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Abstract

Key message:
Overview of seed size control.
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Keywords (separated by '-')	Seed development - *Arabidopsis* - Seed size - Seed coat - Endosperm
Footnote Information	Communicated by Silvia Coimbra. A contribution to the special issue ‘From Gametes to Seeds’.

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REVIEW

Networks controlling seed size in *Arabidopsis*

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Abstract

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Keywords Seed development · *Arabidopsis* · Seed size · Seed coat · Endosperm

Introduction

Increasing seed production is a key goal to meet world demand and consumption of agricultural crops, for food and feed in emerging economies. In this context, the study of the molecular mechanisms controlling seed formation becomes essential for plant scientists as seed size is a major component of seed yield (Adamski et al. 2009). Thus, advances in the basic knowledge about seed development in the model species *Arabidopsis thaliana* are of key relevance for the rational design of genetically engineered traits in relevant agronomic crop species that could complement and improve upon traditional breeding systems (Varshney et al. 2009; Langridge and Fleury 2011; Feuillet et al. 2011; Becker et al. 2014).

Arabidopsis seed development (see Fig. 1) starts after a double-fertilization event (for a complete seed development review, see Nowack et al. 2010; Becker et al. 2014). During the first fertilization event, the zygotic embryo is generated by the fusion of the egg cell and one sperm cell. The second fertilization event, which triggers the development of the triploid endosperm, starts with the fusion of the central cell of the embryo sac with the second pollen sperm cell (endosperm development is reviewed by Lafon-Placette and Köhler 2014). The two biparentally derived fertilization products (the embryo and the endosperm) are encased by the maternal sporophytic tissue (the seed coat), which is derived from the ovule integuments (seed coat development has been reviewed recently by Khan et al. 2014; Figueiredo and Köhler 2014). The seed coat represents a protective layer that prevents damage from external factors such as UV radiation, toxic chemicals and pathogens, as well as impeding germination until conditions are favorable (Haughn and Chaudhury 2005). Furthermore, the seed coat plays a major role in controlling communication between the two generations (reviewed by Bencivenga et al. 2011).

In spite of the influence of several abiotic factors on plant growth and development, such as temperature, light and day length, the final size of plant organs is reasonably

Communicated by Silvia Coimbra.

A contribution to the special issue 'From Gametes to Seeds'.

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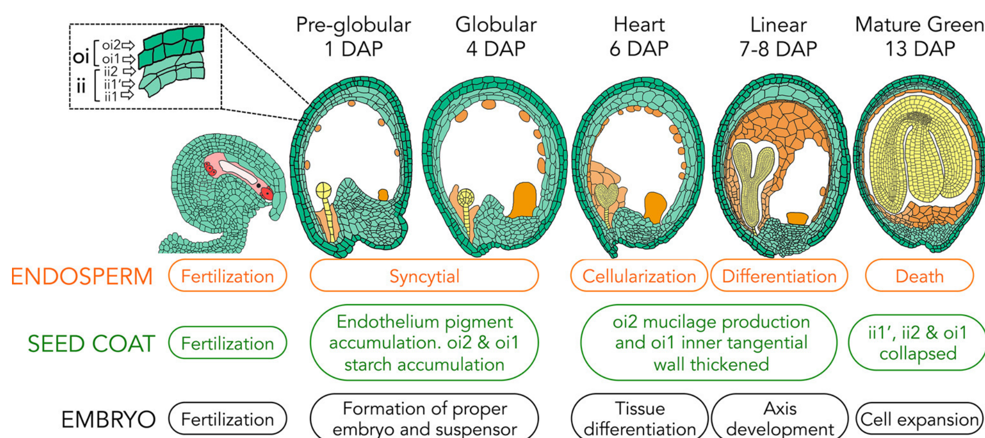

Fig. 1 Schematic representation of seed development in *Arabidopsis*. Diagrams of an unfertilized ovule and five stages of seed development from the preglobular (1 day after pollination—DAP) to mature *green* (13 DAP) stage. Embryo developmental stages were adapted from Le et al. (2010). Detail of the five seed coat layers according to Appelhagen et al. (2014). Seed coat developmental events are specified by Beeckman et al. (2000). Endosperm development is presented according to Lafon-Placette and Köhler (2014). Drawings are not to scale. Abbreviations and color code: *oi* outer integument, *ii* inner integument, *DAP* days after pollination. *green* seed coat, *orange* endosperm, *yellow* embryo

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constant within a given species (Tsukaya 2006), indicating that it is mainly the genetic seed developmental plan which determines the rate of growth until the seed reaches a predetermined mass and final size (Conlon and Raff 1999; AQ2 Day and Lawrence 2000). *Arabidopsis* seed size is mainly attained either during the rapid proliferation and growth of the endosperm (Boisnard-Lorig et al. 2001) and proliferation of the seed coat cells. These events span from fertilization to 6 days after pollination (DAP) of seed development (Fig. 1). From 7 to 13 DAP, there is a residual increase in seed volume occurring when the embryo expands at the expense of the endosperm. At this point, seed growth is limited by the seed coat that acts as a constraining physical barrier (Fang et al. 2012). Thus, to understand the whole mechanism governing seed size, it is essential to unveil both the mechanisms of endosperm and integument growth and development, as well as the interplay existing between the developmental programs of these structures.

In the last decades, many key regulators of seed size have been identified (reviewed by Kesavan et al. 2013—summarized in Table 1). However, there are still major gaps in knowledge regarding seed size and the available data are still fragmentary and need to be assembled into a global and coherent picture (see Fig. 2). This review provides a summary and an update of the different pathways controlling seed size in *Arabidopsis*. We analyzed seed size regulation in *Arabidopsis*, focusing on different functional categories in order to better describe them singularly. This includes mechanisms underlying the developmental processes of (A) the endosperm, including genomic imprinting and parent-of-origin effects, and (B) the seed coat/integuments. Moreover, we discuss (C) the cross talk between endosperm and seed coat and the role of (D) hormone synthesis and perception in determining seed size.

Endosperm development

Successful seed development requires the synchronized growth of the endosperm, the embryo and the seed coat (Fig. 1). Coordinated growth and development between these structures is reached through exchange of signals whose nature is still unknown. The profound morphological changes that characterize seed coat development could start only if the endosperm undergoes its developmental program, as embryo development by itself is not sufficient to stimulate seed coat growth and differentiation (Nowack et al. 2007; Hehenberger et al. 2012). However, it was demonstrated that central cell nuclei could start to proliferate even in the absence of karyogamy between central cell and sperm nucleus (Guitton et al. 2004). The failure of karyogamy in the central cell has been shown to impair endosperm development causing seed abortion (Aw et al. 2010). Interestingly, viable seeds can also be produced in the presence of homoparental diploid, as opposed to triploid, endosperm (Nowack et al. 2006, 2007). In *cdka;1* mutants, pollen fertilizes only the egg cell, not the central cell due to karyogamy failure (Aw et al. 2010). If *cdka;1* pollen is used to fertilize the *medea* (*mea*) mutant, in which the endosperm proliferates without fertilization (Kiyosue et al. 1999), full embryogenesis and viable plants are produced in the presence of diploid endosperm (Nowack et al. 2007). Endosperm development has four phases (Fig. 1): syncytial, cellularization, differentiation and death. The syncytial phase is characterized by a series of

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Table 1 List of *Arabidopsis* seed size-regulating genes and their functions

Gene	Locus	Mutants characterized	Effect on seed size	Expression patterns		Functional category	Reference(s)
				Ovule (prefertilization)	Seed		
ARF2	AT5G62000	*arf2-6; arf2-7; arf2-8*	+	Y	Embryo	Transcription factor	Okushima et al. (2005), Schruff et al. (2006)
IKU1	AT2G35230	*iku1*	–	Y	Endosperm	VQ motif protein	Garcia et al. (2003), Wang et al. (2010)
KLU	AT1G13710	*klu-2; klu-4*	–	Y		Cytochrome P450 oxygenase	Adamski et al. (2009)
TTG2	AT2G37260	*ttg2-1*	–	Y	Seed coat, endosperm	Transcription factor	Johnson et al. (2002), Dilkes et al. (2008)
STK	AT4G09960	*stk-1; stk-2*	–	Y		Transcription Factor	Pinyopich et al. (2003), Mizzotti et al. (2012)
FER	AT3G51550	*fer-4*	+		Seed coat	Receptor kinase	Yu et al. (2014)
AHK2	AT5G35750		+	Y	Developing siliques, embryo	CK receptor	Higuchi et al. (2004), Riefler et al. (2006)
AHK3	AT1G27320	*ahk2-5 ahk3-7 cre1-2*[a]			Developing siliques, embryo	CK receptor	Nishimura et al. (2004)
CRE1/AHK4	AT2G01830			Y	Developing siliques, embryo	CK receptor	Riefler et al. (2006), Nishimura et al. (2004)
FIS2	AT2G35670	*fis2*	POE	Y	Endosperm	Chromatin remodeller	Luo et al. (2000)
DA1	AT1G19270	*da1-1*	+	Y	Embryo	Ubiquitin receptor	Li et al. (2008)
IKU2	AT3G19700	*iku2-3*	–	Y	Endosperm	Receptor kinase	Garcia et al. (2003), Luo et al. (2005)
MINI3	AT1G55600	*mini3-1*	–		Endosperm, embryo	Transcription factor	Luo et al. (2005), Li et al. (2013)
CKX1	AT2G41510	*35S:AtCKX1*[b]	+	Y	ND	CK biosynthesis	Werner et al. (2003)
CKX5	AT1G75450	*ckx3 ckx5**	+	Y	ND	CK biosynthesis	Bartrina et al. (2011)
CKX3	AT5G56970	*35S:AtCKX3*[b]	+	ND	ND	CK biosynthesis	Werner et al. (2003)
MYB56	AT5G17800	*myb56-1*	–		Developing seeds	Transcription factor	Zhang et al. (2013)
SHB1	AT4G25350	*shb1-D*[c]	+		Endosperm, embryo	SYG1 homologous protein	Zhou et al. (2009)
AP2	AT4G36920	*ap2-11*	+		Seed coat, endosperm and embryo	Transcription factor	Ohto et al. (2005), Kinoshita et al. (2004)
EOD3	AT2G46660	*eod3-ko1*	–	ND	ND	Ubiquitin ligase	Fang et al. (2012)
MET1	AT5G49160	*met1-6*	POE		Endosperm, embryo	DNA methylation	Xiao et al. (2006)
CYP78A9	AT3G61880	*cyp78a9-ko*	–		Developing seeds	Cytochrome P450 oxygenase	Fang et al. (2012)
EOD1	AT3G63530	*eod1-2*	+	Y	Embryo	Ubiquitin ligase	Li et al. (2008), Xia et al. (2013)
UBP15/SOD2	AT1G17110	*ubp15-1*	–	Y	NS	Ubiquitin protease	Du et al. (2014)
GOA	AT1G31140	*goa-1*	+	Y	Developing siliques, seed coat, embryo	Transcription factor	Prasad et al. (2010), Erdmann et al. (2010)
CYP72C1	AT1G17060	*shk1-D*[d]	–		Developing siliques	Cytochrome P450 oxygenase	Takahashi et al. (2005)
DET2	AT2G38050	*det2*	–	Y		BR biosynthesis	Fujioka et al. (1997), Jiang et al. (2013)

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Table 1 continued

Gene	Locus	Mutants characterized	Effect on seed size	Expression patterns		Functional category	Reference(s)
				Ovule (prefertilization)	Seed		
BRI1	AT4G39400	*bri1-5*	−		Developing seeds	BR signaling	Jiang et al. (2013), Hategan et al. (2014)
ABA2	AT1G52340	*aba2-1*	+		Developing siliques	ABA biosynthesis	Cheng et al. (2014)
ABI5	AT2G36270	*abi5-2*	+		Developing siliques, dry seeds	Transcription factor	Finkelstein and Lynch (2000), Cheng et al. (2014)
MEA	AT1G02580	*f644/mea*	POE	Y	Developing siliques, embryo	Chromatin remodeller	Grossniklaus et al. (1998), Kiyosue et al. (1999)
DA2	AT1G78420	*da2-1*	+	Y	NS	Ubiquitin ligase	Xia et al. (2013)
AGL62	AT5G60440	*agl62-2*	−		Developing seeds	Transcription factor	Hehenberger et al. (2012)

The effect of specific gene mutations on seed size is described as positive (+) or negative (−) as reported in the literature

Y yes, *NS* not specified, *ND* not detected, *POE* parent-of-origin effects

[a] Only double or triple mutant presented seed size phenotype

[b] Overexpression line

[c] Gain of function

[d] Activation line of *CYP72C1*

divisions of the triploid nuclei without cytokinesis (Boisnard-Lorig et al. 2001) and parallels the maximal phase of seed growth (Garcia et al. 2005). After eight rounds of syncytial mitoses, the cellularization process starts, initially from regions surrounding the embryo and proceeding toward the chalazal region. Cellularization is followed by a differentiation of functional tissues, and eventually most endosperm cells die during seed maturation. The timing of endosperm cellularization correlates with the end of the main stage of seed growing; therefore, the size attained by the endosperm syncytium appears to be a major determinant of seed size (Boisnard-Lorig et al. 2001). Consequently, precocious endosperm cellularization results in small seeds, while delayed endosperm cellularization causes the formation of enlarged seeds (Scott et al. 1998; Garcia et al. 2003; Berger et al. 2006). A number of mutations have been described that impair proper endosperm development and the timing of endosperm cellularization. The existence of three redundant pathways that control endosperm cellularization has been recently proposed (Kang et al. 2013). The first pathway regulates endosperm cellularization through the action of APETALA 2 (AP2) and the MADS-box transcription factor AGL62 (Kang et al. 2008). The second endosperm cellularization pathway includes members of the Polycomb group (PcG) proteins and their targets (discussed below). The third pathway is the *IKU* pathway.

The *AP2* pathway controlling endosperm cellularization has only two members previously linked to seed size determination: *AP2* itself and *AGL62*. *AP2* encodes the founding member of the plant-specific family of transcription factors that contain an AP2/EREBP (ethylene-responsive element binding protein) DNA-binding domain (Jofuku et al. 1994; Okamuro et al. 1997; Riechmann and Meyerowitz 1998). AP2 is involved in a great variety of developmental processes, including endosperm cellularization. *ap2* mutant seeds undergo an extended endosperm proliferation stage, associated with a delay in cellularization (Ohto et al. 2009). Additionally, the abnormal endosperm development in *ap2* mutants resulted in other seed defects, such as enlarged embryos that show increased cell number and cell size (Jofuku et al. 2005; Ohto et al. 2005). AP2 has also been associated with seed coat development and integument-endosperm cross talk, as we discuss below. The other member belonging to this pathway is *AGL62*, a type I MADS-box transcription factor. The expression level *AGL62* correlates with endosperm cellularization in a dosage-dependent way, suggesting that it represents a key regulator of endosperm cellularization and consequently of seed size determination. Accordingly, the *agl62* mutants have precocious endosperm cellularization and a small seed phenotype (Kang et al. 2008; Kradolfer et al. 2013), while increased *AGL62* expression correlates with a delay

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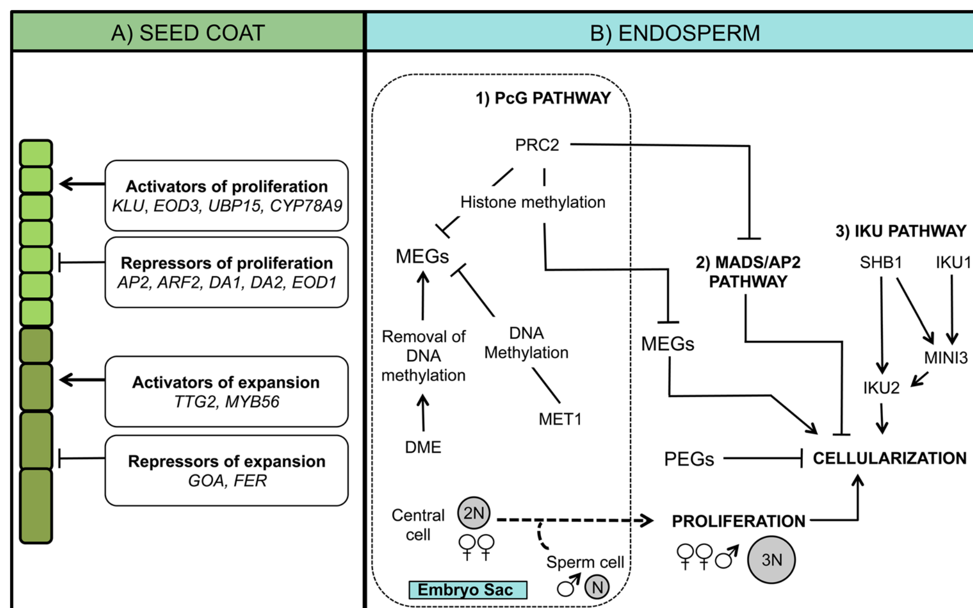

Fig. 2 Model indicating the pathways determining seed size in *Arabidopsis*. The model illustrates the main networks and/or key regulators characterized in the literature, based on their role in development of the **a** seed coat or **b** endosperm. **a** Seed coat. Genetic pathways involved in the activation/repression of cell proliferation and cell expansion during seed coat development, thus controlling seed size in a maternal way. Four functional categories (boxes) are indicated based on previous characterization studies. **b** Endosperm. Schematic representation of factors that influence endosperm cellularization and, therefore, seed size. One of the mechanisms involved in parents-of-origin effects includes activation of DME in the central cell and simultaneous repression of MET1, resulting in hypomethylation of MEGs, and consequently their preferential expression over PEGs in the endosperm. The expression of MEGs is furthermore controlled by PRC2 action through histone methylation. The two additional pathways (MADS/AP2 and IKU) that regulate the timing of endosperm cellularization are indicated. *Lines ending in arrowheads* indicate positive transcriptional regulation, and *lines ending in bars* indicate repression of expression

178 or a complete absence of cellularization (Erilova et al. 2009; Tiwari et al. 2010). Interestingly, *AGL62* expression is under negative control of the FIS-PRC2, an indication that the timing of endosperm cellularization is epigenetically controlled (Hehenberger et al. 2012).

183 The second pathway controlling cellularization of the endosperm involves the PcG protein complex and its imprinted genes. Imprinting and its relation with seed size control will be discussed in a separate section of this review.

188 The IKU pathway is probably the best-described pathway for endosperm cellularization. The genes *HAIKU1* (*IKU1*) and *IKU2* have been shown to be key regulators of seed size in *Arabidopsis* via control of the transition from syncytial phase to the cellularization phase of the endosperm (Garcia et al. 2003). *IKU1* encodes a protein containing a VQ motif (Wang et al. 2010), while *IKU2* encodes a leucine-rich repeat kinase (Luo et al. 2005). *iku1* or *iku2* mutant plants show reduced proliferation of the endosperm, as well as a precocious cellularization process, leading to reduced seed size (Garcia et al. 2003). Another member of the IKU pathway is *MINISEED3* (*MINI3*), a WRKY class transcription factor that regulates the endosperm cellularization process (Luo et al. 2005). *mini3* mutant plants phenocopy *iku1* and *iku2* small seed phenotypes, due to precocious cellularization of the endosperm. In addition, the small seed phenotype of *mini3* mutant is ascribable to reduced cell expansion in the seed coat and reduced cell proliferation that results in a smaller embryo compared with wild type (Garcia et al. 2003; Luo et al. 2005). Genetic and mutant analyses indicate that *IKU1*, *IKU2* and *MINI3* are likely to participate in a single pathway, with *IKU1* regulating both *MINI3* and *IKU2*, and *MINI3* regulating *IKU2* (Luo et al. 2005). Apparently, *MINI3* could positively regulate *IKU2* by binding to the putative W-box identified in the *IKU2* promoter. Seed size of the double mutants *iku2-1 mini3-1* is similar to the seed size of homozygous mutant alleles of each single locus (Luo et al. 2005).

217 Recently, it has been reported that short hypocotyl blue 1 (SHB1) binds to the promoters of *IKU2* and *MINI3* (Zhou et al. 2009; Kang et al. 2013). *SHB1* encodes a nuclear SYG1-homologous protein (Kang and Ni 2006) that is recruited by MINI3 to activate the *IKU2* and *MINI3* expression, and probably other genes required for endosperm development, stimulating the process of endosperm cellularization (Kang et al. 2013). *SHB1* was first described to be involved in hypocotyl development (Kang and Ni

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2006) and later as a regulator of endosperm proliferation and the timing of cellularization. The gain-of-function overexpression mutant *shb1-D* displayed an enlarged seed size phenotype associated with a delay in endosperm cellularization (Zhou et al. 2009).

Thus, independent networks act as key regulators of endosperm growth, by controlling endosperm proliferation and cellularization with a major impact in final seed size (Fig. 2). Further investigation is required to identify all the molecular players in these pathways and to determine whether they share downstream targets.

Genomic imprinting and parent-of-origin effects

In plants, genomic imprinting has been observed primarily in the endosperm (Bauer and Fischer 2011) and rarely on the embryo (Jahnke and Scholten 2009). Imprinting of a specific allele depends on the presence of an epigenetic mark on the corresponding locus (reviewed by Ferguson-Smith 2011). It has been proposed that imprinted genes regulate the transfer of nutrients from the sporophyte to the developing progeny. In particular, maternally expressed genes (MEGs) function to equally allocate nutrients to all seeds, while on the other hand paternally expressed genes (PEGs) function as growth factors that allow their own offspring to extract the maximum amount of nutrients from the mother. Therefore, increased PEGs activation determines the formation of larger seeds (Haig and Westoby 1989).

Epigenetic modifications performed on genetically identic alleles lead to parent-of-origin specific expression. Of particular importance is the balance of methylation between maternal and paternal alleles in the central cell. Removal of DNA methylation relies on the enzymatic activity of DEMETER (DME) (Kinoshita et al. 2004; Gehring et al. 2006), and DNA methylation depends on the enzyme DNA methyltransferase 1 (MET1) (Hsieh et al. 2011; Jullien et al. 2012). *DME* is expressed in the central cell in the embryo sac (Choi et al. 2002) and in the vegetative cell of the pollen grain (Schoft et al. 2011). This leads to specific DNA hypomethylation of the maternally inherited genome. Previous studies showed that altering DNA methylation in a parental-specific manner via MET1 resulted in variation in seed size (Xiao et al. 2006). When crossing *MET1::RNAi* pistils with wild-type pollen, the result is production of enlarged F1 seeds. Meanwhile, reciprocal crosses generated smaller F1 seeds, as expected from the presence of hypomethylated paternal genome (Adams et al. 2000; Luo et al. 2000; Xiao et al. 2006). Thus, the methylation status of both the maternal and paternal genome directly influences seed size.

The second major mechanism involved in imprinted expression of a subset of genes relies on PcG proteins. PcG proteins are pivotal regulators of cell identity that act as transcriptional repressors in multimeric complexes (Schuettengruber and Cavalli 2009). Among these, the PRC2-complex catalyzes the trimethylation of histone H3 on lysine 27 (H3K27me3) and has been implicated in controlling endosperm development. Specifically, the FIS-PRC2 (*fertilization-independent seed-Polycomb repressive complex 2*), which comprises the different subunits encoded by *MEDEA* (*MEA*), *fertilization-independent seed 2* (*FIS2*), *fertilization-independent endosperm* (*FIE*) and *multicopy suppressor of IRA1* (*MSI1*), acts in the central cell of the female gametophyte and in the endosperm, targeting DNA hypomethylation sites (Weinhofer et al. 2010). The FIS-PRC2 mainly represses the expression of maternally inherited (and hypomethylated) alleles. Seeds with mutations in *mea*, *fis2* or *fie2* show endosperm proliferation even in the absence of fertilization, but also prolonged endospermal proliferation and absent or delayed cellularization if fertilization occurs (Grossniklaus et al. 1998; Kiyosue et al. 1999; Makarevich et al. 2008). The phenotypes of these mutants imply that PCR2 complexes promote fast endosperm differentiation after fertilization, thus directly acting on a pathway that greatly influences seed size (Fig. 2).

Finally, it is necessary to mention that perturbation of the relative dosages of the maternal and paternal genomes, typical in the case of interploidy crosses, directly affects endosperm development and seed size (Garcia et al. 2003; Luo et al. 2005; Kang et al. 2008; Zhou et al. 2009; Wang et al. 2010). The defects and low endosperm viability often observed in seeds of interploidy crosses (as in the case of wheat) can be explained in terms of maternal or paternal genome excess, i.e., an imbalance between MEGs and PEGs, and its effect on endosperm growth (Haig and Westoby 1991). However, the negative effects on seed development of interploidy crosses are reduced in *Arabidopsis*, in which both paternalized (PEGs excess) and maternalized (MEGs excess) seeds show the expected alteration from wild-type size, but show normal endosperm viability. This mitigated effect is probably due to the high rate of self-pollination that is characteristic of this model species (Scott et al. 1998).

The role of the seed coat in seed size determination

The *Arabidopsis* seed coat derives from the ovule integuments, formed by a set of five cell layers in mature ovules (Fig. 1). Two cell layers derive from the outer integument (oi) and three from the inner one (ii). The outer integument consists of two cell layers (oi1 and oi2), and the inner integument consists of three cell layers (ii1, ii1′ and ii2) (Beeckman et al. 2000; Kunieda et al. 2008). The

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innermost layer of the inner integument, ii1, named the endothelium (Beeckman et al. 2000), is in direct contact with the endosperm cells.

The seed coat deeply influences seed size, highlighting a fundamental role for seed maternal tissues in the control of this aspect of seed yield. The seed cavity (the space enclosed by the seed coat) increases in volume after fertilization, partly due to the independent developmental plan of the seed coat and partly as the result of the interplay between the seed coat and the endosperm (Ingouff et al. 2006; Roszak and Köhler 2011). After fertilization, the cells belonging to the different seed coat layers predominantly experiment intense expansion activity but still undergo division activity (Garcia et al. 2005). Both cell division and expansion cease at 6 DAP (Du et al. 2014). Before fertilization, the female gametophyte (embryo sac) seems to have only a moderate importance in generating the signals to stimulate the integuments' proliferation (Ingouff et al. 2006); this was proven by demonstrating that mutants defective in embryo sac formation, such as *sporocyteless* (*spl*), are still able to develop integument to some extent (Yang et al. 1999). Numerous studies have identified genes involved in *Arabidopsis* ovule integuments and seed coat development, and some of them have provided a functional characterization of seed size contribution. In particular, seed size mutant phenotypes showing a clear maternal inheritance are mainly due to an alteration of cell proliferation or elongation in the seed coat. The control of these two pathways will be discussed separately.

Factors controlling integuments cell proliferation

A key player in the control of cell cycle and expansion in *Arabidopsis* is *auxin response factor 2* (*ARF2*), which encodes a B3-type transcription factor of the *ARF* family (Li et al. 2004). *ARF* genes take part in auxin-related responses and recognize specific AuxRE (auxin response elements) consensus elements on target genes (Ulmasov et al. 1999). Among the different ARF proteins, ARF2 is thought to act as a transcriptional repressor, exercising a negative control over cell proliferation and expansion (Li et al. 2004; Okushima et al. 2005; Schruff et al. 2006). In particular, different *arf2* loss-of-function mutants exhibit abnormal flower morphology and enlarged seeds in comparison with the wild type (Okushima et al. 2005), a phenotype characterized in detail in the case of *arf2-9*, which presented more cells in the seed coat compared with wild-type seeds. The result of the increased volume of the seed cavity in *arf2-9* is that seeds are 46 % heavier than the wild-type seeds, showing in some cases additional cell layers in the seed coat (Schruff et al. 2006). A further confirmation that *ARF2* is important for the maternal control of seed size comes from the maternal inheritance of *arf2-9* phenotype observed in the reciprocal crosses with wild-type plants (Schruff et al. 2006). Besides enlarged seeds, the *arf2-9* mutant also has a significant reduction in fertility due to improper flower development (Schruff et al. 2006). Reduced fertility often correlates with increased seed weight (Harper et al. 1970; Ohto et al. 2005). However, this is not occurring in the *arf2-9* mutant, since the hypothesis of the large-seed phenotype as an indirect effect of the seed size/seed number trade-off was later refuted in a subsequent study (Hughes et al. 2008). In fact, the defects in the floral morphology of the *arf2-9* mutant were overcome by expressing *ARF2* under the promoter of *APETALA1* (*AP1*). The *pAP1::ARF2 arf2-9* plant improved the fertility, retaining the enlarged seed size phenotype of the original *arf2-9* mutant, thus showing the pivotal role of ARF2 in seed development.

Another negative regulator of cell division is the transcription factor AP2, whose role in endosperm development has been described above. Interestingly, the increased cell proliferation observed in *ap2* is under maternal control and affects both the seed coat and the endosperm (Jofuku et al. 2005; Ohto et al. 2005). Notably, *AP2* expression is negatively regulated by miR172 during flower development (Chen 2004), while *ARF2* is negatively regulated by transacting small-interfering RNA (tasiRNA) (Williams et al. 2005). Similarly, it was reported that mutation in the gene *miR159* results in seeds smaller than wild type (Allen et al. 2007). The two known targets of *miR159* that are expressed in developing seeds, *MYB33* and *MYB65*, have no described function in the seed. However, they are responsible for the *mir159ab* seed phenotype, as the quadruple mutant *mir159ab myb33 myb65* showed a reversion of the seed traits (Allen et al. 2007). Taken together, these results provide evidence of a fundamental role for post-transcriptional regulation via small RNAs in the control of seed size.

Cytochrome P450 KLUH, encoded in *Arabidopsis* by *CYP78A5*/*KLU*, is a regulator of organ size (both leaves and floral organs) as well as of plastochron length (Anastasiou et al. 2007; Wang et al. 2008). It has also been shown that *KLU*, expressed prior to fertilization in the inner integuments of the ovule, acts as a maternal positive regulator of seed size. *klu-2* seeds have a reduced number of cells in the outer layers of the seed coat in comparison with wild type, with the result that *klu-2* seeds are 13 % lighter than seeds of wild-type plants. The opposite phenotype was observed in *KLU*-overexpressing plants, whose seeds are 11 % heavier (Adamski et al. 2009). *KLU* seems to act independently of previously described integument cell proliferation factors as *AP2* and *ARF2*, because seeds of the double mutants *klu arf2* and *klu ap2* were an intermediate seed size between those of the respective single mutants (Adamski et al. 2009).

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In *Arabidopsis*, the importance of ubiquitin pathway in the determination of seed size has been widely investigated over the last decade. Several members involved in this pathway have been identified (reviewed by Li and Li 2014) for their role in maternal control of seed size. Among them, *DA1* and *DA1-related* (*DAR*) encode for plant-specific ubiquitin receptor protein. While single mutants *da1-ko* and *dar1-1* do not exhibit variation in seed size in comparison with wild type, the double mutant *da1-ko dar1-1* produces larger seeds. Another mutation in the *DA1* sequence (a single arginine-to-lysine aminoacidic change at position 358, the d*a1-1* mutant) results in plants producing seeds with increased cell proliferation in the seed coat, a phenotype also observed in 35S::DA1^{R358K}. This suggests that the mutated DA1 protein might act antagonistically with native DA1 or DAR (Li et al. 2008). *DA2* and *enhancer of DA1* (*EOD1*) encode proteins with E3 ubiquitin ligase activity and are also negative regulators of seed size, as shown by the enlarged seeds of single mutants *da2-1* and *eod1*. They may act synergistically with *DA1*, as observed by the enhanced seed size of *da1-1 da2-1* and *da1-1 eod1* double mutants in comparison with *da1* mutant (Xia et al. 2013). *EOD3* encodes cytochrome P450 CYP78A6. The gain-of-function mutant *eod3-1D* proved to be a dominant enhancer of the *da1-1* seed size phenotype, while on the contrary *eod3-ko* produced smaller seeds than wild type (Fang et al. 2012). *CYP78A9* encodes for another cytochrome P450 and is the most closely related gene to *EOD3,* with whom it might act synergistically in promoting the size of the seed coat. This is implied by the additive small seed phenotype observed in *eod3-ko cyp78a9-ko* double mutants in comparison with the single mutants (Fang et al. 2012). *Ubiquitin-specific protease 15* (*UBP15*)/ *suppressor of DA2* (*SOD2*) encodes for a de-ubiquitinating enzyme acting downstream of *DA1* (Li et al. 2008; Du et al. 2014). The *ubp15* mutant produces small seeds, while the overexpression line of *UBP15* results in larger seeds. This is likely due to a positive effect on cell proliferation in maternal integuments of ovules and developing seeds.

It has been suggested that *da1-1* acts independently of *ARF2* and *AP2*, as the seed phenotype of the double mutants *da1-1 ap2* and *da1-1 arf2* is additive in comparison with the one of the single mutants (Li et al. 2008).

Factors controlling integuments cell elongation

A reduction in cell elongation is observed in the loss-of-function mutant *transparent testa GLABRA 2* (*TTG2*). In the *ttg2* mutant, cell elongation in the integuments is affected, possibly because of the increased physical constraint of the cell walls, or possibly because of disruption of the developmental pathways for elongation. Endosperm development is also affected, probably as a consequence of the defects in integument cells (Garcia et al. 2003, 2005). Developing seeds produced by the double mutant *ttg2 iku2* display extremely reduced size in comparison with the single mutants *ttg2* and *iku2* seeds (Garcia et al. 2005). The combination of *ttg2* and *iku2* mutations prevents integument cell elongation and growth of the endosperm more severely than in each single mutant. The double homozygous mutant displays a cumulative phenotype combining the maternal effects of *ttg2* with the endospermal effect of *iku2* (Garcia et al. 2003, 2005). The additive reduction in integument cell division and elongation, endosperm growth and seed size when *iku2* and *ttg2* mutations are combined, indicates that each mutation acts in distinct genetic pathways, but has common effectors. In parallel, reduction in the endosperm volume is more evident in the double mutant relative to the single mutants. To achieve the size of the integument, dictated by the size of the syncytial endosperm, integument cells regulate elongation, not cell proliferation. Integument cell elongation plays a key role in the coordination of size between the endosperm and the integument. Accordingly, *TTG2* would modulate the competence of the integument cells to elongate via a maternal integument elongation-dependent pathway (Garcia et al. 2005).

Another positive regulator of seed size in *Arabidopsis* is the R2R3 MYB transcription factor, MYB56, which maternally affects seed development by regulating seed size and shape (Zhang et al. 2013). The loss-of-function mutant lines of *MYB56* generate smaller seeds, while overexpression of *MYB56* generates larger seeds compared with wild type. *myb56* endothelial cells are smaller and more rounded. Apparently, the role of MYB56 is locally dependent since its altered expression on the endothelial layer affects cell size but not cell number; however, in the two layers of the outer integument, *MYB56* controls only cell number but not the cell size (Zhang et al. 2013). *MYB56* affects seed size in a regulatory pathway probably independent of other seed coat development regulators such as *TTG2*, *KLU*, *GORDITA* (*GOA*) and *DA1*, because these genes show no expression changes in a *myb56* mutant background (Zhang et al. 2013).

SEEDSTICK (*STK*) and *Arabidopsis B-sister* (*ABS*) are two MADS-box genes that act together to control the formation of one layer of the seed coat, the endothelium, during seed development (Mizzotti et al. 2012). *STK* controls ovule identity redundantly with *SHATTERPROOF1* (*SHP1*) and *SHP2*. In addition, *stk* single mutant produces smaller seeds (Pinyopich et al. 2003) with respect to wild type, whereas *abs* mutant has no size difference (Nesi et al. 2002). The double mutant *stk abs* completely lacks endothelium development and manifests a high level of sterility, due to both ovule and seed abortions (Mizzotti et al. 2012). Another MADS-box transcription factor involved in seed

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coat development is *GOA*. A loss-of-function mutation in *GOA* causes an increase in the seed size when compared with wild type, due to an impact on cell expansion processes, during fruit and seed development (Prasad et al. 2010; Erdmann et al. 2010).

Very recently, a new actor in the integument development was described, the plasma membrane receptor kinase FERONIA (FER) (Yu et al. 2014). FER has been demonstrated previously to be involved in inhibiting pollen tube elongation (Escobar-Restrepo et al. 2007) and promoting cell elongation in leaves and root hairs (Guo et al. 2009; Duan et al. 2010). *FER* is highly expressed on the integuments of developing seeds, but it was not detected in embryo or endosperm (Yu et al. 2014). *FER*-null mutants develop seed that are 40–60 % larger than the wild type. At 2 DAP, the outer integument of *fer-4* contained larger cells and no differences in cell number from the wild type. The authors concluded that *FER* inhibits the elongation of seed coat cells (Yu et al. 2014). This conclusion is supported by the fact that FER controls cell elongation in root hairs in response to auxin through recruitment of RHO GTPases (ROP/RAC) to promote or inhibit cell elongation. ROP/RAC signaling pathway regulates several cell responses, such as polarized growth and differentiation (Duan et al. 2010; Yu et al. 2014). In the female gametophyte, FER is a receptor of rapid alkalinization factor (RALF), a small peptide whose overexpression or external application promotes cell wall alkalinization and growth inhibition. The FER–RALF interaction causes the phosphorylation of the H^+-ATPase AHA2. AHA2 phosphorylation may have an effect on the cell wall levels of reactive oxygen species (ROS), changing the balance between the ROS promoting/inhibiting cell wall relaxation state (reviewed in Wolf and Höfte 2014). In this way *FER* could, at least partially, control the cell wall's capacity to elongate. However, further research has to be done to fully understand the role of *FER* in seed development.

Endosperm–integument cross talk

Endosperm and integument growth and development are tightly coupled. As mentioned above, seed coat development influences endosperm proliferation and the timing of cellularization (Fig. 1). At the same time, the endosperm performs a key nourishing function and provides signals to coordinate seed maturation (Berger et al. 2006).

Two models have been proposed to explain the cross talk between endosperm and the seed coat and its role in controlling seed size. The 'integument size-restriction model' suggests that the expansion of the integument cells represents a physical constraint to the size of the seed cavity, restricting the size of the embryo. As a result, this volume reduction increases the concentration of the factors triggering the cellularization process (Garcia et al. 2005; Doughty et al. 2014).

In the second model, identified as the 'cellularization signaling model' (Fig. 3), the interplay between seed coat and endosperm is mediated by a signal that moves between integuments and endosperm. Flavonoids (proanthocyanidins [PAs]) represent excellent candidates for the signal that triggers the endosperm cellularization process since they are synthesized in the endothelium. The accumulation of flavonoids is initiated after fertilization in the endothelium (Debeaujon et al. 2003). The relevance of flavonoids in seed size control emerged from the fact that many flavonoid biosynthetic pathway mutants show alterations in

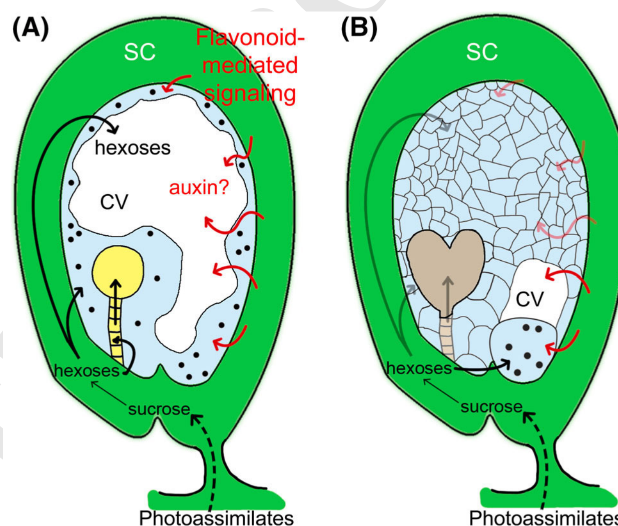

Fig. 3 Schematic representation of endosperm–seed coat cross talk in *Arabidopsis* according to the 'cellularization signaling model.' Seed coat layers are not shown for clarity. *Black circles* represent the endosperm nuclei at syncytial stage. **a** During early seed development (globular stage embryo—4 DAP), the endosperm progresses from the syncytial to cellularized stage. In this suggested model, transport of a cellularization signal between the integuments and the endosperm would be controlled by flavonoid biosynthesis. Adding support to this thesis, several mutants defective in the flavonoid biosynthesis pathway with reduced seed size were found to display a precocious endosperm cellularization (Scott et al. 2013). Hexose concentrations may also play an important regulatory role driving growth of the endosperm, since a higher hexose/sucrose ratio may stimulate mitotic activity and promote cellular proliferation leading to a greater seed size (Ohto et al. 2005). During the early stages of seed development, sucrose is actively transported into plant "sink" tissues like seeds and enters the seed coat via the vascular bundle of the funiculus (*black arrows*). Sucrose is cleaved in the seed coat and the resultant hexoses are used by developing embryo and endosperm. Signaling mechanisms originated in the seed coat (*red arrows*) may enter to the syncytium from the seed coat and later reach the embryo. This could be done directly from the syncytial endosperm, or indirectly via the suspensor. **b** The accumulation of these signals triggers the endosperm cellularization process at later stages of seed development (heart stage embryo). Abbreviations: *SC* seed coat, *CV* central vacuole

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599 the timing of the endosperm cellularization process (Scott et al. 2013; Doughty et al. 2014). Furthermore, it has been reported that flavonols could interact with the phosphoglycoprotein (PGP) auxin transporters PGP1, PGP4 and PGP19 (Peer and Murphy 2007). Flavonoids inhibit PGP-mediated polar auxin transport (Terasaka et al. 2005), which in fact may cause a rapid change in auxin concentration that results in delay/triggering of the endosperm cellularization process (Doughty et al. 2014), thus affecting seed development and seed size.

Another type of candidate molecules that could mediate the seed coat–endosperm cross talk are the polysaccharides. Nutrients from the phloem have to be unloaded from the seed coat into the endosperm and the embryo. The processing of sucrose follows distinct biochemical pathways, such as biosynthesis of cell wall polysaccharides and storage reserves. Thus, maternal tissues are major sites of sugar translocation and partitioning and are hence considered key determinants of sink strength and seed biomass yield. Since sugar metabolism and transport can be highly compartmentalized in seeds (Morley-Smith et al. 2008), even small differences in hexose/sucrose ratio can have dramatic effects on seed development and storage metabolism. For instance, *AP2* seems to modulate the nutritional supply from maternal tissues by changing the ratio of hexose to sucrose during seed development, opening the possibility that *AP2* may also control seed mass through its effects on sugar metabolism (Ohto et al. 2009).

The role of hormone synthesis and perception in determining seed size

As stated before, the complex structure forming the developing seed requires the coordination in growth of multiple tissues and cells with different patterns of proliferation and differentiation. This coordinated growth demands a precise spatiotemporal organization that can be achieved thanks to the synthesis and perception of signals in different seed tissues. This sophisticated communicative system between seed compartments is crucial not only to regulate their balance in growth, but also to control the progression of the whole developmental process within each tissue. The function of hormones in this communicative role to coordinate seed development has been well characterized by studies performed on hormone-deficient and hormone-insensitive mutants of *Arabidopsis*. Several hormonal pathways such as brassinosteroids, cytokinins, auxins and abscisic acid have been already proposed to play a crucial role in seed development (Sun et al. 2010). In this last part of the review, we provide a global panorama of the regulation of seed development by phytohormonal stimuli, emphasizing their impact on seed size (for a review of hormones controlling seed development, see Locascio et al. 2014).

Key role of brassinosteroids in seed size regulation

The function of brassinosteroids (BR) in seed development has been well characterized by studies of BR-deficient and BR-insensitive mutants in several species such as *Arabidopsis*, *Oryza sativa*, *Pisum sativum* and *Vicia faba* (for a review, see Jiang and Lin 2013). At the cellular level, low endogenous concentrations of BR have been shown to exert a positive effect on cell elongation; meanwhile, saturating levels of BR lead to the opposite effects with reduced cell elongation (Fujioka et al. 1997; Turk et al. 2003). Brassinosteroids are required for proper plant growth and deficiencies in their synthesis, and signal transduction pathway leads to severe dwarfed phenotypes (Fujioka et al. 1997). An *Arabidopsis* dwarf mutant overexpressing the P450 monooxygenase gene *CYP72C1* (*shk1-D*) showed a reduction in endogenous BR levels and produced smaller seeds than the wild type, probably due to an effect on cell elongation (Takahashi et al. 2005). A similar small seed phenotype was reported in the *DWARF5* (*DWF5*) loss-of-function mutant. *DWF5* encodes a sterol reductase gene involved in the BR biosynthesis pathway (Choe et al. 2000). The weak BR-deficient mutant *de-etiolated 2* (*det-2*), in which seed size was rescued by exogenous BR application, and the BR-insensitive mutant (*brassinosteroid-insensitive 1*) *bri1-5* produced smaller seeds than wild-type seeds.

The mechanism of BR regulation of seed size is twofold: 1) expanding the seed cavity and endosperm volume, promoting embryo development and 2) controlling integument cell length (Jiang et al. 2013). BR regulates embryo and endosperm development through the brassinazole-resistant 1 (BZR1) transcription factor which controls the IKU pathway by binding to the promoter regions of *SHB1* or *IKU1*, or alternatively through binding to the promoter of *IKU2* (Jiang et al. 2013).

On the other hand, evidence supporting BR control of seed size by regulating integument development comes from the significant decrease of integument cell length in *det2* (Jiang et al. 2013) and from the mutant *arf2*, which develops larger seeds due to extra integument cell divisions (Schruff et al. 2006). *ARF2* is a direct target of BZR1, and its transcription is negatively regulated by BR (Jiang et al. 2013). Thus, it seems that BR might regulate seed size through BZR1 binding and repressing *ARF2* promoter to positively regulate the integument development (Jiang et al. 2013). As a result, *ARF2* has been proposed to mediate the cross talk between auxins and BR. BIN2, a 697

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kinase regulated by BR, phosphorylates ARF2 in vitro. Apparently, this phosphorylation would allow the detachment of ARF2 from DNA, inhibiting its transcriptional repression activity (Vert et al. 2008). The proposed scenario establishes that BR affects BIN2 target specificity promoting a change from BRZ1/BES1 to ARF2. The presence of auxin and/or BR will determine an increment or persistence of the target genes expression (Krizek 2009). Interestingly, the *fer* mutants are hypersensitive to BR (24-epibrassinolide), suggesting that *FER* can act as a critical modulator of the brassinosteroid signaling pathway during hypocotyl development (Deslauriers and Larsen 2010). Deciphering the relation between *FER* and BR promises to be very interesting to better understand seed size determination. Last but not least, BR can act as global regulator, acting at the same time over both integuments, endosperm, and embryo development through BZR1 binding to the *AP2* promoter (Jiang et al. 2013).

The role of auxins in communication

At the cellular level, auxin is involved in many processes, including pattern formation, cell division and cell expansion (Vandenbussche and Van Der Straeten 2004; Leyser 2005). In addition, auxins exert a key role during the first steps of seed development (Hamann et al. 2002; Friml et al. 2003; Jenik and Barton 2005; Cheng et al. 2007; Wabnik et al. 2013). Schruff and colleagues proposed that *ARF2* is a general repressor of cell division in many aerial organs of the plant by controlling expression of *CYCD3;1*, a D-type cyclin involved in cell cycle entry, and *AINTEGUMENTA* (*ANT*), a transcription factor involved in organ growth and cell division control (Klucher et al. 1996; Schruff et al. 2006).

Cytokinins

Several studies have highlighted the importance of cytokinins (CK), together with auxin, in promoting growth by cell division, development and differentiation (Bishopp et al. 2011; Vanstraelen and Benková 2012). High levels of CK are present during early seed development in many species (Yang et al. 2002). In *Arabidopsis*, limited information comes from a few reports (Werner et al. 2003; Garcia et al. 2005; Day et al. 2008) and CK function has not yet been exhaustively characterized. Studies performed on the genetics of CK production have shown that during early stages of seed development transcriptional changes are mostly associated with effects of the hormone on the development of endosperm and seed coat. These data reinforce the idea that the control of seed size would involve a cross talk occurring between maternal and zygotic tissues (Garcia et al. 2005). Transcriptome analysis of the endosperm at 4 DAP revealed an overrepresentation of CK biosynthetic and response genes, supporting the hypothesis that the predominant role of CK is in cell proliferation of the early endosperm (Lur and Setter 1993; Day et al. 2008). Overexpression of two cytokinin oxidase dehydrogenases (*CKX1* and *CKX3*) produced larger seeds with larger embryos. The enlargement found in these transgenic seeds is attributable to increases in cell number and size (Werner et al. 2003). Larger seeds were also produced by the triple mutant of the CK receptor genes *arabidopsis histidine kinase 2* (AHK2), AHK3 and cytokinin response 1/AHK4 (CRE1/AHK4). In this case, an increase of almost two times the seed size was reported, when compared with wild-type seeds, due to an enlargement of the embryo size, with approximately 15 % greater cell number and 30 % greater cell size. Reciprocal crosses with wild-type plants suggested that the increase found in seed size was likely to be regulated by maternal and/or endospermal genotypes (Riefler et al. 2006).

Recently, it was concluded that the control of endosperm size by the IKU pathway is regulated by the cytokinin catabolic pathway through the activation of *CKX2* (*cytokinin oxidase 2*) by MINI3 (Li et al. 2013). CKX2 is also co-regulated by maternal genome dosage and methylation, and both phenomena suppress *CXK2* transcription. These data establish a link between hormonal and epigenetic factors in the regulation of seed size in *Arabidopsis* (Li et al. 2013).

Abscisic acid

The predominant role of abscisic acid (ABA) regulation involves key processes occurring during the maturation stages of seed development. Key aspects of this development are accumulation of storage compounds in the embryo, seed dormancy, and the inhibition of precocious germination (McCarty 1995; Finkelstein et al. 2002; Kanno et al. 2010). ABA biosynthesis exhibits two peaks during seed development: Initially biosynthesis is induced in the embryo and then levels accumulate to a second peak during the late maturation stage, where it is thought that ABA mainly originates from the maternal tissues (Finkelstein et al. 2002; Finkelstein 2004). ABA has been proposed to act mainly as an endosperm development regulator since the mutants *abscisic acid-deficient 2* (*aba2*) and *abscisic acid-insensitive 5* (*abi5*) develop larger seeds than the wild type (Cheng et al. 2014). *ABA2* encodes a dehydrogenase/reductase involved in ABA biosynthesis (González-Guzmán et al. 2002), and *ABI5* encodes a transcription factor involved in ABA signaling (Brocard et al. 2002). Interestingly, *aba2* mutants have delayed endosperm cellularization. The model of action suggests that endogenous ABA levels in the seed

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are raised by ABA2 action, resulting in an enhancement of *ABI5* transcription. ABI5 negatively regulates *SHB1* expression by directly binding to its promoter region. Therefore, ABA regulates proper endosperm development and cellularization processes in a SHB1-dependent way (Cheng et al. 2014). ABA slowly induced *DA1* expression, but other growth regulators such as jasmonic acid, auxin, CK, BR, gibberellins or glucose failed to induce its expression. It therefore seems that the mechanism that restricts proliferative growth under the control of *DA1* control could include ABA signaling (Li et al. 2008).

Future perspectives

Unraveling seed development and its genetic control is important due to the critical role of seeds as a food source for mankind and livestock, as well as the growing interest in seeds as a renewable source of energy. Recently, genomic-based research and other modern technologies have made it possible to identify most of the genes involved in seed development, providing a vast amount of information that could be used in the engineering and design of transgenic crops. However, there are many gaps in the field regarding the functional characterization and determination of the biological relevance of these genes in model species. Unveiling a complete and accurate map of the process remains a major challenge for plant biologists. Achieving these goals will require not only the integration of multiple disciplines including proteomics, metabolomics and functional genomics, but also the development and improvement of automatized computational tools to analyze complex datasets. A comprehensive analysis of large-scale datasets will provide the required tools to enhance the nutritional quality of seeds and also to increase resistance to adverse environmental conditions and/or biological attacks. A second major challenge for plant genomics will be finding an integrative and rational way to apply that information to crop species to improve their agronomic performance. This could be achieved either by using the basic knowledge arising from studies of *Arabidopsis*, or by using the tools and techniques refined with *Arabidopsis* (or other model species), to generate and analyze extensive datasets for important crop species.

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