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Short title:

Gibberellins and ATS in ovule development

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Title:

Gibberellins regulate ovule integument development by interfering with the transcription factor ATS

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One sentence summary

Gibberellins negatively regulate integument growth in ovules through destabilization of the DELLA-ATS protein complex

Author contribution

MD.G. performed most of the experiments; D.V. carried out protein-protein binding assays; R.S. analyzed DELLA mutants; MA.P-A. performed mutant seed analysis; MD.G. and MA.P-A. conceived the project, analyzed and interpreted data, and wrote the article with contributions by all the authors.

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ABSTRACT

Gibberellins (GAs) are plant hormones that regulate most plant life cycle aspects, including flowering and fruit development. Here we demonstrate the implication of GAs in ovule development. DELLA proteins, negative GA response regulators, act as positive factors for ovule integument development in a mechanism that involves transcription factor ATS. The seeds of the *della global* mutant, a complete loss-of function of DELLA, and the *ats-1* mutant are remarkably similar, with a round shape, a disorganized testa, and viviparism. These defects are the result of an alteration in integuments that fail to fully develop and are shorter than in WT plants. *ats-1* also shows some GA-related phenotypes, e.g. higher germination rates and early flowering. In fact, *ats-1* has elevated GA levels due to the activation of GA biosynthesis genes, which indicates that ATS inhibits GA biosynthesis. Moreover, DELLAs and ATS proteins interact, which suggests the formation of a transcriptional complex that regulates the expression of genes involved in integument growth. Therefore, the repression of GA biosynthesis by ATS would result in the stabilization of DELLAs to ensure correct ATS-DELLA complex formation. The requirement of both activities to coordinate proper ovule development strongly argues that the ATS-DELLA complex acts as a key molecular factor. This work provides the first evidence for a role of GAs in ovule and seed development.

INTRODUCTION

Gibberellins (GAs) are plant tetracyclic diterpenoids that play a major role in diverse key developmental processes throughout the plant life cycle, including seed germination, stem and root elongation, flowering, and fruit development (Sun, 2010; Gupta and Chakrabarty, 2013). The master regulator in GA signaling is the DELLA protein, a subfamily of the plant-specific GRAS family of transcriptional regulators (Sun, 2010). Bioactive GAs are perceived by receptor GID1s to allow GA-GID1-DELLA complex formation, which results in structural changes in the DELLA protein that trigger recognition and binding with F-box proteins, polyubiquitination and subsequent degradation by the 26S proteasome (Sun, 2011). Therefore, while DELLA proteins act as plant growth repressors, GAs promote growth and development by the rapid degradation of DELLA proteins. Accordingly, lack of DELLA activity in different mutants of *Arabidopsis* (Cheng et al., 2004; Feng et al., 2008), tomato (Jasinski et al., 2008; Bassel et al., 2008), or rice (Ikeda et al., 2001) results in a constitutive GA response. On the contrary, the mutant alleles of the DELLAs that lack the amino domain DELLA, like *gai-1* (Peng et al., 1997) or pRGA:*GFP-rgaΔ17* (Dill et al., 2001) of *Arabidopsis*, and *Slr1-d* in rice (Asano et al., 2009), encode proteins that cannot be degraded, which results in constitutive DELLA activity and GA response blockage. Unlike most analyzed plant species that encode a single DELLA protein, the *Arabidopsis* genome encodes five DELLAs: GA-INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3. Each one performs distinct, but also overlapping, functions in repressing GA responses (Sun, 2011).

DELLA proteins function as central nodes that integrate hormonal and environmental cues to modulate transcriptional patterns, which finally regulate growth and development (Daviere and Achard, 2016). DELLAs lack a canonical DNA binding domain, and thus mediate the transcriptional regulation of the target genes involved in the GA response throughout the direct physical interaction with transcription factors (TFs) and other regulatory proteins (Vera-Sirera et al., 2015; Daviere and Achard, 2016). DELLA-TF binding can be divided into two major mechanisms. DELLAs can bind to either TFs or other transcriptional regulators to block their function, or bind to TF linked to the promoter of target the genes, modulating their transcriptional activity (Daviere and Achard, 2016). The list of potential DELLA interactors has rapidly increased and provided direct evidence for not only the mechanism of crosstalk between

GAs and other hormones, but also for environmental clues to modulate adequate plant growth and response to environmental conditions (Marin-de la Rosa et al., 2014, 2015; Daviere and Achard, 2016).

GAs and DELLAs are key players during fruit development (Vivian-Smith and Koltunow, 1999; Dorcey et al., 2009; Fuentes et al., 2012). Under normal conditions, the unfertilized pistils do not develop into fruits due to low GA levels. Upon fertilization, GA metabolism is activated which, in turn, triggers GA signaling and fruit development (Hu et al., 2008; Rieu et al., 2008; Dorcey et al., 2009). DELLAs are negative factors in early fruit development steps. In *Arabidopsis*, loss of function of at least four out of the five DELLAs promotes facultative parthenocarpic fruit growth (Dorcey et al., 2009; Fuentes et al., 2012). DELLAs also control other aspects during fruit development, such as endocarp degradation and lignification, which are respectively delayed or advanced in the loss of function mutant of the GA receptors GID1 or in the *della global* mutant, (lacking all five DELLAs; thereafter known as *global* mutant) (Dorcey et al., 2009; Gallego-Giraldo et al., 2014).

Despite all these data, very little is known about the role of GAs during early pistil ontogeny, except for valve margin differentiation (Arnaud et al., 2010). ALCATRAZ (ALC) is a bHLH protein involved in the formation of the dehiscence zone in the valve margin, required for fruit shattering and seed dispersal. DELLAs block ALC activity in the specification of the separation cell layer in the dehiscence zone by means of direct DELLA-ALC protein binding.

No previous evidences have implicated the GAs in ovule initiation and formation, a key developmental process that occurs during early pistil ontogeny. Ovules emerge from the placental tissue as finger-like primordia at stage 8 of flower development (Smyth et al., 1990; Modrusan et al., 1994). Later, three different regions can be distinguished along the proximal-distal axis: the funiculus, which attaches the ovule to the placenta, the chalaza, and the nucellus, which encloses the megaspore mother cell. Integuments develop asymmetrically from the chalaza surrounding the nucellus. The outer integument totally overgrows the inner integument in the mature ovule. Upon fertilization, cells in both the outer and the inner integument undergo a transformation process, which gives rise to the testa or seed coat (Western et al., 2001; Haughn and Chaudhury, 2005). Therefore, the structure and morphology of the mature testa depend on the correct initiation and growth of integuments. Consequently,

regulation of integument development can have vast effects on the final testa structure and composition.

Numerous genes involved in integument patterning and growth have been identified (Sieber et al., 2004; Battaglia et al., 2009; Kelley and Gasser, 2009). One such gene is *ABERRANT TESTA SHAPE* (*ATS*, *At5g42630*) (McAbee et al., 2006), which encodes a KANADI (KAN) TF, previously named KAN4. ATS provides boundary maintenance and promotes the laminar growth of the inner ovule integument. Loss of the ATS function in the *ats-1* mutant leads to the fusion of the inner and outer integuments that grow as a unit to produce a single fused structure. As a result of this fusion, *ats-1* seeds are abnormally rounded and variable in size (Leon-Kloosterziel et al., 1994; McAbee et al., 2006).

Here we describe the implication of GAs as negative factors in integument growth during ovule ontogeny, and how DELLAs are required for correct integuments formation. DELLA activity would be mediated by its direct interaction with the KANADI TF ATS/KAN4. Our data suggest that the ATS-DELLA complex is a key molecular factor for regulating the transcription of the genes involved in ovule integument growth. The positive role of DELLAs and the detrimental effect of constitutive GA signaling on ovule development contrast with the traditional view of the DELLAs as negative growth regulators. This is the first evidence for the implication of DELLAs in the development of ovules. We propose a molecular model of the function of DELLAs and ATS during integument grow.

RESULTS

DELLAs participate in seed formation

Constitutive GA signaling in *Arabidopsis* multiple DELLA loss-of-function mutants gives rise to fruits with fewer seeds and shorter in length than those in L*er* (WT) plants (Cao et al., 2005; Dorcey et al., 2009). To gain a better picture of the role of GAs in seed development, we thoroughly analyzed the number and morphology of the seeds in the *global* mutant (the quintuple *gaiT6 rgaT2 rgl1-1 rgl2-1 rgl3-1* mutant). All the assays were carried out in fruits from emasculated flowers pollinated with WT pollen to ensure that only maternal effects were tested. The seed number of the *global* mutant lowered by 60%, while fruit length shortened only by 30%, which resulted in lower seed density per fruit (Fig. 1, A and B). In many species, including *Arabidopsis*, fruit size correlates with seed number (Cox and Swain, 2006), which suggests that the facultative parthenocarpy of the *global* mutant, due to constitutive GA signaling, may account for the further elongation of these fruits and lower seed density. While mature fruits of WT plants had seed-filled siliques, the fruits of the *global* mutant had many aborted seeds (Fig. 1C). More importantly, the *global* mutant mature seeds were irregularly round in shape and smaller than the WT seeds (Fig. 1, C-E). The *della quadruple* mutant of *GAI*, *RGA*, *RGL1* and *RGL2* showed a similar phenotype (shorter fruits, fewer and rounded seeds, and parthenocarpy), which indicates that the function of these four DELLA proteins were required for proper seed development, and that RGL3 was not involved (Supplemental Fig. S1; Dorcey et al., 2009). In contrast, any of the four triple *della* mutants of GAI, RGA, RGL1, and RGL2 showed parthenocarpy or defects in seed morphology, which would suggest that both processes share a common genetic basis (Supplemental Fig. S1).

The testa of the *global* mutant seeds was defective (Fig. 1D). The epidermal cells in the testa are characterized by polygonal structures with a central elevation or columella. In contrast, the epidermal cells of *global* mutant seeds clearly lacked this structure and were less regular in shape. These modifications may diminish the mechanical restriction to embryo growth and collaborate with GA constitutive signaling in the viviparism occurrence (germination inside the silique) in *global* mutant seeds (Fig. 1E). Alteration in the testa and seed shape of the *global* mutant did not interfere with seed viability, which indicates that embryo development is not altered (see below for seed germination assays). All defects in the *global* mutant seeds are exclusively

Figure 1. Gomez et al.

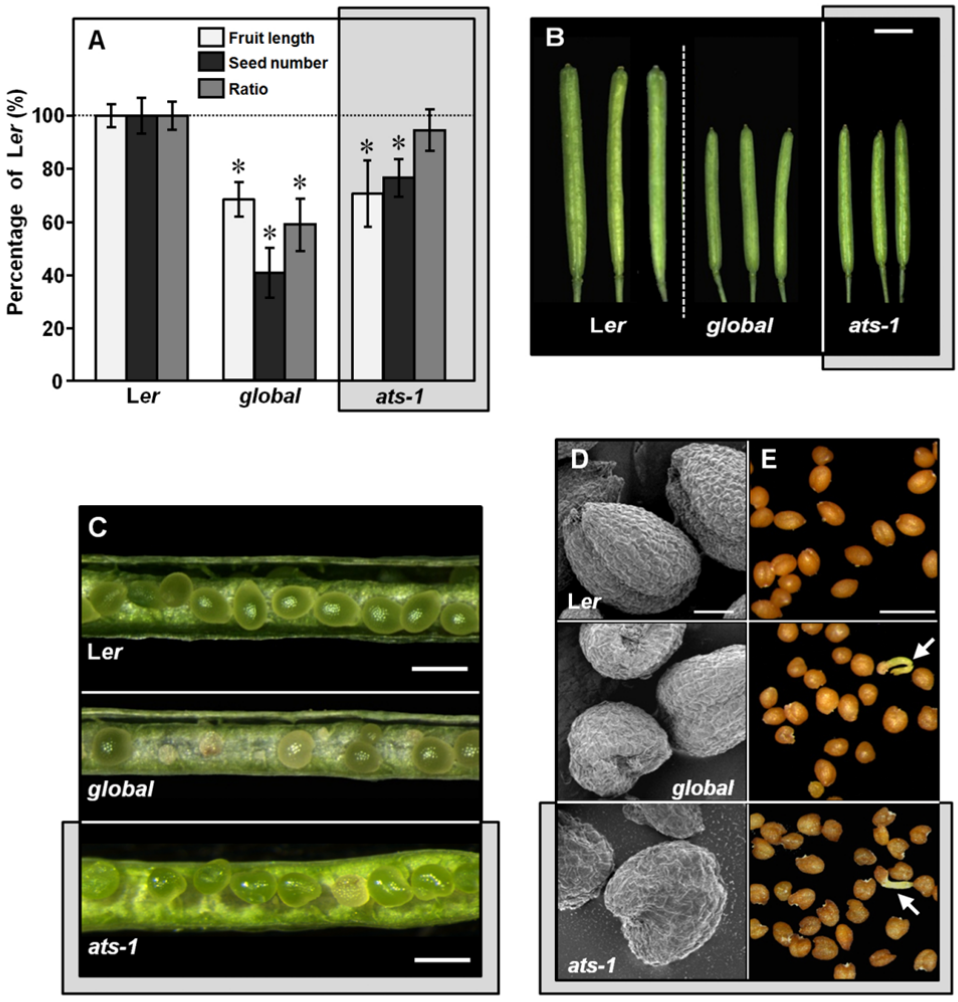

Figure 1. Constitutive GA signaling in the *della global* mutant causes seed defects and reduced fertility, similarly to *ats-1*.
A, Silique length, seed number, and length/width ratio in fruits of L*er* and the *global* and *ats-1* mutants at 12 days-post-anthesis (dpa). Data are the mean and standard error (SE) of three independent experiments, each one from at least 50 fruits. The significant differences (Student's t-test analysis) between Ler and mutants are marked with asterisks (* p-value < 0.01). **B-C,** Images of whole (**B**) and open (**C**) 8-dpa fruits of L*er*, and the *global* and *ats-1* mutants. **D-E,** Images of mature seeds of L*er*, and the *global* and *ats-1* mutants, taken by SEM (**D**) or stereomicroscope (**E**). The white arrow marks the viviparous seeds of the *ats-1* and mutants.
All the images in each category have the same magnification. Scale bars represent 2 mm in B, 500 µm in C and E, and 100 µm in D.

attributable to maternal tissue as all the assays were carried out in emasculated flowers that were hand-pollinated with WT pollen.

DELLA activity during seed formation was also tested using the gain-of-function *gai-1* mutant. The *gai-1* seeds showed apparently unaltered seed morphology, but were 25% larger than those of the WT, due to an increase along the major axis (Fig. 2, A-C), in contrast to the *global* seeds that were 25% shorter in length than WT seeds

Figure 2. Gomez et al.

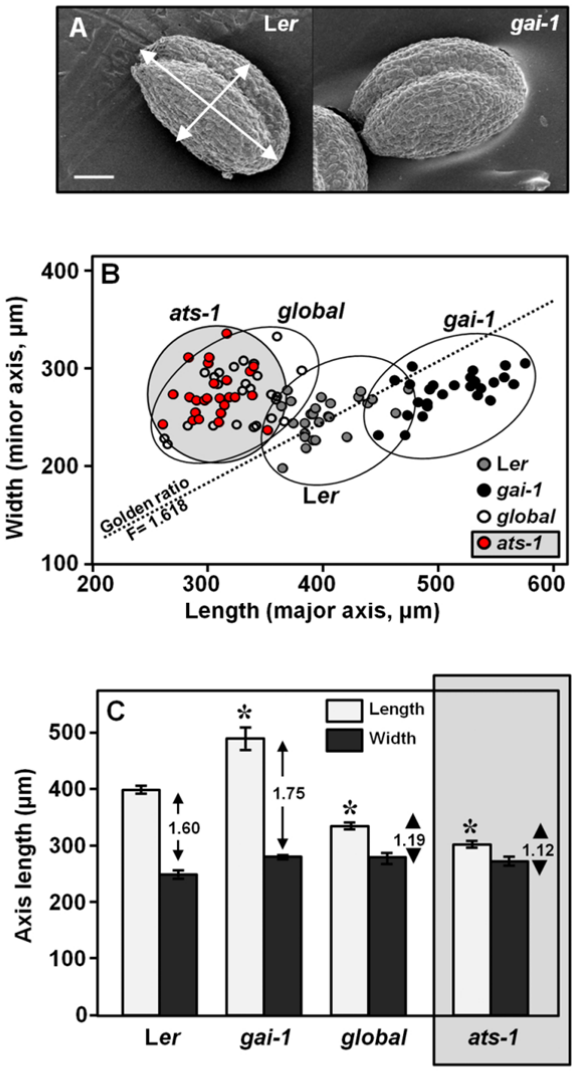

Figure 2. DELLAs and ATS regulate seed size and shape.
A, SEM images of mature seeds of L*er* and the *gai-1* mutant. Length and width (major and minor axis, respectively) are market with white arrows in Ler seed. **B,** Size distribution of mature seeds of L*er* and the *gai-1*, *global* and *ats-1* mutants, represented as length (major axis) and width (minor axis). Golden ratio is represented by a line at the 1,618 ratio. **C,** Seed length and width of L*er* and the *gai-1*, *global*, and *ats-1* mutants. Data are the mean±SE of three biological replicas, each one from 25-30 seeds. Significant differences (Student's *t*-test analysis) between L*er* and mutants are marked with asterisks (* p-value < 0.01). The ratio between seed length and width is indicated.

(Fig. 2, B and C). No significant defects were detected in the width of seeds from the *global* or *gai-1* mutants. It has been reported that length and width of *Arabidopsis* WT seeds follow the Golden ratio (Cervantes et al., 2010), also known as the divine proportion, a mathematical constant (Φ=1.618) frequently found in many biological-based geometries. The length/width ratio of the WT seeds was 1.60, which came very

close to the Golden ratio. In contrast, the ratio in the *gai-1* and *global* mutants was 1.75 and 1.19, respectively, which reflects the enlarged and shorter seed shape caused by the gain- and loss-of function of the DELLA functions. These results indicate that DELLA activity is required for proper seed growth and development, mainly on the proximal/distal axis.

The *della global* mutant has defects in integument development

The testa is differentiated from ovule integuments (Beeckman et al., 2000). Therefore, the majority of mutants with structural defects in the testa are affected in integument development. Figure 3 shows ovule and seed development in the *global* mutant and WT. At early ovule development, no clear distinction was noticed (Fig. 3A), but altered growth of integuments was observed at stage 3-II in the *global* mutant (Schneitz et al., 1995). Integuments were shorter, with no clear distinction among their different cell layers. As a consequence, at anthesis, only two cell layers in each integument were formed, and the outer integument did not cover the nucellus, displaying an altered shape (Fig. 3C). In contrast to the *global* mutant, a WT ovule at anthesis is formed by two layers of cells in the outer integument and three layers of the inner integument; the outer integument has overgrown the shorter inner integument around the embryo sac. Developing seeds of the *global* mutant clearly showed unusual testa development, with three-four cell layers, unlike the five well-defined cell layers of a WT seed, with full or partial endothelium layer, depending on the severity of phenotype (Fig. 3, D and E). Therefore, the altered ovule development of the *global* mutant is the most possible cause of the abnormal seed morphology.

The ovule and seed phenotypes of the *global* mutant resemble those of the *ats-1* mutant

Similar alterations in mature ovules to those in the *global* mutant were observed in the null *aberrant testa shape-1* (*ats-1*) mutant (Leon-Kloosterziel et al., 1994). *ATS*/*KAN4* (*At5g42630*), encodes one of the four KANADI gene family members (Hawker and Bowman, 2004), a subset of the GARP (from GOLDEN2, ARR-B Class, Par1 proteins) family of putative TF genes which play roles in leaf polarity and expansion, and also in ovule development (Eshed et al., 2001; McAbee et al., 2006).

To facilitate comparisons, the data from *ats-1* are shown along with data from the *global* mutant in Figures 1, Figure 2 and Figure 3. The siliques of *ats-1* are shorter

Figure 3. Gomez et al.

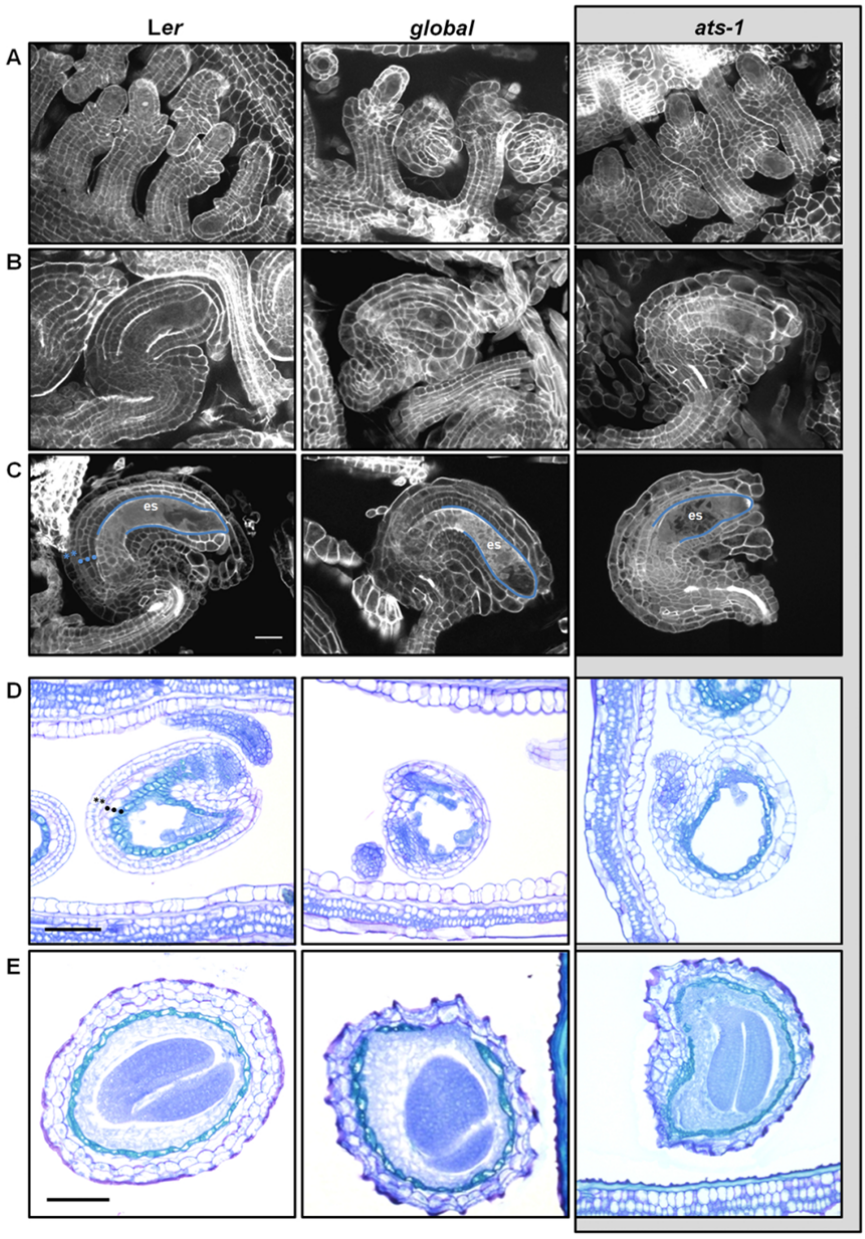

Figure 3. DELLAs and ATS regulate ovule integument development.
A-B, Images of L*er*, *global* and *ats-1* ovules at stage 2-III (A) and 3-II (B) (Schneitz et al., 1995).
C, Images of ovules at anthesis. The shape of the embryo sac (es) is marked by blue lines.
D-E, Transversal section of fertilized ovules at 3 dpa (D) or 7 dpa (E).
The outer and inner integument layers of WT in C and D are indicated with asterisks and dots, respectively. Scale bars represent 50 µm in A, B and C and 100 µm in D and E.

and contain fewer seeds that WT, but unlike the *global* mutant, *ats-1* showed a proportional reduction of seed number and silique length, resulting in a ratio similar to WT (Fig. 1, A and B) as *ats-1* does not display parthenocarpy (Vivian-Smith et al., 2001). The *ats-1* siliques also contained aborted seeds, with defects in the testa and viviparism (Fig. 1, C-E), and were smaller, globular in shape, and had a length/width

ratio of 1.12, (Fig. 2, B and C). Moreover, the ovules of *ats-1* at anthesis resembled to those of the *global* mutant, with short integuments that did not cover the embryo sac at the micropyle and fewer integument layers (Fig. 3C). Unlike *global* mutant, *ats-1* outer and inner integuments were fused, with no separation space between them from early developmental stages (Fig. 3A) (Leon-Kloosterziel et al., 1994; McAbee et al., 2006). In contrast, at stage 3-II, integuments of *ats-1* arrested growth and did not extend beyond the embryo sac, similarly to the *global* mutant (Fig. 3B). Defects in testa during seed development of both *global* and *ats-1* were also very similar (Fig. 3, D and E), Although both *global* and *ats-1* mutants showed different ovule integument development in early stages, we pursued the study of ATS role in the GA regulation of ovule growth based on two key observations: ATS was a potential binding protein to the DELLAs (Marin-de la Rosa et al., 2015) and *ats-1* displayed typical GA-related phenotypes.

ats-1 displays GA signaling phenotypes

We have shown that the ovule and seed defects of *ats-1* closely resembled those of the *global* mutant. Interestingly, *ats-1* also showed GA-related phenotypes (Fig. 4). The *ats-1* plants had similar alterations in germination and flowering to those observed in plants with a constitutive GA signaling response. GAs play a central role in germination by promoting testa breakage and facilitating radicle protrusion. Loss of DELLA activity in the *global* mutant plants led to both light- and GA-independent seed germination, with RGL2 being the predominant repressor of seed germination (Lee et al., 2002; Cao et al., 2005). The *ats-1* mutant seeds showed higher germination rate compared to those of the WT, but lower than those of the *global* and *rgl2-1* mutants (Fig. 4A). However, the *ats-1* seeds germinated at a similar rate to the null *rgl2-1* mutant in the presence of the GA inhibitor paclobutrazol (PCB). Lack of ATS function overcame the seed germination inhibition produced by the stabilized DELLA proteins by PCB, which indicates that ATS plays a role as a repressor of germination. Finally, the alterations in ovule and seed morphology observed in the *ats-1* and *global* mutants did not affect embryo viability as the germination rates were high, especially in PCB.

GAs regulate flowering time and stem elongation, especially under short days (Galvao et al., 2012; Porri et al., 2012). Coinciding with a possible GA-related phenotype caused by the loss-of-function of ATS, the flowering time of the *ats-1* plants advanced particularly in short days (Fig. 4B). The *ats-1* mutant plants also flowered

Figure 4. Gomez et al.

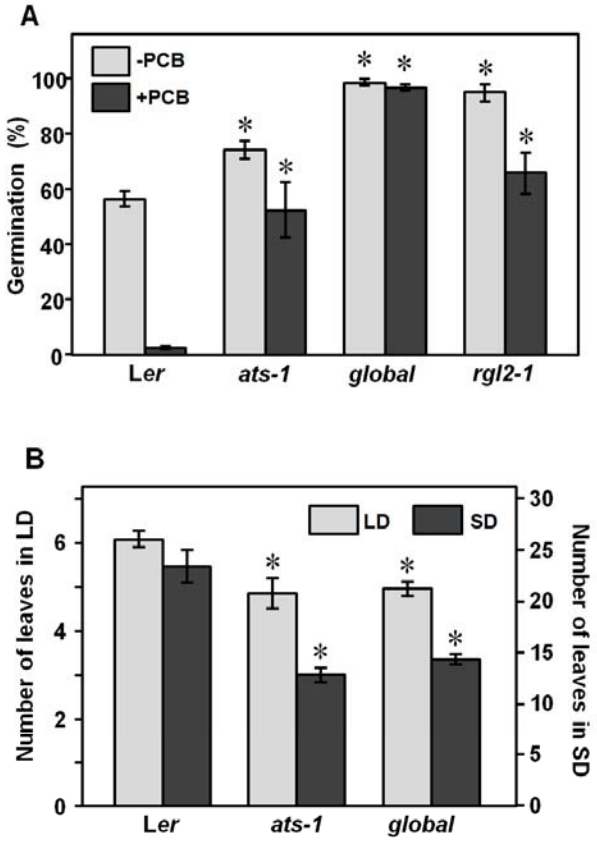

Figure 4. *ats-1* shows higher seed germination and early flowering.
A, Germination rate of L*er* and the *ats-1*, *global*, and *rgl2-1* mutants. Germination was scored at 3 days in the absence (-PCB) or presence of 1 µM of PCB (+PCB). **B,** Flowering time of L*er* and the *ats-1* and *global* mutants. Flowering was scored as the number of leaves in plants grown in long (LD) and short (SD) days. Significant differences (Student's *t*-test analysis) between L*er* and mutants are indicated by an asterisk (*, p-value < 0.01). Data are the mean±SE of three biological replicas, each one from 80-100 seeds in A and 30-40 plants in B.

slightly earlier than the WT under long days. Early flowering of *ats-1* was very similar to that of the *global* mutant (Fig. 4B). Taken together our data suggest that ATS and DELLA may participate in germination and flowering, and also in ovule/seed formation, through a common molecular mechanism. These analysis uncovered new functions of ATS, beyond ovule and seed development reported previously. Interestingly, there is a discrepancy between the known *ATS* expression pattern and the spatial distribution of phenotypes, which would be further explained by possible

indirect effects, non cell autonomy of ATS function, or the lack of a comprehensive determination of the *ATS* expression.

ATS inhibits GA biosynthesis

We hypothesized that an increase in GA content in *ats-1* could be the reason for the GA-related phenotypes described above. Direct quantification of the GA forms that belonged to the non-13-hydroxylated pathway, which contributes mainly to the biosynthesis of the bioactive GA_4, supported a role of ATS in inhibiting GA biosynthesis (Fig. 5A). Levels of GA_4 increased 3-fold in *ats-1* compared with the WT, while levels of intermediates GA_{12} and GA_{24}, and the inactivated form GA_{51}, were also significantly increased. Furthermore, expression of GA biosynthesis genes *GA20ox2*, *GA3ox1*, and *GA3ox2*, but not *GA20ox1*, was up-regulated in *ats-1* (Supplemental Fig. S2). Next we studied the expression pattern of *GA3ox1*, which catalyses the last step in the biosynthesis of bioactive GA_1 and GA_4 (Talon et al., 1990). Expression was significantly increased in developing ovules of the *ats-1* mutant (Fig. 5B). The increased *GA3ox1* expression in *ats-1* likely led to increase bioactive GAs, which finally might promote the alteration in ovule morphology. To analyze whether the increased GA content affected DELLA protein stability in ovules, we tested the RGA levels using the *pRGA:RGA-GFP* reporter construct (Silverstone et al., 2001). While RGA was located in the chalaza and integuments of developing ovules, levels were decreased in the *ats-1* mutant (Fig. 5C). Furthermore, RGA-GFP levels were also decreased in both secondary roots, where ATS is also expressed (Hawker at al., 2004), and the primary root (Supplemental Figure S3). In conclusion, our data indicated a role of ATS in repressing GA synthesis; low GA levels would stabilize DELLAs to properly coordinate growth and development. The increased GA biosynthesis in *ats-1* would result in DELLA degradation and the deregulation of GA-controlled processes, including germination, flowering time, or ovule development.

DELLAs interact with ATS

DELLAs function as transcriptional regulators, but do not encode any known DNA-binding domain. This role is exerted through their interaction with TFs. KAN1, a related KANADI gene family member, has been recently described as a putative DELLA interacting protein (Marin-de la Rosa et al., 2015). Therefore, a plausible

Figure 5. Gomez et al.

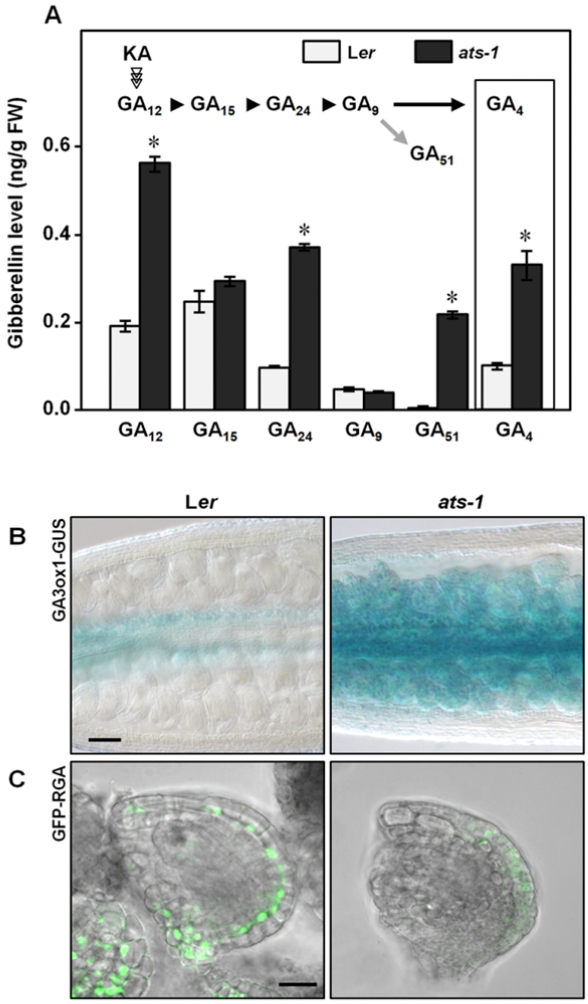

Figure 5. GA levels increased in *ats-1*.
A, The levels of GAs in the GA_4 pathway in L*er* (gray) and *ats-1* (black). The GA_4 synthesis pathway is indicated: KA, ent-kaurenoic acid; black arrow head, GA20oxidase; black arrow, GA3oxidase; gray arrow, GA2oxidase. Significant differences (Student's t-test analysis) between L*er* and *ats-1* are indicated by an asterisk (*, p-value < 0.001). Data are the mean±SE of three independent samples, expressed as ng of GA per g of FW. **B,** Expression of GA biosynthesis gene *GA3ox1* by reporter pGA3ox1:GA3ox1-GUS in ovule primordia at stage 3-I of L*er* and *ats-1*. **C,** GFP-RGA protein stability in ovule primordia of L*er* and *ats-1* at stage 3-III. Scale bars represent 100 µm in B, and 20 µm in C and D.

hypothesis would be that ATS/KAN4 could also interact with the DELLA proteins. To address this hypothesis, we performed a yeast two-hybrid assay using truncated versions of GAI and RGA, which prevent the auto-activation of reporter genes (de Lucas et al., 2008) and ATS. The *ATS* locus encodes at least two splice variants: ATS.1, the longest

Figure 6. Gomez et al.

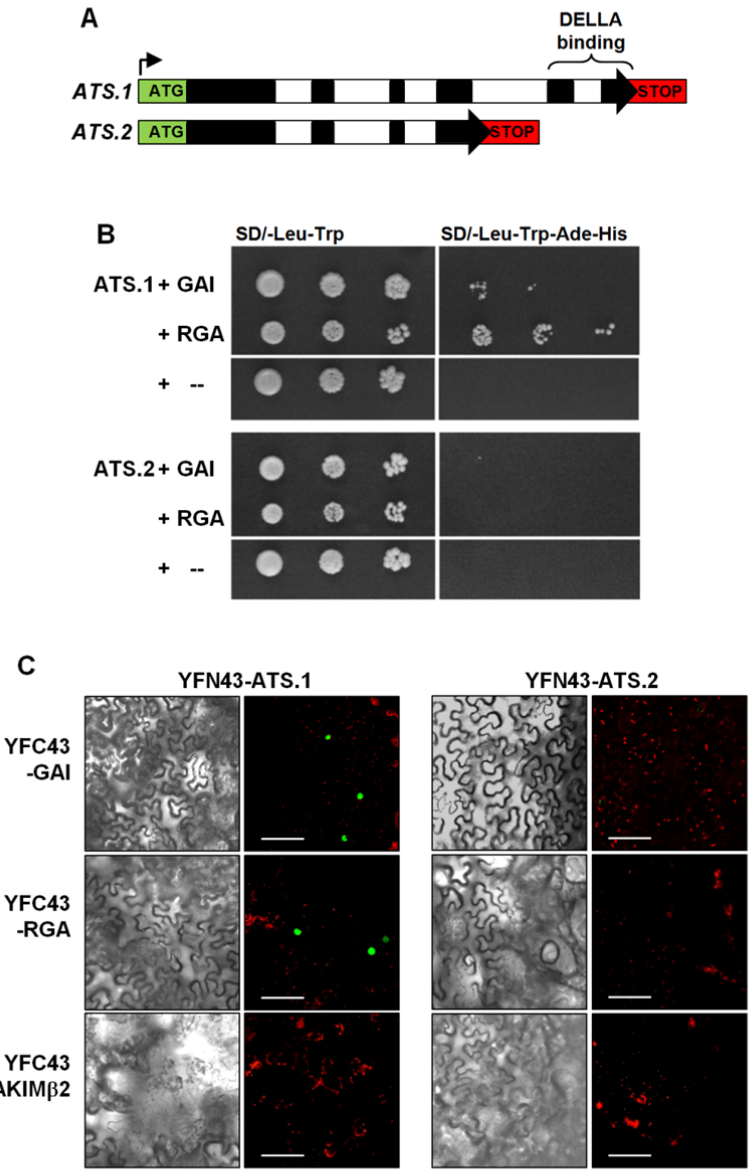

Figure 6. ATS directly binds to DELLAs.
A, Gene structure of the ATS.1 and ATS.2 splicing variants. The putative domain required for binding is localized in the 5 and 6 exons of ATS.1. **B,** Yeast two hybrid assay. DELLAs GAI and RGA fused to Gal4 DNA-BD were tested with ATS.1 and ATS.2 full-length ORFs fused to Gal4 DNA-AD. Diploids were grown in the SD/-Leu-Trp and SD/-Leu-Trp-His-Ade medium. **C,** BiFC assay. Full length GAI and RGA in pYFC43 were assayed with ATS.1 and ATS.2 in pYFN43. AKIMβ2 in pYFC43 was used as a negative control (Belda-Palazon et al., 2012). Left panels are the bright field image and right panels are the merged image of chlorophyll and YFP. Scale bar represent 100 µm.

with 6 exons, and ATS.2, which lacks the last two exons (Fig. 6A) (Gao et al., 2010). Interaction assays demonstrated that only ATS.1 was able to interact with GAI and RGA (Fig. 6B). No interaction was observed for truncated version ATS.2, which indicated that the last 67 amino acids at the carboxyl end of ATS.1 are an absolute

requirement for DELLA-ATS binding, and either act as the binding domain or stabilize the DELLA-ATS interaction. This domain does not encode for any known canonical protein motif. On the other hand, we also confirmed that GAI and RGA could also bind to KAN1 (Supplemental Fig. S4). We examined the subcelular localization of the GAI-ATS and RGA-ATS interactions by Bimolecular Fluorescence Complementation (BiFC) and found that the reconstructed YFP protein was observed only in the nuclei of epidermal cells that co-expressed YFP-ATS.1 and YFP-GAI or YFP-RGA (Fig. 6C and Supplemental Fig. S5). As in the yeast assay, ATS.2 did not bind to GAI or RGA in plants. This further confirmed the DELLA-ATS interaction *in vivo.*

If DELLA proteins regulate integument development it is necessary that they are expressed in these tissues, along with ATS. All four DELLAs *GAI*, *RGA*, *RGL1* and *RGL2* were expressed in developing ovules (Fig. 7B), but with gene-specific patterns: *GAI* expression was located mainly in the funiculus, but also in integument primordia and in the center of the nucellus; *RGA* and *RGL1* were expressed in the funiculus and integument primordia; and *RGL2* was expressed preferentially in the nucellus but also in the integument and funiculus. Therefore, the four DELLAs that were genetically involved in ovule development seemed expressed in the integuments during development, in the same tissues where *ATS* is expressed (Supplemental Fig. S6A) (McAbee et al., 2006), as well as in other tissues within the ovule.

ATS expression in integuments during ovule development is not regulated by GAs.

We also interrogated whether *ATS* and *DELLAs* regulate each other at the transcriptional level. First, we tested that there were no differences in the expression of ATS in the WT and *global* ovules (Supplementary Fig. S6A). In both cases, *ATS* expression was observed in the abaxial cells of the inner integument and in the adaxial cells of the outer integument in developing ovules, as it was previously reported (McAbee et al., 2006). The qPCR analysis revealed that *ATS* was expressed at similar levels in both inflorescences and seedlings of the *global* mutant and WT (Supplemental Fig. S6B), which confirmed *in situ* hybridization data. On the other hand, the expression

Figure 7. Gomez et al.

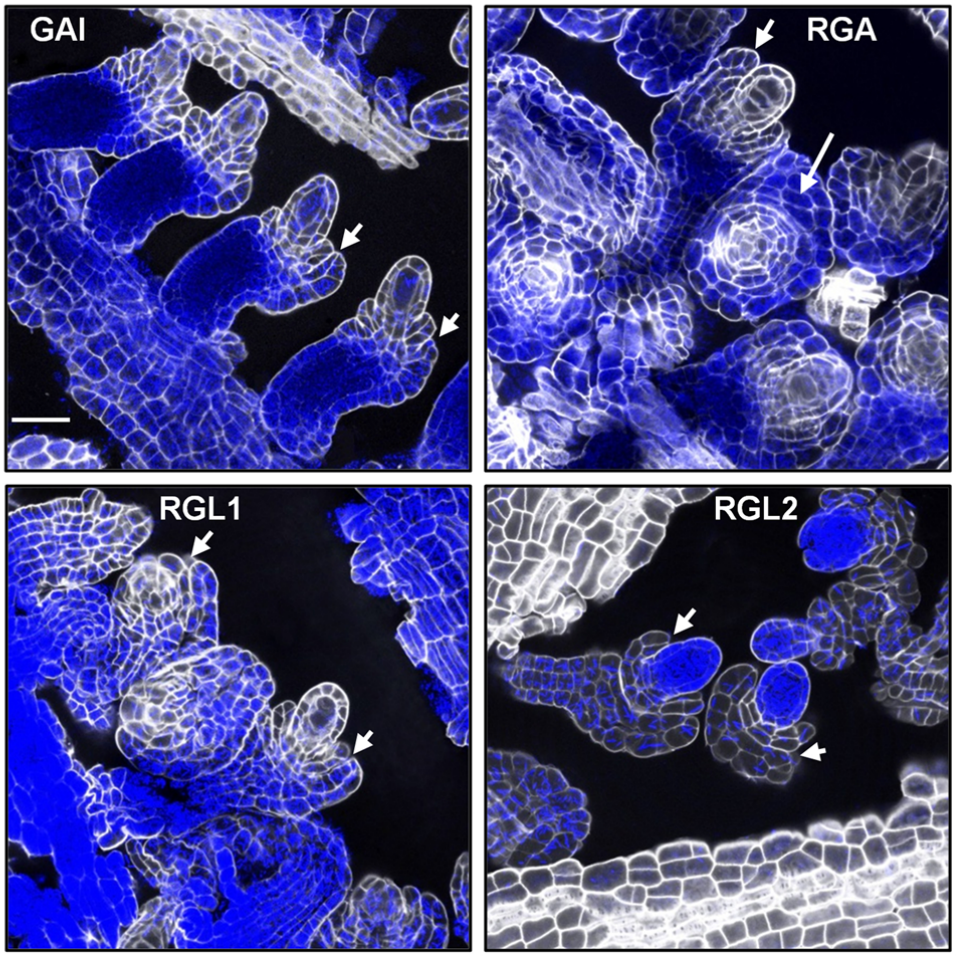

Figure 7. DELLAs are expressed in ovule integuments. Expression of DELLAs by GUS assay coupled with mPS-PI staining. *GAI*, *RGA* and *RGL1* were detected with transcription lines pGAI:GUS, pRGA:GUS, and pRGL1:GUS (Gallego-Giraldo et al., 2014). Expression of *RGL2* was monitored using the *rgl2-5* allele (Lee et al., 2002). Scale bar represents 20 µm.

of DELLA genes *GAI* and *RGA* was not significantly affected in either the seedlings or inflorescences of *ats-1* (Supplemental Fig. S6C). We conclude from these results that DELLA proteins would not be involved in the regulation of *ATS* expression and that ATS would not regulate DELLA gene expression. Therefore, it seems that no reciprocal transcriptional regulation exits between ATS and DELLAs.

Both ATS and DELLA functions are required for correct ovule development

The similar phenotypes of the loss-of-function of ATS and DELLA and the physical interaction showed herein suggested that presence of a protein complex participated by both proteins could be a strict requirement for correct ovule development. Regardless of the interaction capacity of ATS to DELLA, elevated GA levels in *ats-1* could be the cause of the described ovule defects; increased GA levels would result in the destabilization of DELLAs and would, hence, promote similar ovule defects to those in the *global* mutant. In this scenario, a dominant DELLA in an *ats-1* background would overcome ovule defects by reestablishing the proper DELLA function. As observed in Figure 8, the gain-of-function of DELLA in the *gai-1* mutant did not rescue the ovule and seed phenotypes of *ats-1*, which supports the notion that both ATS and DELLAs have to be present to promote integument differentiation. While *gai-1* showed elongated seeds, the double *gai-1 ats-1* had round seeds that were similar in shape and length/width ratio to those from *ats-1* (Fig. 8). In addition, the seeds of the double *ats-1 gai-1* showed viviparism, as in the single *ats-1* and the *global* mutant (Fig. 8A, inset). In contrast, the overall seed size of *gai-1 ats-1* was somewhat larger than those from the single *ats-1* (Fig. 8, B and C), which indicated that the gain-of-function of GAI may promote cell elongation in both the major and minor axes of seeds (Vivian-Smith and Koltunow, 1999). The fact that the dominant *gai-1* was unable to overcome ovule defects in *ats-1* implies that the ATS-DELLA physical protein interaction may be the molecular mechanism by which GAs play a role in integument development.

Figure 8. Gomez et al.

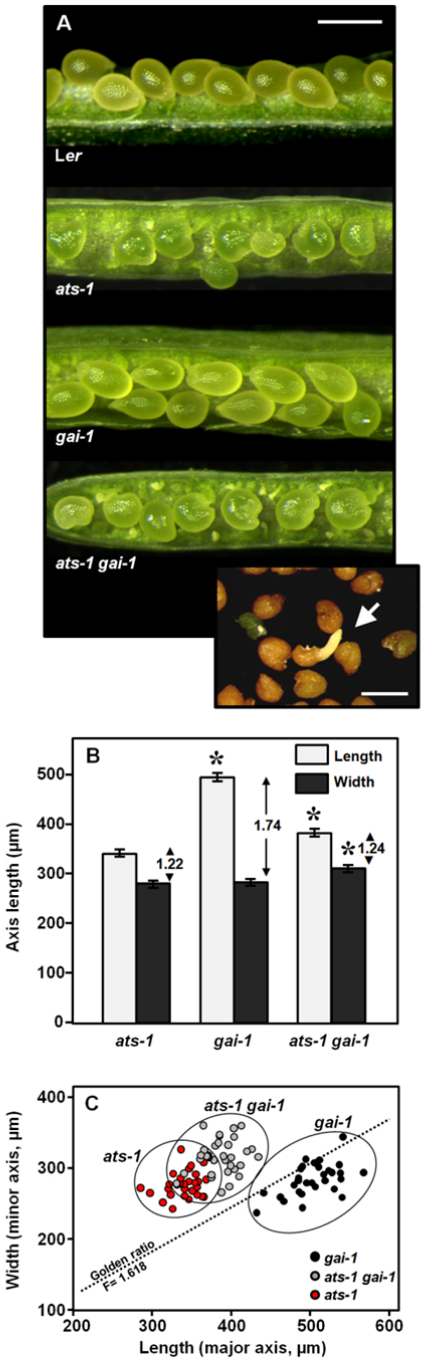

Figure 8. Gain-of-function of DELLA activity does not rescue the *ats-1* mutant defects in seed development.
A, Images of 8-dpa fruits from L*er* and the *ats-1*, *gai-1*, and double *ats-1 gai-1* mutants. The white arrow marks a viviparous *ats-1 gai-1* seed. Scale bars represent 500 µm. **B,** Seed length and width of *gai-1*, *ats-1*, and double *ats-1 gai-1*. Data are the mean±SE of three biological replicas, each one from 25-30 seeds. Significant differences (Student's *t*-test analysis) are marked with asterisks, and *ats-1* is taken as a reference (* p-value < 0.01). The seed length and width ratio is indicated. **C,** Size distribution of the mature seeds of the *ats-1*, *gai-1* and double *ats-1 gai-1*, represented as length (major axis) and width (minor axis). Golden ratio is represented by a line at the 1,618 ratio.

DISCUSSION

In this study we report the role of GAs in the control of integument development during ovule ontogenesis. Our data indicate that DELLA proteins play an essential role

Figure 9. Gomez et al.

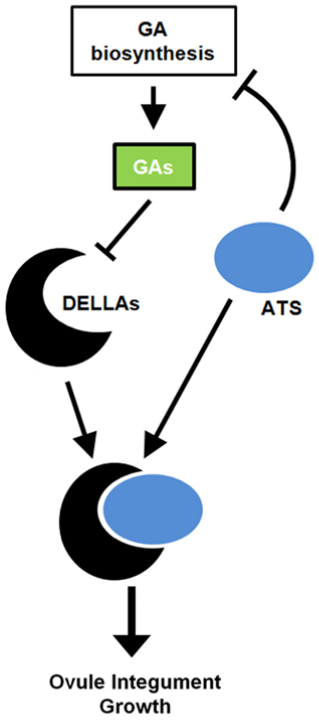

Figure 9. Model for the ATS and DELLA interaction in the control of ovule development. ATS and DELLA directly bind in ovule primordia to mediate proper ovule development, probably by regulating the expression of the genes required for ovule development. In addition, ATS inhibits GA biosynthesis, which promotes low GA levels and the stabilization of DELLAs to facilitate the formation of the protein complex.

in the correct formation of ovule integument, while constitutive GA signaling has detrimental effects. The similarity of the *global* and *ats-1* mutant phenotypes strongly suggests that both DELLAs and ATS participate in integument growth during late stages of ovule ontogeny via a common molecular mechanism (Fig. 9). ATS and DELLAs interact to coordinate proper ovule growth. This function would require the presence of both proteins in a complex, and would explain why the absence of either of them in the *global* or *ats-1* mutants results in similar ovule and seed phenotypes. In line with this, GA biosynthesis repression by ATS may promote the stabilization of DELLAs, which would strengthen the protein complex (Fig. 9). The fact that *ats-1* but not *global* shows fused integuments from early stages, would indicate that the role of ATS in this phase of development would not require the activity of DELLAs. Likewise, DELLAs would also have roles in ovule and seed development in other tissues where ATS is not active.

DELLAs can modulate transcription by binding to TFs in the promoter of target genes (Marin-de la Rosa et al., 2015; Daviere and Achard, 2016). It has recently

406 reported that RGA was localized at *cis* elements that are potential binding motifs of the
407 GARP-G2 TFs, that include ATS/KAN4 and KAN1 (Franco-Zorrilla et al., 2014;
408 Marin-de la Rosa et al., 2015). These data strongly suggest that ATS could recruit
409 DELLAs to the promoters of the ATS target genes involved in integument growth
410 through direct protein-protein interaction. In addition, the binding ATS and DELLA in
411 ovule development resembles that of DELLAs and other GARP genes, the type-B
412 ARABIDOPSIS RESPONSE REGULATORS (ARRs) in root growth. In this case RGA
413 binds to ARRs in the promoters of cytokinin-regulated genes, acting as transcriptional
414 co-activators (Marin-de la Rosa et al., 2015). This molecular mechanism depends on the
415 necessity of the simultaneous presence of DELLAs and ARRs to restrict root meristem
416 growth and to promote photomorphogenesis, similarly to the DELLA-ATS function in
417 ovule integument. RGA selectively induces the expression of ARR1 in the meristem
418 (Moubayidin et al., 2010), which may contribute to facilitate ARR1-DELLA complex
419 formation to arrest meristem growth. In contrast, the expression of DELLAs is not
420 regulated by ATS, or vice versa. In the case of the integument, repression of GA
421 biosynthesis by ATS would stabilize DELLA proteins and favors the ATS-DELLA
422 complex formation.

423 ATS also interacts with ETT/ARF3 to define the boundary between the
424 integument primordia in the ovule (Kelley et al., 2012). The loss-of-function ATS or
425 ETT resulted in common defects in ovule integument development and seed shape,
426 probably by altering auxin distribution in ovule primordia. Interestingly, DELLAs can
427 also bind to ETT (M. Blazquez and D. Alabadi, unpublished), which points out to a
428 multicomplex protein DELLA-ATS-ETT that could coordinate ovule growth and
429 development.

430 Early seed germination within siliques was observed in the *global* and *ats-1*
431 mutants. It is well known that GAs and ABA play antagonistic roles in seed
432 dormancy/germination by means of a dynamic balance between the synthesis and
433 catabolism of these two hormones (Gutierrez et al., 2007; Holdsworth et al., 2008;
434 Weitbrecht et al. 2011). On the other hand, reduced dormancy could be a direct
435 consequence of the diminished mechanical resistance in the testa caused for the loss-of
436 function of the DELLA-ATS complex. It is difficult to discriminate whether viviparism
437 is merely the consequence of testa defects or the result of the constitutive GA signaling
438 (*global*) or elevated GA levels (*ats-1*). However, viviparism was still observed in the
439 double *ats-1 gai-1*, which has blocked the GAI-dependent GA signaling, suggesting that

defects in the testa of *ats-1* are potentially responsible for the reduced dormancy. *ats-1* also showed early germination and advance flowering, similarly to the *global* mutant. Whether these phenotypes are the effect of the elevated GA levels or due to a specific role of ATS-DELLA, as it was observed in ovule development, needs further study.

In conclusion, we have shown that the correct control of GA/DELLA levels is necessary to achieve proper integument development and to generate a normal testa in mature seeds. Constitutive GA signaling by diminished DELLA function results in changes in the ovule/seed shape due to alterations in integuments/testa, which have consequences in both fertility and seed dormancy.

MATERIALS AND METHODS

Plant material assays for fruit-set, parthenocarpy, germination and flowering.

Seeds were surface-sterilized in EtOH, plated onto MS media (Murashige and Skoog, 1962), incubated at 4°C for 4 days in complete darkness, and transferred to a growth chamber at 22°C in long day photoperiod (16/8h) for 10 days. Seedlings were transferred to soil (a mix of peat moss, vermiculite and perlite, 2:1:1) and grown in a growth chamber at 22°C in long day photoperiod.

ats-1 was obtained from the NASC. The *della global* mutant (*quintuple gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1*) (Feng et al., 2008) was obtained from SW Deng (Yale University, US). Triple DELLA mutants *gai-t6 rga-24 rgl1-1*, *gai-t6 rga-24 rgl2-1*, *gai-t6 rgl1-1 rgl2-1*, and *rga-24 rgl1-1 rgl2-1* were obtained by genetic cross from single mutants. The pDELLA:GUS lines have been previously reported (Gallego-Giraldo et al., 2014). The pRGA:GFP-RGA (Silverstone et al., 2001) and pGA3ox1:GA3ox1-GUS lines (Mitchum et al., 2006) were obtained from T-p. Sun (Duke University, USA).

Fertility was assayed in hand-emasculated flowers 1 day before anthesis and hand pollinated with WT pollen at anthesis. Fruits were collected at maturity (14 days post-anthesis, dpa), seed number was counted and silique length was measured with a digital caliper. The ratio (seed number vs. silique length) was determined. Parthenocarpy was assayed in hand-emasculated flowers, which were not pollinated.

Germination was assayed in 2-3-week old seeds, plated in MS, stratified for 3 days at 4°C in the darkness. Germination was scored 3 days after incubation at 22°C as radicle emergence through the testa. Flowering was assayed under long-day (LD, 16/8h) and short-day (SD, 8/16h) conditions. After stratifying for 3 days at 4°C in the darkness,

seeds were germinated directly in soil and grown at 22°C. The total number of leaves before flowering was scored.

Gene expression analysis by qPCR

Three-day old seedlings and inflorescences were used. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) and genomic DNA was eliminated with DNase I (Qiagen). cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and qPCR was carried out with the SYBR® GREEN PCR Master Mix (Applied Biosystems) with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems), as previously described (Dorcey et al., 2009). Expression levels were calculated according to the expression of the constitutive *EIF4A1* (*At3g13929*) and data were normalized by the ΔΔCt method. The primers for the splicing version ATS.1 (Forward 5'-CAACTCATCACACTAAGGACAATGAAG-3', which spanned the intron between exon 5 and 6, and Reverse oligo 5'-CCAAATTGAGATGAATGTTGGTATCC-3') were tested for efficiency. The primers for GAI, RGA, and GA biosynthesis genes were previously described (Dorcey et al., 2009; Gallego-Giraldo et al., 2014).

Scanning Electron Microscopy (SEM)

Seeds were mounted on SEM stubs attached to the specimen holder of a CT-1000C cryo-transfer system (Oxford Instruments), and frozen in liquid N_2. The frozen samples were transferred to the cryo-stage of a JEOL JSM-5410 scanning electron microscope, sublimated at -85°C, and sputter coated with a film of gold. Finally, samples were observed at incident electron energy of 10 kV.

Histological procedures

The pGA3ox1:GA3ox1-GUS (Mitchum et al., 2006) and pDELLA:GUS lines (Gallego-Giraldo et al., 2014) were used for the GUS assay and the histological procedures basically as previously described (Carbonell-Bejerano et al., 2010). The $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ concentrations were adjusted for each line to obtain optimal signals (5 mM for pGAI:GUS and pRGA:GUS; 2 mM for pRGL1:GUS, and 4 mM for *rgl2-5*, and 50 μM for pGA3ox1:GA3ox1-GUS). After GUS staining, samples were either cleared with chloral hydrate or stained following a modified pseudo-Schiff propidium iodide (mPS-PI) technique (Truernit et al., 2008). Images were captured with

a microscope Eclipse E600 (Nikon) equipped with Nomarski interference optics or with a ZEISS LSM 780 confocal microscope. GUS staining was visualized in the confocal with a MBS T80/R20 dichroic (561 nm and 545-570 nm excitation and reflexion, respectively). PI staining was excited at 561 nm and detected at 580-660 nm.

For thin sectioning in Figure 3D and E, fruits were fixed overnight in 4% (w/v) p-formaldehyde in 0.1 M sodium phosphate, pH 7.2, with 0.05% (v/v) of Tween 20 at 4°C and dehydrated in ethanol. Samples were then infiltrated in Technovit 7100 resin, sectioned in a Reichert Jung Ultracut E microtome at 3 μm, and stained in 0.02% Toluidine blue as described in Gomez et al. (2004).

Quantification of GAs

GAs were quantified basically as described by Seo et al. (2011). Three-day-old seedlings were extracted with 80% methanol-1% acetic acid, and extracts were passed consecutively through HLB (reverse phase), MCX (cationic exchange) and WAX (ionic exchange) columns (Oasis 30 mg, Waters). GAs were separated using reverse phase UPHL chromatography (2.6 μm Accucore RP-MS column; ThermoFisher Scientific) with a 5-50% acetonitrile gradient with 0.05% acetic acid at 400 μL/min. GAs were analyzed by electrospray ionization and targeted-SIM in a Q-Exactive spectrometer (Orbitrap detector; ThermoFisher Scientific). [17,17-^{2}H] GA_4, GA_9, GA_{12}, GA_{15}, GA_{24}, GA_{34} and GA_{51} were used as internal standards for quantification.

***In situ* RNA hybridization**

Inflorescences were embedded, sectioned and hybridized as described elsewhere (Weigel and Glazebrook, 2002; Gomez et al., 2011). The partial clone for ATS.1, corresponding to the complete codifying sequence, was cloned in the pGEM-T easy vector (Promega). Sense and antisense probes were synthesized using the SP6 and T7 RNA polymerases, respectively.

Yeast two hybrid.

Full-length ORFs were first cloned in pGEM-T (ATS.1 and ATS.2) or pBSK (KAN1), and transferred into pGADT7 (Clontech) by EcoRI-ClaI digestion-ligation. The M5-truncated versions of cDNA for GAI and RGA (de Lucas et al., 2008) in pGBKT7 (Clontech) were obtained from S. Prat (CNB, Spain). Plasmids pGADT7 and pGBKT7 were introduced into the Y187 and the Y2HGold yeast strain, respectively.

Both strains containing the corresponding plasmids were mated o/n at 28°C and diploids were selected in the SD media supplemented with His and Ade. The interaction was assayed on minimal media plates without either Leu/Trp or Leu/Trp/Ade/His in 10-fold serial dilutions for 5 days at 28°C. Empty vectors were used as negative controls.

BiFC assay.

The BiFC assay was carried out basically as described in Belda-Palazon et al. (2012). The full-length ORFs of ATS.1/ATS.2 and GAI/RGA were cloned in pCR8 and transferred by GATEWAY into the YFN43 and the YFC43 plasmid, respectively. As a nonspecific control, cDNAs for AKINβ2 in pYFC43 and AKIN10 in pYFN43 were used (Ferrando et al., 2001). Fluorescence was observed 2-3 days after infiltration at 488 nm under a ZEISS LSM 780 confocal microscope.

ACKNOWLEDGMENTS

We wish to thank Dr. T-p. Sun (Duke University) for the pGA3ox1:GA3ox1-GUS and pRGA:GFP-RGA lines, Dr. S.W. Deng (Yale University) for the *global* mutant, Dr. S. Prat (CNB, Spain) for the M5-truncated versions of GAI and RGA in pGBKT7, and Dr. A. Ferrando (IBMCP, Spain) for the BiFC plasmids. We also thank Dr. I. Lopez-Diaz and E. Carrera (hormone quantification facility, IBMCP, Spain) for the quantification of GAs, and Ms. M. Gascon, C. Fuster and M.A. Argomániz for technical assistance.

565 **FIGURE LEGENDS**

566

567 **Figure 1. Constitutive GA signaling in the *della global* mutant causes seed defects**
568 **and reduced fertility, similarly to *ats-1*.**

569 **A,** Silique length, seed number, and length/width ratio in fruits of L*er* and the *global*
570 and *ats-1* mutants at 12 days-post-anthesis (dpa). Data are the mean and standard error
571 (SE) of three independent experiments, each one from at least 50 fruits. The significant
572 differences (Student's *t*-test analysis) between L*er* and mutants are marked with
573 asterisks (* p-value < 0.01). **B-C,** Images of whole (**B**) and open (**C**) 8-dpa fruits of L*er*,
574 and the *global* and *ats-1* mutants. **D-E,** Images of mature seeds of L*er*, and the *global*
575 and *ats-1* mutants, taken by SEM (**D**) or stereomicroscope (**E**). The white arrow marks
576 the viviparous seeds of the *ats-1* and *global* mutants. All the images in each category
577 have the same magnification. Scale bars represent 2 mm in B, 500 μm in C and E, and
578 100 μm in D.

579

580 **Figure 2. DELLAs and ATS regulate seed size and shape.**

581 **A,** SEM images of mature seeds of L*er* and the *gai-1* mutant. Length and width (major
582 and minor axis, respectively) are market with white arrows in L*er* seed. **B,** Size
583 distribution of mature seeds of L*er* and the *gai-1*, *global* and *ats-1* mutants, represented
584 as length (major axis) and width (minor axis). Golden ratio is represented by a line at
585 the 1,618 ratio. **C,** Seed length and width of L*er* and the *gai-1*, *global*, and *ats-1*
586 mutants. Data are the mean±SE of three biological replicas, each one from 25-30 seeds.
587 Significant differences (Student's *t*-test analysis) between L*er* and mutants are marked
588 with asterisks (* p-value < 0.01). The ratio between seed length and width is indicated.

589

590 **Figure 3. DELLAs and ATS regulate ovule integument development.**

591 **A-B**, Images of L*er*, *global* and *ats-1* ovules at stage 2-III (**A**) and 3-II (**B**) (Schneitz et
592 al., 1995). **C,** Images of ovules at anthesis. The shape of the embryo sac (es) is marked
593 by blue lines. **D-E,** Transversal section of the fertilized ovules at 3 dpa (**D**) or 7 dpa (**E**).
594 The outer and inner integument layers of WT in A and D are indicated with asterisks
595 and dots, respectively. Scale bars represent 50 μm in A, B and C and 100 μm in D and
596 E.

597

598 **Figure 4. *ats-1* shows higher seed germination and early flowering.**

A, Germination rate of L*er* and the *ats-1*, *global*, and *rgl2-1* mutants. Germination was scored at 3 days in the absence (-PCB) or presence of 1 μM of PCB (+PCB). **B,** Flowering time of L*er* and the *ats-1* and *global* mutants. Flowering was scored as the number of leaves in plants grown in long (LD) and short (SD) days. Significant differences (Student's *t*-test analysis) between L*er* and mutants are indicated by an asterisk (*, p-value < 0.01). Data are the mean±SE of three biological replicas, each one from 80-100 seeds in A and 30-40 plants in B.

Figure 5. GA levels increased in *ats-1*.

A, Levels of GAs in the GA_4 pathway in L*er* (gray) and *ats-1* (black). The GA_4 synthesis pathway is indicated: KA, ent-kaurenoic acid; black arrow head, GA20oxidase; black arrow, GA3oxidase; gray arrow, GA2oxidase. Significant differences (Student's *t*-test analysis) between L*er* and *ats-1* are indicated by an asterisk (*, p-value < 0.001). Data are the mean±SE of three independent samples, expressed as ng of GA per g of FW. **B**, Expression of GA biosynthesis gene *GA3ox1* by reporter pGA3ox1:GA3ox1-GUS in developing ovules at stage 3-I of L*er* and *ats-1*. **C,** GFP-RGA protein stability in integuments of L*er* and *ats-1* at stage 3-III. Scale bars represent 100 μm in B, and 20 μm in C and D.

Figure 6. ATS directly binds to DELLAs.

A, Gene structure of the ATS.1 and ATS.2 splicing variants. The putative domain required for binding is localized in the 5 and 6 exons of ATS.1. **B,** Yeast two hybrid assay. DELLAs GAI and RGA fused to Gal4 DNA-BD were tested with ATS.1 and ATS.2 full-length ORFs fused to Gal4 DNA-AD. Diploids were grown in the SD/-Leu-Trp and SD/-Leu-Trp-His-Ade medium. **C,** BiFC assay. Full length GAI and RGA in pYFC43 were assayed with ATS.1 and ATS.2 in pYFN43. AKIMβ2 in pYFC43 was used as a negative control (Belda-Palazon et al., 2012). Left panels are the bright field image and right panels are the merged image of chlorophyll and YFP. Scale bar represent 100 μm.

Figure 7. DELLAs are expressed in ovule integuments.

Expression of DELLAs by GUS assay coupled with mPS-PI staining. *GAI*, *RGA*, and *RGL1* were detected with transcription lines pGAI:GUS, pRGA:GUS, and pRGL1:GUS (Gallego-Giraldo et al., 2014). Expression of *RGL2* was monitored using the *rgl2-5* allele (Lee et al., 2002). Scale bar represents 20 µm.

Figure 8. Gain-of-function of DELLA activity does not rescue the *ats-1* mutant defects in seed development.

A, Images of 8-dpa fruits from L*er* and the *ats-1*, *gai-1*, and double *ats-1 gai-1* mutants. The white arrow marks a viviparous *ats-1 gai-1* seed. Scale bars represent 500 µm. **B,** Seed length and width of *gai-1*, *ats-1* and double *ats-1 gai-1*. Data are the mean±SE of three biological replicas, each one from 25-30 seeds. Significant differences (Student's *t*-test analysis) are marked with asterisks, and *ats-1* is taken as a reference (* p-value < 0.01). The seed length and width ratio is indicated. **C,** Size distribution of the mature seeds of the *ats-1*, *gai-1*, and double *ats-1 gai-1* mutants, represented as length (major axis) and width (minor axis). Golden ratio is represented by a line at the 1,618 ratio.

Figure 9. Model for the ATS and DELLA interaction in the control of ovule development.

ATS and DELLA directly bind to mediate proper ovule development, probably by regulating the expression of the genes required for ovule development. In addition, ATS inhibits GA biosynthesis, which promotes low GA levels and the stabilization of DELLAs to facilitate the formation of the protein complex.

Supplemental Data

Supplemental Figure S1. Parthenocarpy and seed defects are characteristic of the *global* and *quadruple*, but not of any of the triple *della* null mutants.

Supplemental Figure S2. Expression analysis of GA biosynthesis genes.

Supplementary Figure S3. Increased GA level in *ats-1* promotes RGA degradation in roots.

Supplemental Figure S4. Binding of GAI and RGA to KAN1 in yeast two hybrid assay.

Supplemental Figure S5. Controls of the BiFC assay between DELLAs with ATS.

Supplemental Figure S6. ATS and DELLAs do not regulate each other at the transcriptional level.

Supplemental Figure S1. Parthenocarpy and seed defects are characteristic of the *global* and *quadruple*, but not of any of the triple *della* null mutants. A, Fruit/pistil length of WT, *global* and the triple mutant combinations of *GAI*, *RGA*, *RGL1* and *RGL2*. Flowers were hand-emasculated and fruit or pistil length was scored at 12 dpa. Data are the mean±SE of three independent experiments, each one from 40-50 flowers. Significant differences (Student's *t*-test analysis) between L*er* and mutants are marked with asterisks (* p-value < 0.01). **B,** Images of 8-dpa fruits of L*er* and the *global*, quadruple, and triple DELLA mutants. **C,** Genotype of the *della* mutants used in this assay. Triple 1 (t1) is *gaiT6 rga24 rgl1-1* with native RGL2; triple 2 (t2) is *gaiT6 rga24 rgl2-1* with native RGL1; triple 3 (t3) is *gaiT6 rgl1-1 rgl2-1* with native RGA; triple 4 (t4) is *rga24 rgl1-1 rgl2-1* with native GAI. Scale bar represents 500 µm.

Supplemental Figure S2. Expression analysis of GA biosynthesis genes.
qPCR expression analysis of *GA20ox1*, *GA20ox2*, *GA3ox1* and *GA3ox2* in 3-day-old seedling of L*er* (light gray) and the *ats-1* (dark gray). Expression was normalized to that of *EIF4A1* (*At3g13929*) in L*er.* Data are the mean±SE of three biological replicas.

Supplementary Figure S3. Increased GA level in *ats-1* promotes RGA degradation in roots.
GFP-RGA protein stability in secondary (**A**) and primary (**B**) roots of L*er* and *ats-1* of 3-day old seedlings. Scale bar represents 30 µm.

Supplemental Figure S4. Binding of GAI and RGA to KAN1 in yeast two hybrid assay.
A-B, Yeast two hybrid assay of the M5-truncated version of GAI, RGA, and the pGBKT7 empty vector (BD, --) against KAN1 (A) or the pGADT7 empty vector (AD, --) (B).

Supplemental Figure S5. Controls of the BiFC assay between DELLAs with ATS.
Upper panel, nonspecific protein AKIN10 in pYFN43 was assayed with RGA in pYFC43. Medium panel, the empty pYFN43 vector was assayed with GAI and RGA in

pYFC43. Lower panel, BiFC assay using empty pYFN43 and pYFC43 vectors. No signal was observed in any plasmid combination. BF, bright field; merge, merged images of chlorophyll and YFP. Scale bar represents 100 μm.

Supplemental Figure S6. ATS and DELLAs do not regulate each other at the transcriptional level.

A, *In situ* mRNA hybridization of *ATS* in the ovules of L*er* and the *global* mutant at stage 2-III. Outer and inner integument cell layers are indicated with black and blue asterisks, respectively. **B,** Expression of *ATS* in 3-day old seedlings and inflorescences of L*er* (light gray) and the *global* mutant (dark gray). **C,** Expression of *GAI* and *RGA* in 3-day-old seedling of L*er* (light gray) and *ats-1* (dark gray). The expression of *ATS* and *DELLAs* in B and C was normalized to that of *EIF4A1* (*At3g13929*) in L*er.* Data are the mean±SE of three biological replicas.

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