterozygous siliques were arrested at the globular stage, indicating that *VDD* plays a role during the early stages of seed formation (see Supplemental Figure 2 online).

To confirm that the female gametophytic defect was caused by the loss of *VDD* activity, we performed complementation experiments using plants heterozygous for the *vdd-1* mutation. Into these plants, we introduced a binary vector carrying the genomic region of the *VDD* gene, which included 1221 bp upstream of the translation start site, the complete genomic coding region, and 389 bp downstream of the stop codon. As already described, the reporter gene construct containing the same *VDD* sequences showed GUS expression in the young ovules and during female gametophyte development, whereas it was not expressed during seed formation (Figure 3). The T-DNA also encodes a visible selection marker (enhanced yellow fluorescent protein [EYFP]) under the control of the strong napin

(I) to **(K)** In situ hybridization experiment performed in the *shp1 shp2* double mutant background.

(I) *VDD* is expressed in young flowers as in wild-type plants.

(J) Ovule primordia in the *shp1 shp2* mutant plants express *VDD*.

(K) The hybridization signal is visible in mature ovules.

(L) and **(M)** In situ hybridization experiment performed in the *stk shp1 shp2* triple mutant background.

(L) *VDD* is expressed in developing stamens and carpels.

(M) and **(N)** *VDD* transcripts are not detected in the ovule primordia **(N)**. No hybridization signal is visible in the carpel-like structures that develop inside the *stk shp1 shp2* mutant carpel.

c, carpel; cls, carpel-like structures; cc, central cell; ec, egg cell; fm, floral meristem; mo, mature ovules; op, ovule primordia; sc, synergid cells; se, sepal.

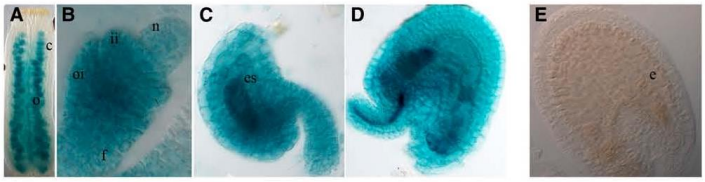

Figure 3. Expression of the *pVDD:VDD-GUS* Reporter Gene.

(A) *GUS* expression (blue color) is detectable during ovule formation.
(B) At stage 12 of flower development, *GUS* transcript is present in the funiculus, integuments, and nucellus.
(C) Within the ovule, the *VDD* promoter drives the expression of the reporter gene in the gametophytic and sporophytic tissues.
(D) The *GUS* reporter gene is transcribed in the mature female gametophyte and in the integuments.
(E) Following fertilization, the selected *VDD* promoter was not active in the developing endosperm and embryo.
c, carpel; e, embryo; es, embryo sac; f, funiculus; ii, inner integument; n, nucellus; o, ovules; oi, outer integument.

seed-specific promoter, allowing visible selection of transformant seeds (Stuitje et al., 2003; Battaglia et al., 2006). Selected T1 transformants were tested for the presence of the complementation construct and the presence of the *vdd-1* allele. Analysis of the siliques in six of these T1 lines showed a reduced seed abortion rate in three plants. Since the T-DNA was hemizygous in T1 plants, we isolated EYFP positive seeds and selected four T2 plants that had only EYFP-positive seeds, indicating that the complementation construct was homozygous and did not segregate. When we analyzed the siliques of these plants, we did not detect abortion due to unfertilized ovules; as expected, we still observed early seed abortion (Figure 4D). Furthermore, segregation analysis of the *vdd-1* allele in plants ($n = 48$) obtained from these seeds showed a segregation ratio of 1:2 (wild type:*vdd-1*/*VDD*), and no homozygous *vdd-1* mutants were identified. This suggests that we successfully complemented the female gametophytic defect caused by the absence of *VDD* activity. Because at the time of manuscript preparation there were no other *vdd* mutant alleles available, we also investigated *VDD*'s role during embryo sac formation by silencing *VDD* in wild-type plants via an artificial microRNA (Schwab et al., 2006) specific for the *VDD* gene. We expressed this artificial microRNA under the control of the *STK* promoter (*pSTK*; Kooiker et al., 2005). Analysis of the siliques of transgenic plants (six siliques for each plant) expressing the *pSTK:amiR-vdd* construct showed ovule abortion due to nonfertilization, whereas we did not observe increased early seed abortion in comparison to wild-type plants (Figure 4E). Since the *pSTK* promoter is active before fertilization, these results support the hypothesis that the *vdd-1* mutation caused the female gametophytic defects. Due to the presence of wild-type VDD activity during seed formation, transgenic plants carrying the *pSTK:amiR-vdd* construct did not show seed abortion (Figure 4E).

Pollen Tube Guidance Is Normal but Fertilization Does Not Occur in *vdd-1* Mutant Ovules

To examine whether the observed fertilization defect arose from defective embryo sac development, DIC microscopy analysis was performed on heterozygous *vdd-1* plants. Interestingly, analysis of unpollinated mature ovules showed that all ovules in heterozygous *vdd-1* plants were morphologically indistinguishable from each other and embryo sacs were composed of seven cells as in the wild type (see Supplemental Figure 2 online).

Subsequently, we analyzed by aniline blue staining whether the *vdd-1* phenotype is due to a defect in pollen tube guidance (Figures 5A and 5B). In wild-type plants, pollen tubes grow along the transmitting tract toward the funiculus and subsequently to the micropyle and into the female gametophyte. When we pollinated *vdd-1* heterozygous plants with wild-type pollen, 86% of pollen tubes successfully reached the micropyle ($n = 545$). These results were not statistically different compared with wild-type plants in which 88% of pollen tubes reached the micropyle ($n = 229$).

Despite the fact that *vdd-1* mutant embryo sacs retained the capability to attract and guide pollen tubes, we investigated whether fertilization occurs once that pollen tube enters the *vdd-1* mutant female gametophyte. Therefore, we pollinated pistils of wild-type and heterozygous *vdd-1* mutant plants with pollen obtained from plants homozygous for the MINISEED3:GUS construct (Luo et al., 2005). As expected, following double fertilization, 90% of the ovules in wild-type pistils expressed the *GUS* reporter gene in the endosperm (Figure 5E). By contrast, ~30% of the ovules in heterozygous *vdd-1* mutant plants did not show GUS activity (Figure 5F), suggesting that fertilization did not occur in these ovules. Since we did not observe pollen tube overgrowth in the *vdd-1* female gametophyte, it is most likely that a subsequent step, such as sperm cell delivery or gamete fusion, is affected in *vdd-1* female gametophytes.

To investigate this further, we analyzed whether the synergid cells degenerated in heterozygous *vdd-1* plants. In wild-type plants, pollen tube entrance in the micropyle is followed by degeneration of one synergid cell, which is revealed by intense fluorescence using confocal laser scanning microscopy (CLSM) (Christensen et al., 1997). As expected, when we pollinated wild-type pistils, high fluorescence was visible in almost all the embryo sacs at 12 h after pollination, suggesting that synergid degeneration occurred (Figure 5C). Interestingly, when we pollinated pistils of *vdd-1* heterozygous plants using wild-type

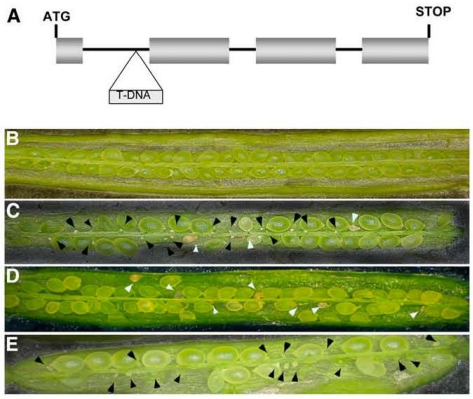

Figure 4. Seed Set in Wild-Type, *vdd-1/VDD* Heterozygous Plants, Complemented *vdd-1* Heterozygous Plants, and *pSTK:amiR-vdd* Plants.

(A) Schematic representation of the *vdd-1* mutant allele. T-DNA is inserted in the first intron, 44 bp upstream the 3′ splicing acceptor site.
(B) Wild-type silique showing full seed set.
(C) Siliques of *vdd-1/VDD* plants containing aborted ovules (black arrows) and aborted seeds (white arrows).
(D) Siliques of *vdd-1/VDD* plants complemented with the genomic region of the *VDD* gene. The complementation construct is able to rescue *VDD* expression in the female gametophyte. Aborted seeds (white arrowheads), but not aborted ovules, are present.
(E) Siliques of wild-type plants transformed with the *pSTK:amiRvdd* construct. Aborted ovules only (black arrowheads) are present.

pollen, we observed frequently no synergid degeneration (Figure 5D), which might explain the observed defect in fertilization.

VDD Affects Accessory Cell Differentiation in the Embryo Sac

To investigate whether the identities of the *vdd-1* embryo sac cells, in particular the synergids, were altered, the expressions of different cell-specific molecular markers were analyzed (Figure 6; see Supplemental Table 2 online). The marker lines were crossed with the heterozygous *vdd-1* mutant and reporter gene expression was analyzed in the F2 generation. The expressions of the egg cell-specific marker EG1 (Gross-Hardt et al., 2007) and a central cell-specific marker (Chaudhury et al., 1997) were not altered in any of the embryo sacs (Figures 6A to 6D), indicating that gametic cell fate was not affected.

When we analyzed accessory cell (synergid and antipodal) specification, we observed an abnormal expression profile for antipodal and synergid cell markers when compared with wild-type female gametophytes (Figures 6E to 6G). The expression of the antipodal cell marker (Yu et al., 2005) was analyzed at 24, 48, and 72 h after emasculation (HAE) in wild-type and *vdd-1/VDD* heterozygous siliques. We analyzed the expression at 24 HAE to exclude the possibility that our observations were influenced by the degeneration of antipodal cells. Unlike previously reported (Murgia et al., 1993; Christensen et al., 1997), we did not observe antipodal cell death prior to fertilization, and they were still clearly present at this time point. In heterozygous plants, at 24 HAE, only 55% of the ovules expressed the antipodal cell marker, whereas we did not detect GUS expression in the remaining ovules (n = 309). Interestingly, at 48 HAE, 6% of the ovules expressed the antipodal cell marker in the synergids instead of the antipodal cells, whereas in 38% of the megagametophytes, we were not able to detect GUS expression (n = 480). The rest of the ovules showed antipodal cell expression. At 72 HAE, the number of megagametophytes that showed GUS expression in the synergids further increased to almost 50% (n = 498) (Figure 6E). At 24, 48, and 72 HAE wild-type sibling plants showed GUS expression in the antipodal cells in 98, 97, and 98% of the megagametophytes, respectively (n = 324, 403, and 456, respectively).

Analysis of the expression of the synergid cell marker line ET2634 (Gross-Hardt et al., 2007) in *vdd-1* heterozygous plants 48 HAE revealed that 32% of the megagametophytes did not express the synergid-specific marker (n = 740). For this marker also, an inversion of the expression was occasionally observed: 4% of the megagametophytes expressed the synergid marker in the antipodals instead of the synergid cells, which was not observed in wild-type plants. In comparison, wild-type sibling plants homozygous for the reporter construct showed GUS expression in 96% of the synergids at 48 HAE (n = 665) (Figures 6F and 6G).

Our data indicate that cell identity of antipodal and synergid cells in the *vdd-1* mutant embryo sac is not correctly specified and/or that their differentiated state is not maintained. Interestingly, the 32% of female gametophytes that did not express the

Table 1. Segregation Analysis of the *vdd-1* Mutant Allele

		Progeny Genotype				n[a] Transmission Efficiency[b]		
Male Genotype	Female Genotype	VDD/VDD	vdd-1/VDD	vdd-1/vdd-1	Total	TE_{male}	TE_{female}	P Value[c]
vdd-1/VDD	*vdd-1/VDD*	92	120	0	212	n.a.[d]	n.a.	0.054
vdd-1/VDD	*VDD/VDD*	151	128	n.a.	279	84.8%	n.a.	0.169
VDD/VDD	*vdd-1/VDD*	207	57	n.a.	264	n.a.	27.5%	2.7e-20

[a]Total number of plants scored.
[b]Transmission efficiencies (TE) as calculated by Howden et al. (1998).
[c]P value obtained using the χ^2 test under the hypothesis of a 1:1 segregation ratio.
[d]n.a., not applicable.

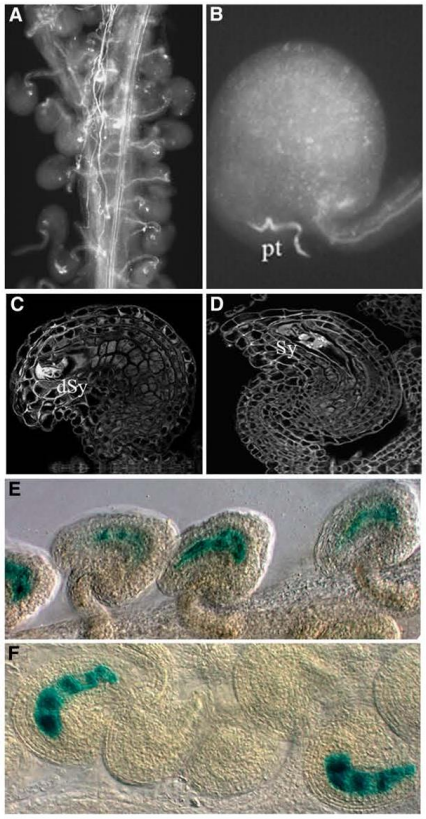

Figure 5. Fertilization Analysis in *vdd-1* Heterozygous Plants.

(A) Pollen tube staining with aniline blue shows that all embryo sacs in the *vdd-1* heterozygous background are reached by pollen tubes.
(B) Detailed image of aniline blue–stained pollen tube reaching the micropyle in the *vdd-1* heterozygous carpel.
(C) CLSM image of a wild-type fertilized female gametophyte. Observation performed 12 h after pollination showed one degenerating synergid in all the embryo sacs. The strong fluorescence signal indicates the degeneration of a synergid cell.
(D) Detailed image of a female gametophyte in the *vdd-1* heterozygous pistil. In this genetic background, not all the embryo sacs show synergid degeneration at 12 h after pollination.
(E) Following pollination of wild-type pistils with pollen carrying the MINI3:GUS reporter construct, all seeds showed *GUS* activity in the developing endosperm.
(F) Polllination of heterozygous *vdd-1* pistils with MINI3:GUS pollen. GUS activity is not observed in all of the ovules.
dSy, degenerated synergid; pt, pollen tube; Sy, synergid.

synergid cell marker corresponds to the percentage of ovule abortion observed in *vdd-1/VDD* heterozygous plants, whereas we found wrong specification of the antipodal cells in almost 50% of the gametophytes in *vdd-1* heterozygous siliques. These data suggest that the fertilization defects described in the *vdd-1* mutant gametophyte correlates with defects in the development or differentiation of synergid cells.

DISCUSSION

VDD Is a Direct Target of the Ovule Identity MADS Domain Transcription Factor Complex

MADS box genes belonging to the *AG* subfamily play a redundant role in the regulation of ovule development (Pinyopich et al., 2003; Brambilla et al., 2007), and their function has been well conserved during the course of plant evolution (Colombo et al., 2008). This was underlined not only by genetic studies, but also through the analysis of protein–protein interactions, which were found to be conserved between distantly related species (Favaro et al., 2002; Ferrario et al., 2003; Immink et al., 2003; Dreni et al., 2007). Despite the efforts to describe the molecular pathways regulated by MADS domain factors during flower development, very few direct target genes have been identified and characterized to date (Lamb et al., 2002; Hepworth et al., 2002; Ito et al., 2004; William et al., 2004; Gómez-Mena et al., 2005; Sundström et al., 2006), and this knowledge is completely missing for the ovule identity MADS domain factors.

Most efforts to identify direct target genes used transgenic plants in which a transcription factor was under external control, allowing the controlled induction of its activity. Subsequent genome-wide transcript profiling shows that genes are significantly changed in expression upon induction (Ito et al., 2004, 2007; Gómez-Mena et al., 2005). Other approaches include the comparison of differences in the transcriptome between wild-type and mutant plant tissues (Zik and Irish, 2003; Wellmer et al., 2004). Only recently, the ability to perform high-throughput sequencing on immunoprecipitated chromatin allowed the description of AP1 and SEP3 binding sites at the genome-wide level and the correlation of SEP3 function with plant hormone signaling pathways in *Arabidopsis* inflorescences (Kaufmann et al., 2009, 2010). Here, we present an alternative method to identify targets of the MADS domain ovule identity factors. This method starts with defining a subset of genes that are expressed in a specific cell type. In this study, we used ovule primordia at a very early stage of development (the 8-cell stage). Because we look here at expressed genes, we exclude the identification of genes that are completely silenced by the ovule identity factors. Subsequently, we selected from this subset of genes only those encoding putative transcription factors. We made this selection because until now most of the MADS domain target genes that have been identified encode transcription factors. The next step was to identify those genes that contain a putative MADS domain binding site. Since these CArG sequences are rather frequent in the *Arabidopsis* genome (Gómez-Mena et al., 2005), we considered only those genes that contain in their genomic region at least two putative CArG boxes within a distance <300 bp. This

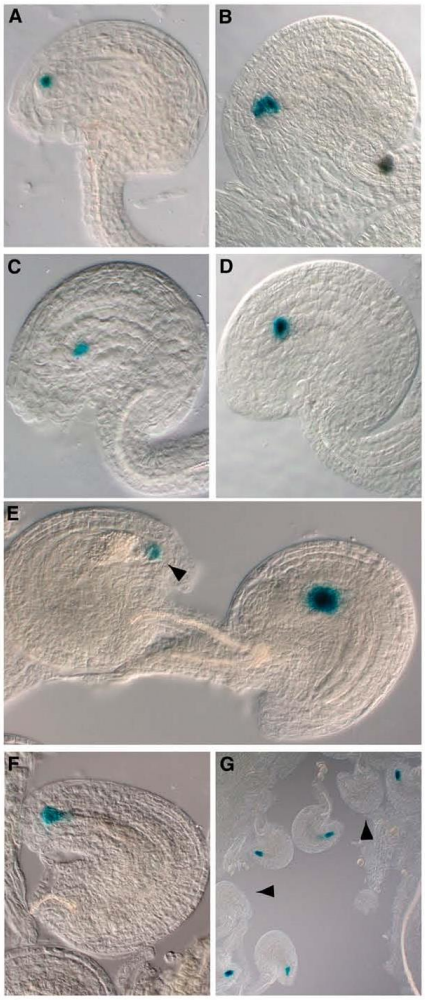

Figure 6. Expression Pattern of Gametophytic Cell-Specific Markers in Wild-Type and *vdd-1* Heterozygous Plants.

Plants homozygous for the gametophytic marker constructs were analyzed 48 HAE if not otherwise indicated.
(A) and **(B)** Egg cell–specific marker expression.
(A) Wild-type plants showed GUS expression in 98% of the female gametophytes (n = 289).
(B) *vdd-1* heterozygous plants showed blue staining in 97% of the megagametophytes (n = 304).
(C) and **(D)** Central cell marker expression in wild-type plants **(C)** (98% of female gametophytes; n = 282) and in the *vdd-1* heterozygous plants **(D)** (99% of megagametophytes; n = 327).
(E) Antipodal cell marker expression in the *vdd-1/VDD* plants 72 HAE. At this time point, *vdd-1/VDD* heterozygous siliques showed GUS expression in the synergids cells (arrowhead) (49%; n = 244). In the remaining 51% of megagametophytes (n = 254), blue staining was visible in the antipodal cells.
(F) and **(G)** Expression profile of the ET2634 synergid cell marker.
(F) In wild-type plants, the ET2634 synergid cell marker is visible in almost all of the mature embryo sacs (96%; n = 665).
(G) In the *vdd-1* heterozygous plants, 32% of the mature embryo sacs (n = 740) did not express the synergid-specific cell marker (arrowheads).

reduced the number of candidate targets to a subset of 15 genes. ChIP experiments using antibodies against STK confirmed 4 of these 15 genes as in vivo targets of STK. Although the criteria used to select these genes may seem a bit arbitrary, the results that we obtained in this study (along with those of other studies) support the model that MADS domain protein complexes often interact with DNA by contacting multiple nearby CArG sequences (Egea-Cortines et al., 1999; Gregis et al., 2008; Liu et al., 2008).

Ovule identity determination in *Arabidopsis* is dependent on the interaction between STK (or SHP1 or SHP2) and the SEP proteins (Favaro et al., 2003; Pinyopich et al., 2003; Brambilla et al., 2007). Therefore, we tested whether the CArG boxes in the *VDD* genomic region that are bound by STK were also interacting with SEP3. Interestingly, CArG boxes 1 and 3, which are directly contacted by STK, are also direct targets bound by SEP3.

This result suggests that SEP3-STK heterodimers could interact with DNA through the formation of tetramers as previously proposed for other MADS box complexes (Theissen and Saedler, 2001). Moreover, our binding analyses indicate that MADS domain protein dimers display affinity for specific CArG elements, since CArG box 2, which maps only 95 bp from CArG 3, is not contacted by the ovule identity protein complex composed of STK and SEP3. It will be interesting to investigate by site specific mutagenesis whether in the absence of a high affinity binding site, like CArG 1 or 3, the STK-SEP3 protein complex is able to interact with the *VDD* promoter using the lower-affinity CArG box 2.

VDD Expression Requires the Activity of the Ovule Identity Factors STK, SHP1, and SHP2

The ability to isolate ovule primordia and to analyze the tissue-specific transcriptome through the combination of laser microdissection and microarray hybridization allowed us to identify which genes are coexpressed with the ovule identity gene *STK*. As already discussed, this approach led to the identification of *VDD* as the first direct target of the ovule identity complex. This gene belongs to a plant-specific transcription factor family, namely, the REM family, which appears to be highly expanded in *Arabidopsis* (Swaminathan et al., 2008; Romanel et al., 2009). Despite the presence of at least 76 *REM* genes in the *Arabidopsis* genome, to date, *VERNALIZATION1* is the only *REM* for which a function has been determined (Levy et al., 2002). This lack of functional information regarding the role of *REM* genes during

plant growth might be due to high functional redundancy within this gene family and/or may be due to the fact that the mutant phenotypes are difficult to observe.

In general, AtGenExpress data (Schmid et al., 2005) shows that most of the REM-encoding genes are tissue-specifically expressed (Swaminathan et al., 2008; Romanel et al., 2009). *VDD* transcripts are present in the same tissues as the ovule identity genes *STK*, *SHP1*, *SHP2*, *AG*, and *SEP* (Yanofsky et al., 1990; Ma et al., 1991; Rounsley et al., 1995; Flanagan et al., 1996; Mandel and Yanofsky, 1998). *VDD* expression studies by in situ hybridization using different mutant backgrounds highlighted the redundancy of the ovule identity factors STK, SHP1, and SHP2 in the regulation of *VDD* expression within the ovule, since the expression of *VDD* is strictly dependent on the activity of the three MADS domain ovule identity factors. Only the absence of all three ovule identity proteins leads to complete absence of *VDD* expression during ovule development. This suggests that the ovule identity complex that binds the CArG boxes 1 and 3 in the *VDD* regulatory region is composed of STK, SHP1, SHP2, and SEP proteins. However, since our ChIP experiments do not include IP experiments using SHP antibodies, we cannot at this point exclude that STK binds preferentially to these sites and that only in the absence of STK, the SHP proteins will replace STK. The hypothesis that STK may be the main player regulating *VDD* expression is supported by the fact that loss of *STK* activity results in a significant reduction in *VDD* expression. Other transcription factors mediate the expression of *VDD* in the inflorescence and floral meristems, suggesting that other MADS domain factors might regulate this gene during other phases of flower development.

VDD Is Required for Cell Differentiation in the Female Gametophyte

Morphological and genetic analyses of plants carrying the *vdd-1* allele showed that this mutation was transmitted through the female gametophyte at a significantly reduced efficiency. This reduction in *vdd-1* transmission is due to a defect in fertilization after the pollen tube reaches the female gametophyte. In particular, pollen tubes correctly find their way along the funiculus into the micropyle and arrest their growth, but fertilization does not occur. Understanding the mechanisms that control the fertilization process represents an intriguing aspect of plant reproductive biology. After entering the micropylar opening, sperm cell discharge is under the control of both male and female gametophytes. In the female, the synergid-specific expression of *FER* is required for pollen tube growth arrest, rupture, and sperm cell release (Huck et al., 2003; Escobar-Restrepo et al., 2007). Continuous pollen tube overgrowth into *fer* mutant female gametophytes is linked to the absence of the signaling cascade regulated by the FER receptor-like Ser-Thr kinase in the receptive synergid cell (Escobar-Restrepo et al., 2007). The recent characterization of the *syl* mutant has indicated that additional embryo sac cells may also be involved in pollen tube reception (Rotman et al., 2008). It is thus possible that aspects of the molecular dialogue during fertilization takes place not only between male and female gametophytes but also among the haploid cells of the megagametophyte. The functional characterization of the *FER* homologs *ANXUR1* (*ANX1*) and *ANX2* allowed the identification of a male signaling cascade necessary to prevent pollen tube rupture before entering the micropyle. A precise molecular dialogue between the *FER*-dependent and *ANX*-dependent signaling cascades may be necessary for proper pollen tube reception (Boisson-Dernier et al., 2009; Miyazaki et al., 2009).

Silencing of the *VDD* gene segregates with defects in embryo sac cell specification and a lack of fertilization. Expression analysis of antipodal- and synergid-specific marker genes showed that the identity of these cells is compromised in plants heterozygous for the *vdd* mutation. The frequency with which we observe the loss of synergid cell identity correlates well with the reduced transmission efficiency of the *vdd-1* allele through the female gametophyte. Therefore, this defect in synergid differentiation explains the lack of fertilization in the *vdd-1* mutant embryo sacs. This is even further supported by the fact that we frequently do not observe synergid degeneration in the *vdd-1* heterozygous mutant.

Until now, most of the female gametophytic mutants affecting pollen tube reception led to the identification of factors that are potentially involved in the signal transduction cascade (Escobar-Restrepo et al., 2007; Boisson-Dernier et al., 2008; Capron et al., 2008). It is not clear how *VDD* fits into this pathway. Since *VDD* encodes a putative transcription factor, it likely regulates the expression of downstream genes involved in the specification of the two accessory cell types, which is reflected by the lack of fertilization in *vdd-1* mutant embryo sacs. The antipodal and synergid cells are located at opposite poles of the embryo sac, and the common origin of these cells can be traced back to the one-nucleate stage of embryo sac development, when the two nuclei migrate to opposite poles (Christensen et al., 1997). Therefore, it might be that *VDD* is already influencing gene expression before the migration of these nuclei, subsequently affecting their specification and differentiation, leading to defects in male gamete discharge. However, it could also be that *VDD* is involved in a pathway that establishes accessory cell identity based on differences in signals at the two poles of the embryo sac. It is important to point out that synergid identity is not completely lost in *vdd* mutant embryo sacs as they are still competent to attract the pollen tube and induce its growth arrest. It is therefore possible that *vdd* mutant synergids have a mixed identity, losing some aspects of synergid function while gaining others, as indicated by the gain of and loss of expression of antipodal and synergid cell markers, respectively. One of our challenges for the future will be to identify the genes that are regulated by *VDD*.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana wild-type (ecotype Columbia) and mutant plants were grown at 22°C under short-day (8 h light/16 h dark) or long-day (16 h light/8 h dark) conditions. The *Arabidopsis stk*, *shp1shp2*, and *stk shp1 shp2* mutants were kindly provided by M. Yanofsky (Pinyopich et al., 2003). Gametophytic cell marker line corresponding to the egg cell (Gross-Hardt et al., 2007), central cell (promoter of the gene At1g02580;

Chaudhury et al., 1997), and antipodal cell (promoter of the gene At1g36340; Yu et al., 2005) marker lines were kindly provided by R. Gross-Hardt. The synergid cell marker line (ET2634) was generated in the lab of U. Grossniklaus. All the gametophytic marker lines analyzed encode for a nuclear localization signal that is in frame with the GUS reporter gene. The MINI3:GUS reporter line was kindly provided by M. Luo. *Arabidopsis* (ecotype Columbia) seeds carrying the *vdd-1* mutant allele were obtained from the Syngenta *Arabidopsis* Insertion Library (SAIL 50_C03) collection (www.Arabidopsis.org/abrc/sail.jsp). T-DNA is inserted in the first intron, 44 bp upstream the second exon.

Laser Microdissection

Young inflorescences from wild-type plants were prepared as previously described (Kerk et al., 2003). Ovules corresponding to stage 8-9 (referred as ovule primordia) were dissected using a Leica laser microdissection system (LMD 6000; Leica Microsystems). The selected cells were cut using a UV laser (337-nm wavelength). The dissection conditions were optimized as follows: L40x objective at power 35 to 45 and speed 3 to 4. Samples from ~30 sections (at least 1000 cells) were collected in a single tube. RNA was extracted from three different tubes. RNA from laser microdissection cells was extracted using the PicoPure RNA isolation kit (Arcturus) according to the manufacturer's instructions. RNA obtained from three independent extractions was pooled and amplified as describe by Van Gelder et al. (1990).

Identification of Putative CArG Sequences

The genomic regions located 3 kb upstream of the ATG, 1 kb downstream of the stop codon and in the exons and introns of the ovule primordia expressed transcription factors were analyzed to identify CArG sequences. The *Transfac* bioinformatic program available at the Biobase website (http://www.biobase-international.com) allowed us to identify perfect CArG boxes, CArG sequences with one mismatch, and AG, AGL15, SHP1, and SEP binding sites deduced by a probability matrix. To restrict the sample further, we selected genes containing two putative CArG sequences within a distance of ~300 bp.

ChIP and Quantitative Real-Time PCR Analysis

ChIP experiments were performed as a modified version of a previously reported protocol (Gregis et al., 2008); a detailed protocol is available in the Supplemental Methods online. STK polyclonal antibody was obtained against the synthetic peptide: NH_2-RTKVAEVERYQHH-COOH. The polyclonal SEP3 antibody was obtained against the following synthetic peptides: NH_2-EVDHYGRHHHQQQQHSQA-COOH and NH_2-SQQEYLKLKERYDALQRCOOH. Antibodies were produce by Primm.

Enrichment of the target region was determined using a Sybr Green Assay (iQ_ SYBR Green Supermix; Bio-Rad). The quantitative real-time PCR assay was conducted in triplicate and was performed in a Bio-Rad iCycler iQ optical system (software version 3.0a). Relative enrichment was calculated normalizing the amount of immunoprecipitated DNA against an *ACTIN2/7* (*ACT2/7*) fragment and against total INPUT DNA. In particular, for the binding of STK to the selected genomic regions, the affinity of the purified sample obtained in the *shp1 shp2* mutant background was compared with the affinity-purified sample obtained in the *stk* single mutant background, which was used as negative control. For the binding of SEP3 to the selected genomic regions, the affinity of the purified sample obtained from wild-type carpel tissue was compared with the affinity-purified sample obtained from wild-type leaf tissue, which was used as negative control.

Fold enrichment was calculated using the following formulas, where Ct.tg is target gene mean value, Ct.i is input DNA mean value, and Ct.nc is actin (negative control) mean value: dCT.tg = CT.i-CT.tg and dCT.nc =CT.i-CT.nc. The propagated error values of these CTs are calculated: dSD.tg = sqrt((SD.i)^2+ (SD.tg^2)/sqrt(n) and dSD.nc = sqrt((SD.i)^2+ (SD.nc^2)/sqrt(n), where *n* = number of replicate per sample.

Fold-change over negative control (actin and wild-type plants) was calculated finding the "delta delta CT" of the target region as follows: ddCT = dCT.tg- dCT.nc and ddSD = sqrt((dSD.tg)^2+ (dSD.nc)^2. The transformation to linear "fold-change" values is obtained as follows: FC = 2^(ddCT) and FC.error = ln(2)*ddSD*FC.

Oligo sequences are as follows: *ACTIN2/7* forward, 5′-CCAATCGTGAGAAAATGACTCAG-3′; ACTIN2/7 reverse, 5′-CCAAACGCAGAATAGCATGTGG-3′); CArG 1 forward, 5′-AACATTGCTTTCTCCTTCCAAA-3′; CArG1 reverse, 5′-CAAAAGGGAGTTCAAGTGAAAGAAC-3′; CArG2 forward, 5′-CTACATTCTACAGACTAGCTAG-3′; CArG2 reverse, 5′-CTAAAAAGACAGCGTCATATTTCC-3′; CArG3 forward, 5′-GGAAATATGACGCTTGTCTTTTTAG-3′; CArG3 reverse, 5′- CAGAAACAGCAATATGCTCGTG-3′.

Expression Analysis

For the microarray hybridization experiment, RNA purified from LCM cells was amplified, labeled, and hybridized on the ATH1 GeneChip at the Affymetrix Microarray Unit at the Molecular Oncology Foundation Institute (IFOM; http://www.ifom-firc.it). Two hybridization replicates were performed to reduce the technical variability; each replicate was measured twice. Expression levels were calculated as an average of the closest three values out of the four measurements. All the samples were normalized together to a per-chip and per-gene median value. Clustering analysis was performed using condition tree clustering on all samples. Similarity was measured using Spearman correlation (GeneSpring version 7.2). Microarray data were normalized and analyzed using GeneSpring software. Our analysis identified 14,575 genes significantly expressed in the ovule primordia with a P value < 0.05 (see Supplemental Data Set 1 online). As reported in the text, we focused our attention on transcription factor encoding genes. For these genes, the q-value was calculated, which allowed us to measure the minimum false discovery rate (FDR) that is incurred when calling that test significant. In our analysis, q-values were measured from the corresponding P values using a freely available database (genomics.princeton.edu/storeylab/qvalue). Q-value estimation was done using a specific FDR level of 0.05, a lambda range from 0.0 to 0.90, and a smoother pi_0 method as main parameters. FDR analysis allowed the identification of 1024 transcription factor genes that are significantly expressed in ovule primordia (Storey et al., 2004) (see Supplemental Data Set 2 online). A comparison of the replicates for each tissue type was done and the mean signal values were ranked, revealing the number of genes that passed the cutoff and furthermore are considered as expressed genes.

Quantitative real-time RT-PCR experiments were performed on cDNA obtained from leaves, flower, and siliques at 3 d after pollination and siliques at 5 d after pollination. Total RNA was extracted using the LiCl method (Verwoerd et al., 1989). DNA contamination was removed using the Ambion TURBO DNA-free DNase kit according to the manufacturer's instructions (http://www.ambion.com/). The treated RNA was subjected to reverse transcription using the ImProm-IITM reverse transcription system (Promega). *VDD* transcripts were detected using a Sybr Green Assay (iQ SYBR Green Supermix; Bio-Rad) with the reference gene *UBIQUITIN*. The real-time PCR assay was conducted in triplicate and was performed in a Bio-Rad iCycler iQ Optical System (software version 3.0a). Relative enrichment of *VDD* transcripts was calculated normalizing the amount of mRNA against a *UBIQUITIN* fragment. Diluted aliquots of the reverse-transcribed cDNAs were used as templates in quantitative PCR reactions containing the iQ SYBR Green Supermix (Bio-Rad). The difference between the cycle threshold (Ct) of *VDD* and that of *UBIQUITIN* ($\Delta Ct = Ct_{VDD} - Ct_{UBIQUITIN}$) was used to obtain the normalized expression of *VDD*, which corresponds to $2^{-\Delta Ct}$. The expression of *VDD* was analyzed by the following primers: *VDD* forward,

5′-TGGATGGAACCAGTTTGTGA-3′, and *VDD* reverse, 5′-CTTCACATC-TTTGTAGATGCTC-3′. The expression of *UBIQUITIN* was analyzed using the following primers: UB forward, 5′-CTGTTCACGGAACCCAATTC-3′, and UB reverse, 5′-GGAAAAAGGTCTGACCGACA-3′.

For in situ hybridization analysis, *Arabidopsis* flowers were fixed and embedded in paraffin as described previously (Huijser et al., 1992). Sections of plant tissue were probed with digoxigenin-labeled *VDD* antisense RNA corresponding to nucleotides 240 to 557. Hybridization and immunological detection were performed as described previously (Coen et al., 1990).

For the GUS assays, gametophytic cell-specific marker lines were used as female and pollinated with pollen obtained from *vdd-1* heterozygous plants to introduce the reporter constructs into the *vdd-1* mutant background. The F2 progeny obtained from self-fertilization of F1 plants heterozygous for the *vdd-1* allele and the reporter constructs were analyzed for GUS expression to identify *vdd-1/VDD* plants homozygous for the reporter constructs. Flowers were emasculated and harvested 48 h following emasculation for GUS staining. MINI3:GUS pattern in the *vdd-1* heterozygous background was analyzed 26 h after pollination. All GUS assays were performed overnight as described previously (Liljegren et al., 2000). Samples were incubated in clearing solution, dissected, and observed using a Zeiss Axiophot D1 microscope equipped with DIC optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

Microscopy

To analyze ovule development in *vdd-1* heterozygous plants, flowers at different developmental stages were cleared and analyzed as described previously (Brambilla et al., 2007).

For the aniline blue staining experiments, *vdd-1* heterozygous plants were emasculated and pollinated 24 h after the emasculation. Pollen tube growth was analyzed 24 h after pollination. Aniline blue staining was performed as described by Huck et al. (2003).

For the synergid degeneration analysis, wild type and *vdd-1* heterozygous flowers were emasculated and pollinated using wild-type pollen 24 h after emasculation. Pistils were fixed 12 h after pollination (hap) and observed by CLSM following the Braselton et al. (1996) protocol. Samples were excited using a 532-nm laser. Emission was selected between 570 and 740 nm.

Plasmid Construction and *Arabidopsis* Transformation

For the *VDD* promoter analysis, wild-type *Arabidopsis* plants (ecotype Columbia) were transformed with a construct containing 1221 bp upstream of the *VDD* translation start site, the complete *VDD* genomic coding region fused to the *GUS* reporter gene, and 389 bp corresponding to the *VDD* 3′ untranslated region. The pBGWFS7 vector (Karimi et al., 2002) was modified to substitute the T35S fragment with the *VDD* 3′ untranslated region sequence. The PCR product obtained with the primer 5′- CCATGGACACCATGACGATGATGATATTTTA-3′ in combination with the primer 5′-GACGTCCCAGAAGAGGCTTATGATA-3′ was cloned into the pBGWFS7 vector using the *Nco*I and *Aat*II restriction sites. Following this, the VDD genomic region was amplified using the forward oligo 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCGAACTTTATTCCG-GATA-3′ in combination with the reverse oligo 5′- GGGGACCACTT-TGTACAAGAAAGCTGGGTCTTCTTTGGAGACTTTCACAC-3′. This PCR product was cloned in the pBGWFS7 vector modified as described above.

For the molecular complementation experiment, a 3-kb genomic region containing the *VDD* gene was amplified by PCR using the oligo Atp1669 (5′-TCTAGACCCGAACTTTATTCCGGATA-3′) located 1221 bp upstream of the ATG and the oligo Atp1670 (5′-TCTAGACCAGAAGAGGCTTAT-GATA-3′) located 389 bp downstream of the stop codon. The obtained PCR product was cloned in the *Xba*I site of the pFLUAR binary vector (Stuitje et al., 2003), which contains the YFP coding sequence under the control of the NAPIN promoter. Constructs were verified by sequencing and used to transform *vdd-1* heterozygous plants using the floral dip method (Clough and Bent, 1998). Transformant seeds were visually selected by fluorescence microscopy.

For the *pSTK:amiRvdd* construct, the genomic fragment corresponding to the *STK* promoter was amplified using the primers Atp1509 (5′-TCTGACGTCAGGCGTTTTTGTTGGGTATGTTCTCAC-3′) and Atp 1508 (5′-TCTGACGTCAGGCATCCTTCATTTTAAACATC-3′) (Kooiker et al., 2005) and was cloned in the binary vector pBGW0 (http://www.psb.ugent.be/gateway) upstream of the recombination Gateway cassette using the *Aat*II restriction site (Invitrogen). The artificial RNA directed against the *VDD* gene was prepared according to the information available at the Web microRNA Designer website (WMD wmd2.weigelworld.org/cgi-bin/mimatools.pl?page=8#experimentalProcedure). Site-directed mutagenesis on a template plasmid containing the MIR319a precursor was performed using the following primers: oligo I (5′-GATTTA-CTAACAGTTTCCACCCTTCTCTCTTTTGTATTCC-3′), oligo II (5′-GAA-GGGTGGAAACTGTTAGTAAATCAAAGAGAATCAATGA-3′), oligo III (5′-GAAGAGTGGAAACTGATAGTAATTCACAGGTCGTGATATG-3′), and oligo IV (5′-GAATTACTATCAGTTTCCACTCTTCTACATATATATTCCT-3′). The obtained PCR product was cloned into the TOPO vector (Invitrogen) and further recombined into the pBGW0 binary vector containing the *STK* promoter. Wild-type plants were transformed using the floral dip method (Clough and Bent, 1998), and transformants were identified through BASTA selection.

PCR-Based Genotyping

Identification of the *vdd-1* mutant allele was performed by PCR analysis using the oligo Atp1220 (5′-GCCTTTTCAGAAATGGATAAATA-GCCTTGCTTCC-3′) on the T-DNA left border and the oligo Atp1219 (5′-CGAAGGAGAGAAGCAGAGATG-3′). The *VDD* wild-type allele was identified using the oligo Atp 1219 in combination with oligo Atp1218 (5′-TGAAGTACCGGCTTCAGAGTC-3′).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: ACTIN2/7, AT5G09810; AGAMOUS, AT4G18960; ANXUR1, AT3G04690; ANXUR2, AT5G28680; APETALA1, AT1G69120; FERONIA, AT3G51550; MINISEED3, AT1G55600; SEEDSTICK, AT4G09960; SEPALLATA1, AT5G15800; SEPALLATA2, AT3G02310; SEPALLATA3, AT1G24260; SEPALLATA4, AT2G03710; SHATTERPROOF1, AT3G58780; SHATTERPROOF2, AT2G42830; VERNALIZATION, AT3G18990; and VERDANDI, AT5G18000.

Author Contributions

L.M.-H. and R.B. are the main contributors to the experimental part of this manuscript. R.B. drafted the manuscript. F.G. performed ChIP and real-time RT-PCR experiments. M.R. performed complementation tests. C.E. and U.G. performed pollen tube guidance experiments and provided advice on experimental approaches. M.M.K. and U.G. provided advice on experimental approaches and helped with writing of the manuscript. The work was generally performed in the group of L.C. who provided funding and supervised the research and the writing of the manuscript.

Supplemental Data

The following materials are available in the online version of this manuscript.

Supplemental Figure 1. Laser Microdissection of Ovule Primordia.

Supplemental Figure 2. *vdd-1* Mutant Female Gametophyte.

Supplemental Table 1. List of the 15 Transcription Factor-Encoding Genes Selected for the ChIP Analysis.

Supplemental Table 2. Cell-Specific Marker Gene Expression.

Supplemental Data Set 1. List of Ovule Primordia Expressed Genes According to Microarray Analysis.

Supplemental Data Set 2. List of Transcription Factors Encoding Genes Expressed in the Ovule Primordia According to Microarray Analysis.

Supplemental Methods.

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VERDANDI Is a Direct Target of the MADS Domain Ovule Identity Complex and Affects Embryo Sac Differentiation in Arabidopsis

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CONCLUSIONS AND FUTURE PROSPECTS

Auxin distribution and *PIN1* correct localization seems to be fundamental for the proper ovule formation and growth. Once the primordium is formed, PIN1 is required to re-establish the auxin gradient in the outgrowing ovule primordia, and PIN1 is mainly localized in correspondence of the ovule tip (Benkovà et al., 2003).

We observed that the lack of CUCs produces less ovules and a placenta where PIN1 is reduced and mis-localized in vesicles. Our data indicate that CUC1 and CUC2 are required for both correct *PIN1* expression and PIN1 localization. The involvement of CUCs in *PIN1* expression is not new. In fact, it was proposed a role for CUC2 in establishing *PIN1* expression during leaf serration formation (Bilsborough et al., 2011). Furthermore, through protoplast assays we show that CUC1 and CUC2 are able to induce *PIN1* expression but we cannot assert that this activation is direct. In order to verify that the CUCs directly regulate the transcription of PIN1 we should performe ChIP experiments.

The cytokinin 6-Benzylaminopurine (BAP) treatment restores the PIN1 reduced expression level, and PIN1 is not localized in vesicles anymore. BAP highly induces PIN1 expression that results ectopically present in all the placenta and primordium cells even after the primordium outgrowth. The BAP treatment is also able to restore the reduction in ovule number observed in the CUCs silencing mutants, but not in the *pin1-5* mutant carpels. It suggests that PIN1 expression is, at least partly, under the control of cytokinins, as already proposed for roots development where cytokinins modulate cell-to-cell auxin transport and auxin level (Ruzicka et al., 2009). It also suggests that cytokinins act downstream of the CUC1 and

CUC2 transcription factors in order to induce *PIN1* expression. An increase in ovule number was also observed in wild-type BAP treated carpels (Bencivenga et al., 2012). What is not clear is how is possible that a so high and ectopic *PIN1* level produced by the BAP treatment can re-organize the auxin flux leading to the formation of new primordia.

It could be interesting to study if this regulatory mechanism is conserved in other organs such as leaves where it was shown that CUC2 controls *PIN1* expression level (Bilsborough et al., 2011).

The BAP treatment cannot restore the drastic ovule reduction observed in carpels in which *ANT*, *CUC1* and *CUC2* are simultaneously silenced. It indicates that ANT might function in a pathway independent to that of CUC1 and CUC2.

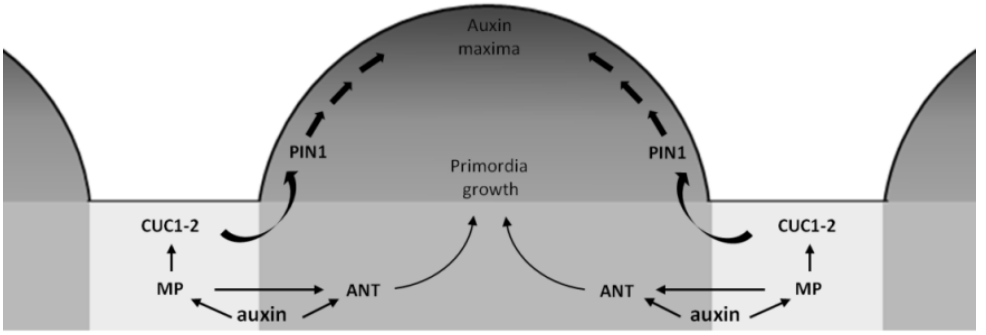

Figure 12 Proposed model of the ovule porimordia pathway

Taking into account these data and the fact that we demonstrate that *ANT*, *CUC1* and *CUC2* are MP direct target of, we propose a model (Figure 12), which integrates all these elements during ovule primordia formation, similar to the one already proposed for leaf serrations development (Bilsborough et al., 2011). MP regulates *ANT*, *CUC1* and *CUC2* expression during early stages of ovule development, and *CUC1* and *CUC2* expression

is necessary for correct *PIN1* expression. Once PIN1 is correctly expressed and localized auxin gradient is established and ovule primordia are formed. Once the primordium is formed, the interaction between auxin and cytokinins provide the right signaling for the ovule development.

In order to further investigate the molecular network involved in ovule primordia formation and development, it would be of high interest investigate which genes are direct target of CUCs. The use of RNAseq approach could allow having a list of putative interesting genes, and it would be intriguing focus the attention on those putative targets that are connected to cytokinins and auxin trafficking in order to integrate the ovule formation network with new elements.

We demonstrated that MP directly regulates *STK* expression during ovule development (unpublished data). It has been shown that STK is involved in many developmental processes like ovule integuments differentiation into seed coat (Mizzotti et al., 2012) and cell expansion during funiculus development (Losa et al., 2009). Furthermore STK, together with the two MADS box transcription factors *SHP1*, and SHP2 redundantly regulate ovule development and, together with the SEP proteins determine ovule identity (Pinyopich et al., 2003; Favaro et al., 2003; Brambilla et al., 2007).

VERDANDI (*VDD*) has been identified as the first direct target of the ovule identity complex in *Arabidopsis thaliana* (Matias-Hernandez et al., 2010). *VDD/vdd* mutants show a reduced fertility due to the fact that synergid cells lose their identity. It is known that synergids play a crucial role in the female-male communication during fertilization process (Higashiyama et al., 2001; Kasahara et al., 2005; Higashiyama et al., 2006; Okuda et al., 2009; Kessler et al., 2011;Takeuchi et al., 2012), but even if we

know that loss of *VDD* function presents defects in synergid identity specification, it is not clear how VDD acts in in this pathway. Investigations in this direction could elucidate the fine mechanism that regulate synergid-pollen tube communication and the fertilization process itself. Since *VDD* encodes a putative transcription factor, it would be particularly interesting identify the genes regulated by VDD that can be involve in the specification of the synergid cells.

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