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Regulation of ovule initiation by gibberellins and brassinosteroids in tomato and *Arabidopsis*: two plant species, two molecular mechanisms

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GAs and BRs in the control of ovule number

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ABSTRACT

Ovule primordia formation is a complex developmental process with a strong impact on the production of seeds. In *Arabidopsis* this process is controlled by a gene network, including components of the signaling pathways of auxin, brassinosteroids (BRs) and cytokinins. Recently, we have shown that gibberellins (GAs) also play an important role in ovule primordia initiation, inhibiting ovule formation in both *Arabidopsis* and tomato. Here we reveal that BRs also participate in the control of ovule initiation in tomato, by promoting an increase on ovule primordia formation. Moreover, molecular and genetic analyses of the co-regulation by GAs and BRs of the control of ovule initiation indicate that two different mechanisms occur in tomato and *Arabidopsis*. In tomato, GAs act downstream of BRs. BRs regulate ovule number through the downregulation of GA biosynthesis, which provokes a stabilization of DELLA proteins that will finally promote ovule primordia initiation. In contrast, in *Arabidopsis* both GAs and BRs regulate ovule number independently of the activity levels of the other hormone. Taking together, our data strongly suggest that different molecular mechanisms could operate in different plant species to regulate identical developmental processes even, as in the case of ovule primordia initiation, when the same set of hormones trigger similar responses, adding a new level of complexity.

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INTRODUCTION

Seeds are extremely important in many ways. Besides their biological roles for the survival of the species, preserving the embryonic life or being the vehicles for dispersal, seeds are the primary basis for human sustenance (Sabelli and Larkins, 2009). Seeds are formed upon the double fertilization of the ovule in the ovary; therefore, seed number depends, among other factors, on the number of viable ovules that are formed.

Ovule development has been extensively studied in *Arabidopsis* at the morphological, genetic and molecular levels (Schneitz et al., 1995; Cucinotta et al., 2014). Ovule primordia are formed in the carpel medial meristem (CMM) as lateral organs from the placenta and follow a well-established developmental process (Schneitz et al., 1995). A key step in ovule development is the determination of the position and number of ovule primordia in the CMM (Cucinotta et al., 2014). CMM and ovule primordia formation are both controlled by regulatory genes as well as by several plant growth regulators, including auxin, cytokinin (CKs), and brassinosteroids (BRs) (Bartrina et al., 2011; Bencivenga et al., 2012; Huang et al., 2013; Galbiati et al., 2013; Cucinotta et al., 2014; Cucinotta et al., 2016; Müller et al., 2017; Reyes-Olalde et al., 2017). Recently, we have demonstrated that gibberellins (GAs) also play an important role in the modulation of ovule number in *Arabidopsis* as well as in tomato and rapeseed (Gomez et al., 2018). In contrast, morphological characterization of tomato ovule development has only been described in *Solanum pimpinellifolium*, a close relative to the commercial tomato *Solanum lycopersicum* (Xiao et al., 2009). *S. pimpinellifolium* ovules grow as a protrusion in the placenta towards the center of the ovary locus in flower buds at floral stage 7, *circa* 9-10 days post floral initiation.

Key elements in the GA signaling are DELLA proteins (coded by five genes in *Arabidopsis* and one in tomato), which repress GA responses (Sun, 2010, 2011). In the presence of GAs, DELLA proteins are degraded via the proteasome, releasing the repression of GA signaling. Degradation depends upon an N-terminal domain of the DELLA protein (the so-called DELLA domain). Gain-of-function varieties of DELLA proteins, such as the *gai-1* allele of *GIBBERELLIC ACID INSENSITIVE* (*GAI*) in *Arabidopsis* (Peng et al., 1997), lack this domain and encode stable proteins that cannot be degraded by GAs, hence promoting a constitutive blockage of the GA response. Interestingly, DELLA proteins are transcriptional regulators that lack a canonical DNA binding domain, and therefore exert their activity by binding to a wide variety of transcription factors (TFs) (Vera-Sirera et al., 2015; Daviere and Achard, 2016), such as ABERRANT TESTA

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SHAPE (ATS/KAN4) to regulate integument growth (Gomez et al., 2016). Regarding ovule initiation, constitutive repression of GA responses in the *gai-1* mutant produces more ovules that the wild type, whereas constitutive GA signaling in null *della* mutants or by GA treatment causes a strong decreased in ovule number in *Arabidopsis* (Gomez et al., 2018). Similar role of GAs in ovule initiaton was confirmed in tomato and rapeseed. Therefore, DELLA protein activity is a positive factor in ovule formation, although the molecular mechanism of DELLA action in the CMM is still unknown.

Most of the BR signaling pathway relies on the BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1) TFs. In the presence of BR, the receptor complex BRI1-SERK3/BAK1 mediates the dephosphorylation of BRZ1and BES1 by a PP2A protein phosphatase, which activates their transcriptional activity upon BR-regulated genes (Belkhadir and Jaillais, 2015; Nolan et al., 2019). BR signaling positively regulates ovule and seed number in *Arabidopsis* (Huang et al., 2012). The *bzr1-1D* mutant (a gain-of-function of BZR1) increases ovule and seed number, while the BR-deficient mutant *det2-1* (Fujioka et al., 1997) produces fewer ovules and seeds. It has been proposed that BRs control ovule number by the effects of BZR1 on the expression of ovule development genes. Among these, expression of *HUELLENLOS* (*HLL*) and *AINTEGUMENTA* (*ANT*) are up-regulated, whereas that of *APETALA2* (*AP2*) is down-regulated by BRs and in *bzr1-1D* plants (Huang et al., 2013). In addition, *ANT* and *AP2* are direct targets of BZR1 (Huang et al., 2013).

GAs and BRs control similar developmental processes. Both hormones promote hypocotyl growth during skoto-morphogenesis in *Arabidopsis* (Tanaka et al., 2003), or participate in the control of cell elongation to determine plant height in rice (De Vleesschauwer et al., 2012; Xiao et al., 2017; Tang et al., 2018). Moreover, mutants deficient in either GAs or BRs display dwarf plant architecture in *Arabidopsis* (Clouse, 2011; Sun, 2011) and tomato (Marti et al., 2006). BR mutants and GA-deficient plants show other phenotypes, such as impaired germination (Unterholzner et al., 2015), reduced hypocotyl elongation, darker green leaves (Marti el at., 2006), late flowering or reduced fertility (Clouse, 2011). The complex interaction between GAs and BRs points out to both a direct regulation of GAs biosynthesis by BRs in *Arabidopsis* (Unterholzner et al., 2015) and rice (*Oryza sativa*; Tong et al., 2014), as well as the direct protein-protein interaction between DELLA proteins and BZR1 in *Arabidopsis* (Bai et al., 2012; Gallego-Bartolome et al., 2012; Li et al., 2012). In the latter, DELLA proteins inhibit BZR1 transcriptional activity by protein-protein interaction, whereas GAs relieve the repression by promoting DELLA

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degradation via the ubiquitin-proteasome mechanism, which allows BZR1 binding to target gene promoters.

In the case of ovule primordia formation in *Arabidopsis*, GAs and BRs act antagonistically; BRs promoting and GAs reducing ovule number. Although it has been proposed that BR action rely on the transcriptional regulation of several TFs, including *ANT*, the mechanism by which GAs participate is unknown. In this work, after showing that BRs also regulate ovule number in tomato, we focused in the possible interaction of GAs with the BR signaling pathway in tomato and *Arabidopsis*. Our findings pointed out two different scenarios, depending on the plant model used. In tomato, BRs regulate ovule number through the inhibition of GA biosynthesis and stabilization of DELLA proteins, which in turn promote ovule initiation. In contrast, GAs and BRs would participate independently in ovule initiation in *Arabidopsis*. Moreover, ANT would not mediate the increment in ovule number by BZR1 or DELLAs. Therefore, caution should be taken when inferring the molecular mechanism from one plant species to another, even though as in this case, the same set of hormones trigger similar responses in the two plant species.

RESULTS

BRs positively regulate ovule initiation in tomato

We had demonstrated that GAs control ovule number in the Micro-Tom (MT) cultivar of tomato (*Solanum lycopersicum*) (Gomez et al., 2018). Ovule number was reduced in GA-treated plants, in the DELLA lost-of-function mutant *procera* (a null allele of *PROCERA*, the only DELLA gene in tomato; Carrera et al., 2012), and in the transgenic line that constitutively overexpresses the GA biosynthetic gene *CcGA20ox1* from citrus. We aimed to test whether BRs also regulate ovule number in tomato. We used the MT variety, which harbors a mutation *dwarf* in the *DWARF4* gene, and the isogenic line carrying the wild type *DWARF* introgressed in MT (MT-*D*, thereafter). *DWARF4* (*CYP85A1*, *Solyc02g089160*) encodes a cytochrome P450 of the BR biosynthesis pathway; hence, the mutation of *DWARF4* in MT causes reduced BR levels and reduced plant height (Marti et al., 2006; Serrani et al., 2010). Taking advantage of these powerful genetic tools, we determined that BRs also control ovule number in tomato (Figure 1a-b). In agreement with the higher BR levels, MT-*D* produced more ovules than MT, implying that BR levels positively correlate with ovule number in tomato. Moreover, both MT and MT-*D* treated

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with BRs produced similar number of ovules per ovary, as the treatment induced an increase in ovule number especially in the MT background, complementing its reduced BRs levels (Figure 1a), as well as alleviating dwarfism. Therefore, we conclude that BRs also contribute to ovule primordia formation in tomato, as it was previously observed in *Arabidopsis*.

BRs control ovule number through the inhibition of GA biosynthesis in tomato ovaries

Next, we aimed to study whether BRs and GAs may interact in the control of ovule number in tomato. For this, we introgressed the *procera* mutation in MT-*D* and compared it with *procera* in the MT background. Interestingly, whereas MT-*D* produced more ovules than MT, no differences in ovule number were observed between MT and MT-*D* harboring *procera* (Figure 1a-b). Reduced ovule number was observed in *procera* plants regardless the presence of normal or low BR levels in MT-*D* or MT, respectively. Similarly, treatment with GAs alone or combined with BRs also suppressed the differences in ovule number in both MT and MT-*D* plants (Figure 1a). These analyses imply that DELLA activity is necessary for the BR-dependent regulation of ovule number in tomato. Therefore, GAs would act downstream of BRs in the control of ovule formation in tomato.

A plausible scenario is that BRs negatively regulate GA levels. Direct determination of GA levels in unpollinated tomato ovaries revealed that MT-*D* had lower bioactive GAs (GA_1 and GA_4) than MT (Figure 2a). Reduction in GA levels can be observed not only in the bioactive GAs but also in most of the precursors and degradation products (Table 1). These data strongly suggest that BRs could increase ovule number by directly down-regulation of GA levels.

Furthermore, we tested whether GA biosynthesis was also altered by BRs. We generated a translational fusion GUS reporter line (*pSlGA20ox1:SlGA20ox1-GUS*) incorporating promoter, exonic and intronic sequences of *SlGA20ox1*, a key gene in the GA biosynthesis pathway (Serrani et al., 2007). GUS activity was mainly localized in the placental tissue in ovaries at 9-10 days before anthesis (dba) (Figure 2b), when ovule primordia emerge (Figure S1). Moreover, BR treatment drastically reduced GUS activity in the placenta (Figure 2b), which is coincidental with the reduced GA levels in MT-*D* and the increase in ovule number observed in MT-*D* and BR-treated plants (Figure 1a). Regulation of *SlGA20ox1* expression by BRs could be mediated by the tomato BZR1; however, canonical Brassinosteroid Binding Responsive Element, previously identified in *Arabidopsis* (He et al., 2005), was not identified in the promoter sequence of the tomato *SlGA20ox1* gene.

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We noticed that the *procera* mutant, which has a deficient DELLA activity, did not alter ovule number in response to BRs (Figure 1a). If increased ovule initiation by BRs was due to the reduction of GA levels, overexpression of the GA20-oxidase activity would confer insensitivity to the BR-mediated decrease of GA levels. Therefore, ovule number reduction would not be observed. As shown in Figure 2c, BRs (comparing MT-*D* vs. MT) did not have a significant effect in ovule number in the *GA20ox1*-OE line that constitutively overexpresses the citrus *CcGA20ox1* gene (Garcia-Hurtado et al., 2012). This result supports the idea that BRs mediate ovule initiation in tomato by decreasing GA levels through the regulation of the *SlGA20ox1* in the placenta.

Hormonal regulation of tomato ovule number by BRs and GAs does not depend on *ANT*

ANT is a TF that has a clear role in ovule primordia formation (Cucinotta et al., 2014). It has been reported that BRs may control ovule number in *Arabidopsis* via the upregulation of *ANT* expression (Huang et al., 2013). Therefore, we analyzed whether BRs or GAs can also regulate *ANT* expression in tomato ovaries. As indicated in the Figure S2, neither BR nor GA exogenous application were able to modify the expression of the tomato orthologous gene *LeANT* (*Solyc04g077490*) in developing ovaries (between 6 to 9 dba), indicating that regulation of ovule number by BRs or GAs in tomato would not rely in changes in the expression of *ANT*.

GAs act independently of BRs to control ovule initiation in *Arabidopsis*

We next tested whether a similar interaction between BRs and GAs during ovule primordia formation also occurs in *Arabidopsis*. For this, we studied the effect on ovule number upon GA application in BR signaling mutants, BR application in GA signaling mutants, as well as the phenotype of the *gai-1* and *bzr1-1D* gain-of-function mutation combinations. As expected, *bzr1-1D* and *det2-1* mutants produced higher and lower ovule number respectively (Figure 3a). Interestingly, GA-treatment produced a similar reduction, approximately 25%, in ovule number in both *bzr1-1D* and *det2-1* mutants, and in the wild type plants (Figure 3a). On the other hand, treatment of wild type plants with 2,4-epibrassinolide (EBR), an active form of BRs, or brassinazole (BRZ), a specific inhibitor of BR biosynthesis, produced an increase or decrease in ovule number in the wild type plant, respectively, which mimicked the changes observed in *bzr1-1D* and *det2-1* mutants (Figure 3b). Importantly the effects of EBR and BRZ treatment on ovule number were similar in the GA mutant *gai-1* and in wild type plants (Figure 3b). These results

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imply that BRs and GAs would act independently in the determination of ovule number in *Arabidopsis*.

Moreover, the *gai-1* and *bzr1-1D* mutations had additive effect when combined (Figure 3c). Whereas *gai-1* or *bzr1-1D* produced a significant increase in ovule number (15-25%) individually, the combined *gai-1 bzr1-1D* mutant showed an additive effect, an increase in ovule number that was around 40%, very similar to the phenotype of the *gai-1* treated with EBR. Consequently, these results reinforce that BRs and GAs would act independently in the determination of ovule number in *Arabidopsis*. Taken together, our data strongly suport a regulatory mechanism of ovule number by BRs that is independent of GAs.

Increased ovule number per pistil could be due to increase in ovary length, which maintains ovule density in the placenta, rather than a specific increase in ovule density with no alteration in ovary length. In the pistil of *gai-1*, the significant increase in ovule number is accompanied by a slight increase in ovary length, resulting in increased ovule density (Gomez et al., 2018). To test whether BRs could also control ovule number independently of ovary length, we determined ovule number, ovary length and the ratio of ovule number to ovary length from the single and double-mutants of *gai-1* and *bzr1-1D* (Figure S3 and Table S1). While constitutive BR signaling in *bzr1-1D* produced a 15% increase in ovule number, it also had a slight increase (9%) in ovary length, resulting in a slight but significant (6%) increased ovule density (ratio of ovule number to ovary length). Similar effect was observed for *gai-1* in Col-0 background. Moreover, the combined *gai-1 bzr1-1D* mutant showed a strong increase in ovule number of about 43% and 15% increase in ovary length, which resulted in a significant increase of 15% in ovule density. Therefore, the activities of both dominant versions of GAI and BZR1 had a similar effect in promoting an increase in ovule number, but they have minor effect in ovary length. The data from this experiment also confirm the additive effect of both mutations in ovule number, ovary length, and ratio (Table S1). Whether the effect of the DELLA and BZR1 activities on ovary length is a direct consequence of the increased ovule number or it occurs via a different molecular mechanism not linked to ovule number is unknown and requires further study.

BRs promote an increase in GA levels in *Arabidopsis* inflorescences

It has been proposed that BRs and GAs coordinate growth by the BR-mediated up-regulation of GA biosynthesis genes to promote an increase of GA levels in seedlings (Tong et al., 2014; Unterholzner et al., 2015). We studied whether BRs also promote a change in GA levels in

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inflorescences. As shown in Figure 4a and Table 2, levels of the GA_4, the main bioactive GA in *Arabidopsis*, were significantly higher in *bzr1-1D* compared to the wild type, whereas GA_1 levels were not affected. Increased GA levels in *bzr1-1D* would be the consequence of the up-regulation of the expression of GA biosynthesis genes, especially *GA20ox3* and *GA3ox1* (Figure 4b). As a result of the increased GA levels, DELLA protein levels were reduced, as observed for the GFP-RGA levels upon treatment with EBR (Figure 4c). Interestingly, the increased GA level of *bzr1-1D* and the destabilized DELLA proteins upon EBR treatment should result in the reduction of ovule number, which was not the case. On the contrary, *bzr1-1D* and EBR treatment produced an increase in ovule number. Based on these data, we consider that the mechanism by which BRs regulate ovule number is not mediated by the regulation of GA levels.

GA-promotion of *Arabidopsis* ovule initiation does not rely on *ANT*

It has also been reported that the molecular mechanism of BRs signaling in ovule initiation is based on the activation of *ANT* expression in the placenta of developing pistils, being *ANT* a direct target of BZR1 (Huang et al., 2013). To further test whether *ANT* also participates in the GA-dependent ovule formation, we studied the regulation of *ANT* expression by GAs and the genetic interaction between *gai-1* and the strong null *ant-4* mutant. Interestingly, qPCR analyses revealed a similar up-regulation of *ANT* expression by both GAI and BZR1 (Figure 5a). Therefore, a plausible scenario is that GAI could mediate ovule primordia initiation by promoting the expression of *ANT*, as it was previously proposed for BRs (Huang et al., 2013). To prove whether an increased expression of *ANT* was sufficient to trigger an increase in ovule number, we generated an *ANT* overexpressing line, using the strong constitutive *35S* promoter. As reported previously (Mizukami and Fisher, 2000), overexpression of *ANT* caused an increase in the size of floral organs and ovules, but it also impaired anther dehiscence and hence fertility (Figure S4). Moreover, the number of ovules in the *35S:ANT* line was not affected, compared to the wild type plant (Figure 5b), as it occurs with other floral organs that were not altered in number by *ANT* ectopic expression (Mizukami and Fisher, 2000). Therefore, the increased *ANT* expression by BRs and GAI does not explain the increase in ovule number observed in *gai-1* and *bzr1-1D*.

Next, we analyzed the spatial expression pattern of *ANT* by *in situ* mRNA hybridization in two opposite GA mutants, *gai-1* and *global della* (null mutant of the five *Arabidopsis* DELLA genes: *gaiT6 rgaT2 rgl1-1 rgl2-1 rgl3-1*), which produced high and low ovule number, respectively (Figure 5c-d). The spatial expression pattern of *ANT* was not altered in either *gai-1* or

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global della mutants, being the expression localized in the placenta tissue, prior to the initiation of ovule primordia formation at stage 8 of pistil development (Elliott et al., 1996; Schneitzet al., 1998). We also analyzed ANT protein levels using a translational fusion ANT-YPet driven by its own promoter in a *pANT:ANT-YPet* transgenic line. The expression of *ANT-YPet* was able to complement the *ant-4* mutant allele. Inflorescences of *ant-4* plants showed defects in flower development like those reported in other *ant* mutants (Elliott et al., 1996; Klucher et al., 1996; Baker et al., 1997), which include reduced number and width of the four whorls (Figure S5a-b), as well as alteration of ovule morphology causing complete sterility (Figure S5c-e) (Baker et al., 1997). Expression of the *ANT-YPet* fully restored normal flower and ovule development, as well as fertility (Figure S5). ANT-YPet was localized in the placental tissue at early developmental stages, prior to ovule initiation, or in ovule primordia (Figure 5e-g). Treatment of inflorescences with GAs or Paclobutrazol (PCB), an inhibitor of GA biosynthesis, did not alter ANT-YPet protein levels (Figure 5e-f). In addition, levels and localization of ANT-YPet did not differ in the *gai-1* mutant and wild type plants (Figure 5g). Overall, our data indicated that neither GAs nor DELLA proteins significantly regulate levels or pattern of *ANT* expression at the mRNA or protein level, despite the small increase in the qPCR observed in *gai-1*.

Finally, we also evaluated whether the *gai-1* mutation requires ANT function to promote the increase in ovule number. *gai-1* was not able to significantly alleviate the developmental deffects caused by *ant-4* mutation in the inflorescences, pistils or ovules (Figure 6a-d) (Elliott et al., 1996; Klucher et al., 1996; Baker et al., 1997). More importantly, *gai-1* was not able to increase ovule number in *ant-4* (Figure 6e). The strong allele *ant-4* caused a 60% reduction in ovule number, and similar reduction was also observed in the *ant-4 gai-1* double mutant. In summary, our findings strongly imply that promotion of ovule primordia emergence by DELLA proteins in *Arabidopsis* is independent of BRs, and does not rely on changes in *ANT* expression. Despite that, ANT activity is required for GA effects in ovule initiation.

DISCUSSION

In this work, we have shown that BRs positively participate in the control of ovule number in tomato, just like they do in *Arabidopsis*. MT-*D* plants, with higher BR levels, produce more ovules than the BR-deficient MT plants, and MT plants treated with BRs increased ovule number. Moreover, in tomato, there is a direct interaction of GAs and BR in the control of ovule

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primordia. BRs would mediate ovule initiation by decreasing GA levels through the downregulation of GA biosynthesis. This causes the stabilization of DELLA proteins that will finally promote ovule primordia initiation. In contrast, *Arabidopsis* both GAs and BRs would regulate ovule number independently. These results led us to reveal two different molecular mechanisms by which BRs and GAs antagonistically control the same developmental process, ovule primordia initiation, in two key model plant species, *Arabidopsis* and tomato. In both species, GAs limit the number of ovules by the degradation of DELLA proteins, which act as positive factors (Gomez et al., 2018). BRs also positively regulate ovule number in *Arabidopsis* (Huang et al., 2013) and tomato (this paper).

Interaction of BRs and GAs to control plant development

GAs and BRs regulate many aspects of plant growth and development. In most cases, both hormones act cooperatively. For example, in the photomorphogenesis-related hypocotyl elongation in *Arabidopsis*, BRs up-regulate the expression of *GA20ox1* and *GA3ox1* involved in GA biosynthesis, which results in increased GA levels (Unterholzner et al., 2015). On the other hand, DELLA proteins bind directly to BZR1, preventing its binding to target promoters, thus blocking BZR1-mediated transcriptional activity (Bai et al., 2012; Gallego-Bartolome et al., 2012; Li et al., 2012). Both mechanisms are not mutually exclusive; as they act simultaneously in a strong feed-forward loop mode, at least in *Arabidopsis*. GAs and BRs also cooperate in the promotion of shoot elongation in tomato (Marti et al., 2006), and the *procera* mutation enhanced growth in plants with low (MT) and high (MT-*D*) BRs content (Carrera et al., 2012).

On the contrary, antagonistic functions of GAs and BRs, similar to those involved in the control of ovule initiation, have also been reported. In rice roots, BRs favored fungal infection, whereas GA treatment enhanced resistance in a concentration-dependent manner (De Vleesschauwer et al., 2012). In this case, BRs promote a reduction of GA levels. More recently, Xiao et al. (2017) stated that the BR-mediated GA repression and growth inhibition in rice is due to the activity of OFP1, which inhibits the expression of the GA biosynthesis genes. In addition to rice, down-regulation of GA levels by BRs have also been reported in tomato, pea and sunflower (Jager et al., 2005; Kurepin et al., 2012; Li et al., 2016).

In tomato ovaries, BRs regulate ovule number through GAs. For example, BRs (comparing MT with MT-*D* or upon BR treatment) can only modulate ovule number in plants with normal GA signaling, but not in plants treated with GAs or in the *procera* mutant, which have

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activated GA response. In tomato unpollinated ovaries, the levels of the bioactive GAs are higher in MT to those from MT-*D*, suggesting that BRs have a negative role in GA biosynthesis through the downregulation of *SlGA20ox1* expression at early phases of ovary development, when *DWARF* is highly expressed (Montoya et al., 2005). The repression of GA biosynthesis by BRs may not be directly controlled by BRZ1, as the canonical Brassinosteroid Binding Responsive Elements of *Arabidopsis* (He et al., 2005) are not present in the promoter of the tomato *SlGA20ox1*. Nonetheless, it is also possible that a tomato-specific *cis*-element, different from the motif described in *Arabidopsis*, could be responsible for the binding of BRZ1 to the *SlGA20ox1* promoter. Taken together, we have clearly demonstrated that, in tomato, BRs control ovule number by reducing the GA levels in the placenta, through the repression of *SlGA20ox1* expression, thus stabilizing PROCERA that would finally promote ovule primordia emergence.

On the contrary, in *Arabidopsis* we have shown that there is no interaction between these hormones during ovule initiation, as GAs reduced ovule number regardless the BR content, or BRs can promote ovule number increase in plants with constitutive or impaired GA signaling (upon GA treatment or in the *gai-1* mutant, respectively). Interestingly, *bzr1-1D* promotes an increase in GA synthesis and, therefore, the destabilization of DELLA proteins in the inflorescences. If BRZ1 would regulate ovule number by promoting GA biosynthesis, a decrease in ovule number in *bzr1-1D* would be observed, which is not the case. Therefore, either the increase in GA levels is not localized in the placenta at ovule primordia initiation, or the additional effects of BRs are stronger and can overcome the effect of GAs. In the latter case, a synergistic effect in the *gai-1 bzr1-1D* would be observed. Since the number of ovules in *gai-1 bzr1-1D* is not synergistic but additive, it is presumed that the increase of GAs observed in the *bzr1-1D* does not localize in the tissue where the ovules are formed, and that there is an independent mechanism of ovule initiation by BRs and GAs in *Arabidopsis*.

Two different molecular mechanisms to control ovule primordia formation by GAs and BRs in *Arabidopsis* and tomato

Based on the data from tomato and *Arabidopsis* presented here, we propose a working model involving GAs and BRs in the determination of ovule number (Figure 7). In *Arabidopsis*, BRs and GAs act independently and antagonistically in ovule initiation, being the activity of both BZR1 and GAI positive factors (Figure 7a). GAI, and probably other DELLA proteins, would bind to unknown TFs to modulate expression of genes that mediate the GA-dependent ovule

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initiation pathway. On the other hand, BRs activate BZR1 activity to promote the formation of the ovule primordia. Although both *gai-1* and *bzr1-1D* showed increase in *ANT* expression in inflorescences, ovule number was not altered in *35S:ANT* plants (discussed below).

In tomato, GAs would act downstream of BRs (Figure 7b). BRs would activate the tomato orthologs of BZR1 or BES1 TFs to inhibit the expression of *SlGA20ox1* gene involved in the GA biosynthesis in flowers and fruits (Olimpieri et al., 2007; Serrani et al., 2007). Sequence analysis of *SlGA20ox1* did not reveal the presence of BR response elements in its promoter, suggesting that *SlGA20ox1* may represent an indirect target of BR signaling during ovary development. Because of the down-regulation of *SlGA20ox1*expression, GA levels were reduced, promoting the stabilization of PROCERA and hence an increase in ovule number. In the absence of PROCERA (either in the *procera* mutant, in plants treated with GAs, or in plants overexpressing the *CcGA20ox1* gene), BRs are not able to regulate ovule number, implying that PROCERA acts downstream of BRs in the regulation of ovule number in tomato. An alternative mechanistic model in tomato can also be foreseen. As it has been reported that *Arabidopsis* BZR1 and DELLA can form a protein complex (Bai et al., 2012; Gallego-Bartolome et al., 2012; Li et al., 2012), tomato BZR1 could bind to PROCERA. Binding would not block BZR1 transcriptional activity as it does in *Arabidopsis*, but instead the PROCERA-BZR1 complex would coordinate the expression of genes involved in ovule initiation. Finally, BRs would also inhibit GA biosynthesis to stabilize PROCERA, which in turn would strengthen the complex.

ANT would not be related to BR or GA pathways in ovule initiation

ANT is a key factor of the genetic control of carpel margin meristem formation, and a master regulator of ovule primordia initiation (Klucher et al., 1996; Galbiati et al., 2013; Cucinotta et al., 2014). In addition to defects in ovule development, *ant* mutants show a strong reduction in the number of ovules per carpel (Elliot et al., 1996; Liu et al., 2000; Azhakanandam et al., 2008; Galbiati et al., 2013). It was proposed that BRs influence ovule development by regulating the transcription of genes such as *HLL*, *AP2*, and *ANT* (Huang et al., 2013), with *HLL* and *ANT* being induced and *AP2* being repressed by BRs. Hence, *AP2* and *ANT* would be direct targets of *BRZ1*, whereas *HLL* is regulated indirectly. Our data, however, clearly indicated that *ANT* is not directly related to the increase in ovule number neither in *bzr1-1D* nor in *gai-1* mutant backgrounds. Although both mutants showed slight but significant increase in *ANT* expression in inflorescences

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by qPCR analysis, GAs did not promote changes in *ANT* expression or ANT-YPet protein levels or distribution in the pistil during ovule initiation. Besides, the constitutive expression of *ANT* in the *35S:ANT* lines did not alter ovule number. This implies that the increase in ANT activity is not sufficient to promote an increase in ovule number, therefore could not be the cause of the increased ovule number in *bzr1-1D*.

Nonetheless, ANT activity seems to be necessary in promoting ovule primordia formation. *gai-1* mutant cannot mitigate the ovule phenotype of *ant-4* since ovule number and ovule development arrest are identical in *ant-4* and in the double mutant *gai-1 ant-4*. Interestingly, it has been proposed that ANT activity, *per se*, is not absolutely required for ovule initiation, as ovule primordia are initiated and continue to develop until the time of integument initiation in *ant* mutants (Azhakanandam et al., 2008). Most probably ANT is required for proper placenta development, being a major regulatory player superimposed to other factors, such the GAs and BRs in ovule primordia initiation.

In summary, our results provide the first detailed analysis of the molecular mechanism of BRs and GAs interaction in ovule initiation in two widely-used plant model systems, *Arabidopsis* and tomato. These two species have completely different interactions between BRs and GAs in ovule initiation. Besides, ANT is probably not related mechanistically with BR or GA pathways in this process. These experimental evidences add one more layer of complexity in the working model of the gene network that governs the determination of ovule number and ovule primordia emergence. Finally, the fact that these two plant species show apparently different mechanism in the regulation of ovule initiation strongly imply that caution should be taken when transfer of knowledge from one model system to another is done without proper examination.

MATERIALS AND METHODS

Plant material assays

Arabidopsis thaliana plants used were in the L*er* or Col-0 backgrounds as indicated. Seeds were sterilized in ethanol and germinated in MS media plates (Murashige and Skoog, 1962) (Duchefa Biochemie, Haarlem, The Netherlands) for 4 days at 4°C in the dark, followed by 7-8 days at 22°C in long day photoperiod (16/8h). Seedlings were then transferred to soil and grown in

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a chamber at 22°C in long day photoperiod (16/8h). All chemicals and oligos were purchased from Sigma-Aldrich (Madrid, Spain) or Integrated DNA Technologies (IDT, Coralville, Iowa, USA), respectively, unless otherwise is stated.

Arabidopsis DELLA mutants were previously described in Gomez et al. (2016). *ant-4* (Baker et al., 1997), *bzr1-1D* (Wang et al., 2002) and *det-2* (Chori et al., 1991) were obtained from the European Arabidopsis Stock Centre (NASC, http://arabidopsis.info/). *pRGA:GFP-RGA* (Silverstone et al., 2001) was obtained from Dr. Tai-ping Sun (Duke University, USA). *gai-1* mutant allele, originally in L*er*, was transferred to Col-0 background by means of three consecutive backcrosses. The combined *gai-1 bzr1-1D* mutant in Col-0 background was obtained by genetic cross between introgressed *gai-1* in Col-0 to *bzr1-1D*.

Plants of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom (MT), MT-*D* carrying the *DWARF* allele (*D*) (Carvalho et al., 2011), the *procera* mutant (Carrera et al., 2012), and GA-overproducing transgenic *CcGA20ox1* plants (Garcia-Hurtado et al., 2012) were used. Plants were grown in the greenhouse at 24°C day and 20°C night. Natural light was supplemented with LED lamps to get a 16h light photoperiod.

Hormonal treatments and ovule number determination

Ovule number in *Arabidopsis* was determined as described in Gomez et al. (2018). GA treatment was applied by watering every other day with 20 µM of GA_4+GA_7 (Duchefa Biochemie) and ovules were counted after two weeks of treatment. EBR and BRZ treatments were carried out by spraying for 5-8 consecutive days with either 2 µM of EBR (2,4-epibrassinolide, Apollo Scientific) or 2 µM of BRZ (TCI Chemicals), all in 0.01% (v/v) Tween 20 as wetting agent. Mock solutions consisted of an equivalent dilution of methanol and Tween 20, as both chemicals are dissolved as 1 mM in methanol. Ovules were determined after 7-10 days of the first treatment. Ovary size was determined in the same pistils used for ovule number determination, from images taken under a stereomicroscope.

Treatments of *pANT:ANT-YPet* plants were carried out by floral dip, immersing the primary inflorescence for 5-10 seconds in either 20 µM of GA_4+GA_7 or 1 µM of PCB (Duchefa Biochemie), all in 0.01% (v/v) Tween 20. Mock solutions consisted of an equivalent dilution of ethanol for GAs or acetone for PCB, both supplementad with Tween 20. After 3h, the inflorescences were harvest and hand dissected to visualize the placenta of developing pistils under a ZEISS LSM 780 confocal microscope.

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Ovule number in tomato was determined as described in Gomez et al. (2018). GA treatment was applied by watering 3 times per week or sprayed every day with 10 µM GA_3 (Duchefa) and 0.1% (v/v) Tween 20 or applied to the roots in the nutrient solution. BR treatment was carried out with a 10 nM solution of New Brassinosteroid with surfactant (TAMA biochemical) by spraying every day. Mock solutions consisted of an equivalent dilution of methanol and Tween 20. Ovules were counted after three weeks of treatment in ovaries of flowers from 9 dba.

Construction of *pANT:ANT-YPet* and *35S:ANT* and plant transformation

pANT:ANT-YPet translational fusion was generated by bacterial homologous recombination system (recombineering) using a modified variety of the pBALU6 (Tursun et al., 2009). Recombineering-based DNA modification was done basically as described in Brumos et al. (2019), using universal adaptors at the 5' and 3' of the recombineering cassette. Oligonucleotides used are described in Table S2. A large genomic fragment containing *ANT* locus (*At4g37750*) in the JAtY57K20 TAC clone was used to introduce a variety of the YPet fluorescent protein at the Ct of *ANT* coding sequence. Modified JAtY57K20 clone with the YPet tag was trimmed at both ends to reduce clone length to stabilize the binary clone and facilitate transformation (Brumos et al., 2019). For the generation of *35S:ANT* lines, the cDNA of the *ANT* gene in the pDONR201vector was obtained from the REGIA collection (Paz-Ares and REGIA Consortium, 2002). The cDNA product was transferred to pMDC32 binary vector by a LR Gateway reaction, and the construct was confirmed by sequencing. Finally, *pANT:ANT-YPet* and *35S:ANT* were introduced in planta by *Agrobacterium*-mediated floral-dip transformation (Clough and Bent, 1998). In the case of *pANT:ANT-YPet*, the selected transgenic line was crossed to *ant-4* to confirm that it complements the *ant-4* phenotypes. In the case of the *35S:ANT*, the phenotype produced by overexpression of *ANT* was similar to that described previously by Mizukami and Fisher (2000).

Construction of the *pSlGA20ox1:SlGA20ox1-GUS* line and tomato plant transformation

The *pSlGA20ox1:SlGA20ox1-GUS* reporter gene is a translational fusion (*pSlGA20ox1-TL-GUS*) comprising the promoter, exonic and intronic sequences of *SlGA20ox1* (*Solyc03g006880*) up to the beginning of the third exon, spliced in frame with the *GUS* reporter gene. A 2598 bp region of *SlGA20ox1* gene consisting of the promoter (from -1387 bp 5′ to the start translation codon) and the primary transcribed region (up to a position 30 bp into the third exon) was

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amplified by PCR from MT genomic DNA, using the primers described in Table S2. This fragment was cloned in frame with the *GUS* gene (containing the SV40 nuclear localization signal) into the *Xho*I and *BamH*I sites of the pBJ60 shuttle vector, from pART7 (Gleave, 1992). The *pSlGA20ox1:SlGA20ox1-GUS* cassette was inserted as a *Not*I fragment into the T-DNA of binary vector pART27 (Gleave, 1992). The construct was introduced into the *Agrobacterium* strain LBA4404 and used to transform MT plants, following the method described in García-Hurtado et al. (2012).

qPCR analysis of gene expression

Gene expression analysis was carried out by qPCR as described in Dorcey et al. (2009) in *Arabidopsis* inflorescences or tomato ovaries at 9-6 dba. Total RNA was extracted with NucleoSpin® RNA Plant (Macherey-Nagel) or RNAqueous-4PCR (Ambion) for *Arabidopsis* and tomato, respectively. cDNA was synthesized using PrimerScript™ 1st strand cDNA Synthesis Kit (TaKaRa). qPCR was performed with the TB Green Premix Ex Taq II (Tli RNase H Plus) (TaKaRa) with a 7500 Fast Real-Time PCR System (Thermo Fisher). The oligonucleotides used (Table S3) 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 genes *UBQ10* (*At4g05320*) in *Arabidopsis* or *Actin-52* (*Solyc10g080500*) in tomato, and the data were normalized by the $\Delta\Delta$Ct method as indicated in each figure legend.

Histological procedures

For the determination of ovule number, tissue sections ovaries from tomato were fixed in 4% (w/v) paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.2. After dehydration in ethanol, the samples were embedded in paraffin (Paraplast Plus; SigmaAldrich). Thick sections (8 μm) were stained with Safranine-Alcian blue solution (a mixture of 2 mL of 0.1% [w/v] Safranine in 50% [v/v] ethanol and 5 mL of 0.1% [w/v] Alcian blue in 50% [v/v] ethanol, diluted in 200 mL of 0.1 M acetate buffer, pH 5.0), viewed with a microscope, and photographed with a spot digital camera (DMX1200F; Nikon).

GUS assay was carried out basically as previously described in Carbonell-Bejerano et al. (2010). The $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ concentrations were adjusted to 2.5 mM to obtain optimal signal. After GUS assay, tomato ovaries were stained following a modified pseudo-Schiff propidium iodide (mPS-PI) technique (Truernit et al., 2008), as described in Gomez et al. (2016).

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Images were captured using a ZEISS LSM 780 confocal microscope with a MBS T80/R20 dichroic (561 nm and 545-570 nm excitation and reflection, respectively). PI staining was excited at 561 nm and detected at 580-660 nm. The same confocal microscope was used to detect *ANT-YPet* with excitation at 514 nm and emission at 517-565 nm.

In situ RNA hybridization

A 516 bp cDNA fragment of *ANT* that excludes the AP2 domain was amplified from inflorescences, using oligos described in Table S2, and cloned into the pGEM-T Easy vector (Promega). Sense and antisense DIG-labeled RNA transcripts were synthesized using the corresponding SP6 and T7 RNA polymerases in the vector. Inflorescences were embedded, sectioned and hybridized as described by Weigel and Glazebrook (2002). No significant signal was detected using the sense probe. Images were obtained using a Nikon Eclipse E600 microscope and a Nikon Digital-Sight (DS-Ri) camera.

Quantification of GAs

Inflorescences of *Arabidopis* of Col-0 and *bzr1-1D* plantswere collected, once flowers at anthesis and 2-3 younger floral buds were removed. In tomato, 6 dba ovaries were collected from MT and MT-*D*. Three biological replicas were harvested and analyzed. Plant material were grinded in a mortar with liquid nitrogen, and 50 mg of frozen material were extracted with 80% (v/v) methanol and 1% (v/v) acetic acid including 17-$^{2}H_2$-labeled GA internal standards (Olchemim) and mixed by shaking for one hour at 4ºC. GA levels were quantified as described in Seo et al. (2011) and Gomez et al. (2018).

Western blot analysis of GFP-RGA

Levels of the GFP-RGA protein were determined from inflorescences of the transgenic pRGA:GFP-RGA line (Silverstone et al., 2001). Upon bolting, adult plants were treated with either mock, GAs (20 µM), EBR (2 µM) or BRZ (2 µM) by spray. Inflorescences were collected 4h later, once flowers at anthesis as well as 2-3 floral buds were removed. Total protein from 50 mg of frozen material was extracted in 1 vol of 2x Laemli buffer (0.25 M Tris·HCl, pH 6.8, 10% [w/v] SDS, 25% [v/v] glycerol, 0.75% [w/v] bromophenol blue). Protein levels were determined with the DC^{TM} Protein Assay method. Total protein (20 µg) were loaded into SDS-PAGE 12.5% (w/v) acrylamide gel and run for 2-3h at 120V. Proteins in the gel were then transferred by

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blotting to a membrane (Amersham™ Hybond™ 0.2 µm PVDF). Anti-GFP mouse Living Colors ® A.v. Monoclonal Antibody (JL8) (TaKaRa) and anti-mouse AmershamECL Mouse IgG (GE Healthcare) antibodies were used to detect GFP-RGA. Signal was detected using SuperSignal™ West FemtoMaximun Sensitivity Substrate and images were recorded in a Fujifilm LAS3000 Imager.

AUTHOR CONTRIBUTIONS

D.B-T., M.D.G., P.T., J.P-R, and M.A.P-A. performed experiments in *Arabidopsis*. J.B., E.C., and I.L-D. performed experiments in tomato. O.R-R. carried out the construct of the *pSlGA20ox1:SlGA20ox1-GUS* line in tomato. J.M.A. helped in the generation of the recombineering ANT-YPet construct. M.D.G. and M.A.P-A. designed the study and wrote the manuscript. All authors commented on the manuscript.

DATA AVAILABILITY STATEMENT

The article's supporting data and materials used or generated in this study can be accessed upon request.

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CONFLICT OF INTERESTS

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

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SUPPORTING INFORMATION

Figure S1. Ovule development in tomato MT.

Figure S2. *ANT* expression is not altered in tomato ovaries.

Figure S3. BRs and GAs control ovule number independently of ovary length in *Arabidopsis*.

Figure S4. Overexpression of *ANT* increases organ size and impairs anther dehiscence in *Arabidopsis.*

Figure S5. Complementation of developmental defects of *ant-4* by the *pANT:ANT-YPet* line of *Arabidopsis*.

Table S1. Both *gai-1* and *bzr1-1D* regulate ovule number and ovary length.

Table S2. Oligo nucleotides used as primers for constructs and probes generation.

Table S3. Oligo nucleotides used for qPCR analysis.

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TABLES

Table 1. Level of GAs in the non and early C-13 hydroxylation pathways (ng·gFW^{-1})

	Non C-*13 hydroxylation*						
	GA_{12}	GA_{15}	GA_{24}	GA_9	GA_{51}	**GA_4**	GA_{34}
MT	1.43±0.1	1.02±0.0	0.92±0.0	2.86±0.0	0.99±0.0	**0.54±0.0**	12.65±0.0
MT-*D*	1.18±0.0*	0.66±0.0*	0.51±0.0*	0.95±0.0*	0.78±0.0*	**0.41±0.0****	7.24±0.1*
	Early C-*13 hydroxylation*						
	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_{29}	**GA_1**	GA_8
MT	5.27±0.1	1.3±0.0	83.77±2.16	1.88±0.2	1.92±0.0	**0.46±0.05**	14.11±0.5
MT-*D*	4.62±0.2*	1.18±0.0*	63.34±0.9*	1.55±0.0*	2.0±0.1*	**0.33±0.0***	7.23±0.2*

*, Significant differences with MT (Student's *t*-test analysis, *P* <0.01).

In bold, the bioactive GAs (GA_4 and GA_1).

Table 2. Levels of GAs in the *Arabidopsis bzr1-1D* (ng·gFW^{-1})

	Non C-13 hydroxylation						
	GA_{12}	GA_{15}	GA_{24}	GA_9	GA_{51}	**GA_4**	GA_{34}
Col-0	4.23±0.29	0.60±0.02	3.42±0.34	5.78±0.37	1.03±0.16	**6.74±0.07**	3.31±0.22
bzr1-1D	3.00±0.12*	0.68±0.05	3.06±0.24	5.05±0.38	0.71±0.05	**11.30±0.90***	4.46±0.30*
	Early C-13 hydroxylation						
	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_{29}	**GA_1**	GA_8
Col-0	0.34±0.03	0.23±0.00	1.51±0.14	0.05±0.00	nd	**0.37±0.04**	0.09±0.02
bzr1-1D	0.28±0.01	0.26±0.03	3.02±0.36*	0.04±0.01	nd	**0.36±0.02**	0.16±0.01*

*, Significant differences with Col-0 (Student's *t*-test analysis, *P* <0.01).

In bold, the bioactive GAs (GA_4 and GA_1). nd, not detected.

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FIGURE LEGENDS

Figure 1. Ovule number in tomato is regulated by BRs in a GA-dependent manner.
(a) Ovule number per ovary in non-treated (Mock), BR-treated (+BRs), the *procera* mutant, GA-treated (+GAs), and BR/GA-treated (+GAs+BRs) plants of the MT and MT-*D* backgrounds. Ovule number was determined from at least six ovaries, and the experiment was repeated three times, with similar results. Data represents mean ± SD. Values were normalized to those in mock-treated MT-*D*. Letters above each box indicate statistical significance as determined by an ANOVA with a Bonferroni post hoc test for multiple comparisons. Data that are not significantly different are marked with the same letter. (b) Images of medial sections of ovaries of MT and MT-*D* plants carring the wild type PROCERA and the *procera* mutant allele. Scale bar represents 200 µm.

Figure 2. BRs repress GA biosynthesis in tomato ovaries.
(a) Levels of bioactive GAs (GA_1 and GA_4) in ovaries of flowers at 5 dba of MT and MT-*D*. GA quantification was determined from three biological samples corresponding to 100 mg FW. Data represents mean ± SD. Asterisks represent significant differences (Student's *t*-test analysis) with the MT-*D* ($P < 0.01$). (b) Expression of *SlGA20ox1* in ovaries of the *pSlGA20ox1:SlGA20ox1-GUS* line at 9-10 dba. Scale bars represent 100 µm. An outline the reporter construct is shown. White boxes represent promoter sequence, black boxes are *SlGA20ox*1 exonic sequence, and blue box is the *UidA* coding sequence (*GUS*). Breaks indicate intronic sequence. (c) Ovule number per ovary in the line overexpressing the citrus *CcGA20ox1* gene (*GA20ox*-OE) in the MT-*D* and MT backgrounds. Ovule number was determined from at least six ovaries, and the experiment was repeated three times, with similar results. Data represents mean ± SD. Values were normalized to those in mock-treated MT-*D*. Letters above each box indicate statistical significance as determined by an ANOVA with a Bonferroni post hoc test for multiple comparisons. Data that are not significantly different are marked with the same letter.

Figure 3. Ovule number is regulated by both BRs and GAs in *Arabidopsis*.

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(a) Ovule number per pistils in non-treated (Mock) or GA_{4+7}-treated (+GAs) plants of Col-0 and in the BR mutants *bzr1-1D* and *det2-1*. (b) Ovule number per pistil in non-treated (Mock), 24-epibrassinolide (EBR)-, or brassinazole (BRZ)-treated plants of L*er* and the GA mutant *gai-1*. (c) Ovule number in *bzr1-1D*, *gai-1*, and the double *bzr1-1D gai-1* mutants. More than 15 pistils from different plants were measured per mutant or treatment, and the experiments were repeated three times with similar results. Data represents mean ± SD. Values were normalized to those in Col-0/L*er* mock. Letters above each box indicate statistical significance as determined by an ANOVA with a Bonferroni post hoc test for multiple comparisons. Data that are not significantly different are marked with the same letter.

Figure 4. BRs up-regulate GA biosynthesis in *Arabidopsis* inflorescences.

(a) Levels of the bioactive GAs (GA_1 and GA_4) in inflorescences of Col-0 and *bzr1-1D*. Significant differences (Student's *t*-test analysis) are indicated (a, $P < 0.001$). Data are the mean ± SD of three independent samples, expressed as ng of GA per g of FW. For representation purposes, the levels of GA_1 were multiplied 10-fold. (b) Expression of GA biosynthesis genes in inflorescences of Col-0 and *bzr1-1D*. qPCR expression analysis was carried out for *GA20ox1* to *GA20ox5* and *GA3ox1* to *GA3ox4* in inflorescences. Expression of *GA20ox4*, *GA20ox5*, and *GA3ox3* was not detected. Expression was normalized to that of *UBQ10* (*At4g05320*) in Col-0. Data are the mean ± SD of three biological replicas. Asterisks represent significant differences (Student's *t*-test analysis) with the Col-0 ($P < 0.05$). (c) Levels of GFP-RGA in plants treated with GAs, EBR, or BRZ. Western blot analysis was carried out in inflorescences of the *pRGA:GFP-RGA* plants treated for 4h with 20 μM GA_{4+7}, or 2 μM of EBR or BRZ. RuBisCo was used as a loading control by Ponceau staining.

Figure 5. Regulation of *ANT* expression by BRs and GAs and ovule number in *35S:ANT* plants in *Arabidopsis*.

(a) Expression of *ANT* in inflorescences of Col-0, *bzr1-1D*, L*er*, and *gai-1*. Expression was normalized to that of *UBQ10* (*At4g05320*) in the corresponding Col-0/L*er* control. Data are the mean ± SD of three biological replicas. (b) Ovule number per pistil in *35S:ANT* plant. More than 15 pistils from different plants were measured per line, and the experiments were repeated three times with similar results. Data represents mean ± SD, and values were normalized to those in Col-0. (c-d) *In situ* mRNA hybridization of *ANT* transcripts in *gai-1*, L*er*, and *global della* mutant

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at early pistil development before ovule primordia initiation and stage 1-I (c) and ovule primordia at stage 1-II/2-I (d). (e-f) Levels of ANT-YPet protein in the placenta (e) or ovules at stage 1-I/1-II (f) of the *pANT:ANT-YPet* line upon 3h-treatment with PCB, mock, or GAs. (g) Levels of ANT-YPet protein in ovules at stage 1-I/1-II of the *pANT:ANT-YPet* line in L*er* or *gai-1* background. In (a) and (b), asterisks represent significant differences (Student's *t*-test analysis) with the Col-0/Ler ($P < 0.01$). Scale bars represent 20 µm in (c), (e), (f), and (g), and 40 µm in (d). O, ovule primordium; P, placenta; V, valve.

Figure 6. Ovule defects in *ant-4* are not alleviated by *gai-1*.

(a-c) Images of inflorescences (a), flowers (b), and pistils at anthesis (c) of L*er*, *ant-4*, *gai-1*, and the double *ant-4 gai-1*. (d) Images of mature ovules of L*er*, *ant-4*, *gai-1*, and double *ant-4 gai-1* mutant. Scale bars represent 2 mm in a and b, 1 mm in c, and 50 µm in d. (e) Ovule number per pistils in L*er*, *ant-4*, *gai-1*, *ant-4 gai-1* +/-, and *ant-4 gai-1* -/-. More than 15 pistils from different plants were measured per mutant or treatment, and the experiments were repeated three times with similar results. Data represents mean ± SD. Values were normalized to those in L*er*. Letters above each box indicate statistical significance as determined by an ANOVA with a Bonferroni post hoc test for multiple comparisons. Data that are not significantly different are marked with the same letter.

Figure 7. Working model for the interaction between GAs and BRs in the regulation of ovule number in *Arabidopsis* and tomato.

(a) Molecular mechanism of regulation of ovule number by BRs and GAs in *Arabidopsis*. (b) Molecular mechanism of regulation of ovule number by BRs and GAs in tomato. See Discussion section for detailed description.

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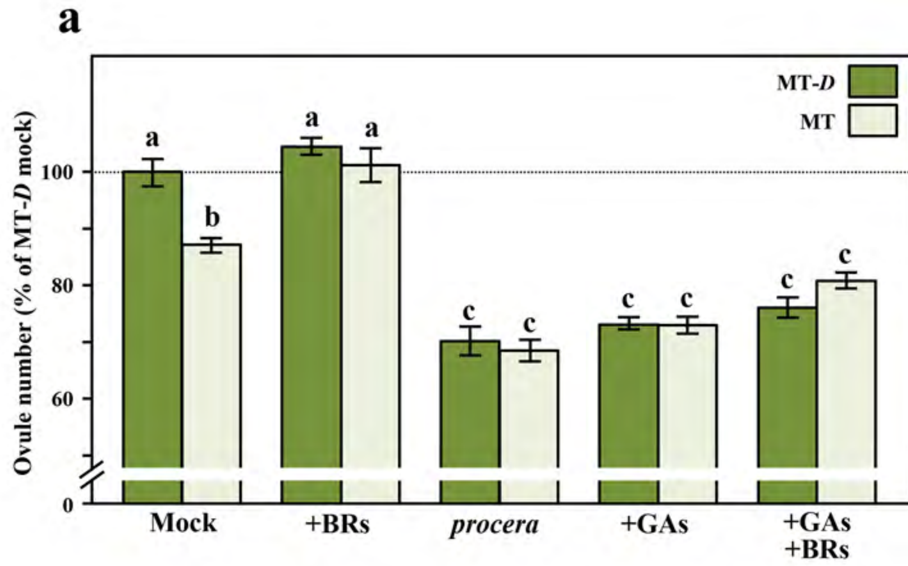

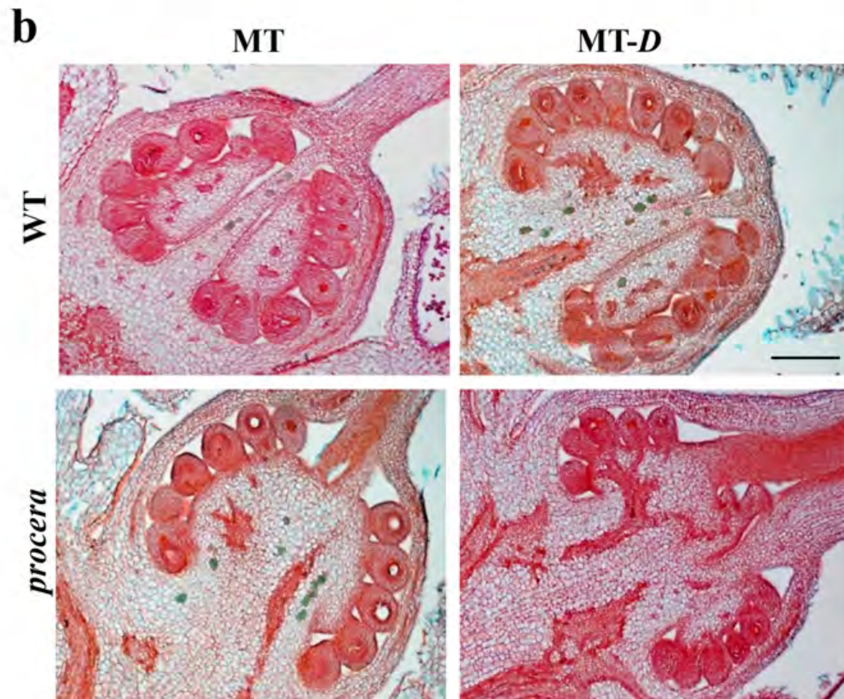

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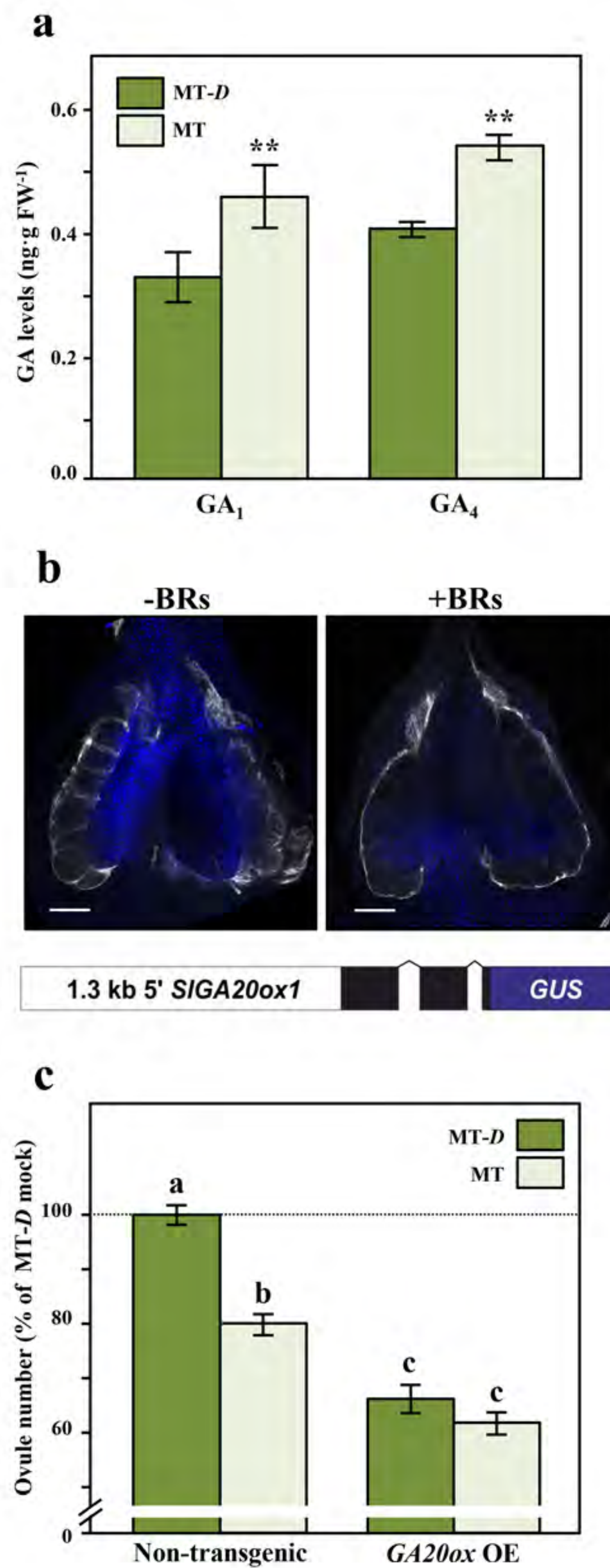

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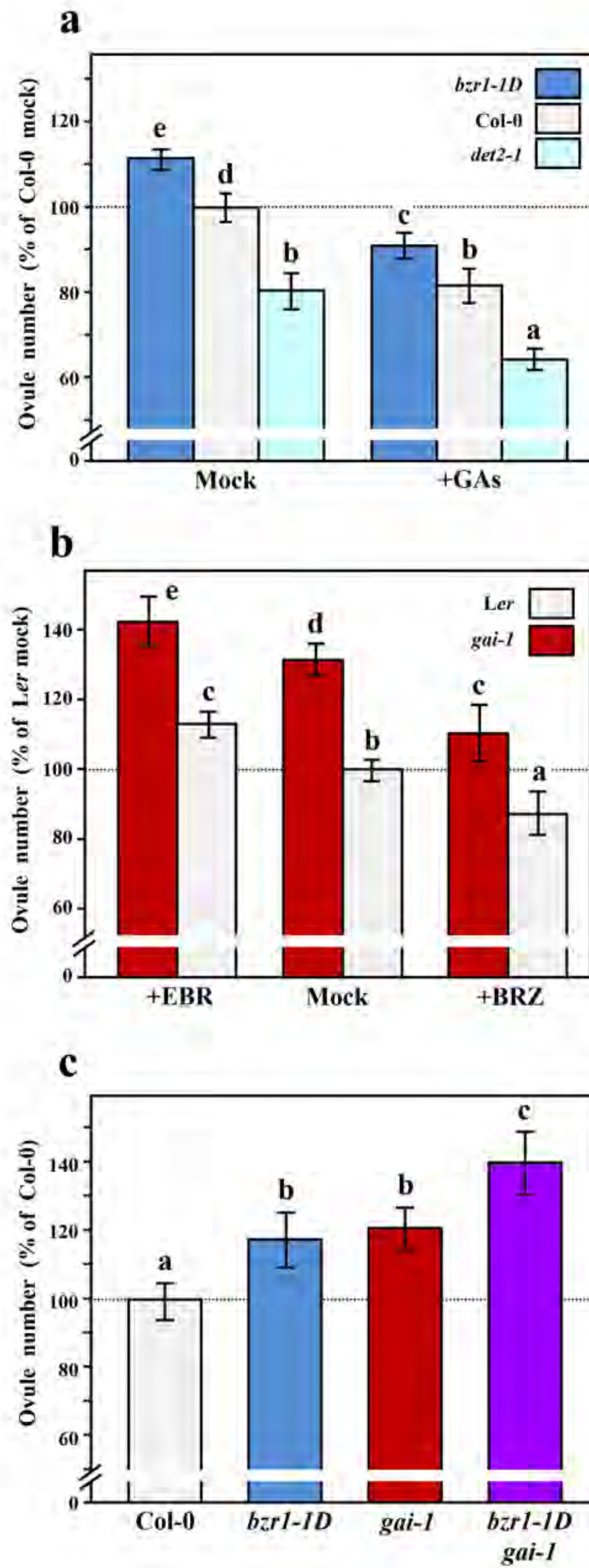

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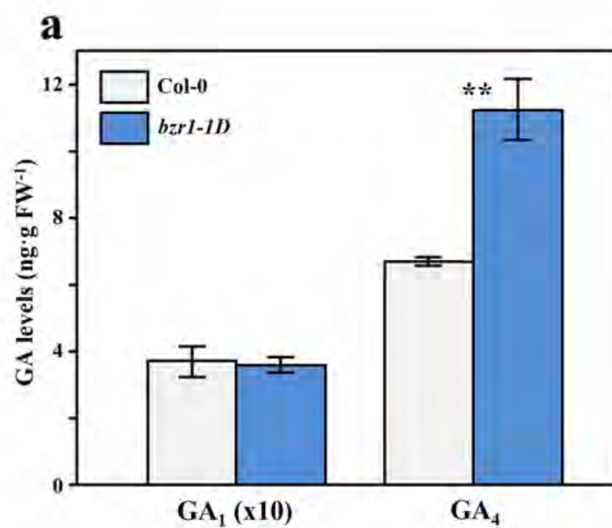

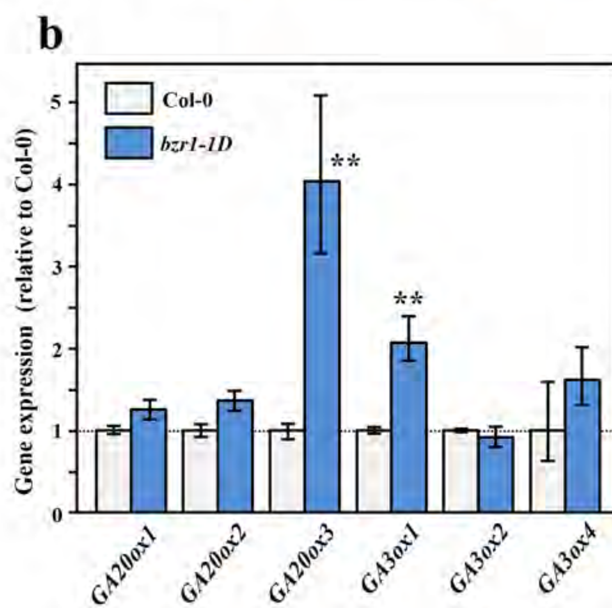

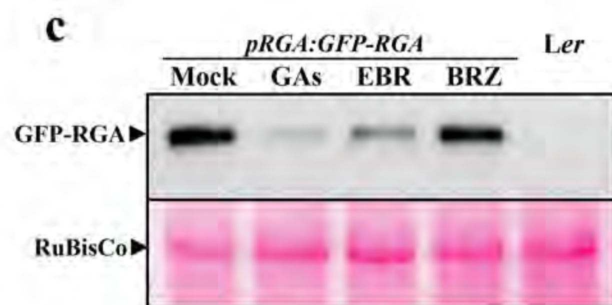

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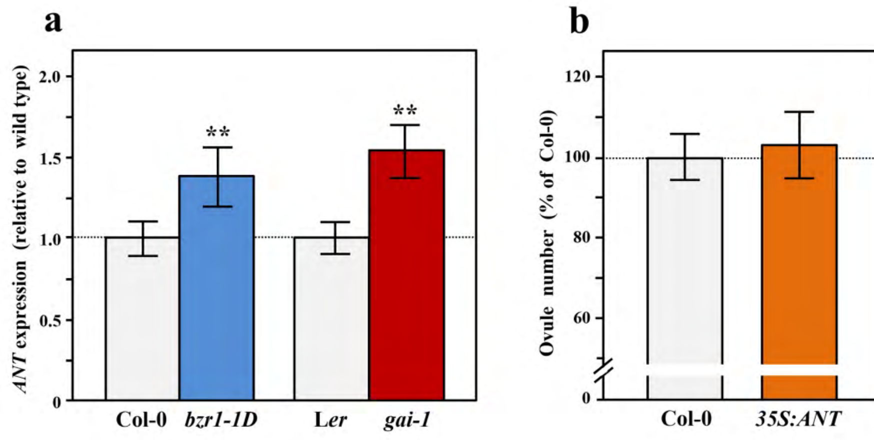

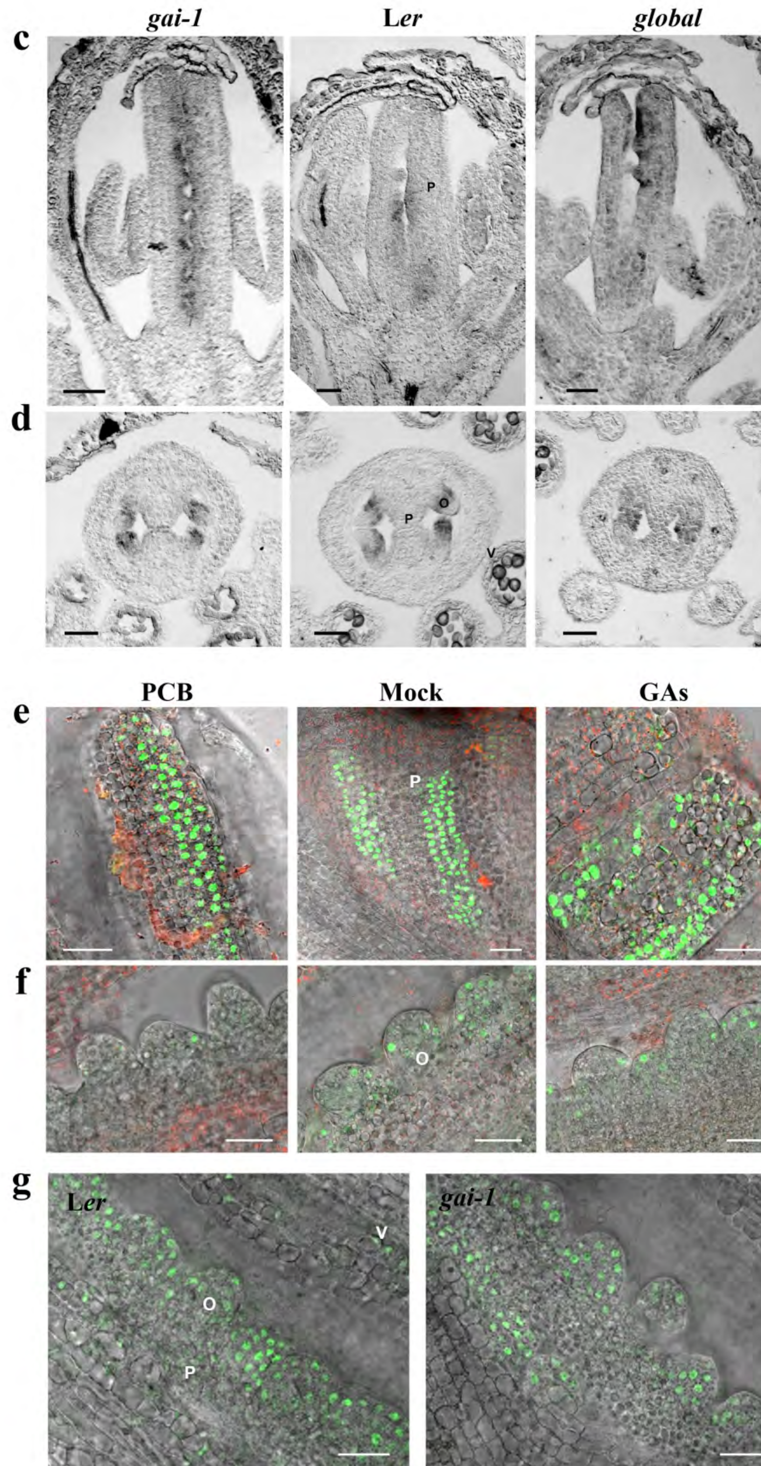

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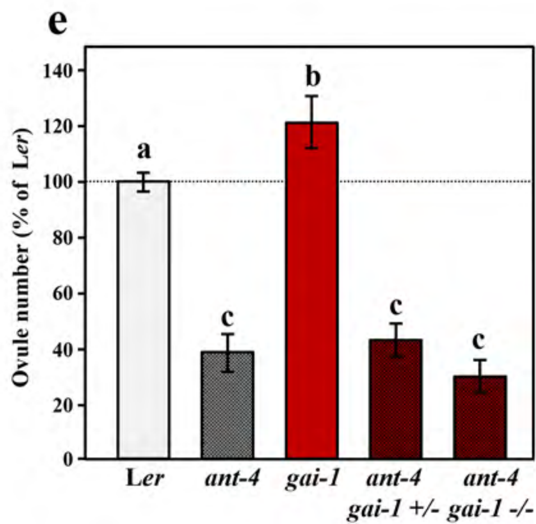
Ler ant-4 gai-1 ant-4 gai-1

a

b

c

d



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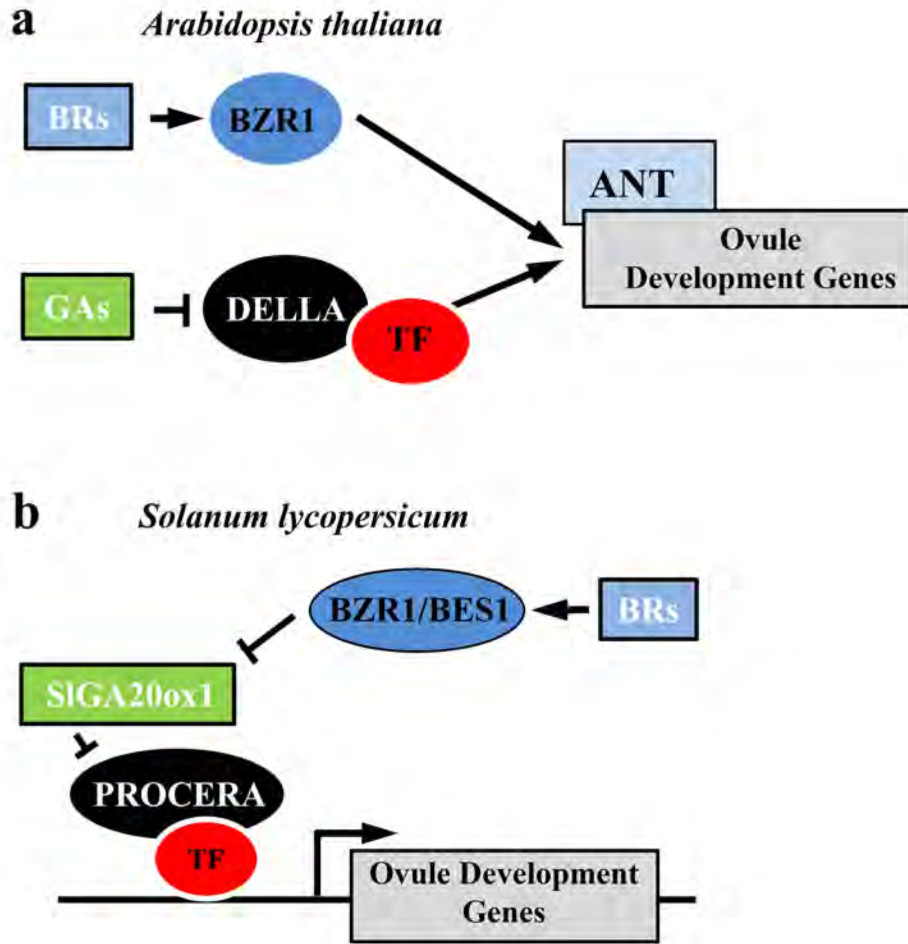

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