Metabolism of deuterium- and tritium-labeled gibberellins in cambial region tissues of *Eucalyptus globulus* stems

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**Summary** Deuterium- and tritium-labeled gibberellins (GAs) were applied to stems of 3-year-old *Eucalyptus globulus* Labill. saplings and 9-month-old potted seedlings. Cambial region tissues surrounding the application point were collected 6, 24 or 48 h later. Twenty-four hours after application of 5 \(\mu\)g of \([\delta^2\text{H}]\text{GA}_{20}\), 7% of the cambial region \text{GA}_{20} pool, 7% of the \text{GA}_1 pool and 58% of the \text{GA}_{29} pool were labeled with deuterium based on selected ion monitoring of purified extracts subjected to gas chromatography–mass spectrometry. The relatively low percent dilution of endogenous GAs by \([\delta^2\text{H}]\text{GAs}\) suggests that the exogenous application of \([\delta^2\text{H}]\text{GA}_{20}\) did not result in substrate overloading, indicating that these conversions probably occur naturally within cambial region tissues. Extracts from similar cambial region tissues fed tritium-labeled GAs were sequentially fractionated by SiO\(_2\) partition chromatography, C\(_{18}\) reversed phase HPLC and Ni(CH\(_3\))\(_2\) HPLC. The radioactivity profiles indicated metabolism of \text{GA}_{20} to \text{GA}_1 and \text{GA}_{29}, \text{GA}_1 conversion to \text{GA}_8, \text{GA}_4 to \text{GA}_3 and \text{GA}_9 to \text{GA}_5. Gibberellins \text{GA}_{13}, \text{GA}_3, \text{GA}_1 and \text{GA}_{29} are C-2β-hydroxylated catabolites of low biological activity, whereas \text{GA}_1 and \text{GA}_4 are probably effectors of growth in the *Eucalyptus* stem and shoot. Evidence for C-13 hydroxylation of \text{GA}_4 to \text{GA}_1, \text{GA}_9 to \text{GA}_4 or \text{GA}_9 to \text{GA}_{20} in the stem was inconclusive. Thus, although \text{GA}_4 and \text{GA}_9 are native to cambial region tissues, \text{GA}_1 is probably not produced from them in significant quantities. We conclude that the early C-13-hydroxylation pathway; i.e., conversion of \text{GA}_9 to \text{GA}_{20} to \text{GA}_1, is the major pathway of \text{GA}_1 biosynthesis.

**Keywords:** cambium, endogenous hormones, gas chromatography–mass spectrometry, high-performance liquid chromatography

**Introduction**

There is considerable evidence that plant hormones are important regulators of cambial cell division and secondary xylem and phloem differentiation, including the mediation of environmental factors that influence wood formation (Little and Savidge 1987, Little and Pharis 1995). Most of this evidence is based on altered cambial activity and wood microstructure following exogenous application of synthetic or natural plant growth regulators. Recently, endogenous hormones in the cambial region of *Eucalyptus* (auxin, gibberellins (GAs)) have been characterized (Hasan et al. 1994, Ridoutt 1995, Ridoutt et al. 1995) and concentrations of several of these native hormones were found to be significantly correlated with xylem fiber differentiation (Ridoutt et al. 1996). One difficulty in interpreting these results has been uncertainty regarding the origin of endogenous hormones in the cambial region and whether metabolism of GAs occurs in cambial region tissues.

Several lines of evidence indicate that cambial region tissues can metabolize GAs. First, all of the GAs produced in the early C-13-hydroxylation pathway, except \text{GA}_{53}, have now been isolated from the cambial region of *Eucalyptus globulus* Labill. (Hasan et al. 1994, Ridoutt et al. 1995). The early C-13-hydroxylation pathway yields \text{GA}_1, a presumed effector of cambial region growth (Ridoutt et al. 1996). Second, the within-tree pattern of variation in cambial region \text{GA}_1 concentrations differs from that of IAA (B.G. Ridoutt, R.P. Pharis and R. Sands, unpublished data), suggesting a different pattern of synthesis and transport for GAs compared with IAA, which is transported basipetally by the cambium and differentiating xylem from expanding buds and young leaves (Little and Savidge 1987). Third, the localized application of trinexapac-ethyl, an acyclohexanedione inhibitor of late-stage GA biosynthesis, to stems of *E. globulus* reduced cambial region \text{GA}_1 concentrations, the latter being significantly correlated with reduced fiber elongation (Ridoutt et al. 1996). Finally, it has been proposed that GA glucosyl conjugates are involved in long-distance GA transport in trees (Sembdner et al. 1994), although GA glucosyl conjugates have not yet been found in the cambial region of *E. globulus*.

Studies with several tree species have shown rapid metabolism of various GAs in leaves and elongating shoots (Wample et al. 1975, Junttila 1993) and during reproductive development (Dunberg et al. 1981, Moritz et al. 1989, Moritz and Odén 1990, Odén et al. 1995). Because the close proximity of the various plant organs in small seedlings makes it difficult to identify where the applied GAs were metabolized (Rood and...
tritium-labeled GAs in isolated plant parts of Salix pentandra L. They reported that, after 24 h, $[^3]H$GA$_{19}$ was metabolized to $[^3]H$GA$_{20}$ and $[^3]H$GA$_1$ in leaves and roots but not internodes. However, $[^3]H$GA$_1$ was metabolized to $[^3]H$GA$_4$ in all plant parts. Wang et al. (1995) showed that shoots of Malus domestica Borkh. (1.40 PBq mol$^{-1}$), $[^3]H$GA$_4$ (1.19 PBq mol$^{-1}$), and $[^3]H$GA$_9$ (> 1 PBq mol$^{-1}$) or $[^3]H$GA$_{20}$. All four of these GAs are native to Eucalyptus spp. (Ridoutt et al. 1995). The cambial region tissues were collected 24 or 48 h after injection by scraping a segment of stem extending for 5 cm above and below the injection point, which included half the stem circumference. These cambial region tissues were immediately scraped, without freezing, into 80% aqueous methanol (MeOH).


In October 1995, tritium-labeled GAs were also applied with a brush to the cambial region tissues of 9-month-old seedlings of E. globulus. At 10 cm above the root collar, a razor blade was used to remove the outer bark from a 1-cm$^2$ area. A 2-mm wide brush was then used to apply 17 kBq of $[^3]H$GA$_1$, $[^3]H$GA$_4$, $[^3]H$GA$_9$, or $[^3]H$GA$_{20}$ in 50 µl of 95% ethanol. One stem was treated with each labeled GA. Cambial region tissues were collected after 24 h as previously described.

**Materials and methods**

**Stem injection of $[^3]H$_2$GA$_{20}$**

We used 3-year-old Eucalyptus globulus seedlings selected in a plantation at Creswick, Victoria, Australia (37°25′ S, 143°54′ E). The trees were approximately 6.5 m in height and 8 cm in diameter (over bark) at 10 cm above ground level. Each stem was free of live branches to at least 2.5 m in height. On September 5, 1993, during rapid spring growth, 5 µg of [17,17-$[^3]H$_2$]GA$_{20}$ (97.8% deuterium enrichment) in 700 µl of 95% ethanol was injected into each of four stems in the region of the cambium at 10 cm above ground level. Cambial region tissues for analysis were harvested 6 and 24 h after injection, in separate trees, by peeling the bark and scraping the soft tissue that underlies the differentiated phloem and adheres to the differentiated xylem. Microscopic analysis indicated that such scrapings consist of vascular cambium having little or no phloem contamination, with differentiating xylem cells that are mainly primary walled (Ridoutt et al. 1995). The segment of stem that was scraped included the injection point and extended for 10 cm above and below the injection point, and for 4 cm circumferentially in either direction. The scrapings were immediately frozen at −70°C, then lyophilized and stored at −20°C until analyzed.


Experiments with tritium-labeled GAs were carried out on 9-month-old seedlings of E. globulus that had been grown in 5-l pots in a greenhouse without artificial lighting or temperature control at The University of Melbourne, School of Forestry, Creswick, Victoria, Australia. The 9-month-old seedlings were approximately 2 m tall and stem diameters at 10 cm above the root collar were 1.2 to 1.5 cm. All branches had been pruned from the lower 1 m of stem. In October 1995, 17 kBq of tritium-labeled GA in 2 µl of 95% ethanol was injected into each stem in the region of the cambium at 10 cm above the root collar. Two stems were injected per GA treatment: $[^3]H$GA$_1$ (1.40 PBq mol$^{-1}$), $[^3]H$GA$_4$ (1.19 PBq mol$^{-1}$), and $[^3]H$GA$_9$ (> 1 PBq mol$^{-1}$) or $[^3]H$GA$_{20}$. All four of these GAs are native to Eucalyptus spp. (Ridoutt et al. 1995). The cambial region tissues were collected 24 or 48 h after injection by scraping a segment of stem extending for 5 cm above and below the injection point, which included half the stem circumference. These cambial region tissues were immediately scraped, without freezing, into 80% aqueous methanol (MeOH).

**Extraction and purification**

The lyophilized cambial region tissues from stems fed with deuterium-labeled GAs were macerated and extracted four times at 4°C with 80% aqueous MeOH. To each extract, 500 Bq [17,17-$[^3]H$_2$]GA$_1$ (1.40 PBq mol$^{-1}$), $[^3]H$GA$_4$ (1.19 PBq mol$^{-1}$), and [1-$[^3]H$]GA$_9$ (> 1 PBq mol$^{-1}$) were added as HPLC internal standards. The solution was then filtered and each filtrate reduced to an aqueous solution in vacuo, adjusted to pH 2.5 and partitioned four times against water-saturated ethyl acetate (EtOAc). The acidic, EtOAc-soluble fraction was dried, dissolved in a minimum volume of MeOH, applied to a DEAE Sephadex A-25 column and eluted with a gradient of H$_2$O:MeOH:acetic acid (Turnbull et al. 1986). Appropriate fractions were pooled, dried on acid-washed celite 545 (Alltech, Baulkham Hills, Australia), then loaded onto a SiO$_2$ partition column, and eluted with formic acid saturated with EtOAc-hexane (95:5, v/v) (Koshioka et al. 1983b). Final separation of the GAs was by C$_{18}$ reversed phase HPLC (see below).

Cambial region tissues from stems that had been fed tritium-labeled GAs were extracted overnight at 4°C with 80% aqueous MeOH containing 0.01% butylated hydroxytoluene, then dried on acid-washed celite and loaded onto a SiO$_2$ partition column. Acidic GAs were eluted with formic-acid-saturated EtOAc-hexane (95:5, v/v), after which the column was washed with excess MeOH to elute the GA glucosyl conjugates (Koshioka et al. 1984b). Each of these two fractions was subjected to C$_{18}$ reversed phase HPLC.

**HPLC**

We used the reversed phased C$_{18}$ HPLC conditions described by Ridoutt et al. (1996), with fractions collected every minute. For this system, Koshioka et al. (1983a) and Pearce et al. (1994) have described approximate retention times (Rts) for many GAs. Following HPLC, the deuterium-labeled GA frac-
Methyl ester derivatives were formed using ethereal diazomethane and trimethylsilyl ether derivatives were formed using bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylsilyl ether derivatives were formed using ethereal diazomethane in dry pyridine, with heating to 70 °C for 30 min. The GC–MS–SIM was performed as described in Ridoutt et al. (1996), using a Fisons Instruments 8000 series (Danvers, MA) GC linked by a direct inlet to the ion source of a Fisons Instruments MD800 mass spectrometer. Data were collected with a dwell time of 50 ms, and the proportion of each GA pool that was [3H] labeled was calculated according to the isotope dilution equation described by Gaskin and MacMillan (1991). For [3H]GA1, GA29 and GA81 m/z 506, 507 and 508 were monitored and for [3H]GA20 m/z 418, 419 and 420 were monitored. The proportions of enriched and unenriched molecules in each sample were calculated based on previously determined normalized intensities for the M+ ion clusters. Additional ions were also monitored to confirm identity.

Results

The GC–MS–SIM analysis showed that, 6 h after stem injection of [3H]GA20 into the cambial region of 3-year-old trees, no deuterium-labeled GA20, GA1 or GA29 was detected. One explanation for the lack of radioactive metabolites is the complete transport of the labeled compounds away from the cambial region. Another possibility is that the [3H]GA20 was not successfully applied to the cambial region tissues. Twenty-four hours after stem injection of [3H]GA20 into the cambial region, 7% of the cambial region GA1 pool and 58% of the GA29 pool was labeled with deuterium (Figure 1).

For the 9-month-old seedlings fed [3H]GAs, the recovery of applied radioactivity was relatively high, with a mean of 13% for the topical cambial region application and 6% for the stem injection method. Cambial region tissues collected 24 or 48 h after injection showed similar radioactivity profiles, with less radioactivity usually being recovered 48 h after application than 24 h after application: implying rapid transport of tritium-labeled GAs away from the point of application.

Radioactive compounds with C18 and N(CH3)2 HPLC Rts characteristic of GA1 and GA8 were recovered from cambial region extracts 24 h after [3H]GA1 was applied topically to cambial region tissues (Figures 2A–C), indicating rapid metabolism of [3H]GA1 to [3H]GAs in the stem. Similar radioactivity profiles were obtained when [3H]GA1 was applied by stem

Figure 1. Gibberellin pools in the cambial region of 3-year-old Eucalyptus globulus saplings 24 h after stem injection of 5 µg of [17,17-3H]GA20. Analysis was by GC–MS–SIM. The amounts of GAs in cambial region scrapings from adjacent scrapings that had not been fed [3H]GA20 were GA20 = 6.3 ng g⁻¹, GA1 = 0.27 ng g⁻¹ and GA29 = 0.47 ng g⁻¹ (GC–MS–SIM data based on stable isotope labeled internal standards as reported in Ridoutt et al. 1995).

Figure 2. Radioactivity profiles after HPLC fractionation of cambial region extracts from 9-month-old Eucalyptus globulus stems 24 h after topical application of 17 kBq (about 10⁹ dpm) of [3H]GA1 to cambial region tissues. The cambial tissue extracts were fractionated by SiO2 partition chromatography and the fraction containing acidic GAs was then separated by C18 reversed phase HPLC (A). The C18 HPLC fractions 25–28 (B) and 17–18 (C) were further separated by N(CH3)2 HPLC. Fractions from the SiO2 partition column containing putative GA glucosyl conjugates were separated by C18 HPLC (D). Radioactivity peaks that correspond with the Rts of known GAs are labeled.
Radioactive compounds with C\textsubscript{18} and N(CH\textsubscript{3})\textsubscript{3} HPLC Rts corresponding with authentic GA\textsubscript{3} and GA\textsubscript{5} were recovered from the cambial region after stem application of [\textsuperscript{3}\text{H}]GA\textsubscript{4} (Figures 3A–C). A small peak of radioactivity was also found corresponding with authentic GA\textsubscript{5} and GA\textsubscript{5} were recovered from the cambial region (Figures 4A–C), indicating metabolism of [\textsuperscript{3}\text{H}]GA\textsubscript{5}. Radioactive fractions with C\textsubscript{18} HPLC Rts of 25 to 36 min did not yield evidence of [\textsuperscript{3}\text{H}]GA\textsubscript{4} or [\textsuperscript{3}\text{H}]GA\textsubscript{5} following N(CH\textsubscript{3})\textsubscript{3} HPLC (data not shown). These unidentified [\textsuperscript{3}\text{H}] peaks could be C-16 diols or other ring A, C or D hydroxylation products. Similar radioactive peaks were found when the [\textsuperscript{3}\text{H}]GAs were applied by stem injection or by topical application to the cambial region.

Most of the radioactivity recovered from the cambial region following injection of [\textsuperscript{3}\text{H}]GA\textsubscript{20} into stems of \textit{E. globulus} seedlings had C\textsubscript{18} and N(CH\textsubscript{3})\textsubscript{3} HPLC Rts corresponding to GA\textsubscript{29} (Figures 5A–B). Smaller radioactive peaks were found at the Rts of GA\textsubscript{20} and GA\textsubscript{29} (Figures 5A, 5C and 5D), indicating metabolism of [\textsuperscript{3}\text{H}]GA\textsubscript{20} to [\textsuperscript{3}\text{H}]GA\textsubscript{29} and [\textsuperscript{3}\text{H}]GA\textsubscript{20} (via [\textsuperscript{3}\text{H}]GA\textsubscript{5}). Collectively, the [\textsuperscript{3}\text{H}] and [\textsuperscript{3}\text{H}]GA\textsubscript{20} feeding experiments confirm that GA\textsubscript{20} is metabolized to GA\textsubscript{20} and GA\textsubscript{29} in the stems of \textit{E. globulus} seedlings and saplings.

Discussion

We obtained evidence that several late-stage gibberellin biosynthetic steps occur in the cambial region of \textit{E. globulus} stems. We showed the metabolism of GA\textsubscript{1} to GA\textsubscript{8}, GA\textsubscript{4} to GA\textsubscript{13}, GA\textsubscript{9} to GA\textsubscript{13}, GA\textsubscript{5} and GA\textsubscript{30} to GA\textsubscript{1} and GA\textsubscript{29} to either 2\beta- or 3\beta-hydroxylation (Figure 6). The gibberellins GA\textsubscript{1}, GA\textsubscript{4}, GA\textsubscript{8}, GA\textsubscript{9}, GA\textsubscript{19}, GA\textsubscript{20} and GA\textsubscript{29} are all native to \textit{Eucalyptus} spp. (Ridoutt et al. 1995).

Junttila et al. (1992) and Junttila (1993) found evidence for the conversion of GA\textsubscript{4} to GA\textsubscript{1} and GA\textsubscript{8} to GA\textsubscript{20} in elongating \textit{Salix} shoots. We were unable to demonstrate C-3\beta-hydroxylation of GA\textsubscript{20} to GA\textsubscript{4} or C-13-hydroxylation of GA\textsubscript{20} to GA\textsubscript{20}, and GA\textsubscript{4} to GA\textsubscript{1} in cambial region tissues of \textit{E. globulus}. We conclude, therefore, that the early C-13-hydroxylation path-
way leading to GA\textsubscript{1} is probably the predominant GA biosynthetic pathway in cambial region tissues of *Eucalyptus* stems. Although GA\textsubscript{4} and GA\textsubscript{9} have been identified by full-scan GC–MS in cambial region extracts of *E. globulus* (Ridoutt et al. 1995), it is unlikely that they are important precursors of GA\textsubscript{1} in the cambial region tissues.

Gibberellin A\textsubscript{1} is thought to be the major biologically active gibberellin in many angiosperms (Junttila et al. 1992, Junttila 1993, Zeevaart et al. 1993, Kobayashi et al. 1994). The finding that the 3\textbeta-hydroxylation of GA\textsubscript{20} to GA\textsubscript{1} occurs in the cambial region tissues of *E. globulus* is therefore of particular interest because this conversion represents an important biosynthetic point of regulation of GA\textsubscript{1} within the stem.

Because the field-grown saplings were about 6.5 m tall and the lower 2.5 m (or more) of the stem was free of live branches, leaves and growing apices on these trees are unlikely to have been sites of labeled GA metabolism. Use of deuterium-labeled GA\textsubscript{20} enabled us to study its metabolism, to identify definitively the metabolites of the exogenous feed by GC–MS–SIM, and to quantify the proportion of the GA pool that was labeled. Stem injection at a single point resulted in 7% of the GA\textsubscript{20} pool (over 160 cm\textsuperscript{2} of stem surface) being labeled with \([\text{\textsuperscript{2}H}\textsubscript{2}]\text{GA}_{20}\). This value lends support to our conclusion that the observed metabolism of \([\text{\textsuperscript{2}H}\textsubscript{2}]\text{GA}_{20}\) to \([\text{\textsuperscript{2}H}\textsubscript{2}]\text{GA}_{29}\) and \([\text{\textsuperscript{2}H}\textsubscript{2}]\text{GA}_{1}\) is representative of naturally occurring GA\textsubscript{20} metabolism in the stem.

An advantage of using high specific activity tritium-labeled GAs to study metabolism is that substrate overloading is unlikely to be a confounding factor. All identifications based on

Figure 5. Radioactivity profiles after HPLC fractionation of cambial region extracts from 9-month-old *E. globulus* stems 24 h after stem injection of 17 kBq (about 10\textsuperscript{9} dpm) of \([\text{\textsuperscript{3}}\text{H}]\text{GA}_{20}\). The extracts were fractionated by SiO\textsubscript{2} partition chromatography and the fraction containing acidic GAs was then separated by C\textsubscript{18} reversed phase HPLC (A). The C\textsubscript{18} HPLC fractions 20 (B), 32–33 (C) and 17–18 (D) were further separated by N(CH\textsubscript{3})\textsubscript{2} HPLC. For N(CH\textsubscript{3})\textsubscript{2} HPLC, the C\textsubscript{18} HPLC fractions from all treatments (i.e., topical and stem injection, 24- and 48-h feeds) were combined to increase total radioactivity. Radioactive peaks that correspond with Rts of known GAs are labeled.

Figure 6. Putative early non-hydroxylation (upper) and early C-13-hydroxylation (lower) pathways of GA biosynthesis in *Eucalyptus globulus*. Bold entries are gibberellins that have been identified as either endogenous GAs or metabolites of endogenous GAs in cambial region tissues of *E. globulus*.
C_{18}HPLC and dimethylamino HPLC Rts should be considered tentative because although GC–MS–SIM could be performed to confirm the identity of the GA present within each radioactive peak, it would not conclusively prove incorporation of the [^{13}C]GA precursor into a specific metabolite. Nonetheless, it seems likely that all of the C-2β-hydroxylated, biologically inactive metabolites of GA_{1}, GA_{2}, GA_{3} and GA_{4} are present in the cambial region of *E. globulus*. The presence of numerous other radioactive peaks, including the highly polar metabolite of [^{3}H]GA_{1} implies that additional, as yet unidentified metabolites of GA_{1}, GA_{2}, GA_{3} and GA_{4} are also present in *Eucalyptus* stems.

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