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IDENTIFICATION OF GIBBERELLINS A_1 AND A_{19} FROM *POPULUS BALSAMIFERA* × *P. DELTOIDES*

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Abstract—Gibberellins (GAs) A_1 and A_{19} were identified from young shoots of a naturally occurring interspecific poplar hybrid (*Populus balsamifera* × *P. deltoides*). Detection of GA-like substances was accomplished by the Tanginbozu dwarf rice microdrop assay after sequential SiO_2 partition chromatography and reversed-phase C_{18} HPLC with internal standards of [^{3}H]GA_1 and [^{3}H]GA_{20}. The radioactive and/or bioactive peaks were subjected to capillary gas chromatography-selected ion monitoring (GC-SIM) with internal standards of [17, 17-^{2}H]GAs A_1, and A_{19}. Identifications were thus based on retention times relative to authentic standards on three sequential chromatographic systems as well as the relative intensities of characteristic ions on GC-SIM. Similar analysis by bioassay and GC-SIM with [17, 17-^{2}H]GA_{20} also suggested the presence of GA_{20}.

INTRODUCTION

Due to their rapid growth rate, hybrid poplars are receiving increasing attention as sources of fibre for pulp and paper and construction composites, or biomass for energy production. Gibberellins (GAs) are known to control shoot elongation in several herbaceous higher plants [1], and evidence suggests similar control in the woody angiosperm, *Salix pentandra* [2]. The exogenous application of gibberellic acid (GA_3) can accelerate poplar growth suggesting a role of GAs in the regulation of shoot growth in hybrid poplar [3]. Additionally, the hypothesis that GAs are involved in the control of heterosis (hybrid vigour) in maize [4, 5] also provides a potential link-up between GAs and shoot growth rate in other plant hybrids.

Prior to an assessment of the possible correlation between GA level and shoot growth rate in hybrid poplars, the native GAs present in the elongating shoot must be identified. Poplars (*Populus*) and willows (*Salix*) are both members of the Salicaceae, and the major native GAs of *Salix pentandra* have recently been characterized as GA_1, GA_{19}, GA_{20}, and GA_{29} [6], all of which are characteristic of the early C-13 hydroxylation pathway.

We have recently demonstrated a relatively rapid procedure for identifying native GAs based on co-chromatography with [^{3}H]-labelled internal standards on sequential liquid chromatography columns followed by co-chromatography with [^{2}H]-labelled internal standards on capillary gas chromatography-selected ion monitoring (GC-SIM) [7]. In the present study these procedures were used to identify the native GAs of hybrid poplar, with particular attention directed towards the presence of biologically active members of the early 13-hydroxylation biosynthetic pathway.

RESULTS AND DISCUSSION

Three major regions of GA-like activity were observed from the stepwise-eluted SiO_2 partition columns (Fig. 1). The first region (I) co-chromatographed with authentic [^{3}H]GA_{20}, the second (II) eluted with authentic [^{3}H]GA_1, and the third region (III) eluted in the MeOH wash where GA glucosyl conjugates will elute.

The SiO_2 partition column region I (Fig. 1) was subjected to reversed-phase C_{18} HPLC and yielded a major bioactive peak which co-chromatographed with authentic [^{3}H]GA_{20} (Fig. 2a, Ib) as well as at least two minor peaks of GA-like activity. SiO_2 column region II (Fig. 1) was resolved through reversed-phase C_{18} HPLC into four peaks of GA-like activity (Fig. 2b), the second of which (Fig, 2b, IIb) co-chromatographed with authentic [^{3}H]GA_1, while the third peak (Fig. 2b, IIc) eluted at the expected R_t of GA_{19} [5, 7, 8].

A GC-SIM analysis of the GA_1-like peak (Fig. 2b, IIb) confirmed the presence of GA_1 (Fig. 3, Table 1). Three of the characteristic ions of GA_1 were observed at the exact R_t of the internal standard [^{2}H](d_2)GA_1, and abundances were reasonably similar to abundances from the internal standard. Additionally, in a separate analysis, five ions characteristic of GA_1 MeTMSi were detected (*m/z* 506, 491, 448, 377, and 305) at the R_t of [^{2}H](d_2)GA_1 MeTMSi (*m/z* 508 also monitored). GC-SIM analyses using deuterated internal standards of the other principal GA-like peak (Fig. 2b, IIc) confirmed the presence of GA_{19} (Table 1). GA_{19} was identified from two separate two-fraction groupings from HPLC peak IIc, which was broad and irregularly shaped, presumably due to the presence of additional biologically active substances (GAs or an inhibitor which could also result in the peak depression (Fig. 2b, IIc)). A second GC-SIM analysis was performed

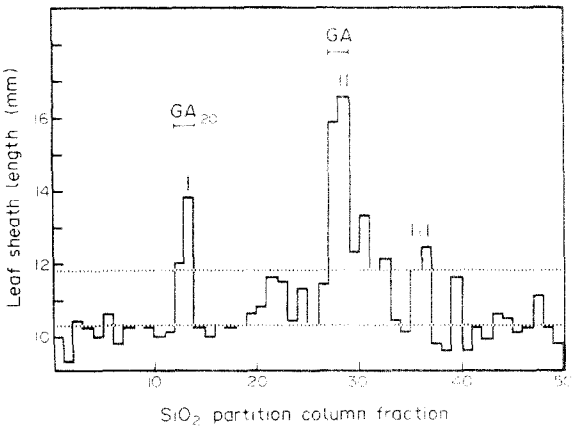

Fig. 1. Elution of GA-like substances as determined with the Tan-ginbozu dwarf rice microdrop assay, from stepwise-eluted SiO_2 partition columns loaded with purified methanol extracts of hybrid poplar shoots. Elution regions of authentic [^{3}H]GAs are shown above the profile of GA-like activity. The lower dashed line represents the leaf sheath length of control seedlings while the upper dashed line represents the response to 10^{-4} μg GA_3 per plant.

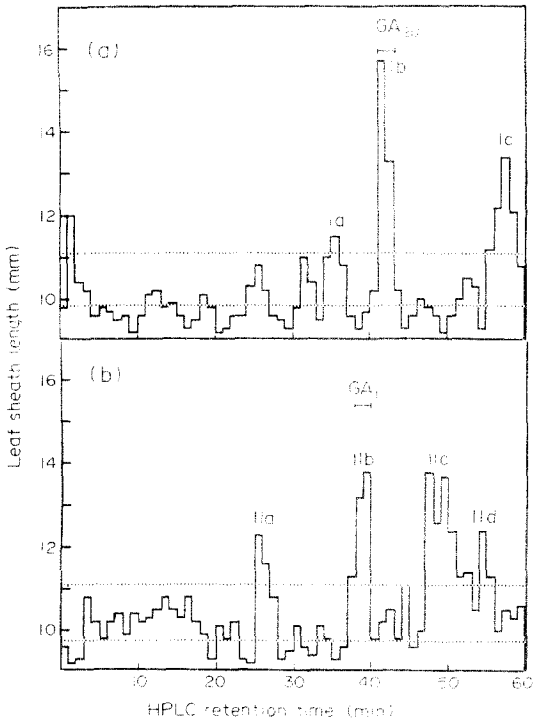

Fig. 2. Elution of GA-like substances as determined with the Tan-ginbozu dwarf rice assay, from gradient eluted reversed-phase C_{18} HPLC columns loaded with (a) region I, or (b) region II, of GA-like activity from SiO_2 columns loaded with extracts of hybrid poplar (Fig. 1). The R_ts of authentic [^{3}H]GA_1 and [^{3}H]GA_{20} are shown above the appropriate profiles of GA-like activity. The lower dashed line represents the leaf sheath length of control seedlings while the upper dashed line represents the response to 10^{-4} μg GA_3 per plant. The two graphs resulted from analyses with separate C_{18} columns and hence, R_ts from the two graphs are not directly transferable.

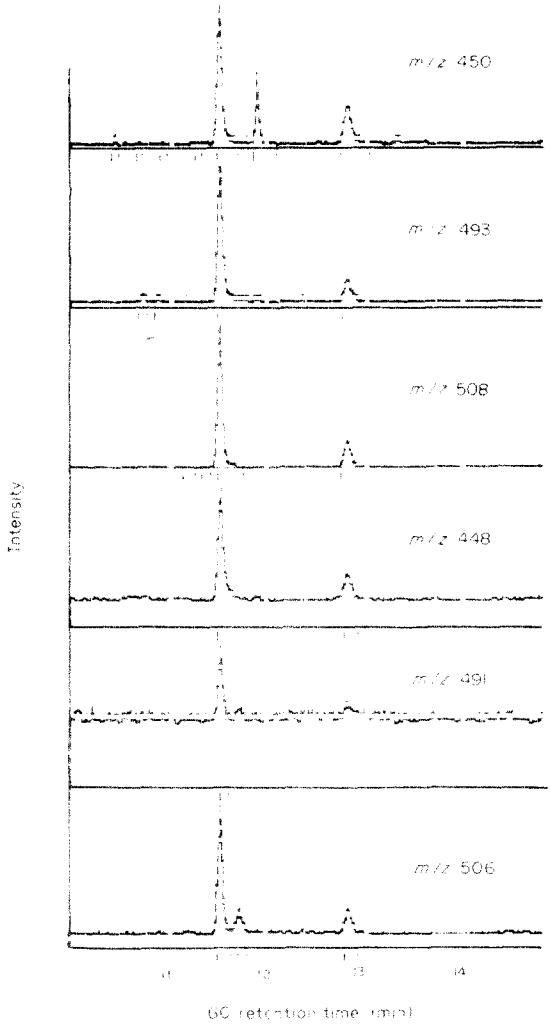

Fig. 3. An example of the capillary GC-SIM profile for authentic [^{2}H](d_2)GA_1 MeTMSi co-injected with putative GA_1 MeTMSi from hybrid poplar. In this figure, the intensity sensitivities were adjusted for the six ions in order to present full scale (FS) deflections for the GA_1 peak. Specific sensitivities for the six plots are: ion 450 (FS = 20), 493 (10), 508 (08), 448 (4), 491 (1), 506 (5).

on a second extract with a GA-like peak chromatographically similar to peak IIc. However, no [^{2}H](d_2)GA_{19} was added prior to derivatization. The presence and relative abundance [9] of the six ions monitored further confirmed the presence of GA_{19} (Table 2).

There was no peak tailing or peak shoulders in the GC-SIM traces for the ions selected (Fig. 3). These peaks contained contributions from both the putative poplar GAs and from the authentic (d_0)GAs associated with the [^{2}H](d_2)GA internal standards (Table 1). The R_ts of the putative poplar GAs on the capillary GC were thus identical to the R_ts of the internal standards. Analysis by GC-SIM of a third major GA-like peak (Fig. 2a, 1b) suggested the presence of GA_{20} although ion ratios were slightly different from those expected. Thus, further analysis of the putative GA_{20} is warranted.

Table 1. Capillary GC-SIM of authentic MeTMSi $[^2H](d_2)GA_1$, GA_{19} or GA_{20} co-injected with similarly derivatized fractions showing GA-like activity after sequential SiO_2 partition (Fig. 1) and reversed-phase C_{18} HPLC (Fig. 2) columns loaded with purified extracts of hybrid poplar

GA	Retention time (min)	Relative abundance of peak (percentage abundance in parenthesis)				
m/z	508	493	450	506	491	448
Putative poplar $GA_1 + [^2H](d_2)GA_1$	11.58 422	41	78	16	1	12
$[^2H](d_2)GA_1$	11.58 422(100)	34(8)	51(12)	1	0	7
corrected intensities for putative poplar GA_1				15(100)	1(7)	5(33)
m/z	464	436	376	462	434	374
Putative poplar $GA_{19} + [^2H](d_2)GA_{19}$	11.43 15	229	223	1	18	18
$[^2H](d_2)GA_{19}$	11.43 16(7)	229(100)	169(74)	0	6	10
corrected intensities for putative poplar GA_{19}				1(8)	12(100)	8(67)
m/z	420	405	377	418	403	375
Putative poplar $GA_{20} + [^2H](d_2)GA_{20}$	10.21 980	140	668	12	2	20
$[^2H](d_2)GA_{20}$	10.21 980(100)	127(13)	570(58)	4	0	12
corrected intensities for putative poplar GA_{20}				8(100)	2(25)	8(100)

Table 2. Capillary GC-SIM of authentic MeTMSi GA_{19} and of similarly derivatived putative GA_{19} from hybrid poplar

	Retention time (min)	Percentage abundance of peak					
m/z		462	434	402	374	345	315
Authentic GA_{19}	12.67	8	100	33	77	38	31
Putative GA_{19}	12.73	8	100	46	77	46	38

The presence of GAs A_1, A_{19}, and probably A_{20} in hybrid poplar suggests that this member of the willow family utilizes the early C-13 hydroxylation GA biosynthetic pathway such as found in maize [1]. Consequently, it is probable that other GAs characteristic of this pathway are also native to hybrid poplar, although either less abundant (e.g. GA_{44}, GA_{53}) or not detected by the dwarf rice GA bioassay (e.g. GA_8, GA_{29}) [10].

The present study demonstrates the application of $[^3H]$ and $[^2H]$-labelled internal standards for sequential chromatographic GA purifications and subsequent GA identification involving vegetative tissue from a woody angiosperm. The final identification of the GA is based on information obtained throughout the procedure. GA detection is achieved with two selective detectors: the GA specific Tan-ginbozu dwarf rice assay and the mass selective detector, monitoring ions characteristic of the GA MeTMSi. The information obtained from the three sequential chromatographic procedures, normal phase SiO_2 partition, reversed-phase C_{18} HPLC, and capillary GC, are additive [11] and coupled with the SIM data, the results are relatively conclusive.

EXPERIMENTAL

Plant material. Stem cuttings of a vigorous, native hybrid of *Populus balsamifera* × *P. deltoides* (also referred to as: *P. x jackii* [12]) were collected from the North Milk River valley in southern Alberta as previously described (clone 24a) [13]. The hybrid was propagated and established in a nursery plot at the University of Lethbridge [13] and on August 10, 1985, after two field seasons in the nursery, elongating shoots of less than 1 cm diameter were harvested, frozen in liquid N_2 and lyophilized.

Extraction and purification. A 1 kg (fr. wt) sample was ground at −20° in MeOH–H_2O (2:8). To allow for the accurate determination of chromatographic R_fs, 1.8 kBq [1, 2-^{3}H]GA_1 (1.21 TBq per mmol, Amersham) and 2 kBq [2, 3-^{3}H]GA_{20} (85 GBq per mmol) [14] were added to the extract. (i.e. 0.5 ng GA_1, and 7.7 ng GA_{20}). The MeOH was removed *in vacuo* at 35°C after the addition of 0.5 M phosphate buffer (pH 8.0). The pH was raised to 9.0 with NaOH and chlorophyll was removed by two extractions with diethyl ether. The pH was then reduced to 8.0 and the buffered aqueous extract was slurried with poly-*N*-polyvinylpyrrolidone and filtered. The pH was then reduced to 3.0 with HCl and the sample extracted with H_2O-saturated EtOAc. After removing the H_2O by freezing and filtration, the EtOAc was subsequently removed *in vacuo* at 35°. The acidic, EtOAc-soluble extract was purified on columns of charcoal–celite (1:1) eluted with acetone–H_2O (8:2). This was followed by stepwise-elution SiO_2 partition chromatography [4, 15] and detection of GA-like activity using a modified [7] dwarf rice cv Tan-ginbozu microdrop assay [16] in serial dilution. The two major biologically active SiO_2 regions were further chromatographed on reversed-phase C_{18} HPLC [5, 8]. Flow and solvent parameters were as previously described except the gradient from 10 to 73% MeOH was run over 60 rather than 30 min. Eighty, 1 min fractions were collected and bioassayed at 3 dilutions (1/200, 1/400, 1/800).

GC-SIM. HPLC fractions showing radioactivity ($[^3H]GA_1$ or $[^3H]GA_{20}$) and GA-like activity or eluting at the expected R_f of GA_{19} [5, 7] were derivatized to the methyl ester using ethereal CH_2N_2 and silylated to the MeTMSi derivative using *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Pierce Chemical Co.). For GC-SIM, a Hewlett-Packard 5790A series Gas Chromatograph and a 5970A series Mass Selective Detector (MSD) fitted with a direct capillary interface for on-column injection were used. The 15 m capillary column was a cross-linked 95% dimethyl–5% diphenyl polysiloxane with a film thickness 0.25 μm and i.d. 0.25 mm (DB-5-15N, J & W Scientific, Inc.). Capillary head pressure was 36 ps and the He carrier gas flow rate was 1.1 ml/min. The GC wa programmed to maintain 60° for 1 min and then rise at 25°/mi up to 250°. The interface was maintained at 280° and the MSI

was operated with the electron multiplier at 2400 V.

To accurately determine capillary GC R_ts and relative intensities of the selected ions, int. stands of 50 ng [17, 17-^{2}H]GA_1, GA_{19}, or GA_{20} were simultaneously derivatized and co-injected with the appropriate HPLC fractions from the purified poplar extracts. The [17, 17-^{2}H]GA_1 and [17, 17-^{2}H]GA_{20} (99.2% enrichment) were prepared from the 17-nor-16-ketones by a modification of the Nozaki procedure [17]. [17, 17-^{2}H]GA_{19} was obtained following the incubation of [17, 17-^{2}H] steviol (similarly obtained from the nor-ketone) in *Gibberella fujikuroi* [18]. For GC-SIM in the presence of [^{2}H](d_2)GAs, six ions were monitored representing three ions characteristic of the endogenous GA and three corresponding ions from the deuterated GA. Ion abundances for the endogenous GAs were corrected for the contribution from the int. stad. GAs [7].

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