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Hormonal Regulation of Ovule Initiation in Arabidopsis

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Abstract
As seed precursors, ovules are fundamental organs during the plant life cycle. Decades of morphological and molecular study have allowed for the elucidation of the complex and intricate genetic network regulating ovule development. Ovule and seed number is highly dependent on the number of ovule primordia that are determined from the placenta during early pistil development. Ovule initiation is positively regulated by the plant hormones auxins, cytokinins, and brassinosteroids, as well as negatively regulated by gibberellins. Each hormone does not act independently; multiple points of hormonal crosstalk occur to coordinately regulate ovule primordia initiation. In this review, we highlight the roles of these hormones and their interactions in the genetic and hormonal network co-regulating ovule initiation in Arabidopsis.

Keywords Ovule development · Plant hormone · Auxin · Brassinosteroids · Cytokinin · Gibberellins

Introduction to Ovule Initiation

In angiosperms, ovules form inside the female reproductive part of the flower known as the gynoecium. In the model plant *Arabidopsis thaliana*, the gynoecium is composed of a single pistil formed by two congenitally fused carpels that emerge from the fourth whorl at the center of the flower. During pistil development, a group of meristematic cells at the lateral margins of the carpels (named the carpel margin meristem, CMM) expands toward the center and gives rise to the septum and placenta. Shortly after, ovules develop by the division of subepidermal placental cells as generating finger-like protuberances regularly spaced along the expanding pistil (Herrera-Ubaldo and de Folter 2022; Simonini and Østergaard 2019). Interestingly, ovule initiation proceeds asynchronously rather than at once along the ovary (Yu et al. 2020). The first group of ovule primordia arise from the placenta, but as the placenta elongates, a second group protrude from the space between the already initiated primordia.

Two key steps of ovule initiation include the establishment of the zone of primordium outgrowth and definition of primordium boundaries, regulated by AINTEGUMENTA (ANT) and the CUP-SHAPED COTYLEDON (CUCs), respectively. ANT belongs to the AP2 family of transcription factors (TFs) and is a key positive regulator of ovule primordia growth (Baker et al. 1997; Elliott et al. 1996; Klucher et al. 1996). *ANT* is expressed in the placenta and the ovule primordia, and *ant* mutations strongly reduce ovule number compared to the wild type without reducing ovary length (Klucher et al. 1996; Liu et al. 2000). CUC1, CUC2, and CUC3 belong to the large plant-specific family of NAC TFs and define key boundaries during plant development (Maugarny et al. 2016), such as the ovule primordia boundaries (Gonçalves et al. 2015; Ishida et al. 2000). Within the pistil, *CUC1* and *CUC2* are first expressed in the adaxial region of the medial wall, then in the CMMs, and later in the septum and placenta (Galbiati et al. 2013; Gonçalves et al. 2015; Ishida et al. 2000; Kamiuchi et al. 2014; Nahar et al. 2012; Takada et al. 2001). Accordingly, gynoecia that lack or have reduced *CUC1* and *CUC2* activity present abnormal CMM and septum development and fewer ovules (Galbiati et al. 2013; Gonçalves et al. 2015; Ishida et al. 2000; Kamiuchi et al. 2014). The expression of *CUC1* and *CUC2* is also regulated post-transcriptionally by *miR164* (Larue

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et al. 2009; Sieber et al. 2007), with plants expressing an *miR164*-resistant version of *CUC2* (but not *CUC1*) showing an increased ovule number (Barro-Trastoy et al. 2022). In addition to *CUC* genes, two secreted peptides, EPFL2 and EPFL9, and their receptors from the ERECTA (ER) family also participate in the spacing of ovules in the developing gynoecium (Kawamoto et al. 2020).

Once initiated, the ovule primordia follow a complex developmental process to become mature, functional ovules. Ovule development has been extensively studied at the morphological level in Arabidopsis, including exhaustive qualitative and quantitative cellular characterization (Christensen et al. 1997; Hernandez-Lagana et al. 2021; Lora et al. 2017; Robinson-Beers et al. 1992; Schneitz et al. 1995; Vijayan et al. 2021, 2022). Once ovule primordia are initiated, the protruded mass of cells is organized to define the nucellus, chalaza, and funiculus during patterning. At later stages, the outer and inner integuments differentiate and grow from the chalaza, megasporogenesis and megagametogenesis occur, and the embryo sac is formed. In this way, at the end of ovule development, the mature gametophyte is protected by the ovule structure. Mature ovules become seeds after fertilization. The zygote and trinuclear central cell found in the embryo sac become the embryo and endosperm, respectively, and the integuments differentiate into the seed coat (Phillips and Evans 2020). Therefore, seed development is closely linked to ovule development before fertilization.

Plant Hormones and Ovule Initiation

Plant hormones are fundamental to coordinate correct plant growth and development, integrating environmental and endogenous signals, and ovule initiation and development are not an exception. In this review, we focus on each of the four main hormones that have been involved in ovule initiation: auxins, cytokinins (CKs), brassinosteroids (BRs), and gibberellins (GAs). We introduce each hormone individually, as well as discuss their pairwise interactions (Fig. 1). While we focus on pairwise interactions, we note that all four hormones act simultaneously in the placenta to coordinately regulate ovule initiation, ultimately affecting the ovule and seed number developed in each fruit. We also pay a special attention to *ANT* and *CUC* genes as hormonal crosstalk hubs in ovule initiation. Finally, we review the data regarding the possible role of other hormones in ovule initiation.

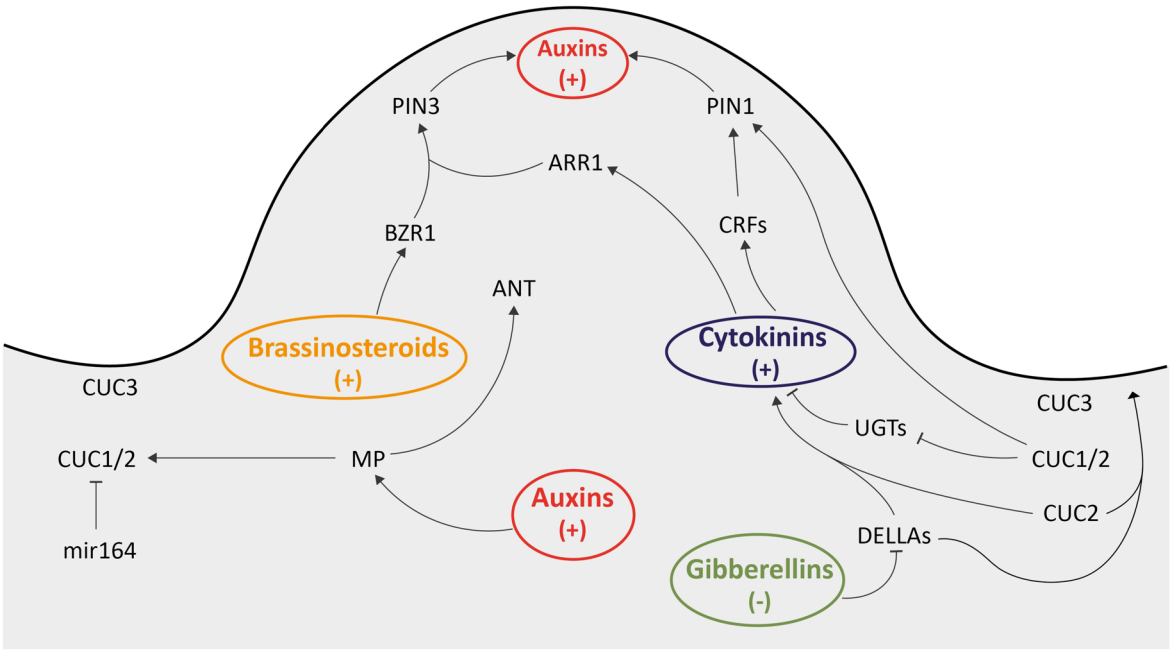

Fig. 1 The current working model for the hormonal regulation of ovule initiation in Arabidopsis. The plant hormones involved are differentiated by color. See text for details. ANT, AINTEGUMENTA; ARR1, ARABIDOPSIS RESPONSE REGULATOR1; BZR1, BRASSINAZOLE RESISTANT1; CRF, CYTOKININ RESPONSE FACTOR; CUC, CUP-SHAPED COTYLEDON; DELLA, DELLA proteins; MP, MONOPTEROS/ARF5 mir164, microRNA164; PIN, PIN-FORMED; UGT, UDP-glucosyl transferases (Color figure online)

Auxins

Auxins play a central role in the coordination of most plant growth processes that shape the plant body via their distribution pattern. Auxin localization is the result of auxin polar transport that relies on the PIN-FORMED (PIN) auxin efflux and other auxin transporter proteins (Hajny et al. 2022), as well as local auxin synthesis (Brumos et al. 2018). Auxin/indole-3-acetic acid (Aux/IAA) repressor proteins and AUXIN RESPONSE FACTORs (ARFs) are major components in auxin signaling. Aux/IAA proteins bind and inhibit the transcriptional activity of ARFs. In the presence of auxin, Aux/IAA proteins are targeted for degradation and ARFs are released to regulate the auxin-dependent transcription of target genes (Leyser 2018).

Auxin accumulation at the ovule initiation site is essential for ovule primordia formation (Fig. 1) (Benkova et al. 2003; Ceccato et al. 2013). Expression of the *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*) gene involved in auxin synthesis occurs in the CMM and the epidermis of the incipient ovule primordia, while the auxin efflux carrier PIN-FORMED1 (PIN1) directs auxin accumulation to the tip of the ovule primordia (Benkova et al. 2003; Nole-Wilson et al. 2010; Yu et al. 2020). The weak *pin1-5* mutant produces pistils with a dramatic reduction in ovule density, suggesting that PIN1 is required for ovule initiation (Bencivenga et al. 2012). In addition, polar auxin transport mediated by PIN3 is key to the asynchronous ovule initiation described above (Hu et al. 2022; Yu et al. 2020). PIN3 is detected in some placental cells prior to ovule initiation (Hu et al. 2022; Larsson et al. 2014), as well as epidermal cells of ovule primordia with its polarity directed toward the tips like PIN1 (Ceccato et al. 2013; Yu et al. 2020). However, unlike with PIN1, a lack of PIN3 slightly reduces ovule density, likely due to a lack of secondary initiated ovules, suggesting that PIN3 is necessary for late ovule initiation (Hu et al. 2022). Regarding auxin signaling, *ARF5/MONOPTEROS* (*MP*) is expressed in the primordia at the early stage 1-I (Schneitz et al. 1995) of ovule initiation, while later expression is restricted to the ovule primordia boundaries (Galbiati et al. 2013).

Auxins integrate the activity of both ANT and CUC proteins in ovule initiation. CUC1 and CUC2 redundantly promote *PIN1* expression and correct PIN1 membrane localization in ovule primordia (Galbiati et al. 2013). Additionally, both *CUC1* and *CUC2* are directly, positively regulated by MP, which also directly induces *ANT* expression (Galbiati et al. 2013; Yamaguchi et al. 2013). ANT has been proposed to regulate the expression of several auxin biosynthesis genes, suggesting a role for ANT in auxin homeostasis, at least in young pistils (Krizek et al. 2020; Nole-Wilson et al. 2010). Another piece of the puzzle is the *ARGOS* gene, which is strongly induced by auxin and regulates *ANT* expression during the control or organ size, suggesting that ARGOS may transduce auxin signals to regulate cell proliferation and organ growth through ANT during organogenesis (Hu et al. 2003).

Little is known regarding the molecular mechanism underlying the effects of auxin or other hormones in ovule formation process. One of the few examples is the possible role of cell wall invertase (CWIN) modulating auxin signaling during ovule initiation (Liao et al. 2020). CWIN activity, which catalyze the hydrolisis of sucrose and are involved in regulating sugar metabolism and signaling, is a positive regulator of ovule initiation in Arabidopsis, as silencing *CWIN2* and *CWIN4* inhibited ovule initiation and induced ovule abortion. Interestingly, the CWIN-originated signals are relayed to the nuclei to regulate expression of genes encoding auxin signaling components and MADS-box TFs, thereby modulating ovule initiation and differentiation (Liao et al. 2020).

Cytokinins

CKs have many roles in plant development that affect several agriculturally important processes, including growth, nutrient responses, and biotic and abiotic stress responses (Kieber and Schaller 2018; Kroll and Brenner 2020). CK biosynthesis, conjugation, inactivation, transport, and signaling result in gene expression changes that regulate CK responses. Key CK-related enzymatic activities include adenylate isopentenyltransferase (IPT, EC 2.5.1.27) and some cytochrome P450 monooxygenase (CYP450) involved in biosynthesis and cytokinin oxidase (CKX; EC 1.5.99.12) that mediates the irreversible degradation of bioactive CK bases and ribonucleosides, and several UDP-glucosyl transferases (UGTs) that catalyze the reversible inactivation of CKs by *O*-glucosylation (Frebort et al. 2011; Hošek et al. 2020; Hoyerova and Hošek 2020). The CK signaling cascade relies on membrane-localized histidine kinase (HK) receptors and type B response regulators (ARR) that results in transcriptional changes of a set of primary response genes (Kieber and Schaller 2018; Kroll and Brenner 2020; Sharma et al. 2022). Another important gene family is the *CYTOKININ RESPONSE FACTORS* (*CRFs*), including the *ERF/AP2* TFs that mediate many CK responses (Kroll and Brenner 2020).

CKs positively regulate ovule number (Fig. 1), as several high-order mutants with reduced CK signaling, such as mutants of the CK receptors, *CRFs* or *ARRs*, present severe reductions in ovule number (Bencivenga et al. 2012; Cucinotta et al. 2016; Reyes-Olalde et al. 2017; Zu et al. 2022). Meanwhile, high CK levels upon exogenous treatments or in multiple *CKX* mutants induce pistil growth and increased ovule density (Bartrina et al. 2011; Cucinotta et al. 2016). However, in *crf* mutants, the ovule number increase with CK treatment is strongly attenuated, revealing the role

of *CRF* genes in ovule initiation (Cucinotta et al. 2016). CUC1 and CUC2 influence CK homeostasis by repressing the expression of *UGT73C1* and *UGT85A3* and pistils from plants lacking both *CUC1* and *CUC2* have lower CK levels (Cucinotta et al. 2018).

Brassinosteroids

BRs are a group of steroid plant hormones with roles in plant growth, development, and stress (Nolan et al. 2019; Planas-Riverola et al. 2019). BRs are perceived by membrane-localized receptors that trigger a phosphorylation relay cascade, regulating the phosphorylation of BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1), two TFs that directly control the transcription of BR-responsive genes (Wang et al. 2002; Yin et al. 2002). In the absence of BRs, BZR1 and BES1 are phosphorylated and inactivated (Peng et al. 2008). In the presence of BRs, BZR1 and BES1 are dephosphorylated and translocated to the nucleus to activate BR responses (Wang et al. 2002; Yin et al. 2002).

BRs are involved in ovule and seed development by regulating their number (Fig. 1), size, and shape (Huang et al. 2013; Jia et al. 2020; Jiang et al. 2013; Modrusan et al. 1994; Schneitz et al. 1998). Plants with low BR levels, such as the BR biosynthesis defective *det2-1* mutant of *DEETIOLATED2*, or plants treated with brassinazole, a BR biosynthesis inhibitor, show a significantly reduced ovule number (Huang et al. 2013). Additionally, loss-of-function mutants of BR receptors *bri1* and the gain-of-function mutant *bin2-1* of *BR-INSENSITIVE 2* have fewer ovules than wild-type plants (Huang et al. 2013; Jia et al. 2020), while the gain-of-function mutant *bzr1-1D* increases ovule number. Therefore, BRs positively regulate ovule number by activating BZR1.

BZR1 regulates the expression of some ovule developmental genes (Huang et al. 2013). BR treatments upregulate *ANT*, *HUELLENLOS* (*HLL*), and *SEEDSTICK* (*STK*) and downregulate *APETALA2* (*AP2*) in inflorescences, as *ANT* and *AP2* are BZR1 direct targets (Huang et al. 2013). ANT promotes ovule primordia growth and integument development (Elliott et al. 1996; Klucher et al. 1996), HLL promotes ovule growth redundantly with ANT (Schneitz et al. 1998), while STK and AP2 affect ovule identity (Modrusan et al. 1994; Pinyopich et al. 2003). Despite BRs upregulate *ANT* expression, 35S-driven overexpression of *ANT* does not increase ovule number (Barro-Trastoy et al. 2020). Previous research suggests that ANT activity is not absolutely required for ovule initiation per se, as ovule primordia are initiated and continue to develop until the time of integument initiation in *ant* mutants (Azhakanandam et al. 2008). Most likely, ANT is required for proper placenta development as a major regulatory player in ovule primordia initiation, in conjunction with other factors, such as BRs.

Gibberellins

GAs are regulators of a range of plant developmental processes (Daviere and Achard 2013), including floral transition, male fertility, ovule morphology, and fruit set (Blazquez et al. 1998; Dorcey et al. 2009; Gomez et al. 2016; Plackett et al. 2011). The GA signaling pathway relies on the degradation of DELLA proteins, which are nuclear proteins of the GRAS family of transcriptional regulators that block GA responses (Silverstone et al. 2001). Bioactive GAs bind to the GID1 receptor to trigger DELLA protein polyubiquitination and degradation. When bioactive GA levels are low, DELLA proteins accumulate and restrain GA responses. DELLA proteins have a conserved C-terminal GRAS domain that is involved in protein–protein interactions and a unique N-terminal regulatory domain that includes the DELLA motif, which is critical for GA-dependent degradation (Vera-Sirera et al. 2016). DELLA proteins lack typical DNA-binding domains; therefore, they act as transcriptional regulators of their target genes by interacting with a variety of TFs and transcriptional regulators (Daviere and Achard 2016; Hernandez-Garcia et al. 2021; Lantzouni et al. 2020; Marin de la Rosa et al. 2014). The Arabidopsis genome encodes five DELLA proteins with partially overlapping functions: GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3 (Dill and Sun 2001; Peng et al. 1997; Silverstone et al. 1998; Wen and Chang 2002). Complete loss of function of DELLA activity causes a constitutive GA response, whereas mutant proteins lacking the N-terminal DELLA motif that cannot be targeted for degradation, such as the *gai-1* allele (Peng and Harberd 1993) and the *pRGA:GFP-rgaΔ17*, *pRGL1:YPet-rgl1Δ17*, and *pRGL2:YPet-rgl2Δ17* lines (Dill et al. 2001; Gomez et al. 2019, 2020), result in constitutive DELLA activity and block the GA-mediated response.

GAs are involved in ovule development from the earliest stages, as evidenced by the presence of various components of the GA signaling pathway and GA metabolism in the placenta and ovules. Specifically, expression of the *DELLA* genes *GAI*, *RGA*, *RGL1*, and *RGL2* is detected in placental tissues and outgrowing ovules (Gomez et al. 2018, 2019, 2020). Genetic evidence identifies GAs as negative regulators of ovule primordia initiation (Fig. 1) (Gomez et al. 2018). During pistil development, constitutive GA signaling in the null mutant *4xdella*, lacking four of the five DELLA proteins in Arabidopsis, produces a reduced number of ovules, with GAI, RGA, and RGL2 playing the largest role (Gomez et al. 2018). A similar ovule number phenotype was also observed in GA-treated Arabidopsis plants, which phenocopy the null *della* mutants. Meanwhile, ovule number increases in the gain-of-function DELLA mutants *gai-1* (Gomez et al.

2018) and *pRGL2:YPet-rgl2Δ17* (Gomez et al. 2019), in the *gid1a gid1b* double mutant lacking GA perception in ovules (Gallego-Giraldo et al. 2014), and in plants treated with paclobutrazol (PBZ), an inhibitor of GA biosynthesis (Gomez et al. 2018). These results indicate that GAs are negative regulators of ovule number by promoting the degradation of DELLA proteins that help regulate ovule primordia formation.

At least in Arabidopsis, the molecular mechanism of DELLA-dependent ovule initiation likely relies on a direct protein–protein interaction with CUC2 to control the expression of downstream target genes (Barro-Trastoy et al. 2022). CUC1 and CUC2 may interact in vivo with GAI in placental tissue and both GAI and CUC2 colocalize in placental cells at ovule initiation. GAI would require CUC2 (but not CUC1) to promote the formation of ovule primordia, since the absence of CUC2 in *cuc2-1* prevents an increase in ovule number in the *gai-1* gain-of-function mutant or in plants treated with PBZ. Moreover, ChIP-Seq analysis identifies several target genes regulated by GAI in a CUC2-dependent manner, some of which have a role in ovule initiation, such as *CKX5*, *CRF2*, *SEP2*, *REM*, and *GRF* genes, providing information about the downstream molecular events controlled by the DELLA–CUC2 complex.

Crosstalk Between Auxins, Cytokinins, Brassinosteroids, and Gibberellins

Rather than acting independently, auxins, CKs, BRs, and GAs act together to establish a complex hormonal regulatory network with multiple crosstalk points to control when, where, and how ovule primordia originate in the placenta during pistil development (Fig. 1; Table 1).

Auxins and Cytokinins

Strong evidence points to an auxin-CK interplay guiding ovule initiation by the direct regulation of *PIN1* expression. CK treatment upregulates PIN1 expression in ovule primordia (Bencivenga et al. 2012; Cucinotta et al. 2016; Galbiati et al. 2013). PIN1 regulation was also observed in mutants of the CK receptors ARABIDOPSIS HISTIDINE KINASES (AHKs) (Bencivenga et al. 2012) and CRFs (Cucinotta et al. 2016). CK treatment does not increase PIN1 expression in a CRF mutant background, suggesting that CK-dependent PIN1 expression is mediated by CRFs (Cucinotta et al. 2016). Moreover, CRFs directly regulate PIN1 expression by binding to a specific cis element in the PIN1 promoter (Šimaškova et al. 2015). In addition, *PIN3* is directly activated by ARR1 or with CK treatments in the medial domain of developing pistils (Reyes-Olalde et al. 2017). These data

Table 1 Hormonal crosstalk during ovule initiation

Pairwise interactions	Nature of interaction	References
Auxin-cytokinin	Auxin localization and auxin maxima are regulated by CKs	Bencivenga et al. (2012), Cucinotta et al. (2016), Galbiati et al. (2013), Reyes-Olalde et al. (2017)
	PIN1 expression is upregulated by CK treatment	
	CRFs directly upregulate the expression of *PIN1*	
	PIN3 is directly activated by ARR1 or CK treatments	
Auxin-brassinostroid	PIN3 function downstream of BRs in late ovule initiation	Hu et al. (2022)
	Auxin response is enhanced with BR and decreased in *bin2-1*	
	PIN3 expression is upregulated in *bzr1-1D*	
	PIN3 expression is downregulated in *bin2-1*	
	BZR1 response is reduced in *pin3* mutants	
Auxin-gibberellin	No direct interaction reported	Barro-Trastoy et al. (2022), Galbiati et al. (2013)
	gai-1 does not alter the auxin maxima	
	PIN1 expression is not regulated upon GA treatments	
Cytokinin-brassinosteroid	BRs and CKs co-regulate expression of *ANT, HLL,* and *STK*	Zu et al. (2022)
	ARR5 and ARR7 expression are upregulated by BR treatments	
	CK response is higher in *bzr1-1D* and lower in *bin2-1*	
	BZR1 protein levels increases by CKs	
	BZR1 can physically bind to ARR1	
Cytokinin-gibberellin	No direct data reported on the direct interplay of CKs and GAs	Barro-Trastoy et al. (2022), Cucinotta et al. (2018)
Brassinosteroid-gibberellin	There is no direct interaction in Arabidopsis	Barro-Trastoy et al. (2020)
	BRs regulate GA levels in in tomato	Barro-Trastoy et al. (2020)
	GA20ox1 expression in ovules is downregulated by BRs	

suggest that the auxin localization distribution and auxin maxima in the ovule primordia could be regulated by CKs.

Auxins and Brassinosteroids

Auxins also interact with BRs to regulate ovule initiation, as the auxin response at the tip of the ovule primordia is enhanced with BR treatments and decreased in *bin2-1* mutant (Hu et al. 2022). Similarly, *PIN3* expression is upregulated in *bzr1-1D* and downregulated in *bin2-1* mutants, although the localization of PIN3 protein levels is not altered in *bin2-1* mutants (Hu et al. 2022). In addition, *pin3* loss-of-function mutants present a reduced BZR1 response in ovule primordia, suggesting that BR-mediated auxin response in ovule initiation depends on PIN3. Ovule number is reduced in *pin3* mutants because late ovule initiation fails, and this phenotype persists in the *bzr1-1D pin3* double mutant (Hu et al. 2022), confirming that PIN3 may function downstream of BRs in ovule primordia.

Auxins and Gibberellins

The role of GAs and DELLA proteins in determining ovule number is likely not related to auxin transport or signaling in the ovule primordia (Gomez et al. 2018). The DELLA gain-of-function *gai-1* mutant does not alter the intensity or spatial distribution of the auxin maximum at the tip of the ovule primordia, monitored by the activity of the DR5 promoter. Additionally, GA treatment does not alter PIN1 expression in the ovule primordia. Despite this, further studies are needed to confirm the independence of the auxin and GA functions in ovule initiation. For example, both *CUC1* and *CUC2* expression are directly activated by MP, an auxin response factor (Galbiati et al. 2013). *MP* shares an expression pattern with *CUC1* and *CUC2* in the placenta before ovule primordia arise and with *CUC2* in ovule primordia boundaries, so *MP* is likely required for *CUC1* and *CUC2* expression during early stages of placenta development and ovule initiation (Galbiati et al. 2013). *CUC1* and *CUC2* promote *PIN1* expression and proper PIN1 membrane localization in ovule primordia (Galbiati et al. 2013), but whether this function of CUC2 requires interaction with DELLA in a protein complex or is DELLA-independent should be explored in future.

Cytokinins and Brassinosteroids

BRs and CKs influence a common subset of ovule development genes, including *ANT*, *HLL*, and *STK* (Zu et al. 2022). The CK response is higher in both the placenta and ovule primordia of *bzr1-1D* and lower in *bin2-1*, while *ARR5* and *ARR7* are upregulated with BR treatments, indicating that BRs enhance CK signaling. In contrast, protein levels of nucleus-localized BZR1 increase in ovules with high CK levels, suggesting that CKs also regulate BR signaling (Zu et al. 2022). BZR1 can physically bind with ARR1, indicating a direct interaction of BR and CK signaling. Moreover, *arr1* loss of mutants have a slightly reduced seed number that is not increased in *arr1 bzr1-1D* double mutants. ARR1 induces both *STK* and *HLL* promoter activities, which is strengthened by BZR1, but high CK levels partially restore *bin2-1* seed number (Zu et al. 2022). Overall, BRs and CKs coordinately regulate ovule and seed number.

Cytokinins and Gibberellins

No research has focused on the direct interplay of CKs and GAs in ovule initiation. However, a possible interaction between GAs and CKs in ovule initiation is supported by GA-mediated ovule initiation, mediated by CUC2 (Barro-Trastoy et al. 2022), and CUC2 affecting CK levels by regulating *UGT* gene expression (Cucinotta et al. 2018). ChIP-Seq data indicate that several CK-related genes are direct targets of the GAI-CUC2 complex, including *CKX5* and *CRF2* (Barro-Trastoy et al. 2022). A plausible scenario depicts the regulation of CK metabolism and signaling by GAs through the activity of the DELLA-CUC2 complex.

Gibberellins and Brassinosteroids

Both GAs and BRs act in opposition in the promotion of ovule primordia formation in Arabidopsis and tomato, but through different mechanisms (Barro-Trastoy et al. 2020). In Arabidopsis, they act independently; GAs reduce ovule number regardless of BR content, and BRs can increase ovule number in plants with constitutive or impaired GA signaling upon GA treatment or in the *gai-1* mutant, respectively. In contrast, in tomato ovaries, ovule initiation relies on a BR-dependent control of GA biosynthesis (Barro-Trastoy et al. 2020). BRs can only modulate ovule number in tomato plants with normal GA signaling, not in plants treated with GAs or in the *procera* mutant (PROCERA is the only DELLA protein in tomato) with a constitutive GA response. In unpollinated tomato ovaries, the levels of bioactive GAs are higher in Micro-Tom (which harbors a dwarf mutation in the *DWARF4* gene involved in BR biosynthesis) than those from Micro-Tom-D (an isogenic line carrying the wild-type *DWARF4* gene), suggesting that BRs reduce GA biosynthesis. This would rely on the downregulation of *SlGA20ox1* expression at early phases of ovary development, when *DWARF4* is highly expressed (Barro-Trastoy et al. 2020; Montoya et al. 2005). In short, BRs control ovule number in tomato by reducing the GA levels in the placenta, through the repression of *SlGA20ox1*, thus stabilizing PROCERA that would promote ovule primordia emergence.

ANT and CUC Genes as Hormonal Crosstalk Hubs in Ovule Initiation

Hormonal crosstalk is more complex than the pairwise comparison we have presented, as the four hormones coordinately regulate ovule initiation. The current working model calls for two key genetic hubs that integrate hormonal regulation during ovule initiation: *ANT* and *CUC* genes. Both ANT and CUC TFs are interconnected with hormone homeostasis and the signaling pathways of auxins, CKs, BRs, and GAs.

CUC1 and *CUC2* are expressed in the CMM, whereas *CUC2* and *CUC3* are expressed at the boundaries between ovule primordia (Galbiati et al. 2013; Gonçalves et al. 2015; Ishida et al. 2000; Kamiuchi et al. 2014; Nahar et al. 2012; Takada et al. 2001; Vroemen et al. 2003). During ovule initiation, *CUC1* and *CUC2* are linked with auxin signaling and transport and CK inactivation (Cucinotta et al. 2018). Both *CUC1* and *CUC2* are directly transcriptionally activated by MP (Fig. 1) (Galbiati et al. 2013). In addition, *MP* shares an expression pattern with *CUC1* and *CUC2* in the placenta before ovule primordia arise and with *CUC2* in ovule primordia boundaries, so MP is likely required for *CUC1* and *CUC2* expression during the early stages of placenta development and ovule initiation (Galbiati et al. 2013). *CUC1* and *CUC2* promote PIN1 expression and proper PIN1 membrane localization in ovule primordia (Galbiati et al. 2013). CK treatments also increase *PIN1* expression (Bencivenga et al. 2012), and *CUC1* and *CUC2* induce CK responses by transcriptionally repressing *UGT73C1* and *UGT85A3* (Cucinotta et al. 2018), suggesting that CKs could act downstream from CUC1 and CUC2 to induce *PIN1* expression (Fig. 1). CUC2 is also linked to GA-mediated ovule initiation by the formation of a complex with DELLA proteins to control the expression of downstream genes that promote ovule initiation (Barro-Trastoy et al. 2022). Among these, several CK genes including *CKX5* and *CRF2* are potential direct targets of the DELLA-CUC2 complex. Therefore, CUCs, particularly CUC2, are hubs that integrate auxins, CKs, and GAs during ovule initiation in Arabidopsis.

ANT, a key positive regulator of ovule primordia and integument development (Baker et al. 1997; Elliott et al. 1996; Klucher et al. 1996), is a direct target of BZR1 (Huang et al. 2013). *ANT* expression is also slightly upregulated by GAI activity in inflorescences of the *gai-1* mutant, although closer examination does not reveal an alteration of *ANT* expression in the placenta of *gai-1* or *global* DELLA mutants or with GA or PBZ treatments (Barro-Trastoy et al. 2020). Overexpression of *ANT* increases the size of floral organs and ovules (Barro-Trastoy et al. 2020; Mizukami and Fischer 2000) but does not induce the formation of more ovules than in wild-type plants (Barro-Trastoy et al. 2020). Therefore, the increased *ANT* expression by BRs and GAI does not explain the increase in ovule number observed in *bzr1-1D* or *gai-1* mutants, respectively. *ANT* is also induced by CKs (Zu et al. 2022), indicating that BRs and CKs may regulate ovule initiation by affecting the expression of a common subset of genes.

In addition, ANT regulates the expression of several auxin genes. First, *ant-8* mutants have reduced expression levels of at least *AUX/IAA1*, *AUX/IAA17*, and *TAA1* in young pistils at ovule initiation (Nole-Wilson et al. 2010). Second, ANT regulates auxin biosynthesis genes in floral development stages 6–7 by directly binding to their respective regulatory regions, suggesting a role for ANT in auxin homeostasis (Krizek et al. 2020). The same RNA-Seq and ChIP-Seq analysis in flower buds revealed that ANT directly targets the expression of *GAI* and *RGA* (Krizek et al. 2020). *ANT* is also a direct target gene of MP (Galbiati et al. 2013; Yamaguchi et al. 2013). However, the nature of this ANT-dependent gene regulation and the expression of *ANT* by MP require further characterization to understand their biological significance in ovule initiation.

Other Hormones Involved on Ovule Initiation

In addition to auxin, BRs, CKs, and GAs, other hormones may also participate in ovule initiation. Analysis of the Arabidopsis ovule transcriptome (Matias-Hernandez et al. 2010) reveals many ethylene-related genes expressed in ovule primordia at early phase of primordia emergence (stages 1-I and 1-II, corresponding to stage 8–9 of flower development), including genes in ethylene biosynthesis such as 1-aminocyclopropane-1-carboxylate (ACC) oxidase (EC 1.14.17.4), ETHYLENE-INSENSITIVE3 (*EIN3*), EIN3-related 1 and 3 (*EIL1* and *EIL3*), ethylene receptors (*ETR1*, *ETR2*, and *ERS2*), several ETHYLENE RESPONSE FACTORs (*ERF1*, *ERF2*, *EFRF4*, *ERF5*, *ERF6*, *ERF7*, *ERF8*, *ERF9*), and other ethylene-responsive genes. The abundance of ethylene genes strongly suggests that ethylene may play a relevant role in ovule initiation. In fact, in grape (*Vitis vinifera* L.), the expression levels of most *ERF* genes were much higher during ovule development in seedless grapes, suggesting a role in ovule abortion related to seedlessness (Zhu et al. 2019). Ethylene also regulates the onset of ovule senescence in unfertilized pistils of Arabidopsis (Carbonell-Bejerano et al. 2011). In contrast to ethylene, only few genes related to jasmonic acid (JA), salicylic acid (SA), or ABA were expressed at early ovule primordia stages (Matias-Hernandez et al. 2010). These include JASMONATE INSENSITIVE1 (*MYC2/JAI1*), genes similar to jasmonic and salicylic acid carboxyl methyltransferases, SA INDUCTION DEFICIENT1 (*SID1*), and ABA INSENSITIVE 5 (*ABI5*) involved in ABA signaling during seed maturation and germination

or transcription factors with binding activity toward ABA RESPONSIVE ELEMENTs (*ABF3* and *ABF4*). Regarding JA, it has been shown that JA regulates stamen development in Arabidopsis but also late ovule development and fertility in tomato through the function of *MYC2*/*JAI1* (Schubert et al. 2019; Huang et al. 2023). In the case of ABA, it was reported a high ovule abortion in F1 plants from the cross of *sdk1-7*, one of the S-domain receptor kinases involved in ABA responses, with a null *abi3-6* (Sankaranarayanan et al. 2020). A more detailed analysis is the specific role of these hormones, and other plant growth regulators in ovule development would be crucial to better understand the complex hormonal network governing ovule primordia emergence.

Conclusion and Future Perspectives

Understanding the molecular mechanisms governing ovule and seed development is vital, as seeds ensure plant reproduction and form the basis of agriculture. Seed number per fruit and seed quality directly depend on ovule initiation and morphogenesis, and fine-tuning these processes could be a valuable crop yield improvement strategy. In this review, we have focused on the hormonal regulation of ovule initiation, with most data generated in the reference species Arabidopsis. Although there is substantial information on the hormonal control of ovule initiation, we are only beginning to fully understand it. Future research should aim to provide a wider and deeper view of the hormonal-molecular mechanisms orchestrating this key developmental process, paying attention particularly to other potential players, such as other hormones and growth regulators not yet directly involved, and cellular mechanisms underlying the effects of phytohormones during ovule initiation. In addition to deepening our knowledge in Arabidopsis, next steps should focus on studying the regulation of ovule development in crop species to determine whether the genetic network governing ovule initiation in Arabidopsis functions totally or partially in other plant species. This would provide two advantages. First, determining how general these gene networks are, i.e., which of these components and their interactions are present and act similarly in all flowering plants and which are species specific. Second, modifying these components to promote the formation of more ovules and seeds would be valuable to increase yield in crops such as legumes or brassicas that rely on seed production for food, feed, and industrial uses. An excellent example is the demonstration that elevated CKs levels in mutants of *CKX* genes in rapeseed produced more flowers, more ovules per pistil, and a significant inclease in seed production (Schwarz et al. 2020). Future work on this direction will allow new strategies to achieve yield enhancement in dicot crop plants.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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