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Rita Adriano Batista

Dissertação

Mestrado em Biologia Molecular e Genética

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To all, a big thank you for everything!

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
CaMV35S	Promoter of the 35S RNA from Cauliflower Mosaic Virus
4-Cl-IAA	4-Chloroindole-3-acetic acid
ABCB	ATP-BINDING CASSETE B
AFB	AUXIN SIGNALING F-BOX
AGL62	AGAMOUS –LIKE 62
APEX	(3-Aminopropyl)triethoxysilane
ARF	AUXIN RESPONSE FACTOR
Aux/IAA	Auxin/INDOLE-3-ACETIC ACID
AUX1	AUXIN RESISTANT1
AuxRE	Auxin Response Element
bps	Base pairs
CLF	CURLY LEAF
Col-0	*Arabidopsis thaliana* ecotype Columbia
DAE	Days after emasculation
DAP	Days after pollination
DAPI	4',6-Diamidino-2-phenylindole
ddH_2O	Double deionized water
DIC	Differential interference contrast
Dicamba	3,6-Dichloro-2-methoxybenzoic acid
DR5	Synthetic auxin response element
EDTA	Ethylenediaminetetraacetic acid
EMF2	EMBRYONIC FLOWER2
EMS	Ethyl methanesulfonate
FCS	Fetal calf serum
FIE	FERTILIZATION INDEPENDENT ENDOSPERM
FIS2	FERTILIZATION INDEPENDENT SEED2

FLC	FLOWERING LOCUS C
GFP	Green Fluorescent Protein
GH3	GRETCHEN HAGEN3
GUS	β-glucuronidase
H3K27me3	Trimethylation of Lysine 27 on Histone 3
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
IAOx	Indole-3-acetaldoxime
IPA	Indole-3-pyruvic acid
KLU	CYTOCHROME P450 KLUH
LAX	LIKE AUX
L*er*	*Arabidopsis thaliana* ecotype Landsberg *erecta*
MADS	Acronym derived from four members of the family: MCM1, AGAMOUS, DEFICIENS and SRF
MEA	MEDEA
MES	4-Morpholineethanesulfonic acid
MINI3	MINISEED3
MS-medium	Murashige and Skoog medium
MSI1	MULTICOPY SUPRESSOR OF IRA1
NAA	1-Naphthaleneacetic acid
NLS	Nuclear localization signal
NPA	1-N-Naphthylphthalamic acid
PA	Protoanthocyanidins
PAA	2-Phenylacetic acid
PAT	Polar auxin transport
PBS	Phosphate buffer saline
PcG	Polycomb group
Pcl-PRC	Polycomb-like PRC

PCR	Polymerase Chain Reaction
PEG	Paternally expressed gene
PGP	P-GLYCOPROTEIN
PHD	Plant Homeo Domain
PHE1	PHERES1
PI	Propidium iodide
PIN	PIN-FORMED
PP2A	PROTEIN PHOSPHATASE 2A
PRC	Polycomb Repressive Complex
pro	Promoter
RT-qPCR	Reverse-transcription quantitative (real-time) PCR
SCF	Skp, Cullin, F-box containing complex
SHB1	SHORT HYPOCOTYL UNDER BLUE1
SWN	SWINGER
TAA1	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS
TAR	TRYPTOPHAN AMINOTRANSFERASE RELATED
TIR1	TRANSPORT INHIBITOR RESPONSE1
Trp	Tryptophan
TTG2	TRANSPARENT TESTA GLABRA2
VRN2	VERNALIZATION2
WT	Wild-type
YUC	YUCCA flavin monooxygenase

Abstract

Seed formation in *Arabidopsis thaliana* requires a coordinated development between embryo, endosperm and seed coat. While the first two structures are originated by a simultaneous fertilization event of egg and central cell, the seed coat is solely of sporophytic origin. Still, initiation of its development is triggered by the presence of the sexual endosperm. In fact, the type I MADS-box transcription factor, AGL62 is necessary to form an endosperm-derived signal that upon fertilization, triggers seed coat development. The nature of such signal is however still unknown. Symplastic connections between endosperm and seed coat are not known to exist, therefore phytohormones, due to their ability to actively cross cell membranes, are good candidates to be involved in this signaling process. Molecular evidence, such as gene expression studies, suggests that auxin is involved in seed coat development. In this study the role of this phytohormone in seed development, and more particularly in seed coat initiation were investigated.

Reporter gene analysis revealed that auxin is present in all tissues of the ovule and seed throughout their development, and suggest that a fertilization-dependent increase in biosynthetic activity may be the initial drive for seed development. Additionally, it was found that auxin has the ability to induce autonomous seed formation, bypassing the developmental repression exerted by PcG proteins, in the absence of fertilization. Also, auxin partially rescued the phenotype of the *agl62* mutant, which fails to develop a seed coat. Moreover, the results obtained here show that auxin is essential for correct cell specification and positioning in the embryo sac. Altogether, these results indicate that auxin has a predominant role in ovule and seed development and that this hormone seems to be involved in the pathway that leads to seed coat formation, most likely not as an exclusive participant.

Keywords

Auxin; seed coat; endosperm; female gametophyte; PcG proteins; AGL62; *Arabidopsis thaliana*

Resumo

Durante o desenvolvimento das plantas observa-se a alternância entre uma fase esporofítica diplóide e uma fase gametofítica haplóide. Em angiospérmicas, ambos os gametófitos unissexuais são constituídos por um pequeno conjunto de células que se encontram envolvidos pela flor. O gametófito feminino forma-se em duas fases distintas: megaesporogénese e megagametogénese e após o desenvolvimento estar completo, o óvulo possui um conjunto de células gametofíticas envolvidas pelo tecido maternal esporofítico – o integumento. A composição final do gametófito feminino inclui uma oosfera, uma célula central formada a partir da fusão dos dois núcleos polares, duas sinérgides e duas antípodas. Na ausência de fertilização, proteínas PcG reprimem o desenvolvimento da semente. Estas proteínas estão organizadas em complexos que inibem a transcrição de vários *loci* através da sua metilação na lisina 27 da histona 3 (H3K27) e cada complexo actua em tecidos específicos durante o desenvolvimento. Assim, no óvulo, o complexo FIS (FERTILIZATION INDEPENDENT SEED) inibe a divisão autónoma da célula central, enquanto que os complexos VRN (VERNALIZATION) e EMF (EMBRYONIC FLOWER) inibem o desenvolvimento do invólucro da semente - o tegumento. A actividade destas proteínas permite portanto que a transição de óvulo para semente aconteça apenas mediante a fertilização.

A fertilização dá-se quando os dois gâmetas masculinos se fundem com a oosfera e a célula central: a oosfera haplóide origina um embrião diplóide, enquanto que a célula central homodiplóide origina o endosperma triplóide. O crescimento simultâneo destas estruturas é essencial já que, apesar de apenas o embrião contribuir para estabelecimento da nova geração, o endosperma é indispensável para nutrir o embrião, assegurando que este possa completar a sua maturação. Para além do embrião e do endosperma, o tegumento da semente, originado a partir do integumento materno, é também essencial, já que mantém a integridade da semente durante o desenvolvimento, protegendo fisicamente os seus conteúdos. Adicionalmente, é também importante para promover a dormência e permitir a dispersão das sementes. Apesar de a fertilização apenas iniciar directamente o desenvolvimento do embrião e do endosperma, o tegumento começa também o seu desenvolvimento após a fertilização, desenvolvimento esse que é coordenado com os restantes componentes da semente. Isto sugere que exista um mecanismo de comunicação entre os tecidos gametofíticos e o tegumento esporofítico. De facto, o uso de mutantes de *Arabidopsis* que apenas produzem um gâmeta masculino – fertilizando unicamente a oosfera ou a célula central – demonstrou que o endosperma é necessário e suficiente para a formação do sinal que inicia o desenvolvimento do tegumento da semente. Para além disto, sabe-se que este sinal está dependente da actividade do factor de transcrição AGL62, expresso especificamente no endosperma. No entanto, a natureza deste sinal ainda é desconhecida.

A comunicação entre endosperma e tegumento parece ser restrita, dado que não existem ligações simplásticas descritas entre estes dois compartimentos da semente. Desta forma, a natureza molecular

do sinal que promove o desenvolvimento do tegumento está limitada a moléculas que consigam atravessar a membrana celular activamente, tais como hormonas. A análise de dados de expressão génica revela que genes de biossíntese de auxina estão sobre-regulados em mutantes de proteínas PcG, que produzem tegumento na ausência de fertilização. Adicionalmente, uma sub-regulação destes genes é observada no mutante *agl62*, cujas sementes não desenvolvem tegumento. Sabe-se também que a aplicação de auxina exógena induz partenocarpia em diversas espécies, incluindo *Arabidopsis,* e recorrendo a genes repórter que respondem à presença de auxina, viu-se que a concentração desta hormona é aumentada pouco após a fertilização. Todos estes dados sugerem a auxina como uma potencial candidata para o sinal que inicia o desenvolvimento do tegumento da semente. Neste estudo pretendeu-se não só melhorar o conhecimento actual sobre o papel da auxina no desenvolvimento dos óvulos e sementes de *Arabidopsis*, como também determinar se esta hormona está envolvida na via de sinalização que promove o desenvolvimento do tegumento.

Os resultados obtidos neste estudo, através de genes repórter e da imunolocalização de auxina, indicam que esta hormona está presente em óvulos, tanto nos integumentos como na célula central. Além disso, a manipulação das vias de sinalização da auxina, permitiu perceber que a presença desta hormona nos integumentos é essencial para a formação do gametófito feminino. A disrupção da sinalização, especificamente nos integumentos, originou defeitos na diferenciação e no posicionamento das células do gametófito feminino e inclusivamente, em alguns dos óvulos, a formação do gametófito não ocorreu, pelo que estas estruturas possuíam apenas os integumentos. Daqui se conclui que a auxina dos tecidos esporofíticos tem um efeito indirecto mas crucial na formação do gametófito feminino.

Após a fertilização, a expressão dos genes de síntese de auxina, *YUC10* e *YUC11,* é induzida e restrita ao endosperma. De modo concordante, a presença de auxina foi detectada no endosperma, bem como no tegumento de sementes em crescimento.

Observou-se também que a aplicação de um composto análogo à auxina, 2,4-D, em óvulos não fertilizados induz a formação de sementes autónomas. Nos óvulos tratados com este composto não só o tegumento se desenvolve, como também ocorre a divisão da célula central, formando assim endosperma assexual. Quando a aplicação deste composto é efectuada no mutante *agl62,* uma recuperação parcial do fenótipo é observada, indicando que a auxina é importante para o desenvolvimento do tegumento.

A aplicação de NPA, um inibidor do transporte polar de auxina que causa a acumulação desta hormona nos locais da sua síntese, produziu resultados semelhantes aos descritos para a aplicação de 2,4-D, o que reforça a ideia de que a presença de auxina, acima de uma determinada concentração, induz a formação de sementes autónomas. Para além disto, e em concordância com os dados obtidos com os genes repórter e a imunolocalização de auxina, o fenótipo resultante da aplicação de NPA indica também que esta hormona é produzida na célula central e nos integumentos dos óvulos não fertilizados, se bem que em menores quantidades do que após a fertilização. É também interessante

notar que a auxina tem a capacidade de ultrapassar a repressão ao desenvolvimento da semente, exercida pelas proteínas PcG.

O mutante *yucca6-2D,* que apresenta níveis aumentados de auxina nos integumentos, e o mutante para as proteínas PcG VRN e EMF, *vnr2-1 emf2-5,* apresentam fenótipos semelhantes: ambos desenvolvem um tegumento autónomo, na ausência de fertilização. A análise dos fenótipos do triplo mutante *yucca6-2D vnr2-1 emf2-5,* e dos níveis de expressão de genes seleccionados nos mutantes *yucca6-2D* e *vnr2-1 emf2-5* revelou que as proteínas PcG e a auxina têm a capacidade de regular processos semelhantes durante o desenvolvimento da semente. Contudo, nem todos os processos em que estão envolvidos serão comuns, já que diferenças na regulação da expressão de genes, bem como relações não-aditivas entre os fenótipos, são observadas nestes mutantes.

Assim, os resultados obtidos neste estudo indicam que a auxina tem um papel fundamental no desenvolvimento dos óvulos e das sementes de *Arabidopsis thaliana.* Por um lado, a presença desta hormona durante a megagametogénese é essencial para a correcta formação do gametófito feminino, por outro, o aumento da síntese de auxina após a fertilização parece ser o factor que comanda e estimula o desenvolvimento da semente, possivelmente através da modulação da actividade das proteínas PcG. O facto de a expressão dos genes de síntese de auxina ser altamente induzida após a fertilização, bem como o facto de esta hormona induzir a formação de sementes autónomas, suportam esta hipótese. Apesar de o sinal que induz o desenvolvimento dos tegumentos ainda não ter sido identificado, os resultados aqui apresentados levam a crer que a auxina tem um papel importante na activação das vias moleculares que promovem o crescimento desta estrutura, quer seja fazendo parte de um sinal complexo criado no endosperma e que se desloca para o tegumento ou, alternativamente, sendo uma consequência directa de um outro sinal distinto.

Palavras-chave

Auxina; semente; tegumento; endosperma; gametófito feminino; proteínas PcG; AGL62; *Arabidopsis thaliana*

1. Introduction

1.1. Ovule and female gametophyte development in *Arabidopsis*

During plant development an alternation between diploid sporophytic and haploid gametophytic generations occurs. In angiosperms both unisexual gametophytes comprise a small set of cells, which are surrounded by the sporophytic tissues. The male gametophyte originates from a microspore mother cell that undergoes a meiotic division to form the haploid microspores, which after two mitotic divisions form the functional male gametophyte (pollen). The female gametophyte, on the other hand, is formed from a megaspore mother cell that also undergoes a meiotic division followed by mitotic divisions, as detailed below.

The complete development of the female gametophyte can be divided in two distinct phases: megasporogenesis (**Fig. 1-1**) and megagametogenesis (**Fig. 1-2**). During megasporogenesis a finger-like protrusion, the ovule primordium, emerges from the placental tissue, where three major areas along the proximal-distal axis can be distinguished (Schneitz *et al.*, 1995): in the proximal end is the funiculus which connects the ovule to the maternal plant and allows communication between the two, followed by the chalaza, from where the integuments derive, and in the distal end, the nucellus. Within the nucellus, a subepidermal cell differentiates into a functional megaspore mother cell that will undergo meiosis and originate four haploid cells. Among these, three degenerate through programmed cell death and only one functional megaspore remains (Christensen *et al.*, 1997)

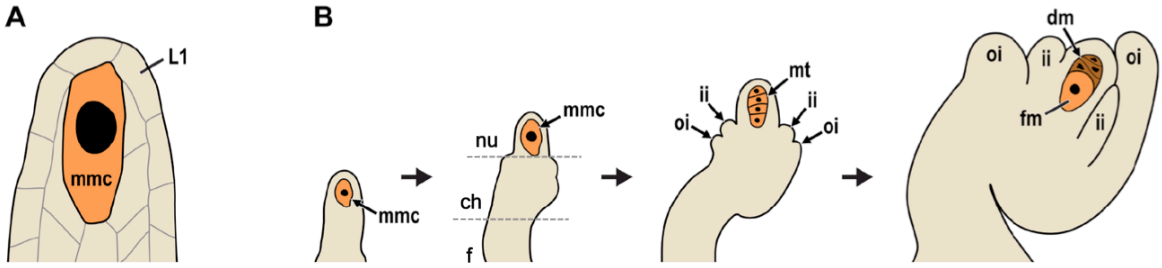

Figure 1-1 Megasporogenesis. (A) Ovule primordium with subepidermal megaspore mother cell. L1 represents the epidermal layer that will be a part of the innermost layer of the inner integument. **(B)** Megasporogenesis starts with a protrusion from the placenta. In the nucellus region the megaspore undergoes meiosis to originate a tetrad of which only the functional megaspore prevails. From the chalaza area of the ovule primordium epidermal cells divide and start formation of the inner and outer integuments. **ch**: chalaza region; **dm**: degenerated megaspores; **f**: funiculus region; **fm**: functional megaspore; **ii**: inner integument; **L1**: L1 epidermal layer; **mmc**: megaspore mother cell; **mt**: meiotic tetrad; **nu**: nucellus region; **oi**: outer integument. Adapted from Drews and Koltunow (2012).

Megagametogenesis is initiated by a succession of mitotic divisions of the functional megaspore that are not followed by cytokinesis. During the first division, a central vacuole is formed isolating the two nuclei on each pole of the coenocyte. Another two rounds of mitosis occur, leading to the formation of a total of eight nuclei. During cellularization, six of these nuclei become surrounded by cell walls whereas two of them, each on one pole, migrate to the center of the female gametophyte and fuse, giving rise to the homodiplod central cell (Drews and Koltunow, 2011). Thus, the final composition of the female

gametophyte comprises seven cells: one egg cell, one central cell, two synergid cells and three antipodal cells (Bajon *et al.*, 1999). Polarization is extremely important so that synergids, central cell and egg cell are maintained close to each other, facilitating the double fertilization event.

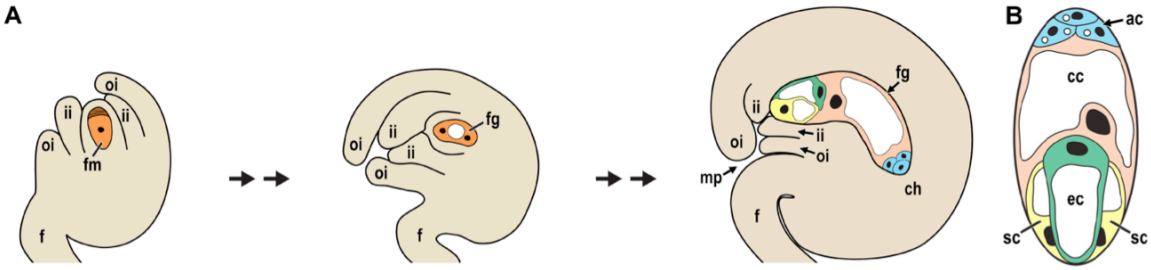

Figure 1-2 Megagametogenesis. (A) The functional megaspore divides, producing a two-nucleated coenocyte, with two nuclei at each pole, separated by a large vacuole. During cellularization differentiation of embryo sac cells occurs. Inner and outer integuments grow, surrounding the female gametophyte, though incomplete growth leaves a small opening, the micropyle. **(B)** Female gametophyte composition. Perpendicular cross-section of that in **(A)**. **ac**: antipodal cells; **cc**: central cell; **ch**: chalazal region of the ovule; **ec**: egg cell; **f**: funiculus; **fg**: female gametophyte/ embryo sac; **fm**: functional megaspore; **ii**: inner integument; **mp**: micropyle; **oi**: outer integument; **sc**: synergid cells. Adapted from Drews and Koltunow (2012).

The sporophytic integuments are derived from the chalazal zone of the ovule primordium and their development starts with the division of the epidermal cells, creating a ring-like structure that gradually envelopes the nucellus, forming the inner integument. Later, outer integument growth starts in a similar fashion, surrounding the inner integument. While the inner integument grows symmetrically, growth of the outer integument is more pronounced on the apical side, causing the ovule to be slightly bent. Two distinct cells layers form both the inner and outer integuments, and the innermost layer of the inner integument undergoes an additional periclinal division. Thus, mature ovules contain five distinguishable cell layers (Robinson-Beers *et al.*, 1992). Nevertheless, the female gametophyte is never fully enclosed by the integuments, since a small opening on the apical side of the ovule, the micropyle, allows the penetration of the pollen tube.

1.2. Double fertilization

The *Arabidopsis* male gametophyte consists of a pollen grain that contains two sperm cells and one vegetative cell. The pollen grain develops in the anthers, and during anthesis it reaches full maturity, allowing its release onto the papillae of the female receptive organ, the stigma. Here, rehydration of the pollen grain triggers the growth of the pollen tube, which is promoted by the vegetative cell. Numerous factors contribute to the guidance of the pollen tube, which grows inside the placental tissue and eventually enters the female gametophyte through the ovule micropyle (reviewed by Drews and Koltunow, 2011). The female gametophyte has a crucial role in ensuring correct guidance of the pollen tube, and synergid cells were shown to be essential to this process (Hulskamp *et al.*, 1995; Higashiyama *et al.*, 2001).

After entering the female gametophyte, the pollen tube contacts with the synergid cells and its growth is arrested. One of the synergid cells undergoes cell death and shortly after, the pollen tube releases the two sperm cells into the cytoplasm of the degenerating synergid. Then, the sperm cells approach and

subsequently fuse with egg and central cells. Both sperm cells have the ability to fertilize either egg or central cell, suggesting that they are equivalent and that this is a random event (Ingouff *et al.*, 2009; Hamamura *et al.*, 2011).

1.3. Seed development

Production of seeds is an evolutionary landmark, which allows plants to endure harsh environmental conditions by suspending their life cycle, and resuming it when more favorable conditions are met (Bentsink and Koornneef, 2008). Seed development is tightly regulated and several specific structures are essential to ensure that a quality seed is produced, so that establishment of the new generation is not compromised (Santos-Mendoza *et al.*, 2008).

The double fertilization event initiates the development of two independent structures – embryo and endosperm – that grow simultaneously (**Fig. 1-3**). Though only the embryo contributes to the next generation, endosperm development is responsible for the nourishment of the embryo, which is essential so that it reaches a mature stage. Besides these two structures, the sporophytic seed coat is also a key element in seed development since it protects the fertilization products from exterior disruptions (Nowack *et al.*, 2010).

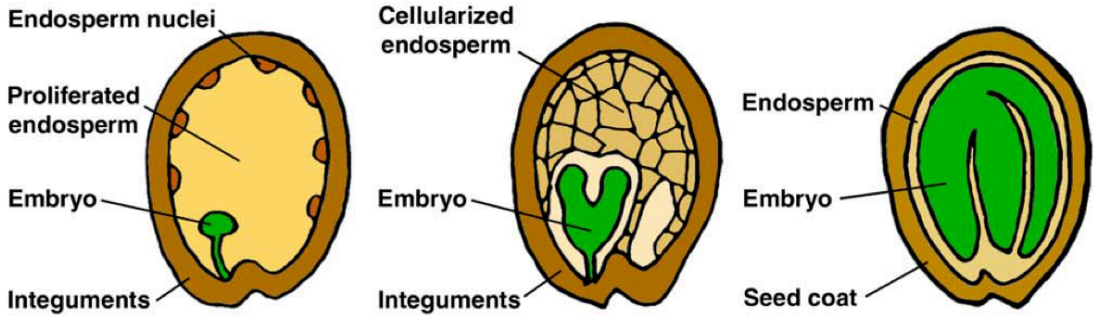

Figure 1-3 Seed development. The double fertilization event initiates embryo and endosperm development, which are accompanied by seed coat growth. In the syncytial phase the endosperm proliferates rapidly which is followed by cellularization. As the embryo grows it consumes the surrounding endosperm and eventually, the seed cavity is completely filled with the mature embryo. Adapted from Sun *et al.* (2010).

1.3.1. Embryo

Embryogenesis starts with a first asymmetric division of the egg cell, originating a smaller apical cell and a larger vacuolated basal cell. The latter undergoes several divisions, forming the embryo suspensor, which supports the embryo and connects it to the maternal tissues. Meanwhile, division of the apical cell leads to its differentiation into the eight-cell proembryo. At this stage, three areas can be defined along the apical-basal axis: the apical tier of the proembryo, which gives rise to the cotyledons and the shoot apical meristem; the lower tier that forms the hypocotyl, root and root apical meristem and finally, the extraembryonic suspensor cells (Möller and Weijers, 2009).

Maturation is achieved through multiple divisions and ultimately, the fully developed embryo fills the seed with its grown hypocotyl and cotyledons. In dicot plants like *Arabidopsis*, this fast growth is supported by the consumption of the nutritious endosperm, which by the end of seed development is

almost absent. At this point, the seed enters the desiccation phase, where the embryo is prepared for dormancy (Bentsink and Koornneef, 2008).

1.3.2. Endosperm

In the majority of flowering plants, endosperm development starts with the syncytial phase, where several rounds of mitotic divisions are not followed by cell wall formation. During the first divisions, endosperm nuclei migrate from the micropylar zone to the chalazal region and subsequent divisions originate specific domains within the syncytium. In these domains, different cytoplasmic and cytoskeletal properties are observed, and division is autonomously regulated. Three areas can be defined: the micropylar endosperm (MCE) that surrounds the embryo, the central or peripheral endosperm (PEN) and the chalazal endosperm (CZE) (Costa *et al.*, 2004; Olsen, 2004).

The following cellularization phase starts first in the MCE, where cell walls are deposited between neighboring cytoplasmic domains that surround each nucleus. The cellularization then spreads to the PEN but not to the CZE. Instead, this domain persists as a syncytium throughout seed development. Mitotic divisions in this domain are not followed by nuclear divisions, and large nuclei form as a result of endoreduplication. The CZE possesses specialized extensions that allow penetration in the sporophytic tissue close to the funiculus, thus it is hypothesized that this specific endosperm domain is responsible for loading the nutrients coming from the maternal vascular bundle (Nguyen *et al.*, 2000).

The cellularized endosperm still goes through additional rounds of division and eventually fills the seed cavity. In dicot plants the endosperm is consumed by the embryo and only its outermost layer, the aleurone, persists (Olsen, 2004).

The endosperm is generated by fertilization of the homodiploid central cell. Thus, the triploid endosperm contains two copies of the maternal genome and one copy of the paternal genome. Maintenance of the correct genome dosage is very important, and changes in the parental genome dosage causes abnormal endosperm development (Scott *et al.*, 1998).

Parent-of-origin-dependent gene expression is an epigenetic phenomenon causing maternal and paternal alleles to be differentially expressed. This phenomenon, also termed genomic imprinting, occurs in the endosperm and probably underlies the sensitivity of the endosperm in response to changes of the parental genome dosage (Schatlowski and Köhler, 2012). One interpretation for this is that maternal and paternal alleles are subject to different evolutionary forces: maternal interests rely on equal distribution of resources among the progeny, whereas paternal interests favor allocation of as many resources as possible into one individual offspring (Haig and Westoby, 1991). Recent studies identified a broad array of imprinted genes in the endosperm, both maternally and paternally expressed, many of which are transcriptional regulators, suggesting that imprinting has a crucial role in regulation of endosperm and seed development (Hsieh *et al.*, 2011; Wolff *et al.*, 2011).

1.3.3. Seed coat

The seed coat has a crucial function in maintaining seed integrity throughout its development by physically protecting the embryo. Furthermore, it is important in promoting dormancy and allowing seed dispersal (Haughn and Chaudhury, 2005).

While integument growth relies mostly on cell division, seed coat growth is mainly achieved by cell elongation, which has a predominant role over cell division in determining final seed size (Garcia *et al.*, 2005; Roszak, 2012). Five cell layers are present in the unfertilized ovule: three belonging to the inner integument, and two to the outer integument. The same layers are present in the early seed (**Fig. 1-4**), but as the seed matures, each layer acquires a specific fate.

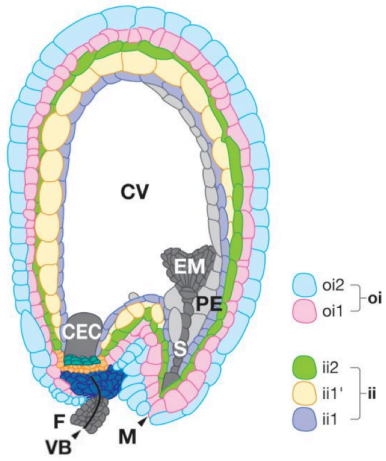

Figure 1-4 Seed coat structure. Shortly after fertilization the seed coat comprises five cell layers. Three layers of the inner integument (**ii**): **ii1**, **ii1**' and **ii2**; And two layers from the outer integument (**oi**): **oi1**, **oi2. CEC:** chalazal endosperm cyst; **CV:** central vacuole; **EM:** embryo; **F:** funiculus; **M:** micropyle; **PE:** peripheral endosperm; **S:** embryo suspensor; **VB:** vascular bundle. Adapted from Lepiniec *et al.* (2006).

Shortly after fertilization, the innermost layer of the inner integument starts accumulating protoanthocyanidins (PA). Later in development PAs are released, oxidized, and spread to the remaining seed coat layers conferring a brown color to the seed (Lepiniec *et al.*, 2006). The other two layers of the inner integument do not differentiate further, go through programmed cell death, and are eventually crushed together by the pressure exerted by the growing embryo (Haughn and Chaudhury, 2005; Nakaune *et al.*, 2005).

In the two layers of the outer integument, an initial deposition of amyloplasts is observed, but as development progresses, each layer diverges in fate. While cells of the subepidermal layer produce thickened cell walls, the epidermal layer secretes high quantities of mucilage to the apoplast. Mucilage accumulation leads to contraction of the cytoplasm that remains confined to the center of the cell. With time, the cytoplasm columns are replaced by cell walls, thus forming the columella, and when all the cell layers collapse, these structures, together with the mucilage, hold the seed shape (Haughn and Chaudhury, 2005). When imbibition occurs, the mucilage is released promoting a moist environment during seed germination (Haughn and Western, 2012).

1.4. Interactions between endosperm and seed coat

Seed growth requires regulatory mechanisms to ensure simultaneous development of embryo, endosperm and seed coat. The seed coat is a sporophytic tissue but initiation of its development is dependent on the fertilization event. This suggests that a signal originated in the embryo or endosperm might be the trigger of seed coat development. The use of an *Arabidopsis* mutant that only produces a single sperm cell showed that only the fertilized endosperm is able to induce seed coat growth (Roszak and Köhler, 2011), revealing that the necessary signal for seed coat growth is generated by the sexual

endosperm. In agreement with that, ablation of the endosperm inhibits seed coat development (Weijers *et al.*, 2003).

In fact, several studies have shown that endosperm development directly impacts seed coat growth. Mutants for genes belonging to the *HAIKU* family (*iku1* and *iku2*) produce seeds with reduced endosperm growth and early cellularization, which results in a decreased seed size (Garcia *et al.*, 2003). Similar phenotypes were described for *mini3* and *shb1* and the proposed pathway suggests that SHORT HYPOCOTYL UNDER BLUE1 (SHB1) is a direct upstream regulator of *IKU2* and *MINISEED3 (MINI3)*, promoting their expression (Zhou *et al.*, 2009). Specific expression of these genes in the endosperm, and absence of maternal sporophytic effects are strong indications that the endosperm regulates seed size.

Another indication that the endosperm regulates seed coat growth comes from the characterization of the *agl62* mutant (Kang *et al.*, 2008; Roszak and Köhler, 2011). *AGAMOUS-LIKE 62* (*AGL62)* codes for a type I MADS-box transcription factor, expressed specifically in the endosperm. Seeds of the *agl62* mutant show early endosperm cellularization and fail to develop a seed coat, despite the presence of dividing endosperm. This suggests that AGL62 is crucial for the formation of the signal that initiates seed coat development.

Several studies have also shown that not only does the endosperm control seed coat growth, but that the reverse also happens. Influence of the maternal sporophytic tissues on endosperm development is illustrated for example, by the *ttg2* mutant. *TRANSPARENT TESTA GLABRA2 (TTG2)* is strongly expressed in all seed coat layers and is known to be a part of the PA and mucilage synthesis pathways (Johnson *et al.*, 2002). Similarly to *haiku* mutants, *ttg2* causes defects in endosperm growth and cellularization and decreased seed coat cell elongation (Garcia *et al.*, 2005). This mutation has a strict maternal sporophytic effect and the defects observed in endosperm growth are a direct consequence of the defective seed coat cell elongation, suggesting that maternal tissues also have a significant impact in seed size control, through interaction with endosperm and regulation of its development.

1.5. Polycomb Group proteins in seed development

Polycomb group (PcG) proteins are chromatin-associated factors that repress transcription of several specific target loci. These proteins were initially identified in *Drosophila*, where they control homeotic genes by ensuring that their expression is restricted to specific tissues and time-points in development, thus being important to maintain cell identity (Jürgens, 1985). PcG proteins are organized in multimeric complexes, of which Polycomb Repressor Complex 1 and 2 (PRC1 and PRC2) are the best characterized ones that are present in multicellular animals and plants (reviewed by Müller and Verrijzer, 2009).

PRC2 proteins are highly conserved in plants and due to genome duplication events the *Arabidopsis* PRC2 complexes have many homologous subunits, with specific and partially redundant functions in distinct phases of development. Transcriptional repression by these complexes is achieved through

association with Plant Homeo Domain (PHD)-finger proteins and subsequent trimethylation of lysine 27 of histone 3 (H3K27me3) at target loci (reviewed by Hennig and Derkacheva, 2009). There are three different PRC2 complexes in *Arabidopsis*: EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN) and FERTILIZATION INDEPENDENT SEED (FIS) and their role in plant development is illustrated in **Figure 1-5**.

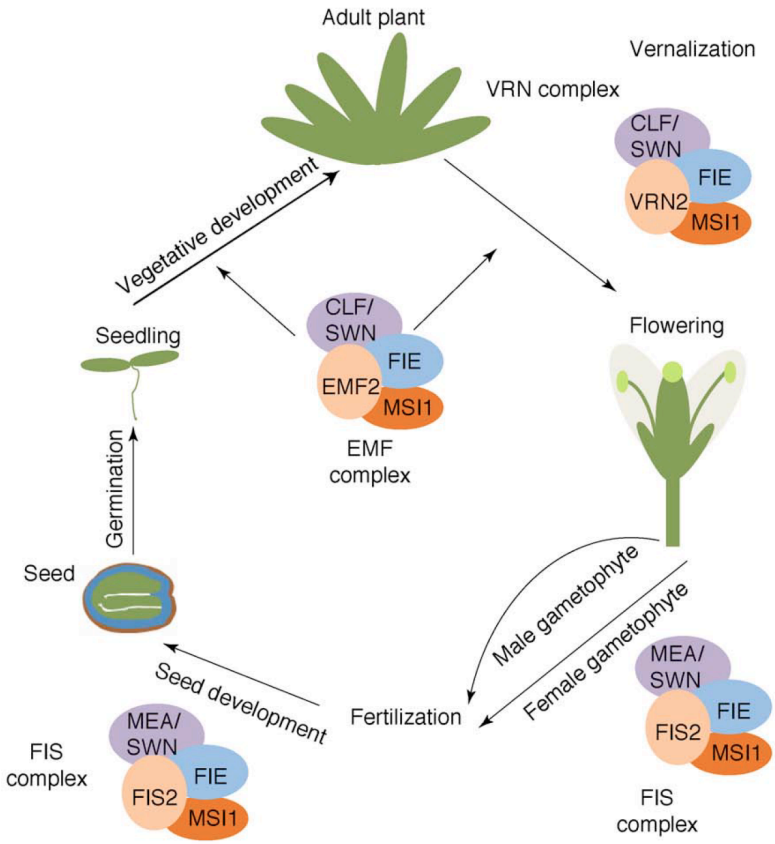

Figure 1-5 PRC2-like complexes in *Arabidopsis*. Composition and roles of each PRC2-like complex in the plant life cycle. The FIS complex (MEA/SWN, FIS2, FIE and MSI1) represses seed development in absence of fertilization and is essential for seed growth. The VRN complex (CLF/SWN, VRN2, FIE and MSI1) controls flowering time through epigenetic silencing of the flowering repressor *FLC*. The EMF complex (CLF/SWN, EMF2, FIE and MSI1) maintains cell differentiation, and represses reproduction by promoting vegetative development. Adapted from Hennig and Derkacheva (2009).

The first subunits of the FIS-PRC2 were identified in a screen for non-sexual seed formation (apomixis) (Ohad *et al.*, 1996; Chaudhury *et al.*, 1997). Mutations in any of the four subunits - FIS2 (FERTILIZATION INDEPENDENT SEED2), MEA (MEDEA), FIE (FERTILIZATION INDEPENDENT ENDOSPERM) and MSI1 (MULTICOPY SUPRESSOR OF IRA19) - lead to the formation of autonomous seeds containing proliferating endosperm and, in some cases, initiation of seed coat development. Additionally, post-fertilization phenotypes, such as non-cellularized endosperm and seed arrest, are also observed (Chaudhury *et al.*, 1997; Kiyosue *et al.*, 1999). This, coupled with the fact that FIS subunits are expressed in the central cell, as well as in the developing endosperm (Luo *et al.*, 2000), indicates that this complex has a crucial role in female gametophyte and seed development. In fact, it is believed that the FIS complex suppresses central cell division in absence of a fertilization event (Ohad *et al.*, 1996; Chaudhury *et al.*, 1997).

Despite the specific action of the FIS-PRC2 in gametophytic tissues, mutations in some of its subunits (FIE and MSI1) have a sporophytic effect and phenocopy to some extent the seed coat initiation phenotype observed in *emf2* and *vrn2* mutants. Both FIE and MSI1 subunits are common to the PCR2

sporophytic complexes EMF and VRN, suggesting a role for these complexes in regulation of seed growth, specifically, by suppressing seed coat development in unfertilized seeds (Roszak and Köhler, 2011).

1.6. Auxin

Auxin is an essential phytohormone that controls multiple processes during plant growth and development. It is known to be a key regulator of critical aspects such as cell division, elongation and differentiation, among others. Thus, auxin responses both on the cellular and on the tissue level have to be tightly regulated, which is achieved by modulating its biosynthesis, downstream signaling and transport.

1.6.1. Biosynthesis

The main active auxin form is free IAA (Indole-3-acetic acid), but other naturally occurring forms, such as PAA and 4-Cl-IAA, as well as synthetic molecules, such as 2,4-D, NAA and dicamba, are also able to generate auxin responses. Besides active forms, the auxin pool is also composed of many inactive auxins that are thought to help maintain homeostasis: these include precursors and storage forms (often auxin conjugated with amino acids, peptides or carbohydrates) (reviewed in Woodward and Bartel, 2005; Korasick *et al.*, 2013).

Synthesis of free IAA occurs via Tryptophan (Trp)-dependent and Trp-independent pathways. Although still little is known about Trp-independent pathways, experimental evidence strongly supports their existence (reviewed by Normanly *et al.*, 2004). Analysis of *trp* mutants, both in *Arabidopsis* and maize, show that these plants have similar free IAA levels when comparing with WT. Also, feeding assays with labeled Trp support the idea that IAA synthesis is not exclusively dependent on Trp presence (Normanly *et al.*, 1993). Still, many components of these pathways remain unknown and further studies are required.

Nonetheless, Trp-dependent pathways are better characterized and three main routes, named according to their main IAA intermediate, are known: indole-3-acetaldoxime (IAOx), indole-3-acetamide (IAM) and indole-3-pyruvic acid (IPA). In *Arabidopsis* the main contributor to free IAA is the IPA pathway, where production of IAA is a two-step process mediated by two different groups of enzymes (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Zhao, 2012). The first step is the conversion of Trp to IPA by Trp aminotransferases: TAA1, TAR1 and TAR2. In the second step, flavin monooxygenases from the YUCCA (YUC) family convert the produced IPA into IAA. Activity of Trp aminotransferases, as well as of YUCs, has been shown to be essential in many developmental processes, such as embryogenesis, lateral root formation, hypocotyl elongation, among others (Cheng *et al.*, 2007; Stepanova *et al.*, 2008). Furthermore, it is proposed that regulation of auxin biosynthesis, essential for vascular and flower tissue formation, can be achieved by spatial and temporal control of *YUC* gene expression (reviewed by Korasick *et al.*, 2013).

1.6.2. Signaling

Several auxin signaling pathways that involve perception of extracellular and cytoplasmic auxin are proposed to exist, but the best understood pathway is the Aux/IAA, localized in the nucleus. This pathway is dependent on the activity of transcriptional repressors belonging to the Aux/IAA family. These repressors have four conserved domains through which they interact with several other regulatory proteins of this pathway (Pierre-Jerome *et al.*, 2013). Domain III and IV, for instance, allow dimerization of Aux/IAAs with Auxin Response Elements (ARF). ARFs are transcription factors that bind to the auxin responsive *cis*-elements (AuxRE) present in the promoters of different auxin responsive genes, and whose transcription is repressed by the formation of ARF-Aux/IAA dimers (Leyser, 2010).

When intracellular levels of auxin increase, this hormone binds to the $SCF^{TIR1/AFB}$ complex. This promotes and stabilizes the interaction of Aux/IAAs (through its domain II) with the TIR1 and AFB subunits of the $SCF^{TIR1/AFB}$ complex (Tan *et al.*, 2007). Through ubiquitination, this complex targets Aux/IAAs for degradation by the 26S proteasome, leaving ARFs free to form ARF-ARF dimers, which in turn, allow transcription of several auxin responsive genes (Leyser, 2010). The common characteristic among those genes is the presence of AuxREs in their regulatory regions, the specific conserved sequence that allows auxin-dependent transcriptional regulation. Based on these sequences, genome-wide studies identified many putative auxin-responsive genes, reinforcing the role of auxin as a powerful regulator of plant development (reviewed in Hagen and Guilfoyle, 2002; Woodward and Bartel, 2005).

1.6.3. Transport

On the whole-plant level, auxin is largely synthesized in shoot apical regions, but since it regulates a wide variety of processes in many other tissues, transport of this hormone is required. In fact, auxin is effectively transported between cells across long distances and directionality of its flow is regulated by polar localization of the transporters, allowing fine-tuning of auxin contents between cells, and establishment of auxin gradients.

Intercellular auxin transport is best described by the chemiosmotic model, which integrates several molecular components known to participate in auxin transport and proposes a physiological basis for auxin movement. Free IAA can enter the cell by passive diffusion, while auxin anions (IAA^-), along with H^+ are imported to the cell by influx carriers of the AUX1/LAX family (Yang *et al.*, 2006). The intracellular alkaline environment promotes IAA dissociation and efflux is achieved through PIN (Petrásek *et al.*, 2006) and ABCB/PGP active transporters (Noh *et al.*, 2001). The asymmetric positioning of both importers and exporters is responsible for the directional flows of auxin. In fact, polar localization of the AUX1 importer, as well as ABCB/PGP exporters is often observed, but PIN positioning on the cellular membrane is the main factor influencing the direction of polar auxin transport. Apical or basal positioning is determined by phosphorylation of these transporters by the PINOID protein kinase, whose transcription is, together with PINs, upregulated by auxin (Benjamins *et al.*, 2001). Furthermore, the eight

members of the PIN family have specific roles and their expression patterns are highly dependent on the developmental stage and tissue in question (Friml, 2010).

1.7. Auxin in ovule and early seed development

Although the role of auxin in ovule and seed development is not fully understood, several studies have supported the idea that this phytohormone is, as in many other developmental processes, essential.

During the first phases of female gametophyte formation, *TAA1* is expressed in the chalazal area of the ovule primordium (Nole-Wilson *et al.*, 2010), where the integuments later arise. Simultaneously, *PIN* expression is detected in the same region, as well as in the nucellus (Pagnussat *et al.*, 2009; Ceccato *et al.*, 2013), along with *YUC* expression (Pagnussat *et al.*, 2009; Bencivenga *et al.*, 2011). In addition, auxin reporter activity (*DR5pro:GUS*) is also strong in the nucellus, suggesting that auxin is important for ovule development, since its onset (Pagnussat *et al.*, 2009; Bencivenga *et al.*, 2011). Furthermore, Pagnussat and colleagues (2009) have reported that female gametophyte cellularization is directly dependent on the establishment of an auxin gradient inside the female gametophyte. This gradient defines the fates of the female gametophyte cells: the highest auxin concentration originates synergids, followed by egg cell and finally, the lowest concentrations originate central cell and antipodals. Hence, this model proposes two essential roles for auxin during ovule development: first it is necessary for induction of embryo sac development, and in a later phase controls gametophyte cell differentiation and specification.

During early seed development, the role of auxin in embryogenesis has been thoroughly investigated and numerous studies show that control of auxin biosynthesis, transport and signaling is absolutely required for correct cell specification and patterning. Furthermore, disruption of auxin-related genes often leads to severe embryo defects (Möller and Weijers, 2009). However, how this hormone specifically influences development of the remaining seed components - endosperm and seed coat - is poorly understood. Analysis of auxin reporter activity suggests that after fertilization, there is an increase of auxin contents in the seed (Dorcey *et al.*, 2009). Also, it is known that in several species, including *Arabidopsis*, application of exogenous auxin leads to parthenocarpic fruit formation (Vivian-Smith and Koltunow, 1999; Dorcey *et al.*, 2009; Pandolfini, 2009). This suggests that auxin might be the fertilization-associated trigger that stimulates seed and fruit development, though further evidence is required to substantiate this hypothesis.

1.8. Aim of the thesis

The synchronized growth of the different seed components requires signaling mechanisms between them and, while the simultaneous fertilization event of both egg and central cell triggers development of embryo and endosperm, the trigger of the sporophytic seed coat development is still unknown. Recent results published by Roszak and Köhler (2011) showed that an AGL62-dependent signal originated in the sexual endosperm is required for seed coat initiation. However, *AGL62* expression is restricted to the

endosperm, excluding this transcription factor from the list of possible mobile signals that, upon fertilization, move to the seed coat and stimulate its development. Moreover, communication routes between endosperm and seed coat are scarce, and it appears that symplastic communication between the two tissues is not possible (Stadler *et al.*, 2005; Ingram, 2010). This restricts the molecular nature of the signal to molecules that can actively cross membranes, such as hormones.

Analysis of gene expression data showed that auxin-related genes are not only upregulated in fertilized seeds when comparing to unfertilized ovules, but are also downregulated in the *agl62* mutant that fails to develop a seed coat (Pawel Roszak, personal communication). In addition, previous studies also support the idea that auxin plays a role in seed and fruit development, since this hormone is able to induce parthenocarpy, and appears to be increased in early seeds. Thus, the major aim of this thesis was to test whether auxin could be the signal triggering seed coat development after fertilization. To achieve this, several experiments were planned to be performed: development of auxin reporter tools that are active in reproductive tissues, determination of the effects of exogenous application of auxin-related compounds in ovules and seeds, and generation of stable transgenic lines that allow modulation of auxin signaling in a tissue-specific manner. Additionally, analysis of the effect of auxin in the *agl62* phenotype, as well as use of auxin overproducing and PcG mutants to identify the molecular relations between auxin and epigenetic regulation of seed development were also planned.

2. Materials and Methods

2.1. Plant material and growth conditions

Seeds were sterilized in 5% commercial bleach and 0.01% Tween-20 for 10 min and washed three times in sterile ddH_2O. Sterile seeds were plated on ½ MS-medium (0.43% MS-salts, 0.8% Bacto Agar, 0.19% MES hydrate and 1% Sucrose; when necessary, the medium was supplemented with the appropriate antibiotics) and stratified at 4ºC in the dark for 48h. Plates were then transferred to a growth chamber (16h light / 8h dark; 110 µmol s^{-1} m^{-2}; 21ºC; 70% humidity) where seedlings developed. After 10 days the seedlings were transferred to soil and grown in a growth chamber (16h light / 8h dark; 110 µmol s^{-1} m^{-2}; 21ºC; 70% humidity).

All the mutant lines, as well as reporter lines used in this project are described in **Table 6-1** and **Table 6-2**, respectively. Double and triple mutants were generated by crossing the corresponding single mutants. Genomic DNA for PCR analysis was extracted as described in Edwards et al. (1991) and genotyping of segregating mutants was performed using the primers described in **Table 6-3**.

2.2. Cloning

All constructs were generated using the Gateway Cloning Technology (Invitrogen) following the manufacturer's instructions. To obtain dominant-negative versions of the selected Aux/IAA proteins, site-

directed mutagenesis, on a highly conserved motif of domain II, was performed. The inserted point mutations were made in order to change the conserved proline of Domain II to a leucine: $IAA5_{P58->L}$; $IAA10_{P53->L}$; $IAA28_{P98->L}$. In the case of IAA32 no site-directed mutagenesis was performed because domain II is not conserved. The coding regions of *IAA5*, *IAA10* and *IAA28* were amplified from cDNA of WT Col-0 plants using the flanking primers in combination with the mutation primers (**Table 6-3**): the right flanking primer (forward) was used with the reverse mutation primer, and the left flanking primer (reverse) was used with the forward mutation primer in two separate PCR reactions. Both PCR products for each *IAA* gene were then combined and a final PCR with the flanking primers was performed, thus originating a single fragment containing the desired mutation and the Gateway adaptors. The mutation primers had opposite directions but were designed to overlap each other (in approximately 10 bps) and the point mutation was present in the overlapping region of both primers. In the case of *IAA32*, only the flanking primers were used and WT Col-0 gDNA was used as a template.

The amplified fragments were then purified from gel, recombined into the donor vector (pDONR221) to create entry clones, and sequenced. Three different destination vectors derived from pB7WG2 (Karimi *et al.*, 2002) were used. These vectors had the *CaMV35S* promoter replaced by the *FIS2*, *PHE1* or *KLU* promoters (the first two vectors were provided by Pawel Roszak and the last one by Duarte Figueiredo). The cassettes including each IAA gene were recombined into the three different destination vectors to generate the following constructs: *FIS2pro:*$IAA5_{P58->L}$*, PHE1pro:*$IAA5_{P58->L}$*, KLUpro:*$IAA5_{P58->L}$*; FIS2pro:*$IAA10_{P53->L}$*, PHE1pro:*$IAA10_{P53->L}$*, KLUpro:*$IAA10_{P53->L}$*; FIS2pro:*$IAA28_{P98->L}$*, PHE1pro:*$IA28_{P98->L}$*, KLUpro:*$IAA28_{P98->L}$*; FIS2pro:IAA32, PHE1pro:IAA32, KLUpro:IAA32.*

2.3. Generation of transgenic plants

Col-0 plants were transformed using the floral dip method described by Clough and Bent (1998) and transformants were selected with the appropriate antibiotics.

2.4. Gene expression analysis

Shortly before anthesis, flowers from WT, *yucca6-2D* and *vrn2-1 emf2-5/+* plants were emasculated. The ovules of 25 emasculated siliques were harvested at 4 DAE in 20µL of RNAlater solution (Invitrogen) and ground for 2 min using a TissueLyser II (Qiagen). Total RNA was extracted using the Qiagen RNeasy kit, followed by DNase I treatment (Qiagen). RNA concentration and quality were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The same amount of RNA for each sample (1µg) was used to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Maxima SYBR Green qPCR Master Mix (Thermo Scientific) was used to perform the qPCR in an iQ5 qPCR system (Bio-Rad). The primers used for the RT-qPCR are described in **Table 6-3**. *PP2A* was used as the reference gene. Relative quantification of gene expression was performed using the Pfaffl method (Pfaffl, 2001).

2.5. Chemical treatments

All chemicals were prepared in a solution containing 5% EtOH and 0,05%Tween-20. Final concentrations of the several compounds were as follows: 2,4-D – 200µM; NPA – 350µM; L-Kynurenine – 200µM and 400µM. In all experiments a mock control was run in parallel.

Flowers were emasculated shortly before anthesis and, at 2 DAE, the chemicals were applied by covering the whole silique with 2µL of the respective solution. The treatment was either done only once, or repeated every 24h until the silique was collected (this is designated as the continuous treatment). A similar procedure was used for pollinated siliques: flowers were emasculated and, at 2 DAE, the siliques were hand pollinated. The solutions were then applied 8h after pollination, as this is the determined time necessary for fertilization to occur (Duarte Figueiredo, personal communication). Treatment of pollinated siliques was also done either once or continuously. The treated siliques were then collected at the specified time-points, dissected, and the ovules or seeds were processed for DIC microscopy, vanillin staining or ovule measurements.

2.6. Microscopy

For DIC microscopy, siliques were opened lengthwise to expose the ovules/seeds, fixed overnight at 4ºC on a EtOH:Acetic Acid (9:1) solution and incubated 10 min with 90% EtOH and 10 min with 70% EtOH. Ethanol was then replaced by clearing solution (66.7g Chloralhydrate; 25g H_2O; 8,3g Glycerol) and samples were incubated overnight. The carpels were then removed and ovules/seeds were mounted in clearing solution.

For GUS staining, siliques were opened lengthwise, incubated in 90% acetone at -20ºC for 1h, washed twice for 15 min in 50mM sodium phosphate buffer and vacuum infiltrated for 20 min in GUS staining buffer (50mM sodium phosphate buffer; 10mM EDTA; 0,1% Triton X-10; 1mM potassium ferrocyanide III; 1mM potassium ferricyanide IV; 1mg/mL X-Gluc). Samples were kept in GUS staining buffer and incubated at 37ºC for the necessary time so that staining became visible, followed by an overnight incubation in clearing solution. Ovules/seeds were removed from the carpels and mounted on chloralhydrate clearing solution.

For vanillin staining, emasculated siliques were opened lengthwise, the carpels were removed and the ovules were mounted in vanillin solution (1% (w/v) vanillin (4-hydroxy-3-methoxybenzaldehyde) in 6N HCl). The slides were observed after 30 min.

All the samples described above were observed on a Zeiss Axioplan microscope or on a Leica DMI 4000 microscope, both equipped with DIC optics and digital cameras.

Confocal imaging was performed on ovules/seeds that were removed from the silique and mounted in PI solution (7% glucose; 0,1mg/ml PI) (Hélène Robert-Boisivon, personal communication). Pictures for ovule measurements were taken in the widest transversal plane, where the ovule appeared bigger. Ovule area was then measured with ImageJ software. A Zeiss LSM 700 or a Leica SP5 was used to image the samples. Excitation and emission wavelengths are detailed in **Table 6-4**.

2.7. IAA Immunolocalization

Siliques were emasculated or pollinated as described above and collected at the specified time points. Ovules or seeds were vacuum infiltrated in 4% paraformaldehyde and incubated overnight at 4ºC, followed by dehydration in a series of acetone dilutions (30%; 50%; 70%; 90%; 100%; each step was repeated once, for 1h, except the last one which was repeated three times). Samples were then embedded in Technovit 8100 (Electron Microscopy Sciences) following the manufacturer's instructions. Sections with 2µm thickness were obtained using a LKB Ultratome III and placed on APEX covered microscope slides. The APEX-covered slides were prepared by submerging them in the following solutions: once in 2% APEX in acetone for 3 min, twice in 100% acetone for 2 min and twice in ddH_2O for 2 min. The slides were then dried at room temperature and stored.

Prepared sections of ovules/seeds were incubated in 10% FCS for 10 min, followed by incubation in primary antibody for 1 h (Mouse Monoclonal Anti-IAA (Sigma-Aldrich); 1/100 dilution in FCS) and a 10 min incubation in 5% FCS. Secondary antibody (Alexa Fluor 488 Rabbit Anti-Mouse IgG (Invitrogen); 1/10 dilution in PBS) was then applied for 45 min and washed with PBS (eight times for 3 min each). Sections were stained with DAPI (1 µg/mL in PBS), washed with PBS (six times for 3 min each), mounted on Mowiol 4-88 mounting medium (1gr Mowiol 4-88; 4mL PBS; 2mL glicerol), and observed on a Leica SP5 confocal microscope.

Controls for the immunolocalization protocol were run in parallel with the standard immunolocalization experiments (**Fig. 6-1**). Two different controls were performed: a negative control, where incubation with the primary antibody was substituted by incubation with 10% FCS solution and a pre-block of the primary antibody with IAA to assess its specificity to this antigen. For the pre-block experiment the primary antibody was incubated overnight at 4ºC with IAA (5 mg/mL) in a ratio of 1:2. This mixture was then diluted 100x in FCS and used in the immunolocalization experiment instead of the primary antibody. The fact that no signal was detected in both controls (**Fig. 6-1**) shows that both antibodies are highly specific to their antigens and that the signal detected in immunolocalization experiments corresponds to IAA presence.

3. Results

3.1. Auxin is present in the integuments of unfertilized ovules

To investigate the presence of auxin in the integuments of unfertilized ovules, plants expressing *GH3.3pro:GUS* were used, and the activity of this promoter was examined (**Fig. 3-1**). This construct was chosen because genes from the *GRETCHEN HAGEN3 (GH3)* family are known to respond to auxin due to the presence of auxin-responsive *cis*-elements (AuxRE) in their promoters (reviewed by Hagen and Guilfoyle, 2002). Promoter expression pattern in emasculated siliques was analyzed and a strong

GUS staining in the funiculus was observed, as well as a patchy staining in the integuments of the unfertilized ovules (**Fig. 3-1A**), suggesting that there is auxin present at these sites, even if in small amounts. To confirm that the *GH3.3* promoter is truly responding to the presence of auxin, emasculated siliques of *GH3.3pro:GUS* plants were treated with the synthetic auxin 2,4-D (**Fig. 3-1C**). As expected, GUS staining is observed throughout the ovule and is much stronger than in the mock treatment (**Fig. 3-1D**), thus confirming that this promoter is indeed auxin-responsive and that it is active in the integuments of unfertilized ovules.

NPA is a polar auxin transport inhibitor that blocks auxin efflux mediated by PIN and PGP proteins (Taiz and Zeiger, 2002). Upon treatment with this compound, it is expected that auxin will accumulate at the site of its production, which makes NPA a very valuable tool to identify these sites. Treatment of emasculated siliques expressing *GH3.3pro:GUS* with NPA led to strong GUS staining in the funiculus, as well as in the inner layers of the ovule integuments (**Fig. 3-1B**), implying that auxin is being produced in these areas prior to fertilization.

IAA immunolocalization on unfertilized ovules also shows that auxin is present in the integuments. It is also interesting to note that the signal is stronger in the funiculus and in the inner layer of the integuments (**Fig. 3-1E**) as it was previously observed in the *GH3.3pro:GUS* ovules.

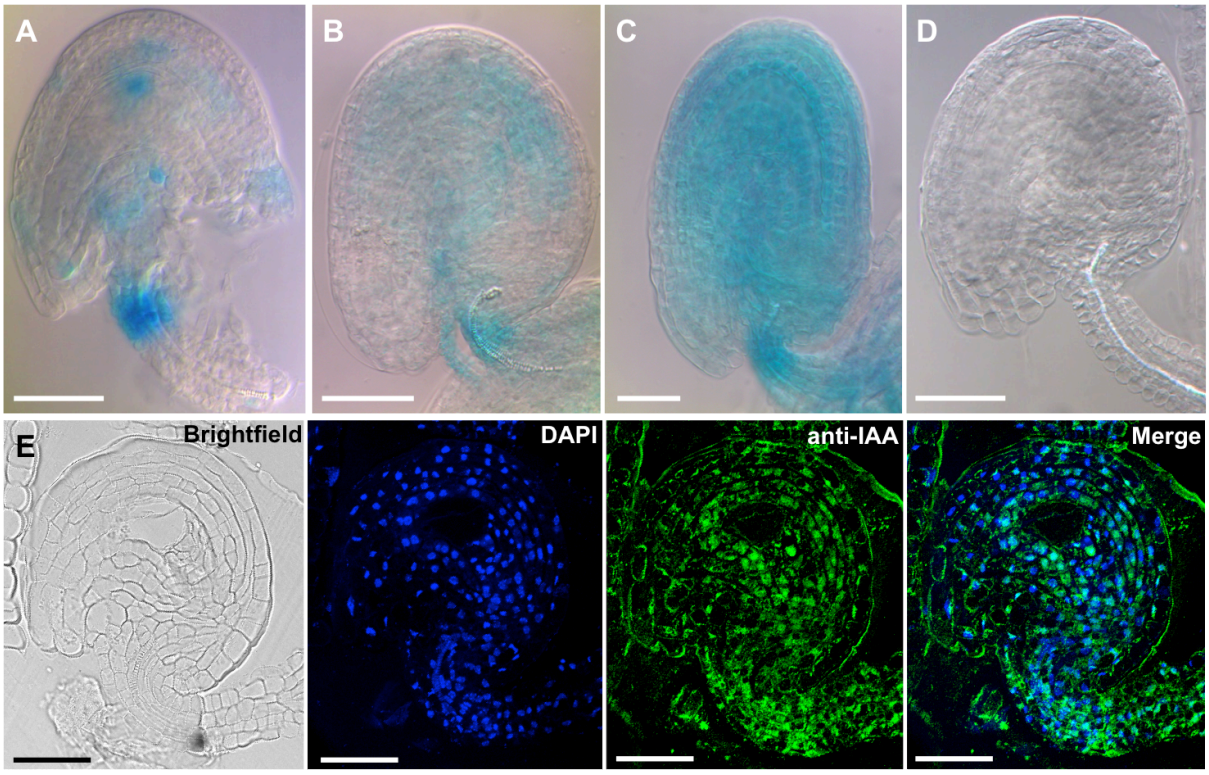

Figure 3-1 *GH3.3pro:GUS* expression pattern and IAA immunolocalization in unfertilized ovules. (A) to **(D)** DIC images of GUS-stained unfertilized ovules of *GH3.3pro:GUS* transgenic plants. Incubation in GUS staining solution was performed overnight in **(A)** and during 3h in **(B)** to **(D)**. **(A)** Ovule at 4DAE. **(B)** Ovule at 4DAE treated with NPA. **(C)** Ovule at 4DAE treated with 2,4-D. **(D)** Ovule at 4DAE treated with mock solution. **(E)** IAA immunolocalization in a 2DAE ovule section, panel shows (from left to right) brightfield, DAPI, anti-IAA and merge between DAPI and anti-IAA. Bars = 50µm.

3.2. *YUC10* and *YUC11* are active after fertilization and auxin is present both in the sexual endosperm and the seed coat

Gene expression data previously obtained in our group (Pawel Roszak, personal communication) indicates that auxin-responsive and auxin biosynthesis genes are upregulated in fertilized seeds compared to unfertilized ovules. This motivated the analysis of reporter lines of auxin biosynthesis genes, namely genes of the *YUC* family. GFP reporter constructs of *YUC6, YUC10* and *YUC11* were used and their expression pattern was analyzed in unfertilized ovules as well as in developing seeds (**Fig. 3-2**). *YUC10* and *YUC11* are not expressed in unfertilized ovules (**Fig. 3-2B** and **3-2D**) however, immediately after fertilization and throughout seed development, they are expressed specifically in the endosperm (**Fig. 3-2A** and **3-2C**). *YUC6* expression was not detected before or after fertilization (data not shown).

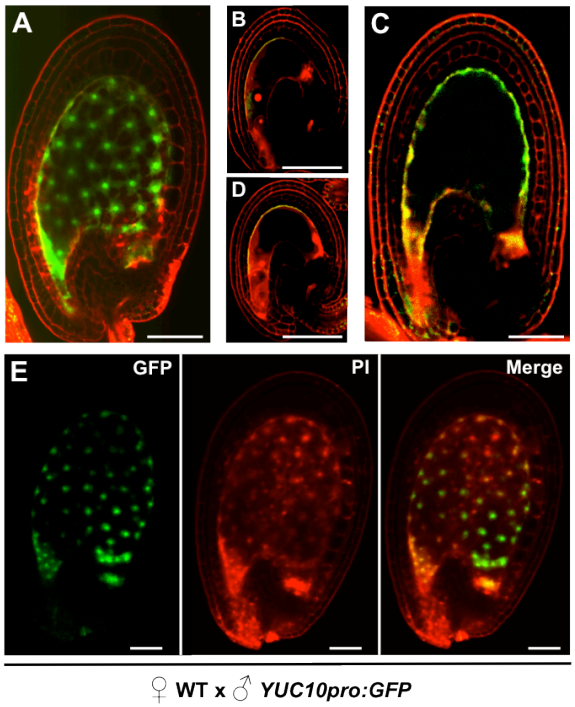

Figure 3-2 *YUC10* and *YUC11* promoter activity in ovules and seeds. Confocal laser scanning microscopy of ovules and seeds of *YUC10pro:GFP* and *YUC11pro:GFP* transgenic lines. **(A)** and **(B)** *YUC10pro:GFP* seed (2DAP) and ovule (4DAE), respectively. **(C)** and **(D)** *YUC11pro:GFP* seed (4DAP) and ovule (4DAE), respectively. **(E)** Seed of WT silique fertilized with *YUC10pro:GFP* pollen at 5DAP. Panel shows (from left to right) *YUC10pro:GFP* signal in green, PI in red and the merge between GFP and PI channels. Bars = 50 µm.

YUC10 was described as a PEG (Hsieh *et al.*, 2011; Wolff *et al.*, 2011). To confirm this, reciprocal crosses between WT and *YUC10pro:GFP* transgenic plants were performed. Indeed, when WT ovules are fertilized with *YUC10pro:GFP* pollen, a strong GFP signal in the endosperm nuclei is detected (**Fig. 3-2E**), which does not happen when this cross is performed in the opposite direction (using WT pollen; **Fig. 6-2**).

Since auxin biosynthesis genes are expressed in the endosperm after fertilization, seeds of *GH3.3pro:GUS* plants were analyzed and IAA immunolocalizations were performed to check weather the observed *YUC* activity correlates with increased auxin contents in the developing seed. **Figure 3-3** shows seeds expressing *GH3.3pro:GUS* in different developmental stages. Initially, the GUS staining is more intense in the funiculus, but also visible in the seed coat (**Fig. 3-3A**). Later in development, the staining becomes much stronger (**Fig. 3-3B**) and specific to the inner

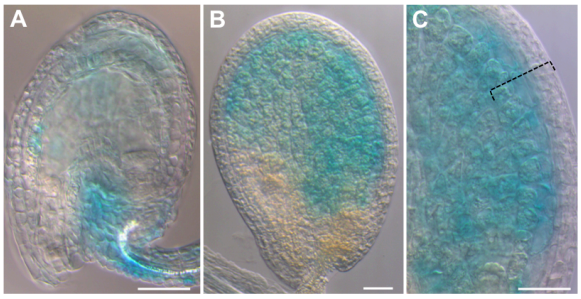

Figure 3-3 *GH3.3pro:GUS* expression pattern in developing seeds. (A) to **(C)** DIC images of GUS-stained *GH3.3pro:GUS* transgenic seeds. **(A)** 1DAP, **(B)** 3DAP, **(C)** seed coat detail of a 3DAP seed. Dashed line delimits the five layers of the seed coat. Bars = 50 µm.

layers of the seed coat (**Fig 3-3C**). Due to technical limitations of the GUS staining method, it was not possible to determine if this promoter is active in the endosperm, since this structure is difficult to image in a non-cleared seed. However, IAA immunolocalizations allowed observation of this structure, as well as of the seed coat, in more detail (**Fig. 3-4**). As it was the case for unfertilized ovules, auxin appears to be more abundant in the innermost cell layer of the seed coat, though it is present in all layers. This pattern is more evident in the early stages of seed development (**Fig. 3-4A**) and it seems that in later stages, the difference in auxin levels between the cell layers is not so pronounced (**Fig. 3-4B** and **3-4C**). As expected, it is also possible to detect a very strong signal coming from the endosperm nuclei in all developmental stages analyzed. This, coupled with *YUC10* and *YUC11* reporter analysis, shows that auxin biosynthesis genes are activated after fertilization in the endosperm, which is correlated with auxin presence in this region.

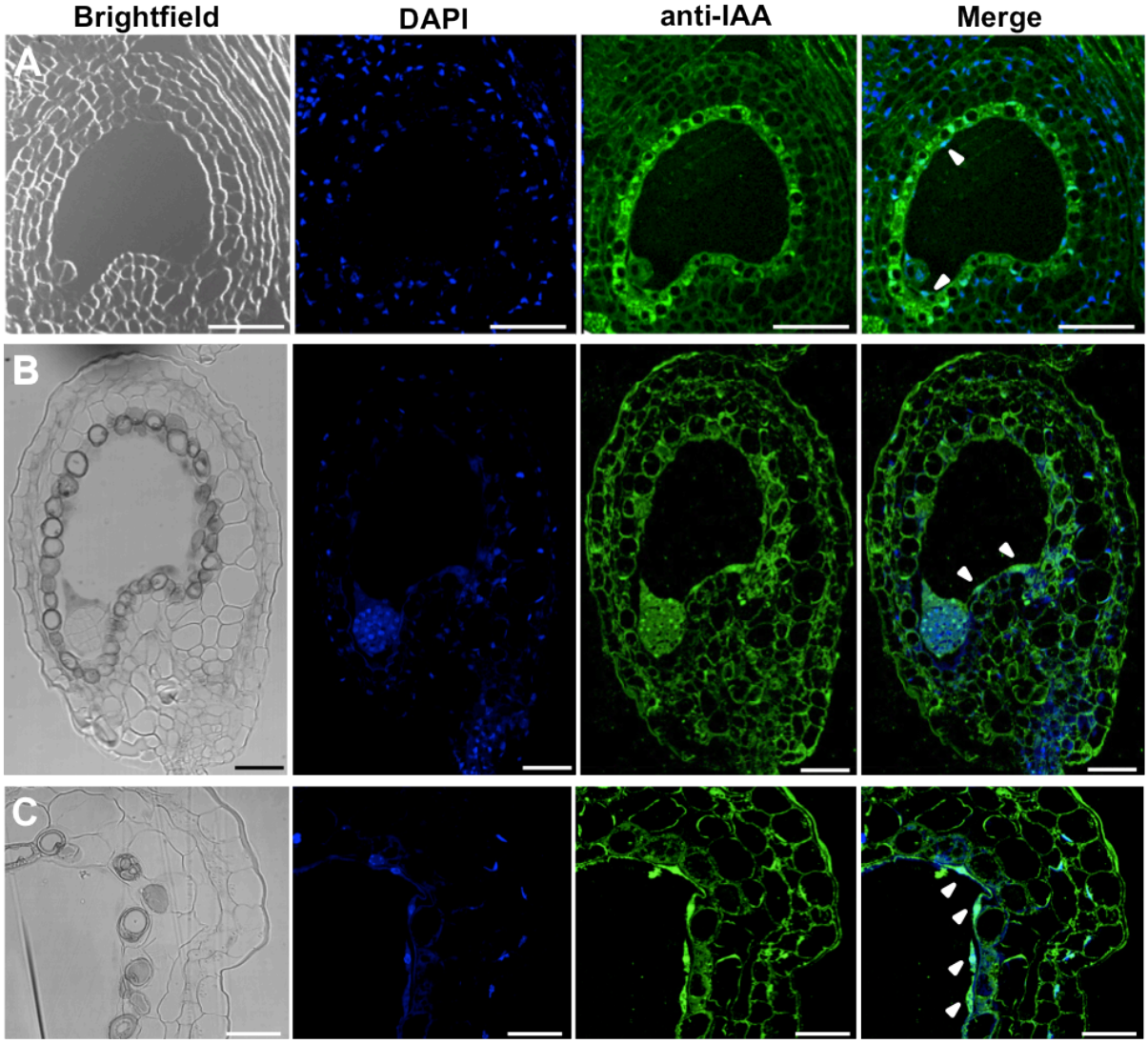

Figure 3-4 IAA Immunolocalization in developing seeds. Confocal laser scanning microscopy images of seed sections. **(A)** 1DAP seed. **(B)** 4DAP seed. **(C)** endosperm and seed coat detail of a 4DAP seed. Panels show (from left to right) brightfield, DAPI, anti-IAA and merge between DAPI and anti-IAA. White arrows indicate endosperm nuclei. Bars in **(A)** and **(B)** = 50 µm. Bars in **(C)** = 25 µm.

3.3. Auxin triggers autonomous central cell division and seed coat development in unfertilized ovules

While applying 2,4-D and NPA to unfertilized ovules expressing *GH3.3pro:GUS,* it was striking that these ovules had a morphology similar to that of young seeds, with an increased size and an apparent seed coat development (**Fig. 3-1**). To further investigate this phenomenon, the same chemical treatments done with the *GH3.3pro:GUS* line were performed with emasculated WT siliques. Interestingly, when WT ovules were treated with either 2,4-D or NPA, a clear initiation of the seed coat was detected, visualized by a strong staining with vanillin (**Fig. 3-5**). This method stains PAs by acid hydrolysis leading to the formation of a red color that can be clearly detected. As PAs are produced when the endothelium starts differentiating, this method can therefore be used to detect seed coat development. Ovules that were positive for vanillin staining were divided into three different categories according to the staining intensity: WT-like (**Fig. 3-5A**), moderate (**Fig. 3-5B**) and strong (**Fig. 3-5C**). While staining was detected in a large number of mock ovules, its intensity was considerably lower when comparing with NPA or 2,4-D treated ovules (**Fig. 3-5D**). It was also clear that the majority of NPA treated ovules developed a moderate staining, while in ovules treated with 2,4-D this staining was much stronger (**Fig. 3-5D**), even comparable to early developing seeds (Debeaujon *et al.*, 2003).

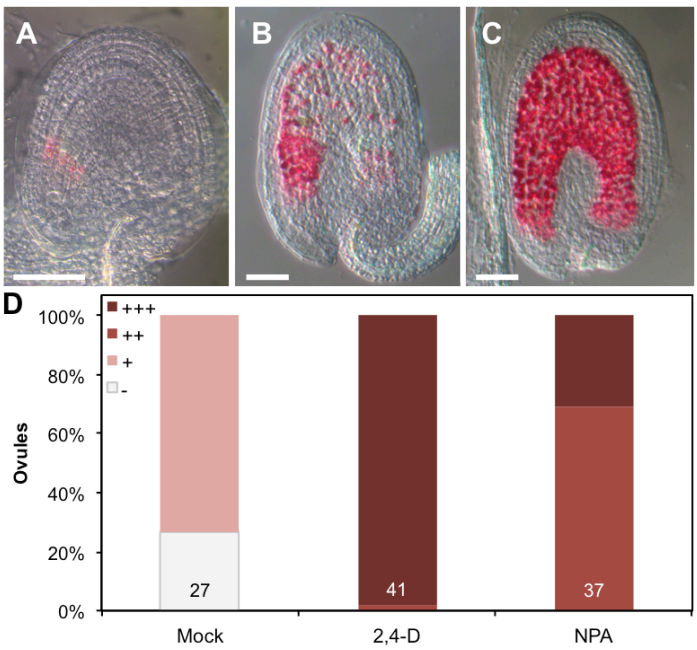

Figure 3-5 Vanillin staining of 2,4-D and NPA treated ovules. (A) to **(C)** DIC images of unfertilized WT ovules at 5DAE. **(A)** WT-like staining, corresponding to the + category. **(B)** moderate staining, ++ category. **(C)** strong staining, +++ category. Bars = 50 µm. **(D)** Percentage of mock, 2,4-D and NPA treated ovules belonging to each staining category. Numbers of analyzed ovules are indicated at the bottom of each bar.

Furthermore, when clearing of these ovules was performed, it was possible to observe autonomously developing endosperm both in the treatment with 2,4-D and NPA (**Fig. 3-6B** and **3-6C**). The induction of central cell division was clear in both cases (61% of the 2,4-D treated ovules and 44% of the NPA treated ovules; **Fig 3-6C**), showing that auxin has the ability to override PcG protein repression of seed development not only in the seed coat, but also in the central cell. Also clear, was the fact that these ovules were much bigger than the untreated ones (2 times bigger with 2,4-D and 1.6 times bigger with NPA; **Fig. 3-6D**). No significant differences in these phenotypes were observed when applying 2,4D and NPA only once or continuously during ovule development (data not shown). Additionally, accumulation of auxin by blocking PAT transport with NPA mimics (although to a lesser extent) the phenotypes

observed in the 2,4-D treatment, thus suggesting that auxin is produced both in the central cell and integuments prior to fertilization.

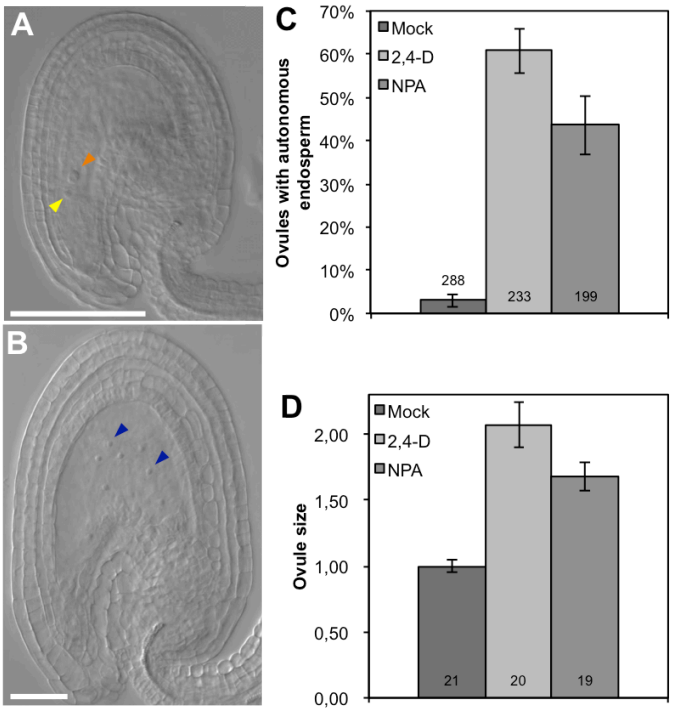

Figure 3-6 Clearings and measurements of 2,4-D and NPA treated ovules. (A) and **(B)** DIC images of cleared ovules at 5DAE. **(A)** Mock. **(B)** 2,4-D. Yellow arrow shows egg cell, orange arrow shows central cell and blue arrows show endosperm nuclei. Bars = 50 µm. **(C)** Percentage of ovules with autonomously developing endosperm in mock, 2,4-D and NPA treatment. Ovules with three or more nuclei were considered as autonomous endosperm **(D)** Area of mock, 2,4-D and NPA treated ovules. Values are normalized to mock treated ovules. Numbers of analyzed ovules are indicated at the bottom of each bar. Error bars represent SD.

3.4. Seed coat development is not affected by inhibitors of polar auxin transport or of auxin biosynthesis

The previous set of data shows that an increase in auxin content triggers endosperm and seed coat development in unfertilized ovules. However, induction of auxin biosynthesis machinery after fertilization was only observed in the endosperm. This might suggest that upon fertilization, auxin is produced in the endosperm and is then exported to the integuments to promote seed coat development. To test this hypothesis, pollinated WT siliques were treated with NPA. Blocking polar auxin transport would elucidate if auxin is transported from the endosperm to the seed coat or instead, produced locally in response to an alternative signal. Clearings of approximately 160 NPA-treated seeds for each time-point (1, 3 and 5 DAP) were performed however, none of these seeds showed deviant phenotypes (data not shown), which would favor the last hypothesis. The auxin biosynthesis inhibitor L-kynurenine was also used to treat pollinated WT siliques and a similar number of seeds were collected and cleared at the above-mentioned time-points, as described for the NPA treatment. This chemical is known to selectively inhibit TAA1/TAR activity (He *et al.*, 2011), two key enzymes of the IPA auxin biosynthesis pathway in *Arabidopsis* (reviewed by Korasick *et al.*, 2013). Similarly to NPA, no effects were detected in seeds treated with this compound (data not shown), which was unexpected since the IPA pathway was shown to be active in early seeds (**Fig. 3-2**).

3.5. The *agl62* mutant phenotype is partially rescued by auxin

Roszak and Köhler (2011) showed that a signal originated in the sexual endosperm is necessary to initiate seed coat development in *Arabidopsis*. This signal is dependent on AGL62 activity and mutants for this MADS-box transcription factor fail to develop a seed coat, which leads to seed arrest. These seeds can either arrest around 3DAP or 5DAP, and are thus called early arresting and late arresting seeds, respectively (**Fig. 3-7**).

Since auxin has the ability to trigger seed coat development in the absence of fertilization, experiments were performed in order to determine if this phytohormone can also rescue the *agl62* phenotype. Therefore, siliques of *agl62/+* mutants were treated with 2,4-D and NPA (**Fig. 3-7A**), and double mutants of *agl62* with the auxin overproducing mutant *yucca6-2D* were analyzed (**Fig. 3-7B**). The *yucca6-2D* mutant is characterized by an increase in auxin biosynthesis in sporophytic tissues that is likely also affecting the integuments. This is caused by an increased expression of *YUC6,* which is under the control of the *CaMV35S* promoter. In both cases, a decrease in the overall percentage of arresting seeds was observed (23% early arresting seeds in the mock treatment vs. 10% in the 2,4-D treatment, 10% late arresting seeds in the mock treatment vs. 2% in the 2,4-D treatment; 25% early arresting seeds in *agl62/+* vs. 14% in *yuca6-2D agl62/+,* 4% late arresting seeds in *agl62/+* vs. 2% in *yucca6-2D agl62/+*). This shows that the signal triggering seed coat development, which is missing in the *agl62* mutant, is partially rescued by auxin. However, the fact that auxin is not able to fully rescue the seed arrest phenotype, indicates that normal development of the seed coat may be dependent on other

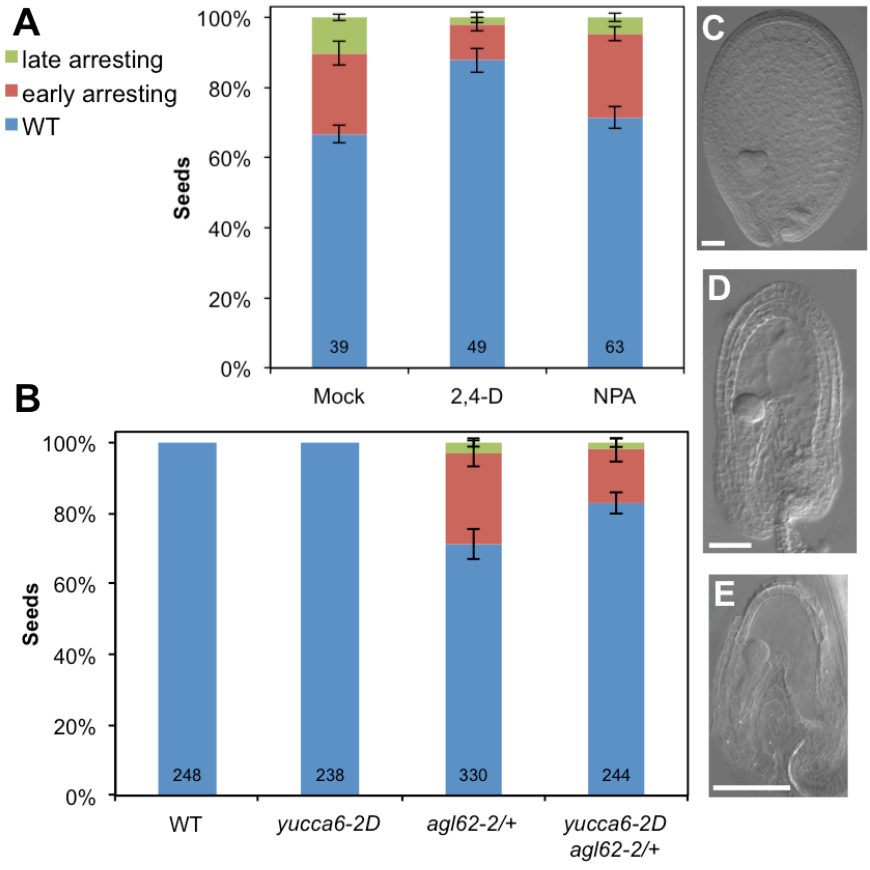

Figure 3-7 Seed abortion phenotype of *agl62-2* plants. Quantification of seed abortion in *agl62-2/+* plants treated with 2,4-D and NPA **(A)** and in the *yucca6-2D agl62/+* double mutant **(B).** Percentage of WT, late arresting and early arrested seeds are indicated by blue green and red bars, respectively. Numbers of analyzed seeds are indicated at the bottom of each bar. Error bars represent SD. DIC images of a cleared WT seed **(C)**, late arresting seed **(D)** and early arresting seed **(E).** Bars = 50 µm.

factors. Moreover, it remains to be tested whether the decrease in seed abortion rates observed in these assays correlates with the recovery of plants homozygous for *agl62*.

Accumulation of auxin in the integuments of WT ovules treated with NPA is sufficient to trigger autonomous seed coat formation. To test if NPA has a similar influence on the *agl62* phenotype, treatments with this chemical were performed (**Fig. 3-7A**). However, no decrease in seed arrest was observed, suggesting that no auxin is being produced in the *agl62* seed or alternatively, that auxin is not produced in a sufficient amount to trigger seed coat development. This is supported by the downregulation of auxin biosynthesis genes observed in gene expression data obtained for this mutant (Pawel Roszak, personal communication).

3.6. Increased auxin in the integuments does not significantly influence autonomous seed development in *fis2* mutants

The PcG mutant *fis2* develops two types of autonomous seeds: one with autonomous endosperm but no seed coat initiation (small seeds, **Fig. 3-8A**) and another with autonomous endosperm that is accompanied by initiation of seed coat development (big seeds, **Fig. 3-8B**). To assess if auxin impacts autonomous seed formation in *fis2*, this mutant was crossed with *yucca6-2D*. Clearings of the single and double mutants revealed that the overall percentage of ovules with autonomous endosperm is similar in *fis2/+* and *yucca6-2D fis2/+*. However, the amount of big seeds is slightly higher in the double mutant

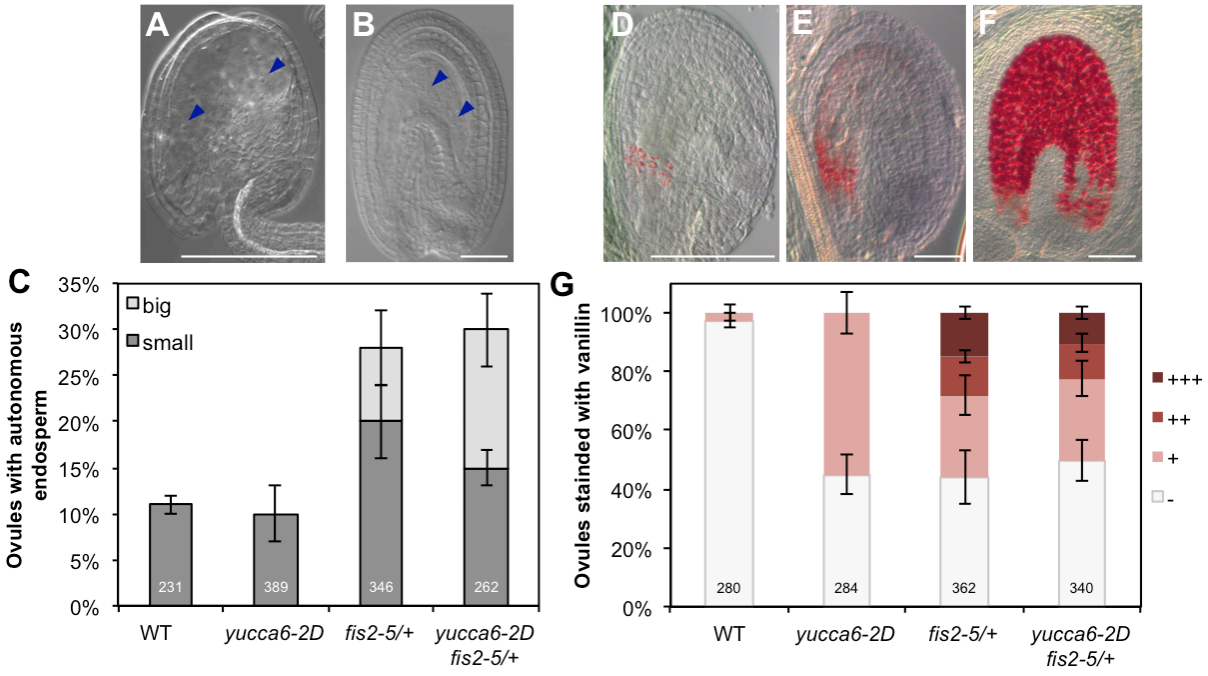

Figure 3-8 Clearings and vanillin staining of *yucca6-2D fis2-5* ovules. (A) and **(B)** DIC images of cleared *fis2-5/+* autonomous seeds at 5DAE. **(A)** small seed **(B)** big seed. **(C)** Autonomous endosperm development in the *yucca6-2D fis2-5/+* double mutant at 5DAE. Dark and light grey bars represent small and big seeds, respectively. Ovules with three or more visible nuclei were considered as positive for autonomous endosperm development. **(D)** to **(F)** DIC images of vanillin stained *yucca6-2D fis2-5/+* ovules at 5DAE. **(D)** WT-like staining, corresponding to the + category. **(E)** moderate staining, ++ category. **(F)** strong staining, +++ category. **(G)** Percentage of vanillin stained ovules belonging to each category. Numbers of analyzed ovules are indicated at the bottom of each bar. Error bars represent SD. Bars = 50 µm.

(**Fig. 3-8C**), which suggests that the presence of increased amounts of auxin in the integuments stimulates seed coat initiation in these autonomous seeds.

Vanillin staining of the single and double mutants was also performed and three categories of staining, according to its intensity, were defined: WT-like (+, **Fig. 3-8D**), moderate (++, **Fig. 3-8E**) and strong (+++, **Fig. 3-8F**). High accumulation of protoanthocyanidins, shown in **Figs. 3-8E** and **3-8F**, are characteristic for autonomous big seeds, therefore one would expect that the increase of autonomous big seeds observed in **Fig. 3-8C** would be reflected in an increase of ovules belonging to the +++ category (**Fig. 3-8F**). Instead, such increase was not observed, and both *fis2/+* and *yucca6-2D fis2/+* seem to have similar amounts of ovules in each staining category (**Fig. 3-8G**). Also, the overall percentage of seeds in the ++ and +++ categories are superior to the number of big seeds reported in **Fig. 3-8C**, suggesting that some of these mutant ovules are initiating seed coat development independently of the presence of autonomous endosperm. In fact, when performing clearing of *fis2/+* and *yucca6-2D fis2/+*, some of the ovules seemed similar to the big autonomous seed represented in **Fig. 3-8B**, but lacked the developing endosperm.

3.7. Reduced PcG function and increased auxin in the integuments lead to autonomous seed coat development

As shown in section 3.3, unfertilized ovules treated with auxin have the ability to bypass the PcG protein repression of endosperm and seed coat development. Overproduction of auxin in the integuments of the *yucca6-2D* mutant mimics the seed coat initiation phenotype described for the sporophytic PcG mutant *vrn2-1 emf2-5/+* (Roszak and Köhler, 2011). Thus, crosses between *yucca6-2D* and *vrn2-1 emf2-5/+* were performed to determine if the phenotypes in these mutants result from the deregulation of the same pathways.

Integuments of WT ovules usually degenerate at 5DAE, however, both *yucca6-2D* and *vrn2-1 emf2-5/+* mutants have preserved integuments at this point (**Fig. 3-9A** and **3-9D**). Besides the preservation of the integuments, both mutants have the ability to initiate seed coat

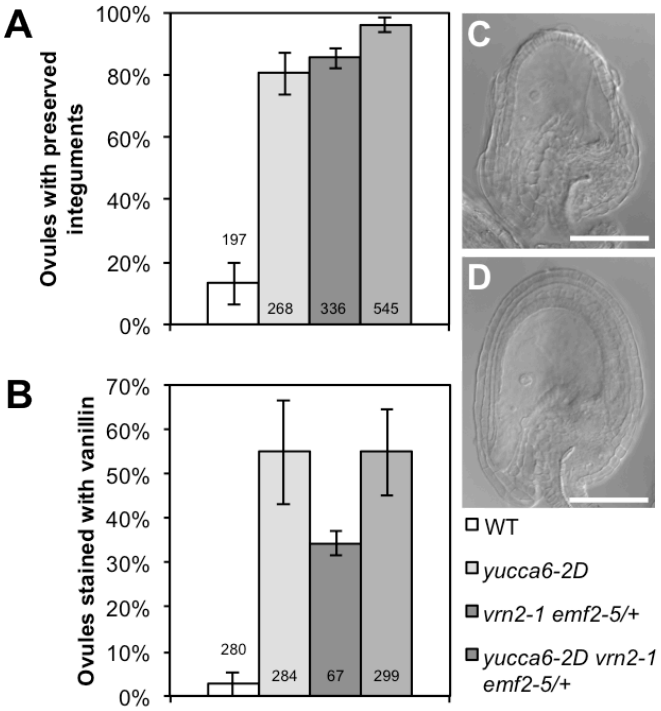

Figure 3-9 Clearings and vanillin staining of *yucca6-2D vrn2-1 emf2-5* ovules. (A) Percentage of ovules with preserved integuments at 6DAE. Differences are statistically significant (χ^2 test) between *yucca6-2D* and the triple mutant ($p<0.001$) and between *vrn2-1 emf2-5/+* and the triple mutant ($p<0.05$) **(B)** Percentage of ovules stained with vanillin at 6DAE. Numbers of analyzed ovules are indicated at the bottom of each bar. **(C)** and **(D)** DIC images of cleared ovules at 6DAE. **(C)** WT, **(D)** *yucca6-2D vrn2-1 emf2-5/+*. Bars = 50 µm. Error bars represent SD.

development as illustrated by vanillin staining of these ovules (**Fig. 3-9B**).

The *yucca6-2D vrn2-1 emf2-5*/+ triple mutant analysis revealed both additive and non-additive interactions, depending on the analyzed phenotype. Thus, preservation of the integuments is slightly enhanced in the triple mutant, with nearly all the ovules having its integuments completely preserved at 6DAE (**Fig. 3-9A**). On the other hand, when analyzing vanillin staining, both the single *yucca6-2D* mutant and the triple *yucca6-2D vrn2-1 emf2-5*/+ mutant have similar amounts of stained ovules (**Fig. 3-9B**). This suggests that loss of sporophytic PcG protein function and increased auxin content in the integuments both participate in the pathway that triggers seed coat development.

In an effort to identify genes differentially regulated between *vrn2-1 emf2-5*/+ and WT ovules, Roszak (2012) compared gene expression profiles of the two samples through microarray analysis. In this study, a set of strongly up and downregulated genes in *vrn2-1 emf2-5*/+ ovules comparing to WT was selected, and the transcript levels of these genes both in *vrn2-1 emf2-5*/+ and *yucca6-2D* ovules were analyzed. This allowed to validate accuracy of the data generated in the microarray and more importantly, to determine if both mutations originate similar deregulation patterns of these genes. Five upregulated (**Fig. 6-3**) and five downregulated genes (**Fig. 6-4**) were selected, which belong to several different categories most of which are hormone related genes (auxin, gibberellin and ethylene – full list of loci and gene names are found in **Table 6-3**). Expression levels for the majority of the analyzed genes followed the trend indicated in the microarray, except in a few cases (**Fig. 6-3** – ATG01520, ATG351600 and AT4G34800) where levels in *vrn2-1 emf2-5*/+ ovules were similar or slightly lower than WT, opposite of what was expected.

While none of the tested genes with increased expression in *vrn2-1 emf2-5*/+ were upregulated in *yucca6-2D* (**Fig. 6-3** – AT4G26280, AT2G44840), all tested genes with decreased expression in *vrn2-1 emf2-5*/+ were also less expressed in *yucca6-2D,* compared to WT. Though additional data are required to generate a final conclusion, these results suggest that loss of PcG function and increased amounts of auxin generate similar responses at the physiological and molecular level, which implies a relation between these two regulatory mechanisms. Despite this, functional overlap is not complete since additive relations, as well as differences in gene expression profiles between the mutants were observed.

3.8. Impaired auxin signaling in the integuments leads to developmental aberrations in the female gametophyte

Modulating auxin signaling in specific tissues of the unfertilized ovule or developing seed is a very useful tool to understand the role of this phytohormone in seed development. To achieve this, several dominant-negative versions of Aux/IAA proteins, which are widely known to be key players in the auxin signaling process (reviewed by Guilfoyle, 2007) were created. There are several described Aux/IAA mutants that have gain-of-function mutations in a specific motif of the conserved domain II. Since this motif allows interaction of Aux/IAAs with the $SCF^{TIR1/AFB}$ complex in the presence of auxin, these

mutations result in dominant or semi-dominant phenotypes (Rouse *et al.*, 1998; Tian and Reed, 1999; Nagpal *et al.*, 2000; Rogg *et al.*, 2001), by blocking downstream auxin signaling. Based on these studies, site-directed mutagenesis to change a specific conserved amino acid in this motif was performed.

Aux/IAA repressors were selected based on their interaction with ARF activators. The goal was to choose a set of Aux/IAA proteins that interact with as many ARF activators as possible, inhibiting auxin-mediated responses. Therefore, four different Aux/IAA repressors that were shown to interact with 16 out of the 22 ARF activators were selected: *IAA5*, *IAA10*, *IAA28* and *IAA32* (interaction data was retrieved from Vernoux *et al.*, 2011, and from http://www.ebi.ac.uk/intact/). Since IAA32 does not possess a conserved domain II (Dreher *et al.*, 2006), site-directed mutagenesis was not performed. It is believed that Aux/IAA proteins that do not have a conserved domain II are not able to interact with the $SCF^{TIR1/AFB}$ complex and consequently, are not degraded. Therefore, they act as a damper of auxin signaling by avoiding extreme oscillations in the transcription of auxin-responsive genes (Sato and Yamamoto, 2008).

The dominant-negative versions of the selected *Aux/IAA* genes were placed under the control of tissue specific promoters, such as *FIS2* (central cell), *PHE1* (endosperm) and *KLU* (ovule integuments). Plant transformations with all these constructs were performed, but only the ones with the *KLU* promoter were so far analyzed.

The *KLU* gene is expressed in leaves and floral organs where it is crucial to promote growth. It is also expressed specifically in the inner integument throughout ovule development (Adamski *et al.*, 2009). The fact that *KLU* expression is not restricted to the ovules, explains the observed phenotypes in other organs, like defects in leaf and flower morphology, pollen development and deficient carpel elongation (data not shown). Very severe phenotypes were also observed on developing ovules and seeds (**Fig. 3-10**). Although different dominant-negative IAA proteins were used, most of the phenotypes were common among the different lines, the most recurrent being a defect in embryo sac formation (**Figs 3-10B** and **3-10C**). In some cases, it was not possible to define a clear boundary between the integuments and the embryo sac (**Fig. 3-10C**), suggesting that this structure is not formed. In other cases, there seemed to be an embryo sac forming but not containing the usual cells found in this structure (**Fig. 3-10B).**

Cell identity and positioning were also altered (**Figs. 3-10D**). It was common to observe ovules containing, what appeared to be, multiple egg cells and/or multiple central cells. Ovules lacking one of these cell types were also present. Observation of the nuclear morphology and positioning in WT ovules is usually sufficient to correctly distinguish the different type of nuclei (**Fig. 3-10A**). However, in these cases it was difficult to do so, and markers for each cell type should be used in the future to achieve a more precise identification, which would contribute to determine the origin of this defect.

Nevertheless, several transgenic plant lines produced WT-like ovules or defective ovules that could nonetheless be fertilized. In *KLUpro:IAA5*$_{P58->L}$ and *KLUpro:IAA28*$_{P98->L}$ transgenic plants, seeds with a normal embryo but collapsed seed coat were observed (**Fig. 3-10F**), as well as seeds with arrested embryo and endosperm development (**Fig. 3-10G**), though in low frequencies. Seed abortion was also

observed in *KLUpro:IAA32* plants, as illustrated by **Fig. 3-10J**. Aborted seeds were clearly brown, shriveled and appeared hollow, but upon observation under the microscope it was visible that seeds contained a developing embryo, endosperm and a collapsed seed coat (data not shown). The cause for seed abortion requires further investigation, since it is necessary to determine if either failure in endosperm proliferation or failure in seed coat development are the origin of this problem. Plants transformed with the *KLUpro:IAA10*$_{P53->L}$ construct produced some seeds with developing endosperm, but no embryo (**Fig. 3-10H**). The most likely cause for this phenotype is that, due to the above mentioned cell specification defect, no egg cell is formed during gametophyte development, and thus only the central cell is fertilized. Among *KLUpro:IAA10*$_{P53->L}$ and *KLUpro:IAA28*$_{P98->L}$ transgenic plants an embryo phenotype was also observed, more precisely in the embryo suspensor (**Fig. 3-10I**), where cell elongation seemed to be reduced compared to WT embryos.

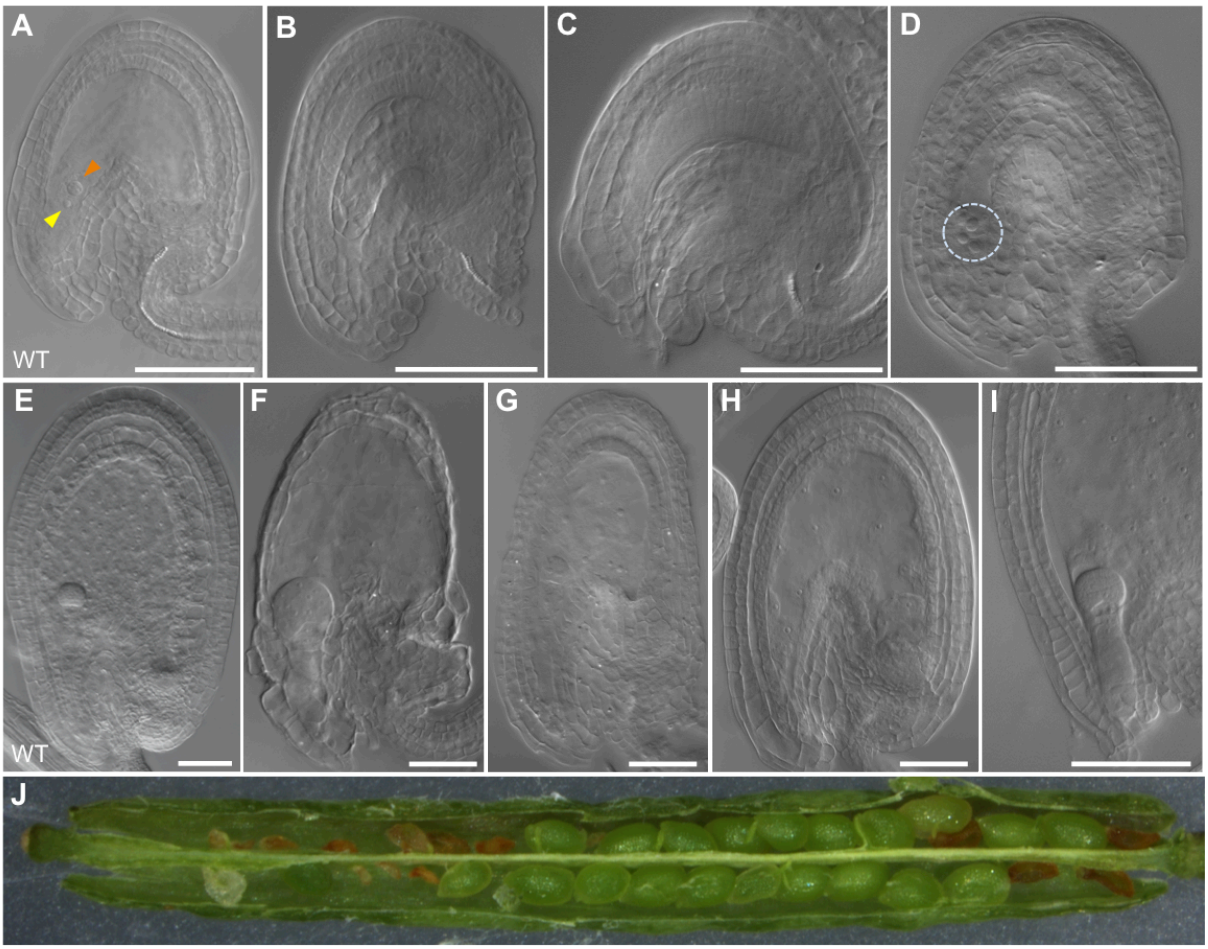

Figure 3-10 Ovules and seeds of dominant-negative Aux/IAA plant lines. (A) to **(J)** DIC images of cleared ovules **(A)** to **(D),** and seeds **(E)** to **(I)** of several different dominant negative Aux/IAA transgenic plant lines and WT. **(A)** WT ovule with visible egg and central cells, indicated by the yellow and orange arrows, respectively. **(B)** Ovule with empty embryo sac. **(C)** Ovule lacking embryo sac. **(D)** Ovule with gametophytic cell specification and positioning defects, gametophytic cells are surrounded by the dashed circle. **(E)** WT developing seed at 4DAP. **(F)** Aborted seed with developing embryo and endosperm, but collapsed seed coat. **(G)** Aborted seed with arrested embryo and endosperm development. **(H)** Developing seed lacking an embryo. **(I)** Embryo suspensor defect. **(J)** Silique of a plant expressing *KLUpro:IAA32* containing green developing seeds and aborted seeds of brown color. Bars = 50 µm.

4. Discussion

4.1. Seed coat development might be triggered by increased auxin production after fertilization

A signal originated by the sexual endosperm has been shown by Roszak and Köhler (2011) to be essential to initiate seed coat development. This signal should be able to cross membranes, since it is believed that there are no symplastic connections between the endosperm and the seed coat (Stadler *et al.*, 2005; Ingram, 2010). A molecule like auxin is a suitable candidate for this signal since it can be transported actively between cells and it triggers a vast array of responses by effectively modulating gene expression and regulating cell expansion, a key process in seed coat development (Garcia *et al.*, 2003; Roszak, 2012).

In fact, auxin is able to trigger seed coat development without fertilization (shown by treatments with 2,4-D and also observed in the auxin overproducing mutant, *yucca6-2D*). It also has the ability to partially rescue the seed abortion phenotype of the *agl62* mutant, indicating that this hormone has a crucial role in the development of the seed coat.

Although the presence of autonomously developing endosperm in WT ovules treated with NPA suggests that auxin is present in the unfertilized central cell, paternal expression of the auxin biosynthesis gene *YUC10* (Hsieh *et al.*, 2011; Wolff *et al.*, 2011; this study) as well as exclusive activation of *YUC11* after fertilization, indicate that production of this hormone is strongly increased after fertilization and, most likely, this increase of biosynthetic activity leads to an increase of auxin amounts in the early seed. It is widely known that physiological responses to auxin, such as cell elongation, are dose-dependent (Taiz and Zeiger, 2002). Elongation of integument cells might be similarly regulated, and the increase in auxin concentration after fertilization might be the trigger of seed coat development. Though in this study it was not possible to correlate increased activity of *YUC* genes with elevated auxin contents in the fertilized seed, future work, such as auxin concentration measurements in ovules and seeds will allow answering this question.

The data presented here suggests that auxin is present in all tissues of the ovule and seed. After fertilization, induction of endosperm-specific biosynthesis genes is observed and before fertilization, *TAA1* expression is specifically detected in the micropylar region of the integuments, which is maintained in the developing seed (Duarte Figueiredo, personal communication). Thus, no clear induction of auxin production in the seed coat after fertilization was detected so far, although results indicate that an increased amount of auxin in the integuments might be the trigger of its transition to a developing seed coat. This suggests that the auxin present in this structure is originated in the endosperm. On the other hand, the lack of deviant phenotypes in developing seeds treated with NPA implies that auxin is not transported from the endosperm to the seed coat, at least through polar auxin transporters. It is however important to mention, that the absence of effects, both in NPA and L-kynurenine treatments, raises the question if these compounds are in fact reaching the developing seeds. Indeed, after fertilization carpel structure is considerably altered and its cells expand, differentiate and thicken (Vivian-Smith *et al.*, 2001),

which might severely affect penetration of the chemicals. Further experiments with higher concentrations and perhaps different application methods should be performed to clarify this issue.

Additionally, studies with auxin transporter mutants and reporter lines of alternative biosynthesis genes, will also help determine if most of the auxin that triggers seed coat development is originated in the endosperm or, if there is an alternative signal, that moves from the endosperm to the seed coat acting there as a positive regulator of auxin biosynthesis genes.

4.2. Seed coat development in fertilized seeds is not exclusively dependent on auxin

This study shows that auxin has an important role in the development of the seed coat. Its ability to rescue the *agl62* mutant phenotype is one clear indication of this. Another indication is its capacity to induce autonomous seed coat development. Despite the fact that application of 2,4-D induces seed coat development in 100% of the treated ovules, the same treatment in *agl62* seeds is not able to fully rescue this mutant's phenotype. Thus, although auxin might take part in the mechanism that triggers seed coat development, it is not its only component. In fact, unfertilized ovules treated with 2,4-D end up collapsing after a few days, even if the supply of auxin is continuous (data not shown). This suggests that auxin is important in stimulating the initial elongation of the integument cells, but that the full development of this structure is dependent on other factors.

AGL62 is thought to be a transcriptional regulator (Kang *et al.*, 2008), thus it is likely to activate several downstream targets upon fertilization, originating a complex regulatory network. Efforts in identifying AGL62 targets and suppressors of the mutant phenotype are being made, which should help clarify which other factors are required for seed coat development.

4.3. PRC2 repression is bypassed by auxin

PcG protein activity, in both central cell and integuments, acts to repress the development of these structures in the absence of a fertilization event. It is known that sporophytic and gametophytic tissues have different regulatory PRC2 protein complexes (reviewed by Hennig and Derkacheva, 2009). Generally, mutants of PRC2 proteins active in both tissues, such as FIE and MSI1, present strong autonomous seed phenotypes, with development of both endosperm and seed coat (Roszak and Köhler, 2011). However, the gametophytic *fis2* mutant forms two classes of autonomous seeds, one of which contains autonomous endosperm and a developing seed coat. This shows that lack of PRC2 repression on the central cell has the ability to trigger pathways that lead to seed coat development. Furthermore, the presence of *fis2* ovules with a developing seed coat but absent endosperm suggests that central cell division is not an absolute requirement for the activation of the seed coat initiation pathways. Indeed, lack of FIS2 repression in the central cell causes this cell to adopt endosperm identity, which is not necessarily followed by endosperm proliferation (Ungru *et al.*, 2008). Thus, it may be that seed coat initiation requires only cell identity change rather then dividing endosperm.

The sporophytic *vrn2-1 emf2-5*/+ mutant also develops a seed coat in the absence of fertilization, reinforcing the role of PRC2 protein activity in regulating seed development. Nevertheless, none of these

autonomous seeds reach late developmental stages, showing that a fertilization event is crucial to drive full seed development.

Interestingly, supply of auxin, either by chemical treatments or through overexpression of an auxin biosynthesis gene, leads to phenotypes similar to the ones observed in PRC2 mutants. Indeed, chemical treatments with 2,4-D have such a strong effect in promoting autonomous seed development, that it can be comparable to *fie* and *msi1* mutant phenotypes (Roszak, 2012). Not only does it induce central cell division, but also initiates seed coat development, which suggests that auxin can modulate PRC2 activity. Moreover, phenotypic analysis of the *yucca6-2D vrn2-1 emf2-5*/+ triple mutant indicates that reduced PRC2 activity and increased auxin levels in the integuments act partly in the same pathways. Gene transcript comparisons between *vrn2-1 emf2-5*/+ and *yucca62-D* also point to a relation between these two factors which are however, not fully redundant.

Since the auxin biosynthesis machinery is activated after fertilization it can be hypothesized that increase of its concentration above a certain threshold counteracts PRC2 activity, which can cause removal of the transcription block at several loci and lead to the development of the seed. Analysis of *PRC2* gene expression, reporter lines and patterns of H3K237me3 are necessary to determine if auxin is a direct repressor of PRC2 activity. Alternatively, auxin downstream signaling might have the ability to regulate genes that are also PRC2 target genes, meaning that both these mechanisms would share common targets.

4.4. Auxin acts non-cell autonomously to regulate female gametophyte development

Auxin is a very important player in plant development and several studies had already suggested a crucial role for this hormone in fruit and seed development (Nemhauser *et al.*, 2000; Pagnussat *et al.*, 2009; Nole-Wilson *et al.*, 2010; Ceccato *et al.*, 2013). In fact, Pagnussat and colleagues (2009) proposed a model of female gametophyte development in which the formation of this structure is controlled by an asymmetric distribution of auxin. Using auxin reporters and cell marker lines this research group found that disruption of auxin synthesis and signaling in the early embryo sac leads to female gametophyte phenotypes similar to the ones observed in this study: cell division, specification and positioning defects. Reporter analysis of *YUC* and *PIN* genes performed by Pagnussat *et al.* (2009) showed a very specific expression of those genes in the micropylar end of the sporophytic tissues. Therefore, these authors suggest that sporophytic auxin might be the initial stimulus that triggers an auxin maximum on the embryo sac, which in turn determines cell fate.

The results presented in this thesis are in agreement with the idea that sporophytic auxin is the initial signal that activates and regulates embryo sac development. Disruption of auxin signaling in the integuments with the aid of the *KLU* promoter reproduces phenotypes of auxin signaling disruption in the female gametophyte reported by Pagnussat *et al.* (2009), which is a good indication that this initial source of auxin is essential to initiate embryo sac development. Nevertheless, to confirm this hypothesis

it is necessary to analyze auxin distribution patterns and to confirm cell identity problems in the Aux/IAA dominant negative lines described here.

Additionally, seed developmental defects, such as seed abortion, were also observed in a few lines. It is known that pre-fertilization activity of some proteins, such as KLU (Adamski *et al.*, 2009) and ARF2 (Schruff *et al.*, 2006), determines the growth potential of the seed. To isolate the cause of the observed defects it is necessary to establish if they originate due to residual *KLU* promoter activity in these tissues, due to faulty auxin signaling in the integuments or alternatively, due to problems in either endosperm or seed coat development. Still, it may be hypothesized that, similarly to KLU and ARF2, auxin presence in the integuments might be essential to ensure the correct development of the fertilized seed.

4.5. Concluding remarks

It is widely acknowledged that auxin is as a key player in numerous aspects of plant development. The results presented in this study are yet another contribution to complement the current knowledge on the role of auxin in ovule and seed development. Analysis of auxin reporters showed that this hormone is present throughout ovule and seed development. Before fertilization, auxin is crucial for female gametophyte development, since it promotes and regulates embryo sac formation and cellularization. Fertilization-dependent activation of auxin biosynthesis machinery in the endosperm, coupled with its ability to induce autonomous seed formation, suggests that increased auxin contents in the early seed may be an important factor driving seed development. Interestingly, a relation between auxin and PcG protein repression was demonstrated, and it seems that auxin might modulate PRC2 protein activity or at least, share several downstream targets with this mechanism, in order to regulate seed development. The nature of the signal that triggers seed coat development is still unknown, however it seems that auxin is an important part of the pathway that promotes seed coat growth, either as part of a complex signal that travels form the endosperm to the integuments, or as a direct downstream target of an alternative signal.

Future studies such as identification of AGL62 targets, central cell and endosperm-specific modulation of auxin signaling, analysis of auxin biosynthesis and transporter mutants, as well as characterization of the relation between PRC2 protein activity and auxin will certainly allow answering these questions.

5. References

Adamski, N.M., Anastasiou, E., Eriksson, S., O'Neill, C.M., and Lenhard, M. (2009). Local maternal control of seed size by KLUH/CYP78A5-dependent growth signaling. *PNAS* **106**: 20115–20.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., *et al.* (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653–7.

Bajon, C., Horlow, C., Motamayor, J.C., Sauvanet, A., and Robert, D. (1999). Megasporogenesis in *Arabidopsis thaliana* L.: an ultrastructural study. *Sexual Plant Reproduction* **12**: 99–109.

Bencivenga, S., Colombo, L., and Masiero, S. (2011). Cross talk between the sporophyte and the megagametophyte during ovule development. *Sexual Plant Reproduction* **24**: 113–21.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P., and Offringa, R. (2001). The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. *Development* **128**: 4057–4067.

Bentsink, L. and Koornneef, M. (2008). Seed dormancy and germination. *The Arabidopsis Book* **6**: e0119.

Ceccato, L., Masiero, S., Sinha Roy, D., Bencivenga, S., Roig-Villanova, I., Ditengou, F.A., Palme, K., Simon, R., and Colombo, L. (2013). Maternal control of *PIN1* is required for female gametophyte development in *arabidopsis*. *PLoS ONE* **8**: e66148.

Chandler, J., Wilson, A., and Dean, C. (1996). *Arabidopsis* mutants showing an altered response to vernalization. *The Plant Journal* **10**: 637–44.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana. PNAS* **94**: 4223–8.

Cheng, Y., Dai, X., and Zhao, Y. (2007). Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. *The Plant cell* **19**: 2430–9.

Christensen, C.A., King, E.J., Jordan, J.R., and Drews, G.N. (1997). Megagametogenesis in *Arabidopsis* wild type and the *gf* mutant. *Sexual Plant Reproduction* **10**: 49–64.

Clough, S.J. and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**: 735–43.

Costa, L.M., Gutièrrez-Marcos, J.F., and Dickinson, H.G. (2004). More than a yolk: the short life and complex times of the plant endosperm. *Trends in Plant Science* **9**: 507–14.

Debeaujon, I., Nesi, N., and Perez, P. (2003). Proanthocyanidin-accumulating cells in *Arabidopsis* testa: regulation of differentiation and role in seed development. *The Plant Cell* **15**: 2514–2531.

Dorcey, E., Urbez, C., Blázquez, M.A., Carbonell, J., and Perez-Amador, M.A. (2009). Fertilization-dependent auxin response in ovules triggers fruit development through the modulation of gibberellin metabolism in A*rabidopsis*. *The Plant Journal* **58**: 318–32.

Dreher, K., Brown, J., Saw, R., and Callis, J. (2006). The *Arabidopsis* Aux/IAA protein family has diversified in degradation and auxin responsiveness. *The Plant Cell* **18**: 699–714.

Drews, G.N. and Koltunow, A.M.G. (2011). The female gametophyte. *The Arabidopsis Book* **9**: e0155.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* **19**: 1349.

Friml, J. (2010). Subcellular trafficking of PIN auxin efflux carriers in auxin transport. *European Journal of Cell Biology* **89**: 231–5.

Garcia, D., Gerald, J., and Berger, F. (2005). Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in *Arabidopsis*. *The Plant Cell* **17**: 52–60.

Garcia, D., Saingery, V., Chambrier, P., Mayer, U., Jürgens, G., and Berger, F. (2003). *Arabidopsis haiku* mutants reveal new controls of seed size by endosperm. *Plant Physiology* **131**: 1661–70.

Guilfoyle, T. (2007). Sticking with auxin. *Nature* **446**.

Gutierrez, L., Mongelard, G., Floková, K., Pacurar, D.I., Novák, O., Staswick, P., Kowalczyk, M., Pacurar, M., Demailly, H., Geiss, G., and Bellini, C. (2012). Auxin controls *Arabidopsis* adventitious root initiation by regulating jasmonic acid homeostasis. *The Plant Cell* **24**: 2515–27.

Hagen, G. and Guilfoyle, T. (2002). Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Molecular Biology* **49**: 373–385.

Haig, D. and Westoby, M. (1991). Genomic imprinting in endosperm: its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Philosophical Transactions of the Royal Society of Biological Sciences* **333**: 1–13.

Hamamura, Y., Saito, C., Awai, C., Kurihara, D., Miyawaki, A., Nakagawa, T., Kanaoka, M.M., Sasaki, N., Nakano, A., Berger, F., and Higashiyama, T. (2011). Live-cell imaging reveals the dynamics of two sperm cells during double fertilization in *Arabidopsis thaliana*. *Current Biology* **21**: 497–502.

Haughn, G. and Chaudhury, A. (2005). Genetic analysis of seed coat development in *Arabidopsis*. *Trends in Plant Science* **10**: 472–7.

Haughn, G.W. and Western, T.L. (2012). *Arabidopsis* seed coat mucilage is a specialized cell wall that can be used as a model for genetic analysis of plant cell wall structure and function. *Frontiers in Plant Science* **3**: 64.

He, W., Brumos, J., Li, H., Ji, Y., Ke, M., Gong, X., Zeng, Q., Li, W., Zhang, X., An, F., Wen, X., Li, P., Chu, J., Sun, X., Yan, C., Yan, N., Xie, D.-Y., Raikhel, N., Yang, Z., Stepanova, A.N., *et al.* (2011). A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in *Arabidopsis*. *The Plant Cell* **23**: 3944–60.

Hennig, L. and Derkacheva, M. (2009). Diversity of Polycomb group complexes in plants: same rules, different players? *Trends in Genetics* **25**: 414–23.

Higashiyama, T., Yabe, S., Sasaki, N., Nishimura, Y., Miyagishima, S., Kuroiwa, H., and Kuroiwa, T. (2001). Pollen tube attraction by the synergid cell. *Science* **293**: 1480–1483.

Hsieh, T., Shin, J., Uzawa, R., Silva, P., Cohen, S., Bauer, M.J., Hashimoto, M., Kirkbride, R.C., Harada, J.J., Zilberman, D., and Fischer, R.L. (2011). Regulation of imprinted gene expression in *Arabidopsis* endosperm. *PNAS* **108**: 1755–62.

Hulskamp, M., Schneitz, K., and Pruitt, R. (1995). Genetic evidence for a long-range activity that directs pollen tube guidance in *Arabidopsis*. *The Plant Cell* **7**: 57–64.

Ingouff, M., Sakata, T., Li, J., Sprunck, S., Dresselhaus, T., and Berger, F. (2009). The two male gametes share equal ability to fertilize the egg cell in *Arabidopsis thaliana*. *Current Biology* **19**: R19–R20.

Ingram, G.C. (2010). Family life at close quarters: communication and constraint in angiosperm seed development. *Protoplasma* **247**: 195–214.

Johnson, C.S., Kolevski, B., and Smyth, D.R. (2002). *TRANSPARENT TESTA GLABRA2*, a trichome and seed coat development gene of *Arabidopsis*, encodes a WRKY transcription factor. *The Plant Cell* **14**: 1359–1375.

Jürgens, G. (1985). A group of genes controlling the spatial expression of the bithorax complex in *Drosophila*. *Nature* **316**: 153–155.

Kang, I.-H., Steffen, J.G., Portereiko, M.F., Lloyd, A., and Drews, G.N. (2008). The AGL62 MADS domain protein regulates cellularization during endosperm development in *Arabidopsis*. *The Plant Cell* **20**: 635–47.

Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. *Trends in Plant Science* **7**: 193–195.

Kim, J.I., Sharkhuu, A., Jin, J.B., Li, P., Jeong, J.C., Baek, D., Lee, S.Y., Blakeslee, J.J., Murphy, A.S., Bohnert, H.J., Hasegawa, P.M., Yun, D.-J., and Bressan, R. a (2007). *yucca6*, a dominant mutation in *Arabidopsis*, affects auxin accumulation and auxin-related phenotypes. *Plant Physiology* **145**: 722–35.

Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinneny, J., Wells, D., Katz, A., Margossian, L., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in *Arabidopsis*. *PNAS* **96**: 4186–4191.

Korasick, D.A., Enders, T.A., and Strader, L.C. (2013). Auxin biosynthesis and storage forms. *Journal of Experimental Botany* **64**: 2541–2555.

Lepiniec, L., Debeaujon, I., Routaboul, J.-M., Baudry, A., Pourcel, L., Nesi, N., and Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. *Annual Review of Plant Biology* **57**: 405–30.

Leyser, O. (2010). The power of auxin in plants. *Plant Physiology* **154**: 501–5.

Luo, M., Bilodeau, P., Dennis, E.S., Peacock, W.J., and Chaudhury, A. (2000). Expression and parent-of-origin effects for FIS2, MEA, and FIE in the endosperm and embryo of developing *Arabidopsis* seeds. *PNAS* **97**: 10637–42.

Mashiguchi, K., Tanaka, K., Sakai, T., Sugawara, S., Kawaide, H., Natsume, M., Hanada, A., Yaeno, T., Shirasu, K., Yao, H., McSteen, P., Zhao, Y., Hayashi, K., Kamiya, Y., and Kasahara, H. (2011). The main auxin biosynthesis pathway in *Arabidopsis*. *PNAS* **108**: 18512–7.

Möller, B. and Weijers, D. (2009). Auxin control of embryo patterning. *Cold Spring Harbor Perspectives in Biology* **1**: a001545.

Müller, J. and Verrijzer, P. (2009). Biochemical mechanisms of gene regulation by Polycomb group protein complexes. *Current Opinion in Genetics & Development* **19**: 150–8.

Nagpal, P., Walker, L., Young, J., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W. (2000). *AXR2* encodes a member of the Aux/IAA protein family. *Plant Physiology* **123**: 563–574.

Nakaune, S., Yamada, K., Kondo, M., Kato, T., Tabata, S., Nishimura, M., and Hara-Nishimura, I. (2005). A vacuolar processing enzyme, ΔVPE, is involved in seed coat formation at the early stage of seed development. *The Plant Cell* **17**: 876–87.

Nemhauser, J.L., Feldman, L.J., and Zambryski, P.C. (2000). Auxin and ETTIN in *Arabidopsis* gynoecium morphogenesis. *Development* **127**: 3877–88.

Nguyen, H., Brown, R.C., and Lemmon, B.E. (2000). The specialized chalazal endosperm in *Arabidopsis thaliana* and *Lepidium virginicum* (Brassicaceae). *Protoplasma* **212**: 99–110.

Noh, B., Murphy, A.S., and Spalding, E.P. (2001). Multidrug resistance–like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. *The Plant Cell* **13**: 2441.

Nole-Wilson, S., Azhakanandam, S., and Franks, R.G. (2010). Polar auxin transport together with *aintegumenta* and *revoluta* coordinate early *Arabidopsis* gynoecium development. *Developmental Biology* **346**: 181–195.

Normanly, J., Cohen, J.D., and Fink, G.R. (1993). *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. *PNAS* **90**: 10355–9.

Normanly, J., Slovin, J., and Cohen, J. (2004). Auxin biosynthesis and metabolism. In Plant Hormones: Biosynthesis, Signal Transduction, Action by P.J. Davies, ed by *Kluwer Academic Publishers: Dordrecht*, pp. 36–62.

Nowack, M.K., Grini, P.E., Jakoby, M.J., Lafos, M., Koncz, C., and Schnittger, A. (2006). A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. *Nature Genetics* **38**: 63–7.

Nowack, M.K., Ungru, A., Bjerkan, K.N., Grini, P.E., and Schnittger, A. (2010). Reproductive cross-talk: seed development in flowering plants. *Biochemical Society Transactions* **38**: 604–12.

Ohad, N., Margossian, L., Hsu, Y.C., Williams, C., Repetti, P., and Fischer, R.L. (1996). A mutation that allows endosperm development without fertilization. *PNAS* **93**: 5319–24.

Olsen, O.-A. (2004). Nuclear endosperm development in cereals and *Arabidopsis thaliana. The Plant Cell* **16**: 214–228.

Pagnussat, G.C., Alandete-Saez, M., Bowman, J.L., and Sundaresan, V. (2009). Auxin-dependent patterning and gamete specification in the *Arabidopsis* female gametophyte. *Science* **324**: 1684–9.

Pandolfini, T. (2009). Seedless fruit production by hormonal regulation of fruit set. *Nutrients* **1**: 168–77.

Petrásek, J., Mravec, J., Bouchard, R., Blakeslee, J.J., Abas, M., Seifertová, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanová, M., Dhonukshe, P., Skupa, P., Benková, E., Perry, L., Krecek, P., Lee, O.R., Fink, G.R., Geisler, M., Murphy, A.S., Luschnig, C., *et al.* (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**: 914–8.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**: 45e–45.

Pierre-Jerome, E., Moss, B.L., and Nemhauser, J.L. (2013). Tuning the auxin transcriptional response. *Journal of Experimental Botany* **64**: 2557–63.

Robinson-Beers, K., Pruitt, R.E., and Gasser, C.S. (1992). Ovule development in wild-type *Arabidopsis* and two female-sterile mutants. *The Plant Cell* **4**: 1237–1249.

Rogg, L., Lasswell, J., and Bartel, B. (2001). A gain-of-function mutation in *IAA28* suppresses lateral root development. *The Plant Cell* **13**: 465–480.

Roszak, P. (2012). Initiation of seed coat development is controlled by Polycomb group proteins and requires a signal from the sexual endosperm. *Doctoral Dissertation no. 20165*. ETH Zurich

Roszak, P. and Köhler, C. (2011). Polycomb group proteins are required to couple seed coat initiation to fertilization. *PNAS* **108**: 20826–31.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M., and Leyser, O. (1998). Changes in auxin response from mutations in an *AUX/IAA gene*. *Science* **279**: 1371–1373.

Santos-Mendoza, M., Dubreucq, B., Baud, S., Parcy, F., Caboche, M., and Lepiniec, L. (2008). Deciphering gene regulatory networks that control seed development and maturation in Arabidopsis. *The Plant Journal* **54**: 608–20.

Sato, A. and Yamamoto, K. (2008). What's the physiological role of Domain II-less Aux/IAA proteins? *Plant Signaling & Behavior* **3**: 496–497.

Schatlowski, N. and Köhler, C. (2012). Tearing down barriers: understanding the molecular mechanisms of interploidy hybridizations. *Journal of Experimental Botany* **63**: 6059–67.

Schneitz, K., Hülskamp, M., and Pruitt, R. (1995). Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue. *The Plant Journal* **7**: 731–749.

Schruff, M.C., Spielman, M., Tiwari, S., Adams, S., Fenby, N., and Scott, R.J. (2006). The *AUXIN RESPONSE FACTOR 2* gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. *Development* **133**: 251–61.

Scott, R.J., Spielman, M., Bailey, J., and Dickinson, H.G. (1998). Parent-of-origin effects on seed development in *Arabidopsis thaliana. Development* **125**: 3329–41.

Stadler, R., Lauterbach, C., and Sauer, N. (2005). Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in *Arabidopsis* seeds and embryos. *Plant Physiology* **139**: 701–712.

Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.-Y., Dolezal, K., Schlereth, A., Jürgens, G., and Alonso, J.M. (2008). TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. *Cell* **133**: 177–91.

Stepanova, A.N., Yun, J., Robles, L.M., Novak, O., He, W., Guo, H., Ljung, K., and Alonso, J.M. (2011). The *Arabidopsis* YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. *The Plant Cell* **23**: 3961–73.

Sun, X., Shantharaj, D., Kang, X., and Ni, M. (2010). Transcriptional and hormonal signaling control of *Arabidopsis* seed development. *Current Opinion in Plant Biology* **13**: 611–20.

Taiz, L. and Zeiger, E. (2002). Plant Physiology, 3rd edition. *Sinauer Associates*, pp. 435–441.

Tan, X., Calderon-Villalobos, L.I.A., Sharon, M., Zheng, C., Robinson, C. V, Estelle, M., and Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**: 640–5.

Tian, Q. and Reed, J. (1999). Control of auxin-regulated root development by the *Arabidopsis thaliana SHY2/IAA3* gene. *Development* **126**: 711–21.

Ungru, A., Nowack, M.K., Reymond, M., Shirzadi, R., Kumar, M., Biewers, S., Grini, P.E., and Schnittger, A. (2008). Natural variation in the degree of autonomous endosperm formation reveals independence and constraints of embryo growth during seed development in *Arabidopsis thaliana*. *Genetics* **179**: 829–41.

Vernoux, T., Brunoud, G., Farcot, E., Morin, V., Van den Daele, H., Legrand, J., Oliva, M., Das, P., Larrieu, A., Wells, D., Guédon, Y., Armitage, L., Picard, F., Guyomarc'h, S., Cellier, C., Parry, G., Koumproglou, R., Doonan, J.H., Estelle, M., Godin, C., *et al.* (2011). The auxin signalling network translates dynamic input into robust patterning at the shoot apex. *Molecular Systems Biology* **7**: 508.

Vivian-Smith, A. and Koltunow, A. (1999). Genetic analysis of growth-regulator-induced parthenocarpy in *Arabidopsis*. *Plant Physiology* **121**: 437–452.

Vivian-Smith, A., Luo, M., Chaudhury, A., and Koltunow, A. (2001). Fruit development is actively restricted in the absence of fertilization in *Arabidopsis*. *Development* **128**: 2321–31.

Weijers, D., Van Hamburg, J.-P., Van Rijn, E., Hooykaas, P.J.J., and Offringa, R. (2003). Diphtheria toxin-mediated cell ablation reveals interregional communication during *Arabidopsis* seed development. *Plant Physiology* **133**: 1882–1892.

Wolff, P., Weinhofer, I., Seguin, J., Roszak, P., Beisel, C., Donoghue, M.T.A., Spillane, C., Nordborg, M., Rehmsmeier, M., and Köhler, C. (2011). High-resolution analysis of parent-of-origin allelic expression in the *Arabidopsis* endosperm. *PLoS Genetics* **7**: e1002126.

Woodward, A.W. and Bartel, B. (2005). Auxin: regulation, action, and interaction. *Annals of Botany* **95**: 707–35.

Yang, C.H., Chen, L.J., and Sung, Z.R. (1995). Genetic regulation of shoot development in *Arabidopsis*: role of the *EMF* genes. *Developmental Biology* **169**: 421–35.

Yang, Y., Hammes, U.Z., Taylor, C.G., Schachtman, D.P., and Nielsen, E. (2006). High-affinity auxin transport by the AUX1 influx carrier protein. *Current Biology* **16**: 1123–7.

Zhao, Y. (2012). Auxin biosynthesis: a simple two-step pathway converts tryptophan to indole-3-acetic acid in plants. *Molecular Plant* **5**: 334–8.

Zhou, Y., Zhang, X., Kang, X., Zhao, X., Zhang, X., and Ni, M. (2009). SHORT HYPOCOTYL UNDER BLUE1 associates with *MINISEED3* and *HAIKU2* promoters *in vivo* to regulate *Arabidopsis* seed development. *The Plant Cell* **21**: 106–117.

6. Appendix

Table 6-1 Mutant lines used in this study.

Name	Locus	Type	Accession	Published
agl62-2	AT5G60440	T-DNA (SALK_022148)	Col-0	Kang et al. (2008)
emf2-5	AT5G51230	EMS	L*er*	Yang et al. (1995)
fis2-5	AT2G35670	T-DNA (SALK_009910)	Col-0	Alonso et al. (2003)
vrn2-1	AT4G16845	EMS	L*er*	Chandler et al. (1996)
yucca6-2D	AT5G25620	T-DNA (SALK_019589)	Col-0	Kim et al. (2007)

Table 6-2 Reporter lines used in this study.

Construct	Accession	Source
YUC6pro:eGFP-GUS	Col-0	Provided by Hélène Robert-Boisivon (Developmental and Cell Biology of Plants, Central-European Technology Institute)
YUC10pro:nls3xGFP	Col-0	Provided by Dolf Weijers (Laboratory of Biochemistry, Wageningen University)
YUC11pro:eGFP-GUS	Col-0	Provided by Hélène Robert-Boisivon (Developmental and Cell Biology of Plants, Central-European Technology Institute)
GH3.3pro:GUS	Col-0	Gutierrez and Mongelard *et al.* (2012)

Table 6-3 Primers used in this study. Sequences in bold are T-DNA-specific primers, in lowercase are Gateway adaptors and underlined nucleotides are point mutations.

Primer description		Sequence (5' – 3')
Genotyping of *agl62-2*	fw	**ATTTTGCCGATTTCGGAAC**
	rv	AAGCATTGTTTCCAAAGGGTGG
Genotyping of *emf2-5* (mutation creates additional MseI site in amplicon)	fw	CGTTTCTCCTAAGCCTGTCG
	rv	TGGTAATGCCCAAAGATACATAAC
Genotyping of *fis2-5*	fw	**ATTTTGCCGATTTCGGAAC**
	rv	AAACCGAACCAGTTTTCATACC
Genotyping of *vrn2-1* (mutation creates XmnI site in amplicon)	fw	TGCGTTCATTAAGTAGGCAACAGAAAATGG
	rv	GAGAAGTAGTTACCTTTGTTTTCTTACAGAAGAGT
Genotyping of *yucca6-2D*	fw	**TCACATCAAAACTAACAATCTCTCTC**
	rv	GGTGACGACGCAGATACGAC
Flanking primers IAA5	fw	ggggacaagtttgtacaaaaaagcaggctCATCAAAGATGGCGAATGAGA

Primer description		Sequence (5' – 3')
	rv	ggggaccactttgtacaagaaagctgggtTCATTCATCCTCTGTTACATGATCT
Mutation primers IAA5$_{P58->L}$	fw	GTTGGCTACCGGTTTGTTCGTA
	rv	AACCGGTAGCCAACCCACAACT
Flanking primers IAA10	fw	ggggacaagtttgtacaaaaaagcaggctAATTTGGGATAATGAATGGTTTG
	rv	ggggaccactttgtacaagaaagctgggtTCTCTACTTACCTACTCCAGCTCCA
Mutation primers IAA10$_{P53->L}$	fw	GGTTGGCTGCCTCTACGGACTTAC
	rv	GTAGAGGCAGCCAACCTACAGCAAC
Flanking primers IAA28	fw	ggggacaagtttgtacaaaaaagcaggctTTAGAAAAATGGAAGAAGAAAAGAGAT
	rv	ggggaccactttgtacaagaaagctgggtGATTTTGGCCAACCTCTCTC
Mutation primers IAA28$_{P98->L}$	fw	GGGATGGCTGCCGGTGAGATCAT
	rv	TCACCGGCAGCCATCCCACCAC
Flanking primers IAA32	fw	ggggacaagtttgtacaaaaaagcaggctCATCAATGGACCCAAACACA
	rv	ggggaccactttgtacaagaaagctgggtTTAAAAGGGAAGAAGAGCATCG
RT-qPCR - AT4G26280 - sulfotransferase family protein	fw	TCGTCGCATCGTACCCAAAA
	rv	TGATGAGAGGAGGTTGTGAGG
RT-qPCR - AT2G01520 - MLP328 (MLP-like protein 328)	fw	CTCGCTGCTGACATGGATGA
	rv	GGAGCAATGTACGTATGAGGGT
RT-qPCR - AT2G44840 - ERF13 (Ethylene Response Factor 13)	fw	AACGGCGTTAACTCACGGAT
	rv	TCGTCGACACTTAACGGCAA
RT-qPCR - AT3G51600 - LTP5 (Lipid Transfer Protein 5)	fw	GAGAGCCTTGGGTTCTCGAC
	rv	CCTGACGGTGTTACAGTTGGT
RT-qPCR - AT4G34800 - SAUR-like (Small Auxin Up RNA)	fw	TGGCGATTAGATTGTCGCGT
	rv	AACCGCAACATGTCCTTTCG
RT-qPCR - AT1G02400 - GA2OX6	fw	CTTTCAAGTTCAGCTCGGCG
	rv	TCGCTAGACTTCTGTCCCCA
RT-qPCR - AT1G30040 - GA2OX2	fw	AAGATGGAAGTTGGGTCGCT
	rv	CTCCCGTTAGTCATAACCTGAAGA
RT-qPCR - AT5G64120 - Peroxidase	fw	CCGGTCCGAACCTCAATCTC
	rv	AGCCTGTTCCTTGAGTGAGAA
RT-qPCR - AT1G28130 - IAA-amido synthase	fw	TCCTCACTAGTTCGGGGACTT
	rv	TCCCTCATCTAGCCCATCCA
RT-qPCR - AT1G68040 - Methyltransferase	fw	TGGTTGTTGGAATGTGTGGGA
	rv	TTCGCTGATCAAACCCTCGG
RT-qPCR - AT1G69960 – PP2A	fw	TAACGTGGCCAAAATGATGC
	rv	GTTCTCCACAACCGCTTGGT

Table 6-4 Excitation and detection wavelengths used for each fluorophore. Emission peaks are indicated between parentheses.

Fluorophore	Excitation (nm)	Detection (nm)
Alexa Fluor 488	514	493-630 (520)
DAPI	405	410-585 (460)
GFP	488	499-543 (508)
PI	514	593-719 (620)

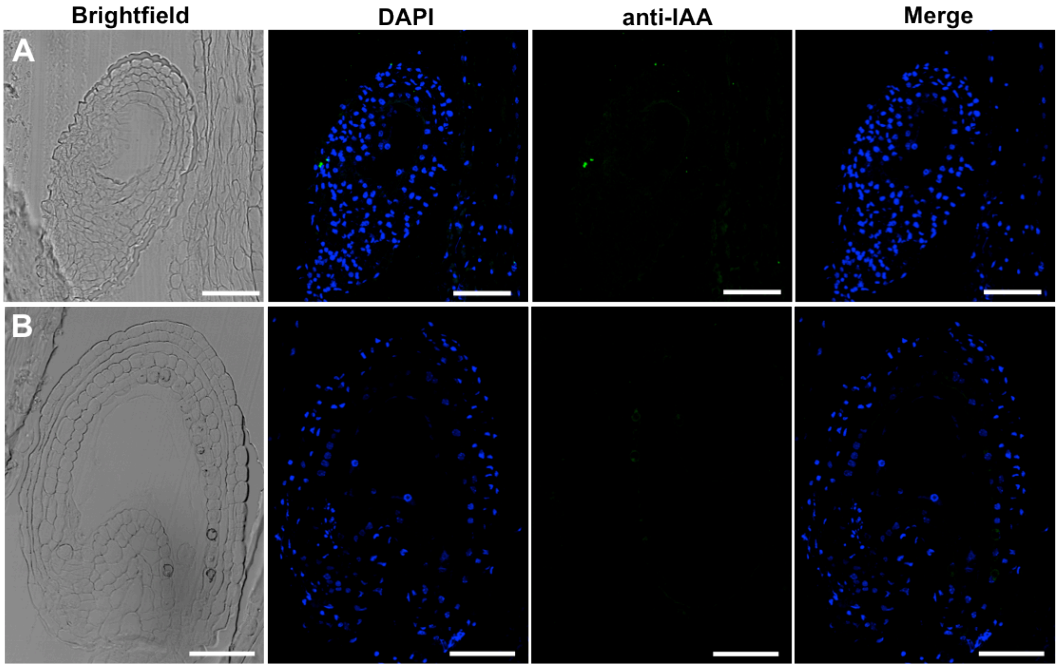

Figure 6-1 IAA immunolocalization controls. Confocal laser scanning microscopy images of seed sections. **(A)** Primary antibody pre-block **(B)** Negative control. Panels show (from left to right) brightfield, DAPI, anti-IAA and merge between DAPI and anti-IAA. Bars= 50 µm.

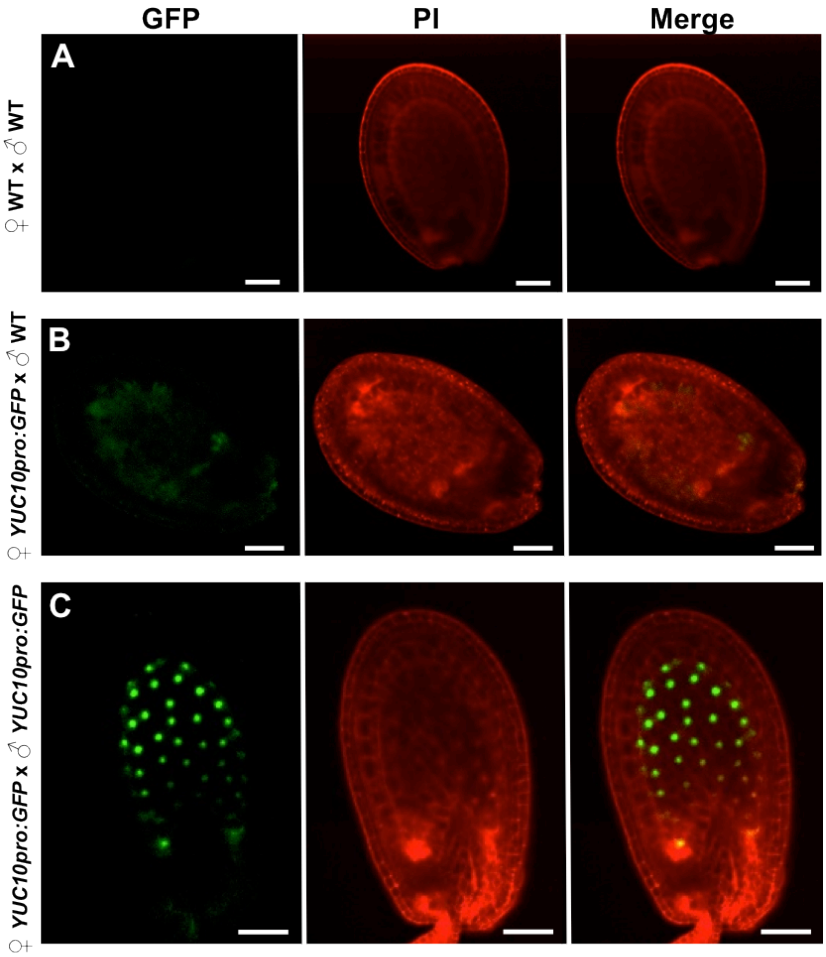

Figure 6-2 Reciprocal crosses between *YUC10pro:GFP* transgenic lines and WT. Confocal laser scanning microscopy of WT and *YUC10pro:GFP* seeds. Maternal and paternal plants used to perform the crosses are indicated on the left side of each panel. Panel shows (from left to right) GFP signal in green, PI in red and the merge between GFP and PI channels. Bars = 50 µm.

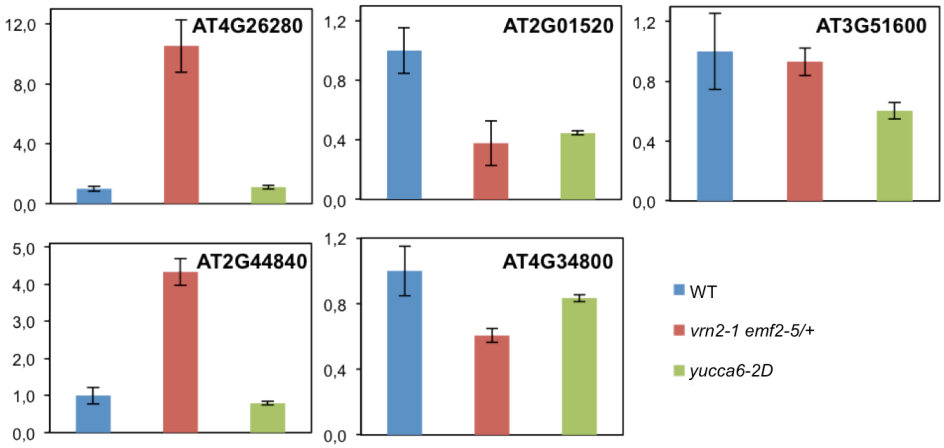

Figure 6-3 Transcript analysis of upregulated genes in microarray data of *vrn2-1 emf2-5* ovules. RT-qPCR performed on WT (blue), *vrn2-1 emf2-5/+* (red) and *yucca6-2D* (green) ovules at 4DAE. Tested genes are indicated in the upper right corner of each graph. Values are normalized to WT. Error bars represent SD.

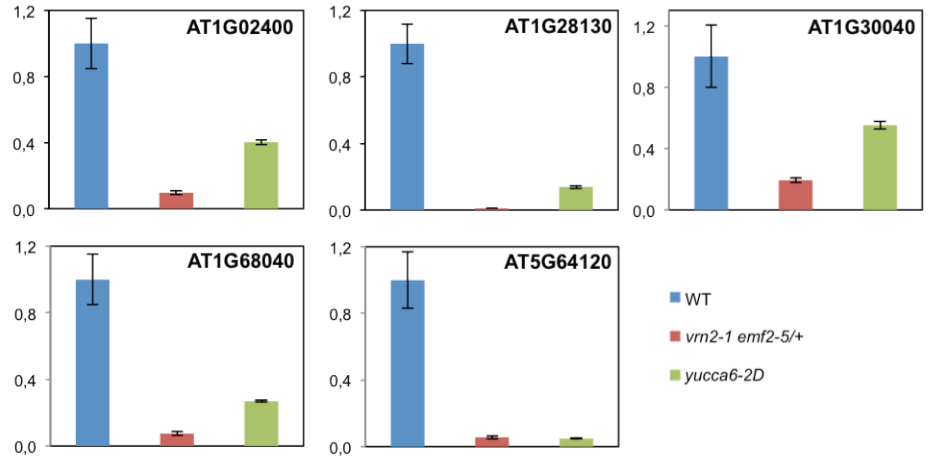

Figure 6-4 Transcript analysis of downregulated genes in microarray data of *vrn2-1 emf2-5* ovules. RT-qPCR performed on WT (blue), *vrn2-1 emf2-5/+* (red) and *yucca6-2D* (green) ovules at 4DAE. Tested genes are indicated at the upper right corner of each graph. Values are normalized to WT. Error bars represent SD.