

Gynoecium size and ovule number are interconnected traits that impact seed yield

Mara Cucinotta¹, Maurizio Di Marzo¹, Andrea Guazzotti¹, Stefan de Folter², Martin M. Kater¹ and Lucia Colombo¹.

¹Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy.

²Unidad de Genómica Avanzada (UGA-Langebio), Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Km. 9.6 Libramiento Norte, Carretera Irapuato-Leon, CP 36824 Irapuato, Gto., Mexico.

Corresponding author: Lucia Colombo, lucia.colombo@unimi.it.

Abstract

Angiosperms form the biggest group of land plants and display an astonishing diversity of floral structures. The development of the flowers greatly contributed to the evolutionary success of the angiosperms as they guarantee efficient reproduction with the help of either biotic or abiotic vectors. The female reproductive part of the flower is the gynoecium (also called pistil). Ovules arise from meristematic tissue within the gynoecium. Upon fertilization, these ovules develop into seeds while the gynoecium turns into a fruit. Gene regulatory networks involving transcription factors and hormonal communication regulate ovule primordium initiation, their spacing on the placenta, and ovule development. Ovule number and gynoecium size are usually correlated and several genetic factors that impact these traits have been identified. Understanding and fine-tuning the gene regulatory networks influencing ovule number and pistil length opens up strategies for crop yield improvement, which is pivotal in light of a rapidly growing world population. In this review, we present an overview of the current knowledge of the genes and hormones involved in determining ovule number and gynoecium size. We propose a model for the gene regulatory network that guides the developmental processes that determine seed yield.

Keywords: gynoecium, hormones, organ boundary, ovule number, ovule primordia, pistil, seed yield.

Introduction

Life on earth is affected by plants in varied ways. Of the estimated 400,000 extant plant species, approximately 94% are seed plants (Govaerts, 2001; Willis, 2017). This demonstrates that seed development and dispersion strategies greatly contributed to the success of this organismal group. The vast majority of seed plants are angiosperms and only a comparatively small number are gymnosperms. Both plant divisions produce ovules; however, only angiosperm species produce flowers and as another selective advantage, each flower produces one or more gynoecia that protect and nourish the ovules. Following fertilization, the gynoecium (or pistil) generally develops into a fruit and ovules develop into seeds.

Depending on the species, the gynoecium consists of one or more carpels that can be fused or unfused (Endress and Igersheim, 2000). The *Arabidopsis* gynoecium consists of two fused carpels (Smyth *et al.*, 1990; Alvarez-Buylla *et al.*, 2010). Along the margins where the carpels fuse, a meristematic tissue, termed the carpel margin meristem (CMM), is formed. The CMM gives rise to the placenta, ovules, septum and the transmitting tract (Reyes-Olalde *et al.*, 2013; Reyes-Olalde and de Folter, 2019). Inside an ovule the female gametophyte develops, which is comprised of three antipodal cells, a central cell, two synergids and an egg cell (Drews and Koltunow, 2011; Bencivenga *et al.*, 2011). Therefore, ovule development is a crucial process during the plant life cycle and has been studied in many species. In recent decades, many reviews on ovule development have been written, demonstrating its importance and the degree of active research in this area (e.g., Reiser and Fischer, 1993; Angenent and Colombo, 1996; Grossniklaus and Schneitz, 1998; Gasser *et al.*, 1998; Bowman *et al.*, 1999; Skinner *et al.*, 2004; Colombo *et al.*, 2008; Shi and Yang, 2011; Endress, 2011; Cucinotta *et al.*, 2014; Gasser and Skinner, 2019; Shirley *et al.*, 2019; Pinto *et al.*, 2019).

To complement existing literature, this review focuses on recent discoveries in ovule development and gynoecium size determination. An overview is provided of the genes and hormonal communication involved in the developmental programs (Fig. 1 and Table 1). Understanding the regulatory networks that determine ovule number and gynoecium size is important as they hugely impact seed yield, and fine-tuning them appears to be a particularly promising strategy for enhancing crop yields.

Placenta development and ovule primordium initiation in *Arabidopsis*

Periclinal cell divisions within the sub-epidermal tissue of the placenta initiate ovule primordium development at stage 9 of flower development (Roeder and Yanofsky, 2006). Subsequently, three layers of primordium cells form a finger-like structure during stage 10, which then differentiates into three regions along the proximal–distal axis: the funiculus, the chalaza and

the nucellus (Schneitz *et al.*, 1995). These three regions undergo distinct but interdependent developmental processes. The nucellus is the site of megasporogenesis, where the megaspore mother cell (MMC) differentiates and locates to the upmost, central and subepidermal position of the digit-shaped ovule primordium (reviewed in Pinto *et al.*, 2019). The chalaza is the region from which the inner and the outer integuments develop, which finally envelop and protect the embryonic sac. The funiculus remains attached to the gynoecium via the placental tissue and this connection is required for the transport of nutrients to the ovule (Fig. 1). For this reason, the placental tissue is fundamental for ovule primordia formation, and for determining their number and maintenance.

In *Arabidopsis*, placental tissue differentiates from the CMM, which is the central ridge of cells that fuse and give rise to the septum. Placental tissue differentiates along the length of the septum adjacent to the lateral walls (Alvarez and Smyth, 2002; Nole-Wilson *et al.*, 2010a; Reyes-Olalde *et al.*, 2013). Communication between transcription factors and hormones is essential to maintain the meristematic activity of the placenta, to determine the sites of ovule initiation and ovule identity, and to establish the distance between two adjacent ovules (Cucinotta *et al.*, 2014). Several genes that are important for placenta development have been described in the literature and reviewed by Cucinotta *et al.* (2014) and Reyes-Olalde and de Folter (2019) and include *AINTEGUMENTA* (*ANT*), *CUP-SHAPED COTYLEDON 1* (*CUC1*) and *CUC2*, *LEUNIG* (*LUG*), *MONOPTEROS* (*MP*) and *PERIANTHIA* (*PAN*) (Fig. 1 and Table 1).

AINTEGUMENTA encodes an AP2 transcription factor (Klucher *et al.*, 1996) and positively regulates organ size via determining cell number and meristematic competence. *Ant* mutants have fewer and smaller floral organs than the wild type. In particular, the *ant-9* mutant is characterised by unfused carpels at the tip of the pistil (Elliott *et al.*, 1996), whereas in *ant-4*, the size of floral organs is reduced (Krizek, 2009). In contrast to these mutant phenotypes, *Arabidopsis* plants that overexpress *ANT* possess larger floral organs than the wild type (Mizukami and Fischer, 2000). Expression of *ANT* is controlled by *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*), an auxin-inducible gene (Hu *et al.*, 2003). When *ARGOS* is overexpressed, floral organs become enlarged, resulting in longer siliques than those of wild type (Hu *et al.*, 2003). This was one of the first pieces of evidence that implicated a key role for auxin in pistil development.

ANT expression initiates in the placenta and is maintained throughout all stages of ovule development, in particular in the chalaza region and in the integuments. The reduced ovule number phenotype of the *ant* mutant is exacerbated when it is combined with other mutations that affect CMM and placenta development, such as *revoluta* (*rev*), suggesting that the activity of the *REV* gene, which encodes a class III homeodomain leucine zipper transcription factor, is also required for placenta formation (Nole-Wilson *et al.*, 2010a). *ANT* interacts with the transcriptional repressor *SEUSS*

(SEU) and simultaneous loss of both protein activities severely affects placenta development and leads to a complete loss of ovule formation. When a weaker *ant-3* allele was combined with *seu-3*, placenta development was maintained but the number of ovules that initiated was reduced to approximately half of that observed in Col-0 wild-type plants (Azhakanandam *et al.*, 2008). Another transcriptional co-regulator involved in gynoecium patterning, is LEUNIG (LUG). Strong *lug-1* and intermediate *lug-3* alleles show a failure in ridge fusion and a reduction in the amount of placental tissue, with a consequent decrease in the number of ovules formed (Liu *et al.*, 2000). The combination of *lug* and *ant* mutations results in gynoecia that are unable to develop ovules (Liu *et al.*, 2000). The loss of ovules in the *ant* and *seu* backgrounds is strongly enhanced by mutations in the *PERIANTHIA* (*PAN*) gene, which encodes a bZIP transcription factor that is expressed in the gynoecium medial ridge, placenta and ovules, where it promotes ovule formation (Wynn *et al.*, 2014).

Similar to *ANT*, factors important for integument growth often affect ovule primordium formation. Two examples are *HUELLENLOS* (*HLL*) and *SHORT INTEGUMENTS 2* (*SIN2*). *HLL* encodes a mitochondrial ribosomal protein and its mutation is associated with smaller gynoecia and a 10% reduction in the number of ovules (Schneitz *et al.*, 1998; Skinner *et al.*, 2001). Shorter gynoecia that bear fewer ovules are also observed in the *sin2* mutant; however, more interestingly, the absence of *SIN2* function leads to an abnormal distribution of ovules along the placenta (Broadhvest *et al.*, 2000), in which the distance between ovules is greater than in the wild type; thus, a reduction in ovule number is caused by a reduction in gynoecium size and by the reduced ability of the placental tissue to initiate ovule primordia. *SIN2* encodes a mitochondrial DAR GTPase and similar to *HLL*, is hypothesised to function in mitochondrial ribosome assembly (Hill *et al.*, 2006). Notably, these two ribosomal proteins, which are targeted to the mitochondria, are necessary for ovule primordium formation, and it has been suggested that impaired mitochondrial function might cause cell-cycle arrest in the placenta and subsequently in the ovule integuments (Broadhvest *et al.* 2000).

127

128 **Complex hormonal communication promotes ovule initiation and determines pistil size**

129 Plant organogenesis requires cells to proliferate, grow and differentiate in a coordinated way.
130 The intercellular communication required during organ initiation is mediated by different
131 phytohormones (Davies, 2004; Vanstraelen and Benková, 2012; Schaller *et al.*, 2015; Marsch-
132 Martínez and de Folter, 2016). As will be discussed in this review, auxins, cytokinins, gibberellins
133 and brassinosteroids all play fundamental roles in ovule primordium formation (Fig. 1).

134 In most auxin-related mutants, defects in gynoecium formation lead to the reduction or
135 absence of placental tissue and the corresponding absence of ovules (reviewed in Balanzá *et al.*, 2006;
136 Cucinotta *et al.*, 2014; Larsson *et al.*, 2013). This phenotype is common to all mutants in which auxin

137 synthesis or transport pathways are compromised, such as *yucca1* (*yuc1*) *yuc4* (Cheng *et al.*, 2006)
138 and *pin1-1* (Okada *et al.*, 1991) or is similar to that following treatment with the polar auxin transport
139 inhibitor, 1-naphthyl phthalamic acid (NPA) (Nemhauser *et al.*, 2000).

140 Polar auxin transport is mediated by the PINFORMED1 (PIN1) efflux transporter and is
141 required to create a zone with an auxin concentration maximum in the placenta, where the founder
142 cells of the ovule primordia will be specified (Benková *et al.*, 2003; Ceccato *et al.*, 2013; Galbiati *et*
143 *al.*, 2013). Subsequently, the orientation of PIN1 within the membrane relocates and redirects auxin
144 flow, establishing a gradient with a maximum at the apices of the formed primordia. In developing
145 organs, auxin distribution can be monitored *in vivo* by imaging a synthetic auxin-inducible promoter,
146 *DR5*. In plants that express GREEN FLUORESCENT PROTEIN (GFP) from the *DR5* promoter,
147 green fluorescence is detected at the apices of the ovule primordia, consistent with PIN1-mediated
148 auxin flow directed to the apex (Benková *et al.*, 2003; Galbiati *et al.*, 2013). The weak *pin1-5* mutant
149 allele can produce some flowers in which the pistils have slightly reduced valves, which on average
150 contain only nine ovules (Bennett *et al.*, 1995; Sohlberg *et al.*, 2006; Bencivenga *et al.*, 2012).

151 Cytokinins (CKs) occupy a central role in the regulation of cell division and cell
152 differentiation. They are positive regulators of ovule formation, as demonstrated by the phenotype of
153 mutants in which CK pathways are altered. In the *ckx3 ckx5* double mutant, the degradation of CKs
154 is compromised and the consequent increase in the levels of these hormones leads to an increased
155 activity of the reproductive meristem (Bartrina *et al.*, 2011). Moreover, the longer than normal
156 gynoecia of *ckx3 ckx5* double mutants contain about twice as many ovules as those of the wild type,
157 indicating an increase in the meristematic capacity of placental tissue (Bartrina *et al.*, 2011). By
158 contrast, reduced ovule formation is observed in mutants in which the synthesis or perception of CKs
159 is compromised. Plants that carry mutations in genes that encode all three CKs receptors, *cytokinin*
160 *response 1* (*cre1-12*) *histidine kinase2* (*ahk2-2*) and *ahk3*, develop five ovules per pistil on average,
161 in addition to showing pleiotropic growth defects (Higuchi *et al.*, 2004; Bencivenga *et al.*, 2012). The
162 AHK2 and AHK3 receptors are expressed throughout ovule development, from the early stages until
163 maturity, whereas *CRE1/AHK4* is expressed in the chalaza region and subsequently in the
164 integuments, suggesting that AHK2 and AHK3 preferentially contribute to ovule primordium
165 formation (Bencivenga *et al.*, 2012). The ovule and gynoecium phenotype of the *cre1-12 ahk2-2*
166 *ahk3-3* triple mutant resembles that of the weak *pin1-5* mutant allele (Bencivenga *et al.*, 2012). This
167 similarity is due to the downregulation of *PIN1* expression in the triple mutant, suggesting that during
168 the early stages of ovule development, CK activates *PIN1* expression. Bencivenga *et al.* (2012)
169 showed that treating inflorescences with the synthetic cytokinin 6-benzylaminopurine (BAP)
170 increases *PIN1* expression in the gynoecium. Strikingly, treatment with BAP causes the formation of

171 on average 20 additional ovule primordia in each gynoecium, which are positioned between the
 172 existing primordia formed before the treatment. This suggests that placental tissue at the boundaries
 173 between ovules maintains meristematic competence. During root development, CK affects auxin
 174 polar transport via PIN1 both at the transcriptional and post-transcriptional levels. In contrast to the
 175 situation in the gynoecium, CK negatively regulates the expression of *PIN1* in the root and control
 176 the endorecycling of PIN1 from the membrane to direct it to vacuoles for lytic degradation (Ruzicka
 177 *et al.*, 2009; Marhavý *et al.*, 2011). In roots, CYTOKININ RESPONSE FACTORS (CRFs),
 178 especially CRF2, CRF3 and CRF6, transcriptionally regulate *PIN1* by binding to its promoter at the
 179 *cis*-regulatory *PIN* CYTOKININ RESPONSE ELEMENT (PCRE) (Šimášková *et al.*, 2015) and
 180 modulate its expression in response to CK. Similarly, CRFs also mediate *PIN1* expression in ovules
 181 in response to CK (Cucinotta *et al.*, 2016). Indeed, *PIN1* expression is reduced in the *crf2 crf3 crf6*
 182 (*crf2/3/6*) triple mutant and cannot be increased by CK treatment. The placenta in *crf2/3/6* is also
 183 shorter, but this is not sufficient to explain the 30% decrease in ovule number as ovule density is
 184 lower in *crf2/3/6* than in the wild type (Cucinotta *et al.*, 2016). Because *PIN1* expression in *crf2/3/6*
 185 was unresponsive to CK application, the mutant was significantly less sensitive to CK treatment than
 186 the wild type with regard to an increase in ovule number and pistil length. Auxin also regulates *CRF2*,
 187 which is a direct target of the Auxin Response Factor (ARF) AUXIN RESPONSE FACTOR
 188 5/MONOPTEROS (ARF5/MP) (Schlereth *et al.*, 2010), highlighting another convergence point
 189 between auxin and CK.

190 Another ARF family member that is required for appropriate apical–basal gynoecium
 191 patterning is ARF3/ETTIN (ETT). The *ett* mutant is characterised by a shorter ovary with an
 192 elongated style and gynophore (Sessions *et al.*, 1997). A similar gynoecium phenotype resulted from
 193 treatment with the auxin transport inhibitor (NPA), suggesting that ETT plays a key role in auxin
 194 signalling along the apical–basal gynoecium axis (Nemhauser *et al.*, 2000). Moreover, ETT restricts
 195 the expression domain of *SPATULA* (*SPT*), which encodes a basic helix-loop-helix (bHLH)
 196 transcription factor (Heisler *et al.*, 2001). Mutations in *SPT* causes a split-carpel phenotype in the
 197 apical part of the gynoecium, leading to a slight reduction in ovule number (Alvarez and Smyth, 1999;
 198 Nahar *et al.*, 2012). *SPT* dimerises with another bHLH transcription factor, INDEHISCENT (*IND*),
 199 to repress the expression of *PINOID* (Girin *et al.*, 2011), which encodes a serine/threonine kinase that
 200 regulates PIN1 polarisation via phosphorylation (Friml *et al.*, 2004). The repression of *PID* by *SPT*
 201 and *IND* allows the formation of a radially symmetric auxin ring in the upper part of the gynoecium
 202 that is required for correct style and stigma development (Moubayidin and Østergaard, 2014).

203 Furthermore, *SPT* interacts with the three closely related bHLH transcription factors
 204 HECATE1 (*HEC1*), *HEC2* and *HEC3* (Gremski *et al.*, 2007) and similar to *ett*, *hec-1 hec-2 hec-3*

205 triple mutants possess an elongated style and shorter ovaries. The HEC proteins and SPT promote
206 auxin transport in concert by activating *PIN1* and *PIN3* expression (Schuster *et al.*, 2015) and also
207 transcriptionally activate the type-A ARABIDOPSIS RESPONSE REGULATORS (ARR-As),
208 which are negative regulators of CK signalling (Schuster *et al.*, 2015). Via this dual action on auxin
209 transport and CK response, HECs and SPT regulate wild-type gynoecium fusion at the apex, and style
210 and stigma development. Furthermore, SPT alone in the medial domain activates the type-B ARRs,
211 especially ARR1, which are positive regulators of CK signalling. The *arr1 arr10 arr12* triple mutant
212 possesses a shorter gynoecium and significantly fewer ovules than the wild type (Reyes-Olalde *et al.*,
213 2017).

214 In addition to auxin localisation, correct auxin signalling is also required for wild-type
215 gynoecium development, as confirmed by a recent study on members of the Small Auxin-Upregulated
216 RNA (SAUR) family, which were initially identified as short transcripts that were rapidly upregulated
217 in response to auxin (McClure and Guilfoyle, 1987). When *SAUR8*, *SAUR10* and *SAUR12* are
218 ectopically overexpressed in Arabidopsis, the gynoecium and resulting siliques are longer than in
219 wild type, suggesting that auxin positively regulates gynoecium length and probably indirectly,
220 silique length (van Mourik *et al.*, 2017). Notably, *SAUR* gene expression increased by 100-fold
221 following combined auxin and brassinosteroid treatment (van Mourik *et al.*, 2017). Brassinosteroids
222 (BRs) are clearly involved in pistil growth and ovule number specification; gynoecia of the enhanced
223 BR-signalling mutant *brassinazole-resistant 1-1D* (*bzr1-1D*) contained not only more ovules than
224 wild type but were also longer. By contrast, BR-deficient mutants such as *de-etiolated 2* (*det-2*),
225 *brassinosteroid insensitive 1* (*bri1-5*) and *brassinosteroid-insensitive 2* (*bin2-1*) developed shorter
226 pistils with fewer ovules (Huang *et al.*, 2013).

227 The involvement of brassinosteroids in gynoecium and ovule development was also
228 confirmed by Nole-Wilson *et al.* (2010), who observed that a reduction in the expression of *CYP85A2*,
229 which encodes an enzyme involved in the final step of brassinolide biosynthesis (Nomura *et al.*,
230 2005), enhances the *seuss* mutant phenotypic disruptions in ovules and gynoecia (Nole-Wilson *et al.*,
231 2010b).

232

233 **CUP-SHAPED COTYLEDON 1 (CUC1) and CUC2 function synergistically with auxin and** 234 **cytokinins**

235 During ovule primordium formation, CK homeostasis requires two NAC-domain
236 transcription factors, CUC1 and CUC2. These are expressed in lateral organ boundaries and function
237 redundantly during organ boundary determination. *CUC1* and *CUC2* are expressed in the septum and
238 placenta, and following the emergence of ovule primordia, *CUC2* expression is restricted to the

239 borders between the ovules (Ishida *et al.*, 2000b; Galbiati *et al.*, 2013; Gonçalves *et al.*, 2015). The
240 *CUC1* and *CUC2* genes are both post-transcriptionally regulated by *miR164* microRNAs (Mallory *et*
241 *al.*, 2004; Laufs *et al.*, 2004). Gynoecia of the *in vitro* regenerated *cuc1 cuc2* mutant as well as of
242 *cuc2-1 pSTK::CUC1_RNAi* plants have reduced ovule numbers. The *cuc1 cuc2* double mutant has
243 on average fewer than 10 ovules per pistil (Ishida *et al.*, 2000a), whereas *cuc2-1 pSTK::CUC1_RNAi*
244 plants, in which *CUC1* was specifically silenced in the placenta and in ovules, showed a 20%
245 reduction in ovule number, but gynoecium length was not affected. In pistils of these plants, ovules
246 were more widely spaced when compared to the wild type (Galbiati *et al.*, 2013). This result was
247 confirmed by silencing *CUC1* and *CUC2* via overexpressing *MIR164A*, which strongly reduced ovule
248 number, indicating a major contribution of *CUC1* and *CUC2* to ovule initiation (Gonçalves *et al.*,
249 2015). The analysis of PIN1-GFP expression in *cuc2-1 pSTK::CUC1_RNAi* plants revealed that
250 *CUC1* and *CUC2* redundantly promote *PIN1* expression and PIN1 membrane localisation in ovules.
251 Treatment with BAP increased *PIN1* expression and complemented the reduced ovule number
252 phenotype of *cuc2-1 pSTK::CUC1_RNAi* plants (Galbiati *et al.*, 2013). Therefore, CK act
253 downstream from or in parallel with *CUC1* and *CUC2* to induce the expression of *PIN1*. Recently, it
254 has been demonstrated that *CUC1* and *CUC2* induce CK responses *in vivo* and function upstream of
255 CK by transcriptionally repressing *UGT73C1* and *UGT85A3*, which encode two enzymes involved
256 in CKs inactivation (Cucinotta *et al.*, 2018). Consistent with this result, the concentration of inactive
257 CKs glucosides was higher in *cuc2-1 pSTK::CUC1_RNAi* inflorescences than in wild-type plants.

258 The expression of *CUC1* and *CUC2* is also linked with auxin signalling: their expression
259 pattern coincides with that of the Auxin Response Factor ARF5/MP (see above) and both genes are
260 downregulated in pistils of the weak *mp-S319* mutant allele (Galbiati *et al.*, 2013). During the early
261 stages of placenta development and ovule formation, ARF5/MP directly transcriptionally activates
262 *CUC1* and *CUC2*, but also *ANT*. The observation that BAP treatment did not complement the ovule
263 number phenotype of *ant-4* suggests that *ANT* functions independently of *CUC1* and *CUC2*. This is
264 further supported by the additive effects on the reduction in ovule number observed in *ant-4 cuc2-1*
265 *pSTK::CUC1_RNAi* plants (Galbiati *et al.*, 2013). Together these data suggest that *ANT* promotes cell
266 proliferation, whereas *CUC1* and *CUC2* regulate CKs homeostasis and auxin transport. Although
267 *CUC3* shares high similarity with *CUC1* and *CUC2*, the *cuc3* mutant was not affected in ovule
268 initiation and number, but together with *CUC2*, *CUC3* promotes ovule separation; this is reflected by
269 the *cuc2 cuc3* double mutant, which produces seeds that result from the fusion of two ovules
270 (Gonçalves *et al.*, 2015). These results suggest that specific *CUC* genes independently promote ovule
271 initiation and ovule separation.

272 In 2009, Lee *et al.* identified LATERAL ORGAN FUSION 1 (LOF1) to be involved in lateral
273 organ separation and to functionally overlap with CUC2 and CUC3. The *LOF1* gene is expressed at
274 the base of ovule primordia and its overexpression results in a wrinkled pistil with an enlarged replum,
275 an amorphous septum and an irregular ovule distribution (Gomez *et al.*, 2011).

276

277 **The role of gibberellins in ovule primordium formation**

278 Gibberellins (GAs) are involved in key developmental processes throughout the plant life
279 cycle, from seed germination in particular, to flowering time (reviewed in Hedden and Sponsel, 2015;
280 Rizza and Jones, 2019), but their involvement in ovule initiation has only recently been demonstrated.
281 In 2018, Gomez and colleagues showed that DELLA proteins, which belong to a subfamily of the
282 plant-specific GRAS family of transcriptional regulators that repress GA-signalling, positively
283 regulate ovule number in Arabidopsis. In addition to DELLA proteins, the GA signalling core
284 includes the GA receptor GID1. When GID1 binds bioactive GA, the GA–GID1–DELLA complex
285 is formed and triggers the polyubiquitination and degradation of DELLA proteins. The *della* triple
286 mutant *gaiT6 rgaT2 rgl2-1* produces fewer ovules than wild type (Gomez *et al.*, 2018). By contrast,
287 the gain-of-function DELLA mutant *gai-1*, which cannot be degraded upon GA sensing, produced
288 more ovules. Consistent with this observation, the double *gid1a gid1b* mutant, which cannot perceive
289 GA, forms more ovules than the wild type, demonstrating a negative correlation between GAs and
290 ovule number (Gomez *et al.*, 2018). The *GAI*, *RGA*, *RGL2*, *GID1a* and *GID1b* genes are expressed
291 in placental tissue and outgrowing ovules. The reduction in ovule number was more dramatic in the
292 *gaiT6 rgaT2 rgl2-1* triple mutant than that in ovary length, resulting in a lower ovule density, whereas
293 the dominant *gai-1* mutant has an increased ovule/placenta ratio, suggesting that GAs predominantly
294 affect ovule initiation and not placenta elongation.

295 Other evidence to demonstrate that DELLA proteins promote ovule formation derive from an
296 experiment in which the expression of the stable mutant protein *rgaΔ17* under the control of the *ANT*
297 promoter in the placenta, resulted in the formation of 20% more ovules than in control lines (Gomez
298 *et al.*, 2018). This effect of GAs on the number of developing ovules was not correlated with auxin
299 signalling or transport, and neither PIN1 localisation nor *DR5* expression was affected by GA
300 treatment or DELLA activity (Gomez *et al.*, 2018).

301 Confirmation of a positive role for *RGL2* in determining ovule number came from the analysis
302 of transgenic lines in which RGL2-dependent GA signalling was blocked by the expression of a
303 dominant version of RGL2 (*pRGL2:rgl2Δ17*)(Gómez *et al.*, 2019). Pistils of *pRGL2:rgl2Δ17* plants
304 contained 10% more ovules than those of the wild type, whereas pistil length did not differ, indicating
305 that the main function of *rgl2Δ17* is to positively promote ovule primordium formation but not

306 placenta elongation (Gómez *et al.*, 2019). Furthermore, Gomez *et al.* (2018) identified
307 *REPRODUCTIVE MERISTEM 22* (*REM22*) and *UNFERTILIZED EMBRYO SAC 16* (*UNE16*) via
308 transcriptomic analysis to be DELLA targets that are positive regulators of ovule initiation. *REM22*
309 is a B3 family transcription factor that is expressed in the placenta (Mantegazza *et al.*, 2014) and
310 increased *REM22* expression in the *rem22-1* enhancer allele significantly increases ovule number.
311 *UNE16* is a transcription factor involved in embryo sac development and the knockdown allele
312 *une16-1* produces fewer ovules. Because *UNE16* expression is regulated by BRs (Pagnussat, 2005;
313 Sun *et al.*, 2010), it represents a potential nexus for crosstalk between GAs and BRs in ovule initiation.
314 The establishment of GA as an important additional component of the ovule regulatory network has
315 introduced an additional layer of complexity to the current model for ovule initiation and it remains
316 to be established how GAs integrate into this model. GAs might function antagonistically to CKs and
317 BRs, which in contrast to GAs, positively regulate pistil size and ovule number.

318 Finally, the *ctr1-1* constitutive ethylene-responsive mutant possesses a shorter gynoecium at
319 anthesis compared to wild type and a delay in the response to GA₃ treatment that induces gynoecium
320 senescence, suggesting that ethylene affects gynoecium size probably via interactions with GA
321 pathways (Carbonell-Bejerano *et al.*, 2011).

322 In conclusion, there is ample evidence for complex interactions between different hormonal
323 pathways that together determine ovule number and pistil size.

324

325 **Ovule number: the ecotype matters**

326 It has been known for twenty years that the number of ovules varies hugely among different
327 *Arabidopsis* ecotypes (diploid accessions) (Alonso-Blanco *et al.*, 1999): for example, the Landsberg
328 *erecta* accession produces 20% more ovules than the Cape Verde Islands (Cvi) accession. Recently,
329 189 *Arabidopsis* accessions from the *Arabidopsis* Biological Resource Center were analysed for
330 differences in ovule number and they display a remarkable degree of variation, ranging from 39–82
331 ovules per pistil (Yuan and Kessler, 2019). The commonly used reference accession Col-0 lies in the
332 middle of the range, with a mean ovule number of 63, which is strongly dependent on experimental
333 growth conditions. Ovule number, in contrast to, for instance, flowering time, does not correlate with
334 geographical origin (Stinchcombe *et al.*, 2004; Yuan and Kessler, 2019). By conducting a genome-
335 wide association study (GWAS) on these 189 accessions, two loci associated with ovule number were
336 identified (Yuan and Kessler, 2019): *NEW ENHANCER OF ROOT DWARFISM* (*NERD1*) and
337 *OVULE NUMBER ASSOCIATED 2* (*ONA2*). Mutation of *NERD1* or *ONA2* leads to a significant
338 reduction in ovule number, with a stronger phenotype in the *nerd1-2* and *nerd1-4* alleles. *ONA2*
339 encodes a protein of unknown function and was not further analyzed. In addition to a reduction in

340 ovule number, *nerd* mutants display additional severe male and female fertility defects. *NERD1*
341 encodes an integral membrane protein mainly localised to the Golgi. Notably, *NERD1* expression is
342 lower in Altai-5 and Kas-2 accessions, which have low ovule numbers (Yuan and Kessler, 2019), but
343 high *NERD1* expression in Altai-5 leads to a significant increase in ovule number. However,
344 overexpression of *NERD1* in Col-0 plants did not affect ovule number, indicating that *NERD1*
345 function in determining ovule number is background-dependent (Yuan and Kessler, 2019).

346 Considerable genetic variation in ovule number was also described for F₁ triploids of different
347 *A. thaliana* genotypes by Duszynska *et al.* (2013), who observed differences in ovule number between
348 genetically identical F₁-hybrid offspring, after crossing parental genome excess lines (2m:1p with
349 1m:2p). These effects can only be explained by epigenetic mechanisms that affect genes controlling
350 ovule number, for example DNA or histone methylation. The analysis of null alleles of *ASH1*
351 *HOMOLOG 2* (*ASH2*), which show a remarkable 80% reduction in ovule number, provided a clear
352 example of the involvement of histone methylation in determining ovule number (Grini *et al.*, 2009).
353 The transcriptional state of the *ASH2* locus remains active during development via H3K36
354 trimethylation (Xu *et al.*, 2008). It will be highly relevant to study the effect of epigenetic
355 modifications induced by biotic and abiotic stresses in determining ovule number. Epigenetic
356 responses to stress are fundamental to create the plasticity required for plant survival, especially
357 considering that plants are sessile organisms. These epigenetic changes can be temporally transmitted,
358 even in the absence of the original stress (Iglesias and Cerdán, 2016). Furthermore, variation in ovule
359 number in response to fluctuations in environmental conditions, such as temperature, can be used to
360 understand the plasticity and inheritability of (epigenetic) adaptation and response to temperature
361 stress. Variation in ovule number under stress conditions is, of course, also highly relevant from an
362 ecological, environmental and evolutionary perspective.

363

364 **Ovule number decreases with ageing**

365 Ovule number varies throughout inflorescence development: early flowers developing on the
366 main inflorescence (from the fifth to the twenty-fifth flower) of *Arabidopsis Ler* plants produced a
367 relatively invariable number of ovules, whereas flowers that developed later had pistils with fewer
368 ovules (Gomez *et al.*, 2018; Yuan and Kessler, 2019). Loss- and gain-of-function mutants of *DELLA*
369 genes showed an increase in ovule number in early and late-arising flowers (Gomez *et al.*, 2018). To
370 minimize age-related variation in their genome-wide association studies, Yuan and Kessler (2019)
371 only counted ovules in flowers 6 to 10 from the main inflorescence.

372 It has been reported for other plant species that flower position as well as size influence ovule
373 number per flower. For example, in Pomegranate, the number of ovules per flower was significantly
374 influenced by flower size, with more ovules being produced in larger flowers (Wetzstein *et al.*, 2013).

375 Overall, when studying changes in ovule numbers it is important to be aware of the possible
376 variation in the different flowers of the plant. Therefore, large numbers will have to be analyzed using
377 thorough statistical analyses, especially for genotypes that show only relative minor changes.

378 379 **A ‘gold mine’ for seed yield improvement within the Brassicaceae**

380 Improving seed yield via the genetic manipulation of crops has historically been a central goal
381 in agricultural research. The enormous body of data, which have been generated and shared by the
382 scientific community over the past decades, represents a true ‘gold mine’ for translational and applied
383 research. The determination of pistil size and ovule number may be considered one of the most
384 straightforward traits that can be enhanced to improve overall seed yield in species characterized by
385 multi-ovulate ovaries and the increasing amount of literature on this topic evidences an active and
386 prolific research field. Although some questions concerning the networks controlling seed number
387 and pistil size remain open, comprehensive knowledge of the phytohormone interactions involved in
388 these pathways is already available and applicable (Cucinotta *et al.*, 2014; Zúñiga-Mayo *et al.*, 2019;
389 Reyes-Olalde and de Folter, 2019).

390 Understanding these developmental processes in *Arabidopsis* can inform promising strategies
391 for knowledge transfer to closely related and agronomically important crops. *Brassica napus*, another
392 Brassicaceae species, commonly known as rapeseed, is an important breeding target, since it is a crop
393 widely cultivated in Europe, Asia, Canada and Australia. It is characterised by an oil-rich seed and
394 its processing provides both rapeseed oil (used as edible vegetable oil or as biodiesel) and a by-
395 product mostly used as cattle fodder (Snowdon *et al.*, 2007).

396 It has recently been demonstrated that *Arabidopsis* and *B. napus* share well-conserved
397 response mechanisms to cytokinin treatment (Zuñiga-Mayo *et al.*, 2018). Strikingly, exogenous
398 cytokinin application causes a reduction in silique length in *B. napus*. However, these shorter siliques
399 contain increased ovule numbers and upon manual pollination, the plants show an increase in seed
400 yield of 18%. Intriguingly, increases in ovule and seed number have also been observed in the
401 offspring of the treated plants, suggesting that the mechanism has an underlying epigenetic basis
402 (Zuñiga-Mayo *et al.*, 2018).

403 An increase in CKs level has also been reported to beneficially affect seed yield in transgenic
404 *B. napus* lines expressing the CKs biosynthetic enzyme isopentenyltransferase (*IPT*) under the A.

405 *thaliana* promoter of the *AtMYB32* gene. An increase in seed yield of up to 23% was obtained in the
406 transgenic lines that were analysed (Kant *et al.*, 2015).

407 CKs homeostasis is mediated by CYTOKININ OXIDASES/DEHYDROGENASES (CKXs)
408 during pistil and silique development in *A. thaliana*. Remarkably, the expression level of CKX genes
409 in *B. napus* is associated with silique length, and RNA-sequencing and qRT-PCR analyses revealed
410 a significantly different expression level of *BnCKX5-1*, *5-2*, *6-1*, and *7-1* in two distinct cultivated
411 varieties with long versus short siliques (Liu *et al.*, 2018). These findings open up promising strategies
412 with which to modulate silique length in *B. napus* by manipulating CKX gene expression.

413 In addition to phytohormones, genetic knowledge from Arabidopsis can be successfully
414 applied to *B. napus* crop improvement. Mutations in the K-box of the Arabidopsis orthologue of
415 *APETALAI* in *B. napus* caused a significant increase in the number of seeds per plant (Shah *et al.*,
416 2018). These generated alleles could conceivably be introduced into a rapeseed breeding programme
417 in field trials.

418 Germplasm of *B. napus* revealed substantial natural variation with respect to seed number per
419 pod. Current rapeseed cultivars produce on average 20 seeds per pod, which is far lower than the
420 maximum observed among the germplasm resources (Yang *et al.*, 2017). Moreover, genetic
421 improvement promises to deliver a massive improvement in seed yield (Yang *et al.*, 2017). The gold
422 mine of knowledge obtained from the closely related species Arabidopsis will certainly be
423 fundamentally important in the exploitation of the encouraging genetic variation potential.
424 Furthermore, it has recently been demonstrated that CRISPR-Cas9 technology can be efficiently
425 applied to precisely induce targeted mutation in rapeseed (Braatz *et al.*, 2017), making it a powerful
426 tool for future genetic improvement. Similarly, existing knowledge could be used to improve other
427 Brassicaceae species, or even non-phylogenetically related species such as soybean.

428

429

430 **Acknowledgements**

431 The authors' work is supported by the H2020-MSCA-RISE-2015 EXPOSEED project and
432 by MSCA-RISE-2014 PROCROP. SDF also acknowledges the Mexican National Council of
433 Science and Technology (CONACyT) grants FC-2015-2/1061 and CB2017-2018 A1-S-10126.
434 Authors would like to thank John Chandler for editing the paper.

435

436

437 **Table 1. Genes involved in determining gynoecium size and/or ovule number.**

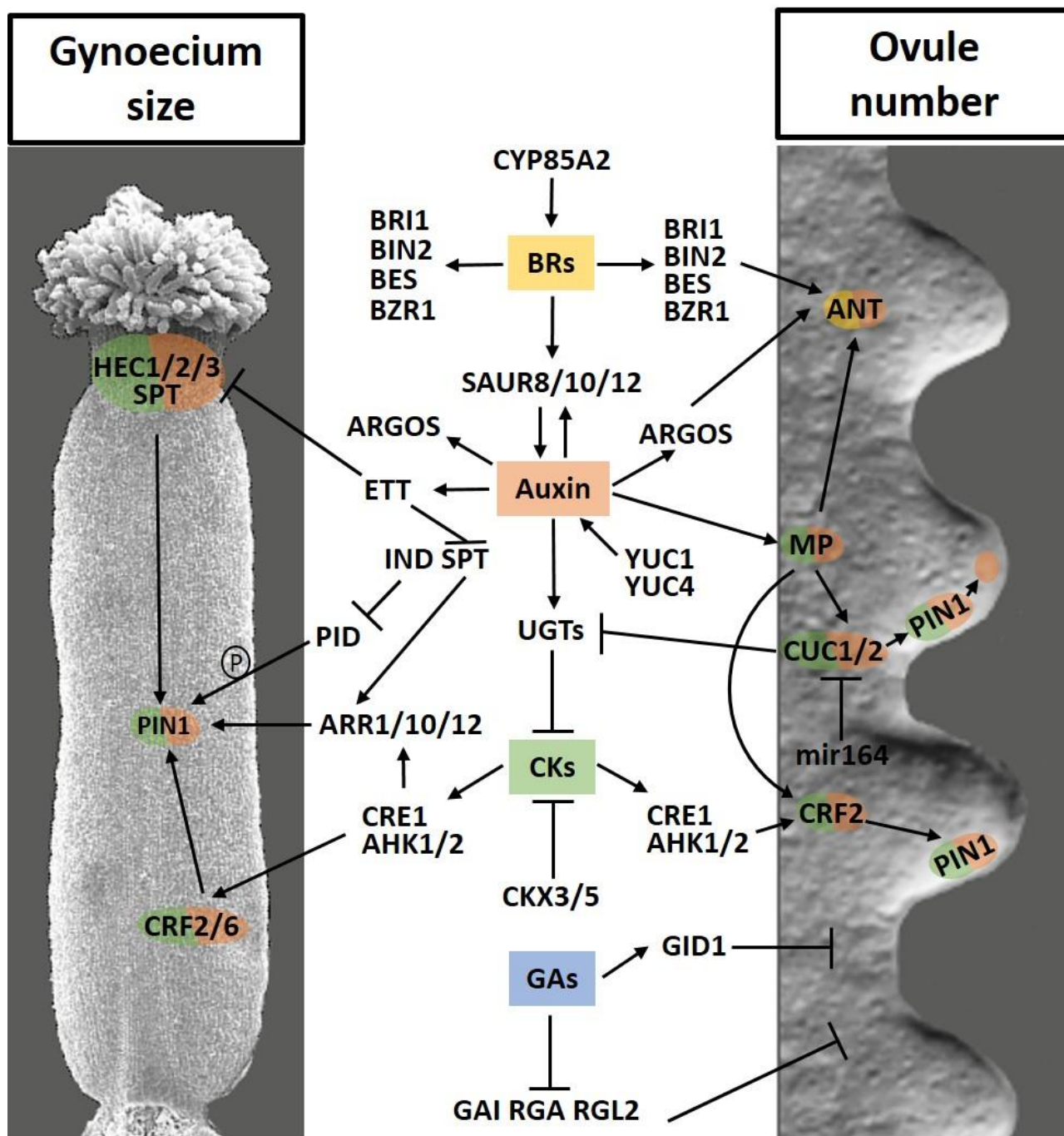
Gene Name	Family or protein type	Gynoecium size	Ovule number	Reference
<i>ANT</i>	AP2 /EREBP transcription factor	<i>ant-9</i> ↓ <i>ant-4</i> ↓ <i>35S::ANT</i> ↑	<i>ant-1</i> ↓ <i>ant-3</i> ↓ <i>ant-4</i> ↓ <i>ant-9</i> ↓	(Elliott <i>et al.</i> , 1996; Liu <i>et al.</i> , 2000; Azhakanandam <i>et al.</i> , 2008; Krizek, 2009; Wynn <i>et al.</i> , 2014)
<i>ARGOS</i>	ARGOS protein	<i>35S::ARGOS</i> ↑		(Hu <i>et al.</i> , 2003)
<i>CRC</i>	YABBY transcription factor	<i>crc-1</i> ↓		(Gross <i>et al.</i> , 2018)
<i>SPT</i>	bHLH transcription factor	<i>spt-2</i> ↓	<i>spt-2</i> ↓	(Heisler <i>et al.</i> , 2001; Alvarez and Smyth, 2002; Nahar <i>et al.</i> , 2012)
<i>ETT</i> (<i>ARF3</i>)	ARF transcription factor	<i>ett-1</i> ↓ <i>ett-2</i> ↓		(Sessions <i>et al.</i> , 1997; Nemhauser <i>et al.</i> , 2000)
<i>HEC1</i> , <i>HEC2</i> , <i>HEC3</i>	bHLH transcription factor	<i>hec1 hec2 hec3</i> ↓		(Gremski <i>et al.</i> , 2007)
<i>ARR1</i> , <i>ARR10</i> , <i>ARR12</i>	Type-B ARR transcription factor	<i>arr1 arr10 arr12</i> ↓	<i>arr1 arr10 arr12</i> ↓	(Reyes-Olalde <i>et al.</i> , 2017)
<i>CRF2</i> , <i>CRF3</i> , <i>CRF6</i>	ERF transcription factor	<i>crf2 crf3 crf6</i> ↓	<i>crf2 crf3 crf6</i> ↓	(Cucinotta <i>et al.</i> , 2016)
<i>PIN1</i>	PIN Auxin efflux carrier	<i>pin1</i> ↓	<i>pin1</i> ↓ <i>pin1-5</i> ↓	(Okada <i>et al.</i> , 1991; Bencivenga <i>et al.</i> , 2012; Cucinotta <i>et al.</i> , 2016)
<i>CKX3</i> , <i>CKX5</i>	CKX Cytokinin oxidase/dehydrogenase protein	<i>ckx3 ckx5</i> ↑	<i>ckx3 ckx5</i> ↑	(Bartrina <i>et al.</i> , 2011)
<i>UGT85A3</i> , <i>UGT73C1</i>	UDP-glucosyl transferase	<i>35S::UGT85A3</i> ↓ <i>35S::UGT73C1</i> ↓	<i>35S::UGT85A3</i> ↓ <i>35S::UGT73C1</i> ↓	(Cucinotta <i>et al.</i> , 2018)
<i>SAUR8</i> , <i>SAUR10</i> , <i>SAUR12</i>	SAUR-like auxin-responsive protein family	<i>35S::SAUR8</i> ↑ <i>35S::SAUR10</i> ↑ <i>35S::SAUR12</i> ↑		(van Mourik <i>et al.</i> , 2017)
<i>BZR1</i>	Brassinosteroid signalling regulatory protein	<i>bzr1-1D</i> ↑	<i>bzr1-1D</i> ↑	(Huang <i>et al.</i> , 2013)
<i>BIN2</i>	ATSK (shaggy-like kinase) family	<i>bin2</i> ↓	<i>bin2</i> ↓	(Huang <i>et al.</i> , 2013)
<i>DET2</i>	3-oxo-5- α -steroid 4-dehydrogenase protein	<i>det2</i> ↓	<i>det2</i> ↓	(Huang <i>et al.</i> , 2013)
<i>BRI1</i>	Leucine-rich receptor-like protein kinase protein	<i>bri1-5</i> ↓	<i>bri1-5</i> ↓	(Huang <i>et al.</i> , 2013)
<i>CYP85A2</i>	Cytochrome p450 enzyme		<i>cyp85a2-1</i> ↓ <i>cyp85a2-2</i> ↓	(Nole-Wilson <i>et al.</i> , 2010b)
<i>SEU</i>	Transcriptional adaptor	<i>seu-1</i> ↓	<i>seu-1</i> ↓	(Nole-Wilson <i>et al.</i> , 2010b)
<i>CTR1</i>	RAF homolog of serine/threonine kinase	<i>ctr1-1</i> ↓		(Carbonell-Bejerano <i>et al.</i> , 2011)

<i>REV</i>	Homeobox-leucine zipper protein		<i>ant rev</i> ↓	(Nole-Wilson <i>et al.</i> , 2010a)
<i>LUG</i>	WD40/YVTN repeat-like-containing domain transcription factor		<i>lug-1</i> ↓ <i>lug-3</i> ↓	(Azhakanandam <i>et al.</i> , 2008)
<i>PAN</i>	bZIP transcription factor	<i>ant pan</i> ↓ <i>seu pan</i> ↓	<i>ant pan</i> ↓ <i>seu pan</i> ↓	(Wynn <i>et al.</i> , 2014)
<i>HLL</i>	Ribosomal protein L14p/L23e	<i>hll</i> ↓	<i>hll</i> ↓	(Schneitz <i>et al.</i> , 1998; Skinner <i>et al.</i> , 2001)
<i>SIN2</i>	P-loop containing nucleoside triphosphate hydrolase superfamily protein	<i>sin-2</i> ↓	<i>sin-2</i> ↓	(Broadhvest <i>et al.</i> , 2000)
<i>YUC1</i> , <i>YUC4</i>	Flavin-binding monooxygenase protein		<i>yuc1 yuc4</i> ↓	(Cheng <i>et al.</i> , 2006)
<i>AHK2</i> , <i>AHK3</i> , <i>CRE1</i>	Histidine kinase		<i>cre1-12 ahk2-2</i> <i>ahk3-3</i> ↓	(Bencivenga <i>et al.</i> , 2012)
<i>CUC1</i> , <i>CUC2</i>	NAC transcription factor		<i>cuc1 cuc2</i> ↓ <i>pSTK::CUC1/RNAi</i> <i>cuc2-1</i> ↓	(Galbiati <i>et al.</i> , 2013)
<i>MIR164A</i>	microRNA		<i>35S::MIR164A</i> ↓	(Gonçalves <i>et al.</i> , 2015)
<i>GAI</i> , <i>RGA</i> , <i>RGL2</i>	GRAS transcription factor	<i>gaiT6 rgaT2 rgl2-1</i> ↓	<i>gaiT6 rgaT2 rgl2-1</i> ↓	(Gomez <i>et al.</i> , 2018)
<i>GID1A</i> , <i>GID1B</i>	alpha/beta-Hydrolase superfamily protein		<i>gid1ab</i> ↑	(Gomez <i>et al.</i> , 2018)
<i>REM22</i>	B3 protein transcription factor		<i>rem22-1</i> ↑	(Gomez <i>et al.</i> , 2018)
<i>UNE16</i>	Homeodomain-like superfamily protein		<i>une16-1</i> ↓	(Gomez <i>et al.</i> , 2018)
<i>NERD1</i>	GW repeat- and PHD-finger-containing protein NERD		<i>nerd1-2</i> ↓ <i>nerd1-4</i> ↓	(Yuan and Kessler, 2019)
<i>ONA2</i>	Unknown protein		<i>ona2</i> ↓	(Yuan and Kessler, 2019)
<i>ASHH2</i>	Hystone-lysine N-methyltransferase		<i>ashh2</i> ↓	(Grini <i>et al.</i> , 2009)

438

439 Table 1. Up- and downwards-pointing arrows represent how mutant phenotype impact either
440 gynoeceium size or ovule number.

441



Legend

Figure 1. Proposed model for the regulation of pistil growth and ovule primordium initiation.

A gynoecium of *Arabidopsis thaliana* is shown on the left while an image on the right depicts ovule primordia; in the centre, the interconnected gene network that regulates the two processes is shown. Auxin, through ETT, regulates gynoecium fusion and elongation by repressing *IND*, *HECs* and *SPT*, which in turn modulate polarisation of the auxin efflux carrier *PIN1* via repressing *PID*. CK positively regulates *PIN1* expression. In particular, the CK response mediated by CRFs and ARR is directly required for pistil elongation and indirectly affects ovule primordium initiation. *CRF2* regulation by

451 MP further integrate the auxin-CK crosstalk. Moreover, MP directly regulates *CUC1* and *CUC2*
452 expression. In turn, CUCs control *PIN1* expression and PIN1 protein localisation, which is required
453 for correct ovule primordium development. CUCs positively influence the CK pathway by
454 transcriptionally repressing the CK-inactivating glycosyltransferase enzymes (UGTs). *ANT*, whose
455 expression is controlled by auxin and BRs, is required for cell division in ovule primordia. *ANT* is
456 also regulated by auxin via MP and ARGOS. BRs signalling also positively affect pistil elongation.
457 GA has a negative effect on ovule number, but its connection with other hormones remains to be
458 addressed.

Bibliography

Alonso-Blanco C, Blankestijn-de Vries H, Hanhart CJ, Koornneef M. 1999. Natural allelic variation at seed size loci in relation to other life history traits of *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences **96**, 4710–4717.

Alvarez-Buylla ER, Benítez M, Corvera-poiré A, et al. 2010. Flower Development. Arabidopsis Book **8**, e0127.

Alvarez J, Smyth DR. 1999. *CRABS CLAW* and *SPATULA*, two *Arabidopsis* genes that control carpel development in parallel with *AGAMOUS*. Development **126**, 2377–2386.

Alvarez J, Smyth DR. 2002. *CRABS CLAW* and *SPATULA* genes regulate growth and pattern formation during gynoecium development in *Arabidopsis thaliana*. International Journal of Plant Sciences **163**, 17–41.

Angenent GC, Colombo L. 1996. Molecular control of ovule development. Trends in Plant Science **1**, 228–232.

Azhakanandam S, Nole-Wilson S, Bao F, Franks RG. 2008. *SEUSS* and *AINTEGUMENTA* mediate patterning and ovule initiation during gynoecium medial domain development. Plant Physiology **146**, 1165–1181.

Balanzá V, Navarrete M, Trigueros M, Ferrándiz C. 2006. Patterning the female side of Arabidopsis: the importance of hormones. Journal of Experimental Botany **57**, 3457–3469.

Bartrina I, Otto E, Strnad M, Werner T, Schmülling T. 2011. Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis*

thaliana. The Plant Cell **23**, 69–80.

Bencivenga S, Colombo L, Masiero S. 2011. Cross talk between the sporophyte and the megagametophyte during ovule development. Sexual Plant Reproduction **24**, 113–121.

Bencivenga S, Simonini S, Benková E, Colombo L. 2012. The transcription factors BEL1 and SPL are required for cytokinin and auxin signaling during ovule development in Arabidopsis. The Plant Cell **24**, 2886–2897.

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115**, 591–602.

Bennett SRM, Alvarez J, Bossinger G, Smyth DR. 1995. Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. The Plant Journal **8**, 505–520.

Bowman JL, Baum SF, Eshed Y, Putterill J, Alvarez J. 1999. Molecular genetics of gynoecium development in Arabidopsis. Current Topics in Developmental Biology **45**, 155–205.

Braatz J, Harloff HJ, Mascher M, Stein N, Himmelbach A, Jung C. 2017. CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). Plant Physiology **174**, 935–942.

Broadhvest J, Baker SC, Gasser CS. 2000. *SHORT INTEGUMENTS 2* promotes growth during Arabidopsis reproductive development. Genetics **155**, 899–907.

Carbonell-Bejerano P, Urbez C, Granell A, Carbonell J, Perez-Amador MA. 2011. Ethylene is involved in pistil fate by modulating the onset of ovule senescence and the GA-mediated fruit set in Arabidopsis. BMC Plant Biology **11**, 84.

Ceccato L, Masiero S, Sinha Roy D, Bencivenga S, Roig-Villanova I, Ditengou FA, Palme K, Simon R, Colombo L. 2013. Maternal control of PIN1 is required for female gametophyte development in Arabidopsis. PloS one **8**, e66148.

Cheng Y, Dai X, Zhao Y. 2006. Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes and Development **20**, 1790–1799.

Colombo L, Battaglia R, Kater MM. 2008. Arabidopsis ovule development and its evolutionary conservation. Trends in Plant Science **13**, 444–450.

- Cucinotta M, Colombo L, Roig-villanova I.** 2014. Ovule development , a new model for lateral organ formation. *Frontiers in Plant Science* **5**, 117.
- Cucinotta M, Manrique S, Cuesta C, Benkova E, Novak O, Colombo L.** 2018. CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 regulate cytokinin homeostasis to determine ovule number in *Arabidopsis*. *Journal of Experimental Botany* **69**, 5169–5176.
- Cucinotta M, Manrique S, Guazzotti A, Quadrelli NE, Mendes MA, Benkova E, Colombo L.** 2016. Cytokinin response factors integrate auxin and cytokinin pathways for female reproductive organ development. *Development* **143**, 4419–4424.
- Davies PJ.** 2004. *Plant Hormones: biosynthesis, signal, transduction, action!* Kluwer Academic Publishers. Dordrecht, The Netherlands.
- Drews GN, Koltunow AM.** 2011. The female gametophyte. *The Arabidopsis Book* **9**, e0155.
- Duszynska D, McKeown PC, Juenger TE, Pietraszewska-Bogiel A, Geelen D, Spillane C.** 2013. Gamete fertility and ovule number variation in selfed reciprocal F1 hybrid triploid plants are heritable and display epigenetic parent-of-origin effects. *The New Phytologist* **198**, 71–81.
- Elliott RC, Betzner AS, Huttner E, Oakes MP, Tucker WQJ, Gerentes D, Perez P, Smyth DR.** 1996. *AINTEGUMENTA*, an *APETALA2*-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. *The Plant Cell* **8**, 155–168.
- Endress PK.** 2011. Angiosperm ovules: diversity, development, evolution. *Annals of Botany* **107**, 1465–1489.
- Endress PK, Igersheim A.** 2000. Gynoecium structure and evolution in basal angiosperms. *International Journal of Plant Sciences* **161**, S211–S213.
- Friml J, Yang X, Michniewicz M, et al.** 2004. A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* **306**, 862–865.
- Galbiati F, Sinha Roy D, Simonini S, et al.** 2013. An integrative model of the control of ovule primordia formation. *The Plant Journal* **76**, 446–455.
- Gasser CS, Broadhvest J, Hauser BA.** 1998. Genetic analysis of ovule development. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 1–24.
- Gasser CS, Skinner DJ.** 2019. Development and evolution of the unique ovules of flowering plants. *Current Topics in Developmental Biology* **131**, 373–399.

- Girin T, Paicu T, Stephenson P, et al.** 2011. INDEHISCENT and SPATULA interact to specify carpel and valve margin tissue and thus promote seed dispersal in *Arabidopsis*. *The Plant Cell* **23**, 3641–3653.
- Gomez MD, Barro-Trastoy D, Escoms E, et al.** 2018. Gibberellins negatively modulate ovule number in plants. *Development* **145**, dev163865.
- Gómez MD, Fuster-Almunia C, Ocaña-Cuesta J, Alonso JM, Pérez-Amador MA.** 2019. RGL2 controls flower development, ovule number and fertility in *Arabidopsis*. *Plant Science* **281**, 82–92.
- Gomez MD, Urbez C, Perez-Amador M a, Carbonell J.** 2011. Characterization of *constricted fruit* (*ctf*) mutant uncovers a role for *AtMYB117/LOF1* in ovule and fruit development in *Arabidopsis thaliana*. *PloS one* **6**, e18760.
- Gonçalves B, Hasson A, Belcram K, et al.** 2015. A conserved role for *CUP-SHAPED COTYLEDON* genes during ovule development. *The Plant Journal* **83**, 732–742.
- Govaerts R.** 2001. How many species of seed plants are there? *Taxon* **50**, 1085–1090.
- Gremski K, Ditta G, Yanofsky MF.** 2007. The *HECATE* genes regulate female reproductive tract development in *Arabidopsis thaliana*. *Development* **134**, 3593–3601.
- Grini PE, Thorstensen T, Alm V, Vizcay-Barrena G, Windju SS, Jørstad TS, Wilson ZA, Aalen RB.** 2009. The ASH1 HOMOLOG 2 (ASHH2) histone H3 methyltransferase is required for ovule and anther development in *Arabidopsis*. *PLoS ONE* **4**.
- Gross T, Broholm S, Becker A.** 2018. CRABS CLAW acts as a bifunctional transcription factor in flower development. *Frontiers in Plant Science* **9**, 1–13.
- Grossniklaus U, Schneitz K.** 1998. The molecular and genetic basis of ovule and megagametophyte development. *Seminars in Cell & Developmental Biology* **9**, 227–238.
- Hedden P, Sponsel V.** 2015. A century of gibberellin research. *Journal of Plant Growth Regulation* **34**, 740–760.
- Heisler MGB, Atkinson A, Bylstra YH, Walsh R, Smyth DR.** 2001. *SPATULA*, a gene that controls development of carpel margin tissues in *Arabidopsis*, encodes a bHLH protein. *Development* **128**, 1089–1098.
- Higuchi M, Pischke MS, Mähönen AP, et al.** 2004. *In planta* functions of the *Arabidopsis* cytokinin receptor family. *Proceedings of the National Academy of Sciences of the United States of America*

101, 8821–8826.

Hill TA, Broadhvest J, Kuzoff RK, Gasser CS. 2006. Arabidopsis SHORT INTEGUMENTS 2 Is a Mitochondrial DAR GTPase. *Genetics* **174**, 707–718.

Hu, Xie, Chua. 2003. The Arabidopsis auxin-inducible gene *ARGOS* controls lateral organ size. *The Plant Cell* **15**, 1951–1961.

Huang HY, Jiang WB, Hu YW, Wu P, Zhu JY, Liang WQ, Wang ZY, Lin WH. 2013. BR signal influences *Arabidopsis* ovule and seed number through regulating related genes expression by BZR1. *Molecular Plant* **6**, 456–469.

Iglesias FM, Cerdán PD. 2016. Maintaining epigenetic inheritance during DNA replication in plants. *Frontiers in Plant Science* **7**.

Ishida T, Aida M, Takada S, Tasaka M. 2000a. Involvement of *CUP-SHAPED COTYLEDON* genes in gynoecium and ovule development in *Arabidopsis thaliana*. *Plant and Cell Physiology* **41**, 60–67.

Kant S, Burch D, Badenhorst P, Palanisamy R, Mason J, Spangenberg G. 2015. Regulated Expression of a cytokinin biosynthesis gene *IPT* delays leaf senescence and improves yield under rainfed and irrigated conditions in canola (*Brassica napus* L.). *PLoS ONE* **10**, 1–18.

Klucher KM, Chow H, Reiser L, Fischer RL. 1996. The *AINTEGUMENTA* gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene *APETALA2*. *The Plant Cell* **8**, 137–153.

Krizek BA. 2009. *AINTEGUMENTA* and *AINTEGUMENTA-LIKE6* act redundantly to regulate Arabidopsis floral growth and patterning. *Plant Physiology* **150**, 1916–1929.

Larsson E, Franks RG, Sundberg E. 2013. Auxin and the *Arabidopsis thaliana* gynoecium. *Journal of Experimental Botany* **64**, 2619–2627.

Laufs P, Peaucelle A, Morin H, Traas J. 2004. MicroRNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. *Development* **131**, 4311–4322.

Liu Z, Franks RG, Klink VP. 2000. Regulation of gynoecium marginal tissue formation by *LEUNIG* and *AINTEGUMENTA*. *The Plant Cell* **12**, 1879–1891.

Liu P, Zhang C, Ma JQ, Zhang LY, Yang B, Tang XY, Huang L, Zhou XT, Lu K, Li JN. 2018. Genome-wide identification and expression profiling of cytokinin oxidase/dehydrogenase (*CKX*)

genes reveal likely roles in pod development and stress responses in oilseed rape (*Brassica napus* L.). *Genes* **9**, 168.

Mallory AC, Reinhart BJ, Jones-Rhoades MW, Tang G, Zamore PD, Barton MK, Bartel DP. 2004. MicroRNA control of *PHABULOSA* in leaf development: importance of pairing to the microRNA 5' region. *The EMBO Journal* **23**, 3356–3364.

Mantegazza O, Gregis V, Mendes MA, Morandini P, Alves-Ferreira M, Patreze CM, Nardeli SM, Kater MM, Colombo L. 2014. Analysis of the arabidopsis *REM* gene family predicts functions during flower development. *Annals of Botany* **114**, 1507–1515.

Marhavý P, Bielach A, Abas L, et al. 2011. Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. *Developmental Cell* **21**, 796–804.

Marsch-Martínez N, de Folter S. 2016. Hormonal control of the development of the gynoecium. *Current Opinion in Plant Biology* **29**, 104–114.

McClure BA, Guilfoyle T. 1987. Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. *Plant Molecular Biology* **9**, 611–623.

Mizukami Y, Fischer RL. 2000. Plant organ size control: *AINTEGUMENTA* regulates growth and cell numbers during organogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 942–947.

Moubayidin L, Østergaard L. 2014. Dynamic control of auxin distribution imposes a bilateral-to-radial symmetry switch during gynoecium development. *Current Biology* **24**, 2743–2748.

van Mourik H, van Dijk ADJ, Stortenbeker N, Angenent GC, Bemer M. 2017. Divergent regulation of Arabidopsis *SAUR* genes: A focus on the *SAUR10*-clade. *BMC Plant Biology* **17**, 1–14.

Nahar MA-U, Ishida T, Smyth DR, Tasaka M, Aida M. 2012. Interactions of *CUP-SHAPED COTYLEDON* and *SPATULA* genes control carpel margin development in *Arabidopsis thaliana*. *Plant and Cell Physiology* **53**, 1134–1143.

Nemhauser JL, Feldman LJ, Zambryski PC. 2000. Auxin and *ETTIN* in Arabidopsis gynoecium morphogenesis. *Development* **127**, 3877–3888.

Nole-Wilson S, Azhakanandam S, Franks RG. 2010a. Polar auxin transport together with *AINTEGUMENTA* and *REVOLUTA* coordinate early Arabidopsis gynoecium development. *Developmental Biology* **346**, 181–95.

- Nole-Wilson S, Rueschhoff EE, Bhatti H, Franks RG.** 2010b. Synergistic disruptions in *seuss cyp85A2* double mutants reveal a role for brassinolide synthesis during gynoecium and ovule development. *BMC Plant Biology* **10**, 198.
- Nomura T, Kushiro T, Yokota T, Kamiya Y, Bishop GJ, Yamaguchi S.** 2005. The last reaction producing brassinolide is catalyzed by cytochrome P-450s, CYP85A3 in tomato and CYP85A2 in *Arabidopsis*. *Journal of Biological Chemistry* **280**, 17873–17879.
- Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y.** 1991. Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *The Plant Cell* **3**, 677–684.
- Pagnussat GC.** 2005. Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. *Development* **132**, 603–614.
- Pinto SC, Mendes MA, Coimbra S, Tucker MR.** 2019. Revisiting the female germline and its expanding toolbox. *Trends in Plant Science* **24**, 455–467.
- Reiser L, Fischer RL.** 1993. The ovule and the embryo sac. *The Plant Cell* **5**, 1291–1301.
- Reyes-Olalde JI, de Folter S.** 2019. Control of stem cell activity in the carpel margin meristem (CMM) in *Arabidopsis*. *Plant Reproduction* **32**, 123–136.
- Reyes-Olalde JI, Zuñiga-Mayo VM, Chávez Montes R a, Marsch-Martínez N, de Folter S.** 2013. Inside the gynoecium: at the carpel margin. *Trends in Plant Science* **18**, 644–655.
- Reyes-Olalde JI, Zúñiga-Mayo VM, Serwatowska J, et al.** 2017. The bHLH transcription factor SPATULA enables cytokinin signaling, and both activate auxin biosynthesis and transport genes at the medial domain of the gynoecium. *PLoS Genetics* **13**, e1006726.
- Rizza A, Jones AM.** 2019. The makings of a gradient: spatiotemporal distribution of gibberellins in plant development. *Current Opinion in Plant Biology* **47**, 9–15.
- Roeder AHK, Yanofsky MF.** 2006. *Fruit development in Arabidopsis*. *Arabidopsis Book* **4**, e0075.
- Ruzicka K, Simásková M, Duclercq J, Petrásek J, Zazimalová E, Simon S, Friml J, Van Montagu MCE, Benková E.** 2009. Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 4284–4289.
- Schaller GE, Bishopp A, Kieber JJ.** 2015. The yin-yang of hormones: cytokinin and auxin interactions in plant development. *The Plant Cell* **27**, 44–63.

- Schlereth A, Moller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jurgens G, Weijers D.** 2010. MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature* **464**, 913–916.
- Schneitz K, Baker SC, Gasser CS, Redweik A.** 1998. Pattern formation and growth during floral organogenesis: *HUELLENLOS* and *AINTEGUMENTA* are required for the formation of the proximal region of the ovule primordium in *Arabidopsis thaliana*. *Development* **125**, 2555–2563.
- Schneitz K, Hulskamp M, Pruitt RE.** 1995. Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue. *The Plant Journal* **7**, 731–749.
- Schuster C, Gaillochet C, Lohmann JU.** 2015. *Arabidopsis* *HECATE* genes function in phytohormone control during gynoecium development. *Development* **142**, 3343–3350.
- Sessions A, Nemhauser JL, McColl A, Roe JL, Feldmann KA, Zambryski PC.** 1997. *ETTIN* patterns the *Arabidopsis* floral meristem and reproductive organs. *Development* **124**, 4481–4491.
- Shah S, Karunarathna NL, Jung C, Emrani N.** 2018. An *APETALA1* ortholog affects plant architecture and seed yield component in oilseed rape (*Brassica napus* L.). *BMC Plant Biology* **18**, 1–12.
- Shi D-Q, Yang W-C.** 2011. Ovule development in *Arabidopsis*: progress and challenge. *Current Opinion in Plant Biology* **14**, 74–80.
- Shirley NJ, Aubert MK, Wilkinson LG, Bird DC, Lora J, Yang X, Tucker MR.** 2019. Translating auxin responses into ovules, seeds and yield: Insight from *Arabidopsis* and the cereals. *Journal of Integrative Plant Biology* **61**, 310–336.
- Šimášková M, O'Brien JA, Khan M, et al.** 2015. Cytokinin response factors regulate PIN-FORMED auxin transporters. *Nature Communications* **6**, 8717.
- Skinner DJ, Baker SC, Meister RJ, Broadhvest J, Schneitz K, Gasser CS.** 2001. The *Arabidopsis* *HUELLENLOS* gene, which is essential for normal ovule development, encodes a mitochondrial ribosomal protein. *The Plant Cell* **13**, 2719–30.
- Skinner DJ, Hill TA, Gasser CS.** 2004. Regulation of ovule development. **16**, 32–46.
- Smyth DR, Bowman JL, Meyerowitz EM.** 1990. Early flower development in *Arabidopsis*. *The Plant Cell* **2**, 755–767.
- Snowdon, Rod, Lühs, Wilfried, Friedt W.** 2007. Genome mapping and molecular breeding in

plants. *Oilseeds* **6**, 54–56.

Sohlberg JJ, Myrenås M, Kuusk S, Lagercrantz U, Kowalczyk M, Sandberg G, Sundberg E. 2006. *STY1* regulates auxin homeostasis and affects apical-basal patterning of the *Arabidopsis* gynoecium. *The Plant Journal* **47**, 112–123.

Stinchcombe JR, Weinig C, Ungerer M, Olsen KM, Mays C, Halldorsdottir SS, Purugganan MD, Schmitt J. 2004. A latitudinal cline in flowering time in *Arabidopsis thaliana* modulated by the flowering time gene *FRIGIDA*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 4712–4717.

Sun Y, Fan X-Y, Cao D-M, et al. 2010. Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in *Arabidopsis*. *Developmental Cell* **19**, 765–777.

Vanstraelen M, Benková E. 2012. Hormonal interactions in the regulation of plant development. *Annual Review of Cell and Developmental Biology* **28**, 463–487.

Wetzstein HY, Yi W, Porter JA, Ravid N. 2013. Flower position and size impact ovule number per flower, fruitset, and fruit size in pomegranate. *Journal of the American Society for Horticultural Science* **138**, 159–166.

Willis, KJ, Royal Botanic Gardens K. 2017. *State of the World's Plants* 2017. Report.

Wynn AN, Seaman AA, Jones AL, Franks RG. 2014. Novel functional roles for *PERIANTHIA* and *SEUSS* during floral organ identity specification, floral meristem termination, and gynoecial development. *Frontiers in Plant Science* **5**, 130.

Xu L, Zhao Z, Dong A, Soubigou-Taconnat L, Renou J-P, Steinmetz A, Shen W-H. 2008. Di- and tri- but not monomethylation on histone H3 lysine 36 marks active transcription of genes involved in flowering time regulation and other processes in *Arabidopsis thaliana*. *Molecular and Cellular Biology* **28**, 1348–1360.

Yang Y, Wang Y, Zhan J, Shi J, Wang X, Liu G, Wang H. 2017. Genetic and cytological analyses of the natural variation of seed number per pod in rapeseed (*Brassica napus* L.). *Frontiers in Plant Science* **8**, 1890.

Yuan J, Kessler SA. 2019. A genome-wide association study reveals a novel regulator of ovule number and fertility in *Arabidopsis thaliana*. *PLOS Genetics* **15**, e1007934.

Zuñiga-Mayo VM, Baños-Bayardo CR, Díaz-Ramírez D, Marsch-Martínez N, De Folter S. 2018. Conserved and novel responses to cytokinin treatments during flower and fruit development in

Brassica napus and *Arabidopsis thaliana*. Scientific Reports **8**, 1–10.

Zúñiga-Mayo VM, Gómez-Felipe A, Herrera-Ubaldo H, De Folter S. 2019. Gynoecium development: Networks in *Arabidopsis* and beyond. Journal of Experimental Botany **70**, 1447–1460.