DELLA proteins positively regulate seed size in *Arabidopsis*

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**Summary Statement**

In Arabidopsis, DELLA protein activity induces cell proliferation in ovule integuments which results in the development of larger ovules and, consequently, larger seeds.

**Abstract**

Human and animal nutrition is mainly based on seeds. Seed size is a key factor affecting seed yield and has thus been one of the primary objectives of plant breeders since the domestication of crop plants. Seed size is coordinately regulated by signals of maternal and zygotic tissues that control the growth of the seed coat, endosperm, and embryo. Here, we provide novel evidence of the role of DELLA proteins, key repressors of gibberellin responses, in the maternal control of seed size. The gain-of-function *della* mutant *gai-1* produces larger seeds due to an increase in the cell number in ovule integuments. This leads to an increase in ovule size and, in turn, an increase in seed size. Moreover, DELLA activity promotes increased seed size by inducing the transcriptional activation of *AINTEGUMENTA*, a genetic factor that controls cell proliferation and organ growth, in the ovule integuments of *gai-1*. Overall, our results point to DELLA
proteins as new players in control of seed size and suggest that modulation of the DELLAn-dependent pathway could be used to improve crop yield.

Introduction

Crop seeds are the main products for human and animal consumption, while also representing a significant source of biofuels (Savadi, 2018; FAO, 2020). For this reason, increasing the yield of seed crops is necessary to ensure both food security and biofuel production. Although seed yield is indeed a quantitative trait with genetic and environmental influences, it mostly depends on seed number and size. Therefore, since the origin of agriculture, plants have been selected for larger seed sizes throughout the course of domestication (Shomura et al., 2008; Fan et al., 2009). Therefore, uncovering the mechanisms regulating seed size is important to enhance the seed yield of crop plants. Over the last few years, investigations in *Arabidopsis thaliana* (*Arabidopsis* hereafter) and crops such as rice, maize, and soybean have contributed to the current knowledge on seed development (Savadi 2018; Li et al., 2019). However, the intricate network of molecular mechanisms and genetic factors controlling seed size is still unfolding.

In angiosperms, seed development begins with the double fertilization of the mature ovule, which triggers endosperm and embryo development (Chaudhury et al., 2001). One of the two sperm cells fuses with the egg cell to form the diploid embryo, while the other fertilizes the diploid central cell to generate the triploid endosperm. After fertilization, the maternal integuments of the ovule, surrounding the developing embryo and endosperm, undergo cell division and differentiation, thereby forming the seed coat (Haughn and Chaudhury, 2005). In *Arabidopsis*, ovule integuments consist of outer and inner integuments. The outer integument is composed of two cell layers (*oi1* and *oi2*), while the inner integument consists of three cell layers (*ii1*, *ii1 ’*, and *ii2*) (Orozco-Arroyo et al., 2015). Thus, seed development is determined by the coordinated growth of the embryo, the endosperm, and the seed coat. In summary, some factors act zygotically, maternally, or both to regulate seed growth, which extends from fertilization to 6 days after pollination (DAP) in *Arabidopsis* (Orozco-Arroyo et al., 2015).
Recent studies have revealed that together with transcription factors (TFs), the ubiquitin pathway (Li and Li, 2014), G-protein signaling (Mao et al., 2010; Li et al., 2012), phytohormones, and growth factors are engaged in the maternal control of seed size in Arabidopsis and rice (Li et al., 2019). The TFs identified in Arabidopsis include TRANSPARENT TESTA GLABRA2 (TTG2; Johnson et al., 2002), belonging to the WRKY family, which induces cell expansion in the ovule integument to promote seed growth (Johnson et al., 2002). In contrast, APETALA 2 (AP2), a member of the AP2/EREBP (Ethylene Responsive Element Binding Protein) family, controls seed size by limiting cell elongation in integuments (Ohto et al., 2005). Another key gene is AINTEGUMENTA (ANT), which encodes an AP2-domain TF with important roles in organ growth control, including the ovule (Klucher et al., 1996). Plants overexpressing ANT give rise to oversized organs, including flowers, ovules, and seeds (Mizukami and Fischer, 2000; Barro-Trastoy et al., 2020). During ovule development, ANT acts maternally to control ovule and seed size (Li and Li, 2015).

Concerning hormones, those implicated in the maternal control of seed size include auxins, brassinosteroids (BRs), and cytokinins (CKs). Auxins regulate most plant growth and developmental processes, with the TFs AUXIN RESPONSE FACTORS (ARFs) acting as key regulators of auxin-mediated gene expression (Chandler et al., 2016). Among the 23 ARF genes in Arabidopsis, ARF2 is known to control seed size by restricting cell proliferation of the ovule integument (Schruff et al., 2006). Thus, auxin signaling appears to set a physical restriction for seed growth and seed size via the ovule integuments. On the other hand, BRs positively regulate seed size by transcriptionally modulating specific seed developmental processes (Jiang et al., 2013). Thus, seeds of the BR-deficient mutant de-etiolated2 (det2) are smaller than those of wild-type (WT) plants due to a reduced endosperm volume and integument cell length. Through the activation of the TF BRASSINAZOLE-RESISTANT1 (BZR1), BRs upregulate the expression of SHORT HYPOCOTYL UNDER BLUE1 (SHB1), MINISEED3, and HAIKU2, which are known positive zygotic regulators of seed size; however, BRs repress AP2 and ARF2, which are negative maternal regulators of seed size (Jiang et al., 2013). Finally, CK-deficient or CK-insensitive mutants display larger seeds than WT plants (e.g., in the triple mutant of the three CK sensor histidine kinases AHK2, AHK3, and AHK4) (Riefler et al., 2006). Genetic analysis has shown that the CK-related
increase in seed size is caused by both the maternal and/or endospermal genotypes (Riefler et al., 2006).

Gibberellins (GAs) are plant hormones involved in a large number of growth and development processes throughout the life cycle of plants (Sun, 2011; Gupta and Chakrabarty, 2013; Gallego-Giraldo et al., 2014; Gomez et al., 2016), including seed growth. Analyses of GA-deficient mutants in tomato, pea, and Arabidopsis suggested that GAs are required for seed development (Groot et al., 1987; Swain et al., 1997). In addition, it has been described that the ectopic expression of a pea GA2oxidase2 (a GA-inactivating enzyme) in Arabidopsis resulted in seed abortion (Singh et al., 2002), whereas the overexpression of some GA-stimulated Arabidopsis (GASA) genes resulted in increased seed weight (Roxrud et al., 2007; Trapalis et al., 2017).

GAs regulate growth and development via the degradation of DELLA proteins, which act as GA signaling repressors. DELLA proteins belong to a subfamily of the plant-specific GRAS family of TFs (Sun, 2010). The bioactive GAs bind to the GID1 receptors, which promotes the binding of the DELLA protein to GID1. This GA-GID1-DELLA complex enables binding with F-box proteins, DELLA protein polyubiquitination, and its 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). DELLA mutant proteins lacking the N-terminal DELLA regulatory domain (17-aa domain), 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 by the GA-GID1 complex, resulting in constitutive DELLA activity and blockage of the GA-mediated gene expression response. Both high GA levels and the loss-of-function of DELLA proteins release GA responses, while low GA levels or GA-insensitive della mutants restrain GA responses (Sun, 2011; Vera-Sirera et al., 2015; Daviere and Achard, 2016). DELLA proteins lack a canonical DNA binding domain and thus mediate the transcriptional regulation of target genes through direct physical interaction with TFs and other regulatory proteins (Vera-Sirera et al., 2015; Daviere and Achard, 2016).

Previous research from our group has suggested that gain-of-function della mutants (gai-1 and rgl2Δ17), which promote GA response blockage, alter seed morphology (Gomez et al., 2016; 2019). In this work, we have characterized the seed phenotype of GA-related mutants and WT plants after pharmacological treatment with paclobutrazol.
(PBZ) to prevent GA synthesis. Our data indicate that DELLA activity promotes the production of larger seeds by increasing the cell number in both the outer and inner integuments of ovules, suggesting that DELLA proteins might maternally control seed size by inducing the increase of cell division in the integuments. The molecular mechanism of the DELLA-mediated control of ovule size could rely on ANT expression. ANT would mediate the DELLA-function in ovule development, being ANT a direct target of GAI. Moreover, ANT overexpression mimics the ovule phenotype promoted by DELLA activity. Tackling the molecular mechanism by which DELLA proteins participate in the regulation of seed size would be useful for designing strategies to increase seed yield in crop plants.

Results

GA-deficient and GA-insensitive Arabidopsis mutants produce large seeds

To determine the role of DELLA proteins in seed growth, we first examined the seed size of the gain-of-function della mutants rgaΔ17, rgl1Δ17, rgl2Δ17, and gai-1 together with the loss-of-function mutants rga24, rgl1-1, rgl2-1, and gaiT6 (Fig. 1A,B). The dry mature seeds of gain-of-function della mutants were significantly larger than those of the WT, except for rgl1Δ17 seeds, which were only slightly larger than WT seeds. gai-1 was associated with the greatest increase in size (approximately 25%), which correlated with an increase in seed weight of approximately 42% (Fig. 1A–C). Interestingly, the seed number per gai-1 fruit did not decrease when compared to the WT, ruling out a possible compensatory mechanism between seed size and number in each fruit (Fig. S1A). The increased seed size is striking considering that gai-1 plants exhibit a severe dwarf phenotype (Fig. S1A,B) (Koornneef et al., 1985; Peng et al., 1997). In contrast, null della mutants, with the exception of rga24, presented a significant decrease in seed size (Fig. 1A,B; Fig. S2A). Despite increasing or decreasing the seed size, DELLA activity did not modify the shape of seeds since the ratios between seed length and width in gai-1 (gain-of-function mutant) and gaiT6 (null mutant) were not different from the WT (Fig. 1B,D).

Thereafter, we examined the seeds of mutants of the GA receptors gid1a, gid1b, and gid1c, which show a lower sensitivity to GAs, and reduced degradation of DELLA proteins. Mutant gid1a and gid1b seeds were larger than the WT and gid1c seeds (Fig.
This is consistent with the fact that both GID1a and GID1b receptors, but not GID1c, are expressed in ovules (Gallego-Giraldo et al., 2014). Finally, we verified that plants treated with paclobutrazol (PBZ), a GA biosynthesis inhibitor that reduces GA levels promoting DELLA protein accumulation, mimicked the seed phenotype of gain-of-function della mutants (Fig. S2C,E). In fact, the seeds of plants treated with PBZ were larger than those of any of the gain-of-function mutants, including gai-1. The greater increase in seed size following PBZ treatment could be due to the simultaneous stabilization of all DELLA proteins. In summary, our observations provide evidence of a positive correlation between DELLA activity and seed size in Arabidopsis. Since gai-1 showed the greatest seed size increase, we focused on the role of GAI by using gai-1 and its corresponding null mutant gaiT6 as genetic tools.

**DELLA proteins act maternally to control seed size**

To determine whether GAI activity controls the seed size as a maternal or zygotic factor, reciprocal crosses between WT plants, gai-1 mutants, and gaiT6 mutants were performed (Fig. 1E). Mature seeds produced by WT plants pollinated by gai-1 or gaiT6 pollen reached the same size as the seeds from WT plants hand-pollinated with WT pollen (i.e., both mutant pollen were unable to modify the seed size) (Fig. 1E). Consistent with these observations, no differences in size were detected in the seeds of gai-1 and gaiT6 plants pollinated with WT pollen when compared to those hand-pollinated with gai-1 and gaiT6 pollen (i.e., WT pollen did not alter the size of seeds in gai-1 or gaiT6 plants) (Fig. 1E). In summary, the effect of the DELLA protein on seed size was only observed when the gai-1 or gaiT6 mutants acted as the maternal plant, regardless of the pollen genotype. This indicates that DELLA proteins control seed size by regulating the growth of the seed maternal tissue (i.e., the ovule integuments or/and the seed coat).

**DELLA activity promotes the cell proliferation of ovule integuments**

After fertilization, the seed coat is formed from the differentiation of the integuments surrounding the ovule. Therefore, we asked whether DELLA protein activity affects early integument development and, consequently, the sizes of ovules and seeds. Indeed, the ovule size in mutant plants was significantly larger (gai-1) or smaller (gaiT6) than those from WT plants (Fig. 2A,B). Similarly, the ovule size of PBZ-treated plants was higher than those in mock-treated plants (Fig. S3A,B). Overall, ovule size phenotypes
were consistent with the seed size of mutants or PBZ-treated plants. To determine how cell proliferation and cell expansion in the integuments in gaiT6 and gai-1 might contribute to ovule size, we analyzed the cell number in the outer oi2 and inner ii1 integument cell layers in both mutants (Fig. 2C). We found that gaiT6 ovules had fewer cells in the oi2 and ii1 layers, which may explain their smaller size. In contrast, gai-1 showed higher cell number in both the oi2 and ii1 layers when compared to the WT. Furthermore, PBZ-treated plants also had more cells in the oi2 layer than mock-treated plants (Fig. S3C), thus mimicking the gai-1 ovules. Next, we examined whether the increase in cell number in gai-1 ovules was maintained during gai-1 seed development (Fig. 2D,E). For this purpose, we analyzed the cell number of the seed coat epidermis (derived from the oi2 layer) of seeds at 4 DAP, considering that cell division is completed in the seed coat at this stage (Garcia et al., 2005). The cell number in the gai-1 seed coat was also higher when compared to the WT, which confirmed that the increase in seed size was due to the increase in cell proliferation during integument development. However, we cannot discard that GAI also controls cell proliferation during seed coat differentiation. Finally, we also determined cell size and observed that although gai-1 has more cells in the integuments, these were smaller than those of the WT (Fig. S4A–C).

DELLA proteins are expressed in developing ovules

According to our data, a plausible scenario is that DELLA proteins in gain-of-function mutants or PBZ-treated plants might accumulate in developing integuments to enhance cell division. In previous works, we reported that GAI, RGA, RGL1, and RGL2 genes were expressed in developing ovules (Gomez et al., 2016) and that stable YPet-rgl1Δ17 and YPet-rgl2Δ17 proteins driven by the respective endogenous promoters were also accumulated in ovule integuments (Gomez et al., 2019; 2020). These data are relevant since DELLA proteins undergo GA-dependent degradation; therefore, gene expression and protein presence do not always overlap. Furthermore, we have now verified that GFP-rgaΔ17 is accumulated in developing ovules (Fig. S5A) using the pRGA:GFP-rgaΔ17 reporter line (Dill et al., 2001). We have also characterized the GAI protein localization during ovule development in detail (Fig. 3). For this, we generated transgenic lines expressing a WT version of the protein fused to 3xYPet (pGAI:GAI-3xYPet) flanked by the 15 and 5 kb genomic regions at the 5’ and 3’ ends of the locus, respectively. In WT ovules, GAI-3xYPet was detected within the nucellus, whereas no
signal was observed in the chalaza, funiculus, or integuments at any developmental stage (Fig. 3A). Using the GA HACR reporter line (Khakhar et al., 2018), we observed that active GAs were distributed throughout integuments, chalaza, and funiculus, while integuments were developing (Fig. 3B). Therefore, the absence of GAI-3xYPet protein in these tissues during ovule development may be due to the accumulation of GAs, which triggers the degradation of DELLA proteins.

We also used the gain-of-function version pGAI:gaiΔ17-3xYPet described previously (Barro-Trastoy et al., 2022), which is identical to the WT version but with a 17-aa deletion of the DELLA domain mimicking that in the gai-1 mutant. In this line, the gaiΔ17-3xYPet protein was clearly visible in ovules throughout development (Fig. 3C–H). At stage 2-I (Schneitz et al. 1995), before integuments are initiated, gaiΔ17-3xYPet was distributed in the funiculus and chalaza, from which the integuments will emerge (Fig. 3C). At stage 2-III, gaiΔ17-3xYPet was also expressed in emerging integuments (Fig. 3D). Localization remained in the same tissues at stages 2-IV and 2-V (Fig. 3E,F) until the ovule was mature (Fig. 3G). Upon fertilization, gaiΔ17-3xYPet was localized in the chalaza and the developing seed coat at 3 DAP (Fig. 3H). Overall, GAI activity could influence seed size through integument growth during ovule development and the initial stages of seed development.

**Expression of B-type cyclins is induced in integuments by DELLA activity**

The increase and decrease of cell number in the integuments of gai1 and gaiT6 ovules, respectively, together with the GAI expression pattern, suggest that GAI can promote cell proliferation in the developing integuments of ovules. To test this, we examined the expression of B-type cyclins during ovule and seed development in the gai-1 mutant. B-type cyclins are expressed shortly before and during mitosis, which makes them suitable markers for mitosis and cell proliferation (Colon-Carmona et al., 1999). Specifically, CYCB1;1 (At4g37490), CYCB1;2 (At5g06150), and CYCB1;4 (At2g26760) were described to be expressed in ovules (Romeiro-Motta et al., 2022). Interestingly, CYCB1;1, CYCB1;2, and CYCB1;4 expression level is increased in siliques at 3 DAP of gai-1 (Fig. 4A). The number of GFP-positive nucleus in ovules of gai-1 increased by 70% when compared to ovules from Ler plants (5.09±1.77 and 3.03±1.31, respectively). Cyclin gene expression was also induced in gai-1 inflorescences (Fig. S6A). Furthermore, using the reporter line pCYCB1;2:Dbox-GFP GFP (Merelo et al., 2022), we observed that CYCB1;2 expression was higher in the developing ovules of gai-1 when compared
to the WT (Fig. 4B–E). 
gai-1 ovules showed the higher expression of CYCB1;2-GFP, with both an increased number of cells and higher signal intensity in the funiculus, chalaza, and integuments (Fig. 4D,E). It should be noted that Figure 4 presents ovules at developmental stages 2-II and 3-I, in which the integuments undergo a high growth rate (Vijayan et al., 2021). These analyses are consistent with an increase in cell proliferation in 
gai-1 ovule integuments.

It should be noted that expression assays using qPCR assays were performed on inflorescences containing developing ovules and on siliques with seeds at an early developmental stage (3 DAP) due to the impracticability of collecting only ovules. Therefore, we also specifically analyzed expression in ovules using confocal microscopy, mainly at the stages where most cell proliferation occurs in integuments.

**DELLA activity positively regulates AINTEGUMENTA**

As described in the Introduction, several transcription and growth factors have been shown to control seed size by regulating cell proliferation in maternal tissues, with ANT being one of them (Klucher et al., 1996; Li and Li, 2015). Interestingly, ANT is also part of a list of bona fide gene targets of GAI obtained in a ChIP-Seq assay using pGAI:gaiΔ17-3xYPet (Barro-Trastoy et al., 2022). ChIP-Seq analyses identified two putative indirect GAI binding sites (thought the interaction with a yet unknown TF), located 3961 and 1256 bp upstream of the transcription start site of ANT (Fig. S7). Moreover, the ovule and seed phenotypes of ANT-overexpressing plants (Mizukami and Fischer, 2000) are similar to those of 
gai-1, with their flowers, ovules, and seeds being larger than those of WT plants (Fig. S8). Thus, it is plausible that the molecular mechanism of the DELLA function relies in the direct transcriptional regulation of ANT during ovule development. To test this hypothesis, we checked the expression level of ANT in 
gai-1 and 
gaiT6 mutants. qPCR assays revealed that ANT expression level is increased in 
gai-1 siliques at 3 DAP (Fig. 4A) and inflorescences (Fig. S6A). A second line of supporting evidence was provided by the pGAI:gai-1-GR transgenic line (Gallego-Bartolome et al., 2011), which expresses a translational fusion between gai Δ17 and the rat glucocorticoid receptor under the control of the GAI promoter. Upon induction of gai-1 by the dexamethasone (DEX) treatment of inflorescences, ANT expression was significantly increased (Fig. S6B). Furthermore, using the reporter line pANT:ANT-YPet (Barro-Trastoy et al., 2020), we observed that ANT expression was clearly higher in the chalaza, integuments, and funiculus of developing 
gai-1 ovules.
when compared to the WT (Fig. 4F,G). Thus, the activation of cell proliferation during ovule development by GAI may involve the increased expression of ANT in this organ, with ANT being a putative direct target of GAI. Taken together, these results suggest that DELLA activity would increase seed size by activating the expression of ANT, which would increase integument cell proliferation and thus ovule size. Unfortunately, no genetic assays could be performed to further confirm these results since ANT null mutants do not produce viable ovules or seeds.

**DELLA proteins do not modify seed structure and composition**

An important question related to the possible use of DELLA proteins as a biotechnological tool to improve seed size in crops is whether embryo development, structure, and metabolic composition are also impacted (i.e., if DELLA proteins are involved in developmental or differentiation processes other than seed growth). Figure 5A shows that WT and gai-1 embryos developed similarly, with the same timing. Also, no differences were detected in the structure of the seed coat, including the mucilage, between gai-1 and WT dry mature seeds (Fig. 5B). That is, the increase in seed size by enhanced DELLA activity does not cause modifications to seed coat structure and morphology. Regarding metabolic composition, we compared the amount of fatty acids, soluble sugars (sucrose, fructose, and glucose), and free amino acids in dry mature seeds from WT, gai-1, and PBZ-treated plants (Fig. 5C). Similar levels of the three types of compounds were detected in all analyzed seeds. Taken together, these data suggest that modulating DELLA activity only impacts seed size, with no alterations on their development, anatomy, or metabolic content.

**Discussion**

Seeds ensure the survival and dispersal of plants and represent the basis of human and animal nutrition. In recent years, significant efforts have been made to understand how seed size is regulated by the signals of maternal and zygotic tissues (Li et al., 2019). Hormones and TFs are integrated into a complex genetic network that regulates seed size in a maternal manner. In this context, auxins, BRs, and CKs have been shown to regulate seed size in *Arabidopsis* (Li and Li, 2015; Orozco-Arroyo et al., 2015; Li et al., 2019). Our work unveils that GAs do not only influence proper seed growth, but that
they are also leading players in the control of seed size via DELLA function in maternal tissues.

The repression of GA signaling (e.g., in the gain-of-function DELLA mutants, gid1 receptor mutants, or in PBZ-treated plants) results in larger seeds, whereas the partial activation of GA responses (e.g., in single loss-of-function DELLA mutants) leads to smaller seeds (Fig. 1). In the case of GAI (the DELLA protein having the greatest effect), stabilization also increased seed weight without affecting its metabolic composition (fatty acid, sugar or amino acid content) or altering seed morphology or the number of seeds per silique (Figs 1, 5). These characteristics make GAI a potentially powerful biotechnological tool for increasing seed crop yield.

It is well established that GAs promote stem, leaf, and root cell proliferation and expansion by inducing the degradation of growth-repressing DELLA proteins (Achard et al., 2009; Daviere et al., 2014; Serrano-Mislata et al., 2017). As a result, mutants that stabilize DELLA proteins, such as gai-1, are dwarf plants. Strikingly increased DELLA activity results in larger rather than smaller seeds, indicating that DELLA proteins have an opposite function in seed development compared to other plant organs. Since DELLA activity positively regulates biotic and abiotic stress resistance while repressing cell division and expansion (Wild et al., 2012; Daviere and Achard, 2016; De Vleesschauwer et al., 2016; Thomas et al., 2016; Shi et al., 2017), we speculate that the DELLA-dependent promotion of seed growth might be part of a similar trade-off mechanism that aims to ensure optimal reproductive development and improve the next generation. Further experiments would be required to confirm this hypothesis.

Through reciprocal crosses between WT plants and gai-1 and gaiT6 mutants, we determined that DELLA proteins induce the growth of the seed from maternal tissue (Fig. 1); that is, they regulate seed size by increasing the integument growth of ovules and developing seeds (Fig. 2). The number of outer and inner integument cells in gai-1 mutant ovules and seeds was significantly higher when compared to the WT, indicating that GAI regulates seed size by promoting cell proliferation associated with an elevated CYCB1;2 activity in the maternal integuments (Figs 2, 4). Since cells in the integuments mainly undergo expansion after fertilization (Garcia et al., 2005), the number of cells in the gai-1 ovule integuments is expected to determine the final size of the seed coat. Furthermore, although mutations that act maternally to produce larger seeds often display a prolonged period of seed coat growth and delayed embryo
development in *Arabidopsis* (Ohto et al., 2009; Fang et al., 2012), gai-1 embryo development was not delayed when compared to the WT (Fig. 5).

Similar to GAI, other genetic factors induce cell proliferation in the integuments, resulting in larger seeds. These include the TFs BZR1 and ANT (Mizukami and Fischer, 2000; Jiang et al., 2013) and the cytochrome P450 proteins KLUH (KLU, CYP78A5) and EOD3 (CYP78A6) (Adamski et al., 2009; Fang et al., 2012). Some of these genetic factors could be GAI targets or interactors during integument development. BZR1 is a well-known DELLA interactor, and GAs and BRs co-regulate many aspects of plant development in both a cooperative and antagonistic manner (De Vleesschauwer et al., 2012; Unterholzner et al., 2015; Barro-Trastoy et al., 2020). Despite this, ovules of the gain-of-function *bzr1-1D* mutant have increased the proliferation and expansion of integument cells (Jiang et al., 2013), which differs from the *gai-1* ovule phenotype in which cells decrease in size (Fig. S4). In addition, ovules of the gain-of-function *eod3-1D* mutant have a similar phenotype to *bzr1-1D* (Fang et al., 2012). It is possible that the control of cell expansion and cell division in the integument by DELLA and BZR1 proteins relies on two different molecular mechanisms (trans-activation versus sequestration). Only ANT and KLU proteins behave similarly to GAI during integument development. Plants overexpressing ANT or KLU show increased integument cell number at the expense of decreasing the cell size similar to the *gai-1* mutant (Mizukami and Fischer, 2000; Adamski et al., 2009). This observation, together with the fact that ANT was identified as a potential target of GAI through a ChIP-Seq analysis (Barro-Trastoy et al., 2022), strongly suggests that ANT may participate in the GAI pathway responsible for the GA-dependent regulation of seed size. In fact, ANT expression significantly increases in the inflorescences of *gai-1* mutant and upon induction with DEX in the 4xdella pGAI:gai-1-GR transgenic line (Fig. 4; Fig. S6). Additionally, ANT-YPet protein level is greater in *gai-1* developing ovules. All these results reinforce the notion that GAI could trigger ANT induction during ovule development to maternally control *Arabidopsis* seed size. This scenario is further supported by the increase of cell divisions in *gai-1* integuments, as reflected by the observed *CYCB1;2* expression, given that the gain of ANT function allows cells to proliferate for a longer period than normal, thereby causing organ enlargement (Mizukami, 2001). Adding an extra layer of complexity, ChIP-Seq experiments revealed that ANT could bind to regions upstream of the *GAI* CDS and that GAI would
be differentially expressed after ANT-GR activation (Krizek et al., 2020); therefore, both genes could co-regulate each other in a positive feedback mechanism. It is most likely that \textit{ANT} expression is not solely regulated by GAI. Previous studies have shown that the B3 domain TFs ARF2 and MATERNAL EFFECT EMBRYO ARREST45 (MEE45) have ANT as a common target (Meng et al., 2015; Li et al., 2021). These TFs exert opposite transcriptional regulatory functions and thus cause inverse effects on seed size. ARF2 binds to the \textit{ANT} promoter and negatively controls seed growth by restricting integument cell proliferation (Schruff et al., 2006), whereas MEE45 directly induces \textit{ANT} expression and thus promotes integument cell proliferation and increases seed size (Li et al., 2021). These findings, combined with the fact that GAI could also be an activator of \textit{ANT}, highlight the multiple signaling pathways that converge at \textit{ANT} for the maternal control of seed development. Based on our findings and the previously described data, we postulate a working model for the mechanism of DELLA function in ovule and seed size (Fig. 6). Notably, GAs negatively regulate DELLA protein stability. DELLA proteins would regulate the expression of \textit{ANT} (and other putative targets) via interaction with an unknown TF(s) that confers target specificity; \textit{ANT} activity would in turn increase cell proliferation. Additionally, through a feedback mechanism, \textit{ANT} would directly upregulate \textit{GAI} by binding to its promoter to ensure a tighter degree of control. Finally, \textit{ANT} expression is also regulated by other TFs such as MEE345 or ARF2.

In most agricultural crops, seeds are the main product for harvesting. Thus, an increase in seed size would be beneficial for improving seed crop yield. In this work, we describe a novel role of DELLA proteins as positive regulators of ovule and seed size. Further efforts would be necessary to fully determine the genetic and molecular mechanisms through which DELLA proteins are involved in seed size control and to address whether elements of this DELLA-dependent pathway could be used to engineer larger seed size in crops.

**Materials and methods**

**Plant materials and growth conditions**

\textit{Arabidopsis thaliana} plants Landsberg erecta (Ler) or Columbia-0 (Col-0) were used as WT lines. All mutants and reporter lines have been previously described. In the Ler
background, we have used gai-1 (Koornneef et al., 1985), gaiT6 (Peng et al., 1997), rga24 (Silverstone et al., 1998), rgl1-1, and rgl2-1 (Lee et al., 2002) mutants, and the lines pRGA:GFP-rgaΔ17 (Dill et al., 2001), 4xdella pGAI:gai-l-GR (Gallego-Giraldo et al., 2014), pRGL2:YPet-rgl2Δ17 (Gomez et al., 2019), pRGL1:YPet-rgl1Δ17 (Gomez et al., 2020), pANT:ANT-YPet (Barro-Trastoy et al., 2020), pCYCB1;2:Dbox-GFP (Merelo et al., 2021), and pGAI:gaiΔ17-3xYPet (Barro-Trastoy et al., 2022). In the Col-0 background, we have used gid1a, gid1b, and gid1c (Griffiths et al., 2006), gai-1 (Barro-Trastoy et al., 2020), 35S:ANT (Barro-Trastoy et al., 2020), and GA HACR (hormone-activated Cas9-based repressor) plants with the PHD6 genotype (Khakhar et al., 2018). We generated the gai-1 ANT-YPet plants by crossing gai-1 with the pANT:ANT-YPet line. F3 homozygous plants were selected by PCR-based genotyping and/or antibiotic or herbicide resistance. All primers used for genotyping are listed in Table S1. Reagents and primers were purchased from Sigma-Aldrich (Madrid, Spain) or Integrated DNA Technologies (IDT; Iowa, USA), respectively, unless otherwise stated.

Seeds were surface-sterilized in ethanol, plated onto ½ Murashige and Skoog medium (Murashige and Skoog, 1962), incubated at 4°C for 3–4 days in darkness, and transferred to a growth chamber at 24°C in a long-day photoperiod (16/8 h) for 7–10 days. Then, seedlings were transferred to soil (a 2:1:1 mix of peat moss, vermiculite, and perlite) and grown in a chamber at 22°C in a long-day photoperiod. PBZ treatment was applied by watering the plants every other day with 1 µM PBZ (Duchefa Biochemie) starting at bolting. A stock solution of 10 mM PBZ was prepared in acetone. DEX was applied onto inflorescences of the line 4xdella pGAI:gai-l-GR by spraying a 5 µM solution, and inflorescences were collected 24 h after treatment. DEX stock solution was made at 10 mM in ethanol.

To conduct reciprocal crosses, the flower buds of Ler, gai-1, and gaiT6 were hand-emasculated 1 day before anthesis, and pistils were hand-pollinated the next day with mature pollen from one of the three different genotypes. Fruits (only one fruit per plant) were collected at maturity and seed size was measured (n≥30 fruits per pollination). All experiments were repeated three times, with similar results.

**Generation of the transgenic pGAI:GAI-3xYPet reporter line**

The pGAI:GAI-3xYPet line was generated by recombineering (Brumos et al., 2020) in a manner similar to the gain-of-function line pGAI:gaiΔ17-3xYPet described in Barro-
Trastoy et al. (2022). All primers used are listed in Table S2. The construct was used to transform Ler Arabidopsis plants by Agrobacterium-mediated floral dipping (Clough and Bent, 1998), and transgenic plants were selected in ammonium glufosinate to obtain homozygous lines segregating as a single locus. To check if GAI-3xYPet behaves like a DELLA protein, the effect of GAs and PBZ on stability of GAI-3xYPet fusion protein was tested in primary roots of seedlings. Transgenic plants were grown on MS plates for 2 days and treated with 100 µm GA$_{4+7}$ or with 100 um PBZ for 24 h and 48 h, respectively (Fig. S5B). YPet fluorescence was observed using confocal microscopy as indicated below. This line will be available upon request.

**Morphological and cellular analysis**

To determine seed size, dry seeds were photographed on white paper under an MZ16F stereomicroscope (Leica) using a DMC6200 digital camera (Leica). The area, length, and width of seeds were measured from the images using ImageJ software (https://imagej.nih.gov/ij/). Seed size was inferred from the obtained images as the medial seed area in a 2D projection. From each genotype, at least 100 seeds from 20 different siliques (one silique per plant between positions 10 and 20 of the main inflorescence) were used. Average seed weight was determined by weighing dry seeds in three batches of 500 seeds each using an AB54 analytical balance (Mettler Toledo). To measure the ovule area, cell number, and cell size, mature ovules from pistils at the anthesis stage were cleared and visualized with confocal microscopy, as indicated below. Figure S9 presents an image illustrating how the ovule size, the cell number in the integuments, and the average cell size (integument length/integument cell number) from the different plants were estimated using ImageJ software.

Statistical tests were performed using GraphPad Prism 8.0.2 and Statgraphics 18.1.13 software, as well as online web statistical calculators (www.astatsa.com).

**Histological procedures**

Seed development was studied using chloral hydrate clearing and differential interference contrast microscopy (DIC). Siliques at different developmental stages were fixed in ethanol:acetic acid (9:1, v/v) for 3 h, washed with 90% ethanol, and then cleared with a chloral hydrate solution (mixture of chloral hydrate:water:glycerol 8:2:1, w/v/v) for at least 3 days. Images were recorded using an Eclipse E600 microscope (Nikon) equipped with DIC optics and a DS-Ri1 digital camera (Nikon).
For the histological analysis of seed coats, dry seeds were fixed overnight at 4°C in 4% (w/v) p-formaldehyde in 0.1 M sodium phosphate (pH 7.2), with 0.05% (v/v) of Tween 20 and dehydrated in ethanol series. Seeds were then infiltrated in Technovit 7100 resin (Heraeus Kulzer), sectioned in an Ultracut E microtome (Reichert Jung) at 3 µm, and stained in 0.02% Toluidine Blue O, as described by Gomez et al. (2004). Images were captured using a DM5000 microscope (Leica).

Confocal laser scanning microscopy
Confocal microscopy was used to study the expression of reporter lines in developing ovules. To measure the ovule area and study the expression of gaiΔ17-3xYPet, samples were previously cleared. For this, inflorescences and mature ovules were fixed in vacuum for 1 h in 4% (w/v) p-formaldehyde in 0.1 M sodium phosphate (pH 7.2). After fixation, samples were cleared with ClearSee solution (Kurihara et al., 2015) for a least 1 week, and then stained with 0.01% (w/v) Calcofluor White (Ursache et al., 2018) before confocal microscope observation. GAI-3xYPet, ANT-YPet, CYCB1;2-GFP, and GA reporter signals were observed in fresh tissues. Images were captured using an LSM 780 confocal microscope (Zeiss). YPet fluorescent protein was observed with excitation at 514 nm and detection at 520–550 nm. Calcofluor White was observed with excitation at 405 nm and detection at 430–480 nm in grayscale. GA HACR ovules were analyzed via the detection of Venus fluorescent protein with excitation at 488 nm and detection at 510–550 nm. The identity of fluorescence signals was confirmed using a λ-scan. For each analysis, several high-quality images were obtained from three biological replicas.

Scanning electron microscopy (SEM)
Dry seeds were mounted on SEM stubs, coated with gold for 90 s in an SCD 005 sputter coater (BAL-TEC), and photographed using a Field Emission Scanning Microscopy ULTRA 55 microscope (Zeiss Oxford Instruments) using incident electron energy of 2kV. The images were acquired at the electron microscopy facility of Universitat Politecnica de Valencia (UPV).
Quantitative RT-PCR (qPCR)

For qPCR analysis, dissected main inflorescences (removing open flowers) and siliques at 3 DAP were collected and flash-frozen in liquid nitrogen. Total RNA was extracted using a NucleoSpin RNA Plant kit (Macherey-Nagel) according to the manufacturer’s instructions. cDNA was synthesized from 1 µg of total RNA using a PrimeScript 1st Strand cDNA Synthesis kit (Takara Bio Inc). The resulting cDNA was diluted 1:9 in ddH₂O and 1 µL of the resulting dilution was used in the qPCR reaction. qPCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR premix ExTaq (Tli RNAase Plus) Rox Plus (Takara Bio Inc). Expression levels were normalized to UBQ10 (At1g05320) (Czechowski et al., 2005) and analyzed by the comparative ΔΔCt method (Schmittgen and Livak, 2008) to the values in the WT or mock-treated plants. Three biological replicates with three technical replicates each were processed. The primers used for qPCR are listed in Table S1.

Metabolite analysis

Soluble sugars and amino acids were analyzed as described in Minebois et al. (2020), using 10 mg of dry seeds per sample. For fatty acid extraction and methylation, 1 mL of 5% H₂SO₄ in methanol, 300 µL of toluene, 5 µl of 1% (w/v) butylhydroxytoluene in methanol, and 5 µl of 1% (w/v) heptadecanoic acid in chloroform as an internal standard were added to 10 mg of seeds. Samples were incubated at 85°C for 90 minutes. Then, 1.5 mL of 0.9% (w/v) NaCl and 1 mL of hexane were added. After vortexing and brief centrifugation, the upper phase (organic) was extracted for injection in the gas chromatograph. The GC-MS conditions used were described in Minebois et al. (2020). Metabolite analyses were performed at the Instituto de Biologia Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) Metabolomics Platform.

Acknowledgments

We wish to thank Dr. P. Merelo (IBMCP, Valencia, Spain) for the pCYCB1;2:Dbox-GFP line. We also thank Ms. C. Fuster for her excellent technical assistance, Ms. M. Gascon for helping in the confocal image acquisition, and Dr. M. Rodriguez-Concepción for his helpful suggestions and comments on the manuscript.
Author contributions
M.D.G. designed the study. M.D.G., I.C., D.B-T., J.S.-M., and P.T. performed experiments. M.D.G. and M.A.P-A. wrote the manuscript. All authors commented on the manuscript.

Competing interests
The authors have declared that no conflict of interest exists.

Data availability statement
All data and resources generated in this study will be freely available upon request.

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References


**Figures**

**Fig. 1.** DELLA proteins act maternally to positively regulate seed size. (A) Seed area of Ler (WT), gain-of-function *della* mutants rgaΔ17, rgl1Δ17, rgl2Δ17, and gai-1, and loss-of-function *della* mutants rga24, rgl1-1, rgl2-1, and gaiT6. (B) SEM images of mature seeds of gai-1, Ler, and gaiT6 plants. (C) Seed weight (mean ± SD of 500 seeds) of Ler, gaiT6, and gai-1 plants. (D) Seed length (Y) and width (X) of Ler, gai-1, and gaiT6 plants. The ratio of length to width (± SD) is shown in gray. (E) Seed area resulting from the indicated reciprocal crosses of Ler with gai-1 and gaiT6. The first genotype was used as maternal parent (ovule) and the second as paternal parent (pollen). In (A), (D), and (E), dots represent data from individual seeds and horizontal lines
represent mean values (n>100 in (A), n=30 in (D), and n≥30 in (E)). Lowercase letters indicate the statistical significance as determined by an ANOVA and a Bonferroni post hoc test for multiple comparisons (P<0.05). Data that are not significantly different are marked with the same letter. In (C), significant differences (Student’s t-test) are indicated (** P<0.01); ns, not statistically significant. The scale bar in (B) represents 100 µm.
Fig. 2. GAI promotes ovule growth by increasing cell proliferation in integuments.
(A) Confocal images of representative mature ovules of Ler, gaiT6, and gai-l plants. (B) Ovule area of Ler, gaiT6, and gai-l plants (n≥20). (C) Cell number in the outer layer of the outer integument (oi2) and the inner layer of the inner integument (ii1) of Ler, gaiT6, and gai-l mature ovules (n≥20). (D) Image of a Ler seed at 4 DAP observed by differential interference contrast (DIC) microscopy. (E) Cell number in the oi2 layer of Ler and gai-l developing seeds at 4 DAP. In (B), (C), and (E), dots represent data from individual ovules or cells and horizontal lines represent mean values. In (B) and (E), significant differences (Student’s t-test) with the corresponding
WT are indicated (** $P<0.01$). In (C), lowercase letters indicate statistical significance as determined by a one-way ANOVA and a Bonferroni post hoc test for multiple comparisons ($P<0.05$). Data that are not significantly different are marked with the same letter. Scale bars represent 20 µm in (A) and 100 µm in (D).
Fig. 3. **GAI is expressed in developing ovules and seeds.** (A) Confocal images of GAI-3xYPet expression in ovules at stage 2-V. (B) GA-regulated reporter (GA HACR; Khakhar et al., 2018) expression in ovules at stage 2-V. Images correspond to an overlay of brightfield and fluorescence micrographs from fresh tissue. (C–H) Confocal images of the expression of gaiΔ17-3xYPet in the funiculus, chalaza, and integuments of developing ovules (C, stage 2-I; D, 2-III; E, 2-IV; F, 2-V), mature ovules (G), and in the seed coat of developing seeds at 3 DAP (H). Ovules were cleared and cell walls were stained with Calcofluor White. The panels show the composite images of Calcofluor
White and the Z-stack projection of YPet fluorescence images. The dotted line defines the shape of the developing ovules. Confocal images are representative of several high-quality images obtained from three biological replicas. ch, chalaza; f, funiculus; ii, inner integument; oi, outer integument; nu, nucellus. Scale bars represent 20 µm.
Fig. 4. **ANT and CYC gene expression is increased in gai-1 ovules.** (A) Relative mRNA levels of CYCB1;1, CYCB1;2, CYCB1;4, ANT, and GA20OX1 (At4g25420) in siliques at 3 DAP of Ler, gaiT6, and gai-1. Data were normalized to UBQ10 (At4g05320) in Ler. Data are the mean ± SD of three replicates. The expression of GA20OX1 was used to validate the result of the qPCR assay since it increases in gai-1 and decreases in gaiT6 (Rieu et al., 2008; Gallego-Bartolome et al., 2011). (B–E) Confocal images of the localization of CYCB1;2-GFP in Ler (B and C) and gai-1 ovules (D and E) at stages 2-II (B and D) and 3-I (C and E). (F and G) Confocal images of the localization of ANT-YPet in Ler (F) and gai-1 ovules (G) at stage 2-IV. Panels show composite images of a brightfield image and a GFP (B–E) or YPet (F and G) Z-
stack projection. The dotted line defines the shape of the ovules. Confocal images are representative of several high-quality images obtained from three biological replicas. ch, chalaza; f, funiculus; nu, nucellus; ii, inner integument; oi, outer integument. Scale bars represent 20 µm.
Fig. 5. *gai-1* seeds show no changes in morphological development or metabolic composition. (A) Embryo development in *Ler* and *gai-1* plants. *Ler* and *gai-1* cleared seeds at 4, 6, 8, 9, and 10 DAP were observed using DIC microscopy. The embryos were colored (orange for *Ler* and green for *gai-1*) to facilitate visualization. (B) Semi-thin sections of *Ler* and *gai-1* dry seeds embedded in resin showing the external mucilage. (C) Content of fatty acids, sugars, and amino acids in dry seeds of *Ler*, *gai-1*, and *Ler* upon treatment with 1 µM PBZ. Dots represent individual values for each of the five biological replicates. One-way ANOVA and Tukey’s multiple comparison tests were performed, and no statistically significant differences were found. Scale bars represent 50 µm in (A) and 100 µm in (B).
Fig. 6. Proposed working model of how DELLA activity regulates ovule and seed size in *Arabidopsis*. GAs mediate DELLA protein degradation, which is a positive factor in the promotion of cell proliferation in integumentary cell layers during ovule development. DELLA proteins interact with an unknown TF to positively regulate the expression of *ANT* (1). DELLA may also interact with other TFs to regulate other target genes (2). *ANT* would upregulate *GAI* directly by binding to its promoter (3). In addition, other TFs like MEE45 or ARF2 can also directly regulate the expression of *ANT* (4). Altogether, the coordinated expression of cyclin and other genes regulate cell proliferation in ovule integuments (5). As a result, the cell number in integuments is increased, which leads to larger ovules and seeds.
Fig. S1. *gai-1* plants exhibit a dwarf phenotype but larger seeds when compared to *Ler* plants, with no change in the number of seeds per fruit. (A) Seed number per silique in *Ler* and *gai-1* plants. (B and C) Images of flowering plants (B) and mature seeds (C) of *Ler* and *gai-1*. Dots represent data from individual siliques, and the horizontal line represents the mean value (n≥15). The difference between pairs was determined by a Student’s *t*-test. ns, not statistically significant. Scale bar represents 500 µm.
Fig. S2. DELLA protein accumulation results in an increase in *della* and *gid1* mutants and PBZ-treated plants have altered seed size. (A) Seed area of *Ler* (WT) and triple *della* mutants *gaiT6 rga24 rgl1-1, gaiT6 rgl1-1 rgl2-1*, and *rga24 rgl1-1 rgl2-1*, and the 4xdella (*gaiT6 rga24 rgl1-1, rgl2-1*). (B) Seed areas of Col-0 (WT) and *gid1a, gid1b, and gid1c* mutants. (BC) Seed area of *Ler* upon treatment with mock or 1 µM PBZ. (CD) Scanning electron microscopy (SEM) images of mature seeds of Col-0 and the *gid1a* mutant. (DE) SEM images of *Ler* seeds upon treatment with mock or 1 µM PBZ. In (A), data are mean values ± SD (n≥100). In (AB) and (BC), dots represent data from individual seeds (n>100) and horizontal lines represent mean values. Lowercase letters indicate statistical significance, as determined by a one-way ANOVA and a Bonferroni post hoc test for multiple comparisons (P<0.05). Data that are not significantly different are marked with the same letter. Scale bar represents 100 µm.
Fig. S3. GA biosynthesis inhibition triggers an increase in ovule size. (A) Confocal images of representative mature ovules of Ler upon treatment with mock or 1 μM PBZ. (B and C) Mature ovule area (B) and cell number in the oii2 layer (C) of mature ovules of Ler upon treatment with mock or 1 μM PBZ (n≥30). Dots represent data from individual ovules (left panel) or cells (right panel), and horizontal lines represent mean values. Significant differences (Student’s t-test) with the corresponding mock are indicated (** P<0.01).
**Fig. S4.** *gai-1* integument cells are more numerous but smaller than those of *Ler*. (A–C) **Whole length** Length (A), cell number (B), and cell size (C) of the oi2 integument layer of mature ovules of *Ler* and *gai-1*. Dots represent data from individual ovules or cells and the horizontal lines represent mean values. Significant differences (Student’s *t*-test) with the correspondent WT are indicated (*P*<0.05, **P**<0.01).
Fig. S5. (A) GFP-rgaΔ17 is present in developing ovules and (B) effects of GA and PBZ treatment on the stability of GAI-3xYPet and gaiΔ17-3xYPet proteins. (A) GFP-rgaΔ17 localization from the pRGA::GFP-rgaΔ17 line in the funiculus, chalaza, and integuments of ovules at stages 2-IV (left panel) and 3-IV (right panel). Panels show the composite image of Calcofluor White and GFP fluorescence. The dotted lines define the shape of the ovules. ch, chalaza; f, funiculus; ii, inner integument; oi, outer integument; nu, nucellus. (B) GAI-3xYPet is localized in the root nuclei (mock), but it is degraded in the presence of 100 μM GA_{4+7} (+GA) or stabilized in the presence of 100 μM PBZ (+PBZ). In contrast, gaiΔ17-3xYPet levels are not altered upon GA or PBZ treatments. Confocal images are representative of several high-quality images obtained from three biological replicas. Scale bars represent 20 μm in A and B.
Fig. S6. *CYCB1;1*, *CYCB1;4*, and *ANT* genes are upregulated when GAI protein is stabilized. (A) Relative mRNA levels of *CYCB1;1, CYCB1;2, CYCB1;4,* and *ANT* in the inflorescences of Ler and gai-1. (B) Relative mRNA levels of *ANT* in inflorescences of the 4xdella pGAI: gai-1-GR line after 24 h of treatment with mock or 5 µM DEX. In (A) and (B), data were normalized to *UBQ10 (At4g05320)* in Ler or mock, respectively. Data are presented as the mean ± SD.
**Fig. S7. GAI could bind to two domains in the promoter region of ANT.** (A) ChIP-Seq profiles for GAI are depicted in two biological replicates for 
$pGAI: gaiΔ17-3xYPet$ (gaiΔ17 rep1 and 2) and $Ler$ (wt rep1 and 2) inflorescences. Regions detected as reproducible peaks are illustrated as orange bars. (B) ANT gene model and chromosome position of the two putative binding sites are shown. Data were obtained from Barro-Trastoy et al. (2022).
Fig. S8. Both gai-1 and 35S:ANT show larger floral organs. (A) Images of Col-0, 35S:ANT, and gai-1 flowers at anthesis stage in lateral (upper images) and zenithal (lower images) views. (B) Cleared mature ovules of Col-0, 35S:ANT, and gai-1. (C) Mature seeds of Col-0, 35S:ANT, and gai-1. Scale bars represent 2 mm in (A), 50 μm in (B), and 500 μm in (C).
Fig. S9. **Illustrative image and diagram of a mature Ler ovule using a confocal image.** The ovule was cleared and the cell walls were stained with Calcofluor White. Ovule area and oi2 layer length were estimated by measuring, in optical sections (as shown), the area enclosed by the orange dashed line and the length of the violet dotted line using ImageJ software. The yellow and white dots label each of the cells in the oi2 and ii1 layers, respectively. The average cell size of the oi2 layer cells (green arrow) was determined by dividing the oi2 layer length by the cell number. Scale bar represents 20 µm.
### Table S1. Primers for genotyping and qPCR.

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### Table S2. Primers for construction of \textit{pGAI:GAI-3xYPet}.

**Recombineering of \textit{pGAI:GAI-3xYPet}**

**Tagging 3xYPet at Ct of GAI**

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**Trimming of genomic clones**

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\(^1\), underlined, sequence of universal adaptors.