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Gibberellins regulate ovule number through a DELLA–CUC2 complex in *Arabidopsis*

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SUMMARY

Ovule development is a key process for plant reproduction, helping to ensure correct seed production. Several molecular factors and plant hormones such as gibberellins are involved in ovule initiation and development. Gibberellins control ovule development by the destabilization of DELLA proteins, whereas DELLA activity has been shown to act as a positive factor for ovule primordia emergence. But the molecular mechanism by which DELLA acts in ovule primordia initiation remained unknown. In this study we report that DELLA proteins participate in ovule initiation by the formation of a protein complex with the CUC2 transcription factor. The DELLA protein GAI requires CUC2 to promote ovule primordia formation, through the direct GAI–CUC2 interaction in placental cells that would determine the boundary regions between ovules during pistil development. Analysis of GAI–CUC2 interaction and co-localization in the placenta supports this hypothesis. Moreover, molecular analysis identified a subset of the loci for which the GAI protein may act as a transcriptional co-regulator in a CUC2-dependent manner. The DELLA–CUC2 complex is a component of the gene regulatory network controlling ovule primordia initiation in Arabidopsis.

Keywords: ***Arabidopsis,*** **gibberellins, ovule, DELLA proteins, GAI, CUC2.**

INTRODUCTION

Ovules are essential for reproductive development as they provide the foundation for seed development that will secure the perpetuation of the plant species. In the last few years, the molecular mechanisms that control ovule formation have attracted interest, and several key pieces of experimental evidence have allowed the construction of models of the molecular mechanism controlling ovule primordia initiation and development (recently reviewed in Barro-Trastoy et al., 2020a; Cucinotta et al., 2020; Erbasol-Serbes et al., 2019; Lora et al., 2019; Pinto et al., 2019). In fact, studying ovule development provides an interesting model for understanding organ formation in general during plant development. In addition, proper ovule initiation and development directly impact the production of seeds and grains, a key component of human and animal diets (Cucinotta et al., 2020; Shirley et al., 2019); hence, a full understanding of the players involved in ovule and seed development will contribute to designing additional approaches to increase crop yield.

In Arabidopsis, ovule primordia arise from the placenta, a meristematic tissue located in the medial domain of the pistil, at stage 8 of floral development (Barro-Trastoy et al., 2020a; Cucinotta et al., 2014, 2020; Vijayan et al., 2021; Yu et al., 2020). This is the first key step of ovule development and depends on the correct determination of ovule primordia position along the placenta. Regulatory genes found to have a role during ovule initiation include *AINTEGUMENTA* (*ANT*), which regulates cell proliferation and promotes ovule primordia growth, and *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2*, which establish the boundary regions between ovule primordia.

Additionally, several plant hormones are also involved, some of them being highly interconnected with *ANT*, *CUC1*, and *CUC2*. PIN-FORMED1 (PIN1) auxin efflux causes auxin accumulation, which is essential to promote ovule initiation (Benkova et al., 2003; Ceccato et al., 2013; Galbiati et al., 2013). Cytokinins (CKs), whose levels are partially controlled by *CUC1* and *CUC2* (Cucinotta et al., 2018), promote *PIN1* expression to allow correct auxin efflux (Bencivenga et al., 2012; Galbiati et al., 2013). Finally, brassinosteroids (BRs) positively influence ovule number, probably through the regulation of *ANT* expression (Huang et al., 2013b).

Recently, we have demonstrated that gibberellins (GAs) negatively modulate ovule number in Arabidopsis, rapeseed (*Brassica napus*), and tomato (*Solanum*

lycopersicum) through DELLA protein activity (Barro-Trastoy et al., 2020b; Gomez et al., 2018, 2019). GAs are a group of diterpenoid compounds involved in many developmental processes such as seed germination, stem and root elongation, flowering, and ovule and fruit development (Gomez et al., 2016; Gupta and Chakrabarty, 2013; Sun, 2011). GA signaling relies on the degradation of DELLA proteins, nuclear proteins that belong to the GRAS family of transcriptional regulators and act as GA signaling repressors.

GA-dependent DELLA degradation is mediated by the N-terminal domain of DELLA (Sun, 2011). Deletion of this 17-aa domain is sufficient to prevent GA-mediated protein degradation, like in the *gai-1* mutant (Peng et al., 1997) allele, which encodes dominant DELLA proteins that constitutively block GA signaling. Both high GA levels and loss-of-function of DELLA proteins release GA responses, while low GA levels or GA insensitive DELLA mutants restrain GA responses (Daviere and Achard, 2016; Hernandez-Garcia et al., 2020; Sun, 2011; Vera-Sirera et al., 2016).

During pistil development, constitutive GA signaling in the null mutant *4xdella*, lacking four of the five DELLA proteins encoded by the Arabidopsis genome (i.e., lacking GA-INSENSITIVE [GAI], REPRESSOR OF GA1-3 [RGA], RGA-LIKE1 [RGL1], and RGL2), produces a decrease in ovule number, with GAI, RGA, and RGL2 being those that have a major role (Gomez et al., 2018). In fact, these three DELLA proteins are expressed in ovule primordia at early developmental stages (Gomez et al., 2018). A similar ovule number phenotype was also seen in GA-treated plants. On the contrary, constitutive blockage of GA responses in the *gai-1* mutant produces more ovules than in wild-type plants (Gomez et al., 2018). Similarly, RGL2 is also involved in ovule initiation, as *pRGL2:rgl2Δ17-YPet* plants, which produce an RGL2 protein version that is not degraded by GAs, also show increased ovule number (Gomez et al., 2019). These pieces of evidence clearly indicate that DELLA proteins are positive factors in ovule formation.

However, the molecular mechanism underlying the regulation of ovule initiation by DELLA proteins remains unclear. Previous analyses suggested that DELLA-mediated ovule initiation does not seem to be related to either auxin or BR pathways in Arabidopsis. On the one hand, neither auxin transport nor the auxin response is altered in the placenta of GA mutants or upon GA treatment (Gomez et al., 2018). On the other hand, genetic analysis revealed that GAs and BRs act independently during ovule initiation (Barro-Trastoy et al., 2020b). Despite these analyses, there is no indication of how DELLA proteins promote ovule primordia formation.

At the molecular level, DELLA proteins are transcriptional regulators that lack a canonical DNA-binding domain, exerting their function by binding to a wide variety of transcription factors (TFs) (Daviere and Achard, 2016; Hernandez-Garcia et al., 2020; Vera-Sirera et al., 2016). Based on this, a plausible hypothesis is that DELLA proteins physically interact with any of the key TFs involved in ovule initiation, such as ANT, CUC1, and CUC2. In fact, CUC2 was found as a putative interactor with the DELLA protein GAI in a yeast two-hybrid (Y2H) screen (Marin-de la Rosa et al., 2014), suggesting CUC2 is a component of the DELLA-mediated ovule initiation mechanism.

CUC1, *CUC2*, and *CUC3* genes encode NAC-domain family TFs with a central and redundant role in organ boundary formation, e.g., in primary and axillary shoots, leaf serration, and floral organs including the gynoecium and ovules (reviewed in Maugarny et al., 2016). In the placenta, *CUC1* and *CUC2* are known to positively regulate ovule initiation, whereas *CUC3* is involved, along with CUC2 in a redundant manner, in ovule separation (Galbiati et al., 2013; Gonçalves et al., 2015; Ishida et al., 2000).

This work aims to uncover the molecular mechanism by which DELLA proteins regulate ovule development. Our data point to CUC2 as the key component of the DELLA-mediated ovule initiation pathway. CUC2 is required for the DELLA protein GAI to promote ovule primordia formation. GAI participates in ovule initiation by its direct protein–protein interaction with CUC2 in cells of the boundary regions between ovules during pistil elongation. Direct analysis of GAI–CUC2 interaction and co-localization in the placenta supports this hypothesis. Furthermore, molecular analysis of the loci at which GAI may act as transcriptional co-regulator in a CUC2-dependent manner identified a subset of genes potentially regulated by the GAI–CUC2 complex and contributing to regulating ovule primordia emergence. Altogether, this analysis allows us to integrate GAs and DELLA proteins in the gene network that governs ovule primordia initiation.

RESULTS

GAI requires CUC2 to mediate ovule number

Previously, a Y2H screen of 1200 TFs from Arabidopsis identified CUC2 as a putative interactor of GAI (Marin-de la Rosa et al., 2014). To address whether CUC2 may mediate DELLA function in ovule initiation, we carried out a comprehensive analysis of ovule number phenotype in *cuc* mutants under the effect of treatments with GA and paclobutrazol (PBZ), an inhibitor of GA biosynthesis that promotes an increase in the levels of DELLA proteins. We also performed analyses of development under the dominant *gai-1* or null *4xdella* backgrounds.

In accord with previous results (Galbiati et al., 2013; Ishida et al., 2000), both *cuc1-1* and *cuc2-1* single null mutants showed a 10% reduction in ovule number (Figure 1(a)), which is consistent with CUC1 and CUC2 being positive regulators of ovule initiation. GA treatment, which reduces levels of DELLA proteins, caused a 20% decrease

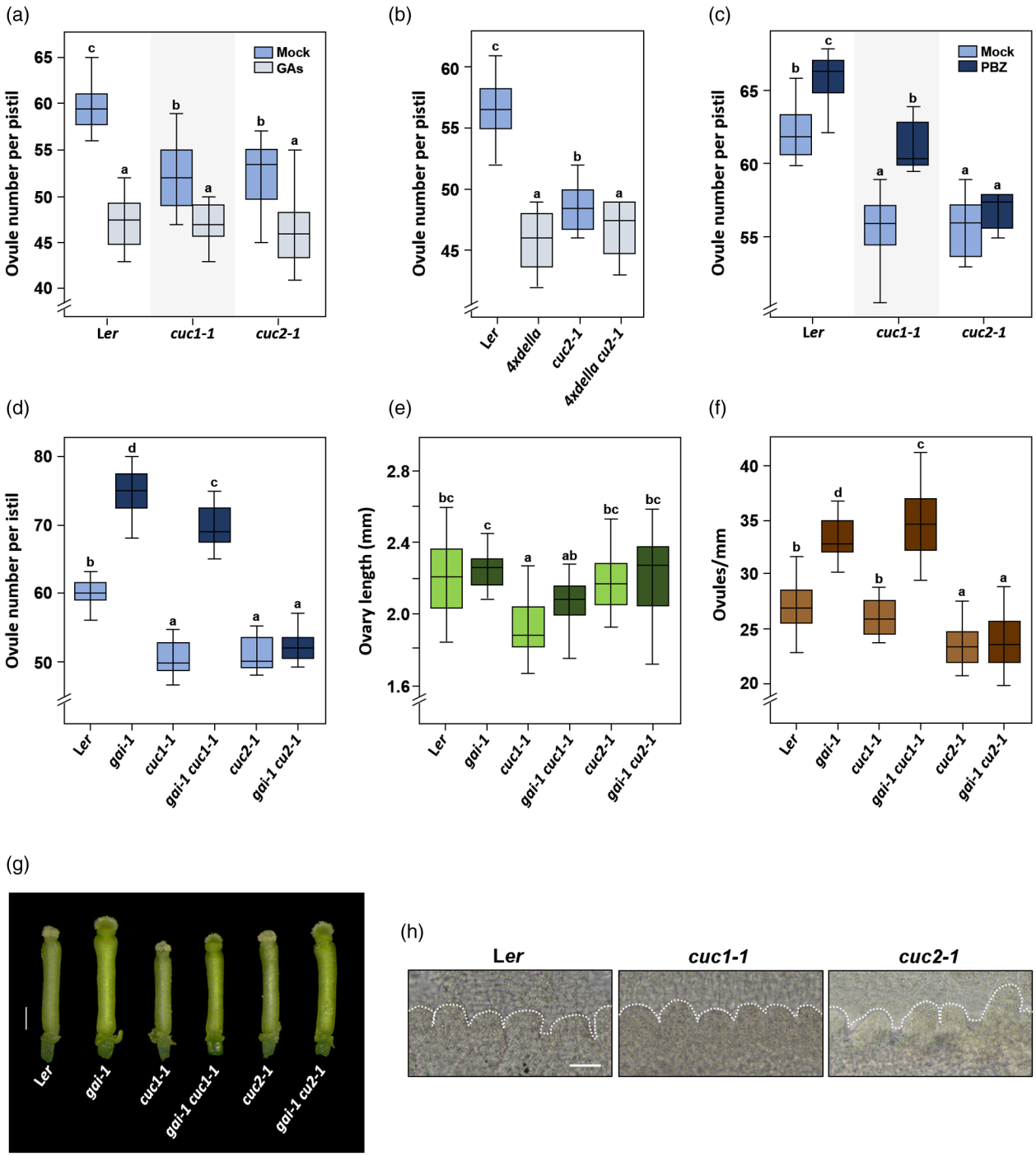

Figure 1. CUC2 but not CUC1 was required for GAI-mediated ovule initiation.
(a) Ovule number per pistil in L*er*, *cuc1-1*, and *cuc2-1* upon treatment with mock or 20 μM of GA_4+GA_7. (b) Ovule number per pistil in L*er*, *4xdella*, *cuc2-1*, and *4xdella cuc2-1*. (c) Ovule number per pistil in L*er*, *cuc1-1*, and *cuc2-1* upon treatment with mock or 1 μM PBZ. (d–g) Ovule number per pistil (d), ovary length (e), ratio ovule number to ovary length (f), and images of mature pistils (g) of L*er*, *gai-1*, *cuc1-1*, *gai-1 cuc1-1*, *cuc2-1*, and *gai-1 cuc2-1*. (h) Images of ovule primordia at stage 1-I from L*er*, *cuc1-1*, and *cuc2-1*. Data are presented as boxplots (n = 10–12 in (a)–(f)). Letters above each box indicate statistical significance as determined by analysis of variance and a Bonferroni post hoc test for multiple comparisons ($P < 0.01$). Data that are not significantly different are marked with the same letter. Scale bars represent 500 μm in (g) and 20 μm in (h).

in ovule number in wild-type plants and a 10% decrease in *cuc1-1* and *cuc2-1* mutants (Figure 1(a)) (Gomez et al., 2018). Therefore, ovule number was similar in wild-type and *cuc* single mutants upon GA treatment. Similarly, *cuc2-1* has no effect on ovule number in the *4xdella* background (Figure 1(b)).

Next, we treated wild-type, *cuc1-1*, and *cuc2-1* plants with PBZ. PBZ treatment slightly increased ovule number in wild-type plants and the *cuc1-1* mutant (Figure 1(c)). Surprisingly, PBZ did not have any effect in *cuc2-1* (Figure 1(c)), indicating that CUC2 but not CUC1 is required by DELLA activity to promote ovule primordia formation. This was confirmed when *cuc1-1* and *cuc2-1* were crossed to *gai-1*; whereas the *gai-1* mutant increases ovule number by around 20% in both wild-type and *cuc1-1* backgrounds, no significant differences in ovule number were observed in the *gai-1 cuc2-1* double mutant compared to *cuc2-1* (Figure 1(d)). These results clearly indicate that GAI activity requires the presence of CUC2 activity to promote the formation of ovule primordia, since *gai-1* was not able to increase the number of ovules in a *cuc2-1* background. It is worth mentioning that whereas *cuc2-1* suppressed the *gai-1* ovule number phenotype, it did not alleviate the overall *gai-1* plant shape phenotype (Figure S1), which is characterized by smaller and darker plants (Peng et al., 1997).

Next, we sought to find out whether the observed differences in *cuc* mutants in the presence of *gai-1* were a direct consequence of changes in ovule number rather than an indirect effect of the alteration of ovary length. Interestingly, the *cuc1-1* mutant developed slightly shorter ovaries (Figure 1(e,g)). As a consequence, the ratio of ovule number to ovary length in *cuc1-1* was not significantly different compared to the wild type, as the ovule number was reduced to a similar extent (Figure 1(f,h)). On the contrary, the length of *cuc2-1* ovaries was similar to those of the wild type (Figure 1(e,g)), which, along with a reduction in ovule number, resulted in a decrease in the ratio of ovule number to ovary length (Figure 1(f,h)). In other words, ovule density in *cuc2-1*, but not *cuc1-1*, was reduced. Finally, ovaries of the *gai-1* single mutant as well as the *gai-1 cuc* double mutant were similar to those of their corresponding control plants (L*er* for *gai-1*, *cuc1-1* for *gai-1 cuc1-1*, and *cuc2-1* for *gai-1 cuc2-1*) (Figure 1(e,g)). Therefore, the ratio was clearly higher in *gai-1* and *cuc1-1 gai-1* but not in *gai-1 cuc2-1* (Figure 1(f)). The parallel analysis of ovule number and ovary length allows us to conclude that both CUC1 and CUC2 participate in ovule number in different ways: *cuc1-1* has an indirect effect on ovule number by reducing ovary length, whereas *cuc2-1* has a direct effect on ovule number. These phenotypes were even clearer in the *gai-1 cuc2-1* mutant, where neither ovule number nor the ratio was altered compared to *cuc2-1*.

To further study the roles of CUC2 and CUC1 in ovule number determination, we counted ovules and scored ovary length in gain-of-function *pCUC1:CUC1m-GFP* and *pCUC2:CUC2m-GFP* lines. *CUC* genes are downregulated by miR164, and these constructs express miR164 cleavage-resistant versions of *CUC1* and *CUC2*, respectively (Baker et al., 2005; Sieber et al., 2007). Both lines developed more ovules per pistil, which is consistent with the positive role of CUCs in ovule initiation (Figure S2(a)). Moreover, in accordance with data from the knockout mutants, *pCUC1:CUC1m-GFP* generated longer ovaries, resulting in the same density of ovules as the wild type, whereas *pCUC2:CUC2m-GFP* did not alter ovary length, resulting in an increased density of ovules in the placenta (Figure S2(b–d)).

Finally, ovule and ovary phenotypes were also tested in higher-order *cuc* mutants in response to GAs and in combination with *gai-1*. The *cuc1-1 cuc2-1* double mutant completely lacks a shoot apical meristem and does not develop shoots and, consequently, inflorescences (Aida et al., 1997; Hibara et al., 2006). As an alternative, we used *cuc1-1 cuc2-1/+* and *cuc1-1/+ cuc2-1* sesquimutants. In *cuc1-1 cuc2-1/+*, a null *CUC1* with only one copy of *CUC2*, we observed a 20% reduction in the number of ovules (Figure S3(a)) and a 10% reduction in ovary length, which resulted in a decrease of ovule density (Figure S3(b–d)). The difference in phenotype observed between *cuc1-1 cuc2-1/+* and the single mutant *cuc1-1* could be due to an additive effect of loss-of-function of *CUC1* regulating ovary length and the loss of one copy of *CUC2*, which further reduced the number of ovules that initiate from the placenta. The *cuc2-1* mutant has been described as potentially semi-dominant (Aida et al., 1997), and, therefore, the loss of one copy of *CUC2* in *cuc2-1/+* may contribute significantly to ovule number reduction. Interestingly, just one copy of wild-type *CUC2* was sufficient to allow *gai-1* to increase the ovule number in the *cuc1-1 cuc2-1/+* background (Figure S3(a)). On the other hand, the loss of one copy of *CUC1* in the *cuc1-1/+ cuc2-1* sesquimutant reduced the ovule number by around 25% but did not affect ovary length compared to *cuc2-1* (Figure S3(a–c)). In the *cuc1-1/+ cuc2-1* background, *gai-1* was not able to increase the ovule number, due to the loss of both wild-type copies of *CUC2*, unlike the case with the single mutant *cuc2-1* (Figure S3(a–c)), which further confirms that CUC2 is an absolute requirement for GAI-dependent ovule primordia formation.

Taken together, these genetic analyses suggest that GAI requires CUC2 activity to directly regulate ovule number, whereas CUC1 indirectly contributes to ovule number determination by regulating ovary elongation, independent of GAs.

GAI interacts with CUC1 and CUC2

Interaction of CUC2 with GAI and with the other four DELLA proteins of Arabidopsis was confirmed by Y2H assays (Figure 2(a)). For this, the M5 truncated versions of DELLA, lacking the DELLA N-terminal domain that confer auto-activation in yeast when fused to the Gal4 DNA-binding domain (de Lucas et al., 2008), were used as baits. To better characterize the GAI–CUC2 interaction, protein domains necessary for protein–protein interactions were mapped (Figure S4). Several deletions of GAI (previously described in Gallego-Bartolome et al., 2012) were tested

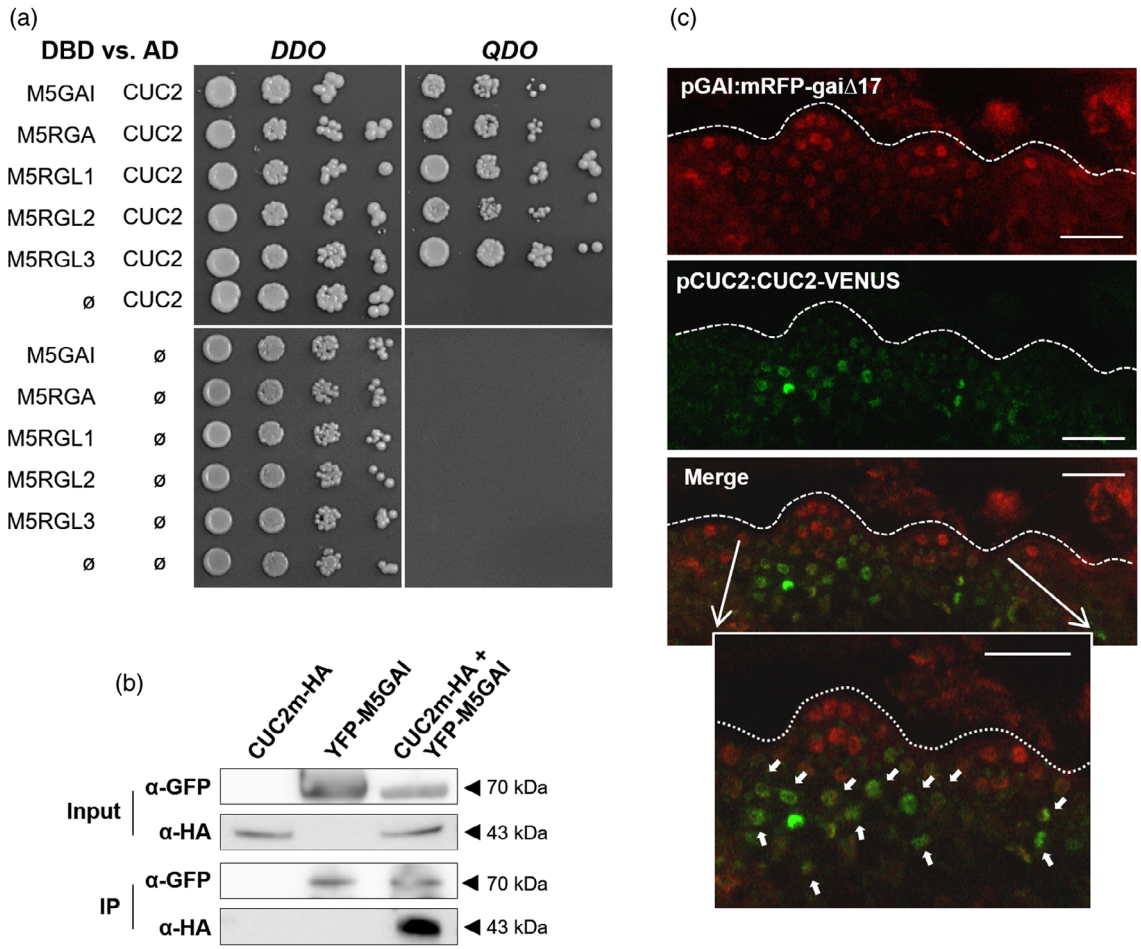

Figure 2. CUC2 binding to DELLA proteins.
(a) Y2H assay of protein interaction between CUC2 and the five DELLA proteins in Arabidopsis. DBD, DNA-binding domain; AD, activation domain; ∅, empty vector. DDO, double dropout (SD/-Leu/-Trp); QDO, quadruple dropout (SD/-Ade/-His/-Leu/-Trp). Tenfold serial dilutions were spotted onto SD media to test/quantify the interactions. M5 truncated versions of DELLA proteins (de Lucas *et al.*, 2008) were used as baits. (b) Co-IP assay of YFP-M5GAI with CUC2m-HA in *N. benthamiana*. Western blot was performed with anti-GFP and anti-HA antibodies to detect YFP-M5GAI and CUC2m-HA, respectively, in both input and IP protein samples. A miR164-resistant version of CUC2 (CUC2m) was used. (c) Co-localization assay in placental tissue of GAI (*pGAI:mRFP-gaiΔ17*) with CUC2 (*pCUC2:CUC2-VENUS*) at stage 1-I of ovule development. White arrowheads point to nuclei where both mRFP-gaiΔ17 and CUC2-VENUS were co-localized. Scale bars represent 20 μm.

against full-length CUC2. First, the DELLA regulatory domain was not involved in the interaction with CUC2, as its removal in the M5GAI version did not affect the interactions in yeast. Second, deletion of the first leucine heptad repeat (LHR1) in GAI (Hauvermale et al., 2012; Vera-Sirera et al., 2016) prevented GAI–CUC2 interaction. However, small fragments of GAI containing the LHR1 did not interact with CUC2, suggesting that the LHR1 is necessary but not sufficient for the interaction (Figure S4(a,c)). On the other hand, serial deletions of CUC2 were tested against the complete GAI protein. CUC2 is characterized by a NAC N-terminal domain divided into five sub-domains that mediate protein interactions and DNA binding (Ernst et al., 2004; Ooka et al., 2003). Serial deletions of these domains showed that elimination of the first NAC sub-domain prevented interaction with GAI, suggesting that this region is important for GAI–CUC2 interaction (Figure S4(b,d)). Finally, the N-terminus of CUC2 containing the five sub-domains is not sufficient to confer binding to GAI. Overall, deletion analysis indicated that full-length CUC2 and at least the GRAS functional domain of GAI are necessary for the formation of the GAI–CUC2 protein complex.

We tested whether CUC1 and GAI interact in a designed Y2H assay. CUC1 showed very little binding to M5GAI (compared to the M5GAI control, which showed residual auto-activation), whereas CUC2 showed strong interaction in the same assay (Figure S5(a)). CUC1–CUC2 and CUC2–CUC2 (forming homo- and hetero-dimers) were used as positive controls (Gonçalves et al., 2015; Rubio-Somoza et al., 2014) (Figure S5(a)).

In planta interaction of both CUC2 and CUC1 with M5GAI was confirmed by protein co-immunoprecipitation (Co-IP) assays in *Nicotiana benthamiana* leaves (Figure 2 (b) and Figure S5(b)). In the assay, CUC2-HA or myc-CUC1 was transiently expressed together with YFP-M5GAI. Both CUC2-HA and myc-CUC1 were co-immunoprecipitated when YFP-M5GAI was pulled down in leaf extracts from plants that were co-infiltrated.

Finally, we also wanted to check if these proteins were co-localized *in vivo* in the placenta of Arabidopsis pistils, which would be key for determining whether the GAI–CUC interaction is a fundamental mechanism for GAI mediation in ovule formation. To test this, we created a *pGAI:mRFP-gaiΔ17* reporter line, which expresses an mRFP fluorescence peptide fused to a stable version of GAI, identical to that in the *gai-1* mutant (Peng et al., 1997). GAI protein expression, monitored with the *pGAI:mRFP-gaiΔ17* line, was localized in the placental cells and primordia at stage 1-I of ovule development (Figure 2(c) and Figure S5(c)), with a similar gene expression pattern to that of the transcriptional line *pGAI:GUS* (Gomez et al., 2016). This line was crossed with *pCUC2:CUC2-VENUS* and *pCUC1:CUC1-GFP* transgenic lines (Gonçalves et al., 2015). Both mRFP-gaiΔ17 and CUC2–VENUS were observed in some nuclei in the placenta and ovule primordia boundaries (Figure 2(c)). Similarly, mRFP-gaiΔ17 and CUC1-GFP also co-localized in some cells of the placenta (Figure S5(c)). In summary, protein–protein interaction and co-localization studies of GAI, CUC1, and CUC2 strongly suggest that these proteins can form a complex in nuclei of placental cells during pistil development.

DELLA and *CUC2* are not reciprocally regulated

At this point, we could formulate a plausible working model in which GA-mediated ovule initiation may rely on the interaction of the DELLA protein GAI with the TF CUC2. To deepen this model, we checked whether DELLA proteins could also regulate *CUC2* expression and *vice versa*. Quantitative PCR (qPCR) analysis indicated that *CUC2* mRNA levels did not significantly change in *4xdella* or *gai-1* inflorescences (Figure 3(a)). In addition, re-analysis of a transcriptomic assay of pistils of *global della* (lacking all five *DELLA* genes) and *gai-1* mutants at stage 8 of floral development (Gomez et al., 2018) revealed that

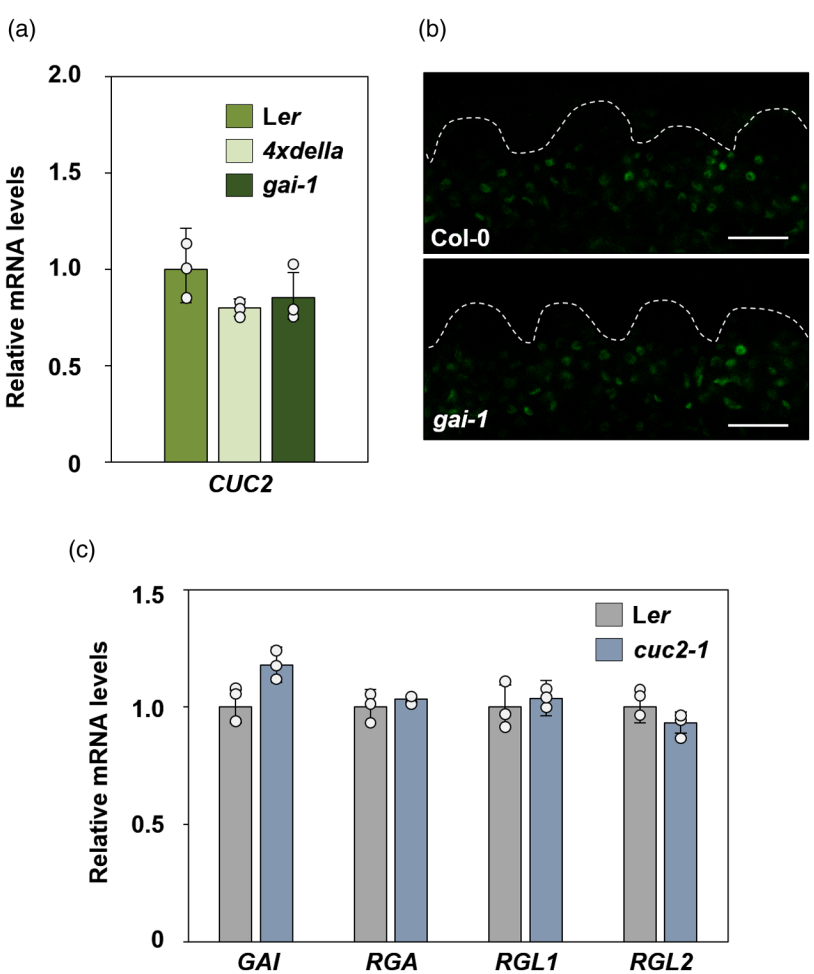

Figure 3. *CUC2* and *DELLA* genes did not transcriptionally regulate each other.
(a) Relative mRNA levels of *CUC2* in inflorescences of L*er*, *4xdella*, and *gai-1*. (b) Confocal microscopy images of ovule primordia at stage 1-I in plants *pCUC2:CUC2-VENUS* in Col 0 (upper panel) or *gai-1* (lower panel). (c) Relative mRNA levels of *GAI*, *RGA*, *RGL1*, and *RGL2* in inflorescences of L*er* and *cuc2-1*. In (a) and (c), qPCR expression analysis was carried out for inflorescences, and data were normalized to *UBQ10* (AT4G05320) in L*er*. Data are the mean ± SD of three biological replicas, and no significant differences (Student's *t*-test) were detected ($P < 0.01$). Scale bar in (b) represents 20 μm.

CUC2 was not differentially expressed. Finally, CUC2 protein levels were not altered in *pCUC2:CUC2-VENUS* reporter lines when crossed with the *gai-1* mutant (Figure 3(b)).

With a similar rationale, we also checked whether CUC2 could regulate the expression of *DELLA* genes. First, mRNA levels of the four main *DELLA* genes involved in ovule development, *GAI*, *RGA*, *RGL1*, and *RGL2* (Gomez et al., 2018, 2019, 2020), did not show significant changes in *cuc2-1* mutant inflorescences (Figure 3(c)). Second, reanalysis of data reported by Cucinotta et al. (2018) of a *cuc2-1 pSTK:CUC1-RNAi* versus wild-type transcriptomic assay revealed that *DELLA* genes were not differentially expressed. Taken together, the data indicate that *GAI* does not regulate *CUC2* expression, and *CUC2* regulates *GAI* at neither the mRNA nor the protein level in the placenta during ovule initiation.

CUC2 does not participate in BR-mediated ovule initiation

It has been shown that *CUC* genes are repressed by BRs (in a BZR1-dependent manner) during organ boundary formation in the shoot apical meristem (Gendron et al., 2012); therefore, CUC expression and boundary formation require reduced BZR1 expression. In contrast, in the placenta both BZR1 and CUC are positive factors for ovule initiation. We asked whether BZR1 may require CUC2 to promote ovule primordia development, as in GAI-dependent ovule primordia formation. To do so, the ovule number was determined in the *bzr1-1D cuc2-3* double mutant in comparison with *bzr1-1D*, *cuc2-3*, and Col-0 wild-type plants (Figure 4(a–c)). First, *cuc2-3* (Col-0 background) reduced the number of ovules by around 10%, like the *cuc2-1* mutant (L*er* background). Second, *bzr1-1D* pistils presented an increase in ovule number of around 20%, however, as previously described (Barro-Trastoy et al., 2020b; Huang et al., 2013b). More importantly, *bzr1-1D* can also increase the ovule number in the *cuc2-3* mutant similar to the situation in wild-type plants. These results clearly suggest that BZR1, and probably BRs, act independently of CUC2 in ovule initiation.

Identification of GAI–CUC2 gene targets in ovule primordia initiation

If DELLA and CUC2 form a protein complex in placental cells, CUC2 would determine the specificity of target genes by its DNA-binding domain, while the DELLA protein (GAI) would transcriptionally co-activate the complex. But what are the direct target genes of the GAI–CUC2 complex? To identify these genes, a ChIP–sequencing (ChIP-Seq) assay of GAI using the *pGAI:gaiΔ17-3xYPet* reporter line in both wild-type (L*er*) and *cuc2-1* genetic backgrounds was performed. The *pGAI:gaiΔ17-3xYPet* plants showed resemblance to the original *gai-1* mutant, indicating that the DELLA deletion in g*aiΔ17* performed as expected. Moreover, gaiΔ17-3xYPet was located in the placenta and ovule primordia cells during early pistil development (Figure S6), as in the *pGAI:mRFP-gaiΔ17* line (Figure 2(c)). The *pGAI:gaiΔ17-3xYPet* line was crossed with *cuc2-1* to allow the identification of GAI targets dependent on CUC2, that is, true GAI–CUC2 complex targets.

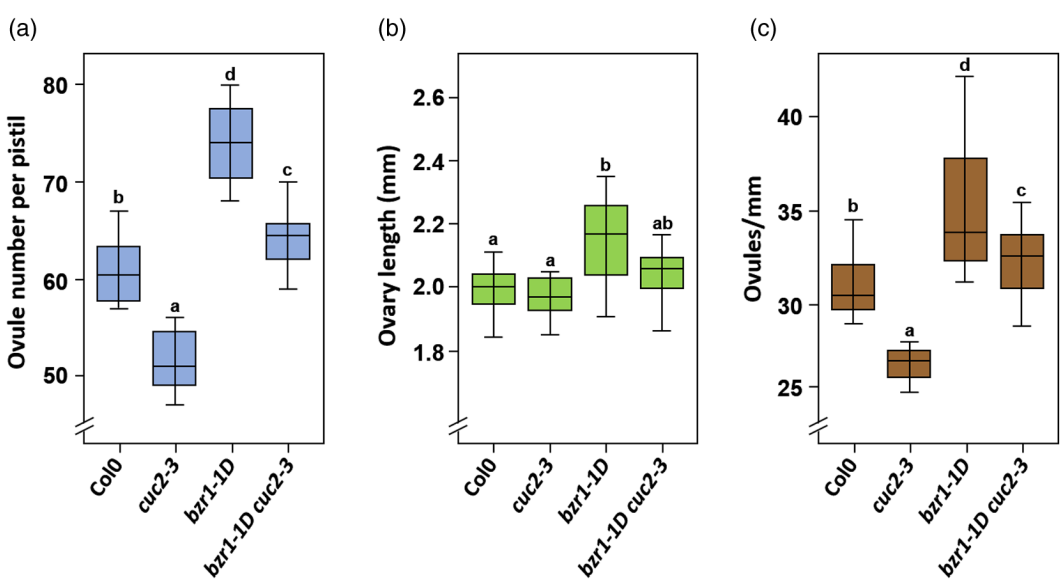

Figure 4. CUC2 did not participate in the BR pathway of ovule initiation.
(a–c) Ovule number per pistil (a), ovary length (b), and ratio of ovule number to ovary length (c) in Col-0, *cuc2-3*, *bzr1-1D*, and *bzr1-1D cuc2-3*. Data are presented as boxplots (n = 10–12). Letters above each box indicate statistical significance as determined by analysis of variance and a Bonferroni post hoc test for multiple comparisons ($P < 0.01$). Data that are not significantly different are marked with the same letter.

In a wild-type L*er* background, ChIP-Seq analyses identified 2194 loci that could be putative indirect GAI binding sites, located 1 kb upstream of the transcription start sites (TSSs) of 2087 genes (Figure S7, Tables S1.1 and S1.2). Cis-element enrichment analysis among these loci highlighted several potential binding motifs, most being canonical DELLA-interacting TF binding sites (Table S1.3), e.g., the bHLH, TCP, bZIP, and MYB families (Hernandez-Garcia et al., 2020; Marin-de la Rosa et al., 2014). Among them, the CUC binding site CG/TTG/ANNNNNNAA/CGNNA, described by the Plant Cistrome project (O'Malley et al., 2016), was also enriched. A subset of 558 GAI-associated genes that had a sequence similar to the CUC binding site was selected. Of these, 381 genes were expressed in ovule primordia, according to Matias-Hernandez et al. (2010). Several Gene Ontology (GO) terms were enriched in this group of genes (Table S1.4), including developmental processes (stamen development and regulation of meristem growth), response to stimulus (cytokinin, gibberellic acid, abscisic acid, and jasmonic acid-mediated signaling pathway), regulation of transcription (DNA-dependent), and metabolic processes (protein amino acid autophosphorylation and jasmonic acid metabolic process).

When ChIP-Seq was performed in the *cuc2-1* background, 1894 genes were identified, with 1886 being common in both wild type and *cuc2-1* and seven present only in the *cuc2-1* mutant (Figure S7(b)). More importantly, 201 out of the 2087 genes identified in the wild type were not present in *cuc2-1*, suggesting that these were CUC2-dependent GAI targets (Table S2 and Figure S7(b)). Of these, 68 genes had a sequence similar to the CUC binding site and 46 were, in addition, expressed in ovule primordia (Table 1). Enriched GO terms in these 46 genes were related to meristem and root development, negative regulation of cellular processes, and regulation of transcription (Tables S2.4 and S2.5). Among this group of genes, putative candidates were those described as participating in ovule development, especially *SEPALLATA2* (*SEP2*), *CYTOKININ OXIDASE 5* (*CKX5*), and *CYTOKININ RESPONSE FACTOR 2* (*CRF2*) (Bartrina et al., 2011; Brambilla et al., 2007; Cucinotta et al., 2016; Favaro et al., 2003).

To confirm that selected genes were indeed direct targets of GAI in a CUC2-dependent manner, we performed ChIP-qPCR analysis to amplify chromatin derived from immunoprecipitation of L*er* and *cuc2-1*. *SCARECROW LIKE 3* (*SCL3*), a well-described direct DELLA target (Yoshida et al., 2014), was used as a control. As shown in Figure 5, *CKX5*, *CRF2*, and *SEP2* displayed lower amplification in *cuc2-1* than in the L*er* background, strongly suggesting that *CKX5*, *CRF2*, and *SEP2* are truly CUC2-dependent GAI targets. On the contrary, *SCL3* displayed high enrichment in both *cuc2-1* and L*er* backgrounds. Taken together, these results support the hypothesis that the DELLA-dependent mechanism regulating ovule primordia formation may reside in the direct DELLA–CUC2 complex, regulating specific target genes, *CKX5*, *CRF2*, and *SEP2* being possible *bona fide* candidates.

DISCUSSION

CUC1 and CUC2 interactions with GAI have different biological significances

The Y2H and Co-IP data in *N. benthamiana* and the co-localization of expression in placental tissue strongly suggest that both CUC1 and CUC2 could interact *in vivo* with GAI in placental tissue. In contrast, genetic analysis of *cuc* mutants reveals that these GAI–CUC interactions may have very different biological significances. On the one hand, CUC2 but not CUC1 would require GAI to promote the formation of ovule primordia; elimination of CUC2 in *cuc2-1* prevents the increase in ovule number by GAI or upon PBZ treatment. On the other hand, CUC1 seems to be involved in pistil elongation, but in this case the effects of CUC1 would not be mediated by GAI. Therefore, most probably the GAI–CUC1 interaction in the placenta is not related to the role of CUC1 in ovary growth. For example, in the *cuc1-1* mutant, ovary length is reduced irrespective of the circumstance tested, such as the presence of *gai-1* or treatment with PBZ and GA. We conclude that the GAI–CUC1 interaction is unlikely to be significant in the control of ovule initiation in a *CUC2* background. More experiments should be designed to elucidate the biological significance of such interaction *in planta*.

CUC and GAs/BRs in ovule initiation

BRs are positive regulators of ovule number; *bzr1-1D* (a dominant mutant of the positive BR signaling regulator BZR1) and *det2-1* (mutant deficient in BR biosynthesis) produce more and fewer ovules, respectively (Huang et al., 2013b). In addition, the role of BRs in ovule number determination is independent of GAs (Barro-Trastoy et al., 2020b). GAs and BRs can negatively and positively modify ovule number in BR and GA signaling mutants, respectively. Moreover, the *gai-1* mutation had an additive effect on ovule number when combined with *bzr1-1D* (Barro-Trastoy et al., 2020). The genetic evidence provided here clearly indicates that GAI but not BZR1 requires CUC2 to promote ovule primordia initiation, which is concordant with both hormones acting independently in Arabidopsis pistils.

Interestingly, BRs are also known to play a role in organ boundary formation in the shoot apical meristem by repressing organ boundary genes like *CUC1*, *CUC2*, *CUC3*, and *LOF1* in a BZR1-dependent manner (Gendron et al., 2012). Moreover, reduced *BZR1* expression in the boundary cells is required for normal *CUC* gene expression and boundary formation. In fact, *bzr1-1D* exhibited similar fusion organ phenotypes as *cuc2-3* and *cuc2-3 cuc3-105* mutants, like fusion of cotyledons, fusion of the cauline

Table 1 Genes identified as GAI binding in a CUC2-dependent manner[a]

AGI code	Name	Annotation	Distance To TSS (bp)	Fold change Rep-1[b]	$-\text{Log}_{10}$ *q*-value Rep-1[b]	Fold change Rep-2[b]	$-\text{Log}_{10}$ *q*-value Rep-2[b]
AT1G01110	IQD18	IQ-DOMAIN 18	−242	3.52	19.40	2.69	21.32
AT1G16720	HCF173	HIGH CHLOROPHYLL FLUORESCENCE PHENOTYPE 173	−208	2.50	8.24	1.79	7.54
AT1G19310		RING/U-box superfamily protein	−56	2.67	9.03	2.11	12.45
AT1G21590		Kinase protein	−445	2.31	5.93	3.13	29.96
AT1G33420		RING/FYVE/PHD zinc finger superfamily protein	−62	2.79	11.56	2.09	9.81
AT1G62870		Hypothetical protein	−243	5.15	39.58	4.41	69.41
AT1G63010	VPT1	VACUOLAR PHOSPHATE TRANSPORTER 1	−790	2.25	5.32	2.90	21.74
AT1G64620	DOF1.8	Plant-specific Dof transcription factor	−463	3.10	12.64	2.37	11.87
AT1G69260	AFP1	ABI FIVE BINDING PROTEIN 1	−349	2.28	5.26	2.23	10.67
AT1G69600	ZFHD1	ZINC FINGER HOMEODOMAIN 1	−427	3.68	19.16	3.39	33.04
AT1G74430	MYB95	MYB DOMAIN PROTEIN 95	−802	2.63	8.47	2.55	14.64
AT1G75450	CKX5	CYTOKININ OXIDASE 5	−749	2.91	10.68	2.12	8.30
AT1G79420	BDR2	BOUNDARY OF ROP DOMAIN2	−646	3.02	11.92	2.34	12.46
AT1G80360	VAS1	REVERSAL OF SAV3 PHENOTYPE 1	−944	1.85	3.31	2.43	15.17
AT2G22840	GRF1	GROWTH-REGULATING FACTOR 1	−269	2.28	5.16	2.97	22.21
AT2G24360	STYK	SERINE/THREONINE/TYROSINE PROTEIN KINASE	−124	3.45	18.91	3.02	30.66
AT2G26520		Transmembrane protein	−61	1.66	2.09	2.92	25.49
AT2G28085	SAUR42	SMALL AUXIN UPREGULATED RNA 42	−899	3.20	13.67	3.22	27.17
AT2G35310	REM23	REPRODUCTIVE MERISTEM 23	−123	3.00	11.82	2.32	11.11
AT2G41550		Rho termination factor	−73	2.54	8.60	1.73	6.71
AT2G47460	MYB12	MYB DOMAIN PROTEIN 12	−953	2.71	8.84	3.49	36.66
AT3G02010		PPR superfamily protein	−632	3.29	14.69	2.90	22.75
AT3G02310	SEP2	SEPALLATA 2	−728	2.19	6.41	2.22	13.15
AT3G03450	RGL2	RGA-LIKE 2	−103	2.49	7.10	2.40	15.12
AT3G07760	WEEP	WEEP	−2	2.86	12.09	2.32	16.96
AT3G12440		Polynucleotidyl transferase, ribonuclease H-like superfamily	−842	2.41	7.10	2.34	14.66
AT3G19930	STP4	SUGAR TRANSPORTER 4	−768	2.57	7.68	2.17	9.44
AT3G22750		Protein kinase superfamily protein	−571	3.39	15.79	4.62	60.50
AT3G24050	GATA1	GATA TRANSCRIPTION FACTOR 1	−255	2.69	8.67	2.58	18.04
AT3G53540	TRM19	TON1 RECRUITING MOTIF 19	−101	3.03	12.01	2.83	21.54
AT3G56370	IRK	INFLORESCENCE AND ROOT APICES RECEPTOR KINASE	−156	2.34	6.32	3.07	28.62
AT3G61260	REM1.2	REMORIN 1.2	−374	2.64	9.29	1.97	8.45
AT3G62000		SAM-dependent methyltransferases superfamily protein	−971	3.18	13.93	2.50	15.37
AT4G00150	HAM3	HAIRY MERISTEM 3	−842	2.62	7.96	2.17	8.98
AT4G13160		zein-binding protein	−43	2.81	9.75	2.72	17.62
AT4G14310		Transducin/WD40 repeat-like superfamily protein	−256	2.34	6.58	1.82	10.19
AT4G15620	CASPL1E2	CASP-LIKE PROTEIN 1E2	−119	2.26	5.28	2.57	17.49
AT4G18890	BEH3	BES1/BZR1 HOMOLOG 3	−561	2.43	6.73	2.62	19.74
AT4G23750	CRF2	CYTOKININ RESPONSE FACTOR 2	−965	2.05	4.48	2.71	22.13
AT4G27830	BGLU10	BETA GLUCOSIDASE 10	−102	2.00	5.06	2.47	19.64
AT4G29040	RPT2a	REGULATORY PARTICLE AAA-ATPASE 2A	−321	2.41	6.76	2.17	9.65
AT4G34640	SQS1	SQUALENE SYNTHASE 1	−37	3.07	14.19	2.57	20.15
AT5G11970		ABC family ABC transporter	−912	2.29	5.57	2.25	13.36
AT5G13240	MAF1	Global repressor of RNA polymerase III	−873	2.60	8.75	2.27	10.39
AT5G49230	HRB1	HYPERSENSITIVE TO RED AND BLUE	−97	2.56	8.32	2.31	15.70
AT5G53130	CNGC1	CYCLIC NUCLEOTIDE GATED CHANNEL 1	−25	2.71	8.84	2.10	7.97

[a]Indirect GAI-binding genes expressed in ovules, according to Matias-Hernandez et al. (2010).
[b]Rep-1, biological replica #1; Rep-2, biological replica #2.

leaves to the main stem, and fusion of stamens (Gendron et al., 2012). In this case, the action of BRs (by BZR1 activity) is to counteract CUC activity in the boundary regions. However, it seems that both *CUC* genes and BRs act as positive regulators of ovule number. Whereas *bzr1-1D* or dominant *pCUC2:CUC2m-GFP* produce more ovules (Barro-Trastoy et al., 2020b; Huang et al., 2013b), the loss of *CUC2* or *DET2* (of BR biosynthesis) results in the production of fewer ovules (Barro-Trastoy et al., 2020b; Galbiati et al., 2013; Huang et al., 2013b; Ishida et al., 2000; this work). This discrepancy in the regulation by BRs and *CUC* genes points to the same molecular components acting

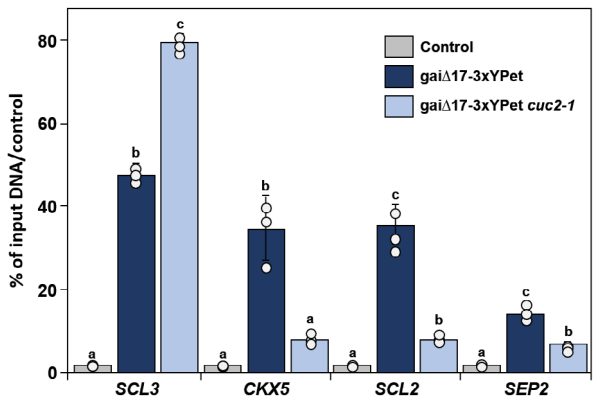

Figure 5. ChIP-PCR analysis of genes that are potentially indirectly bound by GAI in a CUC2-dependent manner.
qPCR amplification of promoter regions of *SCL3*, *CKX5*, *CRF2*, and *SEP2* in immunoprecipitated chromatin using anti-GFP from inflorescences of control L*er*, *pGAI:gaiΔ17-3xYPet*, and *pGAI:gaiΔ17-3xYPet cuc2-1* plants. Data were normalized using the Percent Input Method. Letters above each box indicate statistical significance as determined by analysis of variance and a Bonferroni post hoc test for multiple comparisons ($P < 0.01$). Data that are not significantly different are marked with the same letter.

through differential molecular mechanisms in these two developmental processes.

Working model of DELLA regulation of ovule primordia initiation

GAs are negative regulators in the formation of ovules, probably through the GA-mediated degradation of DELLA proteins (Gomez et al., 2018). Therefore, DELLA activity is a positive factor for ovule primordia emergence. In addition, in Arabidopsis DELLA activity does not alter auxin or BR pathways of ovule initiation, and DELLA seems to act independently of ANT in the placenta (Barro-Trastoy et al., 2020b; Gomez et al., 2018). On the other hand, DELLA proteins are transcriptional regulators that bind to different TFs, which provide the specificity for DELLA actions in plant growth and development (Hernandez-Garcia et al., 2020; Vera-Sirera et al., 2016). Based on the data presented here, as well as our previous data, a working model that includes DELLA proteins in the gene network that participates in ovule primordia determination can be proposed (Figure 6). The data conclusively point to CUC2 as the DELLA protein interactor that mediates the role of DELLA in ovule primordia determination. Genetic and molecular analyses indicated that CUC2 is required for GAI and probably other DELLA proteins to promote the formation of more ovules. In this case, DELLA acts as a co-activator of CUC2, which would provide the specificity to regulate a subset of genes that are responsible for ovule initiation. Therefore, genes regulated by the DELLA–CUC2 complex should contain the canonical cis-regulatory element of CUC in their promoter regions (O'Malley et al., 2016).

Among specific genes identified by genomic analyses, those differentially regulated by CUC2 provide good candidates to finally mediate ovule initiation. Future analysis of such genes with a specific role in ovule initiation may provide biotech tools to modify ovule and seed production by avoiding the detrimental effects on plant development that occur when altering higher-hierarchy regulatory genes, such as those from the *DELLA* or *CUC* families. *CUC2* is a gene described as a determinant of the boundary regions; therefore, a DELLA–CUC2 complex should determine, through the transcriptional regulation of target genes, the boundary domain between ovule primordia to limit cell proliferation therein. The higher DELLA–CUC2 activity in *gai-1*, in *pCUC2:CUC2m-GFP*, or upon PBZ treatment should promote the formation of more boundaries, which ultimately results in more ovule primordia (Figure 6(a)). On the contrary, null *DELLA* or *CUC* mutants (*4xdella* or *cuc2-1*, respectively, and other mutant combinations) and GA treatment result in absence of the complex activity and hence reduced ovule number (Figure 6(b–d)).

GAI–CUC2 targets ovule primordia determination genes

At the molecular level, GAI protein is recruited by CUC2 to the promoters of target genes, where it acts as a transcriptional coactivator. ChIP-Seq analysis points to several candidate target genes regulated by GAI in a CUC2-dependent manner. Some of these have been described to have a role in ovule initiation, such as *CKX5*, *CRF2*, and *SEP2*, providing a clue about the downstream molecular events controlled by the DELLA–CUC2 complex. *CKX5* encodes an enzyme that catalyzes irreversible degradation of CKs. It was noted that the double loss of *CKX5* and *CKX3* induces a drastic increase in ovule number (Bartrina et al., 2011). For its part, the *crf2 crf3 crf6* triple mutant, which is less sensitive to CKs, presented a reduced ovule number (Cucinotta et al., 2016). The DELLA–CUC2 complex may transcriptionally regulate these two CK-related genes in a different manner to finally promote ovule initiation.

Another candidate gene is *SEP2*, belonging to the SEP MADS-box protein family (including SEP1 to 4) that are needed to properly establish ovule identity (Brambilla et al., 2007; Favaro et al., 2003). *SEP2*, together with another MADS-box gene, *AGAMOUS* (*AG*), defines carpel identity in the fourth whorl of the floral meristem (Thomson and Wellmer, 2019). Lack of both activities in an *ag ap2* double mutant produced aberrant flowers with ectopic carpelloid structures instead of sepals that could also develop ectopic ovules; some of them converted into carpelloid structures themselves (Pinyopich et al., 2003).

RGL2 was also identified as a putative CUC2-dependent GAI target by ChIP-Seq analysis. It has been reported that DELLA proteins can regulate the expression of other DELLA genes in Arabidopsis (Gallego-Bartolome et al., 2011). In addition, RGL2 has a role in the control of ovule

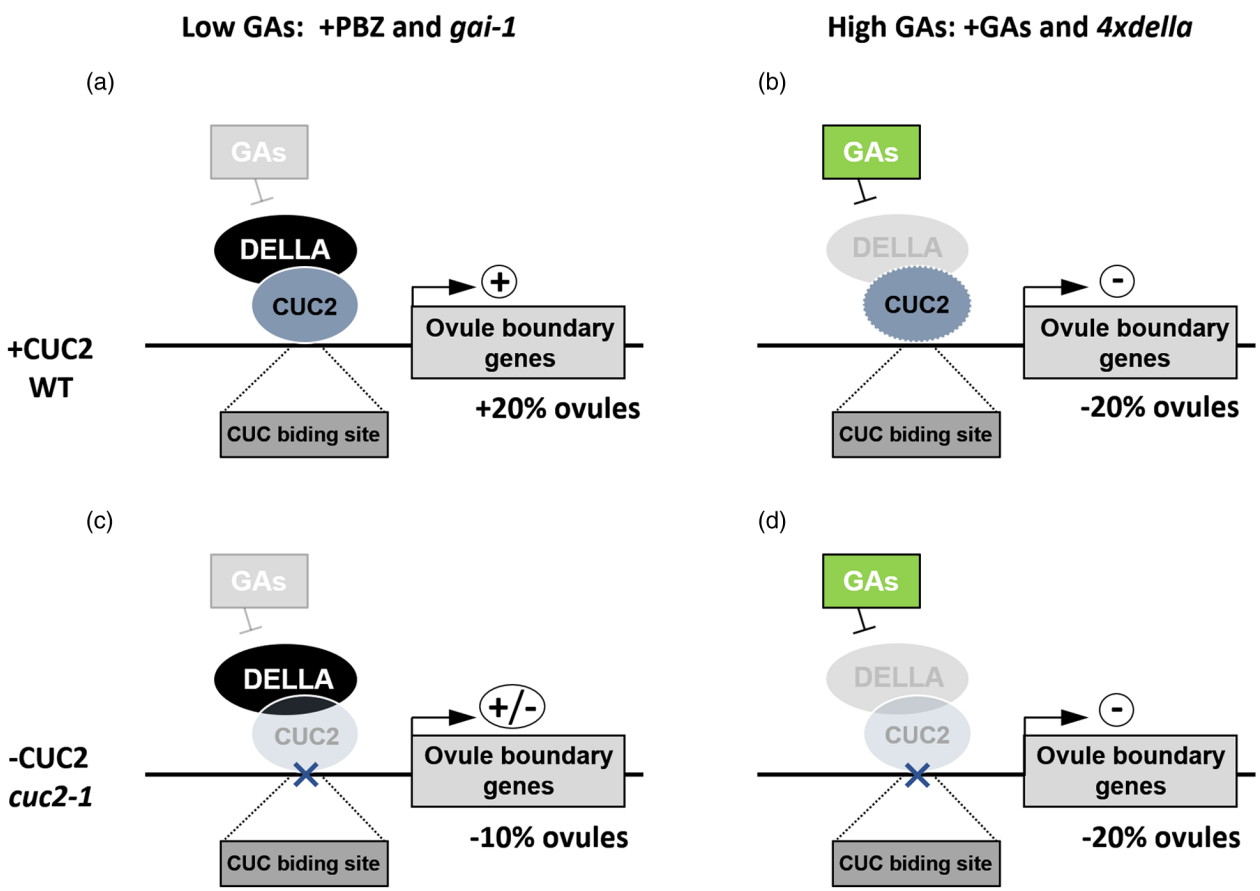

Figure 6. Proposed working model of how GAI–CUC2 mediates ovule number in Arabidopsis.
(a) When GA levels are low, in either PBZ-treated or *gai-1* mutant plants, DELLA protein levels are high, which favors the formation of the DELLA–CUC2 complex, which regulates the expression of target genes involved in ovule initiation, promoting an increase in ovule number by 20%. (b) When GA levels are high, upon GA treatment or in the *4xdella* mutant, DELLA protein levels are low and the DELLA–CUC2 complex is not formed, resulting in a 20% reduction of ovules. (c–d) In contrast, in the *cuc2-1* mutant background, the DELLA–CUC2 complex is not formed either in the presence of high (c) or low (d) DELLA protein levels, and ovule number is reduced consequently.

number (Gomez et al., 2019). Therefore, it could be plausible to foresee a mechanism of GAI regulating *RGL2* during ovule initiation. Experimental analysis of this hypothesis, as well as the possible role of other DELLA proteins, will provide further details on the complex mechanism governing ovule initiation. Furthermore, the three DELLA proteins GAI, RGA, and RGL2 play a significant role in ovule number and show expression in ovule primordia at early stages of development (Gomez et al., 2018). Elucidation whether RGA and RGL2 also participate in ovule number determination by their interaction with CUC2 requires further analysis.

Other genes that have been described to play a role in ovule development are *REPRODUCTIVE MERISTEM* 23 (*REM23*) (Mantegazza et al., 2014), *GROWTH-REGULATING FACTOR* 1 (*GRF1*), and other TF-encoding genes in the major families like *MYB12*, *MYB95*, *ZFD1*, *ZF family*, *GATA TF1*, and *Dof*. Interestingly, *GRF1* has been recently found to be involved in the DELLA pathway of the cold stress response (Lantzouni et al., 2020). The nine-membered GRF TF family, including *GRF1*, are DELLA interactors and also targets of DELLA-modulated transcription after exposure to cold stress. In this case, this DELLA–GRF regulatory module participates in the cold stress response by regulating a subset of target genes.

Finally, comparison of ChIP-Seq data from GAI-3xYPet and those from RGA-GFP by Serrano-Mislata et al. (2017), both performed in inflorescences, revealed a high degree of overlap. As many as 35% of genes identified with GAI were also a target of RGA, including several CUC2-dependent genes, like *RGL2*, *CRF2*, and *SEP2*. Further characterization of these and other candidates will be needed to identify those genes that are regulated by the DELLA–CUC2 complex, acting downstream of GAs in the control of ovule primordia formation.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis plants had L*er* or Col-0 backgrounds as indicated. Seeds were surface sterilized in ethanol and incubated in 1/2

Murashige and Skoog (MS) medium plates (Murashige and Skoog, 1962) for 4 days at 4°C in the dark followed by 7 days at 22°C under a long-day photoperiod (16/8 h). Seedlings were then transferred into soil (a 2:1:1 mix of peat moss, vermiculite, and perlite) and grown in a chamber at 22°C under a long-day photoperiod (16/8 h).

Dominant mutants *gai-1* (Peng et al., 1997) and *bzr1-1D* (Wang et al., 2002), loss-of-function mutants *4xdella* (*rga-t2 gai-t6 rgl2-1 rgl1-1*) (Cheng et al., 2004), *cuc1-1*, *cuc2-1* (Aida et al., 1997), and *cuc2-3* (Hibara et al., 2006), and lines *pCUC1:CUC1-GFP* (Gonçalves et al., 2015), *pCUC2:CUC2-VENUS* (Heisler et al., 2005), *pCUC1:CUC1m-GFP*, and *pCUC2:CUC2m-GFP* (Sieber et al., 2007) were previously described. All mutant combinations were generated by genetic crosses. F3 homozygous plants were selected by PCR genotyping of genomic DNA and/or antibiotic or herbicide resistance. All used primers and PCR conditions are listed in Table S3.

GA and PBZ treatments and determination of ovule number and ovary length

GA and PBZ treatments were applied by watering the plants every other day with 20 μM of GA_4+GA_7 (Duchefa Biochemie, Haarlem, Netherlands) or 1 μM PBZ (Duchefa Biochemie), respectively, starting at bolting. Stock solutions of GA and PBZ at 10 mM were prepared in absolute ethanol or acetone, respectively. Primers and chemical products were purchased from Sigma unless otherwise stated.

Ovule number was determined in pistils of flowers at stage 12 between positions 10 and 20 of the main inflorescence by hand dissection of each pistil under an SMZ-1270 Nikon stereomicroscope (Gomez et al., 2018). Ovary length was determined in each pistil from images taken with a digital camera attached to the stereomicroscope before ovule number determination using ImageJ software (Schindelin et al., 2012). At least 12 pistils were used for each genotype and treatment per assay. Pistil images were captured with a Leica MZ16 F stereomicroscope.

Histological procedures and confocal laser scanning microscopy

Ovule primordia morphology was studied using chloral hydrate clearing and differential interference contrast light microscopy according to Weigel and Glazebrook (2002). Confocal laser scanning microscopy was used to detect and image the distribution of the proteins translated from the *pCUC1:CUC1-GFP*, *pCUC2:CUC2-VENUS*, *pGAI:gaiΔ17-3xYPet*, and *pGAI:mRFP-gaiΔ17* plasmids in stably transformed lines in dissected stage 8 flowers (floral and ovule stages as described by Smyth et al., 1990 and Schneitz et al., 1995). A Zeiss LSM 780 confocal microscope was used with excitation at 488 nm and detection at 510–530 nm for proteins fused to GFP, YPet, or VENUS and excitation at 561 nm and detection at 598–640 nm for mRFP.

Yeast two-hybrid assays

A synthetic DNA fragment corresponding to the full-length *CUC2* coding sequence (CDS) was ordered from IDT (Integrated DNA Technologies, Coralville, IA, USA) and cloned into the pDONR^{TM}221 entry vector (ThermoFisher Scientific; New York, NY, USA). For DELLA–CUC2 Y2H analysis, *CUC2* CDS was then transferred into the pGADT7 prey vector (Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France) by Gateway and introduced into the yeast strain Y187 (Clontech). The M5-truncated versions of DELLA protein cloned in the pGBKT7 bait vector (Clontech) in the yeast strain Y2HGold (Clontech) (de Lucas et al., 2008) were used.

For CUC2–CUC1, CUC2–CUC2, and GAI–CUC1 interactions, synthetic full-length miR164-resistant versions of *CUC2* (*CUC2m*) and *CUC1* (*CUC1m*) CDSs, identical to those in Baker et al. (2005) and Sieber et al. (2007), were cloned into pDONR^{TM}221 and moved into pDEST22 and/or pDEST32 vectors (ThermoFisher Scientific). Deleted versions of CUC2 were amplified by PCR from the synthetic *CUC2m* CDS, cloned into the pDONR^{TM}221 entry vector, transferred into the pDEST32 prey vector (ThermoFisher Scientific), and introduced into the yeast strain Y187. Primers used for deletion and cloning are listed in Table S3. The complete *GAI* cDNA cloned into the pDEST22 bait vector and introduced into the yeast strain Y2HGold and the *GAI* deletions cloned into the pDEST32 prey vector (Gallego-Bartolome et al., 2012) in the yeast strain Y187 were obtained from D. Alabadi (IBMCP, Spain).

Y2H screening was performed via mating. Both strains containing the corresponding plasmids were mated overnight at 28°C, and diploids were selected on synthetic dextrose (SD)/-Leu/-Trp. Yeasts were then plated onto SD/-Leu/-Trp/-Ade/-His (Clontech protocol) or SD/-Leu/-Trp/-Ura/-His (ThermoFisher Scientific protocol) in 10-fold serial dilutions to test interactions. Empty vectors were used as negative controls.

Generation of constructs and transgenic plants

Both *pGAI:gaiD17-3xYPet* and *pGAI:mRFP-gaiD17* lines were generated by recombineering (Brumos et al., 2020) using the JAtY clone JAtY51J10 from the JIC (JAtY library, https://abrc.osu.edu/stocks/number/CD4-96) in the pYLTAC17 vector, which contains the *GAI* locus (AT1G14920). All procedures for removal of the DELLA domain in GAI and tagging with 3xYPet or mRFP were basically as described in Gomez et al. (2019, 2020). All primers used are listed in Table S3. Final constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 to transform L*er* Arabidopsis plants by floral dipping (Clough and Bent, 1998). Transgenic plants were selected in ammonium glufosinate and T3 homozygous lines segregating as a single locus were selected.

qPCR analyses

For qPCR assays, dissected main inflorescences were collected when the plant developed 5–10 flowers and flash-frozen in liquid nitrogen. RNA was extracted with the NucleoSpin RNA Plant kit (Macherey-Nagel, Dueren, Germany); cDNA was synthesized from 1 μg of total RNA with the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Saint-Germain-en-Laye, France); and qPCR was performed as described in Dorcey et al. (2009) in a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Sci., Waltham, MA, USA) with SYBR premix ExTaq (Tli RNaseH Plus) Rox Plus (Takara Bio Inc.). Primers for qPCR amplification are listed in Table S3. Expression levels were normalized to *UBQ10* (AT1G05320) (Czechowski et al., 2005) and analyzed by the comparative ΔΔCt method (Schmittgen and Livak, 2008) to the values in the wild type.

Co-IP assays

Construct *35S:myc-CUC1m* was prepared by transferring the *CUC1m* CDS from pDONR^{TM}221 to the pEarleyGate203 vector (Earley et al., 2006) following the Gateway method. For *35S:CUC2m-HA* preparation, a synthetic full-length *CUC2* CDS fused to an HA-tag sequence at the 3′ end was cloned into the pDONR^{TM}221 entry vector and transferred to the pEarleyGate100 vector. Both constructs were introduced in *A. tumefaciens* strain C58 and tested against *35S:YFP-M5GAI* (Blanco-Touriñan et al., 2020). The different combinations of *A. tumefaciens* C58 cells

carrying vectors and the p19 silencing suppressor were infiltrated with a solution of 10 mM MES, 10 mM $MgCl_2$, and 1 mM acetosyringone in 4-week-old *N. benthamiana* leaves for transient co-expression. After 3 days, infiltrated leaf sections were frozen in liquid nitrogen and homogenized in extraction buffer containing 25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA (pH 8), 150 mM NaCl, and 1× cOmplete EDTA-free protease inhibitor cocktail (Roche, Darmstadt, Germany).

Total proteins were quantified by Bradford assay (Bradford, 1976). An aliquot of 160 μg of total proteins was denatured in Laemmli buffer (125 mM Tris-HCl [pH 6.8], 5% SDS, 12.5% glycerol, and 0.375% bromophenol blue) and set aside to be used as input controls. An aliquot of total proteins (800 μg) was incubated with anti-GFP-coated paramagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 2 h at 4°C and loaded into μColumns (Miltenyi) for immunoprecipitation. Immunoprecipitated proteins and inputs were separated by 12% SDS-PAGE, transferred to a PVDF membrane (GE Healthcare, Chicago, IL, USA), and immunodetected with anti-HA-HRP (3F10, 1:5000; Roche), anti-c-myc (9E10, 1:1000; Roche), anti-GFP (JL8, 1:5000; Clontech), and anti-mouse IgG HRP (NXA931, 1:10000; GE, Healthcare). Chemiluminescence signals were detected with SuperSignal™ West Femto (ThermoFisher Scientific) and imaged with a LAS-3000 imager (Fujifilm, Tokyo, Japan).

ChIP, ChIP-Seq, and ChIP-PCR analyses

For the ChIP assay, 1.8 g of Arabidopsis inflorescences was collected from the main stem when it developed 5–10 flowers. Open/anthesis flowers, as well as two to three older floral buds, were removed before flash-freezing in liquid nitrogen. Two independent biological replicates for each genotype were recollected from *pGAI:gaiΔ17-3xYPet, pGAI:gaiΔ17-3xYPet cuc2-1*, and non-transgenic L*er* plants. The chromatin extraction and *in vitro* double crosslinking were performed as previously described (Milhinhos et al., 2019). The chromatin was then sonicated using a Bioruptor (Diagenode, Seraing, Belgium) for nine cycles (30 sec on/30 sec off each). A 100-μl aliquot of each sample was kept as input. For immunoprecipitation, samples were incubated with anti-GFP (ab290; Abcam, Cambridge, UK) and magnetic Dynabeads™ Protein A (ThermoFisher Scientific). Finally, chromatin was eluted, proteins were digested with Proteinase K, and DNA was purified with phenol:chloroform:isoamyl alcohol and precipitated with ethanol. Illumina Nextera library preparation and sequencing were carried out by the CRG Genomics Core Facility (Barcelona, Spain).

For ChIP-Seq analysis, reads from all different libraries were cleaned with CutAdapt and mapped to the TAIR10 Arabidopsis genome with Bowtie2 (Lagmead and Salzberg, 2012). Data were sorted and indexed with SAMtools (Li et al., 2009). MACS2 (Zhang et al., 2008) was used for peak calling and to calculate fold enrichments and *q*-values using inputs as a control to detect enriched peaks in the IPs. Peaks from two biological replicates with a false discovery rate of <0.01 were intersected using BEDTools (Quinlan and Hall, 2010), and only peaks that exhibited intersection beyond the two replicates were selected, obtaining 6554 peaks (Table S1.1). Peaks were then attributed to gene models within 1 kb upstream of the TSS using PAVIS2 (Huang et al., 2013a). ChIP-Seq data were visualized using the IGV Browser (Robinson et al., 2011). GO enrichment of the genes obtained was performed using AgriGo (Tian et al., 2017). To detect enriched sequence motifs and for *de novo* motif discovery, MEME-ChIP (http://meme-suite.org/tools/meme-chip) (Machanick and Bailey, 2011) was used in discriminative mode.

ChIP-qPCR was performed in the same manner as the qPCR analyses, using the primers listed in Table S3. Enrichments were determined using the Percent Input Method (ThemoFisher Scientific).

AUTHOR CONTRIBUTIONS

DB-T performed most experiments. MDG analyzed GAI spatial localization by confocal microscopy. NB-T participated in the ChIP analysis. DB-T, MDG, NB-T, PT, and MAP-A wrote and commented on the manuscript. MDG, PT, and MAP-A designed the study.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests in this research and the data derived from it.

DATA AVAILABILITY STATEMENT

All data and resources generated in this study will be freely available upon request. Raw sequences (fastq files) from the ChIP-Seq analysis have been deposited in the Gene Expression Omnibus (GEO) database, with accession number GSE178414 (access reviewer token ilaxiqesxlurnal at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178414).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *cuc* mutations did not alter vegetative development caused by *gai-1*.

Figure S2. Dominant CUC2 caused increases in ovule number, whereas dominant CUC1 promotes pistil elongation.

Figure S3. Ovule number of the *cuc* sesquimutant in combination with *gai-1*.

Figure S4. Serial deletions of GAI and CUC2 in Y2H binding assays.

Figure S5. Interaction of CUC1 and GAI.

Figure S6. Localization of *gaiΔ17-3xYPet* in placental cells at early stages of floral development.

Figure S7. Localization of the ChIP-Seq peaks within the corresponding mapped genes and number of genes identified.

Table S1. Analysis of ChIP-Seq data using anti-GFP on *pGAI:gaiΔ17-3xYPet* inflorescences.

Table S2. Analysis of ChIP-Seq data using anti-GFP on *pGAI:-gaiΔ17-3xYPet cuc2-1* inflorescences.

Table S3. Oligonucleotides used in this work.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data are available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178414.

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