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Abbreviations: CaMV, cauliflower mosaic virus; CSLM, confocal scanning laser microscopy; FM, functional megaspore; GA, gibberellin; Ler, Landsberg erecta; MMC, megaspore mother cell.
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RESEARCH PAPER

Gibberellin-mediated RGA-LIKE1 degradation regulates embryo sac development in Arabidopsis

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Abstract

Ovule development is essential for plant survival, as it allows correct embryo and seed development upon fertilization. The female gametophyte is formed in the central area of the nucellus during ovule development, in a complex developmental programme that involves key regulatory genes and the plant hormones auxins and brassinosteroids. Here we provide novel evidence of the role of gibberellins (GAs) in the control of megagametogenesis and embryo sac development, via the GA-dependent degradation of RGA-LIKE1 (RGL1) in the ovule primordia. YPet-rgl1Δ17 plants, which express a dominant version of RGL1, showed reduced fertility, mainly due to altered embryo sac formation that varied from partial to total ablation. YPet-rgl1Δ17 ovules followed normal development of the megaspore mother cell, meiosis, and formation of the functional megaspore, but YPet-rgl1Δ17 plants had impaired mitotic divisions of the functional megaspore. This phenotype is RGL1-specific, as it is not observed in any other dominant mutants of the DELLA proteins. Expression analysis of YPet-rgl1Δ17 coupled to in situ localization of bioactive GAs in ovule primordia led us to propose a mechanism of GA-mediated RGL1 degradation that allows proper embryo sac development. Taken together, our data unravel a novel specific role of GAs in the control of female gametophyte development.

Keywords: Arabidopsis, DELLA, development, embryo sac, gibberellin, megagametogenesis, ovule, RGL1

Introduction

Ovule development is a key process in the perpetuation of plant species, as it ensures the correct formation of the female gametophyte and the subsequent embryo and seed development upon fertilization. Ovule primordia initiation and growth have been studied mainly in the model species Arabidopsis (Schneitz et al., 1995, 1997; Lora et al., 2016; Pinto et al., 2019; Cucinotta et al., 2020), for which detailed developmental stages have been defined (Schneitz et al., 1995).

Ovule primordia, composed solely of diploid cells, emerge from the placental tissue as finger-like protrusions from the placenta in the medial domain of the developing ovary. Successive cell divisions give rise to three prominent domains along a proximal–distal axis: the funiculus, which connects the ovule to the placenta; the chalaza in the central domain, which gives rise to the inner and outer integuments; and the nucellus in the distal region, which produces a single germline cell, the megaspore mother cell (MMC), the progenitor of a
single haploid functional megaspore (FM) (Pinto et al., 2019). At early phases of ovule development, two cell layers, epidermal and subepidermal, can be distinguished in the nucellus. The most distal cell in the subepidermal layer will become the germinal upon differentiation into an archespore cell that later expands to form the MMC (stage 2-I). Meiosis of the MMC produces four haploid megaspores of which only one remains as the FM (stage 3-I). Once established, the FM undergoes megagametogenesis, a series of transformation processes to generate the mature female gametophyte or embryo sac. This developmental process includes three rounds of mitotic divisions, reorganization of nuclei along the embryo sac, vacuole biogenesis, as well as cellular differentiation to ensure female gametophyte fertilization and, therefore, plant reproduction.

Several plant hormones have been shown to be essential for mitosis progression and vacuole formation during the formation of the female gametophyte. In Arabidopsis, mutations in several genes cause mitotic arrest at different embryo sac developmental stages (Serbes et al. 2019). These include (i) PIN-FORMED1 (PIN1), AUX1, and LIKE AUX1 (LAX1) genes, which mediate transport of auxin from the sporophytic tissue into the embryo sac; (ii) YUCCA8 (YUC8) and TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) genes, necessary for auxin synthesis; and (iii) CYP851, which encodes a brassinosteroid synthesis enzyme. Therefore, auxin and brassinosteroid phytohormones are necessary for proper female gametophyte development.

We have reported that gibberellins (GAs) play a major role in both ovule primordia initiation (Gomez et al., 2018, 2019) and ovule development (Gomez et al., 2016). In both cases, constitutive GA signalling impairs these processes. DELLA proteins, a family of plant-specific GRAS transcriptional regulators, are central components of the GA signalling pathway, acting as negative regulators that block a large array of GA-mediated developmental processes essential for the plant life cycle (Sun, 2011; Davière and Achard, 2013, 2016; Hedden and Sponsel 2015; Vera-Sirera et al., 2015). Upon binding to the GA receptor GID1, active GAs mediate the polyubiquitination and the 26S proteasome–dependent degradation of DELLA proteins. Therefore, GAs act by modulating the degradation of DELLA proteins. At low levels of GA, DELLA proteins are stable, allowing the GA response to be blocked, whereas GA synthesis mediates DELLA removal and allows GA responses to take place.

The so-called DELLA domain lies in the N-terminal part of the protein (Dill et al., 2001; Vera-Sirera et al., 2015), and removal of this domain results in a stable GA-resistant protein that constitutively blocks the GA response. Whereas most plant species encode only one or two DELLA proteins, the Arabidopsis genome encodes up to five DELLA genes: GAI (GA-INSENSITIVE, At1g14920), RGA (REPRESSOR OF GAI1-3, At2g01570), RGL1 (RGA-LIKE1, At1g66350), RGL2 (At3g03450), and RGL3 (At5g17490). The presence of multiple DELLA proteins raises an important question regarding the degree of functional redundancy versus specificity of each DELLA in Arabidopsis (Gallego-Bartolomé et al., 2010; Sun, 2011; Vera-Sirera et al., 2015). During ovule development, several DELLA proteins have been shown to act redundantly as positive factors. GAI, RGA, and RGL2 participate in ovule primordia initiation, and GAI, RGA, RGL1, and RGL2 co-ordinately regulate integument development (Gomez et al., 2016, 2018). On the other hand, the GA receptor GID1 has been implicated in the regulation of the fusion of central cell nuclei in the female gametophyte just before fertilization (Gomez et al., 2018) and in the correct differentiation of a single MMC (Ferreira et al., 2018).

Genetic and molecular tools are key for correctly assigning function to a particular gene. In the case of the DELLA genes, gain-of-function mutant alleles have been fundamental to uncovering their molecular and physiological function. These mutants were generated by removing the conserved DELLA domain to prevent GA-dependent protein degradation, and these truncated genes were then expressed under the control of the corresponding endogenous promoter, as is the case of gai-1, GFP-ngaΔ17, or YPet-rgl2Δ17 (Koorneef et al., 1985; Peng et al., 1997; Dill et al., 2001; Gomez et al., 2019). No similar line has been available for RGL1, however. Wen and Chang (2002) reported a dominant RGL1 line carrying a deletion of the DELLA domain, similar to that of gai-1, whose expression was controlled by the cauliflower mosaic virus (CaMV) 35S promoter. Plants expressing the 35S:rgl1Δ17 construct were dark green, dwarf, with underdeveloped and stunted flowers. The use of CaMV rather than an endogenous promoter impedes conclusion on whether the phenotypes observed are truly related to the activity of the native RGL1 protein.

To get a deeper insight in the role of RGL1 in ovule development, we generated translational fusion lines that express YPet-tagged versions of either the native RGL1 (pRGL1:RGL1-YPet) or a dominant version with a 17-aa DELLA domain deletion (pRGL1:YPet-rgl1Δ17), both controlled by endogenous RGL1 regulatory sequences. These lines provide bona fide tools to study the participation of RGL1 in a wide variety of plant developmental processes regulated by GAs, and to uncover new unknown functions. Here we confirm that RGL1 controls organ elongation, as in the inflorescence stems, flower whorls, and siliques. Moreover, RGL1 participated in the control of ovule development, by impairing the formation of the embryo sac. Interestingly, dominant versions of GAI, RGA, or RGL2 did not show embryo sac defects, pointing to RGL1 as the only DELLA protein that acts as a specific negative regulator of embryo sac development. Finally, in situ accumulation of bioactive GAs in ovule primordia correlated with YPet-rgl1Δ17 expression. In summary, GAs participate in the control of female gametophyte development via the GA–mediated degradation of RGL1 in the ovule.

Materials and methods

Plant material

Arabidopsis plants from the Landsberg erecta (Ler) genetic background were used. Dominant mutants gai-1, GFP-ngaΔ17, and YPet-rgl2Δ17 were described previously (Peng et al., 1997; Dill et al., 2001; Gomez et al., 2019). The rgl1-1 null mutant was obtained from the Nottingham Arabidopsis Stock Center (www.arabidopsis.info). GA hormone-activated Cas9-based repressor (HACR) plants (Khakhar et al., 2018)
Bacterial media were supplemented with 25 µg ml⁻¹ kanamycin plus 10 µg ml⁻¹ tetracycline located at 66.8 kb of the 80.3 kb genomic fragment.

JAtY50E24 from the JIC (JAtY library, https://abrc.osu.edu/stocks/(2020). Briefly, both constructs were generated using the JAtY clone combination technology (recombineering), basically as described in Brumos et al. (2020). The ampicillin resistance gene was then re- moved by FRT-mediated recombination, and constructs were confirmed by sequencing.

For the elimination of the DELLA domain in pRGL1:YPet-g1AΔ17, first an RPDSL-Amp cassette was introduced via conjugation into Agrobacterium tumefaciens GV3101 (pMP90) strain (Zhao et al., 2011) and Ler Arabidopsis plants were transformed by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected in ammonium glucosinate, and T3 homozygous lines segregating as a single locus were selected.

Histological procedures

Ovule morphology was studied using chloral hydrate clearing and differential interference contrast light microscopy according to Weigel and Glazebrook (2002). Images were recorded using a Nikon Eclipse E600 microscope equipped with a Nikon DS-Ri1 digital camera. The number of ovules with a wild-type (WT)-like shape or mild and severe defects in embryo sac development was determined from a sample of 875 mature ovules of emasculated flowers from 16 YPet-g1AΔ17 pistils, each from an individual plant.

For histological analysis of ovule development, Ler and YPet-g1AΔ17 inflorescences were fixed overnight in FAE (5% (v/v) formaldehyde, 10% (v/v) acetic acid, 50% (v/v) ethanol), dehydrated in a 50, 70, 90, and 100% (v/v) ethanol series, embedded 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). Images were captured with a Leica DM5000 microscope.

In situ RNA hybridization

Arabidopsis inflorescences were embedded in paraffin, sectioned, and hybridized as described by Gomez et al. (2018). The RGL1 template was amplified (forward primer: GAATCAAGCAGATCTGTGAGG; reverse primer: CCTTCTATTGCGCTGACCCTG) and cDNA was cloned into the pGem-T Easy vector (Promega). Sense and antisense probe were synthesized using the corresponding SP6 and T7 RNA polymerases in the vector. Control experiments were performed with sense probes and no significant signal was detected. Images were recorded using a Nikon Eclipse E600 microscope equipped with a Nikon DS-Ri1 digital camera.

Results and Discussion

Construction of pRGL1:RGL1-YPet and pRGL1:YPet-g1AΔ17

Translational fusions of YPet with RGL1 and a dominant version g1AΔ17 were generated from a genomic clone by bacterial homologous recombination technology (recombineering), basically as described in Brumos et al. (2020). Both constructs were generated using the JAtY clone JAtY50E24 from the JIC (JAtY library, https://abrc.osu.edu/stocks/number/CD4-96) in the pYLTA17 vector, which contains the JAtY50E24 from the JIC (JAtY library, https://abrc.osu.edu/stocks/number/CD4-96) in the pYLTA17 vector, which contains the JAtY50E24 from the JIC (JAtY library, https://abrc.osu.edu/stocks/number/CD4-96). The RGL1 template was amplified (forward primer: GAATCAAGCAGATCTGTGAGG; reverse primer: CCTTCTATTGCGCTGACCCTG) and cDNA was cloned into the pGem-T Easy vector (Promega). Sense and antisense probe were synthesized using the corresponding SP6 and T7 RNA polymerases in the vector. Control experiments were performed with sense probes and no significant signal was detected. Images were recorded using a Nikon Eclipse E600 microscope equipped with a Nikon DS-Ri1 digital camera.

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CSLM) was used to analyse the development of the different cellular layers that make up the YPet-g1AΔ17 ovules. For this, inflorescences were fixed with 4% paraformaldehyde for 1 h with vacuum treatment. After fixation, the samples were washed twice for 1 min in 1× phosphate-buffered saline, moved to ClearSee solution (Kurihara et al., 2013) and cleared for 1 week at room temperature. After clearing, the inflorescences were stained with CalcoFluor White as described by Urach et al. (2018). To detect and image bound CalcoFluor White, we used a Zeiss LSM 780 confocal microscope with excitation at 405 nm and detection at 425–475 nm. The distribution of RGL1–YPet and YPet-g1AΔ17 proteins during ovule development was studied with the same confocal microscope, with excitation at 514 nm and emission filters set to 520–540 nm. Finally, the in situ localization of bioactive GAs in the GA HACR plants were analysed by the detection of Venus fluorescent protein with excitation at 488 nm and detection at 510–530 nm. The identity of fluorescence signals was confirmed with a λ-scan.
for details). In addition, a gain-of-function allele of RGL1 (pRGL1:YPet-rgl1Δ17) was generated by deleting the 17-aa DELLA domain (DELLVVLGYKVRKSSDMA) located at position 32–48 of the YPet–RGL1 protein also by recombineering (Supplementary Fig. S4); elimination of this domain should prevent GA-mediated degradation of the YPet–rgl1Δ17 protein. After trimming both genomic clones to improve stability during plant transformation, the final constructs included genomic sequences 10 kb upstream and 5 kb downstream of the RGL1 locus (Supplementary Fig. S5), which potentially contain all the regulatory regions, providing a reliable expression pattern likely to reflect that of the native gene. Transgenic plants were generated for both pRGL1:RGL1-YPet and pRGL1:YPet-rgl1Δ17 constructs. Different lines for each construct showed similar phenotypes; therefore, single lines (thereafter RGL1-YPet and YPet-rgl1Δ17) were selected for further analysis.

RGL1–YPet is degraded by GAs, but YPet–rgl1Δ17 is GA-resistant

The stability of the RGL1–YPet and YPet–rgl1Δ17 fusion proteins was analysed in primary roots of 4-day-old seedlings upon GA treatment (Fig. 1). Both RGL1–YPet and YPet–rgl1Δ17 were located at the cell division zone of the primary root, the levels of the dominant YPet–rgl1Δ17 being much higher than those of the protein containing the DELLA domain. In addition, tagged proteins were located in the nucleus of the root cells as was previously reported for RGL1 and other DELLA proteins (Silverstone et al., 2001; Fleck and Harberd, 2002; Wen and Chang, 2002; Gonzalez et al., 2019). Moreover, treatment with GAs promoted a strong degradation of RGL1–YPet, whereas levels of the dominant version YPet–rgl1Δ17 remained nearly identical to those of the untreated plants. Therefore, the dominant GA-resistant version, YPet–rgl1Δ17, blocked RGL1-dependent GA signalling.

Strikingly, whereas nuclear-localized RGL1–YPet protein can be degraded by GAs, no RGL1 protein degradation was observed using a green fluorescent protein (GFP)-fused RGL1 protein under the control of the strong CaMV 35S promoter (Wen and Chang, 2002). This discrepancy may reflect the differences in promoter activities. Similar to the 35S:GFP-RGL1 line, degradation of the 35S-driven GAI–GFP fusion protein by GAs was also not detectable (Fleck and Harberd, 2002).

YPet-rgl1Δ17 plants uncover RGL1-dependent growth functions

We generated YPet-tagged versions of RGL1 that include the 16.5 kb genomic region around the RGL1 locus, including 10 kb of the promoter and a 5-kb downstream region that most probably directs the expression of the fusion proteins in a similar manner to the native RGL1. In addition, the dominant YPet–rgl1Δ17 protein was GA-resistant, blocking the RGL1-dependent GA-mediated development. Therefore, the phenotypes of the dominant line are most probably the consequence of specifically blocking RGL1-dependent GA responses, uncovering the functions of RGL1 in plant development.

At the vegetative level, YPet-rgl1Δ17 plants showed delayed flowering and reduced plant height with shorter floral stems (Fig. 2A–C). Delayed flowering was most evident under SD conditions (i.e. 8 h–16 h regimen) when plants flowered after more rosette leaves were produced (Fig. 2A). Under LD conditions (16 h–8 h regimen), YPet-rgl1Δ17 plants flowered 4 d later than the WT, with the same number of rosette leaves. Adult plant architecture was also modified by YPet-rgl1Δ17. These plants showed dwarfism, partial loss of apical dominance, and increased shoot branching (Fig. 2B, C). In addition, YPet-rgl1Δ17 plants evidenced a darker green colour compared with Ler. In terms of reproductive development, YPet-rgl1Δ17 plants also showed morphological alterations, including compact inflorescences due to shorter flower pedioles, and reduced floral size by the shortening of all four floral organs (Fig. 2A–D).

We next studied the expression of RGL1 using the YPet-tagged lines. RGL1–YPet protein was not detected in the different tissues analysed by CSLM, with the exception of the root tip, possibly due to its low abundance, as endogenous bioactive GAs would trigger its degradation to enable organ growth and development. Stable YPet–rgl1Δ17 protein was clearly visualized in a large variety of tissues, however. Therefore, localization of YPet–rgl1Δ17 protein was used to infer the expression pattern of RGL1 during floral organ development. Overall, reduction of floral organs was correlated with expression of YPet-rgl1Δ17 (Fig. 3E–G). The chimeric protein was detected in sepals and petals, especially in the lamina. Expression was also apparent in the stamens, both in filaments at early stages and in anthers throughout development. Therefore, the limited size of floral organs is most probably due to blockage of growth imposed by the dominant YPet–rgl1Δ17 protein. These flower phenotypes were stable throughout plant development.

The data reported here support the participation of RGL1 in flowering, stem elongation, and floral organ development. Wen and Chang (2002) reported similar but enhanced phenotypes in a 35S:rgl1Δ17 line, which overexpresses a dominant version of RGL1 driven by the strong constitutive CaMV 35S promoter. These included severe dwarfism, dark pigmentation, and delayed flowering. But there were also remarkable differences between the 35S:rgl1Δ17 (Wen and Chang, 2002) and...
YPet-rgl1Δ17 phenotypes. For example, in the 35S line, expression of rgl1Δ17 in rosette leaves led to a strong reduction in rosette size similar to the GA-deficient ga1-3 mutant. In contrast, no major defects in rosette leaves were observed in YPet-rgl1Δ17 plants, which suggests that native RGL1 expression in the rosette is very low. The differences in the phenotype penetrance between 35S:rgl1Δ17 and pRGL1:YPet-rgl1Δ17 lines are most probably caused by the different promoter used: the strong ectopic expression driven by the constitutive 35S promoter, compared with the RGL1 endogenous regulatory sequences in the pRGL1:YPet-rgl1Δ17 line.

An important issue regarding the role of the DELLA family in Arabidopsis is the degree of overlapping versus specific roles of each particular gene in the control of GA-mediated developmental processes (Sun, 2011). The participation of the different DELLA proteins in several developmental processes has been uncovered by using single and multiple loss-of-function mutants in different combinations (reviewed in Vera-Seréa et al., 2015). An analysis of the phenotypes of plants upon RGA–RGL2 promoter switching suggested that functional diversification of DELLA proteins relies mainly on changes in their gene expression patterns rather than on their molecular function (Gallego–Bartolomé et al., 2010). Therefore, temporal and spatial expression patterns of the different DELLA proteins may be the major contributor to their functions in development. In view of this, it is critical to use their endogenous regulatory sequence to get bona fide information regarding the role of RGL1, as is used in the case of the pRGL1:YPet-rgl1Δ17 line.
Seed number is reduced in YPet-rgl1Δ17 plants

GAs participate in the regulation of ovule primordial formation (Gomez et al., 2018) and in ovule integument development (Gomez et al., 2016). We used YPet-rgl1Δ17 plants to study the contribution of RGL1 to the regulation of ovule initiation and integument development but also to uncover new roles of this protein in ovule and seed development.

First, we scored ovule number, ovary length, and the ratio of ovule number to ovary length in YPet-rgl1Δ17 plants and compared these with the Ler WT (Fig. 4A). Expression of YPet-rgl1Δ17 caused a small reduction in the number of ovules per pistil, but had a stronger effect in reducing ovary length, leading to an increase in ovule density within the ovary. As ovule initiation and pistil development take place at the same time, the ovule number alterations observed suggests that YPet-rgl1Δ17 mainly blocks ovary valve elongation, resulting in smaller pistils, similar to the shortening of other floral organs. The increased ovule density is probably due to an effect of YPet-rgl1Δ17 in ovary shortening, rather than a direct effect in ovule primordia formation. In consequence, mature ovules in YPet-rgl1Δ17 plants appeared to be closer to each other with folded or stretched funiculi that allow ovules to occupy less space within the ovary (Fig. 4B). Moreover, these ovules have severe alterations in morphology, mainly the total or partial loss of the embryo sac. Interestingly, normal and altered ovules were present side-by-side in the same pistil, without bias towards any particular ovary region (apical or basal). This phenomenon is further examined in the next section.

Mature YPet-rgl1Δ17 plants showed a strong reduction in fertility, with fruits that were much shorter than those in Ler (Fig. 4C). When quantified, seed number was reduced by 60% when compared with a control Ler plant (Fig. 4D). Reduced fruit size may be a direct consequence of reduced seed content, but also to the blockage of valve elongation during silique development.

The mild reduction in ovule number was not the major cause for reduced fertility in YPet-rgl1Δ17 plants (Fig. 4A, C). To understand whether the YPet-rgl1Δ17 defect in seed-set was due to maternal and/or paternal causes, a reciprocal cross-pollination assay was carried out. For this, pistils of Ler and YPet-rgl1Δ17 plants were pollinated with either Ler or YPet-rgl1Δ17 pollen and the amount of seed set was determined. As
Specific role of RGL1 in embryo sac development

shown in Fig. 4E, fertility defects in YPet-rgl1Δ17 plants were of maternal origin. Fruits from Ler plants pollinated with either Ler or YPet-rgl1Δ17 pollen produced a similar number of seeds. In contrast, pistils from YPet-rgl1Δ17 plants always produced fewer seeds, regardless of the pollen origin (Ler or YPet-rgl1Δ17). Although expression of RGL1 in YPet-rgl1Δ17 plants was also detected in anthers (Fig. 3E, F), no significant defects in pollen were observed, as fertility was identical between fruits pollinated with either Ler or YPet-rgl1Δ17 pollen regardless of the pistil genotype.

Similar to Ypet-rgl1Δ17, plants expressing YPet-rgl2Δ17 also had reduced fertility, but here this was caused mainly by defects in stamen development (Gomez et al., 2019). Therefore, both lines are essential to uncover the differential roles of RGL1 and RGL2 in fertility: whereas RGL1 has a major role in maternal fertility and pistil/silique elongation, RGL2 is a major player in male fertility, with only a marginal role in silique elongation.

RGL1 impairs embryo sac development

Fertility defects in YPet-rgl1Δ17 plants were of maternal origin, but were not caused solely by the reduced ovule number (Fig. 4A), pointing to ovule defects as the major cause for the reduced seed-set (Fig. 4B). To get a deeper insight into the role of RGL1 in ovule development, ovules in YPet-rgl1Δ17 plants were dissected by CLSM and light microscopy techniques.

Ovules in YPet-rgl1Δ17 and Ler developed similarly, both morphologically and temporally, until the formation of the FM (Fig. 5). Both Ler and YPet-rgl1Δ17 ovules showed cytokinesis marks inside the nucellus at stage 2-V (according to Schneitz et al., 1995), indicating that meiosis of the MMC had occurred and tetraspores were formed (Fig. 5A, D). At stage 3-I, the three non-functional spores degenerated (Fig. 5B, E), and only the FM remained in Ler and YPet-rgl1Δ17 ovules (Fig. 5C, F). These observations indicate that the process of megasporogenesis occurred properly in YPet-rgl1Δ17 plants. In contrast, from stage

Fig. 4. Ovule and seed number was altered in YPet-rgl1Δ17 plants. (A) Ovule number per pistil, ovary length, and the ratio of ovule number to ovary length in flowers at anthesis of Ler (light grey) and YPet-rgl1Δ17 (dark grey) plants. (B) CLSM images of representative mature ovules of Ler (upper panel) and YPet-rgl1Δ17 (lower panel) plants. Asterisks mark long funiculi; arrowheads mark altered ovules in YPet-rgl1Δ17. (C) Images of mature self-pollinated fruits of Ler and YPet-rgl1Δ17 plants. (D) Number of seeds from self-pollinated fruits of Ler and YPet-rgl1Δ17 plants. (E) Number of seeds from cross-pollinated fruits of Ler and YPet-rgl1Δ17 plants. Data are represented as boxplots; n=10–12 in (A) and n≥30 in (D, E). Letters above each box indicate statistical significance as determined by an ANOVA and a Bonferroni post hoc test for multiple comparisons (P-value<0.01). Data that are not significantly different are marked with the same letter. Scale bars represent 50 µm in (B) and 2 mm in (C). (This figure is available in colour at JXB online.)
3-I on, the embryo sac development was impaired (Fig. 6). We scored the number of altered ovules in YPet-rgl1Δ17 plants and found that approximately 52% of mature ovules had a WT-like female gametophyte containing an egg, two polar, and two synergid nuclei (Fig. 6D–F), very similar to those in Ler plants (Fig. 6A–C). The remaining 48% of YPet-rgl1Δ17 ovules showed severe defects in embryo sac development (Fig. 6H, I, K, L). However, the percentage of altered ovules per pistil ranged approximately from 30 to 80%, showing a large range of penetrance of phenotype (see Supplementary Fig. S6). These defects were clearly visible at stage 3-III, pointing to a role for YPet-rgl1Δ17 in altering the correct differentiation of the FM after stage 3-I, probably interfering with ovule development starting at the first mitotic division.

The defects in YPet-rgl1Δ17 ovules were not homogeneous, since approximately 50% of the defective ovules retained a residual embryo sac (Fig. 6H, I) while the other 50% suffered a complete loss of the embryo sac (Fig. 6K, L). Therefore, the proportion of phenotypes among YPet-rgl1Δ17 mature ovules was approximately 50% WT-like, 25% with mild defects, and 25% with severe defects (total loss of embryo sac). Moreover, the reduced embryo sac usually contained a smaller number of nuclei than Ler ovules (Fig. 6I, L; compare with Fig. 6C), which impedes fertilization. In addition, we also observed ovule primordia with a premature loss of nucellar tissue in YPet-rgl1Δ17 plants (stages 3-II and 3-III, see asterisks in Fig. 6M and arrows in Fig. 6D, G, J).

In Arabidopsis, the embryo sac growth displaces the nucellar tissue starting from the micropyle (Schneitz et al., 1995). This process is clearly observable from stage 3-IV where the nucellar tissue is seen laterally (Fig. 6A). In Ler mature ovules, the nucellus is nearly completely resorbed except for a group of cells at the base of the embryo sac (Fig. 6B). Upon resorption of the nucellus, a cuticle layer surrounds and separates the embryo sac from the inner integument (Fig. 6B) (Schneitz et al., 1995; Beeckman et al., 2000). The cuticle is an auto-fluorescent hydrophobic barrier formed by cutin, which later separates the maternal tissue from endosperm in fertilized ovules (Coen et al., 2019). In YPet-rgl1Δ17 plants, defective ovules showed a premature degradation of nucellar tissue, which led to alterations in embryo sac shape (Fig. 6G–L), or ovules with a fragile embryo sac cuticle that led to rupture and release of the内容 of the sac at stage 3-IV or 3-V, as observed in Fig. 6N. This event would explain the existence of mature YPet-rgl1Δ17 ovules without an embryo sac or, instead, disorganized cell remains (Fig. 6K, L). It should be noted that YPet-rgl1Δ17 ovules presented a characteristic triangular shape, especially pronounced in those without embryo sac, possibly due to an elongation of the cells of the endothelium (innermost layer of inner integument) (Fig. 6E, H, K).

So far, no evidence of similar defects in embryo sac development has been reported for other dominant mutants of GAI, RGA, and RGL2. As can be observed in Supplementary Fig. S7A–E, gai-1, GFP-rgaΔ17, and YPet-rgl2Δ17 plants showed mature ovules with normal embryo sac. Moreover, a comparison of ovule and seed number in Ler and all four dominant mutants confirmed that only YPet-rgl1Δ17 showed a strong reduction of seed number, whereas ovule number was
Fig. 6. Embryo sac development is impaired in YPet-rgl1Δ17 plants during megagametogenesis. (A–L) Images of ovules of Ler (A–C) and YPet-rgl1Δ17 (D–L) plants at stages 3-III (A, D, G), stage 3-IV (J), or mature ovules (B, C, E, F, H, I, K, L, N). (M, N) Images of ovules of YPet-rgl1Δ17 at stage 3-II (M) or mature ovule (N). In (D) a normal (left) and an abnormal (right) ovule is shown. Images were captured by CLSM, except (C, F, I, L), which were captured by differential interference contrast light microscopy. Scale bars represent 20 μm in all panels. Arrows in (D, G, J) point to the cuticle that separates inner integument and developing gametophyte. Asterisks in (G, J, M) mark the degenerated nucellar epidermis. Arrowhead in (N) points to embryo sac content being released from the ovule. In (C, F, I, L), dotted lines define the mature embryo sac, and synergids, polar nuclei, and the egg cell are colour-coded (as indicated in (C)). cu, cuticle layer; ec, egg cell; en, endothelium; ep, nucellar epidermis; nu, nucellar tissue; pn, polar nuclei; syn, synergids. The cuticle layer is auto-fluorescent. (This figure is available in colour at JXB online.)
not reduced to the same extent (see Supplementary Fig. S7F). Finally, the loss-of-function mutant rgl1-1 or a silenced line (rgl1D17-R) that behaves as a loss-of-function phenotype of RGL1 does not show defects in plant development, including fertility (Lee et al., 2002; Wen and Chang, 2002).

As RGL1 acts as a repressor of embryo sac development, it would be expected that lack of RGL1 activity in rgl1-1 should not result in any defect in ovule development. These data strongly suggest that the DELLA role in embryo sac development is RGL1-dependent and -specific. Our data clearly reveal that GAs have a role in the control of embryo sac development, which is mediated solely by RGL1. In YPet-rgl1Δ17 plants, stable YPet-rgl1Δ17 should block downstream events essential for embryo sac formation, probably shortly after the first mitotic division of the FM.

Localization of YPet–rgl1Δ17 correlates to ovule defects

As in the floral organs, RGL1–YPet protein was not detected during ovule development, and therefore the expression pattern of RGL1 was inferred by visualizing YPet–rgl1Δ17 protein by CSLM. The expression profile of YPet-rgl1Δ17 correlates with ovule phenotypes (Fig. 7; Supplementary Fig. S8). During early pistil development, YPet-rgl1Δ17 was expressed at high levels in the pistil, valve, and placenta, and it was slightly detected in ovule primordia at very early stages of development (stage I-1) (Fig. 7A). Soon after, expression could be localized in the funiculus, chalaza, and nucellar epidermis of ovule primordia at stage 2-11, but it was excluded from the germline cell in the centre of the distal portion (Fig. 7B). YPet-rgl1Δ17 expression increased in developing ovules and started to be detected in the integument primordia at stage 2-IV (Fig. 7C). Finally, expression was clearly detected in the mature ovule at anthesis (Fig. 7D). The protein localization data, obtained with the YPet-rgl1Δ17 line, were supported by the expression of the RGL1 gene during ovule development by in situ mRNA hybridization (Supplementary Fig. S9). To determine the expression of YPet-rgl1Δ17 in different cell layers of mature ovules with defects in embryo sac development, cleared ovules were examined by CSLM (Fig. 7E). Expression was detected in the funiculus, chalaza, and endothelium layer, and in other integument cell layers at a lower level. Level of YPet-rgl1Δ17 expression correlates to ovule defects (Supplementary Fig. S10); in WT-like ovules, expression was lower than in those with severe defects. The highly fluorescent layer between the endothelium and the impaired embryo sac corresponds to the cuticle (Fig. 7E, F). Cutin deposition was also detected in ovules in which no embryo sac was observed (Fig. 7F). As cutin deposition around the nucellus takes place upon mitosis of the FM, the presence of this layer in YPet-rgl1Δ17 ovules with severe phenotypes suggests that these ovules underwent megagametogenesis and developed a weak embryo sac that later ruptured.

Taken together, expression and phenotype analysis indicate that YPet-rgl1Δ17 affects embryo sac development from the neighbouring cells. In Arabidopsis, genetic studies have proposed that the development of the FM (megagametogenesis) and embryo sac (megagametogenesis) depends on information from surrounding diploid cells (Yang et al., 2010; Lora et al., 2016; Pinto et al., 2019). Before the appearance of the integuments, NOZZLE/SPOROCYTELESS (NZZ/SPL) (Yang et al., 1999) and WUSCHEL (WUS) (Lieber et al., 2011) participate in coordination to regulate the differentiation of the MMC. Interestingly, these genes are expressed in the nucellar epidermis, but influence the haploid FM development, suggesting that they would act non-cell autonomously in the control of female germline progression.

For example, NZZ/SPL is required to regulate the expression of PIN-FORMED 1, an auxin efflux transporter, in the nucellar epidermis to modulate auxin fluxes to the MMC (Bencivenga et al., 2012; Pinto et al., 2019). Another example is CYP78A5/KLUH (KLU), a gene involved in chromosome pairing during female meiosis, although it is expressed at the base of the nucellus in the region initiating the inner integument. Possibly, KLU performs this function through the production of a mobile signal that diffuses from these tissues to the surrounding cells (Zhao et al., 2014). Moreover, analysis of a set of key genes necessary for integument development, which include AINTEGUMENTA (Klucher et al., 1996), INNER NO OUTER (Villanueva et al., 1999), KLU (Zhao et al., 2014), and BELL1, SEEDSTICK, and SHATTERPROOF 1 and 2 (Battaglia et al., 2008), also supports non-cell-autonomous signalling. The phenotypes of the corresponding mutants demonstrate that these genes not only control integument identity but that they also play a role during megasporogenesis, since embryo sac maturation is impaired. Recently, it has been reported that the cis-expression of the transcription factor FUSCA3 in the integuments severely impairs embryo sac development (Wu et al., 2020). Finally, ARGONAUTE5 (AGO5), an effector of small RNA (sRNA) silencing pathways, is required to promote megagametogenesis in the FM (Tucker et al., 2012). AGO5 is expressed in the inner integument and nucellar epidermis and is thought to participate in embryo sac development by transmitting an sRNA into the FM, repressing movement of a protein or metabolite from the nucellar epidermis or by indirectly influencing nucellus development. All this evidence suggests that inter-regional signalling is important during megagametogenesis.

The data shown here suggest that RGL1 protein could behave like these genes, specifically, like AGO5. Based on the effect of the GA-resistant YPet–rgl1Δ17 protein, we hypothesized that RGL1 activity alters proper embryo sac development after the megaspore has been developed, although it is only expressed in integuments and the nucellar epidermis. It is well known that the function of DELLA proteins, including RGL1, lies in their ability to establish protein–protein interactions with a multitude of regulatory proteins, mostly transcription factors (Davière and Achard, 2013, 2016). Upon binding, the DELLA modifies the DNA-binding capacity or the transcriptional activity of their interactor proteins. A plausible scenario is that RGL1 could bind and block a key transcription factor that is necessary for the correct development of the embryo sac, by impeding transcriptional activity towards its target genes. This mechanism has been well described previously for other developmental processes (Davière and Achard, 2013; 2016). For example, DELLA proteins interact
with BRASSINAZOLE RESISTANT 1 (BZR1) to inhibit its DNA-binding ability, thereby blocking BZR1-mediated transcriptional activity during hypocotyl elongation (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). Therefore, during ovule maturation in WT plants, GAs must mitigate the action of RGL1 in integuments and the nucellus by promoting its degradation, via the ubiquitin–proteasome pathway, to allow adequate gametophyte development.
Bioactive GAs are located in developing ovules

The abnormal embryo sac development observed in YPet-rgl1Δ17 plants is probably the result of the RGL1-dependent blockage of the normal developmental programme that the megasporge undergoes during ovule development. Therefore, in normal ovules, GAs would be present in the developing ovule to degrade RGL1 (and probably other DELLA proteins) and allow normal growth and development. To visualize the presence of bioactive GAs in the ovule primordia, we used plants transformed with a GA sensor (GA HACR) based on the GA-sensitive RGBA that targets a Venus reporter protein (Khakhar et al., 2018). In these plants, endogenous bioactive GA distribution is visualized as a Venus fluorescence signal in confocal microscopy. At stage 2-III of ovule development, fluorescence could be observed in the large central nucleus of the megaspore mother cell, and in the surrounding tissues (Fig. S8A), including the nucellar epidermis, where RGL1 was also detected (Fig. S8B). So far, this is the first observation of active GAs inside the ovule primordia, which supports the participation of GAs in ovule development.

Conclusions

Taken together, the data reported here uncover a new role of GAs in the coordinated control of ovule development, in particular the events that take place from the first rounds of mitotic division of the FM, and allow us to propose a working model (Fig. 9). RGL1 specifically represses normal development of the FM, as the GA-resistant YPet-rgl1Δ17 protein in the nucellar epidermis and integuments caused a partial or complete ablation of the embryo sac. On the other hand, bioactive GAs are detected throughout the ovule primordia development, including the nucellar epidermis and the MMC. In Ler plants (Fig. 9A), GAs mediate the degradation of endogenous RGL1, which allows the correct megagametogenesis. In contrast, in YPet-rgl1Δ17 plants (Fig. 9B), stable YPet-rgl1Δ17 protein is not degraded, impairing embryo sac development. Finally, YPet-rgl1Δ17 may also have a local effect in the nucellar epidermis, causing a weakening of epidermal cells that facilitates the release of the embryo sac content, visible in ovules with severe defects. Further studies should be carried out to find out exactly at what point RGL1 alters megagametogenesis. Regardless of this, our data suggest that RGL1 in the integuments and nucellar epidermis regulates genes involved in the progression of the FM mitotic cycle, nuclear positioning inside the embryo sac, expansion of the central vacuole, or the final cellularization, including proper nucellar epidermis degradation, processes that are necessary for correct megagametogenesis and embryo sac maturation. The identification of the RGL1 target genes and interactors during megagametogenesis would be key to unravel the molecular mechanism underlying the role of GAs in the control of ovule development.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Scheme of the construction of pRGL1:RGL1-YPet and pRGL1:YPet-rgl1Δ17 lines by recombineering strategy.

Fig. S2. Detailed scheme of the generation pRGL1:RGL1-YPet construct from YaT50E24.

Fig. S3. Detailed scheme of the generation pRGL1:YPet-RGL1 construct from YaT50E24.

Fig. S4. Detailed scheme of the 17-aa deletion of the DELLA domain in pRGL1:YPet-RGL1 construct.

Fig. S5. Detailed scheme of the final trimming of modified YaT50E24 clones.

Fig. S6. Variable penetrance of embryo sac defects in pistils of YPet-rgl1Δ17.

Fig. S7. Defects in ovule development are specific to YPet-rgl1Δ17.

Fig. S8. YPet-rgl1Δ17 expression during ovule development.

Fig. S9. In situ RNA hybridization shows that RGL1 is expressed in ovules during development.

Fig. S10. Correlation of the level of expression of YPet-rgl1Δ17 with ovule phenotype.
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Author contributions

MDG performed most of the experiments and analysed and interpreted data; DB-T carried out the analysis of ovule number and fertility; CF-A obtained the transgenic lines; PT contributed with the analysis of ovule phenotype; JMA designed the cloning strategy for the generation of the transgenic lines; MAP-A conceived the project, generated the constructs, analysed and interpreted data, and wrote the article with contributions from all co-authors.

Data availability

The data and material supporting the findings of this study are available from the corresponding author (MAP-A) upon request.

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