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Ovule initiation: the essential step controlling offspring number in *Arabidopsis*

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ABSTRACT

Seed is the offspring of angiosperms. Plants produce large numbers of seeds to ensure effective reproduction and survival in varying environments. Ovule is a fundamentally important organ and is the precursor of the seed. In *Arabidopsis* and other plants characterized by multi-ovulate ovaries, ovule initiation determines the maximal ovule number, thus greatly affecting seed number per fruit and seed yield. Investigating the regulatory mechanism of ovule initiation has both scientific and economic significance. However, the genetic and molecular basis underlying ovule initiation remains unclear due to technological limitations. Very recently, rules governing the multiple ovules initiation from one placenta have been identified, the individual functions and crosstalk of phytohormones in regulating ovule initiation have been further characterized, and new regulators of ovule boundary are reported, therefore expanding the understanding of this field. In this review, we present an overview of current knowledge in ovule initiation and summarize the significance of ovule initiation in regulating the number of plant offspring, as well as raise insights for the future study in this field that provide potential routes for the improvement of crop yield.

Keywords: asynchronous initiation of ovule primordia, early and late ovule initiation, placenta elongation, plant hormones, seed number per fruit

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INTRODUCTION

Angiosperms, or green flowering plants, are the largest species group in the plant kingdom, comprising 416 families and more than 360,000 species (Willis, 2017). Seeds are the offspring of angiosperms. Plants produce many seeds to guarantee that their offspring will survive successfully in varying environments. Regulation of seed number is a complex process. In general, the more seeds, the better the odds of survival for the species. However, in plants characterized by multi-ovulate ovaries, limited ovary space and nutrition might affect seed quality if a plant produces too many seeds. Although increased seed number might negatively influence seed development and seed weight, as long as fertility is not defective, these seeds still contribute to the effective offspring number, and thus still benefit plant production. A balance between seed quantity and quality is therefore required (McGinley and Charnov, 1988; Sadras, 2007). Popular food crops and oil crops, such as cereals, canola, and beans, are

angiosperms that produce seeds as their major harvest product. Seed yield depends on seed number and seed weight. The effective seed number is determined by seed organogenesis and seed development. Increasing seed number benefits seed yield as long as seed weight is not severely limited (Jeong et al., 2012; Hu et al., 2022a). Balance seed number and weight are important, and optimization may be different in crop yield and plant production.

Ovules serve as precursors of seeds, and initiation of ovule primordia is the beginning of seed formation. In other words, ovule initiation provides prerequisite for new life of plant offspring. Ovule initiation determines the maximal possibility of ovule number per flower and greatly influences the seed number per fruit in plants characterized by multi-ovulate ovaries, even affecting fruit size, which makes ovule number an important agronomic trait. In the second green revolution, as scientific disciplines have become increasingly diversified, we need a more complete understanding of the mechanisms by which genetic and environmental variation modify grain yield and composition, so that specific quantitative and qualitative targets can be identified (Wollenweber et al., 2005). To achieve this, we must draw on insights from plant genomics, physiology, agronomy, and plant modeling (Wollenweber et al., 2005). An in-depth understanding of the genes regulating ovule primordia initiation will provide a reference for precision breeding.

The process of ovule primordia initiation encompasses fundamental scientific questions, including cell fate determination, cell differentiation, ovule primordium identity, ovule boundary identity, and ovule protrusion. Investigating the complete process and regulatory mechanism of ovule primordia initiation has both scientific significance and potential applications. Most studies of ovule primordia initiation have been performed in the model plant *Arabidopsis thaliana*, which has multi-ovulate ovaries. Such research in *Arabidopsis* may provide clues for improving the seed yield of similar cruciferous and leguminous crops.

This review summarizes research progress in our understanding of ovule initiation in *A. thaliana*, including: (i) the rules for initiating multiple ovules and their regulatory mechanisms; (ii) integration of phytohormones in regulating ovule initiation; and (iii) signals regulating ovule boundary formation during ovule initiation. We also introduce new opinions on the significance of multiple-step initiation of ovule primordia and suggest directions for future studies and crop yield improvement.

BEFORE OVULE INITIATION

The development of *Arabidopsis* flowers, from the emergence of floral primordia to seed formation, is divided into 20 stages (Smyth et al., 1990). Gynoecium primordia appear at floral stage 5, with the gynoecium of *Arabidopsis* formed by fusion of two carpels (Smyth et al., 1990; Bowman et al., 1991a). At floral stage 7-8, a region possessing meristematic activity called the carpel marginal meristem (CMM) forms in the medial region of the pistil (Smyth et al., 1990; Bowman et al., 1991a). The CMM further differentiates to form four rows of placentae, and the ovule primordia initiate from the sub-epidermis of the placentae (Bowman et al., 1991a; Sessions, 1997; Bowman et al., 1999; Cucinotta et al., 2014). Specification and formation of placentae are essential for the initiation of ovule primordia (Bowman et al., 1999; Reyes-Olalde et al., 2013; Cucinotta et al., 2014). Some transcription factors regulating placenta development and ovule identity have been reported (Colombo et al., 2008; Reyes-Olalde et al., 2013). Loss-of-function mutants of these transcription factors display both defective placenta formation and abnormal ovule identity/reduced ovule number, indicating that these genes function in placenta formation and ovule initiation (Colombo et al., 2008; Reyes-Olalde et al., 2013). MADS-box genes specify floral organ identity (Theißen, 2001; Rijpkema et al., 2010). Among these genes, the Class C gene *AGAMOUS* (*AG*) is involved in carpel and ovule identity; *ag* single mutants lack carpels (Bowman et al., 1989; Yanofsky et al., 1990; Bowman et al., 1991b). In addition, the AP2-like transcription factor *AINTEGUMENTA* (*ANT*), an important gene regulating placenta development, is expressed in all lateral organ primordia and is also highly expressed in placentae (Elliott et al., 1996; Klucher et al., 1996). Carpel fusion fails to occur in the *ant-1*, *ant-3*, *ant-4*, and *ant-9* single mutants and these plants produce a reduced number of ovules with an increased ovule boundary (Elliott et al., 1996; Klucher et al., 1996). Several transcription factors have subsequently been identified that are functionally redundant with *ANT* in placenta formation, including *LEUNIG* (*LUG*) (Liu and Meyerowitz, 1995; Liu et al., 2000), *REVOLUTA* (*REV*) (Otsuga et al., 2001; Nole-Wilson et al., 2010a), *SEUSS* (*SEU*) (Franks et al., 2002; Azhakanandam et al., 2008), *ANT-LIKE* (*AIL*) (Nole-Wilson et al., 2005; Krizek, 2009), *YABBY* genes (Nole-Wilson and Krizek, 2006; Colombo et al., 2010), and *PERIANTHIA* (*PAN*) (Das et al., 2009; Maier et al., 2009; Wynn et al., 2014). Mutations of these genes produce more dramatic abnormalities of placenta formation when combined with *ANT* mutations (Liu et al., 2000; Azhakanandam et al., 2008; Krizek, 2009; Nole-Wilson et al., 2010a; Wynn et al., 2014). Furthermore, members of the MADS-box family defined as Class D genes, such as *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*), and *SHP2*, are involved in ovule identity and development (Theissen et al., 2000; Pinyopich et al., 2003). Some of the ovules in the *stk shp1 shp2* triple mutant are converted into either leaf-like or carpel-like structures (Pinyopich et al., 2003). Some regulators affecting integument development also affect ovule primordia initiation, such as *HUELLENLOS* (*HLL*) and *SHORT INTEGUMENTS 2* (*SIN2*) (Schneitz et al., 1998; Broadhvest et al., 2000). *HLL* encodes a mitochondrial ribosomal protein whose mutation is associated with a 10% reduction in ovule number (Schneitz et al., 1998; Skinner et al., 2001). *SIN2* encodes a mitochondrial DAR GTPase. *sin2* mutants display a shorter gynoecium, fewer ovules, and abnormal ovule distribution along the placenta

(with greater distances between ovules than in the wild type) (Broadhvest et al., 2000; Hill et al., 2006). Genome-wide association studies reveals a novel regulator of ovule number and fertility, *NEW ENHANCER of ROOT DWARFISM* (*NERD1*), which encodes an integral membrane protein. *nerd1-2* and *nerd1-4* mutants have fewer ovules than wild-type plants, and overexpression of *NERD1* leads to an increase in ovule number (Yuan and Kessler, 2019). These genes' functions in placenta formation and ovule initiation can be grouped into developmental regulation of ovule initiation, which are different from the hormonal regulation that mainly influence ovule initiation.

Regulation of placenta formation has been well summarized by Reyes-Olalde et al. (2013), and will not be described in detail in this review. However, it is necessary to point out that placenta formation and ovule initiation appear to be tightly connected since severe mutants of the above genes have both impaired placenta formation (even abnormal floral organs) and ovule initiation (Reyes-Olalde et al., 2013). Weak alleles produce a normal placenta but reduced ovule number, indicating that ovule initiation in those mutants is not substantially disturbed (Reyes-Olalde et al., 2013). Ovule number might be considered to reflect ovule initiation; however, their properties are not exactly the same. Previous work has also identified several hormonal signals that regulate placenta formation and ovule number, which are similar to weak mutants of development-related genes (Bencivenga et al., 2012; Cucinotta et al., 2014; Marsch-Martínez and de Folter, 2016; Deb et al., 2018; Cucinotta et al., 2020; Qadir et al., 2021; Yang and Tucker, 2021); and other hormonal signals involved in ovule number regulation but not in placenta formation (Bartrina et al., 2011; Huang et al., 2013; Gómez et al., 2018), these studies provide further evidence that influencing ovule number does not equate to disturbing ovule initiation.

We propose several possibilities for explaining the combined phenotypes of placenta development and ovule initiation. First, the genes specifically regulating ovule initiation have not yet been identified. Some genes play essential roles in several processes including ovule initiation. Loss-of-function mutants of these genes display severe phenotypes before ovule initiation, while phenotypes associated with ovule initiation are masked by defective development in prophase. Second, the genes specifically regulating ovule initiation (both known and unknown genes) show functional redundancy, and scientists have not identified the gene combinations sufficient and necessary for ovule initiation. Demonstration of the genes essential for ovule initiation will require identification of single or multiple mutants with normal placenta but without any ovules. Third, the identity of the placental tissue is required for regular initiation of ovule primordium because the ovule primordia arise from the placenta tissue, which has been well summarized by the previous reviews (Reyes-Olalde et al., 2013; Cucinotta et al., 2014). In

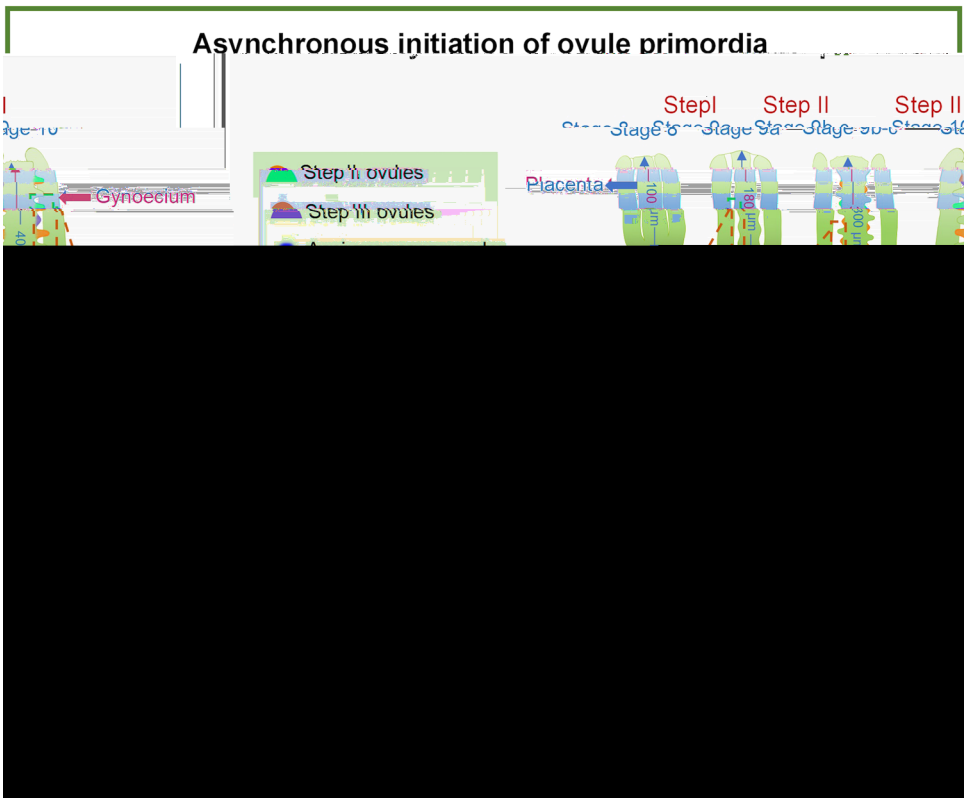

Figure 1. Schematic illustrations of asynchronous initiation of ovule primordia in *Arabidopsis*
At floral stage 8, there are no ovule protrusions on the placenta. At early stage 9 (stage 9a), four to six ovule primordia (small-bump shape) protrude in each placenta in the meantime. At middle-late stages 9 (stage 9b-c), as the placentae elongate, the boundaries between each two initiated ovules (now growing to dome-shape) expanded and new ovule primordia initiate in the boundaries. When the first two rounds of initiated ovules grow to a similar finger shape, sporadic new ovule primordia are initiated in the larger boundaries of the placenta at stage 10. The length of pistil at each stage has been marked on the pistil, and the number below represents ovule number in each placenta in the above stage, respectively. During the ovule initiation, the dynamics of auxin distribution and auxin response peaks depend on the dynamics of PIN1 polar localization.

stage 2-I) (Schneitz et al., 1995), with only minor differences in the number and shape of ovules at each placenta (Yu et al., 2020). Collectively, the ovule primordia in the same placenta initiate asynchronously, and two rounds of ovule initiation at floral stage 9 contribute around 90% of the total ovule number (Yu et al., 2020; Hu et al., 2022b).

Early and late ovule initiation

At stage 10, the initial two groups of ovule primordia gradually grow to a similar size and shape, entering the subsequent developmental process almost simultaneously. At this time, the placentae continue to grow, resulting in elongation of the boundaries between growing ovules. Some boundaries between two existing ovules are larger than others, being sufficient for initiation of new primordia. A small number of new ovule primordia (small-bump shaped, ovule developmental stage 1-I) (Schneitz et al., 1995) initiate sporadically (Hu et al., 2022b). The new ovule primordia are easily identified in stage 11 (dome shape, ovule stage 1-II) (Schneitz et al., 1995) since older ovules have initiated integument primordia (Hu et al., 2022b). Different from the two regular rounds of ovule initiation in stage 9 (Yu et al., 2020), initiation of new ovule primordia on these larger boundaries occurs sporadically (Schneitz et al., 1995; Hu et al., 2022b). The statistical analysis illustrates that around 10% of ovules are initiated at stage 10 (Hu et al., 2022b). The two rounds of regular ovule initiation at stage 9 are defined as early ovule initiation (ovules of step I and step II in Figure 1), which is the main process of ovule primordia initiation and contributes 90% of the total ovule number (Hu et al., 2022b). Sporadic ovule initiation at stage 10 is defined as late ovule initiation (ovules of step III in Figure 1), which is an extra process of ovule primordia initiation and contributes 10% of the total ovule number (Hu et al., 2022b). Since new ovule initiation depends on boundary size, this extra process of ovule primordia initiation may not occur if the environment is unfavorable.

The individual functions and the crosstalk of plant hormones in regulating ovule initiation

Plant organogenesis is regulated by the vital activities of cell proliferation, growth, and differentiation; the intercellular communication required for this process is mediated by different phytohormones. It is well known that auxin plays an essential role in ovule initiation. Other hormones, such as

cytokinin (CK), brassinosteroid (BR), and gibberellin (GA), are also involved in regulating ovule initiation and contribute to gynoecium size and ovule number (Bartrina et al., 2011; Huang et al., 2013; Gómez et al., 2018). These hormones can work both independently and through crosstalk with each other to influence ovule initiation (Cucinotta et al., 2014; Marsch-Martínez and de Folter, 2016; Deb et al., 2018; Cucinotta et al., 2020; Qadir et al., 2021; Yang and Tucker, 2021). The molecular mechanisms by which individual hormones regulate ovule initiation, as well as some crosstalk between hormones, have been well summarized in previous reviews (Cucinotta et al., 2014; Cucinotta et al., 2020; Barro-Trastoy et al., 2020b; Qadir et al., 2021; Yang and Tucker, 2021). Here, we briefly introduce the individual functions of auxin, CK, BR, and GA in ovule initiation, and discuss some very recent progress in understanding auxin regulation of asynchronous ovule initiation and the integration of different hormones regulating ovule initiation (Figure 2).

The process of ovule primordia initiation is somewhat similar to the process of lateral organ primordia initiation in the shoot apical meristem (SAM) (Schwabe, 1984; Kuhlemeier and Reinhardt, 2000; Cucinotta et al., 2014). The well-studied phytohormone auxin is known to undergo dynamic changes in polar transport and signal responses around the SAM; this dynamic determines the formation of new primordia in the SAM and is responsible for the regular arrangement of multiple lateral organs (Reinhardt et al., 2000; Heisler et al., 2005; Sassi and Vernoux, 2013; Kuhlemeier, 2017; Reinhardt and Gola, 2022). Analysis of auxin-related mutants has demonstrated that auxin is also involved in regulating placenta formation and ovule primordia initiation. Most auxin-related mutants produce abnormal gynoecia and consequently show abnormal placenta development and corresponding ovule deletion. The most abundant form of auxin in plants, indole-3-acetic acid (IAA), is catalyzed by YUCCA (YUC) flavin monooxygenases from indole-3-pyruvic acid (Cao et al., 2019; Morffy and Strader, 2020). Multiple mutants of the *YUC* gene family such as *yuc1 yuc4*, *yuc1 yuc2 yuc4*, *yuc1 yuc4 yuc6*, and *yuc1 yuc2 yuc4 yuc6* exhibit severe developmental defects in inflorescence morphogenesis and gynoecium formation, resulting in failure of ovule formation (Zhao et al., 2001; Cheng et al., 2006). The auxin efflux transporter PINFORMED 1 (PIN1) single mutant *pin1-1* exhibits abnormal gynoecium structure with no ovules in the ovary (Okada et al., 1991). Valves of weak *pin1-5* mutant gynoecia are significantly reduced and often fused along one of the margins, containing on average only nine ovules per carpel (Sohlberg et al., 2006; Bencivenga et al., 2012). Treatment of early gynoecia with 100 μM *N*-1-naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport, produces a similar phenotype of abnormal placenta and ovule deletion as *pin1-1* (Nemhauser et al., 2000; Larsson et al., 2014). When auxin is perceived, AUXIN RESPONSE FACTOR (ARF) proteins regulate transcription of auxin-responsive target genes in a positive or negative manner (Leyser, 2018). ARF5/MONOPTEROS (MP) and its downstream targets are involved in floral development and ovule initiation; failure of CMM development in the gynoecium of the *MP* weak allele mutant *mpS319* leads to failure of placenta and ovule formation (Hardtke and Berleth, 1998; Alonso et al., 2003; Galbiati et al., 2013; Cucinotta et al., 2021). Another ARF family member required for apical-basal gynoecium patterning is ARF3/ETTIN (ETT) (Sessions et al., 1997; Nemhauser et al., 2000). *ett* gynoecium phenotypes are

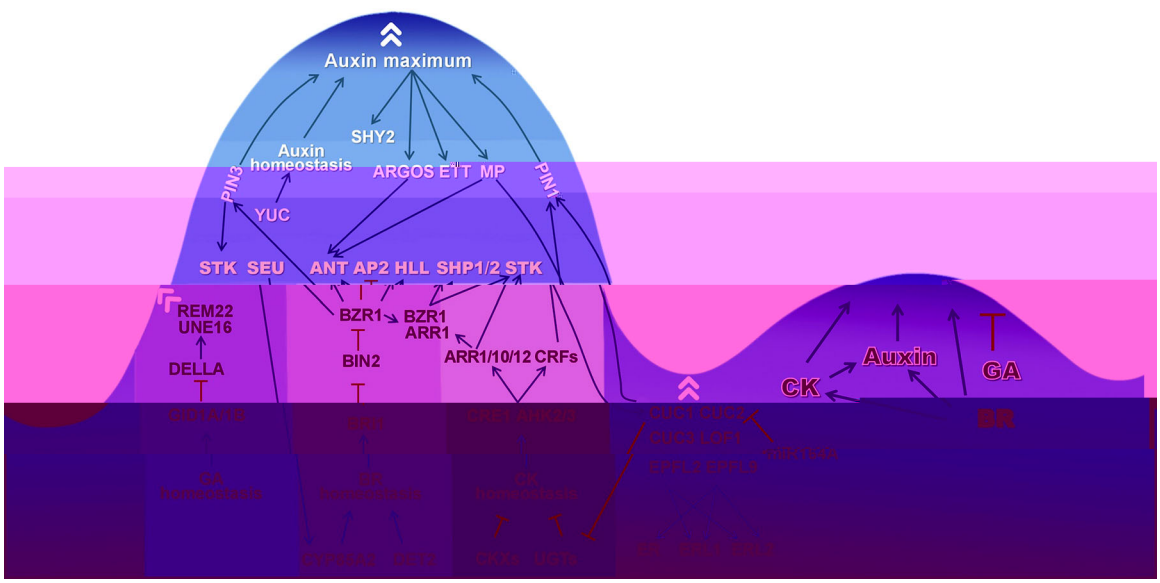

Figure 2. Signaling network during ovule primordia initiation and ovule boundary formation
An integrated gene network for the regulation of ovule primordia initiation and ovule boundary formation. Four types of phytohormones (Auxin, BR, CK, and GA) signals are separated by different background colors. The regulators that function in ovule boundary formation are shown under the ovule boundary (the middle one). The blue arrows indicate positive regulation, the brown lines indicate negative regulation.

dependent on allele-strength and involve aberrant development of tissues in place of the ovary (Sessions et al., 1997). Moreover, transgenic plants overexpressing the auxin-inducible gene *ARGOS* produced 20% more seeds in each silique compared with control plants (Hu et al., 2003). By contrast, *ARGOS* antisense plants contained fewer seeds per silique (Hu et al., 2003). INDOLE-3-ACETIC ACID INDUCIBLE 3 (IAA3)/SHORT HYPOCOTYL 2 (SHY2), a member of the auxin/indole-3-acetic acid inducible (Aux/IAA) family, is a repressor of auxin (Guilfoyle et al., 1998; Soh et al., 1999). SHY2 interacts with ARFs, thereby preventing the activation of auxin-responsive genes (Guilfoyle et al., 1998; Mockaitis and Estelle, 2008). *shy2-2* (gain-of-function) mutants display short carpels and short siliques (Kim et al., 1996; Li et al., 2020). Given that auxin is involved in multiple processes of plant organogenesis and growth development, the phenotypes of auxin-related mutants are often very drastic, with abnormal floral organ morphology occurring before ovule primordia initiation; the ovule phenotypes of the above mutants might therefore result from indirect effects of abnormal floral development and direct effects of abnormal ovule initiation.

PIN1 expression in placental cells and ovule primordia is required for auxin transport in placenta cells where ovule primordia will be specified and creating an auxin concentration maximum at the apex of ovule primordia (Benková et al., 2003; Ceccato et al., 2013; Galbiati et al., 2013; Yu et al., 2020). Before initiation of ovule primordia (floral stage 8), PIN1 is expressed evenly in placental cells. Expression of PIN1 in several clusters of placenta cells gradually increases and polarizes in the upper and lower sides of cell membranes. PIN1 next shows a distinct polarity at the lateral cell membrane of placental cells, leading to a change in direction of placental cell division (from transverse to periclinal), which represents the position where ovule primordia will protrude (Yu et al., 2020). As ovule primordia are initiated from the placenta (floral stages 9-10), PIN1 polarity gradually directs toward the apices of ovule primordia, accompanied by formation of auxin concentration maxima (Larsson et al., 2014; Yu et al., 2020; Hu et al., 2022b). After initiation of ovule primordia, PIN1 in epidermal cells reverses polarity, with auxin transport being directed away from the primordia and back toward the placental regions to form a new auxin concentration maximum on the boundary between adjacent ovules and initiate new ovule primordia (Yu et al., 2020). Treatment with low concentrations of NPA at floral development stage 8 strongly inhibits ovule primordia initiation, despite normal development of the placenta. Treatment at stage 9 strongly inhibits initiation of new ovule primordia despite normal development of the placenta and initiated ovules (Yu et al., 2020). Therefore, polar transport and dynamic distribution of auxin between the placenta and ovule primordia are important for the initiation of new ovule primordia, further demonstrating that ovules on placentae are not initiated simultaneously but asynchronously (Yu et al., 2020). A computational model incorporating auxin signaling, placenta growth, and ovule initiation has been built to ideally mimic the asynchronous initiation of ovule primordia at stage 9 (Yu et al., 2020).

Interestingly, ovule number at late stage 9 is slightly but significantly lower than the final ovule number (Hu et al., 2022b). Further observation reveals larger boundaries between some pairs of existing ovules when these are entering the next developmental step after stage 9, allowing new auxin concentration maximums to form and thus initiating a small number of new ovules at floral stage 10 (Hu et al., 2022b). PIN3 is the main regulator during late ovule initiation, since the loss-of-function mutant *pin3* loses this process (Hu et al., 2022b). Pistils of *pin3* mutants are shorter than those of the wild type at floral stages 10 and 11 (Hu et al., 2022b), and ovule/seed density of *pin3* is reduced than that of wild type (Jiang et al., 2020; Hu et al., 2022b). PIN3 is first detected in several placental cell clusters that develop into ovule primordia but accumulates later than PIN1 (Larsson et al., 2014; Hu et al., 2022b). After protrusion of ovule primordia, PIN3 localization gradually shifts toward the epidermal cells of the ovule tips (Hu et al., 2022b). Spatiotemporal expression of PIN3 overlaps with that of PIN1 during gynoecium development to mediate auxin flow, implying that PIN3 and PIN1 probably overlap in regulating ovule initiation (Yu et al., 2020; Hu et al., 2022b). Most importantly, PIN3 is detected in new ovule primordia and the medial region of the pistil at floral stage 10, implying that PIN3 participates in regulation of late ovule initiation and pistil growth during late ovule initiation (Hu et al., 2022b).

Auxin response factor MP integrates the auxin signaling required for ovule primordia formation to regulate expression of transcription factors such as *ANT*, *CUP-SHAPED COTYLEDON 1* (*CUC1*), and *CUC2* (Galbiati et al., 2013). Expression of *STK* is up-regulated upon treatment with auxin analogues but down-regulated in *pin3* mutants (Hu et al., 2022b). Meanwhile, overexpressing *STK* rescues *pin3* phenotypes, suggesting that STK participates in PIN3-mediated late ovule initiation (Hu et al., 2022b). These findings suggest crosstalk between development signals and hormone signals.

CK plays a central role in the processes of cell division and cell differentiation (Skoog and Miller, 1957). Mutations in CK hydrolase (CYTOKININ OXIDASE, CKX) result in reduced CK degradation and increased CK levels (Werner et al., 2003; Galuszka et al., 2007). The *ckx3 ckx5* double mutant displays increased gynoecium length and approximately twice as many ovules as the wild type (Ashikari et al., 2005; Bartrina et al., 2011). Notably, sextuple *ckx3 ckx5* mutants showed an increased CK concentration and produced more flowers with gynoecia containing 32% more ovules in oilseed rape (*Brassica napus* L.), indicating increased CK level enhances crop yield of oilseed rape (Schwarz et al., 2020). In the triple CK receptor mutant *cre1-12 ahk2-2 ahk3*, an average of only five ovules are formed per gynoecium (Higuchi et al., 2004; Bencivenga et al., 2012). UDP-GLUCOSYL TRANSFERASE 85A3 (UGT85A3) and UGT73C1 catalyze the reversible

inactivation of zeatin-type CKs by *O*-glucosylation (Hou et al., 2004). *ugt85a3* mutants show a significant increase in ovule and seed number but no significant difference in pistil length, indicating the increased ovule density. Overexpression of *UGT85A3* or *UGT73C1* causes a significant reduction in the number of ovules and seeds (Cucinotta et al., 2018). The type-BARABIDOPSIS RESPONSE REGULATORs (ARRs) are a family of positive regulators of CK signaling (Yokoyama et al., 2007); the *arr1 arr10 arr12* triple mutant displays a shorter gynoecium and significantly lower ovule/seed numbers (Reyes-Olalde et al., 2017; Zu et al., 2021). These studies demonstrate that CK promotes ovule initiation.

Previous work has demonstrated that BR signal promotes ovule initiation and positively regulates ovule number (Huang et al., 2013). The BR-deficient mutant *det2* and BR-insensitive mutants *bri1-5* and *bin2-1* have shorter gynoecium and significantly lower ovule number (Chory et al., 1991; Noguchi et al., 1999; Li and Nam, 2002; Huang et al., 2013; Yu et al., 2020), while the BR-signal-enhanced mutant *bzr1-1D* displays increased gynoecium length and significantly higher ovule number and density (Wang et al., 2002; Huang et al., 2013; Yu et al., 2020). Analysis of the molecular mechanism revealed that BR induces the important transcription factor BZR1 to regulate transcription levels of the downstream ovule-initiation regulators *ANT*, *HLL*, and *APETALA 2* (*AP2*), promoting ovule initiation (Huang et al., 2013). CYP85A2 is key enzyme for BR biosynthesis (Kim et al., 2005) and the *seu cyp85a2* double mutant displays a significant reduction in ovule number relative to either single mutant, and the *CYP85A2* expression is decreased in *seu* inflorescence (Nole-Wilson et al., 2010b).

GA negatively regulates ovule initiation, leading to abnormal ovule number (Gómez et al., 2018; Barro-Trastoy et al., 2020a). In plant cell, bioactive GA is perceived by GIBBERELLIN-INSENSITIVE DWARF 1 (GID1) receptors (Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006). The double mutant *gid1a gid1b* forms more ovules than wild-type plants (Griffiths et al., 2006; Gómez et al., 2018). DELLA proteins, the negative regulators of the GA signaling pathway (Ueguchi-Tanaka et al., 2007; Sun, 2010), promote ovule initiation and positively regulate ovule number (Gómez et al., 2018; Barro-Trastoy et al., 2020a). Gynoecia of the DELLA triple mutant *gaiT6 rgaT2 rgl2-1* are shorter, with inhibited ovule initiation (Lee et al., 2002; Peng et al., 2002; Gómez et al., 2018). Mutants with enhanced DELLA protein activity, *gai-1* and *YPet-rgl2Δ17* (Peng et al., 1997; Gómez et al., 2019), display enhanced ovule initiation and increased ovule number (Gómez et al., 2018; Gómez et al., 2019; Barro-Trastoy et al., 2020a). More importantly, the ratio of ovule number to ovary length is increased in *gai-1* and *gid1a gid1b* mutants but decreased in the *gaiT6 rgaT2 rgl2-1* mutant (Gómez et al., 2018). Transcriptomic analysis of pistils of *gai-1* and *global* mutants revealed two TFs, *UNFERTILIZED EMBRYO SAC 16* (*UNE16*) and *REPRODUCTIVE MERISTEM 22* (*REM22*) is up-regulated in *gai-1* (Gómez et al., 2018). Knockdown alleles *une16-1* and enhancer allele *rem22-1* showed a decreased and increased ovule number (Gómez et al., 2018). Interestingly, reduced GA in soybean (*gmga3ox1*) leads to lower seed weight but higher seed yield by increasing seed numbers in (Hu et al., 2022a). These data demonstrate negative regulation of GA during ovule initiation (Gómez et al., 2018). Current evidence suggests that GA regulation of ovule initiation is not related to auxin or BR in *Arabidopsis* (Barro-Trastoy et al., 2020a). Above all, auxin, CK, BR, and GA all influence both ovule number and ovule density, indicating these hormones promote ovule initiation and increase ovule number not only through enlarging placenta size, but also through other mechanisms. As we know, inflorescence meristem size has great impact on flower primordia number, and larger flower primordium leads to more flower organs (Clark et al., 1993, 1995), suggesting larger placenta could produce more ovules. The enhanced ovule density indicates the active signals of ovule initiation.

The integration of auxin, CK, BR, and GA is involved in regulating ovule initiation. CK interacts with auxin signals to regulate ovule number. When inflorescences are treated with the synthetic CK 6-benzylaminopurine (Miller, 1979), expression of *PIN1* in the gynoecium is increased, resulting in an average increase of 20 ovules per gynoecium. This indicates that CK activates *PIN1* expression during ovule initiation (Bencivenga et al., 2012). CK activates *PIN1* expression in ovule primordia through CK RESPONSE FACTORS (CRFs); the triple mutant *crf2 crf3 crf6* displays reduced *PIN1* expression, shorter placentae, decreased ovule number, and reduced ovule density (Rashotte et al., 2006; Cucinotta et al., 2016).

BR interacts with auxin signals to regulate ovule initiation. Exogenous auxin applications partially rescue the shortened carpel length of *bin2-1* mutants, while a lack of *SHY2* activity increases the carpel length of *bin2-1* mutants (Li et al., 2020). During ovule initiation, exogenous BR application enhances the auxin response in ovule primordia (Yu et al., 2020). Expression of *PIN3* is down-regulated in *bin2-1* and *dwarf4* and up-regulated in *bzr1-1D*, with the ovule number of the *bzr1-1D pin3* double mutant close to that of the *pin3* mutant, suggesting that PIN3 is required for BR-mediated ovule initiation (Hu et al., 2022b).

The BR-signal-enhanced mutant *bzr1-1D* and the CK-signal-enhanced mutant *ckx3 ckx5* display increased seed number per fruit, suggesting that BR and CK signals positively regulate initiation of ovule primordia (Bartrina et al., 2011; Huang et al., 2013). Our current research illustrates that simultaneously enhancing BR and CK signals is more effective in promoting ovule primordia initiation than separately increasing either BR or CK signals (Zu et al., 2021). During ovule initiation, BR and CK activate each other's signaling levels (Zu et al., 2021). The BR-induced transcription factor BZR1 interacts with the CK-induced transcription factor ARR1, and BR enhances the level of interaction between BZR1 and ARR1, causing enhancement of ARR1 to target and induce downstream positive regulators of ovule initiation, thus promoting ovule initiation and increasing the number of ovules and seeds (Zu et al., 2021). Enhanced CK signal

partially rescues defective ovule initiation in BR-deficient or -insensitive mutants, but enhanced BR signal cannot rescue defective ovule initiation in CK-deficient plants, suggesting that BR regulation of ovule initiation acts partially through CK signals (Zu et al., 2021). BR appears to regulate ovule initiation through multiple mechanisms (Huang et al., 2013; Yu et al., 2020; Zu et al., 2021; Hu et al., 2022b).

Although GA and BR act independently and antagonistically in *Arabidopsis* (Barro-Trastoy et al., 2020a), GA reportedly interacts with BR signals to regulate ovule initiation in tomato. GA acts downstream of BR, and BR promotes ovule initiation through down-regulating GA biosynthesis, which provokes stabilization of DELLA proteins (Barro-Trastoy et al., 2020a).

Effect of organ boundary formation and organ separation genes on ovule primordia initiation

In addition to developmental signals and hormonal signals regulating ovule initiation, some conserved genes regulating organ boundary formation and organ separation also play important roles in ovule initiation and ovule number. The NAC-like transcription factor CUP-SHAPED COTYLEDON (CUC) family is not only important for the identity and initiation of lateral organs in the SAM but also involved in regulating CMM formation and activity (Ishida et al., 2000; Takada et al., 2001). Both *cuc1 cuc2* double mutant and *cuc2-1 ProSTK::CUC1-RNAi* plants have significantly reduced ovule number, whereas the *cuc2 cuc3* double mutant produces fused ovules to form fused seeds, demonstrating that different *CUC* genes are involved in regulating ovule initiation and ovule separation, respectively (Ishida et al., 2000; Galbiati et al., 2013; Kamiuchi et al., 2014; Gonçalves et al., 2015). *CUC1* and *CUC2* are also involved in the regulation of polar auxin transport and CK homeostasis during ovule initiation (Galbiati et al., 2013). Auxin transcriptionally activates the expression of *CUC1* and *CUC2* by activating expression of *MP*, while CUC1 and CUC2 also promote the expression of *PIN1* and localization of its protein on the membrane (Galbiati et al., 2013). Indeed, the reduced ovule number in *cuc2-1 ProSTK::CUC1-RNAi* plants can be rescued by CK treatment (Galbiati et al., 2013). CUC1 and CUC2 induce CK responses by transcriptionally repressing *UGT73C1* and *UGT85A3* in the pistil (Hou et al., 2004; Cucinotta et al., 2018). Besides, the *CUC1* and *CUC2* genes are both regulated by miR164 (Laufs et al., 2004; Mallory et al., 2004), *CUC1* and *CUC2* expression was silenced in the *2x35S::MIR164A* line, resulting in a strong reduction in ovule number (Gonçalves et al., 2015).

The MYB family transcription factor LATERAL ORGAN FUSION 1 (LOF1) is involved in multiple lateral organ separation and functionally overlaps with CUC2 and CUC3 (Lee et al., 2009). The *LOF1* gene is also expressed at the base of ovule primordia, where its overexpression leads to gynoecium crinkling, enlarged placentae, abnormal septa, and irregular ovule distribution (Gomez et al., 2011). The small secreted peptides EPIDERMAL PATTERNING FACTOR-like 2 (EPFL2) and EPFL9 (Hara et al., 2009) and their receptors ERECTA-LIKE 1 (ERL1) and ERL2 (belonging to the LRR receptor kinase family) (Torii et al., 1999; Shpak et al., 2004) were recently found to be involved in regulation of ovule spacing (Tameshige et al., 2016; Kawamoto et al., 2020). The EPFL9-controlled signaling pathway in the carpel wall promotes silique growth through the LRR receptor kinases ER, ERL1 and ERL2, which express in the carpel wall, while EPFL2 is involved in controlling the initiation and equidistant spacing of ovule primordia through ERL1 and ERL2 in the carpel wall and ovule boundary, with mutations in *EPFL2* resulting in shorter gynoecia and siliques and irregularly spaced ovules (Kawamoto et al., 2020).

The prerequisite for ovule initiation is cell differentiation of ovule primordia and ovule boundaries. Which cells differentiate first remains unclear. Ovule primordia cells might differentiate first, with other cells turning into boundary cells, or the other way around. Alternatively, the two types of cells might differentiate at the same time, maintaining a tight connection. Double marker lines for ovule primordia and boundary cells will provide clues to this fundamental question.

AFTER OVULE INITIATION

After ovule initiation, ovule development enters subsequent processes, which are also important for effective offspring number (Figure 3). Female gametogenesis occurs in the nucellus and can be divided into two processes, megasporogenesis and megagametogenesis (Webb and Gunning, 1990; Schneitz et al., 1995; Christensen et al., 1997). Development of sporophytic integuments and the female gametophyte in the ovule must be coordinated (Acosta-García and Vielle-Calzada, 2004; Yadegari and Drews, 2004; Wang et al., 2008). The developmental events and stages of ovule and female gametophyte have been classified by Robinson-Beers, Schneitz, and Christensen, respectively (Robinson-Beers et al., 1992; Schneitz et al., 1995; Christensen et al., 1997).

During megasporogenesis, a sub-epidermal cell at the distal end of the ovule primordium forms the archesporial cell, which differentiates directly into the megaspore mother cell (MMC) (Webb and Gunning, 1990; Jiang and Zheng, 2021). After the MMC enlarges, development of the inner and outer integuments is initiated (Webb and Gunning, 1990; Schneitz et al., 1995; Christensen et al., 1997). The MMC then undergoes meiosis, giving rise to four haploid megaspores (the tetrad); tetrad formation is accompanied by integument extension toward the apex of the nucellus (Webb and Gunning, 1990; Schneitz et al., 1995). Subsequently, three of the megaspores degenerate, leaving chalazal-most megaspore named the functional megaspore (Webb and Gunning, 1990; Modrusan et al., 1994; Schneitz et al., 1995). The outer integument envelops the nucellus and the inner integument during this stage (Webb and Gunning, 1990;

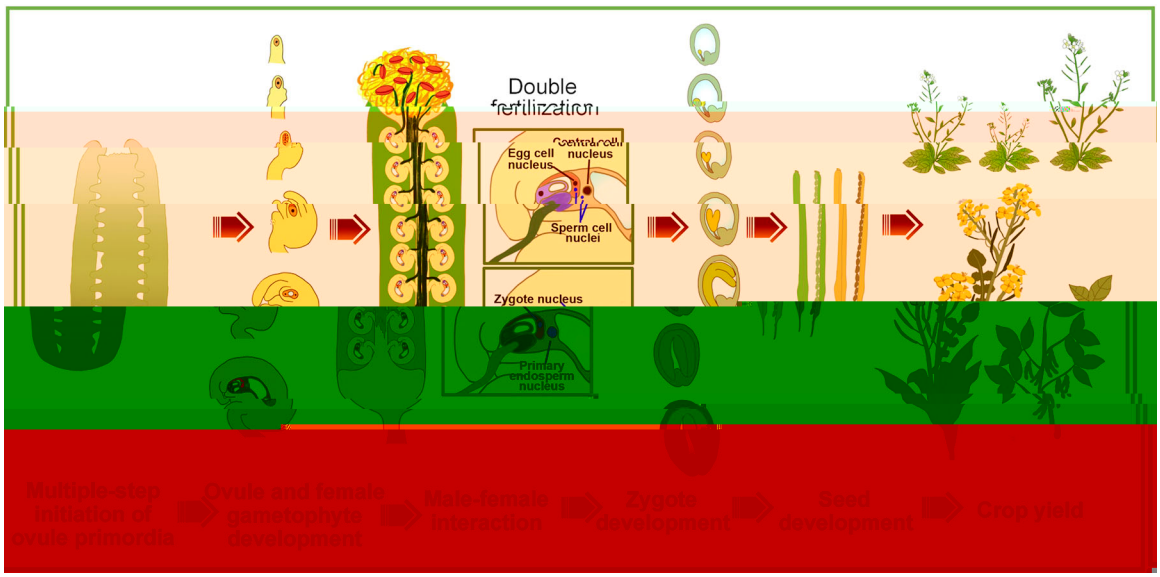

Figure 3. Ovule initiation is an important control for plant offspring number and crop yield
Stages and processes of ovule development are indicated below each illustration. Ovule initiation determines the maximal of ovules and has a great impact on seed number per fruit and seed yield. The normal development of subsequent processes guarantees that the promotion of ovule primordia initiation will enhance plant offspring number and contribute to crop yield.

Schneitz et al., 1995). The regulatory mechanism of integument initiation and growth, MMC identity, meiosis, and programmed cell death of megaspores is summarized by previous publications (Colombo et al., 2008; Drews and Koltunow, 2011; Jiang and Zheng, 2021).

During megagametogenesis, the functional megaspore enlarges and then undergoes three rounds of mitosis to produce eight nuclei; the inner and outer integuments gradually grow to enclose the nucellus, and the funiculus, sporophytic cell-layers, and embryo sac curve to form anatropous ovules (Schneitz et al., 1995; Christensen et al., 1997). The cellularization process occurs during the third mitosis in the embryo sac; during and after that, one nucleus from each pole migrates toward the center of the developing female gametophyte and then fuses (Mansfield et al., 1991; Murgia et al., 1993; Schneitz et al., 1995; Christensen et al., 1997). These events result in a seven-celled embryo sac consisting of four cell types: two synergid cells (SCs, *n*) and one egg cell (EC, *n*) at the micropylar end; one central cell (CC, *2n*) in the middle; and three antipodal cells (ACs, *n*) at the chalazal end (Mansfield et al., 1991; Murgia et al., 1993; Schneitz et al., 1995; Christensen et al., 1997; Yadegari and Drews, 2004). The ACs eventually disappear or undergo programmed cell death before fertilization of the female gametophyte (Murgia et al., 1993; Schneitz et al., 1995; Christensen et al., 1997). Genes functioning in female gametophyte development have been summarized in previous reviews (Colombo et al., 2008; Yang et al., 2010; Drews and Koltunow, 2011). It has been known for a long time that there are two to three developmental stages of the female gametophyte (embryo sac) in the same gynoecium (Christensen et al., 1997). We recently demonstrated that asynchronous development of the female gametophyte results from asynchronous initiation of ovule primordia (Yu et al., 2020). Although the morphology of the first two rounds of ovules is consistent by stage 10 and these ovules start development at almost the same time, female gametogenesis and female gametophyte development maintain asynchrony until the ovules mature (Yu et al., 2020).

After the flower opens, the anthers dehiscence to release pollen grains, which adhere to the stigma; the pollen grains need to hydrate and germinate (Gu et al., 2005; Liang and Zhou, 2018; Zhong and Qu, 2019; Zhou and Dresselhaus, 2019; Liu et al., 2021). Pollen tubes must penetrate through the surface of the stigma (papilla cell) and traverse the transmitting tract in the style (Zhu et al., 2018; Adhikari et al., 2020). Frequent signaling mediated by peptides and receptor kinases plays an essential role in guaranteeing effective guidance of pollen tubes to ovules (Wang et al., 2016; Xiao et al., 2019; Zhong and Qu, 2019; Kim et al., 2021; Zhong et al., 2022). When a pollen tube successfully arrives at a micropyle, it must enter the SC through the micropylar aperture. Its growth is then arrested, ruptures and two sperm cells are released (Duan et al., 2014; Ge et al., 2017; Adhikari et al., 2020). The sperm cells fuse with EC and CC, respectively (so-called double fertilization) (Adhikari et al., 2020). The regulatory mechanisms of pollen germination, growth, guidance, reception, and rupture of the pollen tube, release of sperm cells, and degeneration of the SC have been summarized in several excellent reviews (Cai et al., 2015; Zhong and Qu, 2019; Adhikari et al., 2020).

After double fertilization, development of the zygote begins. In *Arabidopsis*, the embryo (*2n*) acquires the basic architecture of the plant through a series of cell divisions, while

the triploid endosperm develops in two steps, a coenocytic stage followed by a cellularization and differentiation stage (Olsen, 2001). Embryo cells go through a period of cellular expansion and differentiation accompanied by the accumulation of storage products (Baud et al., 2002; He et al., 2021; Hou et al., 2021). Finally, the embryo becomes metabolically quiescent and tolerant to desiccation (Baud et al., 2002). These processes are well summarized in previous reviews (Lafon-Placette and Köhler, 2014; Dresselhaus and Jürgens, 2021; Verma et al., 2022; Wang et al., 2022).

The developmental processes occurring inside ovules contribute to seed formation and also impact regulation of plant offspring number as well as seed yield of crops. As long as subsequent developmental processes are successful, promoting ovule initiation will lead to increased number of effective offspring. Most positive regulators of ovule initiation, including developmental signals (*ANT*, *HLL*, *LUG*, *SEU*, *SIN2*, *STK*) and hormone signals (auxin, CK, and BR), also positive regulate subsequent ovule development processes and increase offspring number (Schneitz et al., 1998; Broadhvest et al., 2000; Liu et al., 2000; Azhakanandam et al., 2008; Gomez et al., 2016; Hu et al., 2018; Jia et al., 2020; Terceros et al., 2020; Cucinotta et al., 2021; Cai et al., 2022). However, the function of GA in ovule initiation and seed development is complicated. DELLAs proteins promote ovule initiation and GA suppresses DELLAs, suggesting that GA negatively regulates ovule initiation (Gómez et al., 2018; Barro-Trastoy et al., 2020a). DELLAs are also required for correct integuments formation (Gomez et al., 2016), but the degradation of DELLAs relieve their repression of the transcriptional activity of downstream regulators, thus facilitating embryo development (Hu et al., 2018). Collectively, the increased ovule initiation caused by active DELLAs and reduced GA could not lead to increased seed number finally (Gómez et al., 2018).

CONCLUDING REMARKS AND

initiation, patterning, emergence, and outgrowth (Péret et al., 2009). Ovule initiation is somewhat similar to leaf primordia initiation from the SAM (Schwabe, 1984; Kuhlemeier and Reinhardt, 2000; Cucinotta et al., 2014), but the placenta is linear (Figure 4). In summary, primordia initiate from subepidermal cells, new primordia are initiated in the boundaries between each two initiated primordia (Schwabe, 1984; Kuhlemeier and Reinhardt, 2000; Heisler et al., 2005; Cucinotta et al., 2014; Yu et al., 2020). Both the ovule primordia and the leaf/branch organ primordia are aligned evenly in the placenta (Cucinotta et al., 2014; Kawamoto et al., 2020; Yu et al., 2020) and surround SAM (Reinhardt and Gola, 2022), which provides samples of regular patterns in plants. Actually, the even alignment of organs/tissues/surface patterns is also in animals, including zebrafish stripes (Asaia et al., 1999), mollusk shells (Meinhardt, 2009), alligator teeth (Kulesa et al., 1996), transverse ridges of the palate (Economou et al., 2012), and feather and hair follicle spacing (Jiang et al., 1999; Sick et al., 2006). The regular patterns are existing in both plants and animals (Kondo and Miura, 2010; Reinhardt and Gola, 2022), which are the exquisite and beautiful parts of the natural world. The reaction-diffusion model (Turing, 1952) has been used to explain several biological patterns (Kondo and Miura, 2010), the similar and different regulatory mechanisms in plants and animals are worth further studying in the future.

Primordia are specified into lateral branch primordia or leaf primordia before initiating from the SAM (Reinhardt et al., 2000). Ovules are initiated from the placentae after ovule primordia are specified. If ovule identity genes are disturbed, leaf-like or carpel-like structures develop in the placenta (Pinyopich et al., 2003). The identity and initiation of ovules, and the similarities and differences in regulation of ovule initiation, leaf initiation, and lateral root initiation are worth studying in more depth.

Ovule initiation is a prerequisite for plants to produce offsp1352.88e0T5i(:)TjET/GS1gsBT8.4682008.46823s7(differeie0T6(genes)29

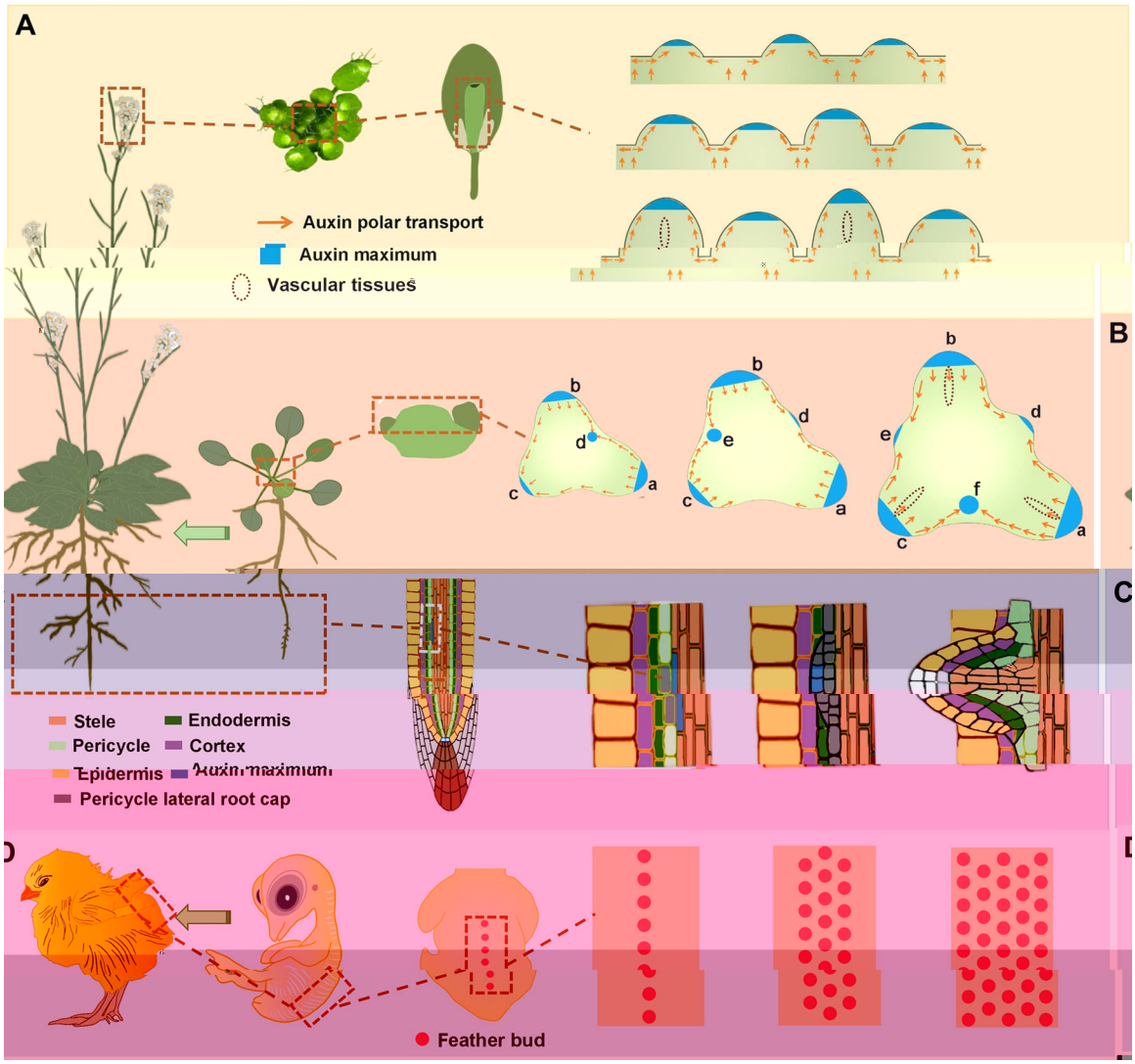

Figure 4. The regular arrangement of different lateral organs

Primordia initiation process varies along different meristem in *Arabidopsis*. (**A**) Ovule primordia initiation. The placenta is linear, the ovule primordia initiate from the placenta and new primordia initiates from the boundary of old primordia. Auxin concentration maximum formed in every primordium. (**B**) Leaf primordia initiation. The Shoot Apical Meristem (SAM) is dome-shaped, the leaf primordia initiate from SAM and new primordia initiates from the boundary of the old primordia. Auxin concentration maximum formed in every primordium (refer to Heisler et al., 2005 and Kuhlemeier, 2017). Branch and flower primordia initiate in a similar way. (**C**) Lateral root primordia initiation. The primary root is linear, the lateral root primordia initiate from the pericycle cells of the primary root. (**D**) The feather bud formation process in chickens. Within mid-dorsum, a single row of buds is initiated in rapid sequence, additional rows are formed consecutively parallel to this primary row until a regular array of buds fills the tract.

stress in different ways. Some plant species complete their life cycle before the onset of drought, so-called “Drought escape”: Plants do not experience drought stress, they flower earlier and produce very few seeds before the soil water depletes or during the dry season (Basu et al., 2016). It is very possible that the reduced seed number is according to inhibited placenta elongation or ovule initiation. Besides, the different extents of stress lead to different responses and adaptions. Under moderate or slight stress conditions, the fundamental growth is not affected, and the plasticity of seed number may relate to the availability of resources (Sadras, 2007). Plants possibly produce fewer offspring to enhance offspring quality for greater survival, which can also be considered a response to unfriendly environments. But under severe stress, both ovule initiation and consequent developmental processes are disturbed. For example, plants exposed to extreme heat during the reproductive process will reduce the number of total seeds and fertile seeds (Bac-Molenaar et al., 2015; Zhang et al., 2017). However, it can't be excluded that the specific stress condition will promote ovule initiation in some plant species since DELLA proteins (the positive regulator of ovule initiation) would be accumulated by stress (Colebrook et al., 2014; Lantzouni et al., 2020). But it may be very difficult to increase seed

number since the normal GA signal is required during seed development. How environmental factors influence ovule initiation worth further investigation. Plants may adapt to stress directly (genes and signals promoting ovule initiation are directly repressed) or indirectly (overall plant growth is suppressed), and the placenta size would be an effective parameter for monitoring environmental conditions. We hypothesize that environmental signals may influence the process of placental growth as well as ovule initiation by integrating hormones or other endogenous signals. The molecular mechanisms by which environmental factors affect initiation of ovule primordia is worth further in-depth study. Multiple-step initiation of ovule primordia provides plants several chances to produce more offspring depending on their internal and external environment, which is important for plant survival and evolution. Understanding the regulatory mechanism of asynchronous ovule initiation provides new clues for improving seed number and yield in crops with multi-ovulate ovaries.

There are still many unknowns surrounding ovule initiation. One important question is how to distinguish the regulation of placenta formation from that of ovule initiation. Identification of mutants with normal placenta but without ovule initiation is crucial for studying the nature of ovule initiation and its specific regulatory mechanisms. We speculate that there may be key genes that have not yet been discovered or some known/unknown genes with redundant roles. Alternatively, the two processes are tightly connected and regulated by the same groups of genes or the same mechanism. Advanced transcriptomic analysis of placental cells through improved single-cell sequencing technology is expected to uncover previously unknown genes or identify gene combinations sufficient and necessary for ovule initiation.

The second question is how do plants stop ovule initiation? It is known that ovule primordia are not initiated after stage 11 in *Arabidopsis* (Hu et al., 2022b). Sporadic ovules initiate at stage 10 while most ovules are developing integuments. Is there a stop signal for ovule initiation, or does ovule initiation just stop when all ovules have developed? Observations suggest that there is enough space in the placenta for additional ovules between the existing ovules since ovaries keep growing during ovule development (Smyth et al., 1990; Hu et al., 2022b). One reasonable explanation is that auxin may accumulate in the developing ovules, with less auxin distributed in the ovule boundaries in the placenta. This question could be easily studied if mutants exhibiting new ovule initiation after stage 11 are discovered. Furthermore, an interesting and attractive question raised following this question is whether there is primary ovule (the first ovule) protruding from the empty placenta. We observed that four to six ovule primordia initiate from the empty placenta at early stage 9, being the first round of ovule initiation. Using marker lines of different key genes, such as WUS, we found that one to two ovules in one placenta displayed the signal first. NPA treatment also illustrated there is one ovule in one placenta in some cases. And the computational modeling illustrates that the primary ovule is initiated from empty placenta (Yu et al., 2020). We hypothesized two possibilities: ovules in the first round are initiated at a similar time, with one ovule being initiated more quickly; or, ovules in the first round are initiated at the same time, with one ovule growing more quickly. However, we cannot exclude that there is primary ovule, which may initiate by the convergence of auxin flows from the stigma and the flower organ junction. Although primary ovule belongs to the first round of ovules, the regulation of primary ovule initiation may differ from that of the other ovules of the first round. Unfortunately, current techniques preclude answering this question since the time window of ovule initiation is too short. The conjecture of primary ovule is worth further studying in the future.

The next question is whether promoting ovule initiation negatively affects subsequent developmental processes inside ovules. Although increasing ovule number dilutes the nutrition available for each ovule and may affect the development of ovules, female gametophytes, and zygotes/seeds, it is unclear whether increasing ovule number leads directly to low-quality seeds and poor yield. Increasing seed number was previously considered to be detrimental to seed yield (Sadras, 2007; Guo et al., 2018) because limited space (in plants with multi-ovulate ovaries) and nutrition. However, several studies in recent years have shown that seed number and weight are not absolutely negatively correlated. Under optimized conditions, seed number and weight can both be increased, dramatically enhancing seed yield (Wu et al., 2008; Bartrina et al., 2011; Zhang et al., 2013). Seed yield will still be enhanced if seed number is greatly increased without significantly affecting seed development or seed weight (Huang et al., 2013). Similarly, there is so far no direct evidence that increasing ovule number leads to aborted ovules and seed. Promoting ovule initiation is definitely an effective way of increasing plant offspring number. For increasing seed yield, the optimal balance for ovule initiation and seed development under different conditions must be determined.

There remain challenges for future study of this field. Ovule initiation is a complicated and delicate process regulated by a combination of developmental and hormone signals. Since the placentae and ovule primordia are extremely small (even larger plants such as *B. napus* have very tiny gynoecia during ovule initiation) and are localized in the innermost layer of multiple tissue layers (sepals, petals, styles, and ovary wall), it is not possible to directly observe the entire process of initiation and development of one ovule *in vivo*. Until technology allowing living observation is established, all conclusions regarding ovule initiation and development will be deduced from observing hundreds and thousands of examples (a common issue for elucidating other developmental processes). Therefore, hypotheses will not be perfect. Furthermore, studying molecular mechanisms requires uniform materials at the same developmental stages for transcriptome and proteome analysis. It is difficult to distinguish the different stages in living *Arabidopsis*. Although some

culture systems allow gynoecia and ovules to grow in specific medium for several days, which is useful for accurately judging stages and observing phenotypes (Sauer and Friml, 2004; Li et al., 2018), these systems need further improvement for studying ovule initiation because the gynoecium at the ovule initiation stage is too small and tender to survive after removal from the plant. The advanced methods of ClearSee, living observation, and *in vitro* gynoecium culture will contribute to study the regulatory mechanism of ovule initiation in the future.

Future research into ovule initiation will focus on the following research goals and agricultural applications. First, to further explore the nature of ovule initiation and its regulatory mechanism and use advanced technology to analyze the expression profiles of placental cells and explore the regulators and mechanisms that promote ovule initiation. Second, to increase seed number and yield in crops with similar multi-ovulate ovaries by promoting initiation of ovule primordia based on research in *A. thaliana*. Finally, to analyze the direct and indirect regulation of *Arabidopsis* ovule primordia initiation by environmental factors, reducing inhibition of ovule initiation and decreasing reductions in seed number and yield caused by transient stress in crops with similar multi-ovulate ovaries.

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CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

W.-H.L. supervised the project and organized this review; W.-H.L. and S.-X.Y. wrote the manuscript; Y.-T.J. draw the concept figures. All authors reviewed and approved of the manuscript.

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