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Seed coat formation: its evolution and regulation

Angel J. Matilla

Department of Functional Biology, Life Campus, Faculty of Pharmacy, University of Santiago de Compostela (USC), 15782-Santiago de Compostela, Spain

Review Paper

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Author for correspondence:
Angel J. Matilla, E-mail: angeljesus.matilla@usc.es

Abstract

In higher plants, the seed precursor (ovule primordia) is composed of three parts: funiculus, nucellus and chalaza, generating the latter one (II) or two (OI and II) protective maternal integuments (seed coat, SC). The appearance of a viable seed requires the coordinate growth and development of the preceding three compartments. Integuments are essentials for seed life as they nourish, protect and facilitate seed dispersion. Endosperm and integument growth and development are tightly coupled. Gymnosperm and angiosperm ovules are commonly unitegmic and bitegmic, respectively. Unusually, ategmy and threetegmy (OI, II and aril) also exist. The expression of the *INO*, *ATS* and *ETT* genes, involved in integument development, seems to have demonstrated that the fusion of OI and II leads to the appearance of unitegmy in higher plants. Likewise, *INO* expression also manifests the conservation of OI during evolution. The molecular control of SC development is constituted by a signalling network with still a multitude of gaps. The fertilization-independent development of the ovule is repressed by the FERTILIZATION INDEPENDENT SEED (FIS), a Polycomb-Repressive-Complex-2 (PRC2). Both endosperm and SC development are tightly linked to PRC2 function. As in many other developmental processes, auxin plays an essential role during ovule and SC development. Auxin transport from the endosperm to the integuments is regulated by AGL62 (AGAMOUS-LIKE 62), the encoding gene of which is specifically expressed in the endosperm to suppress its cellularization. In the absence of AGL62 (i.e. *agl62* mutants), auxin remains trapped in the endosperm and the SC fails to develop (i.e. seed abortion). This update shows that auxin biosynthesis, transport and signalling play a predominant role and seem to be absolutely required in the pathway(s) that lead to SC formation, most likely not as a unique hormonal component.

Introduction

The ovule, contemporaneous with the origin of leaves, is the plant reproductive and lateral organ where the fertilization, zygotic embryogenesis and finally the development of seeds, takes place (Galbiati *et al*., 2013). Ovule diversity has a relatively stable feature in evolution (Endress, 2011a). In Arabidopsis, the ovule is constituted of the funiculus (which transports nutrients, via the phloem, from the mother plant), the nucellus (which originates the female gametophyte) and the chalaza (CZSC; originates both outer (OI) and inner (II) integuments). The seed is the reproductive entity of flowering plants constituted by the embryo and the endosperm, which are two fertilization products surrounded by an entirely maternal (diploid) and non-fertilized covering named the seed coat (SC) (Olsen, 2004; Ingram, 2010; Endress, 2011a,b). After fertilization, the integuments give rise to the SC, which in angiosperms comprises the testa and tegmen, derived from the OI and II, respectively. Contrary to the maternal SC, essential loci are expressed in the endosperm from the paternal genome immediately after fertilization (Aw *et al*., 2010). Thus, by using *cdka1* mutants (CYCLIN DEPENDENT KINASE A1, also known as CDC2) the failure of karyogamy (i.e. fusion of two haploid nuclei during sexual reproduction) was achieved in the central cell, preventing the incorporation of the paternal genome and endosperm development. Does this mean that the inheritance of integuments is maternally controlled (Berger *et al*., 2006)? In short, the SC does not take part in the fertilization process and it is derived from the ovule integument(s) after having undergone some of the most striking known embryogenic differentiation processes (Radchuk and Borisjuk, 2014; Coen *et al*., 2017; Coen and Magnani, 2018). However, the factor(s) triggering integument development are yet to be described in detail. As discussed below, it is suggested that the starting signal(s) is generated in the fertilized central cell/endosperm and the phytohormone auxin could be a strong candidate (Figueiredo *et al*., 2016; Figueiredo and Köhler, 2018). The signalling pathway initiated by the growing endosperm is required for the formation of all integuments. This process proceeds through the action of AGL62, an AGAMOUS-LIKE MADS-box transcription factor (TF) specifically expressed in the syncytial endosperm (Roszak and Köhler, 2011; Figueiredo *et al*., 2016). Plants lacking AGL62 show a

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premature endosperm cellularization and a seed-lethal phenotype. In response to this endosperm key signal, the SC cell layers (integuments) undergo a rapid phase of cell division and expansion, and follow different cell fates (Haughn and Chaudhury, 2005). In contrast, the elimination of the SC or nucellus is triggered by the endosperm (Xu *et al*., 2016). All Arabidopsis integuments were thought to respond homogeneously to the fertilization of the central cell and to grow in a coordinated way with the endosperm (Coen *et al*., 2017). However, it was recently demonstrated that ii1', a highly vacuolated sub-epidermal cell layer from Arabidopsis, has a unique developmental program (Coen *et al*., 2017). This interaction between the endosperm and integuments determines the final size of the seed (Li and Li, 2015; Orozco-Arroyo *et al*., 2015). Accordingly, (i) defects in SC growth (e.g. *transparent testa glabra 2*; *TTG2*) may negatively impact endosperm growth (García *et al*., 2005); (ii) deterioration of endosperm proliferation has a harmful effect on SC development (García *et al*., 2003, 2005; Luo *et al*., 2005); and (iii) increased integument cell proliferation (e.g. *megaintegumenta/auxin responsive factor2*) results in enlarged seeds with more abundant endosperm (Schruff *et al*., 2006).

Integuments are the layers of tissue that usually cover the ovule, enveloping the nucellus and forming the micropyle (i.e. a small opening through which the pollen tube penetrates for ovule fertilization) at the seed apex. In Arabidopsis, the beginning of seed integument development is characterized by an increase in mitotic activity after fertilization, followed by a sharp decline leading to a complete absence of cell division 4 days after pollination (DAP) (García *et al*., 2005; Locascio *et al*., 2014). Likewise, after fertilization the integument cell layers display expansion growth that is not homogeneous (Coen *et al*., 2017). Integument II arises first, followed closely by the OI (Endress, 2011a). This event is supported by the fact that the absence of the II is always accompanied in known mutants by the absence of the OI. However, the micropyle only crosses integument II in the most ancient angiosperm *Amborella trichopoda* (Tobe *et al*., 2000). Among other important peculiarities, the integuments provide and constitute the cavity for the growth of both embryo and endosperm after fertilization (Roszak and Köhler, 2011; Lafon-Placette and Köhler, 2014; Li and Li, 2015). As SC growth proceeds by a process driven mostly by endosperm cell elongation, it is most likely that auxin synthesized in the fertilized central cell/endosperm and then exported to the integuments, is the trigger for SC formation (Locascio *et al*., 2014; Figueiredo *et al*., 2016). The SC grows from the CZSC and protects and ensures the effective response of the seed to both biotic and abiotic factors to reach seed maturity and facilitates the balance between seed dormancy and germination (Radchuk and Borisjuk, 2014). Taken together, once the SC, the endosperm and the embryo are completely differentiated, the storage compounds accumulate, desiccation tolerance is acquired, and, finally, primary seed dormancy is triggered (Graeber *et al*., 2012; Harada and Pelletier, 2012; Smÿkal *et al*., 2014; Chahtane *et al*., 2017).

Accordingly, angiosperm evolution kept the SC as a multifunctional and key organ for viable seeds (Linkies *et al*., 2010). That is, the SC is not only a mechanical and chemical protective envelope, but also a dynamic tissue that plays an indispensable role in a diverse set of processes such as maintenance of the balance between dormancy and germination, imbibition and nutrition of the endosperm and growing embryo (Beeckman *et al*., 2000; Graeber *et al*., 2012; Vogiatzaki *et al*., 2017; Carrillo-Barral *et al*., 2018). In *Arabidopsis thaliana*, sugars are unloaded to the SC via the funicular phloem, which is symplastically connected to the integument OI (Stadler *et al*., 2005). Thus, three members of the SWEET transporter family are directly responsible for the translocation of sucrose through the integuments (Chen *et al*., 2015). The CZSC is the zone where the vascular vessels end in many seeds, including Arabidopsis. Therefore, CZSC represents an area of nutrients unloading and an important interface connecting the maternal and filial tissues (Patrick and Offler, 2001; Stadler *et al*., 2005; Vogiatzaki *et al*., 2017). It is not surprising then that a considerable number of genes involved in water, sugar or amino acid transport are more expressed in the maturing zone of CZSC than in the distal SC (Obroucheva, 2013; Khan *et al*., 2014). However, *A. trichopoda* lacks vascular bundles in the integuments (Tobe *et al*., 2000). The absence of plasmodesmata between SC and filial tissues indicates that they are apoplastically connected (Patrick and Offler, 2001; Zhang *et al*., 2007). Apart from this, the SC exerts its germination-restrictive action most of the time by its mechanical resistance to radicle protrusion (Rodríguez-Gacio *et al*., 2012; Carrillo-Barral *et al*., 2018; Steinbrecher and Leubner-Metzger, 2018). In this context, *A. thaliana* SC mutants affected in testa shape and pigmentation, and which display reduced dormancy, confirms the importance of the seed envelope in the control of dormancy and germination, and highlight the importance of the testa as a restriction to radicle emergence (Debeaujon *et al*., 2000; Bentsink and Koornneef, 2008; Rodríguez-Gacio *et al*., 2012; Steinbrecher and Leubner-Metzger, 2018). On the other hand, the requirement for gibberellins (GAs) in *A. thaliana* seed germination is determined by the SC characteristics, embryonic growth potential and embryonic abscisic acid (ABA) (Urbanova and Leubner-Metzger, 2016; Nonogaki, 2019). Inhibition of ABA catabolism delays germination by inhibiting SC softening in Arabidopsis (Lee *et al*., 2010). Interestingly, this last feature has been confirmed in distantly related species. Together, the degradation of the SC surrounding the micropylar area makes the protrusion of the elongated embryonic axis more feasible. Recently, it has been shown in Arabidopsis that endosperm cell expansion is a key component of germination (Sánchez-Montesino *et al*., 2019). All of these elongation and weakening processes require the hydrolysis of cell wall (CW) components for seed germination to occur (Holdsworth *et al*., 2008; Linkies *et al*., 2009; Lee *et al*., 2010; Rodríguez-Gacio *et al*., 2012). In addition to GAs and ABA, auxin appears to be involved in CW softening. Auxin is well known to induce changes in CW mechanical properties and CW synthesis. Auxin-induced organ outgrowth requires demethylesterification of pectins, which causes CW loosening (Braybrook and Peaucelle, 2013). This pectin degradation process inhibits, *inter alia*, endosperm cellularization in Arabidopsis (Wolf *et al*., 2015; Batista *et al*., 2019).

Unlike the gymnosperms, which only have one single integument, angiosperms have two (i.e. OI and II) which can be either two- or multi-cell layered (Endress, 2011b; Coen *et al*., 2017). As the seed matures, each layer acquires a specific fate. Thus, the outermost cell layer of the OI (oi2) produces mucilage, and usually forms a dead covering layer in mature seeds (Debeaujon *et al*., 2007; Huang *et al*., 2011; Western, 2012; Tsai *et al*., 2017). Mucilage accumulation leads to contraction of the cytoplasm that remains confined to the centre of the cell. After a while, the cytoplasm columns are replaced by CW components, thus forming the columella. Once all cell layer structures have crashed, the seed shape will hold (Haughn and Chaudhury, 2005). When imbibition occurs, the mucilage is apoplastically released,

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promoting a moist environment during seed germination (Western, 2012). However, the inner cell layer (oi1) may remain viable (Figueiredo and Köhler, 2014), whereas ii2 and ii1' undergo programmed cell death (PCD) during seed development (Domínguez and Cejudo, 2014). In Arabidopsis, the integument II has three layers and the innermost named endothelium (ii1) contacts directly with the endosperm cells and synthesizes, shortly after fertilization, flavonoid-derived tannins called proanthocyanidins (PAs) (Francoz *et al.*, 2018). Later in development PAs are released, oxidized and spread to the remaining SC layers conferring a brown colour to the seed (Lepiniec *et al.*, 2006). Regarding the release of PAs, recent studies have demonstrated that these tannins are stored within the vacuole. Therefore, PAs and/or their precursors must be shuttled across the tonoplast. The elimination of the *AHA10* gene, involved in encoding a putative P-type ATPase H^+ pump in Arabidopsis SC, disrupts the production of PAs resulting in pale SC phenotypes with increased levels of PA precursors (Baxter *et al.*, 2005; González *et al.*, 2016). It is noteworthy that PAs also sometimes act as germination inhibitors (Ma *et al.*, 2015; Shah *et al.*, 2018). PAs inhibit seed germination by affecting ABA, GA and ROS regulatory genes (Jia *et al.*, 2012). The absence of the endothelium results in seed abortion (Mizzotti *et al.*, 2012). Although no endothelium exists in the ancient *A. trichopoda*, the micropyle-adjacent cells are usually tanniniferous (Tobe *et al.*, 2000). On the other hand, seed development relies on a coordinated interaction between the outer environment, the SC, the embryo and the endosperm (Radchuk and Borisjuk, 2014). Thus, the environmental cues and flow of nutrients from the mother plant to the endosperm and embryo must pass through the SC (Chen *et al.*, 2015). All this trafficking is controlled at genetic, epigenetic, metabolic and hormonal levels in order to direct seed development (Haughn and Chaudhury, 2005; Köhler and Makarevich, 2006; Köhler and Kradolfer, 2011; Figueiredo and Köhler, 2014). However, it is not yet known how this complex trafficking network is controlled. In addition, during seed development, maternal tissues enclosing the embryo (i.e. nucellus, SC in dicots, pericarp in monocots and nucellar projections) undergo a progressive degeneration or, where appropriate, disappearance by PCD (Domínguez and Cejudo, 2014; Bastos-Lima *et al.*, 2015; Lu and Magnani, 2018; Matilla, 2019). Based on the above and current progress (e.g. Villanueva *et al.*, 1999; Debeaujon *et al.*, 2000; Yamada *et al.*, 2003; Haughn and Chaudhury, 2005; Reinheimer and Kellogg, 2009; Lee *et al.*, 2010; Roszak and Köhler, 2011; Liu *et al.*, 2013; Locascio *et al.*, 2014), this update emphasizes the evolution of the SC, its biochemical characteristics and genetic control of its development by auxin, adding to the understanding of the roles of the SC in higher plants.

Evolutionary alterations of the seed coat: unitegmy and bitegmy

Cells from both OI and II undergo prompt growth and differentiation once the ovule has been fertilized (Haughn and Chaudhury, 2005). These processes are coordinated with the endosperm (Coen *et al.*, 2017). The OI grows faster than the II and extends beyond the apex of the nucellus first (Souza-Caetano *et al.*, 2018). The II grows as a radially symmetrical structure to envelop the terminal nucellus whereas OI grows asymmetrically. OI shows a curvature that is distinct from II, due to the faster cell division rate in the abaxial than adaxial side (Fig. 1). After a time of fertilized ovule development, II and OI

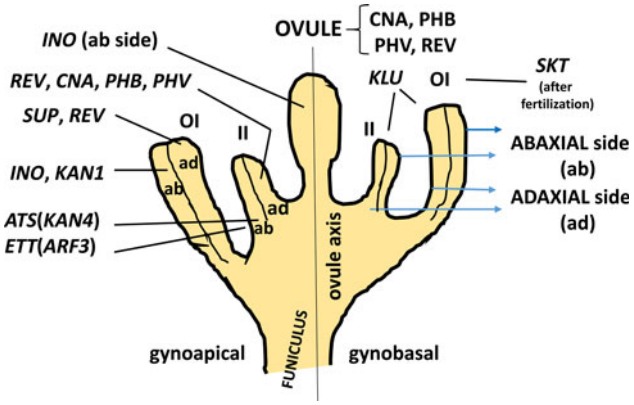


Fig. 1. The spatial distribution of genes known in Arabidopsis to regulate seed integument formation and ovule development. More details are listed in Table 1. ATS, aberrant testa shape; CNA, corona; ETT, ettin; INO, inner no outer; KLU, P450 kluh; PHB, phabulosa; PHV, phavoluta; REV, revolute; SKT, seedstick; SUP, superman.

form the SC (Skinner *et al.*, 2004; Colombo *et al.*, 2008). The SC of different species undergoes similar phases of development in relation to the endosperm and embryo. Nevertheless, the SC and endosperm develop first in legumes (Weber *et al.*, 2005). However, SC characteristics, such as composition, structure and thickness have evolved to adapt to the environment of the mother plant. Regarding SC thickness, this property is considered as a stable character and affects seed germination, dormancy and mortality (Linkies *et al.*, 2010; Endress, 2011a). Data obtained from a large number of species belonging to diverse families indicate that the SC is different in architecture and thickness (Beeckman *et al.*, 2000; Coen and Magnani, 2018). Thus, the ovule can be covered in three ways. The first is the ancestral and more common bitegmic ovule covered by II and OI, respectively. Among other species, this is the case in *Arabidopsis thaliana* (Coen *et al.*, 2017), *Prunus persica* and *P. armeniaca* (Lora *et al.*, 2015) and *Oryza sativa* (Endress, 2011b). It is striking that the ancient *A. trichopoda* has also bitegmic ovules (Tobe *et al.*, 2000). However, the origin and homology of the OI is not clear (Doyle, 2006; Doyle *et al.*, 2008) and therefore the existence of OI in bitegmic angiosperms remains elusive. However, the II of angiosperms is probably homologous to the only integument in gymnosperms and their ancestors (Reinheimer and Kellogg, 2009). The second way is the unitegmic ovule as the earliest Paleozoic seeds (Gerrienne *et al.*, 2004), Solanaceae (Skinner *et al.*, 2016), *Prunus incisa* (Lora *et al.*, 2015), *Arabidopsis thaliana* mutant *aberrant testa shape* (*ats*) (McAbee *et al.*, 2006), Asterids (e.g. Petunia and *Solanum lycopersicum*) and Rosaceae (Endress, 2011b) and more common gymnosperms. The ovule is surrounded by only one integument in all these and other species (Gasser and Skinner, 2019). The unitegmy existing in extant gymnosperms is probably homologous to that of ancestral seed plants. Strikingly, although phylogenetic analyses suggest that bitegmy is the ancestral condition in angiosperms, unitegmic ovules have arisen independently several times during angiosperm evolution (Endress, 2011b; Gasser and Skinner, 2019). However, the reason(s) for the presence of a single integument in some angiosperms is unknown. Although there are many studies on unitegmy, it is not clear if its appearance in angiosperms was the result of the loss of one of the two integuments or, alternatively, was the fusion of OI and II due to the presence of intercalary growth between II and OI (Colombo *et al.*, 2008; Lora *et al.*,

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2015). McAbee *et al.* (2005) were able to show in *Impatiens* that the bitegmic to unitegmic transition was the result of congenital fusion of OI and II. The pattern of expression of *INO* orthologs was conserved in bitegmic and unitegmic species. Thus, INO shows almost identical expression in the outermost layer of Arabidopsis and tomato, despite poor similarity between promoter sequences (Skinner *et al.*, 2016). Genetic analyses in Arabidopsis suggest that integumentary fusion can be achieved through a lack of boundary formation between OI and II (Colombo *et al.*, 2008; Kelley *et al.*, 2012). This interpretation is confirmed by the analysis of molecular markers of boundary formation in *Prunus* bitegmic and unitegmic ovules (Lora *et al.*, 2015; Table 1). Moreover, the expression analyses in Santalales ategmic ovules, revealed that integument molecular markers are expressed at the distal end of the ovules (Brown *et al.*, 2010). These data have been interpreted as the fusion of an integument remnant to the nucellus. Regarding the fusion process, based on previous data and their own, Coen and Magnani (2018) point out two different and non-exclusive mechanisms: (a) total or partial lack of boundary formation between II and OI; and (b) intercalary growth between them. Finally, the third way for ovule coating is the ategmy found in some species of the order Santalales in which the integuments may be missing or poorly developed (Brown *et al.*, 2010). Remarkably, some taxa have a third envelope that originates from the funiculus, which becomes a fleshy structure and covers the SC (Endress, 2011a,b). This additional envelope is called the aril and occurs, *inter alia*, in *Passiflora* L., *Nymphaea* L. and gymnosperm *Taxus baccata* (Coen and Magnani, 2018). In this case the two integuments inside of the aril become dry and hard. Secondly, the rice seed modifies the number of integuments after fertilization; the reason(s) for this second process is still unknown (Krishnan and Dayanandan, 2003).

Given that the general morphology of the ovule is fairly well conserved in angiosperms and that these plants have two integuments (i.e. II and OI) and gymnosperms only one (i.e. II) (Gasser and Skinner, 2019), it is of interest to know what gymnosperm gene information disappeared and which remained and is exclusive for flowering plants (see next section and Table 1). Due to the absence of contrasting data in gymnosperms, it is necessary to compare ancient angiosperms, such as Amborella with recent angiosperms. The Amborellaceae family is the most ancient of angiosperms and consists of a single genus and a single species, Amborella, which has two-cell-layered OI and II. There are some reports that the II of angiosperms is considered a homologue to the single integument of gymnosperms (Reinheimer and Kellogg, 2009). In relation to this, some peculiarities about the evolution of SC thickness have been reviewed recently (Coen and Magnani, 2018). All these and other data may attempt to reveal why gymnosperms possess only II and why in many angiosperms II is covered by an OI or even also by a third layer called the aril. However, unless it is essential, evolution does not seem willing to increase the number of organs. An interesting reason to justify the presence of OI could well be to reinforce the protection of both ovule and seed. However, why then did II not become thicker and more resistant? An explanation could be that SC thickness is genetically considered a stable character (Endress, 2011a). Thus, it can be concluded that the origin and the homology of the OI is at present uncertain. Current hypotheses support the origin of OI from a leaf-like structure called the cupule, which was present in groups such as the glossopterids and *Caytonia*, possible sisters of angiosperms (Doyle, 2006). If that was the case, there must be a genetic homology between these leaf-like structures and the current OI. That is, homologous genes should be expressed in homologous structures (i.e. cupule and OI). Data of *YABBI* and *KANADI* (see Table 1) seem to be congruent with this evolutionary hypothesis (Gasser and Skinner, 2019; Jaramillo and Kramer, 2007). Separate origins for both OI and II are also supported at the molecular level as the control of both II and OI development occurs through different genes (Arnault *et al.*, 2018). In conclusion, given the small number of species studied until now, to explain why the appearance of OI is necessary, it is imperative to expand the range of angiosperm species to those evolutionarily close to gymnosperms and study gene expression and genetics in both wild-type and deficient mutants in key genes clearly involved in the appearance and development of OI.

Gene expression during SC development

It is well known in Arabidopsis that the development of abaxial and adaxial parts (Fig. 1) of both OI and II depends on distinct sets of genes (Arnault *et al.*, 2018). A series of them involved in the development of the ovule and, very specifically, in the growth and development of II and OI are shown in detail (Table 1). Among the listed genes, *INO*, *ANT*, *WUS*, *ETT* and *KAN* deserve attention for their roles in the initiation and differentiation of integuments and subsequent SC appearance. However, it is not known which of these genes were already present in early plants and which evolved later. This fact is evolutionarily significant and requires further investigation.

INNER NO OUTER (*INO*) is a TF of the *YABBY* gene family sufficient in Arabidopsis for OI initiation at the CZSC level (i.e. gynobasal side of the ovule; Fig. 1) and OI asymmetric growth. That is to say, *INO* is a positive regulator of OI growth and *ANT* works as a negative regulator that helps to establish the spatial pattern of *INO* expression (Villanueva *et al.*, 1999). Otherwise, *ANT* may promote OI development by facilitating II development and through the positive regulation of *INO* (Skinner *et al.*, 2016). Conservation of the specific expression pattern and function of *INO* orthologs in OI has been observed in all other angiosperm species in which *INO* has been examined (Yamada *et al.*, 2003, 2011; McAbee *et al.*, 2005; Lora *et al.*, 2011; Skinner *et al.*, 2016). In other words, *INO* was conserved in bitegmic and unitegmic species, indicating a likely conservation of *INO* gene function between Rosids and Asterids. Because *INO* is expressed only in the OI, it might serve as a marker for OI identity in the unitegmic process. Unfortunately, very few species of basal and higher angiosperms have been studied to date in order to explain the exact role of *INO* through evolution. However, expression studies in the basally diverging angiosperm *Nymphaea* suggest that *INO*'s tissue-specific role has been conserved from early stages of angiosperm evolution (Yamada *et al.*, 2003, 2011). Likewise, genetic analyses show that *LUG*, *SEU* and *STK* contribute to the growth of the OI (Bao *et al.*, 2010; Mizzotti *et al.*, 2014). Recently, it was confirmed that *INO* interacts with both *LUG* and *SEU* co-repressors and with the co-activator *PRZ1* (Simon *et al.*, 2017). Integrating the above data, *INO* has a bifunctional nature as it acts as both a repressor and activator of target genes. For more information about *INO* molecular properties, see Table 1. The similar expression patterns of the putative ortholog of *A. thaliana* ovule development gene *INO* was found inside intermediate integuments of *Impatiens* (i.e. an Ericales genus), supporting the hypothesis of integument fusion in this species with

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Table 1. Some genes and proteins involved in regulation of seed integuments and ovule development

Gene	Function	Mutant	Phenotype
AGAMOUS-like MADS-box 62 (*AGL62*)	Specifically expressed in endosperm. Required for promotion of nuclear proliferation. Suppresses cellularization during the syncytial phase of endosperm development. Is a direct target gene of *PRC2*	*agl62-2*	Premature endosperm cellularization. Seed lethal phenotype
AINTEGUMENTA (*ANT*)	Central role in regulating growth and cell number of integuments. This TF promotes initiation and growth of ovule integuments in CZSC. Determines the site of II growth. Encodes an APETALA2-like (AP2) TF. *ANT* has been detected in integuments of some unitegmic gymnosperms and in the surface layers of ategmic ovules	*ant* *ant stk*	Nude ovules. Reduced number of ovule primordia with respect to wild type. Over-expression of *ANT* in both Arabidopsis and tobacco resulted in large seeds. *ant stk* lacks integument development as in *ant*
SHORT INTEGUMENTS-2 (*SIN-2*)	Involved in cell division during integument development of the ovule	*sin-2*	Reduced integument growth in ovules
BELL1 (*BELL1*)	Controls integument morphogenesis and is involved in the acquisition of chalaza identity. Its expression is a pre-requisite for that of *INO. CAN*, *PHB* and *PHV* cooperatively collaborate with *BELL1* to confine *WUS* expression to the nucellus in Arabidopsis ovules. *CAN*, *PHB* and *PHV* establish the boundary between nucellus and chalaza	*bell-1*	Ovule fails to develop II. The OI is abnormal. A necklace appears in place of the integuments
WUSCHEL (*WUSCHEL*)	This TF regulates the induction of integument initiation from the nucellus. WUS and ANT work in connection to induce integument formation. Both TFs seems to act in independent pathways. SPL/NZZ negatively regulates the expression of *ANT* and *BELL-1* in the nucellus, while positively regulating *WUS* expression in which WUS acts downstream of SPL. However, *SPL/NZZ* expression in the chalaza is activated by ANT and BELL-1. Thus, WUS is activated by the TF SPL/NZZ in the ovule and promotes the expression of *WINDHOSE* (*WIH1/2*)	*wus-1*	Cannot produce integuments. Over-expression of *WUS* leads to appearance of additional integuments
KANADI (*KAN*)	Abaxial identity of ovules. Involved in integument differentiation. *KAN-1* and *KAN-2*, together with *ATS*, direct the growth of OI development. *KAN* is also present in *Selaginella* and *Physcomitrella*, suggesting an ancestral function of this gene family	*kan-1* and *kan-2*	Limited OI growth
ABERRANT TESTA SHAPE (*ATS/KAN4*)	Works redundantly in delimitating *INO* expression. Expressed in abaxial side of II and promotes its growth. Interacts with DELLA. Involved in differentiation of OI and II. UNICORN is an *ATS* interactor. *ATS* acts with *REVOLUTA (REV)* to restrict *INO* expression and OI growth	*ats*	OI and II develops to form a single thicker integument. Abnormal seed formation (e.g. heterogeneous seed size). Seeds phenotypically similar to *ett* mutant
YABBY (*YAB*)	Specific to seed plants. Abaxial identity in ovules. Promotes the expression of WUS-related TF in the margin		
INNER NO OUTER (*INO*)	*INO* is the only *YABBY* gene expressed in the abaxial side of ovule. Essential and sufficient for formation and asymmetric growth of OI. *INO* requires *ANT*. OI development depends of the interactions of *INO* with the co-repressors *LEUNIG* (*LUG*) and *SEUSS* (*SEU*; a co-repressive partner of *LUG*), and also with the co-activator *ADA2b/PROPORZ1* (*PRZ1*). *ADA2b* and *LUG* function independently to promote OI growth. *INO* can act as repressor or activator of target genes. Abaxial side of *Amborella*	*ino*, *seu*, *ada2b*, *lug* *ada2b lug*	Absence or abortive OI. Is phenotypically wild type. Similar deficiency in OI growth and extension. These mutations are epistatic to the *ino* mutation Are additive and synergistic in the double mutant
SUPERMAN (*SUP*)	Essential for suppressing adaxial growth of OI. Interferes with *INO* promoting ovule bilateral symmetry	*sup*	Equal OI growth on both sides of the ovule
SEEDSTICK (*STK*) (*AGL11*)	Controls, together with *SHP1* and *SHP2*, ovule integument identity. *STK* also controls PA metabolism in the seed coat and represses the	*stk-shp1-shp2* *stk*	Integuments transformed into carpelloid structures. *ANT* expression is not altered.

(*Continued*)

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Table 1. (*Continued.*)

Gene	Function	Mutant	Phenotype
	expression of seed coat regulators (e.g. *TT8*). Upon fertilization, *SKT* is expressed in the OI. *STK* is the only *Arabidopsis* gene known to control funiculus development. *STK* and *Arabidopsis B-sister* (*ABS*) act together to control formation of the endothelium	*stk-abs*	Smaller seeds Ovule and seed aborted. Lacks endothelium
TRANSPARENT TESTA (*TT*)	Regulation of PA biosynthetic process in seed coat. Expressed mainly in the ovule. It is a MADS BOX TF	*tt*	Impaired in flavonoid synthesis and accumulation in the seed coat
TRANSPARENT TESTA 16 (*TT16*)	Acts upstream of the PA biosynthetic pathway. Transcriptional control of PA synthesis in endothelium development. Necessary for PA synthesis with the exception of the chalazal-micropylar area. Control of endothelium differentiation and development. *TT16* and *STK* are redundant in promoting ii1' formation in the ovule. Promotes nucellus elimination and inhibits cell division	*tt16*	Accumulates PAs in the chalaza and micropyle while PAs are absent from the endothelium
TRANSPARENT TESTA GLABRA (*TTG*)	TTGs are regulatory proteins of seed development. TTG2 is a TF of the WRKY family expressed strongly in seed integument and a low level in the endosperm. *TTG2* is involved in maternal control of seed size. It is an activator of cell expansion in the seed coat	*ttg2* *ttg2-iku2*	Cell elongation of the integuments is damaged. Defective for mucilage deposition and PA synthesis (yellow seeds). Endosperm growth is limited and *ttg2* is sporophytic recessive. Prevents integument cell elongation more than in each single mutant
FERTILIZATION INDEPENDENT SEEDS (*FIS*)	Suppresses autonomous seed formation and represses endosperm development prior to fertilization. It prevents seed development without fertilization. Belongs to Polycomb-repressive Complex (PRCs) of proteins. Wild-type and *fis* mutant seeds are similar in seed coat development. *FIS2* is paternally imprinted and only expressed during early endosperm development	*fis*	Can bypass the fertilization requirement and initiates seed development in the absence of fertilization. In *fis2*, the repression of seed coat development remains active and autonomous endosperm division is not accompanied by seed coat development. *fis2-5* lacks endosperm cellularization and embryo growth
ETTIN (*ETT*)	*ETT* belongs to the Auxin Response Factor (ARF3) family. ETT-ATS complex is essential for integument development. Loss of either gene resulted in congenital fusion of the OI and II and altered seed morphology. *ETT* expression occurs in the boundary of the OI and II and is altered in unitegmy. *ATS* and *ETT* are co-expressed. *ARF4* is not required for integument development. Auxin induces modulation of *ETTIN*	*ett* *ett-3* pETT::ETT$^{2C\text{-}S}$ *arf2-9*	Reduced ovary development. Similar phenotype to *ATS* (unitegmic ovule). Aberrant ovule integument morphology. Abnormal overgrowth of OI and closure of funiculus. M-ore cells in the seed coat than the wild type (i.e. increased seed cavity)
CYTOCHROME P450 78A5 (*CYP78A5/KLU*)	*KLU* is expressed prior to fertilization in the OI. It is a maternal positive regulator of seed size. Its expression in non-cell autonomously stimulates cell proliferation in the seed coat	*klu-2*	Reduced number of cells in OI (i.e. lighter seeds II than the wild type). Seeds heavier when *KLU* is over-expressed
FERONIA (*FER*)	Plasma membrane receptor kinase involved in integument development. Markedly expressed alone in integuments of developing seeds. It is a repressor of cell expansion in the seed coat	*fer-4*	Larger cells in the OI but no difference in cell number compared with wild type

bitegmic (e.g. *I. hookeriana*), unitegmic (e.g. *I. walleriana*) and intermediate (e.g. *I. balsamina*) integument morphology (McAbee *et al.*, 2005). On the other hand, the authors indicate that the role of *INO* has been conserved between two divergent angiosperms, the rosid Arabidopsis and the asterid *Impatiens* (McAbee *et al.*, 2005). Likewise, an ortholog of Arabidopsis *INO* is similarly expressed in genus *Nymphaea* (*NaINO*), a diverging angiosperm (Yamada *et al.*, 2003).

On the other hand, the WUSCHEL (WUS) TF is necessary to prevent *INO* expression in developing II (Sieber *et al.*, 2004). Thus, ectopic *WUS* expression in the CZSC resulted in the formation of a single II and multiple OI (Gross-Hardt *et al.*, 2002; Sieber *et al.*, 2004). *WUS* is strictly expressed in the nucellar region with the highest level of expression coincident with integument initiation (Gross-Hardt *et al.*, 2002). It is likely that both *ANT* and *WUS* expression need to occur at a distance on the surface of the ovule to induce integument initiation (Skinner *et al.*, 2016), but it is a possibility that still requires thorough confirmation. However, what is clear is that *ANT* and *WUS* are essential for the formation of both OI and II. In the case of OI development, *ANT* and *WUS* interact with *INO* which positively affects *ANT* expression (Skinner *et al.*, 2016). Given that the over-expression of *WUS* results in supernumerary integuments (Sieber *et al.*, 2004), the dynamics of the expression of this TF could explain both the origin of II and the variable number of integuments observed in seeds. This variation fluctuates from bitegmy to the supernumerary integuments of taxa nested within otherwise unitegmic clades (Yang, 2004;

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Friis *et al*., 2011), to third integuments in ancestrally bitegmic clades (Endress, 2011b).

ARF3/ETTIN (ETT) is a master regulator of development and morphogenesis of the female reproductive structure (Simonini *et al*., 2016). *KANNADI* (*KAN4*/*ATS*) and *ETT* are expressed in the abaxial side of the II (Fig. 1) and act to remove auxin from the zone between the two integument primordia (Kelley *et al*., 2012). In certain species of unitegmic *Prunus*, expression of the *ETT* ortholog was absent from the CZSC and II (Lora *et al*., 2015). It was proposed that the loss of this expression represents a possible evolutionary mechanism for the reduction from two integuments to one in these species (Lora *et al*., 2015). In Arabidopsis, there is a clear division of functions between the II and OI, as *ATS* is the principal *KAN* family member expressed in II, while the role of *ATS* is played redundantly by *KAN1* and *KAN2* in OI (McAbee *et al*., 2006). *KAN* is also present in *Selaginella* and *Physcomitrella*, suggesting an ancestral function of this gene family (McAbee *et al*., 2006).

Taken together, it can be concluded that actual differences are ancient, and already existed in early flowering plants (Arnault *et al*., 2018). The evolution to unitegmy from bitegmy in angiosperms is not uniform. In unitegmic basal angiosperms and some derived groups it probably evolved mainly by the loss of the OI. However, in derived eudicots (e.g. Asterids) it is mainly by incorporation of the OI into II (Endress, 2011a). Taking the *ATS* task as an example (Table 1), it was demonstrated that loss of this function in the *ats-1* mutant leads to the fusion of II and OI that grow as a unit to produce a single fused structure (McAbee *et al*., 2006; Dorcey *et al*., 2009; Arnault *et al*., 2018). Interesting studies in unitegmic *Prunus* have demonstrated that expression of an *ETT* ortholog was absent from the chalaza and II. This loss of expression may be of evolutionary interest (i.e. reduction from two integuments to one in this Rosaceae). However, *ETT* is expressed in II in bitegmic species (Lora *et al*., 2015).

Auxin, a new player involved in SC formation and development

Given the great difficulty in isolating plant zygotes, the molecular and hormonal processes that take place as a result of fertilization are scarcely known. Nevertheless, the use of sophisticated methodologies is helping now to clarify it (Brunoud *et al*., 2012; Liao *et al*., 2015; Schon and Nodine, 2017; Robert *et al*., 2018). It is very likely that the onset of transcription and the activation of translation occur in the endosperm and the zygote (Aw *et al*., 2010). One of the events that sustains this assertion derives from the fact that the fertilized endosperm development is required to induce the autonomous initiation of SC (García *et al*., 2003; Aw *et al*., 2010; Hands *et al*., 2016; Figueiredo and Köhler, 2011, 2018). Therefore, removal of the endosperm inhibits SC development (Weijers *et al*., 2003) as developing endosperm produces molecular signals which are addressed to the integuments to initiate their formation (Ingram, 2010). Thus, endosperm and SC coordinate their growth through a cross-talk signalling pathway that was first identified in the study of the maternally acting *TTG2* (García *et al*., 2005; Ingram, 2010). The *ttg2* mutant shows premature arrest of endosperm development and reduced seed size (García *et al*., 2005; Ingram, 2010). Besides, mutations in genes belonging to the *HAIKU* family (*iku1* and *iku2*) produce seeds with reduced endosperm growth and early cellularization, which results in decreased seed size (Garcia *et al*., 2003). On the other hand, studies of endosperm development indicate that the presumed TF AGL62 (i.e. an AGAMOUS LIKE MADS-box domain protein) plays a determinant role in the development of central cell and/or endosperm (Figueiredo and Köhler, 2018). The expression pattern of *AGL62* and the phenotype of *agl62-2* (endosperm cellularizes prematurely and shows retention of auxin) suggest that AGL62 functions during the syncytial phase by suppressing the expression of genes required for endosperm cellularization (Kang *et al*., 2008). Another indication that the endosperm regulates SC growth comes from the characterization of the *agl62* mutant (Kang *et al*., 2008; Roszak and Köhler, 2011). Seeds of the *agl62* mutant show early endosperm cellularization and fail to develop a SC, despite the presence of a dividing endosperm. This suggests that AGL62 is crucial for the formation of the signal that initiates seed coat development. On the other hand, several studies have also shown that not only does the endosperm control SC growth, but that the reverse also happens. So, *TRANSPARENT TESTA GLABRA2* (*TTG2*) is strongly expressed in all SC layers and is known to be a part of the PA- and mucilage synthesis pathways (Johnson *et al*., 2002). The *ttg2* mutation causes defects in endosperm growth and cellularization and decreased SC cell elongation (Garcia *et al*., 2005). Furthermore, the *AGL62* expression pattern in *fis* mutant seed (i.e. endosperm cell is diploid) suggests that endosperm cellularization is triggered by suppression of *AGL62* at the end of the syncytial phase and that this suppression is mediated by the Fertilization Independent Seed (FIS) Polycomb Repressive Complex 2 (i.e. FIS-PRC2; Kang *et al*., 2008; Roszak and Köhler, 2011). The evolutionarily conserved Polycomb-Group (PcG) proteins are chromatin-associated factors contributing to the maintenance of transcriptional repression (Grossniklaus and Paro, 2014; Laugesen *et al*., 2019). The PcG are organized in multimeric complexes of which Polycomb Repressor Complex 1 and 2 (PRC1 and PRC2) are the best characterized. These proteins control a multitude of developmental transitions in plants (Derkacheva and Henning, 2014; Laugesen *et al*., 2019). The fertilization-independent development of the ovule is repressed by FERTILIZATION INDEPENDENT SEED (FIS), a PRC2 complex. In particular, the FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) FIS, both expressed in the nucellus and SC, act sporophytically to repress the differentiation of the integuments (Roszak and Köhler, 2011; Xu *et al*., 2016). FIS represses seed development in the absence of fertilization and is essential for seed growth. After fertilization of the central cell, the endosperm initiates a signal through the action of *AGL62*, relieving the *FIS* mediated repression and leading to the differentiation of the ovule integuments into the SC (Roszak and Köhler, 2011).

Figueiredo *et al*. (2016) suggest that auxin is the putative fertilization signal that coordinates endosperm and SC development. The important role of *AGL62* is demonstrated by the fact that once the PRC2 repression is relieved, *AGL62* leads to differentiation of the ovule integuments into SC (Roszak and Köhler, 2011). Thus, *AGL62* expression controls the timing of cellularization (Hehenberger *et al*., 2012) and seeds lacking this putative TF fail to initiate formation of the integuments (Roszak and Köhler, 2011; Coen *et al*., 2017). In short, the development of endosperm and SC is tightly linked to PRC2 function. In contrast, the initiation of embryo development is probably controlled by other repressive pathways unrelated to PRC2 (Roszak and Köhler, 2011). Although some components of the PRC2 complex show evolutionary conservation, the identity of the imprinted genes

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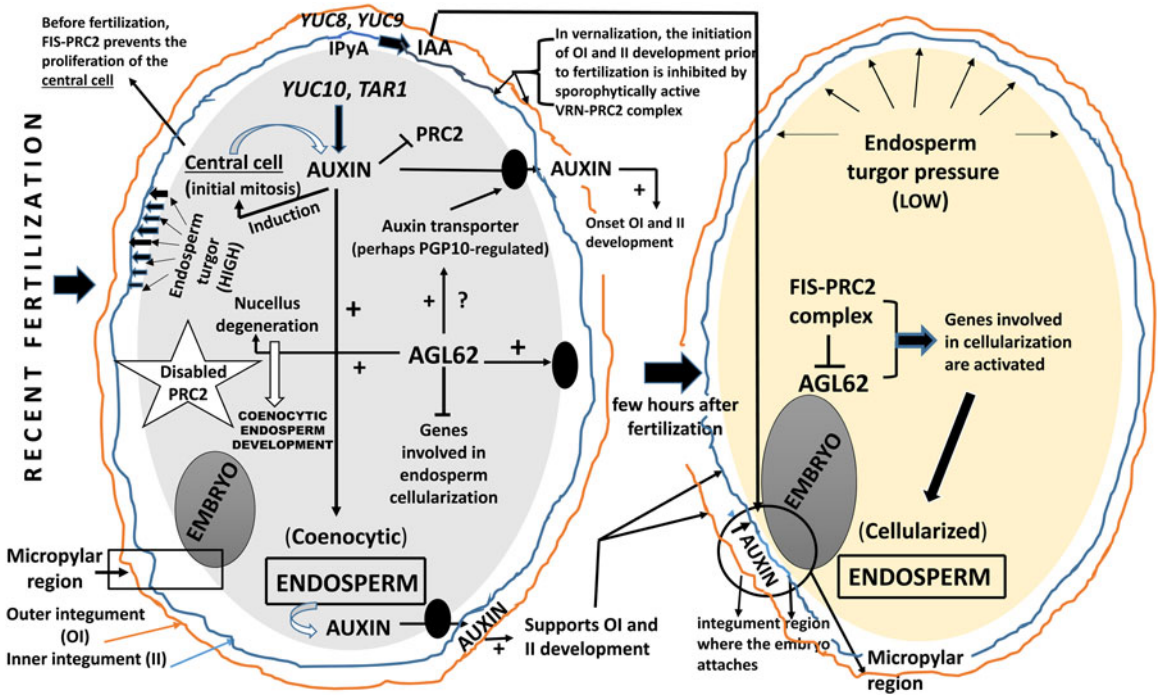


Fig. 2. Current status of the knowledge of auxin intervention in ovule fertilization and post-fertilization. It is important to note the mediation of AGL62 and PRC2 in the development of seed integuments and endosperm. AGL62, Agamous-like 62; IPyA, indolepyruvic acid; PRC2, polycomb-repressive complex 2; TAR, tryptophan aminotransferase-related; VRN, vernalization; YUC, YUCCA flavin monooxygenase.

encoding PRC2 components is not conserved (e.g. in cereal endosperm; Tonosaki and Kinoshita, 2015). Genomic imprinting is an epigenetic phenomenon rendering maternal and paternal alleles specifically expressed dependent on their parent of origin (Gehring, 2013). PRC2 blocks the SC development and this blocking seems necessary to be suppressed upon ovule fertilization. Thus, PRC2 must be strongly disabled following fertilization (i.e. phase of syncytial endosperm), whereas during cellularization of the endosperm negatively affects AGL62 (Figueiredo *et al.*, 2019). Recently, a subset of *AGL62* genes was subjected to FIS-PRC2-dependent maternal imprinting or FIS-PRC2-independent paternal imprinting, indicating a dual role for FIS-PRC2 in the regulation of its target genes during early endosperm development (Zhang *et al.*, 2018). Recently, Creff *et al.* (2015) have shown that during the onset of fertilization, endosperm-derived pressure is perceived in the innermost cell layer of the OI which subsequently undergoes thickening of its inner CW (for more details, see Fig. 1).

Until now, the role of phytohormones in zygotic embryogenesis mainly refers to the study of the eudicot *Arabidopsis thaliana*. Auxin involvement in the formation of the seed is now undisputed (Smit and Weijers, 2015) but very little is known about auxin biosynthesis and homeostasis, polar auxin transport and response during early embryogenesis in monocots. However, some of these features seem to be conserved in both monocot and dicot seeds (Robert *et al.*, 2018; Robert, 2019). At the beginning of the investigation of the possible intervention of auxins in SC formation, it has been shown that the female gametophyte cellularization is directly dependent on the establishment of an auxin gradient inside the gametophyte (Figueiredo *et al.*, 2019). This gradient defines the fates of the female gametophyte cells. Thus, the highest auxin concentration originates synergids, followed by egg cell and finally, the lowest concentrations originate the central cell and antipodals (Pagnussat *et al.*, 2009). In addition, female gametophyte development requires both localized auxin biosynthesis and auxin import from the sporophytic ovule (Panoli *et al.*, 2015). Although auxin is sufficient to initiate autonomous endosperm development in *A. thaliana*, by inducing the first few divisions of the central cell (maximal auxin response in the seed of monocot maize; Chen *et al.*, 2014), auxin alone is not sufficient to form a fully differentiated and cellularized endosperm (Figueiredo *et al.*, 2015). Recently, ample molecular evidence has emerged, suggesting that once fertilization has become effective and consolidated, auxin biosynthesis in the endosperm drives the development of its own endosperm and is responsible for the start of the appearance of the SC (Dorcey *et al.*, 2009; Figueiredo *et al.*, 2015; Smit and Weijers, 2015). It was also demonstrated that auxin synthesized in the developing endosperm is transported into the integuments to support SC growth (Figueiredo *et al.*, 2016) (Fig. 2). This hypothesis was evidenced by the authors since (i) auxin rapidly accumulates in the integuments after fertilization; (ii) impaired endosperm auxin signalling, but not auxin synthesis, did not impair SC formation; and (iii) ectopic auxin production in the central cell initiates SC development without fertilization. Auxin exerts its early physiological task directly in the zone where the integuments are beginning their formation (Figueiredo *et al.*, 2016; Figueiredo and Köhler, 2018). However, the mechanism of this transport is still not clear.

By contrast, the possibility of an auxin being transported from SC to endosperm was recently also demonstrated (Chen *et al.*, 2014). Interestingly, fertilization causes restriction of auxin export through the funiculus, resulting in the spread of auxin throughout the integuments (Larsson *et al.*, 2017). It is appropriate to highlight that endosperm auxin synthesis is a feature which is evolutionarily conserved (Hatorangan *et al.*, 2016; Sun *et al.*, 2017). At present, the possibility of bidirectional transport of auxin is

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not ruled out. On the other hand, it is feasible that transport of auxin to integuments is necessary for the abolition of the PRC2 task on the integuments, then allowing the intervention by GAs and production of PAs in the endothelium (Lepiniec *et al.*, 2006; Dorcey *et al.*, 2009; Doughty *et al.*, 2014; Figueiredo and Köhler, 2018). However, it is still unknown how auxin intervenes in the regulation of the FIS-PcG complex (i.e. FIS-PRC2) during fertilization. Existing data suggest that the repression of the autonomous endosperm formation by PRC2-mediated control of auxin biosynthesis seems to be a conserved feature in angiosperms (Luo *et al.*, 2009; Tonosaki and Kinoshita, 2015; Figueiredo and Köhler, 2018). Finally, it cannot be not ruled out that some mobile signal external to the endosperm contributes to the development of both endosperm and integuments. It is speculated that this signal could be the auxin from the pollen grain (Fig. 2). Finally, in a recent study related to auxin dynamics during the onset of embryo growth in Arabidopsis, Robert (2019) provided some outstanding evidence: (i) the expression of the auxin biosynthetic machinery and the auxin signal output are differentially regulated during ovule maturation and pollination; (ii) an increased activity of the auxin production via the indole-3-pyruvic acid (IPyA) pathway is a major contributor of auxin accumulation in the integuments after pollination; (iii) maternal auxin production in the integuments surrounding the embryo contributes to early embryo development; (iv) presumably, the integuments provide auxin directly to the embryo, while the endosperm-derived auxin does not have a notable role; and (v) in the maternal tissues of Arabidopsis (dicot) and maize (monocot) there is a similar increase in auxin response despite the remarkable difference in the organization of their ovules. Summarizing this section, different localization of auxin accumulation, signalling and activity coordinates embryo, endosperm and SC development. However, there are many gaps in clarifying the regulation of auxin synthesis and distribution during the life of the unfertilized ovule and subsequent embryogenesis, specifically the fertilized ovule with both coenocytic and cellularized endosperm. In other words, all recently obtained results suggest that the distribution of auxin to different intracellular or extracellular compartments is strongly controlled. This control by still unknown genes, is necessary to carry out the signalling depicted in Fig. 2.

Concluding remarks and future perspectives

The unravelling of SC regulation is an important objective of modern plant molecular biology. A promising challenge for the future is to translate basic research into practical applications in crops. In particular, the application to a selection of species of agricultural interest and the study based on the molecular regulation of the source–sink relationship that constitutes the SC and endosperm, are two main reasons to explore the SC at several -omics levels. Deepening the study of the expression of integument master regulator genes in higher plants would help better address the nature and evolution of SC components. Recent progress in the genetic and molecular mechanisms of SC development has been summarized in this update. Several genetic frameworks are emerging. Although the role of auxin in seed development is not fully understood, several studies have supported the idea that this phytohormone is essential. However, how auxin 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 content in the seed. Also, it is known that in several species, including *Arabidopsis thaliana*, application of exogenous auxin leads to parthenocarpic fruit formation. Given the importance of ABA in the seed maturation process, and in the induction and loss of dormancy, a study of the ABA–auxin inter-relationship at the various levels is timely.

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