UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXVII Ciclo

AUXIN-CYTOKININ CROSSTALK DURING OVULE PRIMORDIA DEVELOPMENT

Mara Cucinotta

PhD Thesis

Scientific tutor: Prof.ssa Lucia Colombo

Academic year: 2013-2014

SSD: BIO/01; BIO/18

Thesis performed at Dipartimento di BioScienze – Università degli Studi di Milano

Contents

The best scientist is open to experience and begins with romance - the idea that anything is possible.

Ray Bradbury

Abstract

Coordinated action of the plant hormones auxin and cytokinins (CKs) is required for all plant developmental process. My Ph.D. project focused on the crosstalk between these two hormones involved in ovule primordia formation, and ovule number determination in Arabidopsis *thaliana*.

Ovules develop as lateral organ from the placenta, a meristematic tissue, and once fertilized, form the seeds; therefore they propagate plant offspring and ultimately determine yield in seed-crop plants.

CKs and auxin are required for ovule initiation and, CKs in particular have a positive role in the determination of ovule number.

I have contribute to propose an interesting model that integrates the role of both hormones with the activity of the transcription factors MONOPTEROS (MP/ARF5), CUP SHAPE COTYLEDON 1 (CUC1) and CUC2. MP responds to auxin and activates *CUC1* and *CUC2*. In turn the CUCs, upstream or in parallel with CKs, control expression and localization of the auxin efflux carrier *PIN1*, which is necessary for the ovule primordia formation. Data obtained by a transcriptomic analysis suggest that CUCs act upstream of the CKs pathway, since they regulate several genes involved in CKs catabolism and response.

Moreover, we identified CYTOKININ RESPONSE FACTOR 2 (CRF2) as a key player of the auxin-cytokinin crosstalk responsible of pistil growth rate and indirectly of ovule number determination. Indeed, *CRF2* is a MP direct target and it promotes *PIN1* expression in response to CKs. All the results obtained have been integrated in the model that describes the molecular control of ovule primordia formation**.**

Part I

State of the art

1. The importance of ovules

Ovule formation is a key event in the life cycle of flowering plants, which main purpose is to form the female gametophyte and develop into seed upon fertilization.

Plants are indispensable for life on earth, since they provide oxygen and food to animals. Human nutrition is mainly based on calories that derived from plants, and in particular from seeds of grain-crops. The two main types of commercial crops are cereals, such as wheat and rice, and legumes, such as bean and soybeans. Given the importance of seeds for our diet, deciphering the mechanism behind their development could help in the improvement of yield and food production, a necessity that is becoming increasingly urgent nowadays. Indeed, world population is continuously increasing and already at the moment, over 805 million people in the world today suffer from hunger. About one person out of nine does not have enough food to lead a healthy and active life (WFP - World Food Programme, www.wpf.org).

For this reason, we focus energies in research the molecular mechanism that control of ovule development, using Arabidopsis *thaliana* as a model species, taking into account that this process seems to be widely conserved in the plant kingdom. Understanding ovule formation and plant reproduction is of great importance for potential biotechnology applications to increase crop yields.

Finally, Arabidopsis ovule provide an excellent and complex system to study processes of organ boundaries establishment, organogenesis, and cell differentiation in plant.

2. Arabidopsis thaliana as a model species

Since the early 1900s Arabidopsis *thaliana*, a dicotyledon belonging to the Brassicacea family, became a genetic model organism for many reasons.

The small size and rapid lifecycle are advantageous for research in laboratories. Arabidopsis can be also easily transformed using Agrobacterium *tumefaciens* to transfer DNA to the plant genome (Clough & Bent 1998). The relatively small size of its genome (about 125 million pairs of nucleotides, in only five chromosomes) has made it an ideal plant for creation of genetic maps and for genome sequencing. The Arabidopsis genome was completed in 2000 (Arabidopsis Sequencing Consortium, 2000), and tremendous progress has been achieved in the analysis of plant development from that moment.

TAIR (www.arabidopsis.org) and NASC (http://arabidopsis.info/) are curated sources that only provide mutant seeds collection but also makes available all the genetic and molecular information arising from the international Arabidopsis research community. Studies done in Arabidopsis have contributed to the improvement of crops because once a gene is discovered in Arabidopsis, the equivalent gene may be found more easily in other plants. For example, researches on Arabidopsis ovule identity gene *SEEDSTICK (STK)* helped in the characterization of its homologous in rice *(OsMADS13)*, soybean *(GmAGL2)* and oil palm (*SHELL*) (Dreni et al. 2007; Xu et al. 2010; Singh et al. 2013). In an opposite way, the function of many genes isolated from crop plants can be better understood via study of their Arabidopsis homologues. For instance, the CKs biosynthetic gene *LONELY GUY 1 (LOG1)* was initially isolated in rice and then deeply studied in Arabidopsis (Kurakawa et al. 2007; Kuroha et al. 2009). Thus, knowledge on Arabidopsis reproductive mechanisms could be very useful in improving crop-yield.

3. The role of Phytohormones

Plant organogenesis is a very fascinating process and it represents a masterpiece of sophisticated control of gene expression. Cell differentiation during organogenesis requires communication between neighbouring cells, which is achieved by chemical signals. In this context, phytohormones play a fundamental role. Long and short-range hormonal signals coordinate development in separate parts of the plant.

Phytohormones includes auxins (AUXs), cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA), ethylene, brassinosteroids (BRs) and jasmonic acid (JA). Each hormone interacts with the others and regulates many aspects of plant growth and response to biotic and abiotic stresses (Vanstraelen & Benková 2012; O'Brien & Benkova 2013).

In particular, the formation and identity of lateral organs is strictly dependent on balancing between auxin and cytokinin. The study of the crosstalk between these two hormones is the main subject of this work.

3.1. Auxins

Auxins were the first plant hormones to be identified, and their fundamental role in all aspects of plant development was immediately clear. Indeed, the term 'auxin' comes from the Greek 'auxein' that means 'to grow' (Thimann & Went 1937). The main auxin functions are: promotion of cell elongation, specification of vascular tissue, initiation of root and leaves, stem cells population maintaining and control of patterning during organogenesis. Auxin is universally present in all plant species, including algae (Yue et al. 2014).

3.1.1. Auxin biosynthesis

There are four native endogenous auxins, but among them auxins indole-3-acetic acid (IAA) is the most abundant. IAA mediates the majority of auxin effects in plants; thus, auxin is often used as synonymous of IAA.

Four interconnected Tryptophan (Trp) -dependent IAA biosynthetic pathways have been proposed; indol-3-acetamide (IAM), indol-3-pyruvic acid (IPyA), indol-3-acetaldoxime (IAOx) and tryptamine (TAM) pathways, each named after the intermediated immediately downstream the Trp (Zhao 2010; Tivendale et al. 2014).

Analysis of IAA overproduction mutants allowed the identification of key enzymes involved in IAA synthesis. For example *YUCCA (YUC)* genes, encode for flavin monooxygenase-like enzymes, which catalyse the rate-limiting step of tryptamine hydroxylation (Cheng et al. 2006). *YUC* genes are essential for the formation of floral organs and vascular tissues, indeed multiple *yucca* mutants display severe defects in floral patterning and vasculature formation (Cheng et al. 2006).

TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1) is conserved in several plant species and it controls the synthesis of IPA (Stepanova et al. 2008). Plants with impaired IPA pathway show typical auxin-related phenotypes, as for instance, reduced apical dominance, infertile flowers and severe vasculature defects in leaves and flowers (Stepanova et al. 2008). IAA can also be produces via tryptophan-independent pathway, but this mechanism is still poorly understood (Normanly et al. 1993).

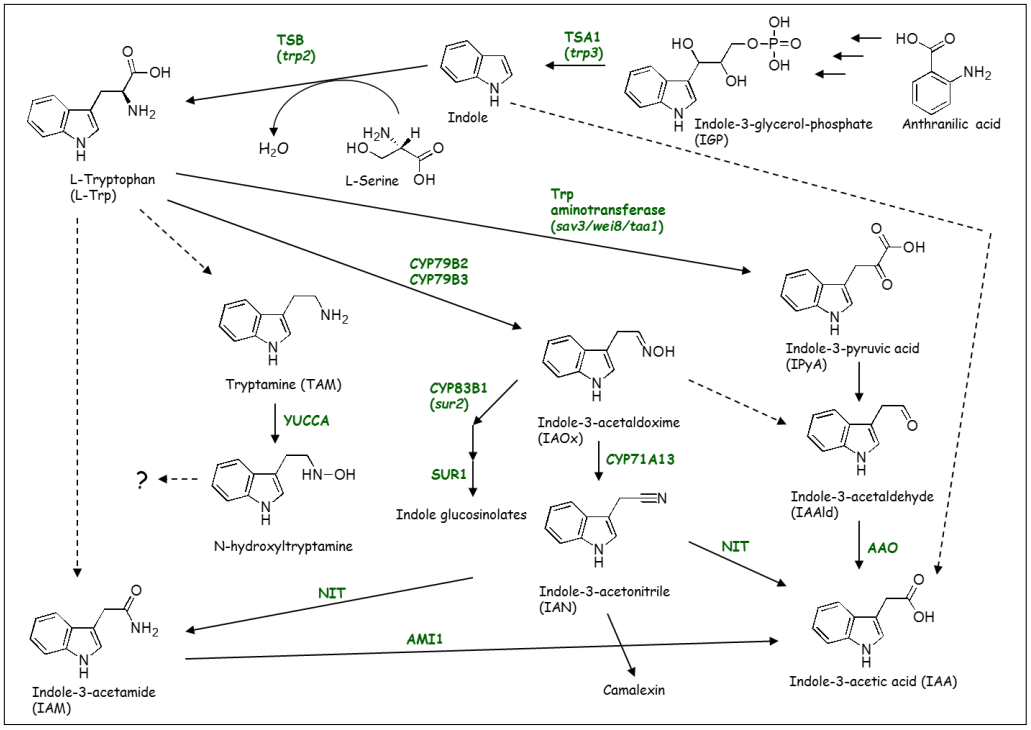

Figure 1:The main auxin biosynthesis pathways in Arabidopsis.

Several pathways are known to exist in the Trp-dependent auxin biosynthesis. In the scheme are reports the enzymes responsible for some of the reactions, whose genes have been identified using molecular or genetic approaches. Picture has been modified from http://hormones.psc.riken.jp/pathway_ck.shtml (RIKEN Plant Hormone Research Network).

3.1.2. Auxin transport

Auxin is mostly produced in young tissues of developing organs, such as shoot apex, emerging leaves and developing seeds.

Then auxin is redistributed throughout all aerial and underground plant body through a fast, non-polar transport in the phloem and a slow, cell-to-cell polar transport. To explain the mechanism behind the unique auxin characteristic of being transported through living cell files, the so called ‘chemiosmotic model’

was formulated in the seventies (Rubery & Sheldrake 1974). This model is based on physical and chemical nature of auxin molecules and on the difference in pH outside and inside a cell. Indeed, the pH inside the cell (pH ~ 7) is less acidic than the pH in the apoplast (pH ~ 5.5), because of protons extruded by H+-ATPases at the plasma membrane. The higher pH outside the cell makes that significant portion of the IAA molecules is proto-associated (un-dissociated). This portion of auxin molecules is charge-neutral and therefore it is able to diffuse through the plasma membrane. Auxin uptake into the cell could be also facilitated by the presence of auxin influx carrier, like AUXIN RESISTANT 1 (AUX1) (Bennett et al. 1996).

Once inside the cell, the molecules are exposed to the more basic pH of the cytoplasm, and they dissociate almost completely in anionic IAA−, which being chemically polar are therefore unable to cross back the plasma membrane. Thus, the transport of anionic IAA− out of the cell requires active membrane transport proteins.

Analysis of *pin-formed 1* (*pin1*) mutant, which clearly showed defects in polar auxin transport (PAT), leaded to the identification of the first auxin efflux carrier in Arabidopsis (Okada et al. 1991; Gälweiler et al. 1998). The PIN proteins transporters function as 'auxin efflux carriers', and by maintaining asymmetric localisation on plasma membrane they control directionality of the auxin flow from cell to cell (Zazímalová et al., 2010). Phenotype of *pin1* is very severe, with needle-like inflorescences and it mimics the one of wild type plant treated with auxin efflux inhibitors. Later on, other seven *PIN* genes have been identified in Arabidopsis. Specifically, *PIN3*, *PIN4* and *PIN7* are required for tropism, root meristem patterning and early embryo development (Jiří Friml et al. 2002; Jiri Friml et al. 2002; Friml et al. 2003; Blilou et al. 2005).

PIN5 and PIN8, respect to the previous described PIN proteins, localize to the endoplasmic reticulum (ER) and have opposite roles in the regulation of intracellular IAA homeostasis (Mravec et al. 2009; Ding et al. 2012). PIN5 presumably mediates auxin flow from the cytosol to the lumen of the ER (Mravec et al. 2009), while PIN8 appears to counteract this activity (Ding et al. 2012). Similar mode of action has been described for a novel family of putative auxin transporters, designated PIN-likes (PILS), which were also found to localized at the ER (Barbez et al. 2012).

Another fundamental aspect of auxin transport is the subcellular trafficking of PINs, fundamental for the establishment of their polarities (Adamowski & Friml 2015). In fact, PINs continuously and dynamically cycle between their polar domain at the plasma membrane and the endosomal compartments. It is known that the endosomal ARF-GEF GNOM, a molecular pathway necessary for the formation of coated vesicles, is required for PINs trafficking events, polar localization and recycling (Steinmann et al. 1999; Geldner 2001).

Each PIN localizes preferentially to specific sides of the cell membranes. For example, PIN1 localizes to the basal side of plasma membrane, while PIN2 to the apical one. This suggests that the protein sequence itself regulates the polarity (Wiśniewska et al. 2006). Moreover, the position polarity is also controlled by the phosphorylation state of the PIN protein. A mutant named *pinoid (pid)*, which exhibits apical-to-basal polarity switch of PIN1, showed phenotypic characteristics resembling those of *pin1* mutant (Bennett et al. 1995; Friml et al. 2004). The *PID* gene encodes for a protein kinase able to phosphorylate PIN1, and phosphorylated PIN1 is targeted to the apical membrane (Friml et al. 2004). Conversely, the PROTEIN PHOSPHATASE 2A (PP2A), responsible of PIN1 de-phosphorylation of PIN1 moved it to the basal membrane (Michniewicz et al. 2007).

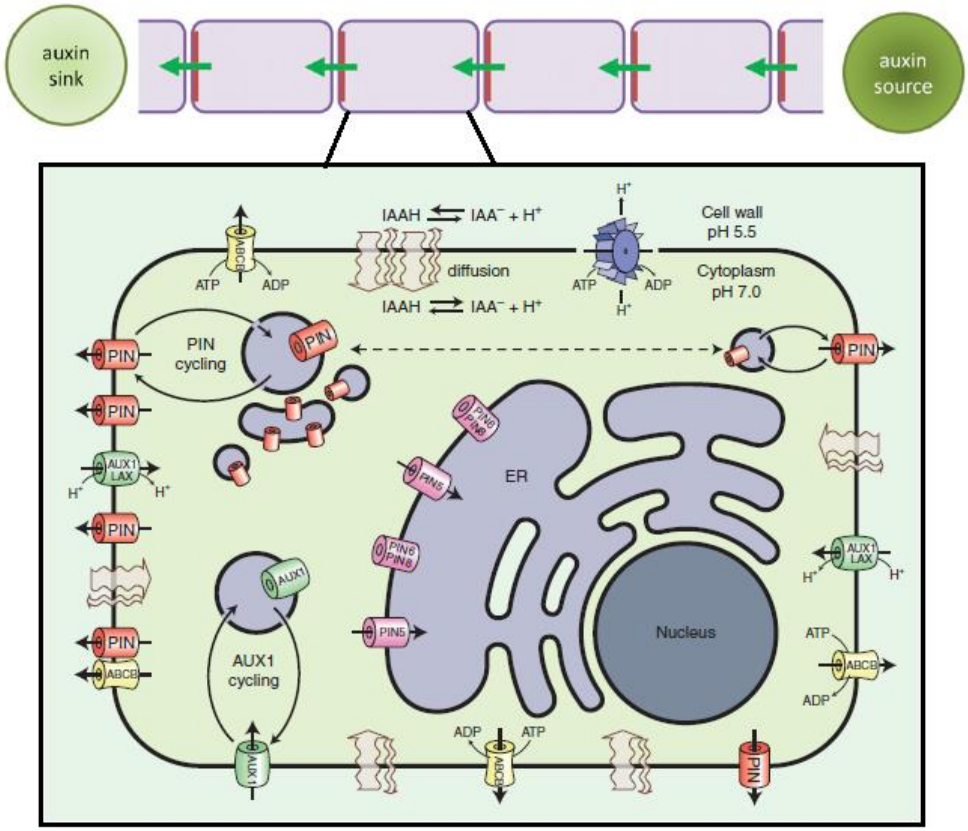

Figure 2: Auxin transport. On the top of the figure is schematically illustrated a canal of auxin flow connecting auxin source to the sink, the lines in red represent PIN polarization. Below, a schematic cell model reports the chemiosmotic model with the difference in pH between the inside and outside of the cell. The scheme also shows organization of the proteins involved in auxin transport: auxin influx facilitator AUX1 is in green, PIN efflux carrier proteins at the membrane are in red (PIN1, 2, 3, 4, and 7), whereas PINs marked in pink (PIN5, 6, and 8) localized at the endoplasmic reticulum (ER). Constitutive protein cycling, and the process of transcytosis are also reported with continuous line and dashed line respectively (Pictures modified from Zazímalová et al. 2010; Adamowski & Friml 2015).

3.1.3. Auxin signalling

Auxin signalling is primarily regulated by the *AUXIN RESPONSE FACTOR (ARF)* gene family products, together with the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins (Quint & Gray 2006). Under sub-threshold auxin concentrations the Aux/IAA proteins heterodimerize with ARF transcription factors, thereby repressing the modulation of ARFs downstream targets (Ulmasov et al. 1997; Reed 2001). In presence of high auxin concentration, the Aux/IAA proteins, a 18 to 35 kD short-lived nuclear proteins, are degraded by the action of an ubiquitin protein ligase complex, called SCF^{TIR1}, in which the F-box protein TRANSPORT INHIBITOR RESPONCE1 is an auxin receptor (Ruegger et al. 1998). Indeed, auxin bind to TIR1 and stabilizes, like a molecular glue, the interaction between TIR1 and the Aux/IAA (Tan et al. 2007). The formation of this complex results in the ubiquitination of Aux/IAA proteins and in their subsequent degradation via the 26S proteasome (Dharmasiri et al. 2005; Tan et al. 2007; Calderon-Villalobos et al. 2010). Most Aux/IAA proteins have four highly conserved domains (I-IV). Domain II is important for the interaction with the SCF^{TIR1} complex and domain III and IV are responsible for heterodimerization with ARFs protein (Reed 2001; Overvoorde et al. 2005). Degradation of Aux/IAA alleviates the repression on ARFs and allows expression and/or repression of specific downstream genes in response to auxin (Rogg & Bartel 2001). Mutations in domain II usually lead to the block of Aux/IAA degradation and result in a constitutive inactivation of ARF functions. Disruption of auxin response often causes dramatic phenotypes, as exemplified by *bodenlos (bdl)/iaa12* gain of function mutation which fails to initiate primary root (Hamann et al. 2002) and solitary root/iaa14 mutation that completely lacks lateral roots (Fukaki et al. 2002).

In Arabidopsis, there are 23 *ARF* genes encoding proteins. ARFs contain a B3 DNA binding domain (DBD), a middle region that could act as activator or repressor domain and, with the exception of ARF3,13 and 17, a carboxy-terminal dimerization domain (CTD) (Ulmasov et al. 1997; Ulmasov et al. 1999; Tiwari et al. 2003; Guilfoyle & Hagen 2007). ARFs bind with specificity to Auxin Response Elements (AuxREs), whose sequence (TGTCTC) was found in promoters of primary/early auxin response genes (Ulmasov et al. 1995; Ulmasov et al. 1997). Recently the structures of DBD of two ARFs were solved and revealed the possibility of ARF to homodimerize through the DBD (Boer et al. 2014). ARF C-terminal III/IV domain structure has been also recently solved, enlightening the possibility of higher order complexes of ARFs and Aux/IAAs (Korasick et al. 2014; Nanao et al. 2014; Wright & Nemhauser 2015). The broad number of TIR, Aux/IAA and ARF genes provides to the plant an extremely large repertoire to modulate cellular responses to auxin. This allows the plant to use auxin in a variety of ways, depending on the needs of the single cells, tissues, organs and on the different conditions.

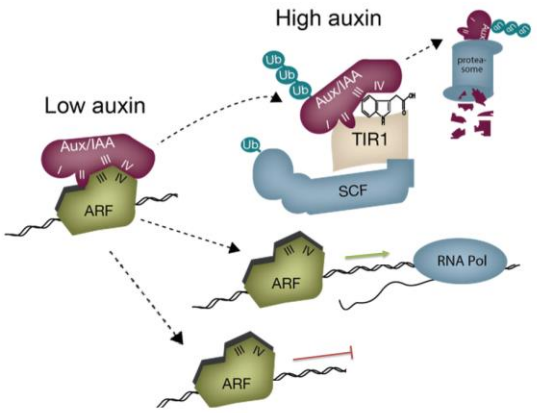

Figure 3: Molecular mechanism of auxin signalling. ARFs bind to auxin-response elements in promoters of downstream targets. When auxin concentrations is low, Aux/IAA repressors associate with ARFs through domains III and IV. When auxin concentration increases, auxin binds to the TIR1 receptor and promotes the interaction of the SCF^{TIR1} complex with Aux/IAAs. This interaction leads to ubiquitination and degradation of the Aux/IAAs. ARFs alleviated from the Aux/IAA repression could modulate the expression of downstream targets. Picture modified from Strader Laborary web page (https://pages.wustl.edu/strader/research).

3.2. Cytokinins

Cytokinins (CKs) were originally identified by their ability in promoting cell division (i.e., cytokinesis) (Miller et al. 1955; Letham 1963). Since their initial discovery, a plethora of CKs biological functions have been observed, including: de novo organ formation from cultured tissues, delayed senescence, cell proliferation and differentiation in shoots, nodulation, light responses and immunity.

Naturally occurring CKs are adenine derivatives carrying either an isoprene-derived or an aromatic side chain at the N^6 terminus. Conventionally, these families are called isoprenoid CKs and aromatic CKs, respectively. Common natural isoprenoid CKs are N^6-(Δ^2-isopentenyl)-adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DZ). Among them, the major derivatives generally are *t*Z and iP as well as their sugar conjugates, but there is a lot of variation depending on plant species, tissue, and developmental stage. Generally *t*Z and iP exhibited higher activities than *c*Z (Sakakibara 2006).

3.2.1. Cytokinins biosynthesis

The first and limiting step in the biosynthesis of CKs is catalysed by the enzyme isopentenyltransferase (IPT). In Arabidopsis seven *IPT* genes encode for adenosine phosphate-isopentenyltransferase (Kakimoto, 2001; Takei et al., 2001). Other two (*IPT2* and *IPT9*) are tRNA isopentenyltransferase which modify a subset of adenine bases on tRNA (Golovko et al. 2002).

The next step involves the cytochrome P450 enzymes CYP735A1/CYP735A2, able to convert iP ribosides to *trans*-zeatin (tZ) (Takei et al. 2004).

In the active forms of CKs both nucleotides are converted into the corresponding nucleobases by a single enzymatic reaction. *LONELY GUY (LOG)* gene, which belongs to the CKs nucleoside 5′ monophosphate phosphoribohydrolases family, has been identified in rice (Oryza *sativa*) as the enzyme responsible of performing the CKs activation (Kurakawa et al. 2007). Nine Arabidopsis thaliana *LOG* genes (*At LOG1* to *LOG9*) were predicted to be homologs to the rice *LOG*, and seven of them codify for proteins with the same enzymatic activities (Kuroha et al. 2009).

3.2.2. Cytokinins catabolism

Plants maintain correct CKs homeostasis trough a fine balance of synthesis and catabolism. CYTOKININ OXIDASE/DEHYDROGENASES (CKXs) are the major enzymes responsible for CKs irreversible degradation (Bilyeu et al. 2001). The Arabidopsis *AtCKX* gene family has seven members (*AtCKX1* to *AtCKX7*) and each one shows distinct patterns of expression and slightly different enzymatic properties (Werner et al. 2003). Some CKX proteins degrade free iP and *t*Z, but others, including CKX1, CKX5 and CKX7, display good affinities towards *c*Z (Gajdošová et al. 2011). The level of active CKs can be also decreased by glucose conjugation and O-glycosylation, which is likely reversible, and N-glycosylation that is thought to be irreversible (Mok & Mok 2001; Hou et al. 2004).

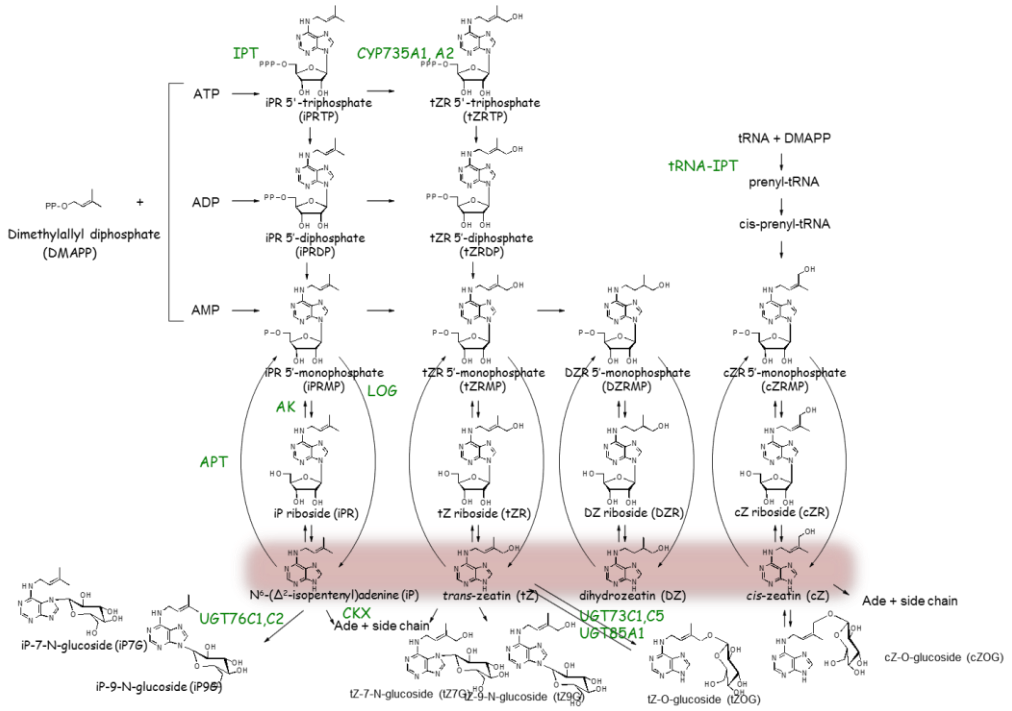

Figure 4: Cytokinins metabolism pathways in Arabidopsis. In the scheme is reported the current model of isoprenoid CKs biosynthesis pathways in Arabidopsis. Adenosine phosphate-isopentenyltransferases (IPTs) preferably utilize ATP or ADP as isoprenoid acceptors to form iPRTP and iPRDP. The CKs nucleotides are converted into the corresponding *tZ*-nucleotides by CYP735A. Then, enzymes encoded by the *LOG* gene family catalyse the conversion of tzRMP into active CKs form. About catabolism, iP and *tZ* can be degraded by CKX to adenine or adenosine. CK nucleobases can be also converted into O-glucoside or N-glucoside by UDP-glucosyltransferase (UGTs). Picture has been modified from RIKEN Plant Hormone Research Network web page (http://hormones.psc.riken.jp/pathway_ck.shtml).

3.2.3. Cytokinins transport

The spatial expression patterns of CKs related genes indicate that CKs can be locally synthesized, then act as an autocrine and/or paracrine signal and finally be catabolized at distant sites (Hirose et al. 2008). Results from studies on grafted mutants showed that CK also behaves as long-distance signal that moves from root to shoot and vice versa (Beveridge et al. 1997). Roots are the major sites of *t*Z production, and *t*Z plays a role as a root-to-shoot acropetal signal. Phloem, instead, predominantly contains iP-type and *c*Z-type CKs, which function as basipetal signal (Corbesier et al. 2003).

Such compartmentalization might be needed for plant cells to recognize the direction of the CKs signal (Hirose et al. 2008). Researchers have not yet clearly identified the transporters that move CKs across the plasma membrane. To date only few purine permeases have been implicated in CKs transport (Bürkle et al. 2003). More recently, an ATP binding cassette transporter, called ABCG14, has been shown to be necessary for movement of *t*Z from root to shoot (Ko et al. 2014).

3.2.4. Cytokinins signalling

CKs signalling is mediated by a two-component signalling pathway (TCS), similar to the TCS system described in bacteria (Stock et al. 2000).

CKs molecules bind to Arabidopsis histidine kinases (AHKs) receptors, which are on the endoplasmic reticulum as well as on the plasma membrane. Arabidopsis genome has three CKs receptors (*AHK2*, *AHK3*, and *CRE1/WOL/AHK4*), which codify for proteins that contain a conserved CKs binding CHASE domain, a histidine kinase domain and a receiver domain.

AHK2, *3* and *4* play positive and partially redundant roles in CKs responses (Hwang & Sheen 2001; Inoue et al. 2001). Arabidopsis mutant plant lacking all three receptors does not respond to CKs in a variety of assays and it show serious growth and fertility problems (Higuchi et al. 2004; Nishimura et al. 2004). CKs binding induces conformational changes in the AHKs that trigger a phosphorylation cascade. In turn, the phosphoryl group (P) is relayed to Arabidopsis histidine phosphotransferase proteins (AHPs). AHPs continuously translocate between the cytosol and the nucleus acting as intermediates in transferring the phosphorylation to the downstream Arabidopsis response regulators (ARRs) (Tanaka et al., 2004). AHP1-5 are functional, positive and partially redundant elements in CKs signalling and the quintuple *ahp1-5* mutant is seedling lethal (Deng et al. 2010). Conversely to other AHPs, AHP6 lacks conserved His residue necessary for the phosphorylation, so that it plays as inhibitor of CKs signalling most likely by competing with other AHPs for interaction with the activated receptors (Mähönen et al. 2006).

In the signal cascade, under the AHPs are the ARRs. In Arabidopsis the 22 ARRs have been divided into two main classes called type-A and type-B ARRs. Both ARR type could be phosphorylated, but only type-B ARRs contain a Myb-like DNA binding domain that gives them property of transcription factors (Imamura et al. 1999). The type-A ARR are rapidly induced by CKs and, by contending with the type-B ARRs for the phosphorylation, function as negative feedback regulators of the cascade (Hwang & Sheen 2001; To et al. 2004). *Type-B ARRs* display overlapping expression pattern in regions where CKs play a significant role, including rapidly dividing cells in the shoot apical meristem and in young leaves (Mason et al. 2004).

More recently, *CYTOKININ RESPONSE FACTORS (CRFs)*, which belong to the AP2 Arabidopsis gene family, have been discovered as new components of the CKs response machinery. CRFs are rapidly re-localized to the nucleus in response to CKs, and their movement required both AHPs and AHKs receptors (Rashotte et al. 2006). Once activated, CRFs together with the type-B ARRs, mediate CKs-regulated gene expression, affecting an overlapping set of target genes (Rashotte et al. 2006).

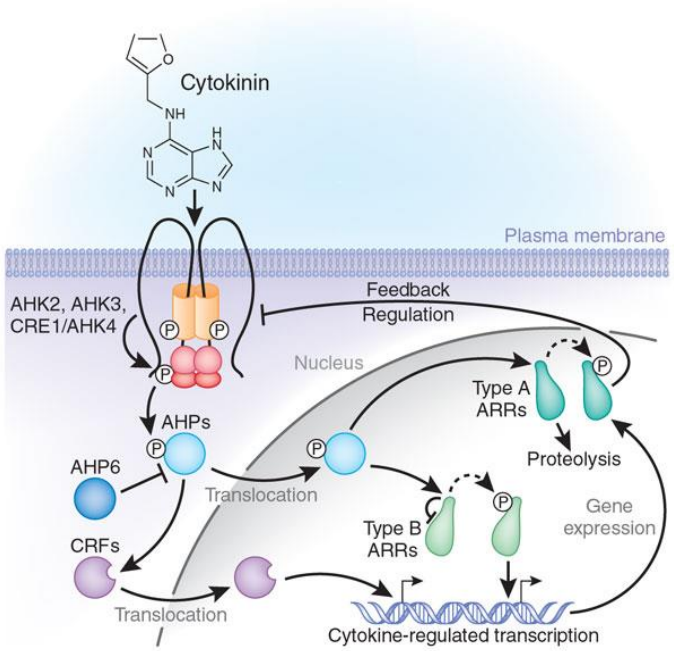

Figure 5: Cytokinin signalling machinery. At the plasma membrane CKs is perceived by the AHK receptors. The signal is then amplified by phosphorylation cascade, which lead to the activation and subsequent nuclear translocation of AHP proteins. AHP proteins transfer the phosphoryl group to type-A and type-B ARR proteins. The former act as repressors of CKs signalling, whereas the latter play as positive transcriptional regulators of CKs-induced genes. CRF proteins are also activated by CKs and, after translocation to the nucleus, they modulate transcription of downstream genes. CRFs and type-B ARRs share many common targets. Figure modified from (Santner et al. 2009).

4. Auxin-cytokinin crosstalk during development, some examples.

As already introduced, auxin and cytokinin coordinate many plant growth and developmental processes. In the last few years significant progresses have been made in elucidating the molecular mechanisms through which these hormones interact (Moubayidin et al. 2009; Perilli et al. 2010; Vanstraelen & Benková 2012; Schaller et al. 2015; Chandler & Werr 2015). In some organ, as in the root, auxin and CKs have an antagonist role, while in other parts of the plant, like in the SAM and female gametophyte they positive cooperate.

In the transition zone of root meristem, CKs activate *IAA3/SHY2*, by the two-component signalling pathway, and leads to down-regulation of *PIN1*, *PIN3* and *PIN7*, and consequent cell differentiation (Dello Ioio et al. 2008). At the same time, auxin mediates IAA3/SHY2 protein degradation through the SCF^{TIR1} ubiquitin–ligase complex, sustaining *PINs* activity and cell division (Dello Ioio et al. 2008). In lateral roots, CKs negatively control lateral root initiation by down-regulating *PINs* expression, thus preventing the establishment of the auxin maximum in the pericycle cells required for lateral root initiation (Laplaze et al. 2007).

At the SAM the general picture of auxin-CKs activity is that, CKs promotes the proliferation of undifferentiated cells, while auxin induce cellular differentiation and organ outgrowth. Auxin stimulates CKs response to control proper SAM activity, acting through ARF5/MONOPTEROS, which represses expression of the negative *type-A ARRs* CKs response regulators (Laplaze et al. 2007; Zhao et al. 2010).

Thanks also to the work of previous colleagues in our laboratory the auxin - cytokinin crosstalk has also been linked to the development of the female gametophyte. It has been proposed that in the sporophytic tissues, formation of auxin and cytokinin maxima regions plays complementary roles in patterning the gamethophyte (Ceccato et al. 2013; Bencivenga et al. 2012). Auxin maxima regions are likely formed through the action of PIN1 and PIN3 (Ceccato et al. 2013). Furthermore, Bencivenga et al., 2012 showed that in the *ahk2-2 ahk3-3 cre1-12*, triple mutant for CKs receptors, integuments initiation was impaired and finger-like ovule structures were observed. Integuments defects in the ovules of the triple receptor mutant were principally caused by a down-regulation of *PIN1* expression (Bencivenga et al. 2012).

My research project was placed in this context, but with a focus on the initiation of ovule primordia and on the determination of their number.
As a part of a special research topic, we reviewed the current knowledge about the hormonal and regulatory pathways that are involved in the formation of the carpel margin meristem and in early stages of ovule development (Cucinotta et al. 2014).

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PLANT SCIENCE

REVIEW ARTICLE
published: 27 March 2014
doi: 10.3389/fpls.2014.00117

Ovule development, a new model for lateral organ formation

Mara Cucinotta, Lucia Colombo and Irma Roig-Villanova*

Dipartimento di Bioscienze, Università degli Studi di Milano, Milan, Italy

Edited by:
Robert G. Franks, North Carolina State University, USA

Reviewed by:
Richard Macknight, University of Otago, New Zealand
Francois Parcy, Centre National de la Recherche Scientifique, France

****Correspondence:***
Irma Roig-Villanova, Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133, Milano, Italy
e-mail: irma.roig@unimi.it

In spermatophytes the ovules upon fertilization give rise to the seeds. It is essential to understand the mechanisms that control ovule number and development as they ultimately determine the final number of seeds and, thereby, the yield in crop plants. In *Arabidopsis thaliana*, ovules arise laterally from a meristematic tissue within the carpel referred to as placenta. For a correct determination of the number of ovules, a precise establishment of the positions where ovule primordia emerge is needed, and a tight definition of the boundaries between ovules is therefore also required. In the last decades, few factors have been identified to be involved in the determination of ovule number. Recently, plant hormones have also been revealed as fundamental players in the control of the initiation of ovule formation. In this review we summarize the current knowledge about both the molecular and hormonal mechanisms that control ovule formation in *Arabidopsis thaliana*.

Keywords: Arabidopsis, ovule primordia, ovule number, development, transcription factors, hormones

INTRODUCTION

Fruits are a major evolutionary acquisition of flowering plants (Angiosperms). They likely evolved to protect the developing seeds and to ensure seed dispersal (Knapp, 2002). Fruits derive mostly from the fertilized mature gynoecium although, especially in fleshy fruits, additional floral components have frequently been recruited. The gynoecium (or pistil), the female reproductive organ, is composed of a single carpel or a number of carpels that are often fused. Carpels are essential for sexual plant reproduction because they house the ovules and upon fertilization the carpel develops into the fruit that protects, nourishes and ultimately disperses the seeds.

In Arabidopsis, the fundamental processes leading to the formation of a complete developed set of ovules can be summarized in a few main steps (**Figure 1**). First of all, the lateral margins of the carpels, containing a meristematic tissue named the medial ridge or carpel margin meristem (CMM), give rise to the placenta, the septum and transmitting track. The CMM formation is known to be controlled by the interaction of genetic and hormonal networks (reviewed by Reyes-Olalde et al., 2013). Once the placenta is formed, some mechanisms, still poorly understood, are needed for the definition of boundary regions that will separate the ovule primordia. The ovule primordia are initiated by periclinal divisions from the subepidermal tissue of the placenta. During the early growth phase of primordia formation a series of predominantly anticlinal divisions take place. Later on, the relatively homogenous mass of cells of the primordium will be organized in three different regions along the proximal-distal axis: the funiculus, the chalaza and the nucellus (**Figure 1**). Within the nucellus, megasporogenesis and megagametogenesis take place, and finally the mature haploid embryo sac is formed. From the chalaza region the two integuments, progenitors of the seed coat, develop, while the funiculus connects the ovule to the mother plant.

In the last decades, several studies have identified genes involved in ovule identity determination and development in different species such as Arabidopsis, Petunia and rice (Bowman et al., 1991; Angenent et al., 1995; Colombo et al., 1995; Angenent and Colombo, 1996; Pinyopich et al., 2003; Dreni et al., 2007). However, the players that determine the number of ovules are largely unknown, due to the difficulties that the studies tempting to genetically dissect ovule initiation and development have encountered. On one hand, many genes that control ovule development are also involved in initiation and growth of other floral organs, masking their effects on ovules. On the other hand, it is difficult to establish if a mutation in a gene causes a reduction in ovule number in mutants that already display an altered gynoecium phenotype.

Nonetheless, understanding the factors that control ovule initiation is of great importance from an agricultural and economical point of view, as the ovule number will determine the number of seeds that develop in a fruit, and thus the crop yield.

With this review on ovule initiation we aim to summarize the current knowledge about the factors and the hormonal pathways that have been identified to be involved in the determination of ovule numbers in *Arabidopsis thaliana*, and the cross-talk between these hormonal and regulatory pathways.

CARPEL MARGIN MERISTEM FORMATION

GENETIC FACTORS CONTROLLING CARPEL MARGIN MERISTEM FORMATION

As already mentioned, the establishment and maintenance of the meristematic tissues of the CMM is inherently correlated to the generation of ovule primordia. CMM development is

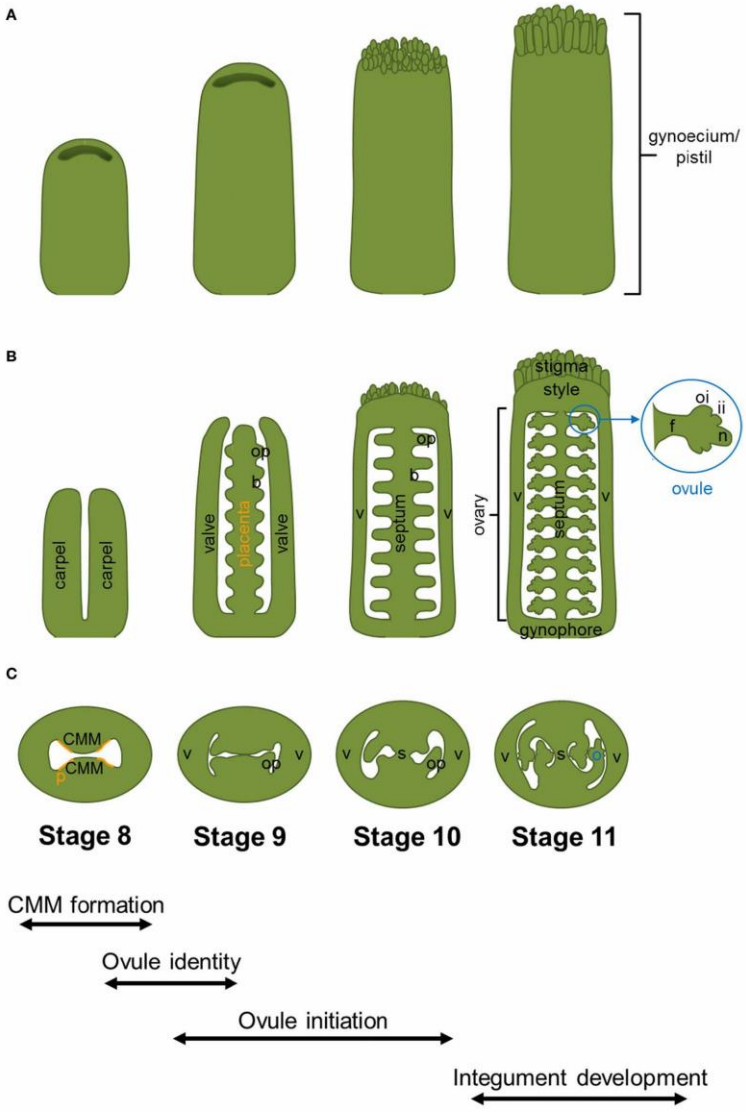

FIGURE 1 | *Arabidopsis thaliana* gynoecium development. Cartoons displaying wild-type gynoecia **(A)**, in longitudinal sections **(B)**, and transversal sections **(C)** from stage 8 to 11. The different stages and processes of gynoecium and ovule development are indicated at the bottom of the figure.

Abbreviations: b, boundary; CMM, carpel margin meristem; f, funiculus; ii, inner integument; n, nucellus; o, ovule; oi, outer integument; op, ovule primordium; p, placenta; s, septum; v, valve. The region of the CMM where placenta is formed is indicated with orange lines.

known to be controlled at the transcriptional level and by hormones as reviewed by Reyes-Olalde et al. (2013). Several single and higher order mutant combinations with strongly reduced carpel marginal tissue development have been described in literature. One of the them is the *aintegumenta* (*ant*) mutant. ANT is a transcription factor that contains two AP2 domains that controls organ initiation and promotes cellular divisions during organ development (Klucher et al., 1996). Interestingly, the *ant-9* mutant has medial ridges that are frequently unfused to each other with a consequent reduction in functional CMM tissue. It has also been reported that the *ant* mutant displays enhanced morphological defects when combined with a mutation in *REVOLUTA* (*REV*), a member of the class III Homeodomain-Leucine Zipper (HD-ZIP III) family. In the *ant rev* double mutant a partial disruption of CMM and placenta development causes the reduced development of ovule primordia (Nole-Wilson et al., 2010).

An unfused carpels phenotype due to the compromised fusion between the two medial ridges was also observed in the mutants for *LEUNIG* (*LUG*), a floral organ identity gene that encodes a glutamine-rich protein with seven WD repeats, typical of transcriptional co-repressors (Liu et al., 2000). Despite this failure in ridge fusion, ovules are formed from the placenta although in a markedly decreased number in both *lug-1* (intermediate-strength allele) and *lug-3* (strong allele) mutants (**Table 1**). The simultaneous loss of *LUG* and *ANT* functions enhanced the defects in flower development in respect to the single *lug* and *ant* mutants. While the double mutant *lug-3 ant-9* did not form any ovules, septum or stigma, nearly 50% of the *lug-1 ant-9* pistils could develop normal medial ridges, that gave rise to partially formed septal tissues, although ovules, stigma and style were never present (Liu et al., 2000) (**Table 1**).

ANT also interacts synergistically with SEUSS (SEU), a transcriptional coregulator functionally similar to LEU, in the control of organ size of the flower. While the *seu-3* single mutant shows on average ovule numbers not significantly different from wild-type Col-0, the double mutant *seu-3 ant-1* results in a complete loss of ovule initiation, caused by severe defects in early gynoecia development. In the weaker allelic combination *seu-3 ant-3*, employing the *ant-3* hypomorphic allele, placenta formation is not compromised but defects such as ovule initiation and gametogenesis are present at later stages (**Table 1**) (Azhakanandam et al., 2008).

Other two players in CMM development are CUP-SHAPED COTYLEDON1 (CUC1) and CUC2, two transcription factors that belong to the NAC transcription factor family. The *cuc1* and *cuc2* single mutants display almost no phenotype, while the *cuc1 cuc2* double mutant completely lacks the shoot apical meristem (SAM) and the cotyledons are fused along their margin forming a cup-shaped structure. These seedlings die a few days after germination (Aida et al., 1997). Studying gynoecium development in the *cuc1 cuc2* double mutant was only possible using plants obtained by *in vitro* regeneration. They presented defects in the formation of the septum and in ovule development (Ishida et al., 2000). A gene that has been described to play a role with *CUC1* and *CUC2* in promoting the formation of carpel marginal structures and thus facilitating septum and ovule development is *SPATULA* (*SPT*), which encodes a basic helix–loop–helix (bHLH)

Table 1 | Ovule numbers phenotype of the mutants cited in these article.

Genotype	Ovule number per fruit	Ovule number per carpel	References
Ler		26.4 ± 1.3	Liu et al., 2000
lug-1		15.4 ± 4.2	
lug-3		14.9 ± 3.1	
ant-9		14.8 ± 3.1	
lug-1 ant-9		0.0 ± 0	
lug-3 ant-9		0.0 ± 0	
Col-0		25 ± 2.0	Azhakanandam et al., 2008
Col-*gl*		21 ± 3.0	
ant-1		12 ± 1.3	
ant-3		20 ± 2.7	
seu-3		23 ± 1.8	
seu-3 ant-1		0.0 ± 0.0	
seu-3 ant-3		13 ± 3.4	
Col-0	55.66 ± 0.83		Nahar et al., 2012
spt-2	48.38 ± 0.61		
cuc1-1 spt-2	36.44 ± 0.59		
cuc2-1 spt-2	34.31 ± 0.49		
Col-0	~ 30		Ishida et al., 2000*
cuc1	~ 31		
cuc2	~ 32		
cuc1cuc2	~ 10		
Ler	51.8 ± 0.6		Galbiati et al., 2013
ant-4	17.8 ± 0.7		
*cuc2-1 ant-4***	20 ± 3		
cuc2-1	41.7 ± 0.9		
pSTK::CUC1_RNAi			
cuc2-1 ant-4	8 ± 1		
*pSTK::CUC1_RNAi***			
pin1-5	8.6 ± 2		
Ler	39.9 ± 1.1		Elliott et al., 1996
ant-9	15.0 ± 0.8		
hll-1	10% less than *wt*		Skinner et al., 2001
hll-3	10% less than *wt*		
Ler	54 ± 4		Broadhvest et al., 2000
sin-2	33 ± 7		
Col-0	48		Bencivenga et al., 2012
cre1-12 ahk2-2 ahk3-3	5.5		
pin1-5	9.35		
Col-0	110		Bartrina et al., 2011***
ckx3-1 ckx5-1	65		
Col-0	52.95		Huang et al., 2012
bzr1-1D	68.06		
bin2	29.07		
det2	52		
WS	46.4		
bri1-5	32.2		
ap2-5	60.4		
bzr1-1D ap2-5	74.8		

(Continued)

Table 1 | Continued

Genotype	Ovule number per fruit	Ovule number per carpel	References
Cvi L*er*	55.5 ± 5.2 66.4 ± 3.9		Alonso-Blanco et al., 1999
ashh2-1, ashh2-2, ashh2-5	80% less than *wt*		Grini et al., 2009

*Mutants presenting defects in the gynoecia or ovule development also reported to be affected at the level of ovule number. *plants regenerated from calli; **Galbiati F. personal communication; ***the number refers to seeds.*

transcription factor. Mutations in *SPT* cause a split carpel phenotype in the apical part of the gynoecium. Moreover, *spt* plants have slightly fewer ovules than the wild type, from which only a small fraction develop into seeds (Nahar et al., 2012). When combined with *cuc1* and *cuc2* single mutants, the average number of ovules decreases. Thus, while the *spt* single mutant shows an average of 48 ovules per carpel, *spt cuc1* and *spt cuc2* present 36 and 32 respectively (**Table 1**), indicating that CUC1, CUC2, and SPT are together required for ovule development. Another mutant that displays an unfused gynoecium at the apex is *crabs claw* (*crc*) (Alvarez and Smyth, 1999). *CRC* encodes a transcription factor of the YABBY family and the characterization of different mutant alleles showed that, besides the failure of the fusion of the stylar region, *crc* mutants present a gradation of phenotypes with wider and shorter gynoecia that contain fewer ovules compared to the wild type (Alvarez and Smyth, 1999; Bowman and Smyth, 1999).

Thereby, the phenotype of ovule reduction that we frequently observe in the mutants defective in medial ridge fusion and thus in CMM formation could be due, at least in part, to their role in regulating cell proliferation in the medial ridges, from which septum and ovules originate.

CMM FORMATION AND THE AUXIN GRADIENT

Auxin is a key hormone for plant development, and it is also fundamental for gynoecium and thereby CMM and ovule development. In the last two decades several studies have demonstrated that local auxin biosynthesis and polar transport are responsible for the correct apical–basal patterning of the gynoecium. The auxin gradient hypothesis supports that high levels of auxin in the gynoecium apical regions control stigma and style formation; medium levels direct ovary formation whereas low levels of the hormone are responsible of gynophore development at the gynoecium base (Nemhauser et al., 2000). Indeed, all mutants in which the auxin synthetic pathway or transport are compromised have a similar severe gynoecium phenotype forming a pistil-like structure with reduction/absence of the valves, expansion of the gynophore and stylar regions and serious vasculature defects (reviewed in Balanzá et al., 2006; Larsson et al., 2013). This phenotype was characterized for the first time in the flowers of *pin-formed1-1* (*pin1-1*), a strong mutant allele of the auxin efflux carrier PIN1 (Okada et al., 1991) and in the *pinoid* mutant, a knock-out line for a serine/threonine kinase that regulates PINs polarity (Bennett et al., 1995). Other examples of mutants with similar pistil-like structure phenotypes are the *yucca1 yucca4* (*yuc1 yuc4*) and *weak ethylene insensitive8 tryptophan aminotransferase related2* (*wei8 tar2*) double mutants, in which local auxin production is impaired (Cheng et al., 2006; Stepanova et al., 2008). Predictably, in most of these auxin-related mutants the severe defects in gynoecium formation lead to a pistil with a reduction or complete absence of ovules and the consequent complete sterility.

Nemhauser et al. (2000) confirmed the importance of polar auxin transport (PAT) in gynoecium development through an experiment in which they used 1-napthylphthalamic acid (NPA), an inhibitor of the auxin transport. They showed that NPA application caused significant loss of ovules. The authors also highlighted that ovules seemed more sensitive to disruption in PAT, with respect to the other tissues of the gynoecium. Indeed, treated carpels were largely devoid of ovules but were still able to produce valves. In 2010 Nole-Wilson and collaborators proposed the connection between ANT and the hormone auxin on the base of the observation that the *ant* mutant is more sensitive than the wild type to alteration in PAT. Moreover, the expression of a subset of auxin-related genes was altered in the *ant* single and *ant rev* double mutant gynoecia, indicating that the morphological defects of the *ant rev* double mutants, at least in part, are due to an alteration in auxin homeostasis in these plants.

Auxin signaling is primarily regulated by the *AUXIN RESPONSE FACTOR* (*ARF*) gene family products, together with the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins. The phenotype of *ARF5/MONOPTEROS (MP)* strong mutant alleles results in an embryo lethal phenotype, while *mp* partial loss of function mutants have normal embryo development whereas that their reproductive development is compromised (Hardtke and Berleth, 1998). In the pistil of the *mpS319* weak allele the CMM does not develop, and placenta and ovules are completely missing (Cole et al., 2009; Galbiati et al., 2013). Interestingly, MP has been demonstrated to directly activate the *ANT, CUC1* and *CUC2* transcription factors encoding genes (Galbiati et al., 2013) Their role as major players in ovule primordia initiation and ovule number determination will be discussed in the following sections.

OVULE IDENTITY ESTABLISHMENT

The ovule cell fate is controlled by the ovule identity genes *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*), that belong to the MADS-box gene family of transcription factors. While in the single and double mutant combinations of these genes there is no detectable ovule phenotype, in the *stk shp1 shp2* triple mutant the ovule integuments are converted into leaf/carpel-like structures (Pinyopich et al., 2003; Brambilla et al., 2007). Moreover, ectopic expression of these MADS box genes results in ovule formation on sepals (Favaro et al., 2003; Pinyopich et al., 2003; Battaglia et al., 2006). *STK*, *SHP1*, and *SHP2* have overlapping expression patterns in the placenta and ovule primordia also with *AGAMOUS (AG)* (Rounsley et al., 1995; Savidge et al., 1995; Theißen et al., 1996; Pinyopich et al., 2003), one of the first identified MADS-box factors that determines stamen and carpel identity (Yanofsky et al., 1990). It has been shown that also

AG plays a role in ovule development by experiments in which the *apetala2* (*ap2*) single mutant was compared with the *ap2 ag* double mutant. Thus, in the *ap2* single mutant petals were mostly absent, while sepals were converted into carpel structures bearing ectopic ovules, some of which were transformed into carpelloid structures. Interestingly, the sepals (or first-whorl organs) of the *ap2 ag* double mutant still presented carpel identity, and the number of ovules converted into carpel structures was significantly higher, indicating that AG activity also contributes to ovule identity establishment (Bowman et al., 1991; Pinyopich et al., 2003). Interestingly, Skinner et al. (2004) suggested that when the functions of *stk, shp1*, and *shp2* were lost in a triple mutant, fewer ovules initiated and ovule development is severely disrupted.

OVULE PRIMORDIA INITIATION

THE ESTABLISHMENT OF THE BOUNDARIES

When new organ primordia are originated in the plant, two different regions, the boundaries and the zone of primordia outgrowth, need to be defined. The organ boundary is defined as the region between the meristem and the developing organ, or, as in the case of ovules, as the region between two adjacent ovule primordia. As Aida and Tasaka nicely reviewed in 2006, the "boundary cells" need to have peculiar characteristics respect to the surrounding cells, usually displaying reduced cell division and expansion. Another important aspect is the arrangement of the plasmodesmata that regulates the movement of transcription factors between cells. For example, the boundaries in the inflorescence meristem seem to restrict the passage of proteins into flower primordia (Wu, 2003).

The boundary-specific regulatory genes play a critical role in orchestrating several morphogenetic and patterning events and their spatial coordination. When this coordination is missing, fusion between organs is the most frequent observed phenotype (Aida et al., 1997). The *CUC* gene family was the first discovered to have a fundamental role in organ boundary establishment. In fact, in the *cuc1cuc2* double mutant embryo the cotyledons do not separate (Aida et al., 1997).

The transcripts of *CUC1* and *CUC2* were detected by *in situ* hybridization in the anlagen placenta and in ovules at stage 1-II and later on, starting from stage 2-I, restricted to the boundary between two ovules (Ishida et al., 2000; Galbiati et al., 2013). As we already mentioned, the study of the gynoecium phenotype of the *cuc1 cuc2* double mutant was only possible on plants regenerated *in vitro*. They showed defects in the formation of the septum and in ovule development; most of the gynoecia having less than 10 ovules (**Table 1**). However, the *cuc1 cuc2* double mutant plants never gave seeds (Ishida et al., 2000). A further demonstration that CUC1 and CUC2 are directly linked to the determination of ovule number in a direct way came from the work of Galbiati et al. (2013). In order to study the ovule phenotype in absence of both *CUC1* and *CUC2, CUC1* was silenced in a *cuc2-1* mutant background using a *CUC1* specific RNAi construct under the control of the ovule-specific *SEEDSTICK* promoter (*pSTK:CUC1_RNAi*) which is already active in the placenta before ovule primordia arise. The analysis of *cuc2-1 pSTK:CUC1_RNAi* plants revealed a reduction in ovule number of 20% (**Table 1**). Furthermore, *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants were generated in order to analyze the possible additive role of ANT to CUC function in the regulation of ovule primordia formation. The *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants displayed a further dramatic reduction in the number of developing ovules (a mean of seven ovule primordia per pistil), while the single mutant *ant-4* and the plants *cuc2-1 pSTK::CUC1_RNAi* showed 20 and 30 ovules per pistil, respectively (**Table 1**). Despite the reduction in ovule number in the different mutant backgrounds, the size of the pistils was not reduced. Therefore, the ovules were more distantly spaced compared to those in wild-type pistils (Galbiati et al., 2013). These studies of the characterization of the *ant* single and *cuc1 cuc2* double mutants, as well as *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants prove that ANT, CUC1 and CUC2 are key players in the control of the number of ovule primordia that develop from the placenta and that they act additively (Elliott et al., 1996; Ishida et al., 2000; Galbiati et al., 2013). All the information about these factors taken together indicates that they work in different ways: while ANT promotes ovule primordia growth, the CUCs play a role in the establishment of the ovule primordia boundaries (**Figure 2**).

CUC3, another putative NAC-domain transcription factor member of the *CUC* family, is expressed in an extensive range of boundaries in adult plants. Besides, the function of CUC3 is partially redundant with that of its homologous CUC1 and CUC2 in the establishment of the cotyledon boundary (Vroemen et al., 2003). Several studies revealed that *CUC* expression is controlled and restricted to the boundaries in several ways. For instance, in the SAM *CUC1* and *CUC2* but not *CUC3* are regulated by *miR164*, which restricts the expression of *CUC1* and *CUC2* mRNAs to the boundary domain (Laufs et al., 2004). In the carpel, in a similar way to the pattern already described

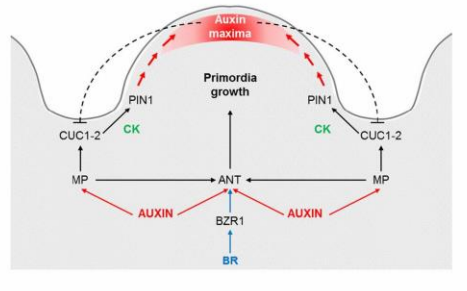

FIGURE 2 | Proposed model for the control of the ovule primordia initiation. Auxin triggers ANT and MP expression, which in its turn is required for *ANT, CUC1* and *CUC2* expression during the early stages of placenta development and ovule primordia formation. *ANT* expression is also regulated by brassinosteroids (BR), specifically being directly regulated by BZR1. ANT controls cell proliferation in the placenta and ovules, whereas CUC1 and CUC2 establish the boundaries and control *PIN1* expression, which is required for primordia formation. Cytokinin (CK) may act downstream of CUC proteins in promoting *PIN1* expression. Once the primordia have formed, auxin accumulates at the edge of the developing ovule. An inhibitory loop of auxins on CUC1 and CUC2, as it is postulated for the leaf serration, could be happening at the ovule boundaries. Adapted from Galbiati et al. (2013).

for *CUC1* and *CUC2, CUC3* expression marks the boundaries between ovule primordia. Therefore, it would be interesting to study also the contribution of *CUC3* in the regulation of defining ovule boundaries.

In 2008, Xu and Shen showed that three different transcription factors, ASSYMETRIC LEAVES 1 (AS1), AS2, and JAGGED (JAG), support normal sepal and petal growth by restricting the expression domain of the boundary-specifying genes *CUC1* and *CUC2*. AS1 and AS2 were already suggested to have roles in boundary control, given that they positive regulate, within the shoot apex, the members of the *LATERAL ORGAN BOUNDARIES* (*LOB*) gene family, a plant-specific family of transcription factors that are expressed in the boundaries (Byrne et al., 2002). *LOB*, the gene that names the family, is expressed at the base of all lateral organs. Interestingly, plants overexpressing *LOB* produced abnormal flowers with reduced floral organs and they were sterile even when fertilized with wild-type pollen (Shuai et al., 2002). Lee et al. in 2009 identified two new MYB transcription factors involved in lateral organ separation: *LATERAL ORGAN FUSION 1* (*LOF1*) and *LOF2*. The single mutant *lof1* exhibits a novel fusion between the axillary stem and the cauline leaf. Additional fusions resulted when *lof1* was combined with *lof2*, *cuc2* or *cuc3*, indicating the existence of overlapping roles for *LOF1, CUC2*, and *CUC3* to control organ separation during reproductive development.

Despite the identification of a number of boundary-specific transcription factors, boundary formation and maintenance is still a poorly understood process, and only CUC1 and CUC2 have been demonstrated to have a role in ovule boundary establishment. The factors that have been described to regulate or interact with the CUCs in a different developmental context could also have a role during ovule initiation, and some of them, like *AS1* and *AS2* are already known to be expressed in the gynoecium and ovules (Xu and Shen, 2008).

AINTEGUMENTA, A MASTER REGULATOR OF PRIMORDIA FORMATION

In Arabidopsis many genes have been described to play roles in the different phases of ovule development, although most of them do not determine directly the number of ovules (Schneitz et al., 1997; reviewed in Shi and Yang, 2011). However, the ANT transcription factor has been described to have a clear role in ovule primordia formation. *In situ* hybridization experiments showed that within the carpel it is expressed in the placenta and in the integuments of the developing ovules. In *ant* plants ovules do not develop integuments and megasporogenesis is blocked at the tetrad stage leading to complete female-sterility (Elliott et al., 1996). ANT is not only required for ovule development but it is also involved in ovule primordia formation. Indeed, in the *ant-9* mutant the number of ovules per carpel is reduced by more than half in respect to the wild-type (**Table 1**). Given that the *ant* gynoecia have the same length as those of wild type, the ovules that do arise in *ant* are more distantly spaced than in wild-type plants (Liu et al., 2000).

In addition to *ANT*, another essential gene for the regulation of ovule primordia outgrowth and for the control of integument formation is *HUELLENLOS* (*HLL*), a gene that encodes a mitochondrial ribosomal protein. Thus, plants presenting mutations in *HLL* display a phenotype similar to *ant* at the level of ovule integuments (Schneitz et al., 1997, 1998). Moreover, *hll-1* and *hll-3* mutant alleles display a reduction of about 10% in the number of ovules, although the authors also describe that *hll* plants display smaller gynoecia, which could contribute to the development of fewer ovules (**Table 1**). The phenotype of the double mutant *hll ant* was more severe at the level of primordia outgrowth however, nothing was described regarding ovule number (Schneitz et al., 1998; Skinner et al., 2001). A similar phenotype to *hll* was observed in the *short integuments 2* (*sin2*) mutants. Apart of an arrest in cell division in both ovule integuments, *sin2* plants presented shorter pistils bearing less ovules than the wild type (**Table 1**). Moreover, the authors describe an abnormal distribution of the ovules along the placenta, being the distance between ovules bigger than in wild-type plants (Broadhvest et al., 2000). Thus, in this particular case the shorter carpel might not be the only cause of reduced ovule numbers. The double mutant *sin2 ant-5* was not different from *ant-5* single mutant, indicating that *ANT* is epistatic to *SIN2* with respect to ovule development. On the contrary, *sin2 hll-1* double mutant had a stronger effect on ovule development than *sin2* or *hll-1* single mutants (Broadhvest et al., 2000). All these experiments taken together indicate that although ANT plays a master role, SIN2 and HLL also contribute to ovule primordia formation.

THE ROLE OF HORMONES IN OVULE PRIMORDIA FORMATION

AUXIN IS REQUIRED FOR OVULE PRIMORDIA FORMATION

As we previously underlined, the boundary region and the primordia formation zone are highly interconnected. It has been demonstrated that a fundamental role of the "boundary transcription factors" is to organize PAT, mediated by PIN proteins, in order to create a zone of auxin maximum where organ founder cells will be selected. Auxin maxima are fundamental for the formation of primordia, and auxin action has been well described for lateral roots (LR) and flower primordia (reviewed in Benková et al., 2003, 2009; Yamaguchi et al., 2013). The directionality of auxin flux depends principally on the polar localization of the PIN proteins. In Arabidopsis there are eight PIN proteins (PIN1-8), from which only *PIN1* and *PIN3* are expressed in the pistil and ovules (Benková et al., 2003; Ceccato et al., 2013). PIN1 protein is localized at the membrane of placenta cells and later on, in the developing ovules, it is restricted to the lateral-apical membranes of nucellus cells. PIN3 is also present in few cells at the tip of the developing nucellus shortly after ovule primordia emergence but, contrary to PIN1, it is not expressed in the placenta cells (Ceccato et al., 2013). PIN-dependent efflux mediates primordium development by supplying auxin to the tip creating an auxin maxima; indeed in plants expressing the *GFP* reporter gene downstream the auxin-responsive *DR5* promoter (*pDR5::GFP*), the GFP signal is detected at the tip of all ovule primordia (Benková et al., 2003). The weak *pin1-5* mutant allele is able to develop some flowers in which the pistils have slightly reduced valves but normal styles and stigmas (Sohlberg et al., 2006). The pistils of the *pin1-5* weak allele have an average of 9 ovules per carpel (**Table 1**) (Bencivenga et al., 2012). In addition, Galbiati et al. (2013) demonstrated that the reduced number

of ovules in *cuc2-1 pSTK:CUC1_RNAi* was caused by a down-regulation of *PIN1* and an incorrect PIN1 protein localization. CUC1 and CUC2 promote *PIN1* expression and localization to correctly form the auxin maximum where primordium will form (**Figure 2**). In the same way, a CUC2-dependent regulatory pathway controlling PIN1-mediated auxin efflux has been described to explain leaf serrations (Bilsborough et al., 2011). Moreover, in the newly formed primordia of the SAM the auxin maxima, in a negative feed-back loop, repress *CUC2* expression and restricts it to the boundaries (Vernoux et al., 2000; Heisler et al., 2005; and reviewed in Aida and Tasaka, 2006; Rast and Simon, 2008). A similar inhibitory loop could control *CUC* expression at the ovule boundaries (**Figure 2**). The phenotype of *cuc2-1 pSTK:CUC1_RNAi* was completely recovered by cytokinin (CK) application, since CK has been demonstrated to increase *PIN1* expression in the ovules (Bencivenga et al., 2012). These experiments evidence a convergence of two different plant hormones in the regulation of ovule primordia formation. In the next paragraph we will delve deeper into the role of CK in the formation and determination of ovule number.

CYTOKININ POSITIVELY REGULATES OVULE NUMBER

CK is an essential hormone for plant growth and development as it has a central role in the regulation of cell division and differentiation. In the last 10 years, several studies have clearly proven that CK has also a significant role during ovule development. As it will be explained in this paragraph, it has been demonstrated that in plants that are defective in the production or perception of this hormone, correct ovule formation is compromised and/or the number of ovule is drastically reduced. CK signaling, which has been recently summarized in a detailed review article (Hwang et al., 2012), is mediated by a two-component signaling pathway: histidine protein kinases (AHKs) work as CK receptors, while histidine phosphotransfer proteins (AHPs) transmit the signal from AHKs to nuclear response regulators (ARRs), which are able to regulate transcription. In *Arabidopsis* the CK signal is perceived by three histidine kinases: *ARABIDOPSIS HISTIDINE KINASE4* (*AHK4*, also known as *CYTOKININ RESPONSE1*, *CRE1*/*WOODEN LEG*, *WOL*), *AHK2* and *AHK3*. These three genes are all expressed in inflorescences, carpels and developing ovules (Higuchi et al., 2004; Nishimura et al., 2004). More precisely, *AHK2* and *AHK3* are expressed during all stages of ovule development, starting from early primordia stages to ovule maturity, whereas *CRE1* expression remains restricted to the chalazal region and later to the integuments of ovules during all the developmental stages (Bencivenga et al., 2012). The single and double mutants of *AHKs* do not present any phenotype at the level of the ovules (Higuchi et al., 2004). However, mutants lacking all three receptors exhibit no perception of CK and present a strong slowdown of shoot and root growth. The resulting miniature plants also show delayed flower induction and impaired fertility (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Thus, the triple mutant *cre1-12 ahk2-2 ahk3-3* do not produce seeds (Higuchi et al., 2004) because the gametophyte arrests at stage FG1-FG2 (Bencivenga et al., 2012). Moreover, a severe reduction in the ovule number, an average of 5 ovule per pistil, was noticed in these triple mutant plants (**Table 1**) (Bencivenga et al., 2012). A similar sterile phenotype was also observed for another allelic combination: the *ahk4-1 ahk2-1 ahk3-1* triple mutant (Nishimura et al., 2004). Differently, Riefler et al. (2006) obtained a weaker triple mutant *cre1-2 ahk2-5 ahk3-7* that self-fertilized and formed few seeds, suggesting that infertility of the histidine kinase triple mutants is a phenotype associated with specific mutant alleles.

Attention has also been given to the importance of CK catabolism. In *Arabidopsis* the irreversible degradation of CK is catalyzed by the oxidase/dehydrogenase (CKX). The *CKX* gene family of Arabidopsis consists of seven members (*CKX1* to *CKX7*), and by *promoter:GUS* fusion constructs it was shown that *CKX1*, *CKX5*, and *CKX6* (At3g63440, previously called *AtCKX7*) are expressed in flower tissues, being *CKX6* the only one reported to be expressed in the carpel and ovules, in particular in the funiculus (Werner et al., 2003). Werner and colleagues engineered transgenic *Arabidopsis* plants that individually overexpressed six different *CKXs* in order to enhance CK degradation. As expected, these plants manifested phenotypes linked to CK deficiency, like delayed vegetative growth and leaf expansion, diminished activity and size of the SAM but increased overall root system. The reproductive development of CK-deficient plants was also altered. In *35S::CKX1* and *35S::CKX3* plants, flowering was strongly delayed and furthermore the fertility of flowers was heavily reduced, partially due to the lack of pollen. *35S::CKX1* and *35S::CKX3* siliques were not filled completely and they formed approximately 8–20 viable seeds, whereas the wild-type siliques harbored up to 60 seeds. Although the number of ovules formed in these plants was not reported in this work, the expression patterns together with the phenotypes in the flowers and fruits indicate once more that CK play a role during reproduction. Moreover, the authors suggest a role for ANT in the observed reduced cell division in the leaves of *ckx* plants. Considering the documented role of ANT in ovule primordia initiation already introduced in this article, it will be very interesting to analyze also its role in the reproductive tissues of these plants.

With an opposite experimental approach, the simultaneous mutations of two *CKX* genes, it was demonstrated that plants with an increased level of CK had an enhanced activity of the reproductive meristem (Bartrina et al., 2011). Indeed, the *ckx3-1 ckx5-1* double mutant produced more flowers due to a larger inflorescence meristem with more cells than the wild type. Moreover, flowers were bigger and so were the gynoecia. Besides, double mutant gynoecia contained twice as many ovules as wild-type ones, indicating an increased activity of their placental tissue. The *ckx3-1* and *ckx5-1* single mutants already developed more ovules than the wild-type, and the flower size and the number of ovules was reflected into the length of the fruits (siliques of *ckx3 ckx5* were 20 mm long compared with the 17 mm of the wild type) and the seed number (110 seeds in the *ckx3 ckx5* mutant siliques compared with an average of 65 seeds in wild-type siliques, **Table 1**) The authors suggested that CKX3 and CKX5 may regulate the activity of meristematic cells in the placenta thus affecting organogenic capacity and ovule primordia formation.

A conclusive evidence about the relationship between the levels of CK and the initiation of ovule formation was obtained from

experiments in which inflorescences were treated with synthetic CK (6-Benzylaminopurine, BAP). The treatment resulted in the formation of new primordia, 20 ± 3 primordia in average in each pistil, positioned between the ovules already formed before the CK application (Bencivenga et al., 2012). An equivalent CK treatment was also able to increase the ovule number in *pSTK::CUC1_RNAi cuc2* plants already described in this review, by acting on the expression and localization of the auxin efflux carrier PIN1 (Galbiati et al., 2013). These results point out the importance of the cross-talk between CK and auxin during ovule primordia formation. However, the hormonal cross-talk is not limited to auxin and CK since very recently it has been demonstrated that also brassinosteroids (BR) play a crucial role in ovule and seed formation by regulating the expression of genes that control ovule development (Huang et al., 2012), as will be explained in the next paragraph.

THE ROLE OF BRASSINOSTEROIDS

BRs are hormones known to control general plant development. More specifically, they have been described as involved in the control of the initiation and formation of reproductive organs (Szekeres et al., 1996; Kim et al., 2005). Huang et al. (2012) found that the BR-deficient and -insensitive mutants have smaller and less seeds, while BR-enhanced mutants have more seeds. The analysis of the number of ovules and seeds and the morphological analysis of the siliques of *det-2* (a BR-deficient mutant involved in BR biosynthesis), *bri1-5* (the mutant for the BR receptor), heterozygous plants for *bin2-1* (a gain of function mutant deficient in BR signaling) and *bzr1-1D* (a BR signal-enhanced mutant) leaded to the conclusion that BR signaling positively regulates ovule number (**Table 1**) (Huang et al., 2012). Specifically, it was found that the transcription factor BRZ1 plays an important role in ovule and seed number determination, depending on its state of phosphorylation/dephosphorylation (more dephosphorylation implying more activity and more ovules and seeds).

By treating plants with BR it was shown that BR influences ovule development through regulating the transcription of genes such as *HLL* and *ANT*, which are redundant in the control of ovule primordia growth as already introduced in this review (Schneitz et al., 1998), and *AP2*, that affects floral organ (including ovule) pattern formation (Modrusan et al., 1994). *HLL* and *ANT* are clearly induced by BR, while *AP2* is slightly repressed by BR. These genes appeared to be targets of BRZ1, and its state of phosphorylation/dephosphorylation influences the expression of these genes. Further analysis indicated that *AP2* and *ANT* are direct targets of BRZ1, while *HLL* is regulated by an indirect way. The analysis of ovule number of *bzr1-1D* and *ap2-5* single mutants and *bzr1-1D ap2-5* double mutant (**Table 1**), together with other molecular proofs, indicate that BZR1 and AP2 play antagonistic effects in ovule number determination, being BZR1 (and HLL and ANT) promoters and AP2 inhibitor of ovule primordia formation (Huang et al., 2012).

A model for ovule primordia formation that integrates the molecular and hormonal networks has been proposed by Galbiati et al. (2013): MP is required for *ANT*, *CUC1* and *CUC2* expression during the early stages of placenta development and ovule primordia formation, being ANT expressed in the ovule primordia, whereas *CUC1* and *CUC2* in the ovule boundaries. CUC1 and CUC2 may be involved in the increase of CKs required for proper *PIN1* expression needed for primordia formation. Once the primordia have formed, auxin accumulates at the edge of the developing ovule. This model can be easily extended with the recently discovered role of the plant hormones BR, which positively regulate the number of ovule primordia, in part by the direct regulation of *ANT* by BZR1 (**Figure 2**).

OTHER MECHANISMS CONTROLLING OVULE NUMBER: THE EPIGENETIC REGULATION

Interestingly, in different Arabidopsis ecotypes (diploid accessions) a variation in ovule numbers can be observed. Alonso-Blanco et al. (1999) found that the Lansberg *erecta* ecotype presents 20% more ovules than the Cape Verde Islands (Cvi) one (**Table 1**). Recently a considerable genetic variation in ovule number was described in selfed F1 triploids of different *A. thaliana* genotypes (Duszynska et al., 2013). Triploids were obtained by crossing a tetraploid L*er*-0 line (used as a male or female parent) with different diploid accessions. Interestingly, it was observed an effect of the parental genome excess (2m:1p *vs.* 1m:2p) in the determination of the total ovule number in genetically identical F1 hybrid offsprings. These were the first parent-of-origin effects on ovule number in reciprocal triploids of plants. The authors postulate that such effects may represent epigenetic effects, because changes in DNA sequence cannot explain mitotically and/or meiotically heritable changes in gene function but they might be due to changes in DNA methylation, for example (Duszynska et al., 2013). Indeed, in Arabidopsis the ASH1 class of proteins, that can methylate lysine residues on histone tails, maintains an active transcriptional state during development. One of its members, ASH1 HOMOLOG 2 (ASHH2), has been described as a controller of reproductive development via H3K36 trimethylation. Plants homozygous for *ashh2* null alleles presented an 80% reduction in ovule numbers when compared to wild-type plants (**Table 1**) (Grini et al., 2009). These data altogether indicate that epigenetics may also play a role in the control of ovule number, and they open up a new interesting field of research.

CONCLUDING REMARKS AND FURTHER PERSPECTIVES

In the past years, several genes such as *AG*, *STK*, *SHP1*, and *SHP2* have been identified as ovule identity genes, and *ANT*, *HLL*, *SIN2*, *INNER NO OUTER* (*INO*), and *SUPERMAN* (*SUP*) as regulators of ovule outgrowth (Elliott et al., 1996; Schneitz et al., 1997, 1998; Broadhvest et al., 2000; Pinyopich et al., 2003). Nevertheless, most of their targets, which might be the genes that determine the correct development of the ovule, remain to be uncovered. Another quite unknown process is the regulation of the ovule primordia initiation. As explained in this review, only a few regulators, such as the transcription factors ANT, CUC1, CUC2, AP2 and the mitochondrial ribosomal protein HLL have been identified (Elliott et al., 1996; Schneitz et al., 1998; Galbiati et al., 2013). The majority of them are transcription factors, and the transcriptional cascades triggered by them, that will determine the regulation of the morphogenetic parameters such as cell division

and expansion, or expression patterns of identity genes of particular organs, are also largely unknown. Therefore, one of the next challenges would be the identification of downstream targets of these transcription factors by genetic or molecular biological approaches, including suppressor/enhancer mutant screenings or RNA-sequencing transcriptome analyses. It is worth to highlight that these regulators are not exclusively transcription factors, but also mitochondrial proteins or chromatin remodeling factors, indicating that a correct ovule initiation depends on a complex genetic and molecular network.

One of the difficulties of the genetic dissection of ovule initiation and development is that many mutations that affect ovule initiation have already pleiotropic effects on earlier stages of the development of the reproductive tissues, causing floral aberrations that may mask their effects on ovules. Thus, many genes that control ovule development are also involved in primordium initiation and growth of other floral organs (Elliott et al., 1996; Schneitz et al., 1998; Alvarez and Smyth, 1999). Moreover, it is difficult to establish if a mutation in a gene causes a reduction in ovule number if this mutant already has an altered gynoecium phenotype (Alvarez and Smyth, 1999; Western and Haughn, 1999; Broadhvest et al., 2000; Liu et al., 2000; Pinyopich et al., 2003; Nole-Wilson et al., 2010; Nahar et al., 2012). The reanalysis of these carpel mutants, measuring the space between ovules, or expressing the ovule number as the ovule number per millimeter of gynoecium, as some authors already presented (Huang et al., 2012), could contribute to resolve this uncertainty. The use of specialized vectors, for instance containing placenta-specific promoters to obtain milder vegetative and/or floral effects of these mutations would help to uncover the role of specific factors in ovule development. Besides, a reverse-genetic strategy using RNA interference or insertional mutants can be used to identify new regulators of ovule numbers determination.

Ovule boundary establishment is still a poorly understood process, and only CUC1 and CUC2 have been demonstrated to play a role (Galbiati et al., 2013). The contribution to the determination of ovule boundaries of the genes that have been described to regulate or interact with the *CUCs* in other organ boundaries would be worth to be analyzed, by means of the study of their patterns of expression and how these are accurately determined. The identification and characterization of single and multiple mutants, as has been done for the *CUC* genes (Aida et al., 1997; Galbiati et al., 2013) is also key to study their roles. Moreover, the analysis of their incidence at the cellular level will help to define the effects on cell behavior (i.e., division or expansion) that these factors could have. It has also been widely demonstrated that hormones play a role in the regulation of ovule primordia initiation, being auxin, CK and more recently also BR identified as the important hormonal players in this process. The crosstalk between these hormones, as Bencivenga et al. (2012) and Galbiati et al. (2013) present in their works, is starting to be revealed (**Figure 2**) and it will be very interesting to investigate in the future how auxin, CK and BR interact. Moreover, it will be important to explore if hormone and gene expression levels are responsible for the variation in ovule numbers described for the different ecotypes (Alonso-Blanco et al., 1999), and to identify QTLs linked to this trait.

Based on the experimental data exposed in this review, a similarity between ovule initiation and the initiation of other lateral organs in the plant can be proposed. The strongest pieces of evidence are the triggering role of auxins and the conservation in the genes that establish the boundaries and promote new organ growth. Although further studies will be needed in order to identify the common and specific players of the different lateral organ initiation processes, conserved modules can be already suggested. In the case of flower primordia initiation, similarly to what happens during ovule primordia formation, the coordinate action of MP and ANT is required. In particular, at the reproductive shoot apex, auxin-activated MP directly induces *ANT*, other two key regulators of floral growth, *LEAFY* (LFY) and *AINTEGUMENTA LIKE-6* (*AIL6*), and probably a forth unknown factor, which together lead the flower primordium initiation (Yamaguchi et al., 2013). Also the factors determining the new organ boundary seem conserved between ovule and flower primordia initiation: the coordinated spatial and temporal action of auxin, PIN transporters and CUC proteins is required (Heisler et al., 2005; Galbiati et al., 2013). If we instead compare the initiation of ovule primordia with the initiation of LR we also find many common players, despite the clear fact that ovule primordia arise from the naked placenta while LR have to pass through several cell layers to emerge. Thus, we find an auxin maxima that precedes organ formation (Benková et al., 2003). Also other hormones, such as BR and CK play a role in both ovule and LR initiation, although CK play opposite roles (it activates ovule primordia formation while inhibits LR initiation) (Werner et al., 2003; Higuchi et al., 2004; Bartrina et al., 2011; Huang et al., 2012; Bianco et al., 2013; Chang et al., 2013). Besides, the participation of IAA/AUX-ARF modules exists in both processes, and MP seems to be a regulator of the two of them (De Smet et al., 2010; Galbiati et al., 2013). Other members of the ARF family, as well as the NAC and the MADS-box transcription factors could be conserved in both processes, as some introductory works seem to indicate (Pinyopich et al., 2003; Moreno-Risueno et al., 2010; reviewed in Benková and Bielach, 2010). Finally, downstream the auxin signaling cascades, the activation of cell cycle genes will take place in order to promote organ growth, as it is starting to be revealed in the case of LR (Rast and Simon, 2008). Thereby, the analysis of the expression of cell cycle genes during ovule primordia formation would be very revealing. Apart of the hormonal and molecular pathways controlling LR formation, the influence of the environmental factors on this process is of extreme importance for the plant. How environment influences ovule primordia formation would be for sure a very challenging topic of research.

With this work we wanted to point out the little specific information available about the factors that control ovule primordia initiation, due to the difficulties to identify mutants presenting defects only in this particular step of ovule formation. Here we propose different experimental approaches to overcome the severity of some mutant phenotypes as well as to investigate these processes from a new point of view. The contribution and conservation of chromatin remodeling changes to the regulation of ovule number is starting to be elucidated and opens an extremely interesting field of research. Moreover, the most recent progresses

in the fields of ovule, flower and root development strongly suggest common hormonal and molecular signals in all these organ initiation processes; such as a crosstalk between auxin and CK and probably also BR and the factors that establish organ boundaries and those that promote new organ outgrowth.

ACKNOWLEDGMENTS

We apologize for references not cited because of space limitations. The authors are grateful to Prof. M.M. Kater for critical reading of the manuscript. Mara Cucinotta was funded by a Ph.D fellowship from the Università degli Studi di Milano, Irma Roig-Villanova was funded by MIUR-Fondo per gli Investimenti della Ricerca di Base-Futuro in Ricerca. The work of Lucia Colombo is supported by MIUR-PRIN 2012.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 January 2014; paper pending published: 21 February 2014; accepted: 11 March 2014; published online: 27 March 2014.

Citation: Cucinotta M, Colombo L and Roig-Villanova I (2014) Ovule development, a new model for lateral organ formation. Front. Plant Sci. 5:117. doi: 10.3389/fpls.2014.00117

This article was submitted to Plant Evolution and Development, a section of the journal Frontiers in Plant Science.

Aim of the project

The aim of my PhD project was to investigate the molecular network that mediates auxin–cytokinin crosstalk during ovule primordia formation and responsible of ovule primordia initiation and ovule number determination.

During the first year, I contributed to unveiling the role of MP, CUC1 and CUC2 in the regulation of *PIN1* expression, required for primordia initiation. To investigate more in detail the functions of *CUCs* and their relation with the hormonal pathways, we decided to perform a genome wide identification of CUCs targets.

The obtained results have reinforced the hypothesis that CK and auxin work in a coordinate manner to control ovule primordia formation.

Therefore, we have characterized the role of the CKs response regulators (CRFs) in pistil and ovule development. Indeed, it has been recently discovered that some of the CRFs control directly *PIN1* expression. CRF2 is also been reported to be target of MP consequently I have performed experiments to clarify the role of MP in the reproductive phase. It is interesting to notice that despite being much studied, MP regulation and its molecular mechanism of action remain still unclear.

Main results

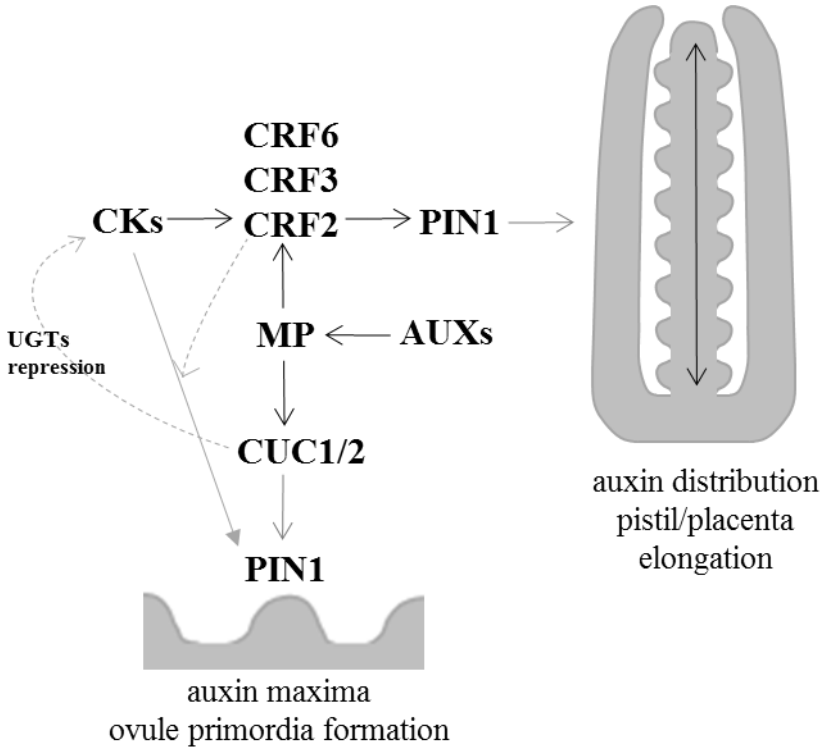
In the first part of my Ph.D. I participated in the work that lead to the first description of the molecular regulation of ovule primordia formation (Galbiati et al. 2013). We demonstrate that *CUC1* and *CUC2* genes have additive effects on ovule number determination, and that they are direct targets of the auxin response factor MP. In particular, I contribute by demonstrating that CUCs, upstream or in parallel with CKs, positive regulate *PIN1* expression, which is necessary for the correct ovule primordia formation.

In order to investigate more in detail the *PIN1* transcriptional regulation by CKs, we analyzed the function of CRF2, a direct regulator of *PIN1* expression in roots. Our results demonstrate that CRF2 is required for *PIN1* expression also during early stages of pistil development and that it plays a role controlling pistil growth rate. Moreover, being *CRF2* also a MP direct target, it arises as a new key regulator of the auxin-cytokinin crosstalk. Mutation in *CRF2* determines a change in pistil length and consequently influences ovule number, feature that makes *CRF2* an interesting candidate for future applications.

Other putative players in the regulation of ovule number were identified by the transcriptomic analysis performed on *cuc2CUC1i* mutant. Indeed, CUCs seems to regulate several genes related to CKs catabolism and response. In particular, preliminary analysis of UGT73C enzymes family, involved in CKs inactivation, gave promising results on their importance in determining ovule number.

Finally, MP role in pistil and ovule development starts to be understood thank to the analysis of *mp-S319* mutant, however further experiments need to be performed to unravel the intricate aspect of the auxin response pathway involved in this developmental process.

The main results obtained could be integrated in the model for the control of pistil growth and ovule primordia initiation.



Proposed model for the control of pistil growth and ovule primordia initiation.

CKs positive regulates *PIN1* expression. In particular, CRFs mediated CKs response is directly required for pistil elongation, and has an indirect effects on ovule primordia initiation. Among the *CRFs*, *CRF2*, being also under the control of MP, further integrate the auxin pathway. MP was demonstrated to directly regulate *CUC1* and *CUC2* expression. In turn, CUCs control *PIN1* expression and PIN1 localization which is required for correct ovule primordia formation. Our work on CUCs targets analysis also suggested that CUCs positive influence CKs pathways, likely by transcription repression of the CKs inactivation enzymes *UGTs*.

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Part II

Contents

the plant journal

SEB Society for Experimental Biology

The Plant Journal (2013) doi: 10.1111/tpj.12309

An integrative model of the control of ovule primordia formation

Francesca Galbiati[1,†], Dola Sinha Roy[1,†], Sara Simonini[1,‡], Mara Cucinotta[1], Luca Ceccato[1,§], Candela Cuesta[2,3], Maria Simaskova[2,3], Eva Benkova[2,3], Yuri Kamiuchi[4], Mitsuhiro Aida[4], Dolf Weijers[5], Rüdiger Simon[6], Simona Masiero[1] and Lucia Colombo[1,7,*]

[1]*Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy,*

[2]*Institute of Science and Technology Austria, Am Campus 1, 3400 Klosterneuburg, Austria,*

[3]*Department of Plant Systems Biology, Flanders Institute for Biotechnology, 9052 Ghent, Belgium,*

[4]*Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916–5 Takayama, Ikoma, Nara 630-0192, Japan,*

[5]*Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703HA Wageningen, The Netherlands,*

[6]*Institut für Entwicklungsgenetik, Heinrich Heine Universität, D-40225 Düsseldorf, Germany, and*

[7]*Istituto di Biofisica, Consiglio Nazionale delle Ricerche (CNR), Via Celoria 26, 20133 Milano, Italy*

Received 24 April 2013; revised 7 August 2013; accepted 8 August 2013.

*For correspondence (e-mail lucia.colombo@unimi.it).

†These authors contributed equally to this paper.

‡Present address: Department of Crop Genetics, John Innes Center, Norwich NR4 7UH, UK.

§Present address: Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Universita' degli Studi di Padova, Viale Dell'Università 16, 35020 Legnaro, Italy.

SUMMARY

Upon hormonal signaling, ovules develop as lateral organs from the placenta. Ovule numbers ultimately determine the number of seeds that develop, and thereby contribute to the final seed yield in crop plants. We demonstrate here that *CUP-SHAPED COTYLEDON 1* (*CUC1*), *CUC2* and *AINTEGUMENTA* (*ANT*) have additive effects on ovule primordia formation. We show that expression of the *CUC1* and *CUC2* genes is required to redundantly regulate expression of *PINFORMED1* (*PIN1*), which in turn is required for ovule primordia formation. Furthermore, our results suggest that the auxin response factor *MONOPTEROS* (*MP*/*ARF5*) may directly bind *ANT*, *CUC1* and *CUC2* and promote their transcription. Based on our findings, we propose an integrative model to describe the molecular mechanisms of the early stages of ovule development.

Keywords: ovule development, *AINTEGUMENTA*, Arabidopsis, *CUP-SHAPED COTYLEDON*, PIN1, MONOPTEROS.

INTRODUCTION

In Arabidopsis, ovules arise from the placenta as lateral organs, and both auxin synthesis and transport play important roles in their formation (Benková *et al.*, 2003). In the *yucca1 yucca4* (*yuc1 yuc4*) and *weak ethylene insensitive8 tryptophan aminotransferase related2* (*wei8 tar2*) double mutants, the local auxin response is compromised, and the pistil that develops lacks ovules (Cheng *et al.*, 2006; Stepanova *et al.*, 2008). A similar phenotype has been described for *mp S319*, a *MONOPTEROS* (*MP*) partial loss-of-function mutation (Cole *et al.*, 2009; Lohmann *et al.*, 2010). *MP* encodes a member of the auxin response factor (ARF) family (Hardtke and Berleth, 1998) that binds, as a monomer or as a dimer, to the promoters of target genes to control their transcription (Ulmasov *et al.*, 1997). Although total loss-of-function *mp* mutants are unable to correctly form embryos, embryo development is unaffected in *mp S319* plants, suggesting that the MP dimerization domain is required for activities that mainly occur in the post-embryonic phase of plant development (Lau *et al.*, 2011).

Further indications that auxin may influence the number of ovule primordia were obtained by characterization of the partial loss-of-function mutant *pin1-5*, which develops pistils that have fewer ovule primordia compared to the wild-type (Bencivenga *et al.*, 2012).

Cytokinins have also been reported to play a role in ovule primordium formation. The *cytokinin response1-12* (*cre1-12*) *histidine kinase2-2* (*ahk2-2*) *ahk3-3* triple mutant

shows a reduction in the cytokinin response, and the number of ovule primordia is drastically reduced (Higuchi *et al.*, 2004). By contrast, the *cytokinin oxidase/dehydrogenase3* (*ckx3*) *ckx5* double mutant, in which cytokinin degradation is affected, develops twice as many ovules as the wild-type (Bartrina *et al.*, 2011). In this regard, it has recently been shown that cytokinin modulates auxin fluxes during ovule development by regulating *PIN1* expression (Bencivenga *et al.*, 2012).

In addition to the auxin and cytokinin signaling pathways, transcription factors such as ANT (Elliott *et al.*, 1996), CUC1 and CUC2 (Ishida *et al.*, 2000) have been shown to be important for ovule primordia formation. In the *ant* single mutant, a reduced number of ovule primordia are formed. Furthermore *ant* ovules lack the two integuments, and the embryo sac arrests at the one-cell stage, suggesting that *ANT* has multiple functions during ovule development (Elliott *et al.*, 1996; Klucher *et al.*, 1996; Schneitz *et al.*, 1997; Losa *et al.*, 2010).

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

In the present work, we studied CUC1 and CUC2 function during ovule development using RNAi-based silencing of *CUC1* under the control of an ovule-specific promoter in the *cuc2* mutant background. By crossing these plants with the *ant* mutant, additive roles for *ANT*, *CUC1* and *CUC2* in the determination of ovule numbers were apparent. CUC1 and CUC2 are also involved in the localization and expression of *PIN1*. In addition, we show that *ANT* is not expressed in the pistils of the *mp S319* mutant, thus confirming that MP controls *ANT* expression during the reproductive developmental phase. *CUC1* and *CUC2* were found to be direct targets of MP. Based on our findings, we propose a model for ovule primordia formation in which MP integrates the auxin signaling required for ovule primordium formation to regulate the expression of the transcription factors encoded by *ANT*, *CUC1* and *CUC2*.

RESULTS

ANT, CUC1 and CUC2 are required for ovule initiation

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

All mutant combinations were morphologically analyzed by differential interference contrast microscopy. For each genotype, we analyzed ten pistils from each of six plants. The down-regulation of *CUC1* due to the specific RNAi was verified using real-time PCR (Figure 1c). The *ant-4* (Figure 1e) and *cuc2-1 pSTK::CUC1_RNAi* mutant plants (Figure 1f) showed reduced ovule number compared to

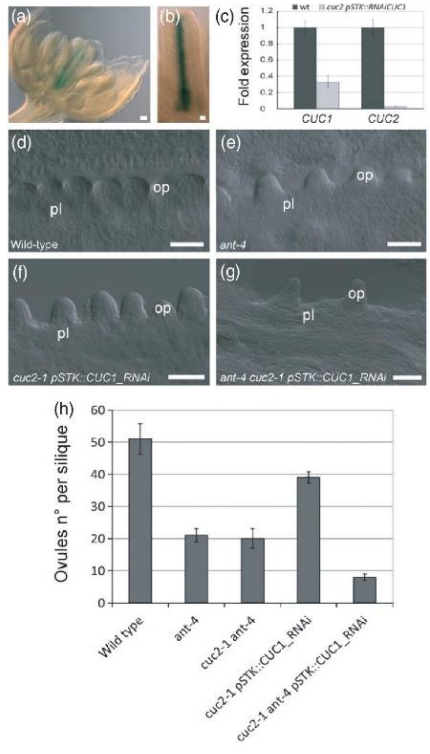

Figure 1. Ovule primordia in wild-type and mutant plants.
(a,b) *pSTK::GUS* in wild-type inflorescence.
(c) *CUC1* and *CUC2* expression levels in wild-type and *cuc2-1 pSTK::CUC1_RNAi* inflorescences.
(d–g) *Arabidopsis thaliana* ovule primordia at stage 1-I in wild-type (d), *ant-4* (e), *cuc2-1 pSTK::CUC1_RNAi* (f) and *ant-4 CUC2-1 pSTK::CUC1_RNAi* (g) plants.
(h) Mean numbers of ovule primordia in wild-type, *ant-4*, *cuc2-1 ant-4*, *cuc2-1 pSTK::CUC1_RNAi* and *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants.
Abbreviations: pl, placenta; op, ovule primordia. Scale bars = 20 μm.

wild-type (Figure 1d), in agreement with previously published data (Elliott *et al.*, 1996; Ishida *et al.*, 2000). The ovules of the *ant-4 cuc2-1* double mutant resembled those of the *ant-4* single mutant (Figure 1h). In the *ant-4 cuc2-1 pSTK::CUC1_RNAi* transgenic plants, we observed a further dramatic reduction in the number of developing ovule primordia (Figure 1g). The *cuc2-1 pSTK::CUC1_RNAi* mutants developed a mean of 30 ovules per pistil, the *ant-4* mutant developed a mean of 20 ovules per pistil, and the *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants developed a mean of seven ovule primordia per gynoecia (Figure 1h), suggesting that *ANT*, *CUC1* and *CUC2* act additively in controlling the number of ovule primordia that develop. Despite the reduction in ovule number in the mutant backgrounds, the sizes of the pistils were not reduced (Figure S1). Therefore, the ovules present in the pistils of the mutants are more distantly spaced compared to those of the wild-type (Table S1).

CUC proteins are involved in controlling *PIN1* expression and are required for ovule formation

A reduction in the numbers of ovule primordia was also observed in the *pin1-5* mutant (Bencivenga *et al.*, 2012) and the *cre1-12 ahk2-2 ahk3-3* triple mutant (Higuchi *et al.*, 2004). Recently, it has been proposed that cytokinin regulates *PIN1* expression during the early stages of ovule development (Bencivenga *et al.*, 2012), and that PIN1 is required to form ovule primordia (Benková *et al.*, 2003; Bencivenga *et al.*, 2012).

To analyze whether the reduction in ovule number observed in the *ant-4 cuc2-1 pSTK::CUC1_RNAi* mutant was due to *PIN1* down-regulation, we crossed the *ant-4/ANT cuc2-1 pSTK::CUC1_RNAi* plants with those containing the *pPIN1::PIN1-GFP* reporter construct (Friml *et al.*, 2003). Analyses of these reporter line plants showed that, during the early stages of pistil development, *PIN1–GFP* is expressed in the ovule primordia before stage 1-I (Figure 2a) and at stage 1-II (Figure 2b) in the plasma membrane of the epidermal cell layer of the ovule primordia. The expression and localization of PIN1–GFP in the plasma membrane in the *ant-4*, *pSTK::CUC1_RNAi* and *cuc2-1* ovule primordia were similar to that observed in wild-type plants (Figure S2). In contrast, in the *cuc2-1 pSTK::CUC1_RNAi* plants, the PIN1–GFP recombinant protein was barely visible and was partially included in vesicles (Figure 2c,d). Furthermore, in *cuc2-1 pSTK::CUC1_RNAi* plants, PIN1 appears to be expressed in all cells of the primordium as well as in the boundary between ovules (Figure 2d). Real-time PCR analysis showed that *PIN1* expression was down-regulated in *cuc2-1 pSTK::CUC1_RNAi* plants compared to *ant-4* single mutants and wild-type plants (Figure 2e). In *cuc2-1* and *pSTK::CUC1_RNAi* mutant plants, the levels of *PIN1* expression are not different compared to wild-type (Figure S3). Furthermore, *PIN1* down-regulation in *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants was similar to that in *cuc2-1 pSTK::CUC1_RNAi* plants (Figure S3). This result suggests that CUC1 and CUC2 may redundantly regulate *PIN1* expression during the early stages of ovule development.

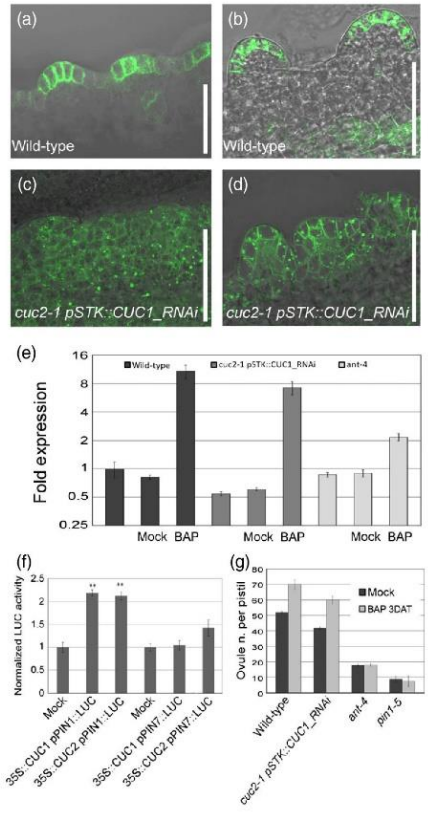

Figure 2. *PIN1* expression in developing wild-type and mutant ovules.
(a,b) Wild-type *pPIN1::PIN1-GFP* ovules at stage 1-I (a) and stage 1-II (b).
(c,d) *cuc2-1 pSTK::CUC1_RNAi pPIN1::PIN1-GFP* ovules at stage 1-I (c) and stage 1-II (d).
(e) *PIN1* expression levels in pre-fertilization pistils of mock- and BAP-treated wild-type, *cuc2-1 pSTK::CUC1_RNAi*, *ant-4* and *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants.
(f) Transient expression of *PIN1::LUC* and *PIN7::LUC* in BY-2 protoplasts. *PIN1* expression is up-regulated by *CUC1* and *CUC2*, while *PIN7* expression remains at basal levels. Transactivation is relative to the normalized luciferase (LUC) activity. Error bars indicate the standard error ($n = 8$ separate transfection events and measurements). Statistical significance between mock and effector constructs (35s::CUC1, 35s::CUC2) was determined by Student's *t*-test (** $P < 0.001$).
(g) Number of ovules in mock- and BAP-treated pistils of wild-type, *cuc2-1 pSTK::CUC1_RNAi*, *ant-4* and *pin1-5* carpels.
Scale bars = 20 μm.

To verify whether CUC1 and CUC2 are able to induce *PIN1* expression *in vivo*, we performed transient expression assays in BY-2 tobacco protoplasts. A significant increase in expression of the *pPIN1::LUCIFERASE* (*pPIN1::LUC*) reporter, when co-transformed with *CUC1* or *CUC2*, further confirmed that the transcription factors encoded by *CUC1* and *CUC2* act as positive regulators of *PIN1* (Figure 2f). In contrast, *PIN7::LUCIFERASE* expression was not affected by either CUC1 or CUC2 (Figure 2f).

To understand whether the reduction in ovule number observed in the *cuc2-1 pSTK::CUC1_RNAi* plants is caused by the down-regulation of *PIN1* expression, we up-regulated *PIN1* expression in the *cuc2-1 pSTK::CUC1_RNAi* pistil. It has been shown that treatment with cytokinin 6-benzylaminopurine (BAP) is able to increase *PIN1* expression in pistils (Bencivenga *et al.*, 2012). We therefore compared the number of ovules between wild-type mock-treated flowers at stage 8–9 and flowers treated with 1 mM BAP at 3 days after treatment. We observed an increase in ovule number in the BAP-treated pistils compared to the mock-treated ones (Figure 2g and Table S2). Similar treatments were performed using stage 8–9 *pin1-5* flowers. In this case, we did not observe new ovule primordia in either the control or BAP-treated plants, confirming that PIN1 is required for the increase in ovule primordia in BAP-treated plants (Figure 2g and Table S2). These results are consistent with those reported by Bencivenga *et al.* (2012). Interestingly, we also observed an increase in ovule number in *cuc2-1 pSTK::CUC1_RNAi* carpels of BAP-treated inflorescences (Figure 2g and Table S2). The increase in ovule numbers in this genetic background (46.0%) was comparable with that obtained in treated wild-type plants (46.3%; Figure 2g and Table S2). To verify whether the phenotypic complementation observed in BAP-treated plants at 3 days after treatment was linked to the restoration of *PIN1* expression levels, we analyzed *pPIN1::PIN1-GFP* levels (Figure S4) and performed quantitative RT-PCR (Figure 2e) in BAP- and mock-treated wild-type and *cuc2-1 pSTK::CUC1_RNAi* plants. This showed that expression of *PIN1* is increased in BAP-treated plants (Figure 2e).

We also tested the effect of cytokinin on ovule numbers in the *ant-4* mutant background. BAP treatment was unable to complement ovule number reduction (Figure 2g and Figure S4) even though PIN1 expression increased after the treatment (Figure 2e), suggesting that ANT may act in a CUC1/CUC2-independent pathway, as is also suggested by the genetic data.

The GFP signal driven by the DR5 promoter is not affected in *cuc2-1 pSTK::CUC1_RNAi* ovule primordia

To analyze whether the auxin response is compromised, we crossed *cuc2-1 pSTK::CUC1_RNAi* plants with the auxin-responsive reporter line *pDR5rev::GFP* (Benková *et al.*, 2003). The GFP signal (reflecting auxin accumulation) was not visible in the placenta or in the ovule primordia at stage 1-I (Figure S5). However, the GFP signal was visible in the mutant ovule primordia starting from stage 1-II (Figure S5). This pattern of expression is identical to that of wild-type plants, suggesting that, although *PIN1* expression is affected in these plants, the auxin maximum is nevertheless established in the ovule primordia.

It has been shown previously that *DORNRÖSCHEN* (*DRN*), which encodes an AP2 domain transcription factor, is transcriptionally regulated by auxin signaling. In the presence of auxin, use of the *DRN* regulatory region to drive GFP expression has been reported to be more responsive than use of the *DR5* promoter (Chandler *et al.*, 2011). Therefore, in an attempt to observe auxin-induced signals earlier during ovule development, we crossed *cuc2-1 pSTK::CUC1_RNAi* plants with *pDRN::GFP* plants. In both wild-type and *cuc2-1 pSTK::CUC1_RNAi* plants, the *DRN* promoter was active from stage 1-I, although we were unable to detect GFP before the ovule primordia were formed (Figure S5). Whilst these experiments do not allow us to verify whether *PIN1* down-regulation in *cuc2-1 pSTK::CUC1_RNAi* plants affects the local auxin distribution in the placenta, we conclude that, once the primordia are formed, the auxin gradient along the ovule axis is not affected.

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

Analysis of the *yuc1 yuc4* double mutant and the *mp* mutant suggests that auxin is required for ovule development (Cheng *et al.*, 2006; Cole *et al.*, 2009; Lohmann *et al.*, 2010). The *mp S319* mutant produces few flowers, all of which have a reduced number of organs, and, importantly, the pistils do not develop ovules (Cole *et al.*, 2009; Lohmann *et al.*, 2010). Morphological analysis shows that, in this mutant, the pistils do not have any carpel margin tissue (Figure S6). The placenta is completely missing and consequently ovules are unable to develop (Figure S6). To explore whether MP regulates *CUC1*, *CUC2* and *ANT* expression in the pistil, we focused our analysis on its expression during the early stages of ovule development. We analyzed *pMP::MP-GFP* plants using confocal laser scanning microscopy. In these plants, GFP is visible in the placenta before ovule primordia are formed (Figure 3a). Once the primordia arise (stage 1-I), GFP expression is observed in the epidermal cell layer of the primordia (Figure 3b). Starting from stage 1-II, MP–GFP is mainly localized in the boundaries between the ovules (Figure 3c), and, from stage 2-I, is localized in the boundary between the nucellus and the chalaza (Figure S7).

Like *MP*, *ANT* (Figure 3d,e), *CUC1* (Figure 3f,g) and *CUC2* (Figure 3h,i) are expressed in the placenta before ovule primordia arise, and in ovules at stage 2-II. However, starting from stage 2-I, *ANT* is expressed in the chalaza and in the integument primordia, whereas *CUC1* and *CUC2*

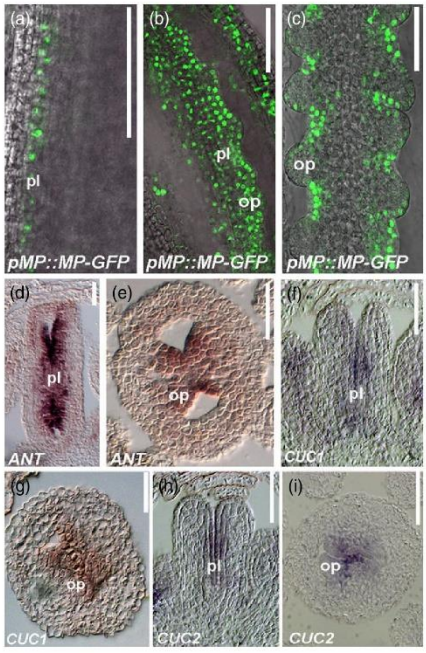

Figure 3. *MP, ANT, CUC1* and *CUC2* expression patterns.
(a–c) The *MP* expression profile was deduced by analyzing *pMP::MP-GFP* plants: signal is observed at carpel meristematic margin before ovule primordia are formed (a), in ovule primordia of stage 1-I (b), and in developing ovules at stage 1-II (c).
(d,e) *In situ* hybridization using a specific *ANT* antisense probe in the wild-type background.
(f–i) *In situ* hybridization with *CUC1* (f,g) and *CUC2* (h,i) antisense probes in the wild-type background. (d,f,h) Stage before ovule primordia are formed; (e,g,i) stage 1-I of ovule development.
Abbreviations: pl, placenta; op, ovule primordium. Scale bars = 50 μm.

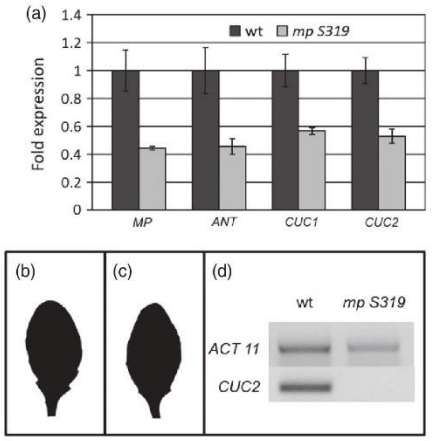

Figure 4. *MP* regulates the expression of *ANT, CUC1* and *CUC2*.
(a) *MP, ANT, CUC1* and *CUC2* expression in *mp S319* mutant pre-fertilization inflorescences compared to wild-type inflorescences.
(b,c) Silhouettes of rosette leaves in wild-type (b) and the *mp S319* mutant (c).
(d) CUC2 expression analysis by quantitative RT-PCR on wild-type and *mp S319* rosette leaf cDNA. Actin was used as the control.

expression is limited to the boundary region between the chalaza and the nucellus, as is the case for *MP* (Figure S7). To study whether MP regulates *ANT, CUC1* and *CUC2* expression, we performed quantitative RT-PCR on pistils of the *mp S319* mutant and the wild-type. This analysis revealed that *ANT, CUC1* and *CUC2* are down-regulated in this mutant (Figure 4a).

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

It has recently been shown that *ANT* is a direct target of MP during floral meristem formation (Yamaguchi *et al.*, 2013), and therefore it is likely that MP may also control *ANT* expression in the pistil. To assess whether MP directly regulates *CUC1* and *CUC2*, we performed chromatin immuno-precipitation (ChIP) experiments using anti-GFP antibodies on chromatin extracted from the pistils of *pMP::MP-GFP mp/mp* plants before fertilization. The MP–GFP fusion protein fully complements the *mp* phenotype, indicating that the MP–GFP protein is biologically functional (Schlereth *et al.*, 2010). Pre-fertilization wild-type pistils were used as a negative control, and *ARABIDOPSIS RESPONSE REGULATOR15* (*ARR15*) and *ANT*, which are direct targets of MP, were used as positive controls (Zhao *et al.*, 2010; Yamaguchi *et al.*, 2013).

Within the genomic regions of *CUC1* and *CUC2*, starting from 3 kb upstream of the ATG start codon to 0.5 kb downstream of the stop codon, several putative ARF binding sites were identified (Figure 5a). Three independent quantitative RT-PCR experiments were performed for each of the three independent immuno-precipitated chromatin samples. This analysis indicates that MP binds to the ARF binding site present in the *CUC1* genomic region (Figure 5b), and also that present in *CUC2* at 34 bp after the start codon

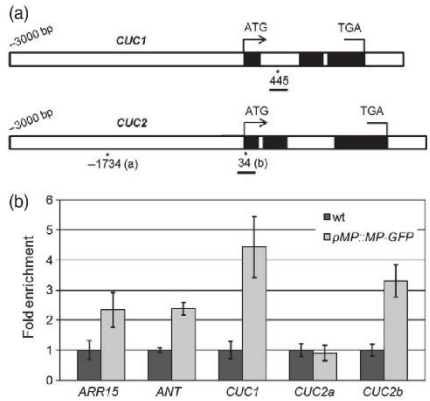

Figure 5. MP directly binds *ANT*, *CUC1* and *CUC2* genomic regions.
(a) Schematic representations of *CUC1* and *CUC2* genomic loci. Black underlining indicates the enriched loci. MP binding was verified by ChIP experiments.
(b) ChIP experiments. Chromatin was extracted from pre-fertilization pistils of *pMP::MP-GFP mp/mp* plants, and wild-type pre-fertilization pistils were used as a negative control. ChIP experiments were performed using anti-GFP antibodies. The propagated error values are calculated as previously reported by Gregis *et al.*, (2013).

(Figure 5a,b). No enrichment was evident in correspondence to the ARF binding site present at 1734 bp upstream of the *CUC2* start site was evident (Figure 5b). These data support the contention that *CUC1* and *CUC2* may be direct targets of MP during pistil development.

DISCUSSION

Ovule primordia formation: an integrative model

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

That *PIN1* is involved in the determination of ovule number was inferred from characterization of the *pin1-5* mutant, as it develops fewer ovules compared to wild-type plants (Bencivenga *et al.*, 2012). According to our data, CUC1 and CUC2 are required for both correct *PIN1* expression and PIN1 localization. However, in *cuc2-1 pSTK::CUC1_RNAi* plants, we did not observe any changes in auxin accumulation during early stages of ovule development. It may be that DR5 is not sensitive enough to detect the changes. Involvement of CUC2 in *PIN1* expression has already been proposed for the formation of leaf serrations (Bilsborough *et al.*, 2011). Although we have shown that CUC1 and CUC2 are able to induce *PIN1* expression *in vivo* using protoplast assays, it is likely that *PIN1* transcriptional activation is indirect. Starting from stage 1-I, PIN–GFP is localized in cells (Ceccato *et al.*, 2013;) in which CUC1 and CUC2 are not expressed, suggesting the involvement of non-cell-autonomous CUC downstream target(s) in the regulation of PIN1 expression.

The reduction in the number of ovules in *cuc2-1 pSTK::CUC1_RNAi* plants is less pronounced than that seen in the *pin1-5* mutant, indicating that factors other than the CUC proteins regulate PIN1 expression at this stage of ovule development. Our study shows that treatment with BAP results in restored ovule numbers in *cuc2-1 pSTK::CUC1_RNAi* plants. However, BAP treatment does not have any effect if PIN1 is mutated, as is the case in *pin1-5* plants, suggesting that cytokinin acts downstream of the CUC1 and CUC2 transcription factors to induce *PIN* expression. It will be of interest to determine whether this regulatory mechanism is also conserved in other organs such as leaves, in which it has been shown that a CUC2-dependent regulatory pathway controls PIN1-mediated auxin efflux (Bilsborough *et al.*, 2011).

The results of BAP treatment suggested that PIN1 expression levels are important for ovule primordia formation, but it is not clear how PIN1 membrane localization is compatible with primordia formation and the observed maintenance of the auxin gradient during ovule development. The observation that BAP treatment cannot complement the *ant-4* phenotype suggests that ANT functions in a pathway that is independent of CUC1 and CUC2. This is supported by the additive effects on the reduction in ovule numbers observed in *ant-4 cuc2-1 pSTK::CUC1_RNAi* plants.

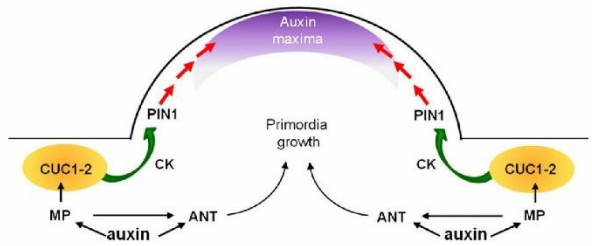

Figure 6. Proposed model.
MP is required for *ANT*, *CUC1* and *CUC2* expression during the early stages of placenta development and ovule primordia formation. ANT controls cell proliferation in the placenta and ovules, whereas CUC1 and CUC2 control *PIN1* expression required for primordia formation. Cytokinins (CKs) may act downstream of CUC proteins in promoting *PIN1* expression. Once the primordia have formed, auxin accumulates at the edge of the developing ovule.

MP is a key player in ovule primordia formation

MP is broadly expressed, and is involved in the transcriptional regulation of several auxin-responsive genes (Cole *et al.*, 2009; Donner *et al.*, 2009; Schlereth *et al.*, 2010). Our data suggest that MP directly regulates the expression of *ANT*, *CUC1* and *CUC2* during the formation of placenta and ovule primordia. Accordingly, expression of these genes in the pistil is reduced in a partial loss-of-function *mp* mutant (*mp S319*). The role of ANT in pistil development has been described previously. For example, in *ant lug* and *ant seuss* double mutants, as well as in the *ant shp1 shp2 crc* quadruple mutant, placenta formation is seriously compromised and ovules are not formed (Liu *et al.*, 2000; Azhakanandam *et al.*, 2008; Colombo *et al.*, 2010; Wynn *et al.*, 2011). We suggest that, in the pistil, MP responds to auxin by activating the expression of *ANT*, which is required for correct pistil and ovule primordia formation. In this regard, it has recently been suggested that MP also regulates *ANT* expression in the floral meristem (Yamaguchi *et al.*, 2013). Furthermore, we suggest that MP may regulate the expression of *CUC1* and *CUC2* in the pistil, which is required for *PIN1* expression.

Due to the lack of placenta tissue in the *mpS319* mutant, it is not possible to discriminate between the effect of MP on ANT and CUC proteins in the placenta with respect to ovule primordia formation, although it is clear that MP is required for *ANT*, *CUC1* and *CUC2* expression in the pistil, as shown by RT-PCR experiments. The ChIP experiments suggest that MP directly regulates the transcription of *ANT*, *CUC1* and *CUC2*, although functional analysis of the binding site is required to verify this suggestion.

Based on our results, we propose a model in which MP regulates *ANT*, *CUC1* and *CUC2* expression during the early stages of placenta and ovule development (Figure 6). The expression of *CUC1* and *CUC2* is necessary for correct *PIN1* expression, which, in turn, is a prerequisite for ovule primordia formation. Once *PIN1* is expressed, an auxin responce occurs in the apex of the nucellus (Figure 6). The high auxin concentration may repress *CUC* genes, as shown previously for *CUC2* in leaves (Bilsborough *et al.*, 2011). This model is very similar to that proposed for the development of leaf serration (Bilsborough *et al.*, 2011). At later stages of development, expression of *MP* and the *CUC* genes is confined to the boundary regions between ovules and the region between the nucellus and the chalaza. Once ovules have formed, the interaction between auxin and cytokinin provides a signaling system for the correct growth and development of ovules (Bencivenga *et al.*, 2012; Marsch-Martínez *et al.*, 2012). Interestingly, our results appear to confirm the importance of cytokinin in controlling ovule numbers (Werner *et al.*, 2003; Kinoshita-Tsujimura and Kakimoto, 2011; Bencivenga *et al.*, 2012), and suggest that interaction between the auxin and cytokinin pathways is required for formation of ovule primordia.

EXPERIMENTAL PROCEDURES

Plant materials

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

Plant treatments

BAP treatment was performed on flowers at stage 8–9 of development, as previously described by Bencivenga *et al.* (2012). We collected treated inflorescences at 3 days after treatment, and counted the numbers of ovules in pistils.

Optical and confocal microscopy

GUS staining was performed overnight as described previously (Liljegren *et al.*, 2000). Siliques and carpels were collected and cleared as described by Yadegari *et al.* (1994). Pistils were observed using a Zeiss Axiophot D1 microscope (http://www.zeiss.com) equipped with differential interference contrast optics. Images were recorded using an Axiocam MRc5 camera (Zeiss) with Axiovision version 4.1.

For confocal laser scanning microscopy, fresh material was collected, mounted in water and immediately analyzed. Confocal

laser scanning microscopy analysis was performed using a Leica (www.leica-microsystems.com) TCS SPE microscope with a 488 nm argon laser line for excitation of GFP fluorescence. Emissions were detected between 505 and 580 nm. Images were collected in multi-channel mode, and overlay images were generated using Leica analysis software LAS AF 2.2.0.

Plasmid construction and Arabidopsis transformation

To construct *pSTK::CUC1_RNAi*, a specific *CUC1* fragment (nucleotides 730–925) was amplified using primers AtP_2916 and AtP_2917, and recombined into RNAi vector pFGC5941 (Karimi *et al.*, 2002) through an LR reaction (Gateway system, Invitrogen, www.lifetechnologies.com). The CaMV 35S promoter of the pFGC5941 vector was removed and substituted by the *STK* promoter (amplified using primers AtP_590 and AtP_591) (Kooiker *et al.*, 2005). This construct was used to transform *ant-4 cuc2-1* plants using the floral-dip method (Clough and Bent, 1998). The primers used are listed in Table S3.

RT-PCR and quantitative RT-PCR analysis

Total RNA was extracted from pistils at stages 8–10 of flower development (Roeder and Yanofsky, 2006) using the LiCl method (Verwoerd *et al.*, 1989). Total RNA was treated using the Ambion TURBO DNA-free DNase (http://www.lifetechnologies.com/it/en/home/brands/ambion, kit and then reverse transcribed using the ImProm-II™ reverse transcription system (Promega, www.promega.com). The cDNAs were standardized relative to *UBIQUITIN10* (*UBI10*) and *ACTIN2-8* (*ACT2-8*) transcripts, and gene expression analysis was performed using the iQ5 Multicolor real-time PCR detection system (Bio-Rad, www.bio-rad.com) with SYBR Green PCR Master Mix (Bio-Rad). Baseline and threshold levels were set according to the manufacturer's instructions. RT-PCR and quantitative RT-PCR primers are listed in Table S1.

Protoplast transfection

Protoplast preparation and transient expression experiments were performed as described by De Sutter *et al.* (2005). Protoplasts were prepared from a BY-2 tobacco cell culture, and co-transfected using a reporter plasmid containing the *Firefly LUCIFERASE* (fLUC) reporter gene driven by the *PIN1* or *PIN7* promoter, together with a normalization construct expressing *Renilla* LUCIFERASE (rLUC) and effector constructs expressing *CUC1* and *CUC2*, respectively, under the control of the CaMV 35S promoter. The reporter construct was generated as follows: the pEN-L4-PIN1-R1 or pEN-L4-PIN7-R1 vector (where the *PIN1* promoter comprises −2098 bp upstream of the *PIN1* coding sequence, and the *PIN7* promoter comprises −1423 bp upstream of the *PIN7* coding sequence) was recombined together with pEN-L1-fLUC-L2 by multi-site Gateway LR cloning with pm42GW7 (Karimi *et al.*, 2007). For the effector constructs, pEN-L1-'ORF'-R2 [where 'ORF' represents *CUC1* genomic DNA (1565 bp) or the *CUC2* coding sequence (1128 bp)] was used to introduce the ORFs into p2GW7 (Karimi *et al.*, 2002) by Gateway LR cloning for over-expression. Two micrograms of each construct were added, and the total effector amount was equalized in each experiment using the p2GW7-GUS mock effector plasmid. After transfection, protoplasts were incubated overnight and then lysed; fLUC and rLUC activities were determined using the dual-luciferase reporter assay system (Promega). Variations in transfection efficiency and technical error were corrected by normalization of fLUC against rLUC activities. All transactivation assays were performed in an automated experimental set-up that involved eight separate transfection experiments, and were performed at least twice.

Chromatin immunoprecipitation (ChIP) assays

ChIP experiments were performed using a modified version of a previously described protocol (Gregis *et al.*, 2008) using the commercial antibody GFP:Living Colors full-length A.v. polyclonal antibody (Clontech, www.clontech.com). Chromatin was extracted from stage 8–10 pistils of *pMP::MP-GFP* plants and from wild-type plants (Col-0) as a control. The DNA fragments obtained from the immune-precipitated chromatin were amplified by quantitative RT-PCR using specific primers (see Table S1). Three real-time PCR amplifications on three independent chromatin extractions were performed. The primers are listed in Table S1. Enrichment of the target region was determined using the iQ5 Multicolor real-time PCR detection system (Bio-Rad) with SYBR Green PCR Master Mix (Bio-Rad). The quantitative RT-PCR assays and the fold enrichment calculation were performed as described previously (Matias-Hernandez *et al.*, 2010).

In situ hybridization

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

ACKNOWLEDGEMENTS

The project and F.G. were supported by the CARIPLO Foundation (project 2009-2990) and COST (European Cooperation in Science and Technology) action HAPRECI (Harnessing Plant Reproduction for Crop Improvement). E.B. and C.C. were supported by the European Research Council through a 'Starting Independent Research' grant (ERC-2007-Stg-207362-HCPO). We thank A.P. MacCabe (Consejo Superior de Investigaciones Cientificas, Valencia, Spain) for critical reading of the manuscript.

SUPPORTING INFORMATION

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

Figure S1. Wild-type and mutant pistil lengths.

Figure S2. *PIN1* expression in wild-type and single mutants.

Figure S3. *PIN1* expression in single *CUC* mutants and the triple *ant-4 cuc2-1 pSTK::CUC1_RNAi* mutant.

Figure S4. PIN1 expression in BAP-treated developing ovules.

Figure S5. Auxin accumulation in developing Arabidopsis wild-type ovules.

Figure S6. Wild-type and *mp S319* pistils.

Figure S7. *MP*, *ANT*, *CUC1* and *CUC2* expression at later stages of ovule development.

Table S1. Mean distances between two ovules in wild-type and mutant pistils.

Table S2. Ovule number in wild-type and mutant pistils after mock and BAP treatment.

Table S3. Sequences of oligonucleotide primers used in this work.

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CYTOKININ RESPONSE FACTOR 2 regulates *PIN1* expression required for pistil development.

Mara Cucinotta[1], Nadia Elisa Quadrelli[1], Eva Benkova[2] and Lucia Colombo[1]

[1] *Dipartimento di BioScienze, Università degli studi di Milano, Via Celoria 26, 20133 Milano.*

[2] *Institute of Science and Technology Austria, Am Campus 1, 3400 Klosterneuburg, Austria.*

* To whom correspondence should be addressed (lucia.colombo@unimi.it)

ABSTRACT

Development of plant reproductive organs, such as pistil and ovules, requires the coordinated action of the hormones auxin and cytokinins (CKs). If auxin pathways are compromised, pistil and ovules develop with severe defects. CK, on its side, plays a prominent role as positive regulator of ovule number.

It has been shown that in the pistil, as well as in roots, the crosstalk between auxin and CKs converges in the regulation of the auxin carrier encoding gene *PINFORMED1 (PIN1)*. Recently, CYTOKIN RESPONSE FACTORS (CRFs), members of the AP2 transcription factors family, have been identified as direct regulators of *PIN1*. Here, we show that the CRF2 is required to regulate expression of *PIN1*, and thus coordinates pistil growth and ovule number determination. Moreover, since *CRF2* is also under the control of MONOPTEROS/ARF5, it perfectly integrates the response to auxin and CKs in the molecular network responsible for pistil development.

INTRODUCTION

In Arabidopsis, the ovules emerge as lateral organs from the placenta, a meristematic tissue, that originates after the fusion of the carpel margin meristem (CMM) (Schneitz et al. 1995; Reyes-Olalde et al. 2013).

Placenta formation and ovule growth require auxin. Reduction of local auxin biosynthesis or transport (by NPA treatment) causes severe defects in pistil development, with a consequent loss of placenta tissue and ovules (Nemhauser et al. 2000; Nole-Wilson et al. 2010). The auxin efflux carrier PINFORMED-1 (PIN1) is one of the main factors modulating auxin accumulation during all phases of ovule development (Ceccato et al. 2013). In the *pin1-5* mutant, pistils are correctly formed but contain only few ovules (Sohlberg et al. 2006; Bencivenga et al. 2012).

CKs positively regulate ovule formation. Mutants with reduced CKs production or perception have a drastic reduction in ovule number and compromised female fertility (Werner et al. 2003; Riefler et al. 2006; Kinoshita-Tsujimura & Kakimoto 2011). On the contrary, when the amount of CKs increases, as in the case of *ckx3ckx5* double mutant, the number of ovules is greater than in wild-type, confirming a clear positive correlation between CKs levels and ovule numbers (Bartrina et al. 2011; Bencivenga et al. 2012).

In root, CKs modulate organogenesis, acting on auxin transport by down-regulation of *PIN1* expression (Ruzicka et al. 2009; Dello Ioio et al. 2012) and PIN1 protein endocytic recycling (Marhavý et al. 2011). Conversely, in the pistil it has been shown that CK treatment strongly increases *PIN1* expression (Zúñiga-Mayo et al. 2014; Galbiati et al. 2013; Bencivenga et al. 2012).

Recently, Simaskova and colleagues (2015) found that CYTOKININ RESPONSE FACTORS (CRFs), closely related members of the Arabidopsis AP2 gene family, directly bind to the *PIN1* promoter.

CRFs modulate *PIN1* expression in response to CKs. A cis-regulatory element in the *PIN1* promoter has been identified, whose removal uncouples *PIN1* transcription from CRF regulation and affects root sensitivity to CKs (Simaskova et al., 2015). Among the *CRFs*, *CRF2* had already been shown to be a targets of AUXIN RESPONSE FACTOR 5/MONOPTEROS (MP) in embryo (Schlereth et al. 2010). Here, we show that CRF2, in concert with other CRFs, directly induces the *PIN1* expression required for the control of pistil size and consequently in the determination of ovule primordia.

RESULTS AND DISCUSSION

CRF2 is a MP target involved in pistil growth and ovule number determination.

CRFs, AP2 family transcription factors, mediate CKs responses in concert with a two-component pathway (Rashotte et al. 2006). *CRF2/TMO3* has been identified in a screen for MP targets and it has been confirmed to be direct target of MP during early stages of embryo development (Schlereth et al. 2010). Using a *CRF2* promoter–nuclear GFP reporter, we showed that *CRF2* promoter is active in the carpel margin meristem (CMM) and in developing valves at developmental stages 8-9 (according to Roeder & Yanofsky 2006), when ovule primordia begin to form (Fig. 1A). The GFP signal is also detected in the CMM when ovules are at stages 2-I (Fig. 1B). *CRF2* also seems to be slightly expressed in the distal part of ovule at stage 2-II (Fig. 1C). The GFP expression under control of the *CRF2* promoter largely overlaps with MP expression (Fig. 1D, 1E and 1F). To study whether MP regulates *CRF2* in the pistil, we checked *CRF2* expression in *mp-S319* weak mutant allele (Cole et al. 2009; Galbiati et al. 2013). We observed a significant down-regulation of *CRF2* messenger in *mpS319* compared to wild-type inflorescence (Fig. 1G).

We then performed chromatin immune precipitation using inflorescence of wild type plants containing pMP::MP-GFP construct. In ChIP assays *CRF2* promoter fragments were enriched using a GFP antibody, when compared to wild-type control (Fig. 1H). The *CRF2* promoter region tested is the same reported by Schlereth et al. (2010). *ARABIDOPSIS RESPONSE REGULATOR15 (ARR15)*, which is direct targets of MP, was used as positive controls (Zhao et al. 2010). To examine the role of *CRF2* in pistil development and ovule formation, we compared pistil length and ovule number of *crf2-2*, *pRPS5a::CRF2* and wild-type Col-0. Measurement of pistil length and ovule counts were performed on ovules at stage 1-II (primordia) and stage 2-I (finger-like protrusions), which corresponds to stage 9 and 10 of pistil development (according to Roeder and Yanofsky, 2006; Schneitz et al., 1995).

At these stages, wild-type Col-0 grown in long day condition have an average of 45 ± 1.85 ovule primordia while 38 ± 1.28 (-15%) ovules are formed in *crf2-2* (Fig. 2A). On the contrary, *RPS5a::CRF2* plants show an average of 53 ± 1.16 ovules, a significant (+17%) increase with respect to the wild-type. Interestingly, the *RPS5a:CRF2* ovule phenotype resembles the one observed after CK treatment, in which new small ovule primordia arise in the space between two previously formed ovules (Fig. S1). Placenta length was also measured, and the results revealed a reduction in placenta length (-11%) in *crf2-2* and an increase (+13%) in *RPS5a::CRF2* respectively compared to wild-type (Fig. 2A and 2B). The percentage of increment and reduction observed for ovule number phenotype directly correlate with the observed changes in the size of the placenta and consequently of the pistil. The differences in pistil growth between wild-type, *crf2-2* and *RPS5a::CRF2* are also maintained after fertilization (Fig. 2C).

Taken together, these results suggest that *CRF2* influences the number of initiated ovule primordia through action on pistil growth rate. Changing CKs output through down- or up-regulation of *CRF2* affects the elongation of the pistils and therefore the number of ovules formed. Excess *CRF2* activity not only induced placenta elongation, but also in the space between two primordia facilitated the emergence of a new ovule, as it is with exogenous CKs treatment. The determination of ovule number depends on the pistil size and on the distance between two primordia initiation sites. In *aintegumenta (ant)* and *cucs* mutants, reduction in ovule number is not dependent upon a variation in the size of the pistil; indeed, in these mutants ovule density is lower and the space between ovules is increased relative to wild type (Liu et al. 2000; Galbiati et al. 2013). In those mutants, the process of primordium formation is compromised. Instead, in the case of mutants with either enhanced brassinosteroid signaling or higher CK content, the increase in ovules number is accompanied by larger pistils (Bartrina et al. 2011; Huang et al. 2013). Surely, the processes of placenta elongation and ovule primordia formation are intrinsically related.

It can be assumed that in a pistil that expands more, the placenta extends and the space required for the formation of new primordia is created. In parallel, the molecular machinery responsible for directing the cell cycle is also strictly required for the formation of the new organ. Taken together, the results on *CRF2* function are consistent with and reconfirm the known CK effect on pistil growth and ovule number control.

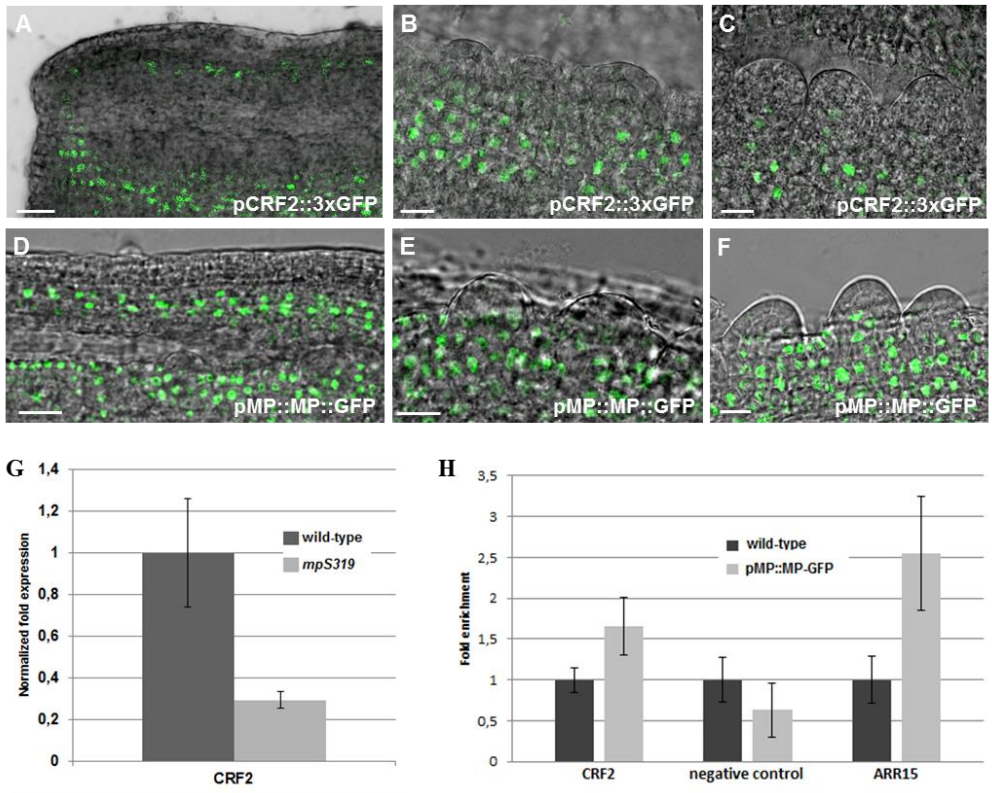
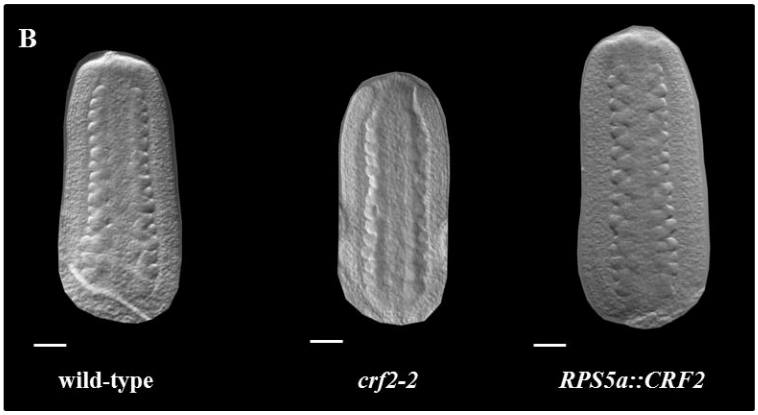


Figure 1: *CRF2* is a direct target of MP and it is expressed at early stages of pistil and ovule development. (A, B, C) CRF2 expression profile was deduced analyzing *pCRF2::3GFP* plants: signal is observed in the placenta and valves of developing pistil (A) at the base of ovule primordia at stage 1-I (B) and at the base and in the forming vasculature of ovules at stage 1-II (C). Similar although more extensive expression pattern was detected for pMP::MP-GFP (D, E, F). (G) *CRF2* expression in *mpS319* mutant pre-fertilization inflorescences compared to wild-type inflorescence. (H) ChIP experiments were performed using anti-GFP antibodies. ChIP assay shows an enrichment in *CRF2* promoter fragment in pMP::MP-GFP *mp/mp* plants compared to wild-type control. As a positive control we used *ARR15*, already well known as a MP target, and as a negative control a region on *CUC1* promoter that was not enriched in Galbiati et al., 2013. The propagated error values are calculated as previously reported by (Gregis et al. 2013).

A

	wild-type	*crf2-2*	*RPS5a::CRF2*
ovule primordia number	45 ± 1,85	38 ± 1,28	53 ± 1,16
placenta lenght	391µm ± 20	350µm ± 20	443µm ± 17



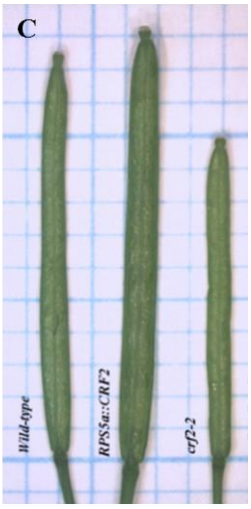


Figure 2: *CRF2* influences pistil length and ovule numbers. (A) Table with number of ovules and placenta length of wild-type, *crf2-2* and *RPS5a::CRF2* pistils. Mean ± standard error is reported. (B) Pistils with visible lines of ovules in wild-type, *crf2-2* and *RPS5a::*CRF2 captured by DIC microscopy. Scale Bars = 50µm. (C) Comparison of mature siliques at the stereo microscopy.

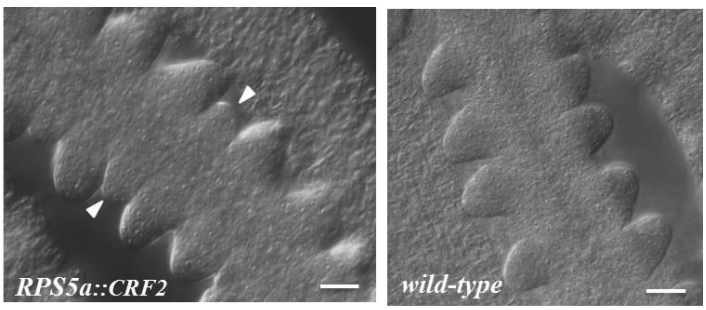


Figure S1: Additional ovule primordia are formed in *RPS5α::CRF2*. DIC microscopy images of ovules primordia lines in *RPS5α::CRF2* and wild-type. White arrows indicate new small ovule primordia formed in *RPS5α::CRF2*.

CRF2 regulates transcription of *PIN1* auxin efflux carrier.

As already mentioned, in the inflorescence CK positively regulates *PIN1* expression, which in turn is needed to form the auxin maxima that lead to ovule primordia development (Galbiati et al. 2013). It has been demonstrated, using protoplast assays, that CRF2 is an activator of *PIN1* (Simaskova et al., 2015). To analyse whether the phenotype observed in *crf2-2* and *RPS5a::CRF2* was due to changes in *PIN1* expression, we performed real-time PCR in the two genetic backgrounds. Results indicated that the level of *PIN1* expression in *crf2-2* is half that of wild-type, while is up-regulated twofold in *RPS5a::CRF2* (Fig. 3A). The expression level of *PIN1* directly correlates with *CRF2* expression level.

Recently, it has been discovered that CRFs contribute to the transcriptional control of *PIN1* through physical interaction with a specific domain in its promoter, named *PIN CYTOKININ RESPONSE ELEMENT* (*PCRE*) (Simaskova et al., 2015). Plant carrying ΔPIN1::PIN1-GFP, in which PCRE1 domain has been removed, allowed us to test if CRF binding to the *PIN1* promoter is required for *PIN1* expression during reproductive phase. In ΔPIN1::PIN1-GFP inflorescences the level of *PIN1-GFP* transcripts is lower than in wild type inflorescences (Fig. 3B). We designed primers to the *GFP* instead of *PIN1* in order to avoid the detection of endogenous *PIN1*. The reduction of GFP expression when under the ΔPIN1 promoter is also visible by confocal microscopy (Compare Fig. 3C, 3D with 3E, 3F).

To determine if *PCRE1* is required for the CKs-mediated *PIN1* expression in inflorescences, we also analyzed *GFP* expression after treatment with synthetic CK 6-Benzylaminopurine (BAP) in both PIN1::PIN1-GFP and ΔPIN1::PIN1-GFP.

Surprisingly, *GFP* expression was two times higher in both ΔPIN1::PIN1-GFP and PIN1::PIN1-GFP (Fig. 3B) with respect to the control (mock treatment) suggesting that other CKs-induced factors promote *PIN1* expression in absence of the *PCRE1* regulatory region. However, *PIN1* expression uncoupled from CRF regulation did not reach the *PIN1* expression level observed in wild-type after BAP treatment, suggesting that CRFs are primarily responsible for CK-mediated *PIN1* expression.

Other well-known positive mediators of CKs signaling are transcription factors of the ARABIDOPSIS RESPONSE REGULATORS-B (ARR-B) family (Hwang & Sheen 2001). Although until now none have been reported to play a role in pistil development, they could be responsible for *PIN1* expression in the absence of the PCRE1 regulatory region. Finally, it is important to remind that in root ΔPIN1::PIN1-GFP is reduced by CKs, which it means that there might be developmental context specific regulation of *PIN*1 expression.

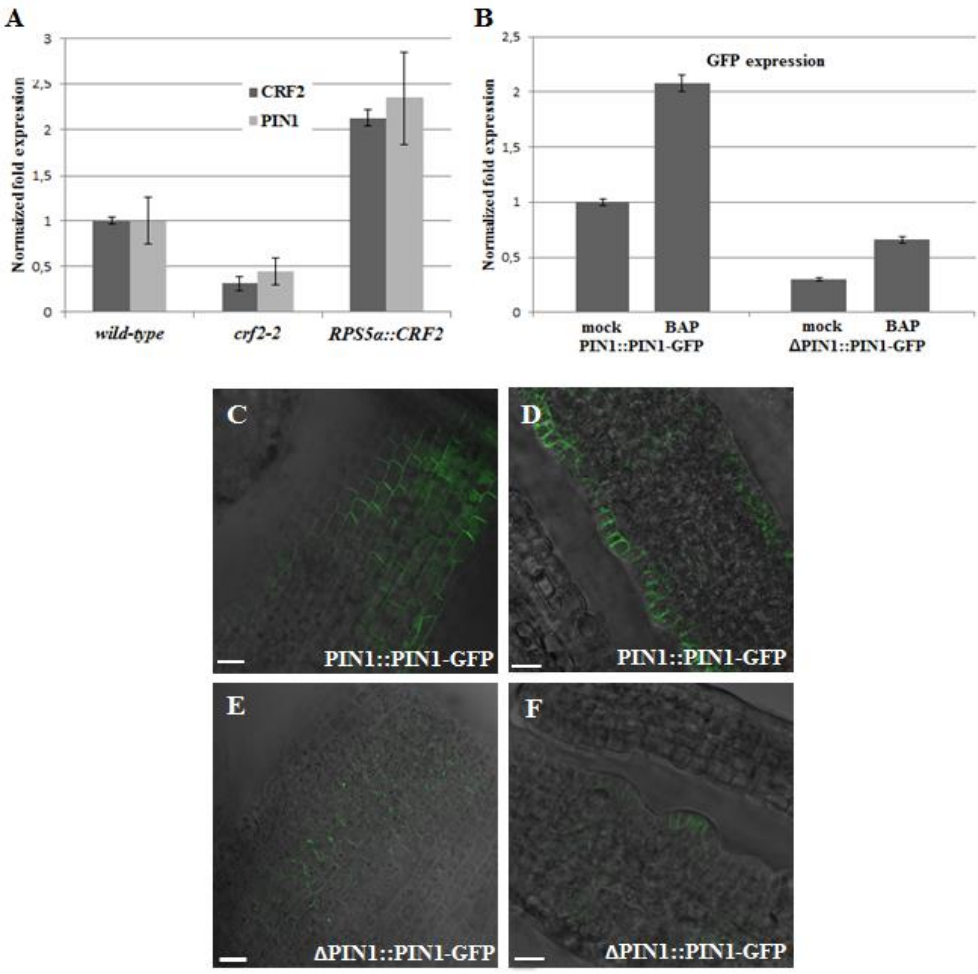


Figure 3: CRF2 regulates *PIN1* expression (A) *PIN1* and *CRF2* expression levels in pre-fertilization pistils of wild-type, *crf2-2* and *RPS5a::CRF2* (B) GFP expression levels in mock and BAP treated pPIN1::PIN1-GFP and ΔpPIN1::PIN1-GFP pre-fertilization pistils. Error bars indicate s.e. based on three technical replicates. The data were normalized with respect to *actin8* and *ubiquitin10* mRNA levels. TConfocal microscope images of pPIN1::PIN1-GFP (C, D) and ΔpPIN1::PIN1-GFP (E, F) in epidermal cells of the valves (C,E) and in ovule primordia cells (D,F). Scale bars = 10 µm.

***CRFs* are required for the determination of pistil size.**

As expected, ΔPIN1::PIN1-GFP in a wild-type background does not show any phenotype, therefore to test the phenotypic effect of the lack of CRF regulatory regions in the PIN1 promoter we introgressed ΔPIN1::PIN1-GFP into the ovule deficient mutant *pin1-5*. Weak *pin1-5* allele exhibits shorter pistil, reduced valves and develops an average of 9 ovules/pistil (Sohlberg et al. 2006; Bencivenga et al. 2012).

PIN1::PIN1-GFP was able to completely complement *pin1* mutants, forming a plant that in all its respects appears wild type, including fertility and seed yield. However, the pistil length of *pin1-5* ΔPIN1::PIN1-GFP plants was drastically reduced (Fig. 4A), the length of the placenta is 233.4 ± 13 and ovule number of 24 ± 2 (Fig. 4A). Importantly, the density of ovules did not change relative to wild type, and the process of ovule primordia formation was not impaired as in *pin1-5* (Fig 4A). Confirmation of the presence of ΔPIN1::PIN1-GFP in the *pin1-5* background is shown in Fig. S2. These results strongly imply that CRFs control the PIN1 expression required to determine the correct size of the pistils, but do not control the PIN function required for ovule primordia formation.

CRF3 and CRF6 are able to bind *PCRE1* regulatory region, and have been confirmed as activators of PIN1 expression (Sismaskova et al., 2015).

For this reason, we decided to investigate the contribution of CRF3 and CRF6 to PIN1 expression during pistil elongation. The triple mutant *crf2 crf3 crf6* produces a plant that mimics phenotypic effects of a lack of CKs (semi-dwarf phenotype, reduced leaves size and shoot growth), as in the case of multiple mutants of CKs receptors (Nishimura et al. 2004). The pistil size in *crf2 crf3 crf6* showed significant reduction with respect to the single *crf2-2* mutant. This strongly suggests that CRF2, 3 and 6 are redundantly involved in pistil elongation.

The ovule number and pistils length phenotype of triple mutants resembled that of *pin1-5* ΔPIN1::PIN1-GFP (Fig.4A), confirming a significant contribution of CRF2, 3 and 6 in direct regulation of PIN1 expression during pistil growth.

The reduction of pistil size observed in CRF mutants could be due to altered cell division or cell expansion processes, or a combination of both. Auxin plays a prominent role in controlling cell expansion. For example, elongation of primary root and hypocotyl required specific auxin transport to determine their expansive growth rates (Spartz et al. 2012; Rayle & Cleland 1992). Interestingly, a reduction in pistil and anther elongation has also been reported for *tir1 afb1 afb2 afb3*, a quadruple mutant that has a compromised auxin signaling pathway (Cecchetti et al. 2008). Understanding of auxin's influence on the cell cycle is still fragmentary; primary evidence indicates that auxin acts on several targets involved in the control of cell cycle (Perrot-Rechenmann 2010). On the contrary, the ability of CKs to promote cell division, in particular through action on D-Type cyclins, was pointed out several years ago (Dewitte et al. 2007; Riou-Khamlichi et al. 1999). In view of this, we propose that *PIN1* expression mediated by CRFs is fundamental to allow correct elongation of the pistil, most likely exerting a dual action on cell division and the cell cycle. The higher number of ovule primordia in *RPS5α::CRF2* is a direct consequence of increased pistil size. Therefore, it is likely that when enough space is created between two ovules, other CKs-dependent factors induce PIN1 expression to create a new auxin maximum. Investigating the molecular machinery involved in the cross talk between auxin and CKs in the coordination of cell division and elongation during pistil and ovule development is an important challenge for the future.

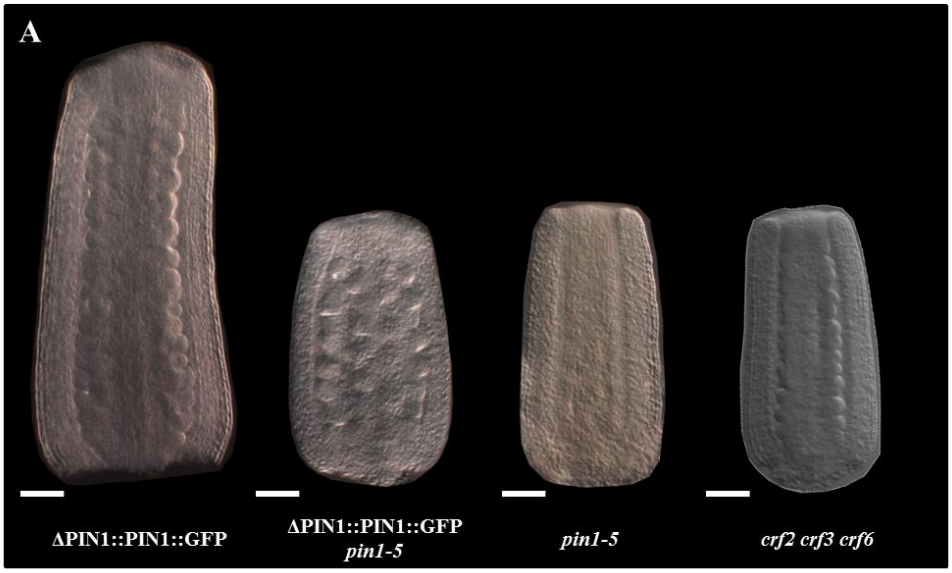


Figure 4: CRF binding to the promoter of PIN1 is required for pistil growth. (a) Pistil with visible line of ovules from ΔpPIN1::PIN1-GFP in wild-type background, ΔpPIN1::PIN1-GFP in pin1-5 background, pin1-5 and crf2 crf3 crf6, captured by DIC, bar = 50µm.

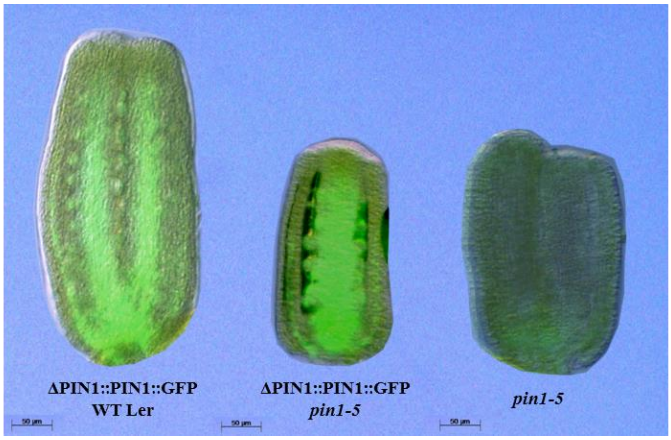


Figure S2: Confirmation of the presence of ΔPIN1::PIN1-GFP in the *pin1-5* , compared ΔPIN1::PIN1-GFP wild-type Ler and *pin1-5* controls. Captured by DIC, bar = 50µm.

CONCLUSIONS

The crosstalk between auxin and CKs is of fundamental importance to the control of ovule number. *PIN1* plays a major role in ovule number determination by controlling pistil size and ovule density. In particular, we confirmed using *crf* mutants that both developmental pathways are PIN1-dependent, however they seem to be regulated independently. Clearly, CRFs are required to mediate CKs signaling to control pistil size, but they do not interfere with ovule primordia density. It will be of great interest to identify CKs-dependent TFs that control *PIN1* expression required for ovule primordia formation. Identification of new key factors involved in ovule number determination is of great importance since this trait directly influences plant productivity, inviting promising application in crop plants.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana wild-type and mutant plants were grown at 22°C under long-day conditions (16 h light per 8 h dark). pRPS5a::CRF2 and pCRF2-3xGFP (Schlereth et al. 2010) were kindly provided by Prof. Dolf Weijers (WER the Netherlands). crf2-2 has been reported as a knock-out mutant allele (Rashotte et al. 2006), PIN1::PIN1-GFP, ΔPIN1::PIN1-GFP, *crf3*, *crf6*, *35S::CRF3 and 35S::CRF6*, all previously described in Simaskova et al., 2015 were obtained from Prof. Eva Benkova (ITS Vienna). *mp S319* (Cole et al. 2009) has been described previously. BAP treatment was performed on inflorescences as previously described by (Bencivenga et al. 2012).

Quantitative RT-PCR analysis

Total RNA was extracted from inflorescence at pre-fertilization stages using the Machery and Nagel RNA total plant kit (www.mn-net.com) and then reverse transcribed using the GeneSpin RT kit (www.genespin.com). The cDNAs were standardized relative to ACTIN2-8 (ACT2-8) and UBIQUITIN10 (UBI10) transcripts, and gene expression analysis was performed using the Bio-Rad iQ5 Multicolor real-time PCR detection system (www.bio-rad.com) with GeneSpin SYBR Green PCR Master Mix (www.genespin.com). Baseline and threshold levels were set according to the manufacturer's instructions. RT-PCR primers are listed in Table Sx.

Chromatin immunoprecipitation (ChIP) assays

ChIP experiments and the fold enrichment calculation were performed as described previously (Galbiati et al., 2013) using the Clontech antibody GFP: Living Colors full-length A.v. polyclonal antibody (www.clontech.com). Chromatin was extracted from pre-fertilized inflorescences of pMP::MP-GFP plants and from wild-type plants (Col-0) as a control. The DNA fragments obtained from the immune-precipitated chromatin were amplified by quantitative RT-PCR using specific primers (Table S1). Three RT- PCR amplifications on three independent chromatin extractions were performed. Enrichment of the target region was determined using the Bio-Rad iQ5 Multicolor real-time PCR detection system (www.bio-rad.com) with Bio-Rad SYBR Green PCR Master Mix (www.bio-rad.com).

Counting ovule number by DIC microscopy

Inflorescences were fixed with Ethanol:Acetic Acid 9:1 overnight, then rehydrated with 70% Ethanol and lastly cleared in a chloral hydrate/glycerol/water solution (8 g: 1 ml: 3 ml) for two hours before dissection under a stereomicroscope. Pistils were observed using a Zeiss Axiophot D1 microscope (www.zeiss.com) equipped with differential interference contrast optics. Images were recorded using an Zeiss Axiocam MRc5 camera with Axiovision version 4.1.We counted only ovules of pistils in which both carpels remained intact after slide preparation and where all four rows of ovules were visible and distinguishable. For each genotype we analysed 5 pistils from plant. The analysis was done for 10 plants for each phenotype.

Confocal microscopy

For confocal laser scanning microscopy, fresh material was collected, mounted in water and immediately analyzed. Laser scanning microscopy analysis was performed using a Leica (www.leica-microsystems.com) SPE microscope with a 488 nm argon laser line for excitation of GFP fluorescence. Emissions were detected between 505 and 580 nm. Images were collected in multi-channel mode, and overlay images were generated using Leica analysis software LAS AF 2.2.0.

Primers for genotyping		
crf2-2	AtP 5303 rev	5'-TGTGCCAGCTGGATAATCCG-3'
	AtP_1220 lbb1	5'-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3'
RPS5α::CRF2	AtP_5541 fw	5'-GCGACGCAAACGATCTTCAG-3'
Primers for expression analysis		
PIN1	RT 509 fw	5'-TGGTCCCTCATTTCCTTCAA-3'
	RT 510 rev	5'-GGCAAAGCTGCCTGGATAAT-3'
CRF2	RT 1902 fw	5'-ACGGAACCGGAGAAAGTTTTG-3'
	RT 1903 rev	5'-GAAACTCGACCTCCCATTATCG-3'
GFP	RT 2017 fw	5'-TGTTCCATGGCCAACACTTG-3'
	RT 2018 rev	5'-AAGTCGTGCCGCTTCATATG-3'
ACT2-8	RT 861 fw	5'-CTCAGGTATTGCAGACCGTATGAG-3'
	RT 862 rev	5'-CTGGACCTGCTTCATCATACTCTG-3'
UBQ10	RT 147 fw	5'-CTGTTCACGGAACCCAATTC-3'
	RT 148 rev	5'-GGAAAAAGGTCTGACCGACA-3'
Primers for qRT-PCR after ChIP		
CRF2	RT 2064	5'-CCGAGGAGTGAGACAGCGTCC-3'
	RT 2065	5'-GCTTCTTCCGCCGTGTTGTAAGTACC-3'
ARR15	RT 446	5'-GAGTAGTCATTGTCAGATAG-3'
	RT 447	5'-GTTAAGATCTTGTGAGGGTC-3'
CUC1	RT 470	5'-CTGTGATAATGTGCTAGATGAG-3'
	RT 471	5'-GTAGAGACTCTGTTTCAGAACC-3'
ACT	RT_045	5'-CGTTTCGCTTTCCTTAGTGTTAGCT-3'
	RT_046	5'-AGCGAACGGATCTAGAGACTCACCTTG-3'

Table S1: Primers used in this study.

Acknowledgments

Mara Cucinotta was funded by a Ph.D. fellowship from the Università degli Studi di Milano and from MIUR-PRIN 2012. The work of Lucia Colombo is supported by MIUR-PRIN 2012. We would like to thank Edward Kiegle for editing the paper.

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Genome wide identification of CUCs targets revealed a link with CKs pathways in the determination of seeds yield.

Authors: Mara Cucinotta[1], Nadia Elisa Quadrelli[1], Simona Masiero[1] and Lucia Colombo[1].

Authos affiliation:[1] Dipartimento di Bioscienze, Università degli Studi di Milano, Via G. Celoria 26 – 20133 Milano, Italy

* To whom correspondence should be addressed (lucia.colombo@unimi.it)

ABSTRACT

Seeds derives from ovules upon fertilization; therefore, the total number of ovules determines the final seeds yield, a fundamental trait in crop plants. Several hormones coordinate the process of ovule formation. In particular, cytokinins (CKs) have a prominent role. Indeed, plants with enhanced CKs content or response form more ovules. Others factors involved in ovule number determination are the transcription factors CUC1 and CUC2. Both CUCs and CKs act by regulating the expression of *PIN1* auxin efflux carrier. PIN1 is required for auxin distribution in the placenta, a crucial step for ovule primordia formation. Hereby, using an integrative genome wide target identification approach, we have identified genes regulated by CUCs that are also involved in CKs pathways. One of them the *UGT73C1* gene, is negatively regulate by the CUCs. The *UGT73C1* codify for an enzyme able to catalysed CKs inactivation. Interestingly, the down-regulation of *UGT73C1* lead to an increased seed set per silique, most likely by increasing the level of active CKs. These results reinforce the connections between CUCs and CKs, and underpin the importance of CKs as a factor determining seeds yield.

INTRODUCTION

Seeds yield enhancement is one of the goals of agriculture to meet the increasing food demand. The number of ovules primarily determines the final number of seeds. Indeed, ovules development into the seeds, upon fertilization. For this reason, it is of great importance to understand the mechanisms that control ovule number setting.

Ovule primordia, emerge as lateral organs, after a series of periclinal cell division, from a meristematic tissues inside the pistil called placenta (Schneitz et al. 1995). Placenta in turn derived from the carpel margin meristem (CMM). The formation of the CMM is known to be controlled by the interaction of genetic and hormonal networks (Reyes-Olalde et al. 2013).

In the last decades, the fundamental role of plant hormones in ovule initiation, in particular of CKs, auxin and brassinosteroids has been pointed out and few transcription factor modulating the hormonal response have been identified (reviewed by Cucinotta et al., 2014).

In this work, we focus the attention on CKs, since their role as positive regulators of ovule number has been clearly established (Bartrina et al. 2011; Galbiati et al. 2013; Bencivenga et al. 2012). Mutants impaired in CKs signalling have a drastically reduced number of ovules (Riefler et al. 2006; Kinoshita-Tsujimura & Kakimoto 2011; Bencivenga et al. 2012). On the contrary, plants with higher CKs content form more ovules. For instances, the total seed yield of *ckx3ckx5*, double mutants for two CKs degradation enzymes, increased by 55% respect the wild-type (Bartrina et al. 2011).. Interestingly, block of CKs degradation, through the use of a specific inhibitor molecules, has been proposed as promising strategy to improve plant productivity also in other plant species (Aremu et al. 2014).

It has recently been shown that *CUP SHAPED COTILEDON1 (CUC1)* and *CUC2* transcription factors encoding genes are part of a network that regulate ovule primordia initiation and number (Galbiati et al., 2013). *CUC1* and *CUC2* were known to be involved in boundary establishment between organs. In the *cuc1cuc2* double mutant the cotyledons are fused (Aida et al. 1997). In the placental tissue, *CUC1* and *CUC2* are broadly expressed, but after primordia formation, their expression is limited to the boundaries between ovules (Nahar et al. 2012; Galbiati et al. 2013). Pistils in which both gene are silenced (*cuc2 pSTK::RNAi-CUC1*) have a 20% reduction of ovules respect to wild-type (Galbiati et al. 2013). In *cuc2 pSTK::RNAi-CUC1* pistil size is not affected but the space between two primordia increases, leading to a reduced ovule density. Galbiati and colleagues also demonstrated that CUCs promote expression of the auxin efflux carrier *PIN1*, which is necessary for auxin distribution in the placenta. CKs are also strong activators of *PIN1* expression in the pistil (Bencivenga et al. 2012), and CK treatment rescues *cuc2 pSTK::RNAi-CUC1* ovule number phenotype by restoring correct *PIN1* expression (Galbiati et al. 2013). This data also suggested that *CUCs* could act upstream of CKs pathways. Here, we report a genome-wide target identification analysis, which allow us to identify genes regulated by CUCs and directly involved in CKs homeostasis and response, whit possible implication in ovule number determination.

RESULTS

It has been proposed that *CUCs* acts upstream CKs pathways since CKs treatment is able to rescue *cuc2 pSTK::CUC1_RNAi* ovule number phenotype (Galbiati et al. 2013). Specific experiments have been conducted to elucidate relationship between *CUCs* and CKs.

To verify whether CUC1 and CUC2 are able to induce CKs response in vivo, we performed transient expression assays in BY-2 tobacco protoplasts. We used as CKs response reporter the synthetic promoter 'two component signalling sensor' (TCS) fused to the luciferase. TCS reflects the transcriptional activity of type-B response regulators (Müller & Sheen 2008). Protoplast that overexpressed *CUC1* or *CUC2* have a significant increase of pTCS::LUC expression, compare to those that overexpress *GUS* (Fig. 1). This result suggest that CUC1 and CUC2 are positive regulators of CK response. We checked induction of *PIN1* by both CUC1 and CUC2, as a positive internal control of the experiment (Galbiati et al. 2013). This data reinforce the idea of a direct link between *CUCs* and CKs, and suggest that CUCs act upstream of CKs.

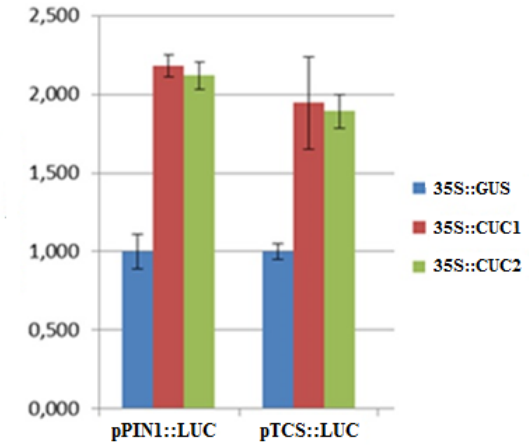


Figure 1: *CUCs* overexpression enhanced CKs response. The graph shows the transient expression of *pPIN1::LUC* and *pTCS::LUC* in BY-2 protoplasts. *TCS* activity, as well as for *PIN1*, is enhanced by *CUC1* and *CUC2* overexpression compared to *GUS* control. Transactivation is relative to the normalized luciferase (LUC) activity. Error bars indicate the standard error (n = 8 separate transfection events and measurements).

Searching for CUCs targets.

In order to identify genes downstream *CUC1* and *CUC2* we performed a transcriptome analysis by RNA-deep-sequencing, comparing wild-type with *cuc2 pSTK::RNAi-CUC1*. Total RNA has been extracted from pre-fertilized pistils at developmental stages 8 – 12 (Roeder & Yanofsky 2006). Then, RNAs were sequenced with Illumina system and the obtained transcriptomic data were analysed with CLC workbench bioinformatics software – see Methods. The bioinformatics and statistical analysis lead to the identification of approximately 500 genes differentially expressed between wild-type and the mutant. In the first eighty position of the down-regulated gene list, we found five genes (*LAS*, *CYP78A5*, *LSH4*, *DPA4* and *PAN*) already known to be downstream of *CUCs* (Aida M. unpublished data). This result positively supports the validity of the data.

Some interesting CUCs candidate targets have been selected for their connection with CKs pathways. The candidates genes are: *UDP-GLUCOSYL TRANSFERASE 73C1 (UGT73C1)*, *UGT73C6*, *WUSCHEL-RELATED HOMEOBOX 9* (*WOX9*, also named *STIMPY* and *HB-3*) and *CLAVATA3/ESR-RELATED 10* (*CLE10*). *UGT73C1* and *UGT73C6* were found to be up-regulated in absence of *CUC1* and *CUC2*, while *WOX9* and *CLE10* were down-regulated.

RNA-sequencing expression data of the four putative CUCs targets have been validate first by qRT-PCR on two different biological replicates for both wild-type and *cuc2 pSTK::RNAi-CUC1* pistils (Fig. 2). We confirmed RNA-seq data results for all the four genes (Fig. 2). *CLE10* and *WOX9* are down-regulated at least by two times in *cuc2 pSTK::RNAi-CUC1*, whereas *UGT73C1* and *UGT73C6* are up-regulated in the mutant compared to wild-type, with a stronger increase for *UGT73C1* (Fig. 2).

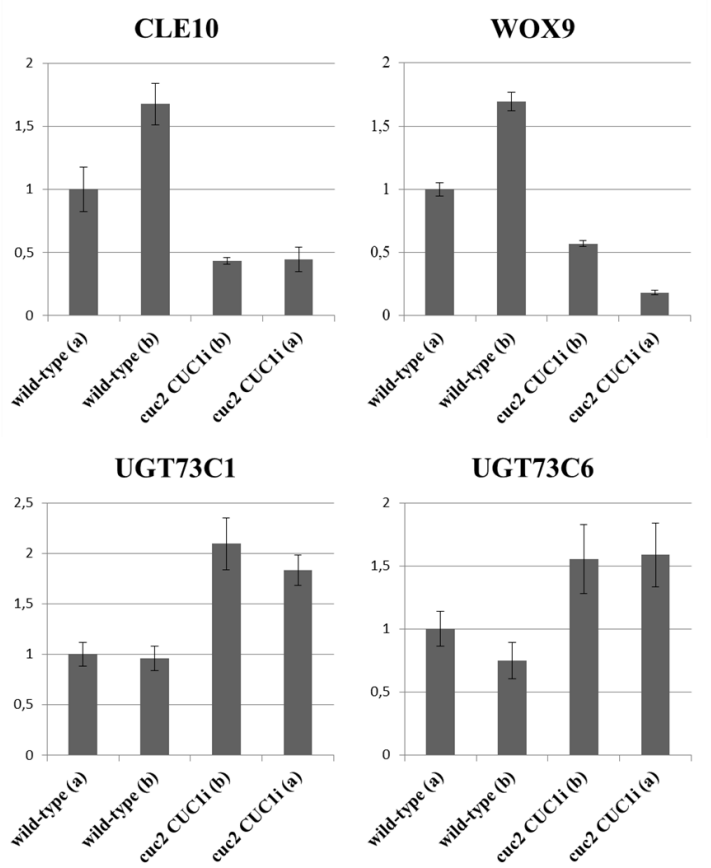


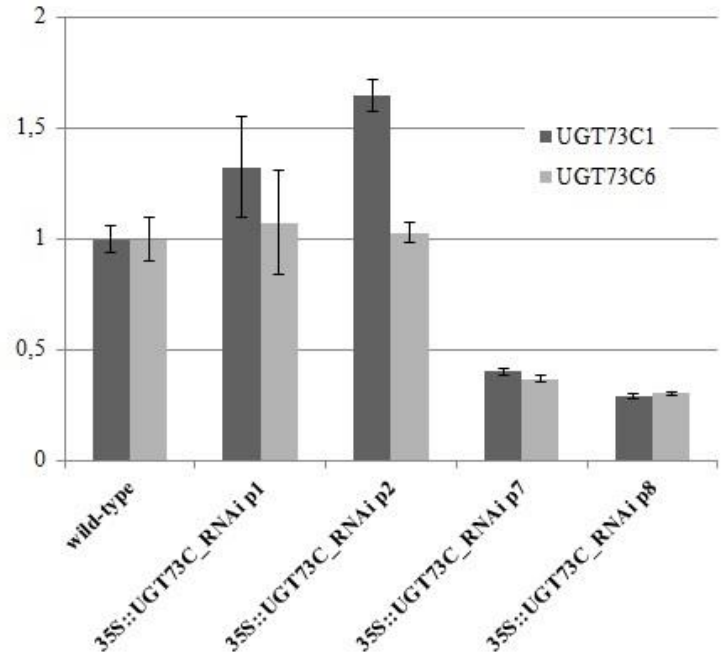
Figure 2: qRT-PCR validation of RNA-seq data for four CUCs candidate targets. Comparison of expression of *CLE10*, *WOX9*, *UGT73C1* and *UGT73C6* between wild-type and *cuc2 pSTK::CUC1_RNAi (cuc2 CUC1i)*. The expression was tested in two different biological replicates, identified as (a) and (b), for both wild-type and mutant background. As obtained in the RNA-seq, also here relative mRNA levels indicate that the expression of *CLE10* and *WOX9* is down-regulated in the mutant, while the one of *UGT73C1* and *UGT73C6* is up-regulated. Gene expression was normalized against *actin8* and *ubiquitin10*. Error bars represent the propagated error value using three technical replicates.

***UGT73C1* and *UGT73C6* are involved in seeds number determination**.

UGT73C1 and UGT73C6 are two enzymes with O-glucosyltransferases activity. They are able to recognize trans-zeatin and dihydrozeatin cytokinins and form O-glucosides (Hou et al. 2004). The CKs glycoconjugates are inactive and are thought to play a role in homeostasis of the hormones (Mok & Mok 2001). O-glucosyltransferase family is comprised of seven genes, six of which clustered in tandem on chromosome 2 (*UGT73C1* to *6*). Their high protein sequence similarity suggest that they evolved from a gene duplication and may therefore have related enzymatic properties (Li et al. 2001).

Plants of *ugt73c1* and *ugt73c6* single mutants do not show any evident phenotype. Also in term of seeds number, they do not differ from the wild-type (data not shown). This is likely due to the high rate of redundancy of the *UGT73C* family. For this reason, we proceeded with an interference approach to down-regulate both *UGT73C1* and *UGT73C6*. *35S::UGT73C1_RNAi* has been cloned using Gateway technology, and transformed in Col-0 plants. We tested expression of *UGT73C1* and *UGT73C6* in several *35S::UGT73C1_RNAi* T1 plants (Fig. 3A). Lines n.7 and n.8 showed the lowest level of both genes compared to wild-type, thus, we selected those lines for further analysis. Preliminary results indicates that in *35S::UGT73C1_RNAi* line n.7 and n.8 the number of seeds increases by approximately 15% compared to wild-type control (Fig. 3B). Moreover, those seeds are more densely spaced since the length of the siliques in both lines does not change respect the wild-type (Fig. 3B). All the seeds counted in *35S::UGT73C1_RNAi* plants were viable, so that from an initial analysis *35S::UGT73C1_RNAi* does not seem to affect plant fertility.

A



B

	seeds number	silique length	seeds/mm
WT Col-0	51,18 ± 0,6	13,31 ± 0,1	3,83 - 3,85
35S::UGT73C_RNAi p7	59,00 ± 1,6	13,50 ± 0,2	4,18 - 4,55
35S::UGT73C_RNAi p8	60,03 ± 1,6	13,00 ± 0,3	4,39 - 4,81

Figure 3: *UGT73C1* and *UGT73C6* down-regulation increases seeds number.

(A) Detection of the expression level of *UGT73C1* and *UGT73C6* through qRT–PCR in wild-type and four different *35S::UGT73C1_RNAi* T1 lines. Gene expression was normalized against constitutively expressed *actin8* and *ubiquitin*10. (B) Results for seeds number counting and siliques length measurement in fifteen siliques for wild-type, *35S::UGT73C1_RNAi* lines n.7 and n. 8 which showed the higher reduction of *UGT73C1* and *C6* expression. Standard error is also reported.

***WOX9*, a player in CKs output, is involved in fruit elongation.**

The second candidate that we include in our analysis was *WOX9*, a *WUS* related transcription factor which is known to mediate CKs response in the shoot apical meristem (Skylar et al. 2010). Dominant gain-of-function allele of *WOX9*, named *stip-D*, develops wavy leaf margins; phenotype that is also reported for leaves overexpressing *CUC2* (Nikovics et al. 2006). *WOX9*, together with several other member of the homeobox family, like *HB-17* and *18*, resulted to be down-regulated in the *cuc2 pSTK::RNAi-CUC1* mutant. Another aspect that makes *WOX9* an interesting candidate is that it is expressed in inflorescence apex, developing carpels and in particular in the placenta (Wu et al. 2005). Indeed, homeobox transcription factors are usually involved in the growth of meristematic tissues, as placenta is.

In order to study the role of *WOX9* during reproductive phase we started phenotypic analysis of both loss of function (*stip-1* and *stip-2*) and activation-tagged (*stip-D*) mutant alleles. Analysis consisted in counting the number of seeds and measuring the length of the siliques.

Plants of *stip-1* and *stip-2* do not present any evident phenotype and they look in general like wild-type. Interestingly, both mutants has longer siliques respect the wild-type, with a more marked effect in the case of *stip-2* (Fig. 4A). Siliques of *stip-2* develops an average of 59,08 ± 1,4 seeds, while the wild-type contains 50,67 ± 0,6 seeds (Fig. 4B), which consist in a significant increase of 16%. Since the seeds are distributes in a longer siliques the final seeds density does not change in *stip-2* compared to wild-type (Fig. 4A, 4B).

To investigate the effect of a WOX9 miss-regulation we analysed the phenotype of dominant gain function s*tip-D.* In *stip-D* mutant the patterning of the pistil is not compromised, but the fruit remains very short after fertilization and it presents a high rate of seeds abortion. In *stip-d*, the siliques develop an average

of 27,5 ± 2,2, considering both those formed and those aborted, and the siliques are about half size respect the wild-type ones (Fig. 4B). Also in case of *stip-D*, decreasing of seeds number correlates with shorter siliques, so that the range of seeds density does not changes compared to wild-type. Taken together these data suggest that *WOX9* have a role in fruit elongation, and that this action influence the final seeds number.

B

	seeds number	silique length	seeds/mm
WT Col-0	50,67 ± 0,6	12,62 ± 0,7	3,98 - 4,08
stip-2	59,08 ± 1,4	15,42 ± 0,95	3,52 - 4,17
stip-D	27,5 ± 2,2	6,8 ± 1,3	3,12 - 5,14

Figure 4: WOX9 controls fruit elongation. (a) Stereo microscopy image show mature siliques of wild-type, stip-1, stip-2 and stip-D. (B) Results for seeds number counting and siliques length measurement in fifteen siliques for each lines. The propagated error values are reported.

DISCUSSION

In this work has been performed a transcriptomic analysis aimed at investigate CUCs downstream genes. Comparing transcription profile of wild-type and *cuc2pSTK::RNAi-CUC1* pistils, by RNA-sequencing, we obtained a list of CUCs candidate targets. We selected those genes that had a link with CKs pathways and we tested their possible implication in ovule number determination.

The first candidates considered were *UGT73C1* and *UGT73C6*, which codify for enzymes able to inactivate CKs by O-glucosylation. Expression data suggested that CUCs act as a repressor of both genes, since they were up-regulated in *cuc2pSTK::RNAi-CUC1*. Down-regulation of *UGT73C1* and *UGT73C6* expression, by RNA interfering, positively influences seeds number. This effect could be explained by the fact that *35S::UGT73C1_RNAi* plants might have a slightly increase of active CKs and so higher activity of the placenta tissue. It can be assumed that, in the placenta, CUCs repress expression of both enzymes in order to increase the level of active CKs and disadvantage the glycoconjugates inactive forms. To complete the analysis, quantification of o-glucoside CKs in *35S::UGT73C1_RNAi* and *cuc2 pSTK::CUC1_RNAi* mutant compared to wild-type needs to be performed.

Another important aspect is that, in *35S::UGT73C1_RNAi* plants, the seeds are more densely spaced but this does not affect their fertility. Even more so, this makes the *UGT73C1* and *UGT73C6* good candidates for future application on plant productivity.

More recently, it has been shown that UGT73C6 could also glucosylate brassinosteroids (BRs), and therefore might modulate BRs homeostasis.

Since also BRs are involved in ovule number determination (Huang et al. 2013; Cucinotta et al. 2014) it is of great interest to investigate the contribution of BRs modulation by UGT73C6 to the phenotype observed in *35S::UGT73C1_RNAi*. Moreover, in *35S::UGT73C1_RNAi*, due to the high sequence conservation, the down-regulation of all the other member of the *UGT73C* family need to be test.

Another interesting CUCs putative target that come out from the analysis was *WOX9*. Our results pointed out a role for *WOX9* in fruit elongation and in seeds number determination. Specifically, *WOX9* seems to inhibit fruit elongation, indeed, in the *stip-1* and *stip-2* mutants siliques are longer than those of the wild-type. On the contrary, overexpression of *WOX9* (*stip-D*), impair fruit development and give rice to very small siliques. Considering that CUCs positively regulate *WOX9*, this result is opposite to what expected on the base on *cuc2pSTK::CUC1_RNAi* phenotype. To clarify this aspect we should analyzed the function of *WOX9* before fertilization, and distinguished it from that after fertilization. Moreover, the exact role of *WOX9* in promoting CK response is still elusive and remain to be clarify. It has been proposed that WOX9 influence the expression of both positive, *CRF2* and *CRF5*, and negative, *ARR5* and *ARR7*, CKs response factors (Skylar et al. 2010b). Lastly, we also identify as CUCs targets, *CLE10*, which encodes for a small peptide able to promote CK signalling (Jun et al. 2010; Kondo et al. 2011). In particular, it has been shown that CLE10 inhibits protoxylem vessel formation by suppressing the expression of *type-A ARR* negative CKs response regulators. The fact that *CLE10* that is activated by CUCs, reconfirm the positive role of CUCs in promoting a positive CKs response. It will be interesting to further study *CLE10* also because it is a small peptide that can be directly apply as a treatment to growing pistils (Kondo et al. 2011).

Moreover, *CLE10* might have a role with *WOX9*, in a typical *WUS-CLE3-like* signalling pathway, to regulate meristematic activity of the placenta (Meng & Feldman 2014).

Taken together, RNA-seq data suggest that *CUC* genes are involved in regulating the expression of genes involved in CKs homeostasis and response, and that their action influence directly or indirectly the final seed set.

Finally, the identification of CUCs targets would implement the knowledge about the molecular network responsible of ovule primordia formation and could have a direct application on plant productivity.

FUTURE PERSPECTIVES

The major future goal of our work is to improve the understanding of the network controlling ovule number. Furthermore it would be of great interest to transfer the knowledge from Arabidopsis to other plant species of agronomical interest. Legumes, for instance, are among the best protein sources in the plant kingdom and their seeds are used for human and animal consumption or for the production of oils. In legumes, the fruits are pods formed by a single carpel, which similarly to the Arabidopsis siliques dehisces at maturity to release the seeds. It should be stressed, however, that the identification of functional equivalents of well-characterized Arabidopsis genes in other plants is a nontrivial task. As already highlighted, the perfect candidate genes will be those whose mutation or induction positively influence ovule numbers without affecting plant fertility. Homologous for these selected gene will be research in leguminous species whose genome is sequenced and for which collections of natural seeds varieties are available, as for example Soybean (Glycine max). In the next years we might verified in cultivar with different seeds number the level of expression of the genes that control ovule number in Arabidopis.

MATHERIAL AND METHODS

Plant material and growth condition

Arabidopsis *thaliana* wild-type (ecotype Columbia, Col-0) and transgenic lines were growth on soil at 22 °C under long day condition (16h light / 8h dark).
cuc2 pSTK::CUC1_RNAi has been already described (Galbiati et al. 2013). *stip-1*, *stip-2* and *stip-D* were previously identify as alleles of *WOX9* (Wu et al. 2005). *35S::UGT73C1_RNAi* were selected on the base of BASTA resistance.

Protoplast transfection

Protoplast preparation and transient expression experiments were performed as described by (Galbiati et al. 2013).

RNA extraction, cDNA library Preparation, and sequencing for RNA-seq

Total RNA was extracted from three biological replicates (0,5 g) from both wild-type and *cuc2pSTK::CUC1_RNAi* mutant pre-fertilization pistils, using the Macherey-Nagel 'NucleoSpin RNA Plant' according to the manufacturer's instructions. RNA integrity were analysed by gel electrophoresis. In order to confirm that *cuc2pSTK::CUC1_RNAi* was a knock out line for *CUC2* and down-regulation line for *CUC1*, expression of both genes were checked by qRT-PCR, as reported in Galbiati et al., 2013. RNA quality validation with Bioanalyzer 2100, sequencing library preparation by TruSeq RNA Sample Prep kit (Illumina Inc.) and sequencing on an Illumina HiSeq2000 (50 bp single-read) have been performed by IGA Technology Services srl (http://www.igatechnology.com/).

Mapping of short reads, quality analysis and assessment of gene expression analysis for RNA-seq

Mapping of short reads, quality analysis and assessment of gene expression were performed as described by (Mizzotti et al. 2014). Evaluation and treatment of raw data was performed on the commercially available CLC Genomics Workbench v.4.7.1 (http://www.clcbio.com/genomics/). The high-quality reads were mapped onto the Arabidopsis genome (TAIR10). Approximately 20M reads of each sample that mapped with ≤2 mismatches were used for further analyses. The fold change and differential expression values between wild type and the *cuc2 pSTK::CUC1_RNAi* mutant was calculated in terms of RPKM of the corresponding transcripts. To obtain statistical confirmation of the differences in gene expression, P values were computed. We applied a threshold value of $P = 0,05$ to ensure that differential gene expression was maintained at a significant level (5%) for the individual statistical tests. Transcripts that exhibited an estimated absolute Fold Change ≥2 (*i.e.* 2 mapped reads per kilobase of mRNA) were determined to be significantly differentially expressed.

Expression analysis

Total RNA was extracted from pistils at pre-fertilization stages using the Machery and Nagel RNA Macherey-Nagel 'NucleoSpin RNA Plant' and then reverse transcribed using the Promega 'GoScript™ Reverse Transcription System'. The cDNAs were standardized relative to ACTIN2-8 (ACT2-8) and UBIQUITIN10 (UBI10) transcripts, and gene expression analysis was performed using the Bio-Rad iQ5 Multicolor real-time PCR detection system with GeneSpin SYBR Green PCR Master Mix. RT-PCR primers are listed in Table S1.

Construction of binary vector and plant transformation.

For the construction of *35S::UGT73C1_RNAi*, DNA fragment containing 148 bp of a region conserved in *UGT73C1* and *UGT73C6* transcripts were amplified using primers AtP_5090 and AtP_5091, and recombined into RNAi vector pFGC5941 (Karimi et al. 2002) through an LR reaction (Gateway system, Invitrogen, www.lifetechnologies.com). This construct was used to transform wild-type Col-0 plants using the floral-dip method (Clough & Bent 1998).

Table 1. Primers list used in this study.

Primers for expression analysis		
UGT73C6	RT 1504 fw	5'-TTTTGTCTTCTGTGTGTTAACGTTCTG-3'
	RT 1505 rev	5'-TCTATCAGGAAAATAAGGAACAATGAAG-3'
UGT73C1	RT 1500 fw	5'-TGCAGATACTAAAAGCCGGTGTG-3'
	RT 1501 rev	5'-CTTCTTTACTCCTTCTTTATCCACCAG-3'
WOX9	RT 1320 fw	5'-CCAATTAGGGTTTCTCTCCGG-3'
	RT 1321 rev	5'-TCCCTCACATTGAACGGTCC-3'
CLE10	RT 1318 fw	5'-CAAGAAACTGGACCAACCGAACTC-3'
	RT 1319 rev	5'-CGTACCTTTGATCAATCTCCGTCG-3'
ACT2-8	RT 861 fw	5'-CTC AGG TAT TGC AGA CCG TAT GAG-3'
	RT 862 rev	5'-CTG GAC CTG CTT CAT CAT ACT CTG-3'
UBQ10	RT 147 fw	5'-CTGTTCACGGAACCCAATTC-3'
	RT 148 rev	5'-GGAAAAAGGTCTGACCGACA-3'
Primers for cloning		
UGT_RNAi	AtP_5090 fw	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC TGGACCTGTTTCCTTGTGCA-3'
	AtP_5091 rev	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC CTAGGCCTAGTCCCAGCTCA-3'

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Part III

Unravelling the role of *MONOPTEROS* during pistil and ovule development.

Authors: Mara Cucinotta[1], Andrea Guazzotti[1], Marta A. Mendes[1], Sara Simonini[1], Francesca Galbiati[1], Elisabetta Caporali[1] and Lucia Colombo[1]*.

Authors affiliation:
[1] Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

* To whom correspondence should be addressed (lucia.colombo@unimi.it)

ABSTRACT

Organs initiation and patterning require precise modulation of auxin signalling, which is primarily mediated by the AUXIN RESPONSE FACTOR (ARF) proteins. Among them ARF5/MONOPTEROS (MP) has been shown to control expression of several key genes needed for pistil and ovule primordia formation. Indeed, in some *mp* mutant alleles, reproductive development is compromised; pistils lack proper patterning and consequently placenta and ovules do not form.

We aim to characterize the network controlled by MP in pistil development. In *mp-S319* the adaxial-abaxial pattern is impaired, therefore we analysed the expression of *KAN2*, *ARF3* and *ARF4* knowing to be important to settled this pattern during pistil development. In the *mp-S319* mutant these genes are up regulated, suggesting that MP is needed for their repression. We propose a model in which MP, interacting with IAAs proteins, negatively regulates the expression of target genes and that this repression is likely achieved through epigenetic modifications.

INTRODUCTION

In plants, all processes of growth and development required the phytohormone auxin. In particular, the initiation of a new organ needs robust patterns of auxin biosynthesis and distribution (Benková et al. 2003; Reinhardt et al. 2003). Then, correct perception of the hormone is fundamental to translate the auxin signal in a precise developmental program (Cole et al. 2009; De Smet et al. 2010; Vernoux et al. 2011).

Auxin signalling is primarily regulated by the AUXIN RESPONSE FACTOR (ARF) gene family products, together with the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins (Quint & Gray 2006). ARFs regulate transcription by binding specific Auxin Response Element (AuxREs) in the cis-regulatory regions of their targets (Ulmasov et al. 1995; Guilfoyle & Hagen 2007). Under sub-threshold auxin concentrations Aux/IAA proteins heterodimerize with ARF transcription factors, thereby repressing the modulation of ARFs downstream targets (Ulmasov et al. 1997; Reed 2001).

ARFs proteins consist of three different domain; a B3 DNA binding domain (DBD), a middle region and a carboxy-terminal dimerization domain (CTD). CTD is required for dimerization with the Aux/IAA (Ulmasov et al. 1997; Ulmasov et al. 1999; Tiwari et al. 2003; Guilfoyle & Hagen 2007).

Genetic studies revealed the fundamental role of ARFs in various developmental processes. Among them ARF5/MONOPTEROS (MP) has been the subject of many researches on embryo development, lateral root formation, vasculature patterning, shoot apical meristem and floral meristem maintenance (Berleth & Jürgens 1993; Hardtke & Berleth 1998; Przemeck et al. 1996; Weijers et al. 2006; Donner et al. 2009; De Smet et al. 2010; Yamaguchi et al. 2013).

Many *mp* mutant alleles are available, the ones considered strong alleles present high percentage of embryo defects, as lack of primary root or formation of a single cotyledon (Hardtke & Berleth 1998; Berleth & Jürgens 1993). What is still not completely clear is if the severity of embryo defects correlate or not with the amount of residual MP function (Odat et al. 2014). Alleles, like *mp-S319, mp-g92* and *mp-T730*, which has intact DBD but impaired middle region and CTD, show low percentage of embryo lethality but they present vascular system defects and their reproductive development is compromised (Przemeck et al. 1996). In *mp-S319,* the weak allele used in this study, the number of flowers are greatly reduced, pistil do not forms valves and placenta and consequently it lacks ovules (Cole et al. 2009; Galbiati et al. 2013).

Several studies have demonstrated that local auxin biosynthesis and polar transport are necessary for correct apical–basal patterning of the gynoecium (Nemhauser et al. 2000; Cheng et al. 2006; Stepanova et al. 2008). Indeed, all mutants in which auxin biosynthesis or transport are compromised present reduction or absence of the valves and expansion of the gynophore and style (Balanzá et al. 2006; Larsson et al. 2013).

To date, the molecular causes of such a severe defect in *mp* gynoecium remain to be clarify. The aim of our work is to investigate the molecular mechanism through which MP integrates the auxin signal and regulates its downstream targets during pistil and ovule formation. In particular, we are interest in clarify the role of MP in ovule development, since it has been shown that MP directly regulate CUP-SHAPED COTYLEDONS 1 (CUC1), CUC2 and AINTEGUMENTA (ANT), key genes for ovule primordia initiation and ovule number setting (Galbiati et al. 2013).

Here, we propose that, during gynoecium and ovule development, MP might function as repressor of downstream targets in regions of auxin minima.

RESULTS AND DISCUSSION

MP expression pattern does not correspond with auxin maxima regions.

In order to investigate the role of MP during early stages of gynoecium and ovule development we analysed in detail pMP::MP-GFP (Schlereth et al. 2010) expression pattern. Since MP function has been reported to be correlated with auxin response we monitored auxin response using DR5v2-ntdTomato reporter (Liao et al. 2015). The recently designed DR5v2 synthetic promoter consists in nine repetition of TGTCGG sequence, identified as MP higher affinity binding site by protein binding microarrays experiments (Boer et al. 2014; Liao et al. 2015). DR5v2 expression encircles the whole apical part of a stage-9 gynoecium, from which style and stigma will develop (Fig. 1A). Auxin maxima is also detected in the gynoecium pro-vasculature (Fig. 1A). The same expression pattern has been shown for the classical DR5 reporter (Larsson et al. 2013). On the contrary, MP-GFP signal is present in the vasculatures but it is completely absent from the gynoecium apical part, as visible from both longitudinal and transversal pMP::MP-GFP gynoecia sections (Fig. 1B, 1C).
Regarding ovule developmental stages, it has been showed that MP-GFP fusion protein is uniformly detected in the naked placenta before that ovule primordia arise (Galbiati et al. 2013). Once the primordia arise, MP–GFP remain broadly express in the placenta, at the base of ovules and in the boundaries between two ovules (Fig. 2A). Later, as ovules protrude forming a finger like structures, GFP signal is detected in the developing ovule vasculature (Fig. 2D). At stage 2-III, as ovule initiates inner and outer integuments, MP localizes in the vasculature, in the inner integument and at the base of nucella (Fig. 2G). Once integuments elongate, MP expression is restricted to the basal part of nucella (Fig. 2L).

Regarding DR5v2, the signal is not detectable in the naked placenta, but it appears in few epidermal cell at the tip of nucella, starting from stage 1–II, and it remain expressed there until stage 2–V (Fig. 2B, 2E, 2H and 2M). Weak DR5v2 signal is also detected in the pro-vascular cells of funiculus and in the chalazal part of ovule from stages 2-III to 2-V (Fig. 2H and 2M). For all the developmental stages taken into account, DR5v2 expression coincides with what reported by previous studies about DR5 (Benková et al. 2003; Ceccato et al. 2013; Galbiati et al. 2013).

More importantly, local DR5v2 signal in ovules clearly coincides with region of auxin accumulation created by PIN1 and PIN3 efflux carrier (Benková et al. 2003; Ceccato et al. 2013).

Unexpectedly, what is evident from this analysis is that in ovules the activity of DR5v2 promoter is detected in the cells where MP is not expressed. Indeed, in the few cells that show DR5v2::ntdTomato signal MP protein is not present. Conversely, where MP is expressed DR5v2 in not detectable (Fig. 2)

From this observation it can be deduced that, in these tissues, MP is not involved in promoting the DR5 expression. On the contrary, protoplast assay experiments clearly showed that MP is a strong activator of DR5 and that, in particular, its Q-rich middle region function as activation domain (Tiwari et al. 2003).

To complete the overall picture of auxin response in ovule, we also investigate the regions of auxin minima. For this purpose, we took advantages of the recently available R2D2 reporter line that allowed semi quantitative read-out of auxin minima (Brunoud et al. 2012; Liao et al. 2015). R2D2 line contains DII-GFP auxin minima reporter and mDII-ntdTomato which lack auxin-dependent degradation (Liao et al. 2015). Co-localization of DII::VENUS and mDII-tdTomato indicate that DII is expressed and not degraded by auxin.

Presence of only mDII revealed region of auxin maxima because despite being expressed DII is quickly degraded (Liao et al. 2015).

Using this tool, auxin minima is detected in the placenta, at the base of ovule primordia and in particular in the boundaries between ovules (Fig 2C and 2F). The distal part of nucella at stage 2-IV is also a clear region of auxin minima (Fig. 2I). Presence of only mDII::GFP signal in the apical part of nucella confirms that this is a point of auxin maxima accumulation (Fig. 2F and 2I). Interestingly, expression pattern of MP and DII-venus are almost overlapping. The fact that MP expression coincides with region of auxin minima, suggests that MP might works where Aux/IAA proteins are not degraded. Among Aux/IAA genes, IAA12/BDL and IAA13 can interact with MP (Weijers et al. 2005). The possibility that, in absence of auxin, MP and BDL have a function together has been already proposed, and in particular that they might work together in a repression complex (Lau et al. 2008). Moreover, it has been demonstrated that BDL and MP interact with the transcription corepressor TOPLESS (TPL) (Krogan et al. 2012; Szemenyei et al. 2008). The hypothesis that MP behaves as a repressor, when it is in complex with Aux/IAA proteins, would explain the absence of DR5 activity in cells where MP is expressed.

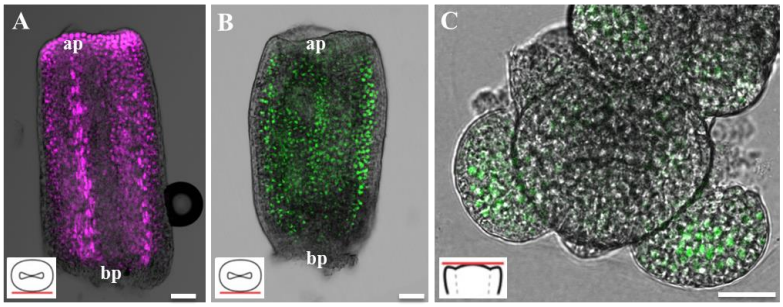

Figure 1: DR5v2:GFP and pMP::MP-GFP expression at early stages of pistil development. (A) DR5v2 is expressed in pro-vasculature and it encircles the apical part (ap) of developing pistil. (B) MP-GFP is expressed in pro-vasculature but not in the gynoecium apical part. (C) Transversal vision from the top confirmed the absence of MP-GFP in the apical part of developing gynoecium. Abbreviation: ap, apical part; bp, basal part. Schematic drawings in bottom left corner indicate the tissue type viewed as in (Larsson et al. 2014). Scale bars, 50 µm.

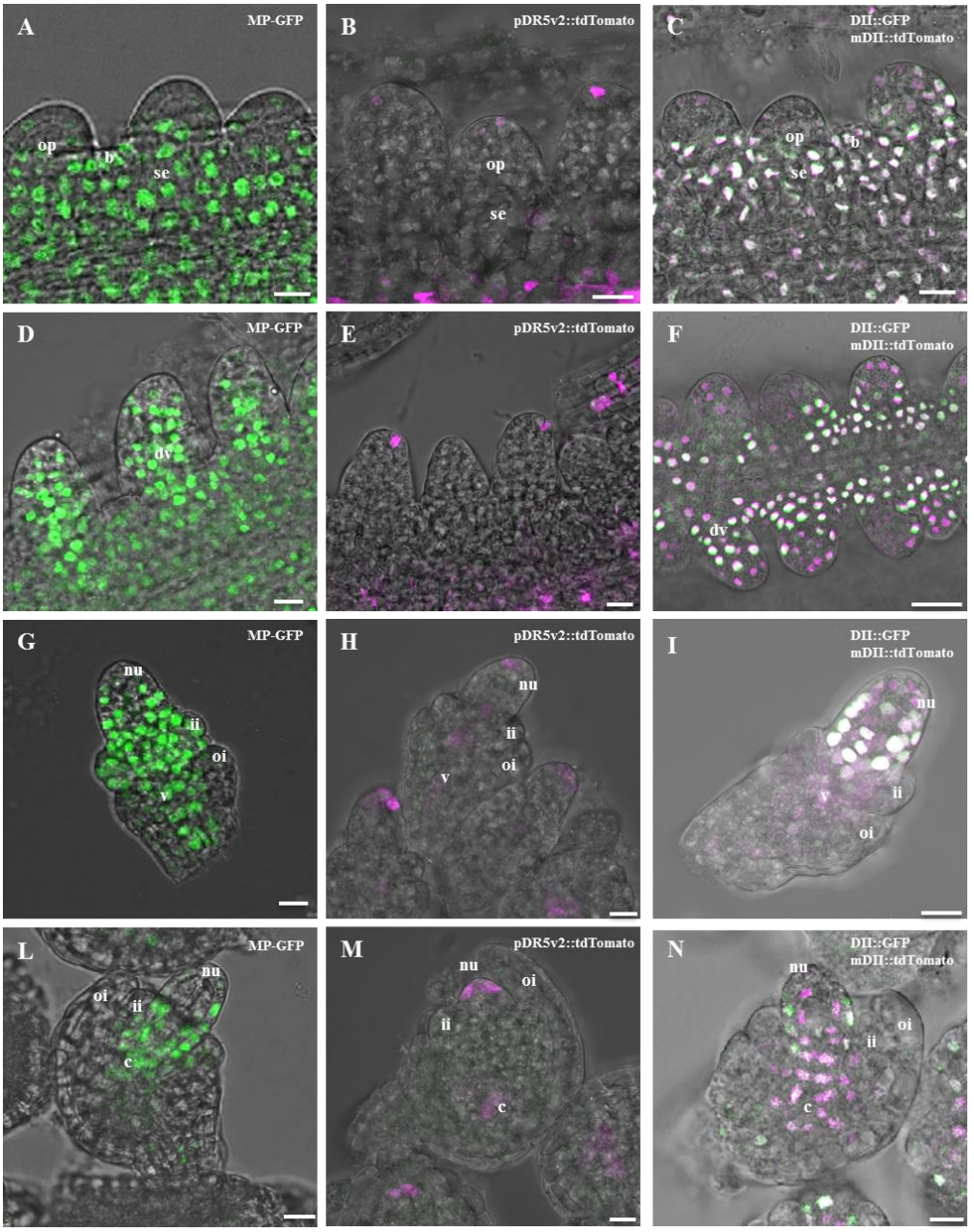

Figure 2: pMP::MP-GFP, DR5v2:GFP and RDR2 expression during ovule development. Reporter lines expression in ovule primordia at stage 1-II (A,B,C), ovules at stage 2-I (D,E,F), ovules initiating integuments at stage 2-III (G,H,I) and ovule at stage 2-V when integuments elongate (L,M,N). Abbreviations: op, ovule primordia; b, boundary; se, sub-epidermal; dv, developing vasculature; nu, nucella; ii, inner integument; oi, outer integument; c, chalaza. Scale bar 10 µm.

MP is involved in pistil development

With the aim of clarify the role of MP during early stages of gynoecium development we performed a detailed morphological characterization of *mp-S319* mutant, which carries a T-DNA insertion at the beginning of the sequence encoding for MP's CTD.

Arabidopsis *thaliana* gynoecium arises as a single primordium, forming an oval growing cylinder. In the adaxial side (inside) of the growing cylinder medial domain develops the carpel margin meristem (CMM) (Smyth et al. 1990; Bowman et al. 1991). In turn, the CMM gives rise to the placenta, ovules, septum and transmitting tract (Reyes-Olalde et al. 2013). Valves and replum differentiate from the abaxial side (outside) of the medial domain (Bowman & Smyth 1999; Roeder & Yanofsky 2006).

Through SEM microscopy, we observed that *mp-S319* emerging gynoecium does not differentiate the abaxial-adaxial and medial-lateral regions. Indeed, it develops as a circular cylinder with a central cavity (comparing Fig. 3A with 3B). DIC microscope images and histological section revealed that *mp-S319* gynoecium lacks all the internal structures, namely placenta, ovules, septum and transmitting tract (Fig. 3F-3I). The other evident effect in *mp-S319* gynoecium is the impaired apical-basal patterning. Stigma develops abnormally while it is not possible to distinguish style, ovaries and gynophore (Fig. 3C-3E). This phenotype is always associated with impaired auxin pathways, in fact it is reported for mutations in the auxin related genes *PIN1*, *PINOID*, *ARF3/ETTIN* and *STYLISH 1* (Okada et al. 1991; Bennett et al. 1995; Sessions et al. 1997; Nemhauser et al. 2000; Sohlberg et al. 2006). The factors responsible of developmental defects observed when this auxin related gene are mutated still need to be investigated.

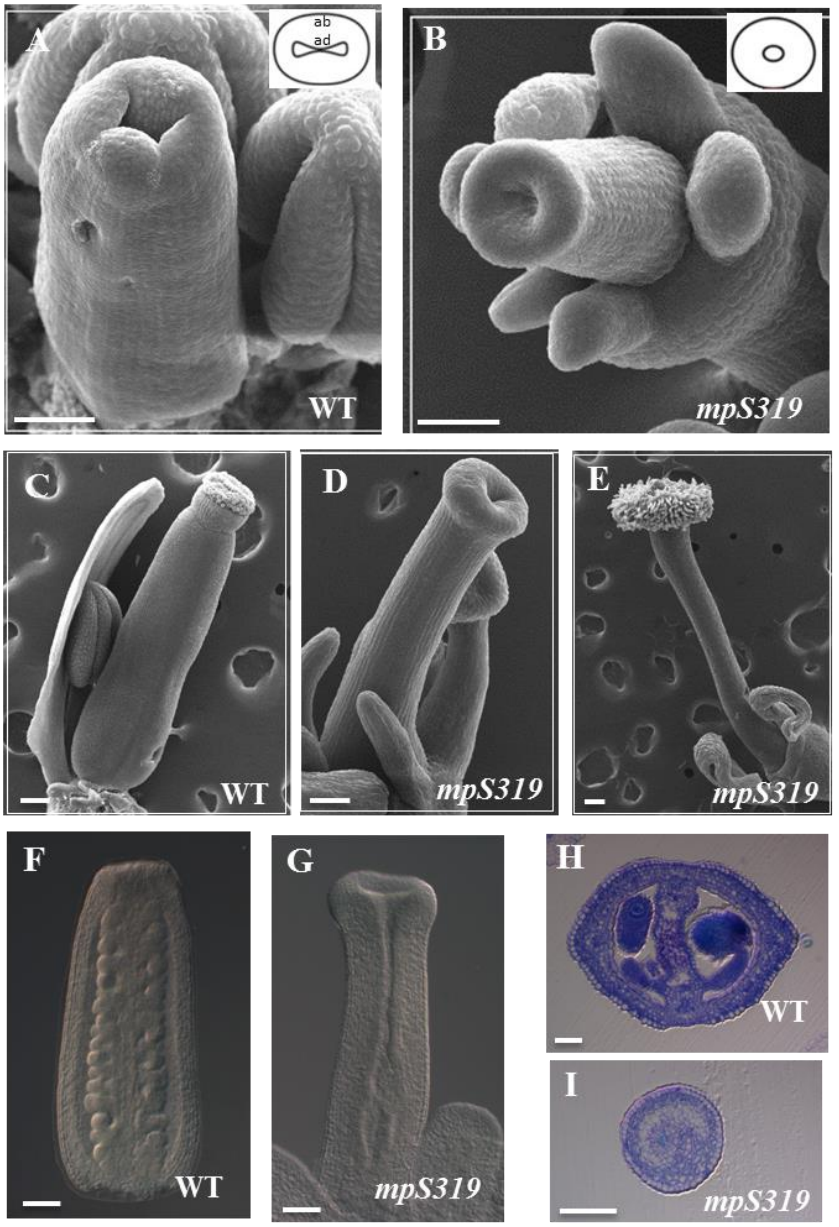

Figure 3: *mp-S319* affects gynoecium patterning

(A,B) A scanning electron microscopic (SEM) of stage 8 wild-type (A) and mp-S319 (B) gynoecia respectively. (C,D) SEM image of a stage 11 wild-type (C) and mp-S319 (D) gynoecia. (D) *mp-S319* mature pistil. (F,G) DIC-microscopy image, taken after clearing protocol, of a stage 11 wild-type (C) and *mp-S319* (D) gynoecia. (H,I) Histological section of a wild-type (H) and *mp-S319* (I) gynoecium at stages 12. Scale bars 100 µm.

Genes controlling adaxial-abaxial fate are deregulated in *mp-S319*

In order to investigate the causes of the radialization of the pistil in *mp-S319* we checked the expression level of several genes known to be involved in the determination of gynoecium patterning.

Adaxial identity in leaves, SAM and gynoecium is primarily conferred by members of the class III homeodomain-leucine zipper (HD-ZipIII) transcription factors *PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA* (Emery et al. 2003; Prigge et al. 2005). On the contrary, abaxial fate specification is mainly carried out by *KANADI* genes, encoding for transcription factors of the GARP family, and several *YABBY* genes (Siegfried et al. 1999; Kerstetter et al. 2001). In parallel with the *KAN* genes, auxin response factor *ARF3/ETTIN* and *ARF4* promote abaxial development (Pekker et al. 2005). Moreover, as already mentioned, mutations of *ARF3* cause severe defects in gynoecium apical–basal patterning, with increased apical regions and strongly reduced ovaries (Sessions & Zambryski 1995; Sessions et al. 1997).

We performed qRT-PCR to evaluate the expression level of these genes in *mp-S319* background. Expression analysis results displayed a strong reduction in the expression of adaxial gene *PHB* and *PHV* in *mp-S319* gynoecium compared to the wild-type (Fig. 4). On the contrary, expression of abaxial gene *KAN2*, *ARF3* and *ARF4* increase in *mp-S319* respect to wild-type (Fig. 4). *KAN2* in particular, is over-expressed up to five time in the mutant than the wild-type (Fig. 4). We do not detected any difference in expression of *REV* and *KAN1*. Up-regulation of abaxial/apical gene fate (*KAN2*, *ARF3* and *ARF4*) and down regulation of the adaxial ones (*PHB* and *PHV*) in *mp-S319* is consistent with the gynoecium phenotype described. *In-situ* hybridization for KAN2 and ARF3 need to be performed to confirm the miss-regulation of these genes in the *mp-S319*.

Furthermore, if *mp-S319* gynoecium defects are caused by miss-regulation of *ARF3* and *KAN2*, *arf3* and *kan2* loss of function mutants in *mp-S319* background should be able to, at least partially, complement *mp-S319* mutant phenotype. We are performing the crosses to test this hypothesis.

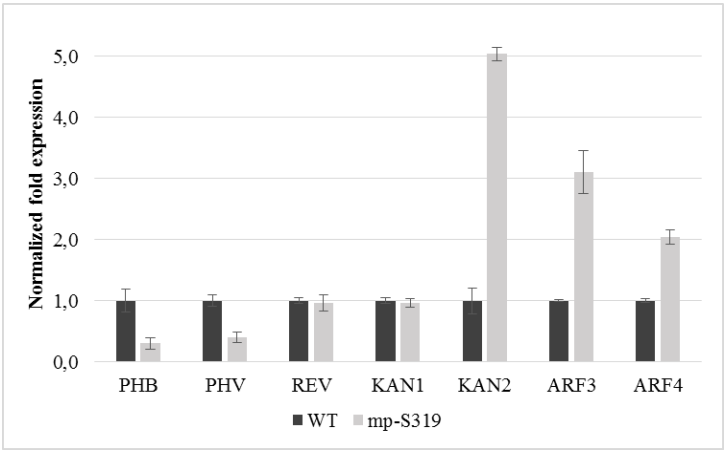

Figure 4: Genes responsible of abaxial-adaxial fate are miss-regulated in *mp-S319*
PHB, *PHV*, *REV*, *KAN1*, *KAN2*, *ARF3*, *ARF4* expression in *mp-S319* mutant pre-fertilized pistils. qRT-PCR results show that *PHB* and *PHV* expression is reduced compared to wild-type, while KAN2, ARF3 and ARF4 expression increase. Error bars indicate s.e. based on three technical replicates. The data were normalized with respect to *actin8* and *ubiquitin10* mRNA levels.

MP overexpression lead to the same gynoecium phenotype of *mp-S139*.

It was already known that in *35S::MP* plants, embryo development is like in the wild type, however plants overexpressing MP produce few and predominantly sterile flowers (Hardtke et al. 2004).

To investigate more in detail this aspect we drive the MP expression under the control of *APETALA1* promoter, which is strongly expressed in inflorescence. We analyzed the phenotype and the MP level of ten different *pAP1::MP* transformant lines. Below, are reported the results for three *pAP1::MP* lines (Fig. 5). The more severe phenotype observed in line n.6 corresponded to the higher level of MP expression (Fig. 5B and 5G).

Pistils of line n.6 have the phenotypical defects described for those of *mp-S319* (compare Fig. 5E with 5F). Line n.11 exhibits an intermediate phenotype, like defect in flower architectures and pistils with reduced valves, and has two-fold MP up-regulation (Fig. 5C). Line n.13, in which flowers and pistils look normal, has wild-type level of MP.

To investigate if the causes of *mp-S319* and *pAP1::MP* gynoecium phenotype correspond, we will investigate by qRT-PCR and *in-situ* hybridization the expression of *KAN2*, *ARF3* and *ARF4*.

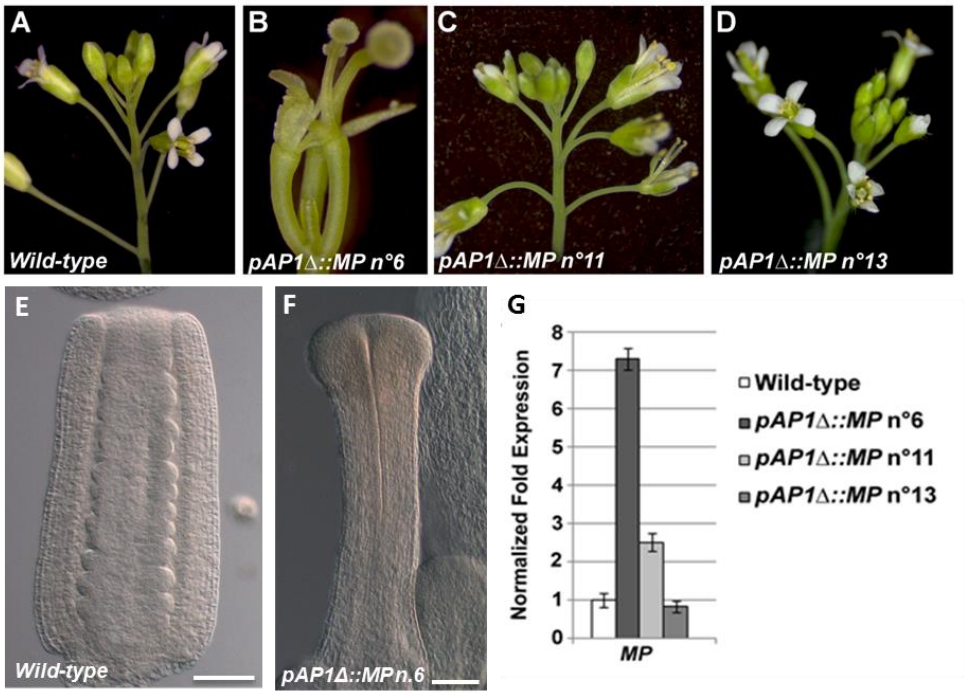

Figure 5: *pAP1::MP* shows the same morphological defects as *mp-S319.*

(A) Stereo-microscopy image of wild-type inflorescence. (B, C, D) Inflorescence of three different lines of *pAP1::MP*. Line n.6 exhibits the strongest phenotype (B), line n.11 has intermediate phenotype (C) and line n. 13 looks like wild-type (D). DIC-microscopy image of wild-type (E) and *pAP1::MP* line n.6 gynoecia at developmental stage 8-9 (F). (G) MP expression analysis, by qRT-PCR, in wild-type and three *pAP1::MP* lines.

Co-expression analysis suggests that MP might be involved in silencing through casiRNA.

To have a general overview of the molecular pathways in which MP is involved we performed a simple co-expression analysis using the ATTED-II tool available online (http://atted.jp/) (Obayashi et al. 2007). Study of co-expression genes relationship could help in the prediction of functionally related genes. The 300 genes highly co-expressed with MP have been grouped on the base of their molecular function. As proof of co-expression analysis validity is the fact that in top positions we found genes already known to be MP direct targets. Specifically they are *ANT*, *TARGET OF MONOPTEROS 5 (TMO5)*, *TMO6* and *HOMEOBOX-8 (ATHB8)*. As expected *MP* is co-expressed with many gynoecium and ovule identity genes. In particular, we have already shown above the positive regulation of MP on *PHV* and *PHB*. In addition, the category of cell cycle was also highly represented.

Interestingly, many players involved in small interfering RNA biogenesis and chromatin remodelling factors are co-expressed with *MP* (Fig. 6 in pink). Among the different small silencing RNA pathways described in plants *MP* co-expressed with *RDR2*, *DCL3*, *HEN1* and *AGO4* which are required for the biogenesis of cis-acting siRNAs (casiRNAs), the most abundant endogenously produced siRNAs in plants (Ghildiyal & Zamore 2009; Holoch & Moazed 2010; Matzke & Mosher 2014).

Biogenesis casiRNAs involved RNA polymerases RDR2 and Pol IV to generate dsRNA precursors, which are then diced by DICER-LIKE 3 (DCL3) to generate 24-nucleotide casiRNAs (Haag et al. 2012). The methyltransferase HEN1 adds methyl modification to the casiRNAs, which then load into ARGONAUTE 4 (AGO4).

HEN1 and *AGO4* promote heterochromatin assembly by targeting DNA methylation and histone modification at the corresponding loci (Zilberman et al. 2003; Boutet et al. 2003). Moreover, *DRD3*, that is also highly co-expressed with *MP*, encodes for the unique largest subunit of nuclear DNA-dependent RNA polymerase V, required for RNA-directed DNA methylation (Kanno et al. 2005). Taken together the bioinformatics co-expression data strongly suggest a link between *MP* and casiRNAs biogenesis pathway.

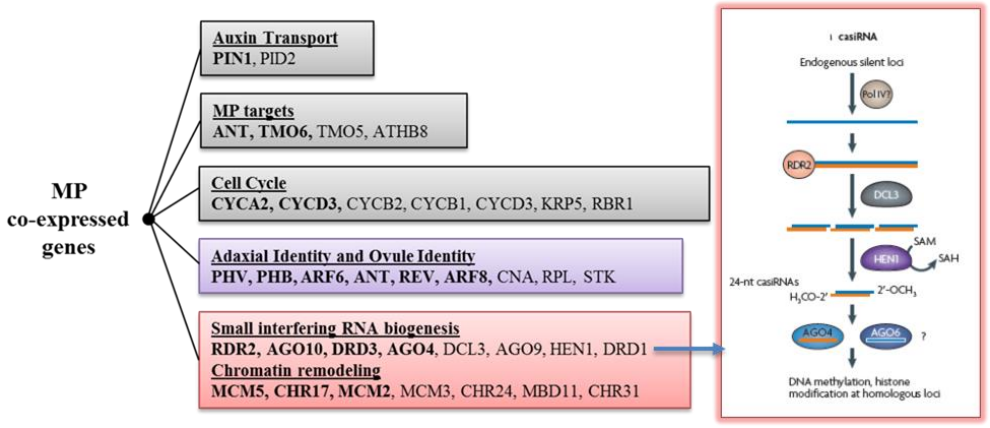

Figure 6: members of casiRNAs pathway are highly co-expressed with *MP*

Schematic representation of genes selected among the 300 *MP* most co-expressed genes and grouped based on their function. The genes were identify using ATTED-II tool. Those in bold are among the top 150 positions. The class of genes involved in casiRNAs biogenesis is particularly represented. In the scheme on the right, modified from (Ghildiyal & Zamore 2009), is represented the molecular pathway involved in casiRNAs biogenesis.

MATERIALS AND METHODS

Plant material and growth conditions

Plants were grown at 22°CC long-day (16 h light/8 h dark) conditions. pMP::MP:GFP *mp-5/mp-5* (Schlereth et al. 2010) and *mp-S319* (Cole et al. 2009) have been described previously. Auxin marker lines DR5v2::GFP and R2D2 (Liao et al. 2015) were kindly provide by Dr. Dolf Weijers.

Scanning electron microscopy

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

Plasmid construction and Arabidopsis transformation

To construct *pAP1::MP*, *MP* genomic fragment was amplified using primers AtP_3628 fw (5'-caccgtgttgagtgtgggagagac-3') and AtP_3629 rev (5'-aaatcggaacaacacatcaaatg-3'), and recombined into vector pB2GW7 through an LR reaction (Gateway system, Invitrogen, www.lifetechnologies.com). The CaMV 35S promoter of the pB2GW7 vector was removed, with restriction enzymes *SacI* and *SpeI* and substituted by the *AP1* promoter. *pAP1* was amplified using primers AtP_3082 fw (5'-ccgagctctcaaaactcaggacgtacat-3') and AtP_3083 rev (5'-ccactagtagctcagactttggtatgaa-3'). This construct was used to transform Col-0 plants using the floral-dip method (Clough & Bent 1998).

Microscopy

To analyse pistil development, flowers at different developmental stages were cleared and analysed as described previously (Brambilla et al. 2007).

Samples were incubated in clearing solution, dissected, and observed using a Zeiss Axiophot D1 microscope equipped with differential interference contrast optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program.

For confocal laser scanning microscopy, dissected pistil were mounted in water and observed with SPE Leica confocal. EGFP was excited at 488 nm and detected at 498–530 nm, tdTomato was excited at 561 nm and detected at 571–630 nm. We used 40x water-immersion objective (numerical aperture = 1.25, pinhole), confocal scans were performed with the pinhole at 1 airy unit.

Images were collected in multi-channel mode, and overlay images were generated using Leica analysis software LAS AF 2.2.0.

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Ringraziamenti

Al termine di questi anni di dottorato desidero ringraziare tutte le persone che mi hanno accompagnato in questo percorso e senza le quali non sarebbe stato possibile realizzare questo lavoro di tesi.

Innanzitutto voglio ringraziare la Prof.ssa Lucia Colombo, per avermi dato la possibilità di lavorare a questo affascinante progetto, e per l'esempio di professionalità e passione per la ricerca che sarà sempre per me un modello da perseguire.

Ringrazio la Dr.ssa Simona Masiero, fonte inesauribile di conoscenze teoriche e pratiche, e la Prof.ssa Eva Benkova, per avermi ospitata nel suo gruppo di ricerca e per aver condiviso idee e conoscenze.

Un grazie di cuore a Chiara, Francesca e Dario perché, anche se le merende sono quasi finite, la vostra amicizia è un tesoro che ha reso speciale questo dottorato.

Ringrazio Marta, amica e collega che con la sua energia mi sprona ad affrontare tutti i misteri di monopteros e delle citochinine . Un grazie ad Andrea, per il suo aiuto ed il suo entusiasmo alla scoperta del magico mondo dell'epigenetica.

Voglio ringraziare tutti i post-doc del gruppo, in particolare Irma, Vicente, Nacho e Sergi per i loro preziosi consigli. Ringrazio Michela ed Elia, per lo scambio di opinioni e le risate. Ringrazio Fra Resentini e Ana Marta due colleghe dolcissime. Ringrazio Stefano e Sara, da cui ho imparato molto all'inizio del mio dottorato. Ringrazio le mie studentesse Nadia e Micol, che oltre ad avermi aiutato nel lavoro mi hanno permesso di migliorare come tutor.

Un grazie gigante a tutta la mia big family, Mamma, Papà, Lalla, Dany, Stefy, Giusy, nonni Matteo e Francesco, zia Lisa, Rosy e Antonio, per aver sempre creduto in me e avermi dato la serenità e l'affetto necessari ad affrontare questo percorso. Ringrazio le mie amiche Giulia e Giulia perché basta una chiacchierata con loro e la giornata migliora.

Ringrazio Marco, per tutto il sostegno e l'amore ricevuto.