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Detection of Endogenous Gibberellins and Their Relationship to Hypocotyl Elongation in Soybean Seedlings

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ABSTRACT

Four gibberellins, GA_{53}, GA_{19}, GA_{20}, and GA_1, were detected by bioassay, chromatography in two HPLC systems, and combined gas chromatography-mass spectroscopy-selected ion monitoring (GC-MS-SIM) in etiolated soybean (*Glycine max* [L.] Merr.) hypocotyls. GC-MS-SIM employed [$^2H_2$]-labeled standards for each endogenous gibberellin detected, and quantities estimated from bioassays and GC-MS-SIM were similar. This result plus the tentative detection of GA_{44} and GA_8 (standards not available) indicates that the early-C-13-hydroxylation pathway for gibberellin biosynthesis predominates in soybean hypocotyls. Other gibberellins were not detected. Growth rates decreased after transfer to low water potential (ψ_w) vermiculite and were completely arrested 24 hours after transfer. The GA_1 content in the elongating region of hypocotyls had declined to 38% of the 0 time value at 24 hours after transfer to low ψ_w vermiculite, a level which was only 13% of the GA_1 content in control seedlings at the same time (24 hours posttransfer). Rewatering seedlings following 24 hours growth in low ψ_w vermiculite resulted in a complete recovery in elongation rate, an increase in GA_1 (20% at 2 hours, two-fold at 8 hours, eightfold at 24 hours), and a decrease in ABA levels (tenfold at 2 hours). Treatment of well-watered seedlings with the GA-synthesis inhibitor tetcyclacis (TCY) resulted in lowered GA_1 levels and increased ABA levels. When seedlings grown 24 hours in low ψ_w vermiculite were rewatered with TCY, recovery of the elongation rate was delayed and reduced, and the decline in ABA levels was slowed. Addition of GA_3 restored the elongation rate inhibited by TCY. Seedlings were growth responsive to exogenous GA_3, and this GA_3-promoted growth was inhibited by exogenous ABA. The data are consistent with the hypothesis that changes in GA_1 and ABA levels play a role in adjusting hypocotyl elongation rates. However, the changes observed are not of sufficient magnitude nor do they occur rapidly enough to suggest they are the primary regulators of elongation rate responses to rapidly changing plant water status.

Hypocotyl elongation rates are greatly reduced when well-watered soybean seedlings are transferred to low ψ_w[3] vermiculite (ψ_w = −0.30 MPa). The inhibition of elongation can be observed within the first hour after transfer, and complete arrest in hypocotyl elongation occurs between 24 and 36 h after transfer. Subsequently, elongation rates partially recover. During the period following transfer, turgor pressure is maintained in the elongating zone (22) due to osmotic adjustment, which occurs during the first 12 to 15 h (20).

Changes in the extensibility of the cell wall could be responsible, in part, for the observed decrease in cell elongation rates. Cell wall extensibility is a function of a complex set of metabolic processes (28), which may be mediated by plant growth regulators (6, 7). For example, ABA, which has been shown to decrease cell wall extensibility (6), accumulates in the hypocotyl zone of elongation of seedlings transferred to low ψ_w vermiculite (2). Its content doubles within 30 min of transfer and continues to increase up to 74-fold by 24 h posttransfer. The initial increase in ABA is temporally inseparable from the start of the growth inhibition and is not due to a turgor loss in the elongating zone. Turgor loss, which is considered to be a signal for induced ABA levels, does occur in other parts of the seedling (22), suggesting that other tissues may serve as the source of the accumulating ABA. In addition, applied ABA has been shown to inhibit hypocotyl elongation in well-watered seedlings (2).

Conversely, applied GA_3, which has been shown to increase cell wall extensibility (14, 15, 29), induces large increases in the elongation rate of stem tissue in intact plants, particularly mutants with low endogenous GA contents (19). In excised oat internodes (15) and lettuce hypocotyls (14), applied GA_3 results in dramatic increases in growth. GA_3 induces growth in lettuce hypocotyls with response times as short as 10 to 15 min without increasing either IAA content or synthesis (14). Studies with dwarf mutants have identified GA_1 as the active GA regulating shoot elongation in maize (24, 27), Brassica (26), rice (see review in Phinney [24]), and pea (13). These findings support a direct role for GA_1 in cell elongation of intact shoots.

The goal of the research reported here was to identify endogenous gibberellins in the elongating region of dark-grown soybean hypocotyls and to relate their quantities to hypocotyl elongation under well-watered and water-limited growth conditions.

MATERIALS AND METHODS

General

To extract, detect, and quantify GAs from the elongating regions of soybean hypocotyls, ^3H-labeled GAs were added to

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[3] Abbreviations: ψ_w, water potential; GC-MS-SIM, combined gas chromatography-mass spectroscopy-selected ion monitoring; GA_x, gibberellin A_x; TCY, tetcyclacis; KRI, Kovat's Retention Index.

extracts as an internal standard to track progress through chromatography and to estimate recovery. After initial purification steps, samples were submitted to HPLC and the resulting fractions bioassayed. Active fractions were grouped, again subjected to HPLC, and these new fractions were bioassayed. Active fractions from this HPLC run were grouped, supplemented with deuterated standard GAs, derivatized, and assayed by GC-MS-SIM. This approach, adapted from Koshioka *et al.* (16), allows the major bioactive GAs to be detected and characterized as to HPLC retention time prior to GC-MS-SIM.

After GAs had been detected by GC-MS-SIM, experiments were conducted to determine whether changes in GA_1 levels occur with changes in plant water status or TCY treatment. For these experiments the GA isolation procedures were streamlined by adding deuterated GA_1 at the time of extraction, conducting a single HPLC separation, grouping the GA_1-containing fractions only and subjecting them to GC-MS-SIM.

Standard Growth Conditions

Soybean seedlings (*Glycine max* [L.] Merr. cv Williams: Illinois Foundation Seed) were dark grown from seed at 29°C, 100% RH, in vermiculite, as previously described (20). Prior to planting, seeds were soaked for 5 min in 1% NaOCl, then rinsed with running tap water for 1 h. For the first 48 h, the seedlings were grown in 0.1 mM $CaCl_2$-saturated vermiculite. Vermiculite was considered saturated when enough solution had been added to allow for the vermiculite itself to be saturated, and a small excess of solution pooled in the bottom 2 cm of the 10-cm-tall growth container. At 48 h, seedlings were transplanted either to a similar saturated vermiculite (ψ_w = −0.01 MPa) or low water potential vermiculite (ψ_w = −0.30 MPa). Water potentials were determined by thermocouple psychrometry using the isopiestic method (5). In recovery (rewater) experiments, seedlings were watered to saturation of the vermiculite with 0.1 mM $CaCl_2$ 24 h after transfer to low ψ_w vermiculite. All seedling manipulation was performed at 100% RH under a green safelight (transmission 475–575 nm).

The elongating zone of the hypocotyl was experimentally determined to be that portion of the hypocotyl between 8 and 23 mm below the hypocotyl-cotyledon axis. Plant hypocotyl elongation was determined by direct measurement of seedlings at timed intervals.

Application of ABA, GA_3, and TCY

(±)ABA (0.1 mM, 1 mM) and GA_3 (0.01 mM, 0.1 mM, 1 mM) (Sigma) were prepared in 25 mM Tris/HCl (pH 6.8), 0.05% (v/v) Tween 20. The same buffer including Tween 20, but without hormones, was used in control treatments. These solutions were applied to seedlings by aerial spraying once every 6 h for the entire test period. Enough solution was applied to moisten the entire exposed portion of the plant. No attempt was made to saturate the vermiculite with the solution.

TCY (5-[4-chlorophenyl]-3,4,5,9,10-pentaaza-tetracyclo-5,4,1,$0^{2,6}$,$0^{8,11}$-dodeca-3,9-diene, BASF, Ludwigshafen, Federal Republic of Germany) was solubilized in 1 mL of acetone, and mixed with 1 or 2 L of deionized distilled water to prepare 5, 50, and 500 μM solutions. Seedlings to be treated with TCY were grown under standard growth conditions for 48 h, then depending on the experiment, were either transferred to vermiculite saturated with one of the TCY solutions, or transferred to low ψ_w vermiculite 24 h before rewatering to saturation with one of the TCY-containing solutions.

GA Extraction and Purification

Hypocotyl segments from the zone of elongation were excised (128 or 256 per treatment), frozen in liquid N_2, and lyophilized. After determination of dry weights, samples were ground to fine powder in a chilled mortar and pestle with the aid of acid-washed sand. The powdered tissue was extracted with 10 mL of ice-cold 4:1 (v:v) CH_3OH:distilled water and approximately 50,000 dpm each of [1,2-^{3}H]GA_1 (31.5 Ci/mmol) and [1,2-^{3}H]GA_4 (31.5 Ci/mmol) (both from Amersham) was added as internal standards for determination of recoveries and chromatographic markers. In the experiments where the time course of GA_1 content was determined after rewatering or transfer to TCY saturated vermiculite, 100 ng of [17,17-^{2}H]GA_1 (synthesized by Prof. L. Mander, Australian National University) was also added during the initial extraction. The tissue residue was then filtered off and reextracted overnight at 5°C with 15 mL of the extraction solvent. The methanolic extracts were combined and adjusted to pH 6.5 to 7.0 with 2 N NH_4OH.

The combined extracts were purified by a procedure modified from Koshioka *et al.* (17). Briefly, they were made up to 40 mL with additional 80% CH_3OH and passed through a 5-g column of Davisil C_{18} (90–130 μm, Alltech). The column was rinsed twice with 10 mL of 80% CH_3OH and the combined 80% eluate diluted with distilled water to equal 60% CH_3OH. The 60% CH_3OH extract was passed through a similar column and the column rinsed twice with 10 mL 60% CH_3OH, followed by 100% CH_3OH. (This latter fraction would contain kaurene and kaurenoic acid, but it was not analyzed further.) In the time course experiments analyzing only GA_1, the second column was omitted and the 80% eluate processed as described below. The 60% CH_3OH (or 80% CH_3OH) column eluate was reduced to rotary flash evaporation to the aqueous phase which was then frozen and lyophilized. The residue was solubilized in a small amount of 80% CH_3OH and dried onto celite with a warm air stream and a hot plate. The celite was loaded onto a 5-g SiO_2 partition column, and GAs were eluted with 70 mL of 95:5 (v:v) formate-saturated $CH_3CO_2C_2H_5$:hexane.

HPLC and Bioassay

The $CH_3CO_2C_2H_5$:hexane fraction was dried on a flash evaporator and prepared for reverse-phase C_{18} HPLC (16). The instrumentation consisted of a model 680 Automated Gradient Controller, a U6K injector, a model 6000A pump, a model 45 pump (Waters), and an ISCO fraction collector. The extract was separated by HPLC on a 3.9 × 300 mm μBondapak C_{18} column (Waters) eluted at 1.5 mL/min with

the following gradient: 0 to 5 min, isocratic 28% CH_3OH in 1% aqueous acetic acid; 5 to 35 min, linear gradient from 28 to 86% CH_3OH; 35 to 36 min, 86 to 100% CH_3OH; 36 to 40 min, isocratic 100% CH_3OH. Fractions were collected every 2 min. The fractions were dried and GA-like biological activity detected with the dwarf rice bioassay (21), modified in that the first leaf sheath length was measured after 48 h. To further purify and separate coeluting GAs, fractions exhibiting biological activity were grouped within samples and separated again by HPLC on a 4.6 × 150 mm 10 μm Nucleosil $N(CH_3)_2$ column (Alltech) eluted isocratically with 0.1% acetic acid in CH_3OH (30). Fractions were collected every 2 min and bioassayed again. Recovery of the [^{3}H]GAs used as internal standards was determined by liquid scintillation spectrometry from aliquots of the resulting fractions.

In the GA_1 time course experiments, the $CH_3CO_2C_2H_5$: hexane fraction was processed by HPLC on the μBondapak column isocratically eluted with 22% CH_3OH in 1% acetic acid. The fractions containing the [^{3}H]GA_1 internal standard (21–23 min) were pooled and taken to dryness.

GC-MS-SIM

Concentrations of endogenous GAs in fractions exhibiting significant biological activity or those corresponding to the expected retention times of biologically inactive GAs (*e.g.* GA_8) were determined by GC-MS-SIM with deuterated internal standards as has been done previously (25, 26). Briefly, 50 ng of the appropriate deuterated GA ([17,17-$^2H_2$]$GA_{1,19,20,53}$ synthesized by Prof. Mander) was added to the vial before derivatization, except for those fractions from the experiments which already contained [^{2}H]GA_1 (time course experiments). The methyl esters were prepared with ethereal diazomethane and, after drying with N_2, freeze-drying, and resolubilizing in 50 μL pyridine, the samples were silylated with 100 μL of *N,N'*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical Co.). Samples were injected on-column on a 12 m, 0.2 mm i.d., 0.33 μm film thickness methyl silicone fused silica capillary column (HP-1, Hewlett-Packard). Hydrocarbon standards, prepared from Parafilm, were coinjected with the sample to determine KRIs (8). The GC (model 5890, Hewlett-Packard) oven, after a 1-min hold at 60°C, was programmed at 15°C/min to 200°C followed by 5°/min to 270°C. Helium head pressure was adjusted to give a linear velocity through the column of 40 cm/s. The GC was directly interfaced to a 5970B Mass Selective Detector (Hewlett-Packard). The MS operating parameters were: interface and source temperature, 280°C; 70 eV ionizing voltage; dwell time, 100 ms. The instrument was regularly tuned with the Autotune program and operated at a photomultiplier voltage 200 mV above the recommended voltage.

For the quantitation and characterization of endogenous GAs, three ions of the endogenous species were measured along with three ions for the deuterated species. Nonlabeled GA standards were not available and, consequently, standard curves could not be constructed. Quantitation was based on the peak area ratios, with endogenous peak areas corrected for any contribution from the deuterated species. For example, the relative abundance of m/z 506 as a percentage of m/z 508 in a standard run of [$^2H_2$]GA_1 is consistently less than 1% (usually undetectable), and in this case no nondeuterated GA_1 is contributed by the [$^2H_2$]GA_1 standard. Because an excess of [$^2H_2$]GA standards was used, contribution of heavy isotopes from endogenous GAs (*i.e.* m/z 508 for GA_1) was negligible and did not significantly affect the quantitation. In all cases, the most prominent ion (usually M^+) of the deuterated and nondeuterated species was used for the quantitation and the other ions for confirmation of identity.

Confidence in the GA data is based on the use of the double internal standard (^{3}H-GA_1 and ^{3}H-GA_4) in each sample, similar results in duplicate experiments (Tables I, II, and III) and uniform trends in the unduplicated time course experiments (Table IV; Fig. 3). Growth data and ABA data are from replicated samples and standard deviations are given.

ABA Determinations

ABA was extracted from plant tissue and analyzed by GLC as previously described (2). An internal standard of (±)-[G^{3}H] ABA (69 Ci/mmol, 0.07 ng ABA, Amersham) was added during the initial homogenization to determine ABA recoveries, which averaged 64%. The final ABA-containing extract was methylated with ethereal diazomethane. Methylated sam-

Table I. *Comparison of Soybean Hypocotyl Samples from a Full Spectrum GA Analysis*

At the time of extraction, 25,435 dpm of [^{3}H]GA_4 and 25,967 dpm of [^{3}H]GA_1 were added as internal standards and chromatographic markers. Biological activity was estimated with the dwarf rice bioassay in serial dilution. Data from one of two similar experiments.

Sample	0 h	24 h Wet	24 h Dry	60 h Dry
Number of segments	256	256	256	256
Tissue dry weight (g)	1.219	1.078	1.594	1.206
Internal standard recovery				
[^{3}H]GA_1	79.7%	91.0%	73.5%	95.9%
[^{3}H]GA_4	42.4%	43.6%	90.3%	41.3%
Total GA-like bioactivity (pg GA_3 equivalent mg dry weight^{-1})	215.5	172.5	55.1	117.0
GA_1-like bioactivity (HPLC fraction 12–16)	33.4	34.9	6.1	38.6

ples were dried under N_2 and dissolved in ethyl acetate with endrin as an internal standard. Quantification of the methyl ester of ABA was accomplished with a Tracor 540 gas chromatograph equipped with a ^{63}Ni electron capture detector and an OCI-3 on-column injector (Scientific Glass Engineering).

Quantitation and confirmation of the identity of ABA was also performed on some samples with the GC-MS system described above. GLC conditions were: Hewlett-Packard HP-1 capillary column as above held isothermally at 80°C for 1 min and then temperature programmed from 80 to 180°C at 25°C/min. At 180°C the rate of increase in temperature was changed to 10°C/min. ABA was quantified in samples and standards by monitoring m/z 190 and m/z 125 (ABA), and m/z 263 (base peak of endrin). Dwell time for both ions was 100 ms.

RESULTS

Endogenous Gibberellins in Soybean Hypocotyl Segments

Significant amounts of biologically active gibberellin-like activity were present in extracts from the elongating region of etiolated soybean hypocotyls. Up to 215 ng GA_3 equivalents per g dry weight were present (Table I). In addition to a peak of activity at 12 to 14 min, which corresponds to the retention time of ^{3}H-GA_1 in this system, a large peak of activity occurred around 22 to 30 min which corresponds to the retention times of GA_{19}, GA_{20}, and GA_{53} determined in separate runs (Fig. 1). The addition of deuterated GA standards was purposefully delayed until after this step so as not to compromise the bioassays, while the amount of ^{3}H-GAs added was insufficient to detect in the bioassay.

Sequential reverse phase C_{18} and Nucleosil $N(CH_3)_2$ HPLC yielded GA-bioactive fractions of sufficient purity to allow GC-MS-SIM. With the corresponding ^{2}H-labeled GA added, four GAs were detected: GA_1, GA_{20}, GA_{19}, and GA_{53} (Table II). Identification is based on similar chromatographic retention times in two HPLC systems, bioactivity on dwarf rice, cochromatography on capillary GC, and similar relative abundances of three characteristic ions on GC-MS. Endogenous levels of GA_{20}-like activity were relatively low, and the third, least abundant, ion (m/z 403) was detected in amounts too low to quantitate. However, the other ions (m/z 418 and 375) were present at the same retention time as [$^2H_2$] GA_{20} at similar abundances. We also noted three characteristic ions for GA_8 and GA_{44} at the expected KRI for each GA (11). Since we did not have standards of GA_8 and GA_{44}, we could not coinject standards with unknowns and compare relative abundances of characteristic ions from the same MS on the same analysis as was done for GA_1, GA_{19}, GA_{20}, and GA_{53} (Table II). For that reason, data are not given for GA_8 and GA_{44}. All of these detected GAs are members of the early 13-hydroxylation pathway.

The full spectrum of GA activity was compared in two experiments with similar results for the pattern of bioactivity (Fig. 1), the percentage of recovery (Table I), the four GAs detected (Table II), and the absence of other major GAs. No evidence was obtained for the presence of other 3-OH, 3,13-OH, or non-OH GAs, including GA_{12}, GA_{15}, and GA_7, which have been identified as endogenous in soybean cotyledons or as metabolites of applied GA_{12}-7-aldehyde in cotyledons (3). Thus, it appears that the early 13-hydroxylation pathway of GA metabolism predominates in dark-grown hypocotyl tissue of soybeans.

Growth Rates, Gibberellin Content, and Water Deficits

Hypocotyl elongation was unaffected when seedlings were transferred to water-saturated vermiculite but was severely inhibited after 24 h in seedlings transferred to low ψ_w vermiculite (Fig. 2). Recovery after rewatering is known to be complete within 30 min (2) and proceeded, thereafter, at a linear rate for at least 24 h (Fig. 2). Growth rate recovers with time in seedlings in low ψ_w vermiculite ([2]; Fig. 2).

Seedling gibberellin content varied both with time and water content of the vermiculite. GA_1 and GA_{19} were the most abundant endogenous GAs in both experiments (Table III). During the first 24 h after transfer, GA_1 levels increased over twofold in seedlings growing in saturated vermiculite, while GA_{19} levels remained constant or declined slightly.

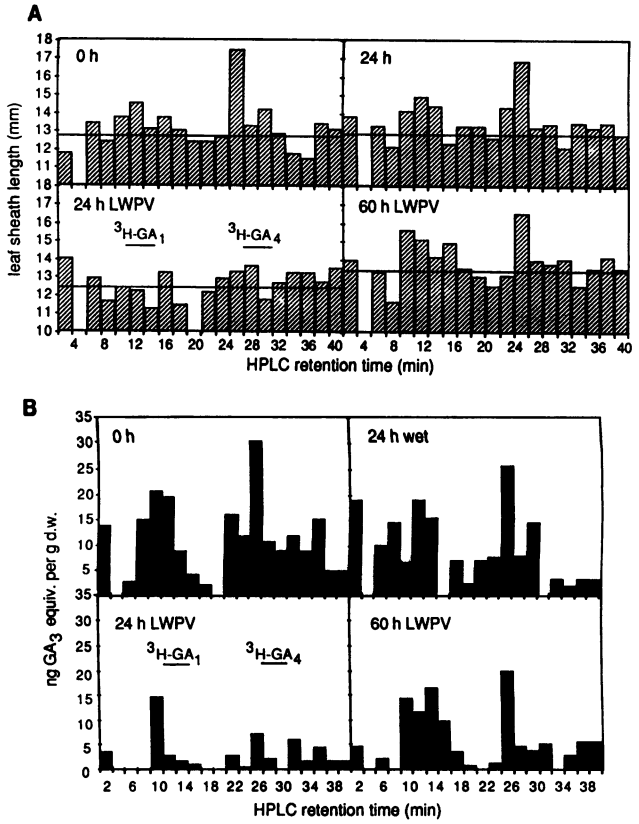

Figure 1. Bioactivity from soybean hypocotyl samples shown in Table I. A), Raw bioassay data; B), same data converted to ng GA_3 equivalents and adjusted for recovery. Bioassay of 1/100 dilution conducted after reverse phase C_{18} HPLC for second full spectrum GA experiment. Retention of ^{3}H-GA_1 was 12 to 14 min and of ^{3}H-GA_4, 28 to 30 min. Grouping for GC-MS combined fractions 2–8, 10–18, 20–32, and 34–40.

Table II. *Ions and Relative Abundances of Endogenous GAs from Elongating Sections of Dark-Grown Soybean Hypocotyls*

Data from two experiments presented in Figure 1 and Table I.[a]

Sample[b]	KRI	Ions (m/z) of [$^2H_2$]GA Internal Standard Abundance (relative abundance)			Ions (m/z) of Endogenous GA Abundance (relative abundance)		
		508	493	450	506	491	448
O h GA_1-like [$^2H_2$]GA_1IS	2673	735467 (100%)	70146 (9.5%)	217335 (29.6%)	656389 (100%)	52741 (8.0%)	115448 (17.6%)
		420	405	377	418	403	375
O h GA_{20}-like [$^2H_2$]GA_{20}IS	2493	643071 (100%)	125756 (19.6%)	572366 (89%)	6292 (100%)	trace	5465 (86.9%)
		464	436	376	462	434	374
24 h wet GA_{19}-like [$^2H_2$]GA_{19}IS	2604	6475 (6.3%)	103108 (100%)	64855 (67.8%)	2120 (2.3%)	93790 (100%)	53670 (57.2%)
		450	418	391	448	416	389
24 h wet GA_{53}-like [$^2H_2$]GA_{53}IS	2507	61775 (100%)	9956 (16.1%)	27980 (45.3%)	13165 (100%)	3544 (26.9%)	8203 (62.3%)

[a] Abundances for the [^{1}H] endogenous GAs have been corrected for the contribution from the [^{2}H] species. Sample names (*e.g.* 0 h GA_1-like) are based on the expected elution of the GA from HPLC and show which internal standard (IS) was used for quantitation. Trace-ion peak present but too small for accurate quantitation. [b] Seedlings harvestd after grown for 24 h in water saturated vermiculite after transplanting (wet) or harvested at the time of transplanting (0 h).

Although there were differences in the absolute amounts of GAs between the two experiments, the trends of changes were similar (Table III).

Growth of soybean seedlings in low ψ_w vermiculite for 24 h lowered the GA_1 content to 38% of the 0 h values and to 13% of the level observed in well-watered seedlings of the same age (Table III). The GA_{19} content also declined during growth in low ψ_w vermiculite. When the GA_1 content was calculated on a per segment basis, as opposed to a dry weight basis, the same trends were observed (Table III).

Time Course of Changes in GA_1 Levels, ABA Levels, and Growth Rates

In order to further characterize the relationship between GA_1 levels and elongation of soybean hypocotyls, additional experiments were conducted involving the GA biosynthesis inhibitor TCY. Both GA_1 and ABA levels were altered by TCY. GA_1 decreased by about one-third from 0 to 8 h and remained stable up to 24 h (Fig. 3). By 48 h, GA_1 had decreased to less than one-fifth of the original value (Fig. 3). GA_1 levels increased in control plants during the first 24 h after transfer to vermiculite without TCY (Table III). ABA levels of seedlings showed an increase 2 h after transfer to TCY and rose to 1200 pg/mg dry weight after 8 to 12 h (Fig. 3). Then at 48 h, ABA declined to a level twofold higher than it was initially. Thus, at 48 h, the growth rate of TCY-treated plants was reduced 30% from controls (Fig. 2), while GA_1 levels were reduced to about one-fifth of their original values (Fig. 3), and ABA levels were doubled (Fig. 3).

When seedlings exposed to low ψ_w vermiculite for 24 h were rewatered, substantial changes occurred in ABA and GA_1 levels. After 2 h, ABA levels decreased 10-fold, but the presence of 50 μM TCY, either with or without added GA_3, blocked much of this decrease (Table IV). GA_1 increased by 20% 2 h after rewatering and more than doubled by 8 h. The increase in GA_1 continued to almost 8-fold at 24 h (Table IV). The general trend was that rewatering increased the growth rate (Fig. 2), decreased the level of ABA (Table IV), and increased the level of GA_1 (Table IV). Another way to evaluate the short-term changes in GA_1 levels following rewatering is to average the data from all the experiments where relevant observations were made. The value for 0 h (four experiments) was 76.2 (±19.3) pg/mg dry weight, while after 24 h in low ψ_w vermiculite the level of GA_1 was 25.3 (±6.49) (three experiments). Two h after rewatering the average was 33.4 ± 12.6 (two experiments). This represents a 32% increase in GA_1 within 2 h after rewatering. Although the standard errors for the averaged 24 h and 2 h post-rewatering observations overlap, the fact that the trend for increased GA_1 levels clearly continues with time (Table IV) suggests that the differences at 2 h are real.

When seedlings were rewatered with 50 μM TCY, they elongated to only 57% of the length achieved by rewatered controls over 20 h (Fig. 2). Rewatering with 500 μM TCY resulted in the same level of inhibition, while rewatering with 5 μM TCY resulted in elongation indistinguishable from controls (data not given). The increase in growth rate of TCY-rewatered seedlings lagged behind that of seedlings given water

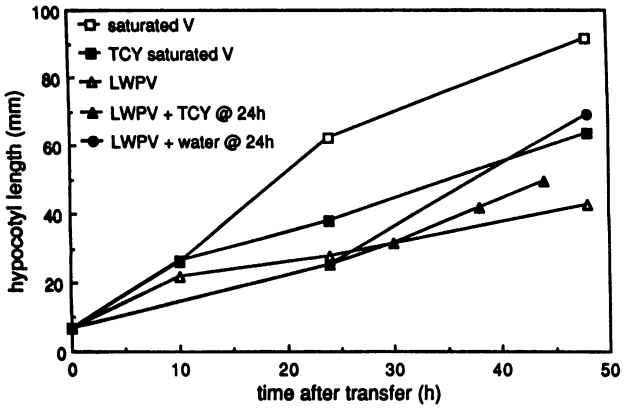

Figure 2. Hypocotyl elongation of soybean seedlings transplanted to water saturated or low ψ_w vermiculite at 48 h after planting (0 h). TCY was applied at 0 h or 24 h. Some plants in low ψ_w vermiculite were rewatered at 24 h. Data averaged from two experiments, four groups of 16 seedlings per data point. Standard error bars same size or smaller than data point symbols.

alone (Fig. 2). TCY reduced the rate of elongation of well-watered seedlings transferred to well-watered vermiculite with TCY, after a lag of at least 10 h (Fig. 2). The addition of 1 mM GA_3 to the 50 μM TCY rewater solution resulted in an elongation rate which was 91% of the control rate over the first 20 h (data not given).

Applied GA_3 and Hypocotyl Elongation

Soybean seedlings (48-h-old) grown under these conditions responded to GA_3. Over 48 h, 0.01 and 1 mM GA_3 promoted elongation by 14 and 25% over control rates (data not given). Applied ABA was capable of reversing this growth promotion (data not given).

DISCUSSION

GC-MS-SIM has been used to detect and quantify GAs when deuterium-labeled, authentic GAs were available as internal standards (25, 26). While more complete information would be available from a full-scan mass spectrum, the information available from a GC-MS-SIM analysis with heavy isotope-labeled internal standards leaves little chance for error. In the present case, reproducible patterns of GA-like bioactivity occurred after HPLC performed under two different sets of conditions. Compounds from these bioactive fractions coeluted with deuterium-labeled GA_{53}, GA_{19}, GA_{20}, and GA_1, and gave the proper relative abundances for three major fragments. Finally, quantitation from the GC-MS data yielded results consistent with the bioassay data. The fact that all of the GAs we recognized occurred in the same early 13-hydroxylation pathway (24) adds to the reliability of the work. Work with maize (24, 27), pea (13), rice (24), and Brassica (26) mutants implicate the early-13-hydroxylation pathway, particularly GA_1 as the regulator of shoot elongation (see review in Phinney [24]).

GA_1 and GA_8 had previously been tentatively identified in immature soybean seeds based on bioassay and HPLC data (18). In addition, Birnberg *et al.* (3) fed radiolabeled GA_{12}-7-aldehyde to soybean cotyledons and identified radiolabeled GA_{12} and GA_{15} in extracts. They also identified GA_7 as endogenous in cotyledons. Of these GAs we found evidence only for GA_1. The fact that we used etiolated tissue, which has been reported to contain higher GA levels than light-grown tissues in *Phaseolus coccincus* seedlings (4), and that we extracted only hypocotyls, may explain both the differences with the results of Birnberg *et al.* (3) and the success we had in detecting endogenous GAs.

Previous work with this system showed that transfer of seedlings from well-watered to low ψ_w vermiculite reduced hypocotyl elongation rates and increased ABA levels (2). In the present study, growth rates and GA_1 levels both declined in response to low ψ_w (Fig. 2; Table III). The rapidity of these changes cannot be determined from the data available. There is an approximately 8-h lag in inhibition of the growth rate below the control (Fig. 2), while the earliest GA analysis was at 24 h (Table III). There was some recovery in both the growth rate of seedlings in low ψ_w vermiculite by 48 h (Fig. 2; [2]) and in GA_1 levels at 60 h (Table III). Previous studies of the effects of water deficits on GA levels have been limited to bioassay and immunoassay data and have produced conflicting results (1, 12).

The dynamics of ABA and GA_1 levels were illustrated by changes in their contents after rewatering and/or treatment with the GA-synthesis inhibitor TCY. Rewatering resulted in a rapid recovery of growth (less than 30 min) (2). There was a rapid, dramatic decrease in ABA content at 2 h (Table IV), while GA_1 increased, but less dramatically, rising 32% in 2 h. The increase in GA_1 levels continued and at 24 h it had increased more than sixfold (Table IV).

TCY applied in the medium to well-watered seedlings

Table III. *Endogenous GA Content of the Elongating Section of Dark Growth Soybean Hypocotyls after Growth in Saturated (wet) or Low ψ_w (dry) Vermiculite*[a]

Sample	pg GA/mg Dry Wt (pg GA/Segment)			
	GA_1	GA_{20}	GA_{19}	GA_{53}
Experiment 1				
0 h	37.7 (0.24)	3.5	29.2	ND[b]
24 h wet	95.5 (0.47)	1.8	32.1	ND
24 h dry	21.3 (0.15)	ND	9.8	ND
24 h dry + 2 h wet	20.8 (0.15)	1.1	7.4	ND
Experiment 2				
0 h	97.0 (0.46)	0.9	80.3	ND
24 h wet	196.7 (0.83)	Trace	132.4	17.8
24 h dry	16.6 (0.10)	ND	Trace	ND
60 h dry	65.2 (0.31)	Trace	ND	ND

[a] After 48 h growth (0 h) seedlings were transferred from saturated (wet) to similar wet or low water potential (dry) vermiculite and harvested after 24 h. At that time one group of seedlings was rewatered and harvested 2 h later (experiment 1) and another group allowed to remain dry until harvested at 60 h (experiment 2). [b] Not detected; trace-ion peak present but too small for accurate quantitation.

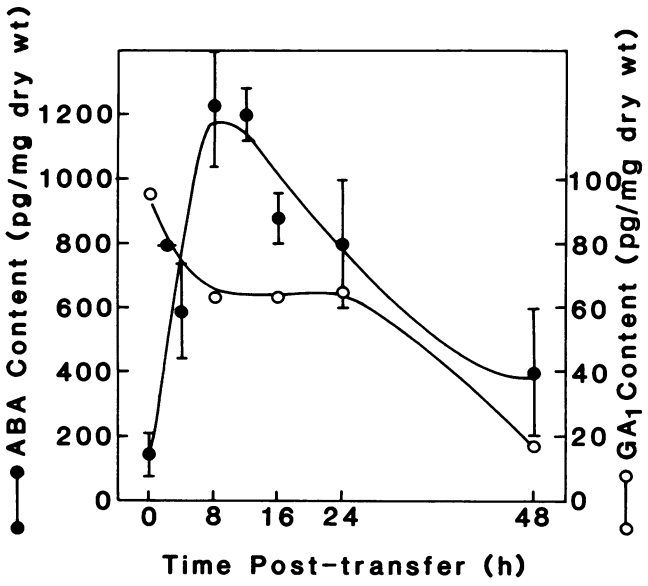

Figure 3. GA_1 and ABA content of TCY treated seedlings. Seedlings grown under standard growth conditions for 48 h were transferred to vermiculite saturated with 50 μM TCY (0 h). Each GA_1 determination (open circles) was made using the elongating section of 128 seedlings. ABA determinations (closed circles) represent the mean value and standard deviations between two repeats using 16 seedlings per measurement. The second ABA data point had a standard deviation smaller than the size of the symbol, and the curve was arbitrarily drawn between the second and third points.

initially increased the ABA level in hypocotyls. This is consistent with reports that TCY inhibits Cyt P-450 mixed function oxidase-mediated reactions, including the breakdown of ABA to phaseic acid (11, 33). Thus, TCY increases ABA levels by sparing its catabolism (Fig. 3). The TCY effect on GA_1 levels was less drastic; a decrease by about one-third was evident at 8 h. TCY blocks the oxidative reactions of GA biosynthesis between *ent*-kaurene and *ent*-kaurenoic acid (9). It inhibits cell elongation (10) and has been shown to cause dwarfed growth of *Agrostemma githago* while reducing the endogenous GA content (32). Assuming that TCY inhibits the recovery of GA_1 levels that occurs in rewatered plants (Table IV) as it does in well-watered plants (Fig. 3), then decreases in GA_1 and increases in ABA levels are consistent with what occurs in seedlings exposed to low ψ_w vermiculite.

While changes in growth rates and hormone levels (GA_1 and ABA) observed here are consistent with a causal relationship, the details of the temporal relationships raise questions. After rewatering, growth rates recover much more rapidly than recovery to well-watered levels of either GA_1 (Table IV) or ABA (2; Table IV). Details of the time course for GA_1 changes when exposed to low ψ_w are not available, but in this case ABA changes appear less rapid than changes in growth rate. From the data presented here and in an earlier report (2), we can tentatively advance one of the following hypotheses: (a) that changes in growth rate following major changes in water relations are not due to changes in GA or ABA levels, and thus these hormones reflect growth rates but do not initiate them, or (b) that changes in growth rates result from a complex interplay between physical pressure for expansion (turgor) and the yield threshold of the cell walls which is, in turn, influenced by hormones. The second explanation is consistent with recent findings on the mechanism of cell growth. Nonami and Boyer (23) found that, even in the presence of positive turgor, the collapse of the ψ_w gradient

Table IV. *Time Course of Changes in ABA and GA_1 Contents of Elongating Segments of Dark-Grown Soybean Hypocotyls following Rewatering*[a]

Time Post-rewater	Rewater Solution	ABA	GA_1
		pg/mg dry wt ± SD	*pg/mg dry wt (pg/segment)*
0 h	Control[b]	3679 ± 409	38 (0.23)
2 h	Water	419 ± 31	46 (0.27)
2 h	50 μM TCY	1767 ± 221	
2 h	50 μM TCY + 1 mM GA_3	1178 ± 254	
8 h	Water		85 (0.39)
16 h	Water		200 (0.79)
24 h	Water		242 (0.96)

[a] Endogenous ABA and GA_1 content was determined for hypocotyl zone of elongation of plants grown under standard growth conditions for 48 h, transferred to low ψ_v vermiculite for 24 h, then rewatered with one of three solutions. Water contained 0.1 mM $CaCl_2$, which is what the tetcyclacis plus GA_3 was dissolved in. [b] Harvested immediately before rewatering of other seedlings.

from the xylem to the expanding cells apparently inhibited elongation. They used the same experimental system used in the present investigation. If similar abrupt changes occur in the ψ_w gradients and levels of turgor upon rewatering, then those changes could explain the initial effects on growth rates. Taylor and Cosgrove (29) have recently shown that GA_3 increases the elongation of cucumber seedlings by accelerating biochemical processes which cause wall relaxation rather than by altering osmotic or turgor pressure. However, these changes take several hours to be expressed and may be related to longer term alterations of growth at the whole plant level by water deficit. In further regard to the second explanation, although we have obtained evidence for changes in GA levels with alteration of plant ψ_w, we do not know whether average concentrations in bulked tissue such as whole hypocotyls parallel physiologically active levels at the active site(s). Thus, a decrease in absolute GA_1 levels by one-third may reflect a greater or lesser change in GA_1 in the target cells. For example, ABA content of leaf epidermal layers increase before ABA accumulation is evident in whole leaves under water deficits (31). More information, in addition to that in this paper, will be needed to clarify the roles of ABA and GA_1 in low ψ_w alteration of growth.

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