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Exogenously Applied GA_4 Is Converted to GA_1 in Seedlings of *Salix*

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Abstract. Gibberellin A_4 (GA_4) is biologically active in *Salix pentandra* and is able to induce stem elongation in seedlings grown under short day (SD) conditions, as well as in seedlings grown under long day (LD) conditions and treated with a growth retardant BX-112. $[^3H_2]GA_4$ and $[^2H_2]GA_4$ were applied to seedlings and leaf and stem explants of *S. pentandra*, and metabolites were studied using HPLC and GC-MS. After application of $[^3H_2]GA_4$ to seedlings of *S. pentandra*, one of the three main radioactive metabolites in the free acid fraction had retention properties similar to GA_1. Using $[^2H_2]GA_4$, this compound was identified by GC-MS with SIM as $[^2H_2]GA_1$ both from short day-grown and BX-112-treated seedlings, as well as in leaf and stem explants. After injection of GA_4 into a mature leaf, GA_1 was mainly found in the elongating stem tissue. Thus, the possibility that the biological activity of GA_4 in *Salix* is due to its conversion to GA_1, cannot be excluded.

Stem elongation in seedlings of *Salix* has been suggested to be dependent on the presence of gibberellin A_1 (GA_1) in the elongating stem tissue (Junttila and Jensen 1988). This suggestion is supported by some recent results. Growth inhibition induced by the novel growth retardant BX-112, which especially prevents 3β-hydroxylation (e.g., conversion of GA_{20} to GA_1) (Kamiya et al. 1991, Nakayama et al. 1990, 1991), was counteracted by GA_1 but not by GA_{20} (Junttila et al. 1991). Recently, this has been shown to be the case with seedlings of *Betula* and *Alnus* as well (Junttila, unpublished observations). Further, GA_9 is biologically active when applied to seedlings of *Salix pentandra* grown under short day (SD) conditions, but under those conditions GA_9 has been shown to be converted to GA_{20} and further to GA_1 (Junttila et al. 1992). GA_9 was almost inactive when applied to seedlings treated with BX-112 (Junttila et al. 1992). Taken together, these results indicate that GA_9 is not active per se in seedlings of *Salix* and that its activity depends on conversion to GA_1.

GA_4, like GA_9, is not a member of the early 13-hydroxylation pathway for biosynthesis of GA_1 (Graebe 1987), a pathway expected to be the main pathway in the vegetative tissue of *S. pentandra*. However, GA_4 is also biologically active in *S. pentandra* and is able to substitite for long day (LD) conditions and induce stem elongation in seedlings grown under SD (Junttila 1981, unpublished results). GA_4 has been suggested to be active per se for stimulation of stem elongation in *Cucumis sativus* (MacMillan 1985). Metabolism of GA_4 has been studied in various systems, and in seedlings of *Phaseolus* (Turnbull and Crozier 1989), *Zea*, *Oryza*, and *Arabidopsis* (Kobayashi et al. 1991), GA_4 is metabolized to GA_1. Results presented herein show that in seedlings of *S. pentandra*, GA_4 is metabolized to GA_1. Thus, the biological activity of GA_4 in *S. pentandra* may be due to its conversion to GA_1.

Materials and Methods

Plant Material and Growth Conditions

Seedlings of *S. pentandra* L., ecotype from 69°39′ N lat., were grown for about 4–5 weeks in fertilized peat at 18°C in 24-h photoperiods (LD, Philips TL 65W/83, 150 μmol m^{-2} s^{-1} within 400–750 nm). Temperature in the phytotron was controlled to +0.5°C, and the water vapor pressure deficit was adjusted to 0.5 kPa. Before application of GA, the seedlings were exposed to a 12-h photoperiod for 10–14 days to induce cessation of elongation growth. BX-112 (calcium, 3,5-dioxo-4-propionylcyclohexane carboxylate; Kumiai Chemical Industry Co., Ltd., Tokyo, Japan) was applied as an aqueous spray at 500 ppm, three times a week before the application of gibberellins. Plants treated

with BX-112 were grown for a 24-h photoperiod. Plants were watered and fertilized as described previously (Junttila 1980).

Application of GA_4

Both [1,2-$^3H_2$]GA_4 (1 PBq mol^{-1} from Prof. R. P. Pharis, Department of Biology, University of Calgary, Calgary, Canada) and [17,17-$^2H_2$]GA_4 (from Prof. L. N. Mander, Research School of Chemistry, Australian National University, Canberra, Australia) were used. GA_4, dissolved in ethanol, was injected (1 μl) into a mature leaf under the adaxial epidermis. Applied amounts of [1,2-$^3H_2$]GA_4 were 0.9 (experiment 1) or 2.4 (experiment 2) MBq/plant, and that of [17,17-$^2H_2$]GA_4 1 μg/plant. There were 10 plants per treatment. Samples were harvested after 48 or 96 h from application. Injected leaf and stem above the injected leaf were collected from treated seedlings. Three separate experiments were done on intact plants, the first using [1,2-$^3H_2$]GA_4 only, the second using both [1,2-$^3H_2$]GA_4 and [17,17-$^2H_2$]GA_4, and the third using [17,17-$^2H_2$]GA_4 only.

In addition to experiments with intact seedlings, the metabolism of [17,17-$^2H_2$]GA_4 was also studied using leaf and stem explants. Samples were collected from plants grown as those used for studies described above. Leaf discs were taken from mature leaves using a cork bore (diameter 10 mm), internodes (8–10 mm) were collected from the second or third internode. Ten leaf discs were used per treatment and they were placed on moist filter paper in petri dishes, and [17,17-$^2H_2$]GA_4 (1 μg/disc) was applied to the adaxial surface in 2 μl ethanol. Internodes, 10 per treatment, were placed into 6-mm diameter glass tubes containing 10 μg [17,17-$^2H_2$]GA_4 dissolved in ethanol and diluted with distilled water to 50 μl. Samples were incubated in light (Thorn EMI, 30 W) or in darkness at 20°C for 20 h.

Extraction and Purification

Samples were harvested directly into cold 80% methanol, homogenized, and extracted in 10–20 ml of 80% methanol. In experiments with intact plants, 10 ml of 0.1 M phosphate-citrate buffer, pH 8, was added to the extracts, after which they were taken to the aqueous phase in vacuo, slurried with insoluble PVPP, filtered, and the pH was adjusted to 2.5. The acidic aqueous extract was partitioned three times against an equal volume of ethyl acetate. The combined acidic ethyl acetate fractions were taken to dryness, dissolved in 0.5 ml of 20% methanol, applied to a 1-g C-18 Sep Pak (Supelco Inc., Bellefonte, PA, USA). GAs were eluted with 7 ml of 80% methanol in 30 mM acetic acid. The eluate was taken to dryness, and dissolved in about 150 μl of 50% methanol in 30 mM acetic acid.

HPLC

Samples were injected onto a radially compressed C-18 cartridge (100 × 8 mm i.d., 10 μm, Waters Rad-Pak system) with a flow rate of 2 ml min^{-1} using a linear gradient of 20–80% methanol in 30 mM acetic acid during 25 min, and then with 80% methanol for an additional 20 min. Two milliliter fractions were collected for 30 min and an aliquot of each fraction was counted for radioactivity. The radioactive fractions, or fraction expected to contain GA_4 or its metabolites, were prepared for analysis by GC-MS.

Table 1. Distribution of radioactivity in seedlings of *S. pentandra* 48 h after injection of [1,2-$^3H_2$]GA_4 into a leaf.

	Radioactivity (MBq)		
Plant part	Acidic EtOAc phase	Water phase	Total
Stem with apex	1.15	0.57	1.77
Leaves	0.56	0.27	0.83
Injected leaf	37.0	1.01	46.6

Plants were grown at 18°C in a 12-h photoperiod. Stem and leaves were collected above the injected leaf.

GC-MS

Fractions for identification of metabolities were taken to dryness, methylated with diazomethane and trimethyl-silylated with MSTFA before analysis by capillary GC-MS. Samples were introduced by splitless injection onto a HP-1 Ultra column (25 m × 0.31 mm i.d., 0.17-μm film thickness, cross-linked methyl silicone) installed in a Hewlett Packard 5890 GC and connected via a direct inlet interface to a Hewlett Packard 5970B Mass Selective Detector. The nominal ionizing voltage was 70 eV. The oven temperature was held at 60°C (2 min) and programmed to 180°C at 30°C min^{-1}, and after 1 min at 180°C further to 240°C at 10°C min^{-1}.

Results

Two days after application of GA_4 into a mature leaf, approximately 28% of the radioactivity was retained in the water phase after solvent partitioning (Table 1). Most of the radioactivity in the acid ethylacetate fraction was retained in the injected leaf; radioactivity found in the stem tissue corresponded to about 3% of the total detected radioactivity in this fraction (Table 1).

Experiments with [1,2-$^3H_2$]GA_4 showed several metabolites both in the injected leaf and in elongating stem tissue (Fig. 1). One of these, mainly present in the injected leaf, co-chromatographed with [1,2-$^3H_2$]GA_4. A radioactive peak with the same retention time as GA_1 was found in all samples, and in stem tissue it was the main peak (Fig. 1C). The most polar radioactive compound in stem tissue had a retention time similar to that of GA_8 (Fig. 1C). There was no significant effect of BX-112 on the conversion of [1,2-$^3H_2$]GA_4 to [1,2-$^3H_2$]GA_1. For example, 4 days after application, the percent radioactivity in injected leaf extracts corresponding to the GA_1 zone was 3.1 and 4.1% from control and BX-112 treated plants, respectively.

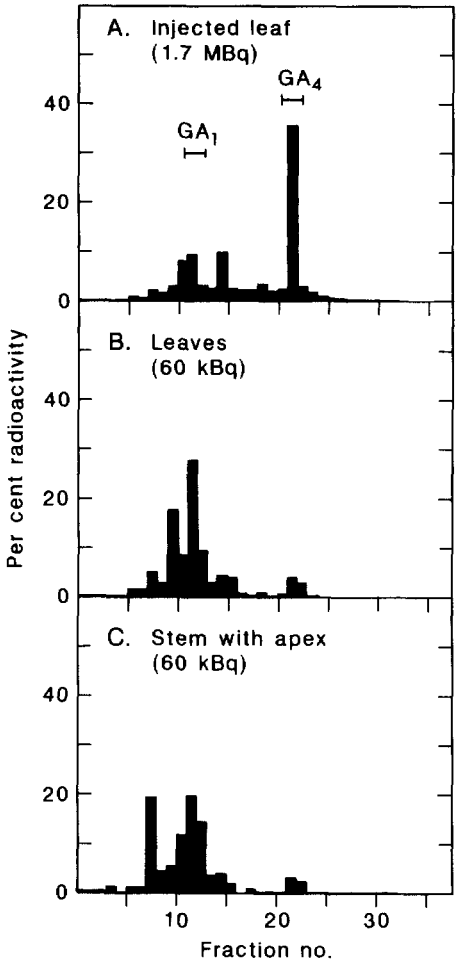

Fig. 1. Distribution of radioactivity in fractions from gradient-eluted reversed-phase C-18 HPLC extracts from stem, leaves, and injected leaf of *S. pentandra* 48 h after injection of [1,2-$^3H_2$]GA_4. Results are expressed as percent of total radioactivity in the chromatographed sample (given in parentheses).

The radioactive fractions from the second experiment were analyzed further by GC-MS. [$^2H_2$]GA_1 was identified with SIM from HPLC fractions 10–12 from both injected leaf and stem tissue extracts (Table 2). The identification of [$^2H_2$]GA_1 with SIM was confirmed in the third experiment. No conclusive identification of the other radioactive peaks could be obtained. However, the molecule ion of deuterated GA_{34} (m/z 508) was detected at the expected retention time for GA_{34} from HPLC fractions 22–24. Identity of the GA_8-like peak (Fig. 1C, HPLC fraction 7) was not confirmed.

Experiments with leaf discs and internode explants showed that both tissues were able to convert [$^2H_2$]GA_4 to [$^2H_2$]GA_1 both in light and darkness. Presence of [$^2H_2$]GA_1 in extracts of explants was confirmed by GC-MS using SIM. Amount of GA_1 in the extracts corresponded to approximately 10% of the amount of GA_4 detected.

Table 2. Principle ions and retention times of methyl ester, trimethylsilyl derivatives of [$^2H_2$]GA_1, and putative [$^2H_2$]GA from seedlings of *S. pentandra*.

		m/z (%)			
Sample	Rt (min)	508	506	450	448
[$^2H_2$]GA_1	25.6	100	2.9	20	3.0
Injected leaf, SD	25.7	100	4.4	22	4.4
Injected leaf, BX-112	25.5	100	ND	19	ND
Stem, SD	25.6	100	11	19	7
Stem, X-112	25.7	100	ND	19	ND

[$^2H_2$]GA_4 was injected into a leaf and samples were collected 96 h after injection. ND, not detected.

Discussion

The present results show that both leaf and stem tissue of *S. pentandra* is able to convert applied [$^2H_2$]GA_4 to [$^2H_2$]GA_1 (Fig. 1, Table 2). Conversion of GA_4 to GA_1 has also been demonstrated in vegetative tissues of *Phaseolus*, maize, rice, and *Arabidopsis* (Durley and Pharis 1973, Kobayashi et al. 1991, Turnbull and Crozier 1989); in pollen of *Pinus attenuata* (Kamienska et al. 1976); and in cell cultures of anise and carrot (Koshioka et al. 1983a,b). Also cell-free preparations from cotyledons of *Phaseolus coccineus* were able to convert GA_4 to GA_1 (Crozier et al. 1991). In epicotyls of *P. coccineus* GA_1 was the first metabolite detected after application of [^3H]GA_4 (Turnbull and Crozier 1989). In addition, GA_{34} is a common metabolite of GA_4 in the studied systems (Crozier et al. 1991, Durley and Pharis 1973, Koshioka et al. 1983a,b, Turnbull and Crozier 1989). GA_{34} is a result of 2β-hydroxylation of GA_4 and is biologically inactive (Reeve and Crozier 1975). Also conjugates of GA_4, GA_{34}, and GA_8 have been identified as metabolites after feeding with [^3H]GA_4 (Turnbull et al. 1986). Presence of GA_{29} (Davies et al. 1985) and GA_8 (unpublished observations) as an endogenous compound in *S. pentandra* and previous metabolic studies (Junttila and Pharis 1987) show that *S. pentandra* tissue is able to carry out 2β-hydroxylation reactions. Thus, both GA_8 and GA_{34} could be expected to be found as metabolities of GA_4. Experiments with tritium-labeled GA_4 indicated the presence of GA_8 among the metabolites in stem tissue (Fig. 1C), but identity of this compound was not confirmed. In the used HPLC gradient elution, GA_{34} will elute close to GA_4 (Jensen et al. 1986), and the molecule ion of

deuterated GA_{34} was found from this HPLC fraction.

Conversion of applied GA_4 to GA_1, and especially the presence of GA_1 in significant higher amounts compared to GA_4 in stem tissue after an injection of GA_4 into a leaf, opens the possibility that GA_4 is not active per se in *S. pentandra,* but that its activity is due to metabolism to GA_1. The present results on the effects of applied GA_4 to *S. pentandra* are comparable to those obtained with applied GA_9 (Junttila et al. 1992). Kobayashi et al. (1991) have suggested that the biological activity of GA_4 in maize, rice, and *Arabidopsis* is due to its conversion to GA_1. On the other hand, GA_4 has been reported to be the active GA for elongation growth in seedlings of *Cucumis sativus* (MacMillan 1985). Studies on rice suggest that 3β-hydroxylation is an important and necessary step in the biosynthesis of gibberellins that promote shoot elongation in rice (Kamiya et al. 1991). Thus, although GA_1 has been suggested to be the primary GA for stem elongation in higher plants (Graebe 1987, Phinney 1984), the possibility that in certain species other GAs also can be active per se has to be considered. Studies with a variety of species are now needed, and especially conifers need attention.

Lack of the effect of BX-112 on the conversion of GA_4 to GA_1 is in accordance with previous results showing that 13-hydroxylation is not affected significantly by this compound (Nakayama et al. 1990). Further, GA_4 is also biologically active in seedlings treated with the free acid of BX-112 (Junttila et al. 1991).

In conclusion, the present results show that applied GA_4 is metabolized to GA_1 in seedlings of *S. pentandra* grown under SD or treated with BX-112. GA_4 is able to antagonize both SD, and BX-112-induced inhibition of shoot elongation. Although GA_4 can be active per se, the present results open for the possibility that its activity is due to a metabolic conversion to GA_1. Further studies, including studies on kinetics of metabolism and translocation of GA_4, are needed to test this possibility.

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