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GENETIC AND EPIGENETIC REGULATION OF OVULE DEVELOPMENT AND PLANT REPRODUCTION IN *A. thaliana*

Andrea Guazzotti

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Scientific tutor: Lucia Colombo

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Contents

SUMMARY

In this thesis, I will present four different manuscripts, all focused on phytohormone signaling pathway and epigenetic mechanisms underneath plant reproduction processes.

Firstly, an insight on cytokinin-auxin crosstalk during pistil development will be presented. In particular, we will focus on the role of CYTOKININ RESPONSE FACTORs in placenta elongation, ovule development and *PIN-FORMED 1* (*PIN1*) transcriptional regulation.

The second manuscript will be focused on uncharted mechanisms of regulation of the *AUXIN RESPONSE FACTOR 5/MONOPTEROS* (*ARF5/MP*), such as multiple translational start site and alternative splicing, introducing two new mutant alleles of *MP* generated using CRISPR/Cas9 technology.

In the third and fourth manuscripts, I will discuss the role of the chromatin remodeler HISTONE DEACETYLASE 19 during ovule primordia formation and pollen tube ovular guidance. In this regard, we will take advantage of innovative techniques such as Fluorescence Activated Cell Sorting coupled to RNA sequencing, to explore transcriptomic changes in *hda19* mutant in ovule-specific sorted cells. In the second manuscript, specifically, we will propose a major role for HDA19 in the regulation of MADS-Box transcription factors during ovule primordia formation process. Besides, in the third manuscript, I will characterize the defective pollen tube growth and guidance in the female sporophyte of *hda19*.

PART I

Abstract

In angiosperms, efficient and successful reproductive strategies have been crucial to achieve evolutionary advantages such as genetic adaptability. However, humans, during crop domestication, selected specific traits for agricultural benefits. Understanding the molecular mechanisms associated to agricultural noteworthy traits, i.e. seed yield, is fundamental for genetic improvement of crops.

On top of that, in the present thesis I will discuss four different projects, all related to plant reproduction process, with a particular focus on phytohormones signaling pathway and epigenetic mechanisms in the model plant species *Arabidopsis thaliana*.

Cytokinin has been widely described as a key regulator of plant development and plant reproduction. Hereby, in the first supplied work, we give a hint on the role of *CYTOKININ RESPONSE FACTORs* in auxin-cytokinin crosstalk during pistil development. Moreover, there will be an insight on unexplored molecular mechanisms of regulation of the *AUXIN RESPONSE FACTOR 5/MONOPTEROS* (*ARF5/MP*), such as multiple transcriptional start site and alternative splicing.

Finally, taking advantage of innovative techniques, such as Fluorescence Activated Cell Sorting coupled to RNA sequencing, we have investigated the role of the chromatin remodeler *HISTONE DEACETYLASE 19* during ovule primordia formation process and pollen tube ovular guidance.

State of the Art

Flowering Plants

Angiosperms diverged from gymnosperms around 250 million years ago (mya) and the first flowering plants are dated 16o mya. Flowers, which contain the reproductive organs of angiosperms, are characterized by an immense variability and are considered as a powerful tool for fitness improvement. Angiosperms are the most diverse group of land plants, with 416 families, 13164 known genera and a total of 295383 species. This is one of the reasons why terrestrial ecosystems are widely dominated by flowering plants.

Flower evolution in angiosperms has been triggered by multiple natural breeding mechanisms, such as wind, insects or bird pollinators.

The flower is the structure where sexual reproduction takes place. During life cycle, plants alternate multicellular haploid and diploid generations. The haploid generation is the gametophyte, whereas the diploid generation is the sporophyte. During sporogenesis, mother cells undergo meiosis and generate haploid spores. The spores undergo gametogenesis, which allows the development of the male and female gametophytes. The fusion between the gametes, produced by the gametophytes, will generate the zygote and the secondary endosperm. The following development of the zygote into the adult sporophyte will complete the life cycle.

Arabidopsis thaliana as model plant species

For all experiments performed in this thesis, I used *Arabidopsis thaliana*, an hermaphrodite dicot species belonging to *Brassicaceae* family, largely used as a model plant in molecular biology and functional genomics.

Its advantages for genetic and molecular biology research purposes are multiple. The relatively small genome of 135 megabase pairs is totally sequenced and fully annotated. The genome encode for around 30000 genes and many of them have been characterized. Molecular and functional characterization of model plant species genes has no direct agricultural application, however, modern breeding has taken advantage of the massive amount of literature available. *Arabidopsis thaliana* has been considered as an excellent model system in genetic and molecular studies for multiple reasons: the small size allows an easy growth in laboratories and greenhouses; easily transformed with *Agrobacterium tumefaciens*; short life cycle; high seed yield production.

Arabidopsis thaliana flowers are formed by four sepals and four petals, six stamens and one pistil. On top of the stamens, the anthers produce and hold pollen grains until the anthesis. The pollen grain is the male gametophyte and contains two sperm cells encased in a vegetative cell. The innermost whorl is the pistil, which is composed by the stigma, the style and the ovary (Fig. 1).

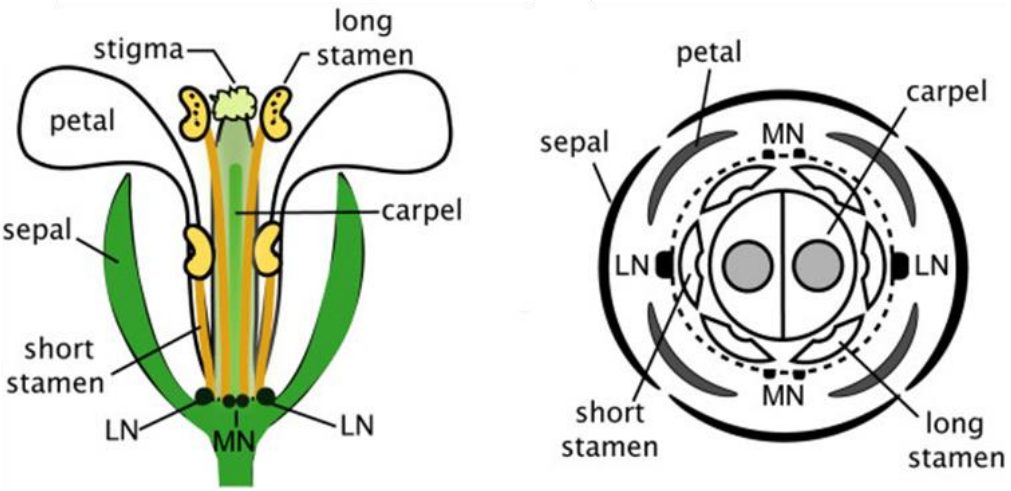

Fig. 1 Schematic representation of the *Arabidopsis thaliana* flower (modified by *Kram* et al., 2009, BMC Plant Biology)

The role of the stigma is to interact with the pollen and let it germinate, if its genotype is compatible with the receptive female reproductive organ. The ovary harbors the ovules, which contain and protect the female gametophyte.

Double Fertilization

After the male and female gametes are properly formed and all the sexual organs have reached the mature stage, reproduction can happen. A fine regulation and an appropriate environment are required for the fertilization to take place and to be successful. The whole process has been named double fertilization since two sperm cells are supposed to fertilize the central cell (2n) and the egg cell (n), giving rise to the triploid cell and the diploid zygote, respectively. The zygote will

then form the embryo whereas the trinucleate cell will develop into the endosperm, whose function is to feed the embryo during its growth.

Pistil and ovule development

The gynoecium, i.e. the pistil, is the female reproductive organ (Fig. 2). In *A. thaliana*, it is composed by two carpels that fuse together during the first phases of development. The lateral margins of the carpels are composed by a meristematic tissue, named carpel margin meristem (CMM). The lateral margins generate three different tissues: the placenta, the septum and the transmitting tract. Just before fusion of the two carpels, ovules arise as organ primordia from the placenta (*Cucinotta* et al., 2014; *Reyes-Olalde* et al., 2013; *Schneitz* et al., 1995). Ovule primordia are formed firstly with periclinal cell division from the sub-epidermal tissue of the placenta, subsequently followed by anticlinal divisions. Three different regions will then compose the developing ovule, the funiculus, the chalaza and the nucellus.

Within the nucellus, female gametophyte development takes place and it will eventually form the embryo sac. Female gametophyte development can be described in two key phases: megasporogenesis and subsequently megagametogenesis (Fig 3) .

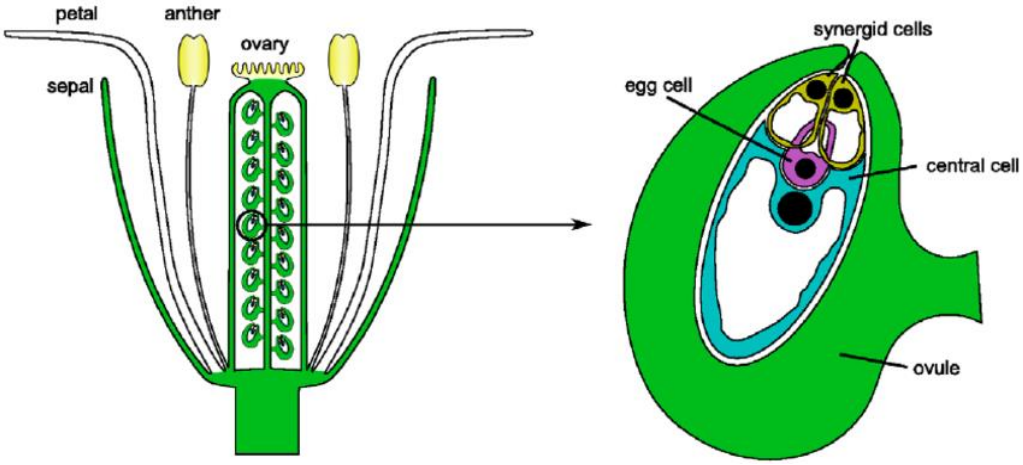

Fig. 2 Schematic representation of the pistil and the mature ovule (modified by *Schmid* et al., 2012, PLOS One)

Initially, one megaspore mother cell (MMC) differentiates into the nucellus and undergoes meiosis. The MMC, thus, generates four haploid megaspores, although after megaspore selection, only the functional megaspore will survive while the other three megaspores undergo apoptosis and degenerate (Fig. 3).

During megametogenesis, the functional megaspore undergoes three mitotic divisions to form the mature female gametophyte (Fig. 3). In Arabidopsis, the female gametophyte is composed by three antipodal cells, one central cell, one egg cell and two synergid cells (Fig. 2).

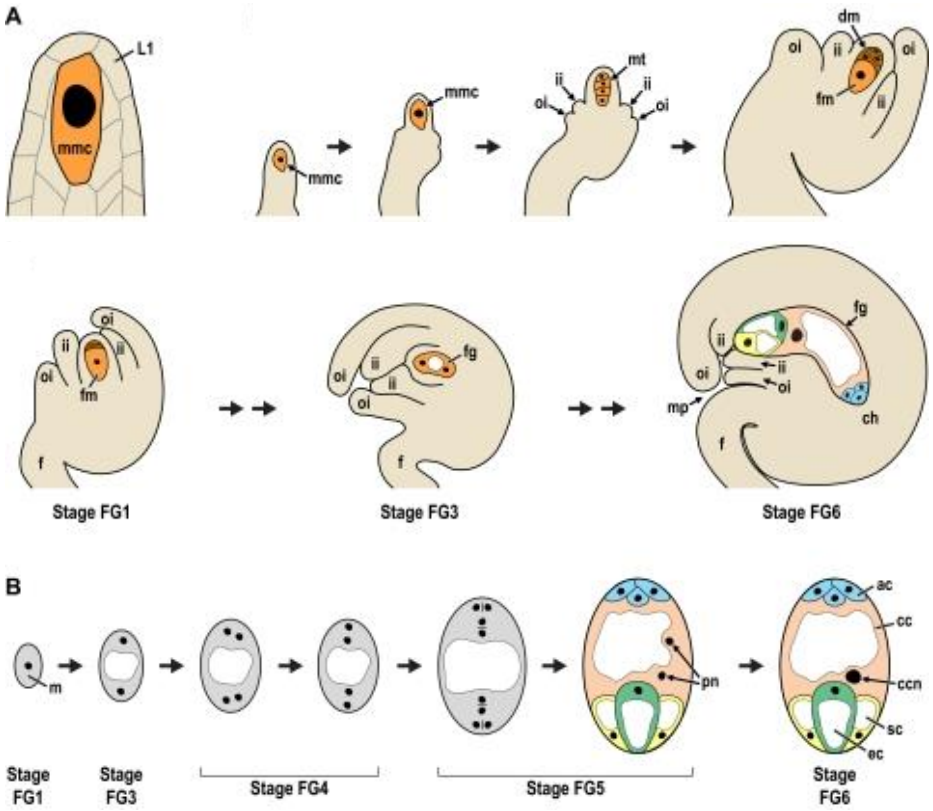

Fig. 3 Ovule and female gametophyte development (modified by *Drews* et al., 2011, Arabidopsis Book)

At the same time of the female gametophyte development, the chalaza region of the ovule primordium will give rise to the integuments. *Arabidopsis thaliana* mature ovules are composed by five integuments, of which the innermost is called endothelium.

Eventually, the funiculus develop independently and will establish a connection between the ovule and the mother plant.

Pollen tube guidance

Double fertilization starts with the pollination of the stigma. The process of pollen tube guidance is extraordinarily important during sexual plant reproduction. The pollen grains stick on the papillae of the stigma and germinate. Subsequently, after germination, the formed pollen tube, which encases the two sperm cells, penetrates the style, goes through it and enters the transmitting tract. Amazingly, pollen tube speed rate, at this stage, is 1 centimeter per hour (in maize) (*Barnabas* and *Fridvalszky*, 1984; *Krichevsky* et al., 2007).

Pollen tube growth in the pistil has been divided in two main phases. The first one is called preovular guidance and is identified as the initial addressing of pollen tubes towards the ovary. In the first phase of pollen tube attraction, the preovular guidance, pollen tubes are attracted indistinctly by the ovary and the key players of the attraction are cells belonging to the female sporophyte, such as style and transmitting tract cells (Fig. 4, Fig. 5, Fig. 6).

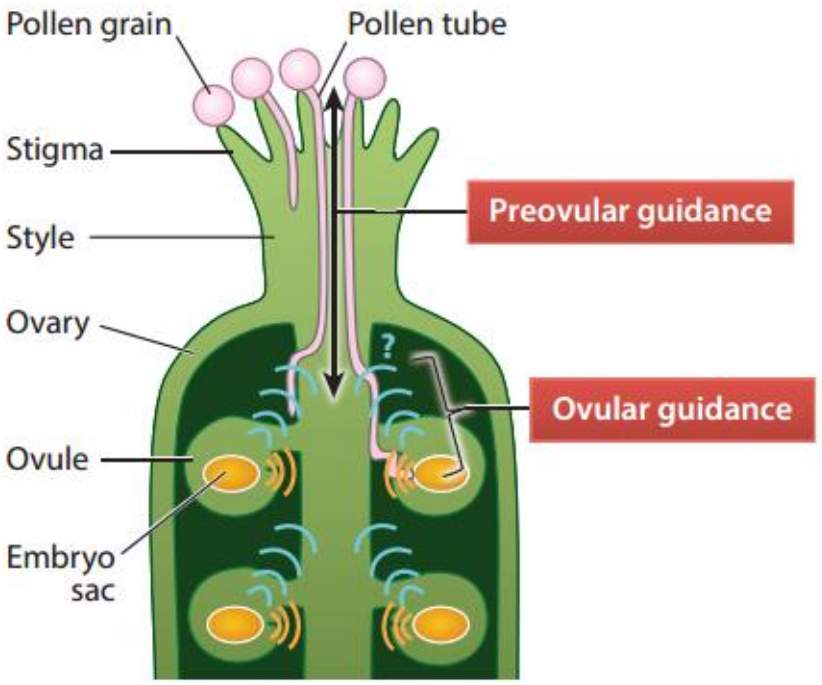

Fig. 4 The mechanism and key molecules involved in pollen tube guidance (modified by *Higashiyama* et al., Annu. Rev. Plant Biol. 2015)

The mechanisms by which the pollen tube grows and targets the ovules is so far blurry and it requires further clarification. However, it is known that a steep calcium gradient on the tip of the pollen tube is required and sufficient for its orientation and the apical elongation. In fact, usual concentration of calcium in cytosol is around 100 nM, whereas in the area nearest to the growing tip (a few micrometers) calcium concentration dramatically rises up to several micromoles (*Holdaway-Clarke and Hepler*, 2003; *Malhó* et al., 1994; *Krichevsky* et al., 2007).

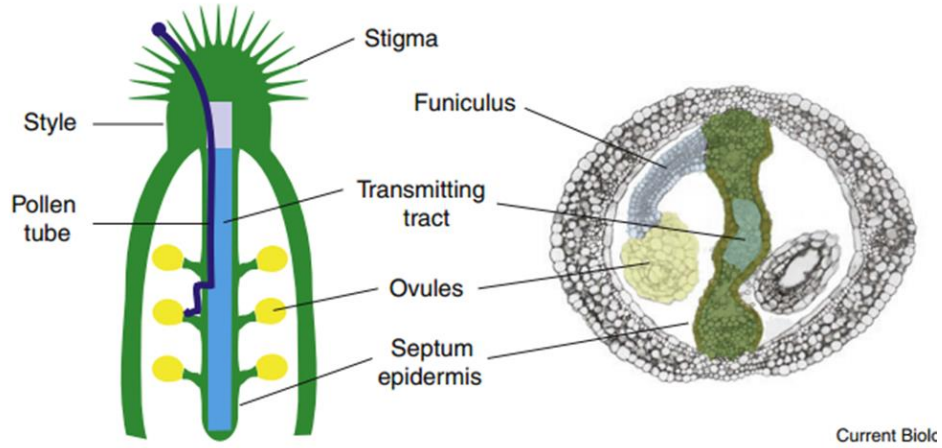

Fig. 5 The transmitting tract (modified by *Crawford* et al., 2008)

The pollen tube grows in the transmitting tissue until it reaches the proximal end of the pistil thanks to the calcium gradient. This process takes about a few hours.

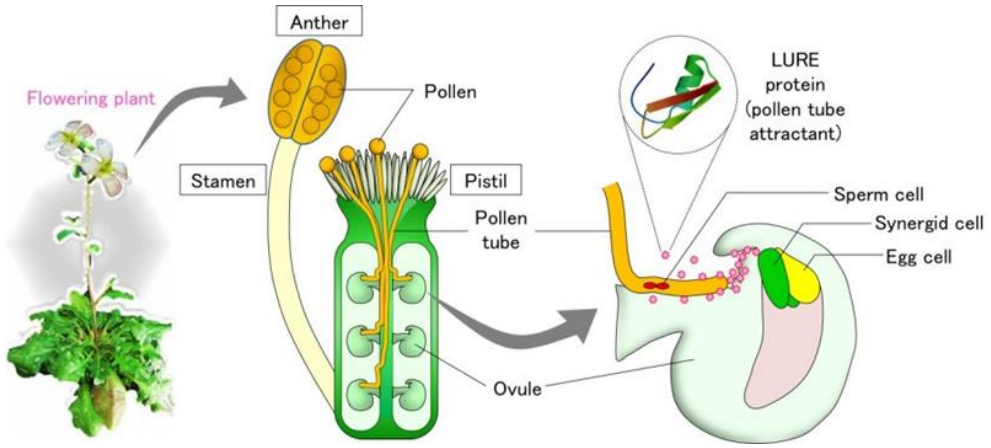

Fig. 6 (Image courtesy of Transformative Bio-molecules, Nagoya University)

Such process is amazingly noteworthy, since the sporophytic tissue is usually associated to the interspecific barriers. In fact, female sporophytic tissues are important for species recognition, self/nonself-recognition and competency of pollen tubes.

It has been indeed demonstrated that genetic removal of transmitting tract, with the subsequent reduction of transmitting tract cell number, decreased interspecific barriers (*Higashiyama* et al., 2015; *Crawford* et al., 2007; *Smith* et al., 2013).

Nonetheless, the mechanisms and the signal molecules of the preovular guidance still remain unclear and need to be elucidated. Chemotropic and mechanical guidance equally contribute to this process.

Known molecules involved in pollen tube preovular guidance are chemocyanin and arabinogalactan proteins, although their role remains blurry.

Other small molecules demonstrated to stimulate pollen tube growth are γ-aminobutyric acid (GABA) through Ca2+-permeable membrane channels of the pollen tube and brassinosteroids (*Ling* et al., 2013; *Palanivelu* et al., 2003; *Yu* et al., 2014; *Vogler* et al., 2014).

Moreover, a CLAVATA3/ESR related peptide of *Arabidopsis thaliana*, CLE45, has been published to push pollen tube growth by interacting with STERILITY-REGULATING KINASE MEMBER 1 (SKM1) and SKM2 (*Endo* et al., 2013).

Eventually, arabinogalactan proteins, as already mentioned above, are proven key players of pollen tube preovular guidance and demonstrated to be in the extracellular matrix of female sporophytic tissues alongside the entire pathway

of pollen tubes (*Coimbra* et al., 2007; *Higashiyama* et al., 2010; *Suarez* et al., 2013).

In the transmitting tract, the pollen tube travels in-between the apoplast, the intercellular space between cell walls (Fig. 5).

After preovular guidance, the pollen tube starts its journey to the ovule micropyle as final destination, through sensing long distance signal coming from the female gametophyte (Fig. 4, Fig. 5, Fig. 6).

So far, little is known about the process of pollen tube deflection towards the ovules. However, recently, it has been proposed a key model for pollen tube guidance based on signal molecules synthesized and secreted by the female gametophyte.

After the pollen tube enters the ovary, the second phase of the pollen tube journey begins, the ovular guidance. The literature about ovular guidance and the mechanisms underneath it are slightly wider and better known in respect to pollen tube activation and preovular guidance.

Chemoattractant molecules necessary for ovular guidance are mostly known to be synthesized by the female gametophyte cells.

As opposed to preovular guidance, during ovular guidance each ovule attracts a single pollen tube and independently addresses its growth and targeting.

A well-known and characterized family of signal peptides crucial for this process are cysteine rich proteins named LUREs, identified in *Torenia fournieri* (Fig. 6).

LUREs are synthesized in the synergid cells under genetic regulation of MYB98. They are present in the ovary in a very close area around the micropyle but also in the funiculus and the septum as demonstrated by immunostaining (Fig. 7).

MYB98 is a transcription factor required for the differentiation of synergid cells. *myb98* mutant has a defective pollen tube guidance and lack of differentiation of the two synergid cells (*Kasahara* et al., 2005).

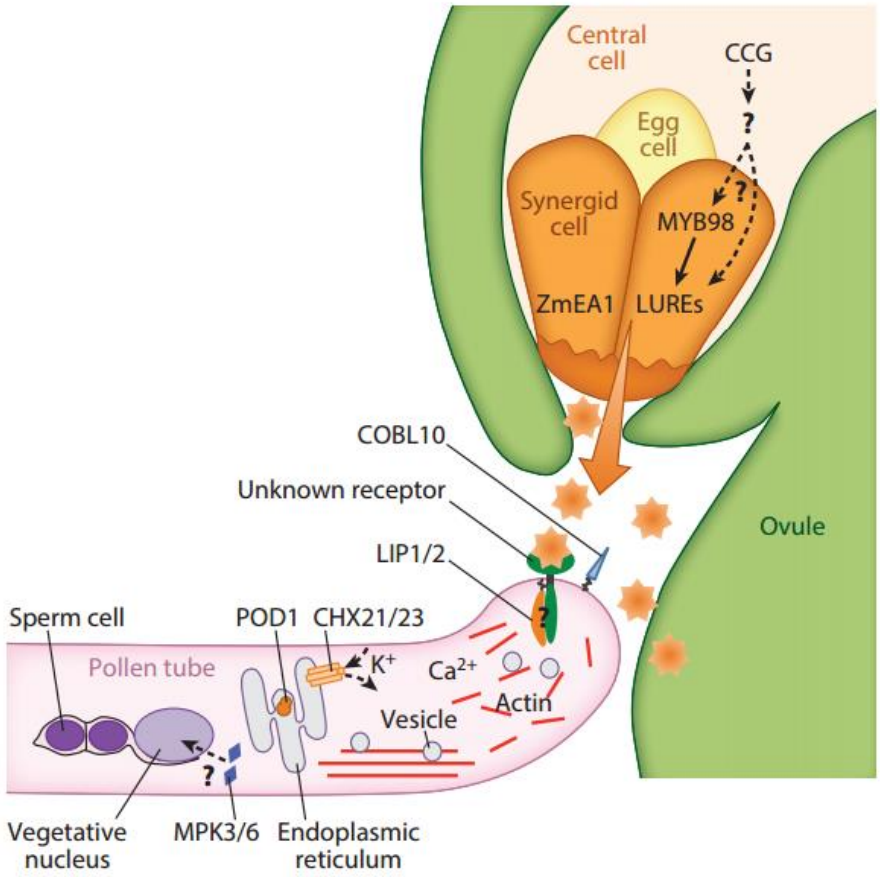

Fig. 7 The mechanism and key molecules involved in pollen tube guidance (modified by *Higashiyama* et al., Annu. Rev. Plant Biol. 2015)

Moreover, egg cell synthesized peptides have been demonstrated to be important for pollen tube attraction in maize (*Màrton* et al., 2005). *Z. mays* EGG APPARATUS 1 (ZmEA1), in fact, is essential for pollen tube attraction in the micropylar region (Fig. 7).

Ovule and seed number

Ovule development is a key and crucial step for plant reproduction and for total seed yield determination. In fact, total seed number is by definition linked and restricted to ovule number, since ovules, upon fertilization, give rise to seeds. The utter ovule primordia number is determined by the total length of the placenta and by the mean distance between them, which can be termed as ovule density. In literature, little is known about ovule primordia development and the key players of this process yet remain to be identified.

Ovules arise as primordia during early stages of gynoecium development. The very first step that originates the ovule formation is the carpel margin meristem (CMM) development in the pistil. The CMM develops in three different structures, the placenta, which will be involved in the ovule primordium initiation, the septum and the transmitting tract, which will be involved in the pollen tube growth and guidance later on.

The placenta is the meristematic tissue where ovule primordia arise in a poorly characterized mechanism which involves auxin and other phytohormones coupled to a fine genetic regulation.

In the past years, by classic forward genetic approach, several mutants which display a reduction in total ovule number have been found.

A remarkable loss of ovule formation has been noticed to be in combination with a defective carpel margin meristem (CMM) development.

For instance, knock out or loss of function alleles for *AINTEGUMENTA* (*ANT*) and *LEUNIG* (*LUG*) (*Klucher* et al., 1996; *Elliot* et al., 1996; *Liu* et al., 2000) have partially disrupted CMM and strong ovule number reduction. ANT is a transcription factor containing AP2 domains which is fundamental and necessary for organ initiation and is a promoter of cellular division during organ development. In severe *ant* mutants, such as *ant-9*, carpels do not fuse properly and total ovule number decreases (*Liu* et al., 2000). The severity of *ant* phenotypes are dramatically enhanced in multiple mutants, such as *ant revoluta* (*rev*), (*Nole-Wilson* et al., 2010), *ant lug* (*Liu* et al., 2000) and *ant seuss* (*seu*) (*Azhakanandam* et al., 2008).

LUG is a floral organ identity gene whose function is transcriptional co-repression. Both intermediate and strong alleles of *LEUNIG* single mutants display defective carpel fusion and significant ovule number reduction (*Liu* et al., 2000). Double mutant *ant leu* is noteworthy severe, as it is not able to form placenta and therefore any ovules.

Further, two NAC transcription factors, CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 have been proven to promote CMM development (*Aida* et al., 1997; *Ishida* et al., 2000; *Galbiati* et al., 2013). Single *cuc1* and *cuc2* mutants do not show any ovule number phenotype, although double mutant *cuc1 cuc2* and either *cuc1* or *cuc2* combined with *spatula* mutation have been shown to have a reduced ovule primordia number (*Nahar* et al., 2012; *Galbiati* et al., 2013).

Key players of ovule initiation process are necessarily plant hormones, such as auxin and cytokinin (*Bartrina* et al. ,2011; *Bencivenga* et al., 2012; *Huang* et al., 2012; *Galbiati* et al., 2013; *Cucinotta* et al., 2014).

The role of phytohormones in plant reproduction and in ovule primordia initiation

As any other developmental process, plant reproduction and flower organs formation needs a fine regulation tuned by phytohormones.

In the present work, there will be a particular focus on cytokinin, auxin and transcription factors involved in the signaling transduction of these two plant hormones.

The role of auxins

Auxins are very important hormones in plants and they have been shown to play a pivotal function in several developmental processes during whole plant life cycle. The most abundant auxin molecule is the indole-3-acetic acid (IAA) and has been isolated for the first time by Thimann (*Went and Thimann*, 1937).

Auxin signal cascade can be divided in three major phases: auxin metabolism, directional auxin transport and signal transduction (*Chapman* and *Estelle*, 2009; *Petràsek* and *Friml*, 2009; *Zhao* et al., 2010; *Ludwig-Muller* 2011; *Hayashi*, 2012).

Auxin biosynthesis and transport are necessary for the establishment of auxin gradients, which are essential to carry out its biological function.

IAA is biosynthesized starting from tryptophan via indole-3-pyruvate (IPA) pathway.

Auxin is directionally transported between cells through symplastic and apoplastic routes, thanks to the large number of auxin influx and efflux carriers (*Grones* and *Friml*, 2015).

Signal transduction and response in terms of transcriptional regulation is the most complex and therefore the less understood pathway in auxin signaling (*Li* et al., 2016).

The key players of auxin-regulated gene expression are the auxin response factors family (ARFs) and the Aux/IAA repressors (*Li* et al., 2016).

ARFs are known to be transcriptional regulators which bind auxin response elements on DNA (AuxREs), present into the promoter of target genes. AuxRE elements are a conserved consensus sequence, which is TGTCTC (*Guilfoyle* et al., 2007) and they are present in the cis-regulatory regions of ARF targets (*Ulmasov* et al., 1995; *Guilfoyle* & *Hagen,* 2007).

AUXIN RESPONSE FACTORs are made by modular domains, which can be considered as separate elements with specific functions.

Although ARFs, in *A. thaliana*, have been grouped in six classes on the basis of the domain composition, all of them have a DNA-binding domain (DBD) at the amino-terminal of the protein. Most of the ARFs has also a middle region (MR) (only ARF23 does not have the MR, fig. 8) which can be an activation or a repression domain (AD or RD) and a dimerization domain (CTD) at the carboxy-terminal of the protein, required for dimerization with the Aux/IAA (*Ulmasov* et al. 1997; *Ulmasov* et al. 1999; *Guilfoyle* et al., 2001; *Tiwari* et al., 2003).

Only ARF3, ARF13, ARF17 and ARF23 do not have the CTD (Fig. 8).

The middle region of ARFs is either rich in glutamine, serine and leucine in case of the Activation Domain or enriched in serine, proline, leucine and glycine residues in case of Repression Domain (*Guilfoyle* et al., 2007).

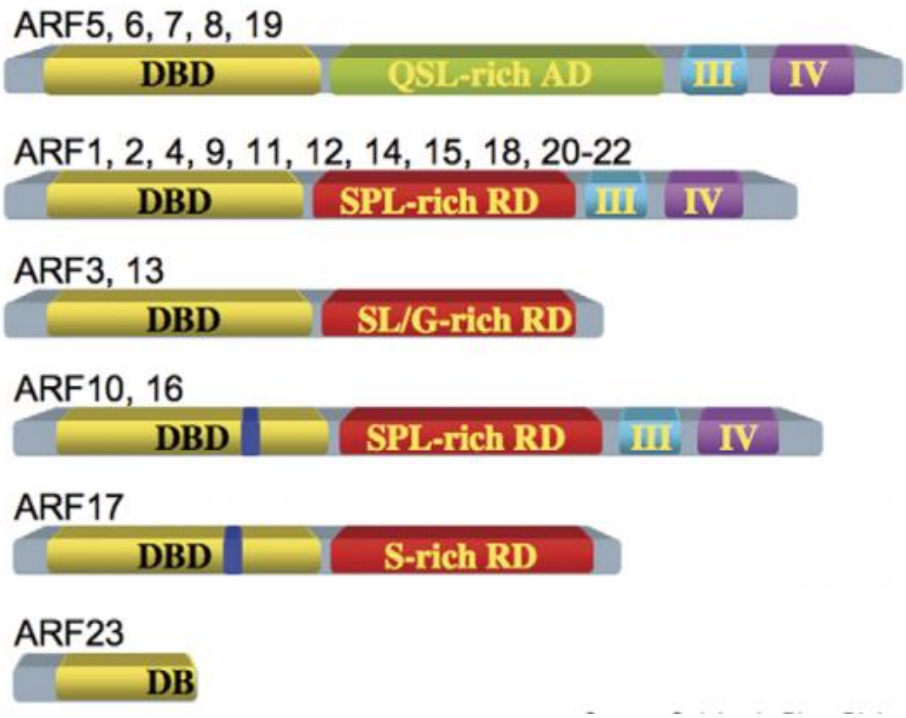

Fig. 8 Composition of ARFs (modified by *Guilfoyle* et al., 2007)

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). In fact, in cells with high concentration of auxin, Aux/IAA repressors are degraded via ubiquitin-

proteasome pathway, whereas in cells with low concentration of auxin, Aux/IAA are free and associate with ARFs, preventing them to become transcriptional activators (*Tiwari* et al., 2001, *Tiwari* et al., 2003; *Wang* et al., 2013).

During the past years, genetic studies have revealed the fundamental role of the 22 annotated ARFs in several developmental processes, spread during all stages of plant life. *ARF5/MONOPTEROS* (*ARF5*/*MP*) has important functions in many aspects of plant development, such as 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).

Although several *monopteros* mutant alleles have been reported in literature, the correlation between the phenotypes and the severity of the mutations appears to be far from being elucidated. Further, the multiplicity of phenotypes observed in the available mutant collection is not sufficient for an easy and clear comprehension of the molecular mechanism controlled by MP. In fact, the mutant phenotypes surprisingly do not correlate with the residual MP function (*Odat* et al, 2014). Claimed strong alleles display high percentage of embryo defects, with lack of primary root or formation of a single cotyledon (*Hardtke* & *Berleth* 1998; *Berleth* & *Jürgens* 1993). Mutant alleles with intact DBD but impaired middle region and CTD, named as *mpS319*, *mp-g92* and *mp-T730*, show low percentage of embryo lethality and vascular system defects (*Przemeck* et al. 1996). In *mpS319*, the number of flowers is greatly reduced, pistil lack valves, placenta and consequently ovules (*Cole* et al. 2009; *Galbiati* et al. 2013).

Role of auxin in ovule primordia formation

Auxin plays a pivotal role in placenta and ovule primordia formation. Impaired auxin biosynthesis or transport causes severe defects in pistil and ovule development (*Nemhauser* et al., 2000; *Nole-Wilson* et al., 2010).

As well as any other organ formation in plants, ovule primordium genesis needs a precise definition of a cell proliferation zone and boundaries at the edge of it (*Cucinotta* et al., 2014). The boundaries are usually featured by a reduced cell division and expansion (*Aida and Takada*, 2006).

A few genes are known to regulate boundary establishment in the very first phases of organ formation, among which the *CUP SHAPE COTYLEDON* (*CUC*) gene family (*Aida* et al., 1997). It has been previously reported how CUC transcription factors are necessary for organ boundary establishment of cotyledons (*Aida* et al., 1997). Indeed, in vitro regenerated double mutant *cuc1 cuc2* show a defective ovule outgrowth and development, reflecting into a dramatic reduction of ovule number (*Ishida* et al., 2000). Moreover, *cuc2* mutant combined with RNA interference of *CUC1* under ovule specific *SEEDSTICK* (*STK*) promoter show less ovule primordia and more distant between each other, indeed ovule density is highly reduced (*Galbiati* et al., 2013).

As widely demonstrated (*Cucinotta* et al., 2014; *Benkovà* et al., 2003; *Bencivenga* et al., 2012; *Galbiati* et al., 2013;), auxin is a key player for ovule primordia formation. Indeed, to emerge, ovule primordium requires the production of an auxin maximum in the placenta, where organ founder cells will be able to initiate the newborn ovule. Auxin maximum is generated via PIN efflux carrier, in particular PIN1 protein, which is specifically localized at the membrane of placenta cells (*Benkovà* et al., 2003; *Ceccato* et al., 2013). On top of that, knock

out allele of *PIN1* causes a reduction of total ovule number per pistil (*Bencivenga* et al., 2012; *Galbiati* et al., 2013).

Although the lack of knowledge, it has been recently proposed, demonstrated and reviewed a genetic model for ovule primordia initiation (Fig. 9) (*Galbiati* et al., 2013, *Cucinotta* et al., 2014).

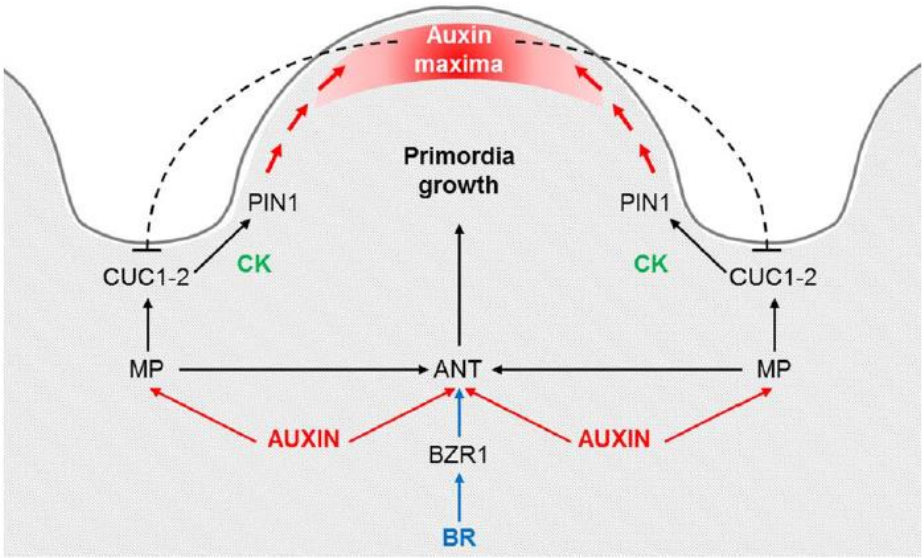

Fig. 9 Proposed model for ovule primordia formation. (modified by *Cucinotta* et al., 2014)

After the primordium arises, SEPALLATA3 (SEP3) together with either SHATTERPROOF1 (SHP1), SHP2 or SEEDSTICK (STK) determine ovule identity fate (*Pinyopich* et al., 2003; *Favaro* et al., 2003).

The role cytokinin in ovule development

Cytokinins (CK) are a broadly expressed class of plant hormones whose primary role is cell growth and differentiation, though they have many other secondary roles in developmental processes, such as ovule development (*Cucinotta* et al., 2014).

After CK biosynthesis, CK hit signal transduction cascade, which is composed by two main pathways: histidine protein kinases (AHKs) and histidine phosphotransfer proteins (AHPs). AHKs perceive CK signal, whereas AHPs convey the signal to the nucleus, where in turns, nuclear response regulators (ARRs) eventually perceive the signal and modulate the response (*Hwang* et al., 2012). Triple mutants of *AHKs* have been shown to cause a drastic reduction in ovule number (*Bencivenga* et al., 2012).

CK oxidase/dehydrogenase are key players of CK catabolism. Double mutant of *CKX*, as *ckx3-1 ckx5-1*, increased the meristem activity with multiple effects on the phenotype. Notably, the meristem activity was boosted also in the placenta. As a consequence, the double mutant displayed pistils with twice as many ovules as wild type (*Bartrina* et al., 2011).

Moreover, inflorescences treated with synthetic CK (6-Benzylaminopurine, BAP) result in an increase in ovule primordia number (*Bencivenga* et al., 2012; *Galbiati* et al., 2013).

Cytokinin has been demonstrated to be meaningfully relevant in ovule primordium formation therefore in determining total ovule number (*Kinoshita-Tsujimura* and *Kakimoto*, 2011; *Riefler* et al., 2006; *Werner* et al., 2003).

The role of epigenetic regulation in ovule formation and function

Plants are a suitable model for studying epigenetic mechanisms. In fact, plants produce new organs during all their life cycle, exploiting self-sustaining stem cells called meristems. Therefore, epigenetic regulation of postembryonic development is much more significant in plant kingdom (*Pikaard* et al., 2017).

Although a full and comprehensive knowledge of all epigenetic modifiers is far from being achieved, major DNA and chromatin remodeler classes have been discovered until now. They can be divided into two major categories, i.e. direct DNA modification and histone post translational modification.

Hereby we will focus on gene transcription epigenetically modulated by chromatin remodelers through histone post translational modification, since we will propose new functions during ovule development and pollen tube guidance for HISTONE DEACETYLASE 19 (HDA19), whose molecular task is transcriptomic repression of its targets.

Histone-modifying enzymes are several and belong to different classes. Usually they act antagonistically and reversibly on a specific aminoacidic residue, very often lysine, located in the histone tails, accessible to the histone-modifying complexes.

Histone acetylation and deacetylation is mediated by histone acetyl-transferases (HAT) alongside histone deacetylases (HDAC).

HATs transfer acetyl group from acetyl-CoA to histone lysine residues. Acetylation of lysines neutralize the positively charged histone tails, which reduce their affinity to negatively charged DNA (*Hong* et al., 2003).

Chromatin with higher lysine acetylation promotes the recruitment of chromatin remodelers that are able to favor the binding of RNA polymerase, therefore transcription and gene activation (*Bannister* and *Kouzarides*, 2011).

Histone acetylation is known to be involved in many key developmental processes of the plant, such as regulation of cell cycle, hormone signaling cascade and flowering time (*Chen* and *Tian*, 2007; *Luo* et al., 2012).

The target of acetylation are N-terminal lysine aminoacids belonging to H3 and H4 histone components. So far, in *A. thaliana*, eleven lysine residues have been identified to be acetylated, six on H3 (K9, K14, K18, K23, K27 and K36) and five on H4 (K5, K8, K12, K16 and K20) (*Berr* et al., 2011; *Mahrez* et al., 2016). These data have to be considered temporary and we can reasonably assume that new residues will be uncovered in the next future.

As well as histone (de)acetylation, histone methylation is a reversible epigenetic mark and includes very large family of genes encoding enzymes involved in this process.

Eventually, also chromatin phosphorylation and ubiquitination are two important post-translational modification marks, although they are less studied.

Nearly all epigenetic modifications influence each other, making the mechanisms underneath epigenetic marks more complex to understand. In fact, they are not only antagonistic but also cooperative.

To date, a few examples of the involvement of epigenetics during ovule development have been given. Indeed, the mutant of *ASH HOMOLOG2* (*ASHH2)* shows 80 % of ovule less than wild type (*Grini* et al., 2009). ASHH2 is a member of the ASH1 protein family, which is known to methylate lysine residues of chromatin histone tails.

Also, *Duszynska* and colleagues demonstrated that selfed F1 triploids of different *A. thaliana* genotypes show variability in total ovule number trait (*Duszynska* et al., 2013). The involvement of parental genome excess in those hybrid plants suggest that epigenetic factors may indeed have a function in ovule formation and total ovule number determination.

Aim of the Project

Two primary final achievements will be discussed.

The first aim will be unveiling and updating the state of the art for signaling transduction of cytokinin and auxin during ovule development with a particular focus on the AUXIN RESPONSE FACTOR 5/MONOPTEROS (ARF5/MP) and the CYTOKININ RESPONSE FACTORs (CRFs).

Hereafter, it will be suggested a new role for epigenetics in the context of ovule development and function through a deep characterization of *histone deacetylase 19* mutant (*hda19*).

All experiments have been performed on the widely known plant model system *Arabidopsis thaliana*.

Main Results

CRFs are required for placenta elongation and ovule development

CYTOKININ RESPONSE FACTORS (CRFs) are encoded by closely related members of the Arabidopsis *AP2* gene family and mediate a large proportion of the CK transcriptional response that functionally overlaps with the B-type ARR-mediated response (*Rashotte* et al., 2006).

We have analyzed *crf2*, *crf3* and *crf6* single, double and triple mutants. Ovule counts were performed on ovules from stage 1-II (primordia) to stage 2-I (finger-like), which corresponds to stages 9 and 10 of pistil development [according to *Schneitz* et al. (1995) and *Roeder* and *Yanofsky* (2006)]. Analysis of single *crf3* and *crf6* mutants, as well as the *crf3 crf6* double mutant, did not reveal any significant difference in ovule number compared with wild type, whereas the single *crf2* and the double *crf2 crf3* mutant showed a small but significant decrease in ovule number.

In the *crf2 crf3 crf6* (*crf2/3/6*) triple mutant, a reduction of 31.68% in ovule number was observed compared to the wild type.

Wild type plants treated with the synthetic cytokinin 6-benzylaminopurine (BAP) yielded 60% more ovules and a 58% longer placenta than untreated plants. The *crf2/3/6* triple mutant treated with BAP produced 19% more ovules and an increase in placenta length of 32%, indicating that the capacity to respond to CKs is strongly reduced in the absence of CRF2, CRF3 and CRF6 activities.

CRFs regulate *PIN1* transcription during pistil growth

In the *crf2/3/6* triple mutant, *PIN1* expression was significantly lower than in the wild type and, interestingly, the level of *PIN1* mRNA in the *crf2/3/6* mutant did not increase upon CK application, suggesting that CRFs are required for CK-dependent *PIN1* expression.

We analyzed plants carrying a *ΔPIN1::PIN1-GFP* construct in which a *PIN1* promoter lacking the *PCRE1* element drives the expression of a fully functional PIN1-GFP fusion protein. Real-time qPCR experiments were performed on *GFP* instead of *PIN1* in order to avoid the detection of endogenous *PIN1*. The level of *PIN1-GFP* transcripts in *ΔPIN1::PIN1-GFP* inflorescences was lower than that in plants carrying the same fusion protein construct under the control of a wild type version of the *PIN1* promoter. The reduction in *PIN1-GFP* expression under control of the *ΔPIN1* promoter was also evident by confocal microscopy in placenta cells and ovule primordia at stages 1-I and 1-II.

On top of that, our results suggest that CRFs are involved in pistil development, particularly in placenta elongation, through *PIN1* transcriptional regulation.

Multiple translational start sites and alternative splicing in *AUXIN RESPONSE FACTOR 5/MONOPTEROS* (*ARF5/MP*)

Using CRISPR/Cas9 technology, we generated two independent T3 lines homozygous *mp* mutant plants named *mpT56* and *mpA54*. The *mpT56* contains a 1-bp insertion in the first exon at the 56th nucleotide from the start codon, while *mpA54* carries a 1-bp deletion in the 54th nucleotide. Both mutations lead to a frameshift and generate premature stop codons. Unexpectedly, homozygous *mpT56* and *mpA54* have wild type phenotype.

We introgressed *mpT56* with *mpS319* (*Cole* et al., 2009) to verify whether *mpT56* was able to produce a functional MP protein. The biallelic *mpS319/mpT56* showed a wild type phenotype, suggesting that *mpT56* allele could complement *mpS319* mutation. Moreover, we have expressed *mpT56* genomic locus under *35S* constitutive promoter. As expected, we obtained 20 transgenic plants with developmental defects similar to wild type *MP* overexpression, whose phenotype also has been previously reported (*Hardtke* et al., 2004)

Surprisingly, the GFP in the *p35S::mpT56-GFP* transgenic plants was not visible. We also excluded the silencing of the endogenous *MP* by co-suppression.

Although the *p35S::mpT56-GFP* mRNA is transcribed, the relative proteins are not detectable. Thus, we considered and investigated alternative splicing events in *MP*.

Through PCR, we identified two mRNA isoforms of *MP*, driven by an intron retention. The intron retention leads to the formation of a premature stop codon in the transcript. The resultant MP protein isoform lacks the CTD domains.

Using optical and confocal microscopy on MP marker lines and in situ hybridization, we were able to detect *MP* expression in the cell with low auxin concentration co-localizing with its own targets, suggesting that MONOPTEROS protein is able to activate transcriptions of its targets in low auxin concentration.

hda19 mutant has defects in ovule development and fertility

Ovules arise from the placenta during stage 8 of pistil development. Ovule count has been made on young siliques after dissection under the stereomicroscope. *hda19-3* mutant allele (in *Col-0*) shows a reduction in total ovule number without

major changes in the pistil length. Whole inflorescences were harvested and treated with chloral hydrate, pistils at stage 8 were selected under the stereomicroscope and then observed under optical microscope. *hda19* ovule primordia are more distant between each other.

Wild type and *hda19* plants were grown either in short day conditions or in long day conditions. As expected and in agreement with previous publications (*Tian* et al., 2003), *hda19* plants showed a more severe phenotype when grown in short day conditions. As described formerly, histone acetylation/deacetylation, and in particular H3K9Ac residue, are chromatin modifications responsive to light (Jang et al., 2011). Similar counts and measurements were performed in a different *hda19* mutant allele (in *Wassilewskija* ecotype). The phenotype appears to be similar in both alleles.

Detailed clearing analysis and confocal microscopy of Feulgen staining (*Braselton* et al., 1996) highlighted a defect in mature *hda19* ovules. In particular, *hda19* ovules show the embryo sac not fully covered by the integuments. This phenotype may be due to either defective integuments growth or to misplacement of the embryo sac.

Moreover, Lugol's Iodine staining pointed out a massive and abnormal starch deposition throughout all the stages of ovule development.

Fluorescence Activated Cell Sorting (FACS)-RNASeq and MADS-Box transcription factors regulation by HDA19

Although *HDA19* mRNA has been shown to be specifically expressed in emerging ovule primordia during early stages of pistil development by in situ hybridization

(*Krogan* et al., 2012), *HDA19* expression levels in flowers have been demonstrated to be very high and spread in all tissues among inflorescence (*Schmid* et al., 2005).

Therefore, we took advantage of recent and successful applied system for tissue-specific wide transcriptomic analysis, such as FACS-RNASeq (*Villarino* et al., 2016).

To be able to sort the ovules and placenta cells we introgressed *pSTK::STK-GFP* (*Mizzotti* et al., 2014) into *hda19* mutant by manual crossbreed. Only the sorted GFP-positive cells were then used for the RNA extraction and sequencing.

STK expression profile has been deeply characterized (*Pinyopich* et al., 2003; *Mizzotti* et al., 2014). Compared to *HDA19, STK* is expressed far less widely among the inflorescence. STK protein is specifically localized in the placenta during early stages of pistil development, in ovule primordia, in the ovule during all stages of development and only in the ovule integuments during later stages of pistil development (*Mizzotti* et al., 2014).

After the introgression of *pSTK::STK-GFP* into *hda19* mutant, STK protein localization has been confirmed to be precisely the same as in the parental *pSTK::STK-GFP*.

The GFP positive cells were, therefore, sorted and used for RNA Sequencing.

Strikingly, all key regulators of ovule primordia initiation (*Galbiati* et al., 2013) have been found to be downregulated in *hda19*, suggesting that all the machinery is therefore transcriptionally repressed.

Notably, the RNASeq included 13 MADS-Box transcription factors mis-regulated in *hda19* mutant.

MADS-Box transcription factor family shows a similar pattern of up-regulation with the only exception of *STK*, *SHP1* and *SHP2*.

Intriguingly, STK, SHP1 and SHP2 are functionally redundant during ovule development, as previously demonstrated (*Pinyopich* et al., 2003). Despite the fact that *stk shp1 shp2* triple mutant displays aberrant ovule development and failure in ovule identity determination, the simultaneous downregulation of these three homeotic transcription factors in *hda19* mutant do not affect ovule identity.

However, *stk shp1 shp2* triple mutant has a consistent and statistically significant decrease in ovule primordia number. Such phenotype is similar to the one observed for *hda19*.

HDA19 mRNA precisely co-expresses with STK protein (*Krogan* et al., 2012; *Mizzotti* et al., 2014) in the placenta during stage 8/9 of pistil development. At this time and exact tissue, ovule primordia arise, and mis-regulation of key regulator of this process may lead to improper organ growth.

FACS-RNA sequencing underlined a massive up-regulation of *SVP*, *FUL* and *AGL42*, together with other 7 MADS-Box transcription factors. To test the direct regulation by HDA19 of *SVP*, *FUL* and *AGL42*, Chromatin Immuno-Precipitation (ChIP) both on *hda19* mutant and wild type control was performed. For the immunoprecipitation, an antibody (Upstate 07-352) recognizing acetylation of lysine 9 residue on histone 3 (H3K9Ac) was used.

H3K9Ac has been addressed as one of the main target for post-translational modification by HDA19 (*Zhou* et al., 2010), although we cannot assume that other lysine residues may be modified.

Real Time qPCR were performed using primers designed in the TSS region, where the acetylation pattern of H3K9Ac has the maximum peak predicted in human cells (*Roh* et al., 2006).

All the three MADS-Box transcription factors tested shows an enrichment of 3 or 4 fold change in respect to the control, indicating that in *hda19* mutant the genomic regions of *SVP*, *FUL* and *AGL42* are more acetylated. Thus, the higher acetylation level in the genomic regions suggests that *SVP*, *FUL* and *AGL42* are direct targets of HDA19.

Starch accumulation in *hda19* ovules

During *hda19* ovule development, we observed an abnormal starch accumulation in the later stages of ovule development.

The RNA Sequencing analysis performed showed a notable number of starch-related genes to be significantly up regulated in *hda19* mutant and, on the contrary, only one gene, *BETA-AMYLASE 7*, was found to be down regulated.

hda19 mutant has defective transmitting tract and pollen tube guidance failure

A second and interesting phenotype has been observed in *hda19 -/-* mutant, that is a partial sterility of the female reproductive organ. In fact, although the shortness of stamens clearly cause an almost complete sterility of *hda19* (*Tian* et

al., 2003) due to the inability of the pollen grains to reach the stigma, hand pollination using either *hda19* pollen or wild type pollen does not recover the phenotype. Moreover, the analysis of fertilized and unfertilized ovules in the pistil results in a random distribution of them. A total 30% of the ovules are fertilized and become seeds, whereas 70% of the ovules are not targeted and reached by pollen tubes and as consequence they do not carry on fertilization.

Aniline blue staining highlighted the retarded growth speed of the pollen tubes in *hda19* transmitting tract. After 12 hours after pollination (HAP), wild type pistils have been entirely penetrated by pollen tubes. Conversely, pollen tubes fail to go across *hda19* pistils and they get stuck halfway in the transmitting tract. The speed rate difference becomes not anymore detectable after 24 hours after pollination. In fact, by that time, all pollen tubes are able to end up to the bottom of the transmitting tract in the mutant.

Intriguingly, by transcriptomic analysis with RNA Sequencing of the *hda19* mutant, we were able to identify transcription factors involved in transmitting tract development, such as *NO TRANSMITTING TRACT* (*NTT*), *HECATE1* (*HEC1*), *HEC2* and *HEC3* down-regulated.

In order to verify that all the gametophytic cells were properly differentiated in the mature ovule of our mutant, we crossed *hda19* with cell identity marker lines. These marker lines are able to express reporter genes such as *GUS* or *GFP* under a promoter specific for each of the seven cell of the female gametophyte.

The different cell identity marker lines show no significant difference in the pattern of expression in *hda19 -/-* background within cells of the antipodal cells, egg cell and central cell. However, when introgressing synergid cell identity marker lines, *pMYB98::GFP* and *pCKX7::GUS* into *hda19* mutant, only 10%

expresses the reporter gene, both *GFP* and *GUS*. In fact, we suggest that not all ovules properly develop synergid cells, thus clarifying the ovule guidance defect. Although little is known regarding the signals for long-distance pollen tube guidance, we hypothesize that the defective synergid cell development in *hda19* might influence this particular pathway. In fact, with aniline blue staining it has been recorded that pollen tubes, along the slower speed rate through the apoplast of the transmitting tissue, also have a high rate of failure in targeting ovules. Pollen tubes that go through the septum and reach the ovary seem not to be properly attracted by the ovule, suggesting that micropylar guidance is not affected in the mutant.

Conclusions and Future Prospects

Plant reproduction is a key developmental process whose mechanisms have been deeply characterized in the past years. However, many questions related to those mechanisms still require detailed answers. Specifically, basic plant biology research aims at uncovering new molecular targets whose knowledge can be applied to improve agriculture and to satisfy human needs.

For this particular reason, there has been a focus on plant reproduction related processes, especially those targeting agriculturally interesting traits, such as seed yield or interspecific barriers during sexual reproduction.

Although hereby just a small step has been done, we can safely conclude that with the work presented we have achieved an advancement of knowledge in the involvement of cytokinin signal transduction during gynoecium development and ovule primordia formation.

In fact, we propose CRFs as master regulators of pistil development, in particular during placenta elongation process. Our findings suggest that PIN1 expression is modulated by CRFs and therefore that cytokinin-auxin crosstalk is fundamental for the pistil growth and related traits such as ovule number.

ARF5/MP is one of the most interesting member of the AUXIN RESPONSE FACTOR family, whose regulation and activity has not yet been fully elucidated. In fact, many different *monopteros* mutant alleles have been described so far, although there is no clear correlation between the severity of the phenotypes and its residual function. In our work, we describe two new mutant alleles generated with CRIPR/Cas9 technology, either carrying an insertion or a deletion at the very beginning of *MP* open reading frame. The two mutant alleles show a wild type phenotype, despite the predicted stop codon formation after 20 aminoacids out of the 902 aa full length MP protein sequence. These results suggest that an alternative translational start site, downstream the annotated ATG, is able to produce a functional MP protein. Besides, we also demonstrate alternative splicing events, causing the retention of the tenth intron of *MP*. The alternative protein isoform lacks the dimerization domain, therefore it is likely not interacting with Aux/IAA proteins. Thus, MP isoform lacking the CTD would be able to positively regulate target genes expression in sub threshold of auxin concentration. Our findings are corroborated by detailed expression pattern characterization of MP, its targets, such as *TARGET OF MONOPTEROS 5* (*TMO5*) and *CUC1*, and auxin minima/maxima reporter lines. *MP* is co-expressed with its targets in cells with low auxin concentration, therefore suggesting a role as a transcriptional activator during ovule development in cells with low level of auxin.

Further, we propose a new role for epigenetic mechanisms of transcriptional regulation, in particular histone (de)acetylation, to determine ovule number determination.

Taking advantage of a pioneering approach, cell sorting through Fluorescence Activated Cell Sorting coupled to deep sequencing analysis, we propose a new role for HISTONE DEACETYLASE 19 during ovule primordia formation, in transmitting tissue development and pollen tube ovular guidance.

Moreover, the data provided with the transcriptomic analysis of *hda19* mutant pinpointed a huge amount of mis-regulated genes encoding cell wall modifier, such as *PECTIN METHYLESTERASEs* and *PECTIN METHYLESTERASE INHIBITORs*.

We assume that the different composition of the apoplast in the transmitting tract of *hda19* could be a possible explanation for the slower growth of the pollen tubes. To address this hypothesis, we are currently phenotyping *hda19* x *p35S::PMEi6*. *hda19* mutant has been crossed with the overexpression of a pectin methylesterase inhibitor, that is claimed to reduce the rate of methylesterification of pectins in the apoplast of the transmitting tract, thus recovering *hda19* phenotype.

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PART II

PUBLISHED PAPER CYTOKININ RESPONSE FACTORS INTEGRATE AUXIN AND CYTOKININ PATHWAYS FOR FEMALE REPRODUCTIVE ORGAN DEVELOPMENT

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RESEARCH REPORT

Cytokinin response factors integrate auxin and cytokinin pathways for female reproductive organ development

Mara Cucinotta[1], Silvia Manrique[1], Andrea Guazzotti[1], Nadia E. Quadrelli[1], Marta A. Mendes[1], Eva Benkova[2] and Lucia Colombo[1,*]

[1]Dipartimento di BioScienze, Università degli studi di Milano, Via Celoria 26, Milano 20133, Italy. [2]Institute of Science and Technology Austria, Am Campus 1, Klosterneuburg 3400, Austria.

*Author for correspondence (lucia.colombo@unimi.it)

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ABSTRACT

The developmental programme of the pistil is under the control of both auxin and cytokinin. Crosstalk between these factors converges on regulation of the auxin carrier PIN-FORMED 1 (PIN1). Here, we show that in the triple transcription factor mutant *cytokinin response factor 2* (*crf2*) *crf3 crf6* both pistil length and ovule number were reduced. *PIN1* expression was also lower in the triple mutant and the phenotypes could not be rescued by exogenous cytokinin application. *pin1* complementation studies using genomic *PIN1* constructs showed that the pistil phenotypes were only rescued when the *PCRE1* domain, to which CRFs bind, was present. Without this domain, *pin* mutants resemble the *crf2 crf3 crf6* triple mutant, indicating the pivotal role of CRFs in auxin-cytokinin crosstalk.

INTRODUCTION

In *Arabidopsis thaliana*, ovules emerge as lateral organs from the placenta, a meristematic tissue that originates after the fusion of the carpel margin meristem (CMM) (*Reyes-Olalde* et al., 2013; *Schneitz* et al., 1995). Placenta formation and ovule growth require auxins. Reduced local auxin biosynthesis or transport causes severe defects in pistil development with a consequent loss of placental tissue and ovules (*Nemhauser* et al., 2000; *Nole-Wilson* et al., 2010). The auxin efflux carrier PIN-FORMED 1 (PIN1) is one of the main elements modulating auxin accumulation during all phases of ovule development (*Benková* et al., 2003; *Ceccato* et al., 2013). Although *pin1-201* does not develop any flowers, in the *pin1-5* mutant the gynoecium has shorter valves and contains a few ovules (*Bencivenga* et al., 2012; *Sohlberg* et al., 2006). Cytokinins (CKs) positively regulate ovule formation and pistil development. Indeed, mutants that have a reduced capacity for CK production or perception exhibit a dramatic reduction in ovule number and pistil size, and compromised female fertility (*Kinoshita-Tsujimura* and *Kakimoto*, 2011; *Riefler* et al., 2006; *Werner* et al., 2003). By contrast, increased CK levels result in a bigger pistil with a greater number of ovules compared with wild type, confirming a positive correlation between CK levels and ovule numbers (*Bartrina* et al., 2011; *Bencivenga* et al., 2012; *Galbiati* et al., 2013). It has been shown that CK treatment positively influences the number of ovules per pistil via a strong increase in *PIN1* expression (*Bencivenga* et al., 2012; *Galbiati* et al., 2013; *Zúñiga-Mayo* et al., 2014).

Conversely, in roots, CKs modulate organogenesis by downregulating *PIN1* expression (*Dello Ioio* et al., 2012; *Ruzicka* et al., 2009) and PIN1 protein endocytic recycling (*Marhavý* et al., 2011).

Cytokinin response factors (CRFs) are encoded by closely related members of the Arabidopsis *AP2* gene family and mediate a large proportion of the CK transcriptional response that functionally overlaps with the B-type ARR-mediated response (*Rashotte* et al., 2006). Recently, *Šimášková* and colleagues (2015) found that CRFs bind directly to the *PIN1* cytokinin response element (*PCRE1*) in

the *PIN1* promoter and thus modulate *PIN1* expression in response to CKs. Deletion of the *PCRE1* cis-regulatory element uncouples *PIN1* transcription from CRF regulation and affects root sensitivity to CKs (*Šimášková* et al., 2015). Here, we show that CRF2, CRF3 and CRF6 redundantly induce the expression of *PIN1*, which is required for ovule development, supporting the crucial and general role of CRF factors as mediators of auxin-CK crosstalk guiding plant organogenesis.

RESULTS AND DISCUSSION

CRFs are required for placenta elongation and ovule development

In the placenta, CKs promote *PIN1* expression, which is needed for the establishment of the auxin gradient that leads to ovule primordia development (*Bencivenga* et al., 2012; *Benková* et al., 2003; *Ceccato* et al., 2013; *Galbiati* et al., 2013). Recently, it has been shown that three members of the CRF family, CRF2, CRF3 and CRF6, directly regulate *PIN1* expression upon CK signalling in roots (*Šimášková* et al., 2015). *CRF2* and *CRF6* promoters were able to drive reporter gene expression in stage 9 and 10 of pistil development, whereas the *CRF3* promoter did not show any activity (Fig. S1). These results are consistent with recently published transcriptomic data of the gynoecial medial domain, which show high expression of *CRF2* and *CRF6* and low expression of *CRF3* (*Villarino* et al., 2016). To investigate whether these three CRFs control *PIN1* expression during early stages of pistil development, we have analysed *crf2*, *crf3* and *crf6* single, double and triple mutants. Ovule counts were performed on ovules from stage 1-II (primordia) to stage 2-I (finger-like), which corresponds to stages 9 and 10 of pistil development [according to *Schneitz* et al. (1995) and *Roeder* and *Yanofsky* (2006)]. Analysis of single *crf3* and *crf6* mutants, as well as the *crf3 crf6* double mutant, did not reveal any significant difference in ovule number compared with wild type, whereas the single *crf2* and the double *crf2 crf3* mutant showed a small but significant decrease in ovule number (Fig. S2). Instead, the *crf2 crf6* double mutant presented ovule numbers comparable to wild type, suggesting a compensatory mechanism between *crf2* and *crf6* (Fig. S2).

Finally, in the *crf2 crf3 crf6* (*crf2/3/6*) triple mutant, a reduction of 31.68% in ovule number was observed with respect to the wild type (Fig. 1A). Wild-type Col-0 plants grown under long-day conditions developed on average (mean±s.e.m.) 46.36±1.24 ovules per pistil whereas 31.67±2.01 ovules were formed in the *crf2/3/6* triple mutant pistils (Fig. 1A).

Placenta length was measured at the same developmental stages. In the wild type, the average length of the placenta was found to be 351±12 µm at stage 9 and 517±12 µm at stage 10, whereas in the *crf2/3/6* mutant it was significantly shorter (269±20 µm at stage 9 and 436±19 µm at stage 10) (Fig. 1B). Ovule density (number of ovules per µm placenta) was also reduced in the *crf2/3/6* mutant (Fig. 1C). CK treatment results in an increase in pistil size and ovule number (*Bencivenga* et al., 2012; *Galbiati* et al., 2013). Because CRFs regulate the transcriptional response to cytokinins (*Rashotte* et al., 2006), we tested the CK response in wild type and the *crf2/3/6* triple mutant. Wild-type plants treated with the synthetic cytokinin 6-benzylaminopurine (BAP) yielded 60% more ovules and a 58% longer placenta than untreated plants (Fig. 1D,E). The *crf2/3/6* triple mutant treated with BAP produced 19% more ovules and an increase in placenta length of 32% (Fig. 1D,E), indicating that the capacity to respond to CKs is strongly reduced in the absence of CRF2, CRF3 and CRF6 activities.

CRFs regulate *PIN1* transcription during pistil growth

To investigate whether the pistil phenotypes observed in the *crf2/3/6* mutant were due to changes in *PIN1* expression, we performed Real Time qPCR experiments. In the *crf2/3/6* triple mutant, *PIN1* expression was significantly lower than in the wild type (Fig. 2A). As previously reported (*Bencivenga* et al., 2012; *Galbiati* et al., 2013), *PIN1* expression was at least twofold higher in BAP-treated wild-type inflorescences. Interestingly, the level of *PIN1* mRNA in the *crf2/3/6* mutant did not increase upon CK application, suggesting that CRFs are required for CK-dependent *PIN1* expression (Fig. 2A).

In roots, CRFs regulate *PIN1* expression by binding the *PCRE1* sequence in the *PIN1* promoter (*Šimášková* et al., 2015); therefore, we analyzed plants carrying a

ΔPIN1::PIN1-GFP construct in which a *PIN1* promoter lacking the *PCRE1* element drives the expression of a fully functional PIN1-GFP fusion protein. Real-time qPCR experiments were performed on *GFP* instead of *PIN1* in order to avoid the detection of endogenous *PIN1*. The level of *PIN1-GFP* transcripts in *ΔPIN1::PIN1-GFP* inflorescences was lower than that in plants carrying the same fusion protein construct under the control of a wild-type version of the *PIN1* promoter (Fig. 2B). The reduction in *PIN1-GFP* expression under control of the *ΔPIN1* promoter was also evident by confocal microscopy in placenta cells and ovule primordia at stages 1-I and 1-II (compare Fig. 2C,D with Fig. 2E,F). Although *PIN1* expression was dramatically reduced (Fig. 2B), PIN1-GFP protein in *ΔPIN1::PIN1-GFP* plants was correctly localized at the membrane of placenta cells (Fig. 2E,F). To understand whether *PCRE1* is the only element in the *PIN1* promoter required for CK-mediated *PIN1* expression in inflorescences, we also analyzed *GFP* expression after treatment with CKs in *PIN1::PIN1-GFP* and *ΔPIN1::PIN1-GFP* plants.

Interestingly, *GFP* expression increased in both *PIN1::PIN1-GFP* and *ΔPIN1::PIN1-GFP* inflorescences compared with the control (mock treatment) (Fig. 2B), suggesting that CRFs might bind to other regions of the *PIN1* promoter besides *PCRE1*. The possibility that other CK-induced transcription factors regulate *PIN1* expression is unlikely as *PIN1* expression remains unchanged in CK-treated *crf2/3/6* inflorescences (Fig. 2A). The same reduction of *GFP* expression in *ΔPIN1::PIN1-GFP* compared with *PIN1::PIN1-GFP* was observed in a second independent *ΔPIN1::PIN1-GFP* line (Fig. S3). Also, in the independent line *ΔPIN1::PIN1-GFP_2*, *GFP* expression increased after BAP treatment, reconfirming the results obtained with line 1 (Fig. S3). These results confirm that CRFs are required to regulate the expression of *PIN1* in the pistil. The possibility of other CRF regulatory regions needs to be investigated as the lack of *PCRE1* does not cause complete CK insensitivity. It is important to recall that in roots *PIN1::PIN1-GFP* expression is reduced by CKs and that *ΔPIN1::PIN1-GFP* is completely CK insensitive (*Šimášková* et al., 2015), indicating that there might be a specific regulation of *PIN1* expression depending on the developmental context.

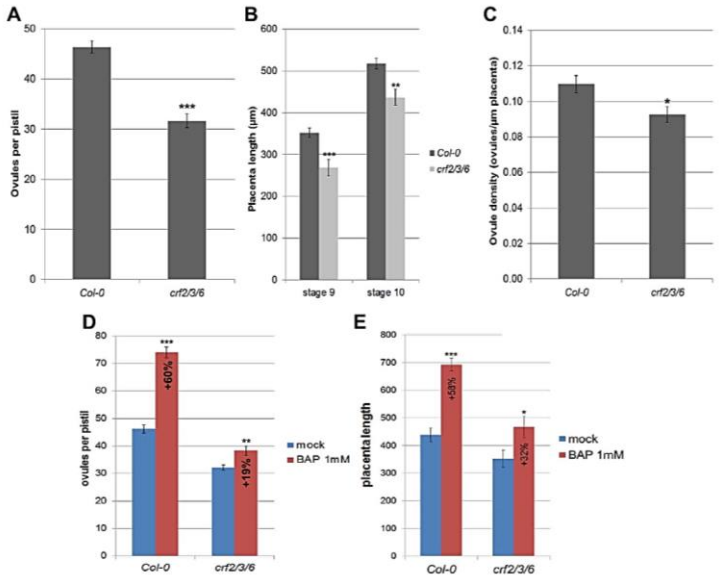

Fig. 1. CRFs influence pistil length and ovule numbers. (A-C) Number of ovules (A), placenta length (B) and ovule density (C) of wild-type (Col-0) and *crf2/3/6* pistils. (D,E) Ovule number (D) and placenta length measurements (E) in mock- and 1 mM BAP-treated wild-type and crf2/3/6 inflorescences 48 h after treatment. Mean±s.e.m. is shown. *P<0.05; **P<0.01; ***P<0.001 (Student's t-test; n=20). Percentage increment is also reported in D,E.

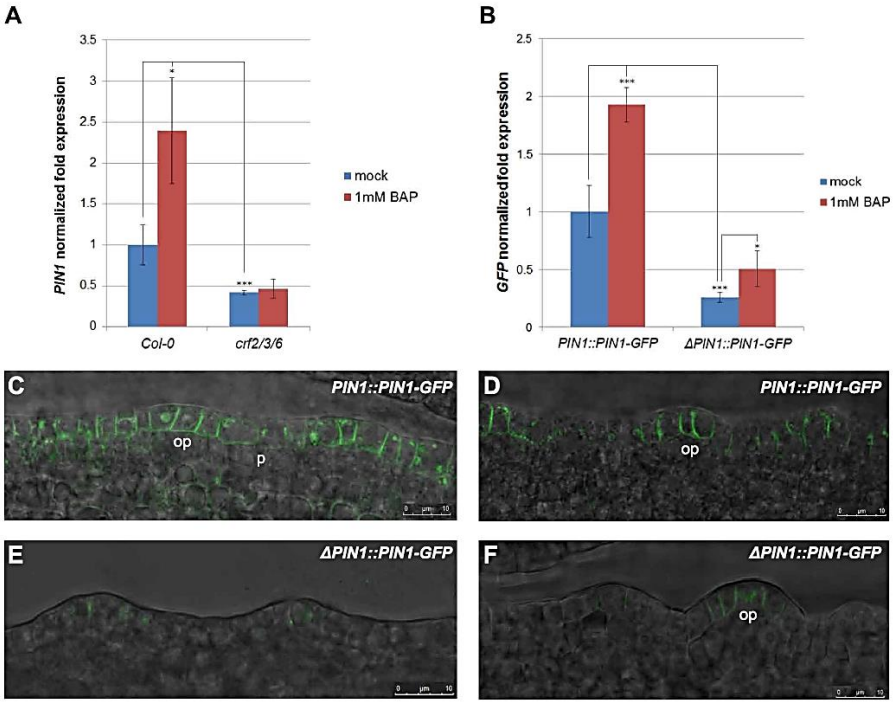

Fig. 2. CRFs regulate *PIN1* expression. (A) PIN1 expression levels in mock- and BAP-treated pre-fertilization inflorescences of wild-type and *crf2/3/6* triple mutant. (B) GFP expression levels in mock- and BAP-treated *PIN1::PIN1-GFP* and *ΔPIN1::PIN1-GFP* pre-fertilization inflorescences. Error bars indicate the s.e.m. based on three biological replicates. *P<0.05; ***P<0.001 (Student's t-test; n=3). Data were normalized with respect to *ACT8-2* and *UBI10* mRNA levels. (C-F) Confocal microscope images of PIN1::PIN1-GFP (C,D) and ΔPIN1::PIN1-GFP (E,F) placenta cells and ovule primordia at stages 1-I (C,E) and 1-II (D,F). Scale bars: 10 µm. op, ovule primordia; p, placenta.

PCRE1 is required for pistil development and ovule primordia formation

Introducing *ΔPIN1::PIN1-GFP* in a wild-type A. thaliana does not lead to any abnormalities in pistil and ovule development (Fig. 3A-C). To examine the functional significance of the CRF regulatory regions in the *PIN1* promoter (*PCRE1*), we introgressed *ΔPIN1::PIN1-GFP* into the *pin1-5* mutant. *pin1-5* is a hypomorphic mutant that has shorter pistils and develops an average of nine ovules per pistil (*Bencivenga* et al., 2012; *Sohlberg* et al., 2006). Confirmation of the presence of *ΔPIN1::PIN1-GFP* construct in the *pin1* mutant is shown in Fig.

S4. *PIN1::PIN1-GFP* completely rescued the *pin1-5* mutant phenotype whereas *ΔPIN1::PIN1-GFP* was unable to rescue the pistil growth phenotype of *pin1-5* (Fig. 3A-C). The placenta length of *pin1-5 ΔPIN1::PIN1-GFP* remained the same as in *pin1-5* (Fig. 3A,B). Placenta length in *pin1-5 ΔPIN1::PIN1-GFP* was similar to that of the *crf2/3/6* mutant (Fig. 3A,B). This suggests that *PCRE1*-mediated transcriptional regulation of *PIN1* is necessary for correct elongation of the pistil. Furthermore, ovule density in *pin1-5 ΔPIN1::PIN1-GFP* (0.0902±0.008 ovules/µm placenta) was similar to that of *crf2/3/6* (0.0926±0.004 ovules/µm placenta). By contrast, *ΔPIN1::PIN1-GFP* did rescue the ovule number phenotype of *pin1-5*, raising the ovule count of *pin1-5* from an average of 8.5±1.7 to 28.67±1.84 (Fig. 3C). These results suggest that *PCRE1*-mediated control of *PIN1* expression is required for determining the correct size of the pistils, whereas it seems to be less relevant for ovule formation. However, it should be taken into account that transcription of *pin1-5* (which encodes a partially functional protein) could be induced by CKs. For this reason, we also analyzed the phenotype of *ΔPIN1::PIN1-GFP* in *pin1-201* mutant. This mutant fails to develop any lateral organs due to a loss-of-function mutation (Fig. S5). Pistil length in *ΔPIN1::PIN1-GFP pin1-201* is similar to that in *ΔPIN1::PIN1-GFP pin1-5* and *crf2/3/6* (Fig. 2A,B). Regarding the ovule number, *ΔPIN1::PIN1-GFP pin1-201* showed a reduction in comparison with *ΔPIN1::PIN1-GFP pin1-5* and *crf2/3/6* (Fig. 3C). The reduction in ovule number highlighted in *ΔPIN1::PIN1-GFP pin1-201* compared with *ΔPIN1::PIN1-GFP pin1-5* might be explained by residual function of the PIN1-5 mutant protein. The analysis of *ΔPIN1::PIN1-GFP* in both *pin1-5* and *pin1-201* allelic backgrounds confirmed that pistil elongation is affected when *PIN1* expression is uncoupled from regulation of CRFs. Finally, we also tested the capacity of *ΔPIN1::PIN1-GFP pin1-5* and *ΔPIN1::PIN1-GFP pin1-201* to respond to CK by checking the number of ovules after BAP treatment. Interestingly, both lines are still able to respond to CK showing an increase in ovule density of 27% and 21%, respectively (Fig. 3D). This result is in agreement with the fact that *PIN1-GFP* expression level increases in *ΔPIN1::PIN1-GFP* after BAP treatment (Fig. 2B; Fig. S3), confirming the importance of CRF-mediated *PIN1* expression for pistil elongation.

The reduction in pistil size observed in *crf* mutants could be due to defective cell division or cell expansion processes or a combination of both. Auxin plays a prominent role in controlling cell expansion. For example, elongation of the primary root and the hypocotyl require specific auxin transport to determine their expansive growth rates (*Rayle* and *Cleland*, 1992; *Spartz* et al., 2012). Interestingly, a reduction in pistil and anther elongation has been reported for *tir1 afb1 afb2 afb3*, a quadruple mutant with compromised auxin signalling (*Cecchetti* et al., 2008). Our understanding of the influence of auxin on the cell cycle is still fragmentary, but primary evidence indicates that auxin acts on several targets involved in the control of cell cycle (*Perrot-Rechenmann*, 2010). On the other hand, the ability of CKs to promote cell division, in particular through their action on D-type cyclins, was described several years ago (*Dewitte* et al., 2007; *Riou-Khamlichi* et al., 1999), and it has been recently been shown that the transcript levels of several cell cycle-related genes were decreased in roots of the *crf1,3,5,6* quadruple mutant (*Raines* et al., 2016). In summary, we propose that *PIN1* expression mediated by CRFs is required for the determination of pistil size. The greater number of ovule primordia in CK-treated pistils correlates with the increased pistil size. Therefore, it is likely that when enough space occurs between two ovules, CRFs and/or other CKs-dependent factors induce *PIN1* expression to create a new auxin maximum.

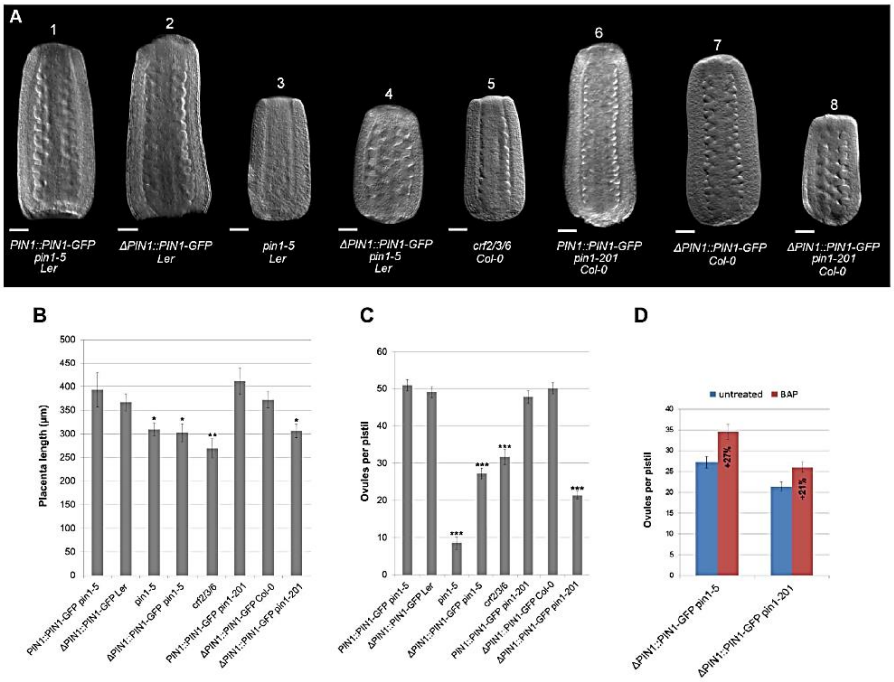

Fig. 3. CRFs regulate *PIN1* expression required for pistil growth. (A) DIC images of pistils with visible lines of ovules in Ler and Col-0 genetic backgrounds. Scale bars: 50 µm. (B,C) Placenta length (B) and ovule number (C) in *PIN1::PIN1-GFP pin1-5* Ler, *ΔPIN1::PIN1-GFP* Ler, *pin1-5* Ler, *ΔPIN1::PIN1-GFP pin1-5* Ler, *crf2/3/6* Col-0, *PIN1::PIN1-GFP pin1-201*, *ΔPIN1::PIN1-GFP* Col-0 and *ΔPIN1::PIN1-GFP pin1-201* Col-0. (D) Ovule number measurements in untreated and 1 mM BAP-treated *ΔPIN1::PIN1-GFP pin1-5* and *ΔPIN1::PIN1-GFP pin1-201* inflorescences 48 h after treatment. Percentage increment is also reported in the graphs. Mean±s.e.m. is shown. *P<0.05; **P<0.01; ***P<0.001 (Student's t-test; n=20).

MATERIALS AND METHODS

Plant materials and treatments

Arabidopsis wild-type and mutant plants were grown at 22°C under long-day conditions (16 h light, 8 h dark) in a greenhouse. *crf2-2* seeds (*Schlereth* et al., 2010) were provided by Dolf Weijers (Laboratory of Biochemistry, Wageningen University, 6703 HA Wageningen, The Netherlands). *PIN1::PIN1-GFP* (*Benková* et al., 2003), *pin1-5* mutant (*Bencivenga* et al., 2012; *Sohlberg* et al., 2006), *pin1-201* (*Furutani* et al., 2004), *crf3-1, crf6-S2, crf3-1 crf6-S2, crf2-2 crf3-1 crf6-S2*,

ΔPIN1::PIN1-GFP, ΔPIN1-GFP pin1-201 and *PIN1::PIN1-GFP pin1-201* (*Šimášková* et al., 2015) lines have been described previously. BAP treatment was performed on inflorescences as detailed by *Bencivenga* et al. (2012).

Quantitative real-time qPCR analysis

Total RNA was extracted from inflorescences at pre-fertilization stages using the Macherey-Nagel Nucleospin RNA Plant Kit and then reverse transcribed using the Promega ImProm-II RT System. Gene expression analysis was performed using the Bio-Rad iQ5 Multicolor RT-PCR Detection System with the GeneSpin SYBR Green PCR Master Mix. *ACTIN 2-8* and *UBIQUITIN 10* were used as reference genes for normalization of transcript levels. RT-PCR primers used in this work were: RT2017fw 5′-TGTTCCATGGCCAACACTTG-3′ and RT2018 rev 5′-AAGTCGTGCCGCTTCATATG-3′ for *GFP*, RT509fw 5′-TGGTCCCTCATTTCCTTCAA-3′ and RT510rev 5′-GGCAAAGCTGCCTGGATAAT-3′ for *PIN1*, RT147fw 5′-CTGTTCACGGAACCCAATTC-3′ and RT148rev 5′-GGAAAAAGGTCTGACCGACA-3 for UBI10, and RT861fw 5′-CTCAGGTATTGCAGACCGTATGAG-3′ and RT862rev 5′-CTGGACCTGCTTCATCATACTCTG-3′ rev for *ACT2-8*.

Counting ovule number by differential interference contrast (DIC) microscopy

Inflorescences were fixed with ethanol/acetic acid (9:1) overnight, rehydrated with 90% and 70% ethanol and cleared in a chloral hydrate/glycerol/water solution (8 g: 1 ml: 3 ml) for at least 2 h before dissection under a stereomicroscope. Pistils were observed using a Zeiss Axiophot D1 microscope equipped with DIC optics. Images were recorded using a Zeiss Axiocam MRc5 camera with Axiovision software version 4.1. Only ovules of pistils in which both carpels remained intact after slide preparation and where all four rows of ovules were visible and distinguishable were counted.

Confocal microscopy

For confocal laser scanning microscopy (CLSM), fresh material was collected, mounted in water and analysed immediately. CLSM analysis was performed using

a Leica 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.C., E.B., L.C.; Formal analysis and investigation: M.C., S.M., A.G., N.E.Q, M.A.M..; Writing - original draft preparation: M.C.; Writing - review and editing: M.C., S.M., A.G, M.A.M, E.B, L.C.; Funding acquisition: L.C.; Resources: E.B., L.C.; Supervision: L.C.

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Supplementary information

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MANUSCRIPT #2

Alternative splicing regulates auxin response during reproductive phase in *Arabidopsis thaliana*

Mara Cucinotta[1], Andrea Guazzotti[1], Alex Cavallieri[1], Marco Bressana[1], Dolf Weijers[2], Martin Kater[3], Veronica Gregis[1] and Lucia Colombo[1*]

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

[2] Laboratory of Biochemistry, Wageningen University, Stippeneng 4, 6708 WE Wageningen, The Netherlands

[3] Dipartimento di Scienze Farmacologiche e Biomolecolari, Universit□a degli Studi di Milano, Via Celoria 26, 20133 Milano,Italy

*Correspondence to: lucia.colombo@unimi.it

†

Abstract

A major player in plant development is the hormone auxin, which modulates the expression of entire sets of target genes through AUXIN RESPON FACTOR (ARF)/AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcription factors combinatorial system. By CRISPR-CAS9 technology, two independent *mp* alleles have been generated with a stop codon just before the DNA Binding Doman (DBD). MP transcription factor is involved in several development processes, by positively regulating target genes in cell with high concentration of auxin. It is also known to be inactivated by AUX/IAA factors when auxin level does not reach a cellular threshold. Hereby we show that

multiple translational start sites and alternative splicing concur in the production of an ARF5/MP protein isoform which is insensitive to AUX-IAA repression system, thus allowing ARF5/MP to positively regulate target genes expression in sub threshold of auxin concentration.

One Sentence Summary

Differential splicing allows fine tune regulation of ARF5/MP activity during reproductive development.

Introduction

RNA alternative splicing (AS) contributes to the formation of protein isoforms with putative different functions and structures. Therefore, AS is a huge source of protein diversity among Eukaryotes (*Blencowe*, 2006). Although in plants AS is still a largely unexplored topic, it has been show that AS regulates several developmental processes such as flowering time (*Macknight* et al., 2002) and stress response (*Capovilla* et al., 2015; *Wang* et al., 2017).

Plant developmental processes require auxin, whose signalling pathway is primarily mediated by AUXIN RESPONSE FACTOR (ARF) proteins together with the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins (*Chandler*, 2016; *Li* et al., 2016). Under sub-threshold auxin concentrations, Aux/IAA proteins heterodimerize with ARF transcription factors, thereby modulating the expression of ARF downstream targets (*Reed*, 2001; *Ulmasov* et al., 1997). ARF proteins

are composed by three different domain: a B3 DNA binding domain (DBD), a middle region (MR) and a carboxy-terminal dimerization domain (CTD), required for dimerization with the Aux/IAA (*Guilfoyle* and *Hagen*, 2007; *Tiwari* et al., 2003; *Ulmasov* et al., 1997; *Ulmasov* et al., 1999). Among ARFs, *ARF5/MONOPTEROS (MP)* has been extensively studied, unveiling its role in several developmental processes such as embryo development, lateral root formation, vasculature patterning, ovule development, shoot apical meristem and floral meristem maintenance (*Berleth* and *Jürgens*, 1993; *De Smet* et al., 2010; *Donner* et al., 2009; *Galbiati* et al., 2013; *Hardtke* and *Berleth*, 1998; *Przemeck* et al., 1996; *Weijers* et al., 2006; *Yamaguchi* et al., 2013). Hereby, we show that *MP* has multiple translation start codon and differential mRNA splicing that allows a fine tune regulation of MP protein activity during reproductive development. The complexity of such regulation reflects the importance of a protein that is essential to many developmental process crucial for plant life cycle.

Results and Discussion

Although many *mp* alleles have been described in the last decades, none of them features a mutation close to the translational start codon. Therefore, in order to target *MP* gene around the first start codon, we have used CRISPR/Cas9 technology, according to *Fauser* et al., 2014 (see Materials and Methods). We generated two independent T3 lines homozygous *mp* mutant plants named *mpT56* and *mpA54*. The *mpT56* contains a 1-bp insertion in the first exon at

the 56th nucleotide starting from the start codon, while *mpA54* carries a 1-bp deletion in the 54th nucleotide (Fig. 1A).

Both mutations lead to a frameshift and generate premature stop codons (Fig. 1B). To determine whether *mpT56* and *mpA54* plants may exhibit the defects previously reported for other *mp* mutant alleles, we compared them to wild type plants grown under the same conditions. As shown, rosette leaves (Fig. 1C), inflorescences of young adult plant (Fig. 1D) and flower (Fig. 1E) of *mpT56* and *mpA54* have no defect. The *mpT56* and *mpA54* siliques are fully fertile (Fig. 1F) and rate of seed germination in Murashige-Skoog medium was comparable to wild type (Fig. S1). Unexpectedly, *mpT56* and *mpA54* plants are indistinguishable from wild type plants. Since *mpT56* and *mpA54* have identical phenotype, we focused our attention and the following analysis on *mpT56* allele.

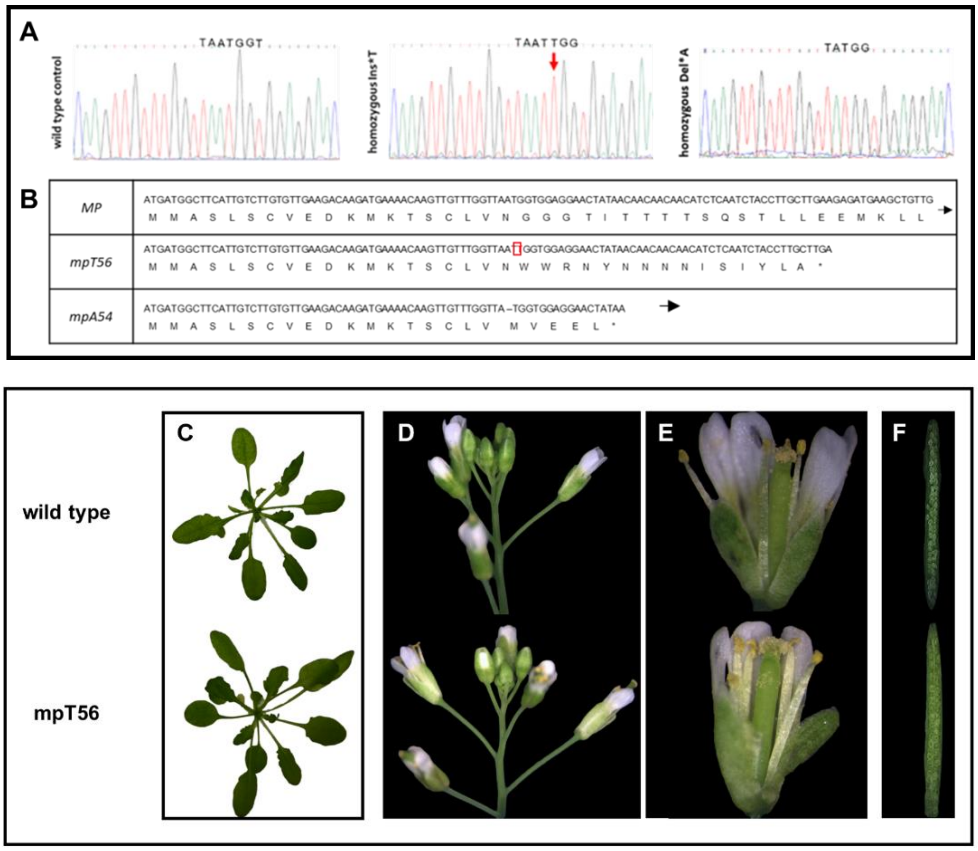

Fig. 1. CRISPR/Cas9 *mpT56* and *mpA54* mutant have wild type like phenotypes. (*A*) Proto-spacer designed for CRISPR/Cas-mediated targeting of *MP* 56 nucleotides after the start codon; A 1-bp insertion was detected in genomic DNA of *mpT56* mutant and a 1-bp deletion in *mpA54*; both mutations create a early stop codon (B). The *mpT56*, *mpA54* and wild type plants display no significant differences at various developmental stages: (C) rosettes, (D) inflorescences, (E) flowers and siliques showing full seed set (F).

MP has putative multiple translation start codons in frame with the first ATG that might be used without compromising the DBD integrity. Therefore, we introgressed *mpT56* with *mpS319* (*Cole* et al., 2009) to

verify whether *mpT56* was able to produce a functional MP protein. The biallelic *mpS319/mpT56* showed a wild type phenotype, meaning that *mpT56* allele could complement *mpS319* drastic phenotype (Fig. 2A, B, C, D).

Moreover, we have expressed *mpT56* genomic locus under *35S* constitutive promoter. As expected, we obtained 20 transgenic plants with developmental defects similar to wild type *MP* overexpression, phenotype also previously reported (*Hardtke* et al., 2004) (Fig 2E).

GFP and *RFP* reporter genes were cloned in frame to the *mpT56*. Surprisingly, the RFP in the *p35S::mpT56-RFP* transgenic plants was not visible (Fig. Suppl. 1).

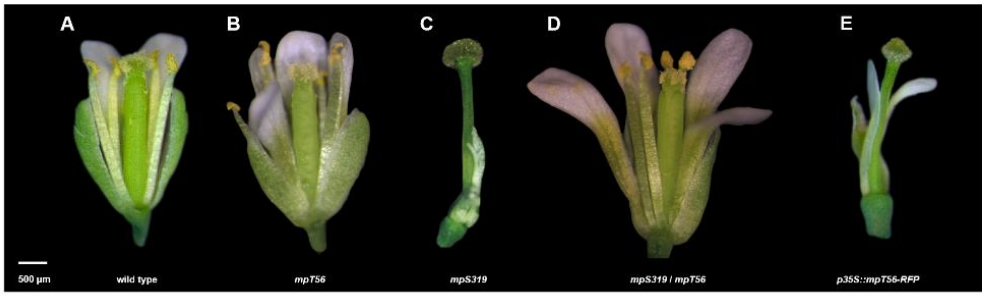

Fig. 2. *mpT56* locus encodes for a functional protein. (A) wild type; (B) *mpT56*; (C) *mpS319*; (D) biallelic *mpS319/mpT56* and (E) *p35S::mpT56-GFP* inflorescence.

We also performed Real Time qPCR on the *MP* 3'UTR, in order to distinguish the endogenous *MP* transcript from the transgenic one. No difference of *MP* transcript was detected, therefore excluding the silencing of the endogenous *MP* by co-suppression, which might lead to the phenotype observed in the *p35S::mpT56-GFP* and *p35S::mpT56-RFP*.

On top of that, these results suggest that a MP functional protein is formed in *mpT56* plants, encoded by a different translation start site respect to the ones previously reported.

Although the *p35S::mpT56-RFP* mRNA is transcribed (Fig. Suppl. 2), the relative protein are not detectable. Thus, we considered and investigated alternative splicing events in *MP*.

To do so, we collected *A. thaliana* inflorescences, extract RNA and retro transcribe cDNA. Hence, we designed specific primers flanking the 10^{th} intron (forward on the 10^{th} exon and reverse on the 11^{th} exon) and used them with either cDNA and genomic DNA (gDNA) as template (Fig. 3, A). Two isoforms were detected in the cDNA reaction, suggesting the coexistence of two mRNA isoforms, due to intron retention. The isoform with the retained intron has been further confirmed through PCR using a forward on the intron and an exon-to-exon junction reverse primer (Fig. 3, B). Such intron retention leads to the formation of a premature stop codon in the transcript. The resultant MP protein isoform would lack the CTD domains (Fig. 3C).

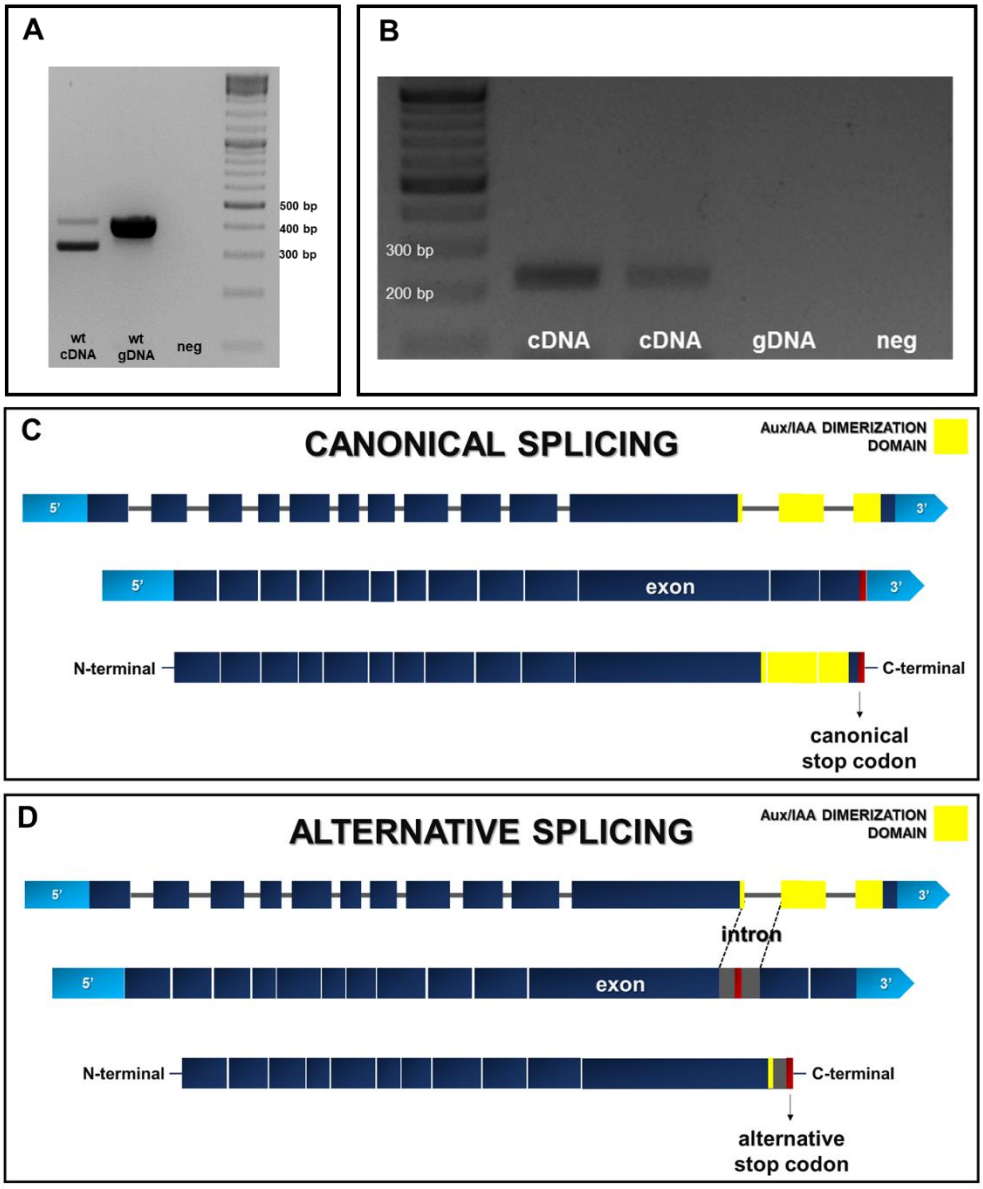

Fig. 3 Detection alternative splicing in *MP*. (A) PCR with primers over intron 10th amplifies two bands (420bp with retained intron and 330 bp without intron) in cDNA sample reaction. (B) Forward primer designed on the retained intron and reverse primer on the exon to exon junction amplifiy in cDNA and not in gDNA. (C) Retention of intron 11th lead to the formation of a premature stop codon. The 'small MP protein' formed lack the CDT.

In ARFs, the CTD domain is required for interaction with AUX-IAA (*Piya* et al., 2014), such as BODENLOS (Hamann et al., 2002). The so formed complexes are able to bind repression complexes (*Szemenyei* et al., 2008; *Wang* et al., 2013).

On top of our findings, we suggest that the MP protein isoform lacking the CTD, free from Aux/IAA regulation, might function as an activator in auxin minima regions.

Although MP extends its function and expression all over the plant life cycle, we decided to focus our attention specifically during ovule development. By in-situ hybridization, we show the transcription of *MP* in early stages of ovule development (Fig.4A,B). Moreover, we characterized the expression profile of two independent reporter lines of MP, i.e. *pMP::MP-GFP*, either with *GFP* fused to the c-terminal end of the protein or with the *GFP* inserted in the middle region thanks to a single endonuclease restriction site (*Schlereth* et al., 2010). Both constructs have been previously shown to complement MP function in severe *mp* mutant alleles *(Schlereth et al., 2010; Dolf Weijers*, personal communication). As already shown (*Galbiati* et al., 2013), *pMP::MP-GFP* (with the GFP in the middle region) displays identical expression pattern of *MP* transcript (Fig. 4A,B and 4D,G).

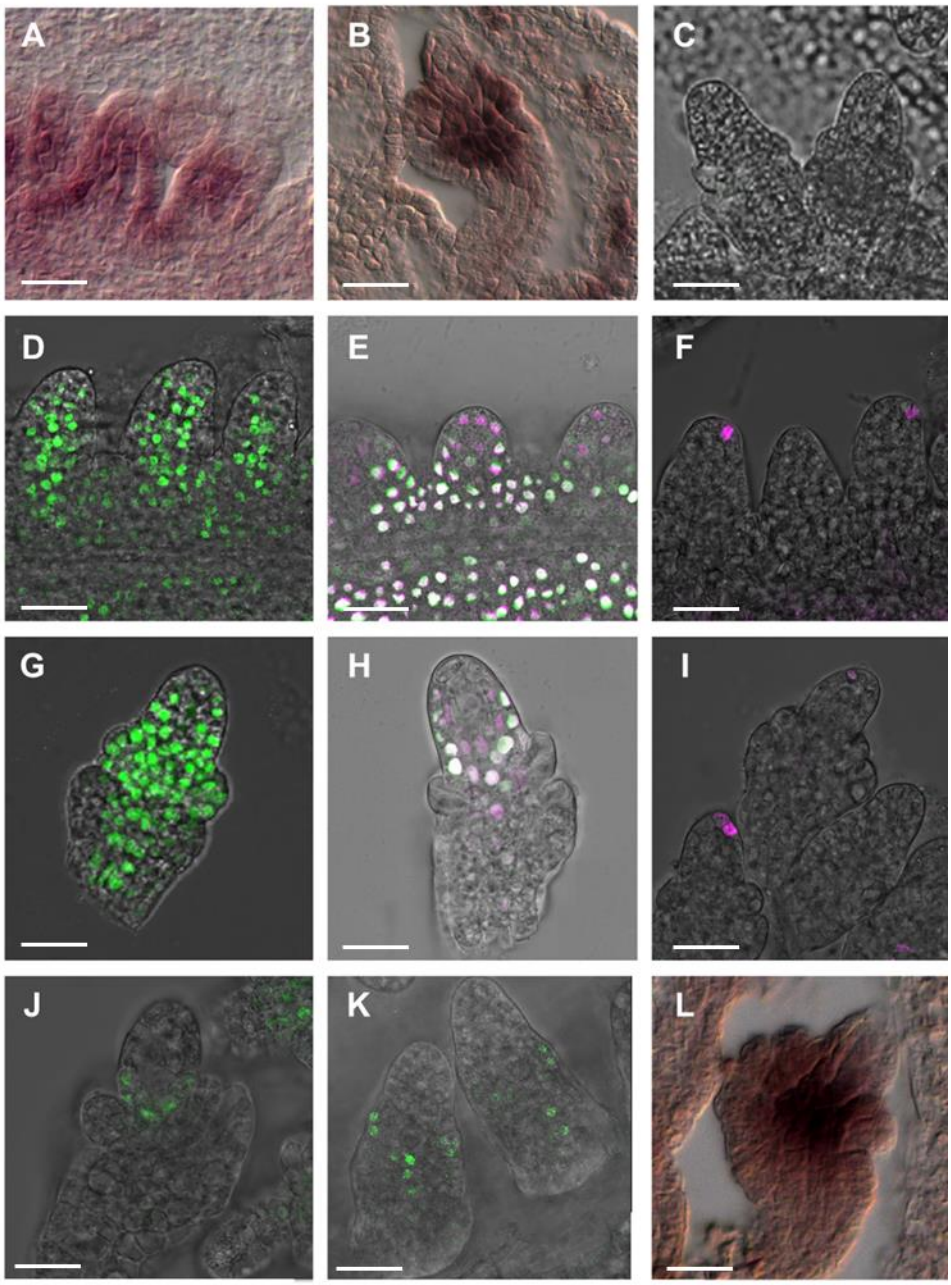

Figure 4. During ovule development *MP* and its targets are expressed in region of auxin minima. (A, B) *MP* expression detected by In-Situ Hybridization in ovules at stages 1-II and 2-II. (C) *pMP::MP-GFP* c-terminal line, no expression is detectable

in ovules. (D, G) pMP::MP-GFP middle region line, in ovules at stage 1-II and 2-III. (E,H) R2D2 (DII::VENUS and mDII::ntdTomato in the same vector) expression in ovules at stage pMP::MP-GFP(MR) in ovules at stage 1-I and 2-III. (F,I) DR5v2 expression in ovules at stages 1-II and 2-V, (J) pTMO5::3xnGFP in ovule at stage 2-III, (K) pTMO3::3xnGFP in ovules at stage 2-I, (L) In-Situ Hybridization of *CUC1* transcript in ovules stage 2-III. Scale bar = 20 µm.

MP transcript and MP–GFP protein are detectable at the base of ovules, in the boundaries between two ovules and in the developing ovule vasculature (Fig. 4D). Once integuments elongate, MP protein signal is restricted to the basal part of nucella (Fig. 4G). Conversely, no clear GFP signal is evident in pMP::MP-GFP C-terminal line, in any ovule developmental stages observed (Fig. 4C).

These observations suggest that, in the context of reproductive development, particularly ovule development, MP function is accomplished by a transcription factor lacking the CTD domain, which is able to activate transcription in cells with low level of auxin. Auxin level has been detected with R2D2 reporter (*Liao* et al., 2015), which measures auxin reduction as an increase of DII::VENUS signal relative to mDII::ntdTomato red signal, thus co-localization of both reporters (white signal in Fig. 4E and 4H) marks region of auxin minima. Besides, expression of DR5v2 (*Boer* et al., 2014; *Liao* et al., 2015) composed by repeated high affinity ARFs binding site, confirms that auxin maxima in ovules is in few epidermal cells at the tip of nucellus, starting from stage 1–II, and it remain expressed there until stage 2–V (Fig. 4F, 4I). Weak DR5v2 signal has also been detected in the chalazal part of ovule from stages 2-V (Fig. 4I).

For all the ovule developmental stages taken into account, auxin minima/maxima detected by R2D2 and DR5v2 match with previously reported studies and agree with auxin pattern produced by PIN1 and PIN3 efflux carrier (*Benková* et al., 2003; *Ceccato* et al., 2013; *Galbiati* et al., 2013). On top of that, in ovules, *MP* is expressed in the cell with low auxin concentration.

Alternative splicing driven *MP* isoform, due to the lack of CTD, in principle should not be bound by AUX-IAA, therefore may preserve the ability to activate direct target genes. Thus, we analysed the expression pattern of known targets of MP during ovule development, such as *TMO3/CRF2*, *TMO5* and *CUC1* (*Galbiati* et al., 2013; *Schlereth* et al., 2010; *Wu* et al., 2015).

TMO5 acts downstream of MP in embryo during root initiation (*Schlereth* et al., 2010), whereas in ovules it is expressed in the chalazal part of the nucella (Fig. 4L). *TMO3*, on the other hand, was also found to be regulated by MP in organogenic region of the reproductive shoot apex (Wu et al., 2015). In the pistil, *TMO3* is expressed at the basis of ovule primordia (*Cucinotta* et al., 2016), in the developing inner integuments and in the ovule vasculature (Fig. 4M). Finally, *CUC1*, which is a MP target during pistil development, is expressed in the boundaries between ovules (*Galbiati* et al., 2013), in the chalazal part of the nucellus, in the developing inner integuments and ovule vasculature (Fig. 4N).

On top of that, *TMO5, TMO3* and *CUC1* are co-expressed with *MP* in low auxin regions, suggesting that, in these cells, MP works as transcriptional activator (Fig 4J-L).

These findings open up to a complete new scenario, since the alternative MP protein isoform is uncoupled from auxin and Aux/IAA regulation. Besides, the lack of CTD does not interfere with MP homo-dimerization. In fact, it has been proved by protein structure resolution that MP-MP binding occurs through the DBD (*Boer* et al., 2014). Moreover, it has been recently proposed and demonstrated that MP is able to recruit SWI/SNF chromatin remodelling ATPases to increase accessibility of DNA, promoting induction of key regulators of flower primordium initiation (*Wu* et al., 2015). In the absence of auxin, Aux/IAA proteins bind MONOPTEROS and block recruitment of the SWI/SNF chromatin remodelling ATPases. Conversely, they recruit a co-repressor/histone deacetylase complex.

Further, we may speculate that the short isoform of MP is able to recruit SWI/SNF also in absence of auxin cues, in order to activate key genes of ovule development in region of auxin minima. However, MP might be regulated by auxin at the transcriptional level, indeed, as recently suggested (*Bathia* et al., 2016), *cis*-elements within the coding region may contribute to the regulation of *MP* expression by auxin.

Eventually, we are obliged to keep in mind that truncated ARF repressors, which are abundant in many plant species, may be able to interact with other truncated ARFs or with ARF repressors. In *Arabidopsis thaliana*, also ARF3, ARF13, ARF17 and ARF23 are truncated, although ARF23 has been described as a pseudogene.

As we confirmed the existence of both *MP* mRNA isoforms co-exist, with or without CTD, but we were not able to detect the two protein

isoforms, it would be fascinating to investigate post transcriptional mechanisms of regulation of *MP*, to further tangle the already intricate *MP* regulation.

Materials and Methods

Plant material and growth conditions

Plants were grown at 22°C long-day (16 h light/8 h dark) conditions. pMP::MP-GFP, *mp-B4149* (*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.

CRISPR/Cas9 system

All the constructs used and the cloning procedure followed are described in *Fauser* et al. 2014 study, that have already demonstrated the possibility to target specific endogenous genes of Arabidopsis, creating stably inherited mutations (*Fauser*, *Schiml*, & *Puchta*, 2014). The expression constructs containing both the Cas nuclease and the single-guide RNA (sgRNA). The CRISPR-P web tool (Lei et al. 2014) was used to identify the appropriate protospacers on the MP genomic sequence, calculating a score that allows to minimize the off-target effects. We expected that the gRNA would bring the Cas9 protein to the MP target site where it will generate double-stranded breaks.

Deletions and insertions will be produced during non-homologous end joining repair of the double-stranded break.

Plasmid construction and Arabidopsis transformation

To construct *p35S::mpT56-RFP*, *MP* genomic fragment was amplified and recombined into vector pB2GW7 (Gateway system, Invitrogen, www.lifetechnologies.com).

Alternative Splicing PCR analysis

RNA was extracted using Macherey-Nagel® Nucleozol, treated with Thermo Fisher Scientific® TURBO™ DNase and subsequently retro-transcribed using Promega® ImProm-II™ Reverse Transcription System. The synthesized cDNA was used for actin PCR to exclude DNA contamination (forward primer CTCAGGTATTGCAGACCGTATGAG and reverse primer CTGGACCTGCTTCATCATACTCTG). PCR for alternative splicing event detection were done using primers couples over intron tenth of *MONOPTEROS* (forward TGCTCAAGATGTCCAGTCGC and reverse CGAGCTTTGTGGGTGAGTTAGTAG) and between the intron and exon-exon junction (forward TGGGTAATGTTTTGACTTGG and reverse CCACAAACTCTTCCCATGGAT)

Microscopy

For confocal laser scanning microscopy, dissected pistil were mounted in water and observed with SP2 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.

In-situ hybridization

Performed as previously published (*Galbiati* et al., 2013)

Supplemental data

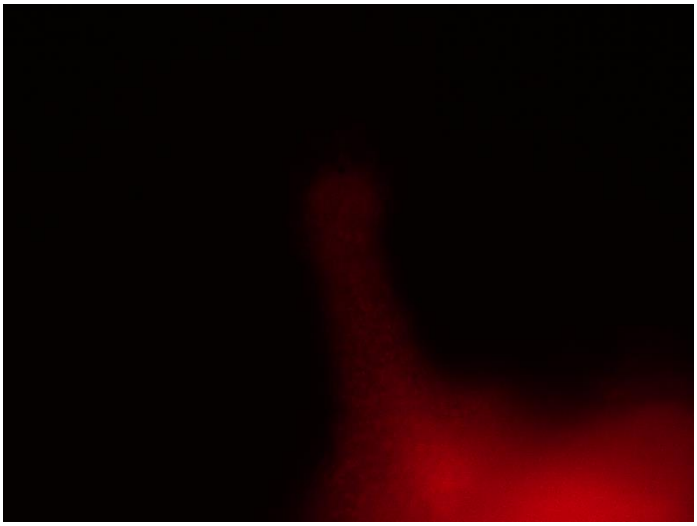

Fig Suppl. 1 Fluorescence signal of 35S::mpT56-RFP. No signal is visible on the pistil.

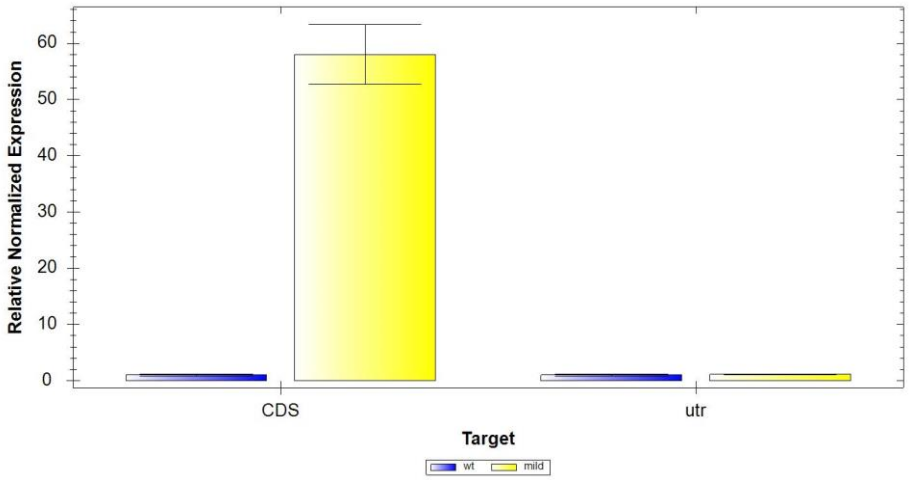

Fig Suppl. 2 Realt Time qPCR analysis of cDNA retro-transcribed from RNA extracted from wild type and 35S::mpT56-RFP inflorescences. The exogenous *MP* mRNA is 55-fold up regulated compared to the wild type (on the left), whereas the endogenous *MP* mRNA is not affected (on the right).

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MANUSCRIPT #3

HDA19: a new role for histone deacetylation during ovule development in *A. thaliana*

Andrea Guazzotti[1], Silvia Manrique[1], Gonzalo Villarino[2], Mara Cucinotta[1], Alex Cavalleri[1], Robert G. Franks[2] and Lucia Colombo[1]*

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

[2] North Carolina State University, Department of Plant and Microbial Biology, Raleigh, NC, 27606, USA

* Corresponding author (@unimi.it)

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Abstract

Seed number is one of the most important crop traits, needed to meet the increasing demand for food, feed, fuel and other plant-derived products. Seed number is linked to ovule number, since ovules, once mature, undergo fertilization and give rise to seeds.

In this work, we discuss a new role for epigenetic driven regulation during ovule primordia formation. We describe, in particular, the involvement of HISTONE DEACETYLASE 19 (HDA19) in the regulation of molecular networks underneath this developmental process. To identify HDA19 putative targets, we have performed a transcriptomic analysis of ovule and placenta cells sorted by Fluorescence Activated Cell Sorting (FACS). We have analyzed the data obtained and

proposed a molecular network controlling ovule number determination.

Introduction

Seed number is an important crop trait and a target for plant breeding related to yield enhancement. Total seed number is linked to ovule number, since ovules, upon fertilization, give rise to seeds.

Although a small number of factors involved in ovule primordia formation are known, the main mechanisms underneath this process still remain poorly characterized.

Ovule primordia arise from a meristematic tissue named placenta during stage 8 of gynoecium development. Ovule primordia number mainly relies on two factors: pistil length, which defines placenta size and the distance between the primordia.

As well as any other organ formation in plants, ovule primordium genesis needs cell division and cell identity determination. The boundaries between two adjacent ovules usually have, conversely, reduced cell division and expansion (*Aida and Takada*, 2006).

A few genes are known to regulate boundary establishment, among which the *CUP SHAPE COTYLEDON* (*CUC*) gene family (*Aida* et al., 1997) are the best characterized. It has been previously reported how CUC transcription factors are required for organ boundary establishment of cotyledons. In *cuc1 cuc2* double mutant or in *cuc2* plants with ovule-specific RNA interference of *CUC1*, ovule density is highly reduced (*Ishida* et al., 2000; *Galbiati* et al., 2013).

Another important transcription factor for ovule primordia formation is AINTEGUMENTA (ANT) (*Elliot* et al., 1996; *Liu* et al., 2000). ANT promotes cell division in the placenta. In fact, *ant* mutants show a dramatic reduction in ovule number, and ovules lack the integuments (*Klucher* et al., 1996).

As widely demonstrated (*Galbiati* et al., 2013; *Cucinotta* et al., 2014), auxin is a key player for ovule primordia formation. The ovule primordium requires the formation of an auxin maximum in the placenta, where organ founder cells will be able to initiate the newborn ovule. Auxin maximum is generated by proper localization of PIN efflux carrier, which specifically localizes at the membrane of placenta cells (*Benkovà* et al., 2003; *Ceccato* et al., 2013). Although *pin1* knock out mutants are embryo lethal, *pin1* allele with defective PIN1 protein causes a severe reduction of total ovule number per pistil (*Bencivenga* et al., 2012; *Galbiati* et al., 2013).

SEPALLATA3 (SEP3) together with either SHATTERPROOF1 (SHP1), SHP2 or SEEDSTICK (STK) determine ovule identity fate. All four abovementioned genes belong to the MADS-box transcription factor family. In the triple mutant *stk shp1 shp2*, ovules lose their identity and they are converted into carpelloid structures (*Pinyopich* et al., 2003; *Brambilla* et al., 2007).

Interestingly, it has been shown that the *HISTONE DEACETYLASE 19* (*HDA19*) is expressed in placenta and ovules (*Krogan* et al., 2012). *HDA19* is one of the most highly expressed histone deacetylase in Arabidopsis and it has been demonstrated to influence several different biological processes and is widely expressed along most

plant organs, including all flower whorls (*Zhou* et al., 2005; *Tian* et al., 2001; *Tian* et al., 2003; *Long* et al., 2006).

In order to find out putative target genes of HDA19 during ovule development, we sorted ovule-specific cells from wild type and *hda19* and did transcriptomic analysis.

Results

hda19 mutant has a reduced total ovule number, impaired ovule development and abnormal starch deposition

According to previous publications (*Tian* et al., 2003), *hda19* mutant alleles have a reduced seed set. To disclose this phenotype, two mutant alleles of *HDA19* have been analyzed in terms of ovule number, pistil length and ovule density. We characterized two mutant alleles, *hda19-3* in Col-0 and a second *hda19* mutant allele in Wassilewskija ecotype.

Whole inflorescences were harvested and cleared with chloral hydrate. Pistils at stage 8 were isolated and observed in order to count ovule number, placenta length and distance between primordia.

Both alleles show a reduction in total ovule number without major changes in the pistil length (Fig. 1 A, B).

As expected and in agreement with previous publications (*Tian* et al., 2003), *hda19* plants showed a more severe phenotype when grown in short day conditions. As described formerly, histone acetylation/deacetylation, and in particular H3K9Ac residue, are chromatin modifications responsive to light (*Jang* et al., 2011).

To check if *hda19* ovule number was affected by photoperiod, wild type and *hda19* plants were grown either in short day conditions or in long day conditions and ovule number was recorded. The number of ovules of *hda19-3* pistils was further reduced in comparison with the wild-type when plants were grown in short day conditions.
The data appear to be consistent in the two different alleles analyzed.

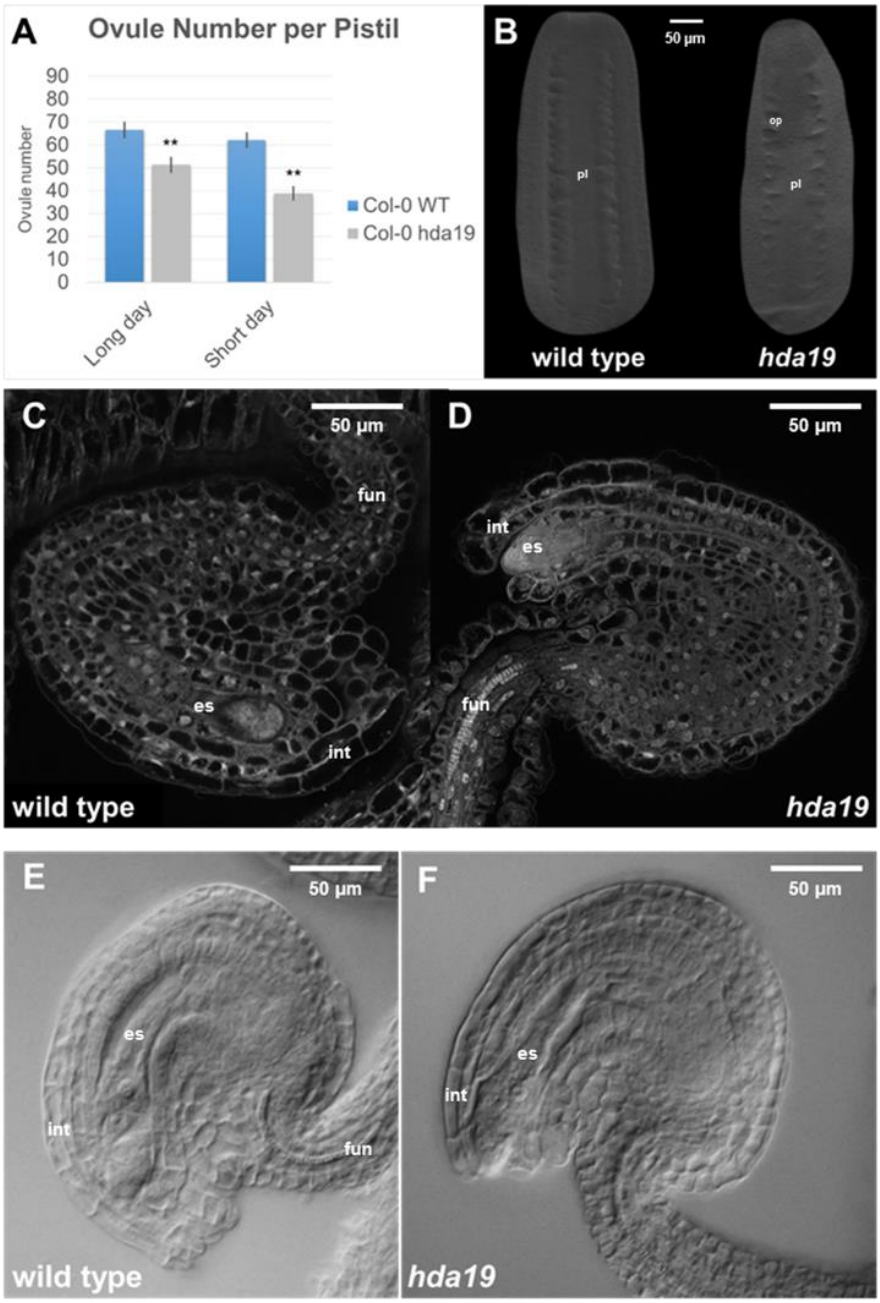

Fig. 1 *hda19* mutant ovule phenotypes. Ovule number, pistil length and ovule density of *hda19-3* and wild type plants. A) Total ovule number of *hda19* and wild type control. Asterisks denote statistical significance by Student's t-test with a p-value=0,05; B) A wild type pistil and a *hda19-3* mutant pistil at stage 8 of carpel development; C) Confocal

microscopy of Feulgen staining mature wild type ovule; D) Confocal microscopy of Feulgen staining mature *hda19* ovule. E) Clearing analysis of mature wild type ovule. F) Clearing analysis of mature *hda19* ovule. The mutant embryo sac appears to be not fully covered by the integuments. pl = placenta, op = ovule primordia, fun = funiculus, es = embryo sac, int = integuments.

Besides reduction of ovule number, *hda19* ovules show a partially exposed embryo sac not fully covered by the integuments. This phenotype may be due to either defective integuments growth or to misplacement of the embryo sac (Fig. 1D;1F).

Fluorescence-Activated Cell Sorting (FACS) of *pSTK::STK-GFP* expressing cells

HDA19 mRNA has been shown to be expressed in emerging ovule primordia during early stages of pistil development (*Krogan* et al., 2012). However, *HDA19* is also broadly expressed in all tissues of the inflorescence (*Schmid* et al., 2005).

In order to study specifically the network affecting ovule development, we collected specific ovule cell types by means of FACS.

We collected inflorescences of *hda19* and wild type plants expressing *pSTK::STK-GFP* (*Mizzotti* et al., 2014). *STK* is first expressed in placental tissue and young ovule primordia (stage 8 of flower development). Later on, during stage 11-12, it is expressed in all ovule tissues including the funiculus (*Pinyopich* et al., 2003; *Mizzotti* et al., 2014).

In *hda19* mutant, STK pattern of expression was similar to the wild type although with a lower intensity (Suppl. Figure 2). Cell sorting

yielded around 80.000 GFP-positive cells. The GFP-positive cells both from wild type and *hda19* have been used for RNA extraction and sequencing.

Transcriptional analysis of wild type and *hda19* sorted cells

RNA sequencing gave a huge list of mis-regulated genes. Among them, a significant number of key regulators of ovule primordia initiation (*Galbiati* et al., 2013) have been found downregulated in *hda19*, suggesting that all the machinery is therefore transcriptionally repressed (Fig. 2).

RNA Sequencing

Gene name	wild type value	*hda19* value	Fold change	P-value	significant
MP	48,8	16,1	**- 3,0**	5,00E-05	yes
CUC1	1,8	0,6	**- 3,2**	0,07195	no
CUC2	15,0	1,5	**- 10,0**	5,00E-05	yes
PIN1	14,6	2,6	**- 5,7**	5,00E-05	yes
ANT	61,7	7,3	**- 8,5**	5,00E-05	yes

Fig. 2 The table represents the RNA Seq data coming from the Fluorescence Activated Cell Sorting. *MONOPTEROS* (*MP*), *CUP-SHAPE-COTYLEDON 1* (*CUC1*), *CUC2*, *PIN-FORMED 1* (*PIN1*) and *AINTEGUMENTA* (*ANT*) are down regulated in the *hda19* mutant.

Transcriptional regulation of MADS-Box genes in *hda19* mutant

According to RNA Sequencing data, 14 MADS-Box transcription factors are significantly mis-regulated in *hda19* mutant.

Notably, all the deregulated MADS-Box genes share a similar pattern of up-regulation with the exception of *STK*, *SHP1* and *SHP2* (Fig. 3), which are down-regulated.

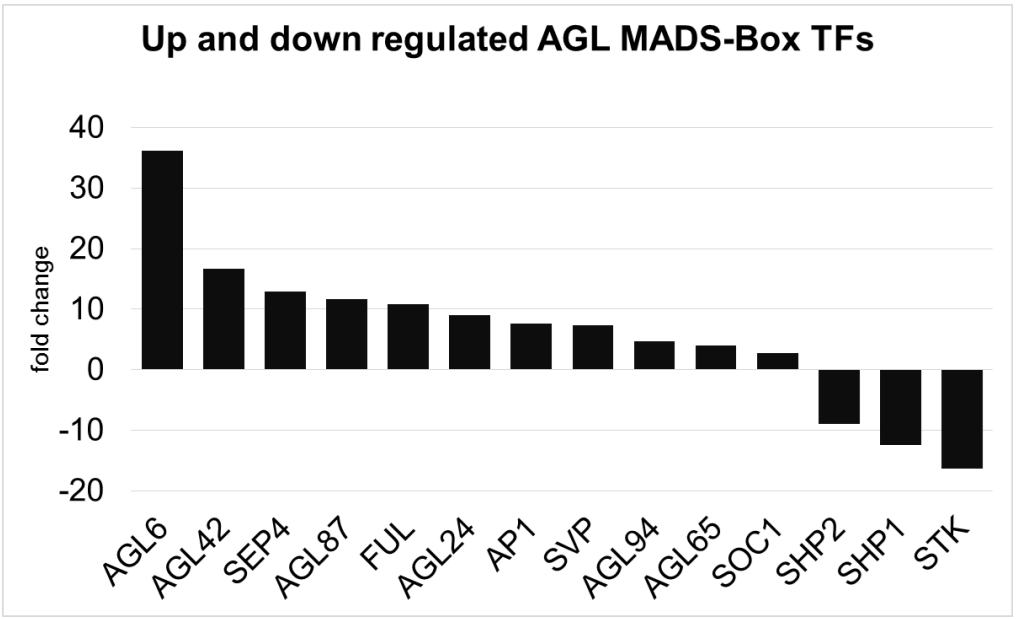

Fig. 3 The table represents the genes belonging to MADS-Box transcription factor family. All of them are up-regulated, with the only exception of *SHP1*, *SHP2* and *STK*, which are down-regulated.

Namely, *STK* is down-regulated by 16-fold and *SHP1* and *SHP2* by around 10-fold (Fig. 4)

RNA Sequencing

Gene name	wild type value	*hda19* value	Fold change	P-value	significant
STK	339,5	20,7	**- 16,4**	5,00E-05	yes
SHP1	67,3	5,4	**- 12,4**	0,00015	yes
SHP2	66,7	7,5	**- 8,9**	5,00E-05	yes

Fig. 4 RNA sequencing results for STK, SHP1 and SHP2.

STK, SHP1 and SHP2 are functionally redundant during ovule development and constitute, together with SEP3, the so-called ovule identity complex (*Pinyopich* et al., 2003). *stk shp1 shp2* triple mutant displays a failure in ovule identity determination. In the case of *hda19* mutant, it seems that despite the significant downregulation, the residual expression of these three transcription factors is enough to allow ovule identity specification and ovule development (Fig. 1).
The *stk shp1 shp2* triple mutant shows a consistent and statistically significant decrease in ovule primordia number. This is in agreement with the localization of STK protein in the placenta during ovule primordia initiation (Fig. 5).

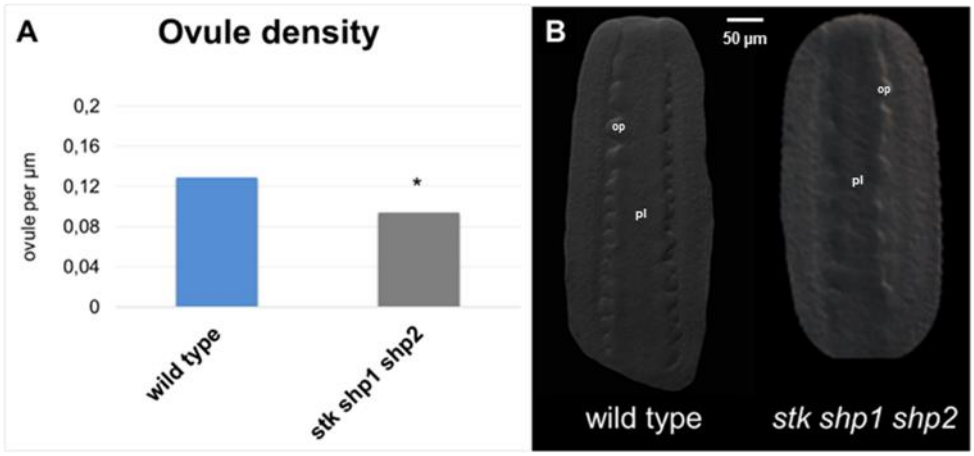

Fig. 5 A) Ovule number per pistil length, also addressed as ovule density, of wild type control and *stk shp1 shp2* triple mutant. B) wild type and stk shp1 shp2 ovule primordia during stage 8/9 of pistil development. op = ovule primordia, pl = placenta.

Thus, our results indicate that the reduction of the levels of *STK*, *SHP1* and *SHP2* might be responsible for the reduced number of ovule

primordia in *hda19* mutant, but the residual activity would be enough to allow ovule development to proceed with only minor defects.
The RNA Sequencing shows an up-regulation of *SVP*, *FUL* and *AGL42* (Fig. 6), together with other 8 MADS-Box transcription factors (Fig. 3). H3K9 is considered the main target for post-translational modification by HDA19 (*Zhou* et al., 2010), although we cannot exclude that other lysine residues may be modified.

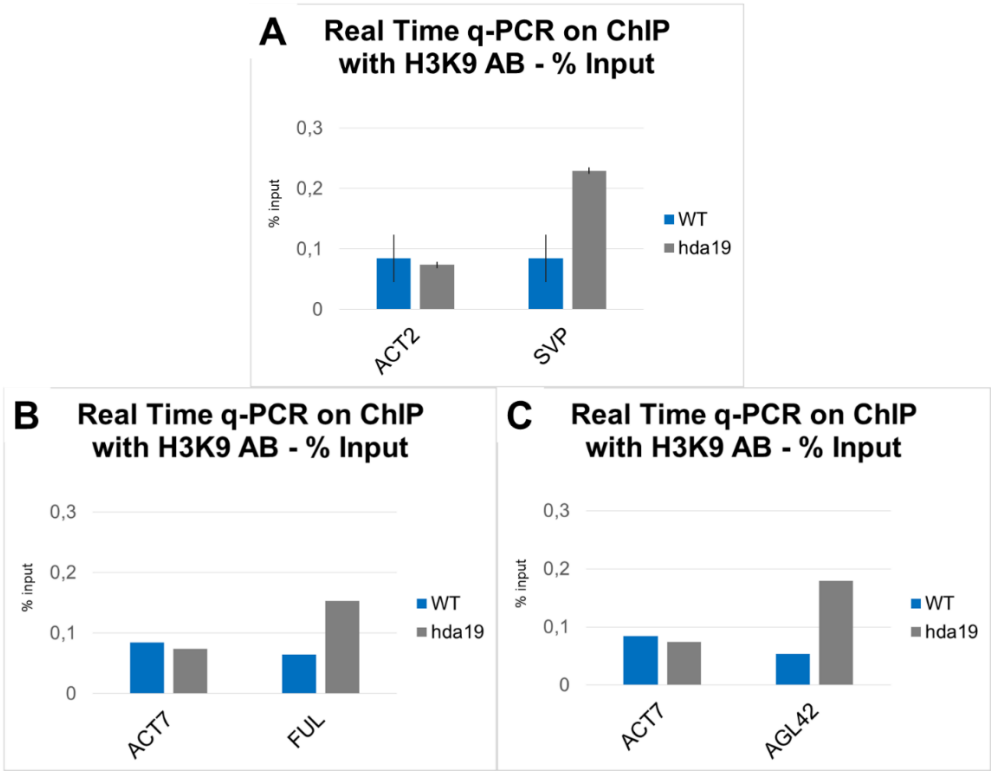

Fig. 6 A-B-C) Real Time qPCR on ChIP amplifying genomic regions of *SVP* (A), *AGL42* (B) and *FUL* (C).

To test the direct regulation by HDA19 of *SVP*, *FUL* and *AGL42*, Chromatin Immuno-Precipitation (ChIP) both on *hda19* mutant and

wild type control was performed (Fig. 6). For the immuno-precipitation, an antibody (*Upstate 07-352*) recognizing acetylation of lysine 9 residue on histone 3 (H3K9Ac) was used. Among the up-regulated MADS-Box genes, we picked up *SVP*, *FUL* and *AGL42* as they are known repressors of *STK* and *SHPs*. In particular, SVP has been demonstrated to bind *STK* promoter and directly regulate it (*Simonini* et al., 2012). On the other hand, FUL is a known repressor of *SHPs* (*Ferrandiz* et al., 2000; *Liljegren* et al., 2004). Furthermore, there are evidences which for AGL42 to bind STK promoter (*Balanzà*, personal communication)

All the three MADS-Box transcription factors tested by Real Time qPCR on ChIP shows an enrichment of 3 to 4 fold respect to the control, indicating that chromatin around *SVP*, *FUL* and *AGL42* loci is more acetylated in *hda19*. This suggests that *SVP*, *FUL* and *AGL42* are direct targets of HDA19 (Fig. 6).

Starch accumulation in *hda19* ovules

During *hda19* ovule development, we observed an abnormal starch deposition. In particular, starch accumulated in the later stages of ovule development.

According to RNA Sequencing data, a considerable number of starch-related genes are significantly up regulated in *hda19* mutant, one the contrary, only one gene *BETA-AMYLASE 7*, was found down regulated (Fig. 7).

RNA Sequencing

Gene name	wild type value	*hda19* value	Fold change	P-value	significant
AMY1, alpha-amylase-like	0,46	3,27	**+ 6,97**	2,00E-04	yes
BAM3, chloroplast amylase	74,72	300,16	**+ 4,01**	5,00E-05	yes
ISA3, isoamylase 3	22,00	68,40	**+ 3,10**	5,00E-05	yes
BAM2, beta-amylase 2	24,87	67,54	**+ 2,71**	5,00E-05	yes
SS4, starch syntase 4	13,87	24,63	**+ 2,49**	5,00E-05	yes
BAM1, beta-amylase 1	82,99	194,83	**+ 2,34**	0,00015	yes
AMY3, alpha-amylase-like 3	28,86	59,15	**+ 2, 04**	0,00055	yes
SBE2.1, starch branching enzyme 2.1	37,46	70.73	**+ 1,88**	0,00065	yes
SS1, starch syntase	42,19	79,58	**+ 1,88**	0,00055	yes
SBE2.2, starch branching enzyme 2.2	33,67	63,17	**+ 1,87**	5,00E-04	yes
BE1, alpha amylase	4,54	8,40	**+ 1,84**	7,00E-04	yes
AMY2, alpha-amylase-like 2	3,27	5,98	**+ 1,82**	0,01365	yes
BAM5, beta-amylase 5	79,35	129,13	**+ 1,67**	0,0111	yes
BAM7, beta-amylase 7	10,22	5,85	**- 1,74**	0,00205	yes

Fig. 7 RNA sequencing results for amylases and starch syntases.

Starch accumulation in the female gametophyte has been previously reported in *stk abs* double mutant (*Mizzotti* et al., 2012).

In *hda19* starch is accumulated in all stages of ovule development (Fig. 8, D and E), with a maximum at FGVII (mature) stage (Fig. 8, F).

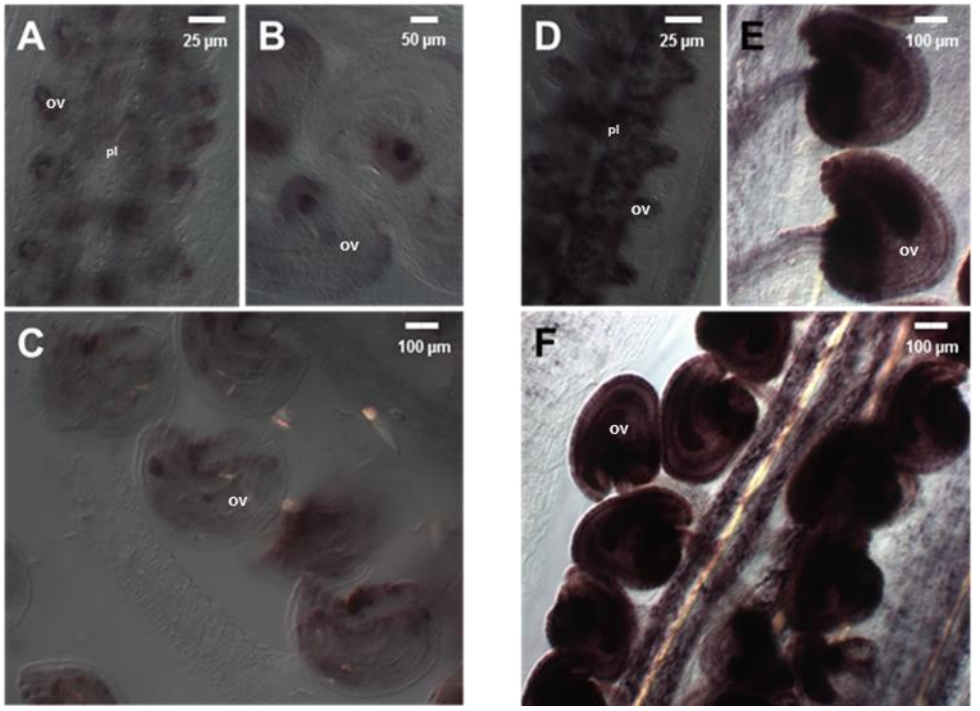

Fig. 8 Lugol's Iodine Staining for starch deposition detection in wild type ovules (A, B, C) and *hda19* ovules (D, E, F) at different stages of development. ov = ovule, pl = placenta.

Discussion

Chromatin remodeling is one of the most important key regulator of the transcription in response to environmental stimuli and stresses. Hereby, we propose histone deacetylation as an epigenetic modification influencing ovule primordium initiation and growth. We suggest that epigenetic regulation driven by HDA19 of *SVP*, *FUL* and *AGL42* may consequently result in *STK*, *SHP1* and *SHP2* down regulation in *hda19*.

In fact, SVP is a negative regulator of *STK* and directly binds *STK* promoter (*Simonini* et al., 2012). Likewise, FRUITFUL is a repressor of

both *SHP1* and *SHP2* (*Liljegren* et al, 2000) and its function as a negative regulator has been recently underlined (*Ehlers* et al., 2016). SVP and FUL, together with a previously reported repressor MADS-BOX transcription factor (*Chen* et al., 2011), AGAMOUS-LIKE 42 (AGL42), may be taken in consideration as candidate repressors of *STK*, *SHP1* and *SHP2* during early stages of ovule development. We may therefore hypothesize that the upregulation of known MADS-Box transcriptional repressors cause a down regulation of *STK*, *SHP1* and *SHP2*. We then suggest that the down regulation of *STK*, *SHP1* and *SHP2* in *hda19* mutant is the cause of ovule primordia reduction, since the triple mutant *stk shp1 shp2* has the same reduction (Fig. 9).

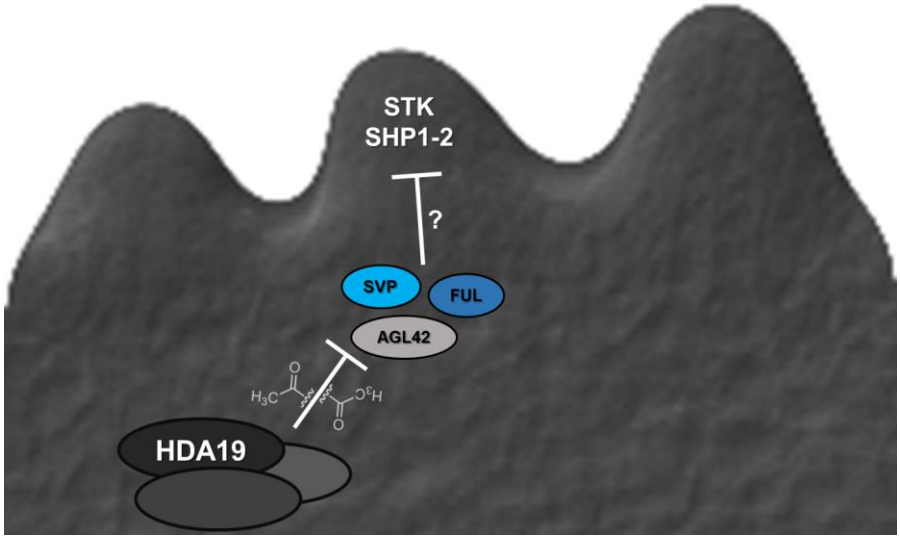

Fig. 9 Epigenetic regulation of ovule primordia growth.

It would be extremely interesting to take advantage of RNA interference to specifically suppress *SVP*, *FUL* and *AGL42* expression

in ovules and placenta in *hda19* mutant, in order to rescue the phenotype.

We cannot exclude other possible mechanisms of regulation by HDA19 in ovule primordia formation, since all the primordium initiation machinery is down regulated, included upstream transcription factors involved in the perception of auxin signaling, such as ARF5/MP, as well as cell cycle regulator ANT.

The *hda19* embryo sac often times remains exposed due to a defect in integuments growth. Fascinatingly, the RNA sequencing analysis showed a massive down regulation of several genes involved in integument development, whose mutants have defects in integuments growth. For instance, as described, *ANT* is down regulated in *hda19*, as well as *INNER NO OUTER* (*INO*) (-21 fold change in *hda19* compared to wild type). On top of that, we may suggest the involvement of HDA19 in epigenetic regulation of maternal tissues of the ovule, such as the integuments.

We have also shown that starch metabolism is severely affected in *hda19* ovules, as a massive accumulation of starch can be detected all over ovule development. It has also been proposed that altered sugar metabolism affects fertilization and in particular female gametophyte development (*Egli* et al., 2010; *Gottwald* et al.,2000; *Feuerstein* et al., 2010; *Niewiadomski* et al., 2005; *Christensen* et al., 2002; *Mizzotti* et al., 2012). It would be particularly exciting to investigate the possible consequences of starch accumulation and morphological defects of *hda19* ovules, such as integuments, during fertilization. In fact, *hda19* female reproductive organ is defective and

causes a partial sterility (*Manuscript #4 HDA19: epigenetic regulation of pollen tube growth and ovular guidance in A. thaliana*).

In conclusion, we have discussed a new role for HDA19 in the epigenetic regulation of ovule development, in particular primordia formation. We also gave clue on MADS-Box transcription factors family regulation driven by HDA19 and, finally, gave a new role for STK, SHP1 and SHP2 in ovule primordia initiation.

Methods

Plant materials and growth conditions

All plants were grown under controlled conditions in the greenhouse, simulating both long day (artificial 16 hours light and 8 hours dark) and short day (artificial 8 hours of light and 16 hours of dark).

hda19-3 mutant allele (SALK_139445) in Col-0 ecotype was provided by SALK Institute, *hda19* mutant allele in Wassilewskija ecotype is Feldmann T-DNA insertion and was kindly donated by prof. Clara Conicella (Institute of Biosciences and Bioresources, National Research Council of Italy, Portici, Italy).

pSTK::STK-GFP line has been previously described (*Mizzotti* et al., 2013) and gently donated by the authors. *stk shp1 shp2* triple mutant has been previously described and generated by *Pinyopich* et al, 2003.

Genotyping

DNA extraction was performed by harvesting young leaves and collecting them in an extraction buffer containing final concentration

of Tris-HCl 200 mM, NaCl 250 mM, 25 mM EDTA and SDS 0,5 %. Precipitation was achieved using with 2-propanol (Sigma) and after pellet reconstitution with bi-distilled water, DNA has been used for PCR-based genotyping. Primers for genotyping used in this study are reported in the table (Suppl. Table 1).

Total ovule number count, sample preparation, Lugol's Iodine Staining and microscopy observation

Total ovule number count was performed with a Leica MZ6 stereomicroscope on young siliques, as a sum of seeds, aborted ovule and aborted seeds. Pistil length has been determined by taking pictures using a Zeiss Axiophot D1 microscope (with Differential Interference Contrast), captured by a Axiocam MRc5 camera (Zeiss) with Axiovision software (version 4.1) and subsequently analyzed with ImageJ Software for the length measure.
Lugol's Iodine Staining has been performed as previously described (*Mizzotti* et al., 2012).

RNA extraction and cDNA synthesis

RNA was extracted from whole inflorescences with Nucleozol®. cDNA was retro synthesized with the kit from Promega®.

Fluorescence Activated Cell Sorting (FACS)-RNASeq

pSTK::STK-GFP line was manually introgressed into *hda19* mutant background by crossbreed and then used for cell sorting, along with the parental *pSTK::STK-GFP* in the wild type background.

For Fluorescence Activated Cell Sorting, closed flowers (stages 1-13) of *pSTK::STK-GFP* and *pSTK::STK-GFP* x *hda19* were manually collected and protoplasts were prepared and sorted as previously described (*Villarino* et al., 2016). For each experiment, inflorescences of around 200 plants were collected. To validate the correct sorting of the cells, in parallel, a similar amount of GFP-negative cells were collected and total RNA was extracted from both samples using the RNAeasy Microkit (Qiagen).

A fraction of the RNA was retro-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen/Life Technologies) and the expression of selected marker genes was checked by quantitative Real-Time PCR. In all cases, GFP-negative samples were depleted of the studied transcript while GFP-positive were enriched, indicating that cells were sorted correctly.

Three validated samples of RNA from wild type GFP-positive cells and 4 samples from *hda19* GFP-positive cells were used for library preparation and RNA. Libraries were sequenced in one lane of the HiSeq2500 Illumina platform with a yield of 250 million reads and an average of 35 million reads per sample. Almost 10 million reads were filtered out and of the 240 million reads remaining, 230 million mapped uniquely (96% of the reads after filtering) and were used for subsequent analysis.

Chromatin ImmunoPrecipitation (ChIP), Real Time qPCR and acetylation level analysis

ChIP has been performed as previously described (*Mizzotti* et al., 2014). Real Time qPCR were performed using primers designed in the

transcriptional start site (TSS) region, as this region has the maximum peak of H3K9 acetylation predicted in human cells (*Roh* et al., 2006). Bio-Rad SYBR Green Master-Mix with Bio-Rad C1000 Thermal Cycler have been used for experiments, Bio-Rad CFX Manager as software for analysis.

Real Time qPCR acetylation level analysis has been performed using % input and background subtraction.

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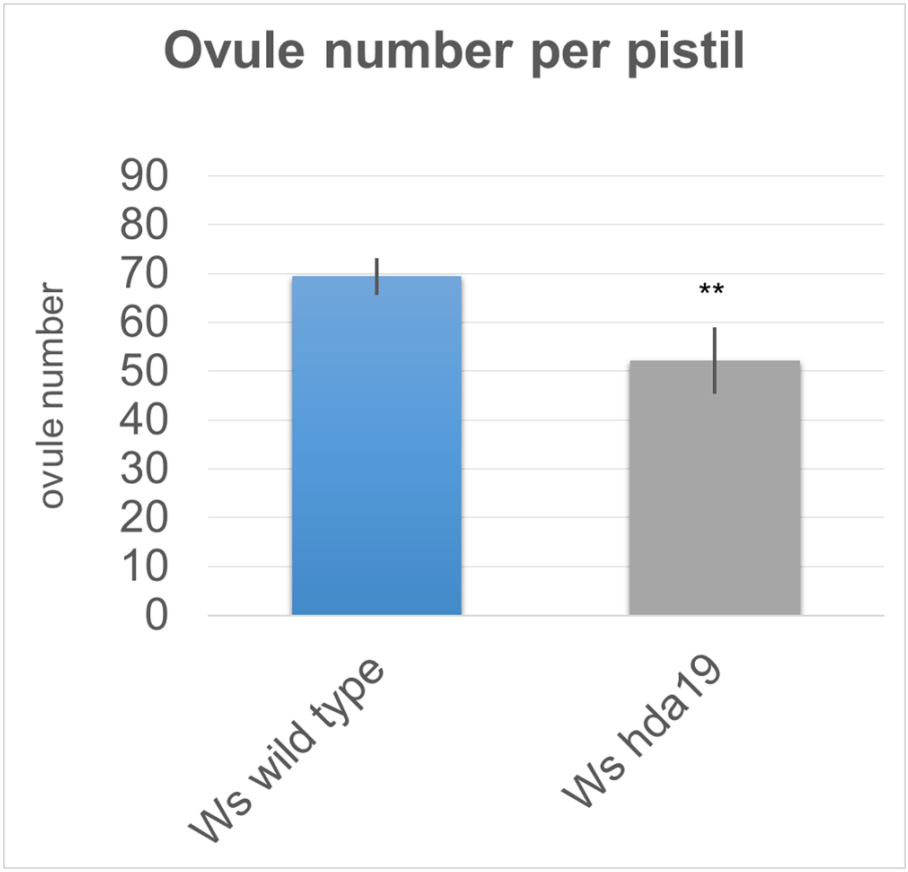
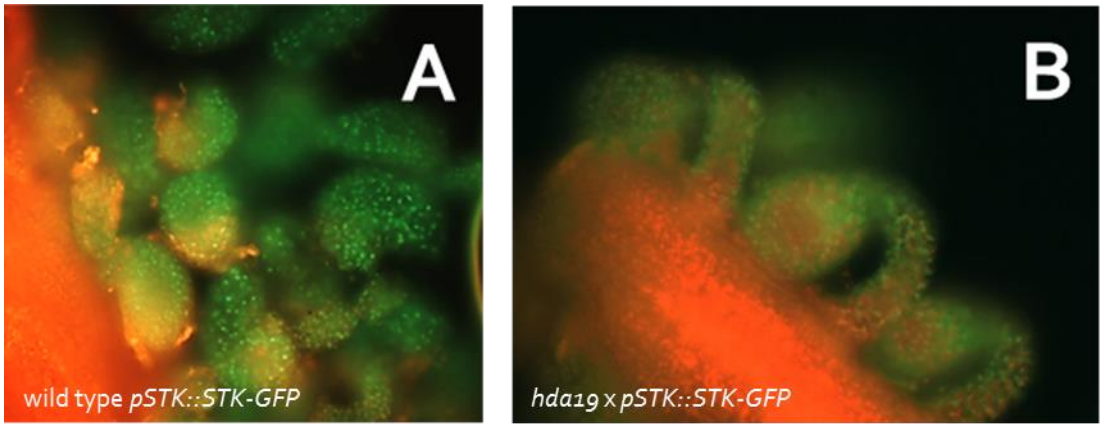
Supplemental data



Suppl. Figure 1. Total ovule number in Wassilewskija wild type and Wassilewskija *hda19* T-DNA insertion line



Suppl. Figure 2. *pSTK::STK-GFP* and *pSTK::STK-GFP* x *hda19* protein localization is specific to placenta, ovules during early stages of pistil development and integuments during late stages of pistil development. A) *pSTK::STK-GFP* "wild type" background GFP expression; B) *pSTK::STK-GFP* x *hda19* GFP expression.

Primers for Genotyping	
hda19 forward SALK_139445	ACTCTCTTCCTTGTCTGCGTG
hda19 reverse SALK_139445	ACCAGACAATGAATCAGCACC
hda19 T-DNA Left Border	TGGTTCACGTAGTGGGCCATCG
stk-2 forward	GGATCCGCCAATAACAACATAAGATCA
stk-2 reverse	GGAACTCAAAGAGTCTCCCATCAG
shp1 forward	GTGACGGAAGGAGGGTTGACG
shp1 reverse	GTCTACTGATGAGTTGTCACTAGG
shp1 T-DNA Left Border	GATGCACTCGAAATCAGCCAATTTTAGAC
shp2 forward	GAGGATAGAGAACACTACGAATCGTC
shp2 reverse	CAGGTCAAGTCAATAGATTCCCTAC

Suppl. Table 1 Primers used for PCR-based genotyping analysis

Primers for Real Time qPCR, ChIP and Expression analysis	
ACT7 Forward ChIP	CGTTTCGCTTTCCTTAGTGTTAGCT
ACT7 Reverse ChIP	AGCGAACGGATCTAGAGACTCACCTTG
SVP Forward ChIP	ATCCCGTTCTCGAAAGATCC
SVP Reverse ChIP	TTCCGGTGGAAGAGAAGATG
AGL42 Forward ChIP	GGTCACTGGCTTGTTTAGGG
AGL42 Reverse ChIP	AGAGAGTTGAGCATCGCAGAG
FUL Forward ChIP	GGTCATTTCAGGGTTGTCGT
FUL Reverse ChIP	CAGCATCGCAGAGAACAGAG
ACT2 Forward ChIP	CTCAGGTATTGCAGACCGTATGAG
ACT2 Reverse ChIP	CTGGACCTGCTTCATCATACTCTG

Suppl. Table 2 Primers used for ChIP Real Time qPCR analysis

MANUSCRIPT #4

HDA19: epigenetic regulation of pollen tube growth and ovular guidance in *A. thaliana*

Andrea Guazzotti[1], Silvia Manrique[1], Alex Cavalleri[1], Ana Marta Pereira[2], Marta Adelina Miranda Mendes[1], Subramanian Sankaranarayanan[3], Tetsuya Higashiyama[3], Elisabetta Onelli[1], Silvia Coimbra[2], Simona Masiero[1] and Lucia Colombo[1]*

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

[2] Departamento de Biologia, Faculdade de Ciencias da Universidade do Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

[3] Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan

*Corresponding author

KEY WORDS: HDA19, pollen tube, transmitting tract, ovule development, synergid cell, female gametophyte, epigenetics

Abstract

Reproduction is a critical event during the life cycle of the plant and it requires a subtle and precise integration of external stimuli and internal signaling. In particular, pollen tube growth and guidance is a process which involves several mechanisms and signals. A crucial role, although not fully understood, during pollen tube growth and guidance is accomplished by the female reproductive organ, the pistil. Hereby, we describe the phenotype of *histone deacetylase 19* (*hda19*) mutant, which shows a dramatic reduction in seed set, due to defective ovular guidance in the female sporophyte. Specifically,

we will discuss the role of HDA19 in transmitting tract development and sporophytic effect on the female gametophyte development.

Introduction

Plant reproduction requires several steps to be accomplished, which are carefully regulated. Double fertilization is influenced by exogenous factors, such as environmental conditions, and unlike other developmental processes, it did not develop escapes for sterility. Its innermost weakness, in fact, reflects with the fact that sterility easily occurs as well as environmental stresses overcome.

Reproduction process in *A. thaliana* has been widely investigated. Final achievement of double fertilization is to generate embryo and endosperm, which are the two essential component of the seed, which represents the next generation and ultimate goal of plant reproduction.

The key players of double fertilization are the male and the female gametophyte. The female gametophyte is composed by seven cells belonging to four different cell types. At mature stage, the embryo sac contains two synergid cells, one egg cell, one central cell and three antipodal cells. The male gametophyte is the pollen grain which is composed by two sperm cells and a vegetative cell. The pollen grain, after landing on the stigma, germinates and penetrates the pistil through the transmitting tract. The pollen tubes grow into the transmitting tract through the apoplast, the space in-between the cells.

The pollen tube grows in the transmitting tissue until it reaches the proximal end of the pistil thanks to a steep calcium gradient, which is required and sufficient for its orientation and the apical elongation (*Holdaway-Clarke* and *Hepler*, 2003; *Malhó* et al., 1994; *Krichevsky* et al., 2007). This process takes about a few hours. However, the final goal of the pollen tube is the ovule. In fact, pollen tubes deflect and get through the septum to reach the ovules. Chemotropic and mechanical guidance equally contribute to the process. Chemoattractant molecules necessary for ovular guidance are known to be synthesized by the female gametophyte cells. Arabinogalactan proteins, as already mentioned above, are proven key players of pollen tube pre-ovular guidance and demonstrated to be in the extracellular matrix of female sporophytic tissues alongside the entire pathway of pollen tubes (*Coimbra* et al., 2007; *Higashiyama* et al., 2010; *Suarez* et al., 2013). Synergid cells have been demonstrated to be key players in pollen tube attraction at short distance (*Higashiyama* et al., 2001). A well-known and characterized family of signal peptides crucial for this process are the cysteine rich protein named LUREs (identified in *Torenia fournieri*) (Takeuchi et al., 2016; *Cheung* et al., 2016). LUREs are synthesized in the synergids under genetic regulation of MYB98. They are present in the ovary in a very close area around the micropyle but also in the funiculus and the septum as demonstrated by immunostaining. MYB98 is a transcription factor required for the differentiation of synergid cells (*Punwani* et al., 2007; *Punwani* et al., 2008; *Takeuchi* et al., 2012). *myb98* mutant has a defective pollen tube guidance caused by failure in the differentiation of the two synergid cells (*Kasahara* et al., 2005).

Although little is known, it has been proposed that the pathway of ovular guidance which attract pollen tubes towards the ovules is a combination of mechanical and chemical contribution, ligand-receptor interactions and further hormonal influence, such as gibberellic acid or cytokinin, as proposed for CYTOKININ OXIDASE/DEHYDROGENASE 7 (CKX7) (*Mendes* et al., 2016)

Taking advantage of genetic approaches, many mutants have been described as interfering with male and female gametophyte development. On the other hand, sporophytic role in male-female gametophytes interaction still remains to be clarified. Despite many studies are present witnessing the obvious fundamental role of female and male gametophyte during reproduction, how the sporophyte is involved in this process remains poorly characterized.

Hereby, we suggest a new role for an epigenetic modifier, HISTONE DEACETYLASE 19 (HDA19) during double fertilization. In particular, we are going to describe in detail the altered pollen tube growth in *hda19* and the influence of the sporophyte on female gametophyte development during pollen tube ovular guidance.

Results

hda19 shows a female sporophytic defect during fertilization

As previously described (*Tian* et al., 2003), stamens belonging to *hda19* mutant are shorter than those of wild type (Fig. 1). Thus, the

siliques of self-fertilized flowers are almost completely sterile and give no seeds (Fig. 1).

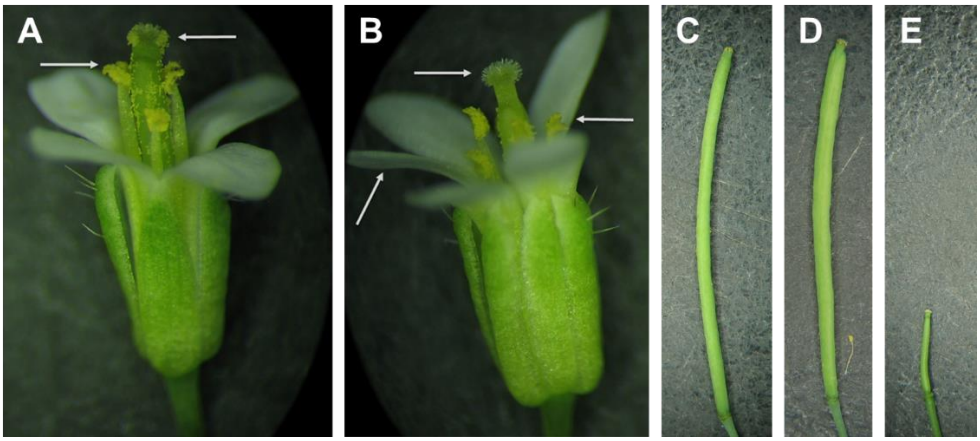


Fig. 1 A) A wild type flower at stage 12 of development; B) *hda19* flower at same stage (12) of wild type. The stamens are too short to ensure a correct pollination of the stigma by the anthers. C) wild type, D) *hda19/+* and E) *hda19 -/-* self-fertilized siliques. White arrows indicate anthers and stigma.

When the pollination is performed manually, either with *hda19* mutant pollen or with wild type pollen, the rescue of the phenotype is not complete, although in comparison with the self-fertilized, 20/30 % of ovules are fertilized and become seeds (Fig. 2). The lack of rescue of the phenotype suggests that the partial sterility of hand-pollinated *hda19* flowers is due to the female reproductive organ. In fact, *hda19* pollen morphologically and functionally does not show any difference compared with wild type pollen (Fig. 2).

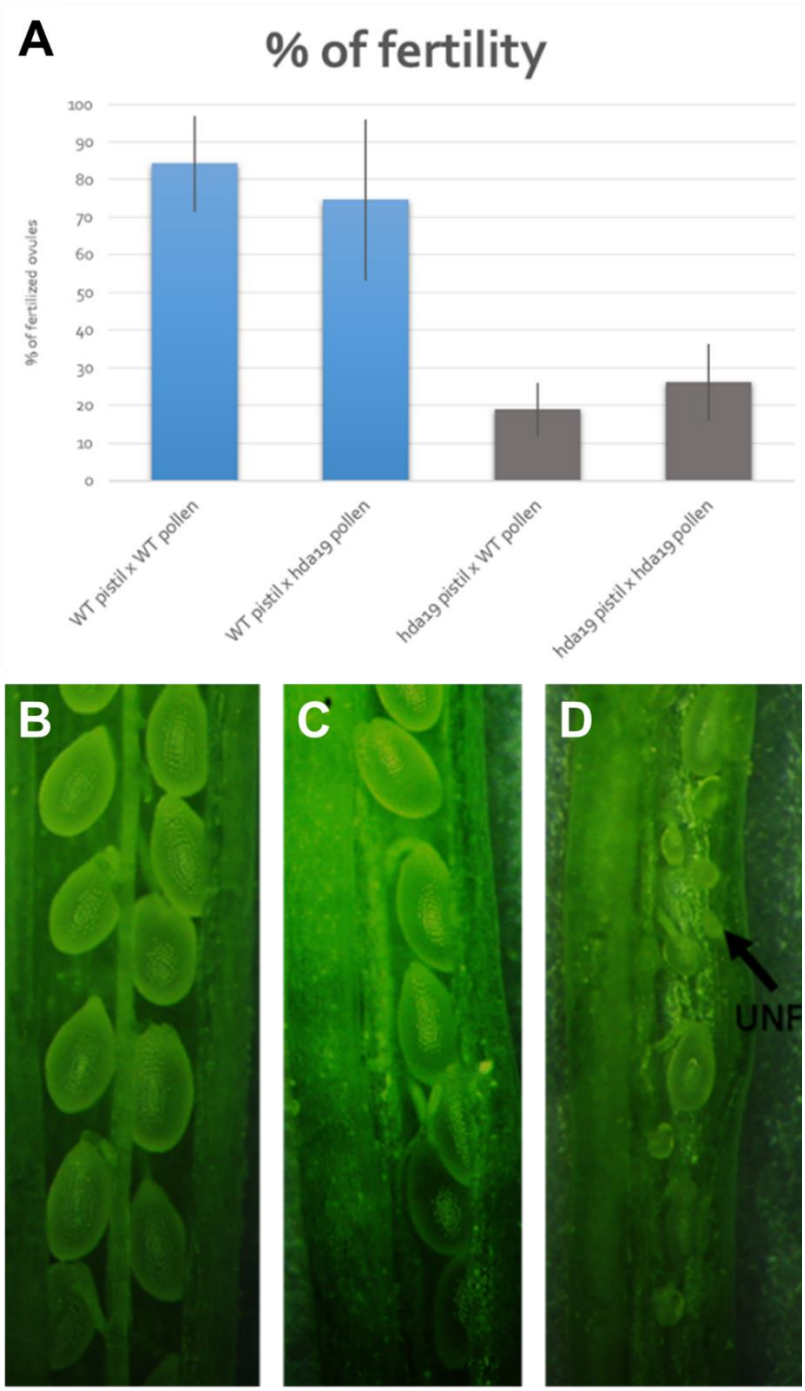


Fig. 2 A) The graph illustrates the fertility rate in the reciprocal backcrosses between wild type pistil with either wild type pollen or *hda19* pollen and *hda19* pistil pollinated with

either wild type pollen or *hda19* pollen; B) wild type silique; C) *hda19/+*; D) hand pollinated *hda19 -/-*. Black arrow indicates unfertilized ovules.

Interestingly, the distribution of fertilized ovules is random and does not correlate with a specific position along the ovary (Fig. 2, D).

hda19 transmitting tract

In order to understand the processes impaired in *hda19* female reproductive organ that causes female sterility, we tracked the pollen tube growth in vivo in *hda19* pistils using the Aniline Blue Staining, that stains callose (Fig. 3)

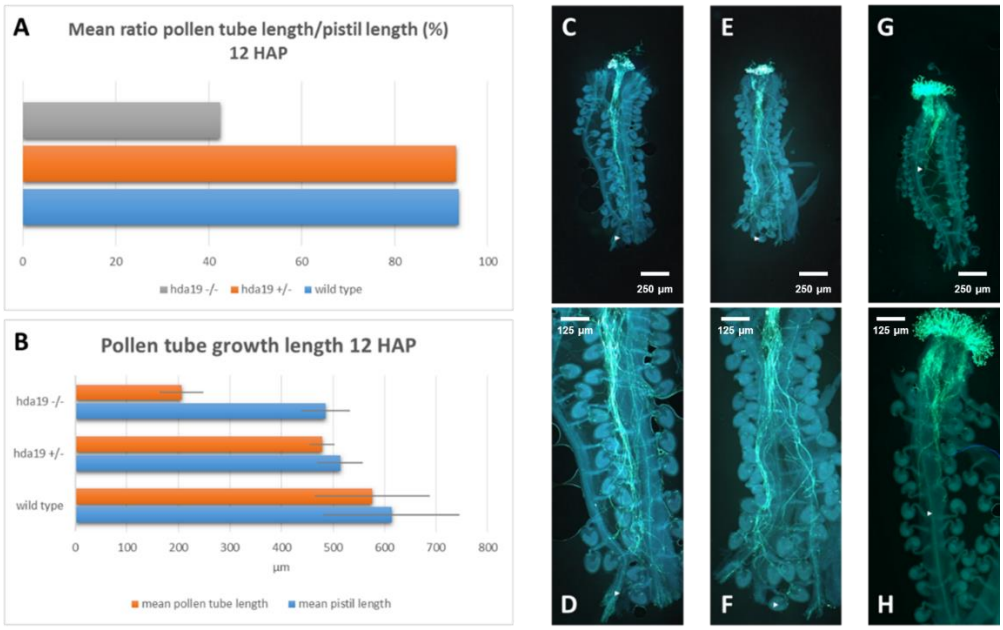


Fig. 3 Aniline Blue Staining 12 hours after pollination and pollen tube growth measurement. C,D Wild type; E,F *hda19* +/-; G, H *hda19* -/-. White arrows shows the farthest point that pollen tubes reach out.

Wild type, *hda19* +/- and *hda19* -/- pistils have been emasculated, pollinated with wild type pollen and fixed 12 hours after pollination (HAP). Pistils were then dissected and observed under the optical microscope after removing the valves.

Pollen tubes reach the proximal end of both wild type and *hda19* +/- pistil. However, in *hda19*, pollen tubes grow with a slower speed rate, as after 12 HAP, they only arrive at half the pistil (Fig. 3, A, B, G and H).

The morphology of the transmitting tissue has been observed with optical microscope after embedding the tissue in LRGold resin.

Transverse sections of either wild type and *hda19* have been made. *hda19* mutant shows a defective carpel fusion which causes an altered final morphology of the transmitting tissue (Fig. 4).

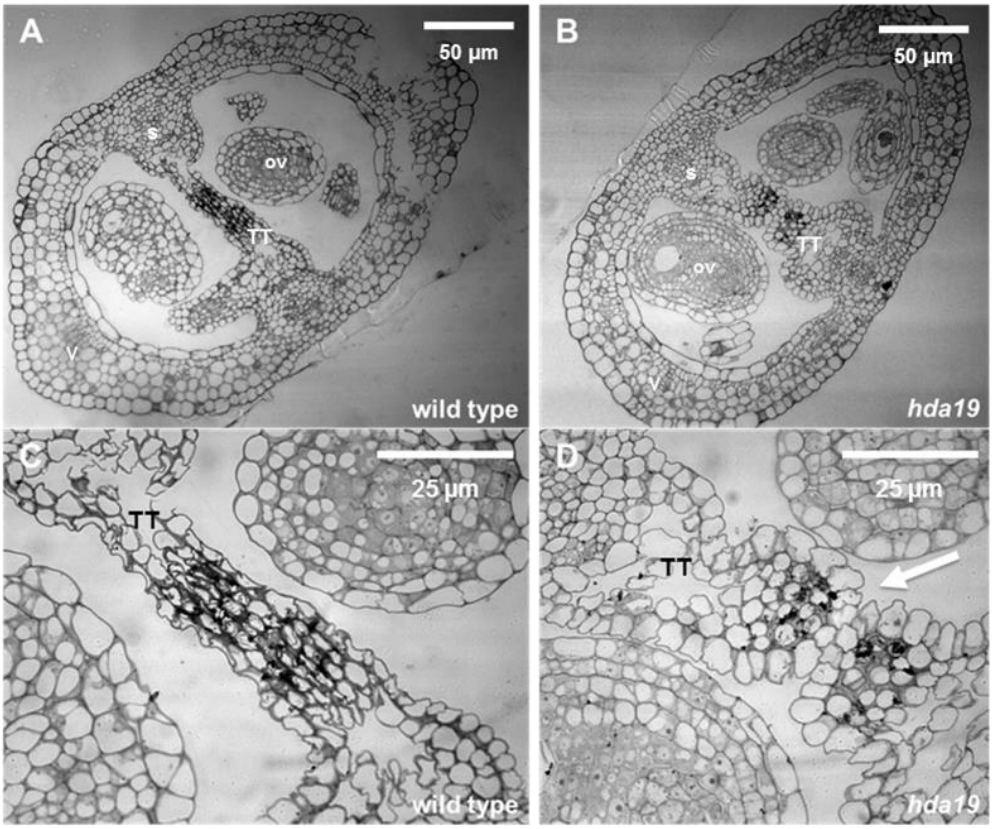
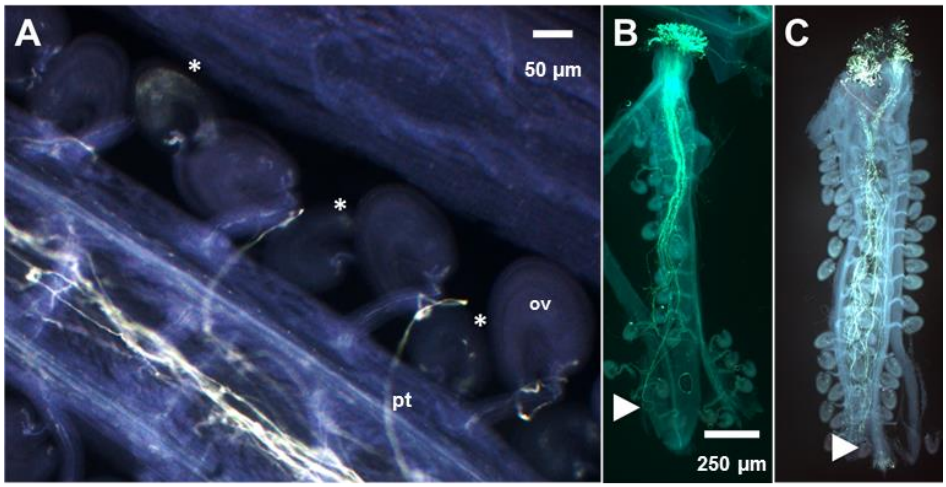


Fig. 4 Transverse section of wild type and *hda19* mature pistil. Detail of transmitting tract of wild type (A, C) and *hda19* (B, D). White arrow points out the morphological difference in *hda19* transmitting tissue. S = septum, ov = ovule, tt = transmitting tract, v = valve.

Although pollen tubes are able to arrive at the proximal end of the transmitting tract in wild type female tissue within 12 hours after pollination, *hda19* pistil needs 24 hours (Fig. 5).



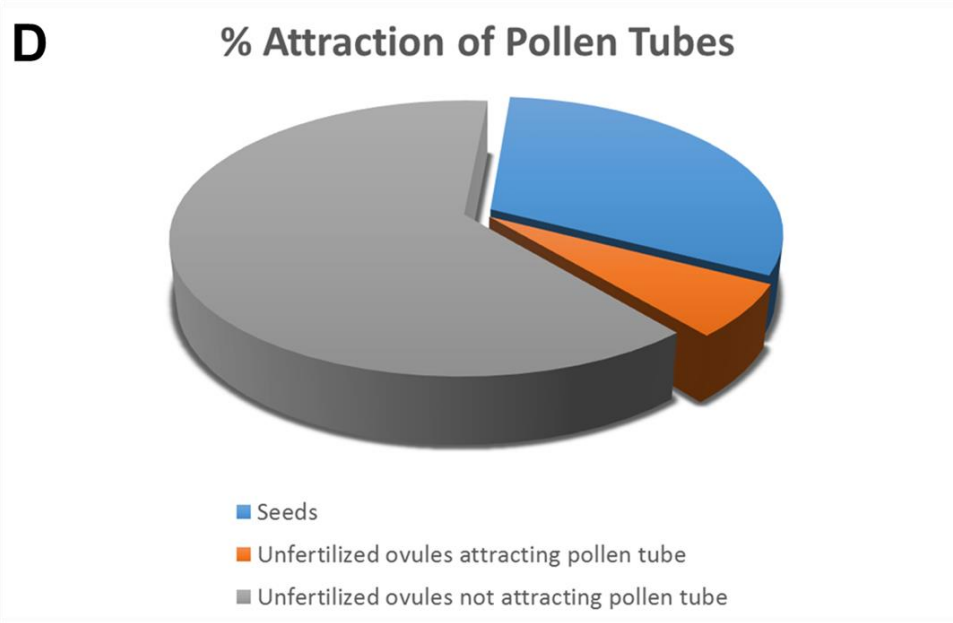


Fig. 5 Aniline Blue Staining 24 hours after pollination. White arrows indicate where the pollen tube arrives at last, white asterisks indicate ovules that do not attract pollen tubes. A) Detail of hda19 -/- pistil pollinated with wild type 24 HAP; B) hda19 -/- pistil pollinated with wild type pollen 24 HAP; C) wild type pistil 24 HAP; D)The pie chart shows the percentage of ovules that attract pollen tubes and successfully perform fertilization (blue), unfertilized ovules that attract pollen tubes but fail in fertilization (orange) and ovules that do not attract pollen tubes (grey). Pt = pollen tube, ov = ovule.

However, even if after 24 hours after pollination pollen tubes are able to get through *hda19* pistil, around 70% of total ovules observed, do not attract pollen tubes (Fig. 5).

Since no micropylar guidance defects have been observed in the heterozygous *hda19/+*, we hypothesize that *hda19* female tissue have either sporophytic defect in ovular guidance or sporophytic effect on the gametophyte, which indirectly causes ovular guidance failure.

hda19 ovule and female gametophyte

As the female gametophyte is needed for pollen tube ovular guidance, we collected previously emasculated pistils and observed ovules.

Pistils were either cleared with chloral hydrate and observed under optical microscope or treated with Feulgen staining for the confocal microscopy.

Both techniques revealed an abnormal development of the integuments, with the subsequent partial extrusion of the embryo sac (Fig. 6). This particular conformation of the ovule does not seem to be fully penetrant. In flowering plants, embryo sac is generally enclosed by the maternal tissue of the ovule, though a few species, such as *Torenia fournieri*, have a protruding embryo sac, not covered by the integuments (*Wu* et al., 1995).

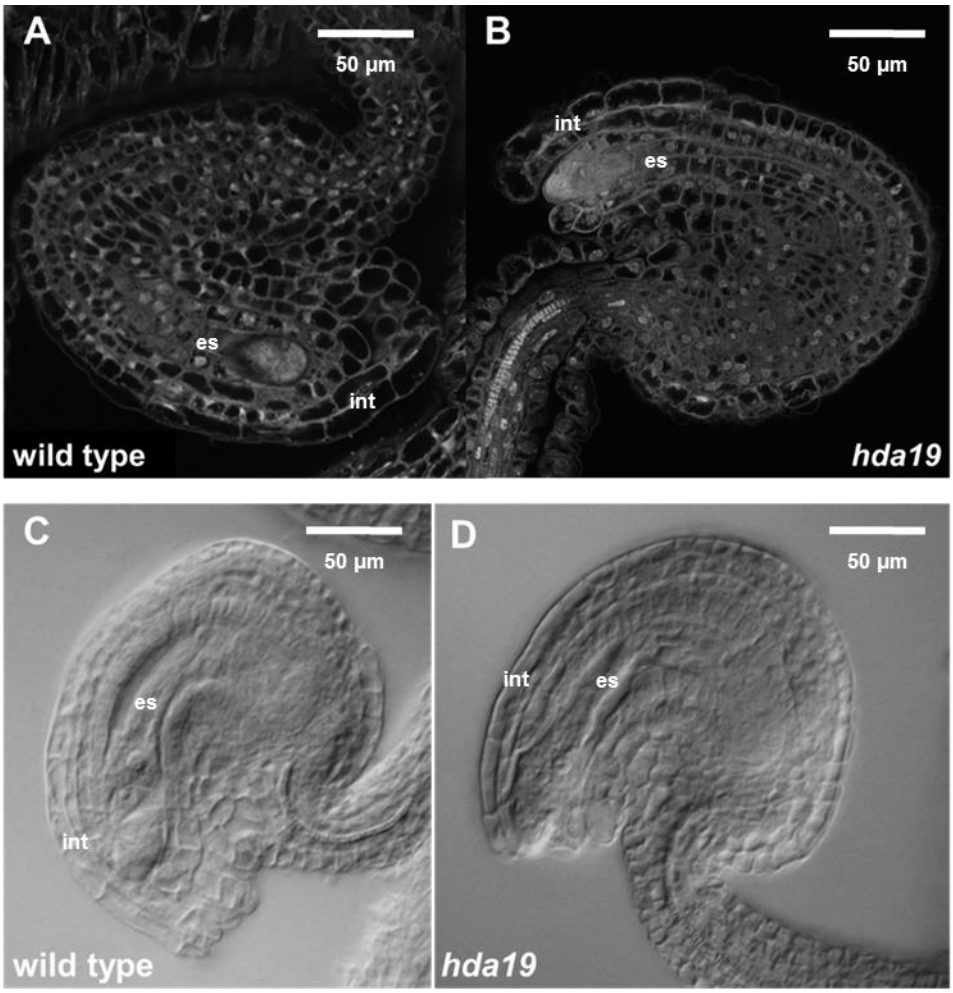


Fig. 6 A) Confocal microscopy of Feulgen staining mature wild type ovule; B) Confocal microscopy of Feulgen staining mature *hda19* ovule. C) Clearing analysis of mature wild type ovule. D) Clearing analysis of mature *hda19* ovule. The mutant embryo sac is not fully covered by the integuments. es = embryo sac, int = integuments.

As briefly mentioned, mature *A. thaliana* female gametophyte is composed by three antipodal cells, one central cell, one egg cell and

two synergid cells. Although their function is not yet fully understood, evolutionary conservation of all the four cell types suggests the fundamental role of each of them. In particular synergid cells and egg cell have been demonstrated to be involved in pollen tube attraction (*Higashiyama* et al., 2002; *Màrton* et al., 2005; *Okuda* et al., 2009).

We generated by introgression *hda19* mutants carrying cell identity marker lines for each female gametophyte cell type. Cell identity marker lines contain a reporter gene downstream promoters active specifically in either antipodal cells, central cell, egg cell or synergid cells and a reporter gene.

Antipodal cell, central cell and egg cell identity marker lines in *hda19* background were correctly localized and expressed in all ovules (Suppl. Figure 1).

For synergid cells, we used two independent cell identity marker lines introgressed in *hda19*: *pMYB98::GFP* (*Kasahara* et al., 2005) and *pCKX7::GUS* (*Mendes* et al., 2016).

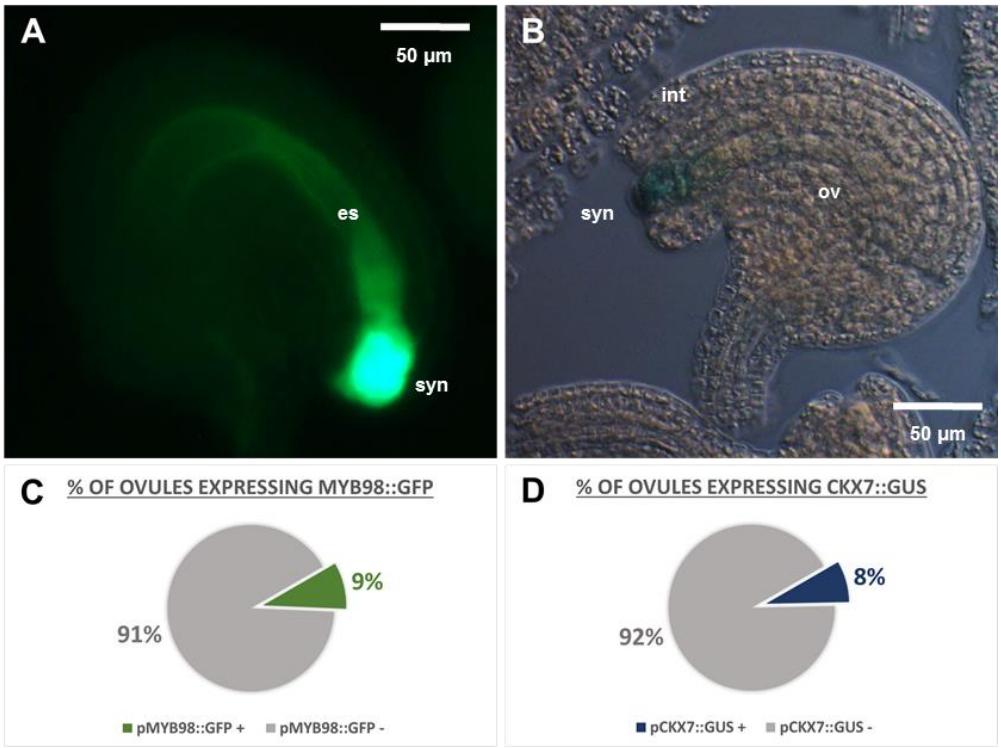


Fig. 7 Synergid cell identity marker lines in *hda19* mutant background. A) A *hda19* ovule expressing *pMYB98::GFP* in the synergid cells; B) *pCKX7::GUS* x *hda19*; The pie charts point out the percentage of detection on *pMYB98::GFP* in *hda19* ovules per single pistil (C) and the percentage of *hda19* ovules per pistil expressing *pCKX7::GUS*. More than 500 ovules for both *pMYB98::GFP* x *hda19* and *pCKX7::GUS* x *hda19* have been used for the analysis. Es = embryo sac, syn = synergid cells, int = integuments, ov = ovule.

Strikingly, we scored less than 10% of *hda19* ovules expressing the *pMYB98::GFP* and a similar result has been recorded by using *pCKX7::GUS*. The analysis has been made on F2 generation on *hda19* homozygous plants carrying the constructs in heterozygosity (Fig. 7).

As two independent cell identity marker lines have been used, we may confidently assume that the majority of *hda19* ovules do not develop synergid cells properly.

Discussion

Most of the known chromatin remodeling systems during plant reproduction relies on meiotically heritable epigenetic marks, both direct DNA methylation and chromatin configuration.

Hereby we suggest the involvement of histone (de)acetylation, particularly H3K9Ac epigenetic mark, during two different processes in double fertilization.

We have shown a defective transmitting tract of *hda19* pistil, which slows pollen tube growth. At the morphological level, *hda19* TT is partially unfused with a less organized structure. Moreover, cell walls appear to be thinner compared with the wild type.

Intriguingly, transcriptomic analysis of *hda19* mutant (Manuscript #3 "*HDA19: a new role for histone deacetylation during ovule development in A. thaliana*") revealed a down regulation of transcription factors involved in transmitting tract development and identity. In fact, NO TRANSMITTING TRACT (NTT), HECATE1 (HEC1), HEC2 and HEC3 shows from 6 to 15 fold down-regulation in *hda19* compared to wild type. Moreover, several genes encoding for cell wall modifiers, such as pectin methylesterases, pectin methylesterase inhibitors and cellulose synthases are mis-regulated

in *hda19*. These enzymes are able to determine the rigidity of the cell wall and a dramatic mis-regulation may cause a severe change in cell wall composition and stiffness. It is widely known that pollen tubes grow towards the proximal end of the pistil through the apoplast of transmitting tissue. An altered composition of the transmitting tract apoplast might therefore influence the pollen tube growth.

In the present work, we also gave a clue on the regulation of the undisclosed interaction between female sporophytic tissue and female gametophyte development. In fact, *hda19* mutant displays a remarkable defect in ovular guidance, likely due to the loss of synergid cell identity in the majority of ovules. Heterozygous *hda19* mutant allele, conversely, does not show any sterility.

On top of that, we may hypothesize that in *hda19* mutant, sporophytic factors involved in the identity determination of synergid cells lose the correct regulation.

Transcriptomic analysis of *hda19* mutant, interestingly, gave us the suggestion of a severe mis-regulation of Arabinogalactan proteins (AGPs) which may be helpful in the future discovery of the factors involved in the sporophytic role during synergid cell identity determination, in the context of *hda19* mutant. Arabinogalactan proteins are a large family of hydroxyproline-rich glycoproteins whose function is mostly unknown. 85 AGPs have been bioinformatically identified (*Showalter* et al., 2010) so far. They are known to be broadly expressed in the female sporophytic tissues during pollen tube growth towards the ovule (*Coimbra* et al., 2008;

Nguema-Ona et al., 2012; *Pereira* et al., 2016). Further, AGPs are predicted to be anchored to the plasma membrane. In the recent years, it has been shown AGPs involvement in several steps of fertilization process (*Coimbra* et al., 2007; *Pereira* et al., 2016; *Dresselhaus* et al., 2016; *Jiao* et al, 2017; *Acosta-García* and *Vielle-Calzada*, 2004; *Demesa-Arévalo* and *Vielle-Calzada*, 2013; *Tucker* et al., 2012; *Tucker* and *Koltunow*, 2014; *Pereira* et al., 2015; *Costa* et al., 2013; *Yang* et al., 2007). AGPs represent new and promising key players of signaling mechanisms during pollen tube growth and guidance.

NTT is down-regulated in *hda19*, as above mentioned. In 2007, *Crawford* and colleagues demonstrated that *ntt* mutant has a reduced staining of polysaccharides and thus speculated about a reduction of AGPs content in the mutant. We may reasonably think that also *hda19* mutant may have such reduction in polysaccharides.

Thus far, only five AGPs (AGP1, AGP4, AGP9, AGP12 and AGP15) have been elucidated in their function during pollen tube interaction with pistil and ovular guidance. None of the 7 *AGPs* up-regulated and 5 *AGPs* down-regulated in *hda19* has a characterized function in these processes. Despite this fact, it would be extremely interesting to deeply investigate the mis-regulated AGPs and their possible role in the defective *hda19* pollen tube guidance.

Methods

Plant materials and growth conditions

All plants were grown under controlled conditions in the greenhouse, simulating both long day (artificial 16 hours light and 8 hours dark) and short day (artificial 8 hours of light and 16 hours of dark).
hda19-3 mutant allele (SALK_139445) in Col-0 ecotype was provided by SALK Institute.

Genotyping

DNA extraction from leaves was performed using Tris-HCl 200 mM, NaCl 250 mM, 25 mM EDTA and SDS 0,5 % extraction buffer and subsequently followed by DNA pellet precipitation using 2-propanol (Sigma). The DNA obtained has been used for PCR-based genotyping.

Aniline blue Staining

Aniline blue staining for pollen tube growth analysis has been performed as previously described (*Mizzotti* et al., 2012).

Microscopy observation

Dissection, observation and fertility assessment was performed with a Leica MZ6 stereomicroscope on young siliques. Pictures of cleared ovules and aniline blue experiments were taken using a Zeiss Axiophot D1 microscope (with Differential Interference Contrast), captured by a Axiocam MRc5 camera (Zeiss) with Axiovision software (version 4.1).

Transmitting tract observation and characterization

Pistils were fixed in cold 2% formaldehyde and 2% glutaraldehyde (in Hepes 50mM, pH 7,4) overnight at 4°C. Samples were repeatedly rinsed in the same buffer, dehydrated with increasing concentrations of methanol and embedded in LRGold resin at -20°C. Semi-fine sections (2µm), obtained using a Reichert Jung Ultracut E microtome, were stained with 1% toluidine blue and observed with a Leica DMRB optical microscope.

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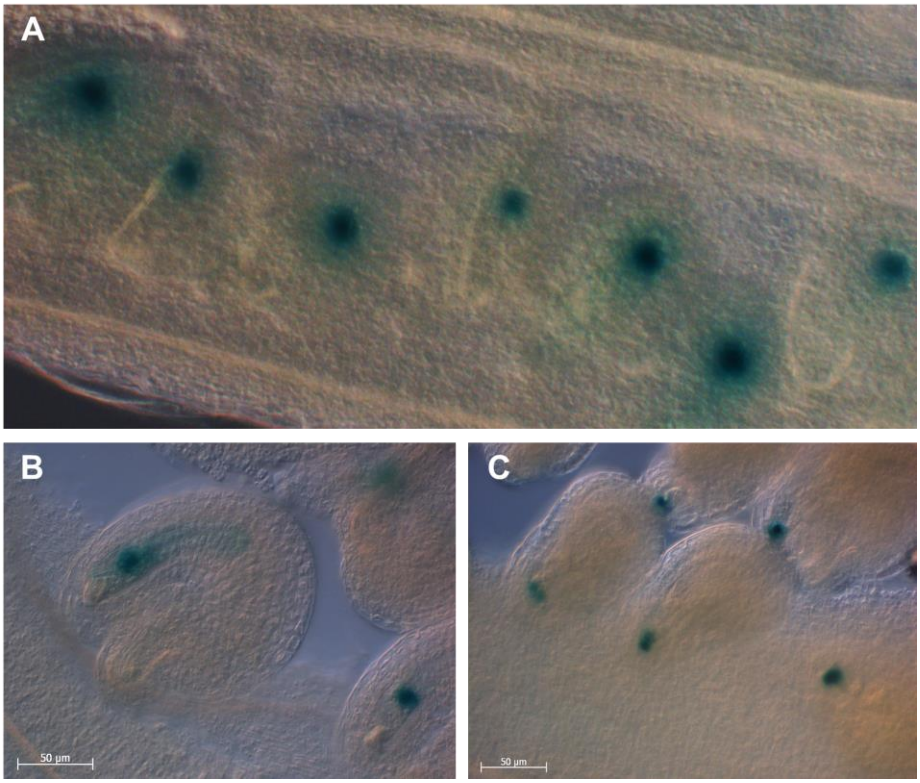
Supplemental Data



Supplemental Figure 1 A) *hda19* x pAC::GUS for antipodal cell identity; B) *hda19* x pCC::GUS for central cell identity; C) *hda19* x pEC1::GUS for egg cell identity.

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