Gibberellin Induces Diploid Pollen Formation by Interfering with Meiotic Cytokinesis[1][OPEN]

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The plant hormone gibberellic acid (GA) controls many physiological processes, including cell differentiation, cell elongation, seed germination, and response to abiotic stress. In this study, we report that exogenous treatment of flowering Arabidopsis (*Arabidopsis thaliana*) plants with GA specifically affects the process of male meiotic cytokinesis leading to meiotic restitution and the production of diploid (2n) pollen grains. Similar defects in meiotic cell division and reproductive ploidy stability occur in Arabidopsis plants depleted of RGA and GAI, two members of the DELLA family that function as suppressor of GA signaling. Cytological analysis of the double *rga-24 gai-t6* mutant revealed that defects in male meiotic cytokinesis are not caused by alterations in meiosis I (MI or meiosis II (MII) chromosome dynamics, but instead result from aberrations in the spatial organization of the phragmoplast-like radial microtubule arrays (RMAs) at the end of meiosis II. In line with a role for GA in the genetic regulation of the male reproductive system, we additionally show that DELLA downstream targets MYB33 and MYB65 are redundantly required for functional RMA biosynthesis and male meiotic cytokinesis. By analyzing the expression of *pRGA::GFP-RGA* in the wild-type Landsberg *erecta* background, we demonstrate that the GFP-RGA protein is specifically expressed in the anther cell layers surrounding the meiocytes and microspores, suggesting that appropriate GA signaling in the somatic anther tissue is critical for male meiotic cell wall formation and thus plays an important role in consolidating the male gametophytic ploidy consistency.

Polyploidization repeatedly occurred in the history of plant evolution, creating a basis for genetic diversification and speciation (Adams and Wendel, 2005). Different mechanisms may contribute to the formation of polyploid species (Ramsey and Schemske, 1998), yet the production of unreduced gametes is considered the main cause of polyploid induction (Bretagnolle and Thompson, 1995). Generally, both pre- and postmeiotic genome duplication events, as well as meiotic restitution, can lead to 2n gamete formation (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). In the tomato *pmcd1* mutant, for example, ectopic defects in cell wall formation during mitotic cell division in meiotic founder cells lead to premeiotic genome duplication and the associated formation of tetraploid meiocytes and diploid pollen grains (De Storme and Geelen, 2013b).

As the main cellular process driving sexual polyploidization, meiotic restitution can be classified into three types of mechanisms: omission of meiosis I or II, alteration in spindle organization, and unsuccessful or incomplete meiotic cytokinesis (Ramanna and Jacobsen, 2003).

Meiotic cell division and gametophytic ploidy stability are strictly controlled at the molecular level, and several factors operating in this process have already been identified (De Storme and Mason, 2014). In Arabidopsis (*Arabidopsis thaliana*), DYAD/SWITCH1 regulates meiotic chromatid cohesion and chromosome structure, and loss of function of this gene has been found to fully convert the reductional meiotic cell division into a mitotic one, eventually yielding clonal, diploid gametes in female sporogenesis (Ravi et al., 2008). The Arabidopsis OSD1/GIG1 and TAM/CYCA1;2 proteins promote the transition of meiosis I into meiosis II, and loss of function of each of them causes omission of the second meiotic cell division, resulting in 2n gamete formation in both male and female gametogenesis (d'Erfurth et al., 2009, 2010). Mutation in *ps* in potato and loss of function of JASON or AtPS1 in Arabidopsis cause an altered spindle configuration in male meiosis II, with parallel, tripolar, and fused spindles giving rise to restituted dyads and triads that contain 2n male gametes (Mok and Peloquin, 1975; d'Erfurth et al., 2008; De Storme and Geelen, 2011). Meiotic restitution may also result from defects in meiotic cytokinesis, either following MI or MII, with either a full or partial elimination of meiotic cell wall formation leading to 2n or polyploid gamete formation (De Storme and Geelen, 2013c). In Arabidopsis, a specific MAPK signaling cascade controls male meiotic cytokinesis (Takahashi et al., 2004; Takahashi et al., 2010).

Loss of function of TES, MKK6, or MPK4 causes defects in male meiotic cell wall formation and eventually leads to the ectopic production of diploid or polyploid male gametes (Spielman et al., 1997; Kosetsu et al., 2010; Zeng et al., 2011). Besides being induced by genetic defects, meiotic restitution and alterations in the consistency of the gametophytic ploidy may also result from environmental stresses, such as heat and cold (Pécrix et al., 2011; De Storme et al., 2012; De Storme and Mason, 2014; Zhou et al., 2015). Although progress has been made in understanding the cellular mechanisms and genetic factors involved in 2n gamete formation, the molecular factors and signaling pathways involved remain largely unknown.

Gibberellins (GAs) are endogenous plant hormones that play an important role in many aspects of plant growth and development, including seed germination, leaf expansion, trichome development, pollen maturation, and floral transition (Debeaujon and Koornneef, 2000; Davière and Achard, 2013; Claeys et al., 2014). The Arabidopsis GA biosynthetic mutant *ga1-3* displays defects in stem elongation, flowering time, and leaf abaxial trichome initiation (Silverstone et al., 1998). Besides mediating somatic tissue growth and development, GA is also involved in the control of reproductive system growth and floral morphogenesis (Fleet and Sun, 2005). GA positively regulates the development of the Arabidopsis stamen and petal by promoting cell elongation and also regulates microsporogenesis and pollen development (Cheng et al., 2004; Plackett et al., 2014). In multiple species, GA signaling regulates both the development and programmed cell death of tapetal cells, which is required for the growth and maturation of the developing microspores. Moreover, GA is also known to operate as a stress signaling molecule upon exposure to abiotic stresses (cold, salt, and osmotic stresses) triggered by either alterations in GA biosynthesis or its downstream signaling pathway. For example, cold stress inhibits root growth and disrupts pollen development by reducing the plant's endogenous GA levels (Achard et al., 2008; Sakata et al., 2014). However, although various significances of GA in both vegetative and reproductive growth and development have been identified, it is unknown whether GA plays a role in the control of meiotic cell division.

The DELLA family, which consists of five members (RGA, GAI, RGL1, RGL2, and RGL3), acts as the main repressor of the GA signaling pathway by negatively regulating the expression of GA responsive genes (Davière and Achard, 2013). The positive effect of GA on plant growth is achieved by stimulating the degradation of DELLAs and hence by counteracting their growth-suppressive effect (Silverstone et al., 2001; Xu et al., 2014). The majority of GA-dependent growth and development processes are mediated by DELLAs and their promiscuous interaction with transcription factors (Fleet and Sun, 2005; Daviere and Achard, 2015). The GA receptor GID1-DELLA complex constitutes an important regulatory pathway in GA signaling. GA stimulates the formation of the GA-GID1-DELLA complex that is a target for ubiquitination and subsequent 26S proteasome (Sun, 2010, 2011).

In barley and rice, GA positively regulates the expression of a specific group of MYB transcription factors, sometimes referred to as GAMYB, by relieving the repressive action of DELLAs (Gubler et al., 2002; Huang et al., 2015). Arabidopsis *MYB33* and *MYB65* are two of the *GAMYB-like* DELLA downstream target genes that are essential for anther development (Millar and Gubler, 2005; Cheng et al., 2009) and are involved in various other development processes (Jin and Martin, 1999; Gocal et al., 2001; Stracke et al., 2001). Several studies have shown that MYB33 and MYB65 are negatively controlled by miRNA159 (Millar and Gubler, 2005; Alonso-Peral et al., 2010; Alonso-Peral et al., 2010), whose expression is suppressed by DELLAs (Achard et al., 2004), suggesting a positive role of DELLA in regulating MYB33 and MYB65.

In this study, we demonstrate that exogenous GA treatment of flowering Arabidopsis plants induces meiotic restitution in male sporogenesis and subsequently generates viable 2n pollen. Using a combination of genetic and cytological analyses, we found that the combined loss of the DELLA transcription factors RGA and GAI causes defective cytokinesis and aberrant callosic cell wall formation at the male meiotic tetrad stage, yielding a subset of diploid, bean-shaped microspores. In agreement with the cytokinesis defects, the formation of the radial microtubule arrays (RMAs) at the end of male meiosis is altered or absent between adjacent nuclei. In addition, combined loss of function of the downstream DELLA targets MYB33 and MYB65 causes similar defects in male meiotic cytokinesis and gametophytic ploidy stability as observed in the DELLA double mutant, suggesting that MYB33 and MYB65 are downstream targets of DELLA in controlling male meiotic cytokinesis. Based on our findings, we propose that gametophytic ploidy consistency depends on a functional GA-DELLA-MYBs regulatory module.

RESULTS

GA₃ Treatment Induces Diploid Pollen Formation in Arabidopsis

To determine the effect of the plant hormone GA on male sporogenesis in Arabidopsis, we sprayed wild-type Col-0 plants with 100 μM GA$_3$ and water. To assess for putative alterations in male spore formation, the ploidy of Arabidopsis pollen was analyzed during the 14 d following GA$_3$ treatment (Fig. 1A). The ploidy level of resulting pollen grains was monitored by assessing pollen diameter, which is a proxy for the gametophytic ploidy level (De Storme et al., 2013). During the first 5 days following GA treatment, pollen grains appeared normally sized, similar to pollen isolated from nontreated control plants. However, from the sixth day (2.61%) to the thirteenth day (0.13%) after GA$_3$ treatment, a variable number of oversized mature pollen grains was found, with the highest frequency of larger

Figure 1. GA$_3$ treatment induces the formation of diploid pollen in Arabidopsis. A, Histogram showing the frequency of larger pollen produced in Arabidopsis Col-0 plants 1 to 14 d after exogenous GA$_3$ treatment (100 μM); see exact numbers in the histogram. B, Histogram showing the frequency of enlarged unicellular-stage microspores yielded 3 to 4 d after GA$_3$ treatment (100 μM). Bright field image of haploid mature pollen grain (C) and unicellular-stage microspore (D) produced in untreated Arabidopsis plants. Enlarged mature pollen grain (E) and unicellular-stage microspore (F) from Arabidopsis plants treated by 100 μM GA$_3$. G and H, Haploid microspore displaying five centromeric GFP dots in control, untreated plants harboring the *pWOX2::CENH3-GFP* reporter construct. I and J, Diploid microspores in *pWOX2::CENH3-GFP* transgenic plants treated by 100 μM GA$_3$, exhibiting two syncytial groups of five CENH3-GFP dots. Scale bars = 10 μm.
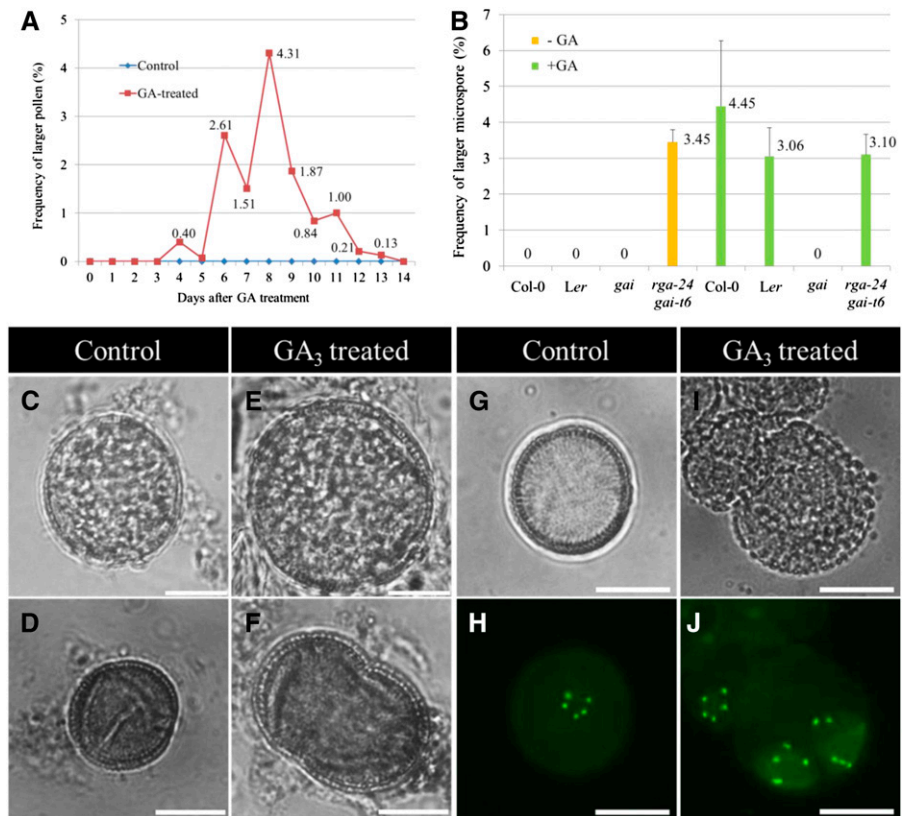
pollen (4.31%) at day 8 post treatment (dpt; Fig. 1, A, C, and E). To monitor whether GA-induced larger pollen resulted from enlarged microspores, spore size at the early microspore stage was analyzed during the first 7 days following GA$_3$ treatment. Three to four days post treatment, a subpopulation of microspores at the unicellular stage (4.45%) appeared larger than similar stage spores isolated from a nontreated control (Fig. 1, B, D, and F). These findings demonstrate that GA induces the formation of larger pollen grains, indicating an increased gametophytic ploidy level and that the GA-induced larger pollen grains originate from a cellular defect occurring before pollen mitosis I.

To determine the gametophytic ploidy level of the GA$_3$-induced enlarged microspores, the chromosome number in male gametes at the unicellular microspore stage was quantified using the *pWOX2::CENH3-GFP* reporter line (De Storme et al., 2016). Under control conditions, unicellular microspores isolated from diploid Arabidopsis plants (2$\times$ = 10) harboring the *pWOX2::CENH3-GFP* construct consistently show five distinct GFP foci, reflecting the haploid chromosome number in their gametophytic nucleus (Fig. 1, G and H). In contrast, the enlarged microspores in GA$_3$-treated plants revealed an increased number of GFP dots, specifically showing 10 distinct foci organized in two spatially separated but syncytial clusters of five GFP dots (Fig. 1, I and J). The occurrence of 10 CENH3-GFP dots provides evidence for a doubled chromosome number, indicating that the enlarged pollen grains are diploid. The specific localization

pattern of the centromeric CENH3-GFP foci in GA$_3$-induced enlarged spores suggests the presence of two syncytial haploid nuclei, indicating that diploid microspores either result from a nuclear duplication event (endomitosis) during early microgametogenesis or, alternatively, from of meiotic nonreduction through defects in meiotic cell wall formation.

Constitutive GA Signaling in the Arabidopsis DELLA *rga-24 gai-t6* Mutant Leads to Diploid Pollen Formation

GA$_3$-mediated activation of the GA signaling pathway generally occurs through degradation of DELLA proteins that mainly act as negative transcriptional regulators of the GA response. To determine whether GA$_3$-induced formation of diploid pollen is mediated by DELLAs, we further analyzed a series of single, double, quadruple, and pentuple null DELLA mutants for alterations in pollen morphology. In contrast to the single *rga-24* and *gai-t6* Arabidopsis mutants, which only produce a very low number of larger pollen grains (Fig. 2A), the double null *rga-24 gai-t6* mutant generates a significant number of larger pollen (3.38%), similar to the frequency of larger pollen grains induced by exogenous GA$_3$ treatment (Fig. 2, A, B–G). Similar frequencies of larger pollen grains were observed in the quadruple *rga-t2 gai-t6 rgl1-1 rgl2-1* mutant (3.30%) and the pentuple *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* mutant
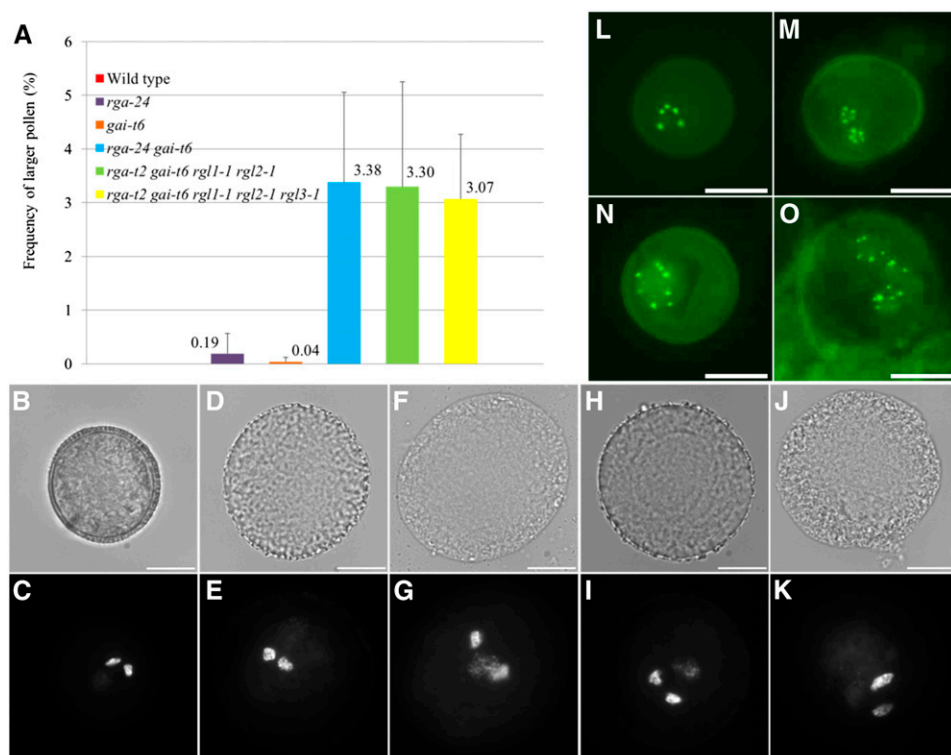
Figure 2. The DELLA double *rga-24 gai-t6*, quadruple *rga-t2 gai-t6 rgl1-1 rgl2-1*, and pentuple *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* mutants produce a subset of diploid pollen. A, Histogram showing the percentage of larger pollen grains produced in *rga-24* and *gai-t6* single and double mutants, *rga-t2 gai-t6 rgl1-1 rgl2-1* quadruple mutant, and in the *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* pentuple mutant. B to K, DAPI staining of mature pollen grains isolated from wild-type L*er* plants (B and C), the *rga-24 gai-t6* double mutant (D–G), and the *rga-t2 gai-t6 rgl1-1 rgl2-1* quadruple mutant (H–K). Unicellular microspores of control plants (L) and the *rga-24 gai-t6* double mutant (M–O) harboring the *pWOX2::CENH3-GFP* reporter construct. Enlarged spores in the *rga-24 gai-t6* double mutant either show 10 (M and N) or 15 (O) centromeric GFP foci. Enlarged GFP dots in picture N result from an overlay of two single GFP foci. Scale bars = 10 μm.

(3.07%; Fig. 2, A, H–K; Supplemental Fig. S1F), indicating that except for *RGA* and *GAI*, the mutations in other DELLA genes (e.g. *RGL1*, *RGL2*, and *RGL3*) do not contribute to the larger pollen phenotype.

To analyze the size and organization of the gametophytic nuclei in the larger pollen grains of the DELLA double and quadruple mutants, nuclei were stained and visualized using 4′,6-diamidino-2-phenylindole (DAPI). The nuclear configuration of the enlarged pollen in both types of DELLA mutants appears highly similar to that of wild-type haploid pollen, displaying one less-condensed vegetative nucleus and two highly condensed sperm nuclei. However, both the vegetative nucleus and the sperm nuclei in *rga-24 gai-t6* and *rga-t2 gai-t6 rgl1-1 rgl2-1* larger pollen appear significantly larger compared to their corresponding counterparts in wild-type haploid pollen, indicating that the enlarged pollen have a higher nuclear DNA content. These findings, together with relative pollen size increase, suggest that the enlarged pollen grains in both types of DELLA mutants are diploid (Fig. 2, D, E, H and I) or triploid (Fig. 2, F, G, J and K). In addition to this ectopic induction of gametophytic instability, it was also noted that all pollen in both DELLA double and quadruple mutants display a defective pollen wall morphology (Fig. 2, D, F, H and J), typically lacking the thick, protein-rich outer cell wall. A similar defect has been observed in the enlarged pollen isolated from wild-type Landsberg *erecta* (L*er*) plants sprayed with 100 μM GA$_3$ (Supplemental Fig. S2), indicating that alterations in GA signaling not only interfere with male gametophytic ploidy stability but also affect processes in pollen wall formation.

The gametophytic ploidy level of the larger pollen in the *rga-24 gai-t6* double mutant was accurately determined by quantifying the chromosome number in early-stage microspores using the *pWOX2::CENH3-GFP*. Contrary to the haploid unicellular microspores from control plants, which consistently show five distinct centromeric GFP dots (Fig. 2L), the oversized microspores in the *rga-24 gai-t6* double mutant harbor either two or three nuclei that each exhibits five distinct GFP foci (Fig. 2, M and O) or alternatively contain one single enlarged nucleus with 10 GFP dots reflecting a fusion of two haploid nuclei (Fig. 2N). These observations demonstrate that the *rga-24 gai-t6* double mutant generates a subset of microspores containing two or more sets of haploid nuclei that subsequently fuse to yield diploid or triploid pollen grains.

To determine the viability of the diploid pollen grains induced by *RGA* and *GAI* mutation, we evaluated cellular activity of mature pollen grains by fluorescein diacetate (FDA) staining (Heslop-Harrison and Heslop-Harrison, 1970; Trognitz, 1991; Abdul-Baki, 1992; Supplemental Fig. S3A–F). FDA staining of both mature haploid L*er* pollen grains (Supplemental Fig. S3, C and D) and enlarged *rga-24 gai-t6* mutant pollen (Supplemental Fig. S3, E and F) showed clear cytoplasmic fluorescence, indicating that haploid, diploid, and polyploid pollen grains were biologically active and thus viable. In addition, the germination ability of the larger pollen grains in the *rga-24 gai-t6* double mutant plants was examined in vitro (Supplemental Fig. S3G–J). Following soiling on pollen growth medium and incubation in the dark for 24 h, enlarged *rga-24 gai-t6* pollen grains germinated and produced pollen tubes (Supplemental

Fig. S3H and J; red dot labels larger pollen grains in J) similar to the control Ler haploid pollen (Supplemental Fig. S3G and I). Altogether, these data indicate that diploid pollen grains produced by rga-24 gai-t6 Arabidopsis plants are viable and may evoke sexual polyploidization events.

GA$_3$ Induces the Formation of Diploid Pollen by Promoting DELLA Degradation

To determine whether GA induces diploid pollen formation through degradation of DELLA, we next examined the GA response of male sporogenesis in the GA-insensitive gai mutant and the DELLA rga-24 gai-t6 double mutant. In contrast to the gai-t6 loss-of-function mutation, the allelic gai mutation is localized in the N-terminal DELLA domain of GAI and thus acts as a gain-of-function mutation, impairing the GA response in multiple GA-mediated processes (seed germination, stem elongation, and onset of flowering), even under exogenous GA treatment conditions (Wilson and Somerville, 1995; Willige et al., 2007). After spraying both wild-type Ler plants and gai mutant plants with 100 μM GA$_3$, the frequency of enlarged uninuclear stage microspore was quantified 4 d post treatment. Under normal growth conditions, gai plants produce normal-sized haploid microspores (Fig. 1B), indicating that an impaired GA response ability in Arabidopsis does not affect male sporogenesis. However, in contrast to GA-treated wild-type Ler plants, which produce a subset of enlarged unicellular-stage microspores (3.06%) (Fig. 1B), no oversized microspores were observed in gai plants after GA treatment (Fig. 1B). As male sporogenesis in gai plants is not responsive to exogenous GA application, these findings demonstrate that GA-induced production of 2n pollen in Arabidopsis requires a functional, targetable GAI DELLA domain and thus indicate that GA-based induction of 2n pollen in Arabidopsis is mediated through GA-based degradation of the DELLA GAI protein.

In support of this, we found that exogenous GA$_3$ treatment of the double rga-24 gai-t6 mutant plants does not enhance the frequency of enlarged uninuclear microspores compared with the untreated rga-24 gai-t6 plants or GA-treated wild-type Ler plants (Fig. 1B), indicating that exogenous GA$_3$ treatment has no additive effect on diploid male gamete formation in the double rga-24 gai-t6 mutant background. Collectively, these data demonstrate that exogenous GA$_3$ treatment induces diploid pollen formation in Arabidopsis by promoting DELLA protein degradation.

Diploid Pollen in rga-24 gai-t6 Mutant and GA$_3$-Treated Wild-Type Arabidopsis Plants Result from a Restitution of Male Meiotic Cell Division

To determine whether GA$_3$-induced formation of diploid pollen is caused by meiotic alterations, male meiocytes were examined at the tetrad stage using orcein

staining (Fig. 3, B–F). Male meiosis in wild-type Arabidopsis plants typically results in balanced tetrads containing four haploid sporocytes (Fig. 3B). Similarly, upon GA$_3$ treatment, the majority of tetrad-stage meiocytes appear normal, showing four equally sized microspores in a meiotic tetrad (Fig. 3C). However, at 36 to 48 h after GA$_3$ treatment, a subset of meiotically restituted triad (Fig. 3, D and E) and dyad microspores (Fig. 3F) were observed, indicating that GA$_3$ interferes with one or more processes during male meiotic cell division. Similarly, 3 or 4 days post GA$_3$ treatment (100 μM), qrt1-2$^{-/-}$ plants (Fig. 3, G–K) were also found to produce a subpopulation of triad (Fig. 3, H and I) and dyad microspore configurations (balanced dyad, Figure 3J and unbalanced dyad, Figure 3K) in agreement with GA$_3$-induced male meiotic restitution. The rga-24 gai-t6 double mutant also revealed a subset of triads (4.71%; Figure 3, N and O) and dyads (Fig. 3M; balanced dyad 0.4% and unbalanced dyad 0.13%), indicating for meiotic nonreduction similar as in GA$_3$-treated plants.

Further nuclear analysis of orcein stained tetrad-stage meiocytes additionally revealed that enlarged sporocytes in restituted rga-24 gai-t6 meiocytes either carry two equally sized nuclei (Fig. 3N) or alternatively harbor one single enlarged nucleus (Fig. 3O), closely according with diploid microspores either showing two distinct groups of five GFP foci (Fig. 2M) or exhibiting one fused nucleus with 10 GFP dots (Fig. 2N), respectively. Further confirmation of binuclear and higher ploidy mononuclear microspores in GA$_3$-treated plants and untreated rga-24 gai-t6 mutant was obtained by DAPI staining of tetrad-stage meiocytes and unicellular-stage microspores (Fig. 3, T–Y). Both the rga-24 gai-t6 mutant and GA$_3$-treated plants show syncytial colocalization of two or more nuclei in tetrad-stage male meiocytes (Fig. 3, U and V) and the unicellular microspore stage (Fig. 3, X and Y).

Collectively, these data suggest that diploid pollen in GA$_3$-treated wild-type plants and in the rga-24 gai-t6 Arabidopsis mutant result from a similar defect in meiotic cell division, with ectopic events of meiotic nonreduction leading to the syncytial colocalization of two or more haploid nuclei in one microsporocyte.

The rga-24 gai-t6 Mutant Displays Normal Male Meiotic Chromosome Segregation

Meiotically restituted dyads and triads generally result from defective meiotic chromosome segregation, omission of either meiosis I or II, or alterations in spindle orientation. To determine which of these cellular processes is affected, male meiotic chromosome spreads of both the rga-24 gai-t6 double mutant and wild-type Ler plants were examined (Supplemental Fig. S4, A–L). In both wild-type and rga-24 gai-t6 mutant plants, four distinct sets of five chromosomes (haploid nuclei) were consistently observed in all telophase II meiocytes, indicating that male meiotic chromosome segregation in the rga-24 gai-t6 double mutant is not affected (Supplemental Fig. S4J). Following nucleation
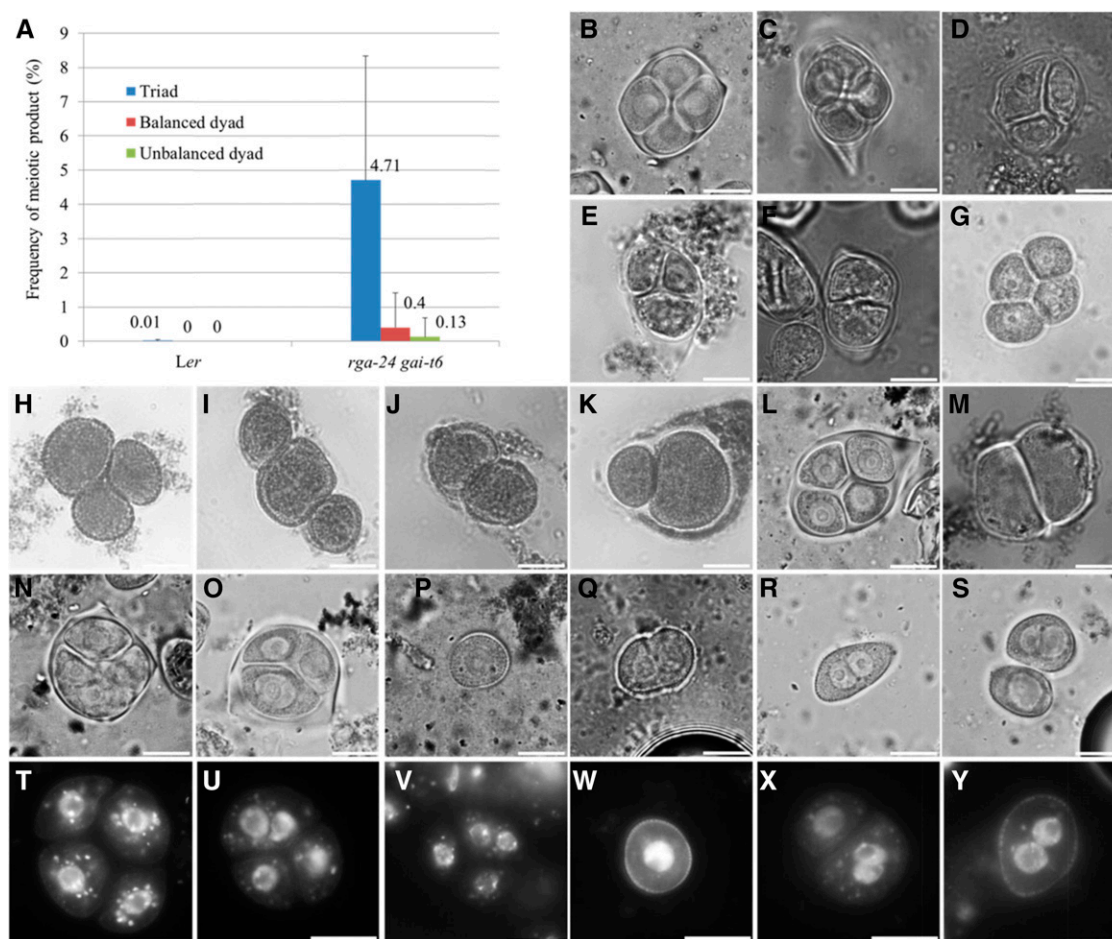
Figure 3. Male meiotic restitution in GA₃-treated plants and the *rga-24 gai-t6* double mutant. A, Histograms showing the frequency of meiotic nonreduction events in the *rga-24 gai-t6* double mutant. Orcein staining of tetrad stage male meiocytes in control (B) and GA₃-treated plants (C–F). Orcein staining of unicellular stage microspores in control (G) and in GA₃-treated *qrt1-2⁻/⁻* plants (H–K). Orcein staining of tetrad stage meiocytes in wild-type L*er* plants (L) and the *rga-24 gai-t6* double mutant (M–O). Orcein staining of unicellular stage microspores in control (P), GA₃-treated plants (Q) and in the *rga-24 gai-t6* double mutant (R and S). DAPI staining of tetrad-stage male meiocytes and unicellular microspores in control (T and W), GA₃-treated plants (U and X), and in the *rga-24 gai-t6* double mutant (V and Y). Scale bars = 10 μm.

at the tetrad stage, haploid nuclei in *rga-24 gai-t6* male meiocytes occasionally display an irregular spatial organization with two or more nuclei in proximity to each other. Under normal conditions, tetrad-stage, wild-type male meiocytes always exhibit two distinct perpendicularly oriented internuclear organelle bands, which physically separate the resulting nuclei in four distinct cytoplasmic domains and additionally determine the spatial positioning of the future cell walls (Supplemental Fig. S4K). In contrast, in the *rga-24 gai-t6* double mutant, some tetrad-stage meiocytes showed a clear absence of the internuclear organelle band between two or more haploid nuclei, typically resulting in the physical clustering of two or more nuclei in one single cytoplasmic domain (Supplemental Fig. S4L). Hence, since the *rga-24 gai-t6* mutant does not show any defect in chromosome segregation during male MI or MII, the cellular defect causing meiotic restitution and diploid pollen formation occurs in a later stage of sporogenesis, that is, after telophase II.

Meiotic Restitution in the *rga-24 gai-t6* Mutant and GA₃-Treated Arabidopsis Plants Results from Defects in Male Meiotic Cytokinesis

Both the normal meiotic chromosome dynamics and the eventual colocalization of haploid nuclei at the tetrad stage suggest that meiotically restituted dyads and triads in the *rga-24 gai-t6* double mutant result from defects in male meiotic cytokinesis. In Arabidopsis male meiosis, the accumulation of intermediate callosic material at the end of MII reflects proper cell wall formation and cytokinesis. Aniline blue staining of GA₃-treated wild-type L*er* plants and DELLA double *rga-24 gai-t6* and quadruple *rga-t2 gai-t6 rgl1-1 rgl2-1* mutants revealed alterations in the deposition of callosic cell walls (Fig. 4). In most cases, GA₃-treated plants (Fig. 4B) and DELLA mutants (Fig. 4, G and L) produce normal tetrads, similar to those seen in wild-type L*er*, typically showing the presence of a distinct cross-shaped callosic cell wall that separates all four

haploid spores (Fig. 4A). However, in GA₃-treated plants, a subset of tetrad-stage meiocytes was found to produce partial or incomplete callosic cell walls at the end of MII, leading to the formation of balanced and unbalanced dyads and triads (Fig. 4, C–E). In this pool of altered meiotic products, a great variation in defect severity was observed, with some cells not forming any callosic wall whereas others produced cell wall stubs or cell plates with small gaps (Fig. 4F). Callose staining revealed similar defects in male meiotic cell wall formation in the DELLA double and quadruple mutants (Fig. 4, H–K and M–P).

Combined DAPI and aniline blue staining on tetrad-stage male meiocytes additionally revealed that aberrations in *rga-24 gai-t6* MII cell wall positioning lead to the colocalization and occasional clustering of two or more nuclei in one single microsporocyte (Fig. 4, R and S). Moreover, in a few rare events, tetrad-stage *rga-24 gai-t6* meiocytes were found to form ectopic callosic cell walls (Fig. 4T), suggesting that one or more processes determining the spatial positioning of cytokinesis and the associated deposition of cell wall components is affected. Collectively, these data demonstrate that meiotic restitution in both GA₃-treated plants and DELLA mutants is caused by defects in male meiotic cytokinesis.

As both the DELLA *RGA* and *GAI* genes are actively transcribed ubiquitously (Silverstone et al., 1998), the question arises whether cell wall formation in other tissues is also affected. To monitor for putative defects in somatic cell wall formation, epidermal cells and associated cell wall patterning of mature *rga-24 gai-t6* petals was assessed using bright field microscopy. Somatic nuclei were concomitantly visualized using DAPI staining. A thorough scanning revealed that *rga-24 gai-t6* petals exhibit normal epidermal cell wall morphology (Supplemental Fig. S5, E–G) and consistently show nuclei of equal size (Supplemental Fig. S5H), similar to wild-type L*er* plants (Supplemental Fig. S5, A–D), indicating that somatic cell wall formation is not affected. Furthermore, propidium iodide staining of root tips did not reveal altered or incomplete cell walls in both wild-type L*er* and *rga-24 gai-t6* mutant plants (Supplemental Fig. S5, I and J). Hence, both the observations in root and petal tissues indicate that *rga-24 gai-t6* somatic tissues do not show any defect in cell wall formation, suggesting that the DELLA proteins RGA and GAI specifically mediate cytokinesis in male meiotic cell division.

Cell Wall Defects in *rga-24 gai-t6* Male Meiotic Tetrads Are Caused by Structural Alterations in the RMA

The positioning of the cell wall and associated deposition of cell wall material, such as intermediate callose, in somatic plant cells is guided by the phragmoplast (De Storme and Geelen, 2013a). In male meiosis, cytokinesis also depends on microtubular phragmoplast-like structures. However, due to their specific spatial intercellular organization, that is, formed at the intersection of microtubules (MTs) emanating from syncytial telophase II nuclei, they are referred to as RMAs (Peirson et al.,

1997; Brown and Lemmon, 2001). Since RMAs are crucial for the formation and positioning of the meiotic cell wall (Otegui and Staehelin, 2004), we next performed immunocytological analysis of the MT subunit tubulin-α in male meiocytes of the *rga-24 gai-t6* double mutant (Fig. 5). In wild-type L*er* plants, TII male meiocytes show a microtubular structure that consists of six RMAs that are organized between the four haploid nuclei (Fig. 5A). Although most *rga-24 gai-t6* male meiocytes show a similar TII cytoskeletal configuration, with distinct RMAs between each of the four haploid nuclei, a subset of TII male meiocytes in the *rga-24 gai-t6* double mutant was found to exhibit clear alterations in the biogenesis and organization of the RMAs (Fig. 5, B–H). In some instances, adjacent nuclei in a single TII meiocyte were found to completely lack the presence of internuclear microtubules, hence causing a physical clustering of haploid nuclei in a triad figure (Fig. 5, B–D). Moreover, besides TII meiocytes that show partial or complete loss of RMA formation, some of the *rga-24 gai-t6* tetrads with regularly separated nuclei display a reduced level of internuclear MT labeling, indicating minor aberrations in RMA biogenesis or MT stability (Fig. 5, E and F). In these slightly altered TII meiocytes, a subset of MTs of the RMA appears bent and exhibits a randomly organized pattern (Fig. 5, G and H). Moreover, fluorescent dots in some cells suggest that microtubules did not elongate properly or were depolymerized (Fig. 5H, arrow), indicating defects in RMA microtubule dynamics.

Overall, the RMA defects observed in *rga-24 gai-t6* TII meiocytes are consistent with the aberrations in male meiotic cell wall formation and form the structural basis for events of male meiotic restitution and the associated production of higher ploidy male gametes.

DELLA Downstream Factors MYB33 and MYB65 Are Redundantly Required for Male Meiotic Cytokinesis in Arabidopsis

MYB33 and MYB65 are downstream factors of DELLA in regulating GA responsive processes (Gubler et al., 2002; Kaneko et al., 2003) and are well known in regulating anther development (Millar and Gubler, 2005). To investigate whether these two MYB factors participate in DELLA-mediated male meiotic cytokinesis in Arabidopsis, we monitored both sporogenesis and gametogenesis in the single and double *myb33 myb65* mutant plants. Both single *myb33* and *myb65* mutant plants produce normal tetrads, haploid microspores, and haploid pollen grains, similar to wild-type Col-0 plants (Fig. 6, A–C, D–F, and G–I). In contrast, double *myb33 myb65* mutant plants were found to produce a subset of meiotically restituted triads together with the associated formation of enlarged spores and mature pollen grains (Fig. 6, J–L). Oversized microspores at the unicellular stage (Fig. 6K) and larger pollen grains (Fig. 6L) occurred at a frequency of 4.35% and 4.64%, respectively. Similar to GA-treated wild-type plants and double *rga-24 gai-t6* mutant plants, mature pollen from the double *myb33 myb65* mutant also shows
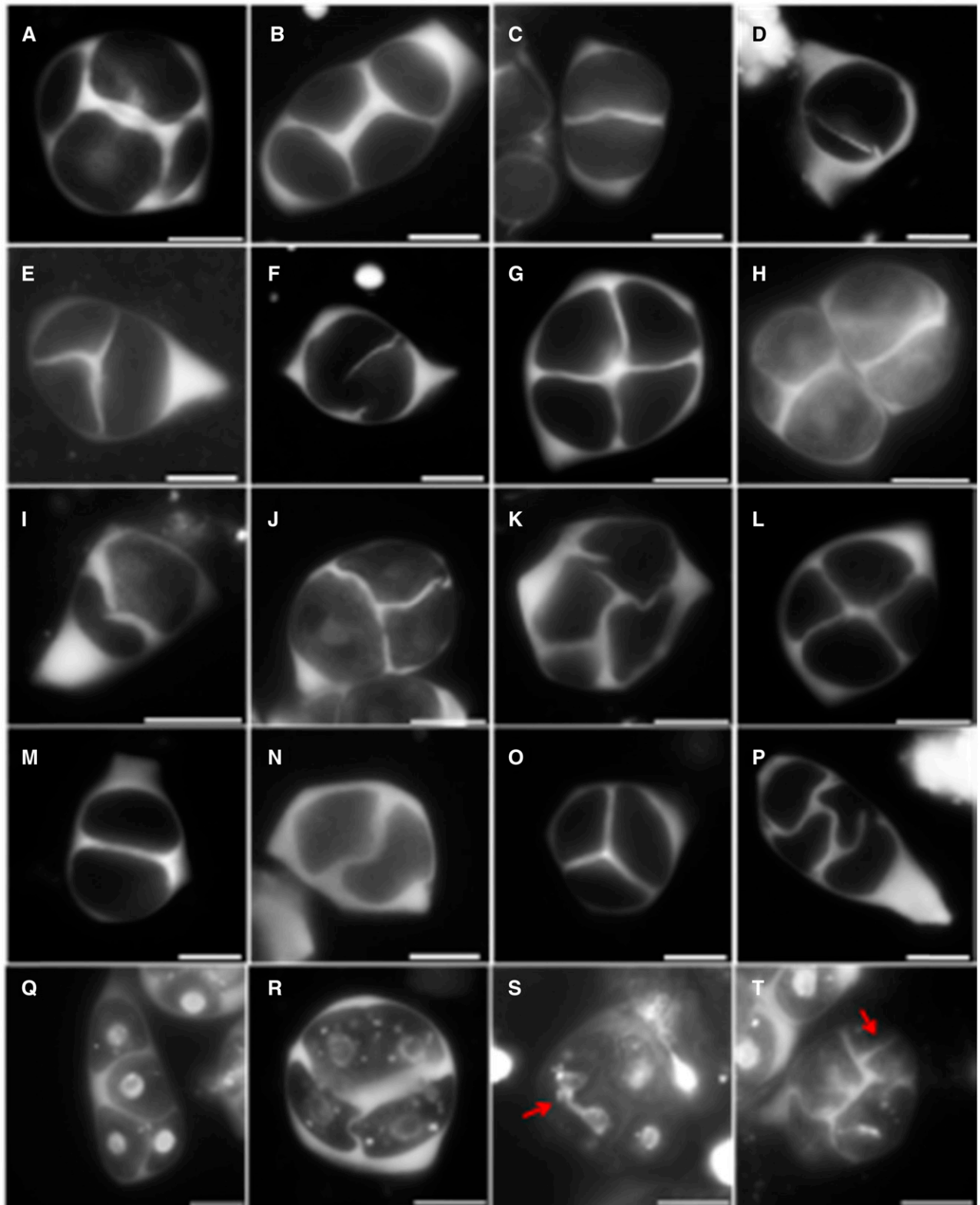
Figure 4. Aniline blue staining reveals male meiotic cytokinesis defects in GA$_3$-treated plants and DELLA double and quadruple Arabidopsis mutants. Aniline blue staining of callosic cell walls in tetrad stage meiocytes of wild-type L*er* plants (A), wild-type L*er* plants sprayed with 100 μM GA$_3$ (B–F), the *rga-24 gai-t6* double mutant (G–K), and the *rga-t2 gai-t6 rgl1-1 rgl2-1* quadruple mutant (L–P). Combined aniline blue and DAPI staining of tetrad-stage male meiocytes in wild-type L*er* (Q) and *rga-24 gai-t6* plants (R–T). Scale bars = 10 μm.
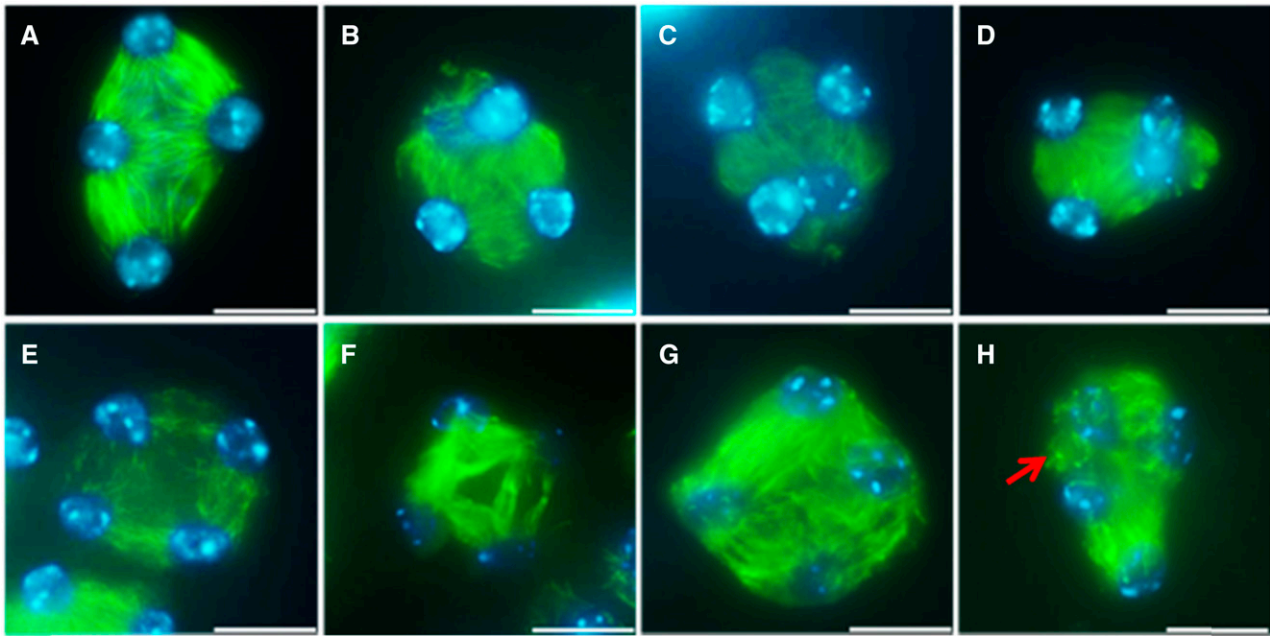
Figure 5. Tubulin-α immunolocalization in TII male meiocytes of wild-type Ler and *rga-24 gai-t6* double mutant plants. Structure of the RMAs in TII male meiocytes of wild-type Ler plants (A) and the *rga-24 gai-t6* double mutant (B–H). Green: α-tubulin, cyan: DAPI. Scale bars = 10 μm.

aberrant pollen wall morphology with lack of the thick protein-rich outer cell wall (Fig. 6L). These data indicate that MYB33 and MYB65 operate redundantly in regulating male sporogenesis in Arabidopsis, with a loss of function of both genes resulting in male meiotic restitution and the associated formation of larger, higher ploidy male gametes.

Aniline blue staining revealed that single *myb33* and *myb65* mutants produce tetrad-stage male meiocytes with regular callosic cell walls (Fig. 7, B and C), whereas male meiotic products in the *myb33 myb65* double mutant display defects in cytokinesis with formation of incomplete or misshapen callosic cell walls (Fig. 7, F–I). Combined aniline blue and DAPI staining further revealed a colocalization of nuclei in the enlarged spore that results from lack of cell wall formation (Fig. 7, J–N). Tubulin-α immunolocalization of *myb33 myb65* TII male meiocytes revealed a complete loss of RMA formation between colocalized nuclei (Fig. 7, S and T) and additionally displayed fluorescent dots in some male meiocytes, suggesting defects in microtubule polymerization or stability (Fig. 7U). MI and MII spindle organization in *myb33 myb65* male meiotic cells appeared normal (Fig. 7, V and W), similar to those in wild-type Col-0 cells (Fig. 7, Q and R) and indicating that other microtubular structures in earlier stages of male meiosis are not affected, allowing normal chromosome segregation. Collectively, these findings indicate that the RMA biosynthesis and/or polymerization stability are affected in the double *myb33 myb65* mutant, leading to defects in male meiotic cell wall formation.

GA-Induced Defects in Male Meiotic Cytokinesis Correlate with DELLA Signaling in the Somatic Anther Tissue

To assess the specific expression pattern of DELLAs during male sporogenesis, we analyzed expression of RGA in the Arabidopsis anther by using a *pRGA::GFP-RGA* marker in the Ler background. To stabilize the GFP-RGA signals in the developing flowers, we sprayed flowering *pRGA::GFP-RGA* plants with 1 mM paclobutrazol and monitored *GFP-RGA* expression/localization 6 hours post treatment using both confocal laser scanning (Fig. 8) and epi-fluorescence microscopy (Supplemental Fig. S6). No GFP signal was detected in the anthers isolated from wild-type Ler control plants (Fig. 8A–D; Supplemental Fig. S6, A–C). Con the contrary, in the plants harboring the *pRGA::GFP-RGA* construct, *GFP-RGA* expression was clearly visible in the anther, showing a distinct dotted pattern that is strictly confined to the somatic tissue surrounding the male meiocytes and/or microspores during anther development (Fig. 8E and Supplemental Fig. S6D, precytokinesis stage; Fig. 8F and Supplemental Fig. S6E, tetrad stage; Fig. 8, G and H and Supplemental Fig. S6F, microspore stage), indicating that RGA is not expressed in the male reproductive tract, that is, in the meiocytes and developing microspores but instead appears specifically expressed in the somatic cell layer, including the tapetum, that surrounds the meiotic and gametophytic cells.

To examine whether exogenous GA treatment reduces *RGA* expression in the tapetal cell layer during male sporogenesis, we next sprayed flowering *pRGA::GFP-RGA*
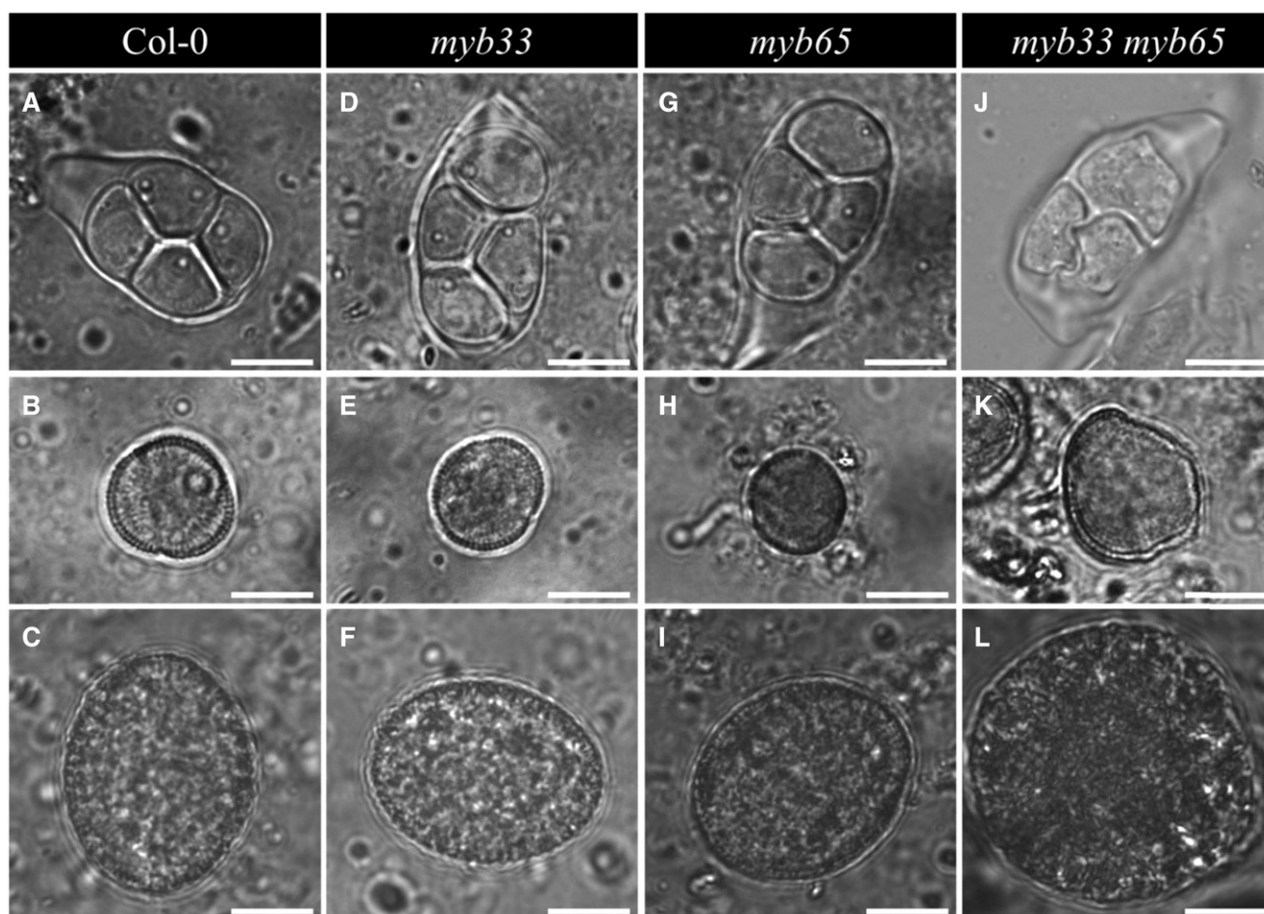
Figure 6. Sporogenesis and gametogenesis in the single and double *myb33 myb65* mutant. Tetrad (A), haploid unicellular stage microspore (B), and haploid pollen grain (C) in wild-type Col-0 plant. Tetrad (D), haploid unicellular stage microspore (E), and haploid pollen grain (F) in single *myb33* mutant plant. Tetrad (G), haploid unicellular stage microspore (H), and haploid pollen grain (I) in single *myb65* mutant plant. Triad (J), enlarged unicellular stage microspore (K), and enlarged pollen grain (L) in double *myb33 myb65* mutant plant. Scale bars = 10 μm.

plants with 100 μM GA$_3$ (24 h post 1-mM PAC treatment, 0.06% Tween 20), and subsequently monitored the RGA-GFP signal 2 h post GA treatment. In the absence of exogenous GA treatment, a distinct pattern of RGA-GFP fluorescent signals was observed in the tapetal cell layer that surrounds the male meiocytes and young microspores during progressive anther development (Supplemental Fig. S7, A–C). However, 2 h post GA treatment, a significant decrease in RGA-GFP signal intensity in developing anthers was observed (Supplemental Fig. S7, D–F), indicating that exogenous GA treatment suppresses the expression of *RGA* in the tapetal cell layer during male meiosis and early gametogenesis.

DISCUSSION

GA-DELLA-MYB Signaling Module Regulates Male Meiotic Cytokinesis in Arabidopsis

In this study, we report that exogenous GA$_3$ treatment induces 2n pollen formation in Arabidopsis by causing

defects in male meiotic cytokinesis. Using genetic studies, we additionally demonstrate that combined loss-of-function of two downstream GA factors, i.e. the DELLA proteins RGA and GAI, causes a phenotype highly reminiscent to external GA treatment, suggesting that the transcriptional regulators RGA and GAI control the expression of proteins that either promote or interfere with regular meiotic cytokinesis. Altogether, our observations indicate that ectopic GA induces male meiotic restitution and 2n gamete formation in Arabidopsis by promoting the degradation of the DELLA RGA and GAI proteins. Cytological studies additionally revealed that combined loss of MYB33 and MYB65, two downstream DELLA targets, leads to similar male meiotic defects and associated formation of enlarged male gametes, suggesting a role of the GA-DELLA-MYB33/MYB65 regulatory pathway in controlling male meiotic cytokinesis in Arabidopsis. Moreover, as the double *myb33 myb65* mutant displays similar RMA defects as the DELLA *rga-24 gai-t6* mutant, we postulate that specific, yet unknown DELLA-MYB downstream factors directly control RMA
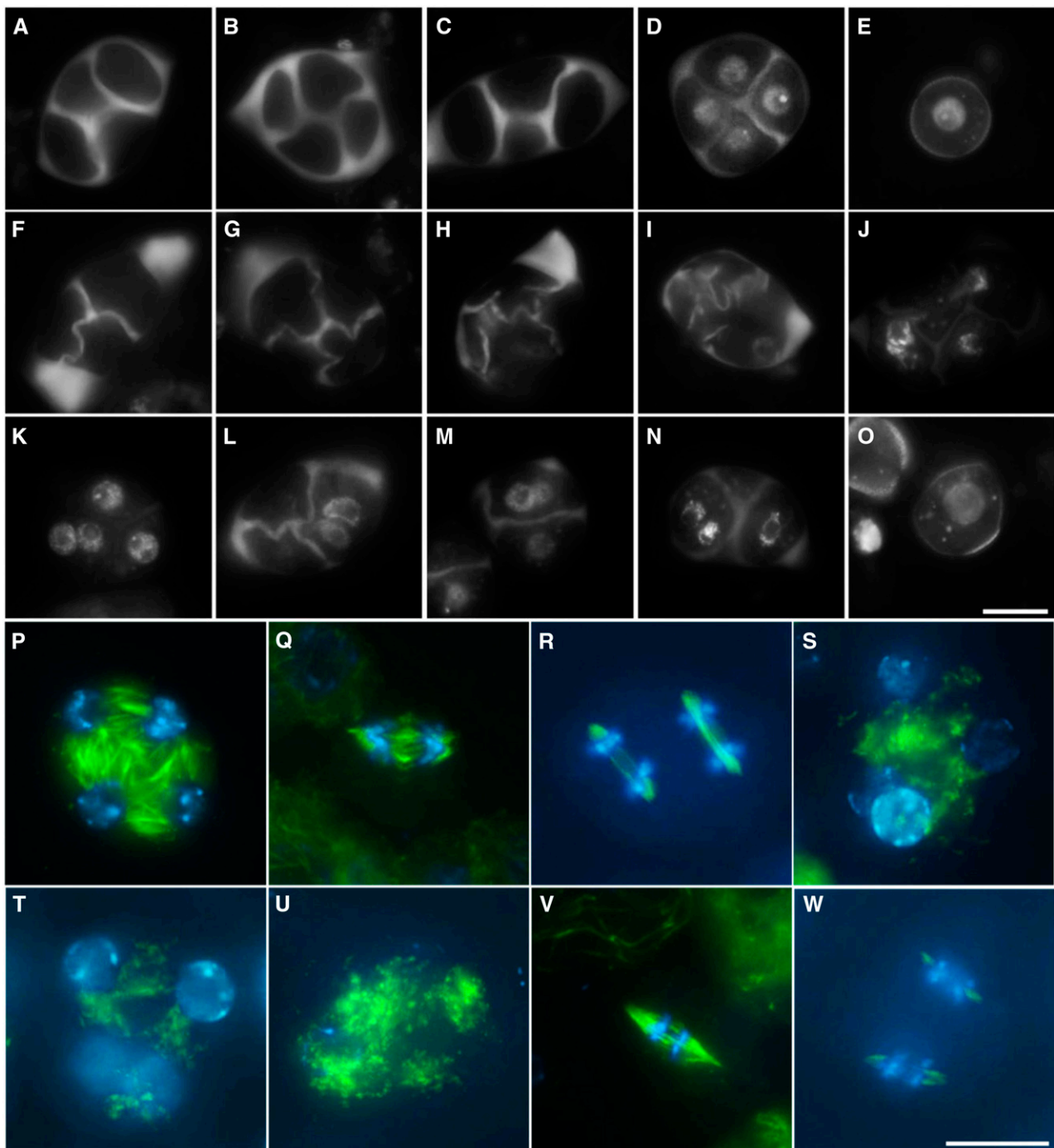
Figure 7. Male meiotic cytokinesis defects in *myb33 myb65* Arabidopsis plants. Aniline blue staining of tetrads in wild-type Col-0 (A) and single *myb33* (B) and *myb65* (C) mutant plants. D, Combined aniline blue/DAPI staining of tetrad-stage male meiocytes in wild-type Col-0 plant. E, DAPI staining of haploid unicellular stage microspore in wild-type Col-0 plants. F to I, Aniline blue staining of defective tetrad stage male meiocytes in double *myb33 myb65* mutant plants. J to N, Combined aniline blue/DAPI staining of defective tetrad-stage male meiocytes in double *myb33 myb65* mutant plants. O, DAPI staining of an enlarged unicellular stage microspore isolated from *myb33 myb65* mutant plant. RMA (P) and MI and MII spindles (Q and R) in wild-type Col-0 plants. Defective RMA (S–U) and normal MI and MII spindles (V and W) in the double *myb33 myb65* mutant. Green: α-tubulin, cyan: DAPI. Scale bars = 10 μm.

biogenesis and associated microtubule dynamics in tetrad-stage male meiocytes. However, the identity of these downstream regulators and their specific mode of

action in controlling male meiotic RMA formation remain largely elusive. Strikingly, in both GA₃-treated wild-type plants as well as all DELLA mutants analyzed,
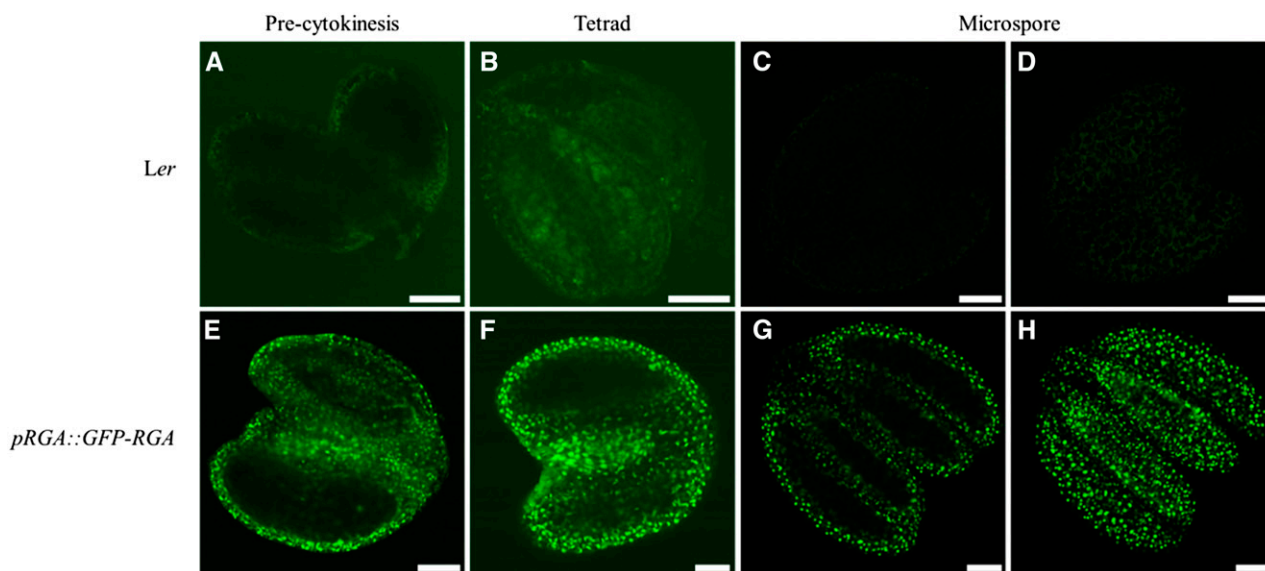
Figure 8. Confocal laser scanning microscopy of *pRGA::GFP-RGA* during different stages of Arabidopsis anther development. Anthers of wild-type L*er* plants at during meiosis (A), tetrad stage (B), and early microspore development (C and D). Anthers of L*er* plants harboring the *pRGA::GFP-RGA* constructs during meiosis (E), tetrad stage (F), and early microspore development (G and H). Scale bars = 50 *μ*m.

cytokinesis defects occurred in only a minority of male meiocytes (around 4%), indicating that male meiotic RMA formation and cytokinesis is largely resilient to GA deregulation. Nevertheless, maintaining ploidy consistency is critical to regular sexual reproduction, with even small deviations in gametophytic ploidy level (e.g. diploid and aneuploid gametes) being highly relevant when considered in an ecophysiological perspective or on a larger evolutionary time scale.

A Role for GA in Integrating Environmental Stress and Reproductive Genome Instability?

During growth and development, plants suffer from various stresses, and the reproductive system is particularly sensitive to adverse environmental conditions (De Storme and Geelen, 2014). Previously, we reported that a short period of severe cold (1–40 h at 4–5°C) induces male meiotic restitution and 2n gamete formation by causing defects in male meiotic cytokinesis. More specifically, low temperatures were found to specifically affect the RMA cytoskeletal network at the telophase II stage, consequently impairing male meiotic cell plate formation and cell wall deposition. In contrast, other microtubular structures in the developing meiocytes, such as MI and MII spindles and the cortical MT array, were not affected by low-temperature stress, as reflected by the regular chromosome segregation dynamics in MI and MII (De Storme et al., 2012). Based on these findings, it is suggested that cold-induced formation of 2n pollen is governed by specific regulators and signaling pathways that specifically interfere with RMA biogenesis and/or MT dynamics during male meiotic cytokinesis.

Here, in this study, we demonstrate that perturbation of endogenous GA levels mimics the cold-induced defects in Arabidopsis male sporogenesis, causing highly similar defects in male meiotic RMA biogenesis and cell wall formation. Based on this similarity, we hypothesize that GA may putatively play an important regulatory role in (cold) stress-induced restitution of male meiosis and associated production of 2n pollen. Previous studies have demonstrated that GA signaling plays a prominent role in the plants' response to cold stress through transient activation of CBF1/DREB1b, a transcriptional regulator controlling downstream cold-responsive genes (Colebrook et al., 2014). However, the putative role for GA in mediating cold-induced 2n gamete formation is probably indirect, since cold has been found to reduce endogenous GA levels, which is incongruent with the observation that increased GA levels induce events of male meiotic restitution. For example, in Arabidopsis seedlings, cold reduces endogenous bioactive GA levels by repressing expression of the GA-biosynthetic GA20 oxidase and stimulating the expression of the GA-catabolizing GA2-oxidase, thereby enabling cellular accumulation of DELLAs and associated reduction of tissue growth (Achard et al., 2008). Similarly, in rice, endogenous GA levels in developing anthers were found to decrease upon exposure to low temperatures (Sakata et al., 2014), suggesting that cold generally reduces endogenous GA levels instead of accumulating it.

Whether low temperature-induced disorders in male meiotic RMA structure are mediated by increased GA levels and whether this occurs in a direct or indirect manner remains to be determined. In plants, microtubules are notoriously sensitive to cold shock, with each

species having a specific critical temperature underneath which MTs become highly unstable and disassemble. However, despite numerous reports of cold-induced effects on MT stability, it is yet unknown how this is mechanistically controlled and whether and how this is regulated at the molecular level. A recent study by Locascio et al. (2013) provides preliminary evidence for mechanistic link of GA signaling and microtubule organization. These authors reported that cortical MT reorganization in elongating hypocotyls strongly depends on the availability of the prefoldin complex in the cytosol and that this availability is regulated by the nuclear-localized DELLA proteins RGA and GAI, which bind and inactivate prefoldin (Locascio et al., 2013). However, despite this mechanistic insight into the regulatory role of DELLAs in cortical MT stability, it is yet unknown whether male meiotic RMA formation is controlled by a similar regulatory mechanism and whether cold stress may interfere with this to cause defects in male meiotic cell wall formation. In both GA-treated plants and the double *rga-24 gai-t6* mutant, we have not observed aberrant organization of cortical microtubules in somatic cells or defects in cell division that could be caused by faulty mitotic cytoskeletal configurations. Hence, the different response of MT structures in somatic and male meiotic cells suggests there may exist different mechanisms by which GA mediates microtubular dynamics in somatic and reproductive tissues, putatively reflecting the differences in the structural organization and molecular regulation of the MT structures in male meiosis (RMAs) and somatic cell division (phragmoplast and cortical MT arrays; De Storme and Geelen, 2013).

Different Roles of GA in Microsporogenesis and Gametogenesis

Several studies demonstrated that GA is required for floral development by repressing the activity of DELLAs (Cheng et al., 2004; Tyler et al., 2004). In the GA-deficient *ga1-3* mutant, male meiocytes display normal MI and MII chromosome segregation and cell wall formation (Cheng et al., 2004), indicating that GA is not required for the meiotic cell division program. This is confirmed in our study by monitoring the sporogenesis of GA-insensitive *gai* mutant. On the other hand, *ga1-3* plants show clear defects in microspore development and pollen maturation because of impaired tapetum development (Cheng et al., 2004; Aya et al., 2009; Mutasa-Göttgens and Hedden, 2009), indicating that GA is essential for microspore development and pollen maturation through its promotive role in tapetum development. Several studies have provided accumulating evidence that GA plays an important role in the developmental regulation of the tapetal cell layer; a nourishing tissue that promotes microspore development and the outer cell wall formation. In rice, for example, impaired GA signaling causes alterations in tapetal cell programmed cell death (Aya et al., 2009), a process that is vital for microspore development through the

supplementation of nutrients, hence causing major gametophytic defects such as spore abortion and male sterility. A conserved family of transcription factors, GAMYBs, that are controlled by the GA signaling pathway have been linked with tapetum functioning and pollen outer cell wall formation (Aya et al., 2011). The GAMYB members MYB33 and MYB65 are redundantly required for the development and persistence of the tapetum cell layer during male reproductive development (Millar and Gubler, 2005; Plackett et al., 2011). In this report, we demonstrate that the combined loss of MYB33 and MYB65 in Arabidopsis not only affects male gametogenesis but also causes defects in male meiotic cell division, similar to the DELLA *rga-24 gai-t6* double mutant, indicating that the GA-DELLA-MYB pathway plays an important role in the developmental control of both male sporogenesis and gametogenesis.

The regulatory role of GA signaling (e.g. DELLAs-MYBs) in male meiotic cytokinesis shown in this study, together with fact that GA plays an essential role in tapetum and microspore development, corroborates with a previous report by Chhun et al. (2007), who reported that GA biosynthesis genes are expressed at a relatively low level in early stages of anther development (i.e. before meiotic cytokinesis) compared with that of GA-signaling genes and that the situation reversely alters at later stages in anther development (Chhun et al., 2007). Although the role of GA in regulating male gametophyte development and maintaining plant male fertility has been widely investigated, our data further clarify that a restriction of GA signaling and associated maintenance of DELLA activity in Arabidopsis is critical for successful male meiotic cell division and gametophytic ploidy stability.

Male Meiotic Cytokinesis: Regulated by GA Signaling in the Surrounding Somatic Tissue?

The GAMYB transcription factor family is conserved in land plants and seems to regulate a range of reproductive processes (Aya et al., 2011). In agreement with GA being involved in the control of tapetum and microspore development, several members of the GAMYB transcription factor family (MYB33 and MYB65 in Arabidopsis, HvGAMYB in barley, and OSGAMYB in rice) have been shown to be expressed in young anthers prior to flower anthesis (Kaneko et al., 2003; Murray et al., 2003; Millar and Gubler, 2005). Although MYB33 transcripts were reported to occur in a wide range of tissues, translational fusions showed that expression of MYB33 is mainly confined to the somatic cell layers of young anthers, likely due to MIR159-controlled restriction of mRNA translation. Lower expression levels were detected in developing microspores (Millar and Gubler, 2005). In this study, RGA expression analysis using a GFP translational fusion indicated that basal RGA levels are very low in young flower buds. Upon treatment with paclobutrazol, however, GFP expression strongly increases in most anther tissues, including the four outer somatic cell layers, except for meiocytes and microspores.

This therefore demonstrates that there is certain level of endogenous GA biosynthesis present in young anther tissues that restricts the expression of the DELLA RGA protein to a certain limit, but up to a level that still allows full completion of male meiotic cell wall formation.

Based on these findings, we conclude that the DELLA-GAMYB module is predominantly expressed in the somatic tissue surrounding the developing meiocytes, playing a putative important role in the appropriate functioning of the tapetum, possibly in cooperation with the other anther outer cell layers. Since expression of DELLAs and GAMYBs in meiocytes and developing microspores is generally low, the observed defects in male meiotic cytokinesis and cell wall formation in corresponding mutants and GA-treated plants may indirectly result from tapetal dysfunctioning rather than from a cell-autonomous male meiotic deregulation of the DELLA-GAMYB module. Our data therefore provide preliminary evidence for molecular communication between the somatic anther cells and enclosed meiocytes/microspores to guide and generate not only pollen outer cell wall maturation but also MT cytoskeletal dynamics and cell wall formation during the final stage of meiosis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0 and L*er* accessions were obtained from the Nottingham Arabidopsis Stock Centre. For GA_3 treatment analysis, the *qrt1-2*$^{-/-}$ mutant (Preuss et al., 1994; Francis et al., 2006) was ordered from Nottingham Arabidopsis Stock Centre. The *gai* mutant was kindly provided by Patrick Achard. For the in vivo gametophytic ploidy determination, Arabidopsis plants containing *pWOX2::CENH3-GFP* (De Storme and Geelen, 2011) were used. The DELLA double mutant *rga-24 gai-t6* and the quadruple mutant *rga-t2 gai-t6 rgl1-1 rgl2-1* were previously described (Achard et al., 2006) and kindly provided by Patrick Achard and Nicholas Harberd. The *rga-24 gai-t6* double mutant plants harboring *pWOX2::CENH3-GFP* were isolated in F2 population from the *rga-24 gai-t6* double mutant intercrossing with *pWOX2::CENH3-GFP* transgenic line, according to the phenotype of *pWOX2::CENH3-GFP* transgenic line and genotyping of *rga-24* and *gai-t6* mutation. The *rga-24* and *gai-t6* single mutants were acquired from F2 population from the *rga-24 gai-t6* double mutant intercrossing with *pWOX2::CENH3-GFP* transgenic line by genotyping *rga-24* and *gai-t6* mutation respectively. The pentuple *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* mutant was kindly shared by M. Höfte. The single and double *myb33 myb65* mutants were kindly provided by Anthony Millar. The *pRGA::GFP-RGA* transgenic plants were obtained from Nicholas Harberd. Primers used for genotyping are listed in Supplemental Table SI.

Seeds were germinated on K1 medium for 6 to 8 d, and then seedlings were transferred to soil and cultivated in growth chambers at 12 h light/12 h night, 20°C, and <70% humidity. Upon flowering, the photoperiod was changed to a 16-h-day/8-h-night regime. For GA_3 treatment, flowering plants were sprayed by water (+0.02% Tween) and 100 μM GA_3 (+0.02% Tween).

GA_3 and PAC Treatment Analysis

To determine the effect of GA_3 treatment on male meiotic products, tetrads and microspores were observed at 36 to 48 h and 72 to 96 h later after GA_3 and water spraying, respectively. To monitor the effect of GA_3 treatment on larger pollen formation, mature pollen grains were analyzed in the 14 d following GA_3 treatment. For *pRGA::GFP-RGA* expression analysis, to stabilize the GFP-RGA signals in Arabidopsis flowers, flowering *pRGA::GFP-RGA* plants were sprayed with paclobutrazol (1 mM, 0.06% Tween 20) and water. Anthers at different developmental stages were isolated to glass slides with a drop of diluted water and analyzed by microscopy.

Pollen Viability Examination

For performing FDA staining, mature pollen grains from newly opened flowers are released in a drop of FDA buffer (2 mg/mL in acetone) on a glass slide, and the fluorescence was observed after 10 min of staining. For pollen germination, mature pollen grains from newly opened flowers are soiled on pollen growth medium (5 mM $CaCl_2$, 1 mM $MgSO_4$, 5mM KCl, 0.01 mM H_3BO_3, 18% Suc, 1.5% agarose, pH at 7.5 modified by 0.1 M NaOH), and the pollen tube is observed after incubation in humid chamber under dark for 24 h.

Cytology

Pollen DAPI staining, callosic cell wall staining, and analysis of the male meiotic products (tetrad-stage analysis by orcein and DAPI staining) was performed as described previously (De Storme et al., 2012). Buds producing significant numbers of mature meiotic products were used for quantification and monitoring assays. DAPI and aniline blue-combined staining of male meiotic products was performed by releasing spores in a drop of DAPI solution (1 mg/ mL; Sigma) and aniline blue solution (0.1% [m/v] in 0.033% K_3PO_4 [m/v]) mixture. Visualization of *pWOX2::CENH3-GFP* constructs was performed by releasing spores in a 0.05 M $NaPO_4$ (pH 7.0) and 0.5% Triton X-100 (v/v) solution. Male meiotic spreads were performed as described previously (De Storme and Geelen, 2011). Visual assessment of petal leaves was performed according to the method of Carland et al. (2002). For observation of the cell wall in root tip, roots were cut from 5-d-old seedlings and mounted on a glass slide with 10 μg/mL propidium iodide solution for 5 min, followed by washing three times using diluted water before observation.

Tubulin Immunolocalization

α-Tubulin immunolocalization analysis was performed as described previously (De Storme et al., 2012).

Microscopy

Both bright-field and fluorescence microscopy were performed using an Olympus IX81 inverted fluorescence microscope equipped with an X-Cite Series 120Q UV lamp and an Olympus XM10 camera. Fluorescence from *pRGA::GFP-RGA* transgenic line and propidium iodide staining was captured using Nikon A1r confocal laser scanning microscope equipped with Axiovision software (LiMiD). Bifluorescent images and Z-stacks were processed using ImageJ. Brightness and contrast settings were adjusted using Photoshop CS6.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Male meiotic restitution and enlarged male gametes in *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* mutant plants.

Supplemental Figure S2. Mature pollen grain in GA_3-treated wild-type L*er* plant.

Supplemental Figure S3. Viability determination of enlarged mature pollen grains in the double *rga-24 gai-t6* mutant plants.

Supplemental Figure S4. The Arabidopsis *rga-24 gai-t6* mutant displays regular chromosome dynamics during male meiosis I and II.

Supplemental Figure S5. Microscopy analysis of cell wall and nuclei in somatic tissues.

Supplemental Figure S6. Expression pattern of *pRGA::GFP-RGA* in Arabidopsis anther.

Supplemental Figure S7. Exogenous GA treatment induces decrease of *pRGA::GFP-RGA* signals in Arabidopsis anther.

Supplemental Table S1. Primers for genotyping.

Liu et al.

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