UNIVERSITA' DEGLI STUDI DI MILANO

Facoltà di Scienze Matematiche, Fisiche e Naturali

Dipartimento di Biologia

DOTTORATO DI RICERCA IN BIOLOGIA VEGETALE

XXIII CICLO

Hormonal Network Controlling Ovule Development

in

Arabidopsis thaliana

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ANNO ACCADEMICO 2010-2011

PREFACE

"If the bee disappears from the surface of the earth, man would have no more than four years to live." (Einstein).

This statement probably made by Einstein points out clearly how humankind‘s existence depends on bees and in general on seed plants sexual reproduction.

Seed formation is dependent on ovule development, so the study of ovule organogenesis can have a big impact on the society.

During my Ph.D., I have focused my research to uncover the molecular network controlling ovule development in *Arabidopsis thaliana.*

This thesis is organized in an introduction part, in which I have described what it is known about ovule development including the role of hormones.

Then, in chapter 1, I have described the research performed to study the role of auxin and auxin polar transport in ovule development.

Chapter 2 is focalized on the study that I have performed to identify and characterized genes involved in auxin biosynthesis during ovule development in *Arabidopsis*.

Chapter 3 described the results obtained manipulating the cytokinin amount in ovule. Cytokinins have an antagonistic role respect to auxin during differentiation and development. I have studied cytokinins pathway using specific markers line and by genetic approaches.

The work proposed in Chapter 4 integrated the studies on role of auxin, cytokinin and transcription factors in ovule development, and a model is proposed to describe the hormonal control of ovule organogenesis.

In the last chapter the final discussion is presented with a general overview of the results and the suggestion of future experiments that could be performed in the near future.

INTRODUCTION

Cross Talk Between the Sporophyte and the Megagametophyte During Ovule Development

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This work was published on Sexual plant reproduction DOI 10.1007/s00497-011-0162-3

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Abstract

In angiosperm ovules two independent generation coexist the diploid maternal sporophytic generation that embeds and sustains the haploid generation (the female gametophyte). Many independent studies on *Arabidopsis* ovule mutants suggest that embryo sac development requires highly synchronized morphogenesis of the maternal sporophyte surrounding the gametophyte, since megagametogenesis is severely perturbed in most of the known sporophytic ovule development mutants. Which are the messenger molecules involved in the haploid-diploid dialogue? And furthermore, is this one way communication or is a feedback cross talk? In this review we discuss genetic and molecular evidences supporting the presence of a cross talk between the two generations, starting from the first studies regarding ovule development and ending to the recently sporophytic identified genes whose expression is strictly controlled by the haploid gametophytic generation. We will mainly focus on *Arabidopsis* studies since it is the species more widely studied for this aspect. Furthermore possible candidate molecules involved in the diploid-haploid generations dialogue will be presented and discussed.

Arabidopsis ovule development: a morphological overview

Ovule primordia arise from the placental tissue and appear as a finger like protrusions. Three elements, the funiculus, the chalaza, and the nucellus, can be distinguished along the proximal–distal axis of the developing ovule providing conspicuous evidence of ovule polarity (Schneitz et al. 1995). The funiculus connects the ovule to the carpel and includes the vascular strand, which channels nutrients through the chalaza to the nucellus and the rest of the developing ovule.

A hypodermal cell at the tip of the nucellus differentiates into the megasporocyte or megaspore-mother-cell. After, meiotic division (which occupies a critical role early in megasporogenesis), the megaspore-mother-cell originates a tetrad of haploid spores. In most flowering plants, including *Arabidopsis thaliana*, three megaspores undergo programmed cell death, however the most proximal one persists forming the functional megaspore), which proceeds into megagametogenesis (Bajon et al. 1999; Schneitz et al. 1995).

The functional megaspore goes through three mitotic divisions forming a mature embryo sac composed of eight nuclei and seven cells: three antipodal cells, two medial polar nuclei, and one egg cell surrounded by two synergids (Mansfield et al. 1991). The development of the female gametophyte of *Arabidopsis thaliana* is a morphologically well-described multistep process (from FG1 to FG7) also known as megagametogenesis (Christensen et al. 1997).

The switch from radial symmetrical to bilateral symmetrical primordia is accompanied by integument initiation and is coordinated with proximal–distal axis development (Balasubramanian and Schneitz 2000; Grossniklaus and Schneitz 1998; Reiser et al. 1995; Schiefthaler et al. 1999; Schneitz et al. 1995). Integuments develop from chalazal epidermal cells. In angiosperms there are species whose ovules have no integuments (ategmic ovules as in *Santales*; Brown et al. 2010), one integument (unitegmic ovules, e.g., Wang and Ren 2008) or two integuments (bitegmic ovules) as in *Arabidopsis thaliana* (Fig. 1). The integuments envelop the nucellus almost completely except for the micropyle. After fertilization the integuments will give rise to the seed coat (Robinson-Beers et al. 1992). According to several authors the integuments arise as a "protective nucellar" tissue (Gross-Hardt et al 2002; Taylor et al 2009), however increasing evidence indicates they are rather involved in communication between the two generations. How this communication occurs and which are the probable messengers is still controversial.

Ovule defective mutants provide a tool to understand ovule development

Several ovule defective mutants have been identified during forward genetic screenings (Bonhomme et al. 1998; Christensen et al. 2002; Feldman et al. 1997; Howden et al. 1998; Pagnussat et al. 2005; Schneitz et al. 1997; Sundaresan et al. 1995) which can be assigned to two major classes: sporophytic and gametophytic mutants (Robinson-Beers et al. 1992).

The ovule sporophytic mutants, described in this review are listed in Table 1 (Baker et al. 1997; Brambilla et al. 2007; Elliot et al. 1996; Gaiser et al. 1995; Klucher et al. 1996; Lang et al. 1994; Modrusan et al. 1994; Reiser et al. 1995; Robinson-Beers et al. 1992; Schneitz et al. 1997). One of the more intriguing mutants described is *bell1* (*bel1*; Robinson-Beers et al. 1992). In *bel1* ovules a large structure appears at the position normally occupied by the integuments (Fig. 1) that consists of epidermal and subepidermal cells that grow above the nucellus and acquire carpel identity as suggested by the transcription of carpel-specific genes (Brambilla et al. 2007). *BEL1* encodes a homeodomain transcription factor (Reiser et al. 1995; Robinson-Beers et al. 1992) that is expressed in the chalaza. *bel1* embryo sacs are unable to develop, since they are blocked at FG1 (Fig. 1) (Christensen et al. 1997; Schneitz et al. 1997). This phenotype indicates that ovule sporophytic maternal tissues exert control on the developing haploid generation (see Table 1).

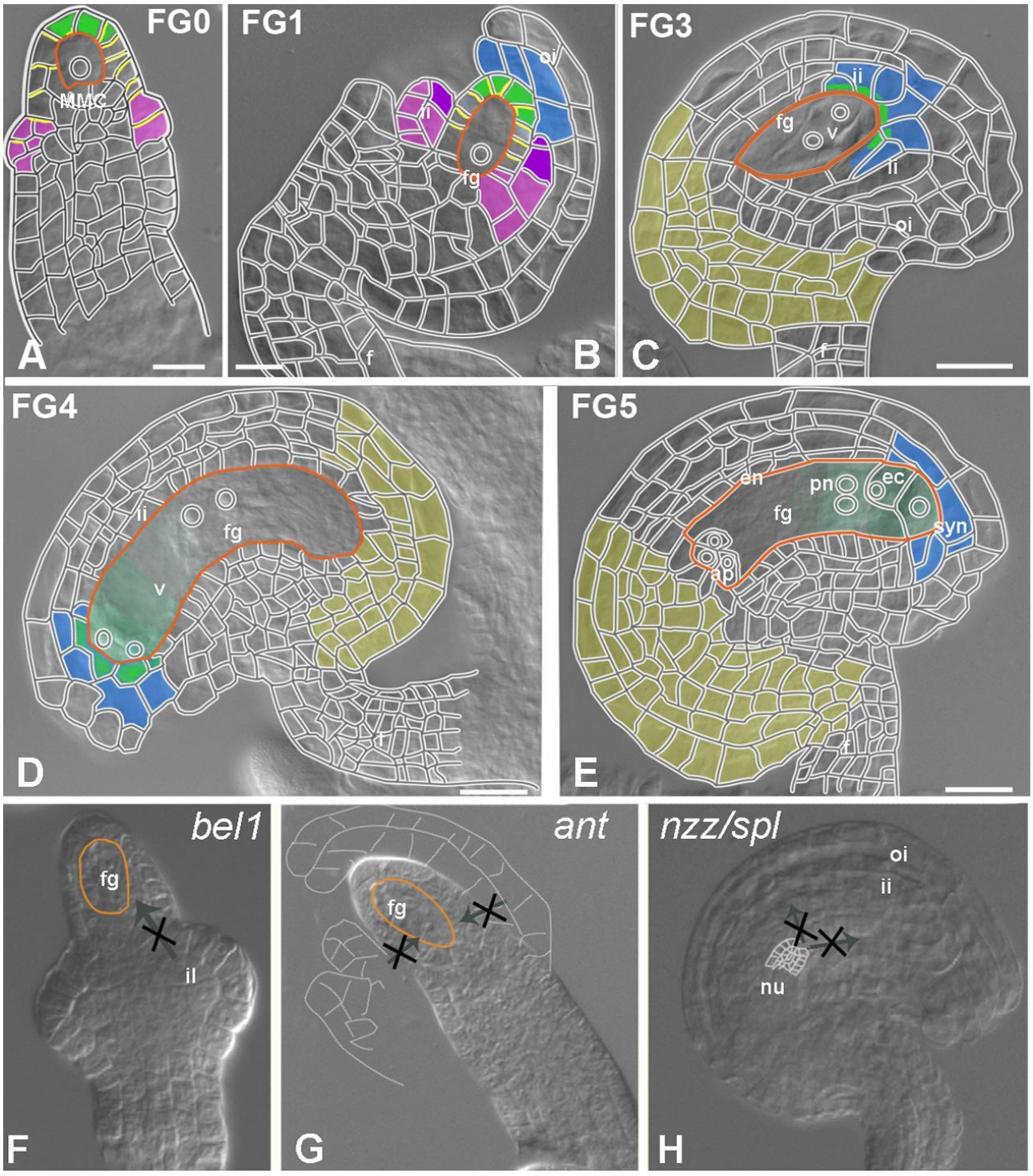

Fig. 1 Wild type ovule and female gametophyte development. *ant, bel1* and *nzz/spl* mutant ovules.

A-E Auxin and cytokinin distribution during wild type ovule formation, the corresponding embryo sac stages are indicated. Expression domains of DR5, *TAA1*, *YUCCA* and *IPT1* are colored in green, pink, blue and yellow, respectively; in violet those regions where *YUCCAs* and *TAA1* are co-expressed. An *orange* line delimits the female gametophyte, whereas yellow lines show PIN1 polarized distribution in nucellar cells. **F** *bel1* mutant ovule, in which the arrested FG1 embryo sac is delimited by an orange line. **G** *ant* mutant ovule without integuments (the absent integuments are drawn in grey). **H** *spl* (*nzz*) mutant ovule at later stages, the integuments developed properly, but not the archeospore (the nucellus is indicated in white). Arrows indicate crosstalk occurring between the female gametophyte and the sporophytic maternal tissues an antipodal cells, cc central cell, ec egg cell, en endothelium, fg female gametophyte, f funiculus, ii inner integument, ou outer integument, MMC megaspore mother cell nu nucellus, sy synergid, v vacuole. Scale bars: 20 µm

The *INNER NO OUTER* (*INO*) mutant description suggests that proper integument formation is also necessary to stimulate megagametogenesis progression. *ino* ovules do not develop the outer integument, however the inner integument seems to develop normally. *ino* embryo sacs are also gametophytically defective, since megagametogenesis cannot proceed after FG5 (Christensen et al. 1997; Schneitz et al. 1997), indicating that both integuments are important in *Arabidopsis* to promote female gametophyte development.

Additionally, *ino* mutant phenotype clearly suggests that the *Arabidopsis* inner and the outer integuments are two tissues differently regulated and with distinct origins, as also supported by paleobotanical evidence (Herr 1995).

The importance of the integument to promote gametophyte formation is clearly exemplified by *aintegumenta* (*ant*) mutant (Fig. 1). The *ANT* gene encodes a putative transcription factor that shares homology with the floral homeotic gene *APETALA2* (*AP2*) (Elliott et al. 1996; Jofuku et al. 1994; Klucher et al. 1996). In *ant*, ovules show extremely reduced or absent integuments (Baker et al. 1997; Elliott et al. 1996; Klucher et al. 1996; Schneitz et al. 1997) and embryo sacs are blocked at FG1 stage, as was described for *bel1* mutant (Fig. 1). Therefore integument defects negatively influence gametophyte development. The precocious expression of *ant* and *bel1* in ovule primordia can explain the observed female gametophyte problems (Elliot et al. 1996; Reiser et al. 1995), pointing towards the existence of transcriptional cascades triggered by ANT and BEL1 (Fig. 1). Nevertheless also in this scenario the maternal tissues exert a strict control on the formation and development the haploid generation. In Table 1 there is a summary list of 20 ovule sporophytic mutants characterized by embryo sac defects.

Clearly, female gametophyte development requires highly synchronized morphogenesis of the maternal sporophyte surrounding the gametophyte. In particular, the inner integument seems to play an essential role in promoting the first steps of megagametogenesis.

Ovule gametophytic mutations that affect embryo sac commitment and formation, and mature female gametophyte functions (pollen tube guidance, fertilization, induction of seed development, or maternal control of seed development) have also been reported (Yadegari and Drews 2004). These mutants are recognized by reduced seed set and distorted segregation ratio, since they are not successfully transmitted through the egg cell. Thus, gametophytic mutations exhibit apparent non-Mendelian segregation patterns and can only be transmitted as heterozygotes.

Table 1 *Arabidopsis* ovule sporphytic mutants that show also gametophytic defects

Gene name	Encoded protein	Ovule phenotype	Reference
ANT (AINTEGUMENTA)	Transcription regulator belongs to the AP2-domain protein	No integument formation	Baker et al. (1997)
BEL1 (BELL1)	Homeodomain protein belongs to BEL homeodomain family	Aberrant structure instead of the integuments	Robinson-Beers et al. (1992)
INO (INNER NO OUTER)	Transcription factor belongs to YABBY protein family	Formation of the inner but not of the outer integument	Gaiser et al. (1995)
ATS (ABERRANT TESTA SHAPE)	Transcription factor belongs to KANADI (KAN) family	The inner and outer integuments are replaced by a single integumentary structure	Leon-Kloosterziel et al. (1994)
LUG (LEUNIG)	Glutamine-rich protein similar to transcriptional corepressors	Protruding inner integument	Schneitz et al. (1997)
TSL (TOUSLED)	Nuclear serine/threonine protein kinase	A protruding inner integument and usually reduced outer integument	Roe et al. (1997)
HLL (HUELLENOS)	Mitochondrial ribosomal protein	Integuments fail to elongate	Schneitz et al. (1997)
UNC (UNICORN)	Not available	Formation of a protrusion at the base of the outer integument	Schneitz et al. (1997)
SUB (STRUBBELIG)	Receptor-like kinase	Aberrant outer integument formation	Schneitz et al. (1997)
BAG (BLASIG)	Not available	Aberrant integuments	Schneitz et al. (1997)
MOL (MOLLIG)	Not available	Integuments with enlarged cells	Schneitz et al. (1997)
LAL (LAELLI)	Not available	Protruding inner integument	Schneitz et al. (1997)
DCL1/SIN1(DICER-LIKE1/ SHORT INTEGUMENT1)	Encodes a Dicer homolog	Short integuments as a result of reduced cell elongation (meiosis does not occur)	Robinson-Beers et al. (1992) Schauer et al. (2002)
ACR4 (ARABIDOPSIS CRINKLY4)	Membrane-localized protein with similarity to receptor kinases	Disorganized integument growth	Gifford et al. (2003)
stk shp1 shp2 (SEEDSTICK, SHATTERPROOF1, SHATTERPROOF2)	MADS-box proteins bind to CArG-box DNA sequence	Integument conversion into carpel-like structure	Pinyopich et al. (2003)
stk shp1 shp2 bel1 (SEEDSTICK, SHATTERPROOF1, SHATTERPROOF2, BELL1)		Integument conversion into carpel-like structure	Brambilla et al. (2007)
er-105 erl1-2 erl2-1/+ ERECTA, ERECTA-LIKE 1, ERECTA-LIKE 2	Receptor-like kinases belong to ERECTA family	reduced cell proliferation in the integuments	Pillitteri et al. (2007)
SIN2 (SHORT INTEGUMENT2)	Mitochondrial DAR GTPase	short or absent integuments	Broadhvest et al. (2000) Hill et al. (2006)
tso1	Putative DNA-binding protein	aberrant integuments	Hauser et al. (1998)
kan1 kan2 (KANADI1, KANADI2)	Transcription factors belong to KANADI (KAN) family	aberrant integuments	Eshed et al. (2001)

Several forward genetic screenings (Bonhomme et al. 1998; Christensen et al. 2002; Feldman et al. 1997; Howden et al. 1998; Pagnussat et al. 2005) have led to the identification of genes involved in megagametophyte development. The gametophytic mutants described to date have a common feature: the ovule sporophytic tissues develop normally. Therefore, it has been proposed that a hierarchy exists in the communication between the two generations, attributing a higher order to the sporophytic maternal tissues.

This concept has also been strengthened by the characterization of the sporophytic mutant *nozzle/sporocyteless* (*nzz*/*spl*). *SPL* was, indeed, one of the first genes discovered to be involved in embryo sac formation (Schiefthaler et al. 1999; Yang et al. 1999). In particular in *spl* mutants, the

nucellus arrests its development before megasporogenesis, but the integuments seems able to develop normally (Sieber et al. 2004) (Fig. 1), suggesting that the nucellus is not necessary for proper integument formation.

Taken together, this genetic evidence indicates that the sporophytic maternal tissues somehow dominate female gametophyte formation, however recently Johnston et al. (2007) proved that the haploid embryo sac is not passively controlled by the sporophyte. Employing a microarray-based comparative approach on *spl* and *coatlique* (*coa*) ovules, both devoid of female gametophyte, 527 genes were found to be up-regulated in the sporophytic tissues, suggesting a mutual coordination between the sporophyte and the gametophyte.

The close and strict connection between the haploid and diploid generations is also provided through plasmodesmata (Bajon et al. 1999), which physically link the functional megaspore and the surrounding nucellus cells. In mature embryo sacs, the three antipodal cells are connected by plasmodesmata to the nucellus and to the central cell (Mansfield et al. 1991). Considered together, the genetic and the morphological data indicate cross-communication between the gametophyte and the sporophyte in the ovule and point to the important role of the chalaza and integuments in mediating the exchange of information necessary for the development of the embryo sac. However, the nature of the messengers i.e. metabolites, small peptides, hormones remains to be elucidated.

The control of the maternal tissues starts very early, before MMC (megaspore mother cell) differentiation. There are maize and rice mutants where multiple cells acquire archeospore fate. The rice *MULTIPLE SPOROCYTE1 (MSP1),* for instance, encodes a leucine rich repeat receptor-like kinase (LRR-RLK) and mediates feedback inhibition from the megasporocyte, thus preventing neighboring cells from entering the germline (Nonomura et al. 2003). *OsTDL1A (Oryza sativa TAPETUM DETERMINANT1*) controls germline specification (Zhao et al. 2008), and it is expressed like MPS1 in the nucellus.

ARGONAUTE 9 (AGO9) and other components of 24-nucleotide small interfering RNA (siRNA) biosynthetic pathways restrict the acquisition of gametic identity by nucellus cells (Olmedo-Monfil et al. 2010). *AGO9* is also not detected in the germline but restricted to neighboring somatic cells, suggesting that non-autonomous movement of siRNAs into the gamete precursors may be implicated in controlling their specification.

The movement of signals both apoplastically (peptide, ligands and hormones) and symplastically (small RNAs and hormones) might decide the fate of somatic cells, inducing them to acquire gametic cell fates. But again which are these signal molecules, and again which are their receptors

or ligands? Old and new literature data are re-evaluating the role of hormones in ovule morphogenesis.

Hormones as messengers in sporophyte-megagametophyte crosstalk

Plant hormones also play fundamental roles in regulation of developmental processes. Hormones integrate information from environmental and endogenous signals into the developmental pathways (Gray 2004) that actively participate in intra- and inter-cellular communication.

Hormones are small molecules derived from various essential metabolic pathways (Santner and Estelle 2009). Several molecules are annotated as plant hormones: abscisic acid (ABA), indole-3-acetic acid (IAA or auxin), brassinosteroids (BRs), cytokinin, gibberellic acid (GA), ethylene, jasmonic acid (JA) and salicylic acid. In the last decade, considerable progress has been made in understanding hormone biosynthesis, transport, perception and response and the identification of many hormone receptors, which highlight the chemical specificity of hormone signaling.

Collectively hormones regulate every aspect of plant life, from pattern formation during development to responses to biotic and abiotic stress.

Recently some laboratories have begun to correlate hormone and ovule formation. Although these studies have focused on the role of just a few hormones, such as ethylene, auxin and cytokinins, in ovule organogenesis these hormones are clearly important to ovule development.

Ethylene

Little information is available about ethylene and ovule development, although genetic and morphological observations suggest an active role for ethylene during ovule development.

For instance, it is has been shown that ovule development is severely compromised in tobacco plants where pistil-specific ethylene production is abolished. In particular, when *ACC (1-aminocyclopropane-1-carboxylate) oxidase*, a key enzyme for ethylene metabolism, is either silenced or inhibited by silver thiosulfate, tobacco ovule development is arrested and megasporocytes are unable to start or complete their formation. An observation that is consistent with the importance of ethylene in gametophytic development is that applications of exogenous ethylene can restore megasporogenesis or megagametogenesis in treated plants (Yang and Sundaresan 2000).

In *Arabidopsis* the ethylene-response mutant *ctr1* (*constitutive triple response1*) shows distorted segregation ratios as a consequence of embryo sac defects (Kieber and Ecker 1994; Drews et al.

1998). The *CTR1* gene encodes a Raf-like Ser/Thr protein kinase involved in ethylene signal transduction (Kieber et al. 1993).

In addition, evidence from orchids supports the close relationship between ethylene and megagametogenesis. Indeed, it has been shown that ethylene biosynthesis inhibitors induce ovary and gametophyte formation (Zhang and O'Neill 1993).

Information regarding ethylene and its role in ovule development is still fragmentary, thus future research will be needed to understand whether ethylene is either mainly involved in specific aspects of ovule gametophyte or sporophytic tissue formation, or if it plays a major role in diploid/haploid generation cross-talk.

Auxin

Auxin is involved in a wide spectrum of functions such as: apical dominance, fruit ripening, root meristem maintenance, hypocotyl and root elongation, shoot and lateral root formation, apical dominance, tropisms, cellular division, elongation and differentiation, embryogenesis, vascular tissue differentiation and all types of organogenesis (Laskowski et al. 1995; Reinhardt and Kuhlemeier 2002; Benkova et al. 2003). Auxin regulates also ovule development; already in 2000 Nemhauser and co-workers demonstrated that transient application of N-1-naphthylphthalamic acid (NPA, an auxin efflux inhibitor) causes significant loss of ovules. These data have been recently confirmed by Nole-Wilson et al. (2010), which associate ovule loss with severe reduction of local auxin biosynthesis. *Arabidopsis* inflorescences treated with NPA show a great inhibition of *TAA1* (*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1*) (Stepanova et al. 2008; Tao et al. 2008; Nole-Wilson et al. 2010), a gene that encodes for a tryptophan aminotransferase and is critical for the synthesis of auxin via the indole-3-pyruvic acid (IPA) pathway (Stepanova et al. 2008; Tao et al. 2008). *TAA1* is expressed in developing ovule integuments as they are initiated (Nole-Wilson et al. 2010) (Fig. 1). Consistently, in *ant* ovules, where integuments are severally affected, *TAA1* expression is highly reduced, thus implying a functional interaction between sporophytic tissues and megagametogenesis via auxin biosynthesis.

Interestingly, Pagnussat and co-workers (2009) have recently reported that auxin gradients control female gametophyte cell identity (Fig. 1). In particular, the distribution of auxin was monitored by DR5-driven expression in the synthetic reporters *DR5::GFP* and *DR5::GUS* (Ulmasov et al. 1997) (Fig. 1). At FG1 stage, the GUS signal was shown to be strong in the nucellus, outside the developing embryo sac, and starting with the FG3 stage GUS staining could be detected inside the forming embryo sac (Fig. 1).

In addition, *DR5::GFP* activity could be detected up to the FG5 stage, with a maximum at the micropylar end of the female gametophyte. After cellularization, the *DR5::GFP* signal was also detectable in all cells of the female gametophyte (Fig. 1). According to the proposed model, such a gradient is formed and maintained by PIN1 (PINFORMED1) and by *in loco* biosynthesis (Fig. 1). Auxin, unlike other hormones, has the unique capability to be transported in a polar way, with PIN-FORMED (PIN) proteins playing a major role as auxin efflux facilitators (Petrasek et al. 2006). With respect to ovule development, PIN1 is initially detected at the FG1 stage and its distribution suggests the presence of an auxin flux from the funiculus to the nucellus, able to establish an auxin maximum at the distal tip of the ovule primordial during the early stages of megagametogenesis (Pagnussat et al. 2009) (Fig. 1). However at later stages, PIN1 is not detected in either the female gametophyte or in the adjacent sporophytic tissues, indicating that the auxin revealed inside the forming embryo sac is locally synthesized

In addition, *YUCCA* (*YUC*) genes, which encode the putative flavin monooxigenases involved in auxin biosynthesis (Cheng et al. 2006), have been shown via GUS assays (*YUC1::GUS* and *YUC2::GUS)* to be transcribed at the micropylar region of the nucellus, outside the embryo sac, from the FG1 stage (Fig. 1). Afterwards they are also expressed at the micropylar pole of the megagametophyte from FG2 stage till cellularization (Fig. 1). Taken together, it appears that *YUCCA* genes are not ubiquitously expressed, but they are rather subject to strict spatio-temporal controls (Zhao et al. 2001; Cheng et al. 2006).

Indeed it is clear that ovules are quite active organs with respect to auxin metabolism and the role of this small molecule with respect to ovule development remains to be fully elucidated.

Cytokinins

Auxin action is counteracted by cytokinins, which promote cell proliferation and differentiation, together with the control of several developmental processes, such as organ formation and regeneration, senescence (Gan and Amasino 1995; Kim et al. 2006), apical dominance (Shimizu-Sato et al. 2009; Tanaka et al. 2006), root proliferation (Werner et al. 2001, 2003), phyllotaxis (Giulini et al. 2004), vascular development (Mahonen et al. 2000), response to pathogens (Siemens et al. 2006), nutrient mobility (Séquéla et al. 2008), and increased crop productivity (Ashikari et al. 2005). Despite their biological importance, the basic molecular mechanisms of cytokinin biosynthesis and signal transduction have been uncovered only in recent years.

In plants it is possible to manipulate cytokinin homeostasis by acting on the control of its synthesis and degradation (Růžička et al. 2009; Werner et al. 2003). For instance, *IPT*

(isopentenyltransferase) gene products are involved in cytokinin biosynthesis and catalyze the rate-limiting step of the biosynthesis pathway, whereas cytokinin catabolism is executed by CKX (cytokinin oxidase) enzymes (Sakakibara 2006).

How cytokinin perception occurs is still not fully understood, but it is known that the signaling pathway is based on His-Asp multi-step phosphorelays that involve histidine kinase (HK)-type receptors, histidine phosphotransfer proteins (HP), and response regulators (RRs). In the widely accepted model cytokinins interact with the cytokinin receptor histidine protein kinases (AHK2, AHK3 and AKH4/CRE1/WOL) (Kakimoto 2003) activating them inducing autophophorylation. Than the phosphoryl group is transfer to the *Arabidopsis* histidine phosphotransfer proteins (AHPs). Once these proteins enter into the nucleus in a phosphorylated state, they donate the phosphoryl group to type-B ARRs (*Arabidopsis* Response Regulator). The phosphorylated type-B ARRs act as transcriptional activators, promoting rapid induction of cytokinin-associated target genes, included also the typeA-*ARR* genes that in turn act as negative regulators of the ARR-B type. (Muller and Sheen 2007).

With respect to ovule development, little information is currently available, since manipulation of cytokinin metabolism severely affects plant fertility. For instance, fertility reduction is induced by over-expression of cytokinin oxidase, which regulates cytokine stability (Werner et al. 2003). Moreover, the loss of function plants for three *Arabidopsis SENSOR HISTIDINE KINASES* genes *AHK2 AHK3 AHK4/CRE1* (Riefler et al. 2006) phenocopy the mutants silenced in the *HISTIDINE PHOSPHOTRANSFER* genes (*AHP1,2,3,4,5*), involved in the cytokinin signaling (Hutchison et al. 2006), showing consequent reduction in fertility associated with the production of larger embryos and seeds. Furthermore, the disruption of *Cytokinin-Independent 1* (*CKI1*), that encodes cytokinin-related kinase also causes gametophytic lethality (Deng et al. 2010; Pischke et al. 2002).

Interestingly, megagametophyte defects could be observed in the *Arabidopsis* mutants *arr7* and *arr15* (*Arabidopsis Response Regulator7* and *15*; Leibfried et al. 2005), although they have not been deeply characterized. The A-type *ARR7* and *ARR15* act in a negative feedback loop of the cytokinin signaling pathway. Moreover, in ovules lacking a functional embryo sac, such as *coatlique* and *sporocyteless* mutants, *ARR7* and other *ARR-A* type genes (*ARR4, ARR5, ARR6*) are over-expressed (Johnson et al. 2007), suggesting a transcriptional gametophytic control of sporophytic cytokinin, which further supports the existence of reciprocal cross talk between these typically haploid and diploid tissues.

Finally, the observation that isopentenyl transferase 1 (*AtIPT1*), involved in cytokinin biosynthesis, is strongly expressed in the chalazal part of the ovule indicates that the source of cytokinins, perceived by the gametophyte, is located in the sporophyte (Fig. 1).

Taken together, the data reported above indicate that, as with auxin, the correct balance of the cytokinins is important for ovule organogenesis, although further analyses are needed to fully elucidate this aspect.

Conclusions

Despite all of the evidence accumulated in the last twenty years (briefly summarized here), more studies will be necessary to completely dissect the molecular basis of crosstalk between the female gametophyte and the surrounding sporophytic tissues. Current information, based mainly on characterizations of ovule mutants, clearly indicate the existence of a cross-talk between the two generations. Recent evidence points out that hormones might be critical controlling molecules involved in this communication, however they more comprehensive study of all the hormones is needed and several questions must still be addressed.

Comparative studies among different species could be a very powerful tool to understand how this communication evolved and how selection has acted upon it over time.

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CHAPTER 1

The role of PIN1 in female gametophyte development.

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Running title PIN1 promotes megagametogenesis

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 determined by the presence of the efflux carrier *PIN-FORMED (PIN),* plays an important role in developmental processes, including ovule development. It has been shown previously that PIN1 is the only efflux carrier expressed in the ovule during the early stage of development.

To study the role of *PIN-FORMED 1 (PIN1)* in *Arabidopsis thaliana* developing ovules we have perturbed *PIN1* expression using a specific RNAi under the control of the *STK* ovule specific promoter. The transgenic plants obtained have a female gametophytic defect that could be rescue by expressing *YUCCA4* under a specific female gametophytic promoter.

Our experiments suggest that *PIN1* expression and cellular localization in the ovule, is essential for correct auxin efflux into the 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 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 partially transported to the ovule from the placenta tissue (Benkova *et al.,* 2003) however it is partially synthesized in loco by WEI 8/TAA1 or YUCCA pathways (Woodward and Bartel, 2005; Pagnussat *et al.,* 2009). The responses to auxin are determined also by the capacity of cells to regulate auxin influx and efflux, thus generating a concentration gradient across cells. The formation of an auxin gradient involves many proteins among which the polar plasma-

membrane localized PIN (PIN-FORMED) proteins. According to accepted and well-documented models the directionality of intercellular auxin transport depends on the polar subcellular localization of transport components such as the PIN proteins (Wisniewska *et al.,* 2006). In *Arabidopsis thaliana* there are eight members of PIN family named PIN1-8 (Teale *et al.,* 2006). However, it has been shown that only *PIN1* is expressed from early stage of ovule development (Benkova *et al.,* 2003). *Pin* mutants are affected in the polar auxin transport (Okada *et al.,* 1991). In particular *pin1* mutant plants are progressively defective in organ initiation and phyllotaxy, which leads to a pin-shaped inflorescence devoid of flowers (Okada and Shimura, 1994; Galweiler et al. 1998). Furthermore, the polar auxin transport inhibitor treatments of wild type plants phenocopy loss-of-function *pin* mutations (Friml *et al.,* 2003). More evidences that PINs act as efflux auxin carrier are given by PINs expression in yeast and mammal cells (HeLa) which showed an increase of auxin efflux, regardless of the fact that these systems do not contain any PIN-related genes nor do they have auxin related signaling or transport machinery (Petrasek *et al.,* 2006).

Our work assigns a new developmental role to polar auxin transport (PAT) in the progression of female gametogenesis since *PIN1* down regulation using ovule specific promoter affect embryo sac formation. 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 is not known. Hereby we discuss the role of the sporophytic PIN1 in the progression of gametophytic generation.

Results

PIN1 localization and auxin distribution in *Arabidopsis* wild-type ovules

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 looked at early ovule development stages to visualize the membrane localization of the PIN1 protein. Besides PIN1, only *PIN3* is expressed in developing

ovules at later stages. PIN3 is expressed in the funiculus starting from ovule at stage FG3 (supplemental data S3).

Immunolocalization experiments (Figure1A) 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 (Figure 1B and 1C). PIN1 was also detected in the inner integument primordia (Figure 1B) and in the funiculus after embryo sac cellularization.

The membrane localization of PIN1 is known to anticipate the formation of auxin maxima (for review see Moller and Weijers 2009) and its position together with its typical basal lateral localisation strongly suggest that auxin fluxes are directed from the epidermal cell layer of the nucellus towards the new forming female gametophyte.

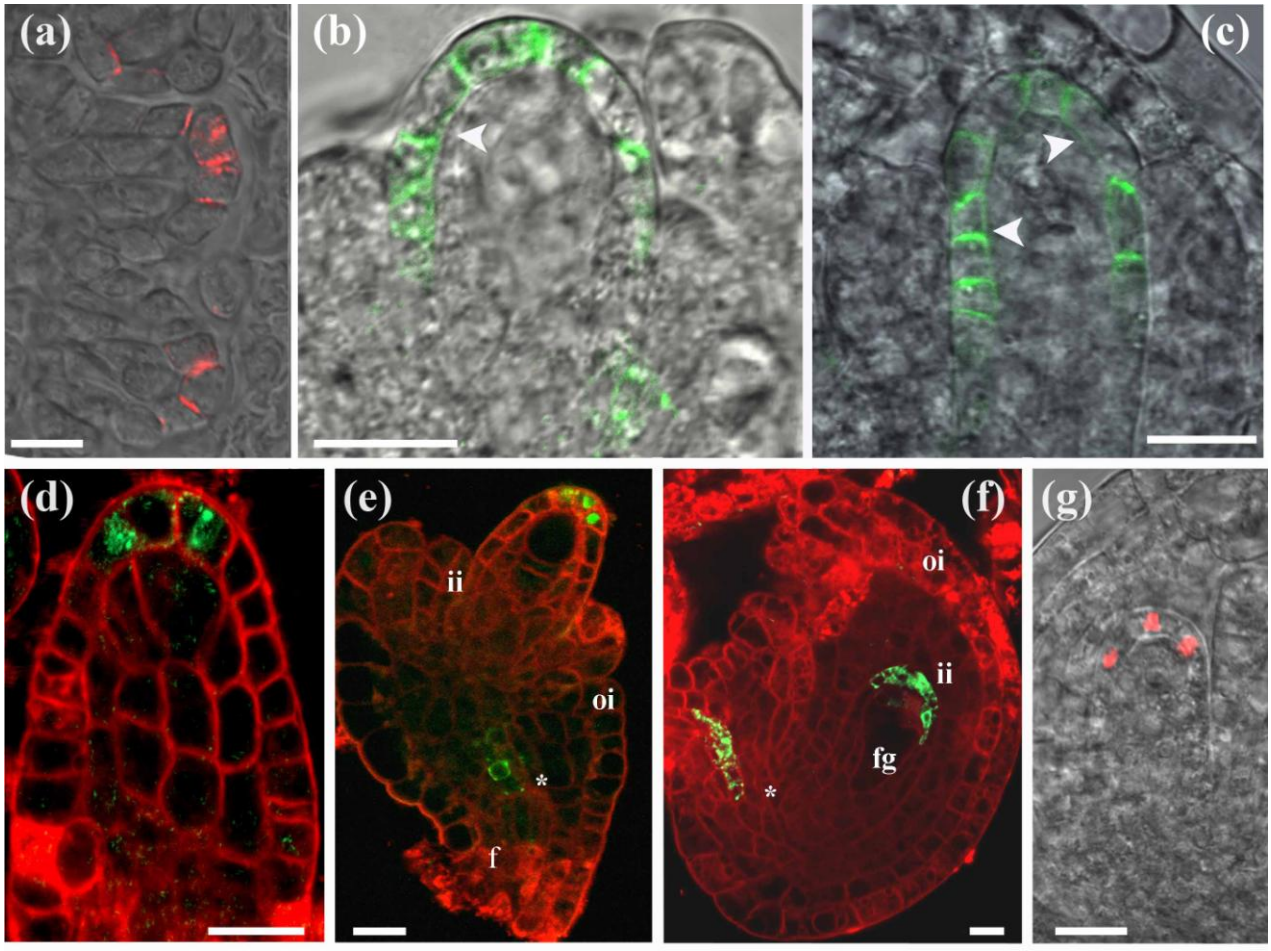

Figure 1 PIN1 expression pattern and auxin distribution in wild-type ovules.

(a) PIN1 immuno-localisation experiments with an anti-PIN1 antibody. PIN1 is detectable in the ovule primordium. **(b)** and (**c)** 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. Wild-type *DR5rev::GFP* (**d** and **f**) and *DR5rev::3XVENUSN7* (**g**). GFP signal is first detected in the epidermis at the distal end of the ovule primordium at FG0 (**d**), such signal persists from FG1 till FG3 (**f**) *DR5rev::GFP* signal is also detected in the forming ovule vasculature (asterisks in **e** and **f).** In **d, e,** and **f** cell membranes were stained with FM® 4-64 FX. fg, female gametophyte; ii, inner integument; oi, outer integument; f, funiculus. Scale bars: 20 µm

To study the distribution of auxin in developing ovules we have used plants carrying the auxin-responsive reporter *DR5rev::GFP* (Friml *et al.,* 2003; Benkova *et al.,* 2003) which provides a convenient tool to study auxin distribution in developing organs (Ulmasov *et al.,* 1997). In wild-type plants the GFP signal appears at the distal tip of the ovule primordium starting from FG0 (Figure1D), and from FG1 auxin is also detected in the funiculus pro-vascular cells (Figure1E). Such pattern is maintained until the nucellus progressively degenerates and is substituted by the endothelium (Figure1F). The localization of auxin maxima in the sporophytic nucellus of stage FG0 and FG1 ovules is further confirmed by analysing *DR5rev::Venus-N7* transgenic plants (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 accumulates at the micropylar pole of the nucellus till FG3 (Figure1G). Using this promoter we couldn't detect any GFP signal inside the developing embryo sacs, in disagreement with Pagnussat and co-workers (2009).

PIN1 plays an important function in the megagametogenesis

To understand the role the PIN1 auxin efflux transporter in the formation of the female gametophyte, we have analysed in detail five *pin1PIN1* heterozygous plants (Gabi-KAT line GK_051A10). In these plants, the seed set is normal and all ovules, half of which contain a *pin1* female gametophyte, were successfully fertilized by wild-type pollen in reciprocal crosses. In agreement with this observation we recovered in the offspring 50% *PIN1/pin1-1* and 50% wild-type plants (85 plants analysed) excluding any gametophytic effects for *PIN1* loss of function. This is not surprising because *PIN1* is not expressed in forming and formed female gametophyte (Pagnussat *et al.,* 2009).

To uncover the role of sporophytic expressed PIN1 we have introduced in *DR5rev::GFP* plants a *PIN1 RNAi* construct under the control of the *SEEDSTICK* (*STK*) ovule specific promoter (Kooiker *et al.,* 2005). *STK* promoter is active in all the stages of ovule development and in all tissues.

Among the 50 *DR5rev::GFP* T_1 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 FG1 stage of megagametogenesis (10 lines) or at the FG3 stage (5 lines) (Figures 2A and 2B) characterised by a high percentage of ovule abortions (Table S1). These data and the observation that *PIN1/pin1-1* 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 due to the reduction of *PIN1* levels, we performed real time-PCR analysis on carpels of the T_2 segregating plants using, as reference genes, either *UBIQUITIN10* or *18S RNA* (Figure 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 (Figure 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 accumulation was not observed in wild-type sister FG5 ovules (Figure 2D).

Starting from early stage of ovule development auxin is locally produced by at least one of YUCCA protein and TAA1. The *YUCCA* (*YUC*) family of flavin mono-oxygenases encodes key enzymes in Trp-dependent auxin biosynthesis (Zhao *et al.*, 2001; Cheng *et al.*, 2006). *TAA1* encode tryptophan aminotransferase, essential for indole-3-pyruvic acid (IPA) branch of the auxin Trp-dependent biosynthetic pathway.

The expression of *TAA1* and *YUCCA4* during ovule development has been analysed using transgenic line expressing reporter genes GUS/GFP under *YUCCA4* or *TAA1* promoters. *YUCCA4* promoter starts to be active at FG2 (Figure 2E, and 2F) whereas *TAA1* promoter is active during early stages of ovule development in the chalaza region and in particular in inner integument primordium (Figure 2G - I). At FG1, GFP driven by *TAA1* is expressed in the inner layer of the inner integument. These observations suggest a ovule local auxin source that start to be active before FG1.

The phenotypes described for *pSTK::PIN1i* transgenic plants was obtained also by an independent experiments where *PIN1* silencing was achieved using an artificial micro RNA (Schwab *et al.*, 2006), under the control of the ovule specific promoter *pDEFH9* (*DEFICIENS like 9, DEFH9*) (Rotino *et al.*, 1997). *DEFH9* is an *Antirrhinum majus* ovule specific gene, and its promoter is active in *Arabidopsis* ovules during all stages as shown in supplemental figure S2.

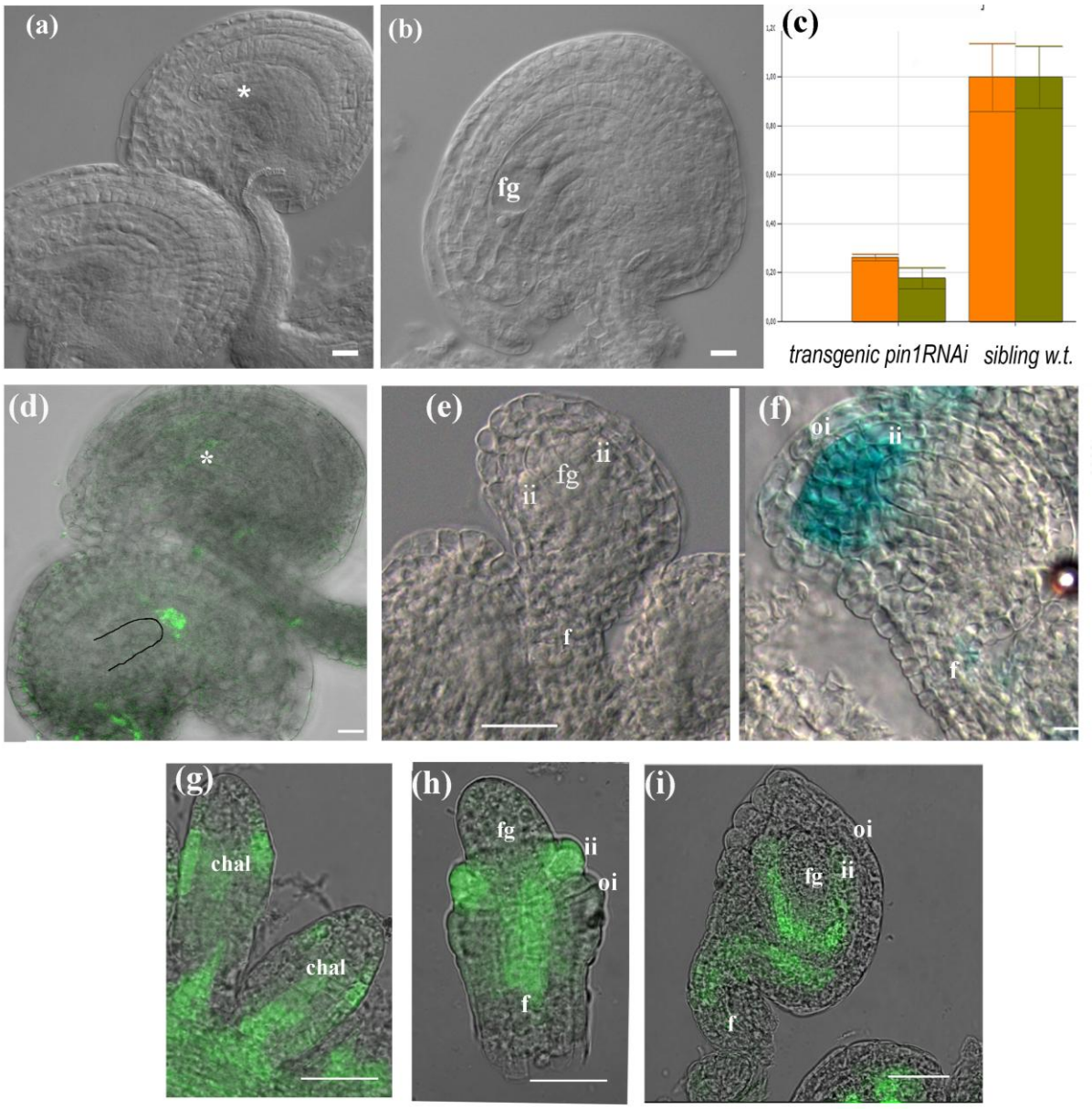

Figure 2. PIN1 down regulation affects ovule development

(a) and (**b)** female gametophyte defects caused by *PIN1* silencing. Female gametophytes a rrest 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)***YUC4* promoter is not active before FG1. (**f)** Since FG1 is active at the tip of the inner integument since. (**g-i**) *WEI8* promoter is active since the arising of the primordia in the chalaza (**g**), and since FG0 in the inner integument and inside of the funiculus (**h** and **i**). fg, female gametophyte; ii, inner integument; oi, outer integument; f, funiculus. Scale bars: 20 µm.

Ectopic expression of *YUCCA4* in the forming embryo sac rescues the early gametophytic defect.

Our data clearly suggest that *PIN1* expression and localization is necessary for female gametophyte development probably due to the establishment of an auxin gradient from the diploid tissues of the

nucellus to the developing gametophyte. However to demonstrate that our hypothesis is correct we are planning to express *YUCCA4* in early stage of female gametophyte under *AGL23* promoter. *AGL23* is a MADS box gene that is expressed starting from FG1 and play an important function during early stage of female gametogenesis (Colombo *et al.,* 2008). In alternative we would like to express *YUCCA4* under STK promoter that is active also during early stages of female gametophyte development. If our hypothesis is correct, the expression of *YUCCA4* in the developing gametophyte could complement the auxin efflux defect due to the down regulation of PIN1 in the transgenic plants. This experiment is in progress.

DISCUSSION

PIN1 promotes early stages of megagametogenesis

In developing ovules, PIN1 membrane localization anticipates the auxin maxima, that is established at the distal part of the ovule primordia. Moreover PIN1 polar localization is in line with auxin accumulation sites. Soon after their formation ovules become able to produce auxin. *In situ* hybridization experiment showed the expression of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1/WEI8*), which encodes an enzyme critical for the synthesis of auxin via the indole-3-pyruvic acid (IPA) (Stepanova *et al.,* 2008; Tao *et al.,* 2008). *TAA1* is expressed within the ovule anlagen and later on in developing ovule integuments (Nole-Wilson *et al.,* 2010). Furthermore we have analyzed transgenic plant containing the *WEI8::GFP* construct showing that *WEI8* promoter is highly active during ovule development. Our data are consistent with the *in situ* hybridization data previously described.

De novo auxin production is highly localized and local auxin biosynthesis is strategic to shape local auxin gradients, essential for proper plant development. The importance of dynamic gradients is strengthened by the tight temporal and spatial regulation of auxin biosynthesis, for instance the expression of *YUC* genes and *TAA1/WEI8* genes is restricted to a small group of discrete cells (Cheng *et al.,* 2006; Stepanova *et al.,* 2008). In later stages of ovule development it has been reported that also female gametophytes can produce auxin due to the expression of *YUC1* and *YUC2* (Pagnussat *et al.,* 2009). According with the model, we propose (Figura 3) the local sporophytic auxin synthesis plays an important function in female gametophytic progression. According to our model the efflux of auxin synthesized in the sporophyte is control by PIN1 efflux carrier which is essential for the progression of female gametogenesis.

In *PIN1pin1* plant the mutated allele segregates in a mendelian fashion, thus excluding PIN1 gametophytic function consistent with the fact that PIN1 is expressed only in the sporophytic tissue of the developing ovule (Pagnussat *et al.,* 2009; this manuscript). Based on the genetic analysis of

PIN1pin1 mutant we have silenced PIN1 under an ovule specific promoter STK that is active in the integuments, in the funiculus and during gametogenesis.

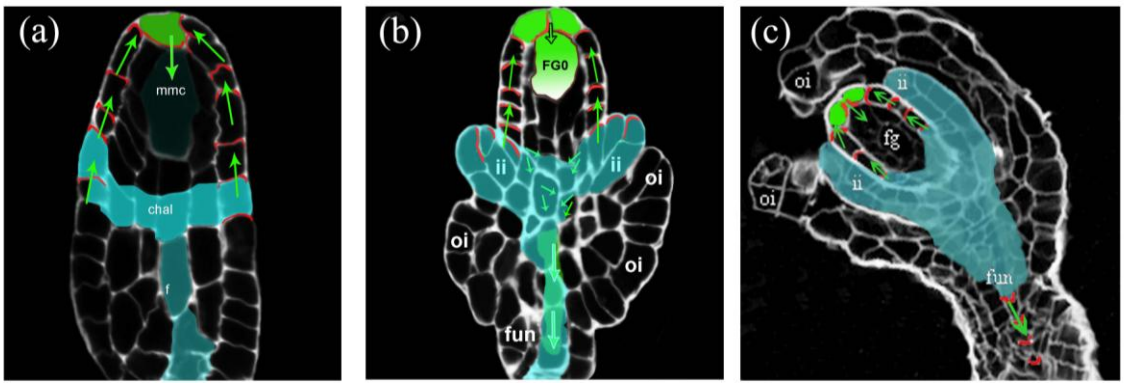

Figure 3 Auxin biosynthesis and transport in ovule

Schematic picture of the ovule at different stages of development (**a**) primordia, (**b**)FG0, (**c**) FG1. Auxin maxima (in green) are detected at the distal tip of developing ovules since the primordium formation (**a**). Arrows indicated possible auxin fluxes, which point towards the forming embryo sac, as strongly suggested by PIN1 (in red) polar localization. At early stages auxin is produce *in loco* by *TAA1* a which expression domains are indicated in blue. mmc, megaspore mother cell; fg, female gametophyte; ii, inner integument; oi, outer integument; f, funiculus.

In ovule at st1-II, PIN1 basal and lateral localization in the nucellar cells (figure 1 F-I) suggests a direct contact with the megaspore mother cell that harbours. This peculiar expression patterns is maintained till FG3, thus we can argue that auxin is directed towards the megaspore mother cell and the new emerging female gametophyte. Moreover our *PIN1* silencing experiments have shown that this auxin flux its extremely important to promote gametogenesis. In agreement with Pagnussat and co-workers we could not visualised any PINs expression and this observations indicate the absence of auxin efflux from the nucellus. The phenotypic analysis of our transgenic lines show that *PIN1* silencing blocks embryo sac development at FG1 or FG3 stage. However just one transgenic line shows defects at later stages. In this transgenic plant the two central cell polar nuclei are unable to fuse, therefore the central cell cannot be fertilised. This defective phenotype supports the idea that *PIN1* is not the major player after FG2. This is also in agreement with the presence of YUC1 and YUC2 inside the later stages forming embryo sacs.

In conclusion, we propose that auxin has an essential role in ovule organogenesis, and that it influences all stages of ovule development. In particular we have proved that the sporophyte and the polar auxin transport are central players in the formation and regulation of auxin gradients.. Our model is supported by genetic and molecular evidences, and it is also very well supported by its auxin maxima position and PIN1 basal-lateral polar localization in the nucellar cells surrounding the archeospore and the forming embryo sacs.

Experimental procedures

Plant materials (*Arabidopsis* lines and NPA treatments)

DR5rev::GFP, *PIN1::PIN1:GFP*, *PIN3::GUS*, *WEI8:GFP* seeds were supplied by J. Friml (University of Gent), *pDR5rev::3XVENUS-N7* seeds by M. Heisler (California Institute of Technology), and *YUCCA4::GUS* from Y. Zhao (University of California at San Diego).

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 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). *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, lower transcript abundances were confirmed by two independent biological experiments and four technical repetitions. Moreover *PIN1* was amplified with two different couple of primers.

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), immunolocalisation and in situ hybridisations

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). T_1 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.

List of the primer used.

Name	**Sequence**	**gene name**
RT 147	CTGTTCACGGAACCCAATTC	RT ubiquitin
RT 148	GGAAAAAGGTCTGACCGACA	RT ubiquitin
AtP0331	TGACGGAGAATTAGGGTTCG	RT 18S

AtP0332	CCTCCAATGGATCCTCGTTA	RT 18S
At590	CTCAGAATTCGTTGGGTATGTTCTCACTTTC	pSTK
At591	GTCACTCGAGTCCCATCCTTCATTTTAAACAT	pSTK
At1104	GATAACGTGGTAGAAGTCCCGCGTCTCTCTTTTGTATTCC	I amiPIN1
At1105	GACGCGGGACTTCTACCACGTTATCAAAGAGAATCAATGA	II amiPIN1
At1106	GACGAGGGACTTCTAGCACGTTTTCACAGGTCGTGATATG	III amiPIN1
At1107	GAAAACGTGCTAGAAGTCCCTCGTCTACATATATATTCCT	IV amiPIN1
At1507	TCTGACGTCAGGCGTTTTTGTTGGGTATGTTCTCAC	pSTK
At1508	TCTGACGTCAGGCATCCTTCATTTTAAACATC	pSTK
At1663	CGAGCTCGCGGCCATGCTAGAGTCCGC	T35S
At1664	CGAGCTCGAGGTCACTGGATTTTGGTTTTAGG	T35S
At2009	TCCGTCTTGTCTTTTCCCACC	PIN1 RNAi
At2208	CACCAAGGTCTCAAGGCTTATCTGCG	PIN1 RNAi
At2398	CGACACTCCCCAACACTCTAG	RT PIN1
At2399	AGCTTAGCTCCACGGTACTC	RT PIN1
At1661	ATATTGACCATCATACTCATTGc	To genotype pin1 (TDNA left border)
At2057	TGTTCAGTCTTGGTCAGTTCC	To genotype pin1
At2058	CTCTTTGGCAAACACAAACGG	To genotype pin1

Acknowledgments

We thank M. Kater and R. Gatta for their help and J. Friml, M. Heisler and Y. Zhao for providing material. Luca Ceccato was supported by a short term EMBO fellowship (ASTF 388.00-2008) and by the Socrates program (contract n°)

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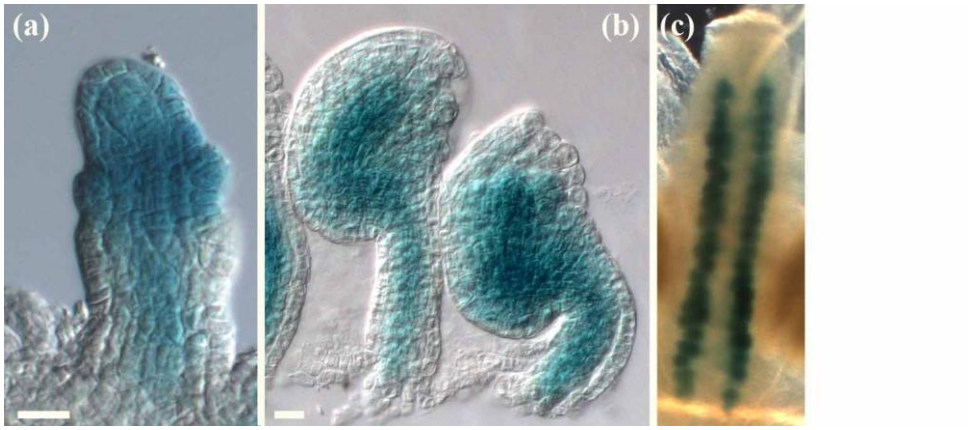
Supplemental material

Table S1

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).

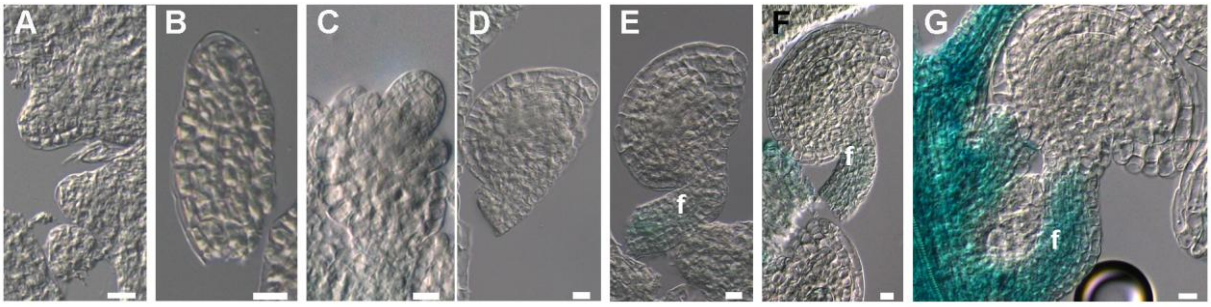
Figure S2



DefH9::GUS ovules

A-B, *DefH9* Gus activity in ovule FG0 (a) , FG1 (b) and in carpel is shown.

Figure S3



PIN3 expression in *PIN3::GUS* ovules

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

CHAPTER 2

The role of local auxin biosynthesis during ovule development in *Arabidopsis thaliana*

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(article in preparation)

Abstract

Auxin is one of the key regulators of ovule development. The auxin is either transported from the carpel to the ovules, or it is locally synthesized by the Trp-dependent auxin biosynthesis pathways.

The local auxin biosynthesis is known to be fundamental to establish and maintain auxin gradients, during organs development however, so far it is unknown which is the function of these biosynthesis pathways during ovule development. In particular, we have studied the role of the auxin pathway mediated by YUCCA proteins, by the analyse of specific *yucca* single, double, triple and quadruple mutants.

Introduction

The identification of key regulators of plant reproductive process has clearly a great importance. In this context the study of the ovules as seeds precursor, have an economic impact, besides the interest to understand the molecular control of - such important and complex structure. The ovule development is characterized by the arrangement of discrete elements along a proximal-distal axis: funiculus, chalaza and nucellus.

Recently it has been proposed that a gradient of auxin is crucial for the megagametophyte cell identity determination (Pagnussat *et al.*, 2009). In this model asymmetric auxin distribution along the nucellus is due to, and maintained by, an active auxin local biosynthesis. In particular it has been reported that *YUCCA1* and *YUCCA2* are expressed during gametogenesis in the developing embryo sac (Pagnussat *et al.*, 2009).

The *YUCCA* genes encode enzymes belong to the flavin monooxygenase and are involved in the triptammine dependent auxin pathway also named TAM. The TAM is one if the five auxin biosynthesis pathways described in *Arabidopsis thaliana* (figure 2.1;Chandler, 2009).

The *YUCCA* genes encode enzymes belonging to the flavin monooxygenase and are involved in the triptammine dependent auxin pathway also named TAM. The TAM is one if the five auxin biosynthesis pathways described in *Arabidopsis thaliana* (figure 2.1) (Chandler, 2009).

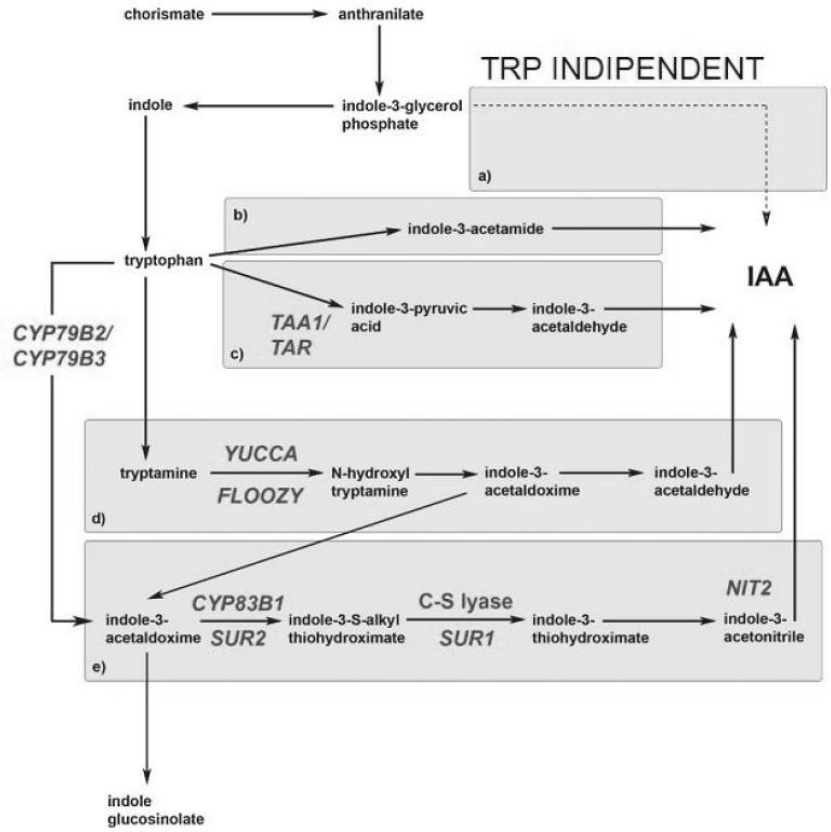

Figure 2.1 Different auxin biosynthesis pathways.

The tryptophan (Trp)-independent pathway and the four main branches of Trp-dependent synthesis via, b: indole-3-acetamide, c: IPA, d: tryptamine or e: indole-3-acetaldoxime. The positions of enzymes encoded by genes that result in a phenotype when mutated are shown (Chandler *et al.*, 2009).

In particular *YUC1*, *YUC2*, *YUC4* and *YUC6* were previously characterized and their involvement in plant development was well documented (Zhao *et al.*, 2001; Cheng *et al.*, 2006). For example, it has been shown that YUCCA1 (YUC1) plays a key role in auxin biosynthesis and the *yuc1-D* dominant gain-of-function mutant showed auxin overproduction as expected (Zhao *et al.*, 2001). The analysis of the *yucca1*, *yucca2*, *yucca4* and *yucca6* loss of function mutants revealed that these four genes play a specific function during development (Cheng *et al.*, 2006). Interestingly while they encode enzymes involved in the same step of the auxin biosynthetic pathway (Figure2.1) only the disruption of certain combinations of four *YUC* genes in *Arabidopsis* causes dramatic developmental defects. The *yucca1*, *yucca2*, *yucca4* and *yucca6* single mutants didn't show phenotype. Among the double mutants, only the *yuc1yuc4* and the *yuc2yuc6* showed a clear phenotype (Cheng *et al.*, 2006). In particular *yuc1yuc4* mutant has a smaller size respect to wild type plant, however the *yucca1yucca4* inflorescence development was reported to be dramatically disrupted. Some flowers have sepal-like organs, while other flowers totally lacked such sepal-like tissue. It was observed stamen-like structures in some of the flowers, and in others flowers two fused carpels with stigma tissue on the top. However in the pistil ovules do not develop.

In the *yuc2yuc6* mutant, the flowers appeared with a normal shape but without pollen. The triple mutant *yuc1yuc2yuc6* and *yuc2yuc4yuc6*, showed a phenotype that resemble the double mutant *yuc2yuc6*, whereas the *yuc1yuc4yuc6* and *yuc1yuc2yuc4 yucca2*, showed a phenotype that resemble the double *yuc1yuc4*. The quadruple mutant *yuc2yuc6yucca4 yucca6* showed strong phenotype affecting all plant development. The plants of the quadruple mutant do not flower (Cheng *et al.*, 2006). Interestingly the developmental defects of these mutants could be rescued by the expression of the auxin biosynthetic gene *iaaM* under *YUCCA6* promoter but not by exogenous application of auxin (Cheng *et al.*, 2006). All these findings suggest the functional redundancy of the *YUCCA* genes but also that the genes, to play their role, should be express in a very specific way and that auxin presence in a precise space-time manner is even more important than its absolute amount (Cheng *et al.*, 2006).

In this manuscript, we have focused on the role of the *YUCCA* genes during ovule development. We have analysed *YUCCA1*, *YUCCA2*, *YUCCA4* and *YUCCA6* expression in the ovules by *in situ* hybridization and by the use of marker genes under specific *YUCCA* promoter. Furthermore, we analysed in details the ovule phenotype in the *yucca* single and double mutants. To study the effects during ovule development of *yucca* multiple mutants, we have create *yucca2 yucca6* transgenic plants in which *YUCCA1* and *YUCCA4* were down-regulated using the STK ovule specific promoter.

The *YUCCAs* are not the only gens involved in auxin biosynthesis expressed in the ovule: *TAA1* and *TAR2* are genes encoding enzymes of the Trp-dependent pathway and they resulted expressed in different stages of ovule development. Interestingly the phenotype of *taa1 tar2* double mutant is very similar to the *yuc1 yuc4*: the flowers have severe organs defect and the pistil develop without ovules (Stepanova *et al.*, 2008). As shown in Chapter 2 we have shown that *WEI8* is expressed during ovule development and probably play an important role in the synthesis of auxin requested for ovule pattering.

RESULTS

To study the role of local auxin biosynthesis during ovule development we have analyzed in details *yucca1, yucca2, yucca4, yucca6* single mutants and *yucca1yucca4* and *yucca2yucca6* double mutants.

At first we have analysed *YUC1 YUC2, YUC4* and *YUC6* expression during ovule development. *YUCCA1::GUS, YUCCA2::GUS,YUCCA4::GUS* and *YUCCA6::GUS* transgenic lines provided by Cheng (Cheng et al 2006) were analysed. We have analysed 10 transgenic plants containing *YUCCA1::GUS* construct and 10 transgenic plants containing the *YUCCA2::GUS* construct.

Unfortunately we were not able to detect GUS expression in none of the analysed plants. We have analysed also 10 transgenic plants containing the *YUCCA4*::GUS contract. The *YUC4* regulatory region is not active during early stages of ovule development whereas GUS expression is detected in the micropylar region of the inner integument, starting from stage FG2 (Figure 2.2 F-G-H). The analysis of 10 transgenic plants containing *YUC6::*GUS construct has shown that *YUCCA6* promoter is active in stamen and style as also described by Cheng and colleagues in 2006 (Cheng al., 2006) but not during ovule development.

The results reported by Pagnussat *et al.*, 2009 are in contrast with our data. In their manuscript they have showed *YUCCA1* and *YUCCA2* expression in the embryo sac starting from FG2. To study in more details the expression of these genes we have performed *in situ* hybridization using *YUCCA6* specific RNA antisense probe. As shown in Figure2.2 (I to K) during early stages of ovule development, *YUC6* resulted expressed in the integument primordia, in the nucellus and in the chalaza, (Figure2.2I, J) whereas in later stages *YUCCA6* seems to be exclusively expressed in the chalaza part of the nucellus (Figure2.2K). *In situ* hybridization experiment using *YUC1* and *YUC2* specific antisense probe will be performed.

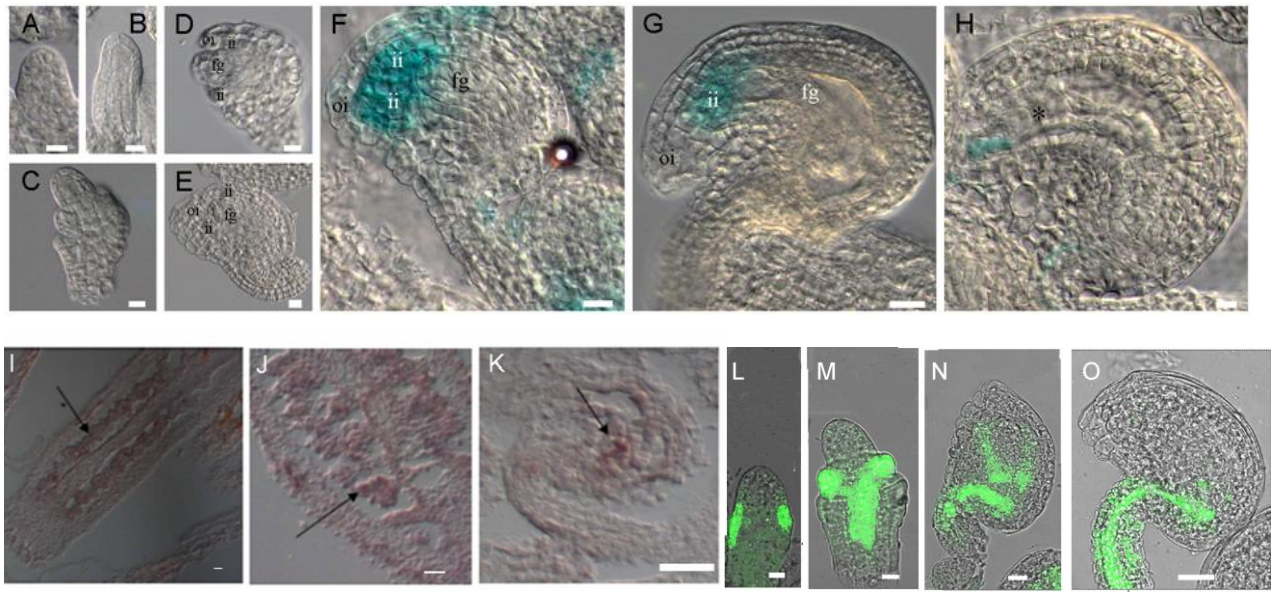

FIG2.2 Expression patterns of auxin biosynthetic genes

A-E: *YUC4* is not expressed before the first stages of megagametogenesis FG1. **F**, **G**: GUS staining is detected at the tip of inner integument ovules. **H**: seed with GUS staining in proximity of the micropylar end of female gametophyte (black asterisk marks the zygote). **I-K**: in situ result with *YUC6* specific probe; **L-O**: WEI8:GFP signal during ovule development. ii: inner integument; oi: outer integument; fg: female gametophyte. Scale bar: 10µm.

Morphologic analysis of *yuc1yuc4* and *yuc2yuc6* double mutants

yuc1yuc4 double mutant showed a strong phenotype in all the inflorescence (Cheng *et al.*, 2006 Figure 2.3A-D). The flowers (90% of them) had a thin carpel lacking of carpel margin meristem

(CMM) tissue including ovules (Figure 2.3E), while a 10% of the flowers showed a pistil composed by four carpels not fused on the tip (Figure 2.3C-D). This abnormal pistil develop CMM tissue and ovules that however, could not be fertilized probably due to the severe pistil aberration that compromised pollen tube growth and perception. We have analysed 100 flowers from which 90 of them have a pistil without CMM and 10 flowers have pistil containing ovules. However, the *yucca1yucca4* double mutant was complete sterile.

The *yuc2yuc6* mutants have flower similar to wild type plants with the exception of the stamens, that resulted shorter respect to wild type stamen (Figure 2.3F). We have pollinated wild type plants with *yuc2yuc6* pollen and we didn't obtain any seeds showing that *yucca2yucca6* pollen is complete sterile. *yuc2yuc6* double mutant plants pollinate with wild type pollen were fertile however, we have obtained only few seed showing that *yucca2 yucca6* double mutants are partially female sterile. To understand which was the female defect in *yucca2 yucca6* double mutant we have performed morphological analyze. We have observed *yucca2 yucca6* (n=200) ovules 30% of which presented morphological defects that could explain the incomplete fertility.

In 10 of the *yucca2 yucca6* ovules on the 200 analyzed, the cells of the gametophyte (Figure2.1G-H) seem to be not proper organized (10 out of 200). To understand more in detail the cause of the partial sterility we have first examined the genotype in a segregating population obtained by selfing *YUC2/yuc2YUC6/yuc6* plants. The double-mutant plants were 25 out of the 413 analysed suggesting the presence of the double-mutant genotype in either male or female gametophytes does not distort the segregation ratio. This finding suggests that the defect in the *yuc2yuc6* gametophyte is due to a sporophytic defect.

To test if the cells in the female gametophyte, maintained the right identity in response to decrease of local auxin biosynthesis we have crossed the *yucca2yucca6* double mutant with gametophytic cell specific *Arabidopsis* transgenic line containing gametophytic cell specific promoter driven GUS reporter gene. The analysis of these plants is in progress.

The sterile phenotype described above does not have complete penetrance so we have decided to silence during ovule development all four *YUCCA* genes by transgenic approach. *yucca2yucca6* double mutant were transformed with construct in which *YUC1* and *YUC4* were silencing by RNAi approach using the ovule specific promoter STK ($_{pro}$*STK* Kooiker *et al.*, 2005). The analysis of the transgenic plants is still in progress.

TAA1, TAR2 gene expression during ovule development

TAA1 and *TAR2* encoding tryptophan aminotransferase are essential for indole-3-pyruvic acid (IPA) in the auxin biosynthetic pathway (Figure 2.2). Analysis of *taa1* and *tar2* mutants have suggested that they play an important function to determine plant fertility (Stepanova *et al.*, 2008).

To study *TAA1*, *TAR2* expression pattern we have analysed transgenic plants containing $_{pro}$*TAA1*:GFP and *TAR2*:GUS constructs.

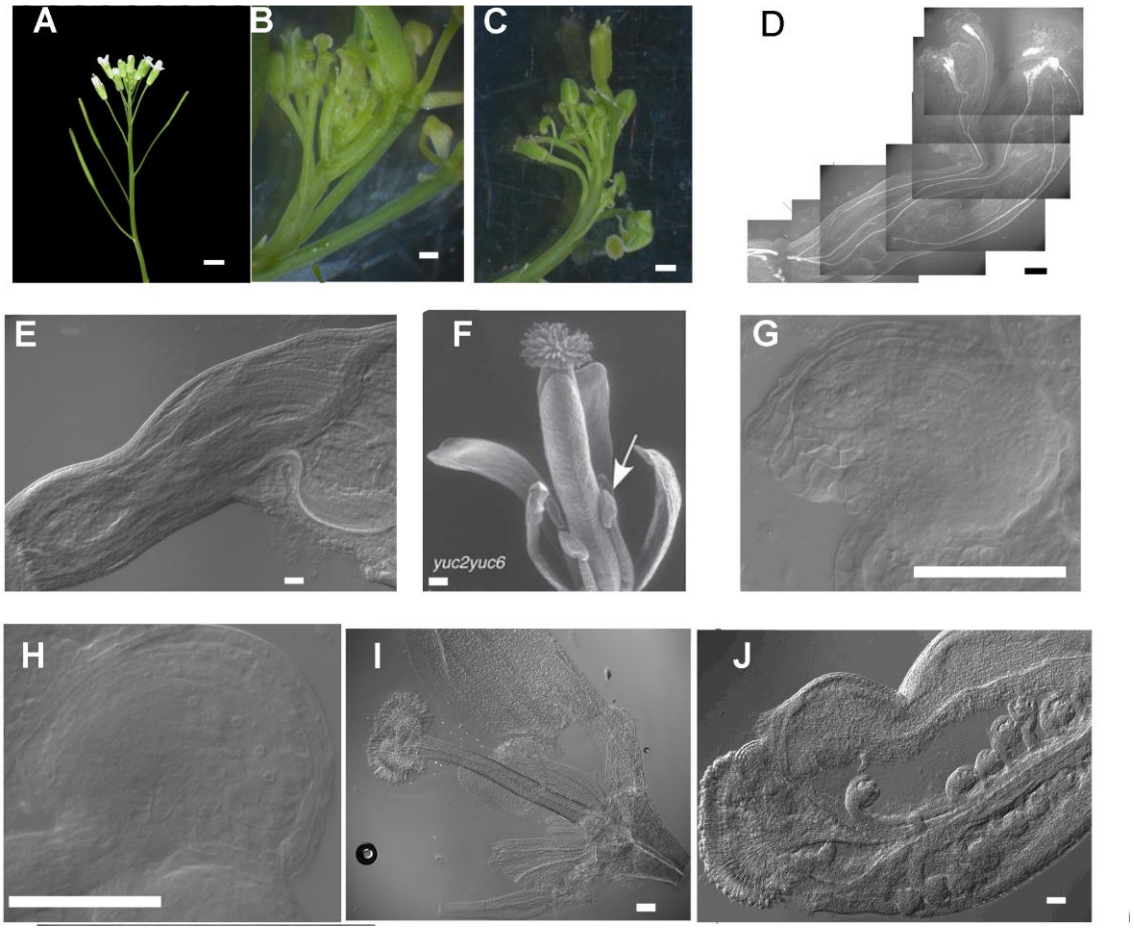

FIG2.3 Mutant for auxin biosynthetic genes
A: wild-type inflorescence; B-C: *yuc1yuc4* inflorescence; D: one of the carpels that brings ovules is showed; E one of the carpel without ovules is showed; F: *yuc2yuc6* flower (Cheng et al,. 2006); G-H: *yuc2yuc6* defective ovules; I-J *wei8tar2* carpels. Scale bar: 40µm.

As shown in Figure 2.2 l-O $_{pro}$*TAA1* is active soon after the ovule primordia formation (Figure 2.2 L) and in later stages it is active in the inner integument (Figure 2.2 M-N). Starting from FG3, GFP expression driven by *TAA* promoter is restrict to the chalaza part of the nucellus (Figure2.2 O).

$_{pro}$*TAR2* does not show activity during ovule development (data not shown). It has been reported that the single mutants *wei8-1(taa1) and tar2-2*, do not have a phenotype (Stepanova *et al.*, 2008) however the *wei8-1 tar2-2* double mutants showed developmental defect including sterility. The

inflorescences of the *wei8-1 tar2-2*, double mutants showed a phenotype very similar to the *yuc1yuc4* mutant (Figure 2.3I-J). Most of the flowers had a pistil without CMM and ovules (Figure 2.2I) while one or two flowers per had more than two carpels containing ovules (Figure 2.2J). If pollinated with wild type pollen the flowers with carpel that brings ovules are able to produce seeds. Morphological analysis confirmed that the few ovules developing *wei8-1 tar2-2* double mutants were normal.

Discussion

The *YUCCA* flavin monooxygenases and the tryptophan aminotransferase are important enzymes responsible for auxin synthesis in *Arabidopsis* and in this work we have studied their role during the ovule development to identify which step of the development depends on *in loco* auxin biosynthesis.

We have considered recent works (Cheng *et al.,* 2006; Stepanova *et al.,* 2008) and we have analysed mutant defective for the auxin biosynthetic enzymes that showed a sterile phenotype: *yuc1yuc4*, *yuc2yuc6*, *taa1tar2*.

Interestingly we have found that the phenotype of *yuc1yuc4* flowers was similar to the phenotype of *taa1tar2*. In the 90% of the flower of these mutants the carpel margin meristem (CMM) fails to form, whereas 10% of the flowers have CMM containing ovules. This indicate that both the pathways of the Trp-dependent way are necessary for the inflorescence formation, but not for the ovule development. The analysis of the expression of these genes showed that at *YUC4* and *WEI8* are expressed in the ovule suggesting that they can be involved in ovule development in a redundant manner with other genes.

YUC2 and *YUC6* encode enzymes with the same function of *YUC1* and *YUC4* (Cheng *et al.,* 2006) but the double mutant *yuc2yuc6* showed a different phenotype: the flowers develop normally but the female gametophyte of the 30% of the ovules was defective. The difference among the genes can be related to their specific expression domain in a precise step that could be more important then the total amount of the auxin.

The identification of the key enzyme required for the megagametophyte formation will be very intriguing.

From the segregation analysis of the double mutant *YUC2/yuc2 YUC6/yuc6*, we have found that defects in the *YUC2* and *YUC6* genes influence the proper female gametophyte formation only in the double homozygotes. The yucca2 yucca6 gametophytic defects described in this chapter are controlled by the sporophyte because there are present only in the double homozygotes.

In contrast with what was recently proposed (Pagnussat *et al.*, 2009), this suggest that the auxin biosynthesis through the *YUCCA* enzymes takes place in the sporophyte (integuments) and it is the sporophyte that controlled the megagametophyte formation. The preliminary expression analysis of the *YUCCA6* confirm this hypothesis, more details analysis is necessary and will be done in near future.

Methods

***Arabidopsis* lines**

yuc1, yuc2, yuc4, yuc6, YUCCA1::GUS, YUCCA2::GUS, YUCCA4::GUS and *YUCCA6::GUS* seeds were kindly supplied by Y. Zhao (University of California, San Diego) (Cheng et al 2006). *wei8-1 tar2-1/+ DR5:GUS* lines were obtained from the NASC Collection is (N16413).

All seeds were harvested directly on soil, kept in a 4°C dark chamber for 2-4 days and then transferred into a permanent light growth chamber (21-22°).

Arabidopsis lines containing a female gametophyte cell specific promoter upstream the reporter gene GUS (Matias-Hernandez *et al.*, 2010) are provided by Rita Gross Hardt.

***In situ* hybridization analysis**

Arabidopsis flowers were fixed and embedded in paraffin. Sections of plant tissue were probed with digoxigenin-labeled *YUC6* antisense RNA corresponding to nucleotides 202 to 414. Hybridization and immunological detection were performed as described previously (Brambilla *et al.*, 2007).

GUS assays

GUS assays were performed on fresh green material. The carpels were dissected from the inflorescences and partially opened. The samples were incubated at 37°C in GUS solution, in darkness, for 1h30’ up to 24h. GUS solution was prepared as described in Vielle-Calzada et al., 2000.

Microscopy analysis

To perform the morphological analysis, inflorescences from wild type and mutant plants were collected, if necessary, the pistils were opened by a needle and covered with some drops of glycerol

20% solution of (samples for CLSM) or chloralhydrate in water (samples for GUS staining analysis, and samples from DpP1, DpPs and SpP1 transformed lines). Finally the cover slip was put on the samples and a slight pressure was applied.

Optical microscopy investigations were performed using a Zeiss Axiophot D1.

Plasmid construction and *Arabidopsis* transformation

To construct *pSTK:: YUC1-4 RNAi YUCCA1* genomic fragments was amplified with At2577 and At2578, 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). The YUCCA1 genomic fragment chose had 99% sequence similarity with a YUCCA 4 DNA fragment. After the cloning, the sequence of recombinant DNA was verified. After that the recombinant plasmid was used to transform *Agrobacterium tumefaciens* according to (Clough and Bent 1998). Finally, *Agrobacterium* containing the construct was used to transform wild-type (*Col-0)* plants using the 'floral-dip' method (Clough and Bent 1998).

Detailed information about pFGC5941 vectors is available http://www.chromdb.org/rnai/vector.

AtP_2577	GGGGACAAGTTTGTACAAAAAAGCAGGCTccatgacactactgaaatgg
AtP_2578	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCActaatattatggagtcg
Atp_590	CTCAGAATTCGTTGGGTATGTTCTCACTTTC
Atp_591	GTCACTCGAGTCCCATCCTTCATTTTAAACAT

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CHAPTER 3

Cytokinins play an important function during ovule development in *Arabidopsis*

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Summary

It has been reported that high level of auxin in the embryo sac is required for the determination of egg cell identity (Pagnussant *et al.,* 2009). Cytokinins are also involved in the control of female fertility however little is known about the cytokinins function during ovule development. In this work, we have studied the cytokinin biosynthesis and perception during ovule development, analysing transgenic plants containing constructs in which promoters of genes involved in cytokinin biosynthesis were used to drive reporter genes.

Furthermore, we have showed that the application of exogenous cytokinin could change embryo sac cells identity. Based on our data and the results already published about the role of auxin in embryo sac cell identity determination, we have proposed and discussed a model that correlates cell identity in the female gametophyte, with the balance between auxin and cytokinin.

Introduction

The *Arabidopsis* female gametophyte development consists in several events starting with the formation of the aploid one-nucleate cell name functional megaspore FM (stage named FG1) (Christensen *et al.,* 1997). The FM, after three mitosis, forms the embyo sac, containing eight nuclei organized in a precise manner (early FG5) (Christensen *et al.,* 1997). After cellularization (late FG5) the female gametophyte is composed of seven cells that have specific identity: two synergids, one egg cell, one central cell with two fused nuclei and three antipodal cells (FG6). The mature female gametophyte (FG7; Christensen *et al.,* 1997) is ready for the double fertilization.

How the cells acquire their identity has been a question of debate since long time but in recent years a series of findings have unlighted this aspect (review by Sprunck and Groß-Hardt). The cells position in the embryo sac determine cells identity. In the *eostre, lachesis, clotho* and *atropos* mutants the gametophytic cells positions are compromised and this correspond to a change of cell identity (Gross-Hardt *et al.,* 2007; Moll *et al.,* 2008; Pagnussat *et al.,* 2007). Indeed the characterization of these mutants have showed that the gametophytic cells can acquire an identity depending on their position (Gross-Hardt *et al.,* 2007; Moll *et al.,* 2008; Pagnussat *et al.,* 2007).

It has been reported that auxin is involved in the determination of the cell identity of the female gametophyte cell (Pagnussat *et al.,* 2009). In the model proposed auxin forms a gradient along the embryo sac with a maximum at the micropylar side and a minimum at the chalazal side. According to this model the female gametophyte cells acquire the identity dependently on their position that

corresponds to a specific auxin concentration. Gradient perturbation causes the identity shift among embryo sac cells. In particular an increase of auxin in the embryo sac through the local expression *YUCCA1* determines the shift of synergids and egg cell into central cell and antipodal cell type. On the other hand when several *AUXIN RESPONSIVE FACTORS* (*ARFs*) are down-regulated with a specific artificial microRNA, the synergids cell turn into egg cells whereas the identity of the central cell and antipodals cells seemed not to be perturbed. It has been suggested that auxin is a morphogene controlling cell identity determination in the embryo sac (Pagnussat *et al.*, 2009).

Cytokinin acts in concert with auxin and the different accumulation of the two hormones is known to be important for the organs fate (Skoog and Miller, 1957).

While metabolism and signal transduction of cytokinin has been elucidated during recent years (Sakakibara, 2006; Werner and Schmulling, 2009 Figure 3.1) little is known about its role in ovule.

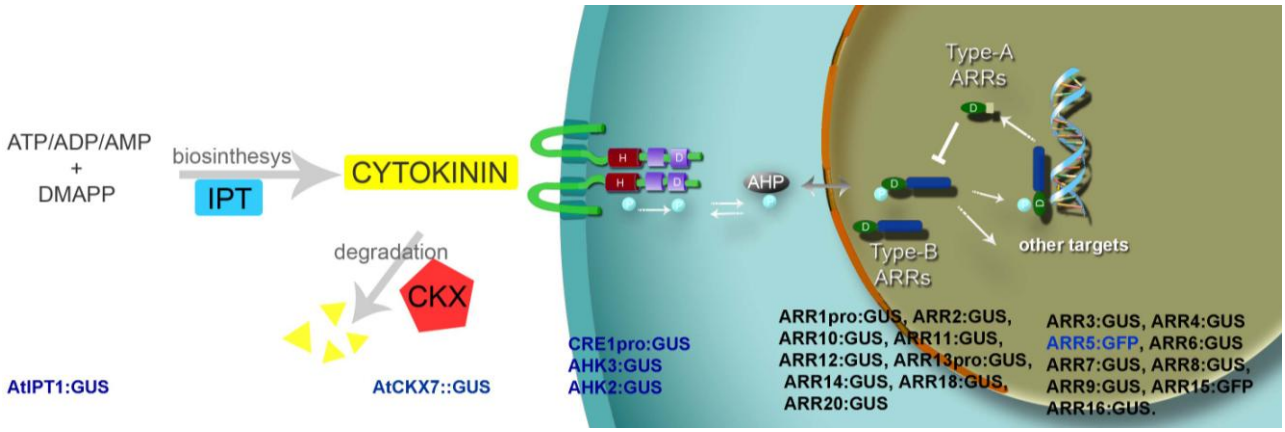

Figure 3.1 Schematic representation of the cytokinin pathway.
The lines analysed are indicated. In blue the lines with activity in ovule, in black the lines without activity in ovule.

It has been reported that there is a direct link between the amount of the cytokinins and plant fertility. Plants that with a reduction of cytokinin production or perception (Miyawaki *et al.*, 2006, Riefler *et al.*, 2006; Werner *et al.*, 2003; Hutchison *et al.*, 2006; Kaori Kinoshita-Tsujimura and Tatsuo Kakimoto 2011) have drastically reduced ovules number.. Furthermore the ovules of these plants show several defects. The *CYTOKININ INDEPENDENT1* (*CKI1*) has been implicated in cytokinin signaling and the *cki1* mutant showed gametophyte defect (Kakimoto, 1996). If the amount of cytokinins increases like in the *ckx3 ckx5* double mutant, the number of primordia increases (Bartrina *et al.*, 2011). Hereby, we propose to investigate the role of several components of cytokinin pathway in ovule development, using transgenic lines containing markers genes controlled by promoters of genes involved in the different steps of cytokinins biosynthesis.

To study the role of cytokinins in embryo sac cell fate determination, we have applied external cytokinins and we have monitored the identity of embryo sac cells, in the treated plants,using specific cell markers. A model has been proposed and discussed about cell identity determination in the female gametophyte through an auxin-cytokinin regulation.

RESULTS

The analysis of cytokinin pathway during ovule development

Cytokinins are a group of molecules composed by adenine derivatives carrying either an isoprene-derived or an aromatic side chain at the N^6 terminus (Sakakibara *et al.,* 2006). One of the most common natural cytokinins is N^6-(Δ^2-isopentenyl)-adenine(iP). The *IPT* (adenosine phosphate isopentenyltransferase) genes encode for enzymes that are the principal responsible of cytokinins synthesis and represent the rate-limiting step of the catalysis of the ATP/ADP (Kakimoto, 2001; Sun *et al.,* 2003, Figure 3.1).

To study the *IPT1* expression during ovule development we have analysed *Arabidopsis* transgenic plants containing the *AtIPT1:GUS* construct (Miyawaki *et al.,* 2004). The *GUS* gene was expressed in the carpel at stage 1-I (Schneitz *et al.,* 1995 data not shown) whereas it started to be detected in the ovule at the beginning of megasporogenesis (FG0, Figure 3.2A). During all the stages of ovule development *AtIPT1:GUS* was found to be active in the nucellus and in the developing female gametophyte (Figure 3.2A to Figure 3.2C). Interestingly in the later stages FG6-FG7, the *GUS* signal was detected in the chalazal region of the embryo sac (Figure 3.2D).

Once that the cytokinins are synthesized, they could be degraded by a pathway catalyzed by the cytokinin oxidase (CKX, Werner *et al.,* 2003). We have analyzed transgenic plants containing the construct *AtCKX7:GUS*. As shown in Figure 3.2E in these plants the GUS reporter gene is expressed in the synergid cells.

Another important component of the cytokinin signal pathway are the receptors *AHK2*, *AHK3* and *AHK4\CRE1*,that are necessary for cytokinin signal transduction (Figure 3.1). These proteins are known to interact with cytokinins to start the multi-step two-component signalling system (Inoue *et al.,* 2001).

To study the expression pattern of these three genes during ovule development we have analyzed transgenic plants containing the *$CRE1_{pro}$:GUS,AHK3:GUS or AHK2:GUS* constructs (Nishimura *et al.,* 2004). Interestingly we have noticed that all three constructs were active in developing ovules.

In particular GUS, driven by *AHK2* and *AHK3* regulatory regions, was expressed through the carpel and in the ovule starting from the ovule primordia to the later stages (Figure 3.2F). *CRE1*$_{pro}$*:GUS* on the other hand showed a specific signal in the chalaza region (Figure 3.2G) of developing primordia. In later stages the GUS expression was restricted to the region of the chalaza and to the inner integument (Figure 3.2H and 3.2I). In the mature ovule *CRE* promoter seems to be active only in chalaza region near the funiculus (Figure 3.2J).

Expression of Cytokinin-Response Genes During the Ovule Development

The cytokinin primary responsive genes (*ARRs*) play a key function in mediated cytokinin response (Figure1) (Perilli *et al.*, 2011). The genes with a clear role during plant development belong to two groups: the ARR B-type involved in a positive cytokinin response, and the ARR A-type that are involved in a negative cytokinin response (Figure 3.1).

To identify *ARR* gene expressed in the ovules we have analysed transgenic plants containing *ARR* promoter or *ARR* genomic region driven GUS reporter gene. In particular eight ARR-B -GUS marker lines were tested: *ARR1*$_{pro}$*:GUS, ARR2:GUS, ARR10:GUS, ARR11:GUS, ARR12:GUS, ARR13*$_{pro}$*:GUS, ARR14:GUS, ARR18:GUS, ARR20:GUS* (Mason *et al.*, 2004, figure 3.1). Expression of GUS gene was found in plant tissues coherent with data previously reported (Mason *et al.*, 2004), however, no GUS activity was detected during ovule development (data not shown).

We analysed also transgenic plants containing ARR-A regulatory regions upstream GUS or GFP genes: *ARR3:GUS*, *ARR4:GUS*, *ARR5:GFP*, *ARR6:GUS*, *ARR7:GUS*, *ARR8:GUS*, *ARR9:GUS*, *ARR15:GUS* and *ARR16:GUS*.

The *ARR5* was the only promoter that has resulted to be active during ovule development. In the developing primordia, GFP signal was found in the region between the chalaza and the nucellus (Figure 3.2K).. However, during the gametogenesis (Figure 3.2L) the GFP expression was detected in the developing embryo sac (FG1 Figure 3.2L, FG3 Figure 3.2M FG4, Figure 3.2N) and after the cellularization GFP expression driven by *ARR5* promoter was restricted in the central cell of the mature gametophyte (Figure 3.2O).

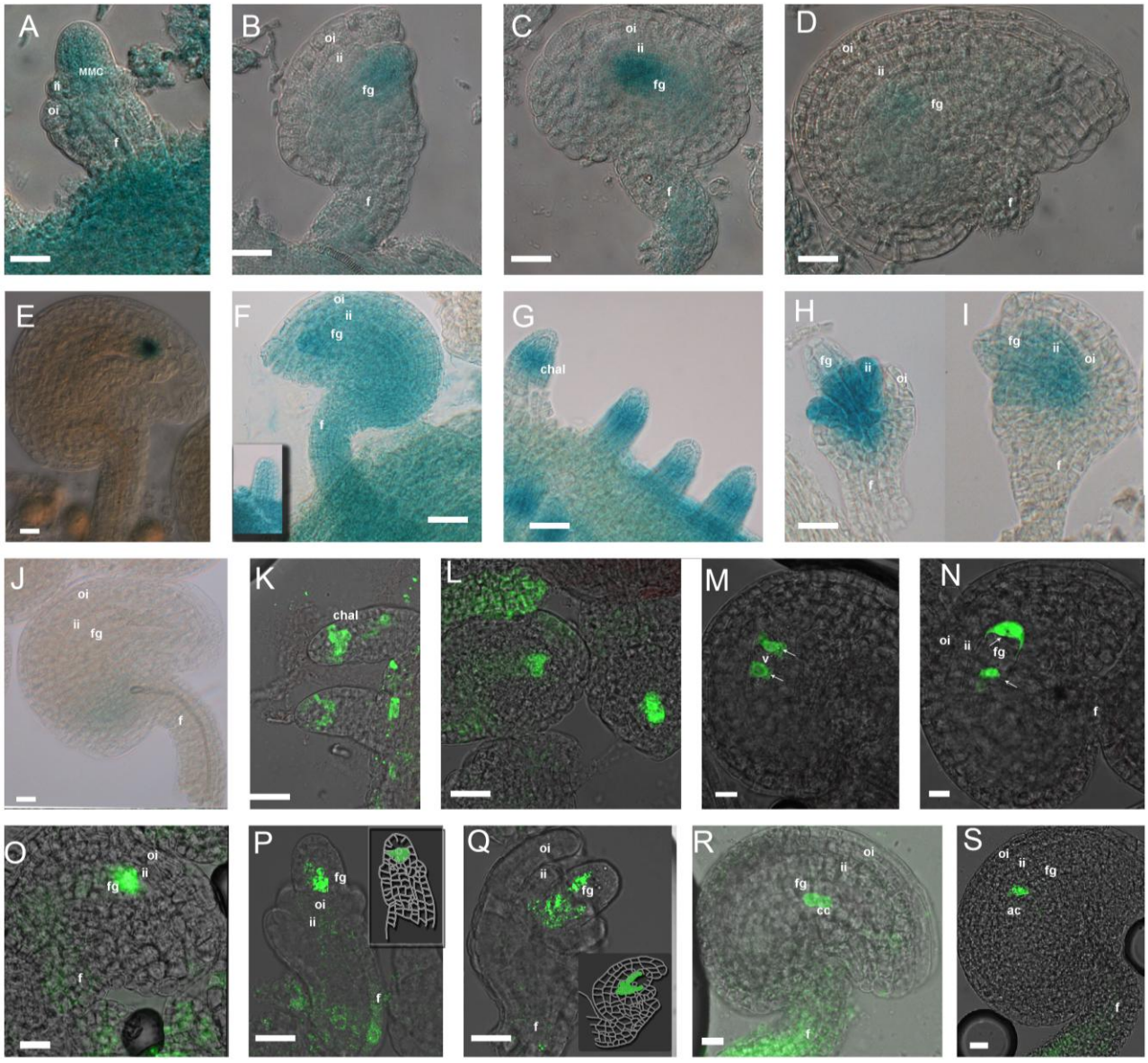

Figure 3.2 Expression Analysis of marker line for cytokinin pathway.

(**A**) to(**D**) GUS expression in*AtIPT1:GUS* plants. GUS staining of FG0 ovule(**A**),FG1(**B**), FG3(**C**) FG7 mature ovule, (**D**), are shown. (**E**) activity of the marker line *AtCKX7:GUS*. (**F**) activity of the marker line *AHK3:GUS* during the arising of the primordia (little picture) and at later stage in an mature ovule (**F**). (**G**) to (**J**) GUS expression in *CREpro:GUS* plants. GUS activity in ovule at early stage of the development, when the primordia arise(**G**), at FG0(**H**) FG1 (**I**) and at later stages FG7 (**J**) are shown. (**K**) to(**O**) GFP expression in *ARR5:GFP* plants. GFP signal in ovule primordia (**K**), in FG1 ovule (**L**) in FG3 ovule (**M**, arrows indicate the two nuclei) in FG4 (**N**, arrows indicate the two couple of nuclei) and in FG7 ovule are shown. (**P**) to (**S**) GFP signal in *TCS:GFP* plants. GFP signal in FG0 ovule (**P**) FG1 ovule (**Q**) FG5 ovule (**R**) and in FG7 ovule (**S**) are shown. chal, chalazal; f, funiculus; fg, female gametophyte; ii, inner integument; oi outer integument; n, nucellus; o, ovules; oi, outer integument. Scale bar: 10µm.

To analyse the presence and the localization of cytokinin in developing ovule we used *Arabidopsis* transgenic plants containing the GFP reporter gene driven by the *TCS* promoter. *TCS* is a synthetic reporter, containing more copies of the B-type *Arabidopsis* response regulator (ARR)-binding motifs and a minimal 35S promoter, and it can be used to detect the presence of cytokinins *in vivo* (Muller and Sheen, 2008). In ovules, at FG0 and FG1 the GFP signal driven by *TCS* promoter was

found in the funiculus and in the region between the chalaza the nucellus, surrounding the basal part of the nucellus (FG0) (Figure 3.2P and FG1 ovule Figure 3.2Q). In the stage FG2 we were not able to detect any signals. Later on a signal was present around the central part of the nucellus and inside it. In the stages FG5 a strong signal appeared in the region of the two polar nuclei that will form the central cell (Figure 3.2R). At FG7 the signal was found in the chalazal part of the gametophyte at the level of the antipodal cells (Figure 3.2S).

The Increasing of Cytokinins Amount in the Female Gametophyte Through BAP Application Modify the Cell Identity

To understand the role of the cytokinin in the female gametophyte development we increased the amount of the cytokinin through exogenous application of a natural cytokinin like N^6-benzylaminopurine (BAP). This type of exogenous cytokinin has been already successfully used for flower meristem studies (Venglat and Sawhney, 1996; D'Aloia *et al.*, 2011).

To confirm that the exogenous cytokinin applied could reach the ovule we have performed the experiments using plants containing *TCS:GFP* and *ARR5:GFP* as positive control. A strong GFP signal due to exogenous cytokinin treatment was detected in the ovule after one day (Figure 3.3A).

Morphological analyses showed that after 48 hours of treatment the cells of the female gametophyte were in the same position as the ones in not treated plants (figure 3.3B).

To test whether cytokinin has a role on female gametophyte cell identity we have repeated the BAP treatment on transgenic plants containing gametophyte cell specific type promoters driven GUS reporter genes.

The expression of the GUS reporter gene under the egg cell-specific promoter EC1 (Gross-Hardt *et al.*, 2007) was analysed after treatment. After 48 hours we have analyzed 512 treated ovules and only 98 of them presented a GUS activity in the embryo sac (near 20%) respect to the 90% (275 out of 312) of the control plants. 17 of the analysed ovules from BAP treated plants showed EC1 promoter active in the egg cell and in one of the synergids (Figure 3.3D to compare with the control 3.3C).

The activity of GUS under the control of the central cell-specific promoter FIS2 (Chaudhury *et al.*, 1997) was analyse after 48 hours of treatment. As in control plant we found signal in the 90% of ovules analysed (280 out of 316) but in 50% the signal was very close to the micropylar end in the region of the egg cell and of the synergids (figure 3.3E , 3.3F and 3.3G).

Analysis of the transgenic plants containing the GUS under a synergid specific promoter ET2634 (Gross-Hardt *et al.,* 2007) treated with BAP revealed that after 48 hours, 312 of the 362 ovules, did not express the GUS reporter gene whereas in the control plants, 260 ovules analysed on 286 showed the GUS expression in the synergids (data not shown).

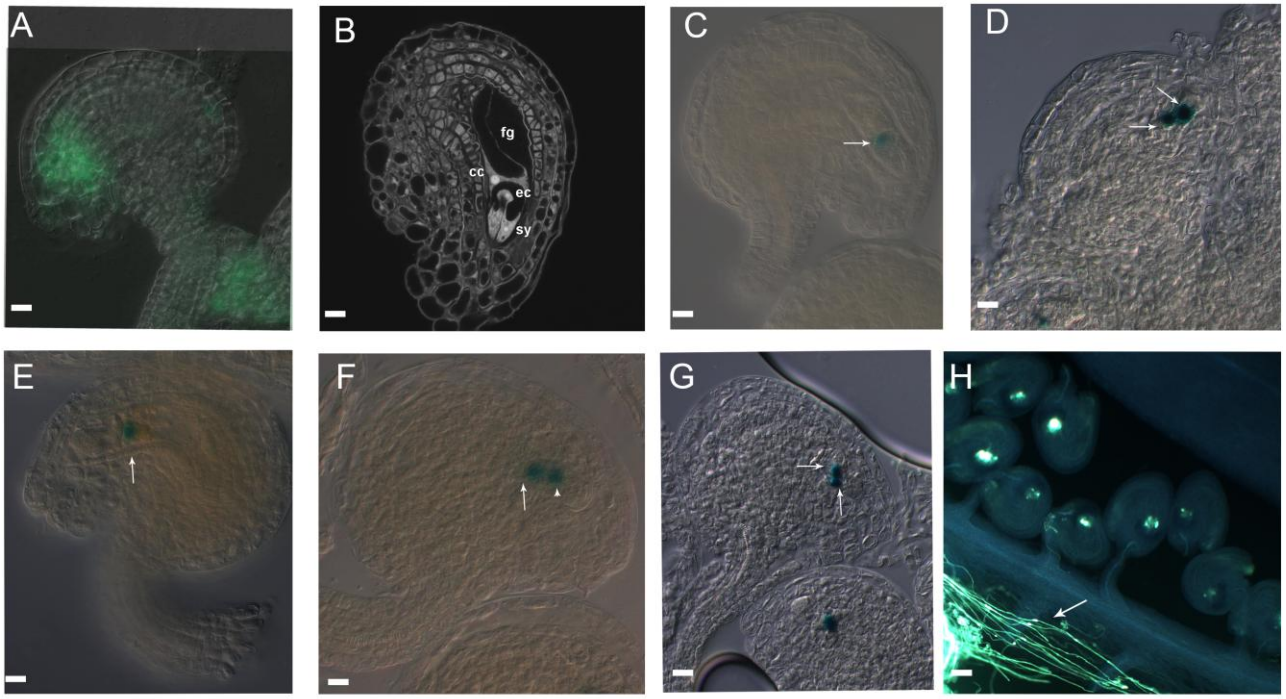

Figure 3.3 Expression Analysis of marker line after BAP treatment.
(**A**) GFP expression in *TCS:GFP* plants after 1 day from the BAP application. (**B**) CLSM image of a BAP treated ovule. (**C**) and (**D**) GUS expression in marker lines for egg cell. (**C**)control plant and (**D**) ovule after 48h of BAP application. (**E**) and **(F**) GUS expression in marker lines for central cell. (**E**)control plant and (**F**) ovule after 48h of BAP application. (**G**) Pollen tube staining with aniline blue shows that pollen tube grow inside the carpel but don't reach the ovule (arrow indicates the pollen tube). cc, central cell; fg, female gametophyte; eg, egg cell; sy synergids. Scale bar: 10µm.

Pollen Tube Guidance Is Affected in BAP Treated Ovules

One of the more important function of the synergid cells is to drive the pollen tube toward the micropyle. To test whether the BAP application is affecting also synergids function and in particular pollen tube guidance, we have fertilized the ovules with wild type pollen after 48 hours of the BAP treatment and we have analysed the pollen tube growth by aniline blue staining. In control plants, pollen tubes grew inside the carpel and penetrate inside the ovules through the micropyle region. When we have pollinated plant treated with exogenous cytokinin with wild-type pollen, the pollen tube grew inside the carpel but only 10% reached the micropyle successfully while the others seemed not to be attracted by the ovule (Figure3.3H) suggesting that also one of the major synergid functions has been affected by exogenous cytokinins treatment.

DISCUSSION

In vitro culture experiments the ratio between auxin and cytokinins determines the tissue fate: when the amount of cytokinin overcomes the amount of auxin the callus differentiates into shoot; whereas high auxin concentration determines root tissue formation. When auxin and cytokinin are in a similar concentration the callus remain in an undifferentiated state (Skoog and Miller, 1957).

Starting from these experiments a lot of work has been done to understand how these two hormones control organogenesis and different model were proposed (Barton, 2010; Galinha *et al.*, 2009). It has been reported that cytokinins in the shoot meristem are important for the meristem maintenance whereas cytokinins promote cell differentiation in roots.

Multistep Cytokinin Signaling Pathway During Ovule Development

Cytokinin performs its function through the multistep signalling and a lot of work shows that its influence on plant development is dependent by the expression of cytokinin receptors and response regulators (Ferreira and Kieber, 2005). Furthermore, the cytokinin effects and amount is dependent on the balance between its biosynthesis and degradation. To study the role of cytokinins during the ovule development we have analysed several components of cytokinins pathway by the analysis of specific marker lines. The results of such analysis have been summarized in Figure 3.4.

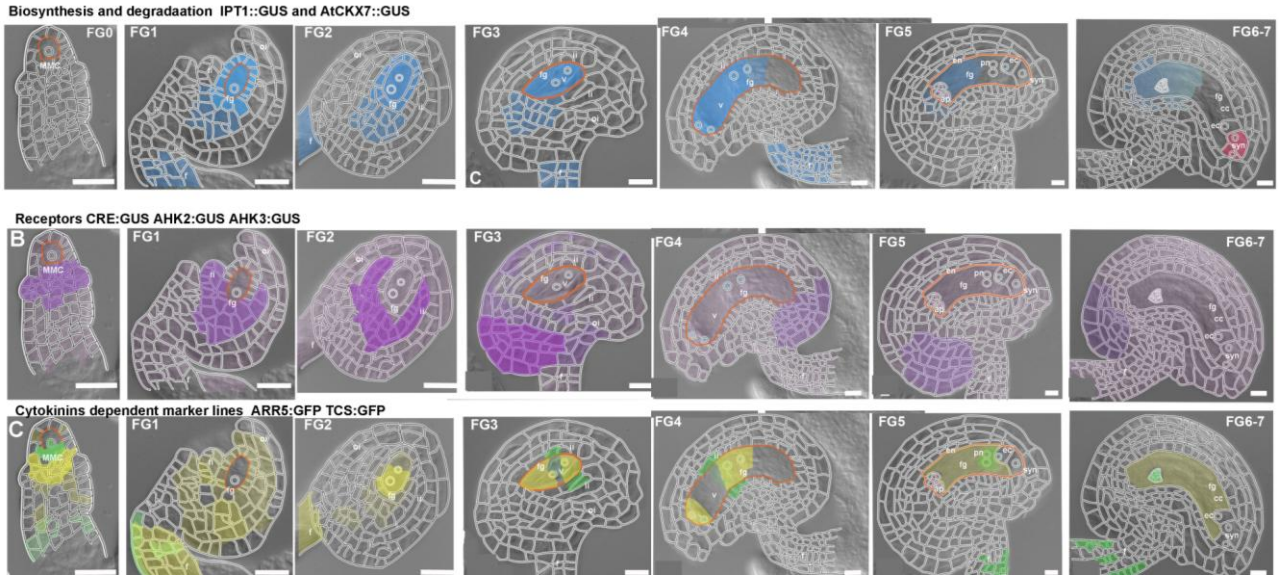

Figure 3.4 Schematic representation of the expression of the marker lines analysed
(**A**) *IPT1:GUS* blue and *AtCKX7:GUS* in pink. (**B**) *CREpro:GUS AHK2:GUS* and *AHK3:GUS*. In Dark violet the expression profile of *CREpro:GUS* and in pale violet the expression profile of *AHK2:GUS* and *AHK3:GUS*. (**C**) *ARR5:GFP* (in yellow) and *TCS:GFP* (in green).

Cytokinin seemed to be synthesized in the funiculus and in the nucellus from the first stage of ovule development. In the latest stages cytokinin synthesis seems to be mainly localized in the chalazal region. In that region we have showed the presences of cytokinin using marker lines sensible to cytokinin presence such as *TCS:GFP* and *ARR5:GFP*. In the mature embryo sac we have shown that cytokinin seems to be specifically present at the level of the central and antipodal cells. Interestingly *CYTOKININ INDEPENDENT1* (*CKI1*), is known to be implicated in cytokinin signalling and necessary for the gametophyte development (Kakimoto, 1996) is also expressed in the central region of the embryo sac. All the regulative regions of the cytokinin receptors resulted to be active in the ovule. However, the activity of the cytokinin oxidase promoter in the synergids, suggests that while the biosynthesis occurs in the chalazal part of the gametophyte, cytokinin degradation could occurs on the other side.

Exogenous cytokinin application induces conversion of micropylar (distal) cell identities to chalazal (proximal) cell identities

In the ovule auxin accumulates at the micropylar region of the nucellus, whereas the cytokinin is synthesized in the chalazal region (figure 3.5A). It has been proposed that an auxin gradient in the embryo sac determine the gametophyte cells identity (Pagnussat *et al.*, 2009).

To study the role of cytokinin in the determination of embryo sac cell identity we applied exogenous cytokinin BAP to increase the amount of the cytokinin in all the embryo sac and to form an artificial cytokinin gradient with a maximum in the micropylar region of the embryo sac. Although this increase of the amount of micropylar cytokinin have shifted the identity of the synergids into egg cell or central cell identity, none identity shift have been observed for the other cell type. This could be explained considering the hypothesize that the embryo sac cell determination could be determine by the auxin/cytokinin ratio as proposed in the model in Figure 3.5B. According to the proposed model, high concentration of auxin and low of cytokinin determines the synergids identity, equals amount of auxin and cytokinin determine the egg cell identity and low amount of auxin and high of cytokinin determines central cell and antipodal cell fate. The increase of the cytokinin amount by BAP treatment, change the ratio between the two hormones as shown in figure 3.5B and consequentially the embryo sac cell identity.

The shift of the identity of the synergids cell might be responsible of the lacking pollen tube attraction observed in the BAP treated plants.

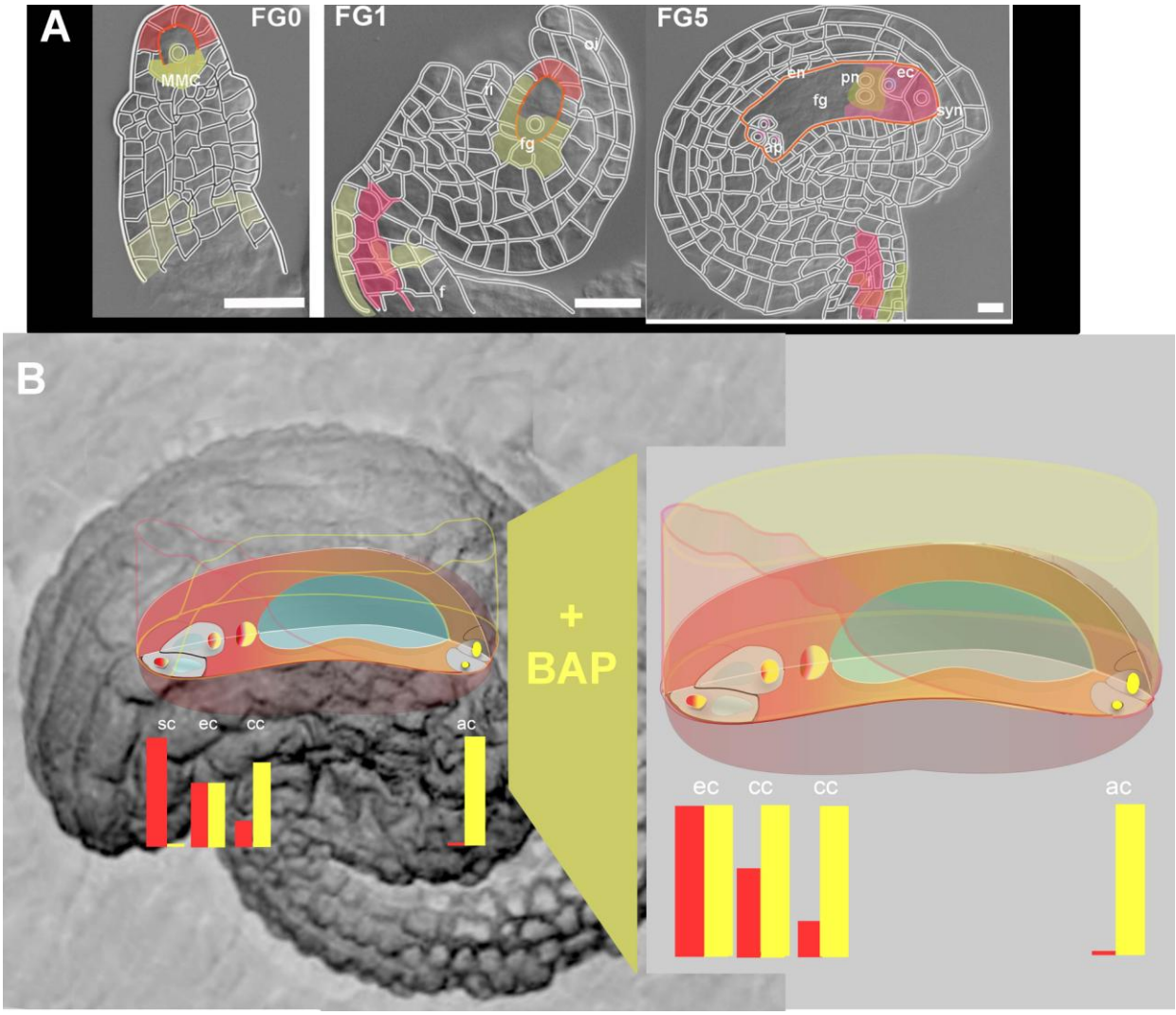

Figure 3.5. **Model for the hormonal control of ovule development.**

(**A**) auxin and cytokinin presence during the three representative stages of the ovule development. (**B**) auxin and cytokinin gradient in the female gametophyte model. On the right is shown how the increase of cytokinin change the gametophyte cell identity. Auxin is indicated in red and Cytokinin in yellow

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (ecotype Columbia) plants were grown at 22°C under long-day (16 hlight/8 h dark) conditions. The *Arabidopsis* lines were obtained from the NASC Collection are: TCS (two-component-output-sensor):GFP (N23900), $_{p}$ARF7:GUS (N24633), $_{p}$ARF19:GUS (N24634) $_{p}$ARF12:GUS (N24635), $_{p}$ARF22:GUS (N24636), PARF2:GUS (N24868), ARR3:GUS(N25259), ARR4:GUS (N25260), ARR5:GUS (N25261), ARR6:GUS(N25262), ARR8:GUS(N25263), ARR9:GUS (N25264), ARR1p::GUS (N25391), ARR2::GUS(N25392), ARR10::GUS(N25393), ARR11::GUS(N25394), ARR12::GUS (N25395), $_{p}$ARR13::GUS (N25396), ARR14::GUS

(N25397), ARR18:GUS (N25398), ARR20::GUS (N25399). *ARR5::GFP* line was kindly provided by Keith Lindsey (Durham University) (Casson et al., 2002). *Arabidopsis* line containing a egg cell specific promoter upstream the reporter gene GUS (Gross-Hardt *et al.,* 2007), the central cell specific promoter upstream the reporter gene GUS (Chaudhury *et al.,* 1997), and antipodal cell specific promoter upstream the reporter gene GUS (Yu *et al.,* 2005 kindly provided by R. Gross-Hardt. The synergid cell marker line (ET2634) was generated in the lab of U. Grossniklaus. All the gametophytic marker lines analyzed encode for a nuclear localization signal that is in frame with the GUS reporter gene.

BAP treatment

N^6-benzylaminopurine (BAP) obtained from Sigma Chemical Co. It was used 10-1 M solution of BAP concentrations Plants were treated once with30µl of a solution of either BAP solution or distilled water (both in 0.05% Tween 20). Solutions were applied either directly onto the inflorescence, and then the plants were covered with a plastic transparent bag for one day.

Microscopy

To analyze ovule development, flowers were emasculated and pollinated using wild-type pollen 24 h after emasculation. Pistils were fixed 12 h after pollination (hap) and observed by CLSM following the Braselton *et al.,* (1996) protocol. Samples were observed using a 532-nm laser. The light emission was selected between 570 and 740 nm.

For the aniline blue staining experiments, treated plants were emasculated and pollinated 24 h after the emasculation with wild type pollen. Pollen tubes growth have been analysed 24 h after pollination. Aniline blue staining was performed as described by Huck et al. (2003).

ACKNOWLEDGMENTS

We thank Tatsuo Kakimoto for providing *IPT1:GUS* line. Lohoman for providing *ARR15:GUS* line and Rita Gross-Hardt for providing the female gametophyte-specific marker lines and Agnieszka Bielach for technical help.

This research was supported by Fondazione Cariplo.

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CHAPTER 4

Cytokinins control *PIN1* specific localization through the homeodomain protein BEL1 during ovule development

Summary

Auxin and cytokinin have been demonstrated to interact to promote and controlled organ initiation (Ruzicka *et al.,* 2009). Here, we proposed that in the ovule, cytokinin controls the auxin flux by regulating the localization of the efflux facilitator PIN1. The correct localization of PIN1 during early stage of ovule primordia formation is responsible to determine auxin accumulation in the nucellus. Our data also suggest that the BEL1 homeodomain protein play an important function in PIN1 localization in these stages of ovule primordia.

Introduction

In *Arabidopsis* auxin is involved in organ formation and it was proved that dynamic gradients of this signaling molecule is necessary for primordium initiation (Benková *et al.,* 2003). The asymmetrical localization of cellular efflux proteins, the PIN proteins, allows the formation of auxin maxima concentration, responsible for the initiation and positioning of organs, such as leaves (Reinhardt *et al.,* 2000; Reinhardt *et al.,* 2003), flowers (Okada *et al.,* 1991; Oka *et al.,* 1998), lateral roots (Laskowski *et al.,* 1995) and ovules (reviewed in Bencivenga *et al.,* 2011, Chapter1 of this thesis). Cytokinins are involved in meristem activity and has been showed that control different process depending on the type of plant tissue (Ferreira and Kieber, 2005; Dello Ioio *et al.,* 2007). Recently it was found that in roots cytokinin negatively control the emerging of secondary root formation regulating PIN expression and consequently changing the auxin pattern along the root (Ruzicka *et al.,* 2009). In this chapter, I have described our studies on the cytokinin function in developing ovules. We have found that the increasing of cytokinin level during ovule development can change the fate of ovule structure and can also induce formation of extra ovules primordia along the placenta. We have proposed that this phenotype induced by higher level of cytokinin, is a consequence of the changing of the localization of PIN1 protein respect to the wild type. We also suggest that this control is mediated by the *BEL1*, an homeodomain transcription factors (Reiser *et al.,* 1995). *BEL1* is expressed in ovule starting from early stage of development (Robinson-Beers *et al.,* 1992; Reiser *et al.,* 1995). In *bel1* mutant a single integument-like structure is formed from the

chalaza region. This integument-like structure enlarges and expressed carpel specific genes (Robinson-Beers *et al.,* 1992; Reiser *et al.,* 1995, Brambilla *et al.,* 2007).

bel1 ovules phenotype resemble the ovules treated with exogenous cytokinin described in Chapter 3. Furthermore, we have shown that also the molecular basis of the ovule phenotype is similar to the one described for the cytokinin induced ovule structure. ChIP experiment analysis will be performed to verify that *PIN1* is a directly target of *BEL1*.

Results and Discussion

Cytokinins are important in the maintenance of the shoot and root meristem, in root elongation and in secondary root formation (Ruzicka *et al.,* 2009). While recent studies have delineated the expression of cytokinin pathway component during ovule development and its role in the megagametophyte (Bencivenga *et al.,* 2011, Chapter 3 of this thesis) so far the role of cytokinin during the ovule development is completely unknown. To understand the influence of cytokinin on ovule development we have manipulated the amount of cytokinin present in this organ.

CK controls ovule formation

To increase the cytokinin amount in ovule, we have applied exogenous cytokinin like N6-benzylaminopurine (BAP). This treatment was already successfully used for flower meristem studies (Venglat and Sawhney, 1996; D'Aloia *et al.*, 2011).

To verify that the treatment have successfully reached the ovules, we have treated 10 transgenic plants containing the construct responding to cytokinins: *TCS:GFP* (Muller and Sheen, 2008). As shown in figure 4.1B after one day from the treatment the GFP signal was detected in all the ovule.

After 4 days from the treatment, one single structure (named CK-IS) grew in replacement of the two integuments. Furthermore, the nucellus development was blocked prior meiosis (Figure4.1D). After four-five days, at the level of the CK-IS structures new ovule primordia seem to be formed (Figure 4.1I). After 10 days from the BAP treatment was possible to recognize a carpel like structure in place of ovule as already reported (Venglat and Sawhney 1996, data not shown). To verify the identity of the CK-IS structures we have performed a detail molecular analysis of treated ovules.

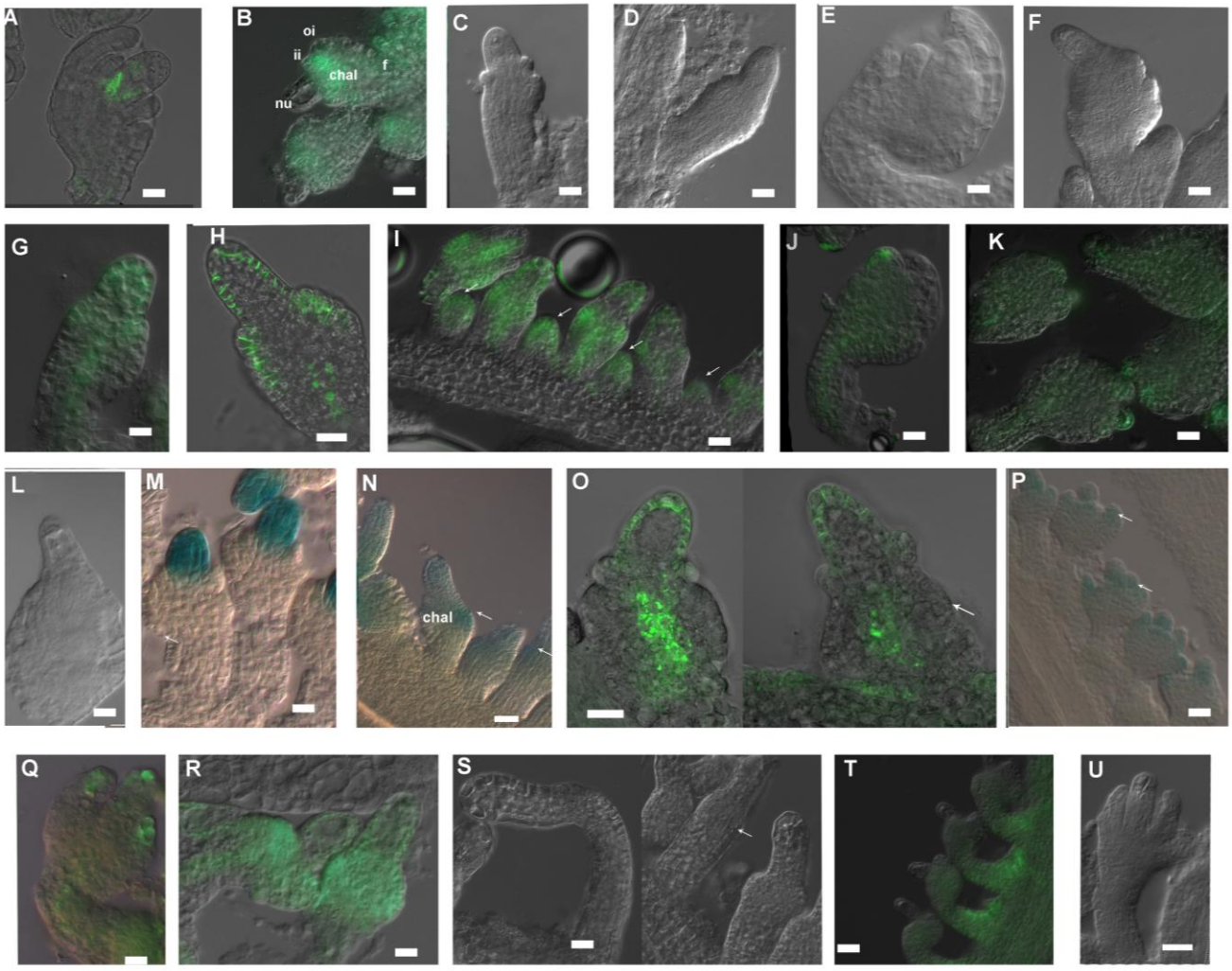

Figure 4.1
TCS:GFP in ovule of control plant (**A**) and *TCS:GFP* signal in BAP treated ovule (**B**) are shown; not treated ovule (**C** and **E**) and ovules after four days from the BAP treatment are shown(**D** and **F**); *PIN1:PIN1:GFP* in control plant (**G**) and *PIN1:PIN1:GFP* after BAP treatment (**H** and **I**) are shown (the arrows in **I** indicate the new primordia formation); *DR5:GFP* signal in not treated ovule (**J**) and in BAP treated ovule (**K**) are shown; ovule treated with NPA (**L**) is shown; *WUS:GUS* activity in not treated ovules (**M**) and in BAP treated ovules (**N**) are shown; *STK:WUS* ovules in *PIN1:PIN1:GFP* are shown (**O**, arrow indicate the new structure induced by *WUS* ectopic expression; ovules of bel1 WUS:GUS are shown (**P** arrows indicate the GUS activity in the *bel1-1* structure); ovules of bel1 *DR5:GFP* are shown (**Q**); ovules of *bel1-1 PIN1:PIN1:GFP* are shown (**R**); ovules of *bel1* treated with NPA are shown (**S**), arrow indicates the block of the formation of the *bel*-structure); ovules of *bel1 TCS:GFP* are shown (**T**); ovule of *STK:BEL1* is shown (**U**); chal, chalaza; f, funiculus; fg, female gametophyte; ii, inner integument; oi outer integument; n, nucellus; o, ovules; oi, outer integument. Scale bar: 10µm.

CK treatment modified PIN pattern localization in ovule.

It has been shown that cytokinin negatively influences secondary root formation (Laplaze *et al.*, 2007) repressing *PIN1* expression (Ruzicka *et al.*, 2009). To test whether the cytokinin have similar effect on *PIN1* expression in the ovule, we have treated with BAP plants containing the construct *PIN1:PIN1:GFP* (Benkovà *et al.*, 2003 and Chapter1 of this thesis).

The control plants showed the GFP signal as previously described (figure 4.1G; see also Benkovà *et al.*, 2003; Chaper1 of this thesis) whereas in the *PIN1::PIN1-GFP* plants, treated with BAP, we

have detected GFP expression not only in the nucellus and in the inner integument but also in the outer integument (Figure 4.1H). The GFP ectopic expression driven by *PIN1* promoter was detected after three days from the treatment, in the epidermal layer of that the abnormal structure developed from the chalaza of BAP ovule (Figure 4.1H). Considering the timing of the signal detection, we could hypothesized that first the *PIN1* is ectopically expressed and later the CK-IS is formed. In the BAP treated ovules, the phenotype is the consequence and not the cause of the *PIN1::PIN1::GFP* ectopic presence.

To prove that PIN1 pattern observed after the BAP treatment determines extra auxin accumulation regions, we have repeated the BAP experiment using plants containing the *DR5::GFP* construct. In the wild type plants, GFP driven by *DR5* promoter was detected at the edge of the developing primordia and in the later stages into the nucellus as already reported (Figure 4.1J), (Benkovà *et al.*, 2003). In BPA treated *DR5:GFP* plants, GFP signal was detected also inside CK-IS structures develop from the chalaza (Figure 4.1K).

To understand whether the BAP treatment induced phenotype is linked to the changing of auxin flux, we have treated 10 plants with the auxin transport inhibitor NPA. Also in this case we have obtained the formation of one big structure in place of the integuments confirming our hypothesis (Figure 4.1L).

We have proposed that the structures that develop from the chalaza region have nucellus identity and the auxin and PIN1 patter observed cause the phenotype (see scheme 4.2).

CK application is sufficient to induce nucellus formation

WUSHEL encodes an homeodomain protein with a fundamental role in meristem activity and in proper integuments formation (Gross-Hardt *et al.,* 2002). It is expressed in the organizer centre and controls non-cell-autonomously the number of stem cells in the niche (Laux *et al.,* 1996; Mayer *et al.,* 1998). Similarly, in the ovule, *WUS* acts non-cell-autonomously from the nucellus to induce integuments growth from the chalaza. *WUS* is the only gene that in ovule has a nucellus specific activity (Gross-Hardt *et al.,* 2002).
To verify the hypothesis that the CK-IS described above is a nucellus-like structure we have treated 10 transgenic plants containing WUS::GUS construct with BAP. In Figure 4.1N it shown that the *GUS* reporter gene is expressed also in the cytokinin induced structure as predicted, confirming our hypothesis.

To investigate whether the CK-IS phenotype is cause by *WUS* ectopic expression, we have expressed *WUS* under the *STK* ovule specific promoter (Kooiker *et al.*, 2005) in the *PIN1::PIN1::GFP* background. All the ovules of the transformed plants (n=9) showed the formation of several short integument like structures from the chalaza region (Figure 4.2O). In this WUS-induced ectopic integument-like structures we haven't detected any *PIN1::PIN1::GFP* activity (Figure 4.2O). This suggests that the *WUS* ectopic expression does not trigger the CK-IS formation.

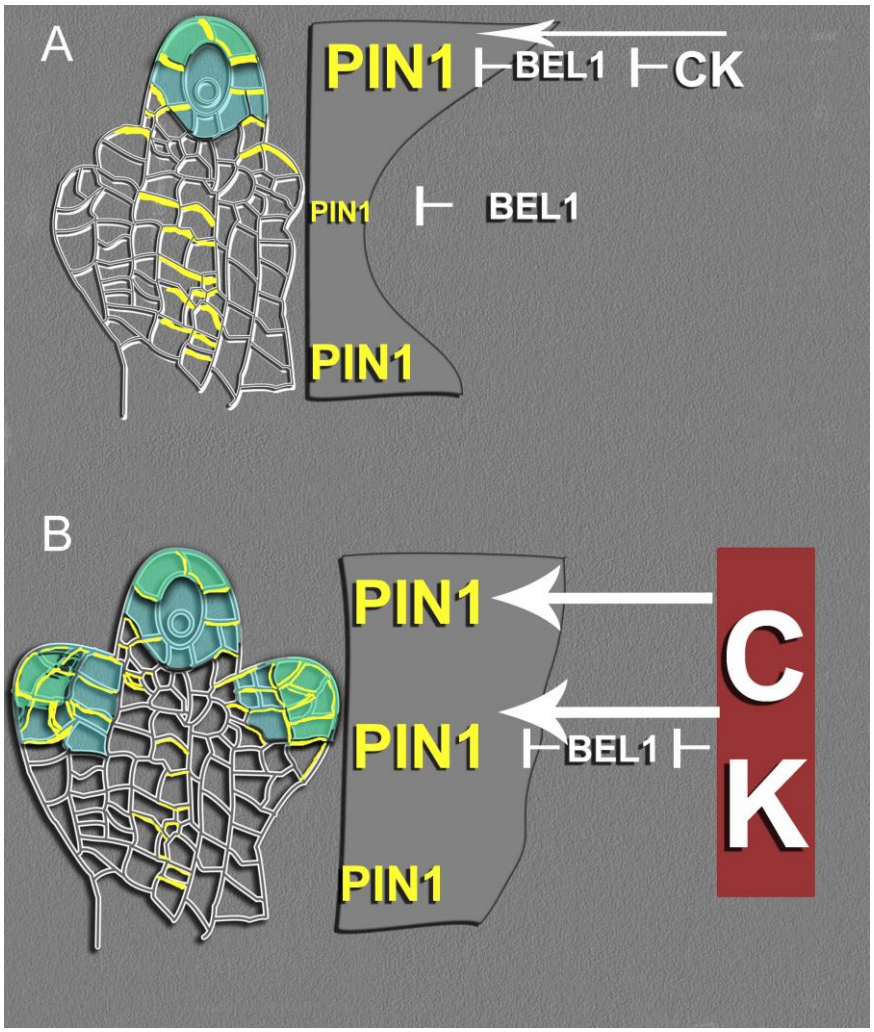

Figure 4.2. Schematic representation of PIN1 regulation by cytokinin
(**A**) In wt, BEL1 repress PIN expression allowing the formation of the integuments. (**B**) When we apply BAP the CK amount increases and represses BEL1. In this way CK induce the PIN1 expression. DR5:GFP signal, WUS:GUS activity and PIN1:PIN1:GFP are shown respectively in green, blue and yellow.

CK influences PIN pattern through the homeobox transcription factor *BEL1*

Cytokinin is known to act through a multistep signalling pathway that starts with the interaction of the hormone with receptors and ends with activation of cytokinins responsive genes that mediated the cytokinin function (Werner and Schmülling, 2009). To identify the molecules that mediated cytokinins effect on the PIN1 pattern, during ovule development, we have analysed mutants with

ovules comparable to the cytokinin-induced phenotype. *bel1* mutant showed the same ovule defects observed after BAP treatment (Robinson-Beers *et al.*, 1992; Reiser *et al.*, 1995, Brambilla *et al.*,2007).

In *bel1* the integuments are replaced by one single structure that at later stages is converted into a carpel like structure (Robinson-Beers *et al.*, 1992, Brambilla *et al.*, 2007).

To compare CK-IS with the structure formed in *bel1* ovules, we analysed BAP treated *bel1* plants containing reporter genes under the control of PIN1, WUS and DR5 promoters (*PIN1:PIN1:GFP*, *DR5:GFP* and *WUS:GUS*).

To compare CK-IS with the structure formed in *bel1* ovules, we analysed *bel1* plants containing reporter genes under the control of PIN1, WUS and DR5 (*PIN1:PIN1:GFP*, *DR5:GFP* and *WUS:GUS*).
As shown in Figures 4.O ,4.P, 4.Q in *bel1* mutant the GFP/GUS reporter genes showed similar expression respect to the cytokinins treated plants. These results have suggested that *PIN1* expression and localization and auxin accumulation in *bel1* ovules is similar to the one described for BPA treated ovules. Furthermore, these observations have suggested that the structure formed in *bel1* in place of the integument has nucellus-like identity as the CK-IS.
Moreover to verify whether the *bel1* mutant phenotype, is caused by the changing of auxin flux, (as in the BAP treated ovule), we have treated 10 *bel1* plants with the auxin transport inhibitor (N-1-naphthylphthalamic acid NPA). After the treatment *bel1* mutant plants had ovule lacking of the integument-like suggesting that this structure is caused by defective auxin flux and accumulation.
Furthermore, these data suggest an involvement of *BEL1* in controlling *PIN1* expression. To test this hypothesis we over expressed *BEL1* in ovule using *STK* ovule specific promoter (Kooiker *et al.*, 2005). We have transformed *PIN1::PIN1::GFP* plants using the construct *STK::BEL1* and we have obtained 60 plants, 10 of which were complete sterile. The analysis of 200 ovules from these 10 plants have showed that over expression of *BEL1* cause the formation of ovules without the nucellus (Figure 4.1U) and with a strong decrease of GFP expression in the ovule, driven by PIN1 promoter (data not shown).

BEL1 ChIP experiment analysis

One of the most important finding of our work is that we have shown that BEL1 regulates *PIN1* expression. To investigate if *PIN1* is a direct target of BEL1 we have decided to performe ChIP experiments using BEL1 polyclonal antibodies.

To test the specificity of BEL1 antibodies, a Western blot experiment was performed. For this experiment we have transformed *E. coli* with *BEL1* inducible expression plasmid and we have used proteins extracts of *E. coli* before and after induction to perform a western blot. Total proteins extract from wild type plant and from *bel1* mutant have been analysed by Western as shown in Figure 4.3. The *bel1* mutant that has been used for this analysis is cause by a stop codon in *BEL* coding sequence. As shown in Figure 4.3 a signal of 85 kDa was detected, in the lane loaded with wild type protein extracts whereas in the lane loaded with *bel1-1* protein extracts, a signal near to 16.5kDa was detected. This size correspond to the BEL1 truncated protein expected in the *bel1* mutant protein extract.

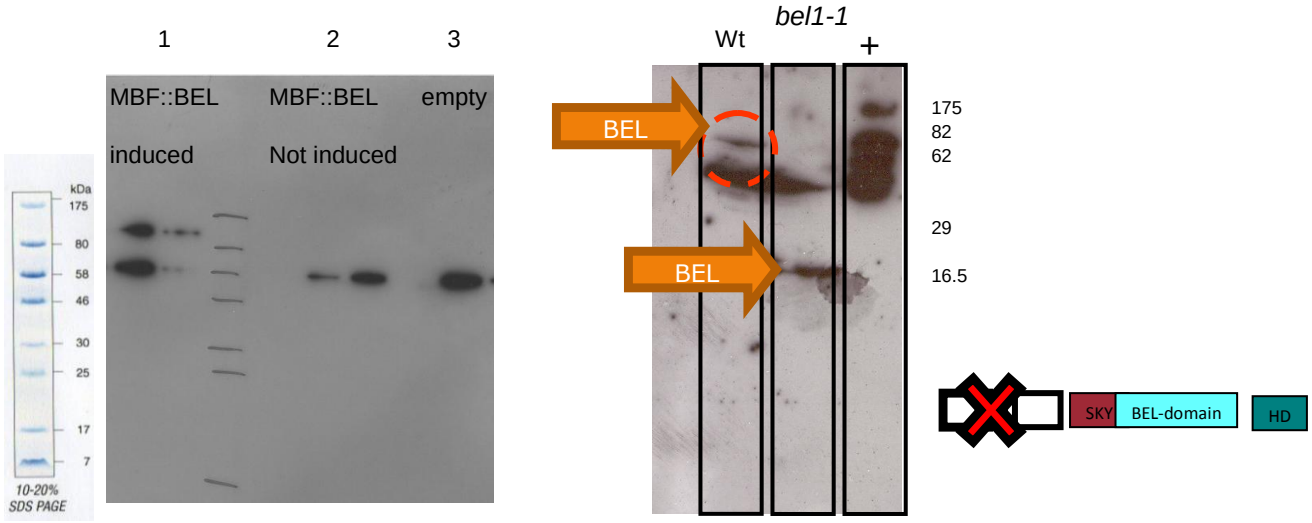

Figure 4.3 Western blot experiments
(**A**)Three type of protein extracts were used. *E.coli* extract after induction of the protein MBF:BEL1, and as a negative control, *E.coli* extract before the induction (2), and *E.coli* extract with a plasmid without the BEL1 gene (3). (**B**) Three type of protein extracts were used. Wild type plant proteins, as a negative control, bel1-1 proteins and as a positive control, *E.coli* extract after induction of the protein GST:BEL (+).

Then chromatin immunoprecipitation (ChIP) experiments were performed using the BEL1 antibody.

We have used flower tissue isolated from the wild type plants and *bel1-3* mutant as negative control, for these experiments. As it is not known the specific binding site of BEL1 on target DNA, we are going to test all the regulative region of *PIN1*. The ChIP experiments are in progress.

Material and methods

Plant Material and Growth Conditions

Arabidopsis thaliana (ecotype Columbia) plants were grown at 22°C under long-day (16h light/8 h dark) conditions. The *Arabidopsis* lines were obtained from the NASC Collection are: TCS (two-component-output-sensor):GFP (N23900). *DR5rev::GFP, PIN1::PIN1:GFP*, seeds were supplied by J. Friml (University of Gent). WUS:GUS seeds were supplied by T.Laux (university of Friburg).

BAP treatment

N^6-benzylaminopurine (BAP) obtained from Sigma Chemical Co. It was used 10-1 M solution of BAP concentrations Plants were treated once with30µl of a solution of either BAP solution or distilled water (both in 0.05%Tween 20). Solutions were applied either directly onto the inflorescence, and then the plants were covered with a plastic transparent bag for one day.

Microscopy analyse

To analyse ovule development, flowers were emasculated and pollinated using wild-type pollen 24 h after emasculation. Pistils were fixed 12 h after pollination (dap) and observed by CLSM following the Braselton et al. (1996) protocol. 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.

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). T_1 seedlings were selected by BASTA. To construct *pSTK::WUS, WUScds* was amplified with GGGGACAAGTTTGTACAAAAAAGCAGGCTC atggcaagagatcagttctatggtcacaat and GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATCAA ACAATATCATGAAGTAATTGAGC,and recombined into the vector pFGC5941 through an LR reaction (Gateway® system, Invitrogen). To construct *pSTK::BEl1*, *BEL1* genomic fragment was amplified with GGGGACAAGTTTGTACAAAAAAGCAGGCTCATGGAGCCGCCACAGCA TCAGCATCATC and GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAACTAGTTCAGA CGTAGCTCAAGAGAAGCGCA, and recombined into the vector pFGC5941 through an LR reaction (Gateway® system, Invitrogen).The 35S was removed and substituted by *pSTK* (amplified

with At590 and At591). 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.

ChIP and Quantitative Real-Time PCR Analysis

ChIP experiments were performed following previously reported protocol (Matias-Hernandez *et al.*, 2008). BEL1 polyclonal antibody was obtained against the first 139aa of the BEL1 starting from the ATG.

Antibodies were produce by Primm.

BEL1 expression in *E.coli*

The *BEL1* open reading frame was amplified with primers 5'_GGGGACAAGTTTGTACAAAA AAG CAGGCTCCATGGCAAGAGATCAGTTCTATG_3' and GGGGACCACTTTGTACAAG AAAGCTGGGTTCAAACAATATCATGAAGTAATTG and cloned by Gateway recombination in pGEX-2T (Amersham Biosciences). The PCR product was digested with EcoRI and SalI and ligated into the pMALC2 vector (New England Biolabs). All the heterologous proteins were induced in the BL21-Gold strain (Stratagene), BEL1-GST was partially soluble at 37°C. For the binding experiments, *Escherichia coli* lysis was obtained in 140 mM NaCl, 2.7 KCl, 10 mM Na2HPO4, 1.8mMKH2PO4, 0.5% Triton X-100, and 5mMDTT, with 1mM PMSF and protease inhibitors.

Western blot analysis

The protein were extracted from wild type flowers and *bel1-1* flowers following previously reported protocol (Vincent *et al.,* 2006) Immuno-blot Analyses were prepared from *Arabidopsis* flowers, fractionated on an SDS-polyacrylamide gradient gel (8%–25% polyacrylamide), and transferred to poly (vinylidene difluoride) membranes (Ihnatowicz *et al.,* 2004). Filters were then probed with BEL1 antibodies signals were detected by enhanced chemiluminescence (Amersham Biosciences).

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CONCLUSIONS

Ovule development is one of the most crucial steps for plant reproduction. The studies on the molecular control of ovule development has an economy impact since it is the first step towards the production of seeds, a important agricultural product. However, studying ovule development also provides a new research ground for the testing of the organogenesis model which has been proposed for other organs. During my Ph.D training period I investigated the molecular pathways controlling ovule development in *Arabidopsis*.

Hormonal control of development

Hormones are small non-peptide molecules derived from various essential metabolic pathways (Santner and Estelle 2009) that control all aspects of plant development. Auxin and cytokinin are the most important hormones whose potential was already clear half a century ago (Skooge and Miller 1957). In fact the ratio between these two hormones is necessary and sufficient determining the fate of plant organ cells.

Recently several evidences have indicated that in ovules both hormones are important for the proper development (see introduction of this thesis). In particular the modulation of auxin concentration with formation of auxin maxima is important for ovule initiation like for the other plant organs (Reinhardt *et al.*, 2000; Reinhardt *et al.*, 2003 ; Oka *et al.*, 1998; Laskowski *et al.*, 1995; Benková *et al.*, 2003). Moreover cell identity determination in the female gametophyte depends on distribution of auxin (Pagnussat *et al.*, 2009).

Cytokinins are known to be important for female fertility but their role during ovule development is unknown.

In the work presented here, the auxin and cytokinin pathways and their role in ovule development have been studied in detail.

Role of auxin during ovule development

To dissect the auxin pathway we have studied in detail auxin biosynthesis, transport and presence during the ovule development (Chapter 1 and 2 of this thesis). Auxin accumulated first at the tip of the ovule primordium, later on accumulation was observed in the nucellus, and inside the funiculus.

To understand how this asymmetric and dynamic accumulation takes place we have studied the auxin biosynthesis and transport during ovule development.

To study auxin transport we have analyzed the distribution of the auxin efflux carrier PIN1 (Teale *et al.*, 2006). Among the eight *PIN* genes, *PIN1* and *PIN3* are the only ones that are expressed in the ovule (Chapter 1 of this thesis and Pagnussat *et al.*, 2009). PIN1 is located initially at the tip of the ovule primordium and then, during the next stages of development in the epidermal layer of the nucellus tip, the inner integument and in the middle part of the funiculus. *PIN3* was found only in the funiculus during later stages of development.

To test the role of the auxin flux during the ovule development, we have silenced *PIN1* specifically in ovules (Chapter1). We found that the embryo sac progression is arrested in plants where we have down regulated *PIN1* in ovules, suggesting that for its development and for the proper nucellus formation auxin accumulation created by a PIN1 mediated flux is essential. Coherently when we arrested the auxin flux by applying an inhibitor of auxin transport, we obtain a similar result.

Transport alone cannot explain the role of auxin in ovules, since local auxin biosynthesis is considered important to provide the hormone in loco (Chandler, 2009). For the local auxin synthesis we studied genes of the *YUCCA* (*YUC*) family of flavin mono-oxygenase enzymes (Zhao *et al.*, 2001) and genes essential for the indole-3-pyruvic acid (IPA) branch of the Trp-dependent auxin biosynthesis pathway (Stepanova *et al.*, 2008). *YUCCA4*, *YUCCA6* and *TAA1\WEI8* are expressed during ovule development. The studies of chapters 1 and 2 are summarized in a model (Figure D.1) that takes in consideration local auxin biosynthesis and auxin flux. We propose that a flux of auxin from the integuments (where it seems to be synthesized in loco by the action of WEI8 and YUC6) towards the nucellus by the action of the PIN1, where the transported auxin accumulates. At later stages, *YUCCA* expression suggests a local auxin biosynthesis in the nucellus.

Moreover we have proposed a model in which both biosynthesis and transport are necessary for ovule organogenesis and for female gametophyte cell identity determination.

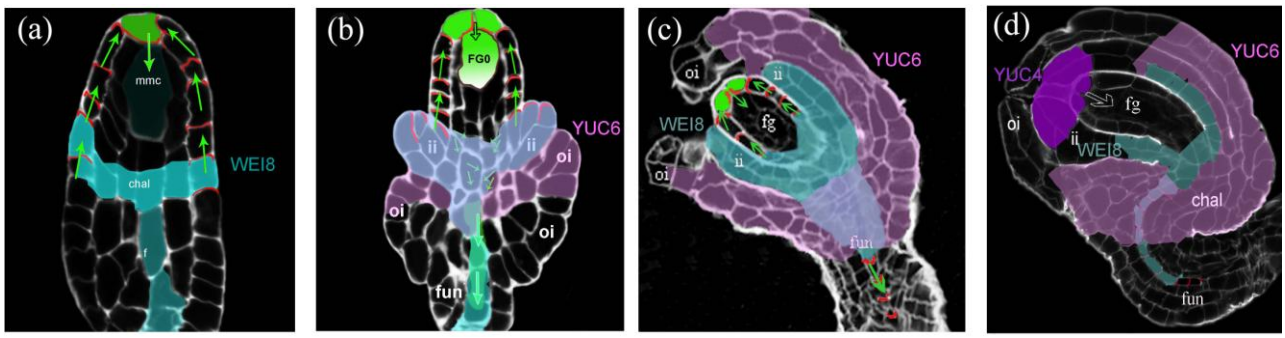

Figure D.1 Schematic representation of auxin biosynthesis and flux in ovule
This model presents the expression pattern of *WEI8* (blue), *YUC6* (pink) *YUC4* (violet) and the activity of *DR5:GFP* (green) during the different stages of ovule development. Primordia (a), FG0 (b), FG1 (c) and FG3 (d). In red is shown the PIN1 disposition and the arrows indicate the auxin flux.

To analyze the importance of local auxin biosynthesis we are characterizing double, triple and quadruple mutants of the genes involved in this process. This work is still in progress.

The role of Cytokinin in ovule development

We have found the expression of *IPT1*, a gene encoding for an enzyme that forms the principal step in cytokinins biosynthesis, inside the nucellus suggesting in loco cytokinin biosynthesis. The expression of *IPT1* persists from early stages to late stages of ovule development. The expression in the synergids of a gene that encodes an enzyme responsible for cytokinin degradation, *AtCKX7*, suggests that there is a strong reduction in cytokinin levels in these gametophytic cells.

The produced cytokinins are perceived thanks to the multistep signalling pathway that starts with the interaction of the cytokinins with the receptors, and their subsequent auto-phosphorylation. We found that all the receptors, *CRE1*, *AHK2*, *AHK3* are expressed in the ovule. In particular, *AHK2:GUS* and *AHK3:GUS* were active in all parts of the ovule, whereas $CRE1_{pro}$*:GUS* activity was initially observed in the chalaza part of the ovule primordia and subsequently in the inner integument and in the basal part of the nucellus. This suggests a sporophytic perception of cytokinin from the ovule and it is coherent with the observation that a phenotype was only found in the *cre1 ahk2 ahk3* triple mutant plants (Riefler *et al.*, 2006), showing a strong redundancy between these genes in the ovule.

The signalling pathway goes on with the transport of the phosphate group and the phosphorylation of the ARR-B type transcription factors that, when activated, induce the transcription of several genes necessary for the response to the cytokinins, including the ARR-A type. We haven't found any ARR-B type genes expressed during ovule development. However, *ARR5* a negative response regulator seems to be expressed in ovules. The analysis of plants containing *ARR5::GFP* and *TCS::GFP* constructs demonstrates the presence of cytokinin perception in the ovule. It is interesting to notice that *CYTOKININ INDEPENDENT1* (*CKI1*), known to be implicated in cytokinin signalling (Kakimoto, 1996; Deng *et al.*, 2011) was found to be necessary for gametophyte development and expressed in the central cell region.

Together all these data suggest that the cytokinins, that are present during all stages of gametophyte development, control the formation of the female gametophyte and might be involved in cells identity determination.

Another interesting observation is that cytokinin is known to act antagonistically to auxin, and auxin is supposed to be important for proper primordia and nucellus formation and in the female

gametophyte of cell identity determination. Based on BAP treatment we could propose a model that we have discussed in Chapter 3. According to this model, female gametophyte development is under the control of both hormones, cytokinin and auxin, and their balance determines the cell fate (Chapter 3 Figure 3.5). A high ratio of auxin/cytokinins determines synergids and egg cell identity whereas a low ratio determines central cell and antipodals identity.

In the last part of my thesis I have shown the progress that I made in the study of the molecules involved in the cross talk between the cytokinin and auxin pathways during ovule development.

We propose a model based on the data that we obtained when characterizing the *bel1* mutant and by hormonal treatment of wild type plants. Our findings are the starting point to understand the cross talk between the two generations (haploid and diploid generation) that coexist in the ovule and a integrated developmental mechanism that is controlled by the interaction between the auxin and cytokinin pathways. Not only the *bel1* ovule phenotype resembles from a morphological point of view ovules treated with cytokinin, but also detailed molecular analysis revealed similarities between these ovules. A future analysis will be performed to test if *PIN1* are directly regulated by the homeodomain protein BEL1.

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APPENDIX

EVOLUTION & DEVELOPMENT 12:1, 61–73 (2010)
DOI: 10.1111/j.1525-142X.2009.00391.x

GRAMINIFOLIA homolog expression in *Streptocarpus rexii* is associated with the basal meristems in phyllomorphs, a morphological novelty in Gesneriaceae

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SUMMARY *Streptocarpus* is a genus showing great variation in vegetative plant architecture and hence provides an attractive system to study the evolution of morphological diversity. Besides species showing an orthodox caulescent plant organization, producing leaves from a conventional shoot apical meristem (SAM), there are species whose body plan is composed of units (phyllomorphs) consisting of a petiole-like structure and a lamina that has the ability of continued growth. The first of these units is the macrocotyledon, derived from the continued growth of one of the two cotyledons by the activity of a basal meristem (BM), whereas further phyllomorphs develop from a SAM-like meristem. We carried out anatomical and morphological studies on the macrocotyledon of *Streptocarpus rexii* showing that the lamina has a bifacial structure, whereas the petiolode is partially unifacial. YABBY transcription factors are known to be involved in organ polarity and also promote lamina growth. We characterized the expression of *SrGRAM*, an ortholog of the *YABBY* genes *GRAMINIFOLIA* (*GRAM*) and *FILAMENTOUS FLOWER* (*FIL*), in *S. rexii* by in situ hybridization and RT-PCR. Gene expression pattern during embryogenesis was found to be conserved between *SrGRAM* and *FIL* from *Arabidopsis*. During subsequent seedling development *SrGRAM* expression in *S. rexii* was closely associated with the activity of the BM of the macrocotyledon and consecutively produced phyllomorphs, whereas it was excluded from the SAM-like meristem. Our results suggest that *SrGRAM* acts in intercalary growth and that an altered regulation of *SrGRAM* may underlay the evolution of the BM in *S. rexii*.

INTRODUCTION

One of the key issues in evolutionary developmental biology (evo-devo) is to decipher the molecular mechanisms that underlie the generation and diversification of evolutionary novelties (Müller and Newman 2005). In this respect, *Streptocarpus* represents a genus of particular interest; besides caulescent species that produce leaves from a conventional shoot apical meristem (SAM), this genus includes acaulescent species that lack an orthodox SAM and therefore have no typical vegetative stem. The vegetative plant body of acaulescent *Streptocarpus* species consists of reiterated units composed of a stalk-region, termed petiolode because of characteristics of shoot and petiole, and a lamina, together termed phyllomorph (Jong and Burtt 1975). Continued growth of these structures is orchestrated from a nexus of three meristems; the persistent growth of the lamina is achieved by a basal meristem (BM) that is located at the proximal end of each lamina lobe, a petiolode meristem (PM) contributes to the extension of the midrib in synchrony with the enlarging lamina and also to the elongation of the petiolode, and a groove meristem (GM), located on the adaxial surface of the petiolode near the junction with the lamina, is the source of additional phyllomorphs and inflorescence primordia (Jong and Burtt 1975). In contrast, conventional dicotyledon leaves are determinate organs because the meristematic activity in their leaf primordia is short-lived (Donnelly et al. 1999; Nath et al. 2003).

Another key feature of *Streptocarpus*, and indeed Old World Gesneriaceae, is the unequal development of cotyledons after germination (anisocotyly) (Burtt 1970; Tsukaya 1997); the first phyllomorph is derived from the continued growth of one of the two cotyledons to form the macrocotyledon, the cotyledonary phyllomorph, by the activity of the BM (Jong and Burtt 1975).

Waites and Hudson (1995) have proposed a model for leaf development in which the juxtaposition of ad- and abaxial cell fates is required for proper lamina outgrowth. Genes of the *YABBY* family are known to be expressed in the abaxial side of leaves and to confer abaxial cell fate. In *Arabidopsis*,

ectopic expression of *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3*, which are both member of this family, is sufficient to cause the development of ectopic abaxial tissues in lateral organs (Siegfried et al. 1999). Occasionally, amphicribal vascular bundles (vascular bundles in which the phloem encircles the xylem) are also observed in the petioles of *FIL*-overexpressing plants (S. Sawa, unpublished observation). Recently, it has been proposed that *YABBY* gene activity, in addition to promoting abaxial cell fate, directs lamina growth in leaves via cell proliferation, an idea that is supported by several findings; loss of *YABBY* activity results in a loss of lamina expansion (Siegfried et al. 1999; Villanueva et al. 1999; Golz et al. 2004) and *YABBY* gene expression correlates with regions of blade growth even when it is ectopically localized (Eshed et al. 2004; Juarez et al. 2004; Gleissberg et al. 2005). More importantly, *YABBY* gene activity was found to be required for the formation of ectopic blade outgrowth observed in *kan1 kan2* mutants. In particular, it has been proposed that at the boundary of *YABBY* gene activity cell division is promoted in domains that span both sides of the boundary. It has thus been proposed that *YABBY* gene activities mediate signaling between the ad- and abaxial leaf domain (Eshed et al. 2004).

It is also suggested recently that YABBY proteins act nonautonomously to affect the meristem, as loss of *YABBY* activity in the periphery of the SAM results in phyllotactic alterations and involves dramatic changes in the expression of *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*), genes expressed in the central zone of SAMs (Goldshmidt et al. 2008).

Here we trace *YABBY* expression in the acaulescent *Streptocarpus* species *Streptocarpus rexii*. Several previous developmental and molecular studies have focused on this species (Harrison et al. 2005; Mantegazza et al. 2007; Nishii and Nagata 2007; Mantegazza et al. 2009) and therefore *S. rexii* provides a well-established system to shed light on the molecular pathway/s involved in the development of anisocotyly and acaulescence. In this paper we focus on the leaf/phyllomorph development showing that the macrocotyledon of *S. rexii* is partially unifacial and that the BM development is associated with an altered expression of the *YABBY* gene ortholog *SrGRAM* (*Streptocarpus rexii GRAMINIFOLIA*), suggesting a role of this gene in the intercalary lamina expansion in *S. rexii*. Furthermore, we report that, when ectopically expressed in *Arabidopsis*, this gene is able to induce *YABBY*-specific phenotypes.

MATERIALS AND METHODS

Plant materials

Plants of *S. rexii* Lindl. (Gesneriaceae; Lindley 1828; RBGE accession number 20030814, Tsitsikamma, Cape Province, SA) were grown in glasshouses at the Royal Botanic Garden Edinburgh and the Botanical Garden of the University of Milan. Voucher specimens are deposited at the herbarium at the Royal Botanic Garden Edinburgh.

Arabidopsis thaliana wild-type plants (ecotypes Ler and Col) and *fil-1* mutant plants (supplied by the Nottingham *Arabidopsis* Stock Centre) were used for plant transformation. *Arabidopsis* seeds were sown on soil in small pots and incubated at 4°C for 3 days. Plants were grown under short-day growth conditions (8 h of light and 16 h of darkness) for 2 weeks and then transferred to long-day conditions (16 h of light and 8 h of darkness).

Microscopic analyses

For light microscopy, *S. rexii* phyllomorphs were either freehand sectioned without fixation or fixed in FAA (3.7% formaldehyde and 5% acetic acid in 50% ethanol) overnight at 4° C. After dehydration in an ethanol series (70–85–95–100%, 1 h each), the fixed samples were embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany), cut at 8-μm thickness and stained with toluidine blue.

For scanning electron microscopy (SEM), samples were fixed in FAA and dehydrated using an ethanol series as above. Following critical point drying and coating with gold, samples were observed with a LEO 1430 scanning electron microscope (LEO Electron Microscopy, Thornwood, NY, USA).

Isolation of *SrGRAM* and sequence analyses

Total RNA was extracted from *S. rexii* lamina tissue using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). First-strand cDNAs were synthesized from total RNA with the SuperScriptIII First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

A *YABBY*-like cDNA fragment was isolated using primers designed to the coding sequence of *GRAMINIFOLIA* (AY451396), the ortholog of *Arabidopsis FILAMENTOUS FLOWER* in *Antirrhinum majus*, because this plant is more closely related to *Streptocarpus* than *Arabidopsis*. The forward primer yab2f (5′-GTGACTGTGAGA TGTGGGCA-3′) was designed to the highly conserved zinc-finger domain and the reverse primer yab2r (5′-GACACACCCACGTTTGCTG-3′) to a conserved sequence region between *GRAMINIFOLIA* and its orthologs in other species (*FIL* AF136538; *TmFIL* AY825263). This cDNA was used as a probe to screen a *S. rexii* cDNA library (*c.* 300,000 pfu) constructed from young lamina using the Uni-ZAP® XR Vector (ZAP-cDNA® Gigapack® III Gold Cloning Kit, Stratagene, La Jolla, CA, USA).

A full-length cDNA sequence was identified and designated *SrGRAM* (GenBank accession number FJ407050).

An alignment of the inferred amino acid sequence of *SrGRAM* with the products of *A. majus GRAMINIFOLIA*, *A. thaliana FILAMENTOUS FLOWER*, and *A. thaliana YABBY3* was created using CLUSTAL W2 (Larkin et al. 2007) at http://www.ebi.ac.uk/Tools/clustalw2. To confirm the homology of *SrGRAM* to *GRAMINIFOLIA*, a dendrogram was reconstructed based on an alignment of *YABBY* amino acid sequences corresponding to both the zinc finger and the YABBY domain as reported in Toriba et al. (2007) (a complete list of accession numbers is given in supporting

information Table S1). A phylogenetic tree was constructed with MEGA 3.1 (Kumar et al. 2004) using the neighbor-joining method (Saitou and Nei 1987). A bootstrap analysis was performed with 1000 replicates.

Isolation of genomic DNA and Southern hybridization

Genomic DNA was isolated from lamina tissues of *S. rexii* as described previously (Chen and Dellaporta 1994). After digestion with restriction enzymes *Xho*I, *Hin*dIII, and *Eco*RV, about 10 µg of DNA per lane was separated by electrophoresis, transferred onto positively charged membranes (GE Healthcare, Little Chalfont, UK), and hybridized at high stringency conditions (65°C) in a Church and Gilbert (1984) buffer. The probe was ^{32}P labeled using a random primed DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). We used a DNA probe obtained by PCR using primers GRAF9 (5′-CATACCTCGATCAGTCAACC-3′) and filR1 (5′-GTTCGTCAATTCCTCGAAC-3′) designed here, that amplified a PCR product (approximately 1400 bp) corresponding to the 5′ region of *SrGRAM*. After washing, the blot was scanned using a Typhoon 8600 imager system (Amersham Pharmacia Biotech, Uppsala, Sweden).

A Southern blot was also carried out at low stringency conditions (55°C) with 10 µg of DNA digested with *Hin*dIII. The DNA probe was obtained by PCR using the primer sets filF1 (5′-GTGGCCATTGCACAAATC-3′) and filR2 (5′-GTGGCTTAG GAAGTTCGTC-3′) (supporting information Fig. S1).

RT-PCR expression analysis

cDNA was synthesized from 1 µg of total RNA extracted from different tissues, that is, the proximal and distal region of 3-month-old laminas, the proximal lamina of flowering phyllomorphs, petiolodes, fruits, and flowers; as described above. Subsequent PCR reactions were carried out using standard protocols and the following gene-specific primer pairs: GRAF9—filR1 for *SrGRAM* and ACTR (5′-GCCAAAGCAGTGATCTCTTTGC TC-3′)—ACTF (5′-CACTCCTGCCATGTATGTCGCTAT-3′) for *SrACTIN*, a constitutively expressed gene that was used as internal standard to normalize the RT-PCR. Both primer pairs were designed to span introns in order to distinguish products amplified from mRNAs from those originating from potential contaminating genomic DNA.

In situ hybridization

Digoxigenin (DIG)-labeled *SrGRAM* RNA was generated using an in vitro transcription kit (Roche Diagnostic GmbH).

Antisense and sense *SrGRAM* probes, lacking the YABBY domain, were obtained by amplifying the 5′ portion of the gene with the primer sets filR1T7 (5′-TAATACGACTCACTATAGG GTTCGTCAATTCCTCGAAC-3′)—GRAF9 and GRAF9T7 (5′-TAATACGACTCACTATAGGGCATACCTCGATCAGT CAACC-3′)—filR1, respectively.

Hybridization and immunological detection were performed as described in Lopez-Dee et al. (1999) with minor modifications. The hybridization was carried out at 50°C overnight. The detection was performed using a DIG-detection Kit (Roche Diagnostics GmbH) with the antidigoxigenin antibody used at a 1:1000 dilution.

Generation of *Arabidopsis* transgenic lines overexpressing *SrGRAM*

The coding sequence of *SrGRAM* was obtained by PCR using the primer set FcsGRA (5′-CACCATGTCCTCTTCATCTG-3′) and RcsGRA1 (5′-GCAGCTTTCTTAATAGGGAG-3′). The PCR products were cloned into the pENTR™/D-TOPO® vector (Invitrogen). This construct served as the entry vector to transfer the coding sequence of *SrGRAM* into the pB2GW7 vector driven by the CaMV 35S promoter. This construct was obtained via the GATEWAY system using the LR reaction (Invitrogen). The vector was introduced into the *Agrobacterium tumefaciens* strain C58C1/pMP90 and transformed into *Arabidopsis*. Transgenic plants were generated by the floral dip method (Clough and Bent 1998) with selection on soil by resistance to the herbicide BASTA. Detection of the *fil-1* allele was performed using the gen145F (5′-CATTAAT ATAATTCCTTGTCACG-3′) and gen145R (5′-ATATAAATTT TTGGGATGTGAG-3′) primer pair. The 147-bp PCR product was digested with *Mse*I when amplified from the *fil-1* allele but not when amplified from the wild-type allele.

RESULTS

Dorsoventrality in the macrocotyledon of *S. rexii*

Four weeks after sowing, seedlings of *S. rexii* had established anisocotyly with the macrocotyledon developing from a BM (Fig. 1A).

The mesocotyl, forming the petiolode of the macrocotyledon, has the function of petiole and stem (Jong and Burtt 1975) and carries the GM (Fig. 1, B and C). The junction between lamina and petiolode is on the adaxial side, where the basal ends of the blade are in close proximity (Fig. 1, B, C, J, and K), almost enveloping the GM (Fig. 1C).

The proximal region of the petiolode (close to the root) is radially symmetric in outline (Fig. 1D) and shows trichomes both in the abaxial and the adaxial side (Fig. 1C). Epidermal margin cells, which in conventional angiosperm leaves form at the leaf edge, including petioles, are absents (Fig. 1, B and C). The vascular system displays a more or less circular arrangement of vascular bundles with phloem surrounding xylem (Fig. 1E).

In more distal region of the petiolode (nearer the lamina), a tissue located toward the adaxial side of the stele proliferates initiating new vascular bundles that will constitute the vascular system of the first phyllomorph (Fig. 1, E and H).

At the level of the GM, vascular bundles in the petiolode display an incompletely closed ring-shaped arrangement and this array continues through the midrib of the lamina (Fig. 1, I–K).

Otherwise, the lamina of the macrocotyledon shows a conventional bifacial architecture, with a single layer of palisade parenchyma located on the adaxial side, whereas spongy mesophyll cells are present in the abaxial region. Additionally, the abaxial epidermal cells are smaller compared with those on the adaxial side (Fig. 1L).

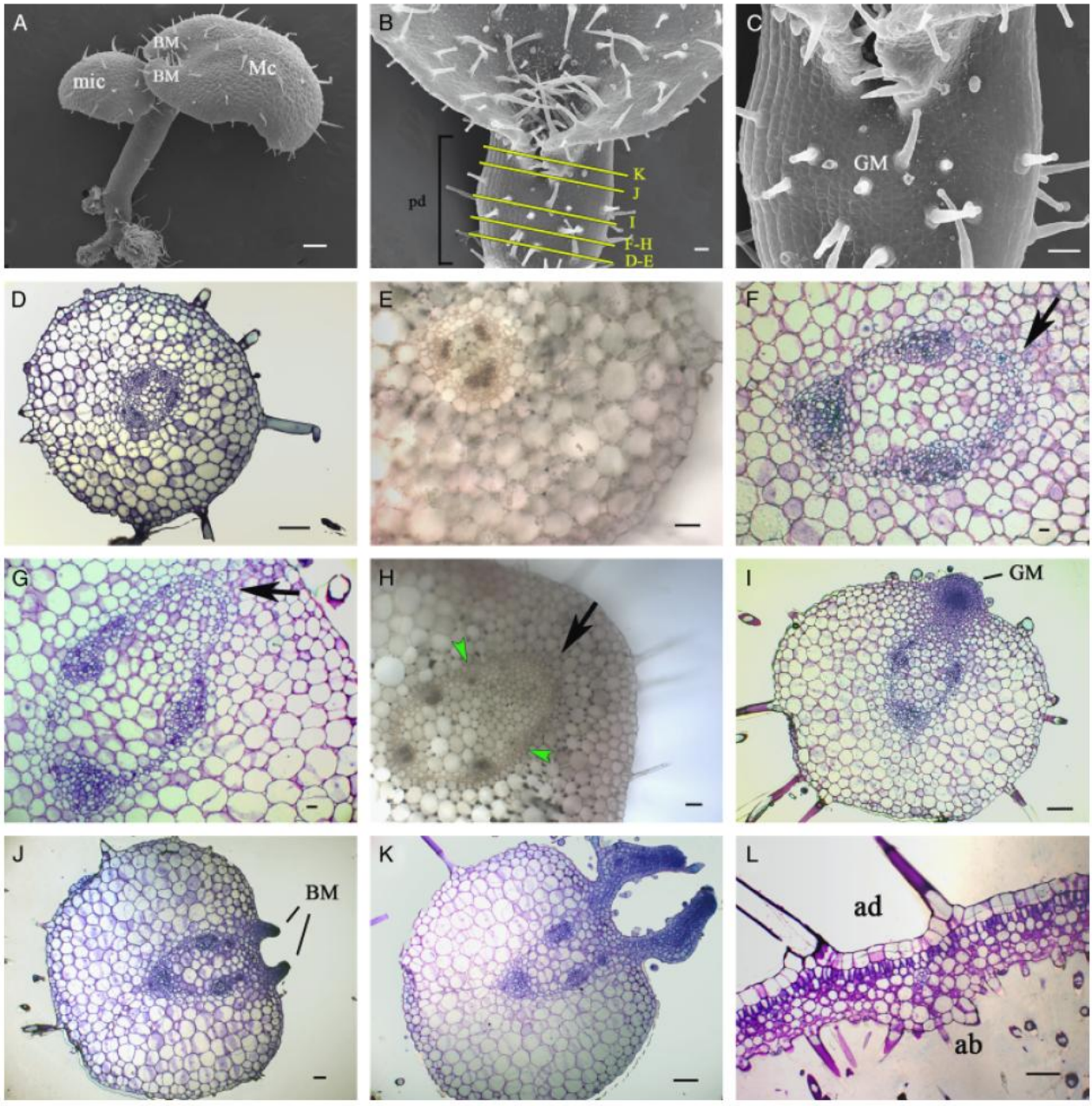


Fig. 1. Dorsoventrality in the macrocotyledon of *Streptocarpus rexii* (A) Scanning electron micrograph (SEM) showing a whole seedling at the anisocotylous stage. Mc, macrocotyledon; BM, basal meristem; mic, microcotyledon. (B) SEM image of a macrocotyledon showing the insertion of the lamina on the adaxial side of the petiolode (pd). (C) Magnification of B showing the developing groove meristem (GM). (D–K) Transverse sections showing the arrangement of vascular bundles in the petiolode of the macrocotyledon taken from positions indicated by lines in B. (D, E) The proximal region of the petiolode appears radial in transverse section (D) and shows vascular bundles arranged in a close ring with phloem surrounding xylem (E). (F–H) A region on the adaxial side of the stele proliferates (arrows), producing new vascular bundles (green arrowheads in (H)) that will constitute the vascular system of the first phyllormorph. (I–K) At the level of the groove meristem (GM) vascular bundles show an arc-shaped arrangement (I), the same pattern is present at the level of the BM (J) and the midrib of the lamina (K). (L) Cross section of the lamina of the macrocotyledon. ad, adaxial; ab, abaxial. Scale bar in (A) = 200 μm; scale bars in (D), (I), (K), (L) = 100 μm; scale bars in (B), (C), (E), (H), (J) = 50 μm; scale bars in (F, G) = 20 μm.

Cloning and characterization of a *YABBY*-like cDNA from *S. rexii*

In order to investigate the role of *YABBY* genes in the development of *S. rexii*, we isolated *SrGRAM* (*S. rexii GRAMINIFOLIA*), a *YABBY* gene by screening a cDNA library of young phyllomorphs. Homology analysis revealed that the deduced amino acid sequence of the protein encoded by *SrGRAM* shared a high degree of identity to GRAM (82%), FIL (62%), and YAB3 (58%). The predicted amino acid sequence consisted of 213 residues and possessed the characteristic features of YABBY proteins, namely a zinc finger-like domain toward the amino terminus, and a YABBY domain toward the carboxyl end (Bowman and Smyth 1999) (Fig. 2A).

Phylogenetic analysis of a range of *YABBY* genes on the conserved zinc finger and YABBY domains placed *SrGRAM* in a well-supported clade with *GRAM*, *FIL*, and *YAB3* (boostrap value = 83%) (Fig. 2B).

Genomic Southern blotting analysis, to estimate the copy number of *SrGRAM* in *S. rexii*, resulted in a single band after hybridizing the probe at high stringency when the DNA was digested with *Xho*I or *Eco*RV, whereas two bands resulted from *Hin*dIII digestion (Fig. 2C). As the probe contained one *Hin*dIII restriction site and no internal *Xho*I or *Eco*RV sites, this hybridization pattern suggests that *SrGRAM* is encoded by a single copy gene in the genome of *S. rexii*.

Low stringency Southern blot hybridization with a probe that partially overlapped the highly conserved zinc-finger domain resulted in six bands (supporting information Fig. S1) suggesting that the *YABBY* gene family comprises at least six members in *S. rexii*. Screening of the cDNA library, from which we isolated the *SrGRAM* gene, allowed us to isolate the homolog of another *YABBY* gene, *PROLONGATA*, a *YABBY5* homolog (data not show), further supporting the presence of a *YABBY* gene family in *S. rexii*.

Ectopic expression of *SrGRAM* in *Arabidopsis*

In order to assess the orthology of *SrGRAM* with the *Arabidopsis YABBY* gene *FILAMENTOUS FLOWER*, *Arabidopsis* wild-type and *fil-1* mutants plants were transformed with the *SrGRAM* cDNA under the control of the CaMV 35S promoter.

The overexpression of *SrGRAM* into wild-type *Arabidopsis* plants ecotype Lansberg *erecta* (Ler) generated different classes of phenotypes. The transgenic plants with severe phenotypes showed wrinkled and purple-colored cotyledons (Fig. 3, B–D). In these transgenic lines SAM development was also affected: some plants showed a complete arrest of SAM development (Fig. 3B) and died after producing two purple cotyledons. However, in some case several leaf primordia developed around the periphery of the SAM (Fig. 3C) that gave rise to wrinkled leaves that curled downwards (Fig. 3D).

The transgenic plants with mild phenotypes produced wrinkled and narrow rosette leaves that curled toward the abaxial side (Fig. 3, E and F). These plants flowered and in some case a limited stem elongation (i.e., bolting) and a large increase in the number of petals, stamens, and carpels was observed (Fig. 3H).

Two transgenic lines exhibited no bolting and produced extra leaves that are severely wrinkled (Fig. 3G).

Similar results were obtained with the overexpression of *SrGRAM* into wild-type *Arabidopsis* plants of ecotype Col (supporting information Fig. S2).

The overexpression of *SrGRAM* into *fil-1 Arabidopsis* mutant plants was able to rescue flower defects of the *fil-1* phenotype. *fil-1* mutant flowers can be divided into two types: type A exhibits floral organs of altered number and shape (Fig. 3I), that is, the number of petals and stamens is decreased and stamens often lack anthers, type B flowers are filamentous structures with or without a sepal-like structure (Fig. 3J). Two transgenic lines produced flowers with a normal number of petals and stamens (Fig. 3, K and L), even if not all the flowers in these transgenic lines recovered a wild-type number of floral organs. The overexpression in the *fil-1* background also resulted in phenotypes that were obtained by *SrGRAM* overexpression in the wt background; that is one transgenic line produced two purple cotyledons and wrinkled leaves (Fig. 3N), one showed no bolting and leaves severely wrinkled (Fig. 3O), and one plant showed limited stem elongation and flowers with supernumerary organs (Fig. 3, P–R).

SrGRAM expression pattern during embryogenesis

The expression pattern of *SrGRAM* was examined by in situ hybridization analysis at successive stages of seed development. In embryos at the late globular stage, *SrGRAM* expression was highest in two domains at the level of the cotyledon anlagen (arrows in Fig. 4A). Because the exact size of cotyledon anlagen is unknown we cannot rule out the possibility that *SrGRAM* expression may be abaxial. Although at the triangular stage, the expression was clearly restricted to the abaxial regions of the emergent cotyledon primordia (Fig. 4B). This expression pattern was continually observed up to the torpedo stage of the mature embryo (Fig. 4, C–E). No expression signal was observed when in situ hybridizations were carried out with the sense probe on the torpedo stage (Fig. 4F).

SrGRAM expression pattern during germination and establishment of anisocotyly

In order to determine whether *SrGRAM* expression is associated with regions of intercalary growth in the macrocotyledon of *S. rexii* we followed *SrGRAM* expression pattern

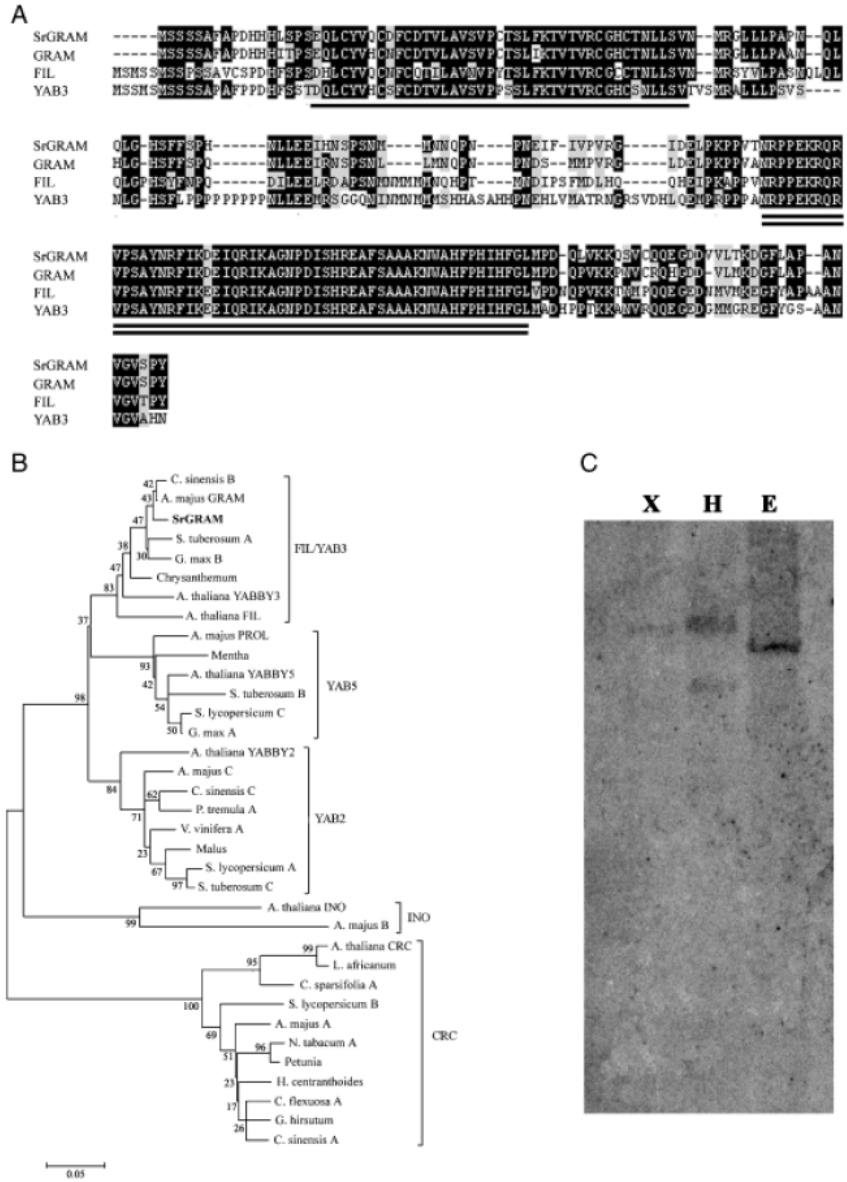


Fig. 2. Molecular characterization of *SrGRAM*.(A) ClustalW2 alignment of SrGRAM, GRAM (*Antirrhinum majus GRAMINIFOLIA* AY451396), FIL (*A. thaliana FILAMENTOUS FLOWER* AF136538), and YAB3 (*A. thaliana YABBY3* AF136540) proteins. Identical residues are highlighted in black and similar residues are highlighted in gray. Dashes represent alignment gaps. The zinc-finger domain is single underlined. The *YABBY* domain is double underlined. (B) Neighbor-joining tree of *YABBY* cDNAs based on both, the zinc finger and the *YABBY* domain. Numbers indicate bootstrap values. The groups shown on the right side indicate the classification from previous studies. (C) Southern-blot analysis of *SrGRAM* in *Streptocarpus rexii*. Each line contains 10 μg of *Xho*I (X), *Hind*III (H), or *Eco*RV (E)-digested total DNA of *S. rexii*.

during macrocotyledon development. Eight days after sowing (DAS), when cotyledons were still equal in size, *SrGRAM* expression was observed only in the basal regions of both cotyledons, and was restricted to the abaxial regions (Fig. 5A).

After the establishment of anisocotyly 29 DAS, a flat GM meristem had developed on the petiolode of the macrocotyledon near the base of the lamina. Longitudinal serial sections of such a seedling showed no *SrGRAM* signal in the GM in sections through this meristem (Fig. 5B), but evidence of

Fig. 3. Phenotypes of *Arabidopsis* plants resulting from ectopic expression of *SrGRAM*. (A) Wild-type *Arabidopsis* plants of ecotype Ler. (B) A 30-day-old transgenic plant with severe phenotype, producing only two purple cotyledons. (C, D) 30-days-old transgenic plants with purple cotyledons exhibiting several leaf primordia (C) and curled leaves (D). (E, F) Transgenic plants with wrinkled leaves that curl downwards. (E) 26-days old. (F) 35-days old. (G) A 52-day-old transgenic plant exhibiting no bolting and wrinkled leaves. (H) A 52-day-old transgenic plant showing limited stem elongation and flowers with more petals, stamens and carpels. (I) A *fil-1* mutant inflorescence showing flowers with abnormal floral organs. (J) Filamentous structures of the *fil-1* mutant (arrows). (K, L) Phenotypes of transgenic *fil-1* plants overexpressing *SrGRAM* (52-days old). Note the lack of filamentous structures (K) and the recovery of floral organ number (L). (M) Wild-type *Arabidopsis* inflorescence. (N) A 36-day-old transgenic *fil-1* plant showing two purple cotyledons and curled leaves. (O) A 52-day-old transgenic *fil-1* plant exhibiting no bolting and curled leaves. (P, Q, R) Transgenic *fil-1* plant with limited stem elongation and flowers showing more petals, stamens, and carpels. (P) 45-days old. (Q) 52-days old. (R) Magnification of (Q).

SrGRAM expression was detected in the macrocotyledon at the level of the BM (Fig. 5C). *SrGRAM* expression was also lacking in the microcotyledon, that is the cotyledon that does not possess an extended BM activity (Fig. 5C).

At 42 DAS, the GM had developed into a bulge. No *SrGRAM* transcripts were detected at the level of the GM (arrow in Fig. 5E) but off-center longitudinal sections showed *SrGRAM* expression in the BM (Fig. 5F). In particular transverse sections revealed that *SrGRAM* expression was localized in the abaxial domain of the BM (Fig. 5, H and I). Later in development, the domed GM had differentiated into a proximal and distal region (Fig. 5, K and L), the latter will form the lamina of the first true phyllomorph. During this stage *SrGRAM* signal was detected only in the distal region of the dome-shaped GM (arrows in Fig. 5, K and L). In the proximal region *SrGRAM* transcripts were not observed.

Later on in development, in situ signals of *SrGRAM* were detectable at the level of the BM of the first phyllomorph (arrows in Fig. 5, N and O), whereas no signal was detected in the GM (arrowheads in Fig. 5, N and O). We also confirmed the lack of *SrGRAM* signal in the distal portion of the lamina of *S. rexii* by using PCR after reverse transcription of RNA (RT-PCR). The RT-PCR, carried out on a nonflowering adult phyllomorph (3 months after initiation), showed *SrGRAM* transcripts in tissues of the proximal region of the lamina that includes the BM. No transcripts were detected in the distal region of the blade (Fig. 6).

Given that the diffuse nature of the PM (Nishii and Nagata 2007) makes it difficult to assess gene expression in this intercalary meristem by in situ hybridization, we also performed RT-PCR on tissues of the petiolode encompassing the PM, and we observed *SrGRAM* amplification (Fig. 6).

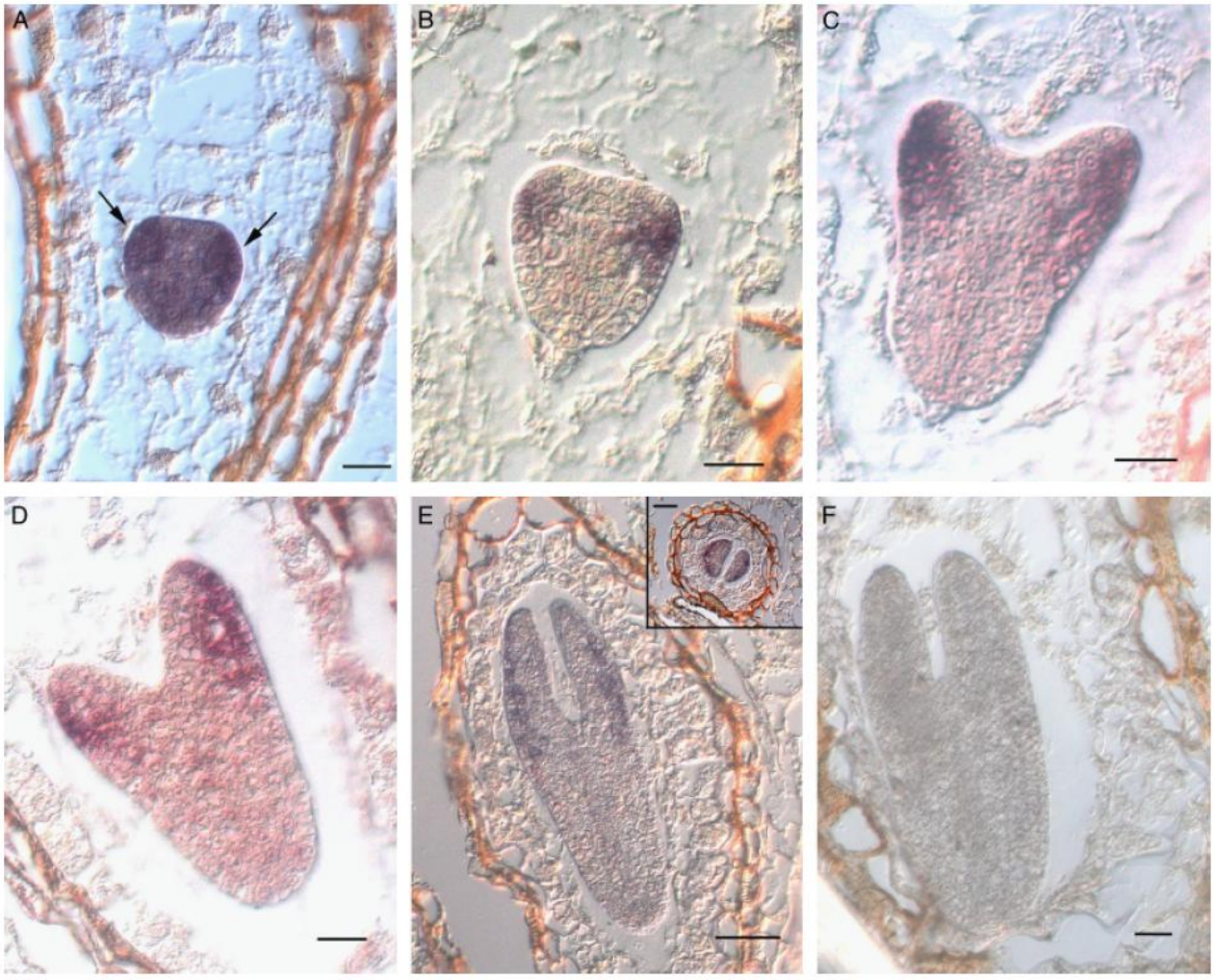
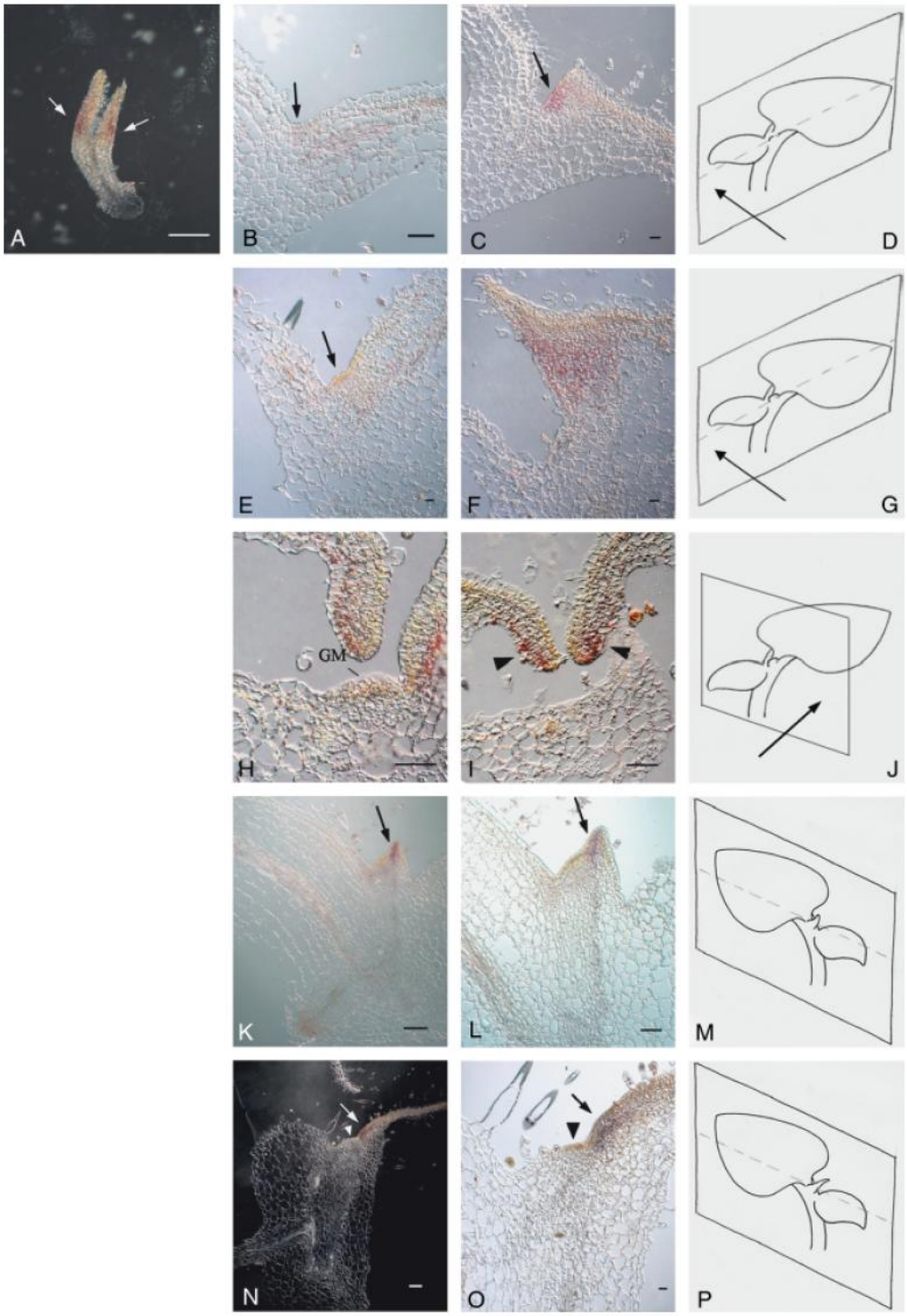


Fig. 4. *SrGRAM* expression pattern during embryo development in *Streptocarpus rexii*. (A) Late globular stage. A higher *SrGRAM* expression is detected in two domains corresponding to the cotyledon anlagen (arrows). (B) Triangular stage. *SrGRAM* is expressed in the abaxial side of the emergent cotyledon primordia. (C, D) Early (C) and late (D) heart stage. *SrGRAM* expression is detected on the abaxial side of cotyledon primordia. (E) Torpedo stage. *SrGRAM* is expressed in the abaxial domain of cotyledons. The *inset* shows the expression of *SrGRAM* in a transverse section through the embryo at the level of the cotyledons. (F) Sense probe control on *S. rexii* embryos. In situ hybridization with the sense probe on the torpedo stages did not label any tissue. Scale bars in (A), (B), (C), (D), (F) = 20 μm; scale bars in (E) = 50 μm.

Fig. 5. *SrGRAM* expression pattern during seedling development in *Streptocarpus rexii*. (A) 8 days after sowing (DAS). Arrows point to *SrGRAM* expression in the proximal abaxial region of the cotyledons. (B, C) 29 DAS. (B) No signal is detected in the flat groove meristem (GM) (arrow). (C) Arrow indicates *SrGRAM* expression in the basal meristem. (E, F) Longitudinal sections through a seedling 42 DAS. (E) No *SrGRAM* expression is visible in the bulged GM (arrow). (F) *SrGRAM* expression is evident in the BM. (H, I) Cross sections through a seedling 42 DAS. (H) The bulged GM does not show any *SrGRAM* signal. (I) *SrGRAM* signal is detected in the abaxial side of the BM (arrowheads). (K, L) Seedling 50 DAS with a domed GM showing proximally on the left a densely cytoplasmatic region lacking *SrGRAM* expression and a distal region showing *SrGRAM* signal (arrows). (L) Magnification of (K). (N, O) Longitudinal section through the first phyllormorph. Arrow indicates *SrGRAM* expression in an adaxial domain of the first phyllomorph, indicating the BM. Arrowheads point to the GM. (O) Magnification of (N). (D, G, J, M, P) Schematic illustrations showing the plane of sectioning; arrows in D, G, and J indicate the directions of cutting. Scale bar in (A) = 200 μm; scale bars in (K), (N) = 100 μm; scale bars in (B), (H), (I), (L) = 50 μm; scale bars in (C), (E), (F), (O) = 20 μm.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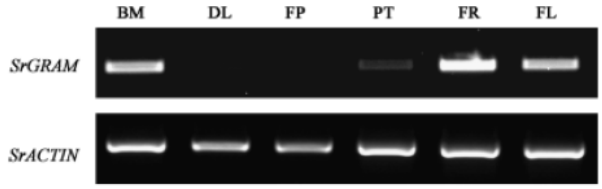

Fig. 6. RT-PCR analysis of *SrGRAM* transcripts in the proximal (BM) and distal lamina (DL) of a 3-month-old phyllomorph, the proximal lamina of a flowering phyllomorph (FP), petiolodes (PT), fruits (FR) and flowers (FL). *SrACTIN* was used as internal standard.

SrGRAM signal was also detected in seeds and flowers by RT-PCR (Fig. 6).

SrGRAM expression in flowering phyllomorphs

During phyllomorph development of *S. rexii* the basal lamina margins appear crinkly and involute. At flowering, the base of the lamina spread out indicating that BM activity, and therefore lamina growth, had ceased (Jong 1978). In order to determine whether cessation of cell division of the BM correlated with the disappearance of *SrGRAM* expression we performed an RT-PCR analysis on the basal region of the lamina of flowering phyllomorphs. As expected no *SrGRAM* transcripts were detected (Fig. 6).

DISCUSSION

Partial unifaciality of the macrocotyledon

As in conventional dicotyledon leaves, the lamina of phyllomorphs of *S. rexii* show an asymmetric arrangement of cell types along the dorsoventral axis. The mesophyll on the adaxial side consists of palisade parenchyma whereas that on the abaxial side consists of spongy parenchyma. In fully bifacial leaves, also the petiole develops a distinct polarity along the dorsoventral axis, in particular with respect to the vascular bundles that are arranged such that xylem is oriented toward the adaxial face whereas phloem lies toward the abaxial side.

In *S. rexii* the proximal region of the cotyledonary petiolode shows vascular bundles arranged in a closed ring with phloem surrounding xylem, which suggests an abaxialization of this region and the stem-like characteristics of this part of the petiolode. In the transition zone between the leaf lamina and the petiolode the arrangement of vascular bundles in an open arc suggests that the petiolode retains a reduced adaxial domain in this region, confirming the findings by Jong and Burtt (1975) for *S. fanniniae* and their view of the petiole-like identity of this region.

Also the course of the leaf margin is suggestive of the partial unifacial nature of the phyllomorph. In bifacial leaves the margins extend continuously from the leaf base to the tip, separating adaxial from abaxial face. The lack of the leaf margins in the proximal region of the petiolode of *S. rexii* confirms the unifacial nature of this region, whereas the close proximity of the basal ends of the blade margin in the distal region of the petiolode indicates that only a narrow domain retains adaxial identity in this region.

The ontogeny of unifaciality

During embryo development *SrGRAM* expression in *S. rexii* parallels that of its ortholog *FIL* in *Arabidopsis* (Siegfried et al. 1999) suggesting that, as for other eudicot *YABBY* genes, *SrGRAM* may play a role in specifying abaxial cell fate. The abaxial expression pattern of *SrGRAM* in embryos and in early stages of seedling development suggests that cotyledons, when still equal in size, are fully bifacial structures, and that the lack of adaxial identity in the unifacial proximal portion of the petiolode of the macrocotyledon arises postgenitally.

Extended BM activity is correlated with *SrGRAM* expression

After the establishment of anisocotyly, *SrGRAM* expression is visible on the macrocotyledon at the position of the BM, the intercalary meristem that enables the continued growth of the lamina, suggesting that *SrGRAM* acts in intercalary lamina expansion in *S. rexii*.

In particular, the *SrGRAM* signal is associated with the abaxial side of the BM. This finding is consistent with the view that *YABBY* activity acts to promote cell division indirectly in regions encompassing both sides of its expression boundary (Eshed et al. 2004).

In the newly formed phyllomorph *SrGRAM* expression is still associated with the BM region. RT-PCR analysis performed on older leaves (3 months after initiation) shows the persistence of *SrGRAM* expression in the proximal region of the lamina, and its absence here in mature, flowering phyllomorphs, where BM activity has ceased. This close association of BM activity and *SrGRAM* expression is a strong support for a role of the gene in continued lamina growth in *S. rexii* in a context of prolonged boundaries of *YABBY* gene expression.

SrGRAM expression is also observed in the petiolode suggesting that *YABBY* expression is likely to be correlated also with the PM, that is the region of intercalary growth of the petiolode.

SrGRAM and GM

Although *SrGRAM* appears to trace the region of BM activity, it is worth noting that signals are absent from the GM. This meristem, despite its unusual topographic location, shows homologies to a conventional SAM, that is a tunica-corpus configuration and the capacity of generating new phyllomorphs and inflorescences (Jong and Burtt 1975). The

lack of *SrGRAM* expression here supports the view that the GM could be regarded as a displaced SAM (see Mantegazza et al. 2007; Nishii and Nagata 2007), as expression analysis in *Arabidopsis* indicated that transcripts of *YABBY* genes are restricted to lateral organ primordia and excluded from the central region of SAMs (Bowman and Smyth 1999; Siegfried et al. 1999). However, in conventional SAMs, *YABBY* expression is present at the SAM periphery (Siegfried et al. 1999). However, in *S. rexii YABBY* expression was not detected in the peripheral regions of the GM.

A recent study has shown that a reduction in *YABBY* expression at the periphery of SAMs results in phyllotactic alterations and stimulates a dramatic change in the expression patterns of genes regulating meristem activity in the central region of SAMs. In particular loss of *YABBY* activity in leaf founder cells results in a considerable expansion of *CLV3* and *WUS* expression (Goldshmidt et al. 2008). Our recent expression analysis of a *WUS*-like gene in *S. rexii* is fully consistent with this finding: in *S. rexii*, *SrWUS* (*Streptocarpus rexii WUSCHEL*) expression is not confined to the organizing center but is detected throughout the GM (Mantegazza et al. 2009). Furthermore, in *S. rexii*, phyllomorph initiation at the GM is irregular and does not follow a phyllotactic order (Nishii and Nagata 2007). In this respect, it would be interesting to investigate other species of *Streptocarpus* that form a more regular, centric rosette (Jong 1978).

SAM versus GM—continuity of the GM

Our findings that during early stages of first phyllomorph initiation, the proximal, densely cytoplasm-rich region of the GM lacks *SrGRAM* signals is very important in the interpretation of the nature of this meristem. It suggests that the cotyledonary GM is not entirely used up in the formation of the first phyllomorph, with a de novo origin of the GM in the new phyllomorph, but demonstrates that the GM is a persistent structure from which subsequent phyllomorphs originate. This is in accord with findings based on developmental analyses by Nishii and Nagata (2007).

Heterologous overexpression

The overexpression of *SrGRAM* in *Arabidopsis* caused phenotypes which are highly similar to those reported for *FIL* overexpression in *Arabidopsis*, that is purple cotyledons and curled leaves (Siegfried et al. 1999), providing strong evidence that *SrGRAM* is a functional equivalent to *FILAMENTOUS FLOWER*.

The rescue of flower defects in *fil-1 Arabidopsis* plants further supports the orthology between *SrGRAM* and *FIL*.

FIL plays important roles in floral meristem development, e.g. control of floral organ number (Sawa et al. 1999). The overexpression of *SrGRAM* in *Arabidopsis* resulted in transgenic lines with supernumerary floral organs suggesting that *SrGRAM* plays a similar role in the control of the correct number of organs within a flower. It is possible to speculate that *SrGRAM* may promote cell proliferation in the floral meristem leading to the development of extra floral organs when ectopically expressed.

YABBY and *KNOX* expression in *S. rexii*

In *Arabidopsis*, mutations in two *YABBY* genes, *FIL* and *YAB3*, result in the derepression of the *KNOX* homeobox genes *SHOOTMERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*), and *KNAT2* in leaves. Therefore it has been proposed that *YABBY* genes mediate the repression of *KNOX* genes in *Arabidopsis* (Kumaran et al. 2002). On the other hand, ectopic *KNOX* expression is not observed in *gram* single mutant leaves of *Antirrhinum*, suggesting that *GRAMINIFOLIA*, the *Antirrhinum* ortholog of *FIL*, and *YAB3*, does not share the same role in *KNOX* repression (Golz et al. 2004).

Mantegazza et al. (2007) showed that *SrSTM1*, the ortholog of *STM* in *S. rexii*, is ectopically expressed in the BM of lamina of *S. rexii*. This partial overlap between *SrGRAM* and *SrSTM1* expression suggest that, as in *Antirrhinum*, *YABBY* activity does not mediate the repression of *KNOX* genes in *Streptocarpus*.

CONCLUSION

We have identified variation in the location and timing of *SrGRAM* expression that correlates with regions of intercalary growth in the lamina of *S. rexii* phyllomorphs. We can speculate that differences in the *cis*-regulatory elements of *SrGRAM* may underlie this altered expression pattern, although alternatively it could be explained by changes in the spatio-temporal expression of upstream factors or differences in the activity of such factors. On the basis of our results, and previous molecular data, one potential upstream factor is *PHANTASTICA* (*PHAN*). Mutations in the *PHAN* locus lead to the ectopic expression in leaf primordia of the *KNOX* gene *HIRZINA*, the ortholog of *STM* in *A. majus* (Tsiantis et al. 1999), and result in the altered expression of the *YABBY* gene *GRAM* in the adaxial part of leaf primordia (Golz et al. 2004). This network may be conserved between *Antirrhinum* of Plantaginaceae and *Streptocarpus* of Gesneriaceae; both families belong to the Euasterid I order Lamiales, and are relatively closely related (Tank et al. 2006).

The development of leaves that are mosaics of needle-like abaxialized tissue and bifacial laminal tissue in *phan* mutants, as in *S. rexii*, with the lamina forming an additional dorsal axis at the junction with needle-like tissue (Waites and Hudson 1995), is also consistent with this suggestion.

Expression analyses of *PHAN* homologs in *Streptocarpus* will therefore be very revealing in understanding the unorthodox growth patterns in this genus.

Acknowledgments

The authors are grateful to Raffaella Mantegazza, Chiara De Luca, and Simona Masiero for their useful advice during this project and to the staff of the Royal Botanical Garden Edinburgh for providing plant material and technical support. Royal Botanical Garden Edinburgh is supported by the Scottish Government Rural and Environment Research and Analysis Directorate. The authors acknowledge financial support of the "Cofinanziamenti progetti di ricerca di interesse nazionale PRIN 2006" and the "European Commission's Research Infrastructure Action via the SYNTHESYS Project" (GB-TAF-3845).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. List of accession numbers of sequences used for the reconstruction of the Neighbor joining tree in Fig. 2B.

Fig. S1. Southern Blot Hybridization carried out at low stringency (55° C). DNA (10μg) was digested with *Hin*dIII and hybridized with a ^{32}P-labelled probe. The probe was obtained by PCR using the primers filF1 (5′-GTGGCCATTG-

CACAAATC-3′) and filR2 (5′-GTGGCTTAGGAAGT TCGTC-3′).

Fig. S2. Phenotypes resulting from the overexpression of *SrGRAM* in wild type *Arabidopsis* plants of ecotype Columbia. (A) Wild type *Arabidopsis* plants of ecotype Columbia. (B) A transgenic line showing narrow leaves that curl toward abaxial sides. (C) A transgenic line producing only two purple cotyledon.