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Gametophyte and sporophyte crosstalk during fertilization in Arabidopsis

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Contents

SUMMARY

Seeds arise from ovules after the double fertilization event. The two sperm cells fertilize both the central cell and the egg cell, giving rise to the triploid endosperm and the diploid embryo, respectively. The ovule integuments, after the fertilization, differentiate into the seed coat. The development of the endosperm, embryo and seed coat is strictly coordinated and a signal should be activated in the sexual endosperm to trigger seed coat development.

The mechanism of the crosstalk between gametophytic and sporophytic tissues, even though intensively studied in the past, is still not well understood. We have been investigating in detail the role of STK in seed development. STK encodes for a MADS-domain transcription factor. The *stk* mutant has smaller seeds and aberrant flavonoid accumulation. Interestingly, combining the latter with the MADS-box mutant *tt16*, also involved in flavonoid accumulation, the endothelium is severely compromised. The double mutant lacks the inner seed coat layer (endothelium) where flavonoids accumulate and this has an effect on endosperm cellularization and the determination of seed size. Moreover, the rate of fertilization is drastically reduced in the double mutant and a massive starch accumulation in the female gametophyte has been noted.

The aim of this work is to understand the role of the endothelium in the process of seed coat development, because of his crucial position; it is indeed the connection layer between the sporophytic and gametophytic tissues of the seed. Moreover, the involvement of STK in the crosstalk between the sporophyte and the gametophyte during early stages of seed development has been investigated.

PART I

Abstract

Arabidopsis thaliana seeds comprise three tissues, which are genetically and functionally distinct. However, in order to ensure a correct seed development, a communication and coordination of them is strictly necessary.

The seed coat is a five-layer tissue surrounding the female gametophyte. In the *stk abs* double mutant only four layers form this tissue: the endothelium, the innermost one, does not differentiate.

Since the endothelium is a sporophytic layer directly in contact with the gametophyte before the fertilization and with the endosperm after the fertilization, we hypothesized a possible role of this layer in the communication between those tissues.

In this work, the role of the endothelium has been investigated, as well as the role of the STK and ABS, the two MADS box transcription factors required for endothelium differentiation.

An additional interesting phenotype of the *stk abs* double mutant is the excessive amount of starch accumulating in the central cell of the female gametophyte. This might causes the partial sterility described for the double mutant.

Combining *stk abs* with the *gpt1* mutant, whose mature ovules do not accumulate starch, we found a partial recovery of the phenotype; this result support the idea that the excess of starch in the double mutant could physically prevent the correct fertilization process.

State of the Art

Seeds, the products of the double fertilization, are at the basis of human nutrition and to satisfy the rapidly increasing global demand of food it is crucial to improve seed number and quality.

Due to both commercial benefit and pursuit of knowledge advancement, scientists have been committing themselves to investigate the genetic and molecular basis of double fertilization in plants.

Taking cues from model plant species, example given by *Arabidopsis thaliana*, researchers tried to widen their point of view, opening up to new model species and cultivated crops.

Research on seed development in the model species *Arabidopsis thaliana* is therefore fundamental because provides the necessary knowledge for translation studies towards crop species.

Fertilization and Seed Development

In all angiosperms, the fertilization process requires the occurrence of perfectly synchronized events in order to ensure the correct fusion between the male and the female gametes, i.e. the sperm cells and the egg and central cells, respectively.

Fertilization in Arabidopsis takes place inside the flower, in which four whorls are distinguished: sepals, petals, stamen and carpels (**Fig. 1**).

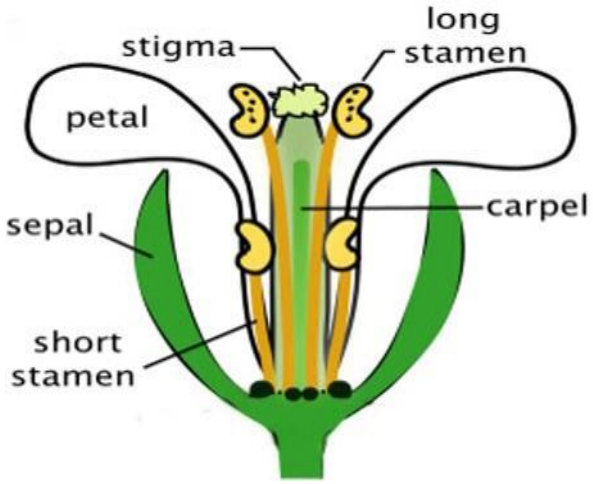

Fig. 1. Schematic representation of the *Arabidopsis thaliana* flower (modified from *Kram et al., 2009*)

Two carpels are fused in the pistil, which is formed by the ovary, the stylus and the stigma. Inside the ovary, the ovules are found. They are the major component of the female reproductive organs in *Arabidopsis thaliana* and they are the progenitors of seeds (*Esau, 1977*). Components of the ovule are the female gametophyte, also known as embryo sac or megagametophyte, and the surrounding five-layer tissue, the ovule integuments. In a mature ovule at anthesis, the integuments are divided in two cell-layered outer integuments and a three cell-layered inner integuments (*Windsor et al., 2000*).

The female gametophyte comprises 7 cells, gathered in 4 different cell types (**Fig. 2**): the egg cell, the central cell (with two polar nuclei), two synergid cells, that attract the pollen tube inside the ovule, and three antipodal cells, which function is still unknown (*Maheshwari, 1950*).

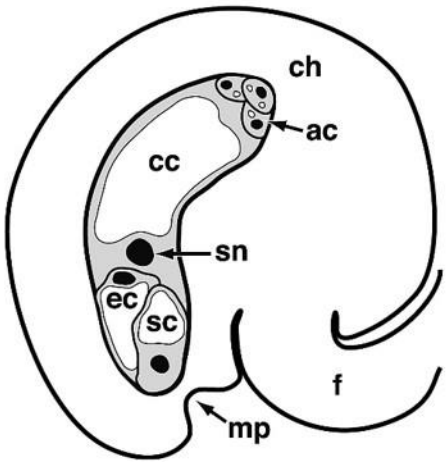

Fig. 2 Schematic representation of an Arabidopsis mature ovule.

Cc: central cell, ac: antipodal cell; ec: egg cell; sc: synergid cell; ch, chalazal region of the ovule; f: funiculus; mp, micropyle; sn, secondary nucleus.

Modified from *Yadegari et al., 2004*

The fertilization process starts when the pollen is deposited on the stigma. The pollen tube is then formed; this grows inside the carpel and makes its way to reach the female gametophyte (**Fig. 3a**).

It enters the embryo sac, chemo-attracted by the synergid cells and grows inside it (**Fig. 3b**).

The receptive synergid cell undergoes cell death soon after. Once inside the embryo sac, the pollen tube content is released, thus letting the fusion between male and female gametes (**Fig. 3c**).

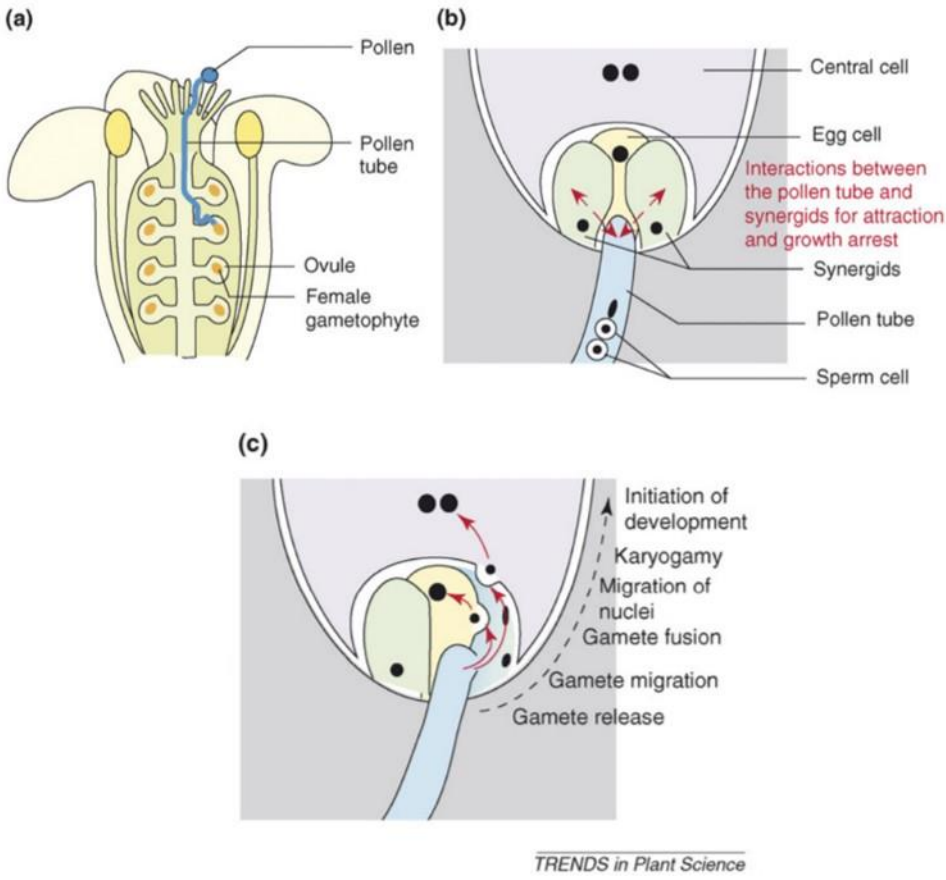

Fig. 3: Schematic representation of double fertilization event. The pollen tube reaches the ovule growing inside the carpel (a). Once in the embryo sac and releases the sperm cells (b) that can fuse with the egg cell and the central cell (c). Modified from *Berger et al., 2008*

After this double fertilization event, the seed tissues are formed (**Fig. 4**). The two sperm cells fertilize both the central cell and the egg cell, giving rise to the endosperm and the embryo, respectively. The ovule integuments, after the fertilization, differentiate into the seed coat: hence the seed teguments are completely maternal.

Seeds consist of three genetically distinct components: the embryo and the seed coat are diploid, while the endosperm is a triploid tissue, in which a double maternal contribute is responsible for the peculiar genome dosage (*Haughn et al., 2005*).

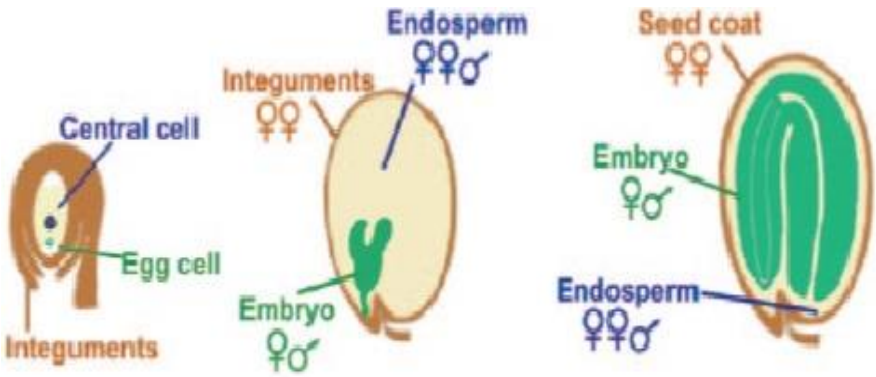

Fig. 4: Seed development in *Arabidopsis thaliana*; central cell fertilization gives rise to the triploid endosperm; the egg cell fuses with the sperm cell to form the diploid embryo whilst ovule integuments differentiate in the seed coat, a completely maternal tissue (Modified from *Sundaresan, 2005*).

Endosperm development

The endosperm arises from the fusion between the central cell, which contains two polar nuclei, and one haploid sperm cell; it is a triploid tissue whose function is to nourish the embryo throughout the whole developmental phase.

Indeed, at the beginning of this process, it occupies the entire seed cavity, but it is slowly used by the embryo for its nutritive necessity. At maturity, the endosperm is completely consumed, leaving only a few cell layers (**Fig 5d**) (*Fang Bai and Settles, 2015).*

Endosperm development starts with a phase of nuclear divisions not followed by cytokinesis; this process of multiple rounds of mitosis, known as syncytial phase, produces a multinucleate cell, a syncytium (**Fig. 5a**). Later, cell walls start to be deposited around each nuclei: this step is known as cellularization (**Fig. 5b**) (*Brown et al., 1998*) and at this point various cells acquire specific functions (**Fig. 5c**). For example, the transfer cells promote the import of nutrients which are stored in the endosperm to facilitate the embryo growth (*Gutierrez-Marcos et al., 2012*).

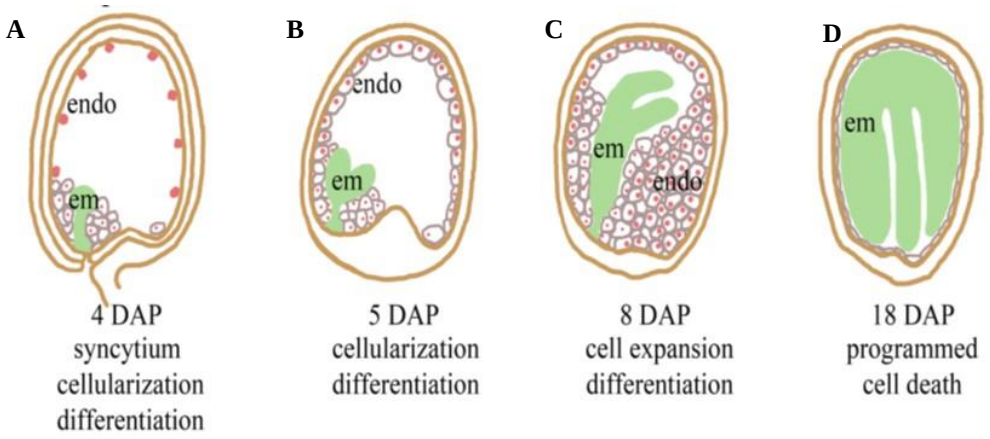

Fig 5: Endosperm development in *A. thaliana*. Early in development, the endosperm is a syncytium (A). Later on, the cellularization occurs (B) and it becomes a store of nutrients (C). In mature seeds, the endosperm is completely consumed by the embryo (D). Modified from *Fang Bai and A. M. Settles, 2015*.

The timing of endosperm cellularization is crucial in the establishment of a correct seed size, sink strength and grain weight.

Precocious cellularization correlates with a reduced nuclear proliferation and a decreased seed size (*Kang et al., 2008*): for example, the *haiku* or *miniseed*

mutant seeds, which cellularization is anticipated, appear smaller compared to the wild type (*Garcia et al., 2003; Luo et al., 2005*).

On the other hand, delayed cellularization means increased nuclear proliferation, thus increased seed size.

Seed coat development

The main function of the seed coat is to protect the embryo from mechanical stresses, pathogen attack and UV damages; it also plays a role in maintaining the dehydrate dormant state of the embryo until proper germination conditions arise and procuring the resources for water uptake at the very beginning of the germination process (*Windsor et al., 2000; Haughn et al, 2005*).

It derives from the differentiation of the ovules integuments as a response to the fertilization step: it's a five layers tissue of specialized maternal cell types.

Two layers originate from the outer ovule integuments (outer integument1 and oi2), the remaining derive from the inner ovule integuments (ii1 and ii2). Soon after the fertilization, integuments cells enter a period of dramatic cell division then followed by cell expansion.

Each layer of the seed coat undergoes a different specialization faith during the development (**Fig. 6**).

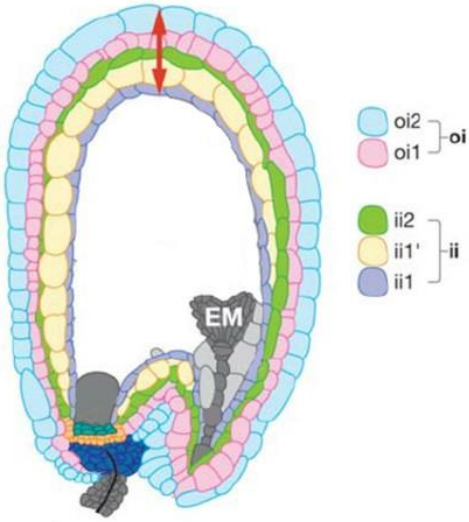

Fig. 6: The testa of Arabidopsis comprises two outers layer and three inner integuments layers. Layers of the seed coat are highlighted in five different colors: the epidermid in blue, the sub epidermid in pink, two inner teguments in green and yellow and the innermost tegument, called endothelium, in purple.

oi: outer integument; ii: inner integument

Modified from *Lepiniec et al., 2006*

The innermost layer of the seed coat, also known as endothelium, is designated to the synthesis and accumulation of proanthocyanidins (PA), also referred to as condensed tannins. They are responsible for the typical brownish color of wild type seeds.

The two other inner integument layers do not proceed further with the differentiation, rather they are squeezed together at the end of the development.

The outer integuments differentiate in the subepidermal layer, characterized by the palisade, a thickened cell wall, and in the epidermal layer (*Lepiniec et al., 2006*).

The specialized cells of the epidermal layer secrete a copious amount of mucilage, a pectinaceous carbohydrate, which facilitates germination and helps seed dispersal, and other polysaccharides that play important roles despite their low abundance (*Voiniciuc et al., 2015c*). After the production of

mucilage, the columella is formed through the deposition of secondary cell wall.

All the five layers undergo cell death at the end of the development; except for the epidermal cells, protected by the mucilage and columella, the other layers are crushed together (*Haughn et al, 2005)*.

Regulation of seed coat development

Embryo, endosperm and seed coat development requires a tight coordination to a successful seed maturation. Thus, these three structures must communicate and cooperate between each other; the cross talk between sporophyte and gametophyte has been extensively investigated recently and several players involved in the regulation of these developmental pathways are now very well known.

The Polycomb group proteins are numbered among the master regulators. They act at the epigenetic level catalyzing the histone H3 trimethylation of the lysine 27 repressing the expression of target genes, thus controlling a wide range of physiological processes (*Hennig and Derkacheva, 2009; Schuettengruber and Cavalli, 2009*).

The Polycomb group proteins have an ancient origin and they are very well conserved among the different kingdoms. They were first identified in *Drosophila melanogaster*: in flies, they play a pivotal role in the regulation of homeotic genes during development (*Duncan, 1982*).

In plants, they exert the same function: they control the switch between the activated and repressed status of target genes in order to regulate developmental pathways.

PcG proteins are organized in complexes based on their target genes and the process which they regulate.

The EMF complex mainly controls the shift from seedling to adult plant (vegetative development) and flowering transition; it is formed by five different components: CURLY LEAF (CLF), SWINGER (SWN), EMBRYONIC FLOWER (EMF2), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (*Yoshida et al., 2001*).

The vernalization response, the competence to flower after a long cold timing, is controlled through Vernalization (VRN) complex that contains VERNALIZATION2 (VRN2), CLF, SWN, FIE and MSI1 (*Wood et al., 2006; De Lucia et al., 2008*).

Instead, MEDEA (MEA) or SWN, FERTILIZATION INDEPENDENT SEED2 (FIS2), FIE and MSI1 are assembled in the FIS complex that regulates female gametophyte and seed development (*Spillane et al., 2000; Köhler et al., 2003a; Wang et al., 2006*). Particularly, they prevent a premature endosperm and seed coat development (*Köhler and Makarevich, 2006*).

Mutations affecting members of these complexes lead to autonomous seed development: *fis2* and *fie* mutants, indeed, present seed-like structures in unfertilized siliques, with a cellularized endosperm and a developed seed coat (*Ohad et al.1996; 1999; Chaudhury et al., 1997*).

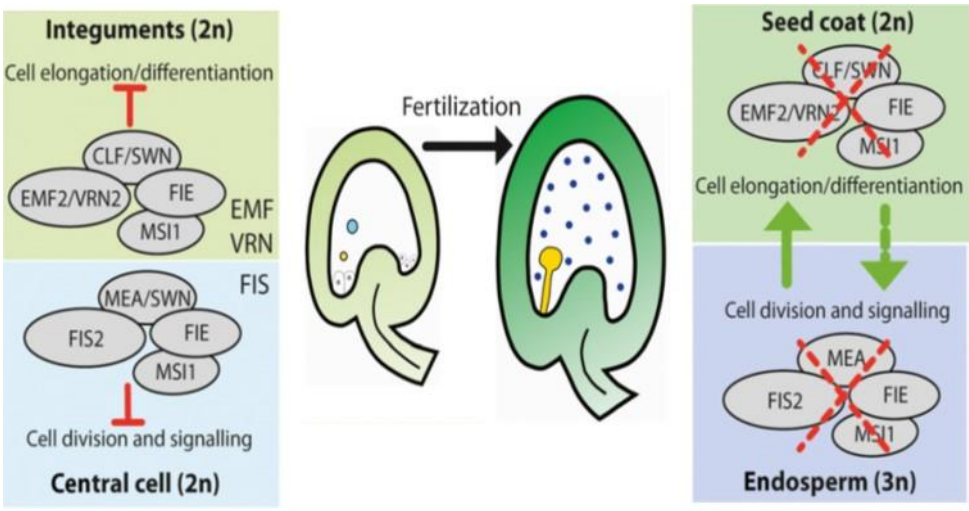

Fig. 7: Schematic illustration of epigenetic regulation of seed coat development by PCR2. In ovule integuments EMF and VRN complex repress their development, whereas in the embryo sac, FIS complex repress endosperm development. When fertilization occurs, the repression carried out by these complexes is repressed; hence, endosperm and seed coat can start to grow. Modified from *Mozgova et al, 2015*

Autonomous seed formation is also evident in *mea* and *swn*, although with a decreased penetrance compared to the *fis2* and *fie* mutants.

However, studies indicate that the number of seed-like structures formed in absence of fertilization undergoes a significant increase in the double mutant *mea swn*, thus suggesting a redundancy between these two PcG proteins (*Wang et al., 2006*).

Roszak and Köhler demonstrated that the *vrn2* mutant ovules initiates seed coat development: indeed, cleared ovules at 6 Days After Emasculation present a well-developed seed coat with 5 layers clearly distinguishable.

As said before, the gametophytic tissues development should be strictly coordinated with the sporophyte growth. The way used to communicate is a

signaling pathway which is triggered in response to the fertilization and release the repression carried out by the Polycomb group proteins.

Several studies were performed in order to unravel the genetic mechanism underneath this signaling pathway.

The role of the sexual endosperm has been demonstrated to be crucial in this mechanism through the use of the *kokopelli* (*kpl*) mutant; the only sperm cell contained in the *kpl* pollen can randomly fertilize either the central cell or the egg cell.

In the study it has been shown that seeds of the *kpl* mutant with embryo but no endosperm do not have differentiated integuments (**Fig 8a**), whereas in *kpl* seeds whose central cell has been fertilized a proper seed coat can be unmistakably identified (**Fig 8b**).

Taken all together, these results suggest that a signal is generated in the sexual endosperm in order to trigger seed coat development (*Roszak and Köhler, 2011*).

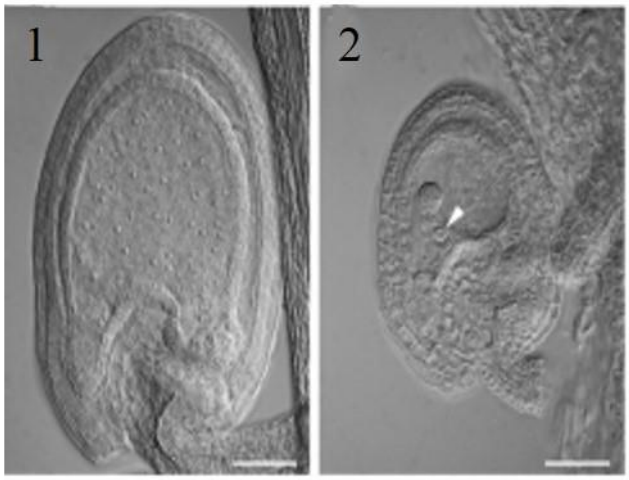

Fig 8: (1) *kpl* mutant showing endosperm but no embryo. (2) *kpl* mutant with a fertilized embryo and a clearly distinguishable unfertilized central cell. Modified *Roszak and Köhler, 2011*

Role of hormones in seed coat regulation

Going deeper in the investigation, scientists found out other elements needed in this complicated signaling cascade.

Phytohormones are known to be involved in the regulation of many growth processes, including seed and fruit initiation (*Fuentes et al., 2012; Figueiredo et al., 2015).*

Auxin is one of the major plant hormones that plays pivotal roles in controlling a huge range of developmental pathways, not only at organs level (such as tissues differentiation), but also on a cellular level (they indeed control cell extension, division and differentiation) (*Guilfoyle and Hagen, 2007; Mockaitis and Estelle, 2008).*

Gibberellins are the pivotal regulators of fruit initiation; indeed, exogenous application of these hormones are able to induce partenocarpy, i.e. fruit development in absence of fertilization (*Gustafson, 1936; King, 1947; Vivian-Smith and Koltunow, 1999; Ozga et al., 2003*).

These two hormones are involved in the repression of the Polycomb Group Protein expression after fertilization, thus regulating seed coat development.

Their accumulation pattern before and after the fertilization has been analyzed through the use of marker lines. Auxin is produced in the endosperm nuclei and in the seed integuments after fertilization, whereas no traces of this hormone can be detected in ovules, excluding a few cell close to the vascular bundle and the antipodal cells (*Figueiredo et al., 2015; 2016*).

Likewise, post-fertilization production of gibberellins has been proved: after the fusion of the gametes, these phytohormones are accumulated in the differentiated integuments (*Figueiredo et al., 2016*).

When auxin and GA are supplied to non-fertilized ovules, a seed coat is evidently discernible, as well as when these two hormones are ectopically produced in the integuments (*Figueiredo et al., 2016*).

In this elaborate cascade, auxin work upstream GA, activating their signaling in the seed coat.

These experiments lead scientists to assume that auxin might be the signal released by the endosperm in order to trigger seed coat differentiation.

AGL62 transcription factor is crucial for the seed coat differentiation process

In wt seeds, auxin needs to be produced in the central cell soon after fertilization and then quickly transported into the integuments. This transportation is mediated by the transcription factor *AGL62*.

AGL62 belongs to the MADS-Box transcription factor family and, to be more specific, it falls in the type 1 group (*Parenicova et al, 2003*). The *agl62* homozygous mutation causes defects in embryo maturation, thus rendering this t-DNA insertion line lethal. Moreover, a precocious endosperm cellularization and a reduced number of endosperm nuclei typify this mutant (**Fig. 9**) (*Kang et al., 2008*).

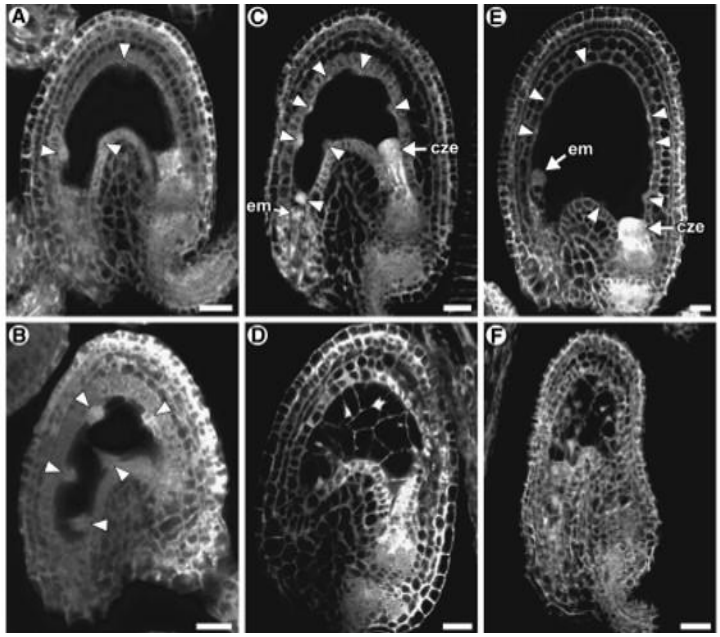

Fig. 9: At 24 h after pollination, both wt (A) and *agl62* seeds (B) contains four to eight endosperm nuclei and the embryo is at the zygote stage. At 36 h after pollination, wt endosperm (C) is still uncellularized whereas in the mutant the cellularization has already begun (D). At 48 h, no cellularization is visible in the wild type (E), *agl62* seeds are partially collapsed (F). Modified from *Kang et al., 2008*.

Through the use of AGL62-GFP marker line, the expression pattern of this transcription factor has been analyzed in details. The fluorescent signal is visible in the endosperm throughout the syncytial phase, from soon after the fertilization till just before the cellularization. The signal is completely absent in the embryo and in the seed coat layers (**Fig. 10**) (*Kang et al., 2008*).

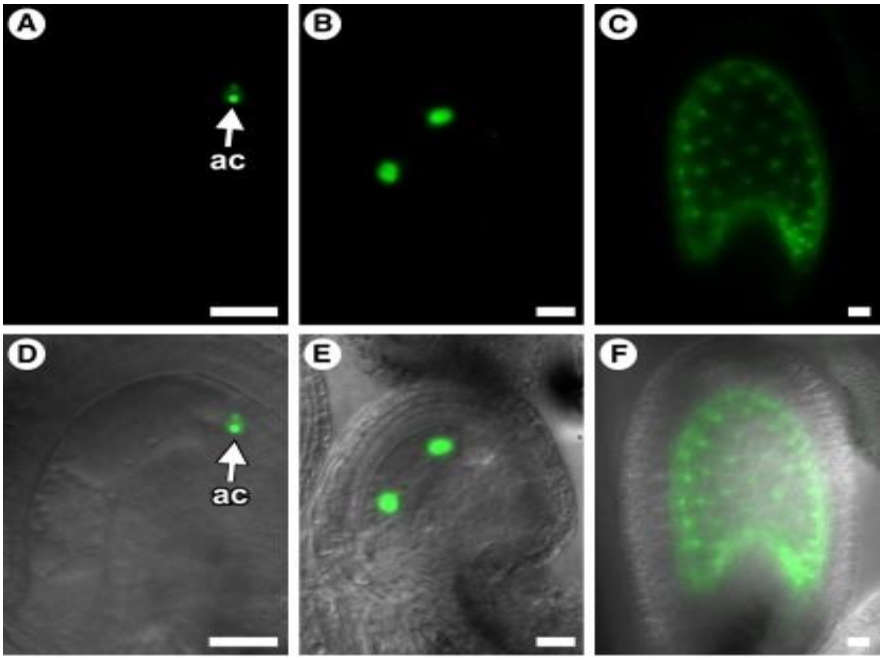

Fig. 10: *AGL62* is expressed in the antipodal cell (A) and (D) of the female gametophyte in ovules and in the endosperm nuclei after the fertilization (B), (C), (E), (F). Bars = 20 μm.Modified from *Kang et al., 2008*

A deeper characterization of this mutant shows that in the homozygous situation seeds initiate embryo and endosperm development but failed to develop a seed coat (**Fig. 11**).

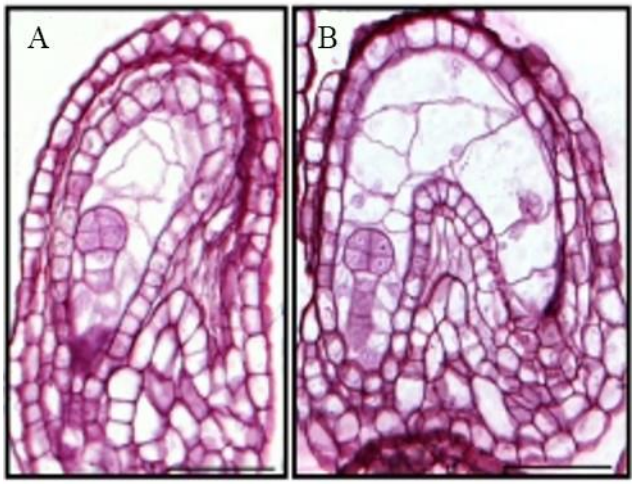

Fig. 11: Sections of *agl62-2/−* seeds at 3 DAP; seed development is arrested in 20 % of *agl62* seeds. Modified from *Roszak and Köhler, 2011*

This suggest that *AGL62* is necessary for the cross talk between seeds tissues.

In literature, several mutants displaying problems in endosperm proliferation have been identified; to name it, *titan2* shows sever defects; indeed, its endosperm cells appear to be giant, compared to the wild type. However, this mutation do not reflect on seed coat growth (*Liu et al., 1998*).

Endosperm proliferation failure *per se* is not sufficient to disrupt integument differentiation.

AGL62 acts in concert with auxin in the induction of central cell proliferation, as demonstrated by auxin treatment in the mutant. When treated with auxin, wt unpollinated ovules undergo autonomous seed development, whereas in the *agl62* mutant the percentage of ovules with a seed-like structure is strongly reduced, thus implying the necessity of this MADS gene in initiating central cell replication (*Figueiredo et al., 2015*).

Published data show that a major auxin accumulation in the endosperm of this mutant, but there is no evidence of auxin presence in the integuments. Moreover, the auxin transporter *PGP10* appears to be heavily downregulated in the mutant background (*Figueiredo et al., 2016)*

The failure in the seed coat differentiation is then due to the failure of auxin transportation in the teguments after fertilization.

As summarized in the picture below, before the fertilization, in the wt ovules integuments the Polycomb group protein are repressing their target gene expression in order to prevent early seed coat formation (**Fig. 12a)**.

When the fertilization occurs, auxin production is activated in the endosperm; *AGL62* transcription factor is necessary for the moving of this hormone in the seed coat (**Fig. 12b**). In this tissue, auxin activates a signaling cascade involving, among others, gibberellins in order to eliminate the repression of the Polycomb group proteins.

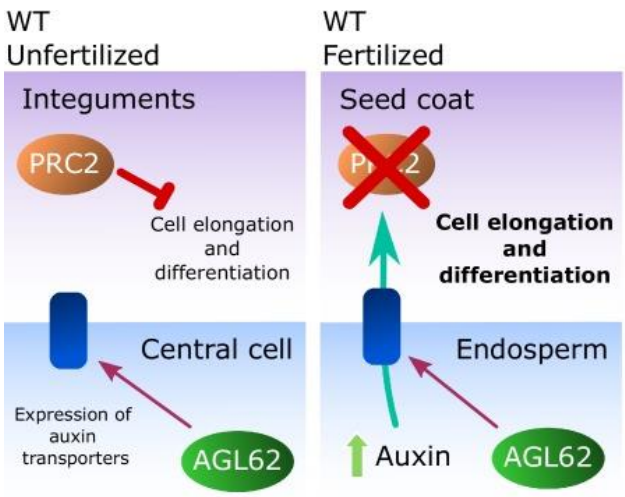

Fig. 12: In a wt ovule, the PRC2 is repressing elongation and differentiation of the integuments (A); when the fertilization occurs, *AGL62* is activating the auxin transport from the endosperm to the seed coat. In this way, the PRC2 repression is repressed, thus letting the seed coat growth (B). Modified from *Figueiredo et al., 2016*.

Loss of AGL62 leads to massive auxin accumulation in the endosperm. The hormone cannot be transferred in the seed coat, where it should release the PRC2 repression. Thus, teguments differentiation cannot be achieved in the mutant. (**Fig. 13**).

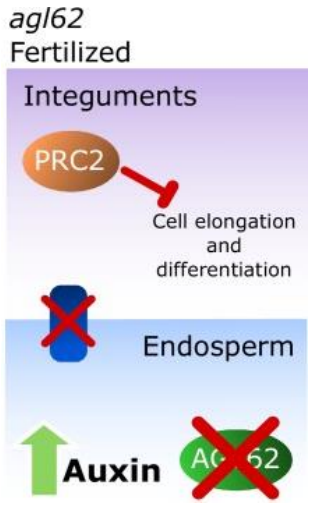

Fig 13: Auxin in the *agl62* background is accumulated in the endosperm and cannot reach the seed coat. The PRC2 are not obstructed in their repression. Modified from *Figueiredo et al., 2016*.

Genetic regulation of seed coat formation

STK is a MADS box transcription factor extensively studied among plant biologists. Redundantly with *SHATTERPROOF1* (*SHP1*) and *SHP2*, it is responsible for the correct determination of ovule identity; indeed, the triple mutant *stk shp1 shp2* present ovules with a leaf-like or carpel-like structure.

The single mutant *stk* shows a variety of phenotype, indicating the importance of this TF in regulating several developmental pathways (*Pinyopich et al., 2003*).

Mutant seeds present an altered shape compared to the oblong profile of wt: they are indeed smaller and rounder (**Fig. 14**).

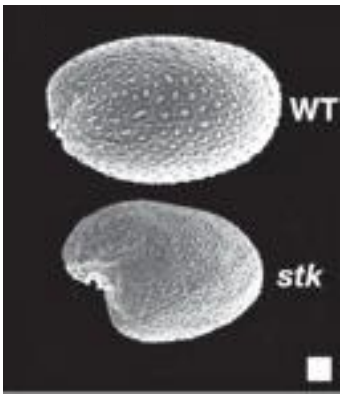

Fig. 14: *stk* has reduced seed size and altered seed shape. Modified from *Pinyopich et al., 2003*

Moreover, the funiculus is longer and its seeds hardly detach from the siliques (*Favaro et al., 2003; Pinyopich et al., 2003*), due to an ectopic lignification of the seed abscission zone (*Balanzà et al., 2016*).

Modulating the expression of *HECATE3*¸ a bHLH transcription factor whose seeds remain attached to the funiculus (*Gremski et al., 2007; Ogawa et al., 2009*), *STK* is fundamental to establish the proper lignification pattern for seed abscission (*Balanzà et al., 2016*).

RNA Seq analysis pointed out the role of *STK* in regulating also secondary metabolism processes; in particular, genes involved in PA biosynthesis were found to be mis-expressed in the mutant background.

Among these, *BANYULS/ANTHOCYANIDIN REDUCTASE* (*BAN*), a branch point enzyme of this pathway, is negatively regulated by *STK* at epigenetic level (*Mizzotti et al., 2014*). To confirm this additional function of this transcription factor, PA staining and metabolomics analysis were performed: in the mutant background, proanthocyanidins are indeed accumulated ectopically.

TRANSPARENT TESTA8 (*TT8*) and *ENHANCER OF GLABRA3*, two seed coat regulators, also popped out from the transcriptome data as up-regulated genes in *stk* mutant. Besides being involved in the anthocyanidins biosynthetic pathway, they are involved in the outer seed coat differentiation (*Baudry et al., 2004*).

A role of STK in regulating the physical properties of seed coat has been hypothesized. AFM technology confirmed this supposition highlighting the increased stiffness of *stk* seed coat compared to the wild type seeds. This phenotype has been linked to the compromised ability of *seedstick* mutant to germinate properly (*Ezquer et al., 2016*).

Because of the importance of this transcription factor in regulating several developmental pathways, ChIP seq analysis were performed in order to elucidate its target genes (*Mendes and Ezquer, unpublished*). Abiding by the topic of seed coat development, STK has been demonstrated to bind the promoter region of *CLF* and *SWN*, members of the PRC2 repressive complex.

Member of the MADS Box gene families, *ARABIDOPSIS BSISTER* (*ABS*), or *TRANSPARENT TESTA 16* (*TT16*), was the first Bsister gene that has been characterized (*Nesi et al., 2002*). The Bsister MADS box genes belong to a clade of genes phylogenetically related to the MADS box genes of class B, expressed preferentially in male reproductive organs.

In contrast, Bsister genes are expressed mainly in ovules and carpel walls; they are specific for the female reproductive organs (*Becker et al., 2002*).

ABS is found to be expressed in buds, flower and immature seeds, in accordance with the previous findings (*Nesi et al*., 2002).

Loss of *ABS* function leads to a failure in the accumulation of PA in the endothelium, the innermost layer of the seed coat, although tannins are present near the chalaza and micropyle zone of the seed, suggesting multiple regulatory strategies of PA accumulation.

The lack of accumulation is due to a misregulation of *BAN* gene, which is, as said before, a key enzyme in the proanthocyanidins regulatory pathways. For this reason, *abs* mutant seeds appear yellow compared to the brownish color of wt seeds.

Moreover, endothelial cells present an abnormal morphology compared to the wild type, as well as cells of the inner integuments. This alteration is already visible before the starting of PA accumulation, indicating that these two phenotypes are not related (*Nesi et al., 2002; Debeaujon et al., 2003*).

The expression pattern of these two transcription factors has been investigated in detail. *In situ hybridization* analysis allowed to localize the position of the mRNA of *STK* and *ABS*; they are both present in endothelial cells of mature ovules (**Fig. 15**) (*Mizzotti et al., 2012*).

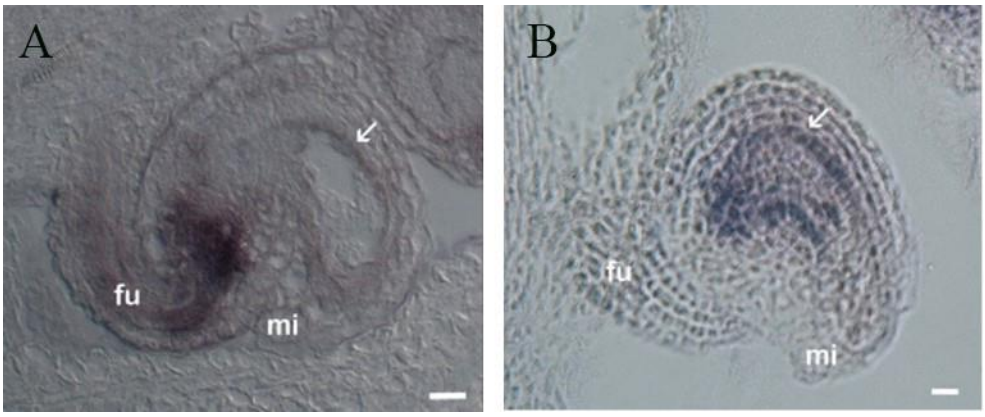

Fig. 15: IN SITU HYBRIDIZATION: *STK* (A) and *ABS* (B) are expressed in the endothelium. Scale bars = 20 μm. Modified from *Mizzotti et al., 2012*.

Furthermore, proteins interaction studies showed that ABS is able to form heterodimers and tetramers with STK or SHP1 and SHP2 (*Kaufmann et al., 2005*).

Given the overlapping expression profiles and the ability to interact between each other, it has been hypothesized a possible cooperation of the two transcription factors in the determination of the innermost layer of the seed coat.

Therefore, the *stk abs* double mutant has been created. The most remarkable phenotype is the complete lack of one of the tegument layers.

In a wild type seeds, five layers are clearly counted, whereas in the double mutant only four are present (**Fig. 16**). Carefully looking at the morphology of the tegument cells in the double mutant, an abnormal shape of those can be noticed.

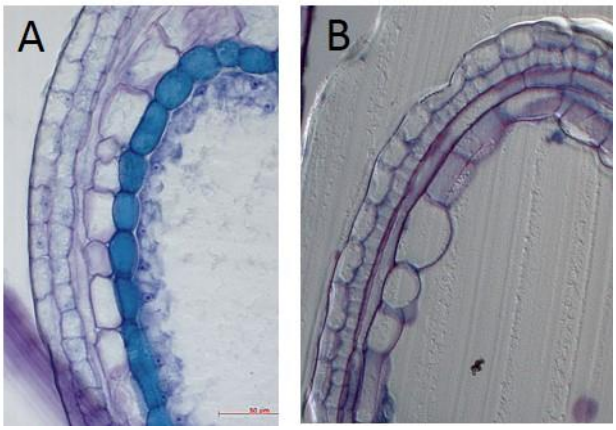

Fig. 16: the wt testa has 5 teguments layers (A) whereas in the *stk abs* only four can be counted (B). *Mizzotti*, unpublished

Moreover, seeds appear smaller compared to the wild type: the seed cavity is drastically reduced, leaving not enough space to the embryo for a correct growth (**Fig. 17**).

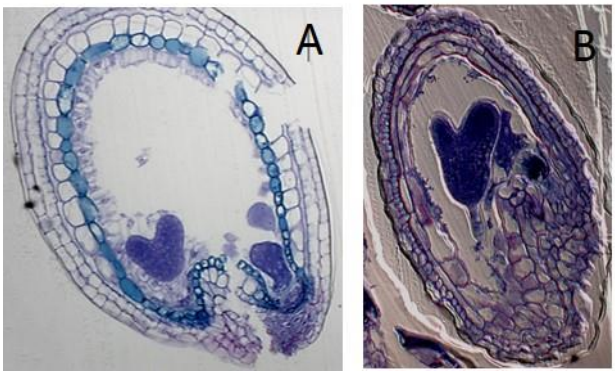

Fig. 17: The morphology of stk abs seed coat cells (B) is altered compared to the wt cell (A). *Mizzotti*, unpublished.

The double mutant also shows reduction in fertility: in a mature wt siliques, a complete seed set is characterized by the development of around 50 healthy seeds; in a *stk abs* silique: ovules abortion and seed arrest are frequently noted (**Fig. 18**).

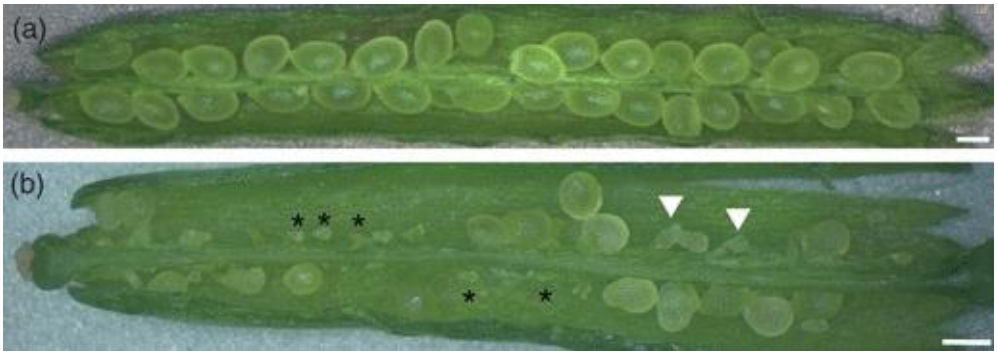

Fig. 18: In a wt mature siliques (A), a complete seed set is formed. In a *stk abs* mutant siliques (B) ovules abortion (in black) and seed arrest (in white) are noted. Scale bar 200µl. Modified from *Mizzotti et al., 2012*.

To date, the reason behind this infertility is still unknown. The correct identity of gametophyte cells has been checked with the use of marker lines. All the seven gametophyte cells in the *stk abs* mutant appear to have a wild type configuration (**Fig. 19**).

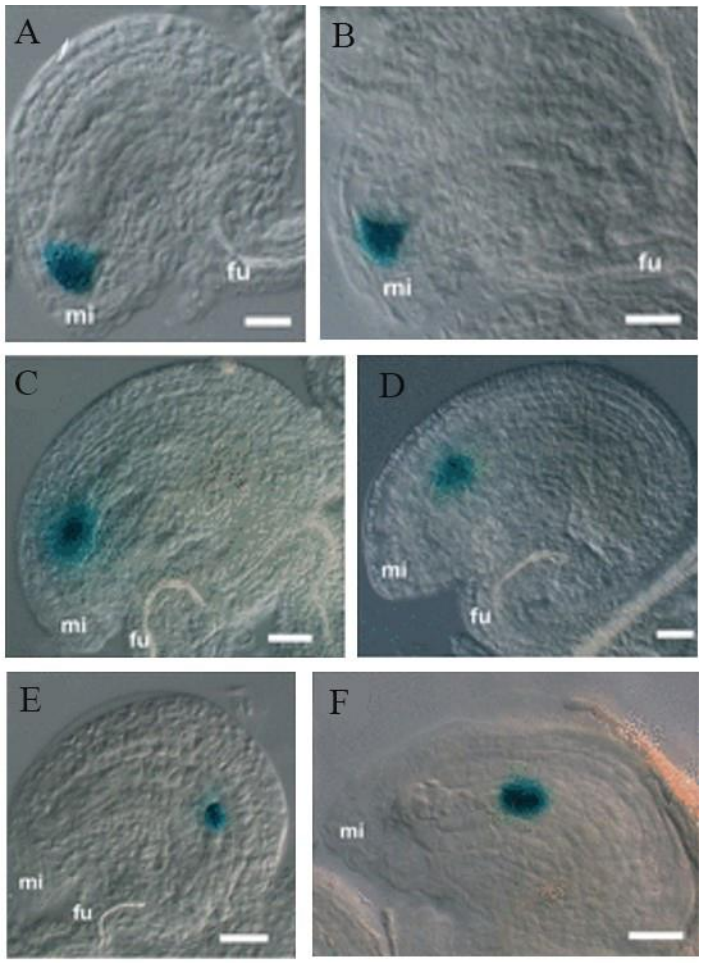

Fig. 19: Synergid marker line x wt (A) and *stk abs* (B); Central cell marker line x wt (C) and *stk abs* (D); Antipodal cells marker line x wt (E) and *stk abs* (F). Scale bar = 20 µm *Mizzotti et al., 2012*

Likewise, the pollen tube penetration and its burst in *stk abs* ovules has been confirmed to occur properly. As shown in the **Fig. 20**, the aniline blue staining confirm that the pollen tube reaches the ovule and enter the micropyle both in the wt and in the double mutant.

The *pLAT52-GUS* transgene was used to evaluate the release of sperm cells inside the ovules, marking the pollen tube cytosol. The GUS signal is present in all the double mutant ovules showing that mutant sperm cells reach the ovule and they are correctly released inside them.

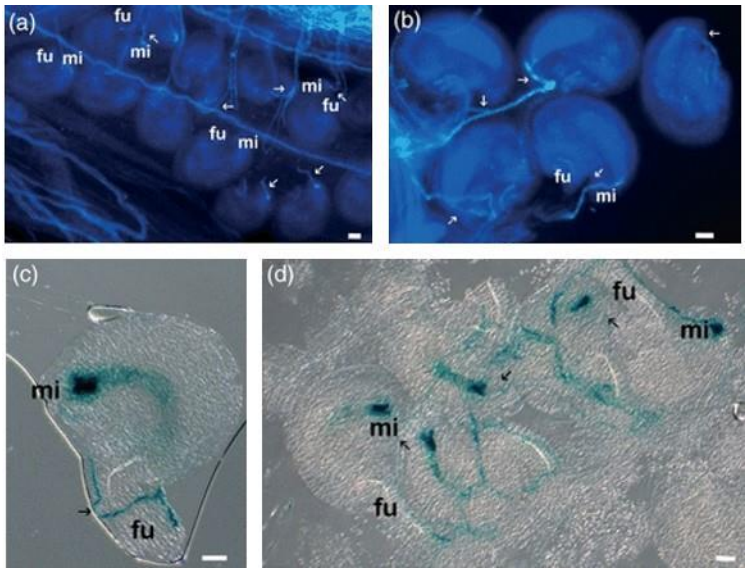

Fig. 20: Pollen tubes arrive and enter the micropyle of wild-type (a) and stk abs (b) ovules. Fertilization of wild-type ovules (c) and *stk abs* ovules (d) with LAT52: GUS pollen. Scale bars = 20 μm Modified from *Mizzotti et al., 2012*.

Beside the reduced seed size and the decreased fertility, this double mutant display an additional interesting phenotype; in the female gametophyte a conspicuous starch accumulation is present compared to wild type (**Fig. 21**) (*Mizzotti et al., 2012*).

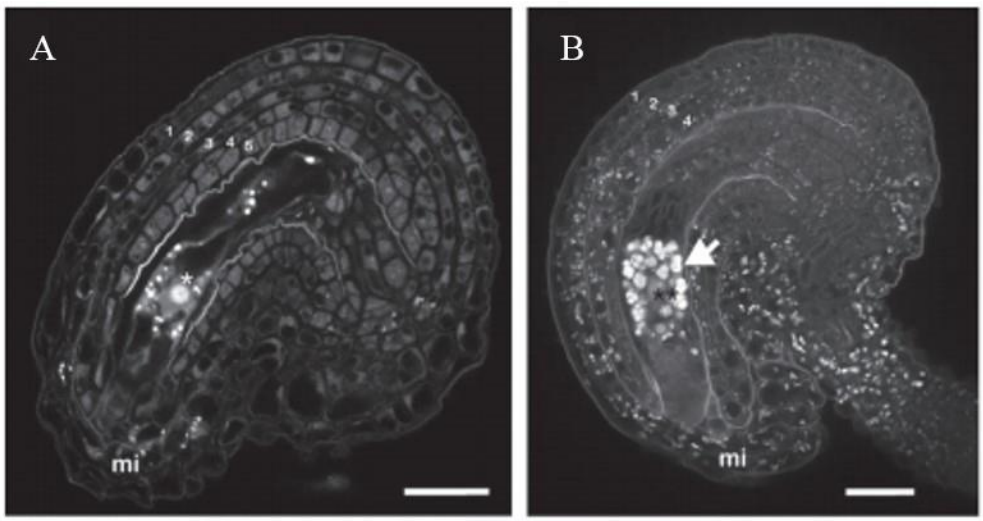

Fig. 21: Confocal images of wt (A) and *stk abs* (B) mature ovules. In the double mutant gametophyte, a massive starch accumulation is indicated by the white arrow. Scale bars = 30 μm. Modified from *Mizzotti et al., 2002*.

Sugars in seed development

As said before, seeds are widely consumed in the human diets because of their composition: they indeed provide the right amount of oil, protein and carbohydrate necessary for an healthy human nutrition.

Numerous studies have been carried out in order to investigate sugar metabolism in plants. This subject has been extensively studied in plants, but little is known about sugar metabolism in seeds.

Despite being an oilseed plant, in Arabidopsis ovules carbohydrates represent a conspicuous part of the embryo weight (*Andriotis et al., 2010*).

Different carbohydrates have different accumulation and degradation pattern during the developmental phases of the seed (*Baud et al., 2002*); starch peaks between 6 and 9 Days After Pollination and then declines abruptly, whereas the hexose highest level is detected at 3 DAP and then diminishes until no more hexose is detected in mature seeds.

The fructose and glucose level are similar to the former; instead sucrose is low till 12 DAP and it is stored in the last phase of maturation.

Composed by glucose units linked by glycosidic bonds, starch is the most abundant storage carbohydrate in plants. The tridimensional structure is organized in two different glucose chains: the branched amylopectin, which account for almost 80% of the total composition, and the linear amylose, which represent the remaining 20%.

It is synthetized in leaves as a product of photosynthesis and its metabolism is widely studied among plant scientist.

However, little is known about the metabolism, accumulation and distribution pattern of this carbohydrate in seeds. Nevertheless, recently this unexplored field of research has gained attention among scientists.

In oilseed species, starch is a transient carbon deposit: after an initial phase of starch accumulation early after fertilization, starch level is slowly dropping as seed maturation goes further; it is indeed used as a carbon source for oil and fatty acid production (*Chen et al, 2015*). In addition, looking at the accumulation and degradation profile of starch, proteins and storage oil in

seeds, it can be noted that the decreasing in starch levels corresponds to the rise of the biosynthesis rate in proteins and oils (**Fig. 22**).

The expression level of genes involved in these compounds metabolism is consistent with the accumulation profile; transcription of genes related to carbohydrate metabolism (highlighted in pink in the figure) peaks in the initial phase of seed development; when their expression starts to diminish, the oil biosynthetic genes are expressed (in green), followed by those related to storage protein production (in blue).

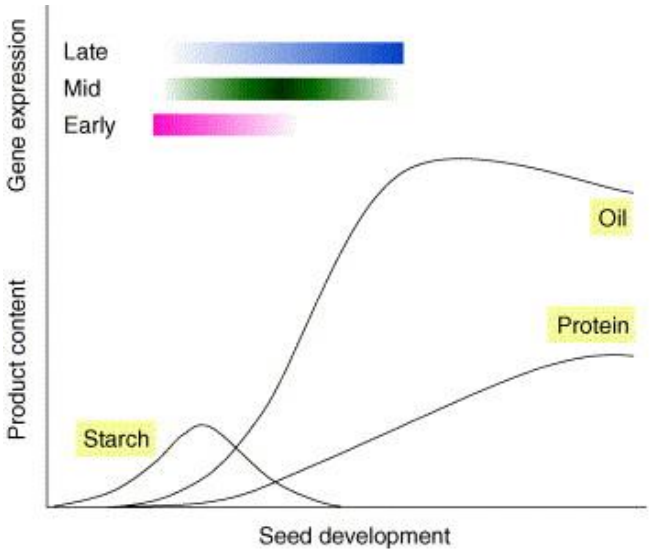

Fig 22: Trend of starch, oil and protein accumulation during Arabidopsis seed development.

Modified from *Hills, 2004*

Starch accumulation can be tracked through the use of iodine staining, which will turn deep purple at the presence of this sugar. Different studies were carried out in order to investigate the pattern of starch accumulation and degradation in Arabidopsis siliques and seeds (*Andriotis et al., 2010; Hedhly et al., 2016*).

Before the fertilization, purple staining is clearly visible in the central cell of mature ovules; the accumulation starts before the fusion between the polar nuclei and increased further after the formation of the secondary endosperm nucleus.

However, in ovules other tissues present starch accumulation: indeed, starch deposits can be seen in the egg cell, in the distal part of the funiculus, in the chalazal proliferating tissue and in the micropylar part of the inner and outer integuments (*Hedhly et al., 2016*).

In developing seeds, the pattern has been studied using the same technique, considering separately the three seed tissues at different time points (4 Days After Pollination, 6, 12 and 16 DAP).

In the embryo, starch accumulation begins at the heart-shape stage, although in very little extent. Later on, between 8 and 10 DAP, this carbohydrate is spotted in two different zones: above the tip of the radicle and at the hypocotyl region below the apical meristem. The cotyledons embryo still exhibits the purple coloration, which, soon after, starts to decline. After 12 DAP, no more starch is visible.

At 8 DAP, starch granules are unambiguously evident in the endosperm. In this tissue, starch peaks at 12 DAP and then starts to weakening till is not visible in mature seeds.

Nevertheless, starch can accumulate in seed coat layers. At 4 DAP, the whole seed coat shows purple coloration, whereas at 6 DAP, the stain is visible only in the outer integuments layers. Its deposition in the testa continues till 12 DAP, but then it start to diminish and no more starch is present at 16 DAP (*Andriotis et al, 2010*).

Carbon is imported in non-photosynthetic tissues via the glucose 6-phosphate/phosphate translocator (GPT). The *Arabidopsis thaliana* genome contains two homologous GPT genes, *AtGPT1* and *AtGPT2*.

Mutation affecting *GPT2* has no obvious effect on growth and development, whereas the mutations *gpt1* are documented to be embryo lethal (*Niewiadomski et al., 2005*).

Nevertheless, a viable homozygous allele has been found: it carries a t-DNA insertion at 19 bp upstream the translational initiation codon. In this *gpt1-3* mutant line, the transcript level are downregulated in tissue-specific manner; precisely, the mRNA is severely reduced at anthesis in carpels compared with the others reproductive organs. Thus starch accumulation is absent in the central cell of mutant plants (**Fig. 23**) (*Hedhly et al., 2016*).

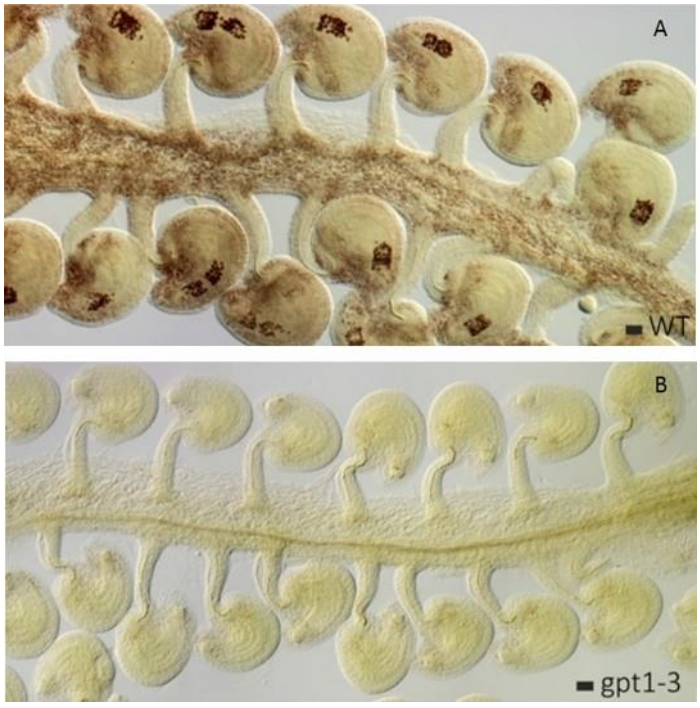

Fig. 23: Iodine staining in wt mature ovule (A) and *gpt1-3* (B) mutant. No starch is accumulated in the central cell of the mutant at anthesis. Scale bar = 20 µm. Modified from *Hedhly et al., 2016*.

Aim of the project

The aim of my project is to understand the genetic and molecular control of early stages of seed development. The mechanism of the crosstalk between the endosperm and the seed integuments has been investigated, since the communication between those tissues is relevant to the establishment of seed size.

The *stk abs* double mutant represents an interesting tool to study molecular mechanism of fertilization and early stages of seed development, because of the absence of this layer.

Moreover, I concentrated on the role of the genes *STK* and *ABS* in this pathway, since they are responsible for the formation of the innermost layer of the seed coat.

A second objective of my work has been related to the reduced fertility of the double mutant. Till now, the reason behind this decreased seed formation has not been elucidated yet.

The *stk abs* double mutant showed a massive starch accumulation in the embryo sac; here we showed that the excess of starch could be partially responsible for the impediment of the fertilization in the mutant.

Main results

Hormones are needed for a correct signaling process between endosperm and seed coat formation

The results presented in this chapter are published in the work of *Figueiredo et al., 2016*; nonetheless, I am going to discuss them as part of my thesis

because they were obtained independently and antecedently the publication of the above mentioned paper.

Several studies demonstrate the importance of hormones in plant growth. In absence of fertilization, applications of phytohormones induce autonomous seed development and fruit elongation, two developmental processes negatively controlled by the PcG complexes. Since these proteins are also involved in the regulation of seed coat development, we wondered if there was a link between hormones and integuments growth.

For this reason, emasculated wt carpels were treated with gibberellins. This exogenous application provokes the differentiation of ovule integuments in seed teguments in around 20% of ovules. This seed coat formation was not accompanied by endosperm development; indeed, a central cell was clearly visible in the above mentioned ovules.

To confirm the identity of the seed coat in treated ovules, vanillin staining was performed. Vanillin staining marks proanthocyanidins, which are accumulated exclusively in specialized cells of endothelium.

As expected, in the treated sample around 20% of ovules presented the typical staining, in accordance with the previous experiment.

Therefore, we can assume that gibberellins are sufficient to trigger seed coat development.

Role of STK in the endosperm-seed coat crosstalk

The crosstalk between endosperm and seed coat is a complex pathway, involving several players. Important regulators of seed development are the Polycomb proteins, which perform their function by histone methylation of

target genes. In particular, the FIS complex represses the endosperm development in absence of fertilization whilst the EMF complex represses seed coat formation (*Mozgova et al., 2015*).

ChIP Seq analysis previously performed in our lab showed that CLF and SWN, members of the EMF complex, are direct target of the *STK* transcription factor. This suggests a possible involvement of STK in the activation of the seed coat differentiation.

At first, we checked the expression levels of members of these PcG complexes by qRT-PCR. *CLF* and *SWN* appears to be heavily up-regulated in the *stk abs* double mutant.

Then, to evaluate a possible cooperation of *STK* in the signalling pathway, the *pSTK*::STK-GFP reporter construct and the p35S::*STK* transgene were introduced in the *agl62*/+ mutant to verify the hypothesis.

AGL62 is a type one MADS Box transcription factors known to be involved in the generation of the signal that induce seed coat development after the fertilization (*Roszack et al. 2011, Figuereido et al., 2015; 2016*).

In the *agl62/*+ heterozygous mutant no changes in the STK expression pattern were observed. When the transgene 35S::*STK* was introduced into *agl62*/+, it partially restored the seed coat phenotype. Interestingly, although seeds appear smaller than the wild type, the seed coat starts to develop.

Taken together, these results suggest us that STK could be involved in the perception of the signal triggered by AGL62, but not sufficient to complement the *agl62* phenotype.

Confocal analysis of *stk abs* double mutant

Given the defects in seed coat development affecting the double mutants, the latter represents a good genetic tool for studying the importance of the endothelium, the innermost layer of the seed coat, in the signalling pathway. Hence, we characterized in detail the *stk abs* double mutant.

This mutant presents smaller seeds compared to the wild type. To understand if this decrease in size was due to a reduced number or a reduced length of the integument cells, confocal analysis were performed.

These two parameters (cell number and cell length) were measured at three different time points: in mature ovules, at 2 Days After Pollination and at 6 DAP.

To sample mature ovules, carpels were emasculated to avoid fertilization and then harvested the day after. Concerning the other stages, the mutants under investigation were hand-pollinated with wild-type pollen and sampled at the desired time point. The same treatment was applied for Col-0 background.

At anthesis, no differences were noticed in any of the mutant under investigation.

Accordingly to our results, a wt ovule contains approximately 40 cells of about seven μm long. In the two single mutants and in the double mutant cell number and cell length was roughly the same observed in the wt condition.

Going further in the development, a problem in the elongation process can be noted in the *stk abs* double mutant as well as in the *stk* single mutant.

Indeed, already at 2 DAP these two mutants integument cells appear to be shorter compared to the wt, whilst the cell number remain constant. On the other hand, *abs* single mutant does not show any variation.

The same results were obtained at 6 DAP: cell number is the identical in all four genotypes considered, whereas cell length is reduced in the *stk* and in the *stk abs* mutant.

Fertilization rate in *stk abs* double mutant

The *stk abs* double mutant showed a drastic reduction in fertility due to defects during ovule and seed formation (*Mizzotti et al., 2011*). To discriminate between ovule abortion and seeds arrest, the reporter construct *pWOX9*::WOX9-YFP was introduced into the mutant. *WOX9* expression is detected in the apical and basal daughter cells after the first zygotic division (*Wua et al., 2007*); this expression pattern was considered as a marker of fertilization.

In *stk abs* double mutant, around 25% of seeds showed fluorescent signal, indicating that fertilization is happening only in a quarter of ovules.

Nevertheless, the reason behind this infertility is still matter of question.

Generation of the *stk abs gpt1-3* triple mutant

A very interesting phenotype related to the *stk abs* double mutant is the massive amount of starch present in the embryo sac around the central cell. (*Mizzotti et al., 2011).*

Since the reason of the reduced fertility of *stk abs* is still unknown, we hypothesized that this huge amount of starch could somehow interfere with a correct fertilization process.

The *gpt1-3* mutant is known in literature for the absence of starch in mature ovules; differently for the other *gpt1* mutant lines, which appear to be embryo-lethal, the *gpt1-3* mutant produces viable seeds because of the tissue specific down-regulation of its transcript (*Hedhly et al., 2016*). Indeed, *gpt1-3* has a mutation in the regulatory region of the *GPT* promoter and this alteration results in a mutant in which the *GPT* is not transcribed in the carpels at anthesis, but it remains expressed in the other reproductive organs.

In order to evaluate a possible disturbance of the excessive amount of starch in the *stk abs* gametophyte for the fertilization process, the triple mutant *stk abs gpt1* was created through genetic crosses.

Partial rescue of seed phenotype in the *stk abs gpt1-3* triple mutant

Phenotypical analysis of *stk abs gpt1-3* triple mutant confirmed the results obtained by the genetic screening.

As already showed before, *stk abs* double mutant presents four tegument layers and an excessive amount of starch in the embryo sac. The *stk abs gpt1-3* triple mutant isolated from the genetic screening displays the typical *stk abs* double mutant phenotype with only four layers of the seed coat and the absence of the starch due to mutation in the *gpt1-3* locus.

A healthy wild type silique at maturity is formed by a complete seed set, which contains around 50 properly developed seeds. In a *stk abs* mature silique, seed maturation is heavily compromised: ovules abortion and seed arrest are frequently noted and only 45% of seeds reach the maturity (*Mizzotti et al., 2011*).

The same experiment was then performed in the triple mutant. In a mature silique, seed arrest with a lower percentage compared with the *abs stk* double mutant. Indeed, 70% of seeds reach the complete maturity.

Subsequently, mature seed dimension was measured using the Smart Grain program. Even though seeds appear still smaller compared to the wild type, a rescue of seed area and seed perimeter is unmistakably evident compared to *stk abs*.

The rescue of seed dimension was confirmed measuring tegument cell length with confocal imaging. The experiment was repeated using the same instruction: the triple mutant was sampled right before the fertilization and at 2 DAP, being hand-pollinated with wt pollen.

Triple mutant mature ovules have the same number of cell of a wt ovule; similarly, cell length was almost the same. At 2 DAP, cell length appear to be decreased in the triple mutant compared to the wild type.

However, even though still smaller compared to the wild type, the triple mutant present tegument cells significantly longer than the *stk abs* double mutant does.

The fertilization rate was checked with marker lines. To evaluate the fertilization of the central cell, the *pMINI3*-GUS reporter lines was used. The signal is absent before the fertilization and became visible in the endosperm nuclei starting from 2 DAP (*Luo et al., 2005*). In the double mutant, only 45% of seeds showed the GUS signal in the endosperm, whereas in the triple mutant the number of fertilized seeds rises at around 60%.

Similarly, the *pWOX9*::WOX9-YFP reporter line enable us to evaluate the fusion between the male and the female gametes and the consequent formation of the embryo.

As said before, only 20% of *stk abs* seeds are fertilized, whereas in the triple mutant the fluorescent signal is detected in around 40% of the analyzed seeds.

Conclusion and future perspectives

Fertilization in *A. thaliana* is a very complicated process in which several developmental steps have to occur correctly. The product of the fertilization is formed by three main structures, genetically and functionally different.

Even though extensively studied, more research are needed to unravel completely this multistep fertilization pathway; the work hereby presented represent an important advancement in the understanding of the players involved.

We have discovered new important function of the endothelium in the crosstalk between endosperm and seed coat.

Moreover, the double mutant used in this work presents defects in fertilization and a huge accumulation of starch in the female gametophyte surrounding the central cell.

We created a triple mutant crossing *stk abs* with the *gpt1* mutant, known in literature for the lack of the carbohydrate in mature ovules.

A rescue in seed size and in the fertilization is clearly achieved, as confirmed with morphological analysis and by the use of marker lines.

These results lead us to speculate that the excess of starch in *stk abs* double mutant is physically obstructing the fertilization.

References

Andriotis, V. M. E., Pike, M. J., Kular, B., Rawsthorne, S., & Smith, A. M. (2010). Starch turnover in developing oilseed embryos. New Phytologist, 187(3), 791–804. http://doi.org/10.1111/j.1469-8137.2010.03311.x

Bai, F., & Settles, A. M. (2015). Imprinting in plants as a mechanism to generate seed phenotypic diversity. Frontiers in Plant Science, 5(January), 1–10. http://doi.org/10.3389/fpls.2014.00780

Balanzà, V., Roig-Villanova, I., Di Marzo, M., Masiero, S., & Colombo, L. (2016). Seed abscission and fruit dehiscence required for seed dispersal rely on similar genetic networks. Development, 143(18), 3372–3381. http://doi.org/10.1242/dev.135202

Baud, S., Boutin, J. P., Miquel, M., Lepiniec, L., & Rochat, C. (2002). An integrated overview of seed development in Arabidopsis thaliana ecotype WS. Plant Physiology and Biochemistry, 40(2), 151–160. http://doi.org/10.1016/S0981-9428 (01)01350-X

Baudry, A., Heim, M. A., Dubreucq, B., Caboche, M., Weisshaar, B., & Lepiniec, L. (2004). TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in Arabidopsis thaliana. Plant Journal, 39(3), 366–380. http://doi.org/10.1111/j.1365-313X.2004.02138.x

Becker, A., Kaufmann, K., Freialdenhoven, A., Vincent, C., Li, M. A., Saedler, H., & Theissen, G. (2001). A novel MADS-box gene subfamily with a sister-group relationship to class B floral homeotic genes. Molecular Genetics and Genomics, 266(6), 942–950. http://doi.org/10.1007/s00438-001-0615-8

Berger, F., Hamamura, Y., Ingouff, M., & Higashiyama, T. (2008). Double fertilization - caught in the act. Trends in Plant Science, 13(8), 437–443. http://doi.org/10.1016/j.tplants.2008.05.011

Brown, R. C., Lemmon, B. E., Nguyen, H., & Olsen, O. A. (1999). Development of endosperm in Arabidopsis thaliana. Sexual Plant Reproduction, 12(1), 32–42. http://doi.org/10.1007/s004970050169

Chaudhury, A. M., Ming, L., Miller, C., Craig, S., Dennis, E. S., & Peacock, W. J. (1997). Fertilization-independent seed development in Arabidopsis thaliana. Proceedings Of The National Academy Of Sciences Of The United States Of America, 94(8), 4223–4228. http://doi.org/10.1073/pnas.94.8.4223

Chen, L.-Q., Lin, I. W., Qu, X.-Q., Sosso, D., McFarlane, H. E., Londoño, A., Samuel, L., Frommer, W. B. (2015). A Cascade of Sequentially Expressed Sucrose Transporters in the Seed Coat and Endosperm Provides Nutrition for the Arabidopsis Embryo. The Plant Cell, 27(3), 607–619. http://doi.org/10.1105/tpc.114.134585

Coen, O., Fiume, E., Xu, W., De Vos, D., Lu, J., Pechoux, C., Lepiniec L., Magnani, E. (2017). Developmental patterning of the sub-epidermal

integument cell layer in Arabidopsis seeds. Development, 144(8), 1490–1497. http://doi.org/10.1242/dev.146274

De Lucia, F., Crevillen, P., Jones, A. M. E., Greb, T., & Dean, C. (2008). A PHD-Polycomb Repressive Complex 2 triggers the epigenetic silencing of FLC during vernalization. Proceedings of the National Academy of Sciences, 105(44), 16831–16836. http://doi.org/10.1073/pnas.0808687105

Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., & Lepiniec, L. (2003). Proanthocyanidin-accumulating cells in Arabidopsis testa: regulation of differentiation and role in seed development. The Plant Cell, 15(November), 2514–2531. http://doi.org/10.1105/tpc.014043

Ezquer, I., Mizzotti, C., Nguema-Ona, E., Gotté, M., Beauzamy, L., Viana, V. E., Dubrulle, N., Costa de Oliveira A., Caporali E., Koroney A.S., Boudaoud A., Driouich A., Colombo, L. (2016). The Developmental Regulator SEEDSTICK Controls Structural and Mechanical Properties of the Arabidopsis Seed Coat. The Plant Cell, 28(10), 2478–2492. http://doi.org/10.1105/tpc.16.00454

Favaro, R., & Pinyopich, A. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. The Plant Cell …, 15(November), 2603–2611. http://doi.org/10.1105/tpc.015123.2

Figueiredo, D. D., Batista, R. A., Roszak, P. J., Hennig, L., & Köhler, C. (2016). Auxin production in the endosperm drives seed coat

development in Arabidopsis. eLife, 5(NOVEMBER2016), 1–23. http://doi.org/10.7554/eLife.20542

Figueiredo, D. D., Batista, R. A., Roszak, P. J., & Köhler, C. (2015). Auxin production couples endosperm development to fertilization. Nature Plants, 1(12), 15184. http://doi.org/10.1038/nplants.2015.184

Parenicová L., De Folter, S., Kieffer, M., Horner, D. S., Favalli, C., Busscher, J., Cook, H. E., Ingram R.M., Kater M.M., Davies, B., Angenent, G.C., Colombo, L. (2003). Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: new openings to the MADS world. The Plant Cell, 15(7), 1538–1551. http://doi.org/10.1105/tpc.011544

Fuentes, S., Ljung, K., Sorefan, K., Alvey, E., Harberd, N. P., & Østergaard, L. (2012). Fruit Growth in Arabidopsis Occurs via DELLA-Dependent and DELLA-Independent Gibberellin Responses. The Plant Cell, 24(10), 3982–3996. http://doi.org/10.1105/tpc.112.103192

Garcia, D., Saingery, V., Chambrier, P., Mayer, U., Ju, G., Lyon, F., & France, D. G. (2015). Arabidopsis haiku Mutants Reveal New Controls of Seed Size by Endosperm 1, D(April 2003), 1661–1670. http://doi.org/10.1104/pp.102.018762.the

Gremski, K., Ditta, G., & Yanofsky, M. F. (2007). The HECATE genes regulate female reproductive tract development in Arabidopsis thaliana. Development (Cambridge, England), 134(20), 3593–3601. http://doi.org/10.1242/dev.011510

Guilfoyle, T. J., & Hagen, G. (2007). Auxin response factors. Current Opinion in Plant Biology, 10(5), 453–460. http://doi.org/10.1016/j.pbi.2007.08.014

Gustafson, F. G. (1936). Inducement of Fruit Development by Growth-Promoting Chemicals. Proceedings of the National Academy of Sciences of the United States of America, 22(11), 628–636. http://doi.org/10.1073/pnas.22.11.628

Gutierrez-Marcos, J. F., Const??ncia, M., & Burton, G. J. (2012). Maternal to offspring resource allocation in plants and mammals. Placenta, 33(SUPPL. 2), e3–e10. http://doi.org/10.1016/j.placenta.2012.08.006

Haughn, G., & Chaudhury, A. (2005). Genetic analysis of seed coat development in Arabidopsis. Trends in Plant Science, 10(10), 472–477. http://doi.org/10.1016/j.tplants.2005.08.005

Hedhly, A., Vogler, H., Schmid, M. W., Pazmino, D., Gagliardini, V., Santelia, D., & Grossniklaus, U. (2016). Starch Turnover and Metabolism during Flower and Early Embryo Development. Plant Physiology, 172(4), 2388–2402. http://doi.org/10.1104/pp.16.00916

Hennig, L., & Derkacheva, M. (2009). Diversity of Polycomb group complexes in plants: same rules, different players? Trends in Genetics, 25(9), 414–423. http://doi.org/10.1016/j.tig.2009.07.002

Hills, M. J. (2004). Control of storage-product synthesis in seeds. Current Opinion in Plant Biology, 7(3), 302–308. http://doi.org/10.1016/j.pbi.2004.03.003

Kang, I.-H., Steffen, J. G., Portereiko, M. F., Lloyd, A., & Drews, G. N. (2008). The AGL62 MADS domain protein regulates cellularization during endosperm development in Arabidopsis. The Plant Cell, 20(3), 635–647. http://doi.org/10.1105/tpc.107.055137

Kaufmann, K., Anfang, N., Saedler, H., & Theissen, G. (2005). Mutant analysis, protein-protein interactions and subcellular localization of the Arabidopsis Bsister (ABS) protein. Molecular Genetics and Genomics, 274(2), 103–118. http://doi.org/10.1007/s00438-005-0010-y

King, G. N. (1947). Artificial parthenocarpy in lycopersicum esculentum; tissue development, 572–581.

Kohler, C., & Makarevich, G. (2006). Epigenetic mechanisms governing seed development in plants. EMBO Rep, 7(12), 1223–1227. http://doi.org/10.1038/sj.embor.7400854

Köhler, C., Hennig, L., Spillane, C., Pien, S., Gruissem, W., & Grossniklaus, U. (2003). The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. Genes and Development, 17(12), 1540–1553. http://doi.org/10.1101/gad.257403

Kram, B. W., Xu, W. W., & Carter, C. J. (2009). Uncovering the Arabidopsis thaliana nectary transcriptome: investigation of differential gene expression in floral nectariferous tissues. BMC Plant Biology, 9(1), 92. http://doi.org/10.1186/1471-2229-9-92

Lepiniec, L., Debeaujon, I., Routaboul, J.-M., Baudry, A., Pourcel, L., Nesi, N., & Caboche, M. (2006). Genetics and Biochemistry of Seed

Flavonoids. Annual Review of Plant Biology, 57(1), 405–430. http://doi.org/10.1146/annurev.arplant.57.032905.105252

Liu, C. M., & Meinke, D. W. (1998). The titan mutants of Arabidopsis are disrupted in mitosis and cell cycle control during seed development. The Plant Journal : For Cell and Molecular Biology, 16(1), 21–31. http://doi.org/10.1046/j.1365-313x.1998.00268.x

Luo, M., Dennis, E. S., Berger, F., Peacock, W. J., & Chaudhury, A. (2005). MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America, 102(48), 17531–17536. http://doi.org/10.1073/pnas.0508418102

McKenzie Duncan, I. (1982). Polycomblike: A gene that appears to be required for the normal expression of the bithorax and antennapedia gene complexes of Drosophila melanogaster. Genetics, 102(1), 49–70.

Mizzotti, C., Ezquer, I., Paolo, D., Rueda-Romero, P., Guerra, R. F., Battaglia, R., Rogachev, I., Aharoni, A., Kater, M.M., Caporali E., Colombo, L. (2014). SEEDSTICK is a Master Regulator of Development and Metabolism in the Arabidopsis Seed Coat. PLoS Genetics, 10(12). http://doi.org/10.1371/journal.pgen.1004856

Mizzotti, C., Mendes, M. A., Caporali, E., Schnittger, A., Kater, M. M., Battaglia, R., & Colombo, L. (2012). The MADS box genes SEEDSTICK and ARABIDOPSIS Bsister play a maternal role in

fertilization and seed development. Plant Journal, 70(3), 409–420. http://doi.org/10.1111/j.1365-313X.2011.04878.x

Mockaitis, K., & Estelle, M. (2008). Auxin Receptors and Plant Development: A New Signaling Paradigm. Annual Review of Cell and Developmental Biology, 24(1), 55–80. http://doi.org/10.1146/annurev.cellbio.23.090506.123214

Mozgova, I., & Hennig, L. (2015). The Polycomb Group Protein Regulatory Network. Annual Review of Plant Biology, 66(1), 269–296. http://doi.org/10.1146/annurev-arplant-043014-115627

Nesi, N., Debeaujon, I., Jond, C., Stewart, A. J., Jenkins, G. I., Caboche, M., & Lepiniec, L. (2002). The TRANSPARENT TESTA16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. The Plant Cell, 14(10), 2463–2479. http://doi.org/10.1105/tpc.004127

Niewiadomski, P., Knappe, S., Geimer, S., Fischer, K., Schulz, B., Unte, U. S., Rosso, M. G., Ache, P., Flugge,U., & Schneider, A. (2005). The Arabidopsis plastidic glucose 6-phosphate/phosphate translocator GPT1 is essential for pollen maturation and embryo sac development. The Plant Cell, 17(3), 760–775. http://doi.org/10.1105/tpc.104.029124

Ogawa, M., Kay, P., Wilson, S., & Swain, S. M. (2009). ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 Are Polygalacturonases Required for Cell Separation during Reproductive Development in Arabidopsis. The Plant Cell Online, 21(1), 216–233. http://doi.org/10.1105/tpc.108.063768

Ohad, N., Margossian, L., Hsu, Y. C., Williams, C., Repetti, P., & Fischer, R. L. (1996). A mutation that allows endosperm development without fertilization. Proceedings Of The National Academy Of Sciences Of The United States Of America, 93(11), 5319–5324. http://doi.org/10.1016/S1369-5266(99)80006-5

Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J. J., Goldberg, R.B., Fischer, R. L. (1999). Mutations in FIE, a WD polycomb group gene, allow endosperm development without fertilization. The Plant Cell, 11(3), 407–416. http://doi.org/10.1105/tpc.11.3.407

Ozga, J. A., & Reinecke, D. M. (2003). Hormonal Interactions in Fruit Development. Journal of Plant Growth Regulation, 22(1), 73–81. http://doi.org/10.1007/s00344-003-0024-9

Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E., & Yanofsky, M. F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature, 424(6944), 85–88. http://doi.org/10.1038/nature01741

Roszak, P., & Köhler, C. (2011). Polycomb group proteins are required to couple seed coat initiation to fertilization. Proceedings of the National Academy of Sciences of the United States of America, 108(51), 20826–20831. http://doi.org/10.1073/pnas.1117111108

Schuettengruber, B., & Cavalli, G. (2009). Recruitment of Polycomb group complexes and their role in the dynamic regulation of cell fate choice. Development, 136(21), 3531–3542. http://doi.org/10.1242/dev.033902

Spillane, C., MacDougall, C., Stock, C., Köhler, C., Vielle-Calzada, J. P., Nunes, S. M., Grossniklaus, U., Goodrich, J. (2000). Interaction of the Arabidopsis Polycomb group proteins FIE and MEA mediates their common phenotypes. Current Biology, 10(23), 1535–1538. http://doi.org/10.1016/S0960-9822(00)00839-3

Sundaresan V. (2005). Control of seed size in plants. Proceedings of the National Academy of Sciences of the United States of America; 102(50): 17887–17888. doi: 10.1073/pnas.0509021102

Vivian-Smith, A., & Koltunow, A. M. (1999). Genetic analysis of growth-regulator-induced parthenocarpy in Arabidopsis. Plant Physiol, 121(2), 437–451. http://doi.org/10.1104/pp.121.2.437

Voiniciuc, C., Yang, B., Schmidt, M., Günl, M., & Usadel, B. (2015). Starting to Gel: How Arabidopsis Seed Coat Epidermal Cells Produce Specialized Secondary Cell Walls. International Journal of Molecular Sciences, 16(2), 3452–3473. http://doi.org/10.3390/ijms16023452

Wang, D., Tyson, M. D., Jackson, S. S., & Yadegari, R. (2006). Partially redundant functions of two SET-domain polycomb-group proteins in controlling initiation of seed development in Arabidopsis. Proc Natl Acad Sci U S A, 103(35), 13244–13249. http://doi.org/10.1073/pnas.0605551103

Windsor, J. B., Symonds, V. V., Mendenhall, J., & Lloyd, A. M. (2000). Arabidopsis seed coat development: Morphological differentiation of the outer integument. Plant Journal, 22(6), 483–493. http://doi.org/10.1046/j.1365-313X.2000.00756.x

Wood, C. C., Robertson, M., Tanner, G., Peacock, W. J., Dennis, E. S., & Helliwell, C. A. (2006). The Arabidopsis thaliana vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. Proc Natl Acad Sci U S A, 103(39), 14631–14636. http://doi.org/DOI 10.1073/pnas.0606385103

Wua X., Chorya J., Weigel D. (2007). Combinations of WOX activities regulate tissue proliferation during Arabidopsis embryonic development. Developmental Biology, Volume 309, Issue 2,

Yadegari, R., & Drews, G. (2004). Female gametophyte development. The Plant Cell Online, 16, 133–142. http://doi.org/10.1105/tpc.018192.S134

Yoshida, N. (2001). EMBRYONIC FLOWER2, a Novel Polycomb Group Protein Homolog, Mediates Shoot Development and Flowering in Arabidopsis. The Plant Cell Online, 13(11), 2471–2481. http://doi.org/10.1105/tpc.13.11.2471

PART II

Manuscript #1

The role of the endothelium during fertilization process in *Arabidopsis thaliana*

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Abstract

Embryo, endosperm and seed coat are the three tissues forming a mature Arabidopsis seed. Their development and differentiation start soon after the fertilization: embryo and endosperm derives from the fusion between male and female gametes, whereas the seed coat comes from the differentiation of the ovule integuments. It is a five-layer tissue surrounding the female gametophyte; the innermost layer of the seed coat is called endothelium and is deputed to the synthesis and accumulation of proanthocyanidins. It has been demonstrated that two MADS Box transcription factors, STK and ABS, collaborate in defining the identity of this layer. Indeed, the *stk abs* double mutant does not differentiate the endothelium and since there is no accumulation of PA, this double mutant seeds appear yellow instead of the typical brownish color of the wild type seeds. Other phenotypes characterize this mutant: a drastic reduction of the fertilization rate and a huge accumulation of starch in the mature ovules surrounding the central cell are noted. In this work we show that this two phenotypes are linked: indeed,

removing the excess of starch in the female gametophyte, a partial rescue in the fertilization rate is actually achieved.

Introduction

Seed development in flowering plants requires the tight coordination of different events. Indeed, embryo, endosperm and seed coat must coordinate their growth and development in order to ensure a correct seed maturation.

These three tissues are genetically and functionally distinct: embryo and endosperm derives from the fusion between male and female gametes and they are a diploid and a triploid tissue, respectively. The seed coat derives from the differentiation of the ovules integuments; hence, it has a completely maternal origin (*Haughn et al., 2005*).

The seed coat is a five layers tissue that surrounds the female gametophyte and protects the embryo from mechanical and chemical damages. Each layer goes through a different specialization faith; at the end of the development, seed coat cells undergo cell death and they are crushed together (*Windsor et al., 2000;* Haughn *et al, 2005).*

The innermost layer of the seed coat is known as endothelium and it is deputed to the synthesis and accumulation of proanthocyanidins. After the fertilization, the oxidation of these molecules confers to seeds the typical brownish color (*Debeaujon et al., 2003*).

In literature, the *stk abs* double mutant is the only mutant with severe defect in endothelium differentiation; indeed, the seed coat has only four layers. No proanthocyanidins are accumulated in those seeds; for this reason, they appear yellow (*Mizzotti et al., 2012*).

SEEDSTIK (STK) and *ARABIDOPSIS BSISTER* (*ABS*) encode for two MADS Box transcription factors which regulates several developmental pathways and metabolic processes.

STK is responsible, together with *SHATTERPROOF1* (*SHP1*) and *SHP2*, for the correct determination of ovule identity. Indeed, the triple mutant present ovules with a leaf-life or carpel-like structure (*Pinyopich et al., 2003*). Furthermore, *stk* seeds are smaller and rounder compared to the wt; moreover, those seeds hardly detach from the siliques (*Favaro et al., 2003; Pinyopich et al., 2003*) because of an ectopic lignification in the seed abscission zone (*Balanzà et al., 2016*).

ABS, or *TRANSPARENT TESTA 16* (*TT16*), was the first Bsister gene that has been characterized (*Nesi et al., 2002*). *ABS* is expressed in buds, flower and immature seeds, therefore only in reproductive organs, in accordance with the typical expression pattern of the Bsister MADS family, exclusive of the female reproductive organs (*Nesi et al*., 2002¸ *Becker et al., 2002*). Mature *abs* seeds appears yellow because of the failure in the accumulation of PA in the endothelium. However, those molecules are present near the chalaza and micropyle zone of the seed, suggesting multiple regulatory strategies of PA accumulation.

These two MADS gene are both expressed in the innermost layer of mature ovules (*Mizzotti et al., 2012*) and they are able to interact between each other (*Kaufmann et al., 2005*). Together, they are responsible for the formation of this seed coat layer.

The defect in the endothelium differentiation in the *stk abs* double mutant causes different phenotypes. The fertilization process is severely compromised; indeed, a mature silique contains nearly half of seeds that

completed the entire developmental process; numerous ovules abortion and seed arrest are present.

The integrity of the seven cells of the female gametophyte has been checked and proved wild type; similarly, the viability and correct delivery of pollen tube has been examined. To date, the reason of this reduction in fertility is still unknown and it is investigated in this work.

Results

Integument cells elongation in *stk* and *stk abs* is impaired

Given the defects in seed coat development affecting the double mutants, the latter represents a good genetic tool for studying the importance of the endothelium, the innermost layer of the seed coat, in the signalling pathway. Hence, we characterized in detail the *stk abs* double mutant.

This mutant presents smaller seeds compared to the wild type (*Mizzotti et al., 2012*). To understand if this decrease in size was due to a reduced number or a reduced length of the integument cells, confocal analysis were performed.

These two parameters (cell number and cell length) were measured at three different time points: in mature ovules, at 2 Days After Pollination and at 6 DAP.

To sample mature ovules, carpels were emasculated to avoid fertilization and then harvested the day after. Concerning the other stages, the mutants under investigation were hand-pollinated with wild-type pollen and sampled at the desired time point. The same treatment was applied for Col-0 background.

At anthesis, no differences were noticed in any of the mutant under investigation (**Fig. 1**).

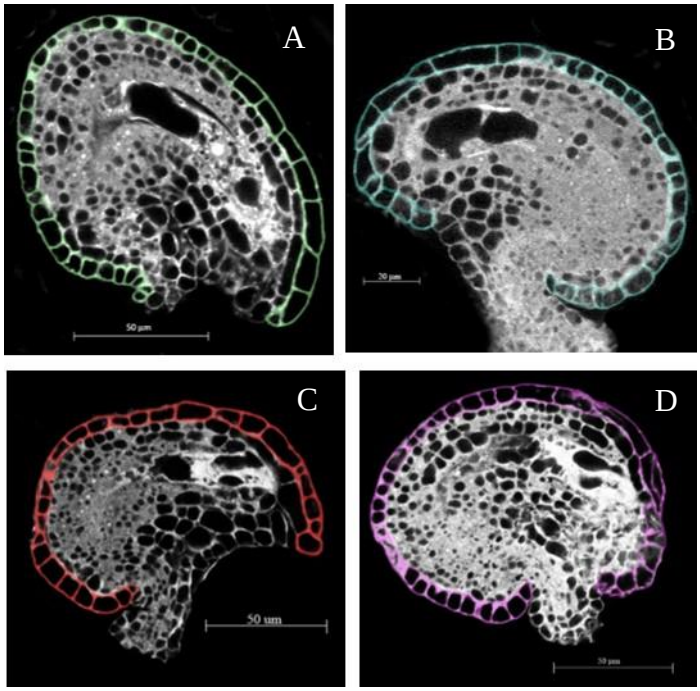
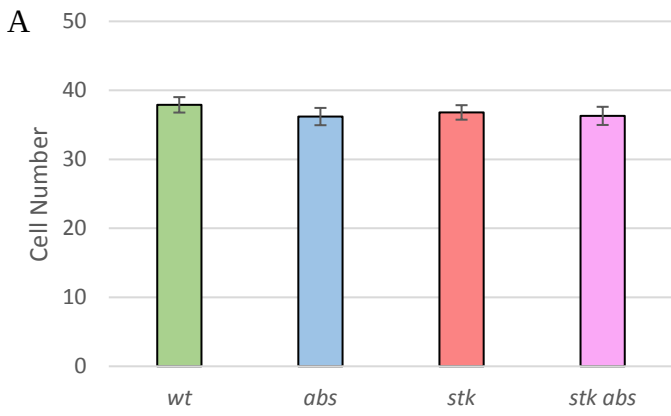


Fig. 1: Confocal analysis of wt (A), *abs* (B), *stk* (C) and *stk abs* (D) ovules at anthesis.

The wt ovule contains approximately 40 cells of about seven µm long. In *abs* and *stk* single mutants and in the *stk abs* double mutant cell number and cell length was roughly the same observed in the wt condition (**Fig. 2**).



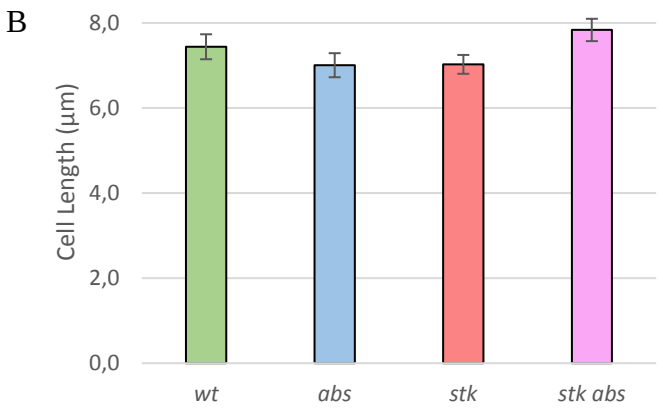


Fig. 2: Cell number (A) and cell length (B) of integuments at 0 DAP. 10 ovules for each genotype were analyzed.

Going further in the development, a problem in the elongation process can be noted in the *stk abs* double mutant as well as in the *stk* single mutant.

Indeed, already at 2 DAP these two mutants tegument cells appear to be shorter compared to the wt, whilst the cell number remain constant. On the contrary, *abs* single mutant does not show any variation (**Fig. 3** & **Fig. 4**).

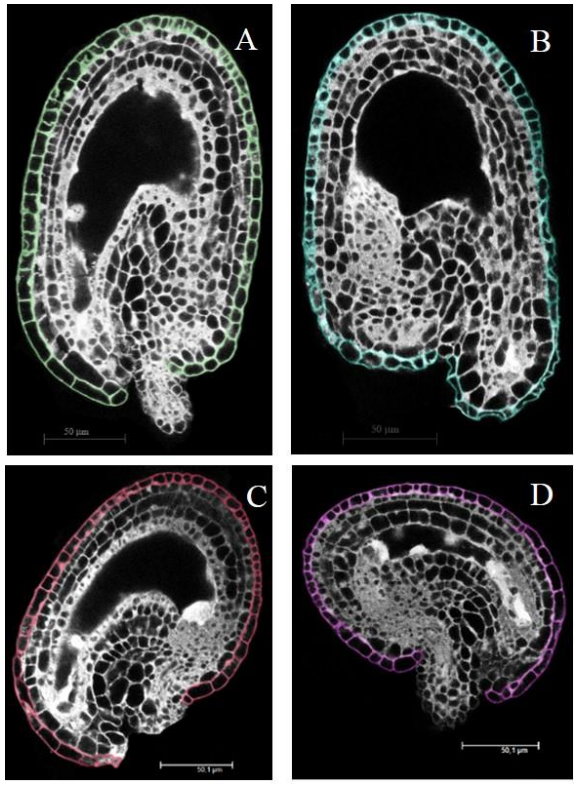
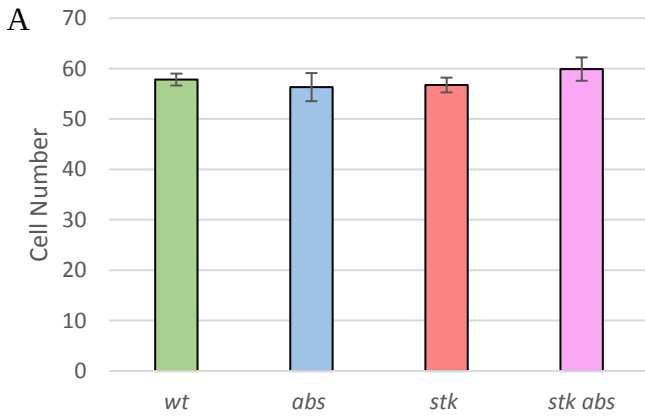


Fig. 3: Confocal analysis of wt (A), *abs* (B), *stk* (C) and *stk abs* (D) ovules at 2 DAP.



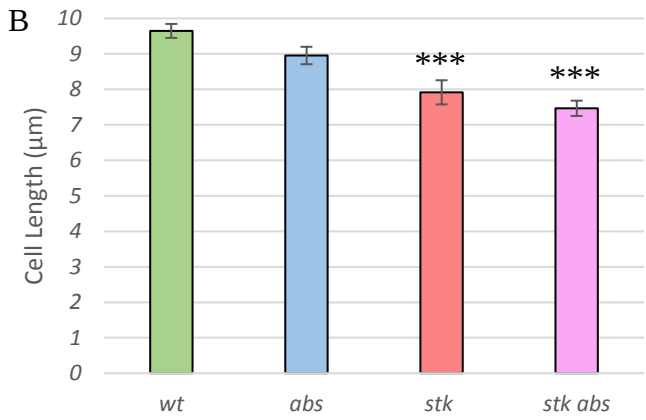


Fig. 4: Cell number (A) and cell length (µm) (B) of integuments at 2 DAP.

The same results were obtained at 6 DAP: cell number is identical in all four genotypes considered, whereas cell length is reduced in the *stk* and in the *stk abs* mutant.

Fertilization rate in *stk abs* double mutant

The *stk abs* double mutant showed a drastic reduction in fertility due to defects during ovule and seed formation (*Mizzotti et al., 2012*). To discriminate between ovule abortion and seeds arrest, the reporter construct *pWOX9*::WOX9-YFP was introduced into the mutant. WOX9 expression is detected in the apical and basal daughter cells after the first zygotic division (*Wua et al., 2007*); this expression pattern was considered as a marker of fertilization.

In *stk abs* double mutant, around 25% of the 500 seeds analyzed showed fluorescent signal, indicating that fertilization is happening only in a quarter of ovules (**Fig. 5**).

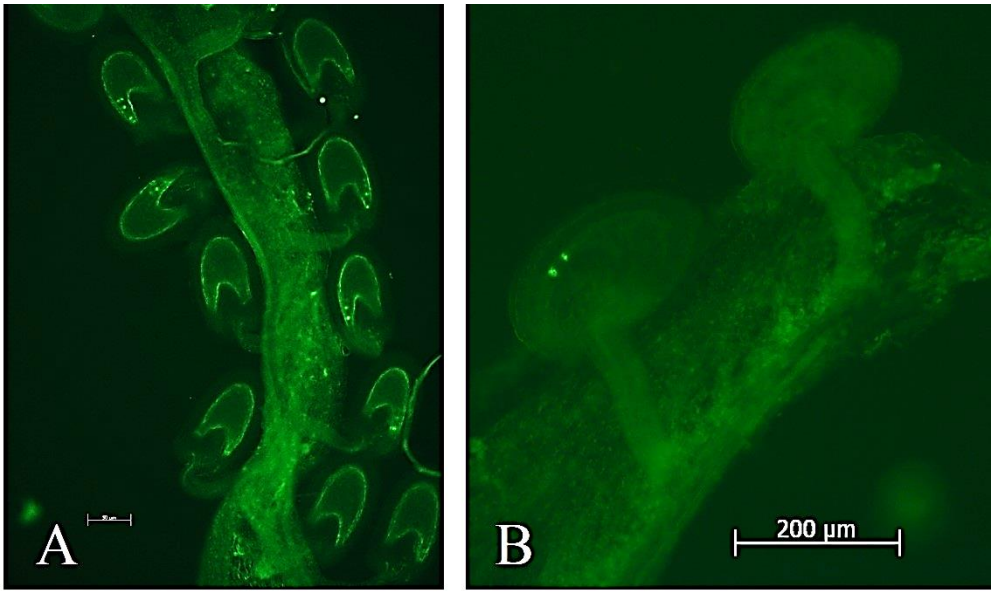


Fig. 5: Introgression of the WOX9- YFP reporter gene in the wt (A) and *stk abs* double mutant (B). In the wt condition, the majority of the ovules are fertilized, whereas in the *stk abs* double mutant the fluorescent signal is visible only in around 25% of seeds.

Generation of the *stk abs gpt1-3* triple mutant

A very interesting phenotype related to the *stk abs* double mutant is the massive amount of starch present in the embryo sac around the central cell (*Mizzotti et al., 2012).*

Since the reason of the reduced fertility of *stk abs* is still unknown, we hypothesized that this huge amount of starch could somehow interfere with a correct fertilization process.

Starch is one the most important storage carbohydrate in plants; even though Arabidopsis is an oilseed plant, starch account for a conspicuous part of the embryo weight.

Carbon is imported in non-photosynthetic tissues via the glucose 6-phosphate/phosphate translocator (GPT). The *Arabidopsis thaliana* genome contains two homologous GPT genes, *AtGPT1* and *AtGPT2*.

Mutation affecting *GPT2* has no obvious effects on growth and development, whereas the mutation *gpt1* are documented to be embryo lethal (*Niewiadomski et al., 2005*).

The *gpt1-3* mutant is known in literature for the absence of starch in mature ovules; differently for the other *gpt1* mutant lines, which appear to be embryo-lethal, the *gpt1-3* mutant produce viable seeds because of the tissue specific down-regulation of its transcript (*Hedhly et al., 2016*). Indeed, it is not transcribed in the carpels at anthesis, but it remains expressed in the other reproductive organs.

In order to evaluate a possible disturbance of the excess amount of starch in the *stk abs* gametophyte for the fertilization process, the triple mutant *stk abs gpt1* was created through genetic crosses.

Partial rescue of seed phenotype in the *stk abs gpt1-3* triple mutant

Phenotypical analysis of *stk abs gpt1-3* triple mutant confirmed the results obtained by the genetic screening.

As already showed before, *stk abs* double mutant presents four teguments layers and an excessive amount of starch in the embryo sac. The *stk abs gpt1-3* triple mutant isolated from the genetic screening displays the typical *stk abs* double mutant phenotype with only four layers of the seed coat and the absence of the starch due to mutation in the *gpt1-3* locus (**Fig. 5**).

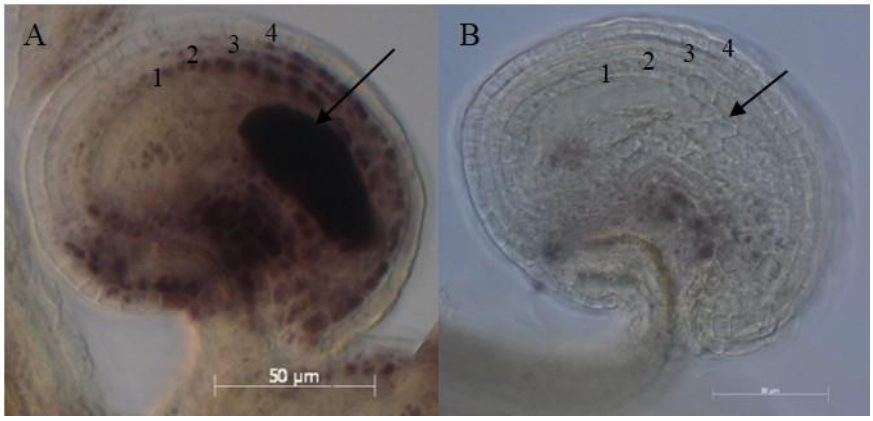


Fig. 5: Lugol staining of a *stk abs* (A) and *stk abs gpt1-3* (B) mature ovules. Scale bar = 50 µm.

A healthy wild type silique at maturity is formed by a complete seed set, which contains around 50 properly developed seeds. In a *stk abs* mature silique, seed maturation is heavily compromised: ovules abortion and seed arrest are frequently noted and only 45% of seeds reach the maturity (*Mizzotti et al., 2011*).

To evaluate a rescue of the abortion in the triple mutant generated, we counted the number of seeds in a mature silique; seed arrest are still existent, but to a lower percentage compared with the double mutant. Indeed, 70% of seeds reach the complete maturity (**Fig. 6** & **Fig 7**).

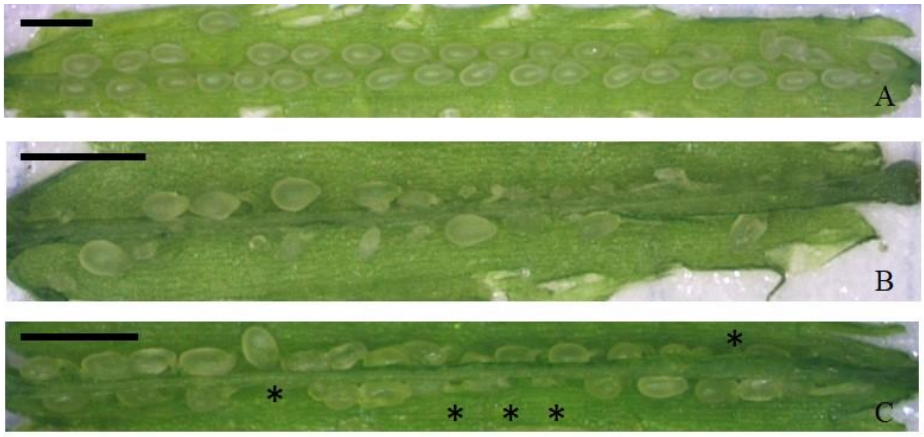


Fig. 6: Stereomicroscope imaging of a wt (A), *stk abs* (B) and *stk abs gpt1-3* (C) mature silique. The black asterisk indicate ovules abortion. Scale bar = 1cm.

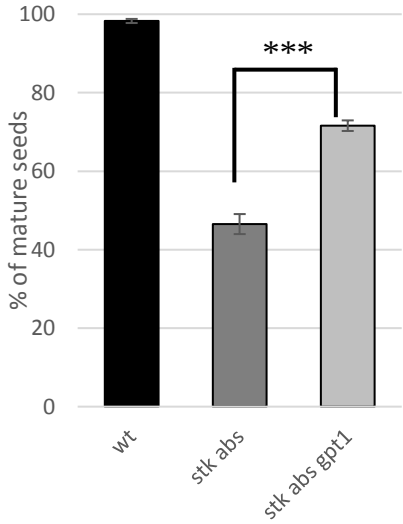


Fig. 7: Statistical analysis of the percentage of mature ovule in the double and triple mutant. p-value <0.05

Subsequently, mature seed dimension was measured using the Smart Grain program. Even though seeds appear still smaller compared to the wild type, a

rescue of seed area and seed perimeter is unmistakably evident compared to *stk abs* (**Fig. 8)**.

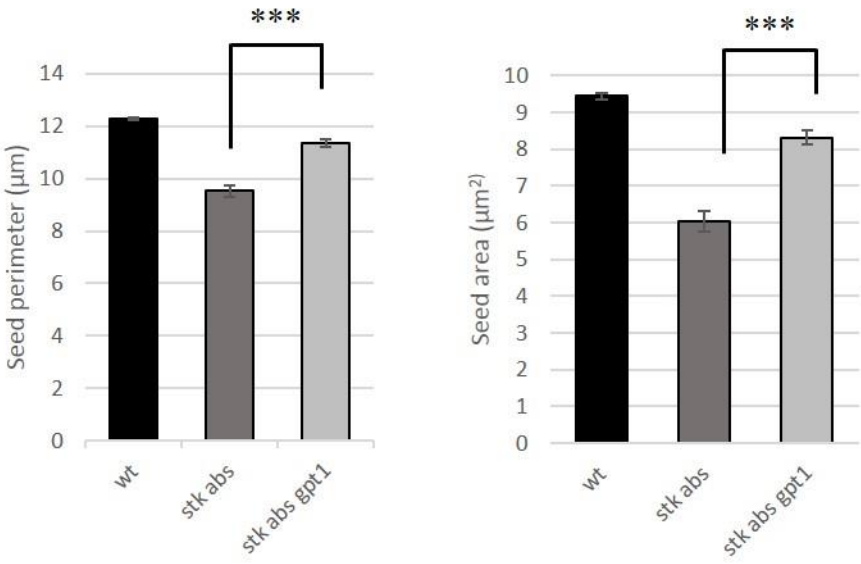


Fig. 8: Seed perimeter (A) and seed area (B) are rescued in the *stk abs gpt1-3* triple mutant.

The rescue of seed dimension was confirmed measuring tegument cells length with confocal imaging. The experiment was repeated using the same instruction: the triple mutant was sampled right before the fertilization and at 2 DAP, being hand-pollinated with wt pollen.

Triple mutant mature ovules have the same number of cell of a wt ovule; similarly, cell length was almost the same. On the other hand, at 2 DAP, cell length appear to be decreased in the triple mutant compared to the wild type.

However, even though still smaller compared to the wild type, the triple mutant present tegument cells significantly longer than the *stk abs* double mutant does (**Fig 9** & **Fig. 10**).

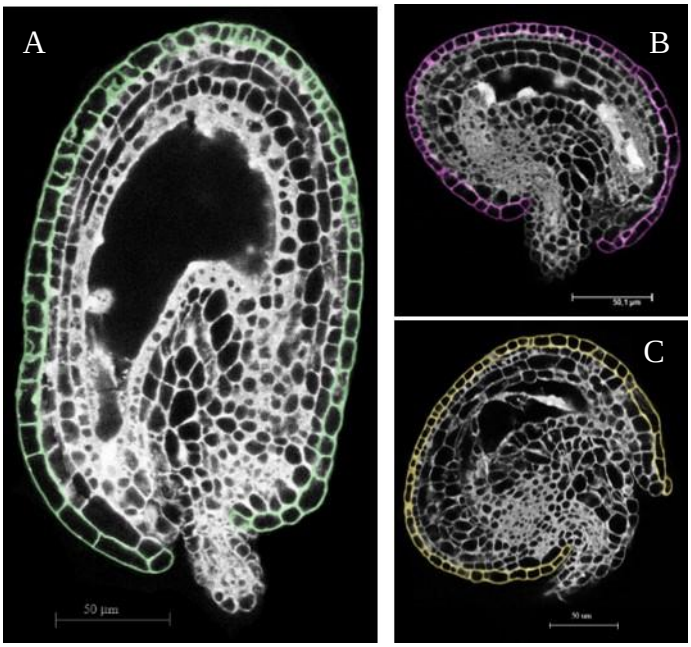


Fig. 9: Confocal images of a wt (A), *stk abs* (B) and *stk abs gpt1* (C) mutant at 2 DAP. Scale bar = 50 µm.

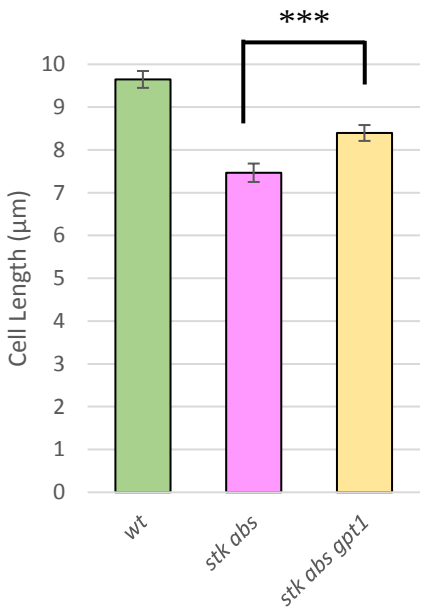


Fig. 10: The *stk abs gpt1-3* seed coat cells, even though still smaller compared to the wt, are significantly longer than the *stk abs* double mutant at 2 DAP.

The fertilization rate was checked with marker lines. To evaluate the fertilization of the central cell, the *pMINI3*-GUS reporter lines was used. The signal is absent before the fertilization and became visible in the endosperm

nuclei starting from 2 DAP (*Luo et al., 2005*). In the double mutant, only 45% of seeds showed the GUS signal in the endosperm, as already published (*Mizzotti et al., 2012*). In the triple mutant, about 900 seeds were analyzed; the number of fertilized seeds rises at around 60% (**Fig. 11**).

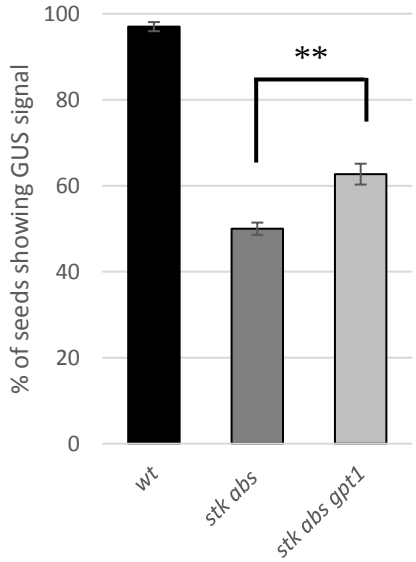


Fig. 11: The fertilization of the central cell occurs in 45% of seeds in the *stk abs* double mutant, whereas in the triple mutant around 60% of seeds show the GUS signal.

Similarly, the *pWOX9*::WOX9-YFP reporter line enable us to evaluate the fusion between the male and the female gametes and the consequent formation of the embryo.

As said before, only 20% of *stk abs* ovules are fertilized, whereas in the triple mutant the fluorescent signal is detected in around 40% of the 600 analyzed seeds (**Fig. 12**).

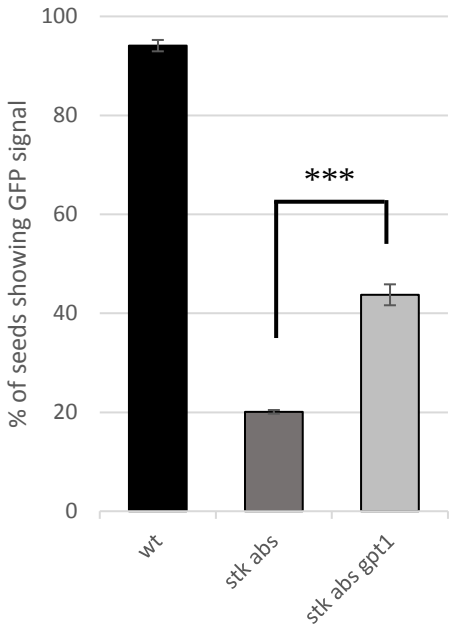


Fig. 12: The fluorescent signal, indicating the zygote formation, is detected in 20% of seeds in the *stk abs* double mutant. The triple mutant, instead, show a fluorescent signal in around 45% of seeds.

Conclusion and future perspectives

Fertilization in *A. thaliana* is a very complicated process in which several decision must been taken correctly. The product of the fertilization is formed by three main tissues, genetically and functionally different.

From the ovules integuments, a seed coat is formed; it is a five layers tissues whose main function is to protect the embryo from mechanical and chemical damages.

Even though extensively studied, more research are needed to unravel completely this multistep fertilization pathway. The work hereby presented represent an important advancement in the understanding the players involved.

Here we propose starch as a physic hindrance to the fusion between gametes. The double mutant used in this work presents defects in fertilization and a

huge accumulation of starch in the female gametophyte surrounding the central cell.

We created a triple mutant crossing *stk abs* with the *gpt1* mutant, known in literature for the lack of the carbohydrate in mature ovules.

A rescue in seed size and in the fertilization is clearly achieved, as confirmed with morphological analysis and by the use of marker lines. These results lead us to speculate that the excess of starch in *stk abs* double mutant is physically obstructing the fertilization.

However, since a complete rescue is not observe, it is highly probable that other reasons lie behind the reduced fertility; the lack of the endothelium could be one of them.

The endothelium is the innermost layer of the seed coat and it is at direct contact with both the sporophytic tissues and the product of fertilization. For this reason, it is crucial for the crosstalk between endosperm and seed coat.

It has been demonstrated that *stk abs* double mutant lacks of the endothelium (*Mizzotti et al., 2012*).

A recent article argues against this interpretation (*Coen et al., 2017*). Technical imaging reconstructed the process of integuments development. The ii1' layer forms at the very end of ovule development; it derives from periclinal division of the ii1, the innermost layer of the integuments.

Hence, as claimed in this work, the *stk abs* double mutant lacks of the ii1' layer, in contrast with the previous belief (*Mizzotti et al. 2012*).

However, the morphological analysis were not corroborated with the use of marker lines, which could have effectively confirmed the identity of the layers.

Moreover, the endothelium is a differentiated tissue, which acquires its identity consequently to the fertilization. PA synthesis and accumulation occur specifically in the specialized cells of the endothelium; these compounds are responsible for the brownish colour of the seed coat.

In the *stk abs* double mutant, no tissue with such identity can be isolated; indeed, those seeds do not accumulate PA and they appear paler than the wild type.

In order to understand a possible involvement of the endothelium in the signalling pathway, we deeper characterized *stk abs* double mutant seed coat cells.

The results obtained confirmed the visual analysis of *stk abs* seeds. They are smaller compared to the wild type because of a reduced length of seed coat cells.

The same conclusion can be stated for *stk* mutant seeds: they are significantly smaller compared to wild type seeds.

The choice of measuring those parameters at 0 DAP has been made for a precise reason; we wanted to exclude that the cause of the diminished seed size was already determined in ovules and demonstrate that the decrease in size is due exclusively to mistakes in the communication between sporophytic and gametophytic tissues after the fertilization. Indeed, at anthesis, all the mutants analysed contains ovules with the same dimensions.

Moreover, as already stated before, seed coat cell possess a totally maternal genome since they are the results of the differentiation of the ovules integuments. For this reason, in order to avoid the effect of the mutation, we decide to hand-pollinate the mutant under investigation with wild type pollen.

Materials and Methods

Plants material and growth condition

Plants were grown in long day condition (16 hours light and 8 hours dark) at 22 °C under controlled condition in the greenhouse. All the lines used were in the Col-0 ecotype.

The *stk-2* allele (accession number At4g09960) contains a transposon element of 74 nucleotides near the splice site of the 3rd intron (*Pinyopich et al., 2003*).

The *abs-6* allele (accession number At5g23260) is a tDNA insertion line; the insertion is localized in the first intron, at 51 nucleotides from the second exon (*De folter et al., 2006*).

The *gpt1-3* allele was kindly given to us by Ueli Grossniklaus (Institute of Plant and Microbial Biology & Zurich-Basel Plant Science Center, University of Zurich, Switzerland); it contains a tDNA insertion at 19 upstream the start codon.

PCR- based genotyping

DNA extraction from leaves was performed with a manual procedure consisting in isopropanol precipitation. For the isolation of the *stk* mutant the oligonucleotides AtP 204 (5'-GCTTGTTCTGATAGCACCAACACTAGCA-3') and AtP 561 (5'-GGAATCAAAGAGTCTCCCATCAG-3') were used.

For the identification of the *ABS* wt allele the oligonucleotides AtP_2456 (5'-TTTCTCCATATTGACCATCATACTCATTG-3') and AtP_2455 (5'-TTAATCATTCTGGGCCGTTGGATCGTTTT-3') were used. For the identification of the mutant allele, the oligonucleotides AtP_2456 and AtP_4756 (5'-ATTTTGCCGATTTCGGAAC-3') were used.

For the identification of the *GPT1* wt allele the oligonucleotides AtP_6348 (5'-TGTCTTAATTACATTTCAAGA-3') and AtP_6349 (5'-ATCATCACCGATCGGATGAG-3') were used. For the identification of the mutant alleles, the oligonucleotides AtP_6348 and AtP_4756 were used.

Confocal analysis

For the integuments morphological analysis, plants were emasculated and hand-pollinated with wild type pollen. Pistils were fixed at anthesis, at 2 DAP and 6 DAP as described by *Christensen et al. (1997).* The samples were imaged using the rhodamine channel on a Leica SP5 confocal laser scanning microscope.

Cytological analysis

For the cytological analysis of mature ovules, Lugol staining was performed. Pistil were sampled and fixed in a solution of acetic acid and absolute ethanol (1:3), cleared with 8N sodium hydroxide and labelled with Lugol's solution. Sample were imaged using a Zeiss Axi-overt 200 DIC microscope.

Central cell fertilization

The analysis of the central cell fertilization was performed pollinating the plants with the *pMINI3*::GUS marker line (*Luo et al., 2005*). Plants were emasculated and hand-pollinated, then sampled at 2 DAP. The GUS staining was performed as described previously by *Liljegren et al., 2000.* Sample were fixed in a clearing solution (acetic acid: ethanol 3:1) and observed using a Zeiss Axiophot D1 microscope equipped with DIC optics.

Egg cell fertilization

The analysis of the egg cell fertilization was performed pollinating the plants with the *pWOX9*::WOX9-YFP marker line (*Wua et al., 2007*). Plants were

emasculated and hand-pollinated, then sampled at 48h after impollination. The samples were observed using a Zeiss Axiophot D1 microscope equipped with DIC optics.

References

Andriotis, V. M. E., Pike, M. J., Kular, B., Rawsthorne, S., & Smith, A. M. (2010). Starch turnover in developing oilseed embryos. New Phytologist, 187(3), 791–804. http://doi.org/10.1111/j.1469-8137.2010.03311.x

Balanzà, V., Roig-Villanova, I., Di Marzo, M., Masiero, S., & Colombo, L. (2016). Seed abscission and fruit dehiscence required for seed dispersal rely on similar genetic networks. Development, 143(18), 3372–3381. http://doi.org/10.1242/dev.135202

Becker, A., Kaufmann, K., Freialdenhoven, A., Vincent, C., Li, M. A., Saedler, H., & Theissen, G. (2001). A novel MADS-box gene subfamily with a sister-group relationship to class B floral homeotic genes.

Christensen, C.A., King, E.J., Jordan, J.R. and Drews, G.N. (1997) Megagametogenesis in Arabidopsis wild type and the Gf mutant. Sex. Plant Reprod. 10, 49–64.

Coen, O., Fiume, E., Xu, W., De Vos, D., Lu, J., Pechoux, C., Lepiniec L., Magnani, E. (2017). Developmental patterning of the sub-epidermal integument cell layer in Arabidopsis seeds. Development, 144(8), 1490–1497. http://doi.org/10.1242/dev.146274

Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., & Lepiniec, L. (2003). Proanthocyanidin-accumulating cells in Arabidopsis testa: regulation of differentiation and role in seed development. The Plant Cell, 15(November), 2514–2531. http://doi.org/10.1105/tpc.014043

De Folter, S., Shchennikova, A.V., Franken, J., Busscher, M., Baskar, R., Grossniklaus, U., Angenent, G.C. and Immink, R.G. (2006) A Bsister MADS-box gene involved in ovule and seed development in petunia and Arabidopsis. Plant J. 47, 934–946.

Favaro, R., & Pinyopich, A. (2003). MADS-box protein complexes control carpel and ovule development in Arabidopsis. The Plant Cell …, 15(November), 2603–2611. http://doi.org/10.1105/tpc.015123.2

Haughn, G., & Chaudhury, A. (2005). Genetic analysis of seed coat development in Arabidopsis. Trends in Plant Science, 10(10), 472–477. http://doi.org/10.1016/j.tplants.2005.08.005

Hedhly, A., Vogler, H., Schmid, M. W., Pazmino, D., Gagliardini, V., Santelia, D., & Grossniklaus, U. (2016). Starch Turnover and Metabolism during Flower and Early Embryo Development. Plant Physiology, 172(4), 2388–2402. http://doi.org/10.1104/pp.16.00916

Kaufmann, K., Anfang, N., Saedler, H., & Theissen, G. (2005). Mutant analysis, protein-protein interactions and subcellular localization of the Arabidopsis Bsister (ABS) protein. Molecular Genetics and Genomics, 274(2), 103–118. http://doi.org/10.1007/s00438-005-0010-y

Liljegren, S., Ditta, G., Eshed, Y., Savidge, B., Bowman, J. and Yanofsky, M. (2000) SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. Nature, 404, 766–769.

Luo, M., Dennis, E. S., Berger, F., Peacock, W. J., & Chaudhury, A. (2005). MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America, 102(48), 17531–17536. http://doi.org/10.1073/pnas.0508418102

Mizzotti, C., Mendes, M. A., Caporali, E., Schnittger, A., Kater, M. M., Battaglia, R., & Colombo, L. (2012). The MADS box genes SEEDSTICK and ARABIDOPSIS Bsister play a maternal role in fertilization and seed development. Plant Journal, 70(3), 409–420. http://doi.org/10.1111/j.1365-313X.2011.04878.x

Nesi, N., Debeaujon, I., Jond, C., Stewart, A. J., Jenkins, G. I., Caboche, M., & Lepiniec, L. (2002). The TRANSPARENT TESTA16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. The Plant Cell, 14(10), 2463–2479. http://doi.org/10.1105/tpc.004127

Niewiadomski, P., Knappe, S., Geimer, S., Fischer, K., Schulz, B., Unte, U. S., Rosso, M. G., Ache, P., Flugge,U., & Schneider, A. (2005). The Arabidopsis plastidic glucose 6-phosphate/phosphate translocator GPT1 is essential for pollen maturation and embryo sac development. The Plant Cell, 17(3), 760–775. http://doi.org/10.1105/tpc.104.029124

Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E., & Yanofsky, M. F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature, 424(6944), 85–88. http://doi.org/10.1038/nature01741

Windsor, J. B., Symonds, V. V., Mendenhall, J., & Lloyd, A. M. (2000). Arabidopsis seed coat development: Morphological differentiation of the outer integument. Plant Journal, 22(6), 483–493. http://doi.org/10.1046/j.1365-313X.2000.00756.x

Wua X., Chorya J., Weigel D. (2007). Combinations of WOX activities regulate tissue proliferation during Arabidopsis embryonic development. Developmental Biology, Volume 309, Issue 2,

PART III

Hormones might induce seed coat differentiation in unfertilized ovules

Phytohormones have been demonstrated to be involved in plant growth and development, including fruit initiation (*Fuentes et al., 2012*) and autonomous seed development (*Figueiredo et al., 2015*). Indeed, exogenous applications of auxin, gibberellin and cytokinin trigger partenocarpy, fruit growth in absence of fertilization (*Fuentes et al., 2012*).

When auxin is supplied to emasculated pistil, central cell replication and seed coat development are induced (*Figueiredo et al., 2015*); similarly, exogenous gibberellins supply induce fruit development in absence of fertilization.

The PcG complexes negatively control these two developmental processes; particularly the FIS complex represses endosperm and seed coat development in absence of fertilization (*Köhler and Makarevich, 2006*).

Given that regulatory pathways are conserved between different organs, we wondered if there was a link between gibberellins and integuments growth during early stage of development. For this reason, we decided to treat unfertilized ovules with GA.

To assess the right experimental procedure and to prove the effectiveness of our treating conditions, we performed a control treatment on emasculated wt carpel and subsequently evaluated fruit elongation in absence of fertilization.

As expected, treatments with a solution of gibberellins 10mM on unfertilized carpels lead to fruit elongation, whereas the control carpels do not elongate in absence of fertilization (**Fig 1**).

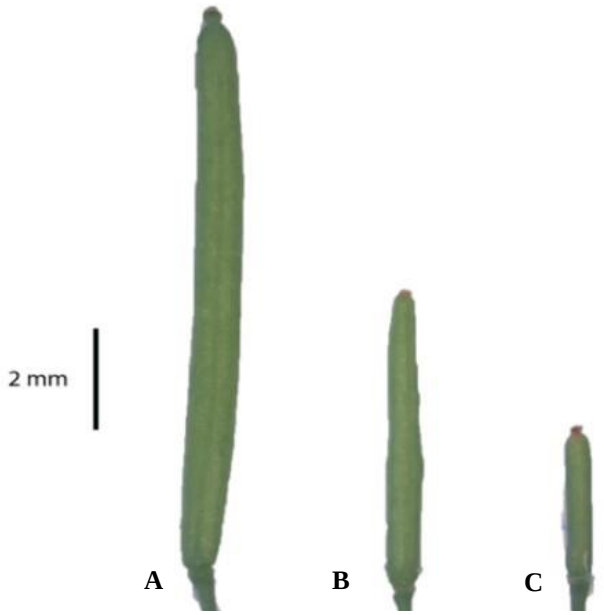


Fig. 1 Stereoscope image of a self-fertilized silique (A), an emasculated carpel treated with GA3 10 mM (B) and an emasculated carpel treated with the control solution (C).

We then evaluated the effect of this exogenous application on mature ovules.

Around 900 ovules were isolated and treated with the hormones solution and the same amount of ovules was treated with the control solution. They were harvested at 3 DAE and cleared in order to evaluate the effect of hormones at teguments level.

Those treatments provoke the differentiation of ovule integuments in seed teguments in around 20% of the treated ovules.

The seed coat differentiation was not accompanied by endosperm development; indeed, a central cell was clearly visible in the above mentioned ovules (**Fig. 2**).

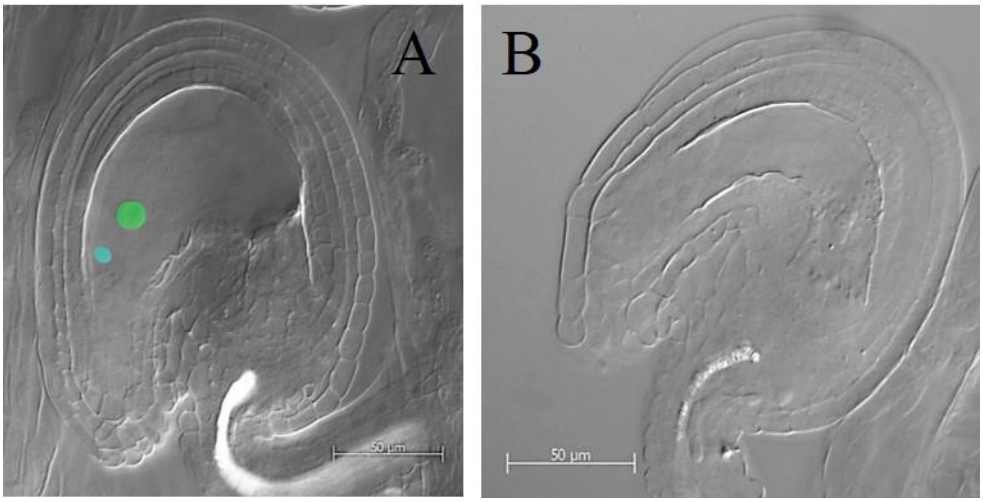


Fig. 2 Images of a cleared unfertilized ovule treated with GA (A) and cleared ovule treated with a mock solution (B). The central cell and the egg cell are colored in green and light blue, respectively.

To confirm the seed coat identity in treated ovules, vanillin staining was performed. Vanillin staining marks proanthocyanidins, which are accumulated exclusively in specialized cells of endothelium.

As expected, in the treated sample around 20% of ovules presented the typical staining, in accordance with the previous experiment (**Fig. 3**).

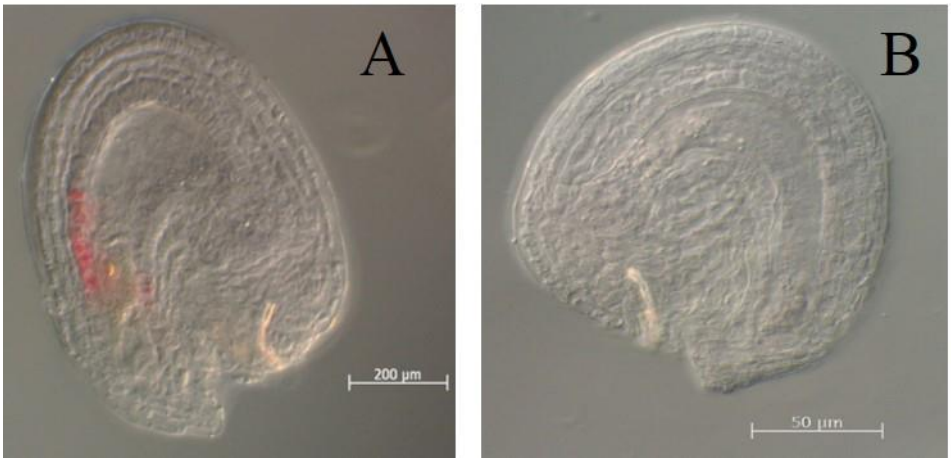


Fig. 3 Vanillin staining of unfertilized ovules treated with GA 10 mM (A) and treated with mock solution (B). The treated ovules showed the typical red coloration, indicating the presence of proanthocyanidins, which accumulate in the differentiated tissues.

Role of STK in the signaling pathway

Gametophytic and sporophytic tissues communicate to coordinate their growth after the fertilization through a complex pathway which involved several factors the MADS box AGL62 (*Roszak and Kohler, 2011; Figueiredo et al. 2015*), hormones (*Figueiredo et al., 2016*) and PcG proteins are some of the known players (*Spillane et al., 2000; Köhler et al., 2003a; Wang et al., 2006*; *Köhler and Makarevich, 2006*).

It has been shown that AGL62 is necessary for the transportation of auxin from the endosperm to the seed coat soon after the fusion between the male and female gametes.

In the seed coats, auxin and gibberellins act together, in order to remove the repression exerted by the PcG proteins, in particular the FIS2 complexes (*Figueiredo et al., 2016*).

ChIP seq analysis previously performed showed that two members of the FIS2 complexes, CLF and SWN, are direct target of STK (*Mendes and Ezquer, unpublished*).

For this reason, we hypothesized a possible involvement of this transcription factor in the signaling pathway.

At first, we analyzed the expression profile of the PcG genes in *stk* and *stk abs* background and we found them up-regulated compared to the wt (**Fig. 4**).

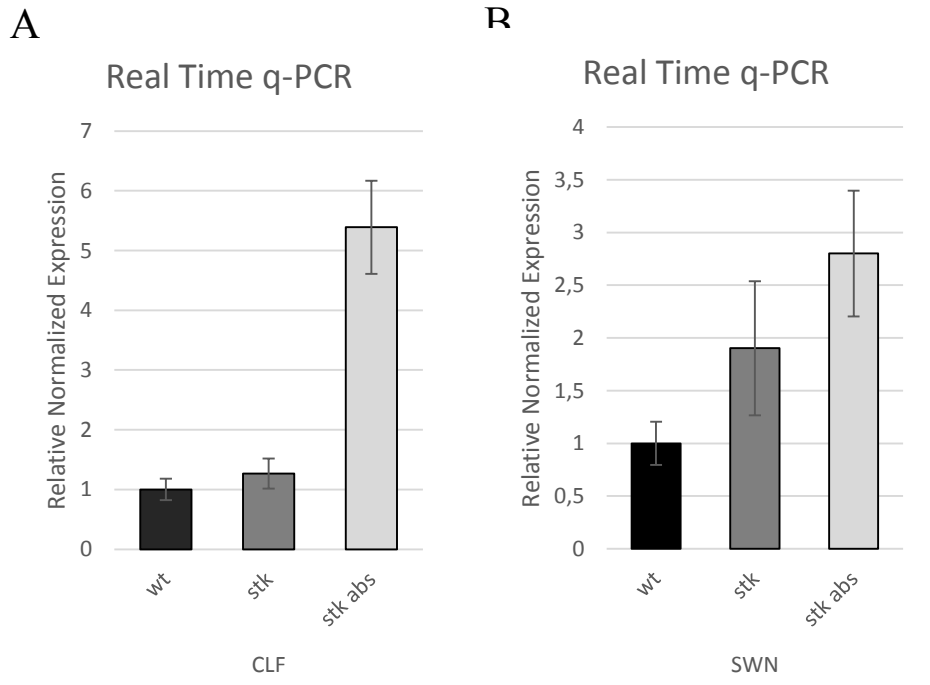


Fig. 4 *CLF* (**A**) and *SWN* (**B**) expression profile in *stk* and *stk abs* mutant background. They appear to be heavily up-regulated in the double mutant.

This finding is in accordance with the phenotype affecting seed size in both mutants. Indeed, *stk* and *stk abs* seeds are smaller compared to the wild type and one explanation could be the PolyComb proteins still exert their repressive function on seed coat differentiation after the fertilization.

To deeper investigate the role of STK in the signaling pathway, we have introduced the 35S::*STK* transgene and the p*STK*::STK-GFP reporter construct in the *agl62*/+ mutant.

When the transgene 35S::STK was introduced into *agl62/+*, it partially restored the seed coat phenotype.

We analysed around 500 heterozygous *agl62/+* seeds at 3 DAP.

As expected, around 20% of them showed the typical *agl62* mutant phenotype, i.e. collapsed seed with no seed coat (**Fig. 5A**), and a small percentage (1.5% of the 500 analysed) arrested their development later with a formed seed coat (**Fig. 5B**).

When the MADS box transcription factors STK is overexpressed in the heterozygous mutant background, the number of *agl62*-like seeds (**Fig. 5C**) strongly decreased (0.8% of n= 550) in favour of small seeds with a developed seed coat (23% of n=550) (**Fig. 5D**).

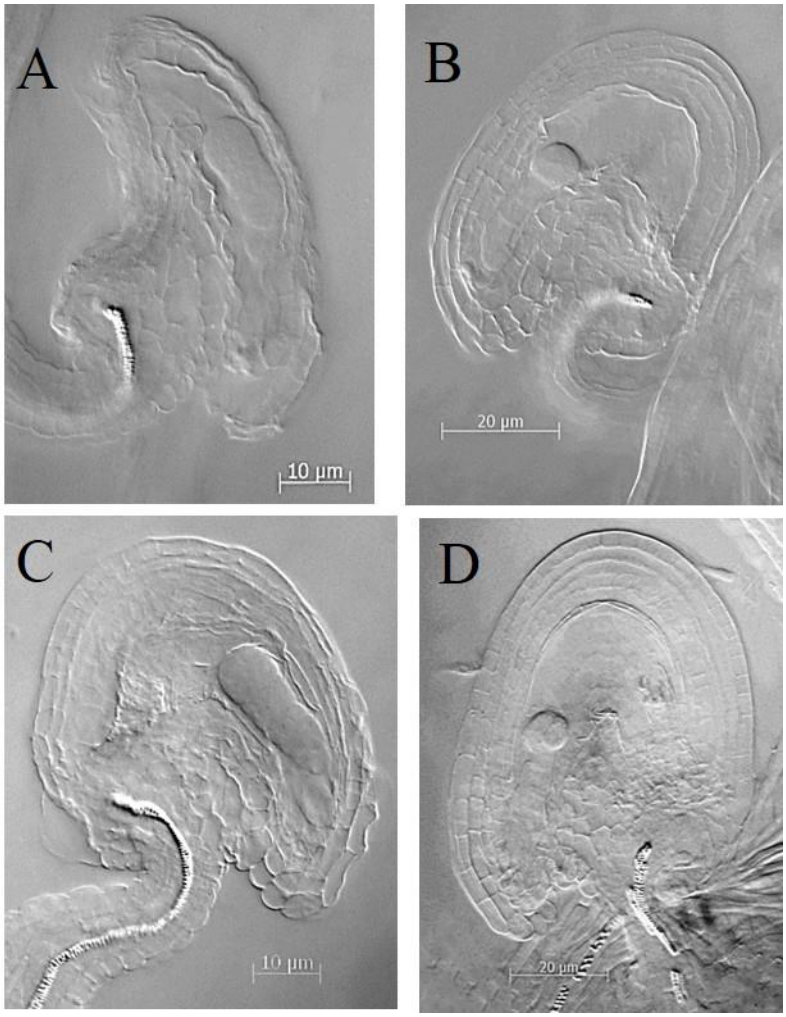


Fig. 5 The *agl62* mutant seeds displaying the typical phenotype; they indeed collapsed and no seed coat is differentiated (A). The penetrance of the *agl62* is incomplete, a small percentage arrest their development later and they present a differentiated seed coat (B). The35S::STK transgene partially restore the *agl62* phenotype: a small percentage of the homozygous seed still present the typical phenotype (C) but the majority present a well-developed seed coat (D).

We have also introduced the p*STK::*STK-GFP reporter construct in the heterozygous mutant in order to evaluate a regulatory role of AGL62 on STK expression: no changes were observed.

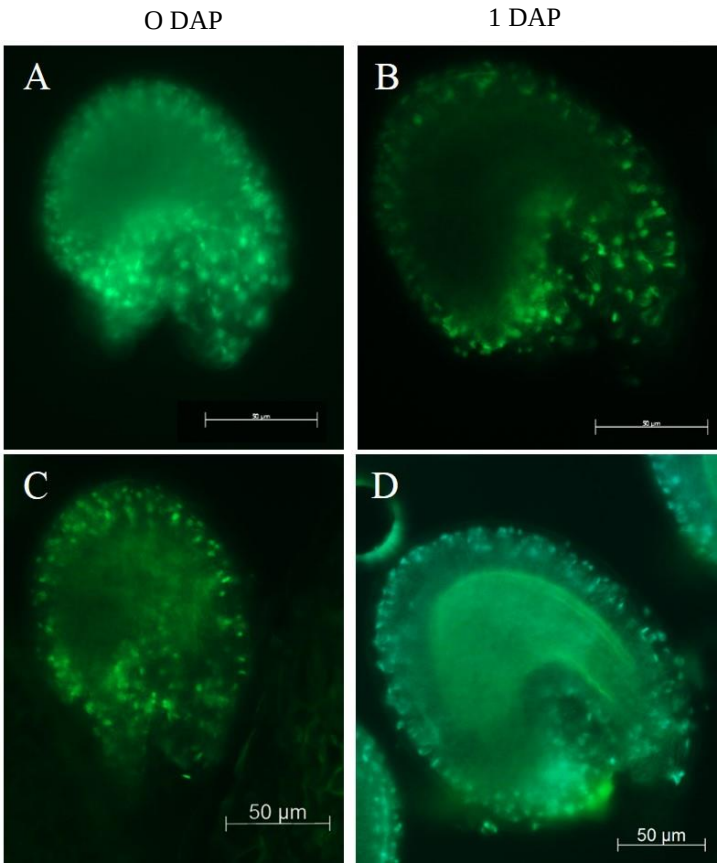


Fig. 6 STK expression in wt (A, B) and *agl62* (C,D) at 0 and 1 DAP. No differences were noted in the fluorescent signal.

Taken together, these results suggest us that STK could be necessary for the perception of the signal triggered by AGL62, but not sufficient to complement completely the *agl62* phenotype.

We hypothesized the necessity of a second factor induced by auxin signaling pathway in the teguments that cooperate with STK in the repression of the PcG proteins after fertilization.

Since auxin has been demonstrated to be the signal that removes the PcG repression (*Figueiredo et al., 2016*), we analyzed the expression profiles of the Auxin Response Factor genes in seeds. The ARF genes are transcription factors which are involved in the regulation of target genes in response to auxin (*Guilfoyle and Hagen, 2007*).

ARF1 was chosen as a good candidate: it resulted as one of the most expressed ARF genes in a wt seed soon after the fertilization (*Kasahara et el., 2016*).

BiFC analysis were done in order to confirm the protein interaction but no YFP signal was detected in tobacco leaves, meaning that this two proteins are unable to interact (**Fig. 7**).

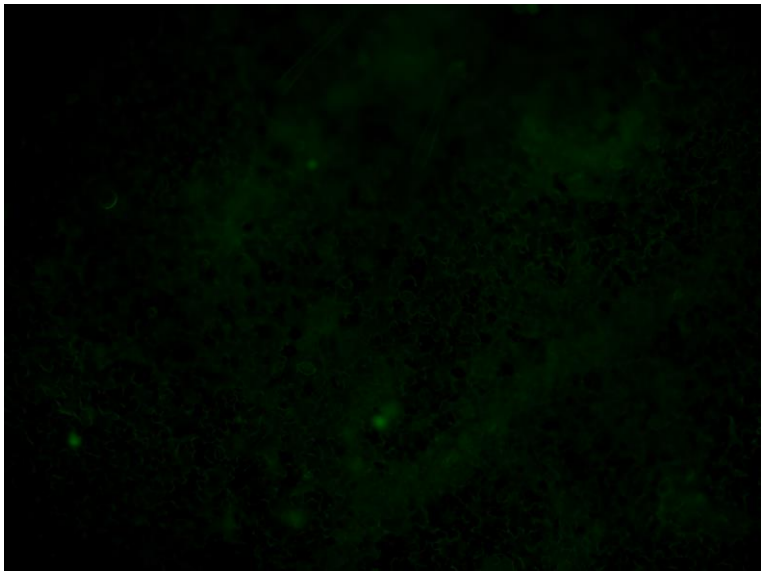


Fig. 7: No YFP signal is visible

References

Figueiredo, D. D., Batista, R. A., Roszak, P. J., Hennig, L., & Köhler, C. (2016). Auxin production in the endosperm drives seed coat development in Arabidopsis. eLife, 5(NOVEMBER2016), 1–23. http://doi.org/10.7554/eLife.20542

Figueiredo, D. D., Batista, R. A., Roszak, P. J., & Köhler, C. (2015). Auxin production couples endosperm development to fertilization. Nature Plants, 1(12), 15184. http://doi.org/10.1038/nplants.2015.184

Fuentes, S., Ljung, K., Sorefan, K., Alvey, E., Harberd, N. P., & Østergaard, L. (2012). Fruit Growth in Arabidopsis Occurs via DELLA-Dependent and DELLA-Independent Gibberellin Responses. The Plant Cell, 24(10), 3982–3996. http://doi.org/10.1105/tpc.112.103192

Guilfoyle, T. J., & Hagen, G. (2007). Auxin response factors. Current Opinion in Plant Biology, 10(5), 453–460. http://doi.org/10.1016/j.pbi.2007.08.014

Kasahara, R.D., Notaguchi, M., Nagahara, S., Suzuki, T., Susaki, D., Honma, Y., Maruyama, D., & Higashiyama T. (2016) Pollen tube contents initiate ovule enlargement and enhance seed coat development without fertilization Sci Adv. 2016 Oct; 2(10): e1600554.

Kohler, C., & Makarevich, G. (2006). Epigenetic mechanisms governing seed development in plants. EMBO Rep, 7(12), 1223–1227. http://doi.org/10.1038/sj.embor.7400854

Köhler, C., Hennig, L., Spillane, C., Pien, S., Gruissem, W., & Grossniklaus, U. (2003). The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. Genes and Development, 17(12), 1540–1553. http://doi.org/10.1101/gad.257403

Roszak, P., & Köhler, C. (2011). Polycomb group proteins are required to couple seed coat initiation to fertilization. Proceedings of the National Academy of Sciences of the United States of America, 108(51), 20826–20831. http://doi.org/10.1073/pnas.1117111108

Spillane, C., MacDougall, C., Stock, C., Köhler, C., Vielle-Calzada, J. P., Nunes, S. M., Grossniklaus, U., Goodrich, J. (2000). Interaction of the Arabidopsis Polycomb group proteins FIE and MEA mediates their common phenotypes. Current Biology, 10(23), 1535–1538. http://doi.org/10.1016/S0960-9822(00)00839-3

Wang, D., Tyson, M. D., Jackson, S. S., & Yadegari, R. (2006). Partially redundant functions of two SET-domain polycomb-group proteins in controlling initiation of seed development in Arabidopsis. Proc Natl Acad Sci U S A, 103(35), 13244–13249. http://doi.org/10.1073/pnas.0605551103