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Auxin and Cytokinin control of female gametophyte development in *Arabidopsis thaliana*

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RIASSUNTO

Il seme contiene e protegge l'embrione oltre a rappresentare l'unità di dispersione della progenie delle piante superiori. I semi sono inoltre fondamentali per l'alimentazione umana e animale e sono quindi oggetto di studi e ricerche per implementarne il numero per pianta oltre che le dimensioni e la qualità. Nelle Angiosperme, della quali fa parte anche *Arabidopsis thaliana*, i semi vengono prodotti a seguito di un processo chiamato doppia fecondazione. Questo processo avviene tra due individui aploidi, il gametofito maschile o grano pollinico e il gametofito femminile o sacco embrionale. I due gametofiti sono racchiusi all'interno degli organi riproduttivi: le antere contengono i granuli pollinici, mentre il gametofito femminile è avvolto dai tessuti sporofitici dell'ovulo che a sua volta si sviluppa all'interno del pistillo. Gli ovuli sono i precursori dei semi e per questo il loro sviluppo, è stato ed è tuttora intensamente studiato. Lo sviluppo dell'ovulo si divide in due fasi: la megasporogenesi e la megagametogenesi. La megasporogenesi inizia quando una cellula somatica acquisisce l'identità di cellula madre della megaspora (MMC), la quale va incontro a divisione meiotica dando origine a quattro spore aploidi. Tre di loro degenerano e quella che sopravvive, si differenzia nella Megaspora Funzionale (FM). La FM durante il processo di megagametogenesi si divide mitoticamente per dare origine al sacco embrionale maturo. A maturità, il gametofito femminile è costituito da sette cellule. Contemporaneamente alla formazione del gametofito femminile, si formano i tegumenti che avvolgono il sacco embrionale. Mutanti difettosi nella formazione dei tegumenti, mostrano anche alterazioni nella formazione e nello sviluppo del gametofito femminile.E' stato dimostrato che gli ormoni svolgono un ruolo importante nello sviluppo del sacco embrionale, tra cui l'auxina e le citochinine, che sembrano avere un ruolo fondamentale in questo processo di organogenesi.

In questa tesi, abbiamo dimostrato che il fenotipo di due mutanti difettivi nello sviluppo dell'ovulo, *ant-4 (AINTEGUMENTA)* e *bel1-1 (BELL1)*, è causato dall'alterazione della distribuzione dinamica dell'auxina, che non solo determina la mancata formazione dei tegumenti dell'ovulo ma anche il mancato completamento della megasporogenesi e conseguentemente la sterilità dell'ovulo. Studiando il potenziale ruolo delle citochinine durante lo sviluppo dell'ovulo abbiamo dimostrato che un'alterazione dei livelli di questo ormone hanno un impatto nel processo di megagametogenesi. In conclusione, auxine e citochinine sono essenziali per la determinazione e sviluppo del gametofito femminile.

ABSTRACT

The seed contains and protects the embryo, and it represents the unit of dispersion of the progeny for the higher plants. The seeds are also essential for human and animal nutrition and therefore, they are the subject of studies and research to implement their number per plant as well as their size and quality. The seeds in Angiosperm, as well as *Arabidopsis thaliana*, are produced after a process called, double fertilization. This process occurs between two haploid individuals, the male gametophyte or pollen grain and the female gametophyte or embryo sac. The two gametophytes are enclosed within the reproductive organs: the anthers carry the pollen grains, whereas the female gametophyte is wrapped by the sporophytic tissues of the ovule that in turn is enclosed within the pistil. Ovules are the precursors of seeds and for this reason, their development which is a tightly regulated process has been studied in details. Ovule development is divided in two phases: the megasporogenesis and the megagametogenesis. The megasporogenesis starts when a somatic cell differentiates in the megaspore mother cell (MMC) that undergoes meiotic division giving rise to four haploid spores. Three of them degenerate and the one surviving differentiates in the Functional Megaspore (FM) and starts to divide mitotically to give rise to the mature embryo sac. At the maturity the female gametophyte consists of seven cells. Simultaneously with the formation of the female gametophyte, the integuments are formed. These structures protect the embryo sac during its development but also provide the female gametophyte of all the signals and factors required for its correct development. Mutants defective in integument formation exhibited also alteration in female gametophyte formation and specification.

Hormones have been shown to play an important role in the control of embryo sac development, including auxin and cytokinins, which appear to play a key role in this organogenesis process.

In this thesis, we've demonstrated that the phenotype of two mutants defective in ovule development, *ant-4* (AINTEGUMENTA) and *bel1-1* (BELL1), is caused by the alteration of the dynamic distribution of auxin, which not only determines the failure to form the integuments but also it leads defects in megasporogenesis-magagametogenesis progression and consequently the sterility of the ovule. By studying the potential role of cytokinins during ovule development, we demonstrated that an alteration in the levels of this hormone has an impact on the process of megagametogenesis. In conclusion, auxins and cytokinins are essential for female gametophyte determination and development.

INTRODUCTION

Ovule development in *Arabidopsis thaliana*

Seeds allow plants to propagate themselves, to maintain biodiversity, and they are also essential in our diets. In Angiosperm, such as *Arabidopsis thaliana*, seeds are produced after a process called double fertilization which occurs between two haploid individuals, the male and the female gametophytes. The gametophytes are respectively enclosed in the reproductive organs: the anthers enclose the male gametophyte or pollen grain whereas the pistils contain ovules which are made of sporophytic tissues that wrap the female gametophyte or embryo sac.

At the maturity the pollen grain contains one vegetative cell and two sperm cells, whereas the female gametophyte is polarized and consists of seven cells distributed along the axis *micropyle – chalaza,* that is determine during ovule development.

Since ovules are the precursor of seeds, they play an important role to guarantee the survival and biodiversity of the plants and therefore their development is a tightly regulated process.

Ovules primordia arise from the placenta (stage 1-I) and elongate as finger-like structures (stage 1-II); along the proximal-distal axis three different regions can be distinguished: the funiculus, the chalaza and the nucellus (Schneitz et al., 1997) (Figure 1). The funiculus connects the ovule to the placenta, allowing the flow of all nutrients required by the developing ovule; above the funiculus, there is the middle region of the primordium, named chalaza, from which two important structures, the inner and outer integuments, are formed. The nucellus, instead, is the most distal region of the primordium, and it is formed by two layers: the outermost layer L1 and inner layer L2, that surround the female germline.

The ovule development is divided in two phases: the megasporogenesis and the megagametogenesis.

The megasporogenesis starts when in the nucellus, a somatic cell differentiates in Megaspore Mother Cell (MMC) (stage 2-I). This cell meiotically divides and produces four haploid spores (stage 2-IV/ V); the most apical three degenerate and the surviving spore near the chalaza, differentiate into Functional Megaspore (FM) (Figure 1).

Meanwhile, at the flanks of the chalaza, integument organogenesis starts (stage 2-II). The development of the inner integument earlier begins than the outer, which starts at the abaxial side of the chalaza (Sieber et al., 2004).

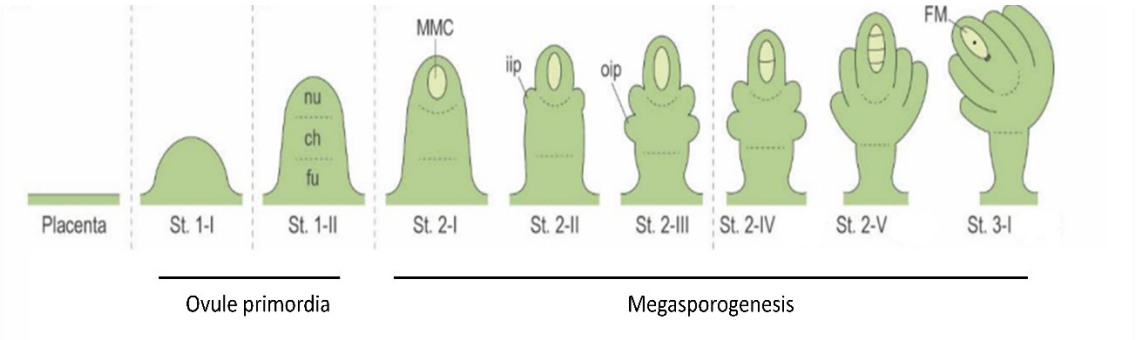


Figure 1 Ovule primordia and megasporogenesis in *Arabidopsis thaliana*. At the stage 1-I ovules arise from the placenta and elongate as finger like structure, in which already at the stage 1-II, three different regions can be distinguished: the funiculus (fu), the chalaza (ch) and the nucellus (nu). Within the nucellus the MMC differentiates (2-I) and undergoes meiotic division (stage 2-IV) giving rise to four haploid spores (stage 2-V). the most apical three degenerate and the one surviving differentiate in Functional Megaspore (FM). Meanwhile, the chalaza starts to develop the inner and the outer integuments (stage 2-II) that protect the nucellus and provide a route for all the signal required for the female gametophyte development. iip, inner integument primordia; oip, outer integument primordia. Adapted from Barro-Trastoy et al., 2020.

Once the FM is specified (stage 3-I), it undergoes a process called megagametogenesis (from stage 3-I until stage 3-V) which consists of three rounds of mitosis followed by cellularization (Figure 2). Meanwhile the two integuments complete their development and fully covered the mature embryo sac, except for a small aperture at the micropyle, that is required for the fertilization process; after fertilization integuments differentiate into the seed coat that protect the developing embryo from the environment.

Integuments arise to protect the nucellus and thus the developing female gametophyte, however, nowadays, evidence suggest that they act also as bridge between sporophytic tissue and the gametophyte, in which can travel signals, like hormones that are involved in embryo sac development.

At the end of megagametogenesis, in the mature female gametophyte can be distinguished two poles: the micropylar and the chalazal pole. Toward the micropyle, there are two synergid cells, one egg cell and just below the central cell which is the only one of the embryo sac to be binucleated (Figure 2). Instead, at the chalazal pole there are three antipodal cells whose function is still unknown.

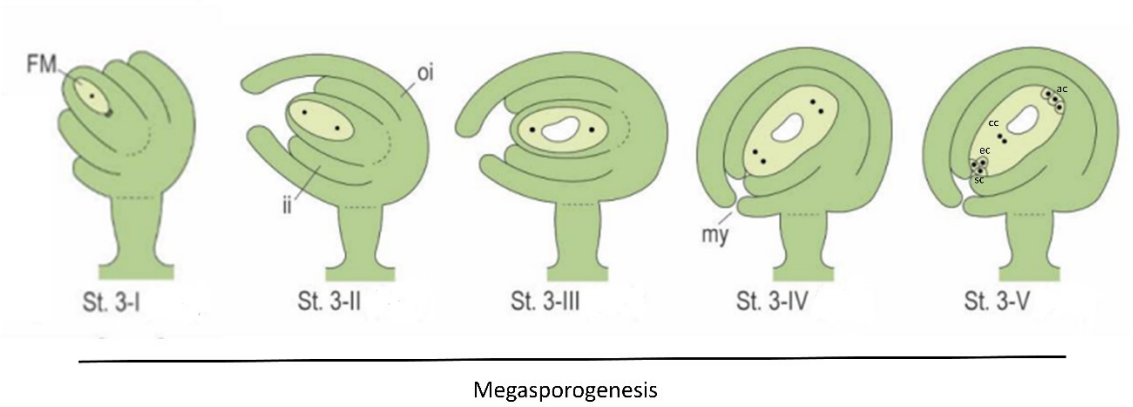


Figure 2 Megagametogenesis in *Arabidopsis thaliana*. Once the FM is specified, it undergoes three rounds of mitotic division (from stage 3-II to stage 3-V) to form the mature female gametophyte. At the maturity, the embryo sac resulted polarized and contains seven cells: at the micropylar pole (my) there are two synergid cells, followed by one egg and one central cell whereas at the chalazal pole, there are three antipodal cells. Meanwhile, the integuments complete their development and almost fully covered the mature embryo sac, except for the small aperture left at the micropyle which is required for the fertilization process. ii, inner integument; oi, outer integument; my, micropyle. Adapted from Barro-Trastoy et al., 2020

The double fertilization process starts when the pollen grain reaches the upper part of the pistil, called stigma. There, the vegetative cell protrudes and forms the pollen tube that grows within the pistil toward the ovule, carrying the two sperm cells (Figure 3A). Once the pollen tube reaches the female gametophyte, it bursts and releases the two sperm cells (Figure 3B). One sperm cell fertilizes the egg cell and gives rise to the embryo, whereas the other sperm cell fuses with the central cell and originates the endosperm, which is the nourishing tissue of the embryo.

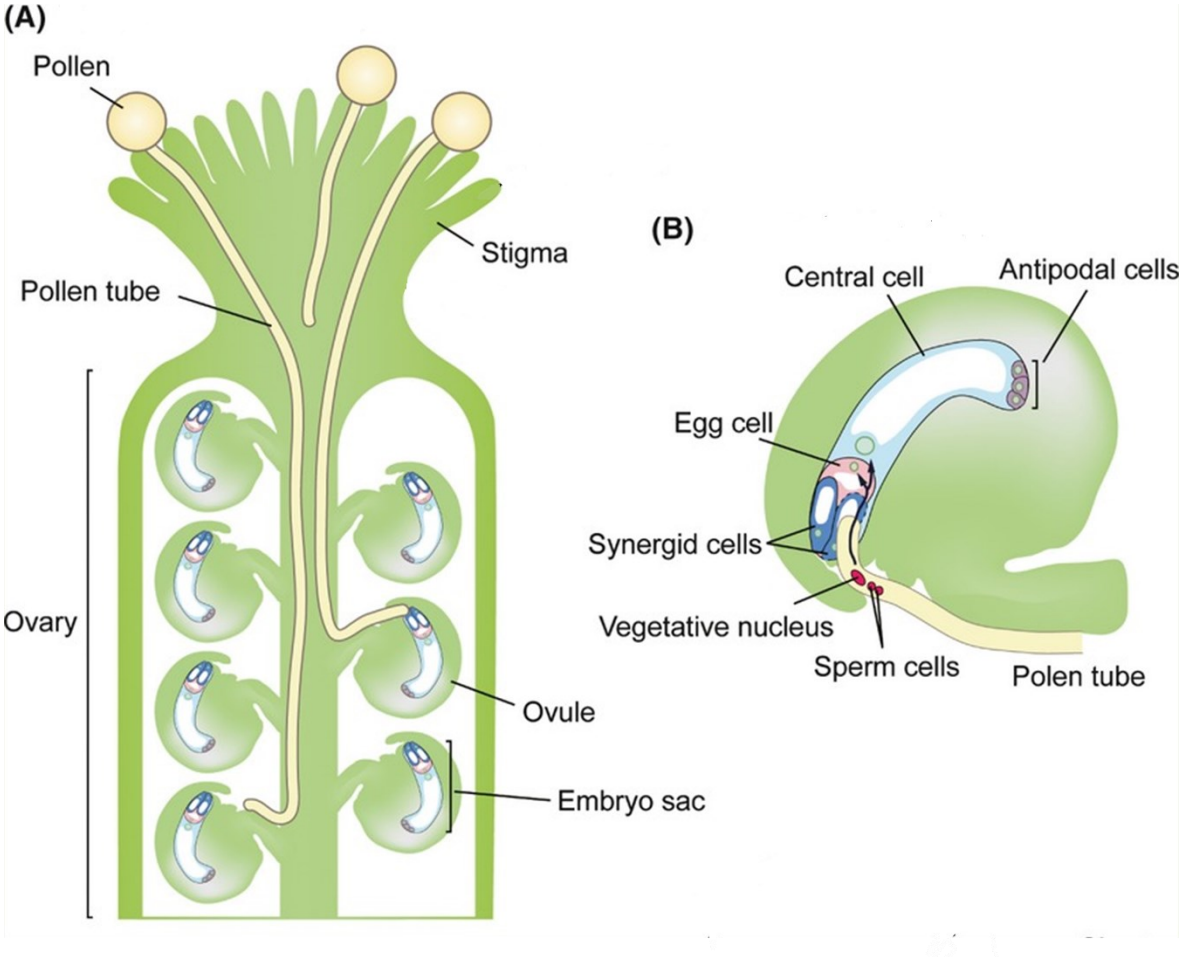


Figure 3 Double fertilization process in *Arabidopsis thaliana*. (A) The double fertilization process starts when the pollen grain reaches the stigma. There it produces a protrusion, called pollen tube which grows through the ovary, within in the pistil, toward the ovules. (B) Once the pollen tube gets in touch with the ovule, one synergid cells degenerate inducing pollen tube burst and the consequently releasing of the two sperm cells within the embryo sac. Adapted from Kurihara et al., 2013

Hormonal control of ovule development

Several factors are involved in the control of ovule development, such as environmental cues, hormones, transcription factors among others.

Hormones act as messengers activating the signalling cascades that are involved in the majority of developmental pathways. During ovule development several hormones have been shown to play fundamental roles, such as brassinosteroid, giberellins, cytokinin and auxin (reviewed in Barro-Trastoy et al., 2020). In particular, my work has focused on the role of cytokinin and auxin.

The role of cytokinin in ovule development

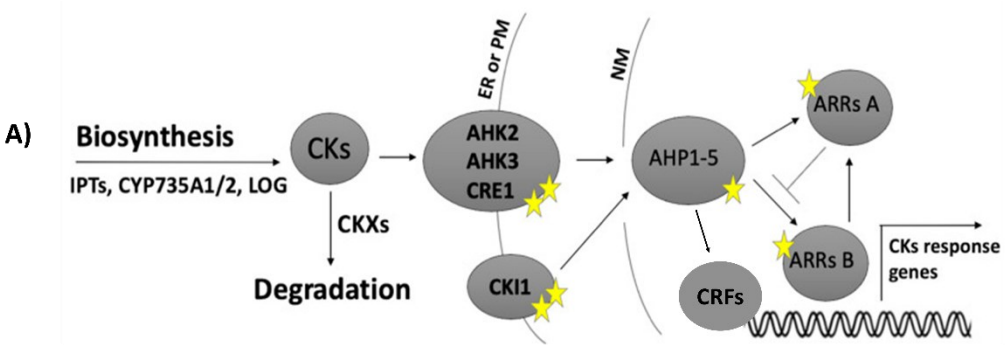
Cytokinins (CKs) play an important role in several physiological and developmental processes in planta: they are involved in the regulation of the proliferation (cell division) and differentiation of plant cells, in the control of delay of senescence, in the regulation of shoot/root growth, vascular, gametophyte, embryonic development and in the response to biotic and abiotic factor (Sakakibara, 2005).

Cytokinin are mainly catalysed by the enzymes isopentenyltransferases (IPTs) that control the rate-limiting step of cytokinin biosynthesis (Takei et al., 2001) (Figure 4A). In *Arabidopsis thaliana*, there are nine genes encoding *IPTs* (*AtIPT1-9*), among them *AtIPT1* gene has been shown to be expressed in developing ovules (Bencivenga et al., 2012; Miyawaki et al., 2004). Instead, cytokinin degradation is operated by CYTOKININ OXIDASE/DEHYDROGENASEs (CKXs) (Figure 4A), and in particular, among the seven CKX existing in *Arabidopsis thaliana*, only three CKXs are expressed within the ovule, *CKX5* (Manrique et al., unpublished) *CKX6* (Werner et al., 2003) and *CKX7* (Köllmer et al., 2014).

Cytokinin are perceived at the membranes by histidine/kinase receptors (HKs) that first autophosphorylate and then transfer the phosphoryl group to intermediate proteins called, phosphotransfers (HPs) that in turn phosphorylate the last acceptors, the Response Regulators (RRs). The RRs can be positive or negative regulators of cytokinin signalling; to the first class belong the type-B RRs, that are also able to bind the DNA and activate the transcription of cytokinin-related genes. By the contrast, type-A RR inhibit the cytokinin signalling (Müller and Sheen, 2008) (Figure 4A).

Another class of cytokinin signalling response factors are the CYTOKININ RESPONSE FACTORS (CRFs), which are member of AP2 gene family and mediate the cytokinin response overlapping with type-B RR (Rashotte et al., 2006).

Three receptors directly bind cytokinin, AHK2, AHK3, AHK4/CRE1, and all of them are expressed in ovule almost ubiquitously (Bencivenga et al., 2012). Only the expression of *AHK4/CRE1* is specifically restricted to the chalaza. Cytokinin signalling can be visualized thanks to the reporter marker line *TCSn::GFP* (Zürcher et al., 2013). Although, cytokinin receptors are widely expressed, the expression of the reporter marker localizes in the chalaza region surrounding the nucella throughout the megasporogenesis. During gametogenesis the expression of *TCSn::GFP* remains at the chalazal pole and in the mature female gametophyte also in correspondence of the central cells (Figure 4B).



B) Cytokinin signalling – *TCSn::GFP*

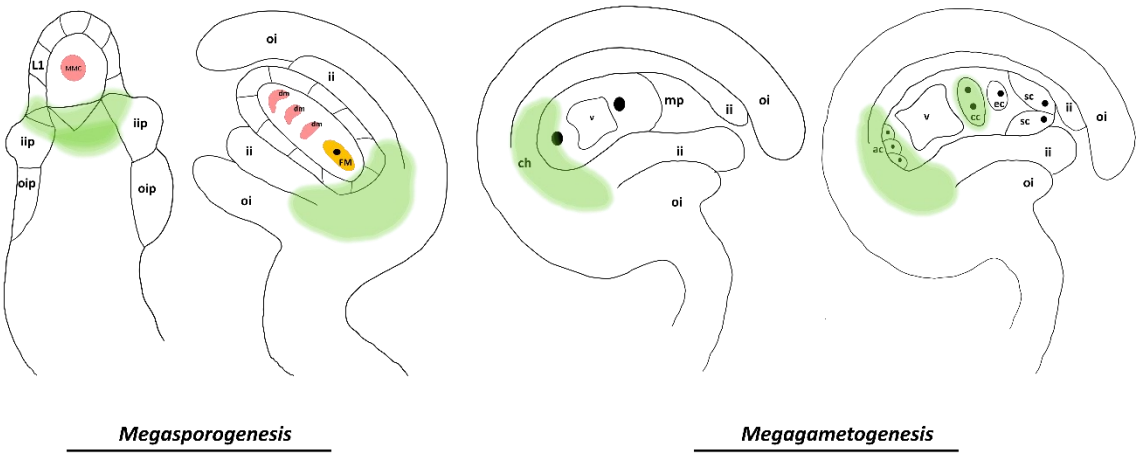


Figure 4 Cytokinin metabolism and signalling in ovule. A) Cytokinin homeostasis is maintained by a balance between biosynthesis (IPTs) and degradation (CKXs). Cytokinin activate a signalling cascade. They are perceived by AHKs receptors that autophosphorylate and

transfer the phosphoryl group to intermediate acceptors, HPs. These last transfer in turn the phosphoryl group to the last acceptors of the signalling cascade, the Response Regulators. In Arabidopsis exist two class of response regulators: type-A are negative response regulators and inhibit the signalling of cytokinin whereas type-B positively affect cytokinin signalling and binding the DNA activate the transcription of cytokinin-related genes. Adapted from Terceros et al., 2020. B) Schematic representation of *TCSn::GFP* expression during ovule development. Cytokinin signalling localized for the entire ovule development in the chalaza region and in the mature female gametophyte also in the central cell. oip, outer integument primordia; iip, inner integument primordia; MMC, megaspore mother cell; oi, outer integument primordia; ii, inner integument primordia; dm, degenerating megaspore; FM, functional megaspore; mp, micropyle; ch, chalaza; sc, synergid cell; ec, egg cell; cc, central cell; ac, antipodal cells; v,vacuole.

The maintenance of cytokinin homeostasis is important for reproductive development indeed, alteration in cytokinin metabolism severely affects plant fertility (review in (Terceros et al., 2020).

For instance,in the double mutant *ckx3ckx5,* in which cytokinin degradation is reduced, that produces a higher number of ovules compares to the wild-type (Bartrina et al., 2011).

By the contrast, fertility reduction was observed in the cytokinin receptors *ahk2 ahk3 ahk4/cre1* triple mutant (Bencivenga et al., 2012; Cheng et al., 2013) unable to specify the FM (Cheng et al., 2013) and/or showing megagametogenesis arrests. In addition, *ahk2 ahk3 ahk4/cre1* ovules fail to develop integuments phenocopy the mutant *pin1-5*, in which auxin transport is affected (Bencivenga et al., 2012). Moreover, defects in integument development have also been observed in wild type ovules exogenously treated with CKs (6-Benzylaminopurine, BAP) (Bencivenga et al., 2012). Notably, the effect of CKs treatment on integument development weren't observed in *ahk4/cre1* single mutant, suggesting that the cytokinin perception in the chalaza mostly depends on AHK4/CRE1 receptor (Bencivenga et al., 2012). Reduction in fertility due to defects in embryo sac development was also observed in mutant defectives for the other components of cytokinin signalling such as the HP (Deng et al., 2010; Liu et al., 2017) or RRs (Cheng et al., 2013). Mutations in HP genes alter gametogenesis and embryo sac cell specification. Indeed, in the *ahp2 ahp3 ahp5* triple mutant, central and antipodal cells lost their identity and gain the egg and/or synergid cells identity (Liu et al., 2017). Another mutant that exhibited alteration in the female gametophyte cells specification is *cki1* mutant. The CYTOKININ INDEPENDENT 1 (CK1) is a kinase, able to activate cytokinin signalling through HPs, independently on the presence of cytokinin (Deng et

al., 2010). It is expressed during the gametogenesis (Yuan et al., 2016) and mutations in *CKI1* lead to female gametophytic lethality (Deng et al., 2010; Hejátko et al., 2003; Pischke et al., 2002). Indeed, in loss of function *CKI1* ovules, the central and the antipodal cell identity is switched to egg cell identity (Yuan et al., 2016). On the contrary, overexpression of *CKI1* altered the specification of the egg cell, and it leads to the formation of unviable embryo-less seeds (Yuan et al., 2016).

Alteration in embryo sac cell identity was also observed in *vdd-1/+* mutant (Matias-Hernandez et al., 2010), in which it has been shown that antipodal and synergid cell identity and/or differentiation are affected. *VERDANDI* encode for a transcription factor belonging to the REM transcription factor family, and it has been shown to control also synergid degeneration during fertilization process (Mendes et al., 2016). In *vdd-1/+* ovules not only the antipodal and synergid cell are mis-specified but also synergid cell persists, instead of degenerating and consequently the fertilization process is impaired. It has been reported that in *vdd-1/+* the *CKX7* promoter is active in 50% of the ovules, suggesting that alteration in cytokinin level (increasing) prevents synergid degeneration (Mendes et al., 2016)

All this evidence supports the importance of chalazal cytokinin signalling, which is required for the correct specification and the development of the female gametophyte.

The role of auxin in ovule development

Auxin controls several physiological and morphological functions in planta such as: apical dominance, fruit ripening, root meristem maintenance, hypocotyl and root elongation, shoot and lateral root formation, tropisms (Laskowski et al., 2006; Benková et al., 2003; Reinhardt and Kuhlemeier, 2002). Moreover, auxin is involved in most growth and developmental processes regulating cell division, elongation, and differentiation (Weijers et al., 2018). One of their prominent functions is to promote organ primordia formation in both shoots (Wang and Jiao, 2018) and (Overvoorde et al.,2010). Auxin, has been also shown to be involved in controlling ovule development, including the formation of the megagametophyte (Pagnussat et al., 2009; Bencivenga et al., 2012; Cucinotta et al., 2016; Huang et al., 2022).

The tryptophan-dependent biosynthesis of auxin mainly depends on two-step pathway controlled by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1) and YUCCA (YUC) enzymes (Mashiguchi et al., 2011) (Figure 5A). In ovules, integuments have been shown to be the source of auxin. Expression analyses of *TAA1* promoter shown that at upon MMC specification, *TAA1* is expressed in the chalaza surrounding the nucellus, in integument primordia and in the funiculus. Once megasporogenesis ends, *TAA1* expression reduces, and it remains slightly visible only in cells surrounding the developing female gametophyte and in the funiculus until stage 3-II (Ceccato et al., 2013). Later, during megasporogenesis, *TAA1* begins again to be expressed in the micropyle throughout megagametogensis (Panoli et al., 2015). Among YUCCA genes, *YUC1*, *YUC2*, *YUC4* and *YUC8* have been shown to be strongly expressed within ovule (Pagnussat et al., 2009; Panoli et al., 2015; Larsson et al., 2017; In particular, it has been reported that *YUC4* is the only YUC gene to be expressed during sporogenesis; *YUC4* promoter is active in ovule already at the stage 2-I in the chalaza, in the inner integuments and in the funiculus (Li et al., 2021). During gametogenesis, *YUC4* expression localize in the funiculus, inner integuments and in the micropyle of developing ovule (Larsson et al., 2017). *YUC1* and *YUC2* are expressed from the beginning of gametogenesis in the micropyle of developing embryo sac (Pagnussat et al., 2009; Ceccato et al., 2013) whereas *YUC8* expression come later at the stage 3- II in the micropyle and persist until the ends of gametogenesis where its expression was observed also in the micropylar region of both integuments (Panoli et

al., 2015). A faint expression in the micropylar region of inner integument of was observed also for YUC5 (Larsson et al., 2017).

Once auxin is synthetized, it is transported in a polar manner, mainly by the auxin polar transporter PIN proteins (Figure 5A). In Arabidopsis there are eight *PIN* genes (*PIN1-PIN8*), among which *PIN1*, *PIN3*, *PIN4*, *PIN7* have been shown to be expressed in ovules (Pagnussat et al., 2009; Ceccato et al., 2013; Wang et al., 2021). PIN1 is already expressed at the apex of the ovule primordium when it arises from the placenta. At the beginning of sporogenesis it localizes in the epidermal layer of the nucellus, in integument primordia and in the funiculus. Then PIN1 expression is restricted to the funiculus during megagametogenesis (Pagnussat et al., 2009, Ceccato et al., 2013). Similar to PIN1, also PIN3 is expressed at early stage of ovule development but its expression is restricted to few cells of the nucellus apex (Pagnussat et al., 2009, Ceccato et al., 2013) whereas during gametogenesis PIN3 is expressed only in the funiculus (Pagnussat et al., 2009, Ceccato et al., 2013). Other two PIN proteins have been shown to regulate auxin transport in the nucellus, PIN4 and PIN7 (Pagnussat et al., 2009, Wang et al., 2021). However, they are expressed in the nucellus in a very specific moment, at the end of megasporogenesis, when the megaspores are degenerating (Wang et al., 2021). Also two auxin influx carrier has been shown to be expressed within ovules; LIKE AUXIN RESISTANT 1 (LAX1) expression was observed in the apex of the nucellus at the beginning of megagametogenesis at the stage 3-I (Panoli et al., 2015) whereas AUXIN RESISTANT 1 (AUX1) is expressed during gametogenesis from stage 3-IV in the micropylar pole of the embryo sac and after cellularization, *AUX1* localizes in the egg cell and synergid cell membranes (Panoli et al., 2015).

Auxin concentration determines a cellular response by a combinatorial system that counts the AUXIN RESPONSE FACTORS (ARFs) and the IAA co-factors (Figure 5A). ARF proteins are characterized by the presence of amino-terminal DNA-binding domain (DBD), a middle region that functions as an activation domain (AD) or repression domain (RD), and a carboxy-terminal dimerization domain (CTD) (Guilfoyle and Hagen, 2007). The DNA binding domain recognized specific sequence in the promoter of target genes, called AuxRE (Guilfoyle et al.,2007) (Figure 5A). In Arabidopsis, there are 23 ARF proteins. It has been shown that several ARFs are involved during ovule development. One of the most well-known and studied is the *AUXIN RESPONSE FACTORS 5/ MONOPTEROS* (*ARF5/MP*) that has been shown to

be important for ovule initiation and development (Galbiati et al., 2013; Cucinotta et al., 2021).

ARF5/MP is dynamically expressed during ovule development (Galbiati et al., 2013; Cucinotta et al., 2021): in particular, it is expressed from stage 2-I throughout ovule development in the chalaza, in the inner integument, funiculus, and in the basal part of the epidermal layer of the nucellus (Cucinotta et al., 2021). Another ARF expressed in the chalaza is *ARF3*, although it has been shown to affect MMC formation (Su et al., 2017). Recently, also *ARF17* has been found to be expressed and to play an important role in MMC specification (Huang et al., 2022).

Usually, the function of ARFs is repressed by AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins (Figure 5A). These proteins are characterized by four domain, domain I-IV (Ramos et al., 2001). The domain III and IV are required to bound ARFs whereas the domain II is of fundamental importance for AUX/IAA auxin-dependent regulation (Ramos et al., 2001). Indeed, auxin, binding to a specific sequence within domain II, acts as molecular glue between AUX/IAAs and the nuclear auxin receptor TRANSPORT INHIBITOR RESISTANT1/AUXIN F-BOX(TIR1/AFB) in SKP1-CULLIN1-F-BOX (SCF) ubiquitin ligase complexes that ubiquitinate AUX/IAA driving their proteolytic degradation (Kepinski and Leyser, 2005) (Figure 5A).

Based on the domain II (DII) of AUX/IAA and on the AuxRE sequence of ARFs, two reporter lines for auxin distribution, *DII-Venus* (Brunoud et al., 2012) and auxin response *DR5* (Ulmasov et al., 1997) were made, although, in 2015, Liao and colleagues improves both reporter lines, named them as *R2D2* (used in this work) and *DR5v2* (Liao et al., 2015) (Figure 5B).

R2D2 relies on auxin-dependent degradation DII domain of AUX/IAA and therefore, its expression can be visualized only in absence of auxin. When ovules arise from the placenta *R2D2* is broadly expressed except for the top of the nucellus where auxin accumulates. In developing ovule, *R2D2* expression localizes in the central part of the chalaza, and in the basal region of epidermal layer of the nucellus (Cucinotta et al., 2021). At the end of megasporogenesis its expression is restricted to the bottom of the nucellus, and in few cells of the chalaza (Cucinotta et al., 2021) (Figure 5B). During gametogenesis *R2D2* is expressed in the chalazal domain of developing ovule (Larsson et al., 2017, Wang et al.,2021) (Figure 5B). By the contrast, *DR5v2* promoter activity depends on the presence of auxin, and it gives indication of auxin response in planta

(Liao et al., 2015). Since ovule formation, DR5v2 expression localizes at the top of the nucellus, where an auxin maximum is formed, and this pattern of expression is maintained throughout the sporogenesis process (Cucinotta et al., 2021) (Figure 5B). Interestingly, at the end of megasporogenesis, it localizes near the degenerating megaspores (Wang et al., 2021). During megagametogenesis, the activity of DR5v2 has been reported first outside and later also inside the developing embryo sac (Pagnussat et al., 2009, Panoli et al., 2015) (Figure 5B).

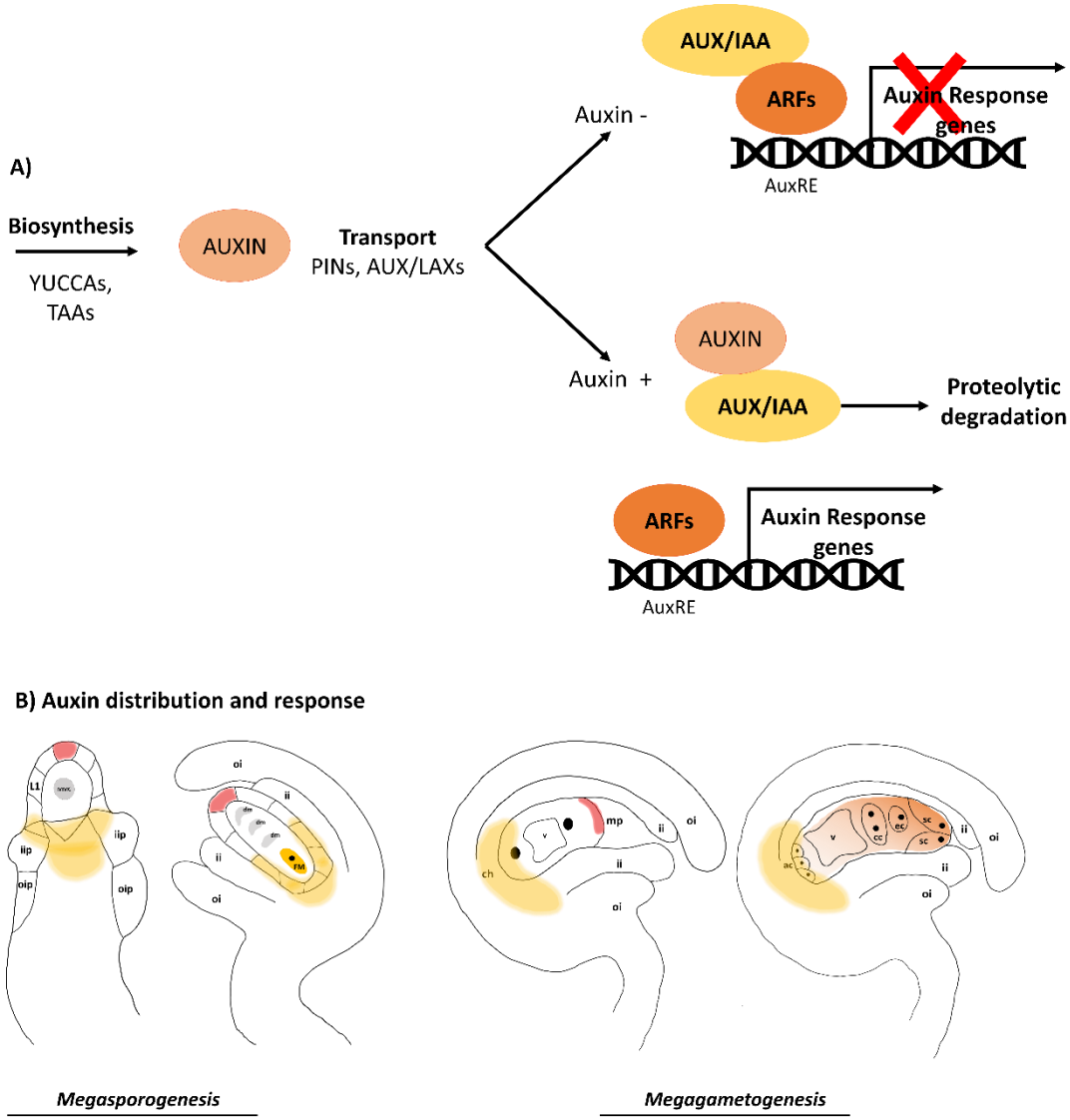


Figure 5 Auxin metabolism, distribution and response A) Auxin is mainly sinthetized by YUCCAs and TAAs enzymes. In planta, auxin is polarly transported by PINs and AUX/LAX1 proteins that allow the distribution and accumulation of auxin within the different cellular compartments. Auxin response depends on two class of proteins, the AUX/IAA and ARFs. In absence of auxin, AUX/IAA repress ARF activity and consequently auxin response. By the contrast, in presence of auxin, AUX/IAA are proteolityc degraded and ARFs can promote auxin

response transcription. B) Schematic representation of auxin distribution and response. During ovule development, two reporter marker line can be used to analyse auxin distribution and response, DR5 and R2D2. During megasporogenesis, auxin accumulates at the top of the nucellus where it creates an auxin maximum (dark orange). During gametogenesis auxin is accumulate at the micropylar and it has been proposed that it forms a gradient within the embryo sac. By the contrast, auxin minimum (yellow) accumulates in the chalaza region of developing ovules. oip, outer integument primordia; iip, inner integument primordia; MMC, megaspore mother cell; oi, outer integument primordia; ii, inner integument primordia; dm, degenerating megaspore; FM, functional megaspore; mp, micropyle; ch, chalaza; sc, synergid cell; ec, egg cell; cc, central cell; ac, antipodal cells; v,vacuole.

The dynamic auxin distribution is required to control proper development of ovules. Indeed, the auxin involvement in ovule development came already in 2000, when Nemhauser and colleagues (Nemhauser et al., 2000) demonstrated that transient application of N-1-naphthylphthalamic acid (NPA, an auxin transport inhibitor) causes significant reduction of ovules number that was observed also in associaton with a severe reduction of local auxin biosynthesis (Nole-Wilson et al., 2010). Indeed, inflorescences treated with NPA show a great inhibition of *TAA1* expression (Nole-Wilson et al., 2010; Stepanova et al., 2008). Ovule number reduction was also observed in *yuc1yuc4* mutant defective in auxin biosynthesis, that cannot develop any reproductive organs (Cheng et al., 2006).

More severe phenotype was observed for the embryo sac of the double mutant *yuc1yuc2* that exhibited mis-positioning of the nuclei within the cells at the micropyle with two cells at the normal position of the egg cell and only one at the synergid position (Panoli et al., 2015). In addition, the 10% of embryo sac contained multiple egg cell, replacing the synergids. Despite these defects the seed set wasn't significantly affected (Panoli et al., 2015). Interestingly, mutation in *YUC8* is gametophytic, and affects the female gametophyte development which results delayed, altered in mitotic division and nuclear migration, similar to other mutants affecting auxin biosynthetic pathway (Panoli et al., 2015). On the contrary, gain of function of *YUC1* extend the micropylar cell identity (in particular synergid and egg cell identity) to the chalaza (Pagnussat et al., 2009).

Also, alteration in auxin transport leads to defect in ovule and embryo sac development. For instance, in the strong mutant allele *pin1-1* flower formation is rare and when produced, flowers contain empty and malformed pistils (Benkova et al., 2003). By the contrast the weaker mutant allele, *pin1-5* can form pistils that, however, have a high reduction in ovule number (Bencivenga et al., 2012). In addition, *pin1-5* ovule exhibited

defects in integument and in the embryo sac development that arrest at early stage of gametogenesis (Bencivenga et al., 2012) similarly to what observed in embryo sac in which *PIN1* is downregulated (Ceccato et al., 2013). Embryo sac arrest at early stage of gametogenesis was also observed in mutant defective for the efflux carrier *AUX1, LAX1* and *LAX2* and analyses on different combination of mutant suggested that AUX1, LAX1 and LAX2 are redundantly required for normal female gametophyte development (Panoli et al., 2015).

Defect in regulation of ARF impaired ovule development. For instance, the ARF5/MP mutant, *mp S319* produces few flowers with a reduced number of organs, and the pistils do not contain ovules (Cole et al., 2009; Lohmann et al., 2010) due to lack of the placenta (Galbiati et al., 2013). Mis-regulation of *ARF3* and *ARF17* leads to supernumerary MMC in the nucellus (Su et al., 2017, Huang et al., 2022). In addition, it has been reported that *ARF17* is able to recover the inability of *nzz/spl* phenopyte to form MMC, by inducing *PIN1* expression (Huang et al., 2022). Instead, ARF3 has been shown to be involved in integument formation, together with ABERRANT TESTA (ATS) (Kelley et al., 2012).

Last, downregulation of ARFs through artificial microRNA, altered female gametophyte cell identities; in fact, synergid cell identity was completely lost and the egg cell identity gained (Pagnussat et al., 2009).

To summarize, auxin distribution is required for ovule and female germline development. In particular, at the beginning of the megasporogenesis, it is required for MMC specification (Becnivenga et al., 2012, Su et al., 2017, Huang et al., 2022) and late in sporogenesis for nucellar degeneration (Wang et al., 2021). During gametogenesis a correct auxin distribution is required for embryo sac development but also for specification of the female gametophyte cells (Pagnussat et al., 2009)

Cytokinin-Auxin crosstalk

It has been shown that hormonal crosstalk is involved in several developmental pathways. For instance, cytokinin-auxin crosstalk is involved in the control of the shoot apical meristem (SAM), where cytokinin promote the expression of meristematic gene *WUSCHEL* (*WUS*) within the organizing centre, that in turn together with AUXIN RESPONSE FACTOR5/MONOPTEROS, (ARF5/MP) represses the negative cytokinin response regulators ARR7 and ARR15 (Zhao et al., 2010). Furthermore, ARF5/MP promotes the expression of the HISTIDINE PHOSPHOTRANSFER 6 (AHP6) which interfere with cytokinin signalling (Gaillochet and Lohmann, 2015).

In the early phases of gynoecium development, cytokinin promotes the expression of auxin biosynthetic genes, *YUC1* and *YUC4* and *PIN7* and repress *PIN3* expression allowing auxin accumulation at the apex (Müller et al., 2017). By the contrast auxin repress cytokinin via AHP6 (Müller et al., 2017).

During ovule formation, two NAC genes, CUPSHPAED COTYLEDONS 1 and 2 (CUC1 and CUC2), have been shown to play an important role in auxin-cytokinin crosstalk (Galbiati et al., 2013). Indeed, ARF5/MP directly binds and activates *CUC1* and *CUC2* expression that in turn are required to induce *PIN1* expression (Galbiati et al., 2013). CUC1 and CUC2 repress the transcription of UGT genes, encoding for enzymes that catalyse the reversible inactivation of cytokinin (Cucinotta et al., 2018). The positive control on PIN1 is exerted also by the cytokinin response factors CRF2, CRF3 and CRF6 that allows the formation of auxin maxima where new ovules are formed (Cucinotta et al., 2016).

The positive control of cytokinin on PIN1 occurs also during megasporogenesis. It has been reported that in the triple mutant *ahk2 ahk3 ahk4/cre1* PIN1 is downregulated (Bencivenga et al., 2012) causing similar ovule phenotype respect to *pin1-5* mutant.

Moreover, it has been shown that cytokinin promotes the expression of *NOZZLE/SPOROCYTLESS (NZZ/SPL)* required for the formation and specification of the MMC and for PIN1 expression in the nucellus (Bencivenga et al., 2012).

During sporogenesis and gametogenesis, auxin and cytokinin are specifically accumulated in opposite domains. Cytokinin are mainly present in the chalaza whereas auxin is accumulated at the top of the nucellus and later accumulates at the micropyle region (Pagnussat et al., 2009). This peculiar distribution of the two hormones is needed for ovule development including also female germline specification.

We focused on unravel the role of cytokinin-auxin crosstalk during megasporogenesis trying to better characterize the molecular pathways controlled by these two hormones.

Chalaza development is required for both integuments and female gametophyte development

It has been reported that the chalaza region is important for the integuments formation but also it affects the female gametophyte development (Bencivenga et al., 2011). Many genes encoding for transcription factors or other type of proteins have been identified as of pivotal importance to form properly integuments.

For instance, the mitochondrial ribosome protein HUELLENOS, has been shown to control development of both integuments. The *hll* mutant ovules fail to develop integuments beyond the initial step of integument primordia formation (Schneitz et al., 1998). The MMC formed tetrads and sometimes binucleated embryo sac is seen although soon after degenerate (Schneitz et al., 1998).

Interestingly, also the homeobox gene *WUSCHEL*, which is specifically expressed in the nucellus, affects and promotes integument development. Indeed, *wus* mutant doesn't develop any integument structures mirroring *ant* mutant (see next paragraph) and the embryo sac never developed. By the contrast, when *WUS* is ectopically expressed in the chalaza, it induces ectopic integuments formation, suggesting that *WUS* is able to promote integument formation in a non-cell autonomously (Groß-Hardt et al., 2002).

Female sterility due to alteration in chalaza/integument development was observed also in *sin1* (SHORT INTEGUMENT/ DICER-LIKE 1) mutant ovules (Robinson-Beers et al., 1992). In *sin1* ovules, the two integuments are initiated but they never covered the nucellus and the embryo sac development is arrested even earlier than formation of the tetrad (Robinson-Beer et a., 1992). Similarly, mutation in SHORT INTEGUMENT 2, which encodes for a mitochondrial DAR GTPase (Hill et al., 2006), affect integument development but also MMC formation. Indeed, in *sin2* ovules, integuments are initiated but their development is arrested shortly after and the megaspores are never formed (Broadhvest et al., 2000).

In 1997, Schneitz and colleagues analysed several sporophytic and megagametogenesis-defective mutants (Schneitz et al., 1997). All of them showed defect in integument formation and/or development affecting the female gametophyte development. For instance, it has been reported that mutation in UNICORN (UCN, *ucn*) affected

integument formation and the embryo sac was usually but not always missing (Schneitz et al., 1997). Semi-sterility, was observed also in another mutant *sub* (STRUBBELIG) in which outer integument is more prominent, often forms protrusions and it doesn't encapsulate neither the nucellus nor the inner integuments. Also, in this mutant the embryo sac development is variably affected as well as for *mog (MOLLIG)* mutant ovules in which both inner and outer integuments displayed balloon-shaped cells (Schneitz et al., 1997).

Instead, in *bag* (BLASIG), *lal* (LAELLI) and *lug* (LEUNIG) mutant ovules, although integuments are initiated and megaspore mother cell originates, the organogenesis of the integuments is never completed and mature embryo sacs are never observed (Schneitz et al., 1997). Similarly, also in *tso1* (*TSO1*) integument and embryo sac development are initiated but both are never fully developed (Hauser et al., 1998).

Reduction in fertility was, also, observed in mutants affecting the expression of *SUPERMAN* (*SUP*) that it is involved in the control of inner integument development (Gaiser et al., 1995). By the contrast, in *ino* mutant, the outer integument development is affected as well as the fertility. *INNER NO OUTER* (*INO*) encodes a YABBY transcription factors which is involved in the control of abaxial-adaxial polarity (Baker et al., 1997; Villanueva et al., 1999).

Also, other genes involved in the control of ovule polarity are also important for integument and female gametophyte development. For instance, the HD-ZIP III, *PHABULOSA* (*PHB*) affects the formation of ovule and the outer integument. Indeed, homozygous *phb-1d* mutant plants are completely sterile and don't form any ovules (McConnell and Barton, 1998). Heterozygous *phb-1d/PHB* plants form ectopic ovules outside the carpel that resemble *ino* mutant phenotype since the outer integument development is compromised (Sieber et al., 2004). Furthermore, PHB collaborates with the other HD-ZIP III, *PHAVOLUTA* (*PHV*) and *CORONA* (*CNA*) in the control of outer integument development (Kelley et al., 2009); in fact in *phb phv cna* the reduced fertility is likely attributable to defects in ovule development since most of them had short integuments (Prigge et al., 2005). Moreover, HD-ZIP III act together with the KANADI gene, *ABERRANT TESTA* (*ATS/KAN4*) in integument morphogenesis (Kelley et al., 2009). In particular, ATS acts with REVOLUTA (*REV*) to restrict expression of *INO* and to promote outer integument growth (Kelley et al., 2009). Loss of function *ats* leads to fusion of inner and outer integuments (Leon-Kloosterziel et al., 1994). Interestingly, it has been shown that ATS physically interacts with ETTIN/ AUXIN

RESPONSE FACTOR 3 (*ETT/ARF3*) to restrict *PIN1* expression and to define integument boundary (Kelley et al., 2012).

It has been reported that also, the ovule identity MADS-Box genes *SEEDSTICK* (*STK*), SHATTERPROOF *1* (*SHP1*), *SHATERPROOF 2* (*SHP2*) are involved in integument development. In the triple mutant *stk shp1 shp2* integuments are converted in carpelloid structures (Pinyopich et al., 2003). In addition, mutation in *STK, SHP1, SHP2* enhanced the phenotype of other two mutants that will be discussed in the next paragraph: *bel1* and *ant4*. Indeed, the phenotype observed in *bel* and *ant* gets worst in the quadruple mutant *bel1 stk shp1 shp2* (Brambilla et al., 2007) and *ant-4 stk shp1 shp2* (Losa et al., 2010).

Recently, also two KNOX genes, *KNAT3* and *KNAT4* have been shown to be involved in integument development via regulation of *INO* (Chen et al., 2023). In *knat3 knat4* integuments don't develop and they are replaced with Integument-like Structures (ILS) and phenocopy *bel* mutant ovules.

All the above cited mutants exhibited integuments defects that leads to alteration in embryo sac development some of them are presented in Figure 6.

In the next paragraph, we focus on the role of two transcription factors, BELL1 (BEL1) and AINTEGUMENTA (ANT) in integument initiation and development.

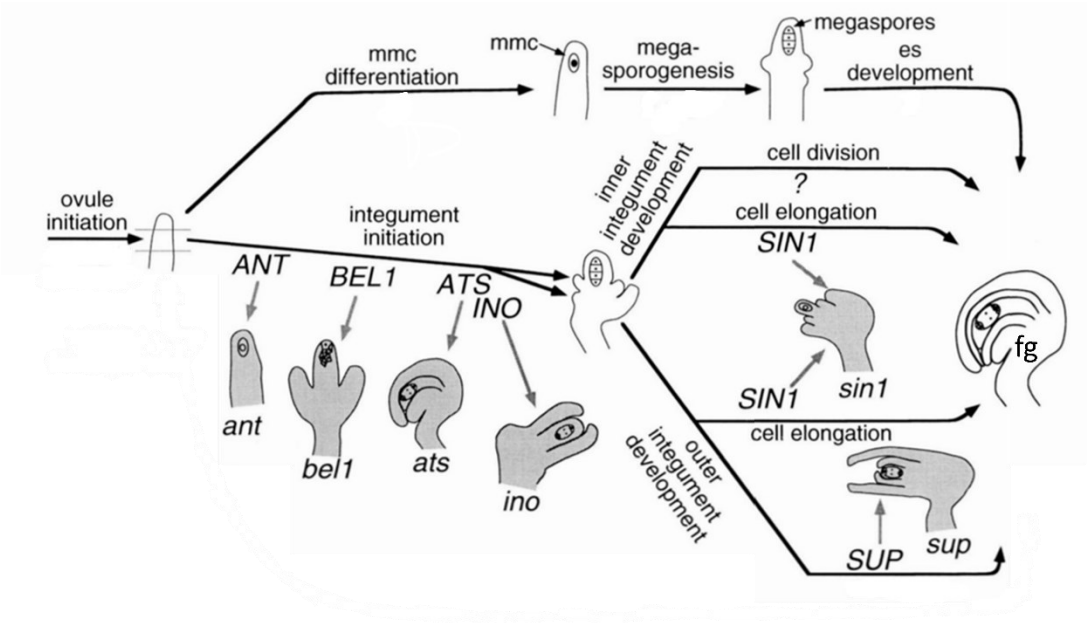


Figure 6 Schematic representation of some genes involved in integument formation. Once ovule arise from the placenta, two main developmental pathways are started. On one side, there is the germline development, that starts with the specification of the MMC and continues to form mature female gametophyte (fg). The second pathway controls the integument development that is required to provide the female gametophyte of all the necessary to correctly develop. Adapted from Baker et al., 1997.

The transcription factors AINTEGUMENTA (ANT) and BELL1 (BEL1)

The transcription factor AINTEGUMENTA (ANT) belongs to the AP2-like family protein, and it is involved in control cell proliferation, organ size, floral organogenesis including floral organ initiation, growth and identity (Elliott et al., 1996; Krizek, 2011a; Mizukami and Fischer, 2000).

Besides other functions, ANT plays an important role in ovule primordia formation (Galbiati et al., 2013) and integument initiation (Baker et al., 1997; Elliott et al., 1996; Klucher et al., 1996). Indeed, *ant* mutant plants form less ovule primordia respect to wild type and *ant* ovules never develop integuments (Galbiati et al., 2013; Baker et al., 1997, Elliot et al., 1996, Klutcher et al., 1996). ANT collaborates with auxin, both to induced ovule primordia and to promote formation of integuments. Indeed, it has been shown that ANT is directly bound by ARF5/MP to promote formation of new ovules. Moreover, it has been reported that in *ant-T* mutant ovules, the promoter of *YUC4* is not actived (Li et al., 2021), suggesting that the auxin and ANT might collaborate to promote integument formation and growth. After all it has been already shown that auxin and ANT collaborate in organ initiation and growth (Krizek, 2011; Krizek et al., 2020).

In the chalaza, ANT repress the *NOZZLE/SPL* (*NZZ/SPL*) (Balasubramanian and Schneitz, 2000) and it is required for activation and maintenance of *INO* expression as suggested by analyses on the activity of INO promoter in *ant-4* ovules (Meister et al., 2004) and by *in situ* hybridization experiment (Balasubramanian and Schneitz, 2002). Interestingly, mutation in *ant* recovered the MMC defect of *nzz/spl* (Balasubramanian and Schneitz, 2000). Indeed, in 60% of *ant nzz/spl* ovules, a MMC was observed although it never developed in a mature embryo sac (Balasubramanian and Schneitz, 2000).

It has been shown that ANT regulates expression of *PHABULOSA* (*PHB*) (Sieber et al., 2004) and this regulation was confirmed by ChIP sequencing analyses from which came out that ANT might directly and positively regulate *PHABULOSA* (*PHB*) and other genes related to auxin biosynthesis such as *TAA1*, and signalling for example, *ARF6*, *ARF11* and *ARF18* (Krizek et al., 2020).

As previously said, ANT regulates cell cycle as well cytokinin; cytokinin seems to positively affect the expression of *ANT*, indeed, in biosynthetic defective mutant

expression of *ANT* resulted highly reduced whereas exogenous treatment of cytokinin rapidly increase *ANT* transcription (Randall et al., 2015).

On the contrary, cytokinin negatively affect *BELL1* (*BEL1*) expression that resulted downregulated after BAP treatment (Bencivenga et al., 2012)

The transcription factor BEL1 has been characterized for its role in integument development (Modrusan et al., 1994; Ray et al., 1994; Reiser et al., 1995; Robinson-Beers et al., 1992). All the allelic mutant variants of BEL1 (*bel1-1, bel1-2, bel1-3, bel1-4, bel1-5, bel1-6, bel1-7, bel1-8,*) are female sterile and integuments are replaced with outgrowths called, integument-like structures (ILS). These structures arise at the stage 2-II, and it has been shown that they first acquired nucellar identity expressing *WUSCHEL* (*WUS*) and later carpel identity as suggested by the ectopic expression of *CRABS CLAW*, a carpel identity gene (Yamada et al., 2019).

BEL1 is required for activating and maintaining *INO* expression. GUS reporter assay revealed that the promoter of *INO* is transiently active in *bel* mutant whereas in situ hybridization couldn't detect any expression of *INO* in *bel* mutant (Balasubramanian and Schneitz, 2002; Meister et al., 2004).

In situ hybridization experiment shown that in the chalaza, *BEL1* restricts the expression of *NOZZLE/SPOROCYTLESS* (*NZZ/SPL*) (Balasubramanian and Schneitz, 2000) but also of *PIN1* which result ectopically expressed in *bel* ovules (Bencivenga et al., 2012).

In addition, BEL1 itself but also collaborating with the HD-ZIP III, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *CORONA* (*CNA*), restricts the expression of *WUSCHEL* (*WUS*) (Yamada et al., 2016)

Although, the two transcription factors, ANT and BEL1 are both involved in control of chalaza identity and development by repressing *NZZ/SPL* and activating *INO*, *ant bel* ovules exhibited *ant* phenotype, suggesting that ANT is epistatic on BEL1. However, it is most likely that they control integument development through different pathways that converges on same targets to control the integument development (Balasubramanian and Schneitz, 2000).

BEL1 belongs to the TALE homeoprotein superfamily which comprises: BEL-like (BELL) and KNOTTED-like (KNOX) homeodomain proteins (Bürglin, 1997; Reiser et al., 1995). BELL proteins are characterized by three domains: at the N-terminal, the SKY domain and the BEL domain, which together form the MEINOX Interacting Domain (MID) and at the C-terminal there is the Homeodomain (HD) (Bellaoui et al.,

2001; Hamant and Pautot, 2010). KNOX proteins have constituted of three domains as well: the MEINOX domain, through which they interact with BELL proteins, the ELK and the HD domains (Hay and Tsiantis, 2010). BELL and KNOX proteins can form heterodimers through the interaction between their respectively, MID and MEINOX domains, whereas HD domain of BELL proteins is required for partner selection and target affinity (Bellaoui et al., 2001) (Figure 7A). It has been shown that, the cellular localization of BELL proteins depends on the interaction with KNOX (Figure 7A); indeed, in the BEL domain there are two sequences called NES (Nuclear Exporting Sequence) that are recognized by nuclear exporting receptors (CMR1, Figure 7B) which re-localizes BELL proteins out from the nucleus into the cytoplasm (Hamant and Pautot, 2010; Rutjens et al., 2009). However, when the heterodimers are formed the two sequences are hidden and the BELL-KNOX complex can be targeted to the nucleus (Bellaoui et al., 2001; Cole et al., 2006; Hamant and Pautot, 2010). The activity of the heterodimers is controlled and repressed by OVATE family proteins (AtOFP) which bind the BELL-KNOX complex and relocate the dimers to the cytoplasm (Hackbusch et al., 2005) (Figure 7B).

Yeast two hybrid assay shown that BEL1 interacts with the KNOX proteins, STM, KNAT1, KNAT2, KNAT5 (Bellaoui et al., 2001) and KNAT6 (Hackbusch et al., 2005), among which only KNAT5 is expressed within ovule (Furumizu et al., 2015). It has been shown that BELL-KNOX heterodimers control several developmental pathways (Hackbusch et al., 2005; Hamant and Pautot, 2010; Hay and Tsiantis, 2010). Indeed, they are involved in SAM and boundary maintenance, leaf shape, phyllotaxis, in the regulation of flowering transition, in fruit and ovule development (Hamant and Pautot, 2010; Hay and Tsiantis, 2010). For instance, the overexpression of *BEL-like HOMEODOMAIN 1* (*BLH1*) as it occurs in *eostre-1* mutant, leads to alteration in nuclear migration and cellularization of the embryo sac that at the maturity presents multiple egg cells at the expense of synergids (Pagnussat et al., 2007). Interestingly, mutation in *KNAT3* (KNOX gene) that has been reported to interact with BLH1 (Hackbusch et al., 2005), partially rescue *eostre* phenotype (Pagnussat et al., 2007), although, neither *BLH1* nor *KNAT3* are expressed in the embryo sac (Pagnussat et al., 2007). Moreover, ectopically expressing under a germline promoter, *BLH1* and four KNAT genes (*KNAT3,4,5,7*) since earlier stage of female gametophyte development, leads to formation of multiple functional MMC, which are able to form multiple embryo sac (Bezodis et al.).

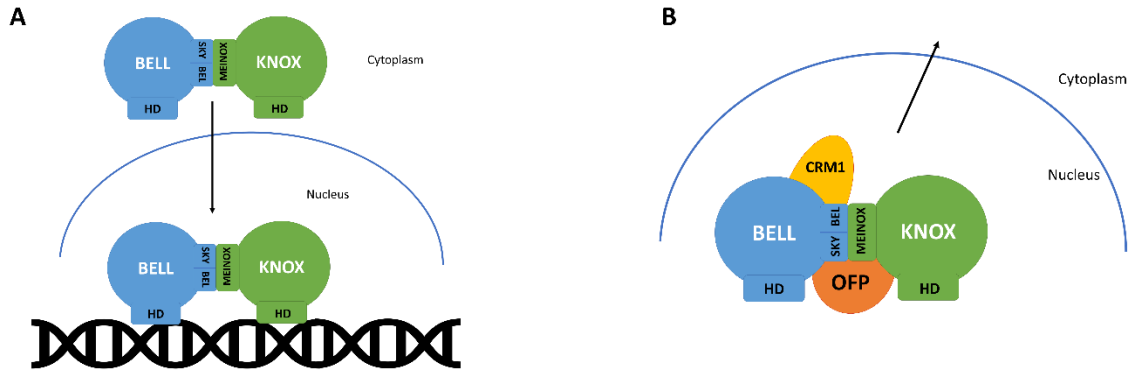


Figure 7 Mechanism of action for BELL and KNOX proteins. A) BELL proteins interact with KNOX proteins through respectively MID (BELL-SKY) and MEINOX domains. This interaction allows the translocation of the complex in the nucleus where they can bind the DNA thanks to their HD domain. B) The regulation of BELL-KNOX complex activity is operated by OFP proteins which bind the complex and re-locate it in the cytoplasm. On BELL protein an additional control is exerted by the nuclear exporting protein (CRM1) that recognized NES (nuclear exporting sequence) sequence within BEL domain.

AIM OF THE PROJECT

Ovule development consists of two phases: the megasporogenesis and the megagametogenesis. During megasporogenesis, female germline is established in the nucellus, and it divides by meiosis producing four haploid spores. Meanwhile, below the nucellus, the chalaza originates the inner and the outer integuments. These structures protect the female gametophyte throughout its development, but they also function as routes for all the signals required for the female gametophyte development and function. The megasporogenesis ends when the most three apical spores degenerate and the only one surviving differentiate in Functional Megaspore (FM). The FM undergoes megagametogenesis which consists of three rounds of mitosis followed by cellularization, at the end of which, the mature female gametophyte is formed.

Ovule development is a tightly regulated process, that involves several transcription factors and hormones. It was already shown that auxin and cytokinin are involved in several development pathways among which also the ovule development. Since hormones act as signal, a correct organogenesis of ovule is required to allow the proper hormone distribution. Indeed, several mutant defectives in integument organogenesis exhibited sterility and alteration in female gametophyte development. For instance, in *ant-4* and *bel1-1* mutants, integuments never properly formed, and a mature female gametophyte never originates. The two transcription factors BELL1 (BEL1) and AINTEGUMENTA (ANT) have been characterized for their role in control of integument organogenesis, however how they can affect the female gametophyte development is still unclear.

To unravel the role of BEL1 and ANT within the complex molecular network controlled by auxin and cytokinin during female gametophyte development, we decided to:

- Analyse the female gametophyte development in *bel* and *ant* mutant ovules.

- Study the cytokinin and auxin signalling pathway in *bel* and *ant* ovules.
- Propose a molecular model controlling female gametophyte development involving *BEL1*, *ANT*, auxin and cytokinin.

To achieve these goals, we first analysed female gametophyte progression in *bel* and *ant* ovules by microscopical analysis, and by observing different marker lines for the female gametophyte specific stages. In addition, we exploited cytokinin signalling and auxin distribution in both mutants, by analysing specific reporter lines.

The analyses and results obtained, are presented in the first two chapters of this thesis.

Instead, in the last chapter, I have unravelled one of the roles of cytokinin during megagametogenesis.

We, have obtained transgenic plants the expressing *IPT1* gene, involved in cytokinin biosynthesis, under the control of *MYB98* promoter that was supposed to be specific for synergid cells. However, we have observed defects in *pMYB98::IPT1* ovule in early megagametogenesis stages due to early MYB98 promoter activation, showing that cytokinin level is of pivotal importance during all megagametogenesis phases. embryo sac on the fertility of the transgenic line, by counting the viable seeds, the aborted seeds and the unfertilized ovules.

RESULTS

Chapter 1
The role of BELL1 during megasporogenesis

Morphological description of *bel1-1* mutant ovules

Ovule development starts when the primordium arises from the placenta (stage 1-I; (Schneitz et al., 1997b) and elongates in a finger-like structure. Soon after, the inner and outer integument primordia develop from the chalaza (stage 2-II) (Figure 8A) and start to grow surrounding the nucellus (Figure 8B). At the end of the megasporogenesis the two integuments almost enclose the female gametophyte (Figure 8C) and during gametogenesis they grow to wrap almost completely the embryo sac leaving only an aperture in correspondence to the micropylar, that is required for the pollen tube to enter into the female gametophyte to fertilize the egg cell and the central cell (Figure 8D)

Until stage 2-II, the development of *bel1-1* ovules doesn't differ from the one observed in wild-type ovule (Figure 8E); however, from stage 2-IV, instead of the two integuments a globular structure starts to form in the chalaza (Figure 8F), that develops in a single integument-like structure (ILS), which arises asymmetrically on both sides of the chalaza (Figure 8G). The ILS expands and acquires first nucellar identity and indeed they were referred as ectopic nucella, and later they convert into carpelloid structures (Figure 8H; (Yamada et al., 2019) as suggested also by the morphological carpelloid shape that they acquire at the stage 3-II. At this stage, all the nucellus start to degenerate (dc, Figure 8H), and consequently *bel* mutant plants resulted female sterile.

The homeodomain transcription factor BELL1 (BEL1) has been described as one of the major factors involved in the control of the chalaza development (Reiser et al., 1995; Robinson-Beers et al., 1992) and its pattern of expression within ovules reflects its function. At the stage 2-I in wild-type ovules, *BEL1* is expressed in the chalaza region in correspondence of the integument primordia (Figure 8I-8J) and later in the developing integuments (Figure 8K-8L). Once the megasporogenesis has been occurred, the expression of *BEL1* is restricted to few cells of the chalaza surrounding the developing embryo sac (Figure 8M-8N).

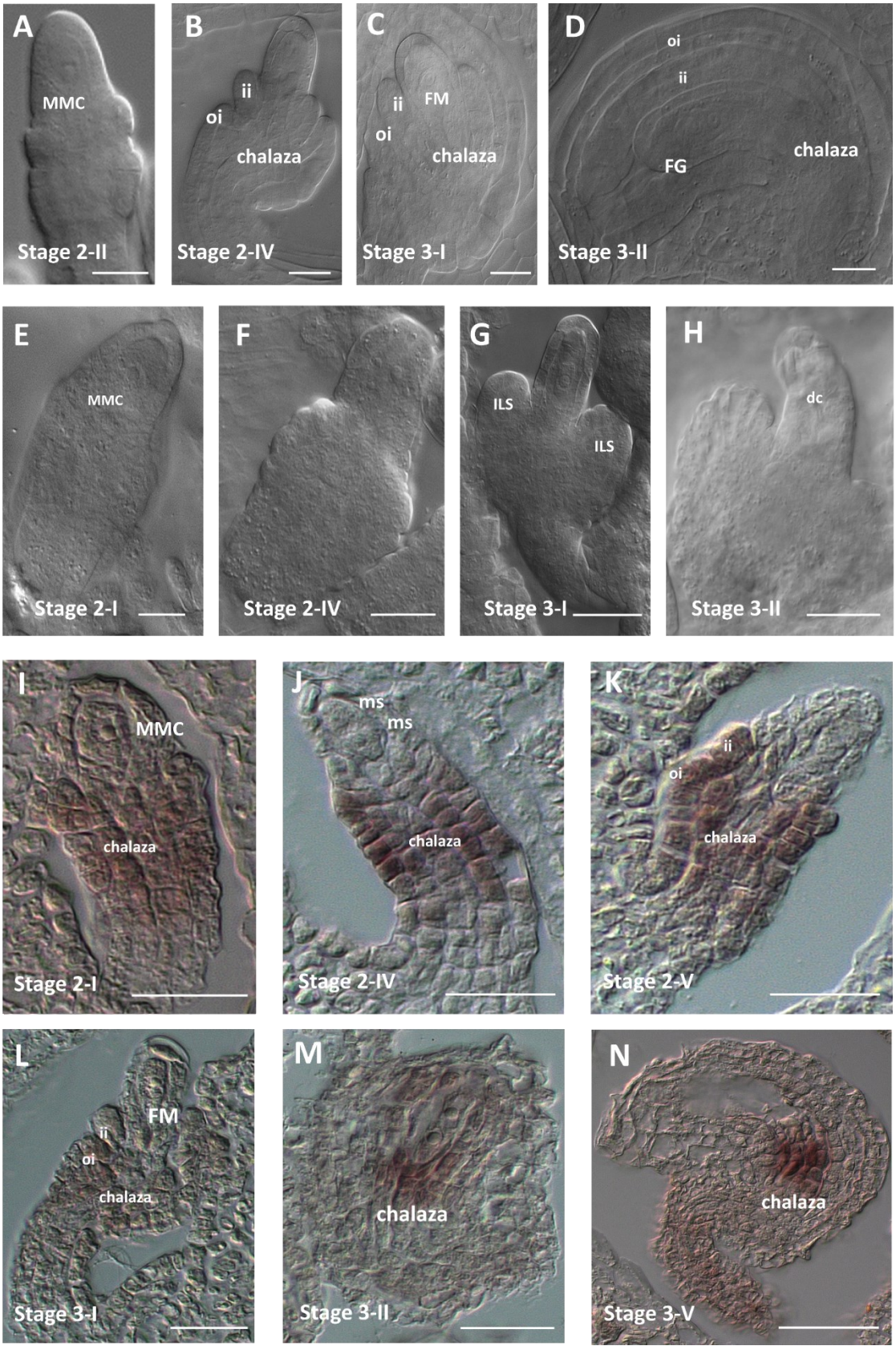


Figure 8 Morphological analyses of *bel1-1* ovules and *BEL1* pattern of expression.
(A-D) Wild-type ovule development. (A) At the stage 2-I one somatic cell differentiates into MMC and undergoes meiotic division to produced four haploid spores. (B) Meanwhile at the chalaza region the inner and outer integument primordia start to arise. (C) Three spores

degenerate and the most chalaza differentiated in FM. (D) The FM undergoes megagametogenesis to produce the mature FG. (E-H) *bel1-1* ovule development. (E) At the stage 2-I, MMC differentiate in the nucellus. (F) Neither the inner nor the outer integument primordia arise from the nucellus of *bel1-1* mutant ovules. (G) Integument like structures (ILS) arise from the chalaza at the stage 3-I of *bel1-1* ovules. (H) All the nucellar content degenerate and the ILS acquired carpelloid shape. At early stage of ovule development (I) BEL1 is expressed in the (J-K) funiculus and in the region where integuments arise (J-K). At the end of megasporogenesis *BEL1* is expressed in the chalaza and in integuments. Later in ovule development *BEL1* is confined to few cells of the chalaza (M-N).
MMC, megaspore mother cell; oi, outer integument; ii, inner integuments; FM functional megaspore; FG, female gametophyte. Scale bar 10 μm

Megasporogenesis in *bel1-1* ovules is impaired

Despite the clear defects in integuments organogenesis of *bel1-1* ovule, morphological analyses revealed that also the female germline development is impaired, suggesting that even if *BEL1* is specifically expressed in the chalaza, it can also affect the nucellus development.

We, therefore, decided to deeper investigate the megasporogenesis and gametogenesis in *bel1-1* mutant ovules.

The megasporogenesis starts when a somatic cell, within the nucellus, expands and acquires the Megaspore Mother Cell (MMC) identity. We analysed the MMC specification in *bel1-1* mutant ovules by analysing the expression of *pKNU::nlsYFP*, marker specific for cell that specifies into MMC (Tucker et al., 2012). In wild-type ovules, *pKNU::nlsYFP* is expressed from stage 2-I in the MMC (Figure 9A) which is localized in the middle of the nucellus. During the megasporogenesis, the megaspores express the marker until stage-2-V, when three of them degenerate.

At the stage 2-I, the 79,9% (143/179 ovules) of *bel1-1* mutant ovules expressed *pKNU::nlsYFP* in the MMC (Figure 9B).

The 6,1% (11/179 ovules) of *bel1-1* ovules expressed *pKNU::nlsYFP* at the late stages of development (Figure 9C), when the single integument like structure have been already formed and they have already acquired the nucellar identity. At this stage, the cell which expressed *pKNU::nlsYFP* seems to be pushed on one side of the nucellus. Although this cell belatedly acquired MMC identity we never observed it undergoing meiotic event or cellular division.

Last, the 14% (25/179 ovules) of *bel1-1* ovules expressed *pKNU::nlsYFP* in correspondence of a nucleus within the ILS that arise from the chalaza region at the late stage of *bel1-1* ovule development, (Figure 9D) supporting the fact that the ILS acquire a nucellar identity and can bear a MMC like nucleus. Although this cell belatedly acquired MMC identity we have never observed that this cell undergoes meiotic event or cellular division

To evaluate whether the MMC of *bel1-1* mutant ovules is able to undergo megasporogenesis process we followed the progression of the process by using Aniline blue staining which allows to mark the callose depositions and by analysing the expression of *ASY3::RFP*, a chromosome axis protein that is required for interhomolog recombination (Yang et al., 2019) which is specifically expressed during

megasporogenesis from stage 2-I to stage 2-V. At the stage 2-I, in both wild-type and *bel1-1* mutant ovules, the callose surrounds the MMC (Figure 9E and 9J). Following the first meiotic division, callose accumulates at the cell wall plate that is formed between the two megaspores (Figure 9F and 9K) and after the second meiotic division another accumulation of callose can be seen at the newly formed cell wall plates (Figure 9G and 9L).

The protein ASY3 is expressed at the condensed chromosome at the stage of MMC, and at the end of the megasporogenesis process it remains attached at the sister chromatids (Figure 9H-I). The pattern of ASY3 expression doesn't change in the mutant ovules compared with the wild-type (Figure 9M-N). So that the megasporogenesis process and all the meiotic machinery are not compromised in *bel1-1* mutant ovules.

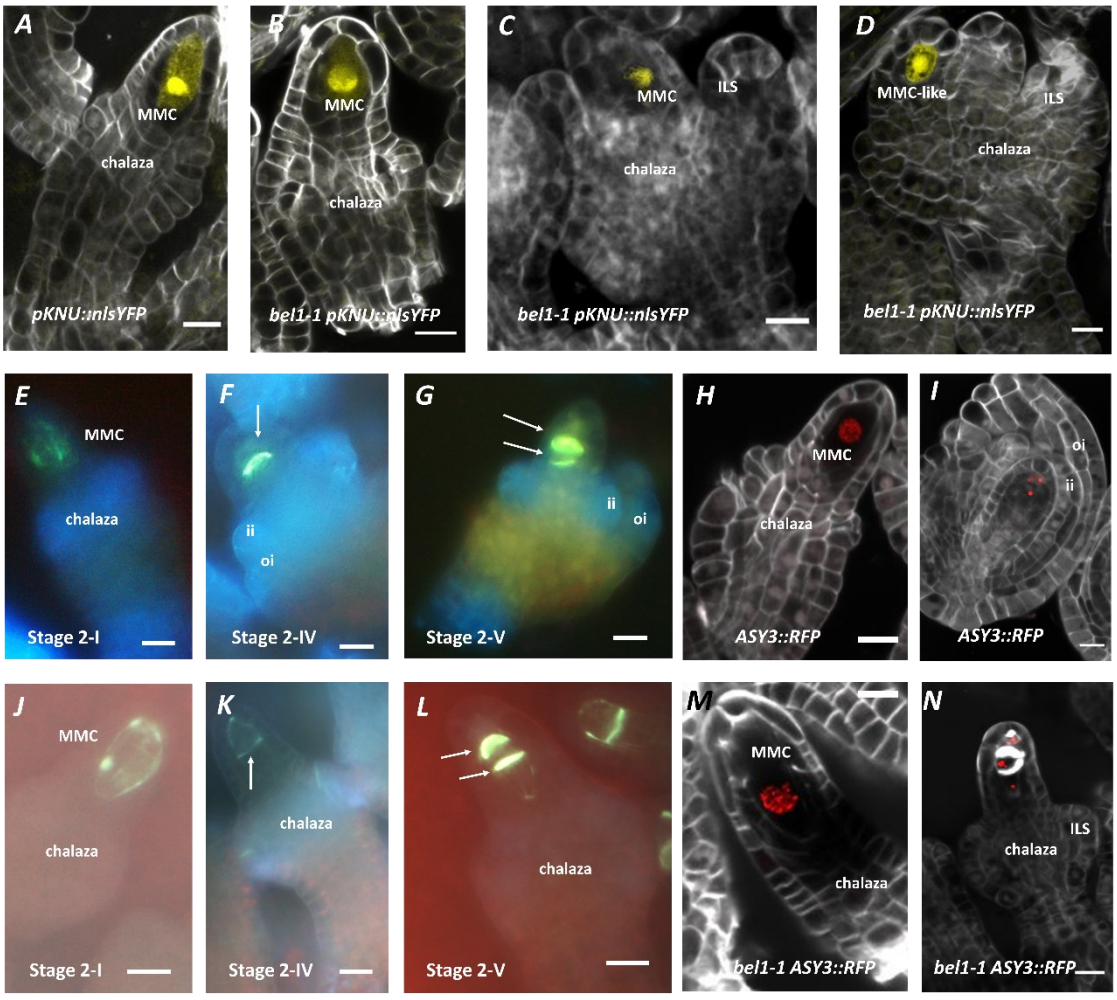


Figure 9 MMC specification and meiotic division in *bel1-1* mutant ovules.
(A) *pKNU::nlsYFP* in wild-type ovule. At the stage 2-I, *pKNU::nlsYFP* is expressed by the specified MMC within the nucellus. (B-D) *pKNU::nlsYFP* in *bel1-1* ovule. (B) *pKNU::nlsYFP* is expressed by the MMC in *bel1-1* at stage 2-I. (C) *pKNU::nlsYFP* is expressed at late stage of *bel1-1* ovule development in a cell pushed on one side of the nucellus. (D) In the ectopic

nucellus of *bel1-1* ovules, one cell can differentiate in the MMC and expresses *pKNU::nlsYFP*. (E-I) Megasporogenesis progression and machinery in wild-type ovule. (E) At the stage 2-I MMC is surrounded by callose that following the meiotic divisions is deposited (F-G) in correspondence of cell wall plates. The MMC expressed *ASY3::RFP*, an chromosome-associated protein, that remain attached to the sister chromatids also after megasporogenesis ends. (J)Also the MMC in *bel1-1* ovules resulted surrounded by callose and (K-L) the same pattern of callose accumulation that we observed in wild-type ovule was seen in *bel1-1* ovules. (M-N) In *bel1-1* ovules, *ASY3::RFP* expression was observed in correspondence of the MMC and also at the end of megasporogenesis as well as in wild-type. MMC, megaspore mother cell; oi, outer integument; ii, inneri integument; ILS, integument like structure. Scale bar 10 μm

The degeneration of megaspores and the functional megaspore specification are altered in *bel1-1* mutant ovules

The megasporogenesis ends when the most three apical megaspores degenerate, and the spore localized at chalaza pole survives and acquires the Functional Megaspore (FM) identity.

To assess whether these two final steps of megasporogenesis occur in *bel1-1* mutant ovules, we analysed the *pLC2::nlsYFP* (Tucker et al., 2012) marker that is specific for the Functional Megaspore.

At the stage 3-I in wild-type ovules, the *pLC2::nlsYFP* is expressed in correspondence of the chalaza spore that differentiates into FM (Figure 10A). After the first mitotic division that characterized the megagametogenesis process, the *pLC2::nlsYFP* is expressed in the two nuclei of the developing gametophyte (stage 3-II; Figure 10B).

In 49,1% (83/169 ovules) of *bel1-1* ovules, the FM identity is acquired by the spore more closed to the chalaza (Figure 10C). However, in most of the *bel1-1* ovules more than one spore survives after meiosis. Indeed, the 17,8 % (30/169 ovules) of *bel1-1* ovules expressed the *pLC2::nlsYFP* in correspondence of one spore, whose localization wasn't the most chalazal (Figure 10D)

In addition, in 12,4% of *bel1-1* mutant ovules (21/169 ovules) the *pLC2::nlsYFP* was expressed at the same time by two spores, supporting the fact that the degeneration of megaspores is compromised in *bel1-1* mutant ovules (Figure 10E)

Interestingly, we observed that in 18,3% (31/169 ovules) of *bel1-1* mutant ovules, the spore that acquired the FM identity can undergo mitotic event before all the nucellar content degenerates (figure 10F). Last, in few cases, only the 2,4% (4/169 ovules), we noticed that *pLC2::nlsYFP* was expressed in correspondence of two nuclei of the embryo sac like structure and by one spore above them, still separated by the cell wall plate (Figure 10G)

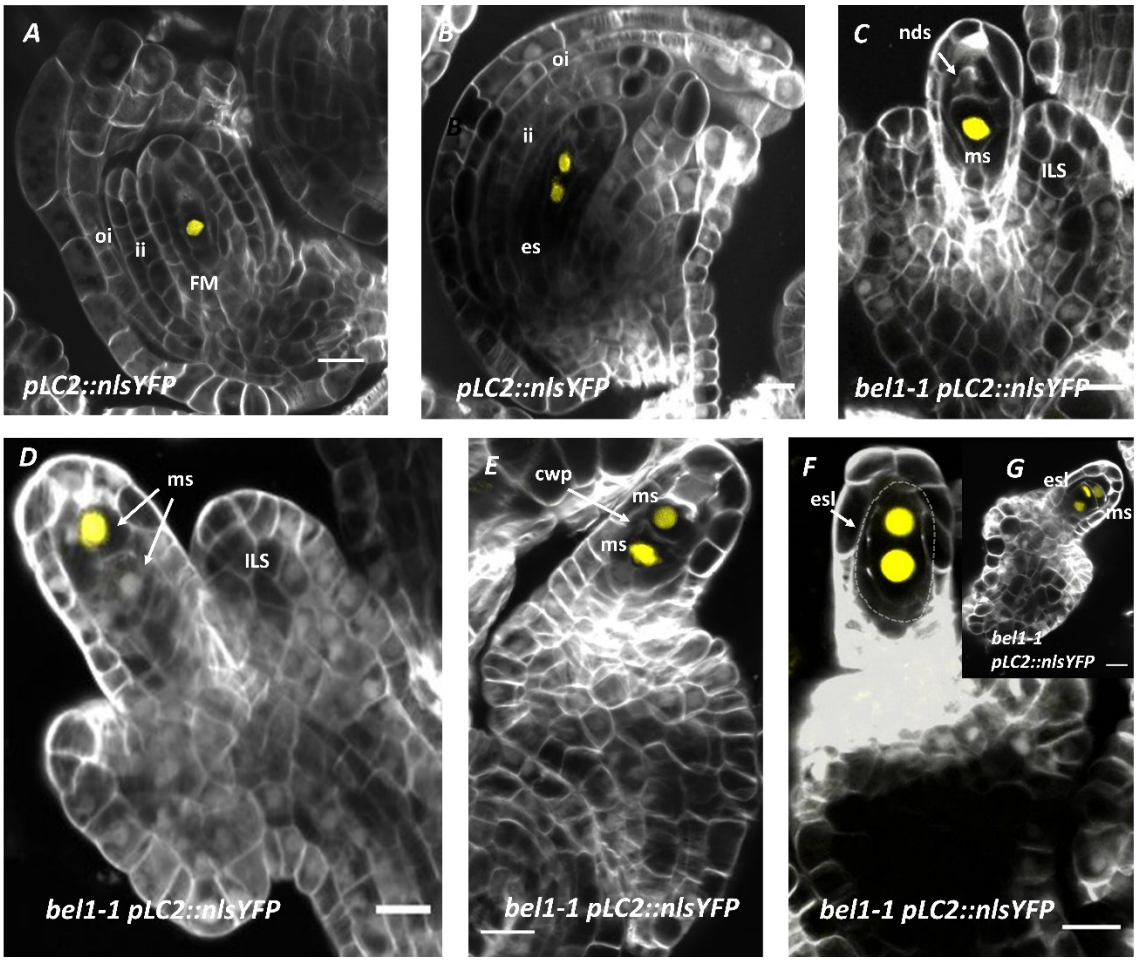


Figure 10 Specification of Functional megaspore in *bel1-1* mutant ovules.
(A-B) *pLC2::nlsYFP* in wild-type ovule. (A) At the stage 3-I, the most chalaza spore specify in FM and expresses *pLC2::nlsYFP*. Once specify the FM starts mitotic divisions and (B) *pLC2::nlsYFP* is expressed by the two nuclei of the embryo sac at the stage 3-II. (C-G) *pLC2::nlsYFP* in *bel1-1* ovule. (C) In less than half bel ovules, the most chalazal spore specify in the FM although one or more than one spores survives. (D) In *bel1-1* ovules the specification of the FM is altered, and the identity of FM is acquired by one 'non-canonical' spore or (E) at the same time by two spores. (F)Also in *bel1-1* ovules, the spore that acquired FM identity divides before all the nucellar content degenerate and in few cases (G) the mitotic division occurs even in presence of the surviving spore. FM, functional megaspore; oi, outer integument; ii, inner integument; es, embryo sac; nds, non-degenerated spore; ms, megaspore; ILS, integument like structure; cwp, cell wall plate; esl, embryo sac like structure.. Scale bar 10 µm

To assess whether the nuclei that acquired the FM identity derived from the meiotic event, we analysed the expression of *pWOX2::CENH3-GFP* (De Storme et al., 2016) in *bel1-1* ovules. This marker line allows to count the number the chromosomes of each nucleus within the megagametophyte from the FM stage (De Storme et al., 2016).

In wild-type ovules each nucleus after the megasporogenesis bears five chromosomes (Figure 11A-B), also in *bel1-1* mutant ovules every nucleus, within the forming embryo sac, have five chromosomes confirming that all the cells showing FM identity derive from the meiotic event. The analyses of this marker line allowed also to confirm that more than one spore survive after meiosis as observed previously in *bel* plants expressing *pLC2::nlsYFP* (Figure 11-C-G)

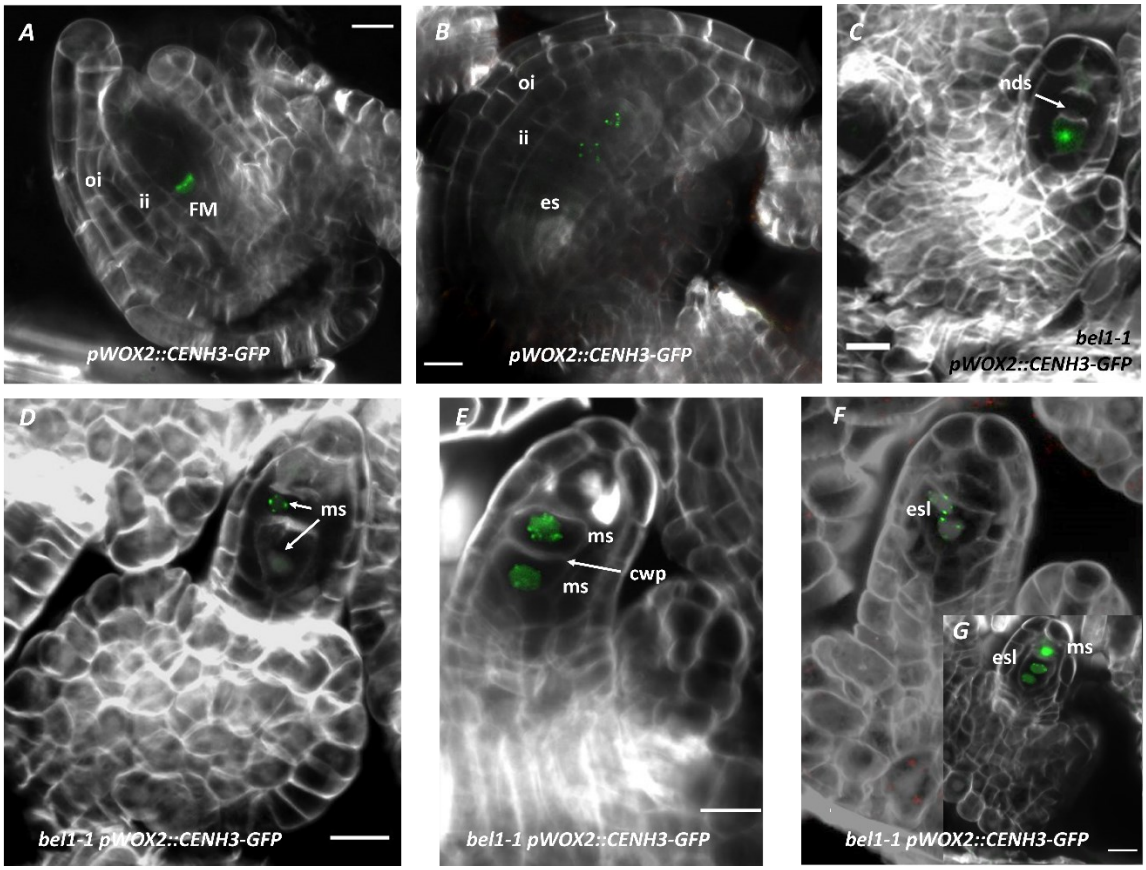


Figure 11 *pWOX2::CENH3-GFP* in wild-type and bel mutant ovules.
(A-B) In wild-type ovules after megasporogenesis the ploidy of FM is a reduced to five chromosomes since it has been produced by meiosis. *WOX2* promoter is active since FM throughout gametogenesis and drives the expression of the centromeric protein of histone 3, *CENH3*, fused with the GFP. (C-G) All the spores produced after megasporogenesis in *bel1-1* present five chromosomes so that all the formed spores derive from meiotic events. In addition, the expression of the reporter marker line in *bel* mutant ovules mirrors the pattern of expression of confirming that the degeneration of megaspores and the specification of FM is impaired in bel ovules. FM, functional megaspore; oi, outer integument; ii, inner integument; es, embryo sac; nds, non-

degenerated spore; ms, megaspore; cwp, cell wall plate; esl, embryo sac like structure. Scale bar 10 μm

Cytokinin signalling in *bel1-1* mutant ovules

It has been reported that cytokinin are involved in the control of ovule development (Bencivenga et al., 2012b; Cheng et al., 2013; Terceros et al., 2020) and negatively affect the expression of the transcription factor BELL1 (Bencivenga et al., 2012b).

To understand whether the defects of megasporogenesis in *bel1-1* ovule could be due to alteration in cytokinin perception or signalling we analysed, first the expression of cytokinin receptors, AHK2, AHK3 and AHK4/CRE1 and then the cytokinin signalling reporter marker, *TCSn::GFP* (Zürcher et al., 2013) in *bel1-1* ovules.

The *AHK2* and *AHK3* are ubiquitously expressed within the ovule (Figure 12A-B, 12E-F) whereas *CRE1/AHK4* is specifically expressed in the chalaza region (Figure 12 I-J) (Bencivenga et al., 2012b; Cheng et al., 2013). In *bel1-1* ovules this pattern of expression remained unchanged and at least the perception of cytokinin within *bel-1* ovules seems to be similar respect to wild type (Figure 12C-D, 12G-H and 12K-L).

Therefore, we moved on studying the cytokinin signalling. As already shown by Zürcher and colleagues, in wild-type ovules the expression of *TCSn::GFP* accumulates throughout the megasporogenesis at the chalaza, just underneath the nucellus (Figure 12M-N). In *bel1-1* mutant ovules, the spatial expression of the marker mirrors the ones observed in wild-type ovules and no changes were observed (Figure 12O-P)

We planned to measure the content of cytokinin by performing immunolocalization analyses in *bel1-1* and wild-type ovules.

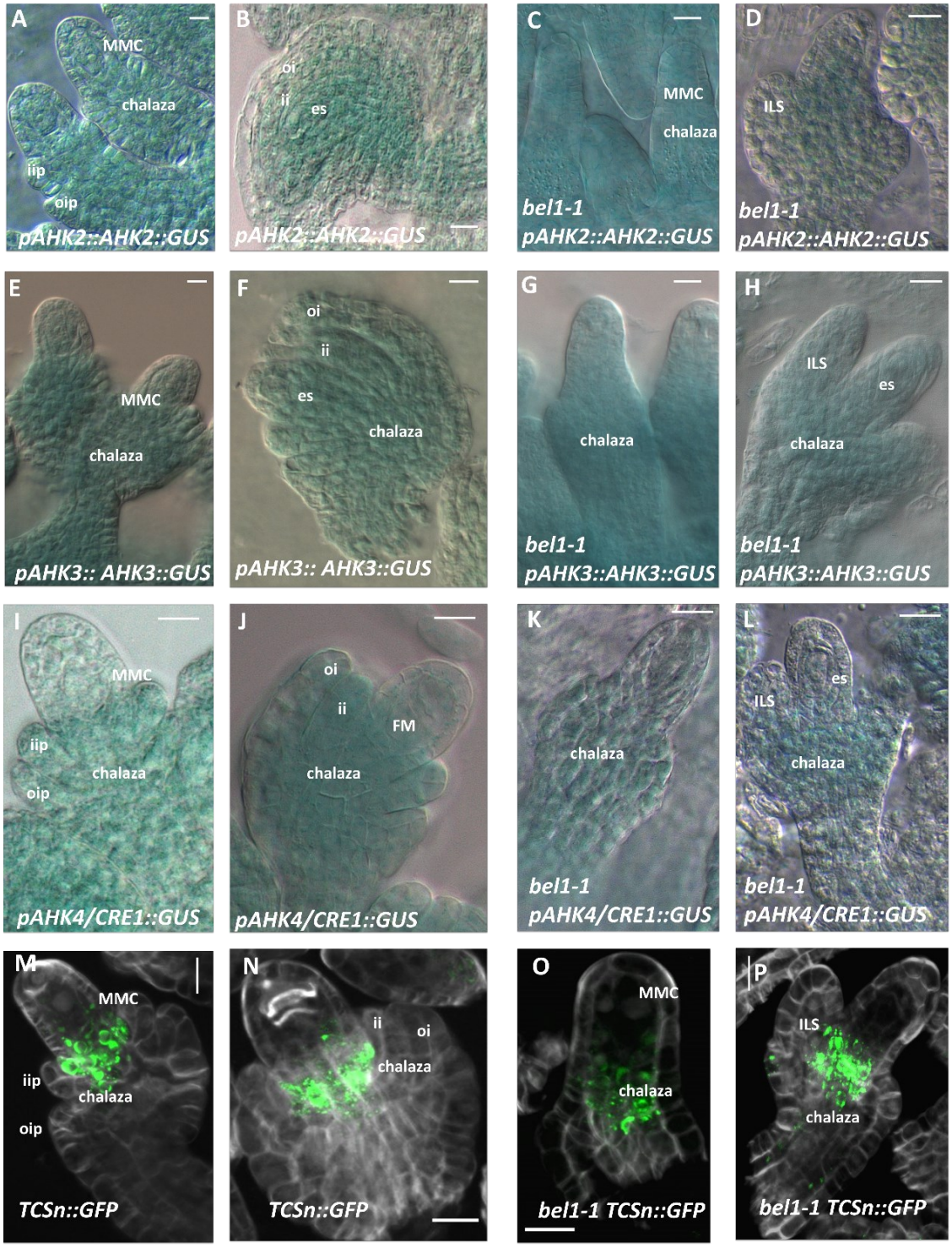


Figure 12 Cytokinin perception and signalling in bel1-1 ovule. (A-B) *pAHK2::AHK2::GUS* in wild-type. Throughout ovule development *AHK2* ubiquitously expressed as well in (C-D) *bel1* ovule. Also *pAHK3::AHK3::GUS* within wild-type (I-J) and *bel1* (K-L) ovules is widely expressed during megasporogenesis. The activity of *AHK4/CRE1* promoter in wild-type (I-J) and in *bel1* (C-D) ovules, localizes in the chalaza below the nucellus, for the entire length of megasporogenesis. The cytokinin reporter line, *TCSn::GFP* in wild-type (M-N) and in *bel1* (C-D) ovules, follows the same pattern of expression. It localizes in the chalaza just underneath the nucellar region, where at the end of the megasporogenesis, the FM specifies. MMC, megaspore

mother cell; es, embryo sac; oi, outer integument; ii, inner integument; ILS, integument like structure; oip, outer integument primordia; iip, inner integument primordia; FM, functional megaspore. Scale bar 10 μm

In addition, we decided to evaluate the expression of three CKs Response Regulators type A: *ARR4, ARR7* and *ARR15*. The response regulators *ARR4* has been already published to be expressed in the ovules (Cheng et al., 2013)and we confirmed this by *in situ* hybridization experiment, as well as, for *ARR7* and *ARR15*. *ARR4*, *ARR7* and *ARR15* belongs to the cytokinin primary response genes, thus their expression is directly dependent on cytokinin (Brandstatter and Kieber, 1998; To and Kieber, 2008; Werner and Schmülling, 2009). In stage 2-I, *ARR4* is expressed in the chalaza and around the MMC (Figure 13A), while at the stage 3-I *ARR4* transcript accumulates preferentially, in the chalaza region and in the integuments (Figure 13B).

ARR7 and *ARR15* are also expressed from early stage of ovule development; at the stage 2-I, *ARR7* is expressed along the epidermal layer of chalaza (Figure 13C) and later it is localized in the integuments (Figure 13D). Instead, *ARR15* is expressed in the nucellus (Figure 13E) at the stage 2-I and in later stages it expressed in the integuments (Figure 13F).

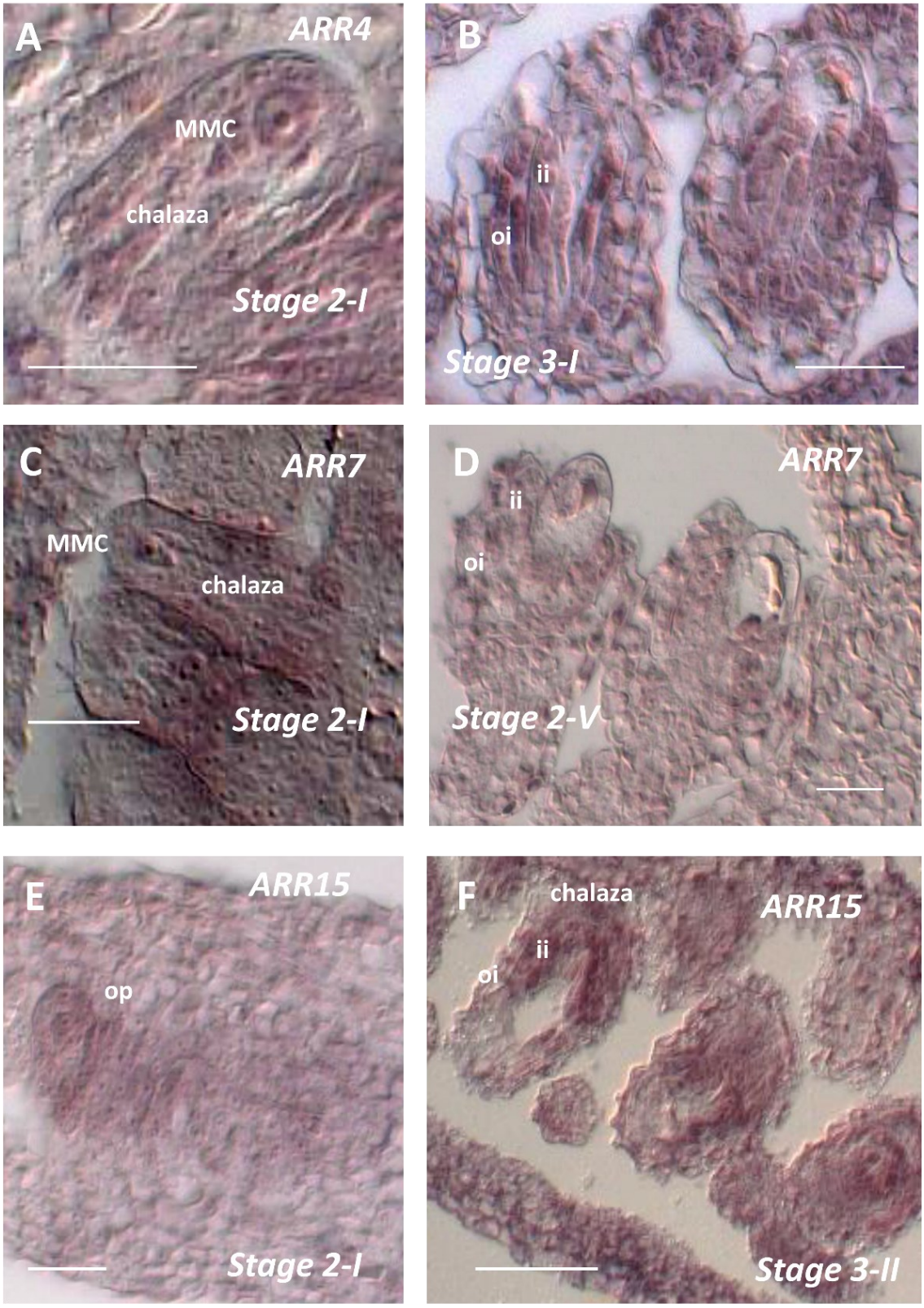


Figure 13 Expression pattern of ARR4,ARR7 and ARR15 within ovule.
The three Response Regulators of Cytokinin signalling are differentially expressed within the ovules. ARR4 is strongly expressed since stage 2-I (A), within the chalaza and in particular in integuments (B). ARR7 is slightly expressed within ovules, (C) at the stage 2-I is expressed in the most outer layer of the nucellus and already (D) at stage 2-V its expression seems to decrease. Instead, (E) ARR15 at the stage 2-I is poorly expressed in the ovule primordia, but

then (F) it accumulates in the chalaza region and along the inner integument of developing ovules. ii, inner integument; oi, outer integument; op, ovule primordia. Scale bar 10 μm

Auxin distribution in *bel1-1* mutant ovules

Auxin homeostasis is important for a correct development of the female gametophyte (Bencivenga et al., 2012b; Ceccato et al., 2013b; Pagnussat et al., 2009). In ovules, auxin is synthetized in correspondence of the chalaza from where it is conveyed at the tip of the nucellus by the auxin polar transporter PIN1 (Ceccato et al., 2013b). This peculiar transport of auxin allows the formation of an auxin maxima at the top of the nucellus. There, thanks to other three auxin polar transporters PIN3, PIN4 and PIN7, the auxin is distributed in the nucellus leading to the progression of the sporogenesis and gametogenesis (Wang et al., 2021).

Previously expression analyses have already shown that *PIN1* is upregulated in *bel1-1* ovules (Bencivenga et al., 2012b); indeed, in wild-type ovule, at the stage 2-II, PIN1 polarly localizes at the cell membrane of the epidermal layer of the nucellus, of the developing integument primordia in the chalaza, and in correspondence of the funiculus (Figure 14A). At the end of megasporogenesis instead PIN1 localizes only in the epidermal layer of the nucellus (Figure 14B). In *bel1-1*, PIN1 localized in the epidermal layer of the nucellus but its expression expands toward the chalaza resulting ectopically accumulated in the region where the integument-like structure that arise from the chalaza (Figure 14C-D).

We observed alteration of expression pattern also for of *PIN3* in *bel1-1* ovules. In wild-type ovules, PIN3 is expressed at the membranes of few cells of the nucellus tip, and from stage 2-V also in the funiculus (Figure 14E-F)

In *bel1-1* ovules, *PIN3* is still expressed at the tip of the nucellus but also in the chalaza, where the ILS arise and this expression pattern is maintained during megasporogenesis (Figure 14G-H)

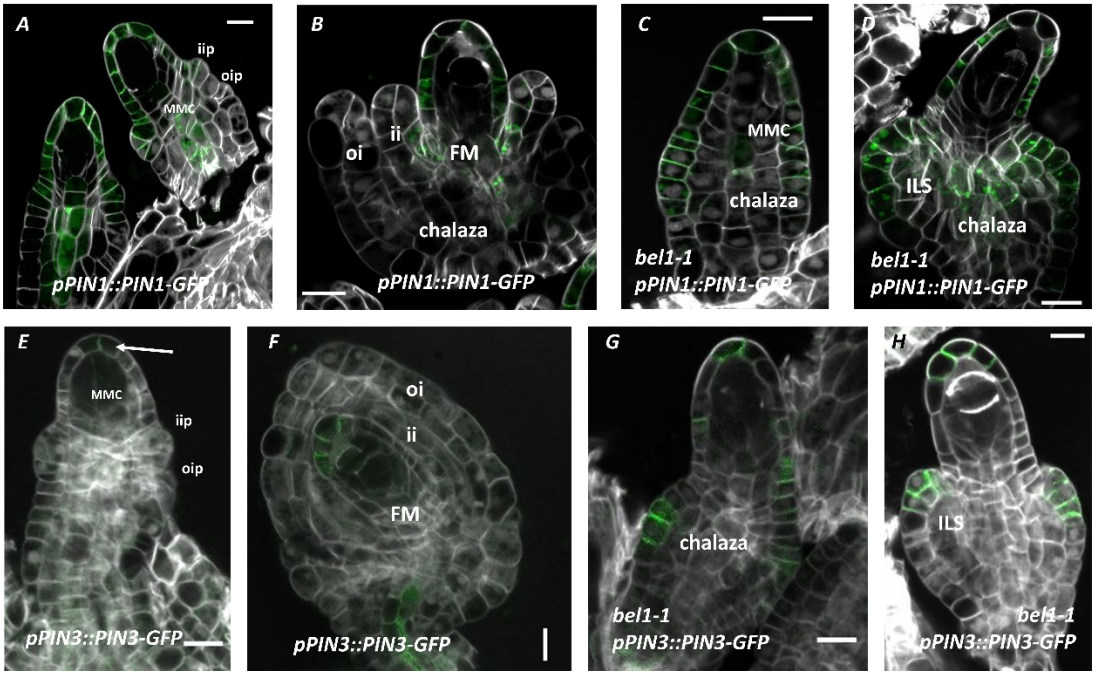


Figure 14 Expression of two auxin polar transporters, PIN1 and PIN3 in *bel1* ovules. (A-D) *PIN1* expression in wild-type and in bel ovules. (A)In wild-type ovules at stage 2-I , *PIN1* localizes at the nucellus, in integument primordia and in the funiculus and at the end of megasporogenesis, (B) it is restricted to few cells of the nucellus. (C) In *bel* ovule *PIN1* is still expressed in the nucellus but its expression expands along the chalaza and (D) at the late stages of *bel* ovule development *PIN1* becomes widely expressed within the chalaza. Similar misexpression was observed also for *PIN3*. (E) At the beginning of ovule development, *PIN3* is expressed in few cells of the nucellus, and (F) at the end of megasporogenesis *PIN3* accumulates also in the funiculus. (G) In *bel* ovules, *PIN3* is still expressed in few cells of the nucellus but also in the chalaza where (H) the ILS arise. MMC, megaspore mother cell; iip, inner integument primordia; oip, outer integument primordia; oi, outer integument; ii, inner integument; FM functional megaspore; ILS, integument like structure. Scale bar 10 μm

Therefore, since both auxin polar transporters PIN1 and PIN3 resulted mis-expressed within *bel1-1* ovules, we wondered whether this misexpression could affect auxin accumulation and/or distribution and thus explained the defect in the last phases of megasporogenesis that we observed in *bel1-1* ovules.

We, therefore, analysed the reporter marker *R2D2* which provides a useful tool to study the auxin distribution (Liao et al., 2015). This reporter marker relies on the auxin-dependent degradation, DII domain of AUX/IAA response factor, therefore, the expression of this reporter marker is visible in the absence of auxin so that in correspondence of what we are used referring to 'minimum' of auxin.

At the beginning of megasporogenesis, *R2D2* is expressed in the chalaza region close to the nucellus (Figure 15A). This pattern of expression is maintained until stage 3-I where beside the expression in the chalaza (Figure 15B), the 75% (102/136) of ovules, expressed the reporter marker in FM (Figure 15C), and in 25% (34/136) of ovules, *R2D2* expression was observed even after the first mitotic division in correspondence of the second nucleus that belonged to the embryo sac (Figure 15D)

In *bel1-1* ovules, at the beginning of the megasporogenesis, the R2D2 expression was similar to the wild-type and localized in the chalaza and closed to the nucellus (Figure 15E-F); at the stage 3-I, the 65,5% (99/151) of *bel1-1* ovules expressed *R2D2* in most chalazal spore that acquired the FM identity (Figure 15G), although the presence of the cell wall plate formed after meiotic division suggests the presence of more than one surviving spores.

In 14,6% (22/151) of mutant ovules, we observed *R2D2* expression in a spore placing in a different position (Figure 15H) and in 5,3% (8/151) of mutant ovules, we observed *R2D2* being expressed in two spores at the same time (Figure 15I).

We also observed *R2D2* expression in two nuclei that seemed to belong to the same embryo sac-like structure, suggesting that a mitotic event had occurred (Figure 15J). Last, similar to what we observed with *pLC2::nlsYFP*, we observed with a low frequency (0,7%, 1/151 ovules) ovule that present *R2D2* expression in two nuclei within the developing embryo sac like structure and in one non-degenerated spore, that is clear separated from the below structure by the cell wall plate formed during meiotic division. (Figure 15K).

We are fully aware that *R2D2* marker line cannot be used for 'FM' identity, however our analyses showed that the defects in megasporogenesis observed in *bel1-1* ovules were due to alteration in auxin distribution, and in particular due to the presence of

auxin minima that interfere with megaspores degeneration and thus the specification of the FM. Indeed, when we compared the frequency of the phenotype observed with the reporter marker line *pLC2::nlsYFP* and *R2D2* (Figure 15L), we noticed that the percentage are similar reinforcing our data.

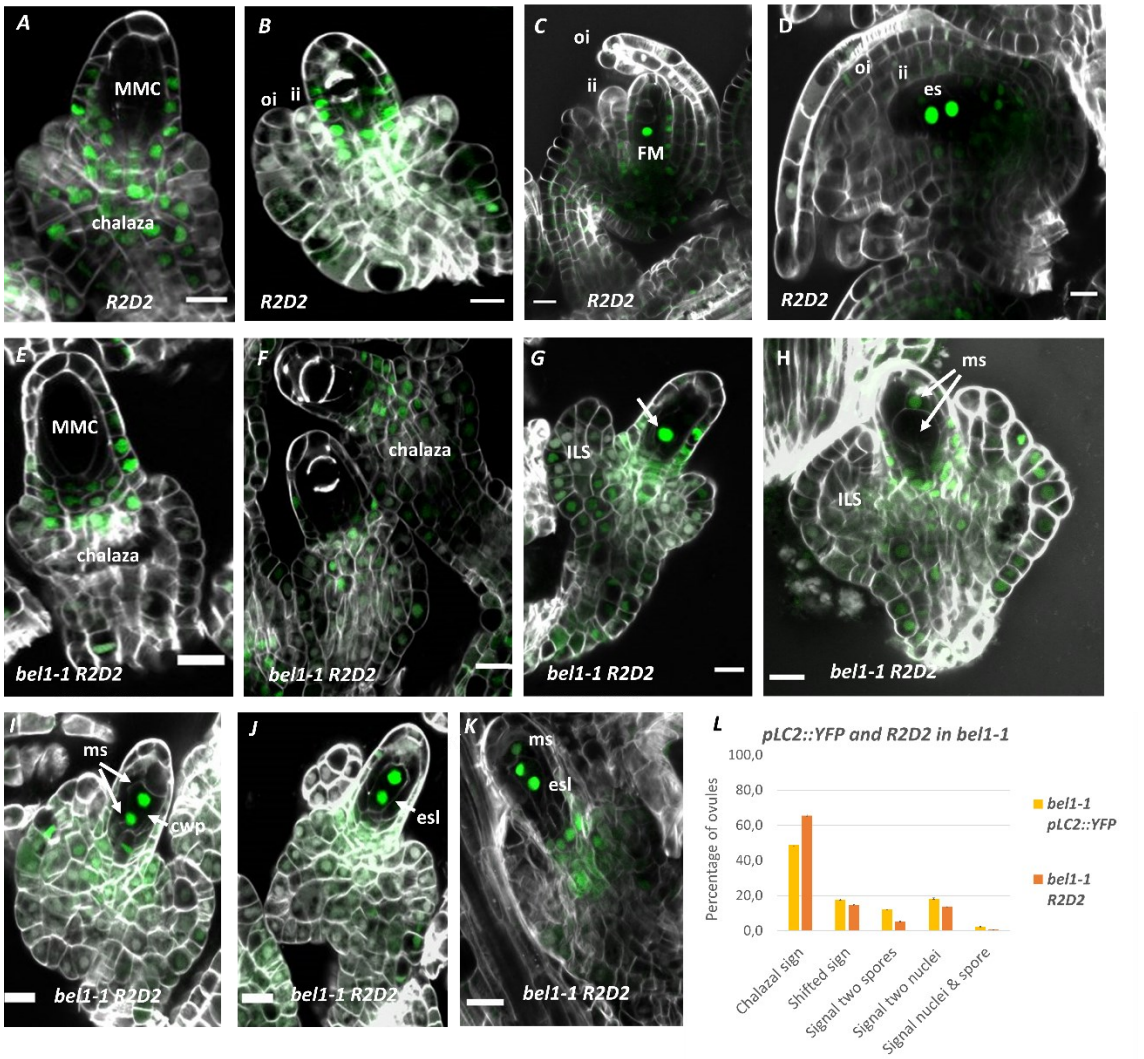


Figure 15 Auxin minima *R2D2* reporter line in *bel* ovules. (A-B) During megasporogenesis, in wild-type ovules, auxin minima formed in the chalaza region and along the basal part of the epidermal layer of the nucellus. (C-D) Once the FM specify, *R2D2* is expressed also in correspondence of the FM and following the first mitotic division in the two nuclei of the embryo sac at the stage 3-II. (E-F) During the first phases of *bel* ovules, the *R2D2* accumulates in the chalaza and in the L1 layer as well observed in wild-type and also in correspondence of the (G) most chalaza spore that is acquiring the FM identity. However, auxin minima reporter line *R2D2* was also seen (H) in correspondence of the spore whose position within the nucellus wasn't the canonical one, or even (I), at the same time in two different spores. In some cases, one of the spores which acquires FM identity (J) undergoes mitotic event even (K) in the presence of a surviving spore above. *R2D2:* the sequence of DII domain of AUX/IAA protein drives the expression of fluorescent protein Venus (green). MMC, megaspore mother cell; oi outer integument; ii, inner integument; FM functional megaspore; es, embryo sac; ILS, integument like structure; ms, megaspore; cwp, cell wall plate; esl, embryo sac like structure. Scale bar 10 µm. (L) Comparison between the frequency of the different phenotypes observed

with *pLC2::nlsYFP* and *R2D2* in *bel1-1* ovules. The frequency of the two reporter marker lines obtained during the expression analyses are similar. Data means (±SE).

It has been reported that the AUXIN RESPONSE FACTOR 5/MONOPTEROS (ARF5/MP) is important for ovule development (Cucinotta et al., 2021; Galbiati et al., 2013). Indeed, MP is involved in the regulation of *CYTOKININ RESPONSE FACTOR 2* (*CRF2*) and *CUP-SHAPED COTYLEDON 1* and *2* (*CUC1* and *CUC2*) that in turn regulates *PIN1* expression in a cytokinin dependent manner in ovule primordia (Cucinotta et al., 2016; Galbiati et al., 2013). Although it was always thought that Auxin Response Factors (ARFs) activates the transcription of their target genes in regions where auxin accumulates, so that in auxin maxima, *ARF5/MP* has been shown that in ovules can regulate its target transcription, in region of auxin minima (Cucinotta et al., 2021).

At the stage 2-I, *pMP::MP-GFP* (Schlereth et al., 2010) is expressed in the chalaza region, and in the basal part of the L1 layer of the nucellus (Figure 16A). Once the integument primordia arise (Figure 16B), *ARF5/MP* become restricted to the inner part of the chalaza and still in the L1 of the nucellus (Figure 16C). In *bel* ovules, *ARF5/MP* expression is similarly to the one observed in the wild-type ovules (Figure 16D-F) and at the later stage of *bel* ovule development, *pMP::MP-GFP* surrounded the ILS as it does with the ‘normal’ nucellus.

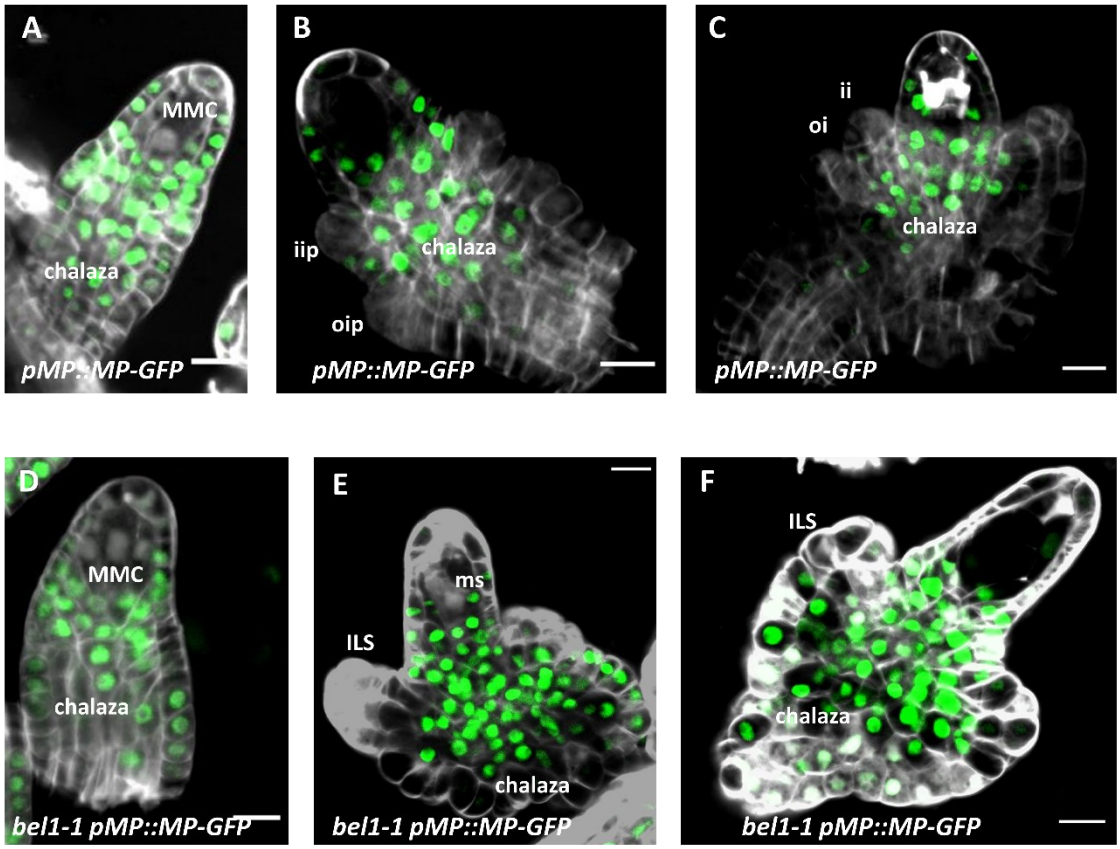


Figure 16 ARF5/MP expression in *bel* ovules.
(A-C*) pMP::MP-GFP* in wild-type ovules is expressed in the chalaza and in the L1 of the nucellus until the end of the megasporogenesis process, except for the inner and outer integuments. (D-F) *pMP::MP-GFP* in *bel* ovules follows the same pattern of expression that we observed in wild-type. MMC, megaspore mother cell; iip, inner integument primordia; oip, outer integument primordia; ii, inner integument ; oi, outer integument; ILS, integument like structure. Scale bar 10 µm

Overexpression of BELL1 within the ovule mimics *bel1-1* mutant phenotype

We have exploited the role of *BEL1* during the megasporogenesis by characterizing the mutant knock-out *bel1-1* line, however we wondered whether also the overexpression of *BEL1* could have some effect on ovule development.

Therefore, we decided to specifically increase the level of *BEL1* within the ovule by driving its expression using *SEEDSTICK* (*STK)* promoter. We, first, assessed that *STK* expression doesn't change in *bel* mutant background by looking at the expression of the reporter marker *pSTK::STK-GFP* (Mizzotti et al., 2014). In wild-type ovules, *STK* is expressed along the epidermal layer of developing ovules (Figure 17A). At the stage 2-III *STK* is expressed in the funiculus, in the chalaza except for the region where the inner integument is forming and in the epidermal layer of the nucellus (Figure 17B). This pattern of expression last until stage 3-II (Figure 17C-D). Similar pattern of expression was also observed in *bel1-1* mutant ovules (Figure 17E-H).

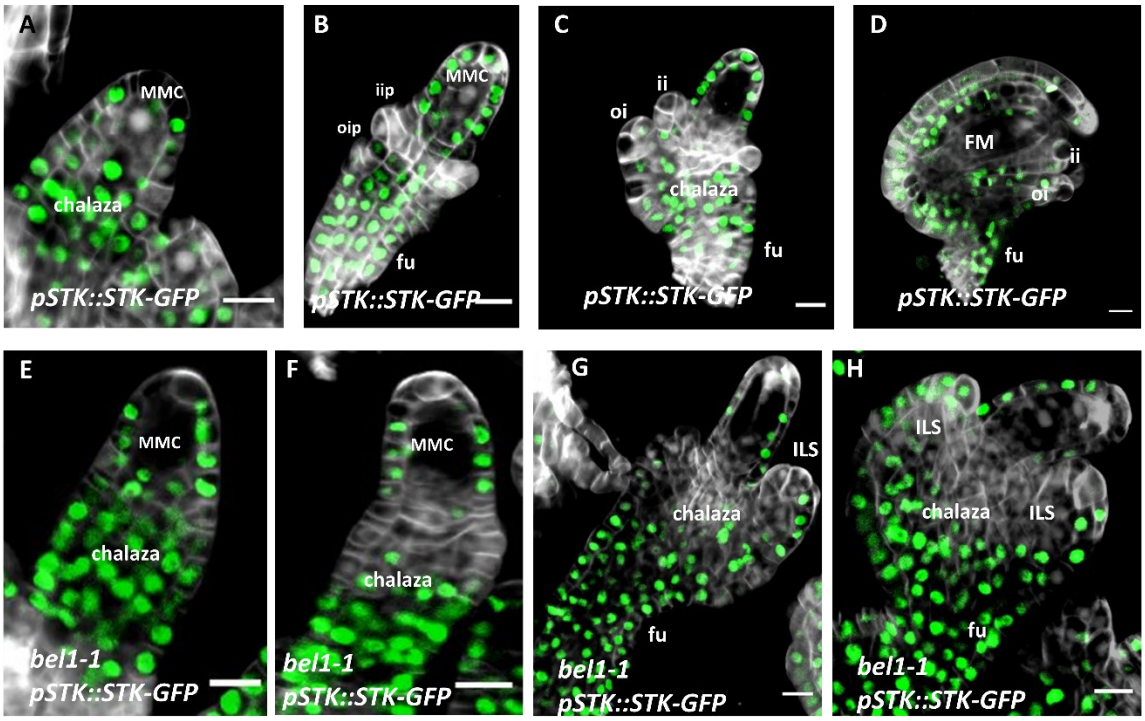


Figure 17 *Pstk::STK-GFP* expression in wild-type and bel1 ovules.
Pstk::STK-GFP expression in (A-D) wild-type ovules localizes in the funiculus, in the chalaza except for the inner integuments and for the region just underneath the nucellus, and in the epidermal layer of the nucellus. The expression of STK in *bel1* ovules (E-F) doesn't change, indeed, it follows the same pattern that we observed in wild-type ovules. MMC, megaspore mother cells; iip, inner integument primordia; oip, outer integument primordia; ii, inner

integument; oi, outer integument; FM, functional megaspore; fu, funiculus; ILS, integument like structure. Scale bar 10 μm

Therefore, since *STK* expression isn't affected in *bel* mutant background we could use it to drive the expression of *BEL1* in wild-type background to mimic an overexpression of *BEL1*. Until stage 2-II, *pSTK::BELL1* ovules developed as wild-type ovules (Figure 18A), but at the stage 2-II, instead of two integuments, one ILS structure arise from the chalaza (Fgiure 18B) and then a second ILS (Figure 18C), like in *bel1-1* ovules. These structures then acquired carpelloid identity (Figure 18D) as it happens in *bel1-1* mutant ovules. We also performed aniline blue staining to follow the progression of megasporogenesis in *pSTK::BELL1* and it seems that the meiotic divisions normally occur (Figure 18E-F) but then the female gametophyte development is arrested. Since from previously analyses, we hypothesised that the defect in megasporogenesis in *bel1-1* mutant ovule were due to alteration in auxin distribution, we speculated that also in *pSTK::BELL1* the defects in ovule development could be due to alteration in auxin distribution, as well. We therefore, analysed the pattern of expression of *pPIN1::PIN1-GFP* (Benková et al., 2003) within *pSTK::BELL1* ovules. Interestingly, the expression pattern of *PIN1* in *pSTK::BELL1* mimics the one observed in *bel1-1* mutant ovules suggesting that the overexpression of *BEL1* leads to a mis-distribution of auxin that avoids the correct ovule development (Figure 18G-H).

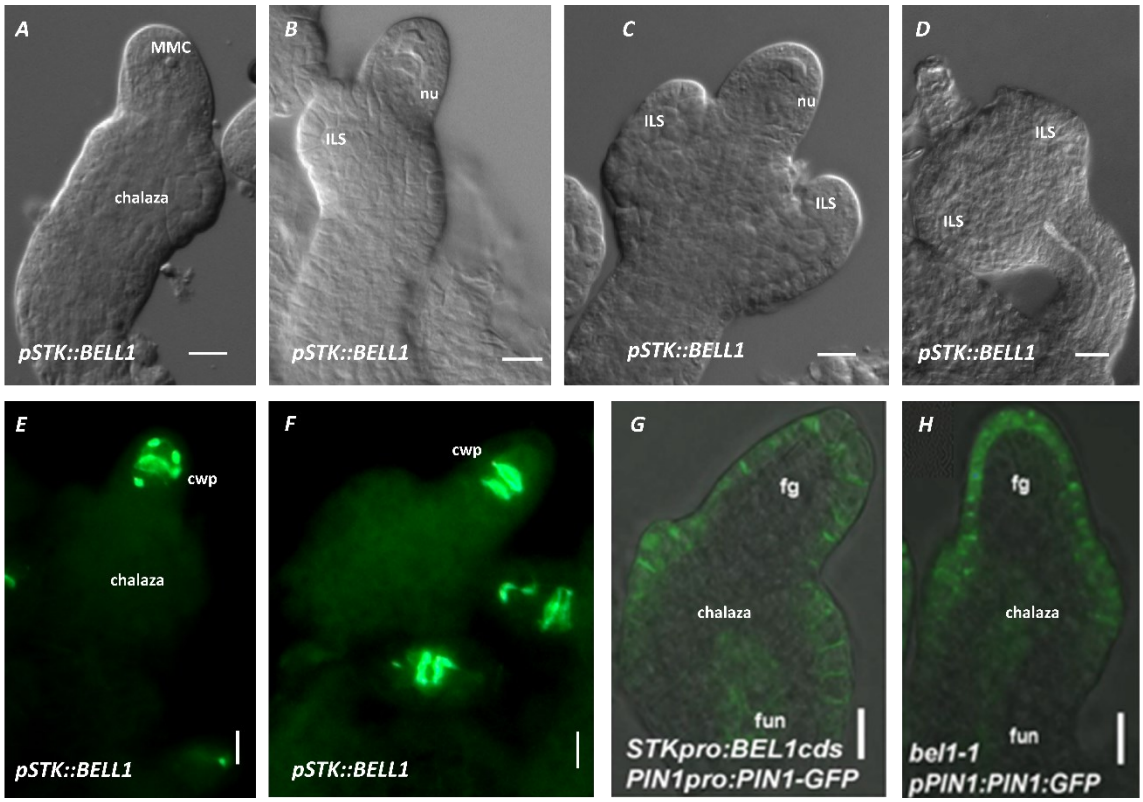


Figure 18 Morphological analyses of *pSTK::BELL1* and *pPIN1::PIN1-GFP* expression in *pSTK::BELL1*. (A-D) *pSTK::BELL1* ovule development: at the stage 2-II (A) a dome-shaped structure arise on one side of the chalaza, but later in the ovule development on the other side, another dome-shaped structure is formed (B), and together they resembles the ILS, observed in *bel* ovules. Subsequently, (C) other structures that has nucellus shape arise from the chalaza and at later stages of *pSTK::BELL1* ovule development, the chalazal structures acquired carpelloid shape. (E-F) Aniline blue staining on *pSTK::BELL1* ovules showed that the meiotic division occurs, so that the megasporogenesis process normally proceed. (G) *pPIN1::PIN1-GFP* expression mirrors the one observed in (H) *bel* ovules, indeed it expands along the epidermal layer of the chalaza. MMC, megaspore mother cell; nu, nucellus; ILS, integument like structure; cwp, cell wall plate; fg, female gametophyte, fu, funiculus. Scale bar 10 μm

To understand the molecular basis of the phenotype it is important to remember that *BEL1* belongs to TALE-HD transcription factors family. These proteins are characterized by a homeodomain HD, and an interaction domain MID formed by the SKY and BEL domains. The interaction domain allows BEL1 to interact with its partner such the KNOX transcription factors as STM, KNAT1, KNAT2 and KNAT5 (Bellaoui et al., 2001b). It has been shown that the interaction between BEL and its partner is required to enter in the nucleus and thus allowing these dimers to play their function as transcription factors (Hay and Tsiantis, 2010) (Figure 19A).

We hypothesised that the BEL1 is required in a stoichiometric balance to allow the correct development of the chalaza. Therefore, the absence of BEL1 in *bel1-1* (Figure 19B) and the overexpression of *BEL* in *pSTK::BELL1* (Figure 19C) interfere with the proper interaction between BEL1 and its partner(s) and thus to the proper localization of BEL in the nucleus.

To evaluate whether BEL1 is able to form homodimers, we performed yeast two hybrid experiment from which we concluded that BEL1 is able to interact with itself, (Figure 19D).

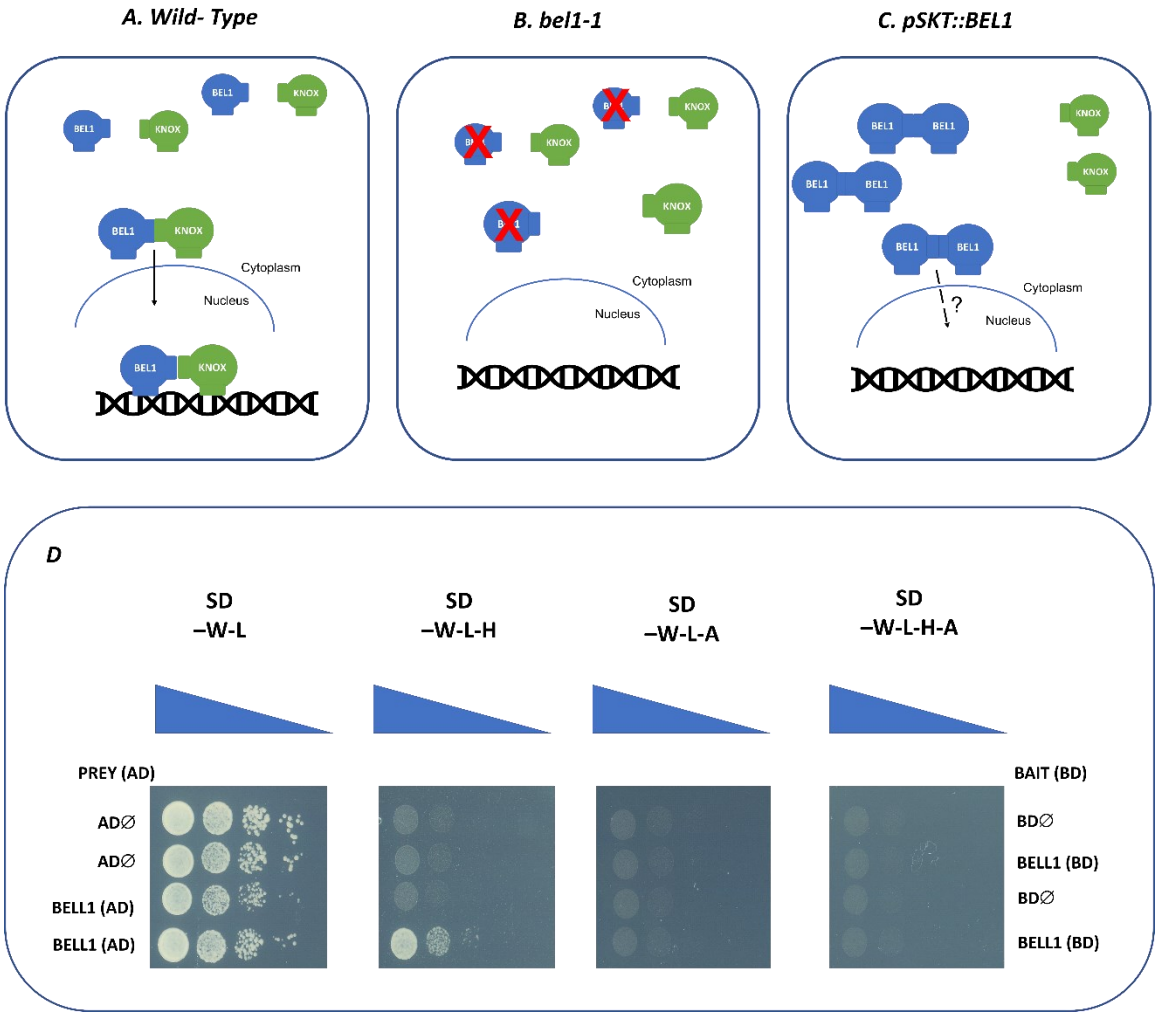


Figure 19 Model of action for BEL1 as transcription factors and Yeast two Hybrid Assay. (A) In wild-type situation, BEL1 interacts with its partner (maybe the KNOX, KNAT5) in the cytoplasm. The interaction allows the two transcription factors to be imported into the nucleus and function as transcription factors in a complex. (B) In bel mutant, the absence of BEL1 prevent the enter into the nucleus also for its partner and their function is inhibited. (C) In the opposite situation, when BEL1 is over-accumulated in the cytoplasm, the capability of BEL1 to form homodimers and the high abundancy of BEL1 itself, prevent BEL1 to interact with its partner, and this lead to obtain the same defects seen in the absence of BEL1. (D) Yeas two Hybrid Assay: BEL1 was tested for its ability to form homodimers, in combination with GAL4 DNA Binding Domain (BD) and the GAL4 DNA Activation Domain (AD). AD Ø and BD Ø empty vectors.
-W -L, permissive medium devoid of Tryptophan and Leucine; -W -L -H, selective medium devoid of Tryptophan, Leucine, Histidine; -W -L -A, selective medium devoid of Tryptophan, Leucine, Adenine; -W -L -H - A, selective medium devoid of Tryptophan, Leucine, Histidine and Adenine.

Upstream and downstream BELL1

It is clear that the defects observed in megasporogenesis in *bel1-1* mutant are due mainly to impaired auxin distribution. The expression of auxin transporter *PIN1* resulted upregulated in *bel1-1* mutant ovules and its expression wrongly localized within the chalaza. The ectopic PIN1 localization might impair the flow of auxin within the ovule and thus leading to defects in the female germline development and specification.

Expression analyses of *pPIN1::PIN1-GFP* in *bel1-1* ovules suggested that in the chalaza BEL1 represses and restricts PIN1 expression domain in the chalaza. To verify this negative regulation of BEL1 on *PIN1* transcription, we performed a Lucifase Assay. In four out of five replicates that were performed, BEL1 resulted acting negatively on the expression of *PIN1* (Figure 20).

We planned to also perform a ChIP experiment on protoplast to assess whether the regulation of *BEL1* on promoter of *PIN1* is direct. This experiment is ongoing and will be performed in collaboration with the group of Prof. Benkova (ISTA, Austria).

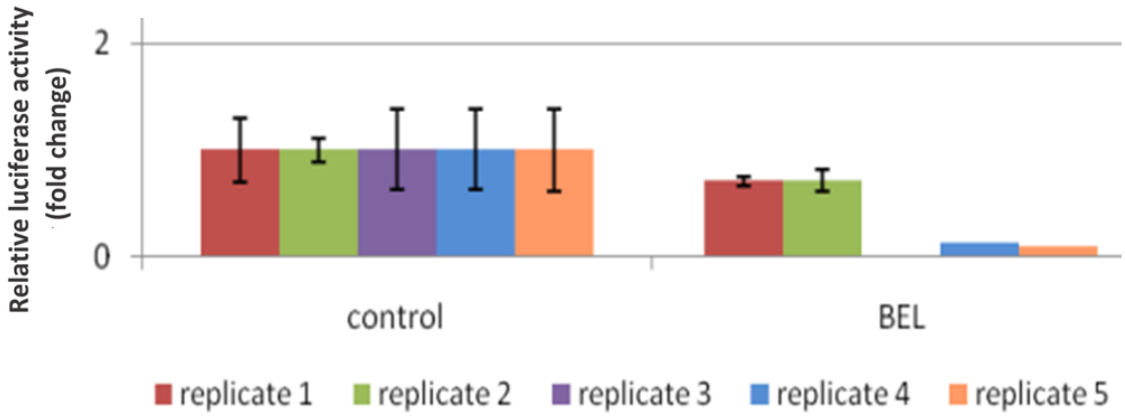


Figure 20 Luciferase Assay for BEL1 activity on *PIN1* promoter. The graph shows Luciferase activity in tobacco BY-2 protoplast transiently transformed with pPIN1::LUC expressing p35S::BEL1. The fold change was normalized on p35S::GUS. Data are means (±SE), n=5 separate transfection events and measurements.

Interestingly, preliminary results regarding ChIP- sequencing analyses on *SEEDSTICK* (*STK*), suggested that, *BEL1* might be a STK target (data not shown)

To verify whether STK was able to directly regulate *BEL1*, we performed an *in-situ* hybridization (ISH) experiment on *stk shp1 shp2* inflorescences, since it is well known that the three MADS box proteins (STK, SHP1 and SHP2) act redundantly in controlling ovule development (Pinyopich et al., 2003). As already described, the expression of *BEL1* within ovule is restricted to chalaza and funiculus (Figure 21A-D). Although from ChIP sequencing analyses *BEL1* resulted as target of STK, from ISH experiment, the expression of *BEL1* didn't change in *stk shp1 shp2* ovules (Figure 21E-H)

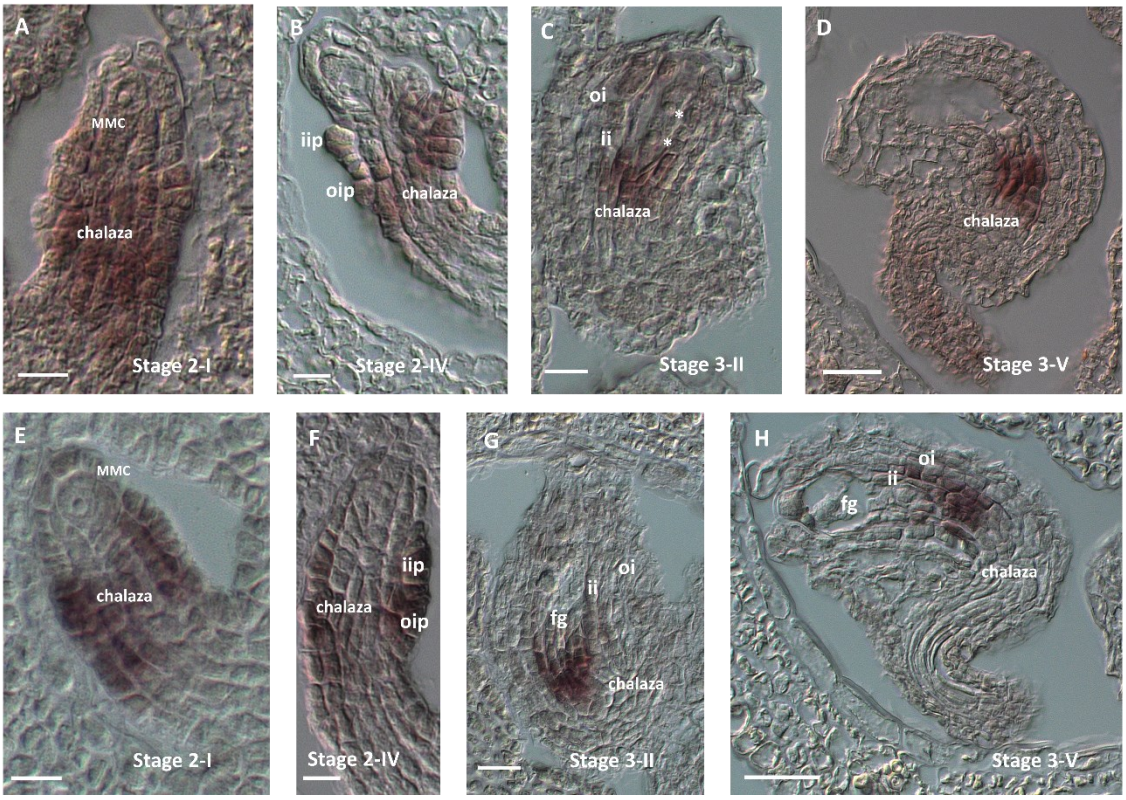


Figure 21 *BEL1* expression in *stk shp1 shp2* ovules.
(A-D) *BEL1 mRNA* is present in the chalaza region, integuments primordia and funiculus. (E-H) The transcripts *BEL1* in *stk shp1 shp2* ovules follow the same pattern of expression observed in wild-type ovules, indeed, they were found in the chalaza, in the integuments. MMC, megaspore mother cell; iip, iner integument primordia; oip, outer integument primordia; oi, outer integument; ii, inner integument; fg, female gametophyte. Scale bar 10 µm

Discussion

The transcription factor *BEL1* has been studied for its role in controlling the integuments development. However, here we proposed a role for *BEL1* in the control of the female germline development. The alteration of auxin homeostasis during ovule pattering in *bel1-1* affects megasporogenesis and consequently the proper specification of the Functional Megaspore.

It has been reported that auxin signalling is required for a proper ovule development (Ceccato et al., 2013b; Pagnussat et al., 2009)and recently Wang and colleagues (2021) demonstrated that the entrance in megagametogenesis is controlled by auxin signal (Wang et al., 2021). We, therefore, speculated that the modification in auxin distribution that we observed in *bel1-1*ovules due to the misexpression of *PIN1* and *PIN3* in the chalaza of mutant ovules cause the megasporogenesis defects. It would be interesting to check whether *BEL1* direct regulates the transcription of *PIN1,* maybe in a complex with its ovule-partner, that we propose to be *KNAT5* because it was published to be one of the *BEL1*interactors (Bellaoui et al., 2001b) and to be expressed within the ovule (Furumizu et al., 2015). In addition, recently, it has shown that two KNOX genes, *KNAT3* and *KNAT4*, are required for integument and ovule development (Chen et al., 2023). Indeed, in the double mutant *knat3knat4* ovules, the morphogenesis of integuments is altered and *BEL1* expression is upregulated. This supported our hypothesis that a KNAT-BEL dimer is required for a proper ovule development. In addition, Chen and colleagues, performed an RNA-sequencing on *knat3knat4,* from which several gene linked to auxin response resulted downregulated, reinforcing our idea that the mis-regulation of auxin pathway alter a proper development of the female germline.

We propose a model by which the interaction between BEL1 and its partner allows the correct regulation of PIN1 and PIN3 in the chalaza which is required to convey the auxin at the tip of the nucellus where it promotes megasporogenesis and proper FM specification (Figure 22).

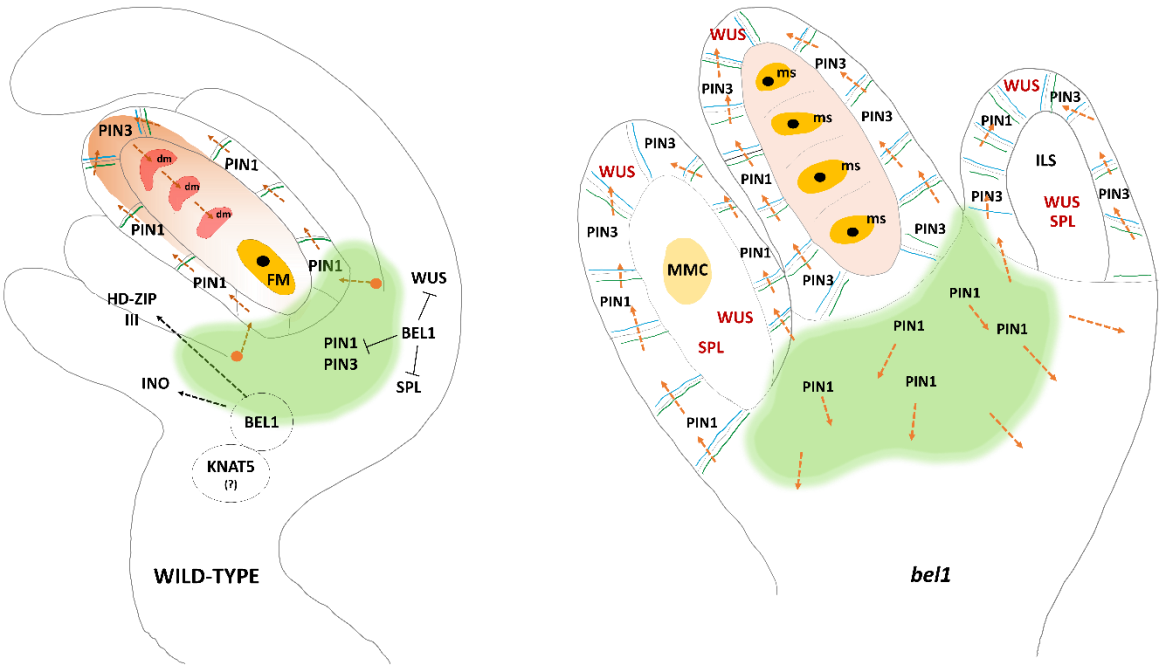


Figure 22 Scheme of FM specification in wild-type and *bel1* ovules. In the chalaza of wild-type ovules, BEL1 restricts the expression of the nucellar genes, *WUS* and *SPL* and the expression of *PIN1* to allow a correct distribution of auxin. Meanwhile, BEL1 promotes integument development activating INO and HD-ZIP III, maybe through the formation of a complex with its partner(s) that we hypothesised to be KNAT5. The control of BEL1 on PIN1 and PIN3 allows the formation of auxin gradient (the shade of orange represents the auxin gradient; dark orange represents auxin maximum, light auxin minimum) which is required for the correct degeneration of megaspores and the correct specification of the FM. Instead, in *bel* ovules the repression of WUS and SPL doesn't occur in the chalaza and therefore instead of developing integument structures, ILS arise in the chalaza that acquire nucellar identity. In addition, the lack of PIN1 and PIN3 repression within the chalaza impairs the auxin distribution within the nucellus (light orange) leading to defects in degeneration of megaspores and in the specification of FM. Cytokinin signalling (green), auxin distribution (orange). Orange point represent auxin source. Dashed orange arrows auxin flow. Dashed black arrows represent positive regulation.

Chapter 2

The role of AINTEGUMENTA during megasporogenesis

Morphological description of *ant-4* mutant ovules

The transcription factor AINTEGUMENTA (ANT) belongs to the APETALA2/ETHYLENE RESPONSE FACTOR APETALA 2 family protein. It has been shown to control cell growth and cell proliferation during organogenesis (Randall et al., 2015, Mizukami and Fisher, 2000) and to promote floral organ and ovule primordia development (Krizek et al.,2011, Krizek et al.,2012, Galbiati et al., 2013). In addition, *ANT* is required for integuments initiation (Elliott et al., 1996; Klucher et al., 1996; Schneitz et al., 1997; Losa et al., 2010) and modulates auxin biosynthesis in ovules regulating *YUC4* (Li YJ et al., 2021).

AINTEGUMENTA starts to be expressed at early stage of ovule development, in the placenta and in ovule primordia (Galbiati et al., 2013). At the stage 2-I it is expressed in the integument primordia and in the chalaza (Figure 23A); during the megasporogenesis process, *ANT* remains expressed in developing integuments, in the chalaza and in the funiculus (Figure 23B-C). At the stage 3-I and throughout the megagametogenesis *ANT* expression is restricted to the chalaza region and to the funiculus (Figure 23D).

It has been identified different mutant allelic variant for *AINTEGUMENTA: ant-1, ant-2, ant-3, ant-4, ant-5, ant-6, ant-7,ant-8, ant-9* (Elliot et al., 1996; Klucher et al., 1996; Baker et al., 1997). All *ant* mutant plants exhibited pleiotropic defects: alteration in floral organs development, such as sepal and petal shape and stamen number, in the gynoecium morphology, that is narrowed and shorter with the stigma that sometimes resulted unfused and female sterility (Elliot et al., 1996; Klucher et al., 1996; Baker et al., 1997). The mutant *ant* ovules present defects in their organogenesis albeit with different penetrance depending on the mutant allele analysed. However, since all the mutant variants of *ANT*, exhibited similar phenotype, we decided to perform all our analyses on *ant-4* mutant plants, which is one of the most severe mutant alleles.

Until stage 2-I, the development of *ant-4* mutant ovules (Figure 23I) didn't differ from wild-type (Figure 23E-H) ovules although the number of ovules within pistil is lower

compared to wild-type pistil (Elliot et al., 1996). However, at stage 2-II, *ant-4* ovules do not develop any integument primordia and going on with ovule development the chalaza cannot develop neither the outer nor the inner integuments from the chalaza (Figure 23J-K) and at stage 3-I the nucellus degenerate (Figure 23L) and consequently ovule development is arrested.

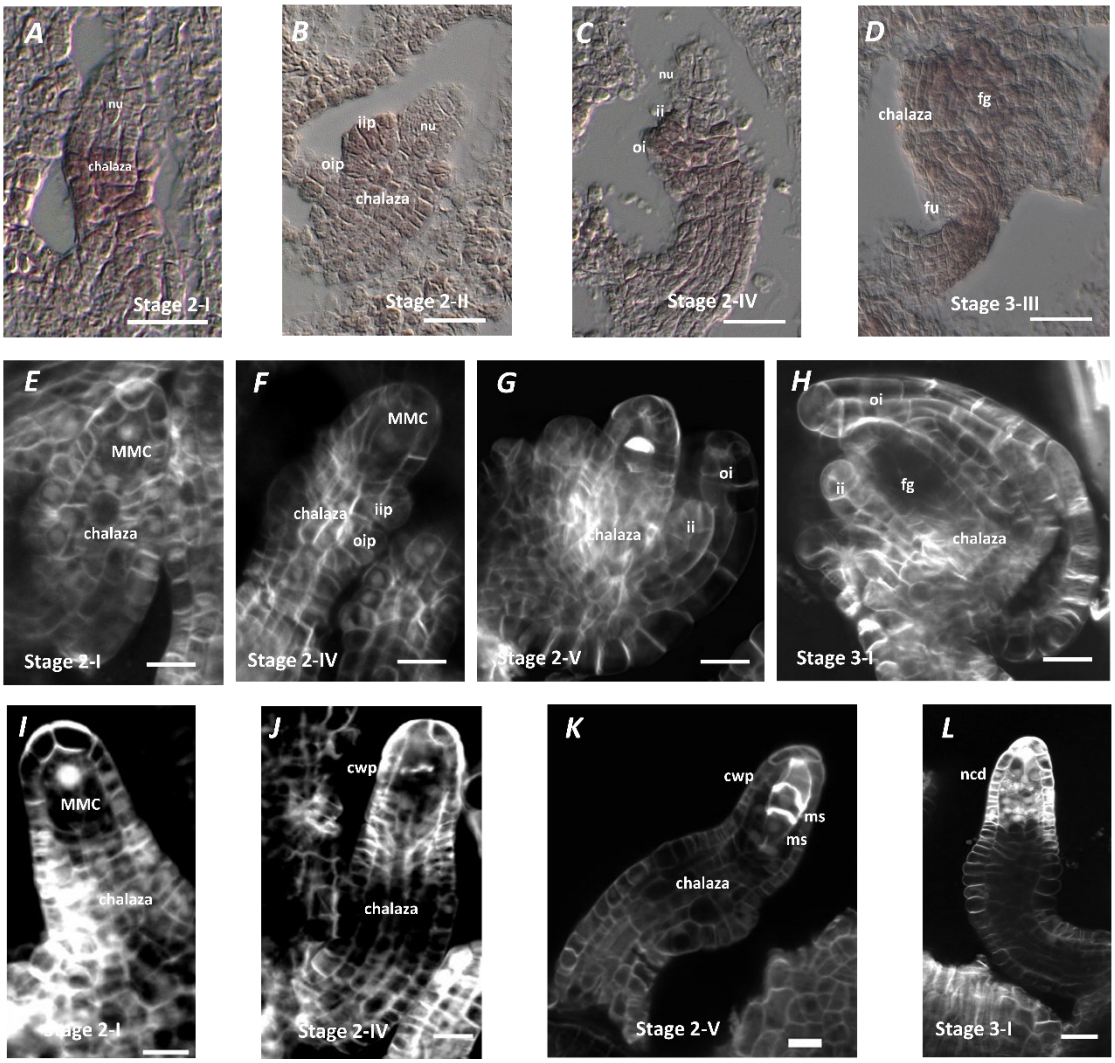


Figure 23 Expression pattern of *AINTEGUMENTA* and morphological analyses of ant-4 ovule development.
At early stage of ovules development (A) *ANT* is expressed in the chalaza, in the (B-C) developing integuments. Later in ovule development (D) *ANT* accumulates in the funiculus and in the chalazal pole of the ovule. (E) Wild-type ovules arise as finger like structure and (F) once the MMC is specified, it undergoes meiotic division (G) meanwhile the chalaza starts to develop integument structures. (H) At the stage 3-I, integument almost completely wrap the developing female gametophyte. (I) Also *ant-4* ovules arise as finger-like structure, (J) however any integument primordia is developed in the chalaza. Indeed, in *ant* ovules, (K) neither the inner nor the outer integument develop and late in *ant* ovule development, all the content of the nucellus start to degenerate. MMC, megaspore mother cell; iip, inner integument primordia; oip, outer integument primordia; oi, outer integument; ii, inner integument; fg, female gametophyte;

cwp, cell wall plate; ms, megaspore; ncd, nucellar content degenerating. (A-D) Scale bar 20 μm; (E-L) scale bar 10 μm

Megasporogenesis in *ant-4* ovules

As we did for *bel1-1* mutant ovules, we decided to study the megasporogenesis process also in *ant-4* mutant ovules, starting from the specification of the MMC.

Therefore we crossed *ant-4* mutant plants with *pKNU::nlsYFP* (Tucker et al., 2012), and analysed their F2 progeny. Surprisingly, we couldn't observe any signal, although for all the analysed plants, we confirmed the presence for *pKNU::nlsYFP* by genotyping.

Despite the fact that the promoter of *KNU* resulted inactive in *ant-4* mutant ovules, at the stage 2-I, one MMC-like cell within nucellus, expressed *ASY3::RFP* (Yang et al., 2019);Figure 24E) as the MMC in wild-type ovules (Figure 24A)at the same stage (Figure 24A) suggesting that in *ant-4* ovules the MMC was specified and prepared to enter into meiosis.

In addition, we assessed the capability of the MMC to undergo meiosis and to complete meiotic division by staining callose with the aniline blue staining. The typical pattern of callose accumulation that is visible starting from stage 2-I until 2-V in wild-type ovules (Figure 24B-D), was observed also in *ant-4* ovules; at the stage 2-I, aniline blue stained callose surrounding the MMC (Figure 24 F), then after the first meiotic division callose deposited in correspondence of the first meiotic cell plate (Figure 24G), and following the second meiotic division the callose was present at the second cell wall plate formed between the two last new-born megaspores (Figure 24H). Therefore, although the promoter of *KNU* was not active, the progression of megasporogenesis seemed to normally occur in *ant-4* mutant ovules.

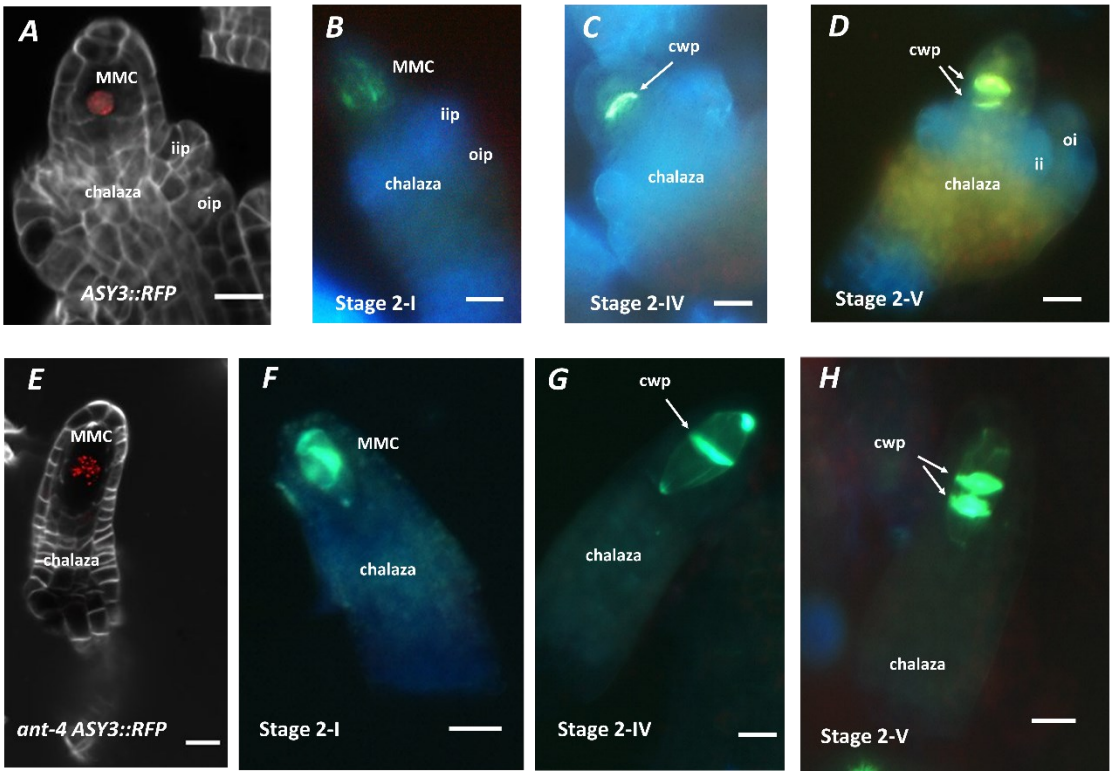
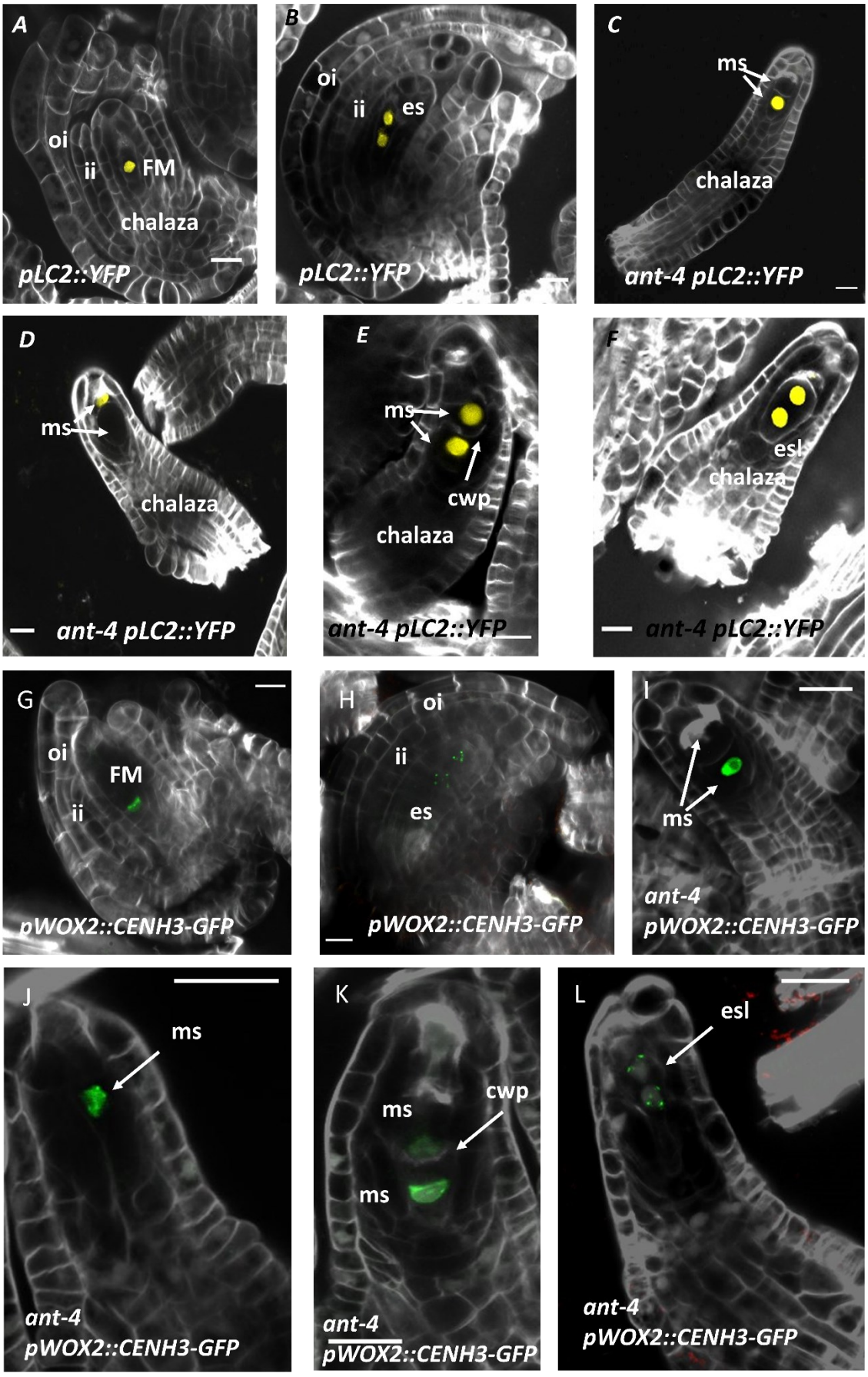


Figure 24 Megasporogenesis process in *ant-4* ovules.
(A)In wild-type ovules ASY3::RFP is expressed in the MMC, that at the beginning of megasporogenesis is surrounded by callose (B) and once it divided by meiosis (C-D), spores are separated from each other by cell wall plates in which it is visible callose accumulation. Also in (E) *ant-4* mutant ovules, at the stage 2-I, *ASY3::RFP* is expressed in the MMC, which is surrounded by callose (F) as aniline bleu staining showed. The MMC can undergo meiotic division, indeed also in *ant-4* ovules the callose is accumulated in correspondence of the cell wall plates (G-H) that divide the megaspores. MMC, megaspore mother cell; iip, inner integument primordia; oip, outer integument primordia; ii, inner integument; oi, outer integument; cwp, cell wall plate. Scale bar 10 µm

The degeneration of megaspores and the functional megaspore specification are altered in *ant-4* mutant ovules.

The degeneration of the three megaspores and the specification of the Functional Megaspore (FM) coincided with the end of megasporogenesis. To analyse the destiny of the four megaspores obtained by MMC meiosis, we compared the expression of the reporter marker *pLC2::nlsYFP* (Tucker et al., 2012) in wild-type (Figure 25A-B) respect to *ant-4* mutant ovules (Figure 25C-F).

Interestingly, also in *ant* ovule the megaspores survived. Indeed, in 47,8% (77/161 ovules) of *ant-4* ovules, the most chalazal spore acquired the FM identity as it occurs in wild-type ovules (Figure 25C). However, the 21,1% (34/161 ovules) of *ant-4* ovules expressed the reporter marker *pLC2::nlsYFP* in one spore of the tetrad whose localization within the nucellus wasn't the canonical one (toward the chalaza, Figure 25D) and even in 30,4% (37/161 ovules) of *ant-4* ovules, two megaspores, still separated by the cell wall plate, expressed at the same time *pLC2::nlsYFP* (Figure 25E) suggesting that both were differentiating as FM. Last, we observed in 4,3% (13/161 ovules) of *ant-4* mutant ovules, that the spore acquiring the FM identity, underwent mitotic division and formed a 'binucleated' embryo sac-like structure (Figure 25F). To confirm that the two nuclei observed in the 'embryo sac' like structure had a ploidy reduction, we analysed also *pWOX2::CENH3-GFP* (De Storme et al., 2016) that allows to count the number of the chromosome within a nucleus, and we confirmed that the nuclei observed contained five chromosome as expected (wild-type Figure 25G-H; ant-4 Figure 25I-L).


A
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C
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chalaza
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chalaza
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ii
pWOX2::CENH3-GFP
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pWOX2::CENH3-GFP
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pWOX2::CENH3-GFP
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pWOX2::CENH3-GFP
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pWOX2::CENH3-GFP

Figure 25 FM specification and *pWOX2::CENH3-GFP* in *ant-4* ovules.
(A) *pLC2::nlsYFP* is expressed in the FM and (B) after the first mitotic division in the two nuclei of the embryo sac. (C) The most chalazal spore of *ant-4* ovule expresses *pLC2::nlsYFP;* arrow points the surviving spore above th*e FM.* (D) The FM identity is acquired by a spore which places in a 'non canonical' position. Arrow indicate the presence of another nucleus below the 'non-canonical spore' (E) Two spores in *ant-4* nucellus divided by the cell wall plates (pointed by the arrow) express *pLC2::nlsYFP*. One spore divided and produced (F) two nuclei expressing *pLC2::nlsYFP* belong to the embryo sac like structure. (G-H) *pWOX2::CENH3-GFP* is expressed from the FM untill the end of the megagametogenesis process. It marks the chromosome that after megasporogenesis are reduced to five. (I-L) *pWOX2::CENH3-GFP* in *ant-4* ovules showed that all the spores that were produced after megasporogenesis derives from meiotic events.In addition, expression analyses of pWOX2::CENH3-GFP in *ant-4* confirmed the phenotype observed with *pLC2::nlsYFP.* FM, functional megaspore; oi, outer integument; ii, inner integument; es, embryo sac; ms, megaspore; cwp, cell wall plate; esl; embryo sac like. Scale bar 10 μm

Cytokinin signalling and Auxin distribution in *ant-4* mutant ovules

As previously mentioned, it is well known that cytokinin are involved in several developmental pathways, such as gynoecium development (Cucinotta et al., 2016b; Marsch-Martínez et al., 2012; Müller et al., 2017), ovule number determination and female gametophyte development (Cheng and Kieber, 2013; Terceros et al., 2020; Yuan et al., 2016b). Cytokinin are also able to promote cell proliferation and mitotic cell cycle, in particular in the SAM by stimulating the mitotic cyclin CYCD3 (Scofield et al., 2013)As well as cytokinin also *ANT* has been shown to promote cell proliferation, and it has been reported in roots, that application of exogenous cytokinin lead to an increasing of 6-fold of *ANT* transcripts (Randall et al., 2015b).

Since both cytokinin and *ANT* are involved in cell proliferation and a relation between them has been previously demonstrated, we evaluated whether cytokinin signalling was affected in *ant-4* mutant ovules.

Therefore, we decided to analyse *TCSn::GFP* (Zürcher et al., 2013) that allows an indirect overview about cytokinin signalling within ovules.

Throughout the megasporogenesis process, in wild-type ovules, *TCSn::GFP* is expressed in the chalaza, just below the nucellus (Figure 26A-C).

In *ant-4* ovules we observed a similar pattern of *TCSn::GFP* expression. Indeed, from the stage 2-I and for the entire megasporogenesis process *TCSn::GFP* signal localized in the chalaza, closed to the nucellus as it was observed in wild-type ovules (Figure 26D-F).

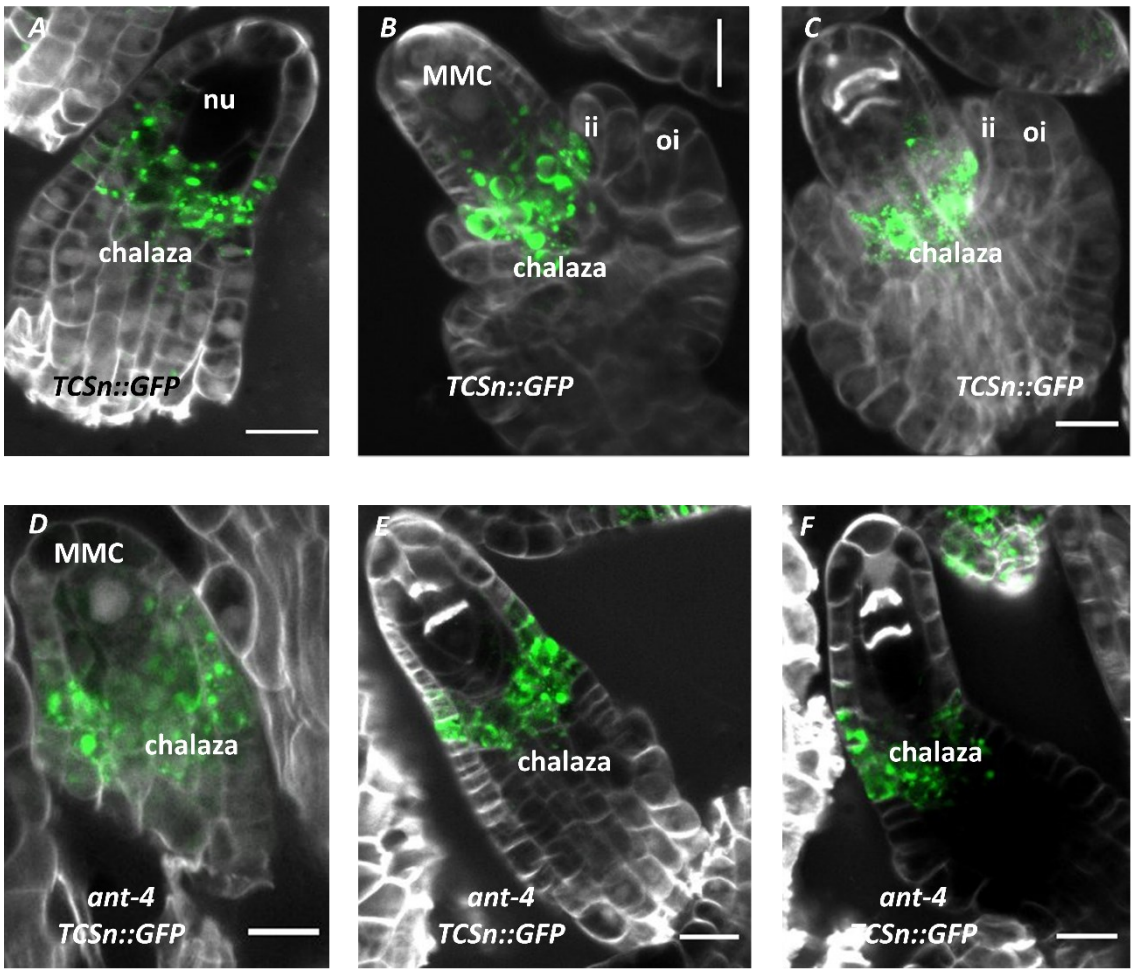

Figure 26 Cytokinin signalling in *ant-4* ovules. (A-C) Cytokinin signalling within the ovules localized in the chalaza region that surrounds the nucellus, throughout the megasporogenesis process. (D-F) In *ant-4* ovules *TCSn::GFP* pattern of expression doesn't change. It places in the chalaza region underneath the nucellus. nu, nucellus; MMC, megaspore mother cell; ii, inner integument; oi, outer integument. Scale bar 10 μm

It has been shown that auxin and *AINTEGUMENTA* collaborate to control floral organ development (Krizek, 2011b; Krizek et al., 2020b). In *ant-T* ovules, the activity of the promoter of *YUCCA4*, required for auxin biosynthesis, resulted highly reduced (Li et al., 2021). For the above cited reasons, we decided to verify whether also in *ant-4* mutant ovules, auxin distribution and/or homeostasis might be altered respect to wild type.

We, therefore, analysed the auxin reporter marker *R2D2* (Liao et al., 2015), which has already described, relies on auxin-dependent degradation domain of the AUX/IAA protein family, that drives the expression of Venus fluorescent protein; so that the signal of this marker would be observed only in absence of auxin.

In wild-type ovules, the expression of *R2D2*, at stage 2-I localized at the chalaza region below the nucellus and at the bottom of the epidermal layer of the nucellus itself (Figure 27A). This pattern of expression persists until stage 3-I when the expression of the fluorescent protein Venus is accumulates mainly at the chalaza region and in few cells of the growing inner integument (Figure 27B). Interestingly, from the stage 3-I, the reporter line *R2D2* marks the Functional Megaspore (Figure 27C) and after the first mitotic division the signal of the fluorescent protein is visible also in both nuclei belonging to the developing embryo sac (Figure 27D).

During the megasporogenesis in *ant-4* ovules, the expression of R2D2 followed the expression pattern observed in the wild-type ovules, accumulating in the chalaza region and in the basal part of the nucellus (Figure 27E-F). However, at the stage in which the FM should acquire its identity, the expression of *R2D2* differs from the one observed in the wild-type (Figure 27G-J).

In 64% (103/160 ovules) of *ant-4* ovules, the expression of the reporter marker was in the chalazal spore, even if the other three spores did not degenerate (Figure 27G).

Indeed, in 15% (24/160 ovules) of mutant ovules, the GFP signal was visible in a spore placed in a non-canonical position (Figure 27H), while 6,3% (10/160 ovules) of *ant-4* ovules, expressed the *R2D2* in two spores at the same time (Figure 27I)

Last, in 14,4% (23/160 ovules) of *ant-4* mutant ovules, we observed two nuclei, expressing the signal of the fluorescent protein, not separated by any meiotic cell wall plate, suggesting that they derived from a mitotic event (Figure 27J).

The percentage of the spore wrongly expressing *R2D2* is similar to that observed with the reporter line *pLC2::nlsYFP* (Figure 27K), suggesting that the alteration in FM specification in *ant-4* ovules is due to defect in auxin distribution.

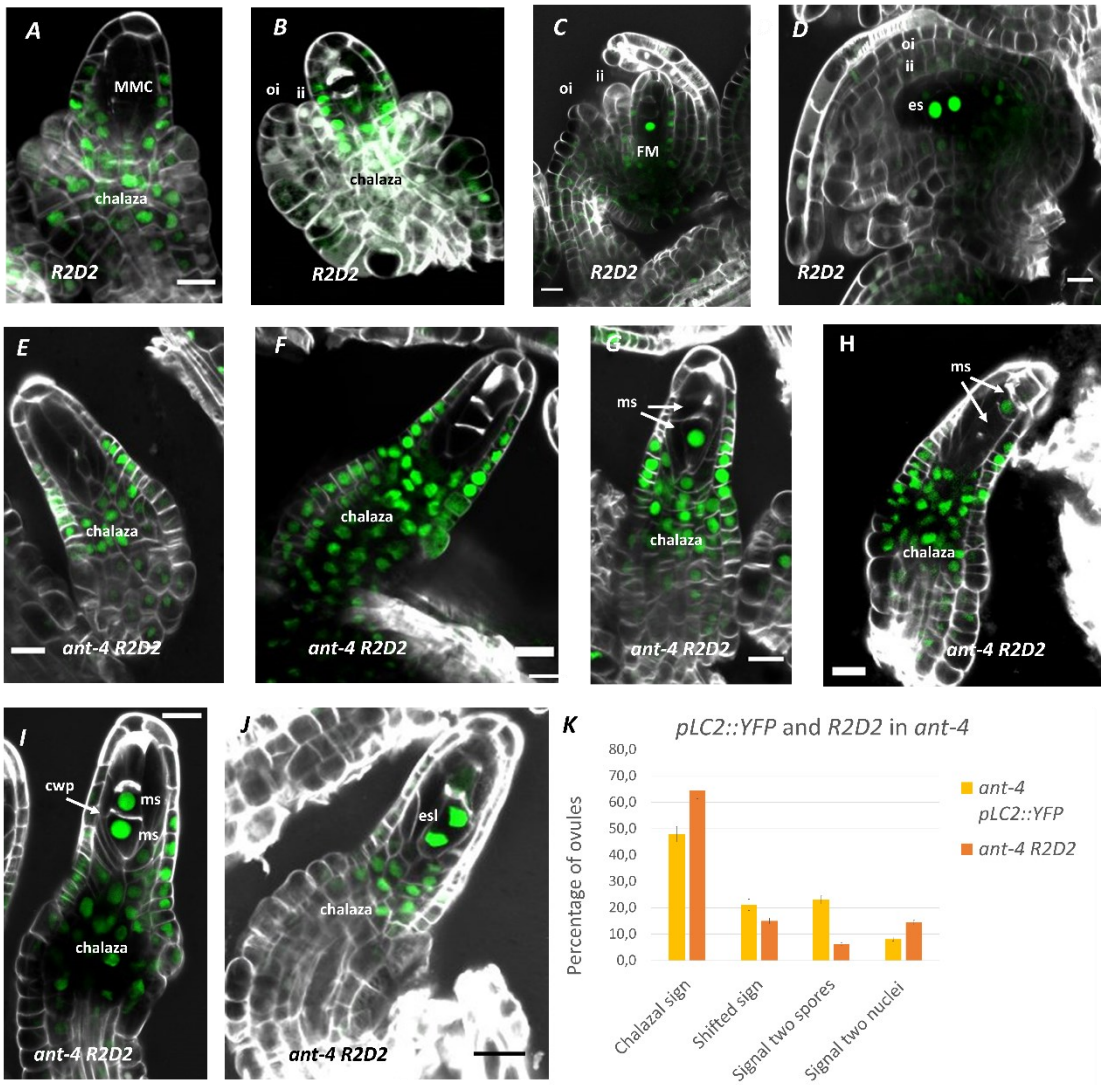

Figure 27 Auxin minima *R2D2* reporter line in *ant-4* ovules. (A-B) During megasporogenesis, in wild-type ovules, *R2D2* is expressed in the chalaza region and along the basal part of the epidermal layer of the nucellus. (C-D) Once the FM specify, *R2D2* is expressed also in correspondence of the FM and following the first mitotic division in the two nuclei of the embryo sac at the stage 3-II. In *ant-4* ovules, during the megasporogenesis division (E-F), *R2D2* accumulates in the chalaza and in the L1 layer as well observed in wild-type. In 60% of ant-4 ovules *R2D2* was seen in correspondence of the (G) most chalaza spore that is acquiring the FM identity. However, the reporter line was also observed (H) in spores whose position within the nucellus wasn't the most chalazal one, or even (I) in two different spores. In some cases, one of the spores which acquires FM identity (J) undergoes mitotic event even. *R2D2:* the fluorescent protein Venus (green) expression is driven by the DII sequence of AUX/IAA protein and its signal truly represents the minimum of auxin, ntd Tomato (red) fluorescent protein is driven by a constitutive promoter which allows to monitor whether the construct of the marker line is properly working. MMC, megaspore mother cell; oi, outer integument; ii, inner integument; FM , functional megaspore; ms, megaspore; cwp, cell wall plate; esl, embryo sac like structure. Scale bar 10 µm. (K) Comparison between the frequency of the different phenotypes observed with *pLC2::nlsYFP* and *R2D2* in *ant-4.* The frequency of the two reporter marker lines obtained during the expression analyses resulted similar. Data means (±SE).

As previously said, the polar auxin transporter PIN1 has been shown to be the major auxin transporter within the ovules. It conveys auxin at the tip of the nucellus to create an auxin maximum required for a correct development of the female germline (Ceccato et al., 2013a; Pagnussat et al., 2009).

Since in *ant-4* mutant ovules, we have observed an alteration in auxin distribution, as we observed for bel1-1, we planned to investigate in more detail whether the absence of the integuments as well as the defects in later stage of sporogenesis were due to alteration in auxin homeostasis.

To study PIN1 expression, we crossed *ant-4* mutant plants with *pPIN1::PIN1-GFP* (Benková et al., 2003) reporter marker line.

Unfortunately, in the F2 population, recombinant lines between *ant/ant pPIN1::PIN1-GFP* were not found suggesting that wild-type allele *ANT* and the *pPIN1::PIN1-GFP* construct were in linkage.

Therefore we will transform *ANT/ant* directly with *pPIN1::PIN1-GFP* construct, to avoid hopefully the linkage problem. .

Discussion

The transcription factor *AINTEGUMENTA* has been studied for its important role in integument development, however, here we proposed an indirect role for this transcription factor in the control of megasporogenesis. The analyses performed on *ant-4* mutant ovules, reinforce the idea that proper female germline development is required and depends on a correct differentiation of the chalaza region and the defects in megasporogenesis observed in *ant-4* ovules seem to be due to alteration in auxin homeostasis and polar auxin transport in the chalaza.

At the beginning of megasporogenesis, ANT promotes integument initiation in the chalaza, by repressing the nucellar gene NZZ/SPL and by controlling auxin biosynthesis through transcriptional activation of *YUCCA4*. During sporogenesis ANT control integument development by promoting the expression of two genes, INO and PHABULOSA (PHB), that are involved in integument development (Balasubramanian and Schneitz, 2002; Sieber et al., 2004). In addition, ANT represses PIN1 allowing the

correct auxin flow within the ovule and thus the correct establishment of auxin gradient within the nucellus required for megasporogenesis / megagametogenesis progression.

In 2020, Krizek and colleagues, performed a RNA-sequencing on the inducible system *ANT-GR* collecting floral buds from stage 1 to 12, on the entire inflorescence(Krizek et al., 2020b). Among the genes that resulted differential expressed, several encode for auxin biosynthesis proteins, such as *TAA1/WEI8, YUC8*, auxin transport *PIN1*, *PIN3* and *PIN7*, and auxin signalling, *ARF6, ARF11, ARF18, SHY2/IAA3*. Interestingly, *PIN1* resulted slightly upregulated whereas *PIN3* and *PIN7* downregulated (Krizek et al., 2020b).

We planned to check the expression of such genes in *ant-4* mutant ovule trying to propose a molecular network for the female germline development, controlled by *ANT* (Figure 28).

Even though, the *TCSn::GFP* pattern of expression didn't differ between wild-type and *ant-4* mutant ovules, it gives us an indirect indication about cytokinin behaviour within ovules. It can be that cytokinin amount increased due to mis regulation of their biosynthesis or contrary due to less degradation, but we cannot perceive this variation because we looked at only downstream the signalling cascade.

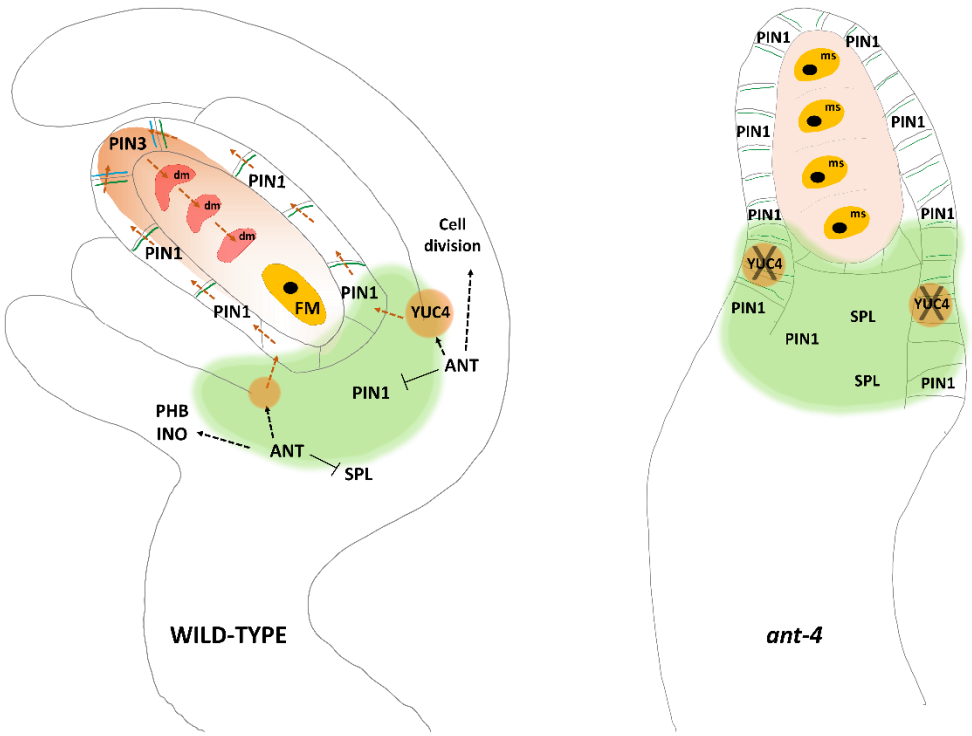

Figure 28 Scheme of FM specification in *ant-4* ovule . In the chalaza of wild-type ovules, ANT restricts the expression of the nucellar gene *SPL* and maybe the expression of *PIN1* to allow a correct distribution of auxin. Meanwhile, ANT promotes integument initiation

controlling auxin biosynthesis via YUCCA4 and integument development activating INO and the HD-ZIP III, PHABULOSA (PHB). In addition, it might be that ANT control also cell cyle machinery. However, the control of ANT on auxin biosynthesis and maybe on PIN proteins, allows the formation of auxin gradient (the shade of orange represents the auxin gradient; dark orange represents auxin maximum, light auxin minimum) which is required for the correct degeneration of megaspores and the correct specification of the FM. Instead, in the chalaza of *ant-4* ovules, auxin biosynthesis is altered as well as the repression of SPL and PIN1 and consequently any integument structure is formed. In addition, the auxin distribution is altered and therefore the degeneration of megaspores and the FM specification don't properly occur. Cytokinin signalling (green), auxin distribution (orange). Orange point represent auxin source. Dashed orange arrows auxin flow. Dashed black arrows represent positive regulation.

Chapter 3

The role of cytokinin during Megagametogenesis

Defects in cytokinin homeostasis alter the progression of female gametophyte development

In the two previous chapters I have described the results I have obtained showing the importance of auxin homeostasis for a correct accomplishment of megasporogenesis. Instead, CKs has been shown to play an important role during megagametogenesis, and perturbing their homeostasis alter embryo sac development and specification (Deng et al., 2010; Yuan et al., 2016).

In addition, our data showed that auxin seems to control the apoptosis of the three megaspores. Another event of apoptosis occurs during fertilization process. Indeed, once the pollen tube arrives at the ovule, it gets in touch with one of the two synergid cells, called receptive synergid, which undergoes programmed cell death. This apoptotic event allows the pollen tube burst and the consequently releasing of the two sperm cells within the embryo sac. It has been reported that *VERDANDI* (*VDD*), a gene encoding for a transcription factor belonging to the REM family, is required for synergids degeneration (Matias-Hernandez et al., 2010). In *vdd-1/+* ovules, the synergids don't degenerate and the fertilization process is compromised (Matias-Hernandez et al., 2010). In addition, it has been shown that in *vdd-1/+* ovules the CYTOKININ OXIDASE/DEHYDROGENASE 7 (*CKX7*) involved in cytokinin degradation, is downregulated (Mendes et al., 2016), and this led to hypothesize that alteration in cytokinin level within synergids prevents their degeneration.

In our laboratory was cloned a CRISPR line for *CKX7*, *ckx7-T*, showing defects in fruit elongation (Di Marzo et al., 2020)but not in female fertility (Figure 29).

Since it has been demonstrated that some CKX genes act redundantly (Bartrina et al., 2011), we decided to investigate whether also CKX7 could act redundantly with another CKXs in synergids apoptosis determination. The most promising was *CKX6* that has

been shown to be expressed in ovules (Werner et al., 2003) and therefore we analysed the double mutant *ckx6-2* (SALK_070071; Bartrina et al., 2011) *ckx7-T.*

However, when we analysed the double mutant silique, we did not observe difference in seed setting in the mutant respects to the wild-type (Figure 29).

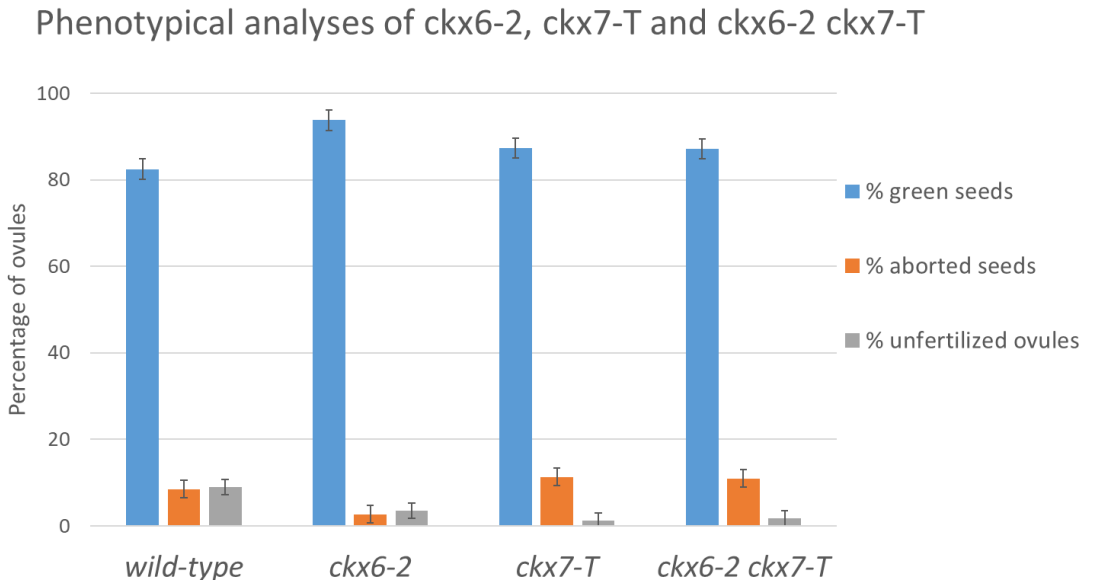

Figure 29 Analyses of silique content in wild-type, *ckx6-2, ckx7-T* and in the double mutant *ckx6-2 ckx7-T.* The percentage of green seeds, aborted seeds and unfertilized ovule doesn't change among the four phenotypes. Data means (±SE).

Since we didn't know how many *CKX* genes were needed to be silenced to get a reproductive phenotype, we decided to use a different strategy.

Therefore, instead reducing the expression of the cytokinin degradation enzymes encoding genes, we have increased the cytokinin level by expressing *AtIPT1* gene, which is required for cytokinin biosynthesis, under the control of *MYB98* promoter, a transcription factors that was shown to be specific for synergid cells (Kasahara et al., 2005).

We obtained twelve *pMYB98::IPT1* T1 plants, that exhibited different level sterility due to the penetrance of the construct (data not shown). However, to investigate better the cause of this sterility we decide to analyse the progeny of these plants.

In contrast to wild-type plants (5,8%, 32/537 ovules) the rate of fertility in T2 plant containing *pMYB98::IPT1* was very low, around 60% (549/934 ovules) of unfertilized ovules (Figure 30).

To confirm that the low rate of fertility was due to female gametophytic defects, we hand-pollinated wild-type pistil with pollen from *pMYB98::IPT1* and vice versa.

The siliques that were produced using *pMYB98::IPT1* pollen on wild-type pistils resulted fertile and only the 11,8% (12/107) of ovule were unfertilized that might be due to hand pollination procedure. On the contrary, when we used wild-type pollen on *pMYB98::IPT1* pistils, the siliques bore 71% (66/93 ovules) of unfertilized ovules (Figure 30). Therefore, we concluded that the low rate of fertility observed in *pMYB98::IPT1* was due to the defects in female reproductive counterpart.

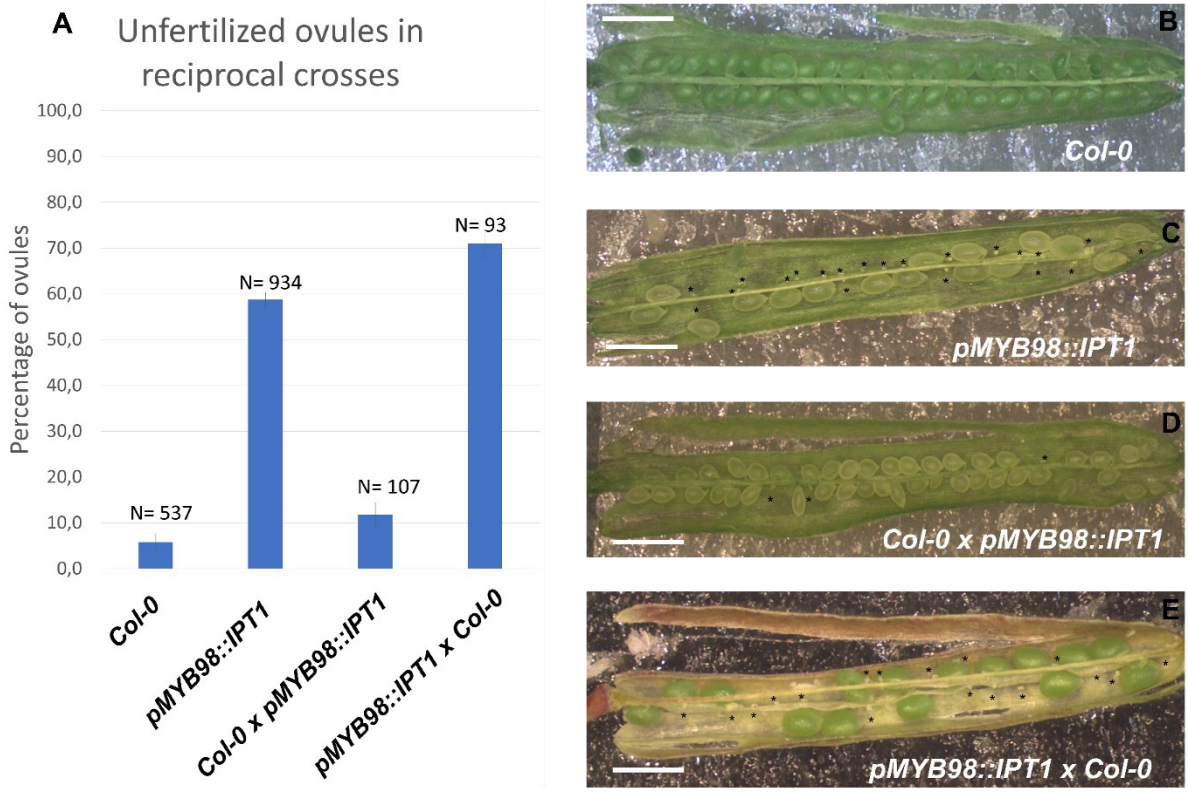

Figure 30 Analyses of unfertilized ovules in silique of *pMYB98::IPT1* compared to wild-type and reciprocal crosses. (A) The graph resume the percentage of unfertilized ovule counted in siliques wild-type, *pMYB98::IPT1*, and in the reciprocal crosses. Respect to the wild-type (B), the siliques of *pMYB98::IPT1*contained a higher number of unfertilized ovules. The reciprocal crosses (D,E) revealed that the high percentage of unfertilized ovule in p*MYB98::IPT1* is due to female sterility. Asterisks mark unfertilized ovules. Data means (±SE). Scale bar 50nm

To understand the cause of the female sterility in *pMYB98::IPT1* plants, we analysed ovule morphology and development by clearing and compared with wild-type (Figure 31A). To synchronize ovule development within pistil we emasculated the flower

before performing morphological analyses. In wild-type pistils, we found that in only the 3,8% (3/67 ovules) of ovules, had arrested gametogenesis (Figure 31B), that was consistent with the percentage of unfertilized ovules and probably due to emasculation process. However the remaining ovules develop normally (Figure 31C)

We expected that sterility defects in *pMYB98::IPT1* plants were due to persistence of synergids. Instead, surprisingly, in *pMYB98::IPT1,* the development of 59,8% (137/230 ovules) of ovules arrested during gametogenesis process at different developmental stages (Figure 31D-F). However, a 40,2% of ovules (93/230 ovules) reached the final FG7 stage (Figure 31G) consistent with the percentage of fertilized ovule that we observed in silique analyses. We expected that sterility in the transgenic plants was due to persistence of synergid cells upon fertilization, instead we observed defects in gametogenesis process. Surprisingly, in 2020 Susaki and colleagues described a more detailed characterization of *MYB98* promoter activity showing that it is active starting from early stage of gametogenesis (Susaki et al., 2020).

Although the role of CKs in synergids degeneration is still opened our data suggested that increasing the level of cytokinin within the embryo sac alters the normal process of megagametogenesis.

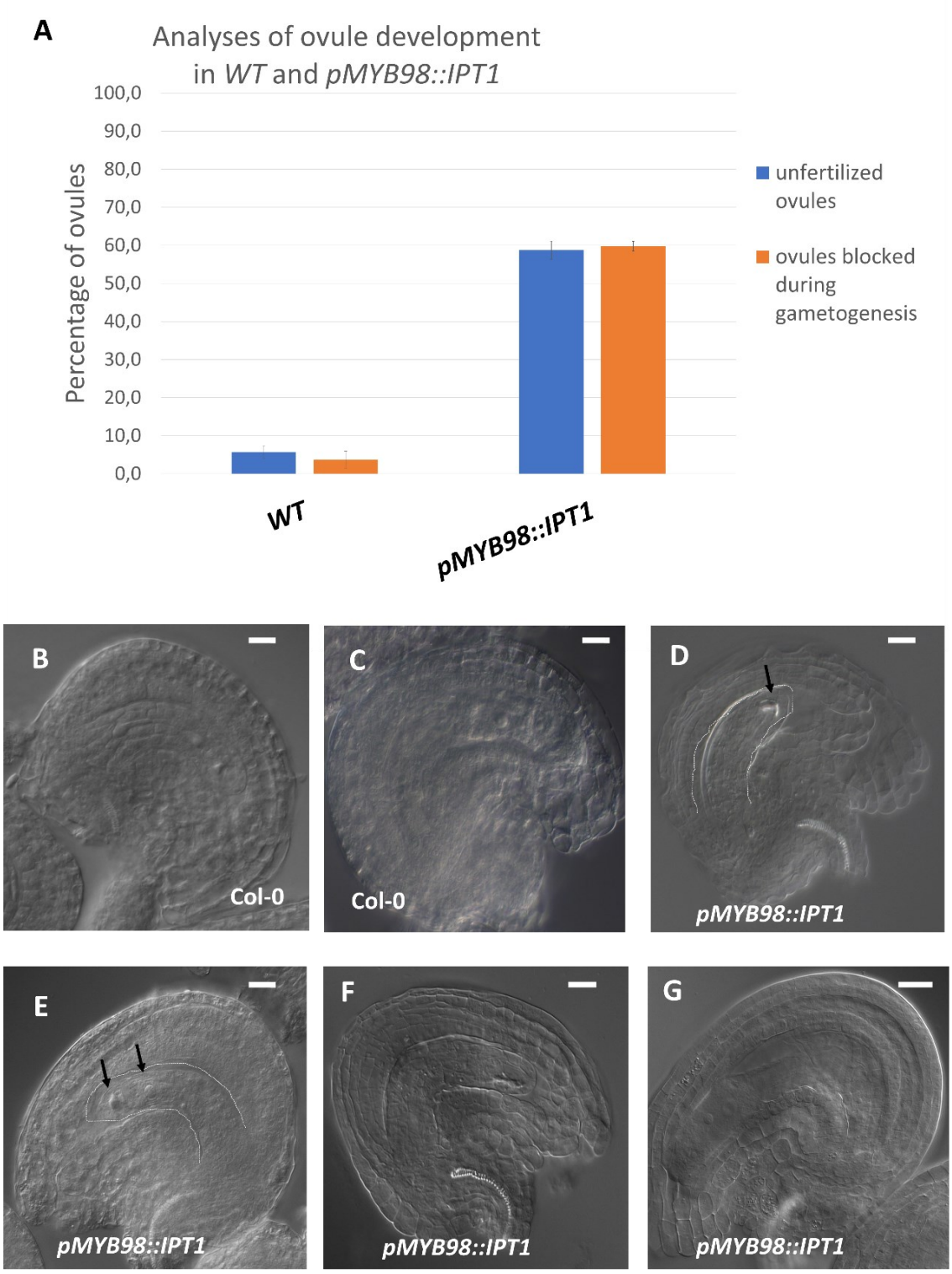

Figure 31 Analyses of ovule development in wild-type and *pMYB98::IPT1*. (A) The percentage of unfertilized ovules and the one of ovules arrested in gametogenesis in wild-type is very low. Instead, in *pMYB98::IPT1*, the percentage of unfertilized ovules and those arrested in gametogenesis are high. Indeed, around 60% of *pMYB98::IPT1* ovule remained unfertilized and the same percentage was observed for those ovule that arrested during gametogenesis. (B) Wild-type ovule arrested in gametogenesis in emasculated flower and (C) a fully developed wild-type ovule. (D-F) Different stages in which *pMYB98::IPT1* ovules arrest. (D) *pMYB98::IPT1* ovule arrested at early stage of gametogenesis; the arrow point to the presence of degeneration (E) *pMYB98::IPT1* ovule arrested at two-nucleated embryo sac. (F) A mature embryo sac of

pMYB98::IPT1 resulted empty. (G) *pMYB98::IPT1* ovule that complete its development. Data means (±SE). Scale bar 10 μm

To confirm that the defects observed in embryo sac development were effectively due to alteration in cytokinin content, we analysed *TCSn::GFP* in *pMYB98::IPT1*, to have an indirect indication whether the transgenic line really led to an increase in cytokinin within the embryo sac.

In emasculated wild-type plants, during gametogenesis, the signal of *TCSn::GFP* accumulates in correspondence of the chalazal region of developing ovules, that at the maturity corresponds to the region that bears the antipodal cells; in addition cytokinin signalling can be visible also from stage 3-VI, in correspondence of polar nuclei of the central cell that start fusing (Figure 32A).

In *pMYB98::IPT1*emasculated plants the expression of *TCSn::GFP*, was present in the chalaza region but also in the apical part of the developing embryo sac (Figure 32B), confirming that modification of CKs homeostasis could indeed the cause of embryo sac development defects observed.

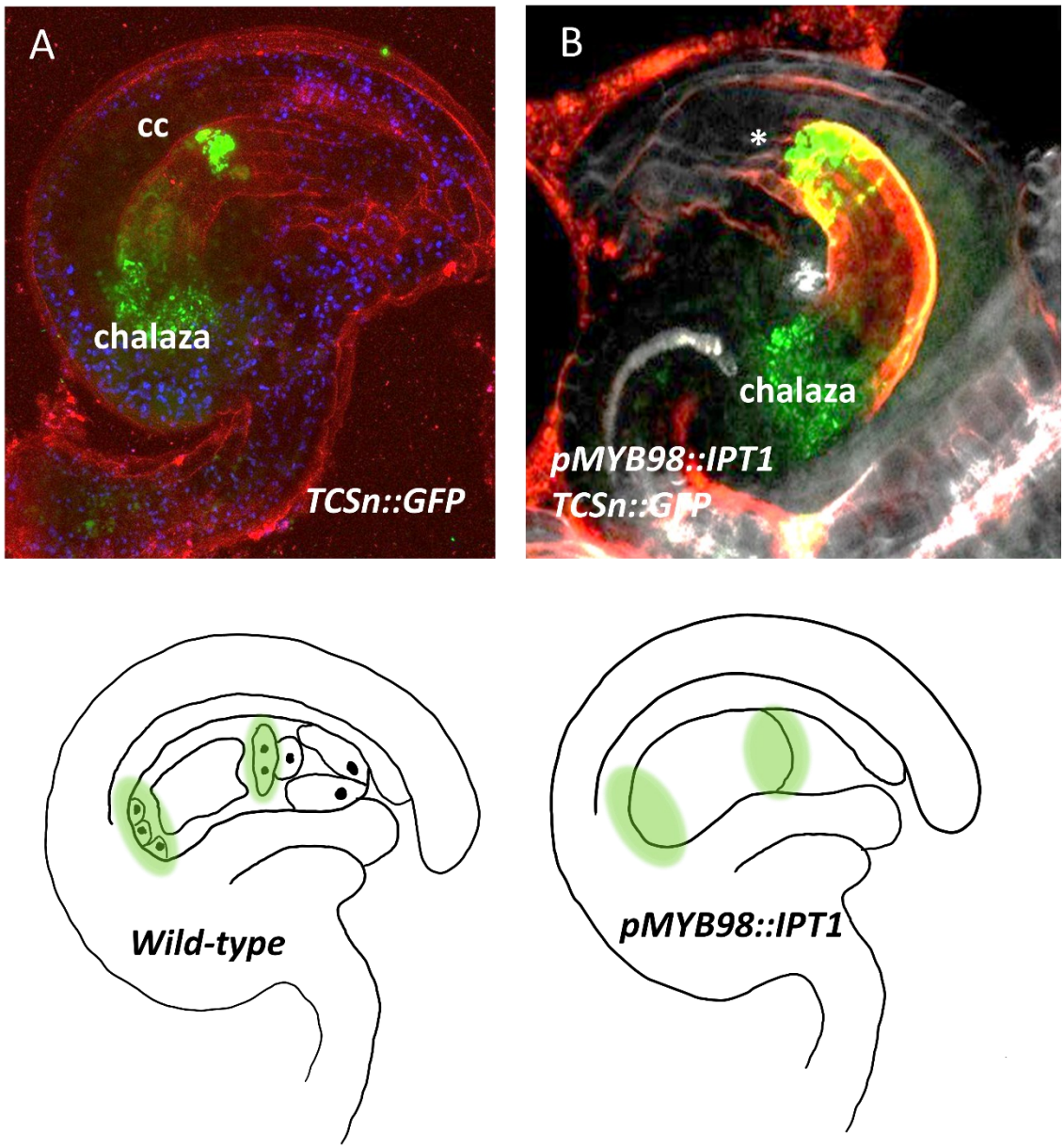

Figure 32 *TCSn::GFP* in wild-type and *pMYB98::IPT1* (A) *TCSn::GFP* at FG7 stage, is expressed in the chalaza and in the central cells. (B) *TCSn::GFP* in *pMYB98::IPT1* in the chalaza but also toward the micropilar pole of the arrested ovules (asterik). Below the graphical representation of the two situations. Scale bar 10 µm

Discussion

Hormones homeostasis is required to allow correct developmental processes. It was already reported that alteration in cytokinin perception (Cheng et al., 2013; Kinoshita-Tsujimura and Kakimoto, 2011) and signalling (Deng et al., 2010; Pischke et al., 2002) led to alteration in female gametophyte cell identity (Yuan et al., 2016). Our results showed that increasing level of cytokinin synthesis leads to sterility which is due to severe impairment of the female gametophyte development. The embryo sac growth was arrested at different stages of gametogenesis. Although, the majority of *pMYB98::IPT1* ovules were defective, the development, 40% of ovules could accomplish their development; this might be due to the penetrance of the construct or to mechanism of compensation that can prevent the increasing of cytokinin biosynthesis.. In addition, it might be interesting to observe the expression of *pWOX2::CENH3-GFP* to analyse the different stages at which the embryo sac arrests, and *FGR7* (Völz et al., 2013), to assess whether the female gametophyte cells correctly acquire their identity.

CONCLUSION

Ovules are the precursor of seeds, and their development requires a tightly controlled network that involves the interaction between environmental cues, transcription factors and phytohormones. Ovules are made of sporophytic tissues that envelop the gametophyte. My work has exploited the important role for the sporophytic chalaza in controlling the female germline development and how this structure can affect the hormonal distribution within the nucellus and ovule, in general. Indeed, a controlled and dynamic spatio-temporal distribution of cytokinin and auxin is required for a proper ovule development and alteration in their homeostasis impair ovule organogenesis and female germline specification and development.

In ovules, auxin is biosynthesized within the chalaza by TAA1 and YUCCA proteins then it is transported by polar transport to the nucellus (Ceccato et al., 2013a). This polar transport guarantees the formation of a local auxin maximum at the top of the nucellus. Down-regulation of *YUCCA4* or *WEI8* as well as *PIN1* have dramatic effects on female gametophyte development (Bencivenga et al., 2012b; Ceccato et al., 2013a; Pagnussat et al., 2009). Once auxin is accumulated at the tip of the nucellus, it is transported across the nucellus thanks to PIN3, PIN4, and PIN7 (Wang et al., 2021). This peculiar transport of auxin allows the formation of a gradient that allows the degeneration of the megaspores and the correct female germline establishment (Pagnussat et al., 2009; Wang et al., 2021).

On the other hand, cytokinin signalling accumulates in the chalaza surrounding the nucellus. Cytokinin perception and signalling defective mutants have been shown to impair specification of the FM and of the gametophytic cells (Cheng et al., 2013; Deng et al., 2010; Pischke et al., 2002; Yuan et al., 2016). In addition, cytokinin and auxin crosstalk has been shown to be involved in several developmental pathways. In SAM for example auxin promotes cytokinin signalling, by inducing the transcription of the *ARF5/MP* that in turn inhibits *ARR7* and *ARR15* negative response regulators of cytokinin signalling (Zhao et al., 2010b). During formation of ovule primordia, crosstalk between auxin and cytokinin converges in the control of *PIN1* to promote ovule formation (Cucinotta et al., 2016a)and again their crosstalk is involved in the establishment of the MMC through the control of *SPL* (Bencivenga et al., 2012b).

The auxin and CKs gradient formed in the different regions of ovule, nucellus and chalaza is required to establish the female germline in megasporogenesis and prepare all the components that allow the correct specification and formation of the mature embryo sac during megagametogenesis.

It will be of great interested to identify the genes that responding to auxin-CKs gradients and consequently drive the proper female germline formation. On this idea, we have evaluated how *BEL1* and *ANT* could be involved in the auxin-cytokinin crosstalk during ovule development.

The two transcription factors BEL1 and AINTEGUMENTA, have been studied for their role in the control of integuments initiation and development. However, even if they are expressed specifically in the chalaza, they affect the development of the gametophyte within the nucellus. In literature, it has been reported that several mutants defective in integument organogenesis exhibited alteration in female gametophyte development (Baker et al., 1997a; Brambilla et al., 2008; Elliott et al., 1996; Gaiser et al., 1995; Klucher et al., 1996; Lang et al., 1994; Reiser et al., 1995; Robinson-Beers et al., 1992). In *bel1-1* and *ant-4* mutant ovules, the organogenesis of the integuments is completely altered and the megasporogenesis is impaired in the degeneration of the megaspores and Functional Megaspore specification. The lack of integuments in the two mutants, is coupled with severe defects in auxin distribution required for the degeneration of the megaspores and for the correct specification of the FM.

In particular, in *bel1-1* ovules, the two polar transporters, PIN1 and PIN3 are upregulated in the chalaza. It might be that BEL1 represses in the chalaza the two *PINs* to allow the correct auxin flow toward the nucellus and at the same time it promotes the transcription of integuments-related genes, such as INNER NO OUTER (INO), PHABULOSA (PHB), PHAVOLUTA (PHV), CORONA (CNA) (Kelley and Gasser, 2009; Yamada et al., 2016) maybe forming a complex with its partner(s).

Interestingly, the overexpression of *BEL1* in *pSTK::BEL1*, phenocopy *bel1* mutant ovules leading to misexpression of *PIN1*. In addition, BEL1 is able to form homodimers, therefore, it was hypothesised that BEL1 is required in a stoichiometric balance within the ovule to allow its function. Low level or high abundance of BEL1 impaired its ability to form functioning complex(es).

In attempt to identify an upstream molecular network controlling *BEL1*, STK came out as a possible candidate. However, *in situ* hyrbidization experiment refuted this hypothesis. It can be that this regulation of STK on BEL1 occurs before ovule initiation

or that SHP1, SHP2 and STK play a redundant role with AGAMOUS in controlling *BEL1* expression (Brambilla et al.,2007).

For what concern *AINTEGUMENTA,* it promotes the initiation of integuments maybe through transcriptional control of the auxin biosynthetic gene *YUCCA4* that in *ant* ovules is downregulated (Li et al., 2021). After all, auxin promotes the formation of lateral organs and integuments can be considered as lateral organs formed from the chalaza (Truernit and Haseloff, 2008). Moreover, *ANT* is epistatic to *BEL* (Baker et al., 1997b), therefore it might be that first ANT initiates integument formation and then BEL1 controls the specification and the growth of integument. However, both transcription factors are involved in the repression of the nucellar gene NOZZLE/SPOROCYTLESS (NZZ/SPL) (Balasubramanian and Schneitz, 2000) and it might be that together repress *PIN1* and *PIN3* expression allowing the correct auxin distribution and the formation of auxin maximum in the nucellus (Figure 33) to guarantee megasporogenesis progression.

If the megaspores degeneration is required for the female gametophyte progression to gametogenesis, another apoptotic event is required to accomplish the fertilization process. Indeed, once the pollen tube arrives at the ovule it gets in touch with one of the two synergid cells degenerate allowing the releasing of the two sperm cells within the embryo sac. It was reported that in *vdd/+* mutant, synergid degeneration was impaired and to test whether alteration in cytokinin levels could prevent degeneration of synergid cells different approaches were used. However, we failed trying to increase cytokinin level specifically within the synergid cells. Indeed, the female sterility observed in the transgenic line *pMYB98::IPT1* was due to defects in gametogenesis progression and around 60% of *pMYB98::IPT1* ovules were arrested at different stage of gametogenesis. These differences could be due to mechanism of compensation or different timing in activation of the promoter. However, expression analyses of the reporter marker TCSn::GFP, suggested that the *pMYB98::IPT1* effectively alter the cytokinin signalling within the embryo sac.

Despite the unexpected results we could highlighted another important role for cytokinin during gametogenesis. Indeed, although it was already reported that they affect embryo sac cell identity, we observed that perturbing cytokinin homeostasis during gametogenesis caused female gametophyte arrest.

To conclude, this work aimed to investigate the molecular network involving auxin and cytokinin and the two transcription factors BEL1 and ANT during female gametophyte specification and development and in particular during megasporogenesis (Figure 33)
In addition, the peculiar distribution of the two hormones within ovule is specifically required to guarantee the apoptotic events during sporogenesis and fertilization process. Alteration in their distribution impaired these two events and the female gametophyte development (Figure 33).

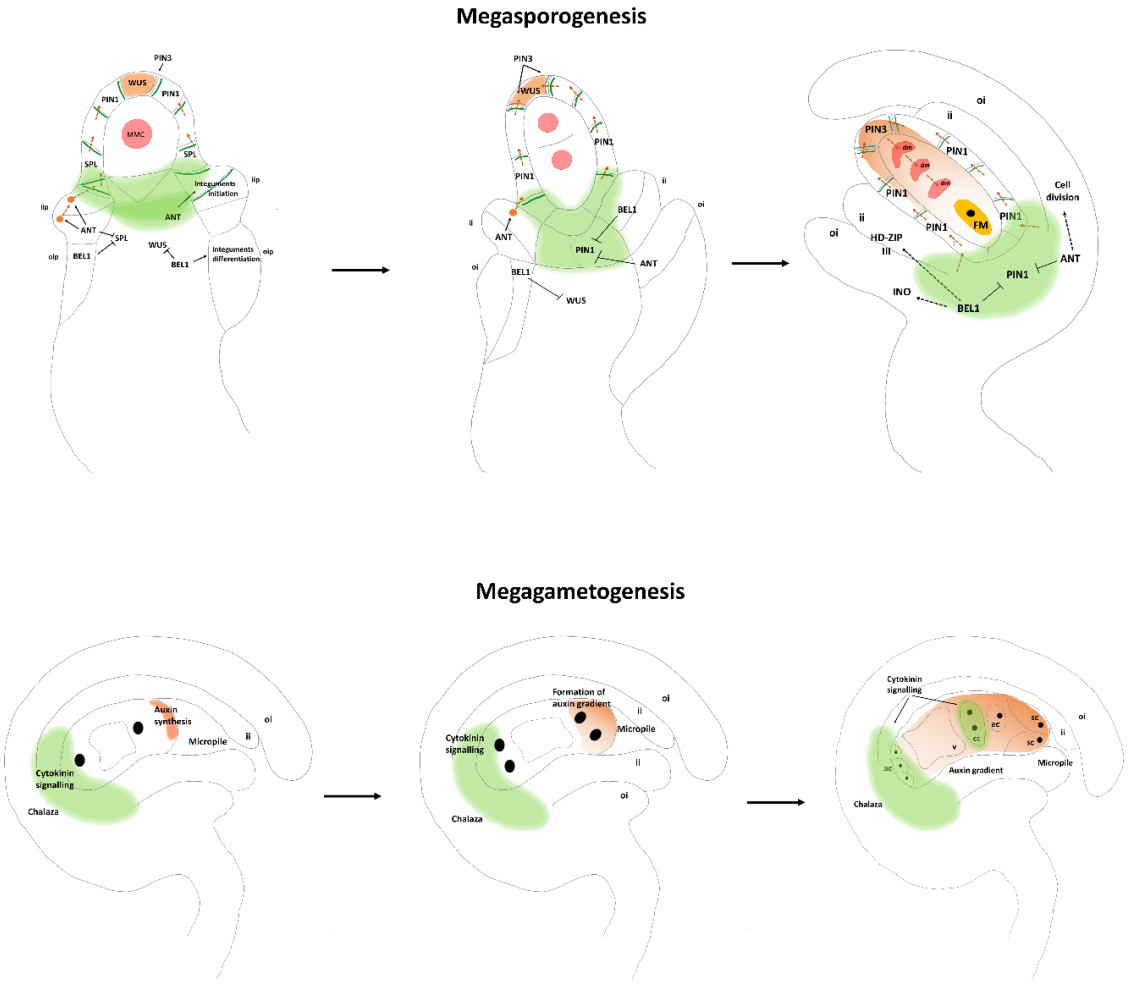

Figure 33 Scheme of Auxin and CKs gradient in megasporogenesis and megagametogenesis in ovules of *Arabidopsis thaliana*. At the beginning of megasporogenesis auxin and cytokinin localize at the opposite pole of the nucellus. In the chalaza, cytokinin signalling (green) is in the region that surrounds the nucellus whereas auxin accumulates at the top of nucellus (orange). In the chalaza are expressed also *ANT* and *BEL1* that are required to initiate and specify respectively the integument primordia. In the chalaza, BEL1 represses *WUSCHEL* (*WUS*) but also *NOZZLE/SPOROCYTLESS* (*SPL*), as *ANT*, that are genes involved in nucellus development. During megasporogenesis the diametrical position of auxin and cytokinin is maintained, and *ANT* and *BEL1* start to restrict the expression of *PIN1* in the chalaza to allow the correct auxin flow toward the top of the nucellus. At the end of the sporogenesis, an auxin gradient is formed within the nucellus, thanks also to the auxin polar

transporters PIN3, PIN4 and PIN7 (here we showed only PIN3). This gradient is required for the degeneration of the megaspores and the FM specification. At the beginning of gametogenesis, CKs still localize in the chalaza whereas auxin biosynthesis genes start to be slightly expressed at the top of the developing embryo sac. However, during gametogenesis CK plays and important role to guarantee the correct specification of the embryo sac cells. For the entire length of gametogenesis CK accumulates in the chalaza and at end of female gametophyte development also in the central cells.

MATERIAL and METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (ecotype Columbia and Landsberg erecta) plants were grown at 22°C under long-day (16-h light/8-h dark) conditions. The *Arabidopsis* lines that were obtained from the European Arabidopsis Stock Centre collection are *bel1-1* (N3090) and ant-4 (N3942).

Reporter marker line *TCSn::GFP* (Zürcher et al.,2013) was provided by Bruno Müller (University of Zurich), *pAHK2::AHK2::GUS*, *pAHK3:AHK3:GUS* and *pCRE1/AHK4::GUS* by Tatsuo Kakimoto (Osaka University), *pMP::MP-GFP*, *pPIN3*::*PIN3-GFP* and *R2D2* marker line seeds were provided by Dolf Weijers (University of Wageningen), *pPIN1*::*PIN1-GFP* seeds was provided by Eva Benkovà (University of ISTA). *ASY3::RFP* by Arp Schnittger (University of Hamburg) , *pKNUnls::YFP* and *pLC2nls::YFP* seeds were provided by Matthew Tucker (University of Adelaide, SA) whereas *pWOX2::CENH3-GFP* (Nico De Storme et al., 2016) by Nico De Storme (University of Leuven).

All the reporter marker lines were fertilized with *bel1-1* and *ant-4* pollen. For all the crosses made, we selected the plants for the presence of the reporter marker and genotyped them to verify the heterozygosity of mutant alleles.

The observed ovule phenotypes were consistent in the F2 and F3 segregating populations indicating that what we observed wasn't dependent on the differences in the ecotype background of our mutants.

Accession Numbers

Sequence data of genes cited in this thesis can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *BEL1* (*AT5G41410*), *ANT* (*AT4G37750*), *AHK2* (*AT5G35750*), *AHK3* (*AT1G27320*), *AHK4/CRE1* (*AT2G01830*), *ARR4* (*AT1G10470*), *ARR7* (*AT1G19050*), *ARR15* (*AT1G74890*), *PIN1* (*AT1G73590*), *PIN3* (*AT1G70940*), *STK* (*AT4G09960*), *MYB98 (AT4G18770), IPT1 (AT1G68460), ARF5/MP (AT1G19850).*

Genotyping

Genomic DNA was extracted from cauline leaves in 400 µl of DNA Extraction Buffer (250 mM Tris-HCl pH 7.5; 25 mM EDTA; 250 mM NaCl) by mechanical homogenization using sterile pestles in 1,50 mL tubes. Samples then were centrifuged at 12500 rpm for 5 minutes. The supernatant was transferred in another tube of 1,50 mL and 360 µl of cold isopropanol were added and the tube mixed by vortex before being centrifuged at 12500 rpm for 20 minutes. The supernatant was discarded and the pellet washed two times with 300 µl of 70% (v/v) ethanol. The pellet then was left drying under hood and resuspended in 50 µl of deionized water.

Genotyping of plants was performed by using 2 µl of DNA as a template for PCR analysis. The PCR was performed in a total volume of 25 µl, containing 5 µl of 5x PCR-buffer (Promega), 2 µl of 2,5 mM dNTPs, 2,5 µl of 10 µM for each primer and 0.1 µl of GoTaq polymerase (Promega). The PCR product was then loaded on a 1% agarose gel.

The *bel1-1* allele (Reiser et al., 1995) contains a C-to-T transition 496 nucleotides after ATG, which once amplified with the primers Atp_407 (5′-GAGAGACATGGCAAGAGATCAG) and Atp_408 (5′-GAGCATGGAGAGCAACTTGG) introduces a BsaAI restriction site. The *bel1-1* allele was identified by BsaAI digestion of PCR products.

The *ant-4* mutant allele (Baker et al., 1997) contains an A-to-T transversion at nucleotide 1342. The *ant-4* allele was identified by XmnI digestion of PCR products amplified with the primers Atp_514 (5′-GGACTGGTAGATATGAAGCTCATCTA-3′) and Atp_515 (5′-GAATCCATGAAGATTGAAGTGAATACT-3′). Primer Atp _515 contains a mismatch (underlined A) that, together with the mutation, produces a restriction site for XmnI.

To identify the presence of the T-DNA encoding *pKNUnls::YFP* we used the following primers: Atp_6044 (5′-GACGCAAACAAACAAAAGCA-3′) and Vep_80 (5′-GTCCATGCCGAGAGTGATC -3′) whereas for *pPIN1:PIN1-GFP* we used: Atp_1481 (5′-TTGTAGTTGTATTCCAACTTGTGG -3′) and Atp_2464 (5′-CCAGTACGTGGAGAGGGAAG -3′).

Analysis by optical and confocal microscopy

To analyse ovule development, flowers at different developmental stages were collected in 9:1 ethanol: acetic acid and incubated at 4°C for an overnight. Then the samples were rehydrated in 90% v/v of ethanol and then placed in ethanol 70% v/v until observation. Ovules were dissected from pistil under Leica stereomicroscope in clearing solution which is composed of 160 g of chloral hydrate (C-8383; Sigma-Aldrich), 100 mL of water, and 50 mL of glycerol. Ovules were observed under Zeiss Axiophot D1 microscope equipped with DIC optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

To analyse callose deposition in ovules, it was performed Aniline Blue staining.

Ovules were dissected from pistil at different stage of development and mounted directly on slide in a drop of 0.005 % (w/v) Aniline Blue solution (Aniline bleu diammonium salt, Sigma Aldrich, catalogue no. 415049).

Sample were observed under fluorescence Axio Imager M2 microscope for viewing under ultraviolet light (DAPI filter; Zeiss Filter set 47: 436 nm/480 nm). Autofluorescence was used to highlight the ovule outline in the dsRED channel (Zeiss Filter set 43: 545 nm/605 nm).

For all GUS assays, samples were collected in acetone 90% (v/v) at -20°C for 20min and then washed three times with water and placed in GUS solution for an overnight at 37°C as previously described (Liljegren et al., 2000). For confocal laser scanning microscopy, dissected ovules were mounted in a staining solution containing 0.1% (v/v) SR2200 (Renaissance Chemicals; stock solution from the supplier was considered as 100% and diluted in water) that was always prepared freshly before use. This protocol allows to mark nuclei and cell wall of samples.Images were taken with Nikon A1 confocal microscopes using 40x water-immersion objective. SR2200 was excited with a 405 nm laser line and emission recorded between 415 and 476 nm (405 nm/415-476 nm). GFP was excited at 488nm and detected at 498-530 nm, Venus excited at 514 nm and detected at 524-540 nm and ndTomato excited at 561 nm and detected at 571-630 nm. Autofluorescence was revealed exciting samples at 561 nm and detected at 640 nn.

In situ Hybridization

Arabidopsis flowers were fixed and embedded in paraffin as previously described by Brambilla et al., (2007). Sections of plant tissue were probed with digoxigenin-labeled for *BEL1* or *ANT* antisense RNA. Hybridization and immunological detection were performed as described previously (Brambilla et al., 2007). The *BEL1* and *ANT* specific antisense probes were amplified respectively with the following primers: AtP_7782 (5'-TAATACGACTCACTATAGGGATGGCAAGAGATCAGTTCTATGGTC -3') AtP_7783 (5'- AAAGACCTTGGCTTGGTCTT -3') and AtP_7844 (5'-TAATACGACTCACTATAGGGCTACAACACCACTCATGAGC -3') AtP_7852 (5'-GATCAAGAATCAGCCCAAGC -3')

Yeast-two Hybrid experiment

For yeast two hybrid experiment, gateway cloning strategy was used. The BEL1 open reading frame was amplified with primers AtP_8028 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTccATGGCAAGAGATCAGTTCTATG -3') and AtP_1081 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCAAA CAATATCATGAAGTAATTG -3') was cloned into pDONR207 (Gentamycin resistance) and/or pDONR201 (Kanamycin resistance) (Life Technologies) and pGBKT7 (BD-Vector; Kanamycin) then into and pGADT7 (AD-Vector; Ampicillin resistance) plasmids.

Saccharomyces cerevisiae strain AH109 was inoculated in 25mL of liquid medium (20 g/l yeast extract, 40 g/l tryptone), containing also 2% (v/v) of glucose, over-night at 28°C. From liquid yeast culture, 2mL were taken and placed in 2ml tubes. Cells were pelleted by centrifuge for 5 minutes at 4000 g. Pellet was washed first with 1 mL of sterile water (centrifuge 4000g for 5 min) and then with 1 mL of 1x LiTE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8, 100 mM LiAc). Pellet was then resuspended in 1 mL of 1x LiTE by pipetting. For each co-transformation, 100 µl of resuspended cells were transferred into new 1,5 mL tubes and combined with 1 µg of each construct (plasmid), 20 µl Carrier-DNA (DNA from fish sperm 2 mg/ml), 20 µl dimethylsulfoxid (DMSO) and 400 µl LiTePEG (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8, 100 mM LiAc, 40% PEG v/v). Each co-transformation were then incubated at 30°C for 30 minutes and at 42°C for 15-20 minutes. Afterward, cells were centrifuged at 9000g for

1min, the supernatant was removed, and cells were resuspended in 100 µl of sterile water. Co-transformations were then plated on SC-Drop-out (-W-L) plates. The plates were incubated at 28 °C for 2 days.

For each co-transformation, few colonies were streaked out and inoculated in 5 ml liquid SC-Drop-out (-W-L) medium overnight.

The OD600 of each transformation was measured and adjust to be the same value for all the co-transformation in a final volume of 1mL. For each transformation, serial 1:10 dilutions were prepared, and 8µl were spotted on different selective plates: SC-Drop-out (-W-L), SC-Drop-out (-W-L-H), SC-Drop-out (-W-L-A) and SC-Drop-out (-W-L-H-A) plates. The SC-Drop-out was prepared with 6.67 g/l yeast nitrogen base (w/o amino acids), 20 g/l glucose, 0.83 g/l Synthetic Complete Drop Out Mix (-W-L/ -W-L-H/ -W-L-A/-W-L-H-A).

Luciferase Assay

Protoplast preparation and transient expression experiments were performed as described by De Sutter et al. (2005). Luciferase assay was performed as previously described in Galbiati et al. (2013).

Plasmid construction and Arabidopsis transformation

To generate the construct *pMYB98::IPT1*, promoter region of *MYB98* was amplified with

AtP_4951 (5'- CAGAGCTCCGGAGATAGTGGCTGAGAGGT – 3') AtP_4952 (5'-CATGATCAGTTCTTGATCACGTGTGAAGATG -3') containing respectively SacI and SpeI restriction sites, and inserted by the restriction enzymes cloning strategy in pB2GW7 (Binary vector with 35S; Spectinomycin resistance / Bar resistance).

The coding sequence of *IPT1* was amplified with AtP_4879 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACAGAACTCAACTTCCACCT – 3') AtP_4880 (5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAATTTTGCACCAAATGCCGC -3') and cloned first in pDONR207 (Life Technologies) and then in pMYB98::pB2GW7 in by using Gateway cloning system. Vectors were electroporated into Agrobacterium tumefaciens GV3101 and *A. thaliana* Col-0 plants

were transformed by using the floral-dip method (Clough S.J., 1998) and selected for BASTA resistance.

List of primers used in this work

Gene	Primers	Sequence (5'-3')	Purpose
BEL1	Atp_407	GAGAGACATGGCAAGAGATCAG	Genotyping
BEL1	Atp_408	GAGCATGGAGAGCAACTTGG	Genotyping
ANT	Atp_514	GGACTGGTAGATATGAAGCTCATCTA	Genotyping
ANT	Atp_515	GAATCCATGAAGATTGAAGTGAATACT	Genotyping
pKNUnls::YFP	Atp_6044	GACGCAAACAAACAAAAGCA	Genotyping
pKNUnls::YFP	Vep_80	TCCATGCCGAGAGTGATC	Genotyping
pPIN1:PIN1-GFP	Atp_1481	TTGTAGTTGTATTCCAACTTGTGG	Genotyping
pPIN1:PIN1-GFP	Atp_2464	CCAGTACGTGGAGAGGGAAG	Genotyping
BEL FW - T7	AtP_7782	TAATACGACTCACTATAGGGATGGCAAGAGATCAGTTCTATGGTC	ISH
BEL RV	AtP_7783	AAAGACCTTGGCTTGGTCTT	ISH
ANT FW - T7	AtP_7844	TAATACGACTCACTATAGGGCTACAACACCACTCATGAGC	ISH
ANT RV	AtP_7852	GATCAAGAATCAGCCCAAGC	ISH
BEL1 CDS	AtP_8028	GGGGACAAGTTTGTACAAAAAAGCAGGCTccATGGCAAGAGAT	Y2H

		CAGTTCTATG	
BEL1 CDS	AtP_1081	GGGGACCACTTTGTACAAGAA AGCTGGGTTCAAACAATATCA TGAAGTAATTG	Y2H
SacI-pMYB98	AtP_4951	CAGAGCTCCGGAGATAGTGGC TGAGAGGT	Gateway cloning
pMYB98-Spe1	AtP_4952	CATGATCAGTTCTTGATCACGT GTGAAGATG	Gateway cloning
IPT1 CDS	AtP_4879	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGACAGAACT CAACTTCCACCT	Gateway cloning
IPT1 CDS	AtP_4880	GGGGACCACTTTGTACAAGAA AGCTGGGTCCTAATTTTGCACC AAATGCCGC	Gateway cloning

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APPENDIX

During my PhD I've contributed to research activities regarding auxin and cytokinin involvement in ovule development, that resulted in review article and a submitted article. For what concern the results presented in this thesis, I'm currently writing an article for the transcription factor BEL1 and its role during megasporogenesis. The results about the transcription factor ANT require additional experiment to better clarify its involvement in the regulation of megasporogenesis. Lastly, we are performing experiment to better characterized the cytokinin involvement during gametogenesis and in synergid degeneration, hopeful to publish our discoveries soon.

Pubblished article

Terceros GC, Resentini F, Cucinotta M, Manrique S, Colombo L, Mendes MA.
The Importance of Cytokinins during Reproductive Development in Arabidopsis and Beyond. Int J Mol Sci. 2020 Oct 31;21(21):8161. doi: 10.3390/ijms21218161.

In this review we've presented the current insights and knowledge regarding cytokinin involvement during ovule, pollen and seed formation in Arabidopsis and crops. (Following attached at the end of this thesis)

Submitted article

Pessino S., CucinottaM., Colono C., Costantini E., Perrone D., Callizaya-Terceros G., Petrella R., AzzaroC., Podio M., Dickinson H., Marconi G., Albertini E., Colombo L., Mendes M.A. AUXIN RESPONSIVE FACTOR 10 insensitive to miR160 regulation induces apospory-like phenotypes in Arabidopsis. (Submitted in iScience)

In this work, we've exploited the role of the AUXIN RESPONSIVE FACTOR 10 (ARF10) in determination of MMC and its entry in meiosis. Alteration in ARF10 regulatory mechanism that involves miR160 leads to phenotypes mimicking apospory (i.e., multiple embryo sac formation with random orientation of cell-identity types and unusual embryo sac morphology). In particular, I've contributed with microscopy analyses and qPCR analyses.

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Review

The Importance of Cytokinins during Reproductive Development in Arabidopsis and Beyond

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Abstract: Fertilization and seed formation are fundamental events in the life cycle of flowering plants. The seed is a functional unit whose main purpose is to propagate the plant. The first step in seed development is the formation of male and female gametophytes and subsequent steps culminate in successful fertilization. The detailed study of this process is highly relevant because it directly impacts human needs, such as protecting biodiversity and ensuring sustainable agriculture to feed the increasing world population. Cytokinins comprise a class of phytohormones that play many important roles during plant growth and development and in recent years, the role of this class of phytohormones during reproduction has become clear. Here, we review the role of cytokinins during ovule, pollen and seed formation at the genetic and molecular levels. The expansion of knowledge concerning the molecular mechanisms that control plant reproduction is extremely important to optimise seed production.

Keywords: cytokinins; reproduction; seed formation

1. Introduction

Cytokinins (CKs) play a crucial role in regulating many aspects of plant growth and development [1–3]. Endogenous CKs are adenine derivatives and are categorised into two groups, based on whether they possess an isoprenoid or aromatic side chain. Isoprenoid CKs are more widespread in nature and include isopentenyladenine (iP), trans-zeatin (tZ)-, cis-zeatin (cZ)- and dihydrozeatin-type derivatives. Generally, tZ and iP exhibit higher activities than cZ, and in addition to their sugar conjugates, tZ and iP are the most abundant forms, although much variation exists, depending on the plant species, tissue and developmental stage [4,5].

The initial and rate-limiting step in CKs biosynthesis is catalysed by isopentenyltransferases (IPTs). *Arabidopsis thaliana* possesses nine *IPT* genes: *AtIPT1*, *AtIPT3* and *AtIPT4–AtIPT8* encode ATP/ADP IPTs, whereas *AtIPT2* and *AtIPT9* encode tRNA isopentenyltransferases, which modifies a subset of adenine bases on tRNAs [6]. The subsequent biosynthesis step involves the cytochrome P450 enzymes CYP735A1 and CYP735A2, which convert CK nucleotides into the corresponding tZ-nucleotides [7,8]. Finally, enzymes encoded by the *LONELY GUY* (*LOG*) gene family activate CKs by converting nucleotides into the corresponding nucleobases [9]. CKs are degraded by CYTOKININ OXIDASE/DEHYDROGENASES (CKXs) and the Arabidopsis *CKX* gene family contains seven members (*AtCKX1* to *AtCKX7*), each of which shows distinct spatio-temporal patterns of expression and slightly different enzymatic properties [10,11]. In addition to degradation, CKs can also be deactivated by glucosylation by UDP-glucosyl transferases (UGTs) [12,13]: N-glucosylation is irreversible, whereas O-glucosylation can be reversed by β-glucosidases (GLUs).

CKs are perceived via a multistep phosphorylation pathway. In Arabidopsis, the three histidine protein kinases (AHKs) AHK2, AHK3 and AHK4/CRE1 function as CK receptors. Upon CK binding, AHK2-4 are auto-phosphorylated and a phosphoryl group is transferred to histidine phosphotransfer proteins AHPs (in Arabidopsis, AHP1–5). AHPs can also be phosphorylated by CYTOKININ INDEPENDENT 1 (CKI1) that acts independently of CKs because it lacks the cytokinin-binding domain [14]. In turn, AHPs transmit the phosphorelay signal to nuclear response regulators ARRs, which modulate the transcription of cytokinin-related genes. Two classes of ARRs exist: nuclear type-B response regulators that act as positive transcriptional regulators, and type-A regulators, which are involved in negative feedback loops [15,16] that also inhibit the transcription of type-B ARRs. In addition, a large proportion of the CK transcriptional response is also promoted by cytokinin response factors (CRFs), a group of closely related transcription factors belonging to the AP2 family [17].

Although CK signalling and perception pathways have been characterised in great detail (Figure 1) and increasingly more studies in recent years have highlighted the importance of CKs during many developmental and physiological processes, how CKs control these developmental processes at the molecular level is still not fully understood. In this review, we discuss the most recent insights regarding the role of CKs during reproductive development and mainly focus on ovule, pollen and seed development in the model species *Arabidopsis thaliana*.

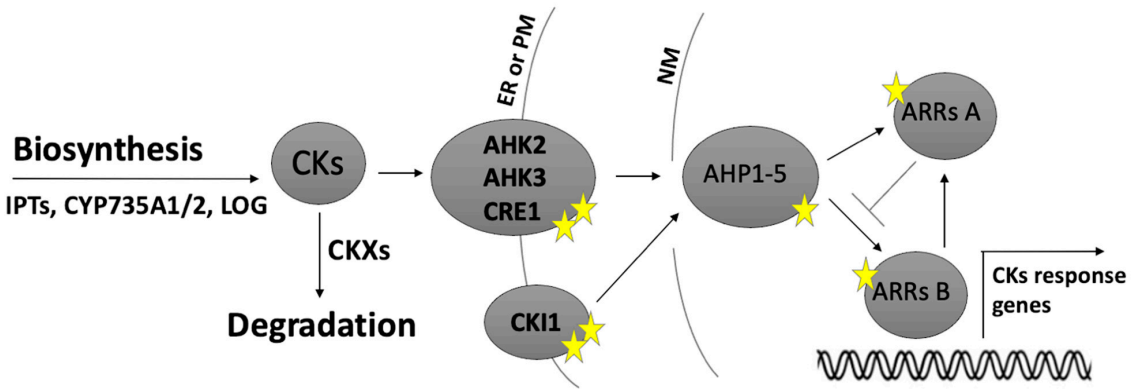

Figure 1. An overview of the cytokinin (CK) pathway from biosynthesis to response in Arabidopsis. CKs are synthesised by isopentenyltransferases (IPTs) and CYP735A1/2 enzymes and converted into active forms by LONELY GUY (LOG) enzymes, whereas cytokinin degradation occurs mainly through CKXs. CKs are perceived by the AHK2, AHK3 and AHK4/CRE1 receptors, which initiate a phosphorylation signalling cascade involving AHPs, which phosphorylate and activate type-B ARRs. Active ARRs then induce cytokinin-responsive genes, such as those encoding the type-A repressor ARRs. AHPs can also be phosphorylated by CKI, a histidine kinase that activates the cytokinin response in the absence of CKs. Stars represent phosphorylation sites. (ER) endoplasmic reticulum; (PM) plasma membrane; (NM) nuclear membrane.

1.1. Cytokinins Play a Pivotal Role in Ovule Patterning and Development

Flowering plants alternate between a diploid sporophytic stage, which constitutes the main body of the plant, and a reduced, haploid gametophytic stage contained within the female and male floral organs. The female gametophyte (FG), or embryo sac, develops within the maternal sporophytic tissues of the ovule, which provides structural support to the female gametes and encloses them until the seed develops, following fertilization [18].

Ovule primordia arise from the meristematic placenta tissue within the gynoecium, and mutants in which the synthesis or perception of CKs is compromised, produce fewer ovule primordia [19]. By contrast, increased levels of CKs in *ckx3ckx5* double mutant lead to an increase in gynoecium size and to the formation of more ovules, due to increased meristematic activity of the placenta tissue [20,21]. Moreover, it has been also reported for other Brassicaceae species that CKs influence ovule number, suggesting that the genetic manipulation of CK metabolism is a potentially powerful tool to enhance

seed yield [19,22]. Intriguingly, however, it was recently demonstrated that high levels of CKs in *ckx7* mutants inhibited fruit elongation [23].

In flowering plants, megasporogenesis begins when ovules arise from the placenta as finger-like structures. Within the ovule primordia, a single subdermal nucellar cell enlarges and differentiates into a megaspore mother cell (MMC) (Figure 2a). The MMC then undergoes meiosis to generate four haploid megaspores, three of which degenerate and the remaining one becomes the functional megaspore (FM) (Figure 2a). During this process, the two inner and outer integuments surrounding the developing female germline develop and after three rounds of mitosis, the FM gives rise to the FG in a process called megagametogenesis (Figure 2a).

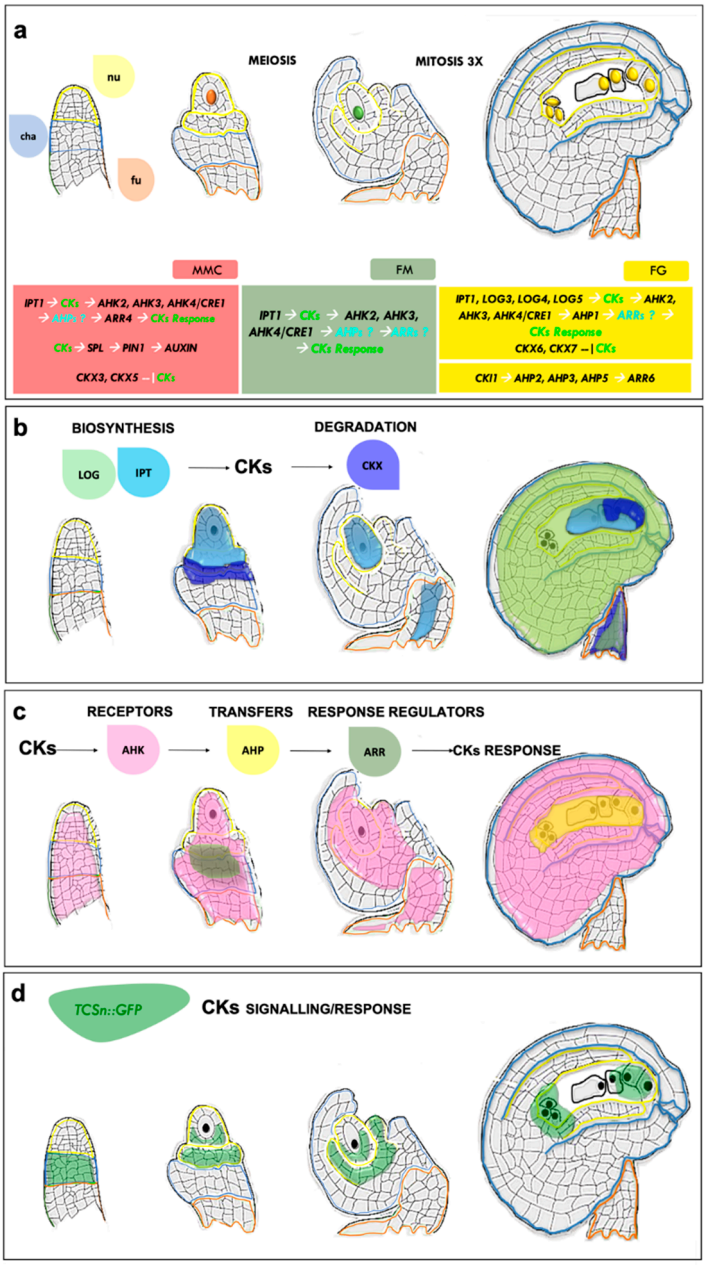

Figure 2. The expression domains of CKs-related genes during ovule development. CKs biosynthesis, degradation and signalling components expression domains and their hypothetical relationships. (**a**) Cartoon of the distinct and sequential phases of ovule development in Arabidopsis, first finger-like structure with three different zones outlined nucella, chalaza and funiculus, then ovule primordium with a megaspore mother cell (MMC) differentiated that enters in meiosis forming the functional

megaspore (after the degeneration of the other 3 spores) that after three rounds of mitosis forms the mature female gametophyte (FG). Below, the distinct relationships among the different CK-related biosynthetic, degradation and signalling components are depicted in coloured rectangles: the MMC stage is highlighted in red, the FM stage is depicted in green and the mature female gametophyte stage in yellow. The hypothetic missing CK-elements are depicted with a '?' and highlighted in light blue. (**b**) CK biosynthesis and degradation related genes expression domains: *pIPT1::GUS* is expressed at MMC and FM stages in the nucellus; at the mature stage of FG, it is active in the synergids and egg cells; the promoter of *LOG3* is active at FG stage in the funiculus, and *LOG4* at the same stage in the mature ovule integuments at the chalazal pole, whereas the activity *pLOG5::GUS* was detected at FG stage. Regarding CK degradation components at MMC stage the mRNA of *CKX5* was detected in the nucellus and chalaza. At FG stage *pCKX6::GUS* is restricted to the funiculus, whereas the *CKX7* promoter is active in synergid cells and CKX7 protein is detected in the synergids and egg cells. In light blue are IPT, in green the LOG and in dark blue the CKX expression domains. (**c**) The expression domains of CK signalling genes. The *AHK1* and *AHK3* promoters are active preferentially at the chalaza pole and only *AHK2* is expressed throughout the whole ovule development. Regarding ARRs activity, *ARR4* is present at MMC stage at the chalazal pole. In pink are depicted the AHK, in yellow the AHP and in green the ARR expression domains. (**d**) CK signalling/response is reported by *TCSn::GFP* expression: *GFP* expression is restricted to the chalazal region from during all stages of ovule development; at FG stage the signal is also present in the micropilar region. The region outlined in yellow is the nucellus (nu), blue represents the chalazal (chal) and the orange corresponds to the funiculus (fu). The ovule drawings were adapted from [24].

CKs are important from the very beginning of ovule emergence and several studies have demonstrated that disruption of CK homeostasis affects all phases of ovule development. For instance, integument initiation in the triple CK receptor mutant *ahk2-2 ahk3-3 cre1-12/ahk4* is impaired and some ovule primordia remain as long finger-like structures, with most of the remaining ovules arresting during gametogenesis [24–26]. Specification of the FM is also affected in this triple mutant, which does not express the *pFM1::GUS* identity marker, and some ovules display a FG that contains only one or two nuclei, or only a central cell-like nucleus, which explains the observed block in gametogenesis [25]. Promoter studies revealed that during ovule development, *AHK3* and *CRE1/AHK4* are preferentially expressed in the chalaza region, whereas *AHK2* is expressed throughout the ovule (Figure 2c) [24,25]. In conclusion, disruption of the three-cytokinin histidine kinase receptors (AHKs) clearly affects ovule integument and FG development, although the effect varies, depending on the *ahk* mutant alleles, and the phenotypes of all mutant combinations are summarised in Table 1. Defects in ovule integument development have also been observed in wild type ovules exogenously treated with CKs (6-*Benzylaminopurine*, BAP), which developed a single structure instead of two integuments. Notably, only the *cre1-12/ahk4* mutant developed two integuments when treated with BAP, whereas integument development remained affected in the other single receptor mutants *ahk2* and *ahk3*, demonstrating a major role for CRE1/AHK4 in the detection of excessive CK levels [24]. The genetic network that controls ovule development involves other two key transcription factors, *BELL1 (BEL1)* and *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*), and the *bel1 spl* double mutant possesses finger-like ovule structures without integuments, similar to those in *ahk2-2 ahk3-3 cre1-12* [24]. The expression of both *BEL1* and *SPL* is regulated by CKs, although CKs negatively affect *BEL1* expression and positively affect that of *SPL*. In addition to a role in integument formation, the fundamental role of *SPL* in MMC initiation has been well characterised and ovules of *spl* fail to produce male and female germlines [27]. Moreover, *SPL* regulates the expression of *WUSCHEL*, which encodes a CK-responsive homeodomain transcription factor initially identified as a stem-cell regulator in the shoot meristem. *WUS* is also required for megasporogenesis and its loss of function leads to defective MMC development [28]. Several studies have demonstrated that CKs control the expression of the auxin efflux carrier gene *PIN-FORMED 1* (*PIN1*), which is required to create the auxin gradient necessary for ovule primordium

formation and integument growth. In particular, *PIN1* expression in the placenta and ovule primordia is mainly mediated by CRFs, because it is reduced in the *crf2 crf3 crf6* triple transcription factor mutant affecting CK signalling [29]. In the chalaza, auxin–cytokinin crosstalk converges on the regulation of *PIN1* by SPL and BEL1. In *spl* mutant ovules, *PIN1* is not expressed, whereas it is ectopically expressed in *bel1*. In *pin1-5*, a few ovule primordia develop as finger-like structures, similar to the ovule phenotype of double *spl bel1* and triple *cre1-12 ahk2-2 ahk3-3* mutants [24].

1.2. CK Perception Influences Female Gametophyte Cell Identity

In wild type Arabidopsis ovules, the FG derives from three rounds of mitosis. The first two rounds occur without cytokinesis and lead to a four-nucleate coenocyte FG with two nuclei at each pole. During a third mitotic division, phragmoplasts and cell plates form between sister and non-sister nuclei; this represents the beginning of the cellularisation process and the FG cells quickly become completely surrounded by cell walls. All these events lead to the formation of a coenocytic, eight-nucleated embryo sac. Subsequent nuclear migration, polar nuclear fusion and cellularisation take place to ultimately produce a seven-celled FG that consists at the micropylar pole of two synergids, one egg cell, one diploid central cell, and three antipodal cells at the chalazal pole [30–32].

The histidine protein kinase CKI1 involved in CK perception is required to specify cell identity in the FG. Experiments with Arabidopsis protoplasts have shown that *CKI1* overexpression leads to the cytokinin-independent activation of the two-component CK signalling pathway [33] and to constitutive activation of the *type-A RESPONSE REGULATOR 6* (*ARR6)* promoter [34]. In addition, CKI1 shares downstream components with the cytokinin signalling pathway, such as the His phosphotransfer proteins AHP2 and AHP3 that act upstream of ARRs in the CK signalling cascade [35]. The mutant alleles *cki1-5* and *cki1-6* cause lethality [36]. As suggested by the genetic heritability of the *cki1-5* and *cki1-6* alleles, half of the FGs in heterozygote plants were defective [36]. Deng and colleagues in 2010 partially rescued the lethal female gametophyte phenotype of *cki1-5* using the *CKI1* promoter to drive the expression of either *IPT8* (biosynthetic gene) or *ARR1* (a type-B Arabidopsis Response Regulator- CK signaling/response), demonstrating that CKI1 is required for cytokinin signalling and for appropriate FG development [35].

More recently, in 2016, a fourth mutant allele, *cki1-9*, was described, whose embryo-sac formation was also compromised [37]. The use of specific FG identity markers showed that both the central cell and the accessory antipodal cells in *cki1-9* lose their identity in favour of egg-cell identity, synergid cell identity was correctly maintained. In *cki1-9* mutants, the ectopic egg cell can be fertilized, the seeds contained zygotes without developing endosperm, consistent with loss of central cell identity [37]. By contrast, ectopic expression of CKI1 under control of the FG-specific (*pAKV*) promoter leads to mis-expression of the central cell marker that became visible in the egg and synergid cells position. The mutant ovules developed seeds with dual endosperm but no embryos [37]. A similar phenotype was observed when *CKI1* was expressed specifically by egg cell- or the synergid cell-specific promoters. The *CKI1-GFP* protein fusion is expressed from the beginning of FG formation and throughout its development. At the one- and two-nucleus stages, CKI1 co-localises with the FG-specific endoplasmic reticulum (ER) marker, *pAKV::TaqRFPer*, in the perinuclear regions. When the nuclei at each pole divide during the second mitotic event to form the four-nucleate FG, CKI1 localisation becomes polarised and is only detected in the ER surrounding the two sister nuclei at the chalazal pole, distinguishing between the nuclei of the chalaza and micropyle [37]. In the mature FG, CKI1 expression is restricted to the central and antipodal cells (Figure 3).

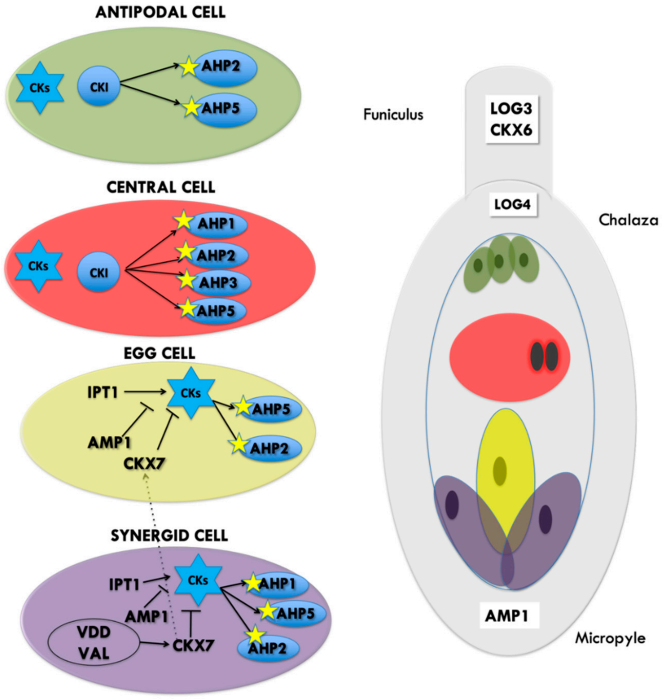

Figure 3. Arabidopsis mature female gametophyte and CKs involvement. The specific localisation and different relationships among CK biosynthesis, degradation and signalling components within the mature FG determine the identities and functions of each cell of the embryo sac. The signalling component *AHP1* is present in the central cell and the synergid cells; instead, *AHP2* and *AHP5* are expressed in all the gametophytic cells and *AHP3* is specifically localized in the central cell. CKI1, cytokinin-independent kinase is specifically expressed in the antipodals and central cells, interacting with the present AHPs. The components involved in CK biosynthesis and degradation genes, *IPT1* and *CKX7*, respectively, are mainly active on the opposite side of the embryo sac, towards the micropyle. Intriguingly, CKX7 promoter activity is detected in synergid cells and the protein in the egg cell. Components of CK biosynthesis and degradation are mainly expressed in the sporophytic tissues surrounding the mature ovule such as *LOG3* that is expressed in the funiculus, as well as degradation components, *CKX6.* Instead, *LOG4* that is expressed in the integuments at the chalaza side. *AMP1* is expressed in the ovule integuments in the synergids and weekly in the egg cell, AMP1 controls the content of CKs. *VDD* and *VAL* REM transcription factors are expressed both in synergid cells and control directly and/or indirectly the expression of *CKX7*. In purple are distinguished synergid cells, in yellow the egg cell, in red the central cell, in green the antipodals and in grey the sporophytic tissues surrounding the female gametophyte. Yellow stars represent the phosphoryl group. (CKs) cytokinins.

As mentioned above, CKI1 shares downstream components with the CK signalling pathway: BiFC protein–protein interaction and yeast two-hybrid assays demonstrated that CKI1 interacts with the CK signaling AHP2, AHP3 and AHP5 and more weakly with AHP1 proteins [38,39]. The *AHP* genes are expressed in the embryo sac (Figure 2c); in particular promoter analysis using nuclear eGFP demonstrated that *pAHP1* is active in the central cell and the synergid cells (Figure 3), *AHP2* and *AHP5* are both expressed in all female gametophytic cells, whereas *AHP3* is specifically expressed in the central cell [40] (Figure 3). Only *AHP2*, *AHP3* and *AHP5* appear to be involved in FG formation. The *ahp2 ahp3 ahp5/+, ahp2 ahp3/+ ahp5, and ahp2/+ ahp3 ahp5* multiple mutants reproduced *cki1-9* mutants, with a strongly reduced sensitivity to cytokinin and reduced fertility and produced gametophytes in which antipodal and central cells identity was lost, and egg-cell fate was acquired [40]. Furthermore, the quintuple mutant siliques were shorter, and seed development was variable, because seed abortion was often observed. However, the mutant seeds produced were larger than those in wild type [41]. The two primary classes of type-A and type-B response regulators (ARRs) respond to CK signalling.

Both ARR types can be phosphorylated, but only type-B ARRs contain a Myb-like DNA binding domain [42]. The type-A ARRs are rapidly induced by CKs and compete with type-B ARRs for phosphorylation, but function as negative regulators of the cascade [34,43] (Figure 1). The quadruple type-B ARR mutant *arr1-3 arr2-2 arr10-2 arr12-1* produces a substantial number of ovules with arrested gametophytes [25], similar to ovules in multiple *ahk* and *cki1* mutants. Type-A ARR double mutant *arr7arr15* also cause female gametophytic lethality, although the cause of this lethality was not investigated [44].

The specification of FG-cell identity also requires two members of the REPRODUCTIVE MERISTEM (REM) family of transcription factors, VERDANDI (VDD) and VALKYRIE (VAL). *VDD* and *VAL* are expressed throughout all stages of ovule development, and their mutation causes a strong defect in synergid-cell specification (Figure 3). The *vdd-1* homozygous mutant is lethal and heterozygous siliques contain a high percentage of unfertilized ovules. A similar phenotype was observed for *VAL-RNAi* transgenic lines. In *vdd-1* heterozygous and *VAL-RNAi* mutants, the identity of the synergids is partially lost, and a low percentage of the synergid cells in *vdd-1/+* ovules express the antipodal cell marker [45,46]. Synergid cells express *CKX7,* whereas the CKX7 protein is also detected in the egg cell [47], which is important for CK degradation, but the activity of *pCKX7::nlsGUS* was absent in approximately half of the *vdd1* heterozygous and *VAL_RNAi* ovules, suggesting that gametophytes that carry these genes knockdown, do not express *CKX7* and probably contain higher levels of active CK. This has been suggested to cause the persistence of the synergids after pollen tube arrival and thereby to affect sperm delivery and fertilization.

Another well-known mutant that exhibits a defect in female gametophyte cell specification is *altered meristem program 1* (*amp1*). *AMP1* encodes a glutamate carboxypeptidase that is important for shoot apical meristem development and phytohormone homeostasis [48] and *amp1-1* and *amp1-2* T-DNA mutant alleles present high levels of CKs [49]. Loss of *AMP1* function in *amp1-10* and *amp1*-13 leads to supernumerary egg cells at the expense of the synergids, enabling the formation of twin embryos [50]. AMP1 is not expressed only in the sporophytic tissue (Figure 3), but also in the synergids and more weakly in the egg cell [50] (Figure 3). Morphological analyses of *amp1/+* heterozygous plants identified twin embryos and supernumerary egg cells only very rarely. This indicates that sporophytic *AMP1* expression is sufficient to prevent the cell-fate change of synergids and suggests that AMP1 might move between cells or be required for the production of a mobile signal necessary for synergid identity [50]. The presence of high levels of CKs and synergid cells that are abnormally specified in *amp1* mutants, is consistent with previous observations for *cki1*, *ahp* and *vdd* and *val* mutants. All the hypothetical interrelationships are depicted in Figure 3.

1.3. Cytokinins Are Involved in Sporophyte–Gametophyte Communication

CKs execute important functions at all stages in ovule development, and particularly during gametophyte cell-fate acquisition. However, not all the components of the CK machinery are expressed in gametophytic cells, but some are also present in sporophytic cells, suggesting that the acquisition of identity within the gametophyte requires CKs-based communication between the gametophyte and sporophyte.

One of the most useful tools available with which to understand CK signalling is the *TCS::GFP* marker developed by Muller and collaborators in 2008, which reflects the transcriptional activity of ARR type-B response regulators to CKs and utilises their DNA-binding motif cloned upstream from the GFP reporter gene [51]. In 2013, Zürcher and colleagues established an improved version of the *TCS* synthetic reporter using an extended version of the ARR type-B DNA-binding motifs that is more sensitive to CK in most tissues analysed [52]. The use of this TCS reporter has enabled CK signalling/response during the different phases of ovule development to be monitored (Figure 2d). The analysis of *TCSn* revealed that at the very beginning of ovule development, *GFP* expression is detected only in the basal part of the ovule that corresponds to the chalaza region, immediately below where the megaspore mother cell MMC differentiates. At the FM stage, the GFP signal is once more

restricted to the sporophyte in the chalazal region of the ovule. The same pattern of expression is observed during mitosis and gametogenesis. At the mature ovule stage, when the FG is completely formed, GFP expression becomes detectable in the micropilar and chalazal poles (Figure 2d) [52]. However, the biosynthetic genes expression pattern suggests that CKs synthesis does not overlap completely with CKs response (Figure 2). In Arabidopsis, the only *AtIPT* gene that is expressed during ovule development is *AtIPT1*, which is expressed in the MMC, companion cells and the surrounding sporophytic nucellar tissues (Figure 2b). During FM stage, following meiosis, *pIPT1* is active within the haploid megaspore, in the surrounding sporophytic cells and funiculus. In the subsequent stages, *pIPT1* expression is detected in the developing FG [24] toward the micropylar pole (Figure 2b).

Among LOG biosynthetic genes at FG stage, *LOG1* is expressed in the transmitting tract within the pistil, whereas *LOG3, LOG4* and *LOG5* are expressed within the ovule. In particular, *pLOG3* is expressed in the funiculus, *LOG4* is present at FG stage in the sporophytic tissues at the chalazal pole (Figures 2b and 3), whereas *pLOG5::GUS* expression is present in the mature ovule (Figure 2b) and *LOG7* was mainly observed in the counterpart of the FG, the pollen [53]. CK transporters play an important role in CK distribution in ovules. In Arabidopsis, the AtPUP proteins are involved in the import of CK from the apoplast to the cytoplasm, and responses to CK signalling during Arabidopsis development are constrained by the transporter PURINE PERMEASE 14 (PUP14), which is expressed in early ovule primordia at the finger-like stage [54]. The expression of *PUP14* is inversely correlated with that of *TCSn* and the loss of *PUP14* leads to ectopic cytokinin signalling. The PUP14 protein localises to the plasma membrane and acts by importing bioactive CKs, thus depleting apoplastic CK pools and inhibiting CK perception at the plasma membrane [54]. AMP1, the glutamate carboxypeptidase mentioned before, might also be an important factor in the CK-based communication between the sporophyte and the gametophyte. In mature ovules, AMP1 is expressed mostly in the integuments, as well as in the synergids [50]. Heterozygous *amp1*/+ plants do not show defects in synergid identity, indicating that sporophytic *AMP1* expression is sufficient to confer synergid identity. Intriguingly, the cell-specific expression of AMP1 in the synergid, egg or even in the central cells can complement *amp1* synergid defects [50], suggesting that synergid specification requires an AMP1-dependent mobile signal that has to reach the synergids, but can be provided by any of the surrounding cells [50]. *amp1* mutants have higher levels of CK synthesis, and contain a 4- to 6-fold increase in Zt and iP CK, respectively, compared to the wild type [49], and the authors suggested that *IPT* genes might be negatively regulated by AMP1. However, Kong and collaborators did not observe differences in *TCSn::GFP* expression in *amp1* ovules [50], suggesting that some components of the CK signalling cascade might be downregulated in *amp1* ovules and prevent an increase in *TCSn::GFP* signal, even in the presence of higher levels of CKs. However, it cannot be excluded that AMP1 might contribute to synergid specification via a mechanism unrelated to CKs, as it has been previously described for the SAM, were AMP1 regulates the stem-cell niche in a CK-independent manner [55] (Huang et al., 2015). In conclusion, the asymmetric and non-overlapping distribution of many CK biosynthesis and signalling components in the ovule and mutants, suggests that CKs are involved in the sporophyte/gametophyte cross-talk required to form a ovule [25,52,56].

1.4. CK Signalling Influences Stamen Development, Anther Dehiscence and Pollen Viability

Male gametophyte development takes place within stamens, which are composed of a filament and an anther. Inside the anthers, the non-reproductive cells differentiate in specialized tissue layers, including the tapetum, that surround the sporogenous cells [57,58]. Here, two distinct and successive developmental phases, microsporogenesis and microgametogenesis, take place and lead to the production of the mature pollen grain (Figure 4). During microsporogenesis, the sporogenous cells, also called pollen mother cell, enter meiosis to generate tetrads of haploid microspores. This stage is completed when the callose wall surrounding tetrads degenerates and individual microspores are released [58,59]. The free microspores then go through two rounds of mitotic divisions. During pollen mitosis I, the microspore divides asymmetrically to produce a large vegetative and a small generative

cell. The control of asymmetric cell division in the first pollen mitosis is essential for the correct cellular patterning of the male gametophyte because each of the resulting two daughter cells possesses unique gene expression profiles that confer their characteristic structures and cell fates [60]. Then, a second mitosis produces two twin sperm cells enabling double fertilization to produce the embryo and endosperm (Figure 4).

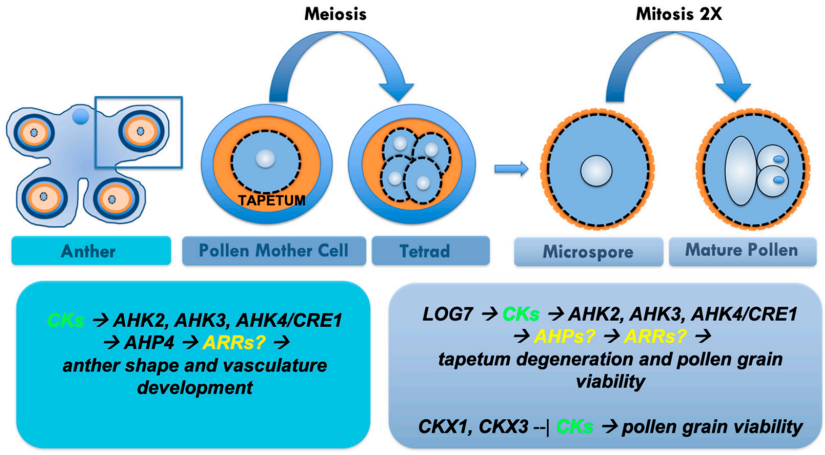

Figure 4. CK biosynthesis, degradation and signalling components during stamen and pollen development. This cartoon depicts the most important phases of pollen development inside Arabidopsis anther. The first step begins when the diploid pollen mother cell enter meiosis and give rise to a tetrad of haploid microspores that subsequently enter two rounds of mitosis: first round is asymmetrical forming the vegetative and germ cells, the second symmetrical only in the germ cell and gives rise to the sperm cells. The involvement of CKs in pollen development starts at the beginning of anther formation because anthers have to acquire a lobed shape and CK receptor mutants exhibit defects in anther shape. Subsequently, CK signalling components AHK2, AHK3, AHK4/CRE1 are also involved in regulating tapetum degeneration that influences ultimately the number of viable pollen grains. Likewise, CK degradation components CKX1 and CKX3 are involved in the viability of pollen grains.

Few studies have analysed the role of CKs during pollen development and function. Arabidopsis and tobacco stamens treated with exogenous CKs produce very short filaments and the anthers do not reach maturity uniformly. Moreover, the small amount of pollen produced is unable to reach the stigma due to the short length of the filaments, causing sterility. If excess CKs inhibit stamen development at the same time as a lack of CK perception, anther dehiscence and pollen viability is impaired [61,62]. The anthers of *ahk2 ahk4* and *ahk3 ahk4* CKs receptor double mutants contain only a small amount of viable pollen grains and do not dehiscence [63]. Similarly, the stamen filaments of the *ahk2 ahk3 cre1/ahk4* triple mutant at anthesis elongated normally, but the anthers were smaller than those in wild type and do not dehisce [26]. The *ahk2 ahk3 cre1/ahk4* triple mutant produced only two- or three-lobed anthers instead of the typical four-lobed structure and the degeneration of the tapetum and the break between the septum and stomium were incomplete. In addition, the vascular tissues of the anther failed to form normally, and the number of pollen grains was reduced. The pollen of the triple mutant was morphologically normal but did not germinate in vitro as efficiently as wild type pollen and could not germinate on wild type stigmas [26]. This inability to germinate might be caused by incomplete maturation, due to incomplete tapetum degeneration [26].

The signalling CK-related gene *AHP4* is predominantly expressed in young flowers and plays an important role in anther formation. Four AHPs (AHP1, AHP2, AHP3 and AHP5) are functionally redundant positive regulators of CK signalling, whereas AHP4 may weakly negatively participate in some CK responses [41]. Plants that overexpressed *AHP4* showed reduced fertility, due to a lack of secondary cell wall thickening in the anther endothecium. Conversely, the anther walls of *ahp4* were more lignified than those in wild type, indicating that AHP4 negatively regulates thickening of the

secondary cell wall of the anther endothecium, and provides insight into the role of CK secondary cell wall formation [64].

Concerning CK degradation, analysis of *pCKX5::GUS* expression showed that *CKX5,* which encodes an apoplastic CKX, is expressed in young developing stamen primordia, and later becomes confined to the central part of developing anthers. Before and during pollination, *CKX5* expression is restricted to the mature pollen grains [11]. An indication of the potential involvement of CKXs in pollen development derives from the analysis of plants that overexpress *CKX1* and *CKX3*, which show reduced pollen production, particularly in early flowers [11]. Very little additional information is available regarding CK-related genes that are specifically expressed in pollen. For example the CK transporter *AtPUP3* expression is restricted to pollen, implicating a potential role for AtPUP3 in the transport of purine derivatives during pollen germination and tube elongation [65], as observed for Petunia pollen [66]. Another pollen-specific gene is the response regulator *ARR2*, which promotes the expression of mitochondrial respiratory chain complex I (nCI), which is upregulated in pollen during spermatogenesis [67]. These data reveal that CKs are essential for stamen development, anther dehiscence and pollen development, but the downstream CK gene network involved in these developmental processes largely remains unidentified. Future studies should include further understanding the crosstalk between CKs and other hormones, such as jasmonic acid and brassinosteroids, and the interactions among hormone signals and environmental cues.

1.5. Cytokinins and Seed Size

Successful fertilization and seed formation require the delivery of two non-motile sperm cells into the female gametophyte by the pollen tube. The first step towards fertilization occurs when the pollen tube comes into contact with the micropyle and the receptive synergid degenerates concomitantly with pollen tube burst, allowing sperm cell nuclei delivery. Subsequently, one sperm nucleus will fuse with the egg cell to form the zygote, and the other will fuse with the central cell to generate the endosperm, forming the seed.

CKs influence also seed development. In addition to the role of CKs in regulating ovule number, which affects seed number, CK levels also affect seed size. The constitutive overexpression of several *LOGs*, CKs biosynthetic genes, including *LOG2, LOG4, LOG5, LOG7* and *LOG8*, leads to the production of larger seeds than those in wild type. When *p35S::LOG4* pistils were crossed with wild type pollen, they produced seeds as large as those resulting from self-pollination, whereas seeds produced from the wild type pistils pollinated with *35Spro:LOG4* pollen were phenotypically wild type, suggesting that CK activity in the female sporophyte controls seed size [53]. However, the effects of CKs on seed size have been mostly observed following the mutation of CKX genes, which catalyse the irreversible degradation of CKs, and several of the seven CKX family members in Arabidopsis have been linked with seed yield. *CKX3* and *CKX5* are involved in the differentiation of reproductive meristems and *ckx3 ckx5* double mutants show delayed meristem activity termination, leading to the formation of more and larger flowers harbouring more ovules that develop into seeds [20]. An apparently contradictory finding regarding seed size control is that low levels of CKs lead to larger seeds, whereas high levels lead to smaller seeds. Thus, the overexpression of *CKX1*, which reduces CK levels [11], leads to larger seeds. *CKX2* is a target of the *HAIKU* (*IKU*) genetic pathway, a leucine rich repeat (LRR) kinase involved in regulating endosperm size. A reduction in the level of CKX2 in *iku2* mutants leads to an increased CK concentration in the endosperm and a concomitant reduction in seed size [68]. Consistent with this finding, CK-insensitive mutants such as the triple receptor mutant *cre1 ahk2 ahk3* [69], the quintuple phosphotransfer protein mutant *ahp1 ahp2 ahp3 ahp4 ahp5* [41], and the triple positive response regulator mutants *arr1 arr10 arr12* [70], also produce larger seeds because these genotypes mimic the absence of CK. This effect of CKX enzymes on seed size has drawn attention as a mechanism to increase crop yields [71,72]; however, the molecular mechanisms downstream of CKs that lead to this phenotype are currently unclear.

1.6. Cytokinins during Reproduction in Crop Species

The engineering of crop plants to obtain greater yields has been a major focus of plant biologists and breeders, with the aim of ensuring food availability for an increasing world population [73]. To increase seed yield in crops, some of the most important traits include ovule number and development, pollen viability, fertility rate and seed size. Research on the model plant Arabidopsis has led to an unprecedented wealth of knowledge on plant development that can guide improvements in crop performance [74].

Brassica napus is a crop plant belonging to the *Brassicaceae* family that is widely cultivated globally due to the edible oil that is extracted from its seeds. It has been recently demonstrated that Arabidopsis and *B. napus* share well-conserved response mechanisms to CK treatment. Exogenous CK application in *B. napus* causes a reduction in stamen length and anthers that do not mature uniformly, such that few produce pollen. However, the pollen was not able to reach the stigma due to the short length of the filament, causing sterility [22]. On the contrary, during maize (*Zea mays*) reproductive development, male-sterile plants can also be obtained following the ectopic accumulation of CKX. Maize plants that ectopically express *CKX1* under the control of a maize pollen- or anther-specific promoter, exhibit rudimentary terminal structures at the apical meristem that lack recognisable male florets or spikelets, and hence a tassel [75]. The exogenous application of kinetin, a synthetic CK that is not degraded by CKX1, and thidiazuron (TDZ), a CKX inhibitor, partially restored male development in transgenic maize plants, demonstrating that CKs execute a crucial role in maize reproduction [75] (Table 2).

In rice (*Oriza sativa*), several mutants that affect CKs have been described; an example is the CK biosynthesis gene *LONELY GUY (LOG)*, which is required to maintain meristem activity and its loss of function causes premature shoot meristem termination, resulting in a severely reduced inflorescence size, an abnormal branching pattern, and fewer floral organs [9]. The *Oslog-3* rice mutant produces flowers that contain six stamens and a slender pistil that lacks an ovule, or no pistil [76] and *Oslog-1* and *Oslog-4* single mutant flowers display a weak phenotype, with only one stamen and no pistil [77] (Table 2). A CRIPR/Cas9 gene-editing approach has been taken to characterise the role of the type-B response regulators (RRs) in CK signalling in rice [78] (Table 2). Mutant phenotypes associated with a decreased activity of rice type-B RRs and CK responses include defects in inflorescence architecture, flower development and fertilization. Triple *rr21/22/23* mutants displayed reduced fertility because of defective stigma development. Anthers from the triple mutant were developmentally normal and produced pollen with a high viability, but the carpels lacked the papillae on the stigma that aid pollen capture, hydration and guidance of the pollen tube towards the ovary, which was probably the basis for the poor grain filling. Moreover, single *rr24* mutants are infertile because of defective anther development [78]. Collectively, these results demonstrate that CK biosynthesis and response are extremely important for the correct development of female and male floral structures. Guo et al. 2018 [79] recently investigated the association between grain number and grain size in rice and showed that *GRAIN SIZE AND NUMBER* (*GSN1*), a protein kinase phosphatase, controls the trade-off between grain size and number. The loss-of-function *gsn1* mutant possesses larger grains than wild type, but less-branched inflorescences, leading to the production of fewer grains than wild type. *CKX2* is upregulated in *gsn1* and consequently, CK levels are reduced during early panicle development, which supports a role for *CKX2*/CKs in controlling grain size and panicle architecture in rice [80]. Similarly, variants of *TaCKX6-D1*, a wheat (*Triticum aestivum*) orthologue of *OsCKX2*, were significantly associated with grain weight but not grain number [72,81] (Table 2). Increasing levels of CK are linked to larger seeds in Arabidopsis, but an appropriate level is required to not affect branching.

Given the high degree of gene conservation among cereals, the current state of knowledge facilitates a more detailed analysis of the development of reproductive structure in crops. Modern genome editing tools could be employed to target and manipulate CK levels to increase seed yield, with the concurrent aim of maintaining quality.

Table 1. Cytokinin-related mutants with reproductive development defects in Arabidopsis.

Gene Name	Family or Protein Name	CK Process	Mutant Line	Phenotype	Ref
LOG2, LOG3, LOG4, LOG5, LOG7, LOG8	cytokinin riboside 5′-monophosphate phosphoribohydrolases	biosynthesis	*35S::LOG2, 35S::LOG3, 35S::LOG4, 35S::LOG5, 35S::LOG7, 35S::LOG8*	Larger and heavier seeds	[53]
CKX1	CKX-cytokinin oxidase/dehydrogenase family	degradation	*35S:AtCKX1*	Few and enlarged seeds	[11]
CKX3	CKX-cytokinin oxidase/dehydrogenase family	degradation	*35S:AtCKX3*	Few and enlarged seeds	[11]
CKX7	CKX-cytokinin oxidase/dehydrogenase family	degradation	*ckx7 ckx7 T*	Shorter fruit	[23]
CKX3, CKX5	CKX-cytokinin oxidase/dehydrogenase family	degradation	*ckx3 ckx5*	Increased production of flowers, longer siliques, more ovules and seeds	[20]
CKX3, CKX5, AHP6	CKX-cytokinin oxidase/dehydrogenase family AHP-Arabidopsis histidine phosphotransfer gene family	degradation signalling	*ckx3 ckx5 ahp6*	More siliques	[20]
AHK4/CRE1, AHK2 and AHK3	AHK-Arabidopsis histidine/kinase receptor family	signalling	*ahk2-1 ahk4-1 ahk3-1 ahk4-1*	No anther dehiscence, small amount of viable pollen	[63]
			cre1-12 ahk2-2tk ahk3-3	Unable to produce seeds, abnormal development of female gametophyte and anthers; defects in tapetum degeneration and reduced number of pollen grains	[26]
			cre1-12 ahk2-2 ahk3-3	Abnormal female gametophyte development	[24]
			ahk2-5 ahk3-7 cre1-2	Reduced seed set, larger seeds	[69]
			ahk2–7 ahk3 –3 cre1–12	Defects in functional megaspore specification, female gametophyte absent	[25]
			ahk2–1 ahk3–1 ahk4–1	Sterile	[63]
CKI1	CKI-cytokinin-independent kinases	signalling	*cki1-5 cki1-6*	Reduced seed set and abnormal embryo sacs	[36]
			cki1-8	Reduced seed set and abnormal embryo sacs	[35]
			cki1-9	Almost sterile, fewer larger seeds. Misspecification of female gametophyte cells	[37]
AHP4	AHP-Arabidopsis histidine phosphotransfer family	signalling	*35S::AHP4*	Anthers lack of secondary cell wall thickening in the anther endothecium	[64]
			ahp4	Anthers more lignified	[64]
AHP1,2,3,4,5	AHP-Arabidopsis Histidine phospotransfer family	signalling	*ahp 1, 2-1, 3/+, 4, 5-1*	Reduced seed set, but larger seeds; arrested during female gametophyte development	[41]
			ahp1,2-2,3,4,5-1	Loss of central cell fate and acquisition of egg-cell identity	[37]
AHP2,3,5	AHP-Arabidopsis Histidine phospotransfer family	signalling	*ahp2-2, ahp3, and ahp5-2*	Unfertilized ovule and seed abortion	[40]
ARR1,2,10,12	ARR-Arabidopsis response regulator type-B	signalling	*arr1–3 arr2–2 arr10–2 arr12–1*	Ovules arrested at FG7, the final developmental stage of the female gametophyte	[25]
ARR1,10,12	ARR-Arabidopsis response regulator type-B	signalling	*arr1-3 arr10-5 arr12-1*	Shorter siliques, larger seeds	[70]
ARR7,15	ARR-Arabidopsis response regulator type-A	signalling	*arr7arr15*	Female gametophyte lethal	[44]
CRF5,6	CRF-cytokinin response Factor	signalling	*crf5 crf6*	Homozygote non-viable	[17]
CRF2,3,6	CRF-cytokinin response Factor	signalling	*crf2 crf3 crf6*	Reduction in ovule number and placenta length	[29]
AMP1	Glutamate carboxypeptidase family		*amp1-1*	Increased cytokinin level	[49]
			amp1-2	Increased cytokinin level	[49]
			amp1-10	Supernumerary egg cell, twin embryos	[50]
			amp1-13	Supernumerary egg cell, twin embryos	[50]

Table 2. Cytokinin-related mutants with reproductive development defects in crop species.

Gene Name	Family or Protein Name	CK Process	Specie	Mutant Line	Phenotype	Ref
CKX1	CKX-cytokinin oxidase/ dehydrogenase family	degradation	Zea Mays	pZmg13::CKX1 pZtap::CKX1	Defects in anther and stamen development	[75]
OsLOG-1	LOG- Lonely Guy	biosynthesis	*Oriza sativa*	Oslog-1	Flowers with only one stamen/no pistil	[77]
OsLOG-3	LOG- Lonely Guy	biosynthesis	*Oriza sativa*	Oslog-3	Flowers with six stamens and a slender pistil lacking an ovule or no pistil/absence of organ differentiation in the ovule founder region	[76]
OsLOG-4	LOG- Lonely Guy	biosynthesis	*Oriza sativa*	Oslog-4	Flowers with only one stamen/no pistil	[77]
OsCKX2	CKX-cytokinin oxidase/ dehydrogenase family	degradation	*Oriza sativa*	Osckx2	Increased number of reproductive organs/enhanced grain yield	[80]
OsRR type-B	ARR-Arabidopsis response regulator type-B	signalling	*Oriza sativa*	rr24	Compromised anther development	[78]
OsRR type-B	ARR-Arabidopsis response regulator type-B	signalling	*Oriza sativa*	rr21/22/23	Compromised pollen capture	[78]
GSN1	GRAIN SIZE AND NUMBER		*Oriza sativa*	gsn1	Upregulation of cytokinin degradation enzyme, *CKX2*. Increase in grain size/ less-branched inflorescence	[79]
TaCKX6-D1	CKX-cytokinin oxidase/ dehydrogenase family	cytokinin degradation	Wheat (*Triticum aestivum*)	TaCKX6-D1 naturally occurring wheat variants		[81]

2. Future Directions

CKs are essential for reproductive success in plants. Currently, most knowledge regarding the role of CKs in reproduction involves the effect of mutation of genes involved either in CK metabolism or signalling (Figure 1, Table 1). To date, only a few non-ARR transcription factors, such as VDD, VAL, SEEDSTICK (STK), SPL and BEL1 [23,24,46] have been implicated to mediate CK responses downstream of CK signalling; therefore, the next step is to elucidate these downstream responses, to understand how CKs regulate specific developmental processes at the molecular level.

Another currently poorly studied aspect is how individual molecular CK species contribute to the biological effect of CKs. Some recent studies have highlighted the importance of specific CK species in eliciting specific biological responses [82], or have linked phenotypes with CK pools present in specific cellular compartments [23]. Due to the difficulty in accessing reproductive tissues, and particularly female reproductive tissues, studying these aspects should be coupled with microdissection and/or sorting techniques. Recently, some studies have quantified CKs in sorted cells [83]. This approach, coupled with more sensitive quantification techniques [84] and the development of biosensors [85], will undoubtedly advance our understanding of the cellular and subcellular distribution of CK species in reproductive tissues and their relationship to their developmental effects.

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