Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7401–7405, August 1993
Plant Biology

Gibberellin A_1 is required for stem elongation in spinach

(growth retardant/gas chromatography–mass spectrometry/photoperiod/quantification)

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Communicated by Bernard O. Phinney, April 20, 1993 (received for review February 10, 1993)

ABSTRACT **The effects of the growth retardants 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate (AMO-1618) and calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate (BX-112) on stem elongation were investigated in the rosette plant spinach (*Spinacia oleracea* L.) under long-day (LD) conditions. Stem growth induced by a LD treatment was prevented by both retardants. The inhibition caused by AMO-1618 was reversed by gibberellin A_1 (GA_1) and GA_{20}, whereas the effects of BX-112 were reversed by GA_1 only. Six GAs (GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, and GA_8) were quantified by gas chromatography-selected ion monitoring using internal standards. Plants treated with BX-112 had reduced levels of GA_1 and GA_8 and accumulated GA_{53}, GA_{44}, GA_{19}, and GA_{20}. The relative levels of four additional GAs (3-epi-GA_1, GA_{29}, GA_{60}, and GA_{81}) were compared by ion intensities only. Relative to GA_{81}, the level of GA_{29} was decreased by BX-112, whereas the levels of GA_{60} and 3-epi-GA_1 were increased. Transfer of spinach from short-day conditions to LD conditions caused an increase in all identified GAs of the early 13-hydroxylation pathway with GA_{20}, GA_1, and GA_8 showing the largest increases. These findings support the position that, of the GAs belonging to the early 13-hydroxylation pathway, GA_1 is the primary GA active *per se* for stem elongation in spinach. The increase in endogenous GA_1 in plants in LD conditions is most likely the primary factor for stem elongation.**

Long-day (LD) rosette plants, such as spinach, grow vegetatively and do not produce stems under short-day (SD) conditions. Upon transfer to LD conditions, the stems elongate and flower formation takes place. LD rosette plants represent, therefore, a class of plants that are physiological dwarfs during the early stages of their life cycle. There is considerable evidence that photoperiodic control of stem elongation in rosette plants is mediated by gibberellins (GAs). Just as GA can restore the normal phenotype in GA-deficient genetic dwarfs (1), so can application of GA to rosette plants under SD conditions overcome the physiological block and cause stem growth (2). Conversely, treatment with inhibitors of GA biosynthesis suppresses stem elongation under LD (3–5). The promotive effect of LD on stem growth appears to be due to enhanced GA biosynthesis (6), which results in an increase in the levels of endogenous GAs (3, 7).

In many higher plants, including spinach (8), the major endogenous GAs belong to the early 13-hydroxylation pathway (GA_{53}, GA_{44}, GA_{19}, GA_{17}, GA_{20}, GA_1, GA_{29}, and GA_8) (9). Mutations in maize (1), pea (10), and rice (11) block the 3β-hydroxylation step, GA_{20} to GA_1. These mutants have played a crucial role in establishing that, for the early 13-hydroxylation pathway, GA_1 is the major GA active *per se* for shoot elongation. The other GAs of the pathway are either precursors to GA_1 or inactive GA metabolites. Because no GA biosynthetic mutants are available in spinach, it has not been possible so far to determine conclusively which GA is intrinsically active. However, the growth retardant calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate (BX-112) and other acylcyclohexanediones are known to inhibit the 3β-hydroxylation step, GA_{20} to GA_1 (12, 13). Also, the growth retardant 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate (AMO-1618), a quaternary ammonium carbamate, inhibits *ent*-kaurene synthetase A, a step early in the pathway (9). We have used these two chemicals to determine whether one or more GAs are active *per se* in spinach. The effects of BX-112 and of photoperiod on the endogenous levels of the GAs are also presented.

MATERIALS AND METHODS

Plant Material. Spinach (*Spinacia oleracea* L., Savoy Hybrid; Harris Seed Co., Rochester, NY) was grown as described (6). SD conditions consisted of 8 hr of light from fluorescent tubes and incandescent bulbs (450 μmol·m^{-2}·s^{-1}) at 23°C, followed by 16 hr of darkness at 20°C. For LD conditions, the 8-hr main light period was followed by 16 hr of weak light from incandescent bulbs (12 μmol·m^{-2}·s^{-1}) at 20°C.

For GA analyses, the plants were cut below the rosette, and older leaves up to the just fully expanded leaf were removed. Plants were harvested at the end of the main light period. Quantitative GA analyses were performed on lots of five plants. Samples were frozen in liquid N_2, lyophilized, and stored at −20°C until analysis. The dry weight per plant was ≈1 g.

Application of Growth Retardants. AMO-1618 was dissolved in distilled water at a concentration of 5 mM. Ten milliliters of this solution was applied per plant via the soil on alternate days. BX-112 was dissolved (20 mg/liter) in distilled water containing 0.05% Tween 20 and 5% ethanol. This solution was sprayed on the experimental plants on alternate days until the leaves were fully wetted.

Application of GAs. Solutions of GAs were prepared in distilled water, containing 0.05% Tween 20 and 5% ethanol. Solutions were applied in 50-μl volumes to the shoot apex and surrounding young leaves on alternate days (when the plants were not treated with BX-112 or AMO-1618), starting on the first LD.

[^{14}C]GA_{20} (1.5 GBq/mmol) was prepared as described (14). A solution containing [^{14}C]GA_{20} was injected into the petioles and midribs of just fully expanded and younger

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Abbreviations: AMO-1618, 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate; BX-112, calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate; GA, gibberellin; LD, long day(s); SD, short day(s); SIM, selected ion monitoring; MS, mass spectrometry.

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leaves. Treatment groups consisted of four control plants and four plants treated with BX-112 for 7 LD. Each group received 13 kBq of [^{14}C]GA_{20}. The plants were harvested after 2 days.

Extraction and Purification. Extraction, removal of pigments, and purification via a charcoal column were performed as described (8). The GAs were eluted from the charcoal column with 80% acetone into a flask containing 10 ml of 1 M potassium phosphate (pH 8.2). The acetone was removed on a rotary evaporator and more 1 M potassium phosphate was added to give a final concentration of ≈0.5 M to enhance partitioning of the more polar GAs into ethyl acetate (15). After adjustment with HCl to pH 2.6, this aqueous phase was partitioned against ethyl acetate [six times, 2:1 (vol/vol)]. The combined ethyl acetate was reduced to a small volume and the extract was applied to a silicic/Celite column, 1:2 (wt/wt). The GAs were eluted with chloroform/ethyl acetate, 3:7 (vol/vol). Five milliliters of water at pH 8.0 was added to the eluate and the organic solvents were removed on a rotary evaporator. The aqueous residue solution was adjusted to pH 8.0 with KOH and the solution was applied to a column of QAE-Sephadex A-25 (3 ml). The column was washed with 3 vol of water at pH 8.0 after which the GAs were eluted with 4 vol of 1 M acetic acid. The eluate was dried on a rotary evaporator and the residue was fractionated with a μBondapak C_{18} column (30 × 0.4 cm). A 30-min gradient of 20–80% methanol in 1% aqueous acetic acid at a flow rate of 2.0 ml/min was used. Appropriate HPLC fractions were combined (GA_8, GA_{29}, GA_{81}, GA_{60}, and 3-epi-GA_1, 7–14 min; GA_1, 15–16.5 min; GA_{20}, 21.5–22 min; GA_{44} and GA_{19}, 23–25.5 min; and GA_{53}, 27–30.5 min), dried with a vacuum centrifuge, methylated, and trimethylsilylated.

Table 1. Comparison of the effects of the growth retardants AMO-1618 and BX-112 and GA_1 and GA_{20} on stem and petiole length of spinach

Treatment	Stem length, cm	Petiole length, cm
Untreated	42.1 ± 4.5	13.1 ± 0.4
AMO-1618	3.1 ± 0.9	5.9 ± 0.2
AMO-1618 + GA_{20}	44.2 ± 3.6	10.2 ± 0.4
AMO-1618 + GA_1	40.9 ± 2.5	10.2 ± 0.3
BX-112	2.4 ± 0.6	8.5 ± 0.2
BX-112 + GA_{20}	3.2 ± 1.2	9.2 ± 0.3
BX-112 + GA_1	41.1 ± 3.9	15.7 ± 0.3

Treatments were as in Fig. 1. Two petioles that developed during the experimental treatments were measured on each plant. Measurements were after 22 days. Results are mean ± SEM for nine plants per group.

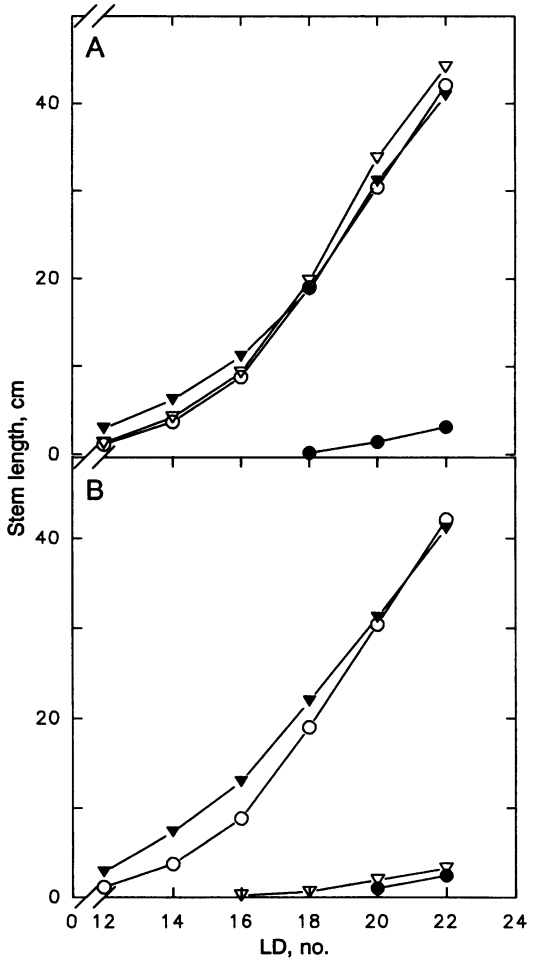

FIG. 1. Time course of stem growth of spinach plants under LD conditions as affected by the growth retardants AMO-1618 (*A*) and BX-112 (*B*) and GA_1 and GA_{20}. The growth retardants were applied every other day, starting 3 days before the plants were transferred to LD. GAs were applied 10 times and were alternated with growth-retardant applications, 2 μg in 50 μl of solvent, for a total of 20 μg per plant and nine plants per treatment. (*A*) ▼, GA_1; ▽, GA_{20}; ○, control; ●, AMO-1618. (*B*) ▼, GA_1; ○, control; ▽, GA_{20}; ●, BX-112.

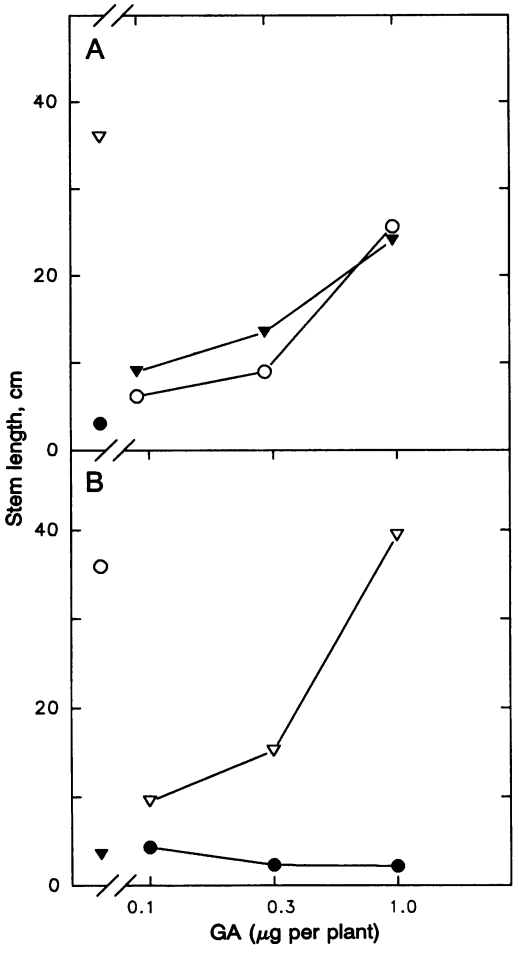

FIG. 2. Reversal of growth inhibition by AMO-1618 (*A*) and BX-112 (*B*) with various doses of GA_1 and GA_{20}. The growth retardants were applied every other day, starting 3 days before the plants were transferred to LD. GAs applied 11 times, alternating with growth-retardant treatments. Measurements were after 22 LD on five plants per treatment. (*A*) ▽, Control; ▼, GA_{20}; ○, GA_1; ●, AM-1618. (*B*) ○, Control; ▽, GA_1; ▼, BX-112; ●, GA_{20}.

Table 2. Effect of BX-112 on GA content of spinach plants sprayed with BX-112, starting 2 days before transfer to LD conditions

GA	GA, ng/g (dry weight)	
	Untreated	BX-112
GA_{53}	11.0	28.1
GA_{44}	15.9	39.9
GA_{19}	72.1	100.6
GA_{20}	23.5	96.5
GA_1	6.7	1.5
GA_8	146.9	14.3

Plants were harvested after 9 LD.

Samples containing [^{14}C]GA_{20} and its metabolites were purified by the procedures described above, but the effluent from the HPLC column was passed through a radioactive flow detector (Flo-One model, Radioanalytic, Tampa, FL), using a 250-μl solid scintillator flow cell. Radioactive peaks were collected, dried, methylated, and again purified by reverse HPLC. The radioactive fractions were dried and trimethylsilylated.

GC-MS. Qualitative GC–mass spectrometry (MS) analyses were performed with a JEOL JMS-AX 505H double-focusing mass spectrometer equipped with a Hewlett–Packard 5890A gas chromatograph. The samples were coinjected with dissolved Parafilm to establish retention times relative to *n*-alkane standards in splitless mode into a fused silica HP Ultra 2 (25 m × 0.32 mm × 0.52 μm film thickness) capillary column as described (8).

GC–Selected Ion Monitoring (SIM). For quantification of GAs by GC–SIM (8), the following GAs were added to the extracts as internal standards: [17,17-$^2H_2$]GA_{53} (99.0% enrichment), [20-$^2H_1$]GA_{44} (95.7% enrichment), [17,17-$^2H_2$]-GA_{19} (94.2% enrichment), [17,17-$^2H_2$]GA_{20} (99.3% enrichment), [17,17-$^2H_2$]GA_1 (99.2% enrichment), and [17-$^{13}C_1$]GA_8 (92.3% enrichment). Four or five prominent ions of each GA were monitored with dwell times of 100 ms. The amounts of GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, and GA_8 were calculated from the peak area ratios 448/450, 432/433, 434/436, 418/420, 506/508, and 594/595, respectively, by the formula in ref. 16, taking into account the abundance of natural isotopes. Labeled standards were not available for 3-epi-GA_1, GA_{29}, GA_{60}, and GA_{81}, so that for these GAs comparison between their relative levels was based on the intensities of the parent ion, M^+ = 506, in their spectra. All experiments were repeated one to three times, but data from only a single experiment are presented.

Table 3. Identification of GA_{60} and GA_{81} as their trimethylsilylated derivatives by GC–MS in extracts from spinach shoots

Material	KRI	Ion *m/z* (relative abundance)
Authentic GA_{60}	2631	506 (100), 491 (16), 477 (5), 447 (14), 416 (3), 375 (47), 357 (3), 313 (5), 238 (6), 207 (17), 194 (22)
Extract	2633*	506 (100), 491 (14), 477 (7), 447 (17), 416 (4), 375 (48), 357 (4), 313 (5), 238 (5), 207 (11), 194 (17)
Authentic GA_{81}	2743	506 (100), 491 (8), 459 (14), 447 (9), 431 (9), 389 (8), 375 (22), 303 (32), 235 (5), 207 (22)
Extract	2743*	506 (100), 491 (8), 459 (8), 447 (5), 431 (5), 389 (5), 375 (36), 303 (21), 235 (4), 207 (14)

KRI, Kovats retention index.
*KRI values were 100 higher than in ref. 8 due to erroneous assignments of *n*-alkane numbers of Parafilm.

Table 4. Comparison of the ion intensities at m/z = 506, the M^+ of the trimethylsilylated derivatives of GA_{60}, GA_{29}, GA_{81}, and epi-GA_1, by GC–SIM analysis in extracts from spinach plants after exposure to 9 LD or from extracts of plants after 9 LD and treated with BX-112

Treatment	Intensity, arbitrary units			
	GA_{60}	GA_{29}	GA_{81}	3-epi-GA_1
9 LD	92 (9)	360 (34)	1071 (100)	59 (5)
9 LD + BX-112	1667 (61)	356 (13)	2718 (100)	989 (36)

Losses were not taken into account and only horizontal comparisons can be made. The same material as in Table 2 was used. Numbers in parentheses are percentages relative to the intensities of GA_{81}.

RESULTS

When AMO-1618 and BX-112 were applied to spinach plants, LD-induced stem elongation was suppressed (Fig. 1). Leaves of plants treated with AMO-1618 had a darker green color than those treated with BX-112. Petiole growth was less inhibited by BX-112 than by AMO-1618 (Table 1). Although the plants treated with growth retardants showed little stem elongation, inflorescences were produced.

In plants treated with AMO-1618, applied GA_1 and GA_{20} were equally effective in overcoming the growth inhibition, but with BX-112, only GA_1 not GA_{20} restored normal stem and petiole growth (Fig. 1 and Table 1). GA_1 (1 μg) applied 11 times to BX-112-treated plants induced stem growth equal to that of the control; similar treatments with GA_{20} gave no growth response. In contrast, GA_1 and GA_{20} were equally effective in reversing the growth inhibition of AMO-1618-treated plants (Fig. 2).

In extracts from spinach plants treated with BX-112, the levels of GA_1 and GA_8 were reduced, whereas the levels of GA_{53}, GA_{44}, GA_{19}, and GA_{20} were increased (Table 2). In addition to the previously identified GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, 3-epi-GA_1, GA_{29}, and GA_8 (8), GA_{60}, and GA_{81} were also identified by full-scan GC–MS (Table 3). The relative levels of 3-epi-GA_1, GA_{29}, GA_{60}, and GA_{81} (internal standards not available) were estimated from the ion intensities at m/z 506 (the M^+ for each of these GAs) by GC–SIM for control plants and plants treated with BX-112. In both extracts, GA_{81} was most abundant; GA_{60} and 3-epi-GA_1 were minor components in extracts of untreated plants. After BX-112 treatment, GA_{60} and 3-epi-GA_1 increased relative to GA_{81}, whereas GA_{29} decreased relative to GA_{81} (Table 4).

All five dihydroxy-GAs (GA_1, 3-epi-GA_1, GA_{29}, GA_{60}, and GA_{81}) present in spinach are metabolites of GA_{20} (Table 5 and Fig. 3). In plants treated with BX-112, no [^{14}C]GA_1 or [^{14}C]GA_8 was detected. The specific activity of each metab-

Table 5. Effects of BX-112 on metabolism of [^{14}C]GA_{20} in spinach plants

GA	KRI	$[M+8]^+$	
		Control	BX-112
[^{14}C]GA_{20} (substrate)	2578	426 (17)	
[^{14}C]GA_{20}	2576	426 (13)	426 (10)
[^{14}C]GA_1	2724	514 (15)	ND
[^{14}C]GA_8	2845	602 (9)	ND
[^{14}C]GA_{60}	2635	514 (10)	514 (6)
[^{14}C]GA_{29}	2739	514 (10)	514 (8)
[^{14}C]GA_{81}	2750	514 (12)	514 (6)
3-epi-[^{14}C]GA_1	2859	514 (16)	514 (11)

GAs were identified by retention times and full-scan GC–MS of their trimethylsilylated derivatives. Ions shown are molecular ions, $[M+8]^+$, of species containing four ^{14}C atoms. The relative abundance is with respect to M^+ (% M^+) of nonlabeled species and is in parentheses. ND, not detected; KRI, Kovats retention index.

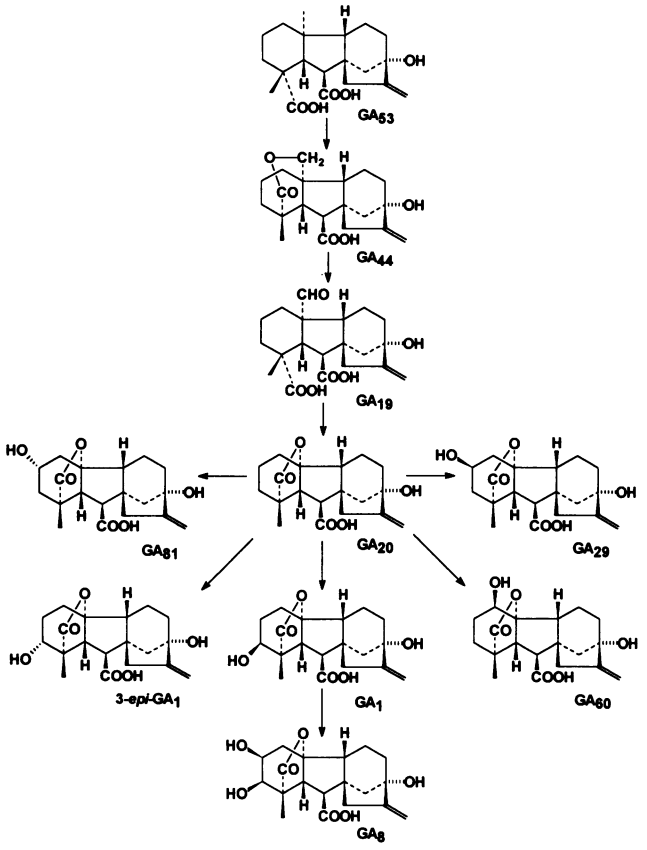

FIG. 3. Last stage of GA biosynthetic pathway in spinach. The growth retardant BX-112 specifically blocks 3β-hydroxylation of GA_{20} to the biologically active GA_1.

olite of [^{14}C]GA_{20} was lower in extracts from BX-112-treated plants than from controls (Table 5).

When spinach plants were exposed to increasing numbers of LD, there was an increase in GA content that was highest for GA_{20} (16.5 times), GA_1 (5.1 times), and GA_8 (14.5 times) (Table 6). With increasing numbers of LD, the level of GA_{29} relative to GA_{81} decreased, whereas the levels of GA_{60} and 3-epi-GA_1 to GA_{81} remained constant (Table 7).

DISCUSSION

LD-induced stem elongation in rosette plants is preceded by extensive cell divisions in the subapical meristem (7). Native GA is apparently a limiting factor for subapical meristematic activity. The evidence presented here shows that 3β-hydroxylation of GA_{20} to GA_1 is necessary for stem elongation in spinach: (*i*) Inhibition of stem growth by BX-112 was reversed by GA_1 only, not by its immediate precursor, GA_{20}. In contrast, growth inhibition by AMO-1618 was reversed by both GA_{20} and GA_1 (Figs. 1 and 2). (*ii*) Metabolism of

Table 6. Changes in GA levels in spinach after exposure to various numbers of LD

GA	GA, ng/g (dry weight)			
	0 LD	4 LD	8 LD	12 LD
GA_{53}	5.9	6.6	7.9	7.1
GA_{44}	2.2	8.8	15.2	17.4
GA_{19}	15.0	27.5	54.2	45.0
GA_{20}	1.4	6.9	18.1	23.1
GA_1	1.0	3.5	3.7	5.1
GA_8	18.0	82.8	153.6	263.9

Stem elongation started after 10 LD.

Table 7. Comparison of the ion intensities at m/z = 506, the M^+ for the trimethylsilylated derivatives of GA_{60}, GA_{29}, GA_{81}, and epi-GA_1, as measured by GC–SIM in extracts from spinach plants exposed to various numbers of LD

LD, no.	Intensity, arbitrary units			
	GA_{60}	GA_{29}	GA_{81}	3-epi-GA_1
0	21 (14)	216 (143)	151 (100)	6 (4)
4	30 (18)	217 (133)	163 (100)	9 (5)
8	95 (16)	501 (87)	578 (100)	20 (3)
12	107 (15)	530 (74)	719 (100)	26 (4)

Losses were not taken into account and only horizontal comparisons can be made. The same material as in Table 6 was used. Numbers in parentheses are percentages relative to the intensities of GA_{81}.

[^{14}C]GA_{20} to [^{14}C]GA_1 and to [^{14}C]GA_8 was inhibited by BX-112 to levels below the limit of detection by GC–MS (Table 5). (*iii*) The levels of GA_1 and GA_8 were lowered by BX-112 while the earlier precursors (GA_{53}, GA_{44}, GA_{19}, and GA_{20}) increased (Table 2). Thus, as was found in studies with rice (13, 17, 18), wheat (12), pea (19), and *Salix* (20), the main effect of BX-112 and other acylcyclohexanediones is inhibition of the 3β-hydroxylation step of GA_{20} to GA_1. This step is also blocked in dwarf mutants of several species: *dl* in maize (1), *le* in pea (10), *dy* in rice (11), and *ga4* in *Arabidopsis* (21).

Besides 3β-hydroxylation of GA_{20} to GA_1, four other hydroxylations of GA_{20} were observed in spinach: 1β-hydroxylation to GA_{60}, 2β-hydroxylation to GA_{29}, 2α-hydroxylation to GA_{81}, and 3α-hydroxylation to 3-epi-GA_1 (Table 5 and Fig. 3). Inhibition of 2β-hydroxylation by acylcyclohexanediones has been reported (13, 22, 23). In the present experiments, GA_{29} decreased relative to GA_{81} after the plants were treated with BX-112 (Table 4), but it does not necessarily follow that the absolute level of GA_{29} was decreased by BX-112, as it was in the case of GA_1 (Tables 2 and 5). Both the levels of GA_{60} and 3-epi-GA_1 were increased by BX-112 relative to GA_{81} (Table 4 and Fig. 3), indicating that 1β- and 3α-hydroxylation were not inhibited by BX-112. The observation that 3-epi-GA_1 and GA_{60} accumulate in spinach treated with BX-112 while stem growth is suppressed suggests that both these GAs have little or no activity in spinach.

During the first days of LD, the levels of GA_{53} and GA_{19} decrease (8). However, in the longer term, there is an overall increase in GA levels (Table 6). It appears that a certain threshold level of GA_1 is required for stem elongation to take place, considering that plants inhibited by BX-112 in LD (Table 2), as well as plants in SD (Table 6), had a GA content that was 4–5 times lower than that of elongating plants. However, the present GA analyses were performed on whole plants, not on meristematic tissue where the active compounds may be concentrated. In *Silene*, much larger differences in GA_1 content were detected in the subapical meristem (7), the site of GA action for stem elongation. The observation that petiole growth was less inhibited by BX-112 than stem growth (Table 1) and that petiole elongation occurs before initiation of stem elongation (4) may indicate that lower GA_1 levels are required for petiole elongation than for stem growth.

We thank Dr. J. MacMillan (Long Ashton Research Station, Long Ashton, U.K.) for providing GA_{60} and GA_{81}, Dr. L. N. Mander (Australian National University, Canberra) for [^{2}H]GAs, Dr. N. Murofushi (University of Tokyo) for GA_{20}, and Dr. B. O. Phinney (University of California, Los Angeles) for [^{13}C]GA_8. BX-112 was kindly supplied by Dr. E. Ishihara, Kumiai Chemical Industry Co., Tokyo. This work was supported by U.S. Department of Energy Grant DE-FG02-90ER20021, by U.S. Department of Agriculture Grants 88-37261-3434 and 90-37304-6469 to J.A.D.Z., and by the

National Institutes of Health Grant RR-00480 to the Michigan State University–National Institutes of Health Mass Spectrometry Facility.

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