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Molecular Network Controlling the Ovule Development in *Arabidopsis thaliana*

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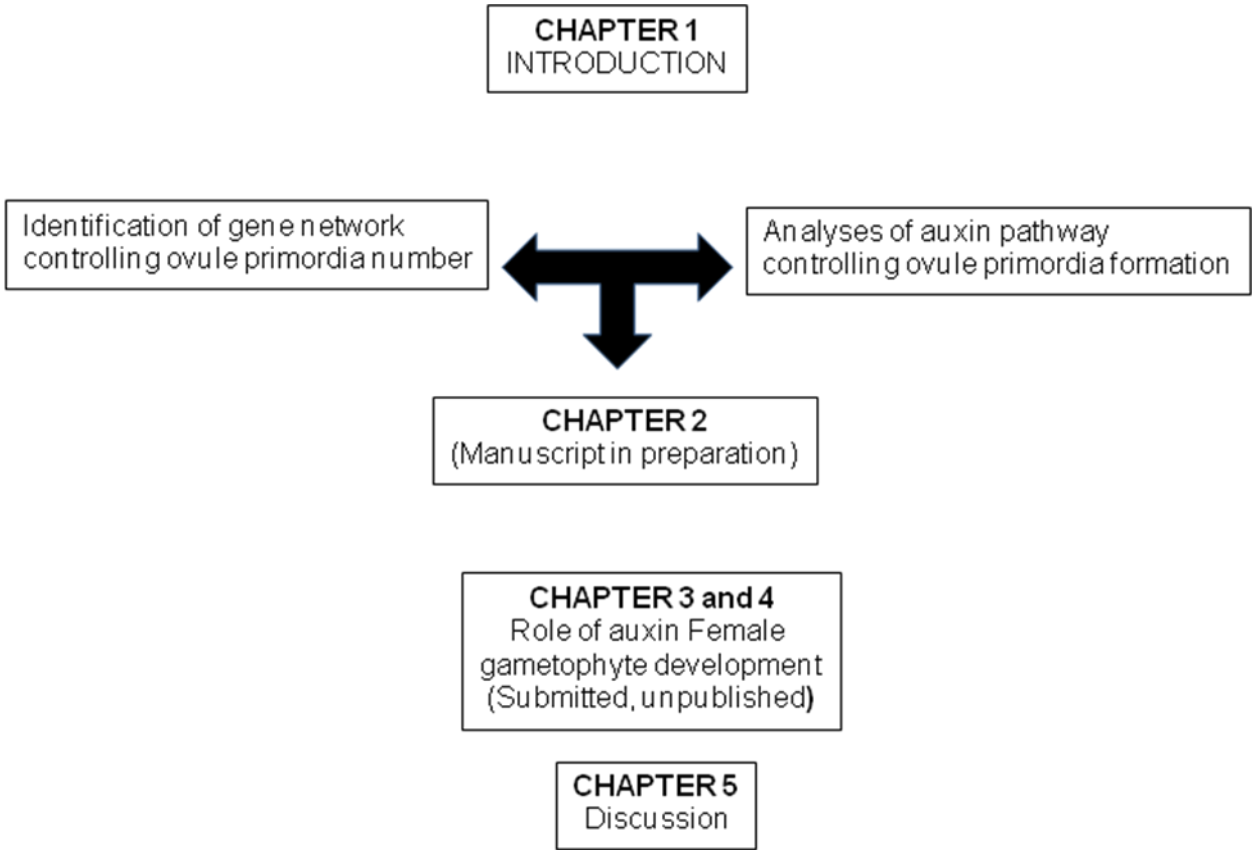


To My Parents and My Beloved Husband Subhro.

Chapter 1

Introduction

Introduction:

Ovules are the essential organ for plant reproduction. Megasporogenesis, megagametogenesis, fertilization and embryogenesis take place within the ovule. In the ovule both sporophytic and gametophytic generation coexist. The analyses of this developmental process provides an excellent tool to understand the molecular mechanism involved in controlling the basic processes like primordia initiation, specification, cell division, expansion and differentiation. This system allows the study of basic developmental process as well as the evolutionary prospect in the same context. In my thesis I have used *Arabidopsis thaliana* as a model system.

Ovule Development in Arabidopsis thaliana:

Ovules are considered as a lateral organ that emerges from the sub-epidermal meristematic tissue of the carpel, named placenta. It derived from the two fused carpels and it is formed at the floral stage 8 (Smyth *et al*., 1990). Each carpel bears two locules with two rows of ovules in each (Schneitz *et al*., 1995). The development of ovule can be divided mainly in 3 phases; primordia formation, megasporogenesis and megagametogenesis.

During stage1 the ovule primordia initiate asynchronously as bulges, and then subsequently appear in row of packed bulges along the placenta. The primordia derived from the periclinal division of the sub-epidermal cells of the placenta. The ovule bulges start to increase in size and give rise to extended protrusions with elongated rectangular cells. As the ovule primordia progressively elongate, they start to differentiate in three zones; the nucellus, the chalaza and the funiculus. In the distal part of the elongated protrusion a file of cells typically forms the nucellus. The hypodermal cells at the tip of the nucellus differentiate into the archesporial cell that becomes prominent and produces the Megaspore Mother Cell (MMC). MMC contains a large nucleus and a vacuole (Figure 1A).

Initiations of integuments take place in the stage 2 of ovule development that represents stage12 of flower development. The chalazal cells undergo periclinal division and form the apical cells of both integuments. At first the Inner integument primordia initiate to develop followed by the formation of the outer integument primordia. The integuments cells divide anticlinally and form two closely connected cell layers. Outer integument grows faster than the inner integument. Outer integument shows distinct curvature due to the faster cell division rate in the abaxial side than the adaxial side. The outer integument grows covering the inner integument and the nucellus more rapidly. Vascular strand starts to develop within the funiculus and that connects the ovules with the carpel.

During stage2, megasporogenesis takes place. The MMC undergoes meiosis and produce four haploid megaspores in the form of linear tetrad or T-shaped tetrad (Reinhardt *et al*., 2000). The three megaspores at the micropylar end of the nucellus degenerate, whereas the megaspore at the chalazal end of the nucellus becomes the Functional Megaspore (FM) (Figure1A-C). The degenerating megaspores are easily distinguishable due to the deposition of callose wall around them. The later stage of the megasporogenesis is named Female Gametophyte stage 1 (FG1, Figure 1C). In this stage, small vacuoles are formed in the forming gametophyte, the inner integument grows half the length of the nucellus and the outer integument surrounds the inner integument but does not completely cover the nucellus (Christensen *et al*. 1997).

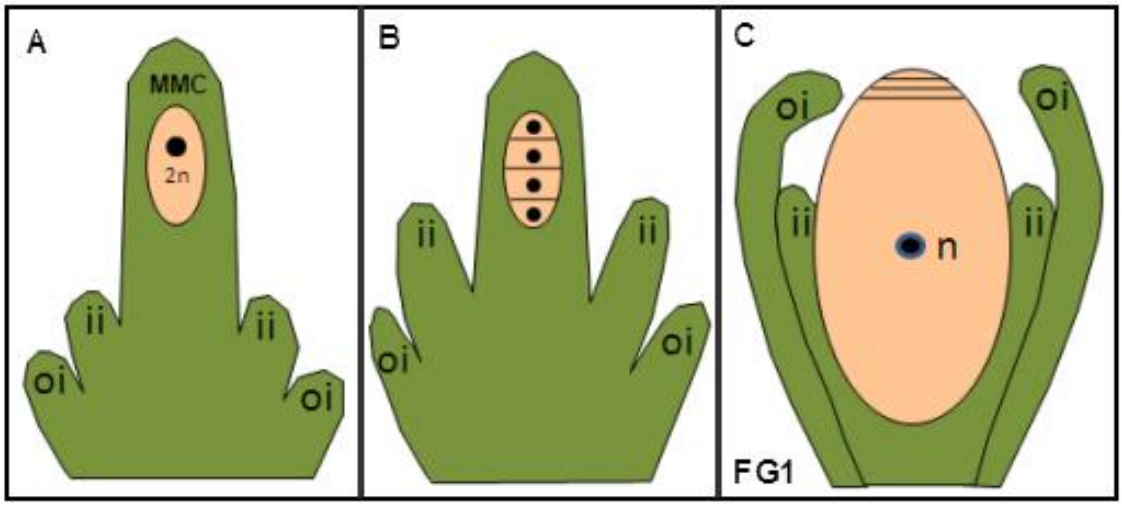


Figure1: The Megasporogenesis in *Arabidopsis thaliana*

A: Stage 2 of ovule development; the two integuments have started to develop the diploid MMC (in cream) occupies almost all nucellus.
B: Stage 2 of ovule development. Four haploid megaspores form in the nucellus.
C: Stage 2 of ovule development; a mononuclear (FG1) embryo sac is formed, while the three megaspores at the micropylar end degenerate.
MMC: megaspore mother cell; ii: inner integument; oi: outer integument; FG: Female Gametophyte.

In the beginning of stage FG2, the functional megaspore undergoes mitosis and gives rise to the two nucleate female gametophyte. Several small vacuoles are present in the developing embryo sac. Continuous expansion of the female gametophyte is observed in this stage. Towards the end of this stage, all the vacuoles start to coalesce in the centre (Figure2B).

In the stage FG3, a central vacuole is formed in the developing embryosac. Central vacuole separates the two nuclei at two pole of the embryo sac. Throughout this stage, degenerated megaspores are still visible. In this stage female gametophyte starts to curved and then both the integument completely enclose the nucellus (Christensen *et al.,* 1997, Figure 2C).

The second mitosis leads to the formation of four nucleate female gametophyte called FG4. In this stage another small vacuole is also present at the chalazal pole. The chalazal nuclei move perpendicular along the chalazal micropylar axis (Christensen *et al.,* 1997). During this stage the endothelium is formed from the inner layer of the inner integument. The endothelium keeps direct contact with almost the embryo sac apart from the chalazal end (Schneitz *et al*., 1995, Figure 2D)

The third and last mitosis produces eight nucleate embryosac with four nuclei at each pole. Two important events occur during this stage. One is the migration of one nucleus from each pole toward the central region of the embryosac and the second event is the cellularization. The cellularization starts during the stage FG5 immediately after the third mitosis and complete before polar nuclei fusion (Figure 2E).

Polar nuclei fuse along the adaxial surface of the female gametophyte during FG6. The mature female gametophyte is composed of three antipodal cells, one big central cell with diploid nucleus, one egg cell and two synergids at the micropylar end (Schneitz *et a*l.,1995, Figure 2F).

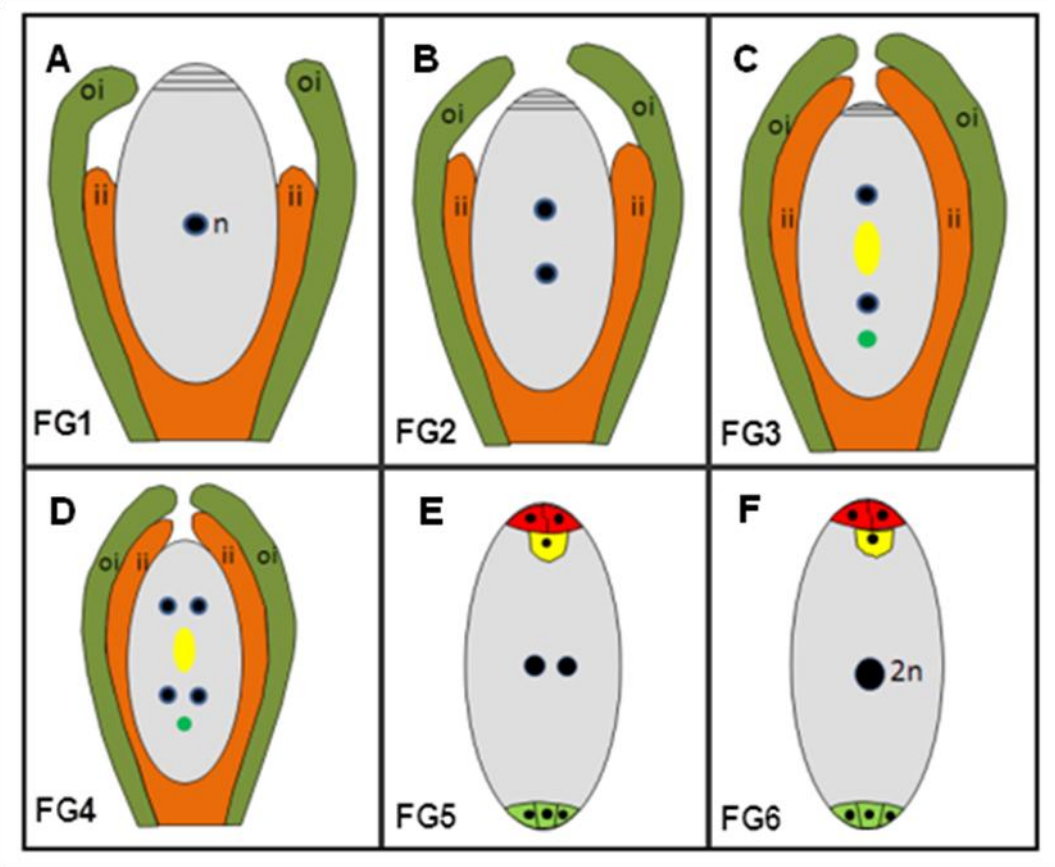


Figure 2: The Megagametogenesis in *Arabidopsis*.

ii (Orange): inner integument; oi (Green): outer integument; n: haploid nucleus; 2n: diploid nucleus.

A: Scheme of a FG1 female gametophyte with the functional megaspore haploid nucleus at the chalazal pole. Degenerating tetrad at the micropylar pole.

B: Scheme of a FG2 female gametophyte with two haploid nuclei.

C: Scheme of a FG3 female gametophyte with one big vacuole (Yellow) separating two haploid nuclei, and one small vacuole (green) toward the chalazal end of embryo sac.

D: Scheme of a FG4 female gametophyte with the big vacuole (in yellow) now separating two groups (four)of 4 haploid nuclei, the small vacuole (in green) toward the chalazal end of the embryo sac.

E: Scheme of a FG5 gametophyte when cellularization ends with the formation of two synergid cells (in orange) and one egg cell (in yellow) at the micropylar pole, one central cell with two haploid polar nuclei at the center, and three antipodal cells (green) at the chalazal pole.

F: scheme of a seven celled FG6 female gametophyte (pre-fertilization) after the polar nuclei fusion has generated one diploid nucleus in the central cell.

During fertilization the pollen tube grows along the funiculus and enters into the embryosac through the micropyle. The pollen tube is attracted by the chemotactic signal that are produced by the female gametophyte (Hulskamp *et al*., 1995, Ray *et al* 1997). As the pollen tube approach to the micropyle one of the synergid cells start to degenerate. It seems that synergid cell degeneration will be complete once the pollen tube penetrates through the degenerating synergid. Once in the synergid, the pollen tube stops growing and burst to release the two sperm cells. One sperm cell fuses with the egg cell form the zygote. Second sperm cell fuses with the central cell to form the first triploid cell of the endosperm.

Molecular Control of Ovule Development

Genes Controlling Ovule Identity:

There are number of mutations that effect the normal ovule development in *Arabidopsis thaliana*. The characterization of these mutants has lead to the identification of several genes involved in the molecular control of the ovule development.

Ovule identity is determined by transcription factor complexes, composed by MADS-box proteins (Brambilla *et al*., 2007, Favaro *et al*., 2003). SEEDSTICK (STK), SHATTERPROOF1 (SHP1), SHATTERPROOF2 (SHP2), SEPALLATA1, 2 or 3 (SEP1,SEP2 or SEP3) are MADS-box transcription factors that could form a complex controlling ovule identity. (Favaro *et al*., 2003).

STK is expressed in the placental tissue, in the ovule primordia, and also in later stages of ovule development. *stk* mutant have seeds that show defective abscission zone formation. Funiculus of *stk* ovules is formed by more and larger cells respect to the wild type (Pinyopich *et al.,* 2003).

SHP1 and *SHP2* single mutant do not have any phenotypic defect. *Shp1shp2* double mutant have carpel lacking of the dehiscence zone (Liljegren *et al.,* 2000). It has been shown that *SHP1* and *SHP*2 are redundant to *STK* in the maintaining the ovule identity (Pinyopich *et al.,* 2003). In the *stkshp1shp2* triple mutant, formation of ovule disrupted completely and ovule integument developed as a carpelloid structure.

Other important MADS-box transcription factor that have an important role in the ovule identity are the *SEPALLATA* (*SEP*). *SEP1 SEP2* and *SEP3* are required to determine the petals stamens and carpel identities (Pelaz *et at*., 2000). Single *sep* mutant have very mild defect, whereas the triple mutant showed indeterminate flower composed of only sepals. Interestingly in the *SEP1/sep1sep2sep3* mutant plants the ovules phenotype is similar to the *stk shp1shp2* triple mutant (Favaro *et al*., 2003). In this mutant the ovules lose

their identity and converted into carpel or leaf like structure. The protein interaction studies revealed that STK, SHP1 and SHP2 along with the SEP are involved in the multimeric complex formation that is controlling ovule identity.

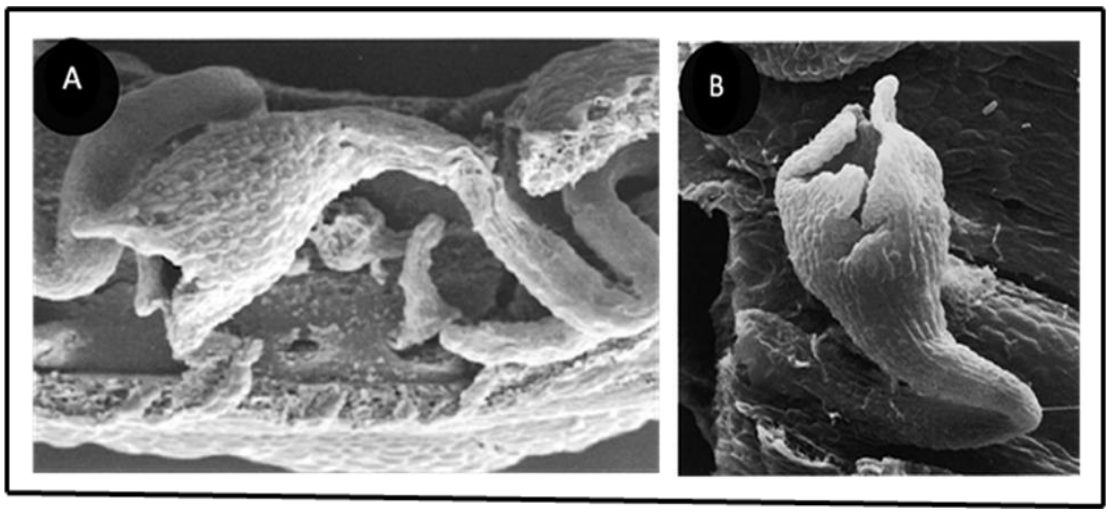


Figure 3: Ovule phenotype of MADS-box gene controlling ovule identity.

A: Ovules of a *SEP1/sep1 sep2 sep3* mutant plant. Ovule development is affected severely. Ovules are transformed into carpel- and leaf-like structures.

B: Ovules of *stk shp1 shp2* mutant plant. Ovule development is affected severely. The phenotype is similar to that shown in (A).

Another gene that is involved in ovule identity determination is *AGAMOUS* (*AG*). *ag* mutant completely lack carpel (Yanofsky *et al.,*1990). When *ag* mutant is combined with *apetala2* mutation, ectopic carpel like structure including ovules are found on the sepal margin (Bowman *et al*., 1991). This phenotype revealed that carpel like structure can develop in the absence of *AG* activity. However in the quadruple mutant of *ag ap2 shp1 shp2* the no carpelloid structure including ovules have been found in the sepals (Pinyopich *et al.*, 2003), suggesting that *SHP1* and *SHP2* are necessary for *AG* independent carpel and ovule formation.

Genes Controlling Ovule Numbers:

One important gene identified by mutants analysis is *AINTEGUMENTA (ANT)* that encodes for an of AP2-like transcription factor. ANT is involved in growth control during lateral organ formation by positively regulating cell proliferation (Mizukami *et al.,* 2000). *ANT* is expressed in all organ primordia. First it expressed in the placenta then in the ovule primordia and later in the funiculus and in the integumants while no *ANT* expression is detected in the nucellus (Elliot *et al.,* 1996). *ant* mutant produce 50% less ovules than wild type and they are completely female sterile (Baker *et al.,* 1997). In the *ant* mutant megagametogenesis does not occur properly (Elliot *et al*., 1996) and embryo sac development is blocked at FG1 stage (Schneitz *et al.,*1997).

Another mutants that presents less ovule primorida is the *cuc1 cuc2* (CUP-SHAPED-COTYLEDON) double mutant that causes a reduced number of ovule primordia and almost complete sterility of the ovules that are formed (Ishida *et al*., 2000). These two genes are essential for maintaining the meristem identity and the proper formation of organ boundaries. It is known that *CUCs* expression are repressed by auxin and their transcripts are cleaved by the auxin induced *micro RNA164* product (Guo et al 2005; Shani et al 2006).

Genes Controlling Ovule Patterning:

During early stages of ovule development primordia are characterized by Proximal-distal (P-D) or radial symmetry. Along the proximal and distal axis three distinct zones can be differentiated. The distal region of the primordium is the nucellus, which bears the mega spore mother cell, the central region is the chalaza, and the proximal region toward the placenta is formed funiculus. The first adaxial abaxial (Ad-Ab) polarity is established in the ovule when the outer integument initiate from the chalaza. The outer integument grows more in the abaxial side respect to the adaxial side and determine the curvature in the developing ovule. Genetic and molecular analyses have identified several genes that are involved in the transition from the radial symmetry of the primordium to bilateral symmetry of the mature ovule.

PHABULOSA (*PHB) and PHABOLUTA* (*PHV*) genes are involved to maintain the adaxial cell fate (Bohmert *et al*.,1998; Lynn *et al*., 1999; McConnel and Barton 1998; McConnel *et al.,* 2001; McHale and Marcotrigiano 1998; Timmernans et al 1998; Waites and Hudson 1995). Those genes are members of *YABBY* and *KANADI* families that encode transcription factors, involved in the abaxial cell fate determination (Bowman 2000a; Esched et al., 1999, Golz and Hudson 1999)

Another important gene controlling the ovule pattern formation is *BELL1* (*BEL1*) that is expressed in the chalaza and in both of the integuments (Reiser et al 1995). *BEL1* encode a homeodomain protein. In the *bel1* mutant an abnormal cell outgrowth takes place from the chalaza region and proper integuments fail to develop (Modrusan *et al*., 1994a; Ray *et al*., 1994; Reiser *et al*.,1995; Schneitz *et al*., 1997). Funiculi of the *bel1* mutant are thicker than the normal which contain more cells than wild type. The more striking phenotype is the abnormal cell outgrowth that forms an integument like structure (Robinson *et al*., 1995). Therefore *BEL1* play an important role in

patterning during ovule development in the crucial developmental step where the transition from the P-D symmetry to the Ad-Ab polarity occurs.

The molecular control of ovule patterning has been partly elucidated with the functional characterization of the *SPOROCYTELESS* (*SPL*) also known as *NOZZLE* (*NZZ*). In the *nzz* mutant nucellus is absent. In the *spl* mutant the outer integument initiate earlier than the inner integument. In *nzz bel1* double mutant chalazal region is absent. The cells in the chalazal region are similar to the funicular cells, which is epidermal in origin. *NZZ* and *BEL1* function redundantly to maintain the chalazal cell specification.

ANT plays an important role not only in ovule primordia formation but also in later stages of ovule development (Krizek 1999., Krizek *et al*., 2000; Leu *et al*., 2000; Mizukami and Fischer 2000; Schneitz *et al.,* 1998). It has been proposed that *NZZ* interact with *BEL1* and *ANT* to control the proximal-distal pattern formation and growth during ovule development (Balsubramanian and Schneitz 2000).

INNER NO OUTER (*INO*) a member of YABBY family plays an important role in Ad-Ab pattern formation during ovule development (Villanueva *et al.,*1990). Outer integument is absent in the *ino* mutant (Baker *et al*.,1997; Schneitz *et al*.,1997). Before the outer integument initiation, *INO* is expressed in cells of the region that will give rise to the outer integument (Villavueva *et al.,* 1999). Therefore *INO* is also involved for maintenance of the ovule pattern formation.

Another important gene that is involved in the pattern formation is the *SUPERMAN* (*SUP)* (Gaiser *et al*.,Sakai *et al*.,1995). In *sup* mutant the outer integument initiate properly but then start to grow equally in the both adaxial and abaxial side. Therefore *SUP* is an important candidate for the maintenance rather than initiation of the Ad-Ab polarity.

The homeobox gene *WUSCHEL* (*WUS)* plays an important role in the transition of ovule patterning. *WUS is* expressed in the nucellus but it was found to be necessary for the integument formation (Gross Hardt *et al.,* 2002).

The ectopic *WUS* expression in the chalaza resulted single inner integument and multiple outer integument formation. Therefore it indicates that *WUS* is enough to promote integument formation non cell autonomously.

The Role of Auxin:

Auxin plays a key role in all aspect of plant development. Auxin influences cell division, cell elongation and cell differentiation. The predominant form of the auxin present in the nature is Indole-3-Acetic Acid (IAA). Auxin is thought to be synthesized in young apical tissues and to transported downward to the mature stem or to the root by the diffusion or polar transport system. Inhibition of polar auxin transport blocks leaf initiation but not the meristem propagation (Reinhardt *et al*., 2000). The local increase in auxin concentration is enough to induce leaf primordia formation. It has been showed that auxin is not produce in the meristem but is transported there from the subtending tissue by the polar auxin transporter.

Auxin also promote lateral root initiation as was demonstrated by exogenous auxin application (Blakely and Evanse., 1979). Auxin accumulates at the position of future lateral root primordia. During later stage of the lateral organ development an auxin gradient is gradually established with maximum at the primordial tip (Benkova *et al.,* 2003)

Auxin move by using two pathways. One is the passive molecular diffusion and another is the active transport from cell to cell manner. These pathways are connected at the level of phloem loading in the leaves (Marchant *et al*., 2002) and phloem unloading in the roots (Swarup *et al*., 2001)

The classical physiological-based model for directional cell to cell auxin transport is the chemiosmotic model (Goldsmith 1977). According to this model a non-dissociated lipophilic form of auxin molecule can enter into the cell from the slightly acidic extracellular apoplast (pH 5.5) by passive diffusion.

AUX1/LIKE AUX1 influx carriers associate their action to the passive diffusion (Swarup *et al.,* 2008). Once inside the cell, auxin cannot diffuse out passively from the cytoplasm due to the higher pH 7. IAA molecules deprotonate, and the hydrophilic auxin anion (IAA^-) which is trapped in the cytosol. The transport of the anionic auxin requires an auxin efflux carrier protein located in the cell membrane.

A major role of the Polar Auxin Transport (PAT) is played by the 8 members of *PIN FORMED* (*PIN*) gene family. In Arabidopsis *PIN1* mediates organogenesis and vascular tissue differentiation, embryogenesis, lateral organ formation (Galweiler *et al*., 1998; Benkova *et al*., 2003; Reinhardt *et al*., 2003). *PIN2* and *PIN3* are involved in root meristem activity (Friml et al., 2002a). *PIN4* and *PIN7* maintain the root patterning and development (Petrasek and Friml 2009). *PIN5* is involved in intracellular auxin homeostasis. (Mravec *et al*., 2009)

PIN1 is the best characterized among all PINs. The pin1 mutant shows structural defect, naked stem due to complete lack of lateral organs. Occasionally flowers with no stamen or carpel, or sometime a pistil like structure is found but devoid of ovules (Okada et al., 1991).

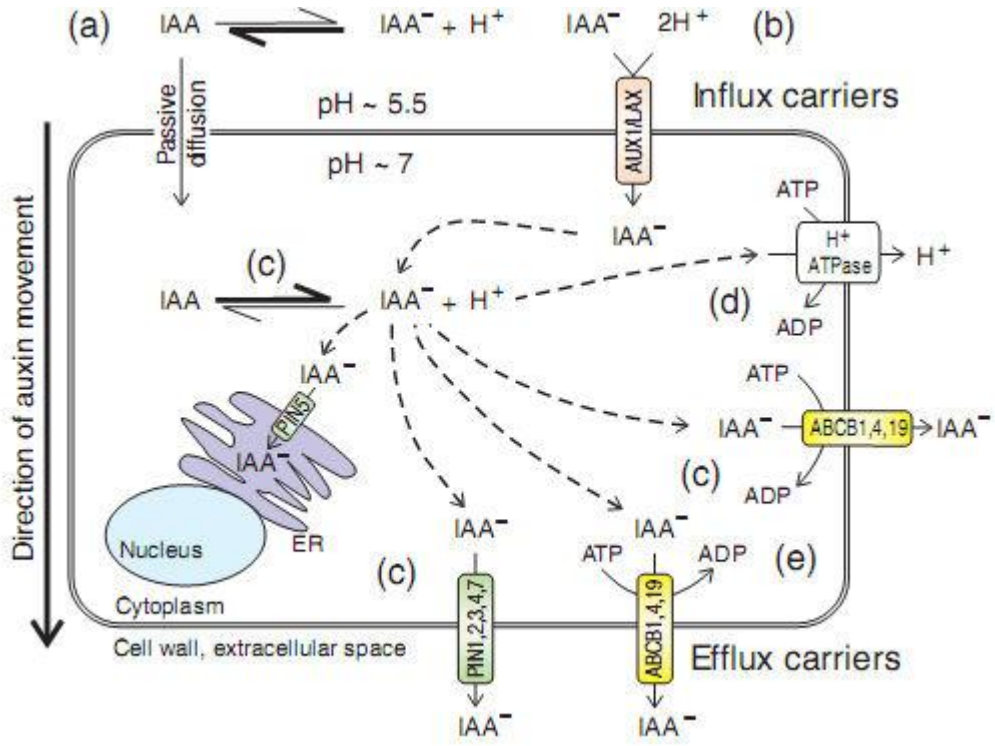


Figure 4: Schematic representation of PAT (from Petrasek and Friml 2009).

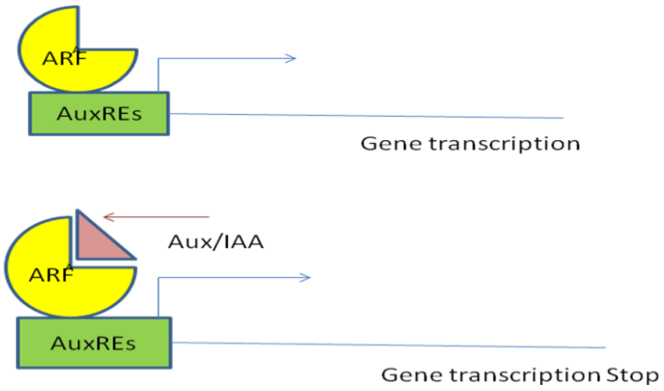
Non-dissociated IAA molecules enter the plasma membrane from slightly acid (ph5.5) extracellular environment by passive diffusion and by AUX1/LAX influx carriers action; the alkaline pH of cytosol turns the majority of IAA molecules into their anionic form (IAA), which can only exit the cell by active transport mediated by efflux facilitators like PIN proteins.

Auxin Signaling Network:

The complex of auxin responses are mediated by two groups of well studied genes. The *Auxin/ Indole 3- Acetic Acid (AUX/IAA*) genes family, that consist of 29 members and *Auxin Responsive Factors* (*ARF*s) gene family with 23 members in *Arabidopsis thaliana*. ARFs control the auxin regulated transcription and they work in collaboration with AUX/IAA proteins. Auxin Response Elements (*AuxREs*) are the binding site of the ARF family of

transcription factors. ARFs bind to AuxREs in the promoter of primary auxin responsive genes and mediating transcription. The activity of ARFs is regulated through the interaction with AUX/IAA repressors. ARFs and AUX/IAA proteins can form dimers through sharing the carboxy-terminal domains (Kim *et al*., Ulmasov *et al*., 1997b.). ARFs recognize AuxREs found in the promoter of *AUX/IAA* early responsive genes (Guilfoyle *et at*.,1998a).The ARF-AUX/IAA complexes are not able to bind the auxin responsive promoters. An increase level of auxin lead to the proteosome mediated degradation of AUX/IAA leaving the active ARF proteins and active transcription of the target genes. Auxin bind to the F-box protein TIR1 (TRANSPORT INHIBITOR RESPONSE 1) receptor this facilitates the interaction and the degradation of AUX/IAA protein. This reaction is catalyzed by an SCF E3 ubiquitin ligase.

AuxREs genes contain the nucleotide sequence TGTCTC (Ulmasov *et al.,* 1997). The auxin responsive *GH3* gene from soybean contains multiple AuxREs. Several copies of AuxREs have been identified from that promoter and cloned upstream of minimal Cauliflower Mosaic virus (CaMv) (Ulmasov *et al* 1997b., Sabatini *et al*., 1999., Friml *et al*.,2003) to form the synthetic auxin responsive promoter, named DR5. The DR5::GUS and DR5::GFP are widely used markers to check the accumulation of auxin *in vivo* (Sabatini *et al.,* 1999; Benkova *et al*., 2003)



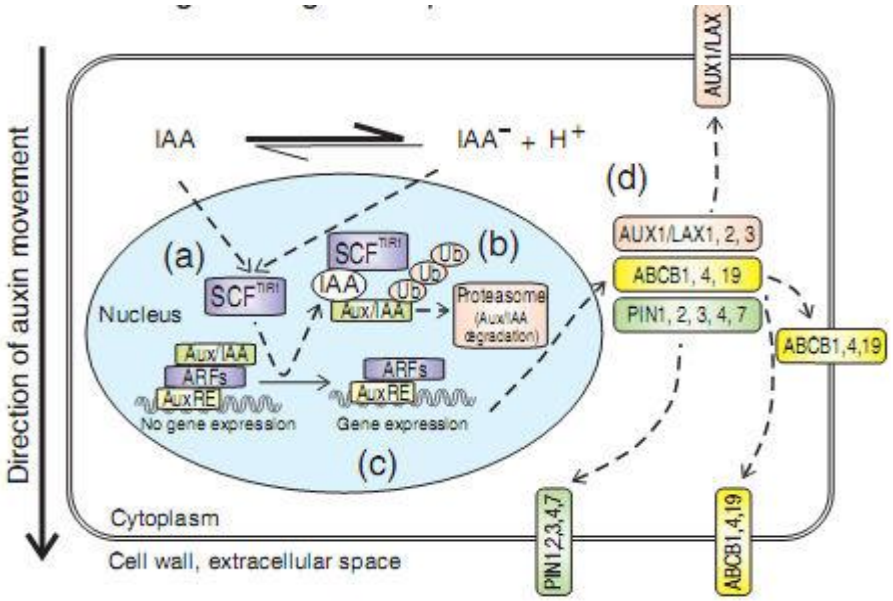


Figure 5: Auxin-regulated gene expression (from Petrasek and Friml 2009).

Intracellular auxin binds to its nuclear receptor (TIR1) which is a subunit of the SCF E3-ligase protein complex.

a: This lead to the ubiquitylation and the proteasome- mediated specific degradation of auxin Aux/IAA transcriptional repressors.

b: Finally, the auxin response factors (ARFs) are de-repressed and can activate auxin-inducible gene expression.

Role of Auxin in Ovule Development:

The first evidence of the involvement of auxin in ovule formation is reported by Benkova and colleagues in 2003. They proposed that in the developing lateral organ primordia, auxin is accumulate in the tip of the primordia by passing through the outer cell layers of the epidermis. From the

tip auxin flow toward the sub epidermal layers and pass through the vascular route (Benkova *et al*., 2003)

Recently it has been shown that gradient of auxin is required to determine the identity of the cell types in the female gametophyte (Pagnussat *et al.,* 2009)

The possible way to investigate the role of auxin in ovule development is to analyze the expression of primary auxin responsive genes in the ovule. One of the genes is *DORNSCHEN* (*DRN*). *DRN* is a transcription factor belonging to the AP2 family. In the ovule *DRN* is expressed in the epidermal cells of their analgen and in the tip of the growing ovule primordia (Kirch et al. 2003; Chandler et al. 2007; Cole et al 2009). *DRN* acts upstream of auxin transport and it also responses to auxin. *drn* embryo shows dislocated diffused DR5 activity and altered polar localization of gene *PINFORMED1* (*PIN1*) (Chandler *et al*., 2007).

MONOPTEROS/ AUXIN RESPONSE FACTOR5 (MP/ARF5) has been now proved to interact in vivo with *DRN* promoter. *MP* binds 2 of 3 AREs in the *DRN* promoter. It provides evidence that auxin, through *ARF5* directly and positively regulate DRN expression in the tip of the cotyledons of torpedo embryos. *MP* action on *DRN* is highly specific. (Cole *et al*., 2009).

REFERENCES:

- **Baker SC, Robinson-Beers K, Villanueva JM, Gaiser JC, Gasser CS.** Interactions among genes regulating ovule development in *Arabidopsis thaliana. Genetics.* 1997. Apr; 145(4):1109-24.
- **Balasubramanian, S. and Schneitz, K. (2000)** NOZZLE regulates proximal-distal pattern formation, cell proliferation and early sporogenesis during ovule development in Arabidopsis thaliana. *Development* 127, 4227–4238.
- **Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D.,Jurgens, G., and Friml, J**. (2003). Local efflux-dependent auxin gradients as a common module for plant organ formation. Cell *115*, 591-602.
- **Blakely, L. M., and T. A. Evans.** 1979. Cell dynamics studies on the pericycle of radish seedling roots. *Plant Science Letters* 14:79-83.
- **Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M. and Benning, C.**(1998). *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO J.***17**, 170-180.
- **Bowman, J.L., and Meyerowitz, E.M.** (1991). Genetic control of pattern formation during flower development in Arabidopsis. Symp. Soc. *Exp. Biol*. 45: 89–115
- **Bowman, J. L.** (2000). The *YABBY* gene family and abaxial cell fate. Curr. Opin. *Plant Biol.* 3, 17-22.
- **Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S., Kater, M. M., and Colombo, L. (2007).** Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in Arabidopsis. *Plant Cell 19*, 2544-2556.
- **Christensen, C. A., King, E. J., Jordan, J. R., and Drews, G. N.** (1997). Megagametogenesis in Arabidopsis wild type and the Gf mutant. *Sexual Plant Reproduction* 10, 49-64.

- **Chandler, J. W., Cole, M., Flier, A., Grewe, B., and Werr, W**. (2007). The AP2 transcription factors DORNROSCHEN and DORNROSCHEN - LIKE redundantly control Arabidopsis embryo patterning via interaction with PHAVOLUTA. *Development 134*, 1653-1662.
- **Cole, M., Chandler, J., Weijers, D., Jacobs, B., Comelli, P., and Werr, W.** (2009). DORNROSCHEN is a direct target of the auxin response factor MONOPTEROS in the Arabidopsis embryo. *Development* 136 (10):1643-51.
- **Elliott, R. C., Betzner, A. S., Huttner, E., Oakes, M. P., Tucker, W. Q., Gerentes, D., Perez, P., and Smyth, D. R. (1996).** AINTEGUMENTA, an APETALA2-like gene of Arabidopsis with pleiotropic roles in ovule development and floral organ growth. *Plant Cell 8*, 155-168.
- **Eshed, Y., Baum, S. F. and Bowman, J. L.** (1999). Distinct mechanisms promote polarity establishment in carpels of *Arabidopsis*. *Cell* **99**, 199-209.
- **Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M. F., Kater, M. M., and Colombo, L. (2003).** MADS-box protein complexes control carpel and ovule development in Arabidopsis. *Plant Cell 15*, 2603-2611.
- **Friml J, Benkova E, Blilou I, Wisniewska J, Hamann T, Ljung K, W oody S, SandbergG, Scheres B, Jurgens G, et al.** 2002. AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis. Cell* **108**: 661–673.
- **Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003).** Effluxdependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature 426*, 147-153.
- **Gaiser, J. C., Robinson-Beers, K. and Gasser, C. S.** (1995). The *Arabidopsis SUPERMAN* gene mediates asymmetric growth of the outer integument of ovules. *The Plant Cell* **7**, 333-345.

- **Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998).** Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science 282*, 2226-2230.
- **Gao, X., Nagawa, S., Wang, G., and Yang, Z. (2008).** Cell polarity signaling: focus on polar auxin transport. Mol Plant *1*, 899-909
- **Goldsmith, M. H. M. (1977)**. The polar transport of auxin. An Rev *Plant Physiol 28* 439–478.
- **Golz, J. F. and Hudson, A.** (1999). Plant development: YABBYs claw to the fore.*Curr. Biol.* **9**, R861-863.
- **Gross-Hardt, M. Lenhard and T. Laux,** (2002) WUSCHEL signaling functions in interregional communication during *Arabidopsis* ovule development, *Genes Dev.* **16** , pp. 1129–1138.
- **Guo, H. S., Xie, Q., Fei, J. F., and Chua, N. H. (2005).** MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. *Plant Cell* 17, 1376-1386.
- **Guilfoyle, T.J.** (1998). Aux/IAA proteins and auxin signal transduction. *Trends Plant Sci*. **3,** 205–207.
- **Hülskamp**, **M., Schneitz, K., and Pruitt, R.E.** (1995). Genetic evidence for a long-range activity that directs pollen tube guidance in Arabidopsis. *Plant Cell* 7: 57–64.
- **Ishida T, Aida M, Takada S, Tasaka M** (2000) Involvement of CUP-SHAPED COTYLEDON genes in gynoecium and ovule development in *Arabidopsis thaliana. Plant Cell Physiol* **41:** 60–67
- **Kim J, Harter K, Theologis A.**1997. Protein–protein interactions among the Aux/IAA proteins. Proceedings of the National Academy of Sciences of the USA94: 11786–11791
- **Kirch, T., Simon, R., Grunewald, M., and Werr, W. (2003).** The DORNROSCHEN/ENHANCER OF SHOOT REGENERATION1 gene of Arabidopsis acts in the control of meristem cell fate and lateral organ development. *Plant Cell 15*, 694-705.

- **Krizek, B. A.** (1999). Ectopic expression of *AINTEGUMENTA* gene results in increased growth of floral organs. *Dev. Genet.* **25**, 224-236.
- **Krizek, B. A., Prost, V. and Macias, A.** (2000). *AINTEGUMENTA* promotes petal identity and acts as a negative regulator of A*GAMOUS*. *The Plant Cell* **12**, 1357-136
- **Kooiker, M. et al. (2005)** BASIC PENTACYSTEINE1, a GA binding protein that induces conformational changes in the regulatory region of the homeotic Arabidopsis gene SEEDSTICK. *Plant Cell* 17, 722–729
- **Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L., and Yanofsky, M.F**. (2000). SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature* 404: 766–770.
- **Liu, Z., Franks, R. G. and Klink, V. P.** (2000). Regulation of gynoecium marginal tissue formation by *LEUNIG* and *AINTEGUMENTA*. *The Plant Cell* **12**, 1879-1892.
- **Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P. and Barton, M. K.** (1999). The *PINHEAD/ZWILLE* gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the *ARGONAUTE1* gene. *Development* **126**, 469-481.
- **Marchant, A. et al. (2002)** AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. *Plant Cell* 14, 589–597
- **McConnell, J. R. and Barton, M. K.** (1998). Leaf polarity and meristem formation in *Arabidopsis*. *Developmen*t **125,** 2935-2942.
- **McConnell, J. R., Emery, J., Eshed, Y., Bao, N., Bowman, J. and Barton, M. K.**(2001). Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots. *Nature* **411**, 709-713.
- **McHale, N. A. and Marcotrigiano, M.** (1998). *LAM1* is required for dorsoventrality and lateral growth of the leaf blade in *Nicotiana*. *Development* **125**
- **Mizukami, Y., and Fischer, R. L. (2000).** Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proc Natl Acad Sci* U S A *97*, 942-947.

- **Modrusan, Z., Reiser, L., Feldmann, K. A., Fischer, R. L., and Haughn, G. W.** (1994). Homeotic Transformation of Ovules into Carpel-like Structures in Arabidopsis. *Plant Cell* 6, 333-349.
- **Mravec, J., Kubes, M., Bielach, A., Gaykova, V., Petrasek, J., Skupa, P.,Chand, S., Benkova, E., Zazimalova, E., and Friml, J.** (2008). Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. *Development 135*, 3345-3354.
- **Mravec, J., Skupa, P., Bailly, A., Hoyerova, K., Krecek, P., Bielach, A., Petrasek, J., Zhang, J., Gaykova, V., Stierhof, Y. D.*, et al.*** (2009). Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature.*
- **Pagnussat, G. C., Alandete-Saez, M., Bowman, J. L., and Sundaresan, V.** (2009). Auxin-dependent patterning and gamete specification in the Arabidopsis female gametophyte. *Science 324*, 1684-1689.
- **Petrasek, J., and Friml, J.** (2009). Auxin transport routes in plant development. *Development 136*, 2675-2688.
- **Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., et al.** (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* 312, 914-918.
- **Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E., and Yanofsky, M. F.** (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature 424*, 85-88.
- **Ray, A., Robinson-Beers, K., Ray, S., Baker, S. C., Lang, J. D., Preuss, D., Milligan, S. B. and Gasser, C. S.** (1994). Arabidopsis floral homeotic gene *BELL (BEL1)* controls ovule development through negative regulation of *AGAMOUS* gene *(AG)*. *Proc. Natl. Acad. Sci. USA* **91**, 5761-5765.
- **Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G. W. and Fischer, R. L.** (1995). The *BELL1* gene encodes a

homeodomain protein involved in pattern formation in the *Arabidopsis* ovule primordium*. Cell* **83**, 735-742.

- **Reinhardt, D., Mandel, T., and Kuhlemeier, C.** (2000) Auxin regulates the initiation and radial position of plant lateral organs *Plant Cell* **12**, 507-518.
- **Reinhardt, D., Pesce, E. R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C.** (2003). Regulation of phyllotaxis by polar auxin transport. *Nature 426*, 255-260.
- **Ray SM, Park SS, Ray A.** (1997) Pollen tube guidance by the female gametophyte. *Development.* Jun;124(12):2489-98.
- **Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., and Scheres, B.** (1999). An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. *Cell 99*, 463-472.
- **Sakai, H., Medrano, L. J. and Meyerowitz, E. M.** (1995). Role of *SUPERMAN* in maintaining *Arabidopsis* floral whorl boundaries. *Nature* **378**, 199-203.
- **Schneitz, K., Hulskamp, M., and Pruitt, R. E.** (1995). Wild-Type Ovule Development in Arabidopsis-Thaliana – A Light-Microscope Study of Cleared Whole-Mount Tissue. *Plant Journal* 7, 731-749.
- **Schneitz, K., Hülskamp, M., Kopczak, S. D. and Pruitt, R. E.** (1997). Dissection of sexual organ ontogenesis: a genetic analysis of ovule development in *Arabidopsis thaliana*. *Development* **124**, 1367-1376
- **Schneitz, K., Baker, S. C., Gasser, C. S. and Redweik, A.** (1998). Pattern formation and growth during floral organogenesis: *HUELLENLOS* and *AINTEGUMENTA* are required for the formation of the proximal region of the ovule primordium in *Arabidopsis thaliana*. *Development* **125**, 2555-2563.
- **Shani E, Yanai O, Ori N** (2006) The role of hormones in shoot apical meristem function. *Curr Opin Plant Biol* **9** 484–489
- **Smyth, D. R., Bowman, J. L., and Meyerowitz, E. M** (1990). Early flower development in Arabidopsis. *Plant Cell* 2, 755-767.

- **Swarup, R. et al. (2001)** Localisation of the auxin permease AUX1 suggest two functionally distinct hormone transport pathways operate in the Arabidopsis root apex. *Genes Dev*. 15, 2648–2653
- **Timmermans, M. C. P., Schultes, N. P., Jankovsky, J. P. and Nelson, T.** (1998).*Leafbladeless1* is required for dorsoventrality of lateral organs in maize. *Development* **125**,2813-2823.
- **Ulmasov, T., Hagen, G., and Guilfoyle, T. J.** (1997a). ARF1, a transcription factor that binds to auxin response elements. *Science* 276, 1865-1868.
- **Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T. J.** (1997b). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell 9*, 1963-1971
- **Villanueva, J.M. et al.** (1999) INNER NO OUTER regulates abaxial adaxial patterning in Arabidopsis ovules. *Genes Dev*. 13, 3160–3169
- **Waites, R. and Hudson, A.** (1995). *Phantastica*: a gene required for dorsoventrality of leaves in*Antirrhinum majus*. *Development* **121**, 2143-2154.
- **Yanofsky, M.F. et al. (1990)** The protein encoded by the Arabidopsis homeotic gene AGAMOUS resembles transcription. *Nature* 346, 35–39

Chapter 2

Interaction among Genetic and Auxin Networks in Ovule Primordia Formation

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Introduction:

A big question in developmental biology is the understanding of the molecular pathways by which the undifferentiated groups of meristematic cells become dedicated to form well-defined organ primordia. In my Ph.d project, the goal is to find out the key regulators that promote the meristematic placenta cells to initiate the developing ovule primordia.

The fundamental processes of primordia initiation depend on the mechanism that determines the position of primordia. Recently the approach has been taken to identify large number of mutants that affecting the number of developing primordia. Analyses of those mutants lead to identify the possible candidate genes, play vital role in primordia formation.

Wild-Type ovule primordia initiate from the meristematic placental tissue of the carpel during stage 8 of the flower development (Smyth *et al.*,1990). Primordia start to elongate, megasporocyte can be recognized in the nucellus during stage 9. The inner and outer integument initiate after the periclinal division of the chalazal epidermal cells during stage 10 of flower development. Megasporogenesis starts during this stage. The growth of integument continues during stage 11 and 12. Asymmetric growth of outer integument leads to cover the inner integument and the nucellus, leaving a small opening micropyle. The megagametogenesis starts during stage 12 of flower development.

It has been shown that the plant hormone auxin plays a central role in determination of position of new ovule primordia. Auxin accumulates in the area, where the primordia are going to arise. In the initiated primordia the level of auxin is highest.

PIN FORMED 1 (PIN1) a putative auxin efflux carrier (Galweiler *et al*., 1998) is important in pattern formation during organ development. After selecting the position of the developing primordia, boundaries are established between two primordia. The cells are specified for proper organ development. *CUP SHAPED COTYLEDON (CUC)* genes are essential to establish the proper primordia boundaries. Very few genes controlling the primordia outgrowth have been identified. Transcriptional regulator AINTEGUMENTA (ANT) is one of them which involved in cell proliferation during early stage of organ formation (Krizek *et al*., 1999.; Mizukami and Fisher., 2000).

AINTEGUMENTA (*ANT*) Controls Ovule Primordia Number:

Several mutants affecting the ovule primordia development has been characterized. Mutant analyses described that *ant* mutant shows defect in integument and female gametophyte development. The initiation of ovule primordia in *ant* mutant occurs in the same floral stage similar to wild type. The number of ovules in *ant* flower is reduced in respect to the wild type flowers. The ovules initiate more distantly spaced from each other than wild type. In the *ant-1* mutant the initiation of integument does not occur. The integument development varied among the different alleles (figure 6B). In the strongest *ANT* allele *ant-4* ovule primordia develop normally. However, instead of the two integument primordia an irregular ridge of cells develops from the chalazal region, called integumentary ridge (Figure 7A). These cells do not under go any further division or expansion. In a weaker allele *ant-5* the integumentary ridge cells under goes further asymmetrical expansion (Figure 7B). In the weakest allele *ant-8* the integumentary ridge grows further and partly it surrounds the nucellus (Figure 7C and D Baker *et al*., 1997). In the ovule of *ant3* the integuments initiation is abnormal (Klucher *et al.,* 1996). Both the integuments develop initially at the one side of the ovule (Figure 6C). The growth of the integument is similar like wild type ovule however the

integuments do not cover completely the nucellus because the size of the integument is shorter and smaller than wild type ovule.

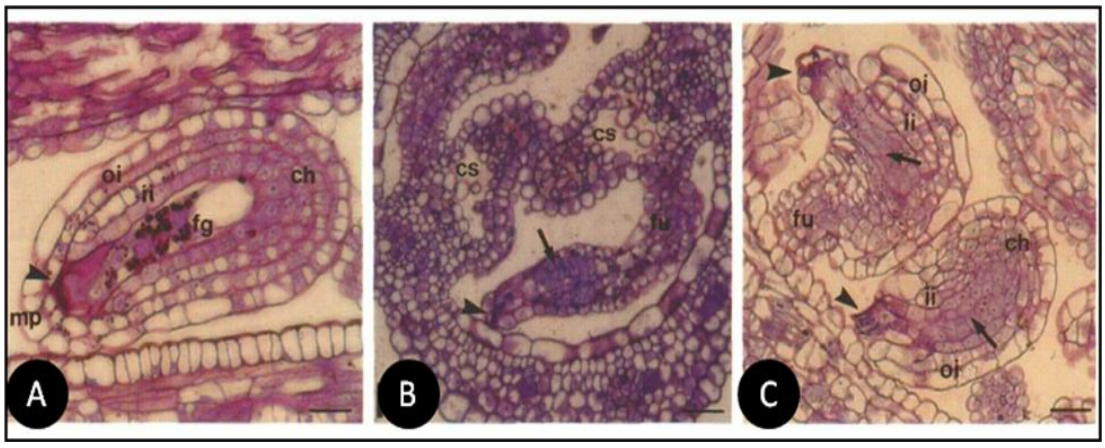

Figure 6: Morphology of ovule in different *ant* alleles (Klucher *et al*.,1996)

A: Wild type mature ovule of stage 13, showing the fully developed inner and outer integuments.

B: *ant1* ovules from a stage 13 with the abnormal proliferation of cells in the nucellus, which is shown by the arrow.

C: *ant3* ovules from a stage13 flower. The arrows indicate the abnormal proliferation of cells in the ovule nucellus.

ch, chalaza; cs, central septum; fg, female gametophyte; fu, funiculus; ii, inner integument; mp, micropyle, ms, megasporocyte; oi, outer integument; ovw, ovary wall.

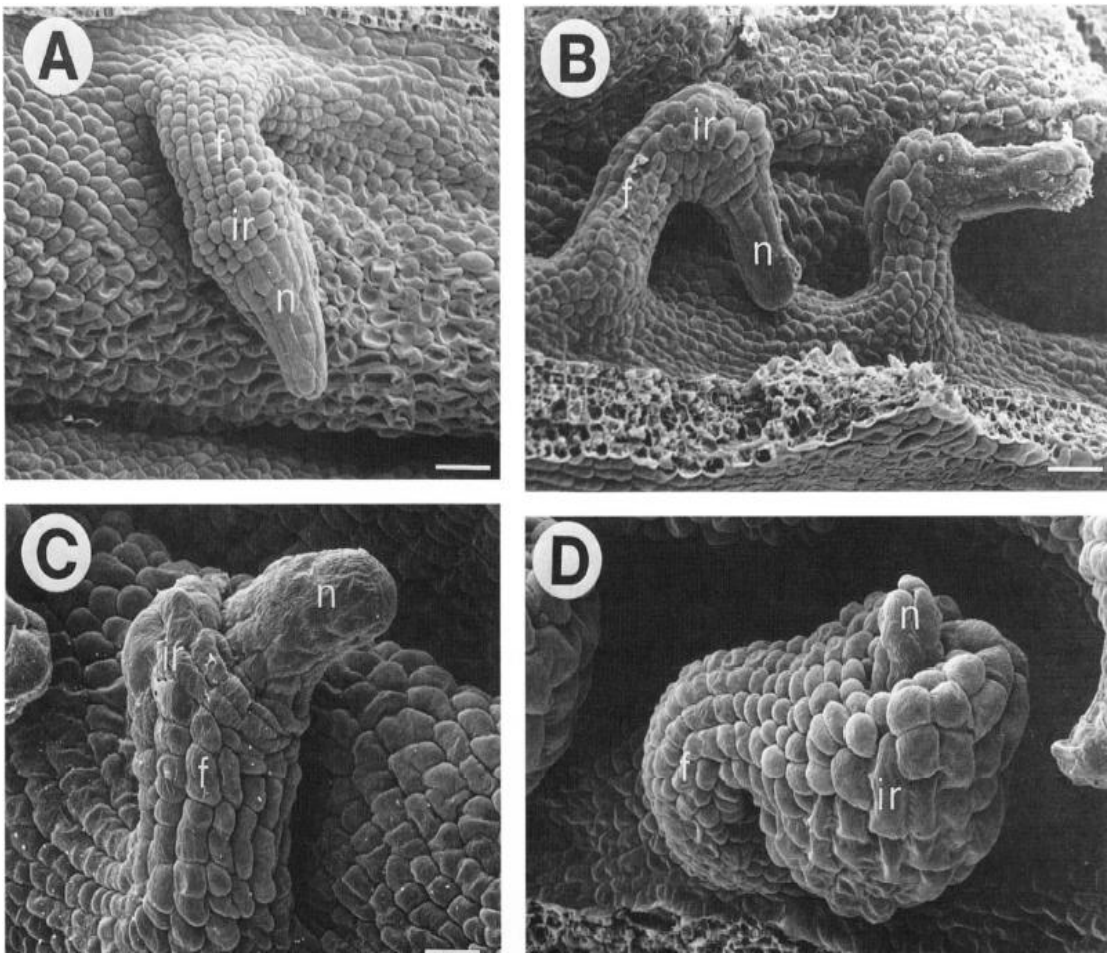

Figure 7: Scanning Electron Micrographs of *ant* Ovules. (Baker *et al*., 1997)

A: *ant-4* ovule, cells of the nucellus and funiculus are clearly differentiated from one another. Integumentary ridge are formed instead of integument primordia.

B: *ant-5* ovule, the integumentary ridge of cells is larger than that of *ant-4* ovule.

C: *ant-8* ovule at stage2, showing integumentary ridge partly surrounds the nucellus.

D: *ant-8* ovule, the integumentary ridge of cells has expanded to almost cover the nucellus.

f, funiculus: ir, integumentary-ridge; n, nucellus.

Another major effect of *ant* ovule is that the embryosac do not form. Megasporocyte develops within the nucellus of the *ant* ovule. According to

Baker et al, meiosis never occurs in the *ant* ovule. But megasporogenesis occurs in *ant3* ovules and tetrad formation has been described also in the *ant-9* ovule (Elliott *et al*., 1996; Klucher *et al.,* 1996). In both cases, the magagametogenesis never occur. Nucellus grows abnormally and produced an irregular proliferation of cells in the space normally occupied by the female gametophyte. *ant* ovule do not produce the seeds, although a small percentage of *ant-3* ovules (0.2% of wild type) generate viable seeds (Klucher *et al*., 1996).

Very strong *ANT* expression is detected in the placenta and in the ovule primordia from the time they arise from placenta (Figure 8A). During early stage of ovule development *ANT* is expressed in the chalaza (Figure 8D), the region from where integuments start to initiate. In the nucellus *ANT* is not expressed. The expression continues in the integuments during the early stage of development and start to decreases when the outer integument continue to grow and covers the nucellus (Elliott *et al*.,1996).

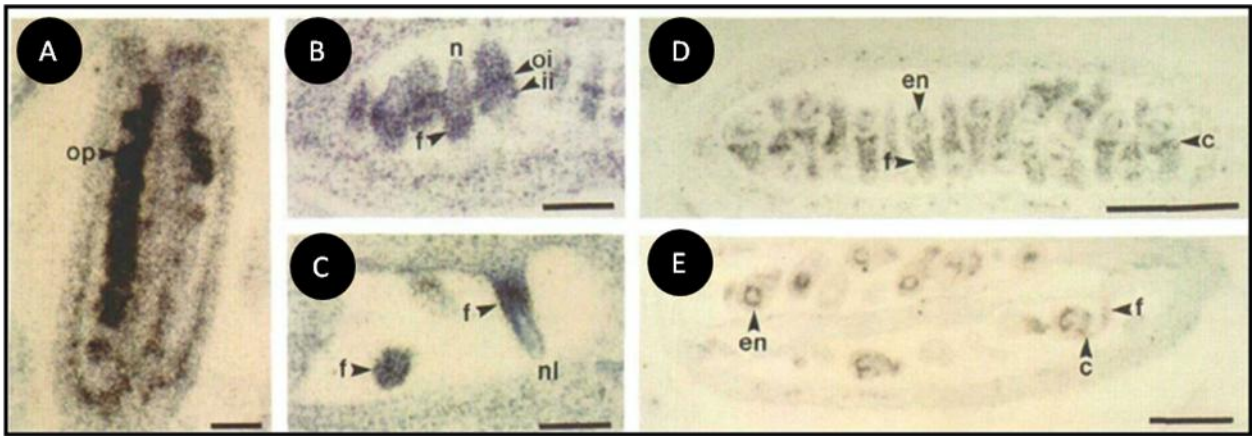

Figure 8: Expression of *ant* in carpel and ovules. (Elliott *et al*., 1996).

A: Longitudinal section of a stage 9 gynoecium in which the ovule primordia are intensely and uniformly labeled.

B: Longitudinal section of wild-type ovules in which the integuments are arising (stage II), showing that labeling is present in the two bands of integument cells and in the adjoining distal region of the funiculus.

C: Longitudinal section of *ant-9* ovules showing reduced but significant labeling in the region of the funiculus close to where integuments would normally have arisen (arrowheads).

D: Longitudinal section of a gynoecium in the medial plane from a stage 12 flower showing that the developing ovules are labeled in the funiculus, in the chalaza at the base of the ovule proper, and in the newly arising endothelium derived from the inner integument.

E: Longitudinal section of a gynoecium of stage 13 flower in which labeling continues in the funiculus, the chalaza**,** and the cup-shaped endothelium.

CUP SHAPED COTYLEDON (*CUC*):

Another group of genes that seems to be important in maintaining the primordia number by establishing the boundary between them is the *CUP SHAPED COTYLEDON* (*CUC*) family. *CUC* genes are involved in establishment of boundaries in the organ primordia. *cuc1 a*nd *cuc2* single mutant looked normal, hardly shows cotyledon fusion in one side and display weak fusion of sepals and stamens in the flowers (Aida *et al*., 1997) but the *cuc1cuc2* double mutant completely lack the shoot apical meristem, the two cotyledons fused along their margin to form a cup shaped structure (Aida *et al*.,1997). These observations indicate that *CUC1* and *CUC2* genes are functionally redundant. *CUC3* function is partially redundant with *CUC1* and *CUC2*. *CUC3* is also involved in the boundary establishment and maintenance (Vroemen *et al*., 2003).

Adventitious shoots regenerated from mutant calli generate the flowers in which the sepals and stamens are severely fused (Aida *et al*., 1997). Plants of *cuc1cuc2* produce fertile pollen, although flowers are sterile (Ishida *et al*., 2000). Carpel of those flowers show defect in septa formation. The number of ovules in the double mutant varied from zero to ten, suggesting that the

initiation of ovule primordia from the placenta in the carpel is inhibited in the *cuc1cuc2* double mutant. *cuc1cuc2* double mutant ovules show a varied degree of phenotype. Few ovules are similar to wild type (Figure 9F). In the ovules integuments grow normally, but in some of the ovules immediately after differentiation outer integument stops to grow further (Figure 9B). In some cases integuments fail to cover the nucellus completely (Figure 9 E). None of the ovules produce viable seeds (Ishida *et al*., 2000).

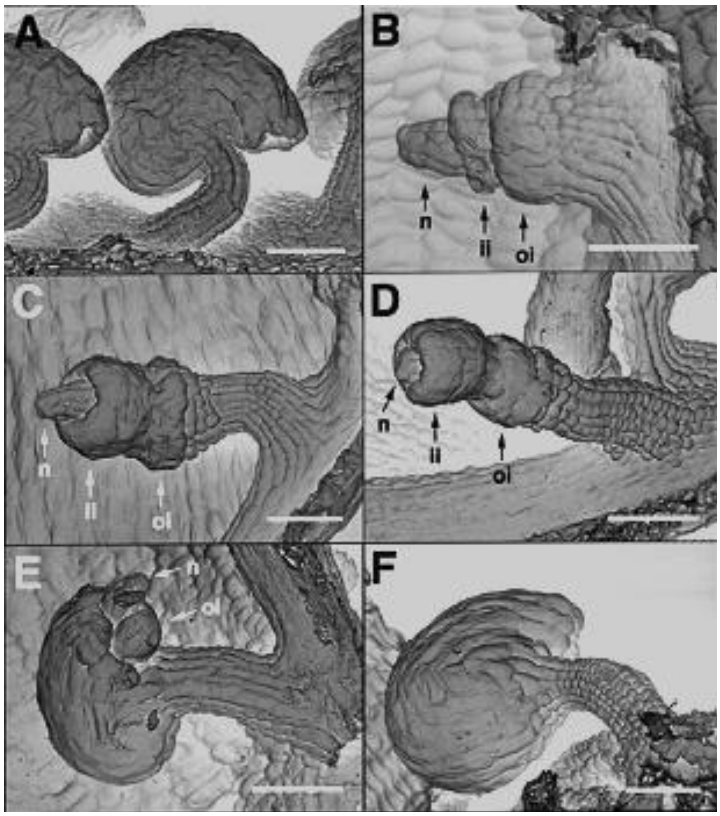

Figure 9: Ovules of the *cuc1cuc2* double mutant. (Ishida *et al.,* 2000)

A: Wild-type ovule at anthesis.

B to **F:** Ovules of the cuc1 cuc2 double mutant.

B: Ovule has a short funiculus and has failed to develop inner and outer integuments soon after differentiation.
C and **D:** Ovules have failed to develop an outer integument soon after differentiation however the growth of the inner integument has occurred. The extent of inner integument growth varies between different ovules.
E: Ovule has developed an outer integument which does not completely cover the nucellus.
F: Ovule is similar to the wild type ovule.
n, nucellus; ii, inner integument; oi, outer integument.

CUC2 is expressed in the boundaries of organ primordia. In the ovule, when the primordia arise from the placenta (Figure11). *CUC2* expression found at the boundaries of the ovule primordia. At the time of integument initiation, the expression of *CUC2* starts within the ovule at the boundary region between chalaza and nucellus.

In the inflorescence *CUC1* is expressed in the boundary region between inflorescence meristem. In the flower it found to be expressed all the boundary region between each floral whorls as well as the boundary region of each whorls (Figure 10). The patten of expression overlapped with *CUC2* (Aida *et al*., 1999., Ishida *et al*.,2000).

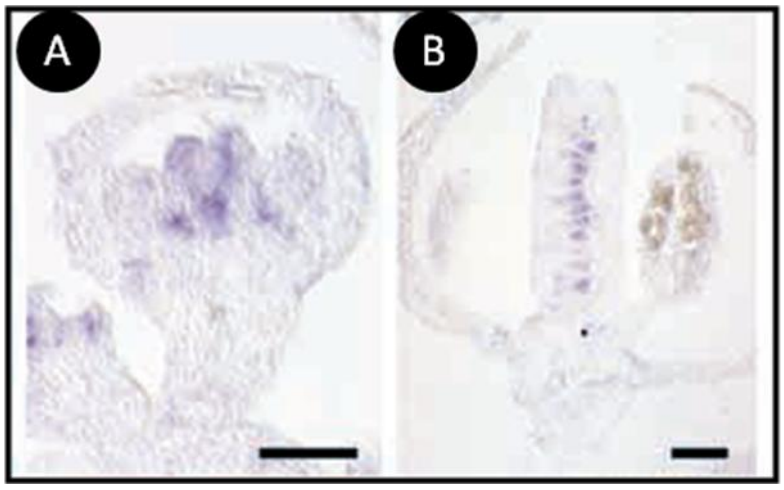

Figure 10: Expression of *cuc1* by *in situ* hybridization (Takada *et al*., 2001).

(A) Longitudinal section through a stage 7 flower.

(B) Longitudinal section through a stage 10-11 flower

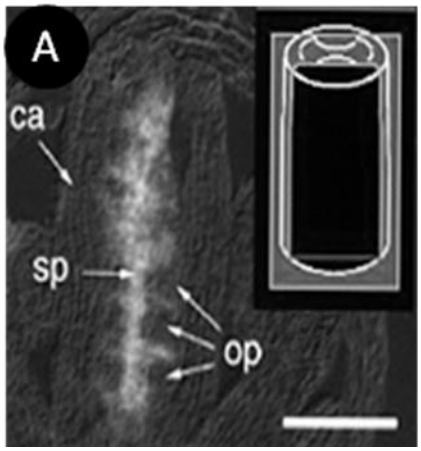

Figure 11: Expression of *CUC2* by *in situ* hybridization. (Ishida *et al*., 2000)

(A) Longitudinal section of gynoecium at late floral stage 8. *CUC2* is not expressed in ovule primordia but at the boundaries of ovule primordia

During embryogenesis several genes play important role to maintain the symmetry of embryo. *CUC1* and *CUC2* expressed in the region between cotyledon primordia and they are involved in cotyledon separation by preventing growth at the boundaries. In the embryo, *CUC1* is expressed starting from the globular stage at two separate region in the apical region. In the embryo of early heart stage *CUC1* is detected between two cotyledon primordia (Takada *et al.*, 2001. Figure12). *CUC2* is expressed starting from the early to mid globular stage of the embryo. In the embryo of heart stage, *CUC2* is expressed in the region between two cotyledon primordia and the expression continues till the torpedo stage of the embryo (Aida *et al*., 1999. Figure13).

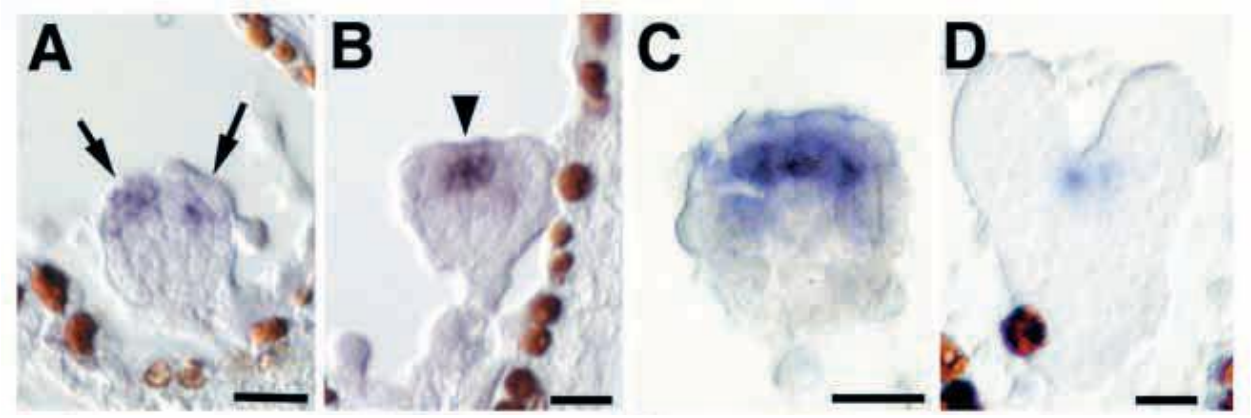

Figure 12: Localization of *CUC1 mRNA in embryos (*Takada *et al.*2001).

(A) Longitudinal section through a globular stage embryo. Arrows indicate *CUC1 expression in* two separate regions.

(B) Longitudinal section through an early-heart-stage embryo. The arrowhead

Indicate the boundary between cotyledon primordia.

(C) Sagittal longitudinal section through an early heart- stage embryo.

(D) Longitudinal section through a late-heart-stage embryo.

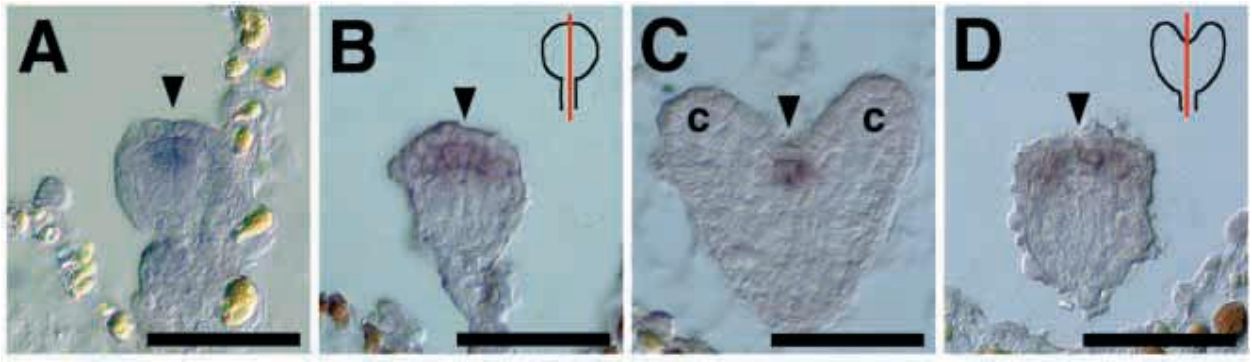

Figure 13 : Localization of *CUC2 mRNA in embryos* (Aida *et al*.1999).

(A) Longitudinal section of mid-globular embryo. Arrowhead indicates the expression of *CUC2.*

(B) Longitudinal section of late-globular embryo.

(C and D) Longitudinal section of early-heart embryo. Arrows indicate the boundary between the two cotyledon primordia.

Besides these genes a limited number of genes also involved in the patterning of embryogenesis. *PIN FORMED1*(*PIN1*) and *MONOPTEROS* (*MP*) have effect on cotyledon development. In *pin1* mutant the cotyledon position, number, and growth is affected (Bennett *et al.,* 1995; Okada *et al*., 1991). In the embryo *PIN1 is* expressed in the early globular stage before the beginning of expression of *CUC1* and *CUC2. PIN1* expression is restricted to the provascular tissue in the embryo axis and cotyledon during heart and torpedo stage of embryo development. The embryo development is also strongly affected in the *mp* mutant. Cotyledons are frequently fused as observed in *cuc1cuc2* double mutant.

PIN1 and *MP* are both related to the plant hormone auxin. PIN1 encode a Auxin efflux transporter (Palme and Galweiler *et al.*, 1999). The *MP* gene encodes a member of AUXIN RESPONSE FACTOR (ARF) gene family (Hardtke and Berleth., 1998) which binds to the promoter of auxin inducible genes (Ulmasov *et al.,* 1997). These two genes expressed normally in the *cuc1cuc2* double mutant embryo. But an abnormal expression of *CUC2* observed in the *pin1* and *mp* embryos, whereas *CUC1* expression domain expanded (Aida *et al*., 2002). This result suggests that *PIN1* and *MP* are important factors to control the *CUC2* expression. It also seems that these two genes *CUC1* and *CUC2* differ in their response to the signaling molecule auxin, involved in primordia patterning (Reinhardt *et al*., 2003) . It shows that different mechanism involved in the specific regulation of *CUC1* and *CUC2.* An additional level of control of *CUC* genes is mediated by the auxin induced *microRNA 164* product (Gue *et al*., 2005).

Role of microRNA in boundary formation during organ initiation:

MicroRNAs (miRNas) are short single stranded ribonucleotide molecules, on average only 22 nucleotides long that regulate gene expression in plants and animals. Several miRNAs were predicted to target the mRNAs encoding transcription factors which are required in organ development (Rhoades *et al*., 2002).. The negatively regulation of boundary specific genes by miRNA, *miR164* has been described previously (Laufs *et al*., 2004; Mallory *et al*., 2004). *miR164* is predicted to target the NAC- domain gene regulation during plant development (Rhoades *et al*.,2002). The mRNA of five NAC-domain genes in *Arabidopsis thaliana* contain *miR164* complementary site with three mismatches to *miR164* (Rhoades *et al.,* 2002). These genes include *NAC1, CUC1, CUC2, At5g61430, At5g07680. CUC1* and *CUC2* are involved in organ separation (Aida *et al*., 1997) where as the function of *AT5g07680* and *At5g61430* have not yet been known. NAC1 is a transcription activator. *NAC1* is induced by auxin and function downstream of TRANSPORT INHIBITOR RESPONSE1 (TIR1) that mediates auxin signals and promote lateral root development (Xie *et al.,* 2002).

Cuc1cuc2 double mutant shows defects in shoot apical meristem formation (Aida *et al.,* 1997). The over expression of *mir164* display altered number of floral organs, fused stamens and sepals which phenocopy the double mutant phenotype of *cuc1cuc2* (Mallory *et al*., 2004). By contrast a defect in vegetative organ development occur in the transgenic plants expressing *miR164* resistant *CUC1* (Mallory *et al.,* 2004a) and *CUC2* (Laufs *et al.,* 2004). An enlarge boundary between sepals found in the transgenic ectopically expressed *CUC2* plants. Alteration of the *miR164* binding site in *CUC2* and the overexpression of *miR164* display opposite effect in the organ development, suggesting that *miR164* regulation are essential for the boundary maintenance in the developing organs. The *NAC1* mRNA level detected in the transgenic plants over expressing miR164 is not changed respect to wild type plants (Laufs *et al.,* 2004).

Results and Discussion:

The aim of this project is to study the genetic and hormone network controlling ovule primordia formation. Reduction in number of ovule primordia was already described in the *ant* mutant (Baker *et al.,* 1996). *cuc1* and *cuc2* single mutant display similar number of ovule primordia like wild type. Double mutant *cuc1cuc2* shows defect in shoot apical meristem formation, two cotyledons fused along their margin to form a cup shaped structure (Aida *et al.,* 1997). Normally it is difficult to get the double mutant plants from the fused cotyledon. Adventitious shoots regenerated from mutant calli form the double mutant flowers, which also display the reduction of number of ovule primordia (Ishida *et al*., 2000). The goal of this work was to investigate the interaction among *ANT*, *CUC1*, *CUC2* genes to determine ovule primordia formation.

With this purpose *cuc1cuc2ant* triple mutant have been obtained by two strategies. *CUC1* and *CUC2* were silenced by using STK ovule specific promoter to drive *miR164* and successively by using an RNAi approach silencing specifically *CUC1* in *cuc2* background.

CUC1 and *CUC2* have been silenced by using miRNAs. The overexpression of *miR164* in the transgenic plants leads to increase the miRNA levels and decrease the target mRNA levels as a result such transgenic plants phenocopy the mutants with deficiencies of target mRNA. In particular the *miR164* has been overexpressed in the ovule using the ovule specific promoter S*EEDSTICK* (pSTK) (Pinyopich *et al.,* 2003; Kooiker *et al.,* 2005) has been used to control the expression of *miR164.*

Plants homozygous for *cuc2* were crossed with *ant4* plants. F2 plants homozygous for both *cuc2* and *ant4* have been analyzed. The double mutants of *cuc2ant* plants have similar phenotype like single mutant *ant4* (Figure 17B and 17C). Morphologically ovules looked like *ant4* ovules, also the number of ovules were reduced 50% respect to the wild type as found in *ant4* carpel. Plants homozygous for *cuc2* and heterozygous for *ANT4* had similar

phenotype like wild type. These plants *cuc2ANT/ant* were transformed by using the construct STK::miR164.

Overexpression of miR164 in ant Mutant Lead to Formation of Reduced Number of Ovules :

T1 plants were selected by BASTA treatment, 60 plants have been analyzed in the T1. Presences of construct in the transgenic plants have been confirmed by PCR. In the transgenic segregation 17 plants *cuc2ANT/ant* showed abnormal seed set compared with wild type. Development of primordia occurred in the same floral stage like wild type. Plants homozygous for both *cuc2* and *ant* morphologically phenocopied *ant4* phenotype (Figure14L and 14F), suggesting that *ANT* is probably epistatic to *CUC2.* In T1 segregation 22 carpels of *STK::miR164cuc2ant* have been analyzed, average 12 ovules per carpel have been detected in these plants. The number of ovules was drastically reduced to *ant* mutant. Plants were female sterile like single *ant4* homozygous plants. These plants were used as male and crossed with the sister plants homozygous for *cuc2* and heterozygous for *ANT4.* T2 plants of these progeny have been analyzed. These plants showed more dramatic phenotype than the T1 plants. In wild type carpel average 52 ovules generally developed from the placenta. But in the transgenic plants homozygous for *cuc2* and wild type for *ANT* showed less number of ovule primordia than wild type plants. Average 40 primordia were present in *STK::miR164cuc2* pistil (Figure14B and 14H, Figure 15). Also the plants homozygous for both *cuc2* and *ant* have shown reduced number of primordia respect to *ant4*. In *ant4* carpel 23 ovule primordia developed, have been counted in average, however in the transgenic plants homozygous for both *cuc2* and *ant4* average 7 ovules have been detected (Figure14E and 14K, Figure 15). Furthermore ovule primordia developed more distantly spaced in *STK::miR164cuc2ant4* respect to wild type carpel (Figure14C and 14L). Presences of more copies of the construct in these segregating plants lead to increase the activation of miRNA level and decrease the *CUC1* and *CUC2* mRNA levels, induced more severe phenotype.

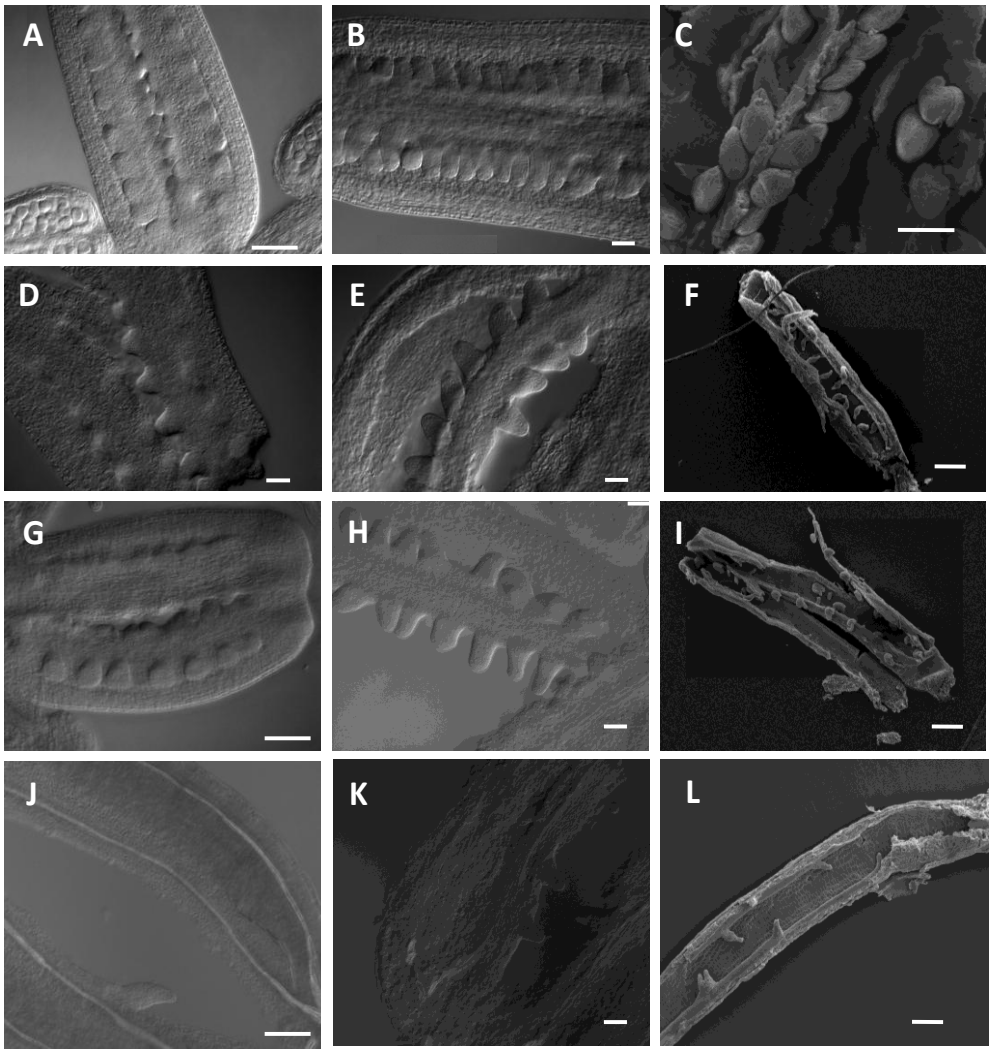

Figure 14: Ovule Primordia bulges and developing ovule primordia of wild type and the transgenic plants.

A: Optical image of wild type carpel, showing ovule primordia bulges.

B: Wild type carpel with developing ovule primordia.

C: SEM image of wild type carpel with mature ovules.

D: Optical image of *ant4* pistil showing the developing ovules.

E: *ant4* ovule primordia. Primordia are more distantly spaced respect to wild type pistil.

F: SEM image of *ant4* pistil with mature ovules.

G: Pistil of *STK::miR164cuc2* showing primordial bulges.

H: Developing ovule primordia of *STK::miR164cuc2* pistil.

I: SEM image of *STK::miR164cuc2* pistil. Ovules are less respect to wild type pistil.

J: Optical image of *STK::miR164 cuc2ant4* pistil. Only one ovule has been detected in the placenta.

K: Developing ovule primordia of *STK::miR164 cuc2ant4* pistil. The spaces between two primordia are more respect *ant4* single mutant.

L: SEM image of *STK::miR164 cuc2ant4* pistil. Randomly very few mature ovules are developed in the carpel. Scale bar: 50µm

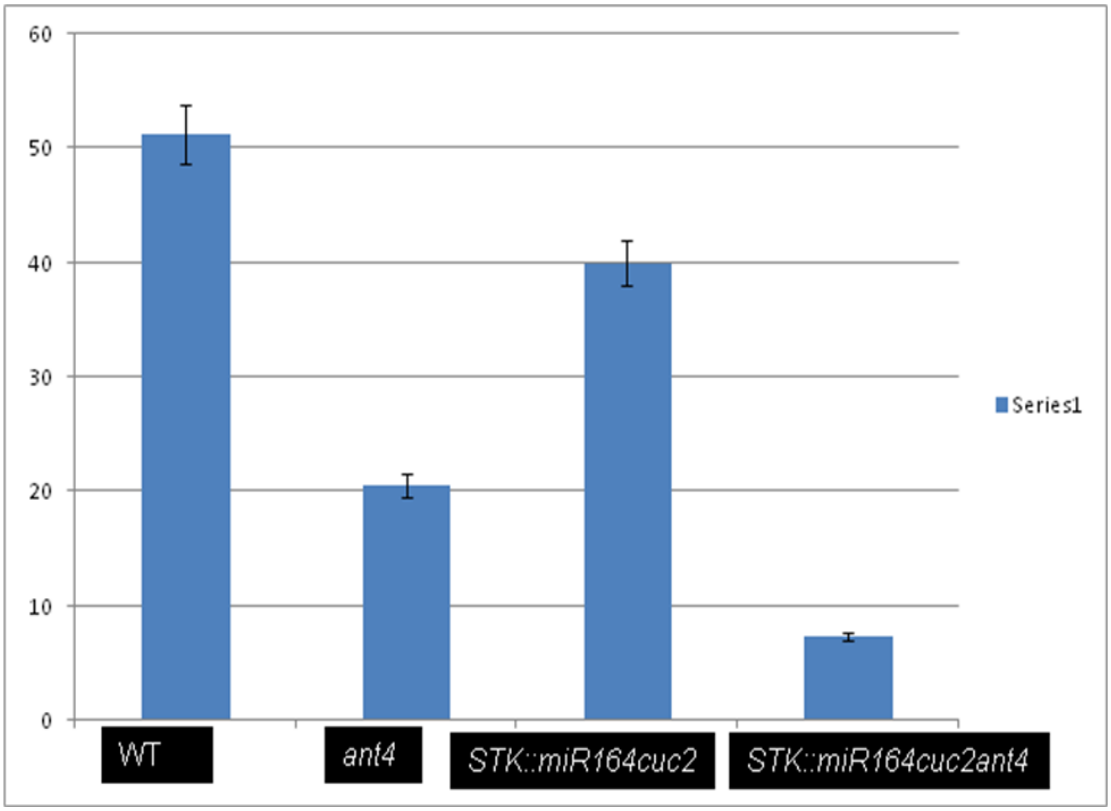

Figure15: Average number of ovule primordia in different genetic background. 45 pistils have been analyzed in each genetic background.

In *Arabidopsis thaliana,* addition to *cuc1* and *cuc2,* other three NAC-domain genes *NAC1, At5g61430, At5g07680* have miR164 complementary site. To investigate the mRNA levels of all these five genes in the transgenic plants real time-PCR analyses have been performed on the transgenic plants containing the *STK::miR164* construct. All the mRNA levels of miR164 target genes were reduced in the transgenic plants compared with wild type plants (Figure 16). As a consequence of this experiment we could not exclude that along with *cuc1* and *cuc2* other three genes may be involved in determination the phenotype described above. To investigate this hypothesis, another strategy has been taken to silence only *CUC1* in the *cuc2 ANT/ant* background. For this purpose, plants homozygous for *cuc2* and heterozygous for *ANT* have been transformed by *CUC1* RNAi construct under the control of *SEEDSTICK* ovule specific promoter (*pSTK::CUC1RNAi).* In the transgenic plants *STK::CUC1icuc2ant4* similar phenotype were obtained as described in the miR164 overexpressing transgenic plants (Figure 17D and 17E), suggesting that other three NAC-Domain genes were not involved for the reduced number of ovule primordia observed previously in the transgenic plants *STK::miR164cuc2ant4*. In the triple mutant *STK::miR164cuc2ant4* very few ovules primordia emerged at the base of the carpel (Figure17E and 17F). Toward the tip of the carpel, placenta remains necked. SEM analyses of the carpel revealed that placental cells developed properly in the triple mutant *STK::miR164cuc2ant4* (Figure 14L).

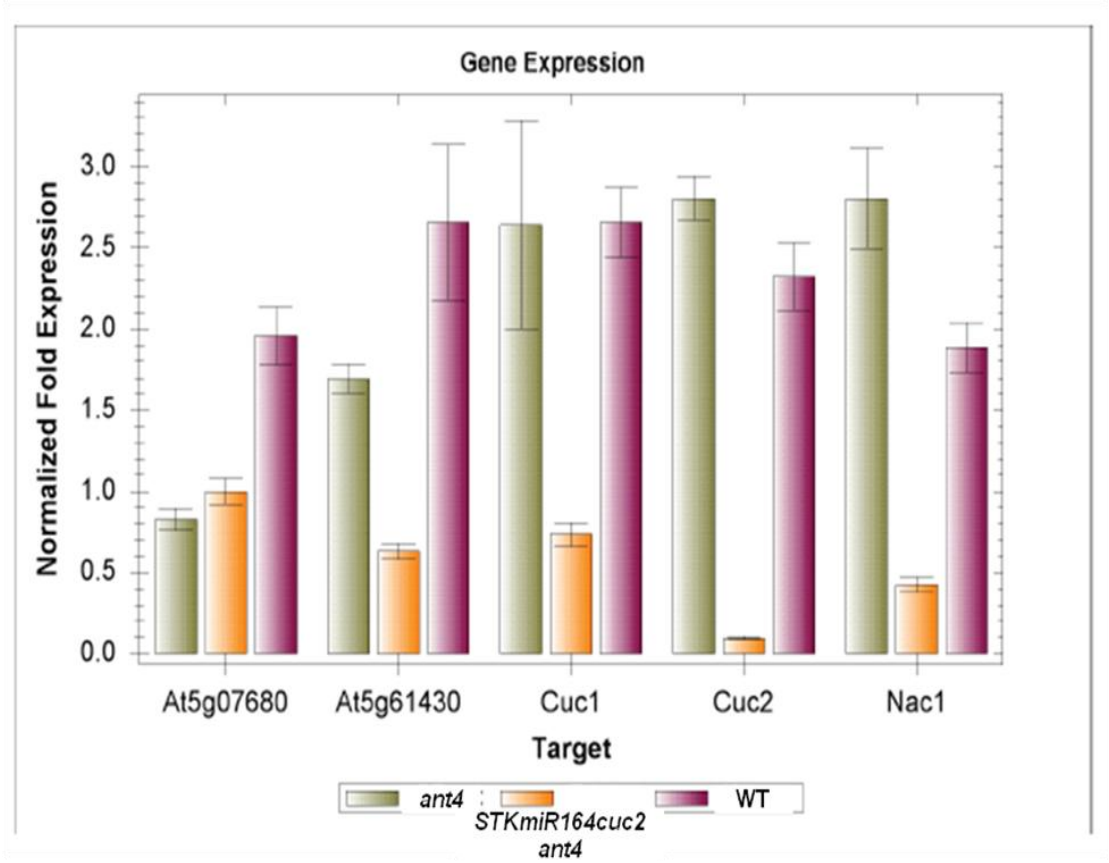

Figure16: Expression of five predicted *miR164* targets in *ant4, STK::miR164cuc2ant4,* and wild type background.

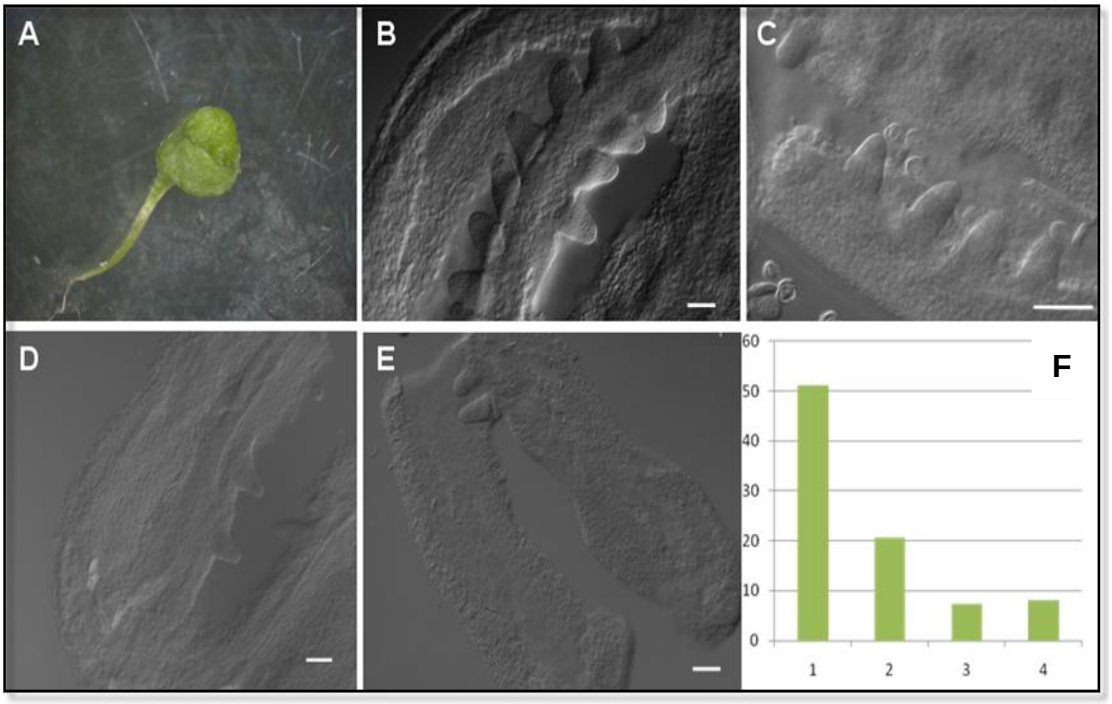

Figure 17: A: *cuc1cuc2* double mutant. Cup shaped cotyledon embryo phenotype.

B: Ovule primordia of *ant4* mutant

C: Ovule primordia of *cuc2ant4* double mutant.

D: Ovule primordia of *STK::miR164cuc2ant4.*

E: Ovule primordia of *STK:: CUC1icuc2ant4*

F: Average number of ovule primordia in different genetic background. The number represented wild type, *ant4, STK::miR164cuc2ant4,* and *STK::CUC1icuc2ant4* respectively. Scale bar: 50µm

Distribution of Auxin and PIN1 in the transgenic plants STK::MIR164cuc2ant4:

Investigations have been done to link between reduction of ovule primordia and the presence of auxin in the transgenic plants. For this purpose the auxin responsive gene promoter of *DORNSCHEN (DRN)* has been studied. Transgenic plants *STK::miR164cuc2ant4* have been crossed with the plants containing *DRN::GFP* construct (Cole *et al.,* 2009). In wild type plants the expression of GFP driven by DRN promoter starts before floral stage 8, in correspondence to the site of future ovule primordia in the carpel (Figure18A, 18B). *DRN* is expressed in the bulges of the developing primordia. Although the development of primordia bulges in the carpel placenta is not a synchronized process and its starts from the bottom of the carpel. In the transgenic plants, the expression of GFP driven by *DRN* promoter starts from the same floral stage like wild type plants. The expression starts in the bulges of the future primordia in the carpel. The *DRN* promoter activity was easily distinguishable by the GFP signal in the placenta in the stage when the ovule primordia start to arise, the expression of GFP varied in different genetic background of the transgenic plants (Figure 18A, 18C, 18E, 18G). Auxin accumulation in the developing primordia of *STK::miR164cuc2ant4* transgenic plants were more diffused respect to wild type carpel. Furthermore the GFP accumulation signal were more distantly spaced between two ovules primordia in the transgenic plants homozygous for both *cuc2* and *ant* in respect to wild type (Figure18A and 18G).

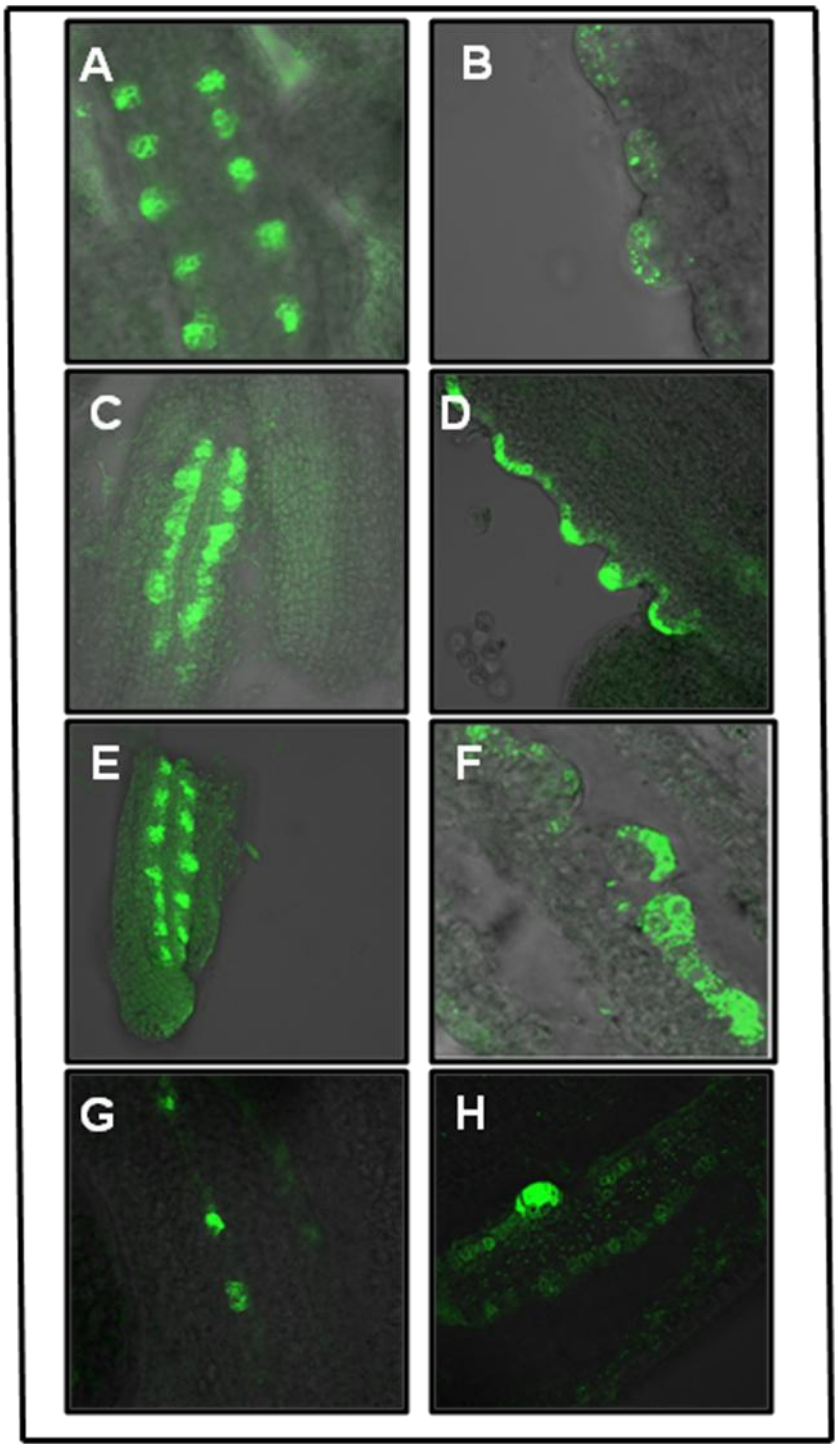

Figure18: Expression of GFP driven by DRN promoter

A: Wild type primordia.

B: Developing primordia of wild type, the DRN activity is localized at the tip of the primordia.

C: *ant4* ovule primordia. Activity of DRN is more diffused than wild type.

D: Developing primordia of *ant4* carpel.

E: Ovule primordia of *STK::miR164cuc2* plants.

F: Developing primordia of *STK::miR164cuc2.* More diffused DRN promoter activity.

G: ovule primordia of *STK::miR164cuc2ant4.* The accumulation of auxin is more distantly spaced.

H: developing primordia of *STK::miR164cuc2ant4.* The number of primordia are reduced respect to wild type.

STK::miR164cuc2 transgenic plants were crossed with *PIN1:: PIN1-GFP* plants (Benkova *et al*.,2003). In wild type the PIN1 localization found in the apical-lateral and the basal membrane cells in the tip of developing primordia (Figure19A and 19B). The altered expression of the *PIN1* has been detected in the transgenic plants. Auxin fluxes in the developing primordia were disrupted strongly. The maintenance of symmetric polarity of PIN1 at the epidermal cells of the developing primordia is completely lost with the vesicles in the transgenic plants, suggesting that the auxin distribution and PIN1flux were strongly disrupted in the ovule primordia of the transgenic plants *STK::miR164cuc2.*

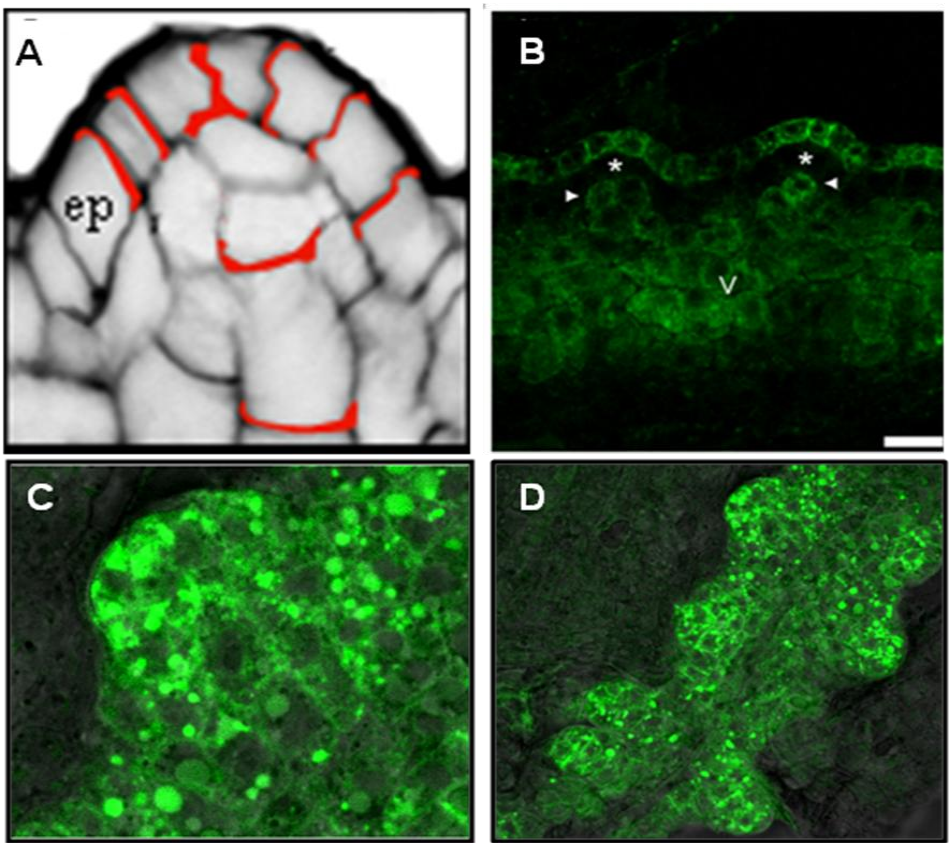

Figure19: .Expression of PIN1:: PIN1:GFP in the wild type and *STK::miR164cuc2* ovules.

A: Shchmatic representation of PIN1 localization in the developing ovule primordia.

B: PIN1 flux in placenta and ovules of the wild type plants.

C: Vesicular accumulation of PIN1 in the single ovule primordia of transgenic plants *STK::miR164cuc2ant4.*

D: PIN1 localization in the placenta and developing ovule of the transgenic plants *STK::miR164cuc2.*

Discussion:

In the *STK::miR164cuc2ant4* transgenic plants, the reduction of ovule primordia number have been observed. These plants were homozygous for *cuc2* and *ant4.* Real time analyses data showed that *CUC1* mRNA level was also reduced in the transgenic plants, suggesting that *STK::miR164cuc2ant4* plants could be considered as *cuc1cuc2ant4* triple mutant ovules. Another experiment where only *cuc1* has been silenced using specific RNAi approach in *cuc2ant4* background to be sure that the other three NAC-domain genes (targets of *miR164)* played no effective role in reducing number of ovule primordia. A reduced numer of ovule primordia have been detected in *ant4* carpel and also in double mutant of *cuc1cuc2,* suggesting that these genes are involved in the maintenance of ovule primordia number. In this study the triple mutant *cuc1cuc2ant* showed very few ovule primordia compared to wild type and also to *ant4* single and *cuc1cuc2* double mutant. The result could suggest an additive role of the three genes to maintain the ovule primordia number. *CUC1* and *CUC2* have a redundant function in establishing boundary specification and *ANT* controls cell division necessary to form a primordia. *CUC3* is not directly regulated by *miR164* although it has been shown that a partial redundancy exists within all *CUC* genes in *Arabidopsis.* The expression pattern of *CUC* genes differ during embryogenesis. Septum formation in the carpel affects more severely in *cuc2* mutation than *cuc1* mutation (Ishida *et al.*, 2000). Also, *CUC2* play greater role in SAM formation compared with *CUC1 a*nd *CUC3* (Vroemen *et al*., 2003), suggesting that although *CUC* genes are redundant in function but they play identical contribution to maintain the boundary. In our study we did not silenced *CUC3.* The few primordia still formed in the triple mutant *STK::miR164cuc2ant4* carpel, could be due to the expression of *CUC3* and its overlapping function with *CUC1* and *CUC2* to establish the boundaries. In *STK::CUC1RANicuc2ant4* also few ovules were detected could be due the approaches we have used that do not results in a complete *CUC1* silencing as shown in Real time PCR experiment.

In the *STK::miR164cuc2ant4* plants the distribution of auxin was disrupted. It showed that the normal auxin accumulation was more diffused than wild type in the region where primordia are going to arise. Also in the transgenic plants auxin accumulates only in those areas where the future primordia is appeared, suggesting that accumulation of auxin lead to develop the organ initiation. The different auxin accumulation could be due to the PIN1 localization defect. In the *STK::miR164cuc2* transgenic plants reduction of *CUC1* and *CUC2* levels lead to a modification of the PIN1 localization in the developing ovule. The PIN1 seems to be included in vesicles instead of proper membrane localization. This could explain the altered auxin accumulation effect. *ANT* is necessary to promote the cell cycle and further development of the primordia. *ANT* could be a target of auxin dependent transcription factor (ARF). It will be next challenge to identify such ARF as a factor that translate auxin signal in ovule genetic network.

Experimental Procedure:

Plant materials:

Plants were grown at 21 °C under short-day (8 h light/16h dark) condition. *ant4* (Baker *et al*., 1997), *cuc1-1 (*Takada *et al*.,2001) and *cuc2-1* (Aida *et al*.,1997) mutant seeds were obtained from Nottingham Arabidopsis Stock Center (http.//nasc.life.nott.ac.uk/). PIN1:: PIN1:GFP were kindly supplied by J. Friml (University of Tubingen, Friml *et al* 2003; Benkova *et al* 2003; Friml *et al* 2002a; Friml *et al* 2002b;). *DRN::GFP* seeds were kindly supplied by Wolfgang Werr (University of Cologne, Cole *et al*.,2009).

Microscopy:

Inflorescences were collected from plants, carpels were opened by a needle and covered with some drops of a solution of glycerol 20% (samples for CLSM) or chloralhydrate in water. Finally a coverslip was put on the samples and a slight pressure was applied.

Optical microscopy:

Optical microscopy investigations were performed using a Zeiss Axiophot D1.

Whole-mount preparation:

Siliques and carpels were dissected using a Leica stereomicroscope. The material was collected to investigate ovule development and was cleared as reported by Yadegari *et al* (Yadegari et al 1994). Inflorescences were fixed in ethanol: acetic acid 9:1 overnight followed by two washes in ethanol 90% and 70%. 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).

Scanning Electron Microscopy:

Samples were prepared and analyzed as described previously (Favaro *et al*., 2003)

In situ hybridization:

In situ hybridization with digoxigenin-labeled antisense RNA was performed as previously described by Masiero et al (2004). *CUC2* probe was prepared as described by Ishida et al (2000).

Confocal Laser Scanning Microscopy (CLSM):

All samples were prepared from fresh material collected the same day of CLSM analysis. Carpels were dissected employing a Leica stereo microscope and covered with few drops of 20% (v/v) glycerol. CLSM analyses were performed using a Zeiss LSM510 Meta Confocal Microscope. GFP was excited by a 488nm wavelength laser and a BP 505-550 nm filter was applied for GFP emission. CLSM pictures were filtered by the Median filter from LSM software, and contrast-brightness.

Plasmid Construction and Arabidopsis Transformation:

All constructs were verified by sequencing and used to transform wild type Landsberg erecta (Ler) plants using the floral dip method (Clough and Bent, 1998). To construct pSTK::CUC1RNAi, a specific CUC1 fragment was amplified with the primer Atp 2916 and Atp 2917 and recombined into the RNAi vector pFGC5941 through a LR reaction (Gateway system, Invotrogen). The promoter 35S was removed and substituted by pSTK (amplified with Atp

590 and Atp 591). For the construct *pSTK::miR164b* the fragment of miR164b was amplified with Atp 2248 and Atp 2249. The destiny vector pBGW (Karimi et al., 2002) was modified introducing the *pSTK* promoter (amplified with Atp 1507 and Atp 1508) and a T35S fragment, amplified with Atp 1663 and Atp 1664. This modified pBGW was used to clone *miR164b.*
Detail information about the pBGW and pFGC5942 vectors are available at http://www.psb.ugent.be/gateway and http://www.chromdb.org/rnai/vector respectively.

Quantitative PCR with reverse transcription

Expression of *CUC1, CUC2, NAC1, At5g07680* and *At5g61430* in *pSTK::miR164cuc2ant* were performed using the iQ5 Multi Color real-time PCR detection system (Bio-Rad). mRNA was extracted-purified using the Dynabeads® mRNA DIRECT^{TM} kit (Dynal AS) starting from 3 mg of carpels, following the manufacturer instructions. The cDNAs were produced using the ImProm-II™ Reverse Transcription System (Promega). All genes specific primers are listed in primer list. Normalisation was performed using UBIQUITIN10 (*UBI10*).

Diluted aliquots of the reverse-transcribed cDNAs were used as templates in quantitative PCR reactions containing the SYBR Green PCR Master Mix (Biorad). The adjustment of baseline and threshold has been done according to the manufacturer's instructions.

Primers:

Primers for Plasmid Construction:

CUC1 RNAi For Atp 2916: 5'-CACCAGCCACGTCACGTCGGTGATG-3'

CUC1 RNAi Rev Atp 2917: 5'-CCGTGAGTGTGTGGCCGTTTA-3'

miR164 For Atp 2248: 5'-CACCTCACGTTTTCAAATATCAAACC-3'

miR164 Rev Atp 2249: 5'-GGTTAGCGAGTATTAGACAGGAGA-3'

STK promoter For Atp 590: 5'-CTCAGAATTCGTTGGGTATGTTCTCACTTTC-3'

STK promoter Rev Atp 591: 5'-ATGTTTAAAATGAAGGATGGGACTCGAGTGAC-3'

35S For Atp 1663: 5'-CGAGCTCGCGGCCATGCTAGAGTCCGC-3'

35S Rev Atp 1664: 5'-CCTAAAACCAAAATCCAGTGACCTCGAGCTCG-3'

STK promoter For : 5'-CTGACGTCAGGCGTTTTTGTTGGGTATGTTCTCAC-3'

STK promoter rev: 5'-GATGTTTAAAATGAAGGATGCCTGACGTCAGA-3'

Primers For In Situ Hybridization:

CUC2 For Atp 2926: 5'-AGCTAGCACCGGAGCCGTCTCCG-3'

CUC2 rev Atp 2927: 5'-GCGTCTCCGGGATGAATTACTGGC-3'

Primers For Quantitative PCR with reverse transcription:

CUC1 For RT 332: 5'-TGCATGAGTATCGCCTTGAC-3'

CUC1 Rev RT 333: 5'-CTTCTTCTTCTGCCGTCACC-3'

CUC2 For Rt 360: 5'-AAAGGAAGAGCTCCGAAAGG-3'

CUC2 Rev RT 361: 5'-AGGAGGAGGAGCAACTGTGA-3'

NAC1 For Rt 328: 5'-TCAGATTTCACCCGAAGGAC-3'

NAC1 Rev Rt 329: 5'-GAGAACTAACCGAGCAACGG-3'

At5g07680 For RT 356: 5'-ATTGGGTGATGCATGAGTATAGGC-3'

At5g07680 Rev RT 357: 5'-ATTCTTCACCATACAACGACAAAACC-3'

At5g61430 For Rt 358: 5'-TCCTGGGTTCAGGTTTCATC-3'

At5g61430 Rev Rt 359: 5'-CTAACCGAGCAACTGAAGCC-3'

REFERENCES:

- **Aida, M., Ishida, T., Fukaki, H., Fujisawa, H., and Tasaka, M.** (1997). Genes involved in organ separation in Arabidopsis An analysis of the cup-shaped cotyledon mutant. *Plant Cell* 9, 841-857.
- **Baker SC, Robinson-Beers K, Villanueva JM, Gaiser JC, Gasser CS.** Interactions among genes regulating ovule development in *Arabidopsis thaliana. Genetics*. 1997. Apr; 145(4):1109-24.
- **Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D.,Jurgens, G., and Friml, J**. (2003). Local efflux-dependent auxin gradients as a common module for plant organ formation. *Cell 115*, 591-602.
- **Clough, S.J., and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735-743.
- **Elliott, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q.J., Gerentes, D., Perez, P., and Smyth,D.R.** (1996) *AINTEGUMENTA*, an *APETALA2*-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. *Plant Cell* **8**:155-168
- **Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M. F., Kater, M. M., and Colombo, L. (2003).** MADS-box protein complexes control carpel and ovule development in Arabidopsis. *Plant Cell 15*, 2603-2611.
- **Friml J, Benkova E, Blilou I, Wisniewska J, Hamann T, Ljung K, W oody S, SandbergG, Scheres B, Jurgens G, etal.** 2002. AtPIN4 mediates sink-driven auxin gradients and root patterning
- **Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998).** Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science 282*, 2226-2230.
- **Guo, H.S., Xie, Q., Fei, J.F. and Chua, N.H.** (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate

auxin signals for Arabidopsis lateral root development. *Plant Cell*, 17, 1376–1386.

- **Ishida T, Aida M, Takada S, Tasaka M** (2000) Involvement of CUP-SHAPED COTYLEDON genes in gynoecium and ovule development in *Arabidopsis thaliana. Plant Cell Physiol* **41:** 60–67
- **Karimi, M., Inze, D., and Depicker, A.** (2002). GATEWAY vectors for Agrobacteriummediated plant transformation. *Trends Plant Sc.i* **7,** 193-195.
- **Klucher, K.M., Chow, H., Reiser, L., and Fischer, R.L.** (1996) The *AINTEGUMENTA* gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene *APETALA2*. *Plant Cell* **8**:137-153.
- **Kooiker, M. et al. (2005)** BASIC PENTACYSTEINE1, a GA binding protein that induces conformational changes in the regulatory region of the homeotic Arabidopsis gene SEEDSTICK. *Plant Cell* 17, 722–729
- **Krizek, B. A.** (1999). Ectopic expression of *AINTEGUMENTA* gene results in increased growth of floral organs. *Dev. Genet.* **25**, 224-236.
- **Laufs, P., Peaucelle, A., Morin, H. and Traas, J.** (2004) MicroRNA regulation of the *CUC* genes is required for boundary size control in*Arabidopsis* meristems. *Development*,
- **Mallory, A.C., Dugas, D.V., Bartel, D.P. and Bartel**,(2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr. Biol.* **14**, 1035 – 1046.
- **Masiero.S, M.A. Li, I. Will, U. Hartmann, H. Saedler, P. Huijser, Z. Schwarz-Sommer and H. Sommer,** *INCOMPOSITA*: a MADS-box gene controlling prophyll development and floral meristem identity in *Antirrhinum*, *Development* **131** (2004), pp. 5981–5990.
- **Mizukami, Y., and Fischer, R. L. (2000).** Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. Proc Natl Acad Sci U S A *97*, 942-947.
- **Palme, K., and Gälweiler, G.** (1999). PIN-pointing the molecular basis of auxin transport. *Curr. Opin. Plant Biol*. **2,** 375–381.
- **Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E., and Yanofsky, M. F.** (2003). Assessing the

redundancy of MADS-box genes during carpel and ovule development. Nature *424*, 85-88.

- **Qi Xie, Giovanna Frugis, Diana Colgan, and Nam-Hai Chua** (2002) *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development**.** *Genes Dev* 14(23): 3024–3036.
- **Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B. and Bartel, D.P.** (2002) Prediction of plant microRNA targets. *Cell*, **110**, 513 – 520.
- **Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D.** (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell* **18**, 1121-1133
- **Smyth, D. R., Bowman, J. L., and Meyerowitz, E. M** (1990). Early flower development in Arabidopsis. *Plant Cell* 2, 755-767.
- **Takada, S., Hibara, K., Ishida, T., and Tasaka, M.** (2001). The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. *Development* **128,** 1127–1135.
- **Ulmasov, T., Hagen, G., and Guilfoyle, T. J.** (1997). ARF1, a transcription factor that binds to auxin response elements. *Science* 276, 1865-1868.
- **Vroemen, C.W., Mordhorst, A.P., Albrecht, C., Kwaaitaal, M.A., and de Vries, S.C.** (2003). The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. *Plant Cell* **15,** 1563–1577.
- **Yadegari, R., Paiva, G., Laux, T., Koltunow, A.M., Apuya, N., Zimmerman, J.L., Fischer, R.L., Harada, J.J., and Goldberg, R.B.** (1994). Cell Differentiation and Morphogenesis Are Uncoupled in Arabidopsis raspberry Embryos. *Plant Cell* **6**, 1713-1729.

Chapter 3

The Role of PIN1 in Female Gametophyte Development:

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Summary

Land plants alternate a haploid generation, which is the gametophyte that forms the gametes, with the diploid sporophytic generation that in seed plants protects the gametophytes. The ovule represents the structure where the two generations coexist: the haploid embryo sac harbouring the egg cell, which is protected by the diploid maternal integument(s). The establishment and regulation of auxin concentration gradients plays an important role in developmental processes, including ovule development. We have analyzed in detail the auxin gradient during ovule development, and to uncover its role we perturbed polar auxin transport (PAT) by silencing the auxin efflux carrier *PIN-FORMED 1 (PIN1)* in *Arabidopsis thaliana* developing ovules. Furthermore we have created transgenic plants, in which the cellular localization of PIN1 is compromised in the ovule due to *CUC1* and *CUC2* down-regulation. Our experiments suggest that the maternally controlled auxin signal, mediated by PIN1 expression and cellular localization in the ovule, is essential for early stages of female gametophyte development.

Introduction

In flowering plants, the gametophytes are comprised of only a few cells embedded within the diploid sexual organs of the flower. The most common polygonum type female gametophyte consists of seven cells and four different cell types (three antipodal cells, two synergid cells, one egg cell, and one central cell) deeply edged by the diploid tissue of the ovule (nucellus and integuments). The formation of the female gametophyte is divided into two main steps, megasporogenesis and megagametogenesis (Schneitz et al., 1995; Christensen et al., 1997). During megasporogenesis, the Megaspore Mother Cell (MMC), easily recognisable for its size in the ovule sub-epidermal layer, undergoes meiosis and produces four haploid megaspores. Three of them will degenerate, while the one that lies at the ovule medial region (the chalaza) turns into a functional megaspore (FM) and this marks the FG1 stage of megagametophyte development. Subsequently, the FM undergoes three consecutive mitotic divisions (FG1-FG4) that lead to the formation of the

mature embryo sac (FG5) (Schneitz et al., 1995; Christensen et al., 1997). Recently it has been shown that auxin plays a key role to determine embryo sac cell fate (Pagnussat et al., 2009). Auxin is an indispensable phytohormone that regulates a wide spectrum of functions such as apical dominance, fruit ripening, root meristem maintenance, hypocotyl and root elongation, shoot and lateral root formation, tropisms, cellular division, elongation and differentiation, embryogenesis, vascular tissue differentiation and many types of organogenesis (Laskowski et al., 1995; Reinhardt et al., 2000; Benkova et al., 2003). However, it is not known if auxin also plays a role in the regulation of early stages of female gametophyte development. The responses of plants to auxin is mainly determined by the capacity of cells to regulate auxin influx and efflux, which generates a dynamic concentration gradient across cells. The formation of an auxin gradient involves many proteins among which the well characterised polar plasmamembrane localized PIN (PIN-FORMED) proteins, which count in *Arabidopsis thaliana* eight members (PIN1-8) (Teale et al., 2006). *pin1* mutant plants are progressively defective in organ initiation and phyllotaxy, which at the end leads to a pin-shaped inflorescence meristem devoid of flowers (Okada and Shimura, 1994; Galweiler et al. 1998). The disruption of auxin transport perturbs the local auxin gradients, which are presumably essential for organogenesis. Our work assigns a new developmental role to polar auxin transport (PAT) in the proper formation of the embryo sac. We show that expression and localization of the PIN1 auxin efflux carrier play an important function in the progression of female gametogenesis since it probably establishes a flux of auxin from the sporophytic ovule tissues towards the arising female gametophyte. Such auxin flux seems to be essential during the early phases of megagametogenesis. The importance of cross talk between the diploid and haploid generations has been reported several times, however the identity of the molecular signal that directs such communication was not known. Here we show that one possible factor involved in this communication might be auxin.

Results and Discussion:

Auxin distribution and PIN1 localization in Arabidopsis wild-type ovules:

To study the role of auxin in the progression of early megagametogenesis, we have analysed auxin distribution in *Arabidopsis thaliana* developing ovules of plants carrying the auxin-responsive reporter *DR5rev::GFP* (Friml et al., 2003; Benkova et al., 2003). In wild-type plants the GFP signal appears at the distal tip of the ovule primordium and starting from FG0, auxin is also detected in the funiculus pro-vascular cells (Fig. 1A and 1D). Such pattern is maintained until the nucellus progressively degenerates and is substituted by the endothelium (de Folter et al., 2006). The localization of auxin maxima in the nucellus of stage FG1 and FG3 ovules was further confirmed by analysing *DR5rev::Venus-N7* transgenic plants (Fig. 1B-1C) (Heisler et al., 2005), where the *DR5rev* synthetic promoter drives three tandem copies of VENUS, a rapidly folding YFP variant fused to a nuclear localization sequence. These analyses clearly show that auxin seems to accumulate at the micropylar pole of the nucellus till FG3 (Fig. 1C and 1D). Auxin distribution, as for other hormones is a balance between production, inactivation and transport. PAT plays a central role to generate a precise and dynamic regulation of auxin gradients. PAT requires a set of carriers that control uptake into the symplast and subsequent efflux into the extracellular apoplast (Morris et al., 2004). In particular PIN proteins are involved in cellular efflux of this phytohormone (Petrasek et al., 2006). Notably auxin distribution within tissues has often been inferred from the analysis of PIN protein orientation across tissues coupled to patterns of auxin accumulation revealed using synthetic auxin-responsive reporter lines (Kieffer et al., 2010). Among the eight Arabidopsis PIN encoding genes, we focused on *PIN1* since it is the only gene expressed during early stages of ovule development as previously reported by Pagnussat and collaborators (2009). In particular we analyzed early stages of megagametogenesis to visualize the membrane localization of the PIN1 protein (Fig. 1E-1G). Also *PIN3* is expressed in developing ovules but at later stages and only in the funiculus (Fig. S1). Immunolocalization experiments

(Fig. 1E) and analysis of transgenic plants containing the *PIN1::PIN1:GFP* construct revealed that PIN1 is localized at the lateral-apical membranes of diploid cells in the nucellus of developing ovules (Fig. 1F and 1G). In particular the presence and the position of PIN1 strongly sustains that auxin is transported into the developing female gametophyte. PIN1 was never detected in the developing embryo sac as also reported by Pagnussat and collaborators (2009).

Molecular Impairment of Auxin Homeostasis:

To uncover the role of auxin in ovule development, we artificially perturbed auxin transport by introducing in *DR5rev::GFP* plants a *PIN1 RNA*i construct under the control of the *SEEDSTICK* (*STK*) ovule specific promoter (*pSTK*), which is active during all stages of ovule development (Kooiker et al., 2005). For this construct we used a fragment of 429 bases of the *PIN1* cDNA (from base 756 to 1185). Among the 50 *DR5rev::GFP* T1 plants containing the *pSTK::PIN1i* construct, 18 were characterised by an abnormal seed set with a significant percentage of their ovules (from 29% to 63%) unable to complete proper development (five siliques for each of these plants were analyzed). Analysis of the F2 population (F1 pollen has been used to pollinate wild-type plants) using BASTA selection showed that 15 out of the 18 plants analyzed, had only one T-DNA copy (or more T-DNA copies in linkage segregating as single locus; data not shown). Optical microscopic analysis showed that ovules from these 15 transgenic plants were blocked at the FG1stage of megagametogenesis (10 lines) or at the FG3 stage (5 lines) (Fig. 2A and 2B) characterised by a high percentage of ovule abortions (Table S1). We have also analysed in detail five plants that are heterozygous for the *pin1* allele (Gabi-KAT line GK_051A10). In these plants seed set is normal thus all the ovules, half of which contain a *pin1* female gametophyte, are all successfully fertilized by wild-type pollen in reciprocal crosses. In agreement with this observation we recovered in the offspring 50% *PIN1/pin1* and 50% wild-type plants (85 plants analysed) excluding any gametophytic effects for loss of

PIN1 function. These data and the observation that *PIN1/pin1* heterozygous plants have normal seed set clearly suggest that the observed gametophytic defects in *pSTK::PIN1RNAi* lines have a sporophytic origin.

To verify that the phenotype was linked to the reduction of *PIN1* levels, we performed real time- PCR analysis on carpels of the T2 segregating plants using, as reference genes, either *UBIQUITIN10* or *18S RNA* (Fig. 2C). This analysis revealed that compared to sibling wild-type plants, *PIN1* transcript levels were reduced by 2 to 5 fold in those *STK:: PIN1i* plants that showed embryo sac developmental defects (Fig. 2C). Interestingly, analysis of the *DR5rev::GFP* reporter in the *PIN1* silenced lines showed that those ovules that were unable to complete megagametogenesis maintained an auxin maxima at the distal edge of the blocked embryo sacs, while such peaks were not observed in wild-type sister FG5 ovules (Fig. 2D). This is probably due to local auxin synthesis combined to the reduced *PIN1* expression that affects auxin transport from the sporophyte into the megagametophyte. The phenotypes described for *pSTK::PIN1i* transgenic plants were confirmed when *PIN1* was silenced through the expression of a *PIN1* specific artificial microRNA again placed under the control of *pSTK* (data not shown). Interestingly only in one line we observed defects at later stages of megagametogenesis, where the two polar nuclei failed to fuse and therefore the central cell was not fertilised. The fact that in most of the *PIN1* silencing lines the ovules that manage to develop further FG2 stage, have almost never defects during later stages of embryo sac development suggests that PIN1 is not the major player after FG2. This might be explained by the fact that *YUCCA* (*YUC*) genes that encode key enzymes involved in auxin biosynthesis (Cheng et al., 2006) are starting to be expressed in the nucellus at the micropilar end of the embryo sac from FG2. This was shown by Pagnussat and collaborators (2009) for *YUC1* and *YUC2* and we show this here for *YUC4* which is transcribed starting from FG2 as shown in Fig. 2F). *YUC1* and *YUC2* are also expressed in the developing embryo sac after FG2 (Pagnussat et al., 2009), thus at these later stages, these *YUCCA* genes are probably able to produce auxin locally and therefore there might be no need for PIN1 to transport auxin from the sporophyte.

Ovules Specific CUC1 and CUC2 Gene Silencing Affects Membrane Localization of PIN1:

It has been reported that *cuc1cuc2* plants, obtained by *cuc1cuc2* hypocotyl calluses are female sterile (Ishida et al., 2000). *cuc1cuc2* ovules often develop integuments, thus suggesting that sterility must be caused by embryo sac defects (Ishida et al., 2000). Furthermore, analysis of the F1 offspring of plants heterozygous for *cuc1* and *cuc2* suggest that the described embryo sac defects are sporophytically controlled (data not shown). Moreover it has been suggested that in *cuc1cuc2* embryos the cup shaped cotyledons phenotype is linked to a defect in auxin gradients in the embryo apical domain (Jenik and Barton, 2005) To uncover a possible molecular link between the *cuc1 cuc2* phenotype in the ovule and the gametophytic defects described in *pSTK::PIN1i* we silenced *CUC1* in *cuc2* homozygous ovules. *CUC1* and *CUC2* are targeted by *miR164* (Mallory et al., 2004), thus we placed *miR164* under the control of *pSTK*, in order to silence *CUC1* exclusively in developing ovules (*pSTK::miR164b*) of the T1 plants containing the construct were selfed to achieve higher expression levels of *miR164*. Analysis of the offspring showed that female gametophyte development was severely compromised (more than 80% of the ovules aborted) and arrested at FG1 and FG3 (Fig. 3A-3B). However some female gametophytes were able to complete their formation although they could not be fertilised by wild type pollen (Fig. 3C). These phenotypes are consistent with the previous description of the *cuc1 cuc2* double mutant (Ishida et al., 2000). Since these transgenic lines are female sterile, their pollen was used to fertilise *PIN1::PIN1:GFP* plants. Surprisingly PIN1 cellular localisation was dramatically affected in the ovules of these transgenic lines. As shown in Fig. 3D, PIN1 is clearly included into vesicles rather than polar localised in the plasma-membrane. PIN proteins are continuously recycled to and from the cell surface via an endosomal compartment in a process that requires the GNOM membrane-associated ARF-GEF (guanine nucleotide-exchange factor on ADP-ribosylation factor G) protein (Steinmann et al., 1999; Geldner et al., 2003). Our results suggest that

in *cuc2 pSTK::miR164* transgenic plants the defect in localization of PIN1 in ovules could prevents proper female gametophyte formation. Our data suggest that *PIN1* expression and PIN1 proper localization is necessary for early stages offemale gametophyte development, probably to establish an auxin gradient from the diploid nucellus
tissues to the developing gametophyte. The next challenge will be to identify the targets of auxin that control these early stages of female gametogenesis.

Experimental Procedures

Plant Materials (*Arabidopsis* lines)

DR5rev::GFP, PIN1::PIN1:GFP, PIN3::GUS seeds were supplied by J. Friml (University of Tubingen), *pDR5rev::3XVENUS-N7* seeds by M. Heisler (California Institute of Technology), and *YUCCA4::GUS* from Y. Zhao (University of California at San Diego). *pin1* (Gabi-KAT line GK_051A10) was provided by K. Palme, *cuc2* seeds were obtained from NASC (id=N3871).

Quantitative PCR with reverse transcription

Expression of *PIN1* in *pSTK::PIN1RNAi* lines was performed using the iQ5 Multi Color real-time PCR detection system (Bio-Rad). mRNA was extracted-purified using the Dynabeads® mRNA DIRECTTM kit (Dynal AS) starting from 3 mg of carpels, following the manufacturer instructions. The cDNAs were produced using the ImProm-II™ Reverse Transcription System (Promega). *PIN1* specific primers are listed in Supplementary information; normalisation was performed using *UBIQUITIN10* (*UBI10*) and *18SrRNA* as internal standards. Diluted aliquots of the reverse-transcribed cDNAs were used as templates in quantitative PCR reactions containing the SYBR Green PCR Master Mix (Biorad). The adjustment of baseline and threshold was done according to the manufacturer's instructions. *PIN1, CUC1* and *CUC2* lower transcript abundances were confirmed by two independent biological

experiments and four technical repetitions. Moreover *PIN1* was amplified with two different couple of primers. Details on oligonucleotides used for PCR-based genotyping and RT-PCR experiments are given in Supplemental Table

GUS assays and whole-mount preparation

GUS stainings were performed as reported by Vielle-Calzada et al. (2000). Developing ovules were cleared according to Yadegari et al. (1994) and 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 (4.1).

Confocal laser scanning microscopy (CLSM) and immunolocalization.

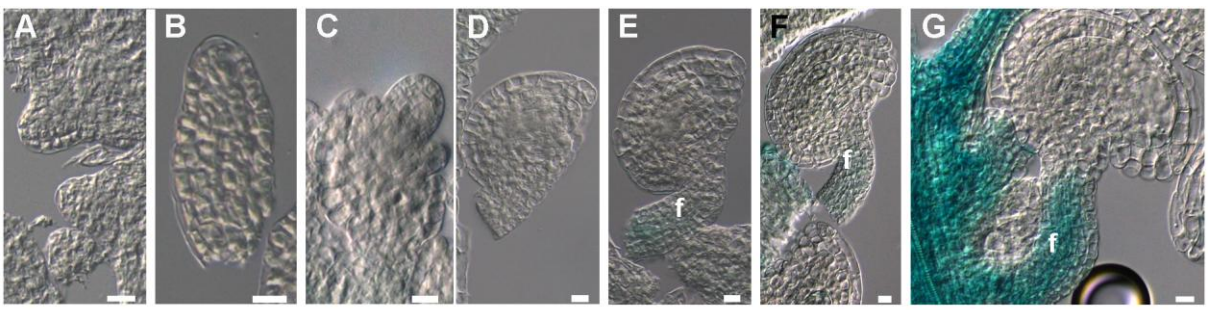
Dissected carpels were mounted with glycerol 20% (v/v). For FM® 4-64 FX application samples were covered by a solution prepared following the manufacturer's instruction (Invitrogen). Samples were incubated 1h-1h30' before observation. The CLSM analysis was performed using a Zeiss LSM510 Meta confocal microscope. To detect the GFP signal a 488nm wavelength laser was used for excitation, and a BP 505-550 nm filter was applied for GFP emission. For FM® 4-64 FX dye application (Invitrogen) an additional BP 575-615 nm filter was used. For the immunodetection experiment a monoclonal anti-PIN1 antibody (Nanotools GmbH) has been used as primary antibody. To detect the signal from the Alexa Fluor® 555 secondary antibody (Invitrogen) a 561nm laser was used for excitation, and a LP 575nm filter was applied for fluorofor emission. For immuno-localisation experiments plant material was fixed as previously described in Brambilla et al. (2007).

Plasmid construction and Arabidopsis transformation

All constructs were verified by sequencing and used to transform wild-type (*Col-0)* plants using the 'floral-dip' method (Clough and Bent, 1998). T1 seedlings were selected by BASTA. To construct *pSTK::PIN1*i a PIN1 fragment was amplified with At2208 and At2009, and recombined into the RNAi vector pFGC5941 through an LR reaction (Gateway® system, Invitrogen). The 35S was removed and substituted by *pSTK* (amplified with At590 and At591). Sequences for the artificial-mRNA against *PIN1* (amiPIN1) were generated following Schwab and co-workers indications (2006). As backbone vector MIR319 was employed. Primers used for amiPIN1 were At1104-07. The destiny vector pBGW (Karimi et al., 2002) was modified introducing the *pSTK* promoter (amplified with At1507 and At1508) and a T35S fragment, amplified with At1663 and At1664. This modified pBGW was also used to clone *miR164b* amplified with At2448 and At2449. Detailed information about the pBGW and pFGC5941 vectors is available at http://www.psb.ugent.be/gateway and http://www.chromdb.org/rnai/vector respectively.

Figures:

Figure S1 related to Figure 1



PIN3 expression in ovules of *PIN3::GUS* plants

A-E, *PIN3* in not transcribed in ovules till FG2 (**E**). Afterwards *PIN3* expression persists exclusively in the funiculus, **F-G.** Funiculus (f). Scale bars: 20µm.

Table S1 related to Figure 2

T1 plant	Defects	Unfertilized ovules	Ovule Abortion (%)	Normal seeds	Abnormal seeds	TOT
1	FG1	52	**29**	126	4	182
2	FG3	80	**63**	67	0	147
3	FG1	110	**42**	150	0	260
4	FG1	54	**34**	104	3	161
5	FG1	40	**32**	86	0	126
6	FG3	50	**43**	64	2	116
7	FG1	35	**32**	69	4	108
8	FG2	82	**41**	115	3	200
9	FG1	38	**30**	85	2	125
10	FG1	125	**42**	170	2	297
11	FG1	82	**51**	74	6	162
12	FG3	125	**44**	160	0	285
13	FG3	80	**31**	170	7	257
14	FG1	75	**59**	48	4	127
15	FG1	117	**45**	142	3	262

Table S1 15 *pSTK::PIN1RNAi* T1transgenic lines showed defective ovule development and seed set. In 10 lines female gametophytes arrest at FG1, in 5 at FG3 (Christensen et al., 1997).

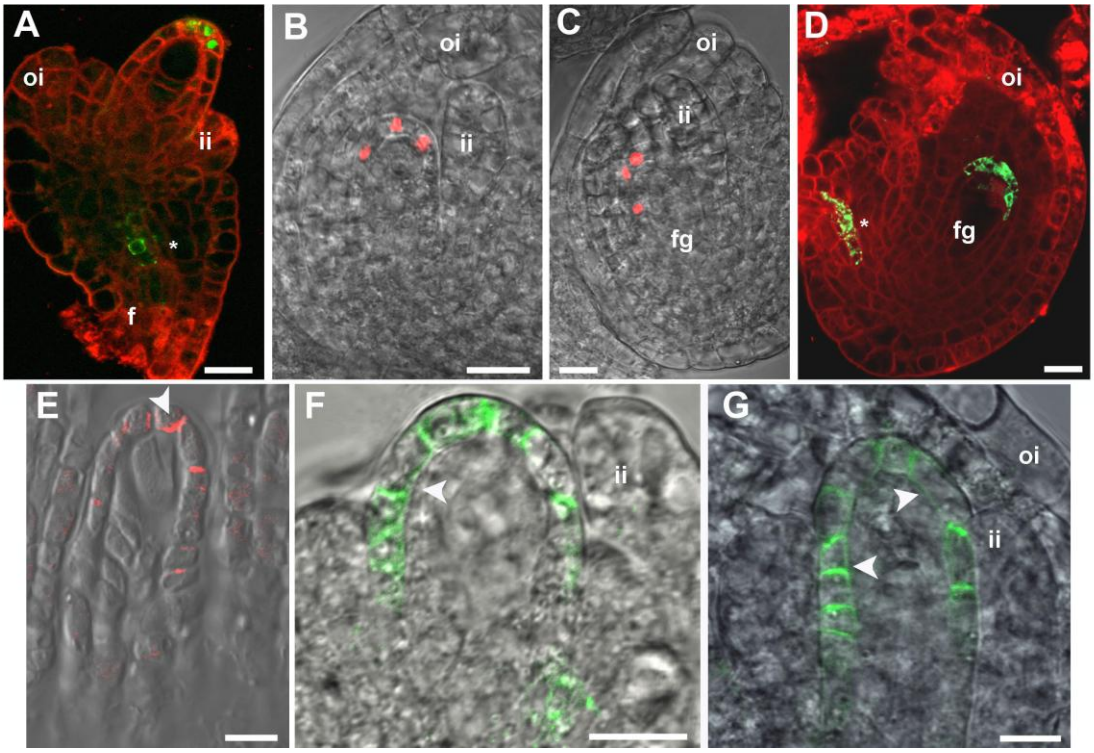


Figure 1: Auxin distribution and PIN1 localization in wild-type ovules.

Wild-type *DR5rev::GFP* (A and D) and *DR5rev::3XVENUSN7* (B and C) ovules were analysed at different developmental stages. GFP signal is first detected in the epidermis at the distal end of the ovule primordium at FG0 (**A**), such signal persists from FG1 till FG2 (**D**). *DR5rev::GFP* signal is also detected in the forming ovule vasculature (asterisks in **A** and **D)**. In **A** and **D** cell membranes were stained with FM® 4-64 FX. **E** PIN1 immuno-localisation experiments with an anti-PIN1 antibody. PIN1 is detected in the apical-lateral membrane of the nucellus in ovule primordium. **F** and **G** CLSM analysis of *PIN1::PIN1:GFP* ovules at stage FG1 and FG2. Arrowheads indicate PIN1 polar localisation, PIN1 is localised at the lateral-apical membranes of the epidermis cells around the nucellus. fg, female gametophyte; ii, inner integument; oi, outer integument; f, funiculus. Scale bars: 20 µm.

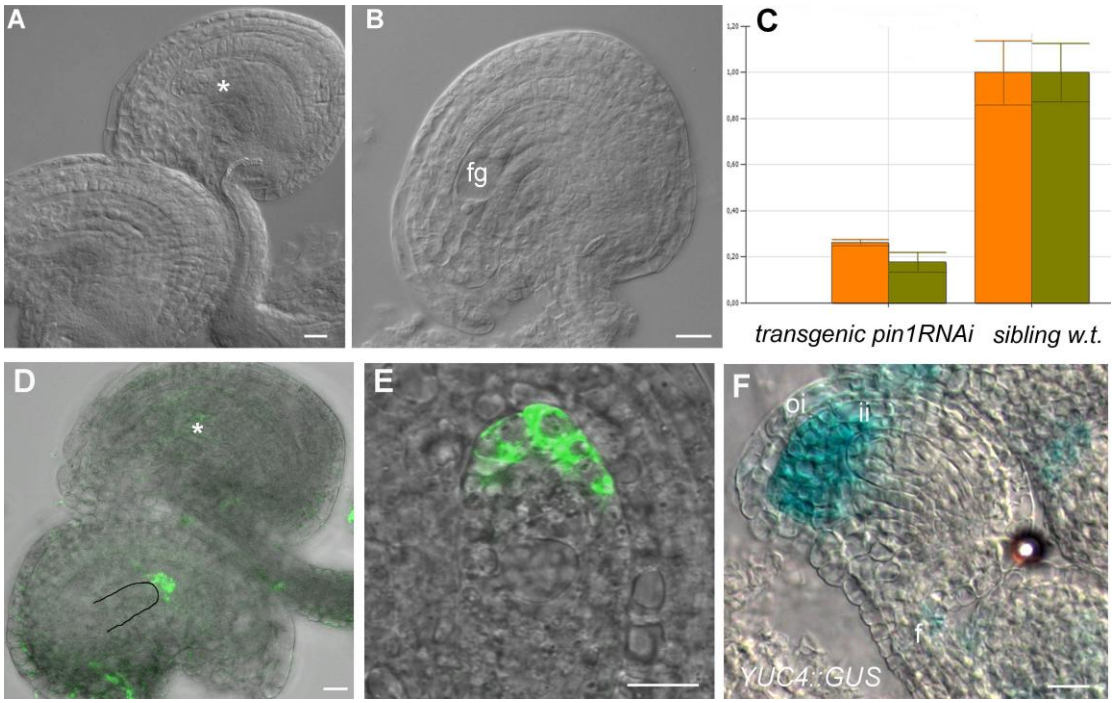


Figure 2: PIN1 down regulation affects ovule development

A and **B**, female gametophyte defects caused by *PIN1* silencing. Female gametophytes arrest their development at FG1 and FG3. **C** Real-time experiment to verify the reduction of *PIN1* expression level in *pSTK::PIN1RNAi*, two couples of *PIN1* specific primers were employed (orange and green). **D**. Auxin GFP signal is still present at the distal edge of a blocked embryo sac in *pSTK::PIN1RNAi* plants, no signal is detected in normal mature embryo sac (asterisk). **E** higher magnification of the arrested ovule shown in **D**. **F**. *YUC4* is detected at the tip of the inner integument since FG1. fg, female gametophyte; ii, inner integument; oi, outer integument; f, funiculus. Scale bars: 20 µm

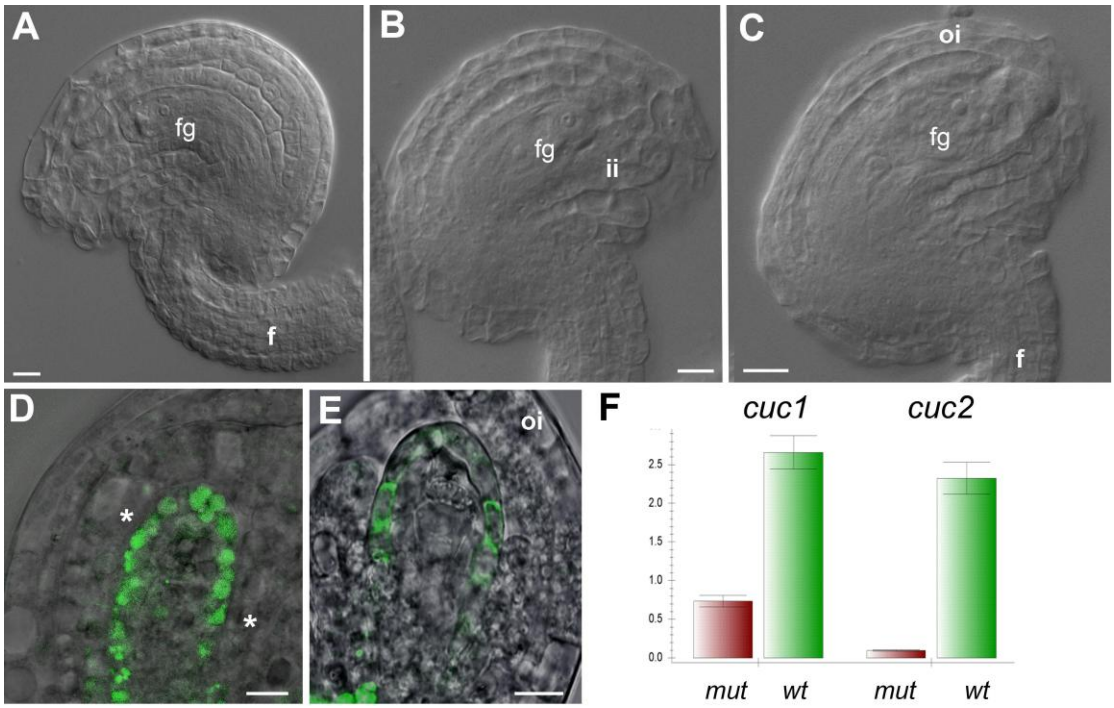


Figure 3

CUC1 down-regulation in *cuc2* ovules causes embryo sac defects

CUC1 was silenced over-expressing *miR164* in Arabidopsis ovules. This induces embryo sac defects which arrests at FG1 (**A**) and FG3 (**B**). Some female gametophytes are able to complete their development but nevertheless cannot be fertilised (**C**) even by wild type pollen grains. In *cuc2cuc2pSTK::micro164b* ovules PIN1 protein appears not any more localised on the plasmalemma, it is rather entrapped in cytoplasmatic vesicles (compare **D** with **E**). **D**, *cuc2cucpSTK::micro164 PIN1::PIN1:GFP*; **E** *PIN1::PIN1:GFP.* In **F** Real time PCR experiments to quantify the reduction of *CUC1* and *CUC2* expression level in transgenic *cuc2* carpels.

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REFERENCES:

- **Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J.** (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**, 591-602.
- **Brambilla, V., Battaglia, R., Colombo, M., Masiero, S., Bencivenga, S., Kater, M. M., and Colombo, L.** (2007). Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in Arabidopsis. *Plant Cell* **19**, 2544-2556.
- **Cheng, Y, Dai, X, and Zhao, Y.** (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*.*Genes Dev.* **20**, 1790–99.
- **Christensen, C.A., King, E.J., Jordan, J.R., and Drews, G.N.** (1997). Megagametogenesis in Arabidopsis wild type and the Gf mutant. *Sex. Plant Rep.* **10**, 49-64.
- **Clough, S.J., and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735-743.
- **De Folter, S., Shchennikova, A. V., Franken, J., Busscher, M., Baskar, R., Grossniklaus, U., Angenent, G. C. and Immink, R. G.** (2006). A B-sister MADS-box gene involved in ovule and seed development in petunia and Arabidopsis. *Plant J.* **47**, 934- 46.
- **Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G**. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature* **426**, 147-153.
- **Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme K.** (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* **282**, 2226-2230.
- **Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A. and Jurgens, G.** (2003). The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* **112**, 219–230.

- **Heisler, M. G., Ohno, C., Das, P., Sieber, P., Reddy, G. V., Long, J.A., and Meyerowitz, E.M.** (2005). Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. *Curr Biol.* **15**, 1899-1911.
- **Ishida, T., Aida, M., Takada, S., and Tasaka, M.** (2000). Involvement of CUP-SHAPED COTYLEDON genes in gynoecium and ovule development in *Arabidopsis thaliana*. *Plant Cell Physiol.* **41**, 60–67.
- **Jenik, P.D., and Barton, M.K.** (2005). Surge and destroy: The role of *auxin* in plant embryogenesis. *Development* **132**, 3577–3585.
- **Karimi, M., Inze, D., and Depicker, A.** (2002). GATEWAY vectors for Agrobacterium mediated plant transformation. *Trends Plant Sc.i* **7,** 193-195.
- **Kieffer, M., Neve, J., and** Kepinski**, S.** (2010). Defining auxin response contexts in plant development. Curr. Opin. Plant Biol**. 13**, 12–20.
- **Kooiker, M., Airoldi, C.A., Losa, A., Manzotti, P.S., Finzi, L., Kater, M.M., and Colombo, L.** (2005). BASIC PENTACYSTEINE1, a GA binding protein that induces conformational changes in the regulatory region of the homeotic Arabidopsis gene SEEDSTICK. *Plant Cell* **17**, 722-729.
- **Laskowski, M.J., Williams, M.E., Nusbaum, H.C. and Sussex, I.M.** (1995). Formation of lateral root meristems is a two-stage process. Development **121**, 3303–3310.
- **Mallory, A.C., Dugas, D.V., Bartel, D.P., and Bartel, B.** (2004). MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr. Biol.* **14**, 1035–46.
- **Morris, D.A., Friml, J. and Zazimalova, E.** (2004). Auxin transport. In Plant Hormones: Biosynthesis, Signal Transduction, Action! *Davies, P.J., ed. Dordrecht*, The Netherlands: Kluwer Academic Publishers, 437–470.
- **Okada, K., and Shimura, Y.** (1994).The *pin-formed* gene. In Arabidopsis, An Atlas of Morphology and Development, J.J. Bowman, *ed. New York: Springer-Verlag*, pp. 180–183.

- **Pagnussat, G.C., Alandete-Saez, M., Bowman, J.L., and Sundaresan, V.** (2009). Auxindependent patterning and gamete specification in the Arabidopsis female gametophyte. *Science* **324**, 1684-1689.
- **Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J.J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanová, M., Dhonukshe, P., Skupa, P., Benková, E., Perry, L., Krecek, P., Lee, O.R., Fink, G.R., Geisler, M., Murphy, A.S., Luschnig, C., Zazímalová, E., and Friml, J.** (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**, 914-918.
- **Reinhardt, D., Mandel, T., and Kuhlemeier, C.** (2000) Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell* **12**, 507-518.
- **Schneitz, K., Hulskamp, M. and Pruitt, R.E.** (1995). Wild-type ovule development in Arabidopsis thaliana, a light-microscope study of cleared whole-mount tissue. *Plant J.* **7**, 731-749
- **Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D.** (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell* **18**, 1121-1133
- **Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C., Paris, S., Gälweiler, L., Palme, K. and Jürgens, G.** (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286**, 316–318
- **Teale, W.D., Paponov, I.A., Palme, K.** (2006) Auxin in action: signalling, transport and the control of plant growth and development. *Nat. Rev. Mol. Cell Biol.* **7**, 847–859.
- **Vielle-Calzada, J.P., Baskar, R., and Grossniklaus, U.** (2000). Delayed activation of the paternal genome during seed development. Nature **404**, 91-94.
- **Yadegari, R., Paiva, G., Laux, T., Koltunow, A.M., Apuya, N., Zimmerman, J.L., Fischer, R.L., Harada, J.J., and Goldberg, R.B.** (1994). Cell Differentiation and Morphogenesis Are Uncoupled in Arabidopsis raspberry Embryos. *Plant Cell* **6**, 1713-1729.

Chapter 4

Role of CUC Genes in Female Gametophyte Development.

Dola Sinha Roy, Francesca Galbiati, Simona Masiero, Lucia Colombo.

Introduction:

Phytohormone auxin is likely to be part of the signaling pathway that regulate the lateral organ development in plants. Auxin is synthesized in the young tissues of the plants and actively transported to site of organ primordia (Davies 2005). The chemical inhibition of polar auxin transport disrupts the lateral organ development causing often an effect on phyllotaxy. Floral meristem fails to develop any lateral organ and resulting a naked pin-like stem (Reinhardt *et al.,* 2000). Inhibition of auxin transport or signaling lead to form several mutants, which lack floral meristem and mimic this effect, For example *pin-formed1* (*pin1*; Okada *et al.,*1991), *pinoid* (*pid;* Bennett *et al.,*1995) and *monopterous* (*mp;* Berleth and Jurgens, 1993; Przemeck *et al*., 1996), are implicated in auxin transport or responses to signaling. The *pin1* mutant phenotype can be rescued by exogenous auxin application (Reinhardt *et al.,* 2000). Also the local application of auxin induced the primordia formation, suggesting that auxin played a role is essential for lateral organ formation.

PIN1 encodes a putative auxin efflux carrier that promotes polar auxin transport (Galweiler *et al*., 1998). By regulating auxin distribution *PIN1* indirectly promote organ formation. The cotyledon positioning, number, growth and separation have been affected in *pin1* during embryogenesis. Similar to *pin1* mutant the *pid* mutant showed a reduction of polar auxin transport in stem and roots (Bennett et al., 1995). Loss of *PID* activity results in an inflorescence phenotype similar to *pin1.* The *pin1 pid* double mutant seedlings completely lacked cotyledons in most severe cases (Furutani *et al*., 2004). Expression of the boundary genes *CUC1* and *CUC2* were slightly expanded in the *pin1 pid* double mutant embryos and it correlated with defect in cotyledon growth (Furutani *et al*., 2004). It has been reported that *PIN1* together with *PINOID* maintain an auxin maxima that is required to control *CUC1* and *CUC2* genes expression during embryogenesis (Furutani *et al*., 2004).

Mutation in *MP* strongly affects embryo development. *mp* mutants showed defects in cotyledon positioning and frequently produced fused cotyledon as observed in *pin1* mutant or in *cuc1cuc2* double mutant. *MP* genes encodes a member of AUXIN RESPONSIVE FACTOR (ARF) gene family, the proteins of this family functionally bind to the promoter elements of auxin inducible genes (Ulmasov *et al.*,1997). It is known that *PIN1* and *MP* maintain the apical patterning of the embryo through regulating the expression of *CUC1 a*nd *CUC2 (*Aida *et al*., 2002).

The unique feature of plant life cycle is the presence of a multi-cellular haploid generation referred to as gametophyte. In Arabidopsis the female gametophyte develops within the ovules which are enclosed within the carpel. During megasporogenesis, the megaspore mother cell under goes meiosis to produce four haploid megaspores, three of which degenerate. The survival megaspore toward the chalazal end is named functional Megaspore (FM). FM under goes three rounds of mitotic division and differentiation develops in to the female gametophyte. The function of the female gametophyte is to produce the haploid gamete, which after fertilization produced the embryo. The mature female gametophyte consist of seven cells including egg apparatus, formed by the egg cell and two synergids, located at the micropylar end. The central cell contains two nuclei. Three antipodal cells located at the chalazal end of the embryosac.

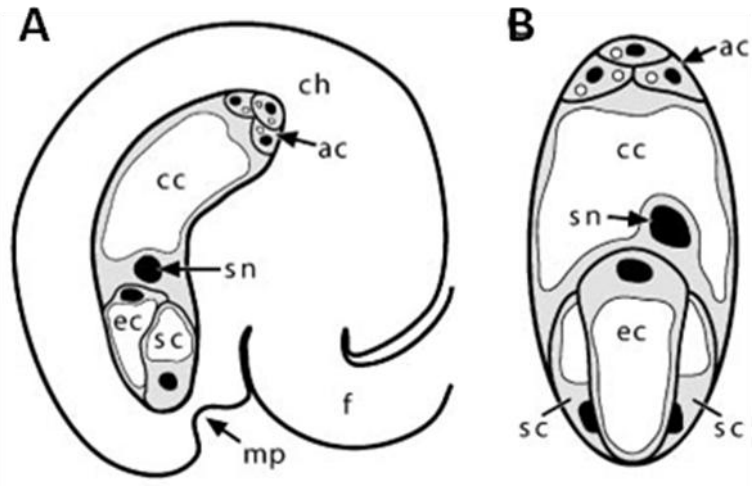


Figure 20: **A:** Ovule.

B: Female Gametophyte.

Abbreviations: ac, antipodal cells; cc, central cell; ch, chalazal region of the ovule; ec, egg cell; f, funiculus; mp, micropyle; sc, synergid cell;

Female gametophyte plays a vital role for the formation of seed. In Arabidopsis recent studies has uncovered many mutation that affect female gametophyte development and function. The work of this project has been reported in chapter 3 where we have shown that PIN1 is involved in female gametophyte development. In this work we wanted to investigate the interaction among *CUC1, CUC2* and the signaling molecule auxin in the progression of female gametophyte development.

Result and Discussion:

Ovule Specific CUC1 and CUC2 Gene Silencing Effect on Embryosac Development:

*CUC1 a*nd *CUC2* are expressed in the placenta and in the developing ovules. *In situ* hybridization showed that before ovule initiation *CUC2* is expressed in the placenta. In later stages *CUC2* is expressed in the nucellus and the integuments. In the mature ovule *CUC2* is expressed in the chalaza.

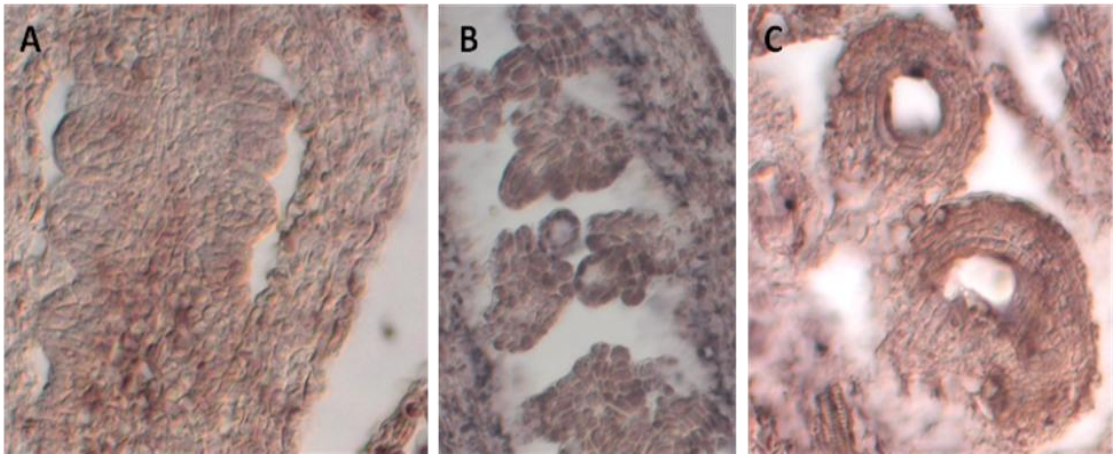


Figure 21: Pattern of *CUC2* mRNA expression.

A: Longitudinal section of the wild type carpel. *CUC2* expression found in the placenta and the area between the primordia.

B: CUC2 is expressed in the integument

C: Transverse section of ovule. *CUC2* expression found in the region of chalaza.

As previously discussed in chapter 2, *CUC1* and *CUC2* transcripts are targeted by micro RNA *miR164* (Mallory *et al*., 2004). To understand the role of *CUC* genes in female gametophyte development we have used the same approach as described in chapter 2. In this study *CUC1 a*nd *CUC2* has been silenced specifically in the ovule by the overexpression of micro RNA *miR164*. Thus ovule specific promoter pSTK has been used to express *miR164* (*STK::miR164*) in the *cuc2 background* to down regulate *CUC1 a*nd *CUC2* genes exclusively in the developing ovules.

As reported in chapter 2, optical microscope study revealed that within the T1 segregation among 60 plants 17 showed abnormal seed set. The sister plants of T1 are crossed to achieve the higher expression level of *miR164.* The offspring of those plants showed significant percentage (around 80%) of several seed set defects mainly due to the ovule abortion (Figure22).

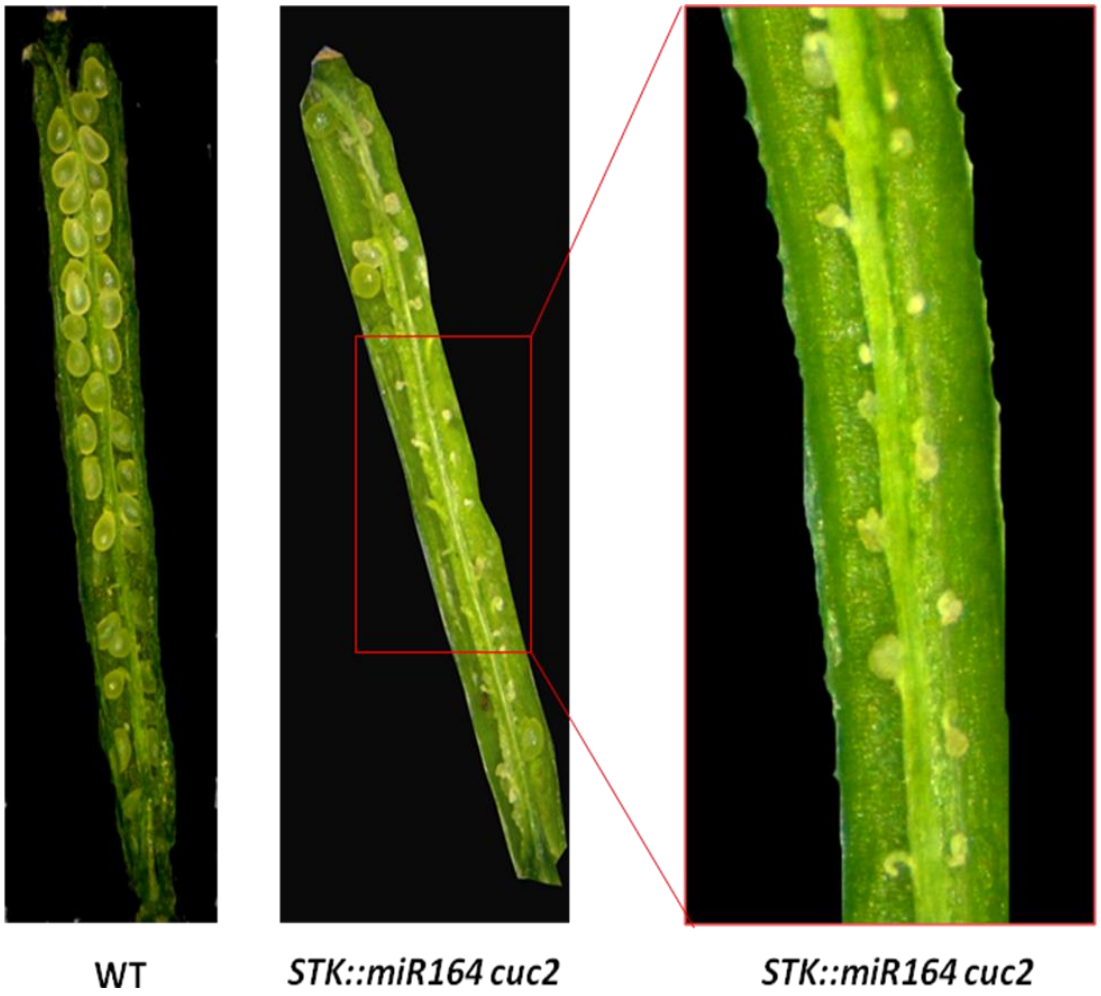


Figure22: Carpels of the *STK::miR164cuc2* and Wild type plants.

In this work we have focused on the phenotype of ovules (reduced in number respect to wild type) in *STK::miR164cuc2* plants. Detail clearing analyses under light microscope showed that in the transgenic plants female gametophyte development were severely arrested in different stages within the same carpel (Figure24). About 80% (96 ovules out of 120) of the analyzed ovules had embryosac arrested in stage FG1 and FG2 of embryosac developments (Figure23). Although some female gametophytes were able to complete their formation but they were not fertilized. In all cases these ovules had the integuments similar to wild type ovules, suggesting that *CUC* genes do not play essential role in the formation of integuments.

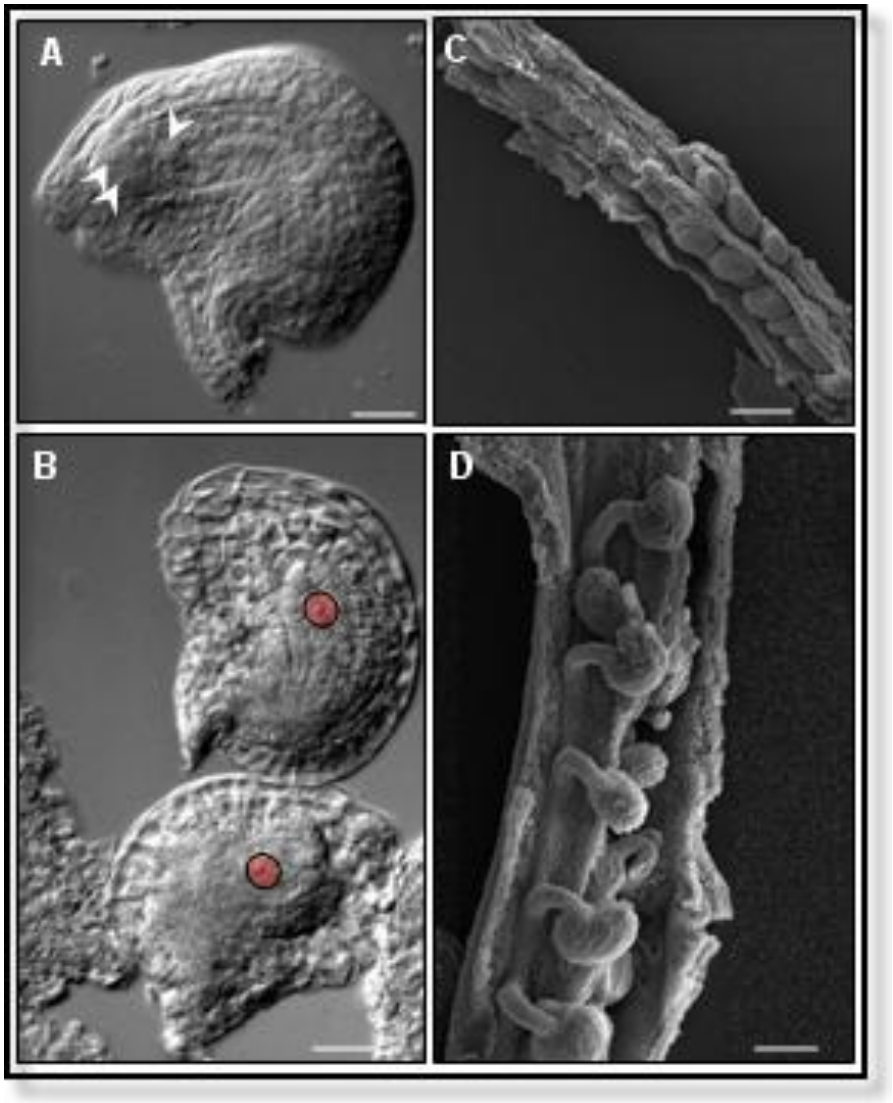


Figure23: Ovules of wild type and *STK::miR164cuc2* carpel.

A: Wild type ovule at FG6 with two synergids and central cell.

B: Mature ovule of *STK:: miR164cuc2* , embryosac blocked in FG1.

C: SEM image of wild carpel, containing mature ovules.

D: SEM images of *STK::miR164cuc2* carpel. Scale bar: 50µm

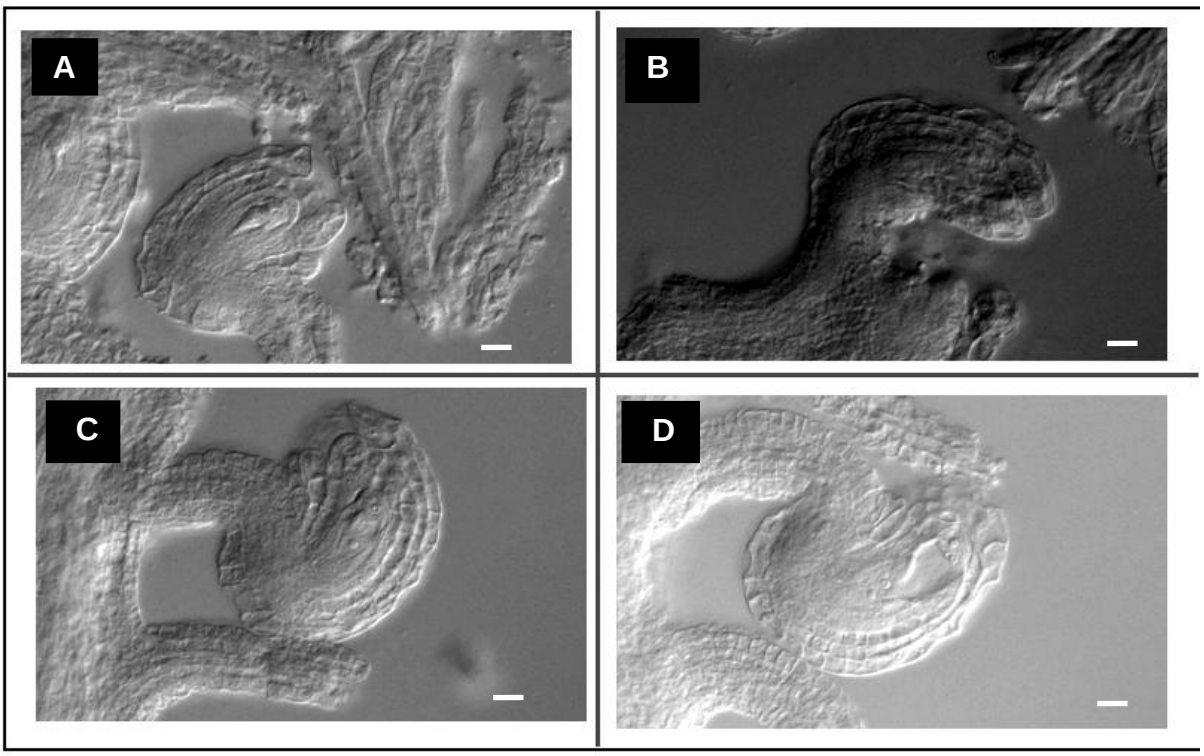


Figure24: *STK::miR164cuc2* ovules with defective female gametophyte at different stages of development, observed in the same carpel.

A: Ovule with female gametophyte blocked at stage of 1 nucleus (FG1).

B: Ovule is much shrunken and do not develop further, blocked in FG1.

C: Female gametophyte blocked at stage of 2 nuclei (FG2).

D: Female gametophyte blocked at stage of 4 nuclei (FG4-5). Scale bar: 20µm

The few ovules of *STK::miR164cuc2* those develope a complete embryosac were not fertilized. Aniline blue staining analyses have been performed to study the defect of pollen tube guidance. In wild type carpel almost 88% (140 out of 160) of the pollen tube reached to the micropyle and enter to the embryosac. When *STK::miR164cuc2* plants were pollinated with wild type pollen only 20% (36 out of 180) pollen tube successfully reached to the micropyle. In the transgenic plants the growth behaviour of pollen tubes

were dramatically different than wild type (Figure26). After the pollen tube emerge on the surface of the septum, it started to grow randomly in all available surface in the ovary wall instead of growing to the funiculus and micropyle of the ovule. The growth of the pollen tube through the ovular micropyle were affected almost 80% (184 out of 230) of the ovules in the transgenic plants *STK::miR164cuc2*. Together with this data, we could conclude that the pollen tube growth on papillar cells and the intercellular growth within the transmitting tract were not affected in the *cuc1cuc2* double mutant transgenic plants. However most of the pollen tubes were unable to enter into the embryosac, and embryosac remained unfertilized.

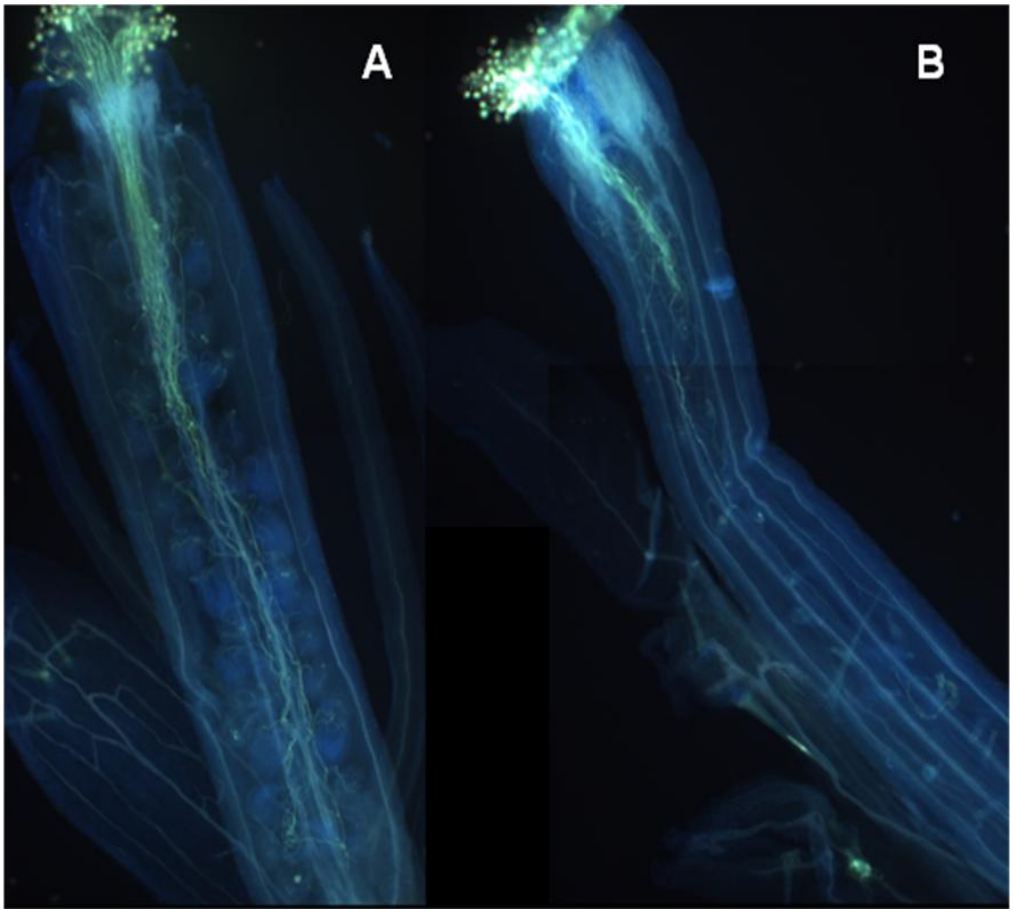


Figure 25: Aniline blue staining analyses in wild type and *STK::miR164cuc2* carpel.

A: Wild type carpel. Most of the pollen tubes reached to the micropyles.

B: *STK::miR164cuc2* carpel pollinated with wild type pollen. Very few pollen tubes reached to the micropyles.

Ovule Specific CUC1 and CUC2 Gene Silencing Effect on PIN1 Localization:

We knew from our previous study that the Pin1 membrane localization was effected in *STK::miR164cuc2* ovule primordia (Chapter2). Furthermore we have shown that PIN1 expression is important for the female gametophyte development (Chapter 3). To understand the effect of *CUC1* and *CUC2* silencing in the ovule development, we decided to study the PIN1localization in the *STK::miR164cuc2* transgenic plants. The pollen of the transgenic plants were used to fertilize the PIN1:: PIN1:GFP plants. In wild type the PIN1 is localized at the lateral-apical membranes of the nucellus in developing ovules (Figure26 A) and at the basal membranes of provascular cells in the funiculus. In the transgenic plants *cuc2* carrying the construct p*STK::miR164* the cellular localization of PIN1 was dramatically affected in the ovules, as shown in figure 26B. PIN1 was included in to vesicular structure in the ovules instead to be localized in the membrane. In the sibling plants not containing the construct *pSTK::miR164* the localization of PIN1 were like wild type ovules, suggesting that alter *CUC1* and *CUC2* expression is important to proper localization of PIN1 in the ovules and is consistent with the phenotype described in plants in which PIN1 was down regulated (Chapter3).

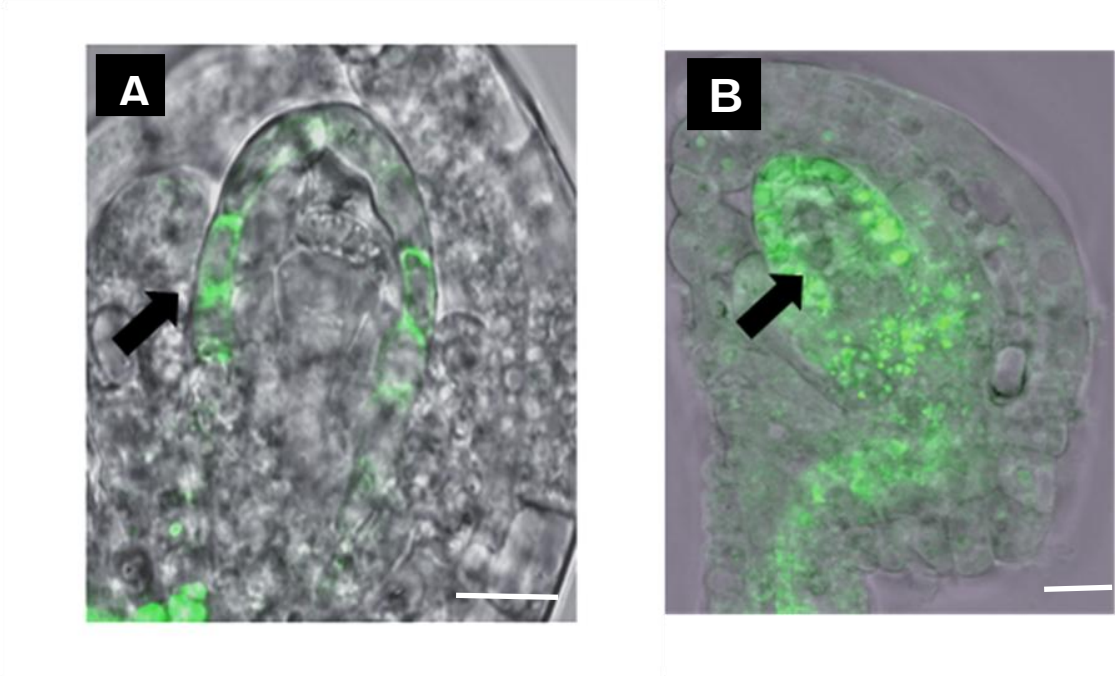


Figure 26: PIN1 localization in Wild type and transgenic plants *STK::miR164cuc2.*

A: wild type ovule at stage FG1. PIN1polar localization detected in the apical cell membrane of the epidermis cells surrounding the embryosac.

B: Ovules of the *STK::miR164cuc2* at stage FG1. Polar localization of PIN1 is disrupted. Pin1 is included in to the vesicular structures.

Discussion:

It has been reported that *cuc1cuc2* plants which derived from the shoot regeneration of double mutant calli are female sterile (Ishida *et al.,*2000). In this project we wanted to correlate the genetic network controlled by *CUC1* and *CUC2* with the hormonal pathway involved in ovule development. Organogenesis is a dynamic process, during this process the changes in auxin distribution must occur rapidly. The sites of organ formation are determined by changes in PIN1 distribution (Reinhardt *et al.,* 2003). Several studies have shown that redistribution of PIN1 protein within a cell following external and developmental cues (Friml *et al*.,2003., Geldner *et al.,* 2001). It is not clear how auxin initiates the organ formation, but after auxin accumulation it activates the genes those involved in organ initiation. Boundary genes *CUC1* and *CUC2* play important role during organogenesis. In *pin1pid* double mutant, it has been shown that *CUC1* and *CUC2,* instead of being expressed in their normal expression pattern, are ectopically expressed causing the cup shaped cotyledon described in *cuc1cuc2* double mutant. It has been suggested *PIN1* and *PID* play a role to control the *CUC1* and *CUC2* expression during embryo development (Furutani *et al*.,2004). The aberrant expression of *CUC* genes in *pin1 pid* double mutant embryo, is correlated with defect in cotyledon growth (Furutani *et al*., 2004). It has been shown that loss of floral meristem in *pin1* mutants and lack of cotyledon in *pin1 pid* double mutant is due to the aberrant or ectopic expression of *CUC* genes (Aida *et al*., 2002; Furutani *et al*., 2004).

From the experiment it is clear that in the transgenic plants *cuc2* containing the construct *pSTK::miR164* have severely affected in female gametophyte development. Light microscopy study revealed that several blocks have been detected in early stage of female gametophyte development. Similar kind phenotype was found when polar auxin transports have been perturb by silencing the auxin efflux carrier *PIN1* in the developing ovules. Also the localization of PIN1 is altered in the ovule of transgenic plants pSTK:: pin1RNAi. Together this data, suggesting that polar auxin transport is an important event for the proper formation of embryosac. The auxin efflux

carrier PIN1 plays an important function to establish a flux of auxin which is necessary for proper development of early stage of female gametophyte. Proper PIN1 localization is required to maintain the auxin maxima in the developing ovules. In the transgenic plants *pSTK::miR164cuc2,* due to delocation of the PIN1 the maintenance of proper auxin maxima has been disturbed which in turn effected on the female gametophyte development. From this data, it is clear that boundary genes *CUC1* and *CUC2* are necessary to maintain the proper PIN1 localization for the development of female gametophyte in the Arabidopsis ovule.

Experimental Procedures:

Microscopy:

Inflorescences were collected from plants, carpels were opened by a needle and covered with some drops of a solution of glycerol 20% (samples for CLSM) or chloralhydrate in water. Finally a coverslip was put on the samples and a slight pressure was applied.

Optical microscopy:

Optical microscopy investigations were performed using a Zeiss Axiophot D1.

Whole-mount preparation:

Siliques and carpels were dissected using a Leica stereomicroscope. Material was collected to investigate ovule development was cleared as reported by Yadegari et al (Yadegari et al 1994). Inflorescences were fixed in ethanol: acetic acid 9:1 overnight followed by two washes in ethanol 90% and 70%. 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).

In situ hybridization:

In situ hybridization with digoxigenin-labeled antisense RNA was performed as previously described by Masiero et al (2004). *CUC2* probe was prepared as described by Ishida et al (2000).

Confocal Laser Scanning Microscopy (CLSM):

All samples were prepared from fresh material collected the same day of CLSM analysis. Carpels were dissected employing a Leica stereo microscope and covered with few drops of 20% (v/v) glycerol. CLSM analyses were performed using a Zeiss LSM510 Meta confocal microscope. GFP was excited by a 488nm wavelength laser and a BP 505-550 nm filter was applied for GFP emission. CLSM pictures were filtered by the Median filter from LSM software, and contrast-brightness.

Aniline blue Staining Experiment:

For the aniline blue staining experiment, the transgenic plants *STK::miR164cuc2* plants were emasculated and pollinated with wild type pollen 24 h after the emasculation. Pollen tube growth was analyzed 16 h after pollination. Aniline blue staining was performed as described by Huck et al. (2003).

Plasmid Construction and Arabidopsis Transformation:

For the construct *pSTK::miR164b* the fragment of miR164b was amplified with Atp 2248 and Atp 2249. The destiny vector pBGW (Karimi et al., 2002) was modified introducing the *pSTK* promoter (amplified with Atp 1507 and Atp

1508) and a T35S fragment, amplified with Atp 1663 and Atp 1664. This modified pBGW was used to clone *miR164b.*

Detail information about the pBGW and pFGC5942 vectors are available at http://www.psb.ugent.be/gateway and http://www.chromdb.org/rnai/vector respectively.

REFERENCES:

- **Aida, M., Vernoux, T., Furutani, M., Traas, J. and Tasaka, M.** (2002). Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the Arabidopsis embryo. *Development* **129**,3965 - 3974.
- **Bennett, S.R.M., Alvarez, J., Bossinger, G. and Smyth, D.R.** 1995. Morphogenesis in pinoid mutants of Arabidopsis thaliana. *Plant J.* 8: 505–520.
- **Davies, P.J. 2005.** Plant Hormones-Biosynthesis, Signal Transduction, Action. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- **Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H.,Hamann,T., Offringa, R. and Ju rgens, G**. 2003. Efflux dependent auxin gradients establish the apical–basal axis of Arabidopsis. *Nature* 426: 147–153.
- **Furutani, M., Vernoux, T., Traas, J., Kato, T., Tasaka, M. and Aida, M.** 2004. PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. *Development* 131: 5021–5030.
- **Galweiler, L., Guan, C., Mu¨ ller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K.** 1998. Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* 282: 2226–2230.
- **Geldner, N., Friml, J., Stierhof, Y. D., Ju¨ rgens, G. and Palme, K**. 2001. Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* 413: 425–428.
- **Huck, N., Moore, J.M., Federer, M., and Grossniklaus, U.** (2003). The Arabidopsis mutant feronia disrupts the female gametophytic control of pollen tube reception. Development 130: 2149–2159.
  Huijser, P., Klien, J., Lonnig, W.E., Meijer, H., Saedler, H
- **Ishida T, Aida M, Takada S, Tasaka M** (2000) Involvement of CUP-SHAPED COTYLEDON genes in gynoecium and ovule development in *Arabidopsis thaliana. Plant Cell Physiol* **41:** 60–67

- **Mallory, A.C., Dugas, D.V., Bartel, D.P. and Bartel**,(2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr. Biol.* **14**, 1035 – 1046.
- **Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y.** (1991). Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *Plant Cell* **3,** 677–684.
- **Reinhardt, D., Mandel, T., and Kuhlemeier, C.** (2000) Auxin regulates the initiation and radial position of plant lateral organs *Plant Cell* **12**, 507-518. *Plant Cell* **12**, 507-518..

- **Reinhardt, D., Pesce, E. R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C.** (2003). Regulation of phyllotaxis by polar auxin transport. *Nature 426*, 255-260.

Chapter 5

Thesis Discussion

GENERAL DISCUSSION:

The main goal of this work is to understand the interaction between genetic and hormonal networks in controlling ovule development starting from the ovule primordia formation. Ovules are very essential organ for plant reproduction. They represent the progenitor of seeds. Ovules are initiated as a small finger like primordia from the internal meristematic surface of the carpel, called placenta. In this project we have identified the major players in the genetic pathway and the regulatory mechanism involved in the ovule primordia formation.

Primordia development is a precisely controlled process which forms a structure of specific size and shape from the undifferentiated group of cells. Several mechanisms involved to maintain this fundamental process. The positioning mechanism is one of the important events, which define where the primordia will arise from the placenta. It was already suggested that auxin plays central role in this process, however genes targeted by auxin is regulated this process were unknown.

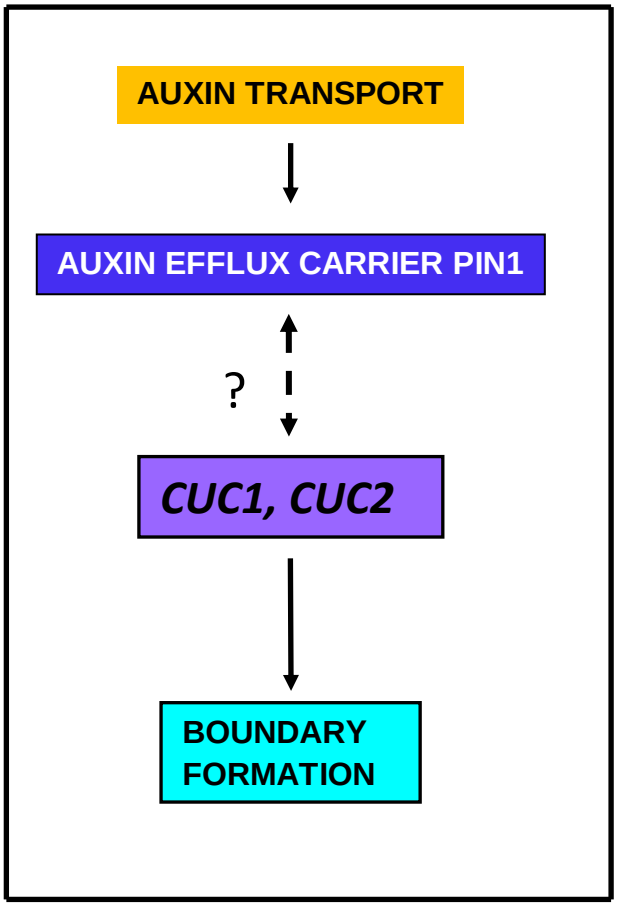
The initiation of new primordia occur where the auxin levels are highest which is surrounding by a zone of auxin depletion (Reinhardt *et al.,* 2003; Stieger *et al.,* 2002). The existing primordia accumulate auxin and prevent neighboring cells to initiate another lateral organ by lower down their auxin concentration (Sachs, 1991). The position of primordia determined by the establishment of boundary, between the primordia. The boundary specific genes *CUC1, CUC2* are required for demarcating primordium boundaries and also to promote meristem formation (Aida *et al*., 1997; Takada *et al*.,2001; Vroemen *et al.*, 2003)

There are genes already known that play vital role in the process of primordia initiation. *AINTEGUMENTA* (*ANT*) is likely to involve in this important process. *ant* mutant display less ovules than wild type. A strong synergistic phenotype has been found in *ant lug* double mutant (Liu *et al.,*

2000). *LEUNIG* (*LUG*) is a putative transcriptional co repressor involved in several developmental processes (Conner *et al*., 2000). The double mutant showed complete loss of septum, placentas and ovules, suggesting that the *ANT and LUG* are necessary for the placental tissue formation to allow the ovule primordia initiation (Liu et al., 2000). The *cuc1cuc2* double mutant carpel which were regenerated from mutant calli, also showed reduced ovule numbers (Ishida *et al.,*2000). In our experiment we have created the transgenic plants *STK::miR164cuc2* that showed fewer ovules than wild type. In the triple mutant *STK::miR164cuc2ant also* showed very few ovules than wild type and also *ant* single mutant. The ovule phenotype of the triple mutant *STK::miR164cuc2ant* phenocopied the *ant* single mutant ovule phenotype, suggesting that *ANT* is epistatic to *CUC* genes once that the ovule primordia are formed.

In our experiment we have ectopically expressed the micro RNA *miR164*, which target the transcript of 5 genes in Arabidopsis including *CUC1* and *CUC2* (Mallory *et al*., 2004). The other three genes targeted by *mir164* are *NAC1, At5g61430, At5g07680.* Whereas the function of *At5g61430, At5g07680* have not yet been known. *NAC1* is the transcriptional activator which is induced by auxin to promote lateral root development (Xie et al., 2002). Real time analyses revealed that mRNA level of all the five genes were reduced in the triple mutant *STK::miR164cuc2ant* carpel. Although Laufs and colleagues in 2004 have reported that no reduction of *NAC1* mRNA level in the inflorescence plants that overexpressed *miR164 (*Laufs *et al*.,2004). They hypothesized that *NAC1* may not be a real target of *miR164* or *NAC1* may not be regulated by *miR164* via mRNA cleavage but through the translational attenuation as described in animals (Aukerman and Sakai 2003; Chen 2004). Xie et al in 2010 has reported that *NAC1* is not expressed in the flowers and siliques. However our data have clearly shown that NAC1 is expressed in pistil and in particular in the ovules.

We have analyzed the auxin accumulation and PIN1 localization in the developing primordia of *STK::miR164cuc2* because we wanted to connect the molecular network with hormonal control pathway. To study the auxin accumulation we have followed the GFP expression driven by DRN promoter. From our data it is clear that auxin accumulate in those regions from where primordia will arise. The higher auxin accumulation leads to establish a lower auxin level in the neighboring region, which in turn determines the position of the developing primordia. In the triple mutant plant *STK::miR164 cuc2ant4* the auxin accumulation zone were more distantly and randomly spaced respect to *STK::miRcuc2* double mutant and wild type plants. This suggests that the position of the developing primordia is determined by the signaling molecule auxin which accumulation is related to *CUC1, CUC2,* and *ANT* expression.



Scheme 1: Auxin transport is necessary to maintain the proper auxin maxima in the developing ovule primordia. Boundary genes *CUC1*, *CUC2*

together with PIN1 are involved in the regulation to maintain the boundary between the ovule primordia.

IAA biosynthesis occurs through the tryptophan–dependent pathway. Recent studies uncovered that YUCCA (YUC) family of auxin biosynthetic genes encoding flavin monooxygenases, which regulate the tryptamine (TAM) pathway of auxin biosynthesis in the shoot apex. The YUC family has 11 members. Although none of the single mutant showed obvious phenotype but several double and triple mutant combinations displayed various defects in the floral development. In the *yuc1yuc4* double mutant pistils are formed without ovules, suggesting that the auxin is necessary to promote primordia formation and which is synthesized by those two YUCCA protein The pin-like structure was also observed in the quadruple mutant, indicating that *YUC* genes are involved in the initiation of flower and also in the flower development through the special and temporal control of auxin biosynthesis (Cheng *et al*., 2007). The feedback mechanism is also necessary for the organogenesis. *pin1* mutation with *yuc1* and *yuc4* led to pin1 defect, almost blocking all the leaf and flower initiation. Similar kind of phenotype also observed in the mutation of *aux1* with *yuc1, yuc2, yuc4* and *yuc6 (*Cheng *et al.,* 2007)*,* indicating that the regulation between the auxin transport and biosynthesis is the important step to maintain the auxin gradients (Weijers *et al*., 2005).

The subcellular localization pattern of PIN1 protein in the specific regions of the meristem revealed the direction of auxin flow and the site of auxin accumulation (Galweiler *et al.,* 1998, Benkova *et al*., 2003; Reinhardt et al.,2003). Auxin accumulation activates the genes, involved in organ initiation. Reduction of polar auxin transport in stem has been detected in the *pid* mutant (Bennett *et al*., 1995). *PID* is a positive regulator of polar auxin transport (Benjamins *et al*., 2001). To investigate the role of *PID* in the ovule development, we are planning to verify the expression pattern of *PID* in the ovules of wild type and in the transgenic plants *STK::miR164cuc2.*

In addition to the effective role of PIN1 in *CUC* genes regulation we have detected the PIN1 regulation by the boundary genes *CUC1* and *CUC2.* In our experiment an alter localization of PIN1 have been detected in the developing ovule primordia where *CUC1* and *CUC2* have been silenced in the ovule. The delocalization of the PIN1 also effect on female gametophyte development in the ovule. Similar kind of phenotype were described when polar auxin transport have been perturb by the silencing of auxin efflux carrier PIN1 in the developing ovule (Ceccato *et al.,* unpublished data). PIN1 play an important role to establish an auxin gradient that control early stage of female gametophyte development. For the maintenance of auxin maxima in the developing ovule, proper location of PIN1 is necessary. In the transgenic plants *pSTK::miR164cuc2,* probably due to the delocalization of PIN1 the regulation of auxin maxima has been disturbed, which resulted in defective female gametophyte. Together with this data we concluded that PIN1 is necessary for the proper development of female gametophyte and the boundary genes *CUC1 a*nd *CUC2* are necessary to maintain the proper auxin localization in the developing ovule.

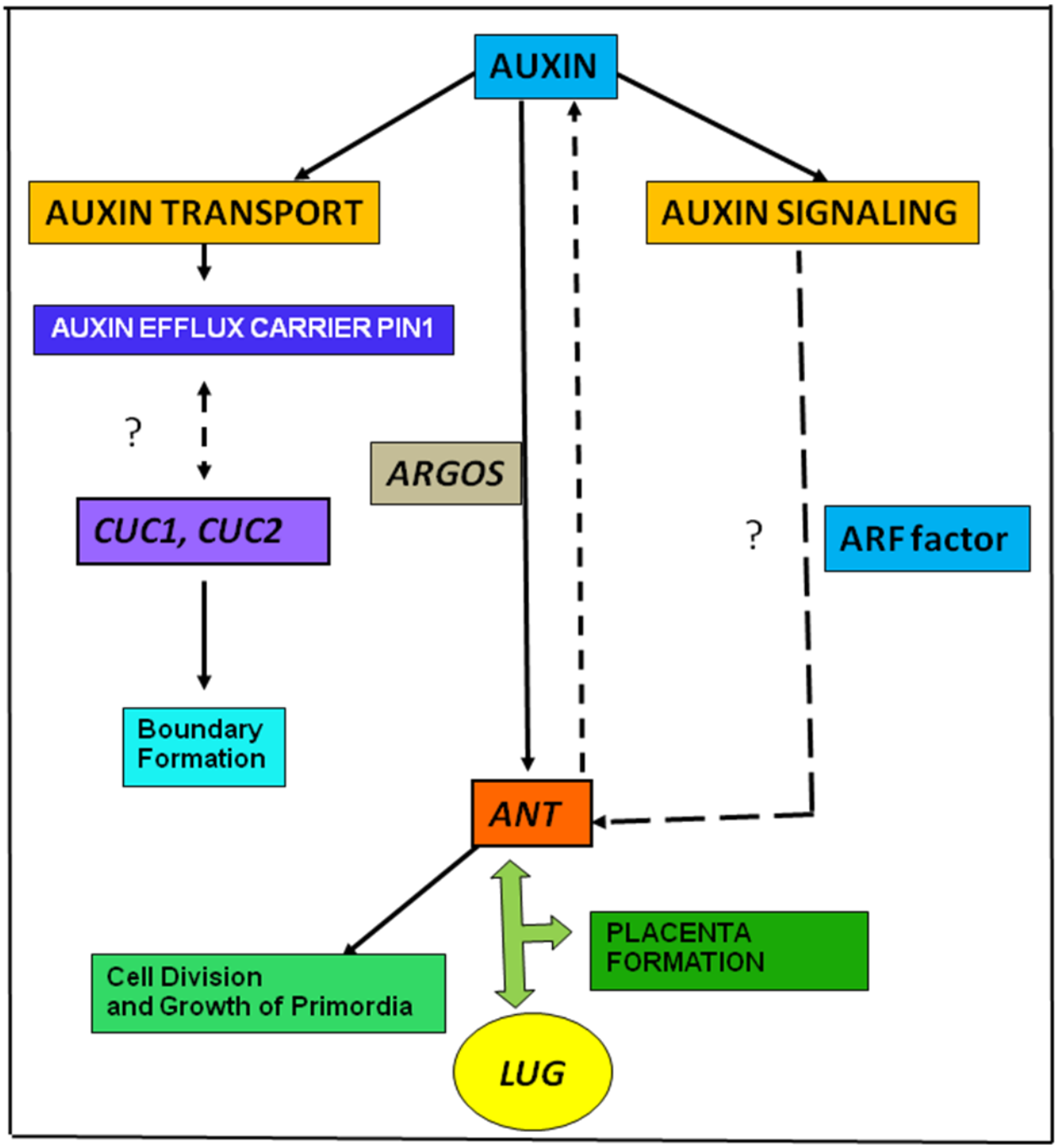
So, with this project we have achieved a better understanding of the regulatory mechanism that are involved in controlling the number of ovule primordia as well as the development of the ovule. Plant hormone auxin, boundary gene *CUC1, CUC2* and PIN1 are involved in this regulatory pathway. We have shown that those genes are involved themselves controlling auxin accumulation through the regulation (directly or indirectly) of the PIN1 membrane localization.

Considering all the analyses and available results of our experiment a model can be proposed for ovule development. During ovule development auxin determine the position of the primordia by maintaining the auxin maxima in the placenta. Auxin in turn activates the *ANT* gene probably through ARF factor (Scheme2). Also it has been shown by Hu colleagues in 2003 that *ANT* function downstream of auxin inducible gene *ARGOS* (Hu *et al*., 2003)*. ANT* promotes the cell cycle and further growth of the primordia. The boundary

genes *CUC1* and *CUC2* play vital role to demarcate the boundary between the primordia.

Further experiment is needed to confirm the regulation of *ANT* by auxin through *ARF* factor. ARFs are transcription factor that modulate the expression of auxin responsive genes (Guilfoyle and Hagen., 2007; Guilfoyle *et al*., 1998). ARFs bind to the TGTCTC auxin responsive eliment (AuxRE) in the promoter of auxin responsive genes (Ulmasov *et al*., 1997). To confirm this hypothesis whether the regulation of *ANT* expression is controlled through the ARF factor, we planned to perform the ChIP experiments with MP:: MP-GFP plants. *MP* is expressed in the placenta of the carpel as *ANT*. If MP binds in vivo to the AuxRE of *ANT*, it proves that *MP* is required for the expression of *ANT* during the ovule primordia development.

Also in the later stage of development auxin play vital role during female gametophyte development. The auxin maxima in the developing female gametophyte are maintained by the boundary gene *CUC1* and *CUC2.* PIN1 is required to regulate the proper auxin flux in the developing ovule.



Scheme2: Auxin determines the position of primordia by maintaining the auxin maxima in the placenta. Auxin in turn activate *ANT* gene probably through the ARF factor. *ANT* promotes the cell division and growth of the primordia. *ANT* together with *LUG* maintain the placental cell formation. Boundary genes *CUC1, CUC2* demarcate the boundary between the developing primordia. *CUC1 and CUC2* maintain the proper localization of

PIN1, in the developing ovule, which is essential for further development of the ovule. The regulation between *CUC* genes and *PIN* is not known.

REFERENCES:

- **Aida, M., Ishida, T., Fukaki, H., Fujisawa, H., and Tasaka, M.** (1997). Genes involved in organ separation in Arabidopsis An analysis of the cup-shaped cotyledon mutant. *Plant Cell* 9, 841-857.
- **Aida, M., Vernoux, T., Furutani, M., Traas, J. and Tasaka, M.** (2002). Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the Arabidopsis embryo. *Development* **129**,3965 - 3974.
- **Aukerman, M. J. and Sakai, H.** (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell* **15**,2730 -2741.
- **Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D.,Jurgens, G., and Friml, J**. (2003). Local efflux-dependent auxin gradients as a common module for plant organ formation. *Cell 115*, 591-602.
- **Cheng Y, Dai X, Zhao Y** (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes Dev **20**1790–1799
- **Cheng Y, Dai X, Zhao Y.** 2007. Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*.Plant Cell **19:** 2430–2439.
- **Conner J, Liu Z (2000)** LEUNIG, a putative transcriptional corepressor that regulates AGAMOUS expression during flower development. Proc Natl Acad Sci U S A. 2000 Nov 7;97(23):12902.
- **Furutani, M., Vernoux, T., Traas, J., Kato, T., Tasaka, M. and Aida, M.** 2004. PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. Development 131: 5021–5030.
- **Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998).** Regulation of polar auxin

transport by AtPIN1 in Arabidopsis vascular tissue. Science *282*, 2226-2230.

- **Guilfoyle, T.J.** (1998). Aux/IAA proteins and auxin signal transduction. Trends Plant Sci. **3,** 205–207
- **Guilfoyle, T. J. and Hagen, G.** (2007). Auxin response factors. *Curr. Opin. Plant Biol.***10**,453 -460.
- **HU, Y**., **Xie, Q., Chua,N.H**., 2003. The Arabidopsis auxin- inducible gene ARGOS controls lateral organ size. *Plant Cell*15, 1951-1961.
- **Ishida T, Aida M, Takada S, Tasaka M** (2000) Involvement of CUP-SHAPED COTYLEDON genes in gynoecium and ovule development in *Arabidopsis thaliana. Plant Cell Physiol* **41:** 60–67
- **Liu Z, Franks RG, Klink VP**.(2000). Regulation of gynoecium marginal tissue formation by LEUNIG and AINTEGUMENTA *Plant Cell*. Oct; 12(10):1879-92.
- **Laufs, P., Peaucelle, A., Morin, H. and Traas, J.** (2004) MicroRNA regulation of the *CUC* genes is required for boundary size control in *Arabidopsis* meristems. *Development*,
- **Mallory, A.C., Dugas, D.V., Bartel, D.P. and Bartel**,(2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr. Biol.* **14**, 1035 – 1046.
- **Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y.** (1991). Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *Plant Cell* **3,** 677–684.
- **Qi Xie, Giovanna Frugis, Diana Colgan, and Nam-Hai Chua** (2002) *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development**.** *Genes Dev*14(23): 3024–3036.
- **Reinhardt, D., Mandel, T., and Kuhlemeier, C.** (2000) Auxin regulates the initiation and radial position of plant lateral organs *Plant Cell* **12**, 507-518.

- **Reinhardt, D., Pesce, E. R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C.** (2003). Regulation of phyllotaxis by polar auxin transport. *Nature 426*, 255-260.
- **Sachs, T.** (1991). Pattern Formation in Plant Tissues. (Cambridge, UK: Cambridge University Press).
- **Smyth, D. R., Bowman, J. L., and Meyerowitz, E. M** (1990). Early flower development in Arabidopsis. *Plant Cell* 2, 755-767.
- **Stieger, P.A., Reihnardt, D., and Kuhlemeier, C.** (2002). The auxin influx carrier is essential for correct leaf positioning. *Plant J*.**32,** 509–517.
- **Takada, S., Hibara, K., Ishida, T., and Tasaka, M.** (2001). The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. *Development* **128,** 1127–1135.
- **Ulmasov, T., Hagen, G., and Guilfoyle, T. J.** (1997a). ARF1, a transcription factor that binds to auxin response elements. Science 276, 1865-1868.
- **Vroemen, C.W., Mordhorst, A.P., Albrecht, C., Kwaaitaal, M.A., and de Vries, S.C.** (2003). The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. *Plant Cell* **15,** 1563–1577
- **Weijers D, Sauer M, Meurette O, Friml J, Ljung K, Sandberg G, Ho oykaas P, OffringaR.** 2005. Maintenance of embryonic auxin distribution for apical-basal patterning by PIN-FORMED-dependent auxin transport in *Arabidopsis*. *Plant Cell*: tpc.105.034637.

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