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A Gibberellin-Deficient *Brassica* Mutant—*rosette*[1]

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ABSTRACT

A single-gene mutant (*rosette* [*ros*/*ros*]) in which shoot growth and development are inhibited was identified from a rapid cycling line of *Brassica rapa* (syn *campestris*). Relative to normal plants, the mutant germinated slowly, had delayed or incomplete floral development, and reduced leaf, petiole, and internode growth. The exogenous application of GA_3 by foliar spray or directly to the shoot tip of *rosette* resulted in rapid flowering, bolting (shoot elongation), and viable seed production. Shoots of *rosette* contained endogenous levels of total gibberellin (GA)-like substances ('Tan-ginbozu' dwarf rice assay) of about one-tenth of that of the normal rapid-cycling line of *B. rapa* which consisted almost entirely of a very nonpolar, GA-like substance which yielded GA_1 and GA_3 upon mild acid hydrolysis. In a normal rapid-cycling *B. rapa* line, the nonpolar putative GA_1 and GA_3 conjugates were present, but additionally, free GA_1 and GA_3 were abundant and identified by gas chromatography-mass spectrometry-selected ion monitoring. The quantities of free GA_1 and GA_3 in the normal line and in *rosette* were quantified by GC-MS-SIM using $[^2H_2]GA_1$ as an internal standard. Fourteen-day-old *rosette* and normal seedlings contained 5.3 and 23.2 ng GA_1 per plant, respectively. At day 21 the *rosette* plants contained 7.7 and 26.1 nanograms per plant of GA_1 and GA_3, while normal plants contained 31.1 and 251.5 nanograms per plant, respectively. Thus, normal plants contained from four to ten times higher levels of total GA-like substances, GA_1, or GA_3, than *rosette*. The *ros* allele results in reduced GA level, yielding the *rosette* phenotype whose delayed germination and flowering, and reduced shoot growth responses indicate a probable role for endogenous GA_1 and GA_3 in the regulation of these processes in *Brassica*.

There are three general experimental approaches that are used to investigate phytohormone physiology. First, and most simply, the exogenous application of the hormone may influence a given process. Exogenous GAs often have a profound effect on higher plants, generally promoting shoot elongation and often altering reproductive development (14). Second, correlations between endogenous levels of the hormone and specific physiological processes are investigated. Third, the biosynthesis, distribution, or action of the hormone can often be manipulated by applying chemicals which interfere with these processes. However, these agents are seldom entirely specific and, hence, other metabolic pathways may be influenced. For example, numerous growth retardant type plant growth regulators have been developed which block kaurene cyclization or other steps leading to GA biosynthesis (17). However, these also frequently block cyclization of other terpenoids and hence, alter levels of sterols and possibly other compounds (2).

A more specific block of GA biosynthesis results from certain single gene mutations (6, 13, 16, 23). These mutations appear to influence only specific steps in GA biosynthesis. and hence complete recovery of the phenotype can result from the application of GA (15). These specific GA-deficient maize, pea, and rice dwarf mutants have been valuable for elucidating the pivotal role of endogenous GA_1 in the regulation of shoot elongation (6, 9, 13, 16, 23). Further, other pleiotropic effects of reduced GA level, such as altered influorescence sexuality in maize (21), are indicated.

A clarification of the role(s) of endogenous GAs and specifically the C-3,13 dihydroxylated GAs, GA_1 and GA_3, in other plants will similarly benefit from the identification and study of GA-deficient mutants. In the present paper, we describe a dwarf mutant of *Brassica rapa* (25, 26) which responds to exogenous GA_3, and quantitatively compare the endogenous GAs of the mutant with those in a normal line using a combination of bioassay and GC-MS-SIM.[2]

MATERIALS AND METHODS

Plant Material

The study involved a normal, rapid-cycling line of *Brassica rapa* (syn *campestris*) (designation: CrGC 51 [formerly CrGC No. 1] base population) and a mutant which was selected from a cross of two accessions of the United States Plant Introduction Service made by P. H. Williams and B. J. Cours (PI 183395 × PI 175079) (designation: CrGC 1-7 [formerly CrGC No. 63] *rosette, ros/ros* [7]). Seed of the two lines was grown in a controlled environment chamber (Conviron 123L, Winnipeg, Manitoba) at 25°C/15°C (day/night), with a RH of 40%. Lighting was provided by cool-white and warm-white fluorescent tubes which emitted 28 μmol s^{-1} m^{-2} PAR as determined with a Li-Cor quantum sensor (Li-Cor Inc., Lincoln, NE). Plants were grown in a 4 × 4 × 11 cm root trainers filled with Metro-mix (W. R. Grace & Co., Ajax, Ontario), a soilless, peat-lite medium, and were fertilized weekly with 0.1

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[2] Abbreviations: GC-MS-SIM, gas chromatography-mass spectrometry-selected ion monitoring; CrGC, Crucifer Genetics Cooperative; EtOAc, ethyl acetate; MeOH, methanol; MeTMSi, methyl ester trimethylsilyl ether; Rt, retention time; SiO_2, silicic acid; a.m.u. atomic mass units.

g 28-14-14 (N-P-K) with added micronutrients (Plant Prod 28-14-14, Plant Products Co. Ltd., Bramalea, Ontario). Containers were watered daily to saturation throughout all experiments.

Response to Exogenous GA_3

Plants of the *rosette* line were grown as described above, and at d 14 after planting 100 ng GA_3 (Fisher, NJ, No. 0-2944) in 10 μL 9.5% ethanol was applied with a microsyringe to each shoot tip of 20 plants. Measurements of plant height to the shoot tip and growth stage (4) were recorded daily.

Table I. *Development of B. rapa Plants and the Mutant rosette*

Developmental Stage (growth stage[a])	Normal	*rosette*
	Days after planting	
Seedling emergence	2	5
Bud (3.1) floral buds visible	12–15	22–28
Flowers open (4.1) anthesis	20–23	>40
Pods present (5.1) (ripening)	26–28	>40

[a] As outlined by Harper and Berkenkamp (4).

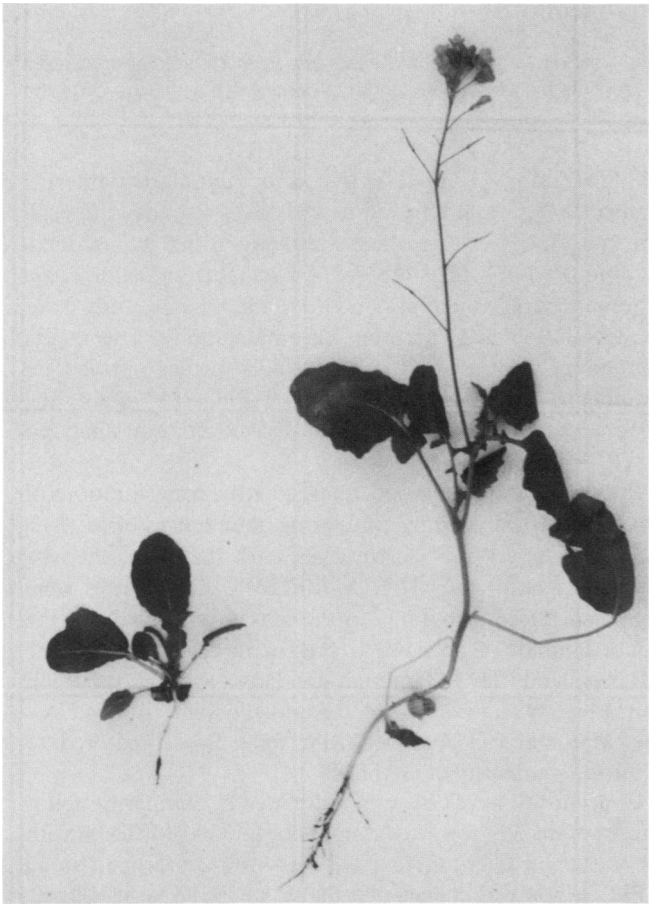

Figure 1. Plants of a normal, rapid cycling line of *B. rapa* (right), and the mutant, *rosette* (left) 28 d after sowing. The background grid is 10 × 10 cm.

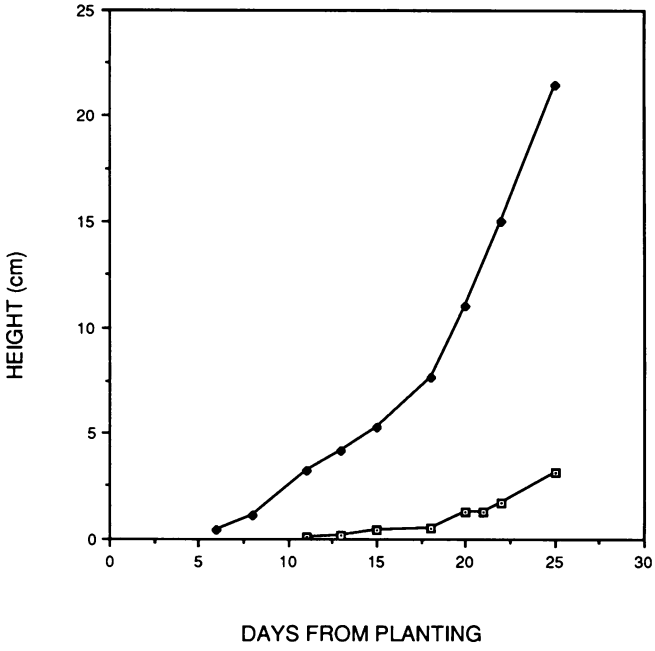

Figure 2. Height to the shoot tip of normal rapid cycling *B. rapa* (♦) and the mutant, *rosette* (□). As an index of variability, standard error values at d 20 were 6 and 1 cm, respectively, for 20 plants.

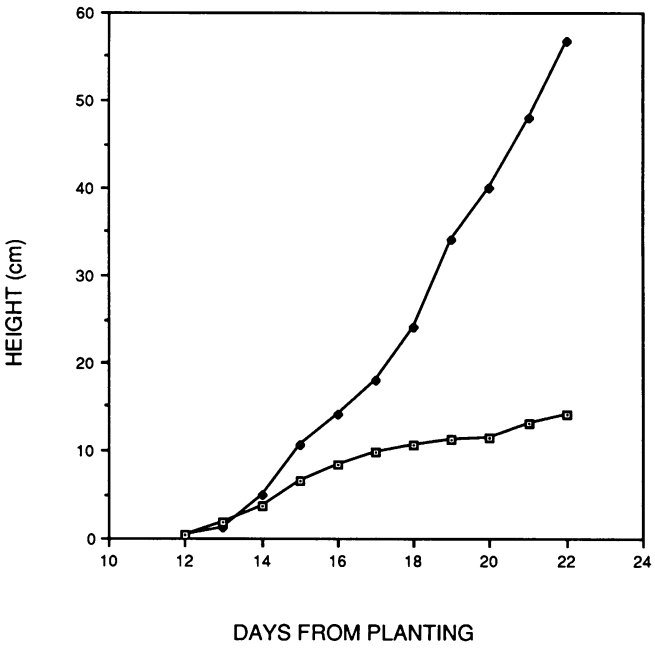

Figure 3. Influence of exogenous GA_3 on height of the *B. rapa* mutant, *rosette*. (□), Control; (♦), GA_3-treated. Standard error values at d 20 were 2 and 3 cm, respectively.

Endogenous GA-like Substances

Plants were grown as described above and at 14 and 21 d after sowing, shoots were harvested for analysis of endogenous GA-like substances as previously described (19, 21). Briefly, shoots from 10 plants were ground in cold 80% aqueous MeOH before vacuum filtration. The MeOH was removed *in vacuo* at 35°C after pH 8.0 phosphate buffer had been added. The aqueous fraction was not slurried with polyvinylpoly-

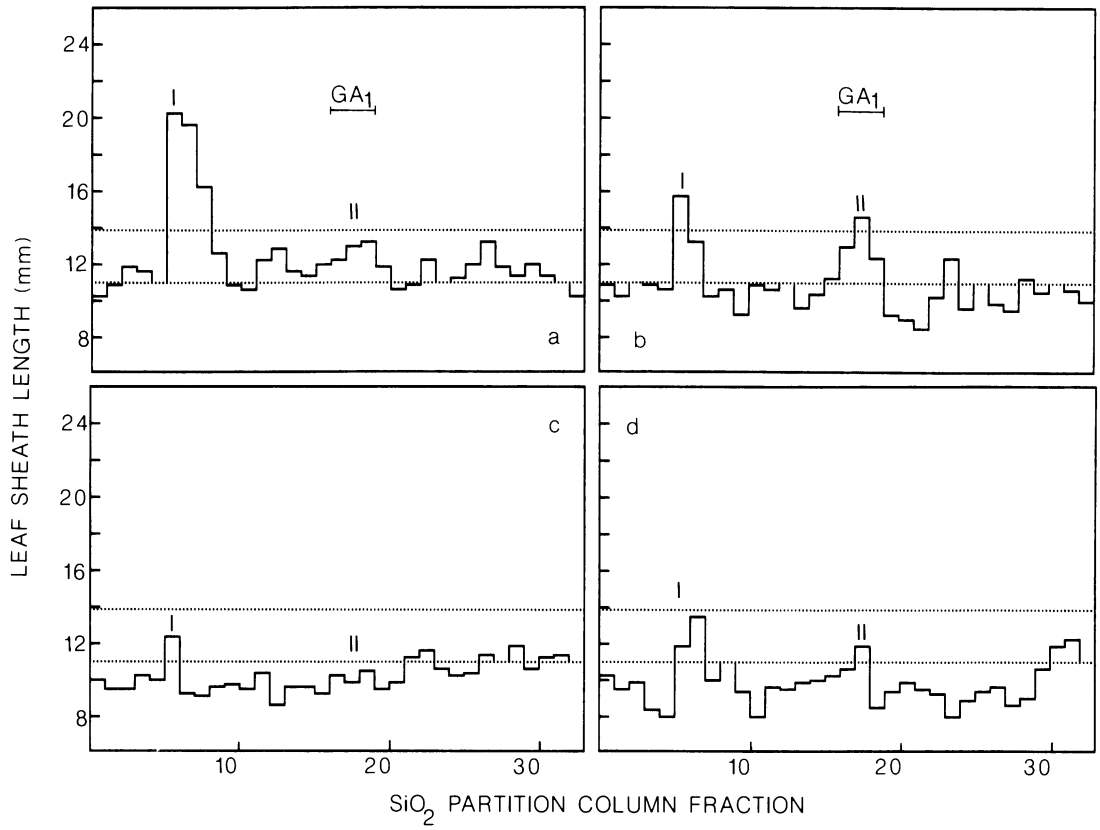

Figure 4. Profiles of GA-like substances from step-eluted SiO_2 partition columns loaded with two replicate extracts from 21-d-old normal, rapid cycling *B. rapa* (a, b) and the mutant, *rosette* (c, d). The lower dashed line represents the leaf sheath length of control rice seedlings while the upper dashed line represents the response to 100 pg GA_3 per rice seedling.

pyrrolidone as is often performed on GA extracts.) The pH of the aqueous residue was reduced to 3.0 with HCl and GAs were extracted three times with water-saturated EtOAc. Water was removed from the EtOAc by freezing and filtration of the ice and the EtOAc was then removed *in vacuo* at 35°C.

The acidic EtOAc-soluble residue was dissolved in 1:1 (EtOAc:MeOH) and loaded onto glass fiber filter discs prior to step-elution SiO_2 partition columns (3, 22). Thirty-three fractions were collected and each was bioassayed at 1/50 and 1/100 dilutions using the 'Tan-ginbozu' dwarf rice microdrop assay (12). Fractions from SiO_2 were dried before bioassay by one of two method: rapid drying under a N_2 stream, or slow air-drying by evaporation in a fume hood.

Bioactive regions from SiO_2 were grouped, then chromatographed on reversed-phase C_{18} HPLC (8). Each HPLC fraction was then bioassayed as above.

Bioactive regions from HPLC were methylated and subsequently silylated to produce the MeTMSi derivative (18). Derivatized extracts were analyzed by GC-MS and GC-MS-SIM as previously described (18, 20). Two regions from the HPLC were analyzed, one from Rt 43 and 44 min which was analyzed by full scan mass spectrometry, and the second of fractions at Rt 28 and 29 min which was analyzed by GC-MS-SIM with monitoring specifically for ions characteristic of GA_2-MeTMSi and GA_3-MeTMSi.

Quantitative Analysis of GA_1 by GC-MS-SIM

Quantities of endogenous of GA_1 and GA_3 in extracts from the normal and *rosette B. rapa* lines were determined by GC-MS-SIM using [17,17-$^2H_2$]GA_1 as a quantitative internal standard (18, 19). Briefly, 50 ng [$^2H_2$]GA_1 was added to each aqueous MeOH extract which was then purified as described. Additionally, 167 Bq [^{3}H]GA_1 (Amersham) was added as a chromatographic internal standard to the glass fiber disc prior to step-elution SiO_2 partition chromatography. The region containing the [^{3}H] was collected and further purified on gradient eluted reversed-phase C_{18} HPLC (8). Again, the region containing [^{3}H] was dried, derivatized, and analyzed by GC-MS-SIM (18, 19).

Endogenous amounts and, consequently, concentrations of GA_1 were determined by comparing abundances of the M+ ion (506 a.m.u.) for GA_1-MeTMSi with that from the M+ ion (508 a.m.u.) for [$^2H_2$]GA_1-MeTMSi. Abundances were corrected for contributions to the 506 a.m.u. ion from the minor amount of protio GA_1 present in accompanying the [$^2H_2$]GA_1 and [^{3}H]GA_1 internal standards (18). Two other ion pairs (491, 493 a.m.u.; 448, 450 a.m.u.) characteristic of GA_1-MeTMSi and [$^2H_2$]GA_1-MeTMSi were also monitored to confirm the identity of GA_1 (18).

Concentrations of GA_3 were estimated by comparing abundances of the M+ ion (504 a.m.u. ion) for GA_3-MeTMSi with the M+ ion (508 a.m.u.) for [$^2H_2$]GA_1-MeTMSi. Since a broad HPLC region was collected at the Rt of [^{3}H]GA_1, it is likely that only minor losses of GA_3 occurred, and further, it is also probable that the losses were similar in *rosette* and normal *B. rapa* extracts.

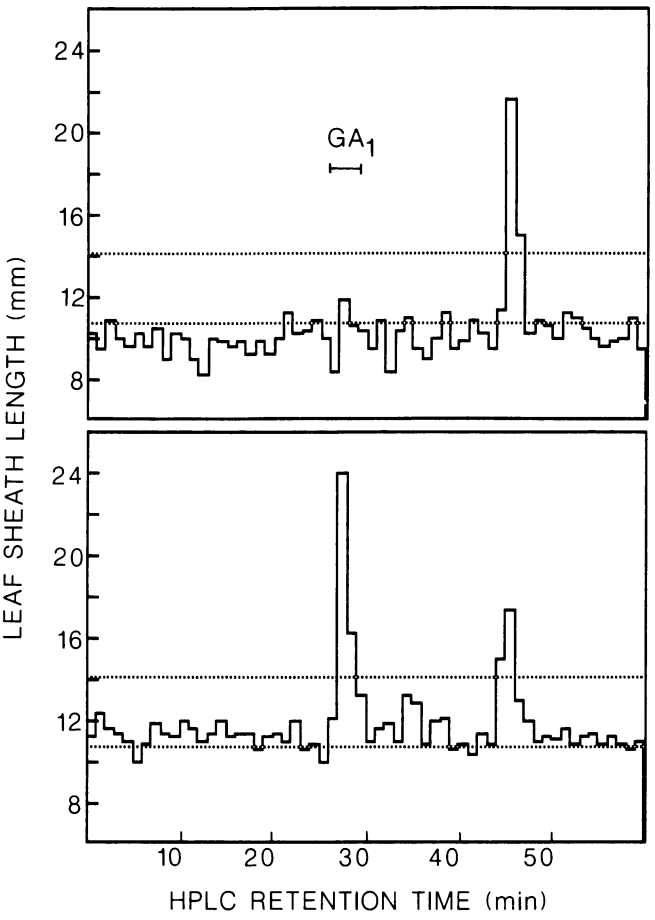

Figure 5. Profiles of GA-like substances from gradient-eluted reversed phase C_{18} HPLC column loaded with SiO_2 fractions 5 and 6 (Fig. 4) from rapid cycling *B. rapa*. The top profile resulted from HPLC analysis after the SiO_2 region was rapidly dried under N_2, while the lower plot resulted from a similar SiO_2 region which was air dried in the presence of formic acid. The lower dashed line represents the leaf sheath length of control rice seedlings while the upper dashed line represents the response to 100 pg GA_3 per rice seedling.

RESULTS AND DISCUSSION

Germination and seedling emergence of *rosette* was slower than that of the normal rapid cycling line (Table I) and some seeds fail to germinate (25). However, imbibition in a GA_3-containing solution overcomes this 'dormancy,' and also accelerates germination of slowly germinating *rosette* seeds (25). Exposure to light, particularly red light, or scarification can also accelerate germination in *rosette* (25). These results are all consistent with a requirement for GA for germination in *Brassica*, and GA is probably involved in the regulation of dormancy and germination of seeds of many other plants including photoblastic (light-requiring) seeds (1).

Morphologically, *rosette* plants were clearly distinguishable from the normal, base population of rapid cycling *B. rapa* (Figs. 1 and 2). The *rosette* plants were shorter, had smaller leaves, and delayed floral development (Table I). In different experiments, shoot size and rate and extent of floral development of *rosette* varied somewhat. It is possible that this variation, as well as the observed variable seed germination, resulted from the seeds containing variable amounts of residual GA_3 which had been exogenously applied to maternal plants to enable seed production.

The application of GA_3 induced bolting (rapid stem elongation) and accelerated flowering in *rosette* seedlings (Fig. 3). Increased elongation was observed within 24 h and anthesis in GA_3-treated *rosette* plants occurred by d 9. The GA_3-treated *rosette* plants were fertilized with pollen from siblings, and viable seeds were collected in this manner (25).

From the normal, rapid cycling line of *B. rapa*, two principal regions of GA-like activity eluted from SiO_2 gel partition columns (Fig. 4, a and b). The first (fractions 5 and 6) eluted prior to $[^3H]GA_{20}$. Very nonpolar GAs (3), such as GA_9, GA_{12}, and GA_{12}-aldehyde elute here.

Analysis of this nonpolar bioactive region from SiO_2 by reversed-phase HPLC confirmed the nonpolar nature of the GA-like substance(s) as it eluted well after authentic $[^3H]GA_{20}$ (Fig. 5, top). This peak from HPLC was derivatized and analyzed by full scan GC-MS but no known GA was detected. Specific SIM scans also failed to detect GA_9, GA_{12}, GA_{12}-aldehyde, GA_{15}, or GA_{15}-aldehyde. Parallel samples were exposed to mild acid treatment by slow air-drying the SiO_2 column fractions in the presence of the formic acid used to saturate EtOAc and hexane eluants. Subsequently, the nonpolar region was resolved into two peaks by HPLC (Fig. 5, bottom). The first peak contained GA_1 and GA_3 as identified by GC-MS (data not presented, similar to Table II). Thus, the nonpolar bioactivity was apparently composed of $GA_{1,3}$ con-

Table II. *Identification of Endogenous GA_1 and GA_3 from Normal, Rapid Cycling B. rapa based on Chromatography of GA-like Activity and Analysis of MeTMSi Derivatives by Gas Chromatography-Selected Ion Monitoring*

	SiO_2 Fraction	HPLC Rt	GC Rt	Constituent Ions (percentage)		
		min				
Authentic GA_1	18	28	20.41	506 (100) 377 (16)	491 (11) 313 (17)	448 (21)
Putative GA_1	18	28–29	20.41	506 (100) 377 (20)	491 (10) 313 (21)	448 (20)
Authentic GA_3	18	28	20.73	504 (100) 370 (14)	489 (8) 208 (104)	445 (7)
Putative GA_3	18	28–29	20.73	504 (100) 370 (27)	489 (8) 208 (98)	445 (9)

Table III. *Endogenous 'Free' GA_1[a] and GA_3 Concentration and Amount in 14 and 21 Day Old Seedlings of Rapid-Cycling B. rapa (CrCG No. 1) and Mutant rosette (CrGC No. 63)*

	Normal	*rosette*	Ratio Normal/ *rosette*
Day 14			
Wet weight per shoot (g)	1.71	0.63	
GA_1 concentration (ng/g wet wt)	13.57	8.40	1.6
Amount GA_1 per plant (ng)	23.20	5.29	4.4
Day 21			
Wet weight per shoot	1.20	0.51	
GA_1 concentration	25.9	15.1	1.7
Amount GA_1 per plant	31.1	7.7	4.0
GA_3 concentration	209.4	51.1	4.1
Amount GA_3 per plant	251.5	26.1	9.6

[a] Determined by GC-MS-SIM using [17,17-^{2}H]GA_1 as a quantitative internal standard.

jugates, but they were not conjugates of a glucosyl nature (8). Further analysis of their nature is continuing.

The second region of GA-like activity from the SiO_2 columns eluted coincidentially with [^{3}H]GA_1 (Fig. 3). Analysis by C_{18} HPLC yielded a single zone of bioactivity coincidential with free [^{3}H]GA_1 (data not presented). These fractions were derivatized and GA_1 and GA_3 were identified by GC-MS-SIM (Table II).

Whereas the normal, rapid-cycling *B. rapa* line contained both the nonpolar putative conjugate(s) of GA_1 and GA_3, the mutant *rosette* usually contained only low levels of the nonpolar precursor-type GA-like region and no detectable free GA_1-like or GA_3-like activity (Fig. 4, c and d). The total amount of concentration of GA-like activity was always lower in *rosette* than in the normal rapid-cycling line (Fig. 4, a and b *versus* 4, c and d). Three separate experiments involving two harvests each (ranging from 7–21 d after seeding) indicated that, on the average, the level of endogenous GA-like substances per plant in *rosette* was about one-tenth of that in the normal line (Fig. 4, and data not presented). Comparisons of concentrations of GA-like activity (*i.e.* activity per unit dry weight) reduced the difference to about one-quarter.

The relative deficiency of GA_1 in *rosette* shoots was confirmed by quantitative analysis of endogenous free GA_1 and GA_3 by GC-MS-SIM using [$^2H_2$]GA_1 as a quantitative internal standard (Table III). At d 14 the normal line contained about 4.4 times the level of free GA_1 per plant as *rosette* although scans for GA_3 were not performed. In a separate experiment at d 21 the normal line contained 4.0 times the level of GA_1 and 9.6 times the level of GA_3 per plant as did *rosette*. Thus, both bioassay estimates and quantitative analyses of GA_1 and GA_3 by GC-MS-SIM are consistent in demonstrating that *rosette* is a GA_1- and GA_3-deficient dwarf mutant.

The observation that a GA_1 and GA_3 deficiency leads to the *rosette* phenotype suggests that C-3,13 dihydroxylated GAs play a regulatory role for stem elongation in *Brassica*, as is the case in the control of shoot elongation of maize, peas, and rice by GA_1 (5, 6, 13, 16, 23). Further, the observation that flowering as well as stem elongation is inhibited in the mutant *rosette*, provides strong evidence that endogenous GAs, and possibly GA_1 and GA_3, play a regulatory role in the control of floral development of *Brassica rapa*. This conclusion is consistent with our earlier work where both stem elongation and flowering in the *B. napus* were correlated with changes of endogenous GA-like substances and endogenous GA_1 (19). This relationship is further supported by the observation that the application of plant growth regulators that are known to block GA biosynthesis prevent stem elongation and also inhibit, delay, or completely prevent flowering in *Brassica* (19). Also, in biennial *Brassica* genotypes, exogenous GA_3 promotes both stem elongation and flowering (10, 24, 27), and increases in endogenous GA-like activity have been found to follow a cold treatment (vernalization) that induces bolting and flowering (11, 28). Collectively, these results provide evidence that endogenous GAs control both shoot elongation and floral development in *Brassica*. Finally, the recognition of this GA-deficient mutant provides a valuable genotype for further molecular and biochemical studies of GA physiology in *Brassica*.

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