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Role of the gibberellin receptors *GID1* during fruit-set in *Arabidopsis*

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SUMMARY

Gibberellins (GAs) play a critical role in fruit-set and fruit growth. Gibberellin is perceived by its nuclear receptors GA INSENSITIVE DWARF1s (*GID1*s), which then trigger degradation of downstream repressors DELLAs. To understand the role of the three GA receptor genes (*GID1A*, *GID1B* and *GID1C*) in *Arabidopsis* during fruit initiation, we have examined their temporal and spatial localization, in combination with analysis of mutant phenotypes. Distinct expression patterns are revealed for each *GID1*: *GID1A* is expressed throughout the whole pistil, while *GID1B* is expressed in ovules, and *GID1C* is expressed in valves. Functional study of *gid1* mutant combinations confirms that *GID1A* plays a major role during fruit-set and growth, whereas *GID1B* and *GID1C* have specific roles in seed development and pod elongation, respectively. Therefore, in ovules, GA perception is mediated by *GID1A* and *GID1B*, while *GID1A* and *GID1C* are involved in GA perception in valves. To identify tissue-specific interactions between *GID1*s and DELLAs, we analyzed spatial expression patterns of four DELLA genes that have a role in fruit initiation (*GAI*, *RGA*, *RGL1* and *RGL2*). Our data suggest that *GID1A* can interact with *RGA* and *GAI* in all tissues, whereas *GID1C*–*RGL1* and *GID1B*–*RGL2* interactions only occur in valves and ovules, respectively. These results uncover specific functions of each *GID1*–DELLA in the different GA-dependent processes that occur upon fruit-set. In addition, the distribution of GA receptors in valves along with lack of expression of GA biosynthesis genes in this tissue, strongly suggests transport of GAs from the developing seeds to promote fruit growth.

Keywords

fruit-set; gibberellin; GA INSENSITIVE DWARF1; DELLA; *Arabidopsis*

INTRODUCTION

Fruit-set is the activation of the developmental program that transforms a pistil into a developing fruit. It is initiated by the fertilization of ovules, which promotes synthesis and signaling of hormones, mainly auxin and gibberellin (GA; Dorcey *et al.*, 2009). These hormones form the initial instructive signals that promote the fruit development program under physiological conditions (Alabadi *et al.*, 2009).

Gibberellins are plant tetracyclic diterpenoids that control a wide range of processes throughout plant development, including seed germination, leaf expansion, stem and root elongation, floral induction, and flower development (Fleet and Sun, 2005; Swain and Singh, 2005; Sun, 2011). Studies also indicate that GAs are key factors for fruit-set and development. GA treatment of unpollinated pistils promotes fruit initiation, probably by mimicking GA production upon ovule fertilization (Vivian-Smith and Koltunow, 1999; Dorcey *et al.*, 2009). In fact, upon pollination, GA biosynthesis genes are up-regulated, and bioactive GA₁ and its precursor GA₂₀ levels increase (Ben-Cheikh *et al.*, 1997). The last steps of GA biosynthesis are catalyzed by GA20-oxidases (GA20ox) and GA 3-oxidases (GA3ox), which convert inactive GA precursors into bioactive GAs (Hedden and Thomas, 2012). Significant progress has been made in defining the roles of both *GA20ox* and *GA3ox* genes during reproductive growth in *Arabidopsis* (Hu *et al.*, 2008; Rieu *et al.*, 2008; Plackett *et al.*, 2012). Expression of most of the *GA20ox* and *GA3ox* genes is induced at fruit initiation, and each gene shows a specific temporal expression pattern; most are up-regulated in the ovules after fertilization (Dorcey *et al.*, 2009). *GA20ox1* and *GA20ox2* play a major role in fertility and fruit growth (Rieu *et al.*, 2008). Reduced fruit length in the double *ga20ox1 ga20ox2* mutant has a maternal origin, pointing out to a defect in GA-dependent fruit elongation in the absence of these *GA20ox* activities. In a similar way, *GA3ox3*, *GA3ox4*, along with *GA3ox1*, have major roles in providing the bioactive GAs during flower development and fruit-set (Hu *et al.*, 2008). *GA3ox4* is involved in promoting seed-dependent fruit elongation. Similar up-regulation of GA biosynthesis genes upon fruit-set has also been reported in other species (Garcia-Martinez *et al.*, 1997; Serrani *et al.*, 2007).

Gibberellin signaling relies on the perception of the hormone by its receptor GA INSENSITIVE DWARF1 (GID1). GID1 was first described in rice as a nuclear localized protein similar to hormone-sensitive lipase family without hydrolase activity (Ueguchi-Tanaka *et al.*, 2005). In *Arabidopsis*, there are three GID1 orthologs (*GID1A*, *GID1B*, and *GID1C*; Griffiths *et al.*, 2006; Nakajima *et al.*, 2006). The active GA binds to GID1 and promotes a conformational change in its N-terminus region (Murase *et al.*, 2008; Shimada *et al.*, 2008). The GA-GID1 complex can then bind to the GA-signaling repressor DELLA proteins, promoting a conformational modification, which allows their recognition by GID2 or SLEEPY1 (SLY1) F-box proteins in rice and *Arabidopsis*, respectively (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003; Griffiths *et al.*, 2006; Willige *et al.*, 2007; Murase *et al.*, 2008; Hirano *et al.*, 2010). The DELLA proteins are then polyubiquitinated and degraded through the 26S-proteasome pathway (Sasaki *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004). Studies using the *sly1* and *gid2* mutants further show that GA-GID1 binding to DELLA can also inhibit DELLA activity without DELLA degradation (Ariizumi *et al.*, 2008, 2013; Ueguchi-Tanaka *et al.*, 2008). Both proteolysis-dependent and -independent mechanisms of GA

signaling call for a close interaction between GID1s and DELLAs. This interaction may occur only if these proteins are temporally and spatially co-expressed. In addition, GID1s and DELLAs showed differential binding affinity *in vitro* (Nakajima *et al.*, 2006 and Suzuki *et al.*, 2009), which may contribute to establishing proper GA-signaling responses in specific tissues during plant development.

The presence of three GID1s in Arabidopsis suggests that each GA receptor may have distinct roles during plant development. In contrast, *gid1* mutant analysis points to partial redundancy. While the single mutants did not show any obvious phenotype, the doubles show partially reduced GA-response phenotypes: *gid1a-1 gid1c-1* is a semi-dwarf and has compromised germination, and *gid1a-1 gid1b-1* shows stamen filament shortening that results in reduced fertility (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006; Iuchi *et al.*, 2007; Voegele *et al.*, 2011). The null mutation in all three genes leads to an extremely dwarf and GA-insensitive plant (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006; Iuchi *et al.*, 2007). Therefore, *GID1A* and *GID1C* play a major role during seed germination (Voegele *et al.*, 2011) as well as in stem elongation (Suzuki *et al.*, 2009). In addition, the role of *GID1A* and *GID1C* is consistent with their spatial expression in stems and seeds.

Fruit-set and subsequent development are controlled by GAs. This study aims to understand the role of GA receptors in Arabidopsis upon fertilization by a combination of expression and mutant phenotype analyses. We show that *GID1A* plays a major role during fruit-set, whereas *GID1B* and *GID1C* have partially redundant function with *GID1A* in seed development and pod elongation, respectively. In addition, the GA-dependent degradation of the endocarp during fruit senescence and maturation, necessary to pod shattering, is controlled mainly by *GID1A*. We also identified distinct co-expression patterns of GID1s and DELLAs, which uncover specific roles of several GID1-DELLA combinations during seed-set and fruit growth.

RESULTS

***GID1* genes are differentially expressed in pistils and fruits**

To investigate the role of each of the *GID1* GA receptors during fruit-set and early fruit development, we first quantified their expression in pistils and fruits, by quantitative RT-PCR (qRT-PCR). mRNAs for the three *GID1* genes were detected in pistils and fruits, with *GID1A* being expressed at higher levels than *GID1B* and *GID1C* (Figures 1a and S1a). The expression of the three *GID1s* increased after anthesis in the unpollinated pistils. However, the expression levels of *GID1A* and *GID1C* remained unaltered, but *GID1B* was decreased upon pollination. The increased *GID1* expression in unfertilized pistils may be due to the feedback mechanism caused by low levels of GAs in this tissue.

To determine whether GID1s are expressed in specific tissues in the pistil, we performed qRT-PCR analysis using hand-dissected ovules + funiculi and valves from unfertilized pistils. Data in Figures 1(b) and S1(b) indicated that *GID1B* and *GID1C* mRNAs were preferentially expressed in ovules + funiculi and valves, respectively, while *GID1A* was expressed throughout the pistil.

GID1 proteins show specific spatial expression patterns

To finely study the localization of GID1 proteins in pistils and fruits, we analyzed their expression patterns using transgenic *Arabidopsis* lines carrying *GID1* promoter:*GID1-GUS* translational fusions (Suzuki *et al.*, 2009). GUS staining in whole pistil at anthesis clearly showed that GID1 proteins are differentially expressed (Figure 2a). GID1A was localized in the ovary and style, but was absent in the stigma. In the ovary, it was expressed in nearly all tissues, with the exception of the central area of the septum, the transmitting track (Figure 2b). In contrast to the broader expression of GID1A, GID1B and GID1C showed restricted expression. GID1B was mainly localized in ovules and funiculi, and it was also expressed at much lower levels in the exocarp and endocarp *a*. GID1B was absent from the mesocarp, stigma or style (Figure 2a,b). Expression of GID1C was localized specifically in the valve and style, but was completely absent in the ovules and funiculi (Figure 2a,b). GID1C is the only GA receptor that showed some expression in stigma (Figure 2a). All three receptors are expressed in the medial and lateral vascular tissues of the pistil.

Expression of GID1A and GID1B in ovules showed slight variations in localization (Figure 2b); while GID1A was localized in all tissues with stronger expression surrounding the embryo sac, GID1B was localized mainly in the chalaza, at the base of the embryo sac. GID1C expression was undetectable in ovules/seeds. In summary, GID1A and GID1C are expressed in valves, while GID1A and GID1B are expressed in ovules. The different spatial and temporal expression of GID1s may reflect their involvement in different GA-mediated processes during fruit-set and development.

RGL1 and RGL2 are differentially expressed in pistils and fruits

DELLAs are involved in the control of fruit-set in *Arabidopsis*. The elimination of four of the five DELLA genes (*GAI*, *RGA*, *RGL1* and *RGL2*) is sufficient to promote facultative parthenocarpy (Dorcey *et al.*, 2009). To study further the role of each DELLA in fruit-set, we analyzed their mRNA levels in pistils and fruits by qRT-PCR. *RGA* and *RGL1* were expressed at higher levels, while *GAI* and *RGL2* were expressed at much lower levels (Figures 3a and S2a). Upon fertilization, the expression of *GAI* and *RGL1* slightly increased, the expression of *RGA* decreased, while no changes in expression were observed for *RGL2*. We also examined the expression of the four DELLAs in hand-dissected pistils to uncover their spatial expression within the pistil. *GAI* and *RGA* were expressed similarly in ovules + funiculi and valves (Figures 3b and S2b), while *RGL1* and *RGL2* were preferentially expressed in valves and ovules + funiculi, respectively (Figure 3b), suggesting their tissue-specific functions in GA-mediated signaling.

DELLAs co-localize with GID1s in ovules and developing seeds

Gibberellin receptors interact, in a GA-dependent manner, with the DELLA proteins, to alleviate DELLA repression on GA signaling. Although the kinetics of GID1-DELLA interaction have been the focus of several reports by *in vitro* assays (Nakajima *et al.*, 2006; Iuchi *et al.*, 2007 and Suzuki *et al.*, 2009), the formation of such protein complexes is only possible by co-localization of GID1s and DELLAs both in time and space. Therefore, to determine which GID1-DELLA complex is mediating GA-dependent fruit-set events, it is important to analyze the tissue-specific expression patterns of DELLAs in pistils, and

compare them with those for the *GID1*s. Toward this goal, we generated promoter-GUS transcriptional fusion transgenic lines for *GAI*, *RGA* and *RGL1* (see Experimental procedures for details). For the study of the spatial expression of *RGL2*, the line *rgl2-5* was used (a translational GUS fusion, Lee *et al.*, 2002; Figure 4).

While *RGA* and *GAI* were expressed throughout the pistil, *RGL1* and *RGL2* showed more restricted expression patterns (Figure 4). *RGA* was detected at high levels in all tissues, but *GAI* expression showed high levels in valves, especially in mesocarp and endocarp cell layers, and in funiculi; weaker *GAI* expression was detected in ovules at anthesis. Interestingly, the expression patterns for *GAI* and *RGA* resembled those for *GID1A*. On the other hand, *RGL1* was expressed mainly in the endocarp, and at lower levels in mesocarp and exocarp (Figure 4), as well as in funiculi at 2 dpa. It was not detected in ovules or seeds. In contrast to *RGL1*, *RGL2* expression was detected in both ovules at anthesis and seeds, including funiculi, but it was totally absent in valves. *RGL1* and *RGL2* showed strikingly similar expression patterns to *GID1C* and *GID1B*, respectively.

Overall, the *GID1* and *DELLA* expression patterns using GUS assays are consistent with the results of qRT-PCR analysis. The comparison of *DELLA* and *GID1* expression patterns in different tissues allowed us to speculate spatial-specific *GID1*-*DELLA* interactions that mediate GA signaling (Figures 2 and 4). In ovules, *GID1A* and *GID1B* would potentially interact only with *GAI*, *RGA* and *RGL2*. In contrast, in valves, *GID1A* and *GID1C* would interact with *GAI*, *RGA*, and *RGL1*.

Mutation of *GID1* genes results in maternal defects in fertility

In this study, we showed that *GID1A* and *GID1C* are expressed in valves, while *GID1A* and *GID1B* are expressed in ovules. These expression patterns suggest that *GID1A* and *GID1C* mediate GA-induced fruit growth, whereas *GID1A* and *GID1B* promote seed development. Consistent with *GID1* expression patterns, the single *gid1a* and the double *gid1a gid1b* and *gid1a gid1c* mutants display compromised fertility (Griffiths *et al.*, 2006). *gid1a* had some decrease in seed number and a slight reduction in fruit length. The double mutant *gid1a gid1c* showed a strong reduction in silique length with a little reduction of seed number. In contrast, the double mutant *gid1a gid1b* had much reduced seed number and silique length; this phenotype was attributed to the shortening of the stamen filament that reduced pollination (Griffiths *et al.*, 2006; Iuchi *et al.*, 2007; Plackett *et al.*, 2011). Fruit growth and fertility is the result of the combinatory effect of male (pollen) and female (ovule and ovary) factors. Considering that *GID1A* and *GID1B* are expressed in ovules, the reduced seed number and silique length phenotype of the double *gid1a gid1b* could also result from reduced seed-set due to defects in the ovule. To test this possibility, we carried out an experiment in which pistils of emasculated WT (Col-0) and *gid1* mutant flowers were hand pollinated with WT pollen. In this way, reduced fertility would only be a consequence of pistil defect. Fruits were harvested at maturity, just before pod opening, and pod length, seed number and the ratio between seed number and pod length for each individual fruit were determined (Figure 5a). Among the single mutants, only *gid1a* showed a slight reduction in silique length and seed content, which resulted in the seed/silique length ratio similar to WT. Double mutants showed different phenotypes (Figure 5a). Similarly to *gid1a*, *gid1a gid1b*

had a proportional but stronger reduction in both silique length and seed number that resulted in a ratio similar to WT. In contrast, *gid1a gid1c* showed the same seed number reduction as in *gid1a* but a strong reduction in silique length, which resulted in a significant increase of seed/length ratio, i.e., a higher seed density or increased packing phenotype in these fruits. *gid1b gid1c* did not show any defects. Almost identical results were obtained with self-pollinated pistils for the three *gid1* double mutants (Figure S3), indicating that the phenotypes of *gid1a gid1b* and *gid1a gid1c* are mainly caused by maternal defects.

Elimination of two out of the three GA receptors in each double mutant combination may cause up-regulated expression of the remaining *GID1* gene, which may alleviate reduced GA-response phenotype. Indeed, expression of *GID1A* in the double *gid1b gid1c* is enhanced in seedlings and pistils (Figure S4), which contributes to normal growth even in the absence of *GID1B* and *GID1C* activity. In contrast, no alteration of the expression of *GID1B* or *GID1C* was observed in either seedling or pistils of *gid1a gid1c* and *gid1a gid1b*, respectively.

There is a close correlation between the final fruit size and the seed number (Cox and Swain, 2006; Dorcey *et al.*, 2009). Therefore, a reduction in seed number with no alteration of the slope of the best fit line of the data (a measure of seed-dependent fruit growth) may reveal defects of seed-set, while a decrease in the slope may reflect impairment of pod elongation, which results in increased packing of seeds in the fruit. To distinguish between defects in seed-set and fruit elongation, we analyzed siliques from the double *gid1* mutants manually pollinated with different amounts of WT pollen, and recorded final fruit length and seed number (Figure S5). The *gid1a gid1b* double mutant showed, as expected, a reduced seed number with no significant alteration of the seed number/fruit length ratio, indicating that the smaller fruit size is due to decreased seed-set. In contrast, *gid1a gid1c* showed a significant decrease in the slope, which suggested that this mutant has defects in pod elongation even in the presence of a significant number of seeds, possibly due to a reduced sensitivity to GAs. Again, *gid1b gid1c* behaved as the WT plant. Finally, without pollination, all three *gid1* double mutants showed similar pistil length as WT (approximately 4 mm).

Consistent with the expression patterns of *GID1*s in the fruit, the phenotypes observed for *gid1a gid1b* and *gid1a gid1c* reflect GA-insensitivity in ovules and valves that causes reduced seed-set and pod elongation, respectively. To determine if the *gid1a gid1c* pistil is indeed insensitive to GA treatment, the response of unfertilized pistils to GAs in double null mutants was recorded (Figure 5b). *gid1a gid1b* and *gid1b gid1c* showed normal GA response; in contrast, the *gid1a gid1c* pistils displayed little or no GA response. Therefore, the reduced fruit growth in *gid1a gid1c* is due to limited GA-mediated pod elongation.

In summary, our data suggest that the three *GID1* GA receptors act partially redundantly in pistil development. The defects observed in the *gid1* double mutants are due to GA perception defects in maternal tissues. The contribution of filament shortening to fertility in *gid1a gid1b* seems to be minor.

Mutation of *GID1* genes promotes alterations at the endocarp

The inner cell layers of the Arabidopsis silique, the endocarp, differentiate during fruit development to facilitate pod shattering and seed dispersal. During fruit development, the endocarp *a*, the adaxial cell layer facing the carpel space, is degraded, along with the lignification of the support cell layer, the endocarp *b*. We previously showed that both processes are mediated by GAs (Dorcey *et al.*, 2009). Early degradation and lignification was observed in GA-treated unfertilized pistils, as well as in the DELLA quadruple mutant (*gai-16 rga-24 rgl1-1 rgl2-1*). To determine which GA receptors participate in this process, we studied pod structure during fruit development in double *gid1* mutants (Figure 6). Morphological analysis suggested that *GID1A* has a major role in the regulation of the differentiation of the inner cell layers of the fruit pod, followed by *GID1C* (Figure 6). Although the single *gid1a* mutant did not show significant alteration, double mutants *gid1a gid1b* and *gid1a gid1c* showed 1 and 2 days delay, respectively, in the degradation of endocarp *a* and the lignification of endocarp *b*. This correlates with the intensity and distribution of their expression: *GID1A* is highly expressed in pods, followed by *GID1C* (Figure 2b). Expression of *GID1B* is also detected, to a much lesser extent, in the endocarp *b*, as well as in the adaxial layer, the exocarp. Even though both *GID1B* and *GID1C* are expressed in the endocarp, degradation and lignification in *gid1b gid1c* was identical to that of the WT plants (Figure 6). The enhanced expression of *GID1A* in all tissues of this mutant may prevent detectable alterations of the process.

Expression of GA biosynthesis genes in ovules and seeds co-localize with *GID1*s

Differential spatial localization of *GID1* proteins suggests that each *GID1* may perceive GAs synthesized in, or transported to, different tissues within the pistil/fruit. Previously, we showed that most of the GA biosynthesis genes have a coordinated temporal expression patterns in ovules upon fertilization (Dorcey *et al.*, 2009). In addition, *GA20ox1* and *GA20ox2*, as well as *GA3ox1*, *GA3ox3*, and *GA3ox4*, have been shown by mutant analyses to have crucial roles in fruit-set and fertility (Hu *et al.*, 2008; Rieu *et al.*, 2008; Plackett *et al.*, 2012). All four *GA3ox* genes are expressed in young developing seeds (Mitchum *et al.*, 2006; Hu *et al.*, 2008). In addition, *GA3ox1* contributes to bioactive GA synthesis in maternal tissues, including replum, funiculus and receptacle (Hu *et al.*, 2008; Arnaud *et al.*, 2010). However, spatial and temporal expression of *GA20ox*s in ovules and developing seeds has not been reported previously. Therefore, we have studied the expression of *GA20ox1* and *GA20ox2* in ovules and seeds with translational fusion GUS lines (Figure 7). *GA20ox1*-GUS (Desgagne-Penix *et al.*, 2005) was expressed only in the pollen and pollen tube (Figure 7a). Expression could also be detected in proximal site of the embryo sac just after fertilization. Interestingly, reciprocal crosses revealed that the *GA20ox1*-GUS activity came from the sperm cells rather than a *de novo* expression in the egg cell. Figure S6(a) shows that *GA20ox1*-GUS activity could be detected in both self-pollinated GUS reporter line and in WT plants pollinated with pollen from the GUS line, but GUS expression was absent when the GUS line was pollinated with WT pollen or in unfertilized pistils. Therefore, GUS activity in the *GA20ox1*-GUS was dependent on the male tissues; *GA20ox1* expression was only detected when pollen was from the *GA20ox1*-GUS line.

GA20ox2-GUS (Frigerio *et al.*, 2006) was expressed in both pollen tubes and seeds (Figure 7b). At 1 dpa, GUS activity was detected in embryo sac, but at 2–3 dpa its expression shifted to the chalazal pole of the developing seed. This change in spatial expression may correlate with its differential expression in male and female tissues at different stages after fertilization. Indeed, as it was observed for the GA20ox1-GUS line, GA20ox2-GUS expression 1d after fertilization in the embryo sac corresponded to male tissue (pollen tube discharge in embryo upon plasmogamy; Figure S6b). On the other hand, expression in the chalaza at 2–3 dpa corresponded to the sporophytic female tissue, as this expression was detected only in the GA20ox2-GUS line, regardless the precedence of the pollen. In contrast, neither GA20ox1 nor GA20ox2 showed any expression in valve (Figure S6).

In summary, GUS assays confirm previous qRT-PCR data indicating that the GAs are mostly synthesized in the developing seeds by GA20ox and GA3ox. With the exception of *GA3ox1* expression in receptacle, replum and funiculus, none of the *GA3ox* or *GA20ox* genes were expressed in the valves. The strong expression of *GID1A* and *GID1C* in valves and their function in GA-induced pod elongation imply that GAs perceived in valves are transported mainly from the seed.

DISCUSSION

Differential expression of *GID1s* in ovules and valves

By qRT-PCR analysis, we observed that all three *GID1s* are expressed in pistils, with *GID1A* expressed at higher levels, followed by *GID1B* and *GID1C*. The predominant expression for *GID1A* reveals its major role in GA perception through the plant, including pistils and fruits. Within pistils, *GID1s* show differential spatial distribution as indicated by our results of qRT-PCR analysis and expression study using *GID1*-GUS transgenic lines. In valves, *GID1A* and *GID1C* participate in GA perception, while in the ovules GA perception is carried out by *GID1A* and *GID1B*, but not *GID1C*. Therefore, distinct *GID1* combinations perceive GAs synthesized in, or transported to, specific tissues of the pistil to regulate growth and development of the seeds and the surrounding pod. Interestingly, most of the *GA20ox* and *GA3ox* genes that control the final steps of GA biosynthesis are only expressed in the developing seeds, but not in the fruit (Hu *et al.*, 2008; Dorcey *et al.*, 2009; this study). The only exception is *GA3ox1*, which is not expressed in young seeds, but is expressed in the replum, funiculus and receptacle of the fruit (Hu *et al.*, 2008). None of the *GA20ox* or *GA3ox* genes are expressed in the valves (Hu *et al.*, 2008; Arnaud *et al.*, 2010; Plackett *et al.*, 2012). Therefore, the bioactive GAs in the valve for promoting fruit elongation are likely transported from the developing seeds and/or from other surrounding tissues (replum, funiculus and receptacle). How bioactive GAs are transported to the valve remains an open question.

GID1s and DELLAs are co-expressed in pistils during fruit-set

Gibberellin signaling occurs through the GA-dependent interaction of *GID1* and DELLA proteins (Sun, 2011). Our spatial expression analysis of DELLAs and *GID1s* suggests that certain *GID1* and DELLA combinations interact *in vivo* to trigger the GA response in pistils. *GID1A* along with RGA, and GAI to a lesser extent, are expressed throughout the pistil. We

propose that GID1A–GAI and GID1A–RGA interactions account for most of the GA-mediated processes in both valve and ovules. In addition, GID1B–RGL2 interaction can also occur in ovules, and GID1C–RGL1 interaction may take place in valves. The latter two interactions may account for tissue-specific GA-mediated processes during fruit-set and development, such as fertility and seed development for GID1B–RGL2 and fruit growth for GID1C–RGL1.

In addition to these co-expression patterns, differential affinity of GID1s towards the DELLAs may also modulate their interactions (Nakajima *et al.*, 2006; Suzuki *et al.*, 2009). For example, *in vitro* binding assays have revealed a strong affinity of RGA–GID1B, RGL2–GID1A, and GAI–GID1B proteins (Suzuki *et al.*, 2009), each pair being co-expressed in ovules/seeds (this study). On the other hand, in addition to RGA–GID1B interaction, RGL1 can strongly interact with GID1A and GID1C, all of these combinations being expressed in the valves. The same *in vitro* assay revealed that RGL1 and RGL2 interact with very low affinity to GID1B and GID1C, respectively (Suzuki *et al.*, 2009). Interestingly, RGL1–GID1B and RGL2–GID1C are not co-expressed in pistils, which may reflect co-evolution of GID1 and DELLA that results in distinct expression patterns and binding affinity. Figure 8 represents the possible GID1 and DELLA interactions in pistils, based on spatial gene expression and mutant phenotype analysis. Based on this model, we analyzed the silique phenotypes (seed number and pod length) of the triple mutants *rga gai rgl2* and *rga gai rgl1*, but did not find significant differences comparing to the WT plants. This could be explained by the presence of GA and GID1s in these mutants. To test the specific roles of different DELLAs in ovule development and pod elongation, expression of gain-of-function *rga gai rgl2* versus gain-of-function *rga gai rgl1* using their endogenous promoters will be necessary.

GID1s have partial redundant function in fertility and fruit growth

Our study showed that the expression patterns of GID1s in developing fruit correlate well with the phenotypes of *gid1* mutants. Lacking functional *GID1A*, which is expressed in nearly all pistil tissues, caused a slight reduction in fertility. Removing both *GID1A* and *GID1B* that are expressed in ovules resulted in reduced fertility. In contrast, mutations in both *GID1A* and *GID1C* that are expressed in the valves led to reduced fruit length.

The reduced silique length and low seed yield were previously correlated with shortening of the stamen filament, which decreased pollination (Griffiths *et al.*, 2006; Iuchi *et al.*, 2007). This is in agreement with the similar expression levels of both *GID1A* and *GID1B* in filaments (Suzuki *et al.*, 2009). However, we found that the double mutant *gid1a gid1b*, which does not have GID1 activity in ovules, showed reduced seed-set even when fertilized by WT pollen. These results indicate that the reduced seed-set of the *gid1a gid1b* mutant is due to defects in maternal tissues, likely because of the absence of the main GA receptors in the ovule (GID1A and GID1B). GID1C expression is not detected in either ovules or funiculi, and it is not induced in the double mutant *gid1a gid1b*. It is intriguing that despite the lack of functional GA receptors in seeds, the double mutant *gid1a gid1b* still produces a significant number of seeds, suggesting the presence of a GA or GID1-independent pathway

in promoting seed-set. However, this hypothesis is difficult to be verified because neither the GA-deficient mutant *gal* nor the triple *gid1a gid1b gid1c* mutant produces fertile flowers.

In contrast to *gid1a gid1b*, *gid1a gid1c* produced shorter silique with little reduction in seed number, even when pollinated with WT pollen, indicating that this phenotype is also due to maternal defects. The reduced fruit length in this *gid1* double mutant is caused by limited GA perception in the silique pod. The double *gid1b gid1c* mutant did not show any fruit defects, indicating that GID1A activity is sufficient for GA perception in the pistil. In addition, *GID1A* expression in *gid1b gid1c* was slightly enhanced in seedlings and pistils (Figure S4), which further contributes to normal growth. Interestingly, expression of *GID1B* or *GID1C* was not induced in either seedling or pistils of *gid1a gid1c* and *gid1a gid1b*, respectively. Previous study showed that expression of *GID1A* and *GID1B* (but not *GID1C*) is controlled by the DELLA-dependent feedback mechanism (Griffiths *et al.*, 2006). The up-regulation of *GID1A* in *gid1b gid1c* may be consequence of this feedback mechanism. However, *GID1B* was not up-regulated in seedlings or pistils of *gid1a gid1c*. Interestingly, up-regulation of *GID1B* in *gid1a gid1c* was described in floral buds, although none of the double mutants showed up-regulation of any of the *GID1s* in the inflorescence stem (Suzuki *et al.*, 2009). All together, these data suggest that the feedback mechanism may be affected by developmental processes.

In summary, our study revealed that GID1A in combination with GID1B and GID1C controls seed-set and fruit growth, respectively. The distribution of GA receptors in valves, along with the lack of expression of late-stage GA biosynthesis genes, strongly suggests GA transportation from the seeds to promote fruit growth. Finally, the co-expression of GID1s and DELLAs in different tissues of the pistil suggests specific roles of each GID1–DELLA combination in the GA-dependent fruit-set and development. Detailed analysis of this interaction and the study of their specific roles in each tissue would be necessary to dissect and understand the GA-mediated molecular mechanisms taking place in pistils and fruits.

EXPERIMENTAL PROCEDURES

Plant material and fruit-set assays

Arabidopsis thaliana plants are in the Col-0 background. *gid1a-1* (SALK_142767), *gid1b-1* (SM_3_30227), and *gid1c-1* (SALK_023529) were described by Griffiths *et al.* (2006). These lines will be mentioned in this report as *gid1a*, *gid1b*, and *gid1c*, respectively. Double mutants *gid1a-1 gid1b-1*, *gid1a-1 gid1c-1*, and *gid1b-1 gid1c-1* were generated by genetic cross and confirmed by genotyping, using the oligos listed in Table S1.

Seeds were surface-sterilized in EtOH and plated onto ½MS (Murashige and Skoog, 1962) media, kept at 4°C for 4 days, and transferred to a growth chamber at 22°C in long day photoperiod (16 h/8 h) for 10 days. Seedlings were then transferred to soil (a mix of peat moss, vermiculite and perlite, 2:1:1) and grown to maturity in a growth chamber at 22°C in long day photoperiod (16 h/8 h).

Fertility was scored using two different approaches: auto-pollinated fruits or fruits from emasculated flowers fully pollinated with WT pollen. Fruits were collected at maturity (12

days post-anthesis, dpa), seed number was counted and silique length was measured with a digital caliper. Ratio (seed number versus length) was determined. Parthenocarpy was assayed by application of GA₃ to unfertilized pistils. Flowers were emasculated 1 day before anthesis and treated the next day with 330 μM GA₃ (Fluka, <http://www.sigmaaldrich.com/>) in 0.01% (v/v) Tween-80, pH 7. Fruit and pistils were harvested 10 days after treatment, and scanned to measure final length with ImageJ software (Abramoff *et al.*, 2004). Experiments were repeated three times with similar results.

Generation of transcriptional DELLA–GUS gene fusions and plant transformation

Transcriptional DELLA–GUS gene fusions were constructed for *RGA*, *GAI* and *RGL1* genes. *RGA* gene fusions were made in binary vector pOCA28. pRGA:GUS contains approximately 7.7 kb of the *RGA* promoter region upstream of the ATG start site fused to the GUS reporter gene. *GAI* and *RGL1* gene fusions were generated using vector pBI101.1. pGAI:GUS contains approximately 4 kb of the *GAI* promoter region upstream of the translational start site fused to the GUS reporter gene. pRGL1:GUS contains approximately 4 kb of the *RGL1* promoter region upstream of the translational start site fused to the GUS reporter gene. For the pRGL2: GUS fusion, the *rgl2-5* allele (Lee *et al.*, 2002) was used. Detailed procedures for making the DELLA–GUS gene fusion constructs are described in Methods S1, using oligos listed in the Table S2. Plant transformations using DELLA–GUS plasmids and isolation of homozygous transgenic lines that contain a single insertion site were conducted as previously described (Hu *et al.*, 2008).

Gene expression analysis by qRT-PCR

Pistils and fruits were harvested at different time points. For the dissection assay, ovules and valves of 1, 2, and 3 dpa unfertilized pistils (from emasculated flowers) were hand-dissected using acupuncture needles under a stereomicroscope. In addition, whole unfertilized pistils of 0 dpa were also harvested as reference. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, <http://www.qiagen.com/>). Genomic DNA was eliminated with 50 units of DNase I (Qiagen) for 15 min at room temperature. cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, <http://www.invitrogen.com/>). qRT-PCR was carried out using the SYBR® GREEN PCR Master Mix (Applied Biosystems, <http://www.lifetechnologies.com/>) in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems), essentially as described in Dorcey *et al.* (2009).

For the quantification of gene expression, cDNAs for each gene were cloned in pGEM-T Easy vector (Promega, <http://www.promega.com/>), using oligos listed in the Table S3. Absolute expression values were calculated basically as described in Whelan *et al.* (2003). Standard curves were generated using serial dilutions of purified plasmid DNA for each gene, ranging from 10⁻⁷ to 10⁻¹⁰. The copy number of plasmid in each dilution was calculated based on the molecular weight and the initial concentration of each construct (Whelan *et al.*, 2003). Log₁₀ of copy number of each transcript were calculated from the standard curve and the value of the threshold cycle (Ct) from the qRT-PCR data. The number of copies of each gene was normalized using the number of copies of *ubiquitin10* (*UBQ10*, At4 g05320; Czechowski *et al.*, 2005) in each sample. Primers (Table S1) were

designed with the Primer Express™ v2.0 software (Applied Biosystems) and tested for efficiency.

Scanning electron microscopy (SEM)

Samples were harvested, mounted on the specimen holder of a CT-1000C cryo-transfer system (Oxford Instruments, www.oxford-instruments.com), interfaced with a JEOL JSM-5410 scanning electron microscope, and frozen in liquid N₂. Samples were fractured, and sublimated at -85°C. Finally, samples were observed at incident electron energy of 10 kV with ×10 to ×100 magnification.

β-Glucuronidase (GUS) histochemical assay and histological procedures

The pGA20ox1:GA20ox1-GUS (Hay *et al.*, 2002) and pGA20ox2:GA20ox2-GUS (Frigerio *et al.*, 2006) transgenic Arabidopsis lines were provided by Dr Hedden (Rothamsted Research Center). The pGID1A:GID1A-GUS, pGID1B:GID1B-GUS and pGID1C:GID1C-GUS transgenic lines (Suzuki *et al.*, 2009) were provided by Dr Nakajima (University of Tokyo). GUS assay and histological procedures were basically as previously described (Carbonell-Bejerano *et al.*, 2010). K₃Fe(CN)₆ and K₄Fe(CN)₆, concentrations were adjusted for each line to obtain optimal signal (5 mM for pGID1A:GID1AGUS and pGID1B:GID1B-GUS, 10 mM for pGID1C:GID1C-GUS, 0.2 mM for pGA20ox:GA20ox-GUS, 5 mM for pGAI:GUS and pRGA:GUS; 2 mM for pRGL1:GUS, and 4 mM for *rgl2-5*). For the detection of GUS activity in thin resin sections, after staining with X-GlcA, samples were dehydrated in a series of 20, 35, and 50% (v/v) ethanol, post-fixed for 30 min in FAE (50% [v/v] ethanol, 5% [v/v] formaldehyde, 10% [v/v] acetic acid), and further dehydrated to 100% (v/v) EtOH and embedded in Technovit 7100 resin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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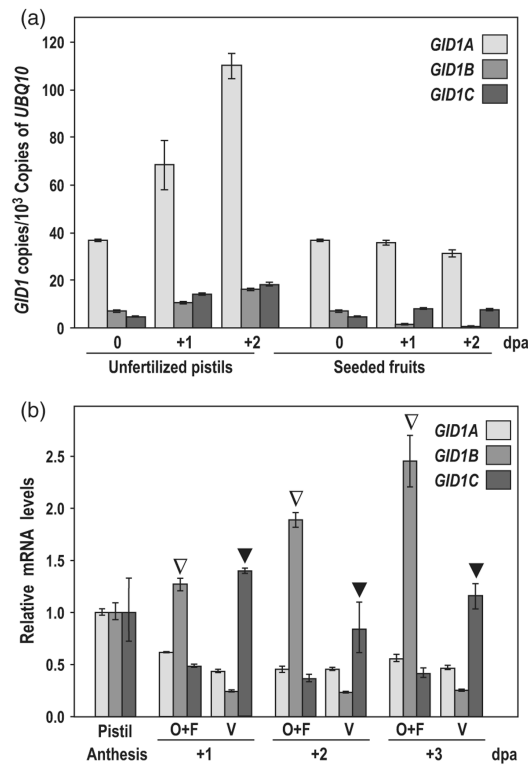


Figure 1.

GID1 genes are differentially expressed in pistils and fruits.

(a) Time-course of *GID1* expression during unfertilized pistil and early fruit development. Expression is represented as the copies of cDNA per 10³ copies of *UBQ10*.

(b) *GID1* expression in ovules + funiculi (O + F) and valve (V) of hand-dissected unfertilized pistils at 1, 2, and 3 dpa. Expression was normalized to *UBQ10* and to the expression in the whole pistil at anthesis. Open and closed triangles indicate preferential expression of *GID1B* in ovules and *GID1C* in valves, respectively. Each experiment was repeated twice using independent samples with similar results (Figure S1). Data are the mean \pm standard deviation (SD) of a single representative experiment. dpa, days post-anthesis.

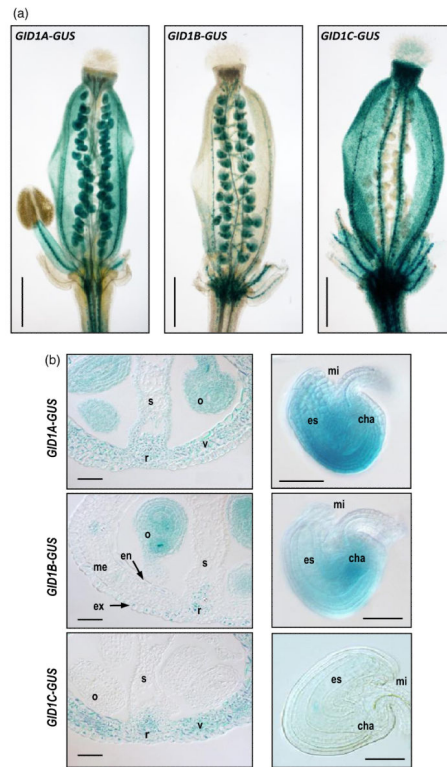


Figure 2.

GID1 expression is differentially localized in tissues of pistils and fruits.

GID1 expression was analyzed using translational fusion lines *pGID1:GID1-GUS* (Suzuki *et al.*, 2009).

(a) Expression in whole pistils at anthesis. Pistils were slightly squeezed between slide and coverslip to differentiate expression in valves and ovules. Scale bar represents 500 μm .

(b) Expression in cross-sections of pistils (left panels) and ovules (right panels) at anthesis. o, ovule; r, replum; s, septum-transmitting track; v, valve, ex, exocarp, me, mesocarp, en, endocarp. mi, micropyle; es, embryo sac; cha, chalaza. Scale bar represents 50 μm .

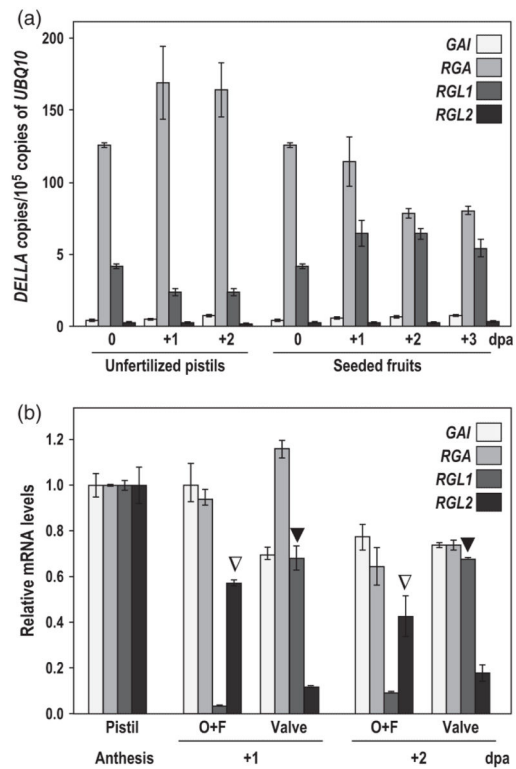
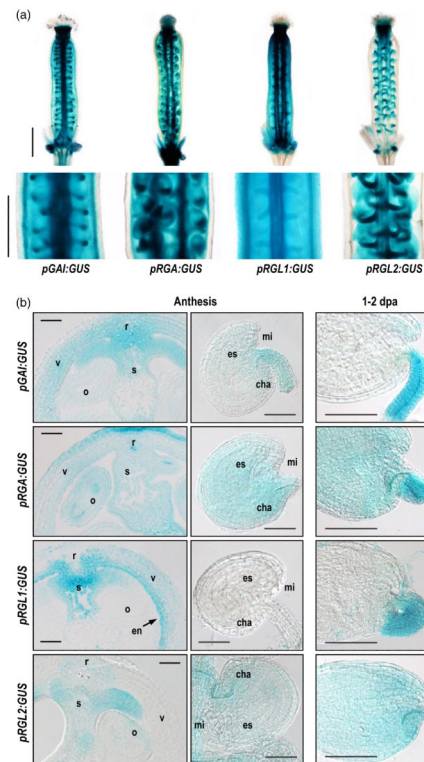


Figure 3.

DELLA genes are differentially expressed in pistils and fruits.

(a) Time-course of *DELLA* expression during unfertilized pistil and early fruit development. Expression is represented as the copies of cDNA per 10⁵ copies of *UBQ10*.

(b) *DELLA* expression in ovules + funiculi (O + F) and valve (V) of hand-dissected unfertilized pistils at 1 and 2 dpa. Expression was normalized to *UBQ10* and to the expression in the whole pistil at anthesis. Open and closed triangles indicate preferential expression of *RGL2* in ovules and *RGL1* in valves, respectively. Each experiment was repeated twice using independent samples with similar results (Figure S2). Data are the mean \pm standard deviation (SD) of a single representative experiment. dpa, day post-anthesis.

**Figure 4.**

DELLA expression is differentially localized in tissues of pistils and fruits.

DELLA expression was analyzed using transcriptional fusion lines *pGAI:GUS*, *pRGA:GUS*, and *pRGL1:GUS* (see Experimental procedures), and mutant line *rgl2-5* for *RGL2*.

(a) *DELLA* expression in whole pistils at anthesis. Scale bar represents 500 μ m.

(b) Expression in cross-sections of pistils and ovules at anthesis (left and middle panels), and seeds at 1 or 2 dpa (right panels). o, ovule; r, replum; s, septum-transmitting track; v, valve, en, endocarp; mi, micropyle; es, embryo sac; cha, chalaza. Scale bar represents 50 μ m.

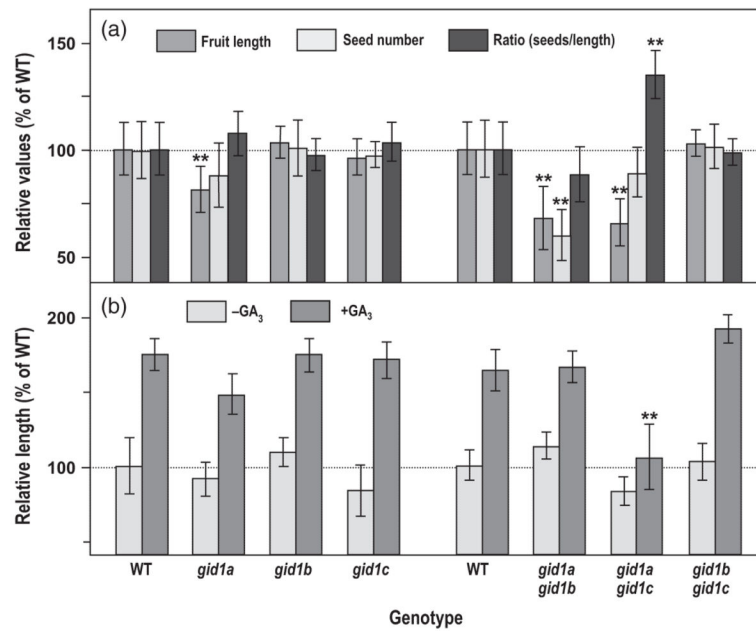


Figure 5.

GID1s control female fertility and pod elongation.

(a) Maternal effect of the *gid1* null mutations. Flowers of *gid1* mutants and wild-type (WT) were emasculated 1 day before anthesis and hand pollinated at anthesis with WT pollen. Mature fruits were individually harvested, and fruit length and seed number were measured. Ratio (seed number versus length) was determined. Values were normalized to the WT.

(b) Gibberellin (GA) response of unfertilized pistils of the *gid1* null mutants as measured by relative pistil length in comparison to untreated WT pistils. Flowers of *GID1* mutants and WT were emasculated 1 day before anthesis; half of the pistils were treated at anthesis with 300 μM GA₃ (+GA₃), and the other half were treated with mock solution (-GA₃). Fruit or pistil length was measured at 10 dpa. Mean and standard deviation (SD) were calculated from at least 50 pistils/fruits per treatment. The experiment was repeated three times with similar results. Significant differences (Student's *t*-test analysis) between the WT and mutants are marked with asterisks (***P*-value < 0.001).

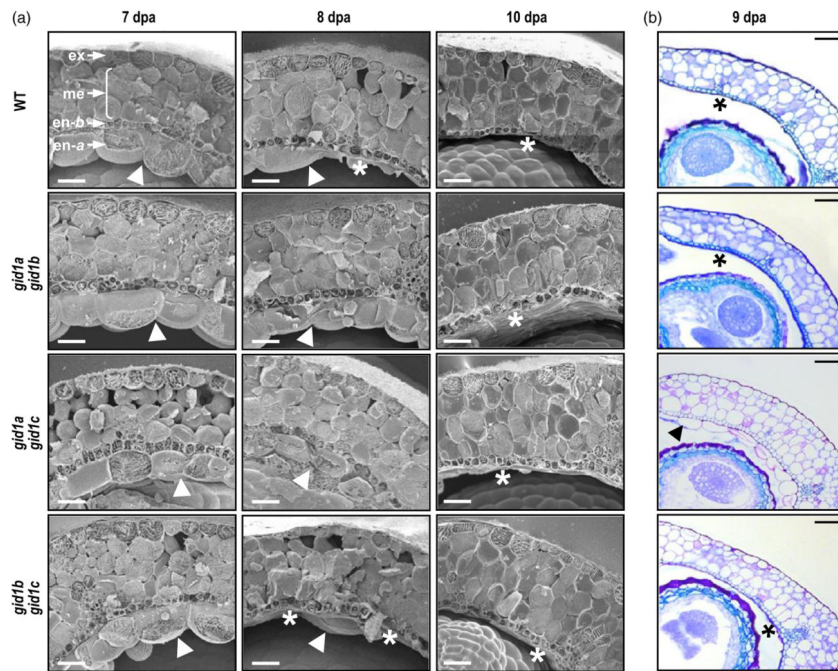


Figure 6.

The *gid1* null mutants show morphological alterations in fruit structure.

(a) Delayed degradation of endocarp a in double *gid1a gid1b* and *gid1a gid1c* mutants.

Transverse cryosections of fruits at 7, 8 and 10 dpa of wild-type (WT) and double mutants are shown. In the left top image, the different tissues are labeled: ex, exocarp; me, mesocarp; en-b, endocarp *b*; en-a, endocarp *a*. Presence or degradation of en-a are indicated by an arrowhead or asterisk, respectively. Scale bar represents 50 μ m.

(b) Delayed lignification of en-b and degradation of en-a in double *gid1a gid1c* mutant.

Transversal sections of fruits at 9 dpa of WT and the double *gid1* mutants are shown. Presence of end-a and delayed lignification of end-b in *gid1a gid1c* mutant is indicated by an arrow head; degradation of en-a and strong lignification of en-b in WT and in *gid1a gid1b* and *gid1b gid1c* mutant is indicated by an asterisk. Scale bar represents 50 μ m.

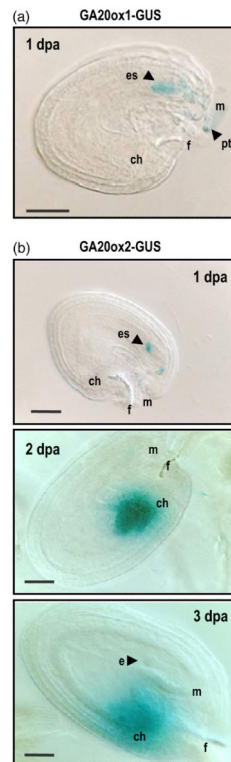


Figure 7.

Expression of gibberellin (GA) biosynthesis genes is located in seeds shortly upon fertilization.

(a) Expression of GA20ox1-GUS (Desgagne-Penix *et al.*, 2005) in seeds at 1 dpa.

(b) Expression of GA20ox2-GUS (Frigerio *et al.*, 2006) in seeds at 1, 2 or 3 dpa. ch, chalaza; e, embryo; es, embryo sac; f, funiculus; m, micropyle; pt, pollen tube. Scale bar represents 40 μm.

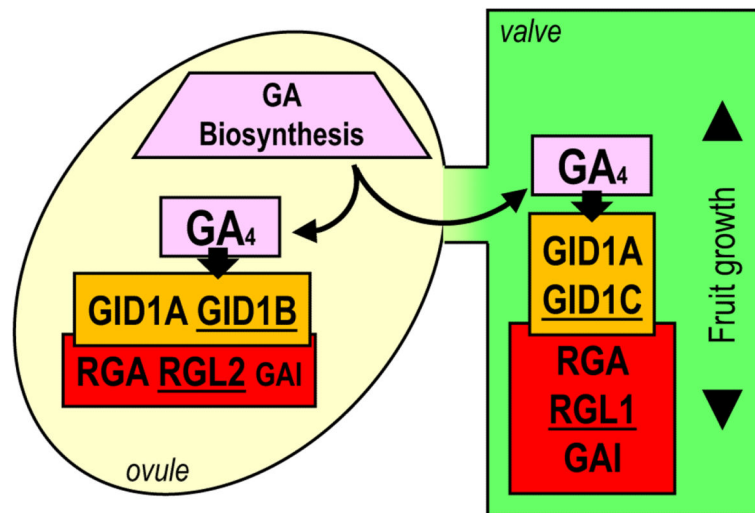


Figure 8.

Model of gibberellin (GA) biosynthesis, perception and signaling in seeds and valves of pistils/fruits.

Bioactive GAs, mainly GA₄, are synthesized in developing seeds by GA20ox and GA3ox activities. In ovules/seeds GAs are perceived by GID1A and GID1B, which interact with RGA, GAI, and RGL2 to promote seed-specific GA signaling. GAs are also transported to the valve and perceived by GID1A and GID1C, which interact with RGA, GAI, and RGL1, to promote GA-specific valve responses (mainly pod growth and degradation of endocarp a). Proteins with tissue-specific expression are underlined.