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COMPARISON OF THE ENDOGENOUS GIBBERELLINS IN THE SHOOTS AND ROOTS OF VERNALIZED AND NON-VERNALIZED CHINESE SPRING WHEAT SEEDLINGS

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Key Word Index—*Triticum aestivum*; Gramineae; Chinese Spring wheat; shoots; roots; vernalization; gibberellins; HPLC; GC-SIM.

Abstract—Endogenous gibberellins (GAs) in Chinese Spring wheat seedlings were isolated by high performance liquid chromatography (HPLC) and identified by combined capillary gas chromatography-selected ion monitoring (GC-SIM). Gibberellins A_1, A_3, A_{19}, A_{20}, A_{44}, and A_{53} were identified in the shoots, A_{19} and A_{20} in the roots. The identification of these 13-hydroxylated GAs demonstrates the presence of the early-13-hydroxylation pathway in wheat seedlings. Based on peak area of total ion response of five characteristic ions by GC-SIM, the approximate levels of GAs in the shoots is $GA_{44} > GA_{19} > GA_1 = GA_3 > GA_{20}$ for the non-vernalized wheat seedlings, and $GA_{44} > GA_{19} > GA_{53} = GA_3 > GA_1 = GA_{20}$ for the vernalized wheat seedlings. The C_{20} GAs, GA_{53}, GA_{44} and GA_{19}, are present in shoots of the vernalized (flowering) wheat seedlings at somewhat higher levels than that in the non-vernalized (rapidly growing) wheat seedlings. Approximate levels of the C_{19} GAs, GA_{20}, GA_1 and GA_3 were lower in the shoots of the vernalized wheat seedlings than in the non-vernalized wheat seedlings. The conversion of GA_{19} to GA_{20} (C_{20} to C_{19} GAs) may be a rate-limiting step in the vernalized wheat seedlings.

INTRODUCTION

Identification of endogenous GAs in developing wheat (*Triticum aestivum* L.) grains has been reported [1, 2]. The GAs identified include GA_{15}, GA_{17}, GA_{19}, GA_{20}, GA_{24}, GA_{25}, GA_{44}, GA_{54}, GA_{55}, GA_{60}, GA_{61} and GA_{62}. Both GA_1 and GA_3 have also been identified in the vegetative tissues of mature [3] and young [4] wheat plants.* The metabolism of [^{3}H]GA_1 in wheat seedlings to material that co-chromatographed with [^{3}H]GA_8 and a conjugate of [^{3}H]GA_8 has been reported [6]. In this communication, we would like to report the identification of GAs in the shoots and roots of vernalized and non-vernalized Chinese Spring wheat seedlings. Most winter wheat cultivars require vernalization of the seedlings for heading, and somewhat atypically, Chinese Spring wheat also requires vernalization for normal heading. The length of the vernalization period affects the number of spikelets of Chinese Spring wheat [7]. The wheat seedlings in vernalized (flowering) condition grow much slower than those in non-vernalized condition. The involvement of GAs in vernalization of plants either directly or indirectly has been shown by the use of exogenous GA application, by the use of various growth retardants and by the examination of endogenous levels of GAs [8, 9].

RESULTS AND DISCUSSION

Fractions from analytical C_{18} HPLC were assayed for GA activity in the Tan-ginbozu dwarf rice bioassay (Fig. 1). Biologically active fractions and fractions eluting at the retention times (R_t) of certain authentic GAs were derivatized and examined by combined capillary GC-SIM (Table 1). The results showed that GA_1, GA_3, GA_{19}, GA_{20}, GA_{44} and GA_{53} in the shoots and GA_{19} and GA_{20}

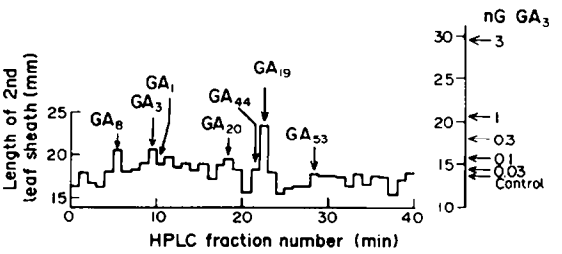

Fig. 1. Tan-ginbozu dwarf rice bioassay of the analytical C_{18} HPLC fractions from the shoots of non-vernalized Chinese Spring wheat seedlings. The bioassay of the shoots of vernalized seedlings, and the roots of both vernalized and non-vernalized seedlings were similar to this figure. For the sample preparation and HPLC conditions, see Experimental.

*After submission of this paper, Dr. Lenton kindly sent a copy of chapter [5] in the book from the Long Ashton Symposium. It includes the information on the identification of GA_1, 3epi-GA_1, GA_3, GA_8, GA_9, GA_{17}, GA_{19}, GA_{20}, GA_{29} and GA_{44} in internodes of wheat and some quantitative data on GA_{20} and GA_1 in internodes and ears.

Table 1. Comparison of GC-SIM data for endogenous GAs in wheat seedlings to GA standards

HPLC Fraction*	Presumptive GA	Kovat's indices	Monitored ions with relative abundances in parentheses				
GA_3 (9.38)†		2696	504 ([M]$^+$, 100)	489 (10)	370 (14)	347 (16)	208 (50)
10 (s, n)‡	GA_3	2694	504 ([M]$^+$, 100)	489 (9)	370 (14)	347 (20)	208 (48)
10 (s, v)	GA_3	2693	504 ([M]$^+$, 100)	489 (8)	370 (11)	347 (20)	208 (43)
GA_1 (10.51)		2669	506 ([M]$^+$, 100)	491 (11)	448 (24)	377 (23)	313 (15)
11 (s, n)	GA_1	2672	506 ([M]$^+$, 100)	491 (10)	448 (24)	377 (30)	313 (9)
11 (s, v)	GA_1	2671	506 ([M]$^+$, 100)	491 (10)	448 (20)	377 (43)	
GA_{20} (18.97)		2490	418 ([M]$^+$, 100)	403 (16)	375 (64)	359 (17)	301 (16)
19–20 (s, n)	GA_{20}	2486	418 ([M]$^+$, 100)	403 (15)	375 (68)	359 (18)	301 (16)
19–20 (r, n)	GA_{20}	2489	418 ([M]$^+$, 100)	403 (18)	375 (64)	359 (15)	301 (13)
18–19 (s, v)	GA_{20}	2489	418 ([M]$^+$, 100)	403 (23)	375 (66)	359 (28)	301 (19)
19–20 (r, v)	GA_{20}	2488	418 ([M]$^+$, 100)	403 (16)	375 (61)	359 (11)	301 (15)
22 (s, n)	GA_{44}	2791	432 ([M]$^+$, 73)	417 (12)	373 (26)	238 (43)	207 (100)
21 (s, v)	GA_{44}	2791	432 ([M]$^+$, 67)	417(11)	373(23)	238(39)	207(100)
GA_{19} (22.41)		2597	434 (100)	402 (40)	375 (65)	374 (76)	345 (37)
23 (s, n)	GA_{19}	2600	434 (100)	402 (39)	375 (65)	374 (78)	345 (38)
23 (r, n)	GA_{19}	2595	434 (100)	402 (38)	375 (61)	374 (71)	345 (34)
22 (s, v)	GA_{19}	2596	434 (100)	402 (40)	375 (62)	374 (77)	345 (34)
23 (r, v)	GA_{19}	2597	434 (100)	402 (35)	375 (57)	374 (68)	345 (32)
GA_{53} (27.81)		2502	448 ([M]$^+$, 100)	389 (64)	251 (60)	241 (53)	235 (45)
28 (s, v)	GA_{53}	2500	448 ([M]$^+$, 100)	389 (63)	251 (54)	241 (45)	235 (39)

*Analytical C_{18} HPLC fraction, 1 fraction/min. GA standards and HPLC fractions were derivatized as MeTMSi for GC-SIM.

†HPLC retention time (min) of the GA standard.

‡s = shoots, r = roots, v = vernalized wheat seedlings, n = non-vernalized wheat seedling.

in the roots are native to wheat seedlings. Our identifications are based not only on combined capillary GC-SIM, and Kovat's retention indices (R_I), but also on R_ts on the analytical reversed-phase C_{18} HPLC. For example, GA_1, GA_{29} and GA_{34} have very similar mass spectra and close R_Is as their methyl ester TMSi ethers [10]. These three GAs can be easily separated by C_{18} HPLC [11] and thus the GA-like substance in Fraction 11 (Fig. 1) is GA_1 not GA_{29} or GA_{34}. Although GA_3 is a commonly used GA standard in our laboratory control experiments and root samples showed no trace of GA_3 and thus its presence in wheat seedling shoot is probably not due to contamination. Gibberellin A_5 is the only GA thus far known to yield GA_3 in higher plants [12, 13], and it is not native (as yet) to wheat. We did not attempt to scan the other GAs native to developing wheat grain [1, 2], because such GA bioassay activity (Fig. 1) was low and we did not know which HPLC fractions should be used to scan for them, since we lacked appropriate standards.

The identification of the 13-hydroxylated GAs listed in Table 1 in both vernalized and non-vernalized wheat seedlings suggests the presence of an early-13-hydroxylation pathway similar to that occurring in corn [14], rice [15] and peas [16]. Gibberellins A_{17} and A_{29}, also found in plants with the early-13-hydroxylation pathway were not detected in the materials examined. Fraction 5 (Fig. 1), where GA_8 should elute, showed too much background in GC-SIM, and the identification of GA_8 by GC-SIM in this fraction was impossible.

Both GA_1 and GA_3 have been previously identified in the vegetative tissues of mature wheat plants [3] and seedlings of the *Rht* lines [4]. Gibberellins A_{15}, A_{17}, A_{19}, A_{20}, A_{24}, A_{25} (tentative), A_{44}, A_{54}, A_{55}, A_{60}, A_{61} and A_{62} have been previously identified in wheat grains [1, 2]. The GAs identified in the present study, except GA_{53}, have thus been previously identified from wheat. The GAs we found in wheat seedlings are in the early-13-hydroxylation pathway in other species [17, 18] with the exception of GA_3. Gibberellin A_8 has yet to be identified in wheat seedlings, however, the conversion of [^{3}H]GA_1 to a labelled compound that co-chromatographed with GA_8 has been reported [6]. Gibberellin A_1 may be dehydrogenated to give GA_3.

The relative amounts of the endogenous GAs in wheat seedlings shown in Table 2 were determined by comparing the peak areas at the R_I of the GAs in the total ion current (TIC) response (5 ions). The relative abundances of the monitored ions were similar to those of the GA standards, however, the relative amounts shown in Table 2 are approximate. The most abundant GA in the shoots was GA_{44}, while GA_{44} was not detected in the roots. The decreasing order of amounts of GAs in the shoots was $GA_{44} > GA_{19} > GA_1 = GA_3 > GA_{20}$ for the non-vernalized wheat seedlings, $GA_{44} > GA_{19} > GA_{53} = GA_3 > GA_1 = GA_{20}$ for the vernalized wheat seedlings. The concentrations of GA_{53}, GA_{44} and GA_{19}, C_{20} GAs of the early-13-hydroxylation pathway, in the shoots of the vernalized wheat seedlings were higher than those in shoots of the non-vernalized seedlings. The concentrations of GA_{20} and GA_1, C_{19} GAs of the early-13-hydroxylation pathway, and GA_3, in the shoots of the vernalized seedlings were lower than those in the non-vernalized seedlings. The higher levels of GA_1 and GA_3 in non-vernalized seedlings were consistent with the accelerated growth of non-vernalized seedlings based on the assumption that these two GAs are effectors for stem elongation in wheat as is the case in maize and pea for GA_1 [19, 20]. The accumulation of C_{20} GAs in the slower-

Table 2. Relative amounts of the endogenous GAs in wheat seedling

Sample	GA identified	Relative amount*
Shoots (non-vernalized)	GA_3	+ +
	GA_1	+ +
	GA_{20}	+
	GA_{44}	+ + + +
	GA_{19}	+ + +
Roots (non-vernalized)	GA_{20}	+
	GA_{19}	+ + +
Shoots (vernalized)	GA_3	+
	GA_1	trace
	GA_{20}	trace
	GA_{44}	+ + + + +
	GA_{19}	+ + + +
	GA_{53}	+
Roots (vernalized)	GA_{20}	trace
	GA_{19}	+ + +

* Measured as the relative area of the total ion current response (5 ions shown in Table 1). Each additional '+' represents an approximate doubling of the amount. Different amounts of shoots and roots were extracted, hence the only valid comparisons are shoots vernalized vs non-vernalized and roots vernalized vs non-vernalized.

growing vernalized plants vs C_{19} GAs in faster-growing non-vernalized plants also suggests that the conversion of C_{20} to C_{19} GAs (GA_{19} to GA_{20}) in the biosynthetic pathways (loss of C_{20} and lactonization) is a rate-limiting step controlling the level of the C_{19} GAs, GA_1 and GA_3, in wheat seedlings.

The build-up of C_{20} GAs in the vernalized seedlings could indicate (a) a partial block of the C_{20} to C_{19} step in vernalized plants, or additionally (b) a rapid increase in overall GA biosynthesis/turnover, the build-up of C_{20} GAs thereby reflecting the increased GA turnover [21]. The turnover rate of GAs could not be measured in this study. The increased levels of GA_1 and GA_3 in non-vernalized seedlings would fit with (a) above, or also fit with a reduced metabolism/catabolism of GA_1 and GA_3, albeit without an increased GA turnover. The levels of C_{20} and C_{19} GAs in roots appear to be affected by vernalization in a similar manner. It would appear that increased levels of GA_1 and GA_3 are not required for normal heading induced by vernalization, vernalization decreased the levels of these GAs in the shoots. Both the vernalized and non-vernalized Chinese Spring wheat seedlings would initiate inflorescences. The role of GA in normal heading of Chinese Spring wheat caused by vernalization is still unknown [8, 9].

EXPERIMENTAL

Plant materials. The seeds (50 g, 1400 seeds) of Chinese Spring wheat (*Triticum aestivum* L.) were aerated in water over night and then sown in vermiculite. For vernalized wheat seedlings (about 12 cm tall, the 3rd leaf just starting to emerge), wheat seeds (50 g) were sown and grown in a growth chamber at 8° and 8 hr photoperiod with light intensity of about 200 μmol/m^2/sec for 28 days [7]. For the non-vernalized wheat seedlings (about 14 cm tall, the 3rd leaf not emerged), wheat seeds (50 g) were sown and grown in growth chamber at 20° and 14 hr photoperiod with light intensity of about 400 μmol/m^2/sec for 8 days. Both the vernalized (28-day-old) and non-vernalized (8-day-old) seedlings were at the same developmental stage (approximately the same weights and heights) and without initiation of inflorescences. The seedlings in non-vernalized condition grew much faster than those in vernalized condition. The seedlings were cut at the surface of the vermiculite to obtain shoots. Roots, excluding seeds, were obtained by washing the vermiculite away and then dried by squeezing the roots wrapped with tissue paper by hand.

Extraction procedure. All glassware was cleaned with chromic acid before use. The shoots of vernalized and non-vernalized seedlings (133 g of each, fr. wt; fr. wt: dry wt; 12:1) were handled separately. The shoots were homogenized with 80% MeOH (4°, 1 l). Extracts were filtered through sintered glass funnels (coarse), and the residual material was stirred in MeOH (1 l) overnight at room temp. The mixtures were filtered, the filtrates were combined and then reduced to an aq. phase by rotary evaporation below 40°. The aq. phases (about 200 ml) were adjusted to pH 8.0 and partitioned against hexane (5 × 100 ml) and then against H_2O-saturated *n*-BuOH (5 × 100 ml; kept for future analysis of neutral conjugated GAs). Finally the aq. phases were adjusted to pH 3.0 and partitioned against EtOAc (5 × 100 ml). The acidic EtOAc fractions were dried *in vacuo* (vernalized, 274 mg; non-vernalized, 343 mg). The extraction procedure for the roots (42 g each) was similar to that of the shoots except that half of the H_2O and solvent were used. For roots, the dried acidic EtOAc fraction weighed 9.5 mg (vernalized) and 28 mg (non-vernalized).

PVPP slurry purification. The acidic EtOAc fractions were dissolved in about 250 ml 0.1 M Pi buffer, pH 8.0. Polyvinylpolypyrrolidone (PVPP, Sigma, St. Louis, MO.) was added to the soln (50 mg/ml of buffer) and the mixtures were stirred for 30 min [22]. PVPP was removed by filtration through a coarse sintered glass funnel. The PVPP was washed with about 50 ml of the buffer. This PVPP slurry treatment was repeated a total of 3 times. The final filtrate was adjusted to pH 2.5 and was extracted with EtOAc (5 × 200 ml). The combined EtOAc extracts were dried. Yields: shoots, vernalized, 142 mg; shoots, non-vernalized, 192 mg; roots, non-vernalized, 10 mg. PVPP purification of the vernalized root extract was not done because its mass (9.5 mg) was such that it could be injected directly onto the analytical C_{18} HPLC.

Preparative C_{18} HPLC. The PVPP purified acidic EtOAc fractions from the shoots were further purified by prep. HPLC using a reversed-phase C_{18} column (1 cm × 25 cm, Spherisorb S5 ODS2, Phase Separations, Inc., Norwalk, CT). The column was eluted with a linear gradient from 35% MeOH (containing 0.05% HOAc) to 100% MeOH (containing 0.05% HOAc) in 40 min at a flow rate of 4 ml/min. The sample was dissolved in 600 μl of the initial eluent and was filtered through a filter unit (pore size: 0.45 μm, material: polypropylene, Millex-HV$_4$ filter unit, Millipore, Bedford, MA) prior to injection. The filtered soln was injected as two separate portions as the prep. column accomodates a maximum of about 100 mg. Fractions of 4 ml were collected and fraction Nos 6–30 were combined and dried (shoots, vernalized, 13.3 mg; shoots non-vernalized, 9.8 mg). These fractions would include GAs ranging in polarity from GA_8 to GA_9. The non-polar GAs, GA_{12} and GA_{12}-aldehyde would, however, be excluded. The extracts from the roots were not subjected to this step. Analytical reversed-phase C_{18} HPLC was used at the last purification step before GC-SIM. The maximum load of the analytical column was about 10 mg and some purification steps were omitted before analytical C_{18} HPLC if the residue was less than 10 mg.

Analytical C_{18} HPLC. The fractions were further purified and separated on a reversed-phase C_{18} column (0.46 cm × 25 cm,

Ultrasphere ODS, Beckmann, San Ramon, CA). The eluent used was a linear gradient from 35% MeOH (containing 0.05% HOAc acid) to 100% MeOH (containing 0.05% HOAc) at a flow rate of 1 ml/min. The sample was dissolved in 250 μl of initial eluent and filtered before injection. Fractions were collected at 1 fraction/min. Each fraction was transferred to a Reacti-vial (1 ml) and was then dried with a N_2 evaporator. The residue was dissolved in 20 μl Me_2CO–H_2O (1:1) and 1 μl of the soln was tested with the Tan-ginbozu dwarf rice bioassay [23] using 5 rice plants for each fraction. The results of the bioassay are shown in Fig. 1.

Derivatization and GC-SIM. Biologically active fractions, fractions eluting at the R_t of certain authentic GAs, or GA standards were dissolved in 100 μl MeOH and methylated with excess ethereal CH_2N_2. The mixtures were dried under N_2 and then under high vacuum. Trimethylsilyl (TMSi) ethers of the methylesters were prepared by the addition of 25 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Pierce Chemical Co., Rockford, IL) at room temp.

The derivatized HPLC fractions and GA standards were analysed with a Hewlett-Packard 5970B quadrupole-based mass selective detector (MSD). The MSD was operated in the scan mode for the GA standards and hydrocarbon series (C_{22}-C_{32}) to obtain full spectra and R_t data for R_I of the available GA standards. The SIM mode and R_I were used for the identification of the GAs present in the analytical C_{18} HPLC fractions of the plant extracts. Samples (2 μl/25 μl in MSTFA) were introduced into the MSD using a HP 5790A GC with a capillary direct interface operated at 280°. A 12 m × 0.2 mm I.D. dimethyl silicone crosslinked column, 0.33 μm film thickness (Hewlett-Packard) was used in these experiments with He as the carrier gas (about 1 ml/min). Column temp. was programmed from 60–180° at 25°/min then 6°/min to the final temperature of 280° in the splitless mode. The injection temperature was 260°. The ionization voltage was fixed at 70 eV, the ion source operated at fixed nominal temperature of 250° and the head pressure was 7.0 psi. Data were analysed using a HP 9816S computer, HP 9133 Winchester Drive and printer. An approximation of the relative amounts of the GAs was obtained by comparing the areas of peaks in the TIC response (SIM). This assumes equal loses during work-up and equal derivatization and that equal amounts of different GAs injected yield approximately equal TIC when ions specified in Table 1 are monitored on SIM. We have shown that the equal amounts of GA_1, GA_3, GA_{19} and GA_{20} yield approximately equal TIC (better than 70%).

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