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Characterization of the Arrest in Anther Development Associated with Gibberellin Deficiency of the *gib-1* Mutant of Tomato[1]

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ABSTRACT

The role of gibberellins in flower bud development was investigated by studying the *gib-1* mutant of tomato, *Lycopersicon esculentum*. This gibberellin-deficient mutant initiates flower buds, but floral development is not completed unless the mutant is treated with gibberellin. Treatment with other plant growth regulators does not induce normal flower development. Development of *gib-1* flower buds, as measured by progress toward anthesis, ceases at a bud length of 2.5 millimeters; however, increase in size of the bud continues. Buds between 2.5 and 3.7 millimeters are developmentally arrested but still are capable of developing normally after treatment with gibberellic acid. Anthers of these developmentally arrested buds contain pollen mother cells that are in the G1 phase of premeiotic interphase. Following treatment of developmentally arrested buds with gibberellic acid, premeiotic DNA synthesis and callose accumulation in pollen mother cells are evident by 48 hours posttreatment, and within 66 hours, prophase I of meiosis- and meiosis-related changes in tapetum development are observable.

GAs[2] are thought to play a significant role in flower development (for reviews, see refs. 9 and 20). In some monocots, and in many dicot species, there is a transitory increase in GA content before anthesis (17, 18, 20). Generally, most of this GA is present in anthers (9, 20). In *Petunia hybrida*, removal of stamens prevents normal corolla growth and differentiation, and treatment with GA can substitute for the presence of stamens (24). GAs are also known to hasten the time to anthesis in many species, and GA biosynthesis inhibitors may delay anthesis (9). Treatment with GA can often overcome the retarding effects of these inhibitors (9). The GA biosynthesis inhibitor (2-chloroethyl)trimethylammonium chloride can cause abortion of flowers in *Pharbitis* if applied 7 d before flower opening (25). Mutants have been described in both dicots and monocots that are male sterile unless supplied with exogenous GA (7, 11, 21).

We are studying the *gib-1* mutant of tomato, *Lycopersicon esculentum*, to further elucidate the role of GA in flower development. This mutant is deficient in GA because its ability to convert geranylgeranyl pyrophosphate to copalyl pyrophosphate is reduced (1). In addition to male and female sterility, the other phenotypes of this mutant are dwarfism and failure of seeds to germinate. Application of GA largely reverses all of these phenotypes (10). In this mutant, initiation of floral meristems and development of all floral organs proceeds normally up to a certain point, but then normal development ceases and flower buds eventually abort. Nester and Zeevaart (19) studied flower development of the *gib-2* tomato mutant. Like the *gib-1* mutant, this mutant is GA deficient, dwarfed, male and female sterile and does not germinate. They showed that meiosis does not occur in *gib-2* flower buds and that degeneration of the tapetal layer followed by degeneration of cells in the microsporangia constitute some of the first observable differences between *gib-2* and wild-type flower bud development.

To characterize the aberrant development of *gib-1* flower buds, experiments were performed to determine (a) the size at which buds reach a block in development, (b) how long buds remain developmentally arrested, (c) to what developmental stage the block corresponds, and (d) what events occur after arrested buds are released from the block by GA_3 treatment.

MATERIALS AND METHODS

Plant Culture

Seeds of the *gib-1* mutant (in the background *Lycopersicon esculentum* cv Moneymaker) were obtained from M. Koornneef, Department of Genetics, Agricultural University, Wageningen, The Netherlands. Wild-type tomato *L. esculentum* cv Moneymaker seeds were from Thompson and Morgan, Jackson, NJ. Mutant seeds were germinated in 5×10^{-5} M GA_3 in Petri dishes and then transferred into 15-cm pots. Growth medium was a mix of one part soil: one part peat: one part perlite. Plants were grown either in a greenhouse or in growth chambers. Greenhouse temperatures during the summer were approximately 30°C during the day and 26°C at night. Growth chamber conditions were 24°C during the day and 18°C at night with a 16-h photoperiod. Chambers contained cool-white fluorescent tubes and incandescent lamps which emitted 400 $\mu E \cdot s^{-1} m^{-2}$ at plant level.

Gibberellin Treatments

Unless otherwise stated, GA_3 was applied directly to flower buds (50 ng/bud) in a solution that contained 50% ethanol,

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[2] Abbreviations: GA, gibberellin; PMC(s), pollen mother cell(s).

0.025% (v/v) Tween-20 (polyoxyethylene-sorbitan monolaurate), and 20 μg/mL GA_3 (Sigma).

Flower Development Study

To determine the relationship between flower bud length and developmental stage, buds were measured and then treated with a solution of GA_3. Individual buds were then marked with colored threads and monitored to determine the duration required to reach the petal reflex stage. The length of the bud was defined as the distance from the receptacle to the tip of the longest sepal. In both wild-type and mutant flower buds, length is a good indicator of chronological age (data not shown). Petal reflex was defined as the day that one petal became reflexed 90° from the axis of the pedicel. This developmental stage was used to score flower development because it is nondestructive and unambiguous. Petal reflex occurs approximately 1 d before anthesis.

Flower bud positions one through four on the cyme were used in these experiments. Bud position does not have a significant effect on the relationship between length and developmental stage for positions one to four (data not shown).

Microscopy

Iron-acetocarmin squashes were used to examine meiotic stages (14). Anthers were removed from flower buds, fixed overnight in 3:1 ethanol:acetic acid and then stored in 70% ethanol at 4°C. The contents of the microsporangia were squashed in iron-acetocarmin and viewed at ×1000 phase contrast with a Zeiss photomicroscope.

The procedure for sectioning was similar to that described by Nester and Zeevaart (19). Buds were fixed for 3 h in 4% (v/v) glutaraldehyde and rinsed in 50 mM Na_2HPO_4, pH 7.2. After dehydration by transfer through a graded ethanol series, buds were gradually infiltrated with L. R. White Resin (medium). The resin was cured in Beem capsules at 60°C for 24 h. Blocks were sectioned on a Sorvall Porter-Blum MT-2 ultramicrotome, and 1.5 μM sections were stained with either toluidine blue [2% (w/v) toluidine blue, 2% (w/v) borate] or analine blue [0.05% (w/v) analine blue, 0.067 M Na_2HPO_4, pH 8.5].

Cytophotometry

To determine the DNA content of individual cells of the anther, 1.5 μm sections were made from appropriately staged anthers and then sequentially stained with mythramycin A and Hoechst 33258. Sections were first stained for 30 min in 0.1 mg/mL mythramycin A, 45 mM $MgCl_2$, 30 mM Na citrate, 20 mM MOPS, 0.1% (w/v) Triton-X100, pH 7.0, and then stained for 1 h in 2 μg/mL Hoechst 33258 and McIlvane's buffer (82 mM citric acid, 36 mM NaH_2PO_4). Sections were then rinsed and mounted in 23 mM $MgCl_2$, 15 mM Na citrate, 10 mM MOPS, 0.05% Triton-X100, 41 mM citric acid, 18 mM NaH_2PO_4, pH 7.0. Fluorescence from the mythramycin A was used to position and focus on nuclei. This fluorescence was obtained by illuminating sections with a mercury lamp, Zeiss HBO 100 W/2, and an exciter-barrier filter set BP 400–440/LP 470 (12). Hoechst induced fluorescence was then obtained with the exciter-barrier filter set G 365/LP 420. Hoechst fluorescence was quantitated using a Zeiss Axioskop equipped with a Zeiss fluorescence cytophotometer.

Because the nuclear diameter of a given cell type depended on the developmental stage observed, fluorescence readings from sections of nuclei were converted to fluorescence per whole nucleus. To accomplish this, the single largest and brightest, isolated nucleus from each microsporangium was chosen for fluorescence quantitations. If it is assumed that nuclei of each cell type are homogeneous in size and shape at each stage examined, the number of nuclei in a microsporangium section is such that there is a 95% probability that the diameter of the nuclear section chosen is within 6% of the true nuclear diameter of that population. Individual fluorescence readings were then converted to whole nucleus readings by dividing by the ratio of the volume of the nuclear section to the total volume of the nucleus ($N = S/(r^2t - t^3/12)/4r^3/3$, where N = whole nucleus fluorescence, S = section fluorescence reading, r = radius of nucleus measured with graduated ocular, and t = thickness of section). This conversion assumes that nuclei are approximately spherical and are homogeneously filled with DNA.

RESULTS

Specificity of GA Response

To determine whether other plant growth substances, in addition to GA, can cause normal flower development in the *gib-1* mutant, flower buds were treated with Ethephon, 2,4-D, naphthaleneacetic acid, indole-3-butyric acid, isopentenyl adenine, BA, kinetin, ABA, GA_3, ABA + GA_3, or Tween-20 alone. Buds were treated directly with 2.5 μL of a solution containing 0.025% Tween-20, 50% ethanol, and the appropriate hormone(s) at 20 μg/mL. Only buds treated with GA_3 or GA_3 + ABA developed normally. In a second experiment, whole plants were sprayed to run off with 0.025% Tween-20 and 5×10^{-5} M Ethephon, 2,4-D, kinetin, GA_3, ABA, GA_3 + ABA, or water. Again, normal flower development occurred only on GA_3 and GA_3 + ABA-treated plants. These results suggest that the block in flower development of the *gib-1* mutant is specifically relieved by GA.

Effect of Flower Age on GA Response

To determine when *gib-1* flower buds lose the ability to develop through anthesis after a single GA treatment, buds ranging in length from 0.2 to 6.3 mm were treated with 50 ng of GA_3, and then their development was monitored. Most buds shorter than 3.7 mm at the time of treatment developed normally (Fig. 1). However, <30% of buds longer than 3.7 mm at the time of treatment developed normally. Those that did not develop normally exhibited sepal elongation and a partial opening of the sepals and petals, but petals were pale yellow and remained reduced in size, and stamens were reduced and sterile. This loss of GA responsiveness was correlated with the appearance of a brown discoloration of the anthers.

Arrest of *gib-1* Flower Development in the Absence of Exogenous GA

At least two different developmental pathways can be envisioned for *gib-1* flower buds that develop in the absence of

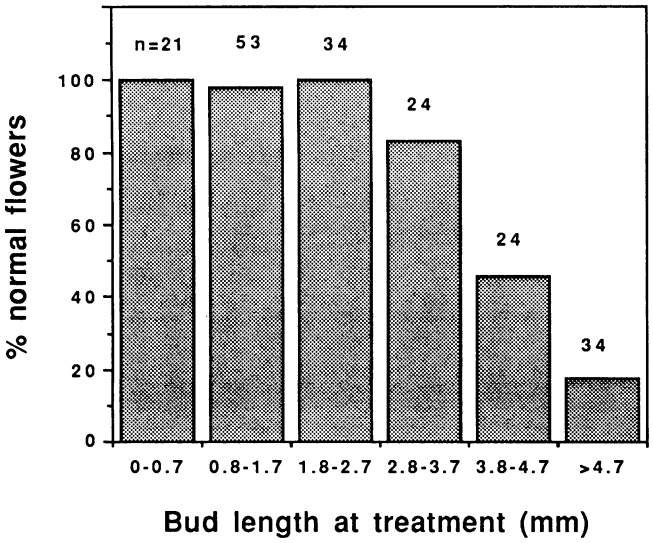

Figure 1. Relationship between bud length and percentage of *gib-1* tomato flower buds that develop normally after GA_3 treatment. Values above each bar, numbers of flowers observed in each size class.

GA. First, buds could reach the point at which GA is required and then in its absence quickly switch to a developmental pathway that results in a loss of responsiveness to GA and ultimately senescence or programmed death. Alternatively, buds could reach a block in development, caused by the absence of GA, and then remain arrested but GA responsive for some time before beginning senescence. In the latter case, GA treatment could rescue developmentally arrested buds to yield normal flowers.

To distinguish between these alternatives, *gib-1* flower buds of different lengths were treated with GA_3, and then, for those buds that developed normally, the number of days required to reach the stage of development petal reflex (see "Materials and Methods") was recorded. *gib-1* buds that are shorter than approximately 2.5 mm display a nearly linear negative relationship between the length of the bud at the time of treatment and the time required to reach the petal reflex stage (Fig. 2A). This suggests that these buds are progressing toward petal reflex and are, therefore, not yet developmentally arrested. Buds that are longer than 2.5 mm, however, take a similar number of days to reach petal reflex (Fig. 2A). This indicates that buds reach a developmental block but remain rescuable by GA_3 for some time after the initial arrest. Together with the data in Figure 1, these results indicate that *gib-1* buds remain developmentally arrested, but GA responsive, from a length of 2.5 to 3.7 mm. This developmental arrest is specific to *gib-1* flower buds. When nearly isogenic wild-type buds are treated in a similar manner, all buds display a negative correlation between bud length and the number of days required to reach petal reflex (Fig. 2B). This correlation exists in wild-type buds both in the presence and absence of applied GA_3.

Determination of the Stage at which *gib-1* Anthers Arrest

Microsporangia of developmentally arrested flower buds (2.5–3.7 mm in length) contain cells with interphase nuclei (Fig. 3A). Following treatment with GA_3, the first visible stage of prophase I of meiosis, synizesis (15), occurred 66 h after treatment (Fig. 3B). By 92 h after treatment, PMCs had undergone both meiotic divisions and were at the quartet stage (Fig. 3C). These data indicate that the *gib-1* mutant is similar to the *gib-2* mutant in that, in the absence of GA, development of anthers is blocked at some point before meiosis.

At the developmental arrest, microsporangia could contain either PMCs or primary sporogenous cells (the precursors to PMCs). To distinguish between these possibilities, we compared the number of cells in microsporangia of developmentally arrested anthers and meiotic *gib-1* anthers. Because we found the same number of cells in sections of both types of anthers (data not shown), and because we saw no mitotic figures in any *gib-1* anthers from buds longer that 2.5 mm, it is likely that developmentally arrested buds contain PMCs in premeiotic interphase.

To determine whether PMCs are arrested at the G1, S, or G2 phase of premeiotic interphase, we determined the DNA content of PMCs, anther wall cells, and tapetal cells in sections of arrested buds, using cytophotometry. There were no significant differences between the DNA contents of these cell types

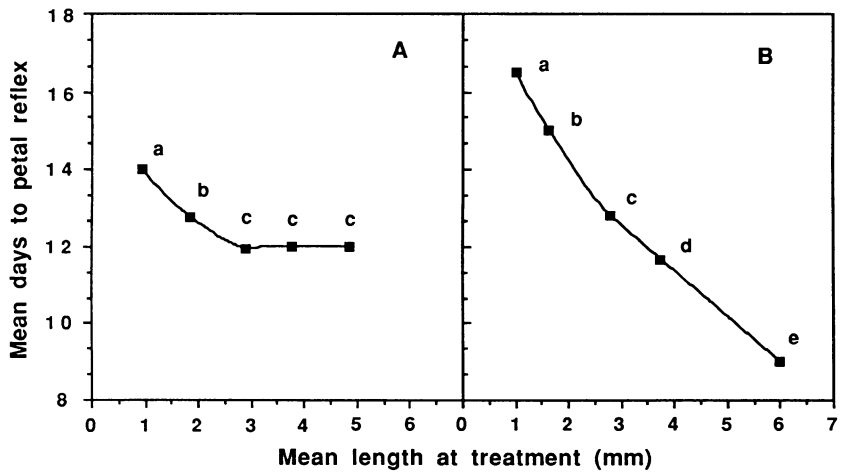

Figure 2. Relationship between flower bud length and the number of days required to reach petal reflex. A, *gib-1* tomato flower buds treated with GA_3; B, untreated wild-type buds. For both *gib-1* and wild-type, bud length was divided into five intervals: 0 to 1.3, 1.4 to 2.3, 2.4 to 3.3, 3.4 to 4.3, and longer than 4.4 mm. The mean number of days to reach petal reflex is graphed against the mean length in each interval. Within each panel, the days to petal reflex means are compared using analysis of variance and Fisher protected least significant difference test. Different letters, 95% probability that these means are not statistically identical.

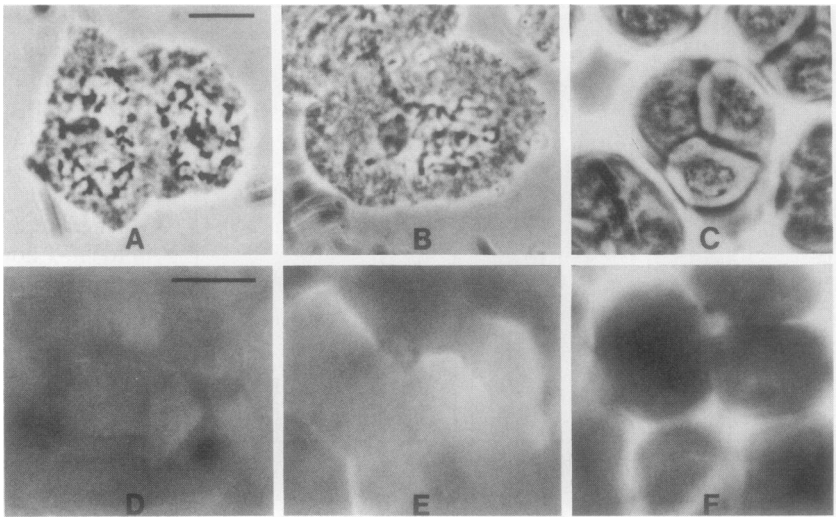

Figure 3. Staining of DNA and callose in *gib-1* tomato anthers. Acetocarmin squashes were used to determine the stage of development of PMCs in developmentally arrested buds (A) or *gib-1* buds treated with GA_3 for 66 h (B) or 92 h (C). Analine blue-induced fluorescence is used to assay for the presence of callose in arrested buds (D) or in buds treated with GA_3 for 48 h (E) or 72 h (F). Bars: A, 10 μM; D, 10 μM. A, B, and C are at the same magnification; D, E, and F are at the same magnification.

at the developmental arrest (Table I). Because in other species the outer tapetum (that portion of the tapetum facing the epidermis of the anther) has a DNA content of 2 C at this stage (13, 16, 23), these data suggest that arrested PMCs have a 2 C DNA content and are therefore in G1. We also determined the DNA content of nuclei in sections of *gib-1* anthers at prophase I of meiosis and at the quartet stage. These stages only occur in GA_3-treated flower buds. The DNA content of developmentally arrested PMCs was significantly different from both meiotic PMCs and young microspores (Table II). If we assume that PMCs in prophase I have a DNA content of 4 C, we can compare fluorescence values to approximate the C value of cells at other stages. When this is done for young microspores a value of 0.9 C is obtained (Table II). Because young microspores have a DNA content of 1 C, this result indicates that the method used here can provide an accurate estimation of the relative DNA content of different cell populations. When the DNA content of arrested PMCs is determined by this method, a value of approximately 2 C is obtained. This again suggests that PMCs are in G1 of premeiotic interphase, but our results do not eliminate the possibility that cells are in early S phase. We also wanted to determine a posttreatment time course for premeiotic DNA synthesis. Therefore, anthers from arrested buds were compared with anthers from arrested buds that had been treated with GA_3 for 2 d. At day 2 after treatment there was a significant increase in DNA content of PMCs (Table I), indicating that the onset of S has occurred by 48 h posttreatment.

The tapetum also exhibits an interesting developmental pattern after arrested buds are treated with GA_3. In tomato, the tapetum is uniseriate and differentiates into an inner tapetum, facing the anther connective tissue, and an outer tapetum, facing the epidermis. A study of the tapetum of wild-type tomato (3) has shown that, early in development, tapetum cells contain a single diploid nucleus. These cells undergo a mitosis that is not followed by cytokinesis (acytokinetic mitosis), to become binucleate cells. After the binucleate state is established, each of the two nuclei then go through one, two, or three cycles of endomitosis, resulting in nuclei with DNA contents of 4, 8, or occasionally 16 C. We have found that at the developmental arrest the inner tapetum is binucleate, and the outer tapetum is uninucleate (Table III). This difference between inner and outer tapetum is not altogether surprising because the tapetum is thought to be of dual origin in most angiosperms (2). In many angiosperms, the

Table I. *Fluorescence of Nuclei in Sections of Anthers after Developmentally Arrested gib-1 Tomato Flower Buds Are Treated with GA_3 for 0, 2, or 4 d*

Fluorescence readings were corrected to fluorescence per whole nucleus (see "Materials and Methods"). C value was calculated by comparing to outer tapetum cells which were assumed to be 2 C. Different letters, 95% significance using analysis of variance and Fisher protected least significant difference test.

Cell Type	Day 0		Day 2		Day 4	
	Fluorescence ± SE	C Value	Fluorescence ± SE	C Value	Fluorescence ± SE	C Value
Inner tapetum	100 ± 14a	1.8	130 ± 10a, c	2.4	440 ± 70e	7.7
Outer tapetum	110 ± 12a	2.0	190 ± 15b, c	3.4	390 ± 38e	6.7
PMC	110 ± 13a	2.0	300 ± 28d	5.3		
Wall cells	120 ± 12a	2.1				

Table II. *Fluorescence from Nuclei in Sections of gib-1 Tomato Flower Buds at Different Stages of Development*

Fluorescence readings were corrected to fluorescence per whole nucleus (see "Materials and Methods"). C value was calculated by comparing to meiotic PMCs which were assumed to be 4 C. Different letters, 95% significance using analysis of variance and Fisher protected least significant difference test.

Stage	Fluorescence ± SE	C Value
Microspores	100 ± 7.7a	0.9
Arrested PMCs	260 ± 12b	2.2
Meiotic PMCs	470 ± 31c	4.0

outer tapetum arises from the parietal layer, and the inner tapetum arises from the connective tissue or less frequently from the sporogenous cells (6). Also, it has been reported that, in other members of the Solanaceae, the inner tapetum is normally more developed than the outer tapetum, and inner tapetum cells often reach a higher level of ploidy than outer tapetum cells (5). In the outer tapetum of *gib-1* anthers, many mitotic figures were observed at 66 h after GA_3 treatment, and by 77 h these cells became binucleate (Table III). The cytophotometric data in Table I show that there is an increase in DNA content of outer tapetum nuclei at 2 d after GA_3 treatment. Presumably, this increase is premitotic DNA synthesis. Interestingly, this occurs at the same time as premeiotic DNA synthesis in PMCs.

By 4 d after GA_3 treatment the DNA content of both inner and outer tapetum nuclei have significantly increased above 4 C (Table I). Also, endomitotic figures were observed in inner tapetal cells 66 h after GA_3 treatment (not shown). Together these data indicate that, by 4 d after GA_3 treatment, inner and outer tapetum nuclei undergo endomitosis to become polyploid.

Finally, we studied the accumulation of callose in *gib-1* anthers. Callose is a glucan that accumulates in the cell walls of PMCs during early stages of meiosis. The presence of callose can be assayed by using the fluorescent dye analine blue (22). To determine a time course for callose deposition, we compared analine blue-induced fluorescence from sections of arrested anthers and sections of anthers from buds that had been treated with GA_3 for 1, 2, or 3 d. We did not detect analine blue-induced fluorescence in PMCs of developmentally arrested buds (Fig. 3D) or in PMCs of buds treated with GA_3 for 1 d (not shown). In contrast, we observed significant fluorescence 2 d after GA_3 treatment (Fig. 3E) and intense fluorescence 3 d after treatment (Fig. 3F). This result indicates that accumulation of callose begins by 48 h after GA_3 treatment.

DISCUSSION

Our results suggest that *gib-1* flower bud development can be divided into three stages. Stage 1 buds (shorter than 2.5 mm) are developing (progressing toward petal reflex); stage 2 buds (2.5–3.7 mm) are developmentally arrested and most (approximately 90%) of these are still able to respond to GA_3; finally, stage 3 buds (longer than 3.7 mm) exhibit a growth response following GA_3 application (mostly sepal elongation), but most do not develop normal anthers. The simplest model to explain these observations is that buds reach a developmental block at 2.5 mm, and then the bud continues to increase in length even though normal development is arrested. Buds remain arrested and GA responsive until they reach a length of 3.7 mm. Following the application of GA_3 to arrested buds, development resumes. Because stage 2 buds are approximately 2.5 to 3.7 mm in length, and because the growth rate of untreated *gib-1* buds is approximately 0.1 mm/d (not shown), buds remain developmentally arrested but responsive to GA_3 for approximately 12 d.

Cytophotometric data indicate that all cells of the developmentally arrested anther have a DNA content of 2 C. Two days following treatment with GA_3, PMCs go through premeiotic DNA synthesis, and outer tapetal nuclei undergo premitotic DNA synthesis. At 66 h after treatment, PMCs are in prophase I of meiosis, the outer tapetum is undergoing a mitotic division to become binucleate and the inner tapetum is beginning endomitotic divisions. Finally, by 96 h after treatment, PMCs have undergone both divisions of meiosis and are at the quartet stage, and inner and outer tapetum cells are binucleate and polyploid.

The observation that, in the absence of exogenous GA, *gib-1* flower bud development arrests before premeiotic DNA synthesis suggests that GA can be thought of as one of the components controlling the transition from mitosis to meiosis. This role for GA in initiation of meiosis is consistent with the observations that GA can overcome flower bud dormancy in *Soldanella minima*, *Saxifraga exarata*, and *Coffea arabica* and that dormant buds of these species are arrested at a premeiotic stage (9). Also, when dormancy in *C. arabica* buds is released by rainfall or irrigation, there is an increase in extractable GA before the rapid increase in the fresh weight of the buds, suggesting a causal relationship between GA levels and the resumption of active growth (4). In tomato, low light-induced flower abortion occurs when anthers are at the PMC stage, and GA plus cytokinin can partially prevent this abortion (8). This again suggests that GA is important for the progression of flower development past this stage. It is also interesting that, in the *gib-1* mutant, observable changes in tapetum development begin at approximately the same time as premeiotic DNA synthesis and only occur after GA_3 treatment. These results suggest either that GA may play a direct role in tapetum and/or PMC development or that the development of these cells is affected by developmental changes occurring in other tissues following GA treatment.

Table III. *Proportion of Binucleate Tapetum Cells Found after GA Treatment of Arrested gib-1 Tomato Flower Buds*

Ratios, number of binucleate cells to the total number of cells observed. Because tapetum cells were observed in thin sections of anthers, the data most likely underestimate the fraction of cells that are binucleate.

	Untreated	48 h	66 h	77 h
Inner tapetum	39/113	12/49	58/166	21/71
Outer tapetum	0/123	0/75	19/140[a]	47/156

[a] In addition to the 19 cells that appeared to be binucleate, there were another 19 cells at this stage that contained mitotic figures.

Although the biology of the *gib-1* and *gib-2* mutants clearly demonstrates that GA is required for meiosis to occur in tomato, this requirement may not be a general phenomenon. GA-deficient mutants in *Zea mays* and *Arabidopsis thaliana* that are male sterile in the absence of applied GA are not blocked before meiosis. Preliminary observations of the *d5* mutant of *Z. mays* and the *ga1* mutant of *A. thaliana* indicate that pollen forms in anthers of these mutants but is not released (data not shown).

We have observed that, under growth chamber conditions, *gib-1* flower buds treated with GA_3 develop synchronously (all anthers in a bud contain PMCs at the same stage of meiosis). We do not believe, however, that this synchrony is caused by the developmental arrest phenotype of *gib-1* buds. Buds treated with GA_3 before the developmental arrest (shorter than 2.5 mm) also exhibit synchronous anther development. In addition, when plants are grown at higher temperatures and higher light intensity in a greenhouse during the summer, arrested buds treated with GA_3 develop asynchronously (a range of meiotic stages in one bud). Finally, wild-type anthers, which normally develop asynchronously, will develop synchronously if plants are grown in dimly lit growth chambers. These observations suggest that the establishment of synchrony or asynchrony depends on plant growth conditions.

The fact that synchrony occurs under certain conditions is of some interest. Synchronous development of PMCs in treated *gib-1* flower buds makes an excellent system to study meiotic stages or other meiosis-related phenomena. The most attractive property of this system is that, when developmentally arrested buds are treated with GA_3, synchrony not only occurs within a single bud but also occurs within a population of buds that are treated simultaneously. We have taken advantage of this developmental synchrony and confirmed that the meiotic sequence proposed for tomato PMCs (15) occurs in *gib-1* buds (data not shown).

The *gib-1* mutant may also prove to be an interesting system to study changes in gene expression that occur after arrested buds are treated with GA. Synchronous development of anthers, and, therefore, synchronous gene expression, may allow detection of transiently expressed low abundant mRNAs that are important in flower development. Furthermore, this system may be useful for isolating genes whose expression is affected by GA and whose gene products are involved in the release of the developmental arrest.

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