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Gibberellin Biosynthesis from Gibberellin A_{12}-Aldehyde in Endosperm and Embryos of *Marah macrocarpus*[1]

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Soluble enzyme preparations from embryos and endosperm of *Marah macrocarpus* (previously *Echinocystis macrocarpa*) were incubated with [$^{14}C_4$]gibberellin($GA)_{12}$-aldehyde, [$^{14}C_4$]GA_{12}, [$^{14}C_1$]GA_9, 2,3-didehydro[$^{14}C_1$]GA_9, [$^{14}C_1$]GA_{20}, and [17-^{13}C,^{3}H]GA_5. Embryo preparations converted GA_{12}-aldehyde, GA_{12}, and GA_9 to GA_4 and GA_7; 2,3-didehydroGA_9 to GA_7; GA_5 to GA_3; and GA_{20} (incompletely) to GA_1 and GA_{60}, but not to GA_3. Endosperm preparations converted GA_{12}-aldehyde and GA_{12} to GA_{15}, GA_{24}, GA_{25}, and GA_9, but, unlike embryo preparations, not to GA_4 or GA_7. However, GA_4 and GA_7 were formed from GA_9 and GA_7 was formed from 2,3-didehydroGA_9. Metabolism of GA_5 to GA_3 and GA_{20} to GA_1 was low. 2,3-DidehydroGA_9 accumulated when GA_9 was incubated with a desalted endosperm preparation. A cDNA clone (M3–8), selected from an embryo-derived cDNA library using a DNA fragment generated by reverse transcriptase polymerase chain reaction, was expressed in *Escherichia coli*. The fusion protein converted GA_{12} to GA_9 (major) and GA_{25} (minor); GA_{53} was metabolized less effectively and only to GA_{44}. Thus, the M3–8 protein is functionally similar to GA 20-oxidases from *Arabidopsis thaliana*, *Spinacia oleracea*, and *Pisum sativum*, but different from that from *Cucurbita maxima* seeds, to which its amino acid sequence is most closely related. mRNA hybridizing to M3–8 accumulated in embryos and endosperm of *M. macrocarpus*, but was absent in vegetative tissues.

The first in vitro studies on GA biosynthesis were conducted with endosperm homogenates from *Marah macrocarpus* (previously *Echinocystis macrocarpa*) following the report (Corcoran and Phinney, 1962) that this endosperm contained very high amounts of bioactive, GA-like compounds. These early biosynthetic studies showed the successive formation of *trans*-geranylgeraniol, *ent*-kaurene, *ent*-kauren-19-ol, *ent*-kauren-19-oic acid, *ent*-7α-hydroxykauren-19-oic acid, and GA_{12}-aldehyde from mevalonic acid (Graebe et al., 1965; Dennis and West, 1967; Murphy and West, 1969; Lew and West, 1971; D. Nakata, unpublished results, cited in West, 1973). No post-GA_{12}-aldehyde metabolic studies with this system were published until Albone et al. (1990) investigated the biosynthetic relationship between GA_4 and GA_7 (see Fig. 1 for structures), the two major C_{19}-GAs in *M. macrocarpus* seeds (Beeley et al., 1975; MacMillan and Gaskin, 1996). Using a cell-free preparation from a mixture of endosperm and embryo, Albone et al. (1990) found that GA_7 was formed from GA_9 via 2,3-didehydroGA_9 and not from GA_4, which is the precursor of GA_7 in cultures of the fungus *Gibberella fujikuroi* (Pitel et al., 1971). The enzyme preparation also converted GA_5 to GA_3 (Albone et al., 1990), although endosperm and cotyledons of *M. macrocarpus* contain much less GA_3 than GA_7 (MacMillan and Gaskin, 1996). The enzyme(s) catalyzing the conversion of 2,3-didehydroGA_9 to GA_7 and of GA_5 to GA_3 was shown to have the characteristics of dioxygenases, requiring Fe^{2+} and 2-oxoglutarate for activity (Smith et al., 1991). It catalyzes an unusual reaction in which loss of the 1β-H is accompanied by rearrangement of the 2,3-double bond to the 1,2-position and hydroxylation on C-3 (Albone et al., 1990). We are currently engaged in the characterization of this novel 2-oxoglutarate-dependent dioxygenase activity and are attempting to isolate the native protein and clone the genes encoding the enzyme(s) using methodology based on PCR.

Both approaches require further information on the location of the GA_7 (GA_3)-forming enzyme in developing *M. macrocarpus* seed and on the nature of other GA-metabolizing dioxygenases present. These questions have been addressed by investigating the metabolism of the following substrates in enzyme preparations from endosperm and from developing cotyledons of *M. macrocarpus*: [1,7,12,18-$^{14}C_4$]GA_{12}-aldehyde, [1,7,12,18-$^{14}C_4$]GA_{12}, 2,3-didehydro[17-^{14}C]GA_9, [17-^{14}C]GA_9, [17-^{14}C]GA_{20}, and [17-^{13}C,^{3}H]GA_5.

This paper describes the results of metabolic studies and initial PCR experiments using embryo-derived RNA, which have led to the isolation and expression of a cDNA encoding a GA 20-oxidase.

MATERIALS AND METHODS

Fruit of *Marah macrocarpus* (formerly *Echinocystis macrocarpa*) were collected in the Santa Monica hills of California. The seeds were cut in one-half longitudinally to bisect the embryo. For the enzyme preparations, endosperm and em-

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Abbreviation: KRI, Kovats retention index.

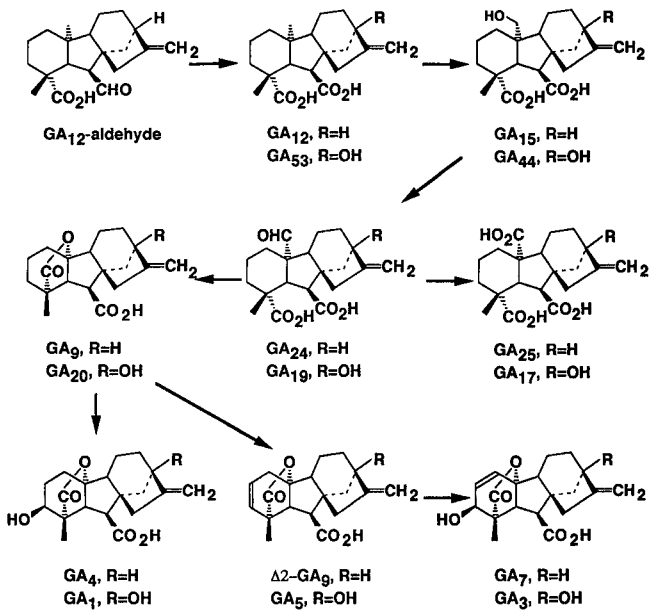

Figure 1. Hypothesized GA biosynthetic pathway from GA_{12}-aldehyde in endosperm and cotyledons of *M. macrocarpus*.

bryos were collected from seeds in which the embryos were 50 to 90% of the seed length, i.e. with a maturity index of 50 to 90 (Blechschmidt et al., 1984). For northern blots, endosperm and embryos from seeds of maturity index 0 to 50, 50 to 75, and 75 to 100 were collected, together with immature and mature leaves and young shoots. All plant material was placed immediately in liquid N_2 and stored at −80°C.

Enzyme Preparations from Endosperm

Frozen endosperm (2.5 g) was homogenized at 0 to 4°C in 2.5 mL of 100 mM Tris-HCl containing 4 mM DTT (pH 7.2 at 30°C), then centrifuged at 35,000*g* for 1 h. A portion of the supernatant (500 μL) was pelleted in liquid N_2 and stored at −80°C for assay as End-1. Another portion (500 μL) was applied to a Sephadex G-25 column (PD-5, Pharmacia) equilibrated with 5 mL of 100 mM Tris-HCl containing 5 mM ascorbic acid and 4 mM DTT, and eluted with 1 mL of the equilibrating buffer to yield End-2, which was pelleted in liquid N_2 and stored at −80°C.

The remainder (4 mL) of the supernatant was treated with 10 mL of saturated ammonium sulfate in 100 mM Tris HCl containing 4 mM DTT. After centrifugation at 35,000*g* for 1 h, the pellet was dissolved in 500 mL of 100 mM Tris-HCl containing 4 mM DTT to yield End-3. From a repeat preparation, End-3, like End-2, was gel-filtered to yield End-4. Both End-3 and End-4 were pelleted in liquid N_2 and stored at −80°C.

Enzyme Preparations from Embryos

Frozen embryos (20 g) were pulverized in liquid N_2, homogenized at 0 to 4°C in 20 mL of 100 mM Tris-HCl containing 4 mM DTT (pH 7.2 at 30°C), and then centrifuged at 35,000*g* for 1 h. The supernatant was pipetted off through paper tissue to remove lipid-like material and treated with 40 mL of saturated ammonium sulfate in 100 mM Tris HCl containing 4 mM DTT. After centrifugation at 35,000*g* for 1 h, the pellet was dissolved in 15 mL of 100 mM Tris-HCl containing 4 mM DTT to yield Emb-1. The cloudy solution was desalted in 2.5-mL aliquots using a Sephadex G-25 column equilibrated with 5 mL of 100 mM Tris-HCl containing 4 mM DTT. The eluate (Emb-2, 3.5-mL aliquots) was pelleted in liquid N_2 and stored at −80°C.

Labeled-GA Substrates

[1,7,12,18-$^{14}C_4$]GA_{12}-aldehyde (7.95 TBq mol^{-1}) and [1,7,12,18-$^{14}C_4$]GA_{12} (7.0 TBq mol^{-1}) were prepared from *R*-[2-^{14}C]mevalonic acid as described by Graebe et al. (1974). The preparation of [17-^{13}C,^{3}H]GA_5 (1.51 TBq mol^{-1}) was as described previously by Fujioka et al. (1988).

2,3-Didehydro[17-^{14}C]GA_9

Dry [^{14}C-methyl]triphenylphosphonium bromide (14.3 mg, 0.04 mmol, approximately 55 MBq) was shaken in freshly distilled tetrahydrofuran (500 μL) in an atmosphere of N_2. One-molar potassium *tert*-butoxide in *tert*-butanol (36 mL, 0.036 mmol) was added (green color), and shaking was continued for 30 min. After centrifugation (1500 rpm for 5 min) the supernatant was added under N_2 gas to solid 2,3-didehydroGA_9 norketone methyl ester (3.3 mg, 0.01 mmol), prepared as described by Albone et al. (1990). The mixture was shaken for 30 min and, after adding a few drops of acetone, was passed through a column (1.0 × 0.5 cm) of silica, which was washed with CH_2Cl_2 (2 × 1.0 mL). The combined filtrate and washings were evaporated to dryness and the residue heated under reflux for 15 h with methanol (500 μL) and 2 M sodium hydroxide (500 μL). The methanol was removed in a steam of N_2 and the aqueous residue, made up to 10 mL with distilled water, was extracted with ethyl acetate (5 × 5 mL) to give a neutral fraction (5.8 MBq), which remained at the origin after TLC on silica gel with ethyl acetate:*n*-hexane:acetic acid (100:100:1, v/v). The aqueous layer was acidified to pH 3.0 with 6 N HCl and extracted with ethyl acetate (5 × 5 mL), which was evaporated to dryness and heated at 70°C for 30 min. An aliquot of the product (7.5 MBq) showed one radioactive spot by TLC (ethyl acetate:*n*-hexane:acetic acid [100:100:1, v/v]) at the same R_F (0.31) as a reference sample of 2,3-didehydroGA_9 (Albone et al., 1990). GC-MS of the product as the methyl ester showed the presence of approximately 50% of 2,3-didehydroGA_9 norketone. An aliquot (5%) was purified by HPLC on a 25- × 0.46-cm, 5-μm column (RLS, Bio-Rad) using a linear gradient from 100% water to 100% methanol over 40 min at 1 mL min^{-1} to yield radiochemically pure 2,3-didehydro[17-^{14}C]GA_9 (310 KBq, 35% yield, based on the starting norketone). It was also chemically pure by GC-MS of the methyl and trimethylsilyl esters, from which a specific activity of 1.75 TBq mol^{-1} was calculated (Bowen et al., 1972).

[17-^{14}C]GA_{20}

[17-^{14}C]GA_{20} was prepared in the same way as 2,3-didehydro[17-^{14}C]GA_9 from the known (Ingram et al., 1984) norketone of GA_{20} methyl ester 13-acetate (3.9 mg, 0.01 mmol). After hydrolysis of the product of methylenation, the acidic fraction was heated at 70°C for 1 h to yield [17-^{14}C]GA_{20} (9.9 MBq, 54% yield), which was shown to be radiochemically pure by TLC (R_F 0.31, silica gel, ethyl acetate:acetic acid, 100:1, v/v) and by HPLC (retention time 27.5 min) on a C_{18} column (see above), and was eluted isocratically with water for 5 min and then with a linear gradient from 100% water to 100% methanol over 45 min. A specific radioactivity of 1.84 TBq mol^{-1} was calculated (Bowen et al., 1972) from GC-MS of the methyl ester trimethylsilyl ether, which showed the absence of GA_{20} norketone.

[17-^{14}C]GA_9

[17-^{14}C]$GA_9$was a gift from Dr. I. Yamaguchi (University of Tokyo) and was purified by HPLC as described above for 2,3-didehydro[17-^{14}C]GA_9. GC-MS of the methyl ester confirmed its chemical purity and gave a specific radioactivity of 2.1 TBq mol^{-1}.

Enzyme Assays

Enzyme preparations (10 μL) were diluted to 90 μL with 100 mM Tris-HCl containing 4 mM DTT (pH 7.2 at 30°C) and added to 5 μL of substrate (approximately 60,000 Bq) and 5 μL of a cofactor solution to yield a final concentration of 4 mM 2-oxoglutaric acid, 4 mM ascorbic acid, 5 mM $FeSO_4$, 2 mg mL^{-1} BSA, and 0.1 mg mL^{-1} catalase. The mixture was incubated at 30°C for 60 min and the reaction was stopped by adding 10 μL of glacial acetic acid and 140 μL of water. The mixture was centrifuged at 3,000 rpm for 5 min and an aliquot (200 μL) was injected by an autosampler (Type 110, Kontron Instruments, Ltd., St. Albans, Herts, UK) onto a guard column (Brownlee RP-18, 5 μm, 3 × 0.21 cm i.d., Anachem, UK). The guard column was eluted with water containing 50 μL of acetic acid L^{-1} (5 mL), which eluted the cofactors, DTT and protein. By means of a switching device (Tracer 670, Kontron) the guard column was connected to a C_{18} column (RLS, Bio-Rad; 5 μm, 250 × 0.46 cm) and an online, solid-scintillant radioactivity monitor (Reeve Analytical, Ltd., Glasgow, UK). The column was eluted with mixtures of methanol and water containing 50 μL of acetic acid L^{-1}. Different elution profiles were used for each substrate as follows: [$^{14}C_4$]GA_{12}-aldehyde and [$^{14}C_4$]GA_{12}, 100% water for 5 min; 75% methanol for 15 min; 75 to 100% methanol, exponential gradient over 10 min; 100% water for 6 min. 2,3-Didehydro[17-^{14}C]GA_9 and [17-^{14}C]GA_9, 100% water for 5 min; 0 to 100% methanol, linear gradient over 30 min; 100% methanol for 8 min; 100% water for 2 min. [17-^{14}C]GA_{20} and [^{13}C,^{3}H]GA_5, 100% water for 5 min; 25 to 62% methanol, linear gradient over 20 min; 62 to 100% methanol, exponential gradient over 20 min; 100% MeOH for 5 min; 100% water for 4 min.

GC-MS Analysis of [^{14}C]GAs

Radioactive fractions from HPLC were evaporated to dryness, dissolved in 200 μL of methanol, and methylated with ethereal CH_2N_2. The methylated fractions were evaporated to dryness and trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoro-acetamide. The methyl ester trimethylsilyl ether-derivatized samples were analyzed by full-scan GC-MS, as described previously (MacMillan and Gaskin, 1996).

Isolation of PCR Clones

Poly(A)$^+$ RNA was prepared from frozen embryos using a method described previously (Phillips et al., 1995). First-strand cDNA was synthesized as described by Phillips and Huttly (1994), except that random hexamer primers were used and the cDNA was purified by phenol-chloroform extraction followed by isopropanol precipitation. The cDNA was used in PCR with degenerate primers: sense primer 5′-GCAAGCTTAA(CT)TA(CT)TA(CT)CC(AGCT)AC(AGCT)TG-3′ and antisense primer 5′-GCGAATTC(AGCT)CC(AGT)AT(AG)TT(AGT)AT(ACGT)AC(AG)AA-3′. Five reactions were performed, each containing 20 ng of cDNA, 2 μg of each primer, 250 μM dNTPs, 10 μL of 10× PCR buffer, and 0.5 unit of *Taq* polymerase (Perkin-Elmer) in a total volume of 100 μL. Reaction mixtures were heated at 95°C for 5 min and then subjected to 40 cycles of 94°C for 1 min, 30°C for 2 min, and 72°C for 3 min, extended by 5 s each cycle. Finally, the reactions were heated at 72°C for 10 min. The pooled products were purified by agarose gel electrophoresis, digested with *Eco*RI and *Hin*dIII, and ligated into pUC19. Ligation products were used to transform *Escherichia coli* strain XL1-Blue, and recombinant clones were identified. After sequencing, three different PCR products encoding dioxygenase sequences were recognized and designated M3, M7, and M9.

Isolation of Full-Length cDNA Clones

A cDNA library was constructed in λZapII from poly(A)$^+$ RNA, prepared as described above, using a DNA synthesis kit (ZAP-cDNA kit, Stratagene) and packaged (Gigapack II Gold, Stratagene). The library of 4.5 × 10^5 recombinant clones was amplified by passage through *E. coli* XL1-Blue and screened with each PCR clone. Five × 10^5 plaques were plated onto ten 10- × 10-cm Petri dishes and allowed to grow overnight at 37°C. Duplicate lifts onto nitrocellulase filters (0.45 μm; Schleicher & Schuell) were probed with single-stranded DNA, labeled by primer extension in the presence of [^{32}P]dCTP (Huttly et al., 1988). Positive signals from congruent plaques were identified by autoradiography and the plaques were cored and rescreened until pure. Inserts were rescued into pBlueScript according to the instructions by Stratagene.

Heterologous Expression and Enzyme Assays

The insert from a full-length cDNA clone (M3–8) that showed high amino acid sequence similarity with GA 20-oxidases was excised with *Bam*HI and *Kpn*I and inserted

into the pTrcHisA expression vector (Invitrogen, San Diego, CA). A 50-mL culture of *E. coli* (TOP10) containing the recombinant plasmid was induced at OD 0.36 (600 nm) with isopropyl-β-D-thiogalactoside (final concentration 1 mM) and then grown at 30°C overnight. Cells were spun down at 4,000 rpm for 5 min and lysed by resuspending in 2 mL of 100 mM Tris-HCl (pH 7.5 at 25°C) containing 4 mM DTT and lysozyme (2 mg), and incubating on ice for 30 min, followed by freeze-thawing. After centrifugation at 15,000*g* for 5 min, 90 μL of the supernatant was incubated overnight at 30°C with [^{14}C]GA_{12} or [^{14}C]GA_{53} (167 Bq, 30 pmol) and cofactors in a total volume of 100 μL, using the assay conditions described above. The identity of products, recovered after HPLC, was determined by GC-MS.

Hybridization to Northern Blots

Poly(A)$^+$ RNA was extracted from frozen tissues as described by Grierson (1992). Aliquots of the RNA (0.5 μg for each tissue, except endosperm at maturity index 75–100, for which only 0.4 μg was available) were separated on denaturing gels (Sambrook et al., 1989) and, after transfer to nylon (Hybond-N, Amersham), probed with the insert from the full-length cDNA clone, M3–8, which had been labeled with ^{32}P by random-primed labeling using a DNA labeling kit (High Prime, Boehringer Mannheim). Blots were hybridized at 42°C overnight, as described by Phillips and Huttly (1994), followed by washing once in 2× SSC, 0.1% (w/v) SDS at 25°C for 10 min, and twice in 0.1× SSC, 0.1% (w/v) SDS at 60°C for 10 min. After autoradiography, blots were stripped by washing twice for 10 min with 0.1× SSC, 0.1% (w/v) SDS at 95°C. The filter was then probed sequentially with a pumpkin GA 20-oxidase cDNA (B11; Lange et al., 1994), M7–3, and the PCR product M9 in the same manner.

DNA Sequence Analysis

The PCR-generated fragments in pUC19 were sequenced by the dideoxynucleotide chain termination method from the universal and reverse sequencing primers using a sequencing kit (T7, Pharmacia). The full-length cDNA clones M3–8 and M7–3 were sequenced on both strands using automated DNA sequencers (Genesis, DuPont, and model 373A, Applied Biosystems, respectively).

RESULTS

Embryo Enzyme Activities

Using the enzyme preparations Emb-1 and Emb-2 under the standard incubation conditions, 2,3-didehydro[17-^{14}C]GA_9 yielded [^{14}C]GA_7 (Fig. 2a), and [^{14}C]GA_9 yielded [^{14}C]GA_7 and [^{14}C]GA_4 (Fig. 2b) as products. [$^{14}C_4$]GA_7 and [$^{14}C_4$]GA_4 were also formed from [$^{14}C_4$]GA_{12} (Fig. 3a) and [$^{14}C_4$]GA_{12}-aldehyde (data not shown). All labeled products were recovered after radio-HPLC and identified by full-scan GC-MS (Table I). There was high dilution of ^{14}C-label in the products from the Emb-1 preparation, but not in the desalted preparation, Emb-2. This dilution is explained by the relatively large amounts of GA_4 and GA_7 present in Emb-1, but not in Emb-2 (MacMillan and Gaskin, 1996).

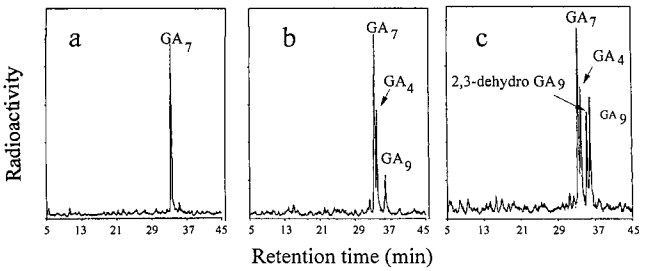

Figure 2. Radio-HPLC profiles from incubation with enzyme preparations from *M. macrocarpus* seeds: a, 2,3-didehydro[17-^{14}C]GA_9 with Emb-1 and Emb-2; b, [17-^{14}C]GA_9 with Emb-1 and Emb-2; and c, [17-^{14}C]GA_9 with End-4. Metabolites were identified by GC-MS (see Tables I and II).

[17-^{14}C]GA_{20} was converted less effectively than was GA_9 (compare Albone et al., 1990). Under the standard incubation conditions, it was only partly converted to [17-^{14}C]GA_1 (41%) and [17-^{14}C]GA_{60} (17%), which were identified by GC-MS (Table I). [17-^{14}C]GA_3 was not formed. However, [17-^{3}H,^{13}C]GA_5 was completely metabolized to GA_3, as was shown by GC-MS (Table I).

Endosperm Enzyme Activities

Incubations with the endosperm preparations End-1 and End-3 gave the same qualitative results. Unlike the embryo preparations, they did not convert [$^{14}C_4$]GA_{12}-aldehyde or [$^{14}C_4$]GA_{12} to [$^{14}C_1$]GA_4 and [$^{14}C_1$]GA_7. The products were the same in both cases and the radio-HPLC profile is shown only for [$^{14}C_4$]GA_{12} (Fig. 3b). The single radio-HPLC peak was a mixture of [$^{14}C_4$]GA_{15} (24%), [$^{14}C_4$]GA_{24} (17%), [$^{14}C_4$]GA_{25} (31%), and [$^{14}C_4$]GA_9 (28%), identified by GC-MS (Table II). Nevertheless, like the embryo preparations, these endosperm preparations completely metabolized [^{14}C]GA_9 to [^{14}C]GA_4, and [^{14}C]GA_7 and 2,3-didehydro[^{14}C]GA_9 to [^{14}C]GA_7. The radio-HPLC profiles were identical to those for the embryo preparations (Fig. 2, a and b), and the GC-MS data (Table II) confirmed the identity of the metabolites.

Preparations End-2 and End-4 were less active, presumably as a result of gel filtration, but gave the same qualitative results as End-1 and End-3 with the substrates [$^{14}C_4$]GA_{12}-aldehyde, [$^{14}C_4$]GA_{12}, and 2,3-didehydro-[^{14}C]GA_9. However, with [^{14}C]GA_9, the intermediate 2,3-didehydro[^{14}C]GA_9 was formed, as well as [^{14}C]GA_4 and [^{14}C]GA_7 (Fig. 2c; Table II).

[17-^{14}C]GA_{20} was converted poorly by the endosperm preparations. For example, it was converted into [$^{14}C_1$]GA_1 in less than 5% yield by End-4. Similarly, [17-^{3}H,^{13}C]GA_5 was metabolized to [^{13}C]GA_3 in only about 25% yield. The products were identified by radio-HPLC only (data not shown).

Isolation of Dioxygenase cDNA Clones

To examine in detail the enzyme activities involved in the conversion of GA_{12} to GA_4 and GA_7, we decided to take

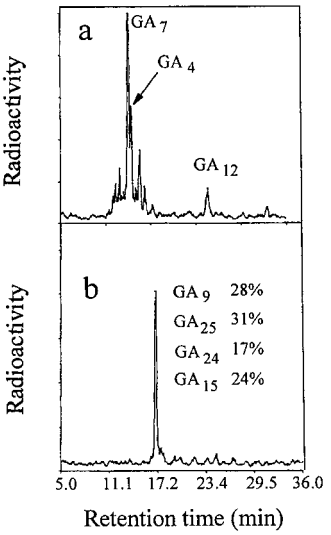

Figure 3. Radio-HPLC profiles from incubations of $[^{14}C_4]GA_{12}$ with Emb-1 (a) and End-4 (b) enzyme preparations from *M. macrocarpus* seeds. Metabolites were identified by GC-MS (see Tables I and II).

a PCR-based approach to isolate dioxygenase cDNAs from developing embryos of *M. macrocarpus*. Degenerate oligonucleotide primers, designed on the basis of conserved regions in the plant dioxygenases, were used to amplify dioxygenase sequences from embryo-derived cDNA. The same primers had been used previously to amplify GA 20-oxidase sequences from Arabidopsis (Phillips et al., 1995). After cloning the PCR products into pUC19 and sequencing, three different dioxygenase sequences were recognized among the products (Fig. 4). The derived amino acid sequences of representative products, designated M3, M7, and M9, were, respectively, 70, 31, and 61% identical to the sequence of a GA 20-oxidase from pumpkin embryos (Lange et al., 1994).

A cDNA library derived from *M. macrocarpus* embryos was constructed in λZAPII and, after amplification, was screened with each of the PCR-derived dioxygenase fragments. Positives, selected with M3 and M7, were plaque-purified and rescued into pBluescript. No positives were obtained after screening with M9. Clones containing inserts of the expected size for full-length dioxygenase cDNAs were partially sequenced to confirm that they encoded dioxygenases. A full-length cDNA clone belonging to each class, M3–8 and M7–3, was fully sequenced and shown to contain the sequence of the respective PCR product. The derived amino acid sequences of both cDNAs contain the regions conserved in the plant dioxygenases (Fig. 5); M3–8 is very closely related to GA 20-oxidases from pumpkin and Arabidopsis, its amino acid sequence being 60% identical (81% similar) with that of the pumpkin enzyme. M7–3 was not closely related to any dioxygenase sequences in the GenBank and EMBL data banks.

Heterologous Expression in *E. coli*

The close sequence similarity between M3–8 and GA 20-oxidases suggested that M3-8 may encode a GA 20-oxidase. This was confirmed by expressing the cDNA as a fusion protein in *E. coli* using the pTrcHis vector. Cell lysates from bacteria transformed with the recombinant plasmid and induced with isopropyl-β-D-thiogalactoside converted $[^{14}C_4]GA_{12}$ to $[^{14}C_4]GA_9$ and a trace of $[^{14}C_4]GA_{25}$ when incubated in the presence of dioxygenase cofactors (Fig. 6, Table III). Under the same conditions, $[^{14}C_4]GA_{53}$ was converted less well than was $[^{14}C_4]GA_{12}$

Table I. *KRI and GC-MS data of the methyl ester trimethylsilyl ether derivatives of metabolites from the substrates [17-^{14}C]GA$_9$, Δ2-[17-^{14}C]GA$_9$, [17-^{14}C]GA$_{20}$, [17-^{3}H,^{13}C]GA$_5$, [^{14}C$_4$]GA$_{12}$, and [^{14}C$_4$]GA$_{12}$-aldehyde, incubated with an ammonium sulfate-precipitated and desalted enzyme preparation from M. macrocarpus cotyledons (seed maturity index, 75–100)*

Identifications are based on a comparison with published data for unlabeled compounds (Gaskin and MacMillan, 1991).

Substrate	Metabolite	KRI	Diagnostic Ions
			m/z
$[^{14}C_1]GA_9$	$[^{14}C_1]GA_7$	2521	195 (25)[a], 224 (100), 225 (56), 284 (23), 358 (17), 386 (8), 418 (9)
	$[^{14}C_1]GA_4$	2498	129 (100), 226 (79), 227 (90), 231 (42), 235 (50), 263 (36), 286 (91), 291 (54), 330 (21), 388 (17), 420 (16)
	$[^{14}C_1]GA_{34}$	2659	203 (61), 219 (35), 225 (44), 231 (55), 290 (14), 315 (24), 389 (9), 418 (11), 461 (5), 508 (100)
Δ2-$[^{14}C_1]GA_9$	$[^{14}C_1]GA_7$	2520	195 (28), 224 (100), 225 (60), 284 (25), 358 (17), 386 (12), 418 (11)
$[^{14}C_4]GA_{12}$	$[^{14}C_4]GA_7$	2522	197 (30), 228 (100), 229 (72), 290 (40), 362 (11), 392 (4), 424 (21)
$[^{14}C_4]GA_{12}$-aldehyde	$[^{14}C_4]GA_7$	2521	197 (43), 228 (100), 229 (46), 290 (23), 362 (12), 392 (8), 424 (10)
$[^{14}C_1]GA_{20}$	$[^{14}C_1]GA_1$	2664	209 (34), 225 (10), 237 (12), 313 (6), 315 (10), 376 (4), 377 (5), 378 (14), 379 (15), 391 (8), 448 (7), 450 (17), 465 (3), 506 (24), 508 (100)
	$[^{14}C_1]GA_{60}$	2572	375 (10), 377 (56), 447 (2), 449 (6), 506 (13), 508 (100)
$[^{3}H,^{13}C_1]GA_5$	$[^{14}C_1]GA_3$	2687	209 (32), 239 (29), 298 (14), 312 (17), 371 (28), 398 (10), 446 (15), 461 (7), 476 (17), 505 (100)

[a] Numbers in parentheses indicate relative abundance as a percentage of base peak.

Table II. *KRI and MS data of the methyl ester or methyl ester trimethylsilyl ether derivatives of metabolites from the substrates [17-$^{14}C_1$]GA_9, Δ2-[17-$^{14}C_1$]GA_9, and [$^{14}C_4$]GA_{12} incubated with the enzyme preparation End-4 from M. macrocarpus endosperm*

Identifications are based on a comparison with published data for unlabeled compounds (Gaskin and MacMillan, 1991).

Substrate	Metabolite	KRI	Diagnostic Ions
			m/z
[$^{14}C_1$]GA_9	[$^{14}C_1$]GA_7	2522	193 (18)[a], 195 (35), 222 (11), 224 (100), 225 (69), 282 (5), 284 (30), 358 (21), 386 (15), 418 (13)
	[$^{14}C_1$]GA_4	2498	129 (100), 226 (54), 227 (68), 231 (25), 233 (17), 235 (41), 263 (22), 286 (71), 291 (44), 328 (22), 330 (17), 388 (17), 418 (3), 420 (10)
	Δ2-[$^{14}C_1$]GA_9	2300	156 (33), 226 (100), 227 (73), 286 (51)
Δ2-[$^{14}C_1$]GA_9	[$^{14}C_4$]GA_7	2517	193 (32), 195 (22), 222 (41), 223 (60), 224 (76), 225 (56), 282 (16), 284 (22), 356 (14), 358 (19), 384 (11), 386 (14), 416 (6), 418 (13)
[$^{14}C_4$]GA_{12}	[$^{14}C_4$]GA_{15}	2608	195 (15), 201 (58), 239 (48), 245 (100), 284 (17), 290 (25), 312 (5), 320 (13), 352 (7)
	[$^{14}C_4$]GA_{24}	2435	225 (35), 226 (28), 227 (20), 231 (100), 232 (86), 233 (64), 254 (13), 262 (31), 285 (11), 286 (17), 292 (35), 293 (37), 294 (31), 314 (16), 322 (44), 342 (3), 350 (12)
	[$^{14}C_4$]GA_{25}	2438	225 (28), 231 (100), 284 (61), 292 (91), 312 (33), 320 (79), 372 (4), 380 (9)
	[$^{14}C_4$]GA_9	2302	226 (35), 230 (42), 270 (34), 276 (100), 292 (22), 298 (18), 306 (59), 338 (8)

[a] Numbers in parentheses indicate relative abundance as a percentage of base peak.

(45% as opposed to almost 100%) and only to [$^{14}C_4$]GA_{44}. *E. coli* transformed with pTrcHis without insert produced no enzyme activity for conversion of [$^{14}C_4$]GA_{12} or [$^{14}C_4$]GA_{53}.

M7–3 was also expressed in *E. coli* using pTrcHis, but the product failed to convert any of the ^{14}C-labeled GA substrates tried: GA_{12}, GA_{12}-aldehyde, GA_9, GA_{15}, GA_{19}, and GA_{20} (data not shown). M7–3 is therefore unlikely to encode a GA-biosynthetic enzyme.

Expression in *M. macrocarpus* Tissues

The content of M3–8 transcript in immature seed tissues at different developmental stages and in vegetative tissues of *M. macrocarpus* was examined by hybridization to northern blots of poly(A)$^+$ RNA (Fig. 7). GA 20-oxidase mRNA was abundant in both the endosperm and embryo, with highest amounts in the younger endosperm (maturity index 0–75%) and older embryos (maturity index 25–100%). No transcript was detected in shoot tissues: mature and expanding leaves, shoot apices, and tendrils. Northern blots were also probed with the pumpkin 20-oxidase cDNA (B11) and with the *M. macrocarpus* DNA sequences M7–3 (full-length cDNA) and M9 (PCR product), but no hybridization was detected (data not shown).

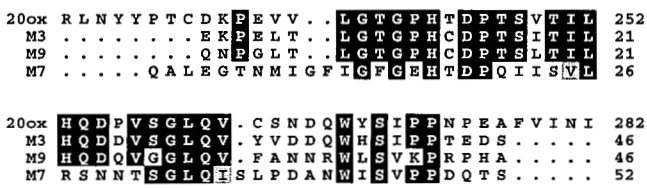

Figure 4. Alignment of the predicted amino acid sequences of the PCR products M-3, M-7, and M-9, amplified from cDNA from *M. macrocarpus* embryos, with that of the GA 20-oxidase from *C. maxima* (20ox). Identical residues are boxed in black; similar residues are shaded in gray.

DISCUSSION

The results of the present metabolic studies are discussed in reference to the hypothesized GA pathways for *M. macrocarpus* seeds shown in Figure 1. For the 13-hydroxylation pathway to GA_1 and GA_3, which are minor endogenous GAs, it was confirmed (Albone et al., 1990) that GA_{20} is not a precursor of GA_3, but that it is a precursor of GA_1 in the embryo and endosperm preparations. It was also shown that the enzyme activity for the conversion of GA_5 to GA_3 is present in both the endosperm and embryo.

For the non-13-hydroxylation pathway, all of the enzymes for the conversion of GA_{12}-aldehyde to the major endogenous GAs, GA_4, and GA_7, are present in the embryo and endosperm. Thus, the individual steps from 2,3-didehydroGA_9 to GA_7 and from GA_9 to GA_4 were shown to occur in cell-free preparations from both tissues. 2,3-DidehydroGA_9 accumulated during the conversion of GA_9 to GA_7 by the endosperm preparation that had been desalted and presumably deactivated sufficiently to allow the intermediate to accumulate. However, a striking difference between the endosperm and embryo preparations is in the metabolism of GA_{12} (and GA_{12}-aldehyde). The embryo preparations metabolized GA_{12} to GA_4 and GA_7, with no accumulation of GA_9 or 2,3-didehydroGA_9. In contrast, the endosperm preparation metabolized GA_{12} only to GA_9 and its C_{20} precursors, despite the fact that the same preparation converted GA_9 efficiently to GA_4 and GA_7. The reason for the inability of the endosperm

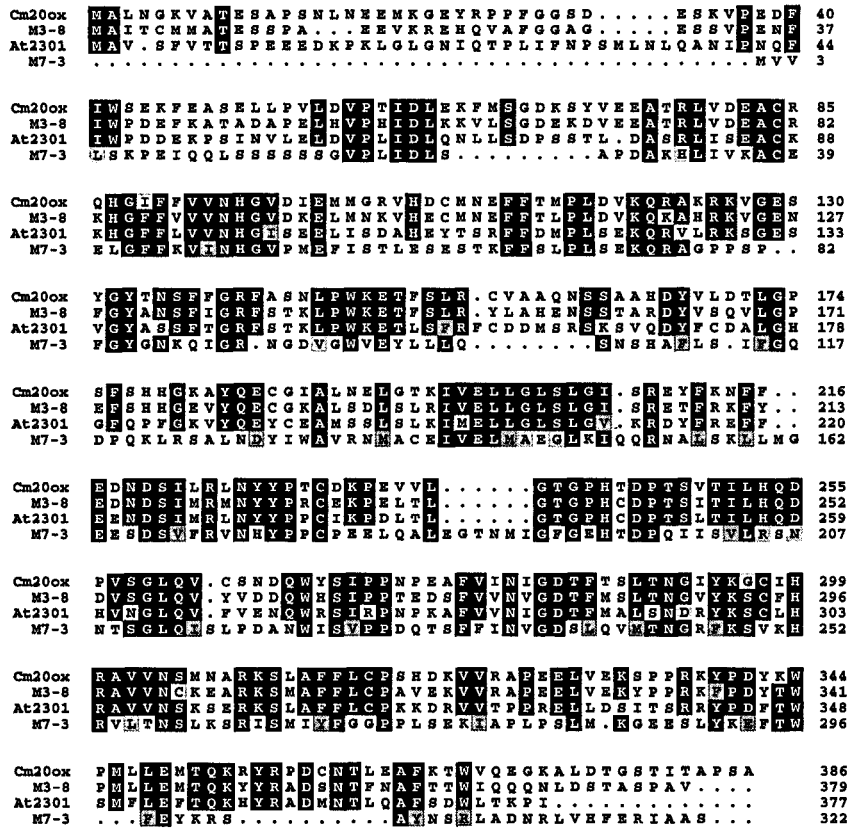

Figure 5. Predicted amino acid sequences of full-length cDNA clones M3–8 and M7–3 aligned with sequences of GA 20-oxidases from *C. maxima* (Cm-20ox; Lange et al., 1994) and *A. thaliana* (At2301; Phillips et al., 1995). Identical residues are boxed in black; similar residues are shaded in gray.

preparation to metabolize GA_{12} directly to GA_4 and GA_7 is unknown.

We have cloned a cDNA encoding a GA 20-oxidase (M3–8) from *M. macrocarpus* embryos and shown it to be expressed at high levels in both embryos and endosperm. The enzyme, prepared as a fusion protein in *E. coli*, mainly converted GA_{12} to GA_9, with GA_{25} as a minor product, and, therefore, has the activity observed in the embryo enzyme preparation. It is functionally similar to GA 20-oxidases that have been cloned from Arabidopsis (Phillips et al., 1995; Xu et al., 1995), spinach (Wu et al., 1996), and pea (Martin et al., 1996), but different from the enzyme present in seeds of the closely related species *Cucurbita maxima* (pumpkin), which produces GA_{25} as the major product from GA_{12} (Lange et al., 1994). Similar to the pumpkin and Arabidopsis enzymes, the *M. macrocarpus* GA 20-oxidase converted the non-13-hydroxylated substrate GA_{12} more readily than its 13-hydroxylated analog, GA_{53}. On the basis of its derived amino acid sequence, the *M. macrocarpus* GA 20-oxidase is more closely related to the pumpkin enzyme (81% similarity) than to enzymes from Arabidopsis (70–73% similarity), despite its close functional relationship with the Arabidopsis enzymes. Hence, the structural differences that determine whether C-20 is lost from the aldehyde intermediate or is oxidized to the carboxylic acid are likely to be subtle. The presence of substantial amounts of GA 20-oic acids in *M. macrocarpus* endosperm (MacMillan and Gaskin, 1996) and the formation of GA_{25} from GA_{12} in relatively high yield in the endosperm homogenates indicates that a 20-oxidase functionally similar to the pumpkin enzyme may also be present in this tissue. If this is the case, however, the enzyme is not a close homolog of the pumpkin 20-oxidase, because cDNA for the pumpkin enzyme failed to hybridize with mRNA from *M. macrocarpus* endosperm.

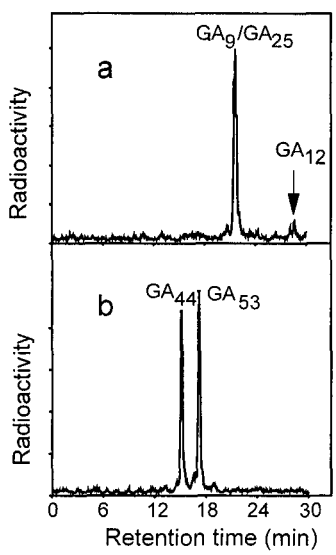

Figure 6. Radio-HPLC profiles from incubations of $[^{14}C_4]GA_{12}$ (a) and $[^{14}C_4]GA_{53}$ (b) with the M3–8 fusion protein. Metabolites were identified by GC-MS (Table III).

Table III. *KRI and MS data of the methyl ester or methyl ester trimethylsilyl ether derivatives of metabolites, identified from the substrates [$^{14}C_4$]GA_{12} and [$^{14}C_4$]GA_{53} incubated with M3-8*

Identifications are based on a comparison with published data for unlabeled compounds (Gaskin and MacMillan, 1991).

Substrate	Metabolite	KRI	Diagnostic Ions
			m/z
[$^{14}C_4$]GA_{12}	[$^{14}C_4$]GA_{25}	2438	231 (100)[a], 290 (66), 292 (88), 312 (23), 320 (56), 380 (18)
	[$^{14}C_4$]GA_9	2299	230 (55), 232 (83), 251 (62), 270 (35), 276 (100), 286 (6), 292 (11), 294 (16), 298 (26), 306 (75), 338 (7)
[$^{14}C_4$]GA_{53}	[$^{14}C_4$]GA_{44}	2778	209 (100), 238 (18), 240 (38), 373 (5), 379 (14), 425 (4), 432 (19), 440 (37)

[a] Numbers in parentheses indicate relative abundance as a percentage of base peak.

M3–8 is not expressed at detectable levels in shoot tissues. It has been found in Arabidopsis that GA 20-oxidase genes form a small multigene family with members expressed in different tissues (Phillips et al., 1995). It is therefore likely that a different 20-oxidase gene is expressed in the vegetative tissues of *M. macrocarpus*. The pumpkin 20-oxidase, which was cloned from embryos (Lange et al., 1994), is also expressed at very low levels in vegetative tissues and mRNA transcripts were detectable only by reverse-transcriptase-PCR (A. Frisse and T. Lange, unpublished data).

Using the PCR approach, we were not able to clone dioxygenases involved in the conversion of GA_9 to GA_4 and GA_7. A second full-length dioxygenase cDNA (M7–3) obtained by this technique does not appear to encode a GA-biosynthesizing enzyme, nor is it expressed in substantial amounts in immature seed tissues. We are therefore currently purifying the enzyme(s) involved in GA_9 metabolism from *M. macrocarpus* embryos as a route to their cDNAs. We hope to determine the number of enzymes required for the conversion of GA_9 to GA_4 and GA_7. By analogy with maize, in which the *D1* gene controls the three steps, GA_{20} to GA_1, GA_{20} to GA_5, and GA_5 to GA_3 (Spray et al., 1996), it is possible that a 3β-hydroxylase-like enzyme catalyzes all three reactions. In that case, the conversion of GA_{12} to GA_4 and GA_7 would require only two enzyme activities.

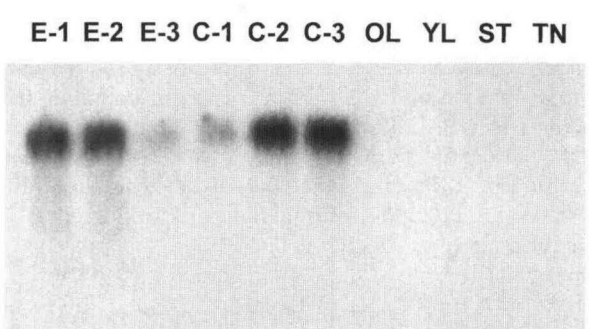

Figure 7. Expression of M3–8 (GA 20-oxidase) in *M. macrocarpus* tissues. M3–8 cDNA was hybridized to northern blots containing 0.5 μg of poly(A)$^+$ RNA extracted from: endosperm (E-1) and embryos (C-1) from seeds of maturity index 0 to 50, 50 to 75 (E-2 and C-2), and 75 to 100 (E-3 and C-3) and from mature (OL) and immature (YL) leaves, shoots (ST), and tendrils (TN). Only 0.4 μg of poly(A)$^+$ RNA was used for E-3.

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