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Identification of Endogenous Gibberellins in the Winter Annual Weed *Thlaspi arvense* L.

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ABSTRACT

Eleven endogenous gibberellins (GAs) were identified by combined gas chromatography-mass spectrometry in purified extracts from shoots of field pennycress (*Thlaspi arvense* L.): $GA_{1,9,12,15,19,20,24,29,44,51,53}$. Traces of GA_8 and GA_{25} were tentatively indicated by combined gas chromatography-mass spectrometry-selected ion monitoring. Comparison of the total ion current traces indicated that GA_{19} and GA_{44} were most abundant, while $GA_{12,15,20,24,29,53}$ occurred in lesser amounts. Only small amounts of $GA_{1,9,51}$ were present. The levels of GA_8 and GA_{25} were barely detectable. Consideration of hydroxylation patterns of the *ent*-gibberellane ring structure indicates two families of GAs: one with a C-13 hydroxyl group ($GA_{1,8,19,20,29,44,53}$) and another whose members are either nonhydroxylated ($GA_{9,12,15,24,25}$) or lack a C-13 hydroxyl group (GA_{51}). This suggests that in field pennycress there are two parallel pathways for GA metabolism with an early branch point from GA_{12}: an early C-13 hydroxylation pathway, leading ultimately to GA_1 and GA_8 and a C-13 deoxy pathway culminating in the formation of GA_9 and GA_{51}.

Field pennycress (*Thlaspi arvense* L.) is a winter annual weed in which the initiation of stem growth is under strong environmental control. Normally, following germination, the plants develop into rosettes. Upon the reception of a thermoinductive stimulus (2–15°C) rapid stem growth is initiated, culminating ultimately in flowering (14).

Previous work from this laboratory has shown that thermoinduced stem elongation in field pennycress is mediated by a change in the endogenous GA[1] status (14). At present it is not known which aspect of the GA status is affected by thermoinductive temperatures. To determine if an alteration in GA metabolism is involved, the nature of the endogenous GAs must be known. In the following paper, we report the identification of 13 endogenous GAs by GC-MS in shoots of field pennycress.

MATERIALS AND METHODS

Plant Material. Seeds of an inbred line of field pennycress, CR_1, were germinated in Petri dishes at 21°C as previously described (14). When the seedlings were 5 to 7 d old, they were transferred to 27 × 27 cm plastic flats (20 per flat) containing vermiculite. The vermiculite was kept moist with one-fourth strength Hoagland solution (14). The seedlings were allowed to grow vegetatively (rosettes) for 6 weeks in the greenhouse, whereupon the plants were transferred to a cold room (4°C) for 4 weeks. During the thermoinductive treatment, the plants received 8 h of dim light daily (20 $\mu E\ m^2\ s^{-1}$) from fluorescent lamps (14). Following the thermoinductive treatment, the plants were returned to the greenhouse for 10 d before harvesting the shoots. The plants at this stage had begun to bolt, *i.e.* initiated stem elongation. The harvested shoots were frozen in liquid N_2, lyophilized, and stored at −15°C prior to extraction. Approximately 1200 shoots yielded 1 kg of freeze-dried plant material.

Extraction and Purification Procedures. Lyophilized shoots, in 100 to 120 g lots, were homogenized twice at low speed for 60 s in 1.5 L of cold 80% (v/v) aqueous acetone with a large capacity Waring Blendor[2] equipped with a cooling jacket maintained at −5°C. The homogenate was filtered and the residue rehomogenized in 1 L of cold 80% acetone as before. This homogenate was then stirred for 1 h at 4°C and filtered. Both filtrates were then stirred separately for 30 min at 4°C with equal amounts of activated charcoal (Darco G-60, Sargent Welch) and Celite. Sixty and 30 g of charcoal were used with the first and second filtrates, respectively. The mixtures were then filtered and the acetone removed under reduced pressure at 35°C.

Free, acidic GAs in the remaining aqueous solution were further purified by preparative reverse phase chromatography similar to that described by Koshioka *et al.* (13). The pH of the solution was adjusted to 6.5 with 6 N KOH and then mixed with an equal volume of methanol. The mixture was passed through a 6.0 × 3.0 cm column of preparative C_{18} reverse phase packing material (55–105 μm; Waters Associates, Milford, MA) that had been previously equilibrated with 50% (v/v) aqueous methanol. The column was then eluted with 100 ml of 50% (v/v) aqueous methanol and the methanol removed under reduced pressure at 35°C.

The pH of the remaining aqueous solution was adjusted to 2.5 with 6 N HCl and partitioned four times with equal volumes of ethyl acetate. The organic phases were combined and dried over anhydrous Na_2SO_4. The volume of ethyl acetate was reduced and the acidic ethyl acetate fraction was subjected to silicic acid adsorption chromatography (15).

The eluate from silicic acid adsorption chromatography was then fractionated by Grad HPLC as described previously (14). Fractions were collected every min from the time of injection and corresponding fractions from 10 HPLC runs (10 extractions)

[1] Abbreviations: GA(s), gibberellins(s); Grad HPLC, gradient eluted C_{18}-reverse phase HPLC; SAP chromatography, silicic acid partition chromatography; Iso HPLC, isocratic C_{18}-reversed phase HPLC; GC-MS-SIM, combined gas chromatography-mass spectrometry-selected ion monitoring; Rt, retention time/volume; MeTMS-GA, methyl ester trimethylsilyl ether of gibberellin; Me-GA, methyl ester of gibberellin; Rt, retention time.

[2] Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

were combined to form two groups of 5 fractions each. The combined fractions were dried and then redissolved in methanol. An aliquot from each of the combined fractions that was the equivalent to about 30 g of dry plant material was tested for the presence of GA-like substances with the d-5 maize bioassay (15).

Fractions with biological activity were further purified by SAP chromatography as described previously (15), except Bio-Sil A (200–400 mesh; Bio-Rad Laboratories, Richmond, CA) was used. Elution of GAs was achieved with a step gradient of increasing concentrations, in 5% increments, of ethyl acetate in hexane. A 30 g dry weight equivalent sample from each step was tested for the presence of GA-like substances by the d-5 maize bioassay.

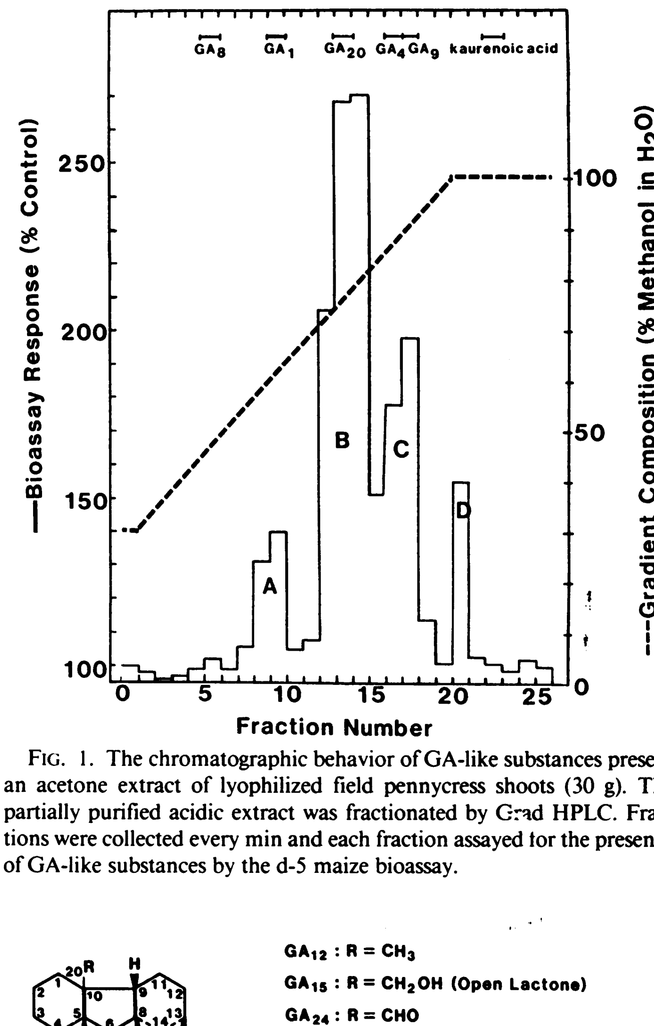

FIG. 1. The chromatographic behavior of GA-like substances present an acetone extract of lyophilized field pennycress shoots (30 g). The partially purified acidic extract was fractionated by Grad HPLC. Fractions were collected every min and each fraction assayed for the presence of GA-like substances by the d-5 maize bioassay.

Final purification was achieved with Iso HPLC. The same HPLC system as described for Grad HPLC was employed, except that a Nova-Pak C_{18} radial compression cartridge (8 mm id., 5 μm particle size; Waters Associates, Milford, MA) was used. The eluting solvents were various mixtures of water and methanol; both solvents contained 1% (v/v) acetic acid prior to mixing. Three solvent systems were used: 35, 50, and 65% (v/v) methanol in H_2O). The residues in fractions resulting from SAP chromatography were dissolved in 25 μl of methanol and then injected. The solvent composition was maintained at one of the three methanol concentrations for 12 min, whereupon the methanol concentration was increased to 100% in 1 min for a total run time of 18 min. The flow rate was 2 ml min^{-1}. Fractions were collected every min from the time of injection and dried. Fractions containing GA-like substances were located by assaying an aliquot with the d-5 maize bioassay as described above.

Derivatization. Fractions resulting from Iso HPLC containing biological activity were methylated in methanol using an excess of ethereal diazomethane. The TMS-ethers of the methyl esters were prepared by adding 100 μl of Tri-Sil (Pierce Chemical Co., Rockford, IL) to dry methylated samples. Just prior to GC-MS, the solvent was removed with a stream of dry N_2 and redissolved in 25 to 100 μl of dry ethyl acetate.

GC-MS. The derivatized fractions were analyzed with a Hewlett Packard model 5992A instrument equipped with a 25 m × 0.31 mm i.d. Ultra-1 fused silica capillary column (Hewlett Packard) coated with methyl silicone (0.52 μm thick). Samples (1–2 μl) were injected in the cool on-column mode with the oven temperature at 150°C. The oven temperature was increased at 10°C min^{-1} to 300°C. The pressure of the carrier gas (He) was 70 kPa. A mass range of 100 to 600 atomic mass units was scanned at a rate of 690 atomic mass units s^{-1}. The ionization potential was 70 eV.

GC-MS-SIM. Derivatized fractions were analyzed by GC-MS-SIM under GC conditions identical to those decribed above for GC-MS. Four ions were monitored for each compound. Dwell times for each ion were chosen based on the relative intensities of the individual ions in the mass spectrum reported in the literature. Weak ions were monitored for 200 ms, while stronger ions were monitored for 50 ms. The SIM response for each ion is reported as a percent of the value of the most abundant ion of the four that were monitored. Other parameters of the mass spectrometer were identical to those described for GC-MS.

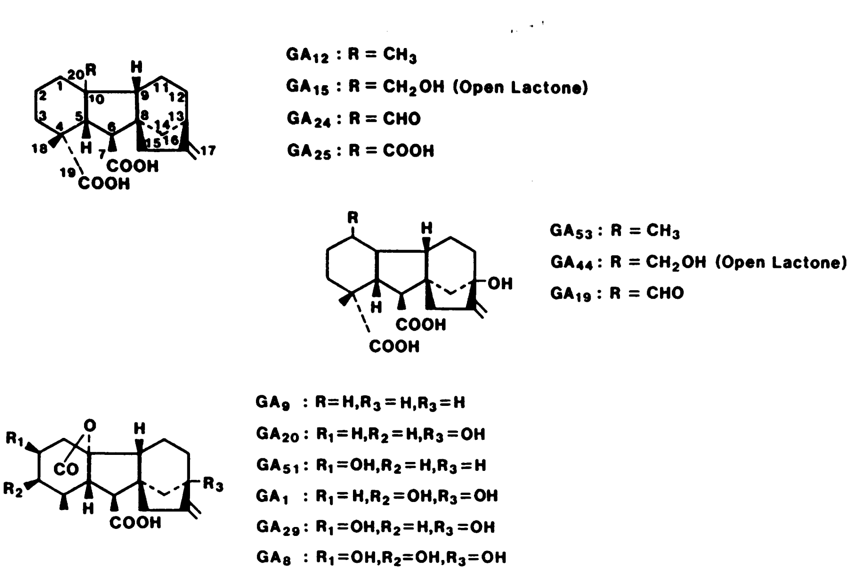

FIG. 2. The structures of the GAs identified by GC-MS and GC-MS-SIM in field pennycress shoots.

RESULTS

Profile of GA-like Substances in Shoot Extracts. The acidic ethyl acetate fraction from an extract of 30 g of lyophilized field pennycress plants was subjected to Grad HPLC and the presence of GA-like substances in each 1 min fraction was determined by the d-5 maize bioassay. Four distinct zones of biological activity were separated by Grad HPLC (Fig. 1). Zone A was the most polar zone with a Rt similar to that observed for the dihydroxylated GAs, GA_1 and GA_3. Zones B and C had similar chromatographic properties as GA_{20} and GA_4, respectively. The Rt of zone D was greater than GA_9, the least polar of the available authentic GAs.

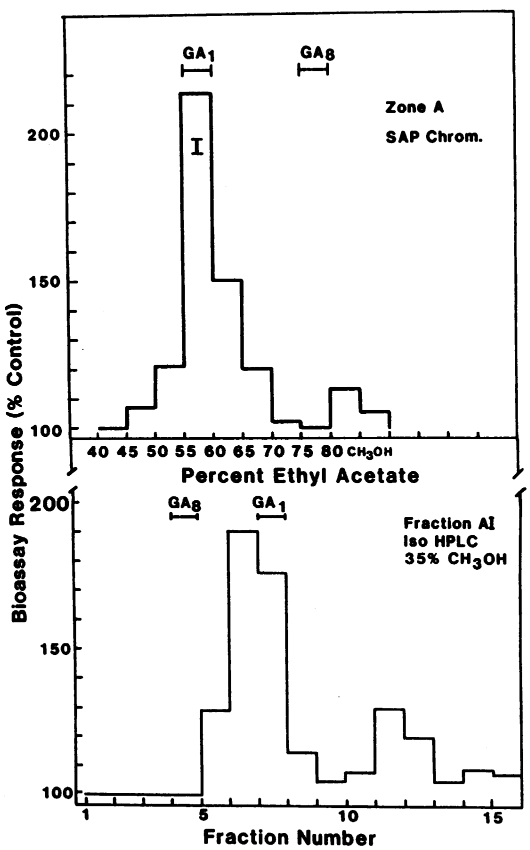

FIG. 3. The behavior of GA-like substances in zone A when subjected first to SAP chromatography (top) followed by Iso HPLC at 35% methanol (bottom). An aliquot from each fraction was tested for GA-like activity with the d-5 maize bioassay as described in the text.

To identify the biologically active GAs that were present in each of the four zones, an extract from 1 kg of lyophilized shoot material was subjected to Grad HPLC, and further purified by SAP chromatography and Iso HPLC. The structures of the GAs identified in each zone are shown in Figure 2.

Zone A. When GA-like substances from zone A were subjected to SAP chromatography, a single peak of biological activity with chromatographic properties identical to GA_1 was eluted with 55 and 60% ethyl acetate (Fig. 3). Biologically active fractions were then chromatographed on Iso HPLC with 35% methanol. Again, the major zone of biological activity was observed that chromatographed with GA_1 (Fig. 3, bottom). When derivatized fractions were analyzed by GC-MS, a compound with the same retention time and similar mass spectrum as MeTMS-GA_1 was detected (Table I). Neither MeTMS-GA_3 nor any other dihydroxylated GAs were detected in the Iso HPLC fraction containing GA_1. A minor peak of biological activity also occurred in Iso HPLC fractions 11 and 12; however, no GAs were detected by GC-MS.

Zone B. Fractionation of zone B by SAP chromatography gave two distinct regions of biological activity: one which co-eluted with authentic GA_{20} (BI) and a more polar one which co-eluted with GA_1 (BII) (Fig. 4, top). Fraction BI could be further separated into two additional regions of biological activity by Iso HPLC (50% methanol) (Fig. 4, middle). The first (fractions 6 and 7) had a Rt nearly identical to GA_{20} and indeed, analysis by GC-MS showed the presence of MeTMS-GA_{20} (Table I). No MeTMS-GA_5 was detected in this fraction. The second peak (fractions 8 and 9) was shown by GC-MS to contain MeTMS-GA_{44} (Table I) (5). None of the other spectra was indicative of any of the known GAs.

Fraction BII chromatographed as a single peak of biological activity when subjected to Iso HPLC with 50% methanol (Fig. 4, bottom). Analysis by GC-MS indicated a single large peak with a mass spectrum closely resembling the mass spectrum of MeTMS-GA_{19} (Table I) (3).

Zone C. SAP chromatography of zone C yielded two peaks of biological activity (Fig. 5, top). CI (25–35% ethyl acetate) chromatographed as a single zone of biological activity when sub-

Table I. *GC-MS Data for Endogenous GAs in Field Pennycress Shoots and for Authentic GAs*

Grad HPLC Zone	Presumptive MeTMS-GA	Time of Scan	Peaks in Mass Spectrum with Relative Abundances in Parentheses						
		min				*m/z*			
A	GA_1	12.9	506 (M^+, 100)	491 (11.1)	477 (3.7)	448 (14.8)	376 (14.8)	208 (31.4)	207 (42.6)
Authentic MeTMS-GA_1		12.9	506 (M^+, 100)	491 (8.8)	477 (2.7)	448 (12.2)	376 (9.5)	208 (8.8)	207 (32.7)
B	GA_{20}	11.1	418 (M^+, 100)	403 (10.7)	375 (38.0)	359 (9.9)	301 (9.9)	208 (9.1)	207 (22.3)
Authentic MeTMS-GA_{20}		11.1	418 (M^+, 100)	403 (11.6)	375 (52.7)	359 (10.1)	301 (14.9)	208 (15.2)	207 (39.0)
B	GA_{44}	13.4	432 (M^+, 20.1)	417 (2.7)	373 (5.3)	251 (2.9)	238 (22.8)	208 (37.6)	207 (100)
B	GA_{19}	12.3	462 (M^+, 8.2)	447 (4.1)	434 (100)	402 (24.7)	374 (53.4)	208 (42.5)	207 (71.2)
C	GA_{15}	12.4	344 (M^+, 12.0)	312 (11.1)	284 (37.3)	240 (23.1)	239 (100)	238 (19.6)	195 (47.6)
C	GA_{24}	10.9	374 (M^+, 4.2)	346 (7.5)	314 (64.4)	286 (45.8)	254 (37.3)	226 (100)	225 (92.9)
C	GA_9	9.8	330 (M^+, 12.5)	298 (78.6)	270 (83.9)	243 (71.4)	227 (64.3)	226 (89.3)	217 (33.9)
Authentic Me-GA_9		9.8	330 (M^+, 10.3)	298 (78.4)	270 (81.9)	243 (78.4)	227 (70.7)	226 (100)	217 (38.8)
C	GA_{53}	11.4	448 (M^+, 29.7)	433 (4.0)	416 (5.0)	389 (8.9)	241 (24.8)	208 (100)	207 (77.2)
D	GA_{12}	9.9	360 (M^+, 3.8)	328 (30.2)	300 (100)	285 (18.9)	241 (50.9)	240 (43.4)	225 (43.4)
A	GA_{29}	13.1	506 (M^+, 100)	491 (11.3)	477 (4.1)	447 (5.2)	303 (26.8)	208 (19.6)	207 (49.5)
C	GA_{51}	11.8	418 (M^+, absent)	386 (36.4)	328 (45.5)	296 (48.5)	284 (100)	268 (60.6)	225 (100)
C	GA_{25}	11.0	404 (M^+, absent)	372 (15.1)	312 (36.0)	285 (23.0)	284 (58.0)	253 (15.1)	225 (100)

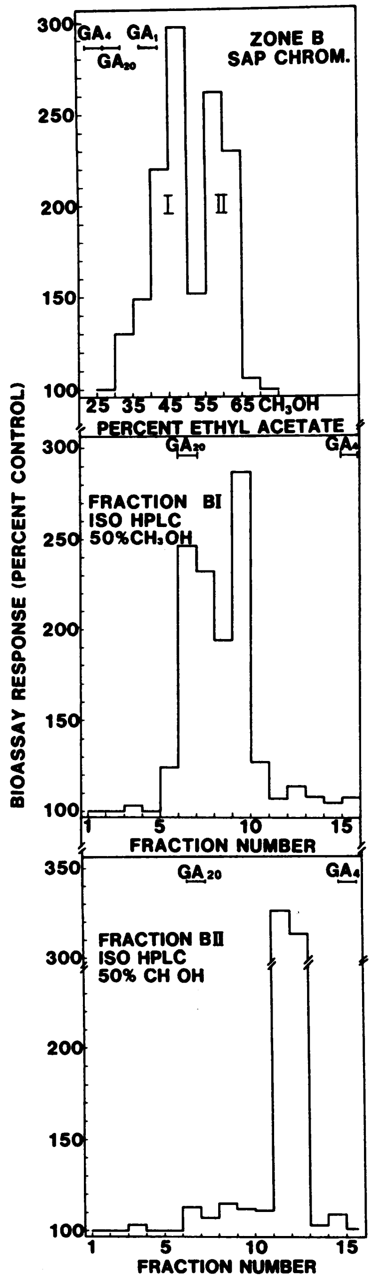

FIG. 4. The behavior of GA-like substances in zone B when first subjected to SAP chromatography (top) and followed by Iso HPLC at 50% methanol (middle and bottom). An aliquot from each fraction was tested for GA-like activity as described in the text.

jected to Iso HPLC at 65% methanol (Fig. 5, middle). Analysis by GC-MS indicated the presence of Me-GA_{15} and Me-GA_{24} as two major peaks in the total ion chromatogram (Table I; 3). In addition, an incompletely resolved trace compound with the same Rt and M^+ as authentic Me-GA_9 was detected (Table I).

Iso HPLC of CII (45–50% ethyl acetate) with 65% methanol gave a single peak of biological activity (Fig. 5, bottom). The presence of MeTMS-GA_{53} was indicated by GC-MS (Table I) (2).

Zone C could also contain GA_4 or GA_7 if present in field pennycress shoots. However, analysis of the appropriate fractions by GC-MS-SIM, which increased instrument sensitivity of detection by about 100-fold, failed to provide any evidence for the existence of either of these two GAs.

Zone D. A single peak of biological activity was detected in zone D following SAP chromatography (Fig. 6). This fraction (25% ethyl acetate) was derivatized and analyzed by GC-MS without any additional purification. Me-GA_{12} was detected (Table I) (3).

Biologically Inactive GAs. All of the above GAs have biological activity in the d-5 maize bioassay. However, it is probable that several biologically inactive GAs also occur in field pennycress. In numerous species, the inactive C-2β hydroxylated GAs $GA_{8,29,51}$ appear to be deactivation products of $GA_{1,20,9}$, respectively (1, 8). Furthermore, GA_{17} and GA_{25}, both with C-20 carboxyl groups, are often found in association with other GAs that have identical hydroxylation patterns (1). Since reference samples of most of these GAs were not available, fractions to be analyzed by GC-MS were chosen based on the reported behavior of a particular GA in SAP chromatography (4) and C_{18}-reverse phase HPLC (10, 12).

Authentic GA_8 eluted in fraction 6 in Grad HPLC and 75% ethyl acetate in SAP chromatography. These corresponding fractions from the plant extract were subjected to Iso HPLC (30%

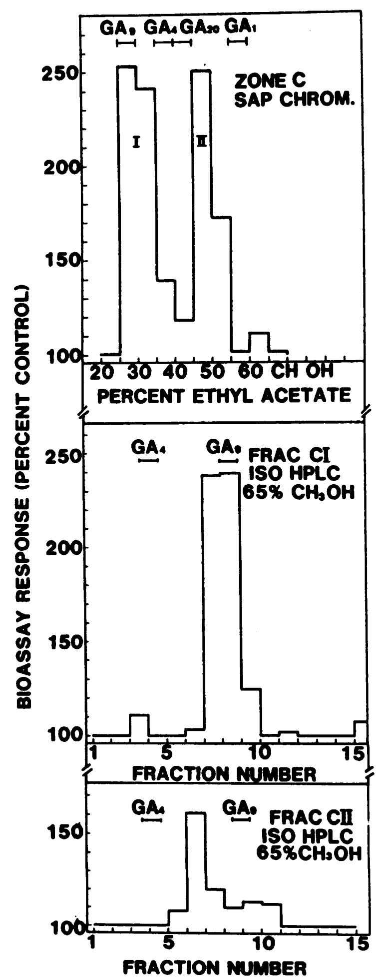

FIG. 5. The behavior of GA-like substances in zone C when first subjected to SAP chromatography (top) and followed by Iso HPLC of 65% methanol (middle and bottom). An aliquot from each fraction was tested for GA-like activity as described in the text.

methanol) and the fraction where authentic GA_8 eluted (fraction 4) collected, derivatized, and subjected to GC-MS. A trace compound was detected with an identical Rt and M^+ (m/z 594) as authentic MeTMS-GA_8. Other diagnostic ions for MeTMS-GA_8 were absent, however. Analysis by GC-MS-SIM showed that the plant compound did contain the important ions with similar relative intensities as authentic MeTMS-GA_8 (Table II), allowing for the tentative conclusion that GA_8 is endogenous to field pennycress.

Although authentic GA_{29} was not available, its behavior in our chromatographic systems could be predicted from the literature. In reverse phase HPLC, GA_{29} reportedly elutes between GA_8 and GA_1 (10, 12). Thus, fractions 7 and 8, from Grad HPLC (zone A), were subjected to SAP chromatography. The fractions eluting with 60 and 65% ethyl acetate were subjected to Iso HPLC (35% methanol). When fraction 5 (GA_1 eluted in fraction 7) was analyzed by GC-MS, a fairly large and well resolved peak was detected that had a mass spectrum very similar to the published spectrum of MeTMS-GA_{29} (Table I) (17).

Chromatographic behavior of GA_{51} should be similar to that of other monohydroxylated GAs (*e.g.* GA_{20}). Indeed, when the fraction resulting from SAP chromatography containing GA_{20} was subjected Iso HPLC (50% methanol), a compound was detected in fraction 13 (GA_{20} eluted in fraction 7) which had a mass spectrum very similar to the published mass spectrum of MeTMS-GA_{51} (Table I) (6) except for the absence of m/z 418, the M^+ of MeTMS-GA_{51}. This was not too surprising because of the small amount of the endogenous compound coupled with the characteristically low intensity of this ion (6). However, analysis by GC-MS-SIM revealed the presence of a small amount of m/z 418 with the identical retention time as the other characteristic ions (Table II).

Analysis by GC-MS of an Iso HPLC fraction eluting 2 to 3 min later than GA_{53} (fraction 6) revealed the presence of a trace compound which had a mass spectrum similar to that published for Me-GA_{25} (Table I) (7). However, m/z 404, the M^+ for Me-GA_{25}, was absent. Further analysis by GC-MS-SIM confirmed the presence of m/z 404 for this compound (Table II), tentatively indicating that GA_{25} is endogenous.

No evidence was obtained, even following GC-MS-SIM analysis, for the presence of MeTMS-GA_{17}.

Relative Concentrations of the Endogenous GAs. An approximation of the relative concentrations of the individual GAs was obtained by comparing the areas of peaks in the total ion current trace. Table III shows the abundance of the endogenous GAs relative to GA_{19}, the most abundant GA. GA_{44} was the second most abundant GA. Modest amounts of $GA_{12, 15, 20, 24, 29, 53}$ were detected. The shoot extracts contained small amounts of

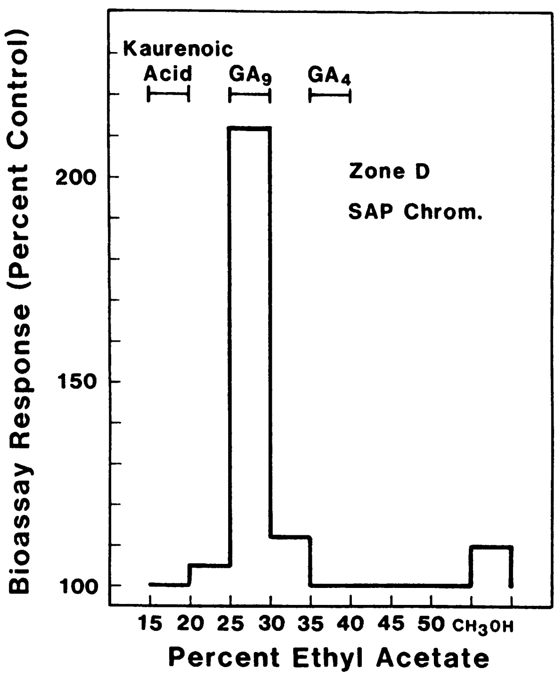

FIG. 6. The behavior of gA-like substances in zone D when subjected to SAP chromatography. An aliquot from each fraction was tested for GA-like activity as described in the text.

Table III. *Relative Amounts of the Endogenous GAs in Field Pennycress Shoots*

Four pluses indicate highest amount, one plus indicates lowest.

GA	Grad HPLC Zone	Relative Amount[a]
GA_8[b]		tr[c]
GA_1	A	+
GA_{29}	A	+++
GA_{19}	B	++++
GA_{44}	B	++++
GA_{20}	B	++
GA_{51}	B	+
GA_{15}	C	+++
GA_{24}	C	+++
GA_{53}	C	++
GA_9	C	+
GA_{25}	C	tr
GA_{12}	D	++

[a] Measured as the relative area of the total ion current response. [b] GA_8 eluted two fractions earlier (e.g. fraction 5) than zone A (fractions 7–9) in Grad HPLC. [c] tr, trace component.

Table II. *GC-MS-SIM Data for Trace Compounds Found in Field Pennycress Shoots and Authentic GAs*

Presumptive MeTMS-GA	Retention Time of Monitored Ions[a]	Ion Monitored with Relative Intensities in Parentheses[b]			
	min	*m/z*			
GA_8	13.6	594 (M^+, 100)	238 (23.5)	207 (49.4)	143 (28.4)
Authentic MeTMS-GA_8	13.6	594 (M^+, 100)	238 (20.5)	207 (28.3)	143 (19.0)
GA_{51}	11.8	418 (M^+, 4.5)	403 (1.7)	284 (100)	268 (74.9)
GA_{25}	11.0	404 (M^+, 1.5)	372 (46.6)	312 (85.7)	284 (100)

[a] Retention times of all monitored ions for a given compound were identical. [b] Ion intensities normalized to the area of the most abundant ion monitored.

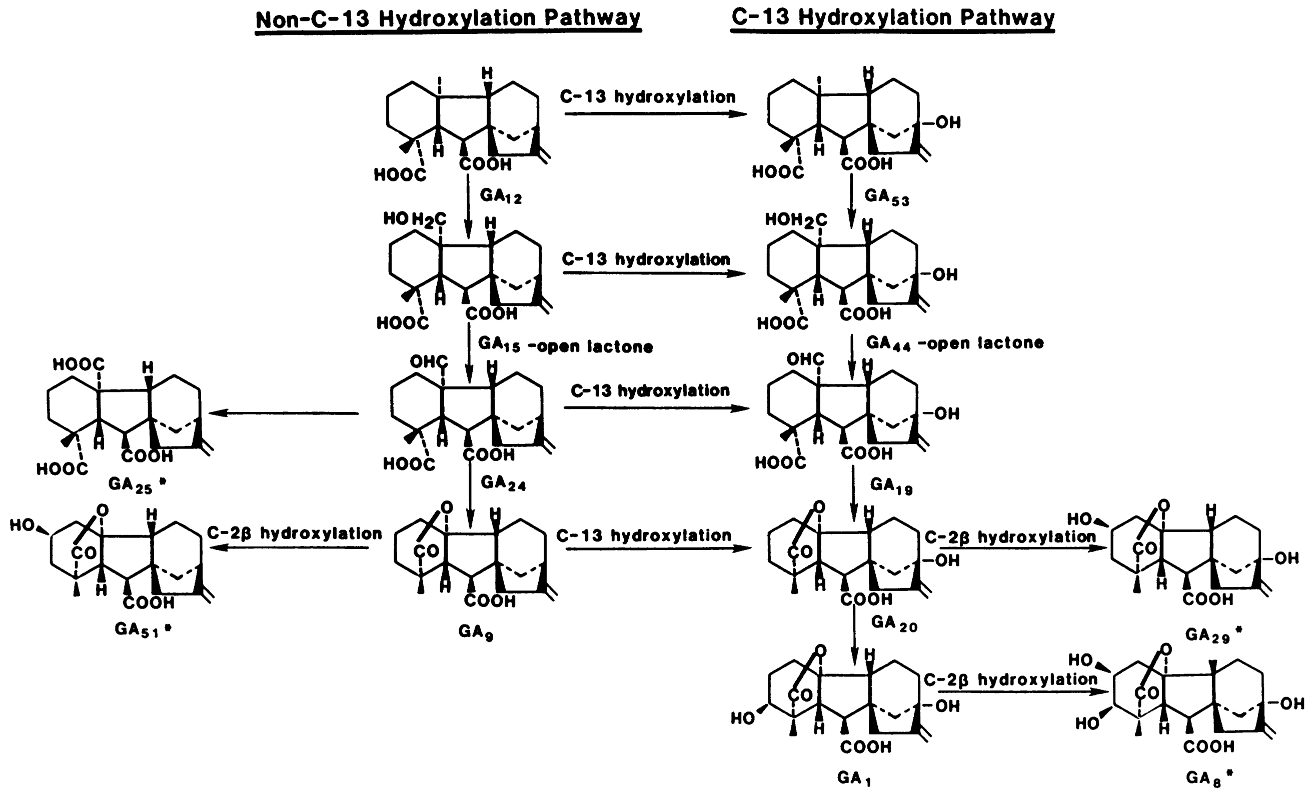

FIG. 7. Possible metabolic relationships between the endogenous GAs identified in field pennycress shoots. * = biologically inactive GA.

$GA_{1,9,51}$, while GA_8 and GA_{25} occurred at levels close to the limits of detection.

DISCUSSION

Two families of GAs have been identified in shoot extracts of field pennycress (Fig. 2). The members of one group share a common C-13 hydroxyl group ($GA_{1,8,19,20,29,44,53}$), while the GAs in the other group are nonhydroxylated ($GA_{9,15,24,25}$) or lack a hydroxyl at C-13 (GA_{51}). This suggests that in field pennycress there are two parallel pathways for GA metabolism diverging from GA_{12}: an early C-13 hydroxylation pathway and a C-13-deoxy pathway. Figure 7 shows the possible metabolic relations between endogenous GAs in field pennycress, based on the observed conversions of GAs in an *in vitro* system derived from immature pea seeds (11), developing pea seeds *in vivo* (6) and intact pea plants (9).

The existence of the early C-13 hydroxylation pathway in field pennycress, a member of the Cruciferae family, demonstrates further the widespread occurrence of this pathway in herbaceous angiosperms. Members of the Gramineae, Leguminosae, Chenopodiaceae, Convolvulaceae, and Caryophyllaceae families have this pathway of GA metabolism as well (1). The C-13 deoxy pathway is less widespread in occurrence but nevertheless occurs in species from the Gramineae, Cucurbitaceae, Rosaceae, and Leguminosae families (1).

The physiological significance of two parallel pathways of GA metabolism is not known at present. In both maize and peas it appears likely that GA_1 is the compound responsible for mediating GA-dependent stem growth (9, 16). The presence of GA_1 in shoot extracts of field pennycress makes it tempting to suggest that cold-induced stem growth in this species is similarly dependent on GA_1 and that other endogenous GAs are biologically active by virtue of their metabolism to GA_1. However, the validity of this hypothesis will be known only when the regulation of GA metabolism is understood and the biological activities of the endogenous GAs in field pennycress are known. Nevertheless, the identification of 13 endogenous GAs in field pennycress provides the necessary starting point from which rigorous metabolism and physiological studies can begin.

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