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RESEARCH ARTICLE

Gibberellins negatively modulate ovule number in plants

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ABSTRACT

Ovule formation is a complex developmental process in plants, with a strong impact on the production of seeds. Ovule primordia initiation is controlled by a gene network, including components of the signaling pathways of auxin, brassinosteroids and cytokinins. By contrast, gibberellins (GAs) and DELLA proteins, the negative regulators of GA signaling, have never been shown to be involved in ovule initiation. Here, we provide molecular and genetic evidence that points to DELLA proteins as novel players in the determination of ovule number in *Arabidopsis* and in species of agronomic interest, such as tomato and rapeseed, adding a new layer of complexity to this important developmental process. DELLA activity correlates positively with ovule number, acting as a positive factor for ovule initiation. In addition, ectopic expression of a dominant DELLA in the placenta is sufficient to increase ovule number. The role of DELLA proteins in ovule number does not appear to be related to auxin transport or signaling in the ovule primordia. Possible crosstalk between DELLA proteins and the molecular and hormonal network controlling ovule initiation is also discussed.

KEY WORDS: Ovule, Seed, Gibberellin, Auxin, *Arabidopsis*, Tomato

INTRODUCTION

Formation of ovules during plant reproductive development is a requirement for seed production to perpetuate the species. The economic impact of seeds is irrefutable, because they are the harvest product of most crops for both human and animal foods and the production of biofuel. In fact, it is estimated that two-thirds of the calories we consume come from fruits and seeds, mainly cereal grains (FAO: The sources of food; www.fao.org/docrep/u8480e/U8480E07.htm). Seed number directly correlates with both the number of ovules and their correct morphogenesis. Therefore, understanding the factors that control ovule initiation and development is important from both an agricultural and an economic point of view.

Ovule initiation and development has been extensively studied in *Arabidopsis thaliana* (*Arabidopsis* hereafter) (Schneitz et al., 1995; Gasser et al., 1998; Skinner et al., 2004; Cucinotta et al., 2014).

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Ovules are derived from the placenta, a specialized meristematic tissue located in the inner side of the carpel margin meristem (CMM), which also gives rise to the septum and the transmitting tract. The ovule primordia emerge from the placenta as finger-like structures during stage 9 of flower development (as defined by Smyth et al., 1990). Primordia later develop into mature ovules in which three basic territories can be distinguished along the proximal-distal axis: the funiculus, which connects the ovule with the placenta; the chalaza, which gives rise to the integuments; and the nucellus, in which the embryo sac is formed. After fertilization, the embryo grows, and the integuments develop into the seed coat, participating in protection of the embryo and seed dissemination.

The number of ovules per ovary is determined by a complex gene regulatory network and hormone signaling pathways, including auxin, brassinosteroids (BRs) and cytokinins (CKs) (Bencivenga et al., 2012; Galbiati et al., 2013; Reyes-Olalde et al., 2013; Cucinotta et al., 2014). During ovule primordia initiation, auxin triggers the expression of the transcriptional regulators AINTEGUMENTA (ANT) and MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5). MP is in turn necessary for ANT, CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 activation during early phases of ovule primordia growth. ANT regulates cell proliferation in the placenta and ovule primordia, whereas CUC1 and CUC2 establish the boundaries between ovules and control PIN-FORMED 1 (PIN1, an auxin efflux carrier) expression to generate an auxin maxima occurring along the tip of the primordia, which promotes its growth (Benková et al., 2003). *ANT* is also regulated by BRs, specifically by the transcriptional factor BRASSINAZOLE RESISTANT 1 (BZR1), and by CKs, which might also act downstream of CUC proteins to promote PIN1 expression.

Gibberellins (GAs) are involved in key developmental processes throughout the life cycle of plants, from seed germination, to stem and root elongation, flowering time and fruit development (Swain and Singh, 2005; Dorcey et al., 2009; Sun, 2011; Gupta and Chakrabarty, 2013; Gallego-Giraldo et al., 2014). The DELLA regulatory proteins belong to a subfamily of the plant-specific GRAS family of transcriptional regulators that act as GA-signaling repressors (Sun, 2010). In the cell, bioactive GAs are perceived by GID1 receptors, allowing the formation of the GA-GID1-DELLA complex, which results in structural changes in DELLA proteins that trigger their recognition by, and binding with, F-box proteins, and their polyubiquitination and subsequent degradation by the 26S proteasome (Sun, 2011). The *Arabidopsis* genome encodes five DELLA proteins (GA-INSENSITIVE, GAI; REPRESSOR OF GA1-3, RGA; and three RGA-LIKE, RGL1, RGL2, and RGL3), whereas tomato or rice encode only one DELLA protein [PROCERA and SLENDER RICE 1 (SLR1), respectively]. Complete loss of function of DELLA activity causes a constitutive GA response, whereas mutant proteins lacking the N-terminal DELLA regulatory domain, such as in the *gai-1* allele or the *pRGA:GFP-rgaΔ17* line of *Arabidopsis* (Peng et al., 1997; Dill et al., 2001), cannot be degraded

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by the GA-GID1 complex, resulting in constitutive DELLA activity and blockage of the GA-mediated gene expression response. DELLA proteins lack a canonical DNA-binding domain and, thus, mediate transcriptional regulation of target genes involved in the GA response throughout the direct physical interaction with transcriptional factors (TFs) and other regulatory proteins (Vera-Sirera et al., 2015; Daviere and Achard, 2016). The list of potential DELLA interactors has increased rapidly over the past few years, providing direct evidence of the mechanism of crosstalk between GAs and other hormones and environmental clues to modulate plant growth (Marin-de la Rosa et al., 2014, 2015; Daviere and Achard, 2016).

Previous studies in *Arabidopsis* and tomato have shown that the lack of DELLA activity by either mutation or GA treatment leads to a decrease in seed number (Dorcey et al., 2009; Fuentes et al., 2012; Carrera et al., 2012). Although there was no evidence involving GAs in ovule development, we recently described a key role of GAs in the control of ovule integument formation (Gomez et al., 2016). DELLA proteins positively regulate integument development in a molecular mechanism that involves the KANADI transcriptional factor ATS (McAbee et al., 2006). ATS and DELLAs bind to each other to allow proper growth of the integument cell layers and, thus, correct ovule development.

Identification and characterization of the elements involved in ovule initiation and development is essential, not only because of the economic implications for plant breeding, but also for a basic understanding of plant development. This is particularly important because ovules are the organs in angiosperm flowers that can be traced back farthest in time, to almost 400 million years ago (Endress, 2011). Here, we provide molecular and genetic evidence that supports the key role of GAs in the determination of ovule and seed number in the reference plant *Arabidopsis* as well as in species of agronomic interest, such as tomato and rapeseed. This evidence is further supported by the ectopic misexpression of a dominant DELLA in the placenta that led to an increase in ovule number. Our data indicate for the first time that DELLA activity in the placenta is important for the correct determination of ovule number during gynoecium development. In addition, our data argue in favor of a scenario in which the role of GAs in ovule number is not related to auxin transport or signaling in the ovule primordia. Possible crosstalk between DELLA proteins and the molecular and hormonal network controlling ovule initiation is also discussed.

RESULTS
DELLA proteins participate in the formation of ovule primordia

The *global della* mutant of *Arabidopsis* (*gaiT6 rgaT2 rgl1-1 rgl2-1 rgl3-1*; *global* thereafter) lacking any DELLA activity, produces fruits with fewer seeds than those in L*er* wild-type (WT) plants (Dorcey et al., 2009; Fuentes et al., 2012). In addition, the *global* mutant produces fertile seeds with altered shape and size resulting from limited integument growth during ovule development (Gomez et al., 2016). Nevertheless, the role of DELLA proteins in integument development do not fully account for the reduced seed number in the *global* mutant. To determine whether the reduced seed number results from reduced ovule number, the number of ovules per ovary in GA-signaling mutants was scored and it was observed that the GA-signaling state affected ovule number (Fig. 1A and Fig. S1A). The *global* mutant produced fewer ovules compared with WT plants. A similar reduction was observed in the quadruple *gaiT6 rgaT2 rgl1-1 rgl2-1* (*quadruple* thereafter) and in the triple *gaiT6 rgaT2 rgl2-1* mutants, which suggests that RGL3 and RGL1 do not have an important role in ovule initiation. In addition, pistils

of *gaiT6 rgl1-1 rgl2-1* had the lower reduction in ovule number, whereas *rgaT2 rgl1-1 rgl2-1* and *gaiT6 rgaT2 rgl1-1* showed an intermediate reduction (Fig. S1A). Based on this phenotypic observations, the quantitative effect of each DELLA protein in ovule number could be represented as RGA>GAI/RGL2>RGL1/RGL3, with RGA being the major player, GAI and RGL2 being intermediate players, and RGL1 and RGL3 with little or no role. Reduced ovule number was also observed in GA-treated plants, which largely recapitulated the null *della* mutant phenotype (Fig. 1B). By contrast, the gain-of-function DELLA mutant *gai-1* produced a significant increase in the number of ovules (Fig. 1A). In line with these observations, the double *gid1a gid1b* mutant, which lacks GA perception in ovules (Gallego-Giraldo et al., 2014), showed a higher ovule number (Fig. 1A). The single *gid1a* or *gid1b* mutants also produced a slight increase in ovule number compared with WT plants (Fig. S1B). In summary, our observations evidenced a close positive correlation between DELLA activity and ovule number per pistil.

Interestingly, ovule number varied throughout inflorescence development. Whereas flowers located at position 5 to 25 at the main inflorescence (from bottom to top) of L*er* plants had a relatively invariable ovule number (around 65 ovules per pistil), flowers that developed later had pistils with a reduced ovule number (Fig. S2). In the *quadruple* mutant, a similar trend was observed. Nevertheless, and more importantly, differences in ovule number between L*er* and the *quadruple* mutant remained constant independently of the flower position along the inflorescence.

To determine whether the observed change in ovule number was a consequence of changes in the length of the placenta, ovule length in genetic backgrounds with altered DELLA activity was scored. The *quadruple* and triple *gaiT6 rgaT2 rgl2-1* mutants developed shorter ovaries, but the reduction in ovary length was less dramatic than the decrease in ovule number (Fig. 1A). Consequently, the ratio of ovule number to ovary length slightly decreased, indicating that ovule density in the developing pistil was reduced (Fig. 1A and C). By contrast, *gai-1* ovaries were slightly longer compared with WT, but produced significantly more ovules, resulting in an increased ratio (Fig. 1A). Ovary length in the *gid1a gid1b* double mutant was also measured and was almost identical to that of WT (Fig. 1A). As a consequence, the ratio of ovule number to ovary length in the *gid1a gid1b* was increased (Fig. 1A,C). Thus, in neither *gai-1*, null *della*, nor *gid1a gid1b* mutants can changes in ovule number be explained by changes in ovary length. These data suggest that the role of GAs in ovule number does not involve changes to the placenta length.

Next, we sought to determine whether seed number correlates with ovule number. To rule out the possibility that mutant pollen has an effect on fertility, WT pollen was used to pollinate mutant flowers. As observed in Fig. 1A, in the *quadruple* and triple *gaiT6 rgaT2 rgl2-1* mutants, there was a reduction in seed number that was proportional to the reduction in ovule number, pointing to ovule number as the limiting factor for fertility in these genetic backgrounds. By contrast, despite developing pistils with a higher ovule number, *gai-1* and *gid1a gid1b* mutants produced fruits with a reduced seed number. In the case of the *gid1a gid1b* mutant, many ovules did not develop into seed (50% seed-set in *gid1a gid1b* mutants compared with 90% seed-set in WT), reflecting defects in either fertilization or early zygote development (Fig. S3A). This phenotype was further studied using the pollen tube-specific marker LAT52 fused to β-glucuronidase (GUS) (*pLAT52:GUS*) (Fig. S3B). In the mutant *gid1a gid1b* pistils, LAT52 pollen tubes failed to fertilize several ovules, which suggests maternal defects in fertilization. In fact, an analysis of the *gid1a gid1b* female gametophyte at the terminal development stage (FG7,
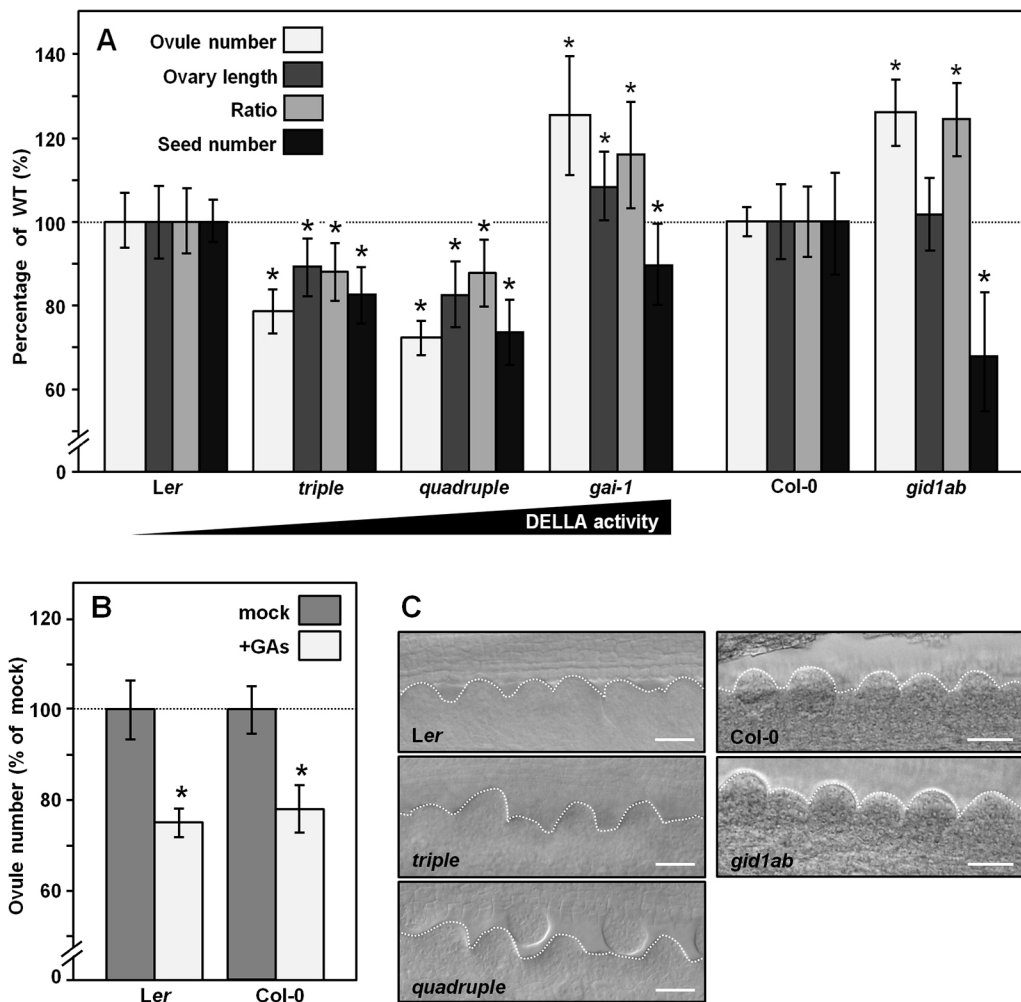
Fig. 1. GAs negatively regulate ovule number. (A) Ovule number per pistil, ovary length at anthesis, ratio of ovule number to ovary length, and seed number in mutants with different DELLA activity: triple *gaiT6 rgaT2 rgl2-1* (*triple*), quadruple *gaiT6 rgaT2 rgl2-1 rgl1-1* (*quadruple*), *gai-1*, and the double *gid1a gid1b* (*gid1ab*) mutants, and in the corresponding WT plants (L*er* or Col-0). Levels of DELLA activity are shown on a black scale below the plot. (B) GA treatment (2×10^{-5} M GA$_4$+GA$_7$) in L*er* or Col-0 plants promotes the reduction of ovule number per pistil. In A and B, more than 15 pistils were measured per mutant or treatment, and the experiments were repeated three times, with similar results. Data represent mean±s.d. Significant differences (Student's *t*-test) with the corresponding WT (L*er* or Col-0) are indicated by an asterisk (*$P<0.01$). Values were normalized to those in the WT. (C) Ovule primordia at stage 1-II of L*er* and Col-0 and in the *triple*, *quadruple* and *gid1ab* mutants. Scale bars: 25 µm.

according to Christensen et al., 1997) revealed that the polar nuclei remained unfused in ~40% of *gid1a gid1b* ovules, unlike the WT, where ovules have fused polar nuclei in a single secondary nucleus (Fig. S3C). In *Arabidopsis*, polar nuclei of the central cell fuse either before or upon fertilization, and several mutants that exhibit unfused polar nuclei at the time of pollination have defects in pollen tube guidance (Yadegari and Drews, 2004).

Misexpression of *rgaΔ17* in placenta increases ovule number

Ovule primordia arise from the placenta during gynoecium development. To test whether the ectopic expression of a DELLA in the placenta is sufficient to increase ovule number, the stable DELLA mutant protein rgaΔ17 was expressed under the control of the *ANT* promoter in the placenta and ovule primordia during early stages of pistil development (Elliott et al., 1996; Balasubramanian and Schneitz, 2000) (Fig. 2). The pOP/LhG4 *trans*-activation approach was used (Moore et al., 2006; Ripoll et al., 2015) with the activator line *pANT:LhG4* and the effector lines *GUS:pOP:GFP* and *GUS:pOP:GFP-rgaΔ17* (Fig. S4). In the pistils of *pANT: LhG4×GUS:pOP:GFP-rgaΔ17* (GUS≪pANT≫GFP-rgaΔ17) F1 plants, ovule number was significantly increased compared with the control cross between *pANT:LhG4* and *GUS:pOP:GFP* (GUS≪pANT≫GFP) or with the parental plants (Fig. 2A). *GUS* expression pattern in GUS≪pANT≫GFP-rgaΔ17 plants confirmed the restricted expression of *rgaΔ17* in the placenta and in ovules during early stages of development (Fig. 2B). These data indicate

that *rgaΔ17* misexpressed in the *ANT* promoter activity domains was sufficient to increase the number of ovules.

In contrast to *gai-1*, the dominant mutant *rgaΔ17* (in the *pRGA: GFP-rgaΔ17* line) produced pistils with fewer ovules (Fig. S1C). A plausible explanation for this is that the stabilization of RGA in nearly all tissues within the pistil (Gallego-Giraldo et al., 2014; Gomez et al., 2016) results in pleiotropic defects in gynoecium development, which is likely to mask its specific effect in ovule initiation.

DELLA proteins also control ovule number in other plant species

To determine whether the role of GA signaling is only circumscribed to *Arabidopsis*, the interaction between GA and ovule number in additional species was analyzed. In the crucifer rapeseed (*Brassica napus* L.), a close relative of *Arabidopsis*, ovule number was significantly reduced upon GA treatment in a dose-dependent manner (Fig. 3A). The *procera* mutant of the solanaceous tomato (*Solanum lycopersicum* L. cv. MicroTom) (MT), which lacks the only DELLA protein in the tomato genome, showed a reduced number of ovules (Fig. 3B). Ovule number was reduced to a similar extent in the GA20ox transgenic tomato line L4 (García-Hurtado et al., 2012), which constitutively expresses a GA20 oxidase of GA biosynthesis and, therefore, accumulates high GA levels. Similar to *procera* and the L4 line, continuous GA treatment of MT plants also reduced ovule number. Overall, our data revealed that GAs have an important
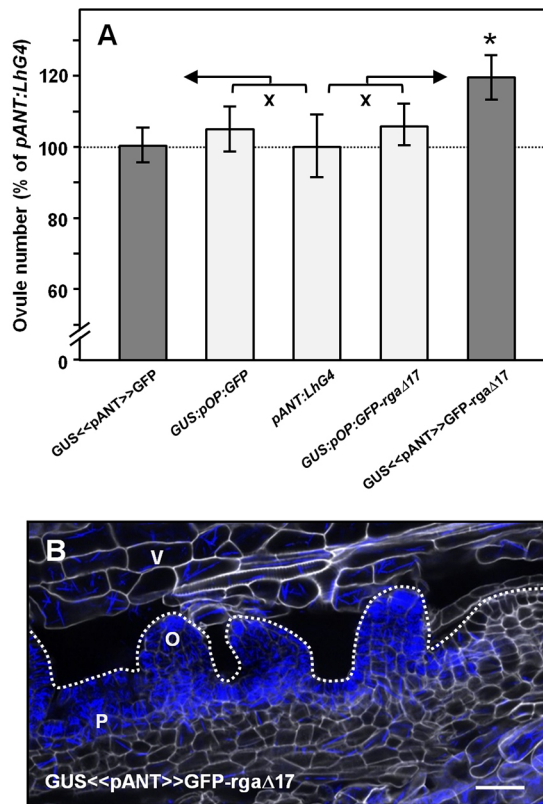
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Fig. 2. Ectopic expression of *rgaΔ17* in the pANT spatial domain promotes increase in ovule number. The pOP/LhG4 *trans*-activation system was used to express the dominant DELLA *rgaΔ17* in the placenta under the control of the *ANT* promoter. (A) Ovule number per pistil in the parental *pANT:LhG4*, *GUS:pOP:GFP* and *GUS:pOP:GFP-rgaΔ17* lines (light gray) and in the F1 generation of their genetic crosses that express GUS and either GFP-rgaΔ17 or GFP in the ANT domain (GUS≪pANT≫GFP-rgaΔ17 or GUS≪pANT≫GFP, respectively) (dark gray) (Fig. S4). More than 15 pistils were measured per line, and the experiment was repeated three times with similar results. Data represent mean±s.d. Significant differences (Student's *t*-test) with the parental line *pANT:LhG4* are indicated by an asterisk (*$P<0.01$). Values were normalized to the *pANT:LhG4* line. (B) GUS expression pattern in the F1 plants from the cross of the activator line *pANT:LhG4* with the effector line *GUS:pOP:GFP-rgaΔ17* (GUS≪pANT≫GFP-rgaΔ17). The GUS assay was carried out in whole inflorescences, and stained using the mPS-PI method. Scale bar: 20 μm. O, ovule primordia; P, placenta; V, valve.

role in controlling ovule initiation and, hence, ovule number, and also highlighted the DELLA proteins as a novel component in the gene regulatory network that governs this key developmental process in plants.

DELLA and *GID1* are expressed in placenta during ovule primordia initiation

A condition to be fulfilled by DELLA proteins as regulators of ovule number is that they should be present in the placenta before ovule primordia are formed. Although ectopic expression of RGA in the placenta was sufficient to promote an increase in ovule number, it is important to determine whether DELLA genes are normally expressed in this tissue. The expression patterns of the DELLA genes during ovule primordia initiation had not been characterized previously. Therefore, we analyzed the expression patterns of three *Arabidopsis* DELLA genes, *GAI*, *RGA* and *RGL2*, and the tomato gene *PROCERA* that are implicated in ovule number control, according to the genetic data from this work (Fig. 4). In accordance with our hypothesis, *GAI*, *RGA* and *RGL2*
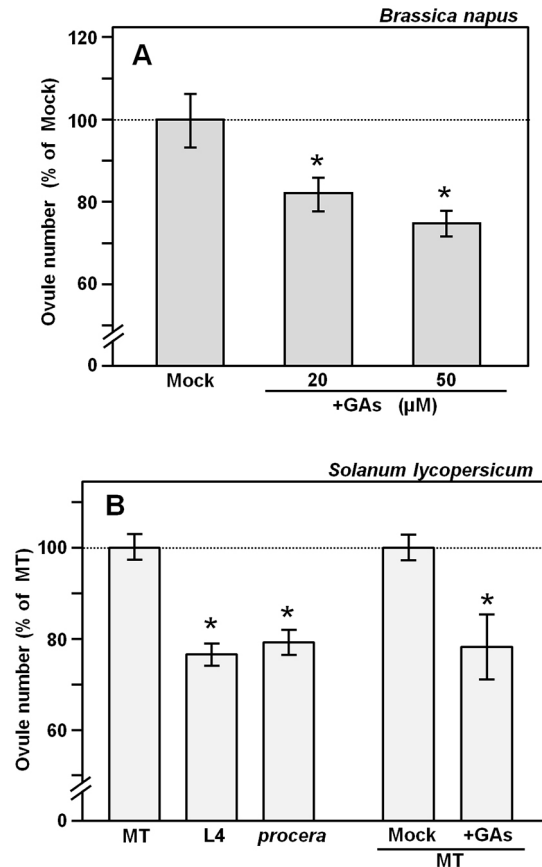
Fig. 3. GAs also regulate ovule number in rapeseed and tomato. (A) Ovule number in mock- and GA-treated rapeseed (*Brassica napus* L.) Topaz DH4079. GAs were applied by watering plants with $2×10^{-5}$ and $5×10^{-5}$ M GA_4+GA_7. More than 20 pistils were used, and experiments were repeated twice, with similar results. (B) Ovule number in tomato MT (*Solanum lycopersicum* L.), in the L4 line overexpressing a *GA20ox1* gene, in the *procera* mutant, and in GA-treated (10^{-5} M) MT plants. Ovule number in tomato was determined from at least six ovaries, and the experiment was repeated three times, with similar results. Data represents mean±s.d. Significant differences (Student's *t*-test) with the WT/mock are indicated by an asterisk (*$P<0.01$). Values were normalized to the corresponding WT or mock plants.

expression was detected in placental tissues and in outgrowing ovules (stages 1-I to 2-I, according to Schneitz et al., 1995) (Fig. 4A-C). Similar to the *Arabidopsis* DELLAs, *PROCERA* was also expressed in placenta and ovule primordia during early stages of development in tomato (Fig. 4D-G).

Similarly to DELLA proteins, both *GID1A* and *GID1B*, which are expressed in ovules and seeds (Gallego-Giraldo et al., 2014; Ferreira et al., 2017), were also localized in the placenta and ovules at stage 1-I of development (Fig. S5). Therefore, a plausible explanation for the ovule number phenotype of the double *gid1a gid1b* mutant is that a lack of GA perception in the placenta results in stabilized DELLA proteins, which in turn promotes an increase in ovule number.

GAs do not alter auxin transport or signaling in ovule primordia
During early stages of ovule formation, an auxin maximum at the tip of the ovule primordia is produced to promote growth. This auxin accumulation has been visualized using the auxin response promoter DR5 (Benková et al., 2003), and is thought to be the result of the activity of the PIN1 auxin efflux carrier located at the membrane of placenta cells (Cucinotta et al., 2014). To determine whether DELLAs promote changes in ovule number
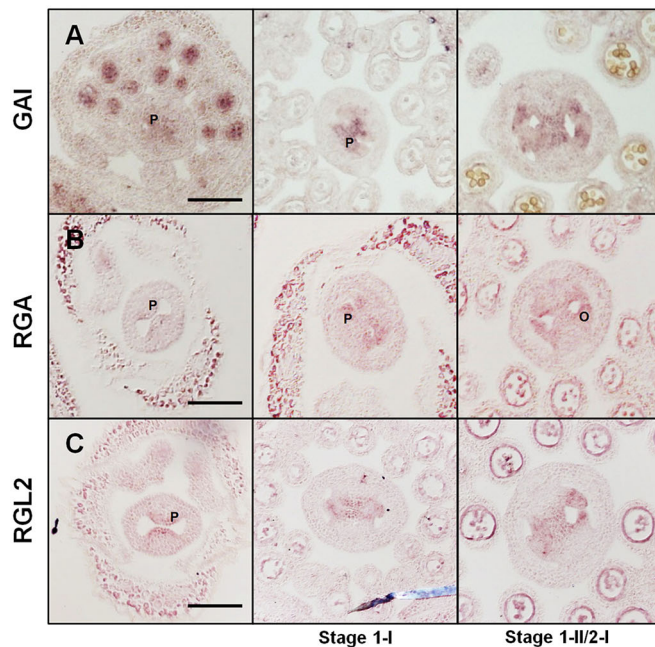
Fig. 4. DELLA genes are expressed in placental tissue. (A-C) Expression of GAI, RGA and RGL2 in placenta and ovule primordia. *In situ* mRNA hybridization was used to detect transcripts of *GAI* (A), *RGA* (B) and *RGL2* (C). Pistils at the developmental stage before ovule primordia initiation (left panels), ovules at stage 1-I (central panels), and ovules at stage 1-II/2-I (right panels). (D-G) *In situ* mRNA hybridization of *PROCERA* in tomato ovaries during early developmental stages. Scale bars: 100 µm. O, ovule primordium; P, placenta.

Fig. 5. GAs do not alter auxin transport or response in ovule primordia. (A,B) Ovule primordia of the *pPIN1:PIN1-GFP* line upon GA treatment, at stage 1-I (A) or 1-II (B) of ovule development. (C) Ovule primordia at early stage 1-II of *pDR5rev:3xVENUS* and *pDR5rev:3xVENUS* lines crossed with *gai-1* mutant. Scale bars: 10 µm.

by altering PIN1 localization or auxin maxima in placenta cells, the PIN1 distribution was examined using a *pPIN1:PIN1-GFP* line upon GA treatment (Fig. 5). In mock-treated plants, expression of the PIN1-GFP signal was located at the membranes of placenta cells during early development of primordia at stages 1-I and 1-II, which correlated with the activation of the *DR5* promoter (Fig. 5A-C). Interestingly, PIN1 reporter expression was not altered by GAs (Fig. 5A,B). In addition, *pDR5:3xVENUS* expression in placenta and ovule primordia was not altered in the *gai-1* mutant (Fig. 5C). Taken together, these data suggest that GAs regulate ovule number independently of auxin distribution.

Differential gene expression in *gai-1* and *global* mutants
To further understand the role of DELLA proteins in ovule number, a transcriptomic analysis of pistils of *gai-1* and *global* mutants was carried out. Whole pistils at stages 1-I and 1-II of ovule development
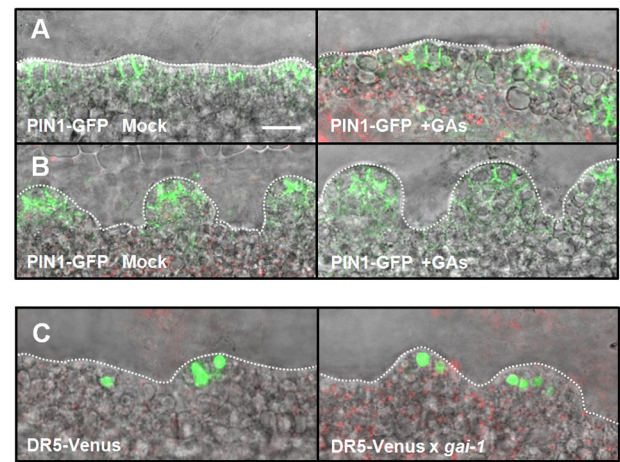
were used (Fig. S6A) as an alternative to the use of isolated placenta during these early developmental stages, which can allow one to test genes expressed in the placenta. A total of 389 differential genes were identified, 235 upregulated and 154 downregulated in the *gai-1* mutant versus *global* mutant with an FDR adjusted $P<0.05$ and fold change ($\log_2$) of >1 (Table S1). Gene Ontology (GO) analysis revealed several categories enriched among the upregulated genes, which included Response to gibberellin stimulus; Response to cold, water deprivation and phosphate starvation; and Regulation of transcription (Table S2).

DELLA proteins act at the transcriptional level by means of direct protein-protein interactions with TFs and other transcriptional regulators. To investigate the molecular mechanism of GA-mediated ovule formation, all 46 differentially expressed TFs were first selected (Table 1 and Table S3), identifying members from all major TF gene families, such as the B3 DNA-binding domain superfamily, along with MYB, basic helix–loop–helix (bHLH), or Homeobox (HB) genes. To further narrow down the list of TFs that could be involved in ovule initiation, those that had also been involved in ovule development based on previous transcriptomic analysis were identified (Table S3). For this purpose, several transcriptomic data were used: developing CMM (Villarino et al., 2016), ovule primordia (stage 8-9 of flower development) (Matias-Hernandez et al., 2010), and ovules at later development stages (Skinner and Gasser, 2009). The majority (38 out of 46; 81%) of the differential TFs were expressed during early ovule development, such as the *REPRODUCTIVE MERISTEM* (*REM*) genes *REM24*, *REM32*, *REM22* and *REM36*, and the *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes *WOX13* and *WOX14*. Other genes in this category included *SCL3*, a DELLA interactor protein that mediates GA-regulated seed germination and root and hypocotyl elongation (Zhang et al., 2011); *SPL4*, induced by GAs in the shoot apical meristem (SAM) upon flowering (Porri et al., 2012); and *UNFERTILIZED EMBRYO SAC 16* (*UNE16*), which is involved in embryo sac development and is regulated by BRs (Pagnussat et al., 2005; Sun et al., 2010).

To test the biological relevance of these genes in ovule number determination, ovule number of null mutants for *COL5* (*col5-1*, SALK_137717C), *REM22* (*rem22-1*, SAIL_714_F09), *REM24* (*rem24-1*, SALK_059706C) and *UNE16* (*une16-1*, SALK_113627C) were determined (Fig. S7A). First, semiquantitative

Table 1. Genes selected in the RNAseq analysis of pistils of *gai-1* and *global* mutants

AGI code	Name	Annotation	TF family	Fold-change (gai-1/global)	P adjusted FDR
At3g03260	HDG8	HOMEODOMAIN GLABROUS 8	HB	6.25	3.86E-17
At1g50420	**SCL3**	SCARECROW-LIKE 3	**GRAS**	2.49	9.46E-07
At1g20700	**WOX14**	WUSCHEL-RELATED 14	**HB**	2.45	5.04E-05
At5g57660	**COL5**	CONSTANS-like 5	**C2C2-CO-like**	2.28	8.28E-07
At3g56770		Basic Helix-Loop-Helix	bHLH	2.26	5.02E-05
At2g24700	**REM32**	REPRODUCTIVE MERISTEM 32	**B3 DBD**	2.25	6.40E-06
At4g25480	**DREB1A**	DEHYDRATION RESPONSE ELEMENT B1A	**AP2-EREBP**	2.23	1.19E-04
At5g53420		CCT motif family protein	**Orphans**	2.22	1.40E-06
At1g35515	MYB8	MYB DOMAIN PROTEIN 8	MYB	2.14	1.24E-08
At4g27310	BBX28	B-BOX DOMAIN PROTEIN 28	Orphans	2.14	3.20E-05
At1g71130	**CRF8**	CYTOKININ RESPONSE FACTOR 8	**AP2-EREBP**	2.12	8.04E-09
At2g18328	RL4	RAD-LIKE 4	MYB-related	2.08	6.95E-04
At3g02980	**MCC1**	MEIOTIC CONTROL OF CROSSOVERS 1	**GNAT**	2.04	1.88E-04
At5g67300	**MYB44**	MYB DOMAIN PROTEIN 44	**MYB**	2.02	2.30E-06
At1g53160	**SPL4**	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 4	**SBP**	2.02	1.34E-09
At2g42150		DNA-binding bromodomain-containing protein	**MYB-related**	2.00	7.77E-04
At3g50410	**OBP1**	OBF BINDING PROTEIN 1	**C2C2-Dof**	1.93	1.94E-04
At1g68670	**HHO2**	HRS1 homolog	**G2-like**	1.93	2.17E-06
At5g67480	**BT4**	BTB and TAZ domain protein	**TAZ**	1.91	1.97E-05
At4g01580	REM8	REPRODUCTIVE MERISTEM 8	B3 DBD	1.85	1.11E-07
At2g16210	**REM24**	REPRODUCTIVE MERISTEM 24	**B3 DBD**	1.83	2.56E-06
At5g63700		Zinc ion binding/DNA binding	**SWI/SNF**	1.78	3.00E-04
At1g05710		Basic helix-loop-helix	**bHLH**	1.76	1.68E-04
At5g13220	**JAZ10**	JASMONATE-ZIM-DOMAIN PROTEIN 10	**Tify**	1.71	3.40E-04
At4g29190	**TZF3**	TANDEM ZINC FINGER 3	**C3H**	1.71	5.98E-04
At1g31760		SWIB/MDM2 domain superfamily protein	SWI/SNF	1.60	5.18E-04
At1g54160	**NF-YA5**	NUCLEAR FACTOR Y SUBUNIT A5	**CCAAT**	1.60	5.99E-04
At5g46760	**MYC3**	JAZ-interacting transcription factor	**bHLH**	1.58	5.54E-05
At3g17010	**REM22**	REPRODUCTIVE MERISTEM 22	**B3 DBD**	1.57	7.50E-05
At1g72830	**NF-YA3**	NUCLEAR FACTOR Y SUBUNIT A3	**CCAAT**	1.56	7.08E-04
At4g14410	**bHLH104**	BASIC HELIX-LOOP-HELIX 104	**bHLH**	1.55	1.12E-05
At3g51880	**HMGB1**	HIGH MOBILITY GROUP B1	**HMG**	1.53	3.61E-04
At1g20693	**HMGB2**	HIGH MOBILITY GROUP B2	**HMG**	1.52	1.01E-04
At4g35550	**WOX13**	WUSCHEL-related 13	**HB**	1.47	3.13E-04
At4g13640	**UNE16**	UNFERTILIZED EMBRYO SAC 16	**G2-like**	1.43	3.19E-04
At1g15340	**MBD10**	METHYL-CPG-BINDING DOMAIN 10	**MBD**	1.41	1.80E-05
At4g40060	**HB16**	HOMEOBOX PROTEIN 16	**HB**	−1.44	2.85E-04
At1g02065	SPL8	SQUAMOSA PROMOTER BINDING-LIKE 8	SBP	−1.50	7.54E-04
At3g46640	**PCL1**	PHYTOCLOCK 1 (PCL1), LUX ARRHYTHMO (LUX)	**G2-like**	−1.51	1.31E-05
At4g14490		SMAD/FHA domain-containing protein	**FHA**	−1.62	1.43E-05
At4g31650	REM39	REPRODUCTIVE MERISTEM 39	B3 DBD	−1.65	1.03E-04
At1g44810	**GPL4**	GEBP-LIKE 4	**GeBP**	−1.85	1.64E-06
At1g07520	**SCL31**	GRAS family	**GRAS**	−1.90	4.83E-04
At5g66270		Zinc finger C-x8-C-x5-C-x3-H	C3H	−2.08	7.32E-04
At4g31620	**REM36**	REPRODUCTIVE MERISTEM 36	**B3 DBD**	−2.41	2.98E-11
At5g17890	**CHS3**	CHILLING SENSITIVE 3	**Orphans**	−10.32	2.71E-26

*DELLA genes have been removed from list. Genes in bold are expressed during ovule development. Green, upregulated genes; red, downregulated genes.

RT-PCR analysis in inflorescences was carried out to confirm that homozygous plants had altered expression of the corresponding gene (Fig. S7B). *rem24-1* was a full knockout, because the *REM24* transcript was not detected in the mutant inflorescences. By contrast, *rem22-1* was an enhancer allele, with a twofold increase in *REM22* expression. Finally, *une16-1* and *col5-1* were knockdown alleles, with expression of *UNE16* and *COL5* that was ~15% of that in Col-0 plants. In addition, expression analysis by quantitative RT-PCR (qPCR) revealed the increased expression of all four genes in the *gai-1* mutant compared with *global* mutant (Fig. S7C), confirming the RNA-sequencing (RNA-seq) data reported here. As can be observed in Fig. 6, whereas *col5-1* and *rem24-1* did not show any alteration in ovule number, *une16-1* and *rem22-1* showed a decreased and increased ovule number, respectively, which is coincident with the altered expression of the corresponding gene (Fig. S7B). Both

UNE16 and *REM22* were upregulated in the *gai-1* mutant compared with the *global* mutant; therefore, decreased and increased ovule numbers in *une16-1* and *rem22-1*, respectively, were consistent with these genes being DELLA targets that act as positive factors in ovule initiation. Further characterization of these and other TFs that are differentially regulated in the pistils of GA mutants at the moment of ovule initiation will allow us to dissect the molecular mechanism of the DELLA-mediated ovule initiation.

DISCUSSION
DELLA proteins act as positive regulators of ovule number
We have shown that GAs negatively regulate ovule number. Pistils of the *gai-1* mutant, with a gain of function of the DELLA GAI, showed a significant increase in the number of ovules. In addition, loss of function of the GA receptors GID1A and GID1B also
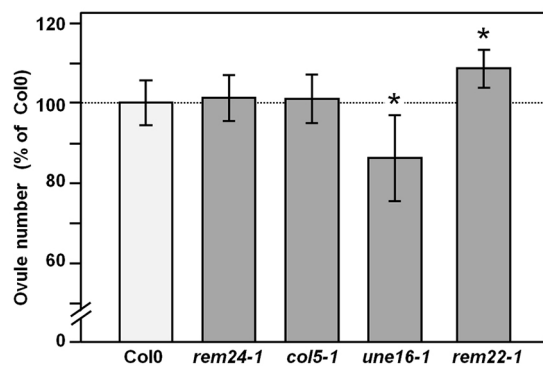
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Fig. 6. Null mutants of *UNE16* and *REM22* show altered ovule number.
Ovule number in Col-0 and in the mutants *col5-1* (SALK_137717C), *rem22-1* (SAIL_714_F09), *rem24-1* (SALK_059706C) and *une16-1* (SALK_113627C). More than 15 pistils were measured per mutant and data represent mean±s.d. Significant differences (Student's *t*-test) with Col-0 are indicated by an asterisk (*$P<0.01$). Values were normalized to those in the WT.

promoted ovule formation, which is likely to be as a consequence of DELLA stabilization in the tissues where ovule initiation takes place (Gallego-Giraldo et al., 2014). Furthermore, the ANT promoter-mediated expression of *rgaΔ17* in the placenta was sufficient to promote an increase in ovule number. By contrast, both loss of function of DELLA activity or GA treatment resulted in a decreased ovule number. Plackett et al. (2014) described reduced fertility in GA-treated plants of *Arabidopsis*, which they associated with a male-sterile phenotype. In tomato, a similar role of GAs was observed, because GA-treated plants or the *procera* mutant form fewer ovules in the ovary. In rapeseed, GA treatments also reduced ovule number in a dose-dependent manner. Based on our observations, the decrease in ovule number upon GA treatment could also contribute to defects in fertility. Although the effects of exogenous application of GA might not necessarily imply a physiological role of GA in ovule initiation, the fact that the *gai-1* and *gid1a gid1b* mutants had more ovules suggests that GA signaling does have a physiologically relevant role in controlling ovule initiation. The mechanism by which the DELLA proteins act in this process is still unknown, but the results of this study support a positive role of DELLA activity in the regulation of ovule initiation in plants. This is of particular interest because it opens a new way to modulate ovule number and, hence, seed number in crops.

Ovule number does not correlate with seed number in DELLA gain-of-function mutants

DELLA promotes the formation of more ovules, but this increase does not correlate with an increase in seeds per fruit. This occurred in both the *gai-1* and the *gid1a gid1b* mutants, which produced between 20% and 30% more ovules, but fewer seeds than the corresponding WT plants. The difference between ovule and seed number observed in *gai-1* and *gid1a gid1b* mutants could be explained by possible pleiotropic defects in pistil development caused by increased DELLA activity or a lack of GA perception that affect fertilization. These maternal defects might vary between *gai-1* and *gid1a gid1b* mutants. In the case of *gid1a gid1b* mutants, a defect in the fusion of central cell nuclei during late stages of female gametophyte development could account for the reduced seed-set (Yadegari and Drews, 2004). It is also known that GID1 participates in ovule development, because plants overexpressing *GID1A* show asynchrony between integument development and the stage of gametogenesis, which could result from either a small delay in megasporogenesis or faster growth of integuments in the transgenic plants (Ferreira et al., 2017).

GAs do not regulate ovule number by interfering with the auxin signaling pathway

Auxins are important for the correct development of the pistil. Auxin polar transport in the apical-basal and the medio-lateral axes is crucial for the formation of the medial ovary tissues, such as the placenta and, therefore, ovule formation (Nemhauser et al., 2000; Nole-Wilson et al., 2010). The role of the auxin efflux transporter PIN1 in ovule number was proposed based on the phenotypic analysis of the weak *pin1-5* mutant allele, which produces fewer ovules per pistil compared with WT plants (Bencivenga et al., 2012). PIN1 is involved in the formation of an auxin gradient along the proximal-distal axis (Benková et al., 2003; Ceccato et al., 2013). Accordingly, a maximum auxin response, monitored by the DR5 promoter, is located in the tip of the ovule primordia (Benková et al., 2003). As shown by the current study, neither expression of PIN1 nor localization of DR5 promoter activity was affected by DELLA activity, suggesting that DELLAs participate in ovule formation independently of the auxin. One plausible scenario is that DELLA proteins regulate early stages of ovule initiation, early on during the determination of the position of ovule primordia in the placenta and, hence, before the establishment of the auxin maxima by PIN1 expression in ovule primordia. In this way, once the position of the primordia is determined, regardless of these being increased or decreased in number in the placenta of GA mutants, the auxin maxima observed would not be altered within each primordia.

Putative genes involved in the GA-mediated ovule initiation mechanism

A relatively large number of TFs were found to be differentially expressed between *gai-1* and *global* mutant pistils, including six *REM* genes. *REM* genes are involved in a variety of growth and developmental processes, and several of them participate in reproductive development (Mantegazza et al., 2014). The group of *REM* genes identified in our assay, *REM8*, *REM22*, *REM24*, *REM3*2, *REM36* and *REM39*, have a role during early stages of *Arabidopsis* pistil development (Mantegazza et al., 2014). Interestingly, *REM39* expression was recently detected in tomato pistils during early stages of development (Ezura et al., 2017). Of particular interest are *REM22*, *REM24* and *REM36*, which are expressed in placenta, coincident with ovule initiation (Gomez-Mena et al., 2005; Mantegazza et al., 2014). Moreover, the null mutant *rem22-1* showed altered ovule number. Taken together, these data suggest that *REM* genes identified in this work are involved in the GA-mediated ovule initiation mechanism in *Arabidopsis*. Other TFs identified in this study were two *WOX* genes. These are members of the ZIP superfamily, which belongs to the HB family, and are key regulators implicated in the determination of cell fate by preventing cell differentiation (Deveaux et al., 2008). *WOX13* expression was detected in the placenta during early stages of pistil development, coincident with ovule primordia emergence (Romera-Branchat et al., 2013). *WOX14* expression has not been previously characterized during ovule development, but its elevated expression in *gai-1* compared with *global* mutants suggests that it has a role during ovule initiation. Another gene that might participate in ovule initiation is *CONSTANS LIKE-5* (*COL5*), a B-BOX zinc finger protein (Khanna et al., 2009) previously implicated in flowering time and flower development (Hassidim et al., 2009). Interestingly, *COL5* is expressed in the ovule primordia and later, during ovule development, at the inner integument (Skinner and Gasser, 2009; Matias-Hernandez et al., 2010). Nevertheless, the lack of phenotype in the null *col5-1* might indicate that this gene either has no role or has a redundant function with other genes during ovule initiation.

Interestingly, *UNE16*, another TF that is involved in embryo sac development (Pagnussat et al., 2005) and is upregulated in *gai-1* mutants, appears to act as a positive factor in DELLA-mediated ovule number determination. The reduced ovule number in *une16-1* is plausible with a target acting downstream of DELLAs in the promotion of ovule initiation. Finally, another differential FT, *CRF8*, encodes a CYTOKININ RESPONSE FACTOR (CRF) (Cutcliffe et al., 2011). It is not known whether CRF8 has an active role during ovule initiation, but it might act as a putative crosstalk node between GAs and CKs in the control of ovule initiation. Understanding the mechanism of GA-mediated ovule initiation will require detailed analysis of the specific role of these genes in ovule formation.

Genetic network regulating ovule initiation: how to fit DELLA proteins into the current model

DELLAs have an important role in modulating ovule number, but how do they do it? Based on the current model for ovule initiation (Galbiati et al., 2013; Cucinotta et al., 2014), several possibilities arise. The model calls for three major regulators: CKs, auxins and BRs.

The activity of the CK response factors CRF2, CRF3 and CRF6 directly promoted an increase in PIN1 expression in the placenta, resulting in an increase in ovule number (Cucinotta et al., 2016). In contrast to this, and as previously stated, GAs or DELLAs do not affect auxin transport (PIN1) or response (activity of the DR5 promoter), suggesting that GAs participate in ovule number independently of CKs. By contrast, Arabidopsis RESPONSE REGULATOR 1 (ARR1) is a TF that positively regulates the response to CKs. DELLA and ARR1 proteins interact physically, which supports a mechanism that explains the antagonistic role of GAs and CKs in different development processes (Marin-de la Rosa et al., 2015), such as ovule initiation (Bartrina et al., 2011; this work). Besides the direct protein-protein interaction of DELLA and ARR1, the antagonist roles of GAs and CKs in ovule initiation could also rely on the repression by GAs of the RGA-mediated expression of *ARR1*, as observed during early stages of root meristem development (Moubayidin et al., 2010).

In the case of the BRs, a complex interaction between GAs and BRs in the regulation of several developmental processes has been reported (Ross and Quittenden, 2016). Recent evidence points to both a direct regulation of GAs biosynthesis by BZR1 (Tong et al., 2014; Unterholzner et al., 2015) and the direct protein-protein interaction between the DELLAs and BZR1 (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). This model explains the synergistic effect of GAs and BRs in several growth and developmental processes, such as the promotion of hypocotyl growth during skotomorphogenesis (Tanaka et al., 2003). However, it appears that GAs and BRs act as antagonistic players in ovule initiation. BR signaling positively regulates ovule and seed number in *Arabidopsis* (Huang et al., 2013), whereas *bzr1-1D* (the gain-of-function allele of BZR1) increased ovule and seed number, and *det2-1* (the null mutant of *DET2* of BR synthesis) produced fewer ovules and seeds. The antagonism of GAs and BRs in ovule initiation resembles that observed during root elongation during fungal infection in rice (De Vleesschauwer et al., 2012). In this latter example, the antagonistic effect of both hormones is thought to result from a cross-inhibitory effect on the reciprocal hormone biosynthesis pathway. Thus, on the one hand, GAs inhibit the BR response and BR biosynthesis genes *OsBLE2* and *OsDWARF2*, whereas, on the other hand, BRs repress *GA20ox3* and induce *GA2ox3* expression. It can be hypothesized that, during ovule initiation, GAs and BRs interact via reciprocal hormonal biosynthesis inhibition, similarly to the mechanism reported in rice.

Together, the results presented here introduce a new role of GAs in ovule initiation and add a new layer of complexity to an important developmental process for plants. Further studies are needed to define the role of the different TFs identified in GA-mediated ovule initiation and to unravel the complex net of hormone interactions controlling this agronomically important process.

MATERIALS AND METHODS
Plant material assays

Arabidopsis plants used were in the L*er* or Col-0 backgrounds as indicated. Seeds were sterilized in ethanol and germinated in Murashige and Skoog (MS) media plates (Murashige and Skoog, 1962) for 4 days at 4°C in the dark followed by 7-8 days at 22°C in a long-day photoperiod (16/8 h). Seedlings were then transferred into soil (a mix of peat moss, vermiculite and perlite, 2:1:1 v/v/v) and grown in a chamber at 22°C in a long-day photoperiod (16/8 h).

della mutant combinations were as described previously (Gomez et al., 2016). The *pGID1A:GID1A-GUS* and *pGID1B:GID1B-GUS* transgenic lines (Suzuki et al., 2009) were provided by Dr Nakajima (University of Tokyo). *pRGA:GFP-rgaΔ17*, *pLAT52:GUS* (N16336), *pPIN1:PIN1-GFP* and *pDR5rev:3xVENUS* seeds were as described previously (Twell et al., 1990; Dill et al., 2001; Benková et al., 2003; Heisler et al., 2005). Null mutants *col5-1* (SALK_137717C, N661168), *rem22-1* (SAIL_714_F09, N862704), *rem24-1* (SALK_059706C, N681160) and *une16-1* (SALK_113627C, N655849) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Plants of tomato (*S. lycopersicum* L.) cultivars of MT and rapeseed (*B. napus* L.) double haploid cultivar Topaz DH4079 were used. Plants were grown in a greenhouse at 22°C (day) and 18°C (night) in an equal mixture of peat moss and vermiculite.

Hormonal treatments, determination of ovule number and fertility assays

In *Arabidopsis*, ovule number was determined in flowers between position 10 and 20 in the main inflorescence by hand dissection of each pistil under a stereomicroscope. For each mutant and treatment, at least 15 pistils were used. GA treatment was applied by watering every other day with 2×10^{-5} M of GA_4+GA_7 (Duchefa Biochemie) starting 2 weeks after transfer the seedling into the soil. Ovary size was determined in the same pistils used for ovule number determination, from images taken under a stereomicroscope. To test the fertility, flowers were hand-emasculated 1 day before anthesis and pollinated with WT pollen at anthesis. Fruits were collected at maturity (12-14 days post anthesis; dpa) and seed number, silique length and the ratio (seed number to length) were determined for each individual fruit as described (Gomez et al., 2016). More than 30 pistils were used for each genotype and/or experiment.

In tomato, ovules were counted in ovaries of flowers from the first bunch 3 days before the day equivalent to anthesis (d-3). Tissue sections of d-3 ovaries were fixed in 4% paraformaldehyde in 100 mM sodium phosphate buffer pH 7.2. After dehydration in ethanol, the samples were embedded in paraffin (Paraplast Plus, Sigma-Aldrich). Thin sections (8 μm) were generated, stained with Safranin-Alcian Blue, and viewed and photographed on a Nikon Eclipse E600 microscope. Ovule number was determined at the medial longitudinal cross-section from at least six ovaries. GAs were applied at 1×10^{-5} M by watering once the plants had two expanded leaves. In rapeseed, GAs were applied at 2×10^{-5} M and 5×10^{-5} M by watering the plants at the beginning of flowering. Ovule counts were carried out by hand dissection under a stereomicroscope of pistils of flowers that developed 2 weeks later.

Fertility in the *gid1a gid1b* and *gai-1* mutants was assayed using the specific pollen marker LAT52. The *pLAT52:GUS* line was used to manually pollinate pistils of *gid1a gid1b*, *gai-1* and Col-0 previously hand-emasculated. Fruits were harvested 48 h after pollination and stained for GUS activity, as described above.

Histological procedures

GUS assays were carried out basically as previously described (Carbonell-Bejerano et al., 2010). The $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ concentrations were adjusted for each line to obtain an optimal signal (2 mM for *pGID1A: GID1A-GUS* and *pGID1B:GID1B-GUS*, and 1 mM for pOP/LhG4 and

pLAT52:GUS). For the *GID1-GUS* and *pLAT52:GUS* lines, samples were dehydrated after GUS staining, cleared with chloral hydrate and photographed with a Nikon Eclipse E600 microscope equipped with a Nikon DS-Ri1 digital camera. Samples of pOP/LhG4 were processed following a modified pseudo-Schiff propidium iodide (mPS-PI) technique (Truernit et al., 2008), as previously described (Gomez et al., 2016). Images were captured using a ZEISS LSM 780 confocal microscope with a MBS T80/R20 dichroic (561 nm and 545-570 nm excitation and reflexion, respectively). Propidium iodine staining was excited at 561 nm and detected at 580-660 nm. To study female gametophyte development in *gid1a gid1b* mutants, pistils of flowers at anthesis were fixed, dehydrated and cleared with chloral hydrate. The images were obtained with a Nikon Eclipse E600 microscope equipped with a Nikon DS-Ri1 digital camera.

In situ RNA hybridization

Arabidopsis and tomato inflorescences were embedded, sectioned and hybridized as described by Weigel and Glazebrook (2002). The *GAI*, *RGA*, *RGL2* and *Procera* templates were amplified, and cDNAs were subsequently cloned into the pGem-T Easy vector (Promega). Oligonucleotides used are described in Table S4. Sense and antisense probes were synthesized using the corresponding SP6 and T7 RNA polymerases in the vector. Control experiments were performed with sense probes and no significant signal was detected. Images were recorded using a Nikon Eclipse E600 microscope equipped with a Nikon DS-Ri1 digital camera.

Expression of *rgaΔ17* under the *ANT* promoter

The pOP/LhG4 system (Moore et al., 2006) was used to express *rgaΔ17* under the *ANT* promoter. Promoter of *ANT* was cloned in the multicloning site of the pBIN-LhG4 vector to generate the activator line *pANT:LhG4*. For the effector lines, *GFP* cDNA was amplified from the pMDC110 vector (Curtis and Grossniklaus, 2003) with oligos GFP-HindIII and GFP-XhoI (Table S4), digested with *Hind*III and *Xho*I and cloned into pH-TOP to obtain *GUS:pOP:GFP*. Next, the *rgaΔ17* cDNA was amplified from genomic DNA of the *pRGA:GFP-rgaΔ17* line (Dill et al., 2001) using oligos rgaΔ17-*Sal*I and rgaΔ17-*Kpn*I (Table S4), digested with *Sal*I and *Kpn*I and cloned into *GUS:pOP:GFP* to generate *GUS:pOP:GFP-rgaΔ17*. Constructs were introduced in *Arabidopsis* Col-0 by *Agrobacterium*-mediated infiltration using the floral dip method (Clough and Bent, 1998). Stable homozygous lines with a single insertion locus were selected by segregation in kanamycin and F1 were generated by genetic cross.

Transcriptome analysis

Twenty pistils of *gai-1* and *global* mutants at stage 8-9 (Smyth et al., 1990) were isolated by hand dissection under a stereomicroscope. RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) according to the manufacturer's indication, and RNA integrity was confirmed by a 2100 Bioanalyzer (Agilent). cDNA was synthesized using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech Laboratories) and amplified with 15 PCR cycles according to the manufacturer's instructions. Sequencing libraries were generated with the Nextera XT DNA Library Prep Kit (Illumina) from 150 pg of cDNA, and libraries were sequenced at the Genomic Core Facility of the University of Valencia (Spain) with the NextSeq (Illumina) platform. Raw reads were analyzed using FastQC report and cleaned with CutAdapt. Low-quality reads were filtered and trimmed. All sequenced libraries were mapped to the *Arabidopsis* genome (Arabidopsis_thaliana.TAIR10.22) with GTF information (Arabidopsis_thaliana.TAIR10.22.gtf) using TopHat (v.2.1). Seqmonk v.1.35 was used to generate a raw count matrix per gene from uniquely mapped reads with the same GTF file used for the alignment step. Statistical assessment of differential gene expression was done either with edgeR (Robinson et al., 2010) or DESeq (Anders and Huber, 2010), and genes with a FDR adjusted $P<0.05$ and fold change ($\log_2$) >1 were finally selected as differentially regulated. Raw data of the RNAseq of *gai-1* and *global* pistils were deposited at SRA-NCBI (PRJNA412799).

Analysis of null mutants of *COL5*, *REM24*, *REM22* and *UNE16*

Genomic DNA was extracted from leaves of Col-0 and the null mutants *col5-1* (SALK_137717C, N661168), *rem22-1* (SAIL_714_F09, N862704), *rem24-1*

(SALK_059706C, N681160) and *une16-1* (SALK_113627C, N655849). Genotyping was carried out by PCR using the oligos described in Table S4. cDNA was obtained as described in Gomez et al., (2016) from inflorescences of Col-0 and the null mutants. Semiquantitative RT-PCR for each gene in the Col-0 and the corresponding mutants was carried out using the oligos described in Table S4, for 28 cycles for UNE16, and 30 cycles for *COL5*, *REM22* and *REM4*. *ACTIN11* (*At3g12110*) was used as a control (Balanzà et al., 2018).

Expression analysis of *UNE15*, *REM22*, *REM24* and *COL5* was carried out by qPCR in hand-dissected pistils of *gai-1* and *global* mutants at stage 8-9 (Smyth et al., 1990). qPCR analysis was carried out as described in Dorcey et al. (2009). Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). qPCR was performed with the SYBR® GREEN PCR Master Mix (Applied Biosystems) with a 7500 Fast Real-Time PCR System (Applied Biosystems). The oligonucleotides used (Table S4) were designed with the Primer Express™ v2.0 software (Applied Biosystems) and were tested for efficiency. Expression levels were calculated according to the expression of the constitutive gene *PP2A* (*At1g13320*) (Czechowski et al., 2005) and data were normalized by the $\Delta\Delta$Ct method to the values in the *global* mutant.

Data availability
Raw data of the RNAseq of *gai-1* and *global* pistils have been deposited in BioProject under accession number PRJNA412799.

Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.163865.supplemental

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