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Isolation and Expression of Three Gibberellin 20-Oxidase cDNA Clones from Arabidopsis

Andrew L. Phillips*, Dennis A. Ward, Scott Uknes, Nigel E. J. Appleford, Theodor Lange, Alison K. Huttly, Paul Gaskin, Jan E. Graebe, and Peter Hedden

IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Bristol, BS18 9AF, United Kingdom (A.L.P., D.A.W., N.E.J.A., A.K.H., P.G., P.H.); Ciba Agricultural Biotechnology, Ciba-Geigy Corporation, P.O. Box 12257, Research Triangle Park, North Carolina 27709–2257 (S.U.); and Pflanzenphysiologisches Institut und Botanischer Garten der Universität Göttingen, Untere Karspüle 2, D-37037 Göttingen, Germany (T.L., J.E.G.)

Using degenerate oligonucleotide primers based on a pumpkin (*Cucurbita maxima*) gibberellin (GA) 20-oxidase sequence, six different fragments of dioxygenase genes were amplified by polymerase chain reaction from *Arabidopsis thaliana* genomic DNA. One of these was used to isolate two different full-length cDNA clones, At2301 and At2353, from shoots of the GA-deficient Arabidopsis mutant *ga1–2*. A third, related clone, YAP169, was identified in the Database of Expressed Sequence Tags. The cDNA clones were expressed in *Escherichia coli* as fusion proteins, each of which oxidized GA_{12} at C-20 to GA_{15}, GA_{24}, and the C_{19} compound GA_9, a precursor of bioactive GAs; the C_{20} tricarboxylic acid compound GA_{25} was formed as a minor product. The expression products also oxidized the 13-hydroxylated substrate GA_{53}, but less effectively than GA_{12}. The three cDNAs hybridized to mRNA species with tissue-specific patterns of accumulation, with At2301 being expressed in stems and inflorescences, At2353 in inflorescences and developing siliques, and YAP169 in siliques only. In the floral shoots of the *ga1–2* mutant, transcript levels corresponding to each cDNA decreased dramatically after GA_3 application, suggesting that GA biosynthesis may be controlled, at least in part, through down-regulation of the expression of the 20-oxidase genes.

The GAs are a family of natural plant and fungal products, a number of which act as plant growth regulators that are involved in a wide range of developmental processes, including germination, stem elongation, flowering, and fruit development (Crozier, 1983). The concentrations of bioactive GAs are important regulators of these processes; this is reflected in the widespread use of growth retardants, which act by inhibiting enzymes in the GA-biosynthetic pathway. These enzymes are thus potential targets for the manipulation of plant development through genetic modification.

The GAs are diterpenoid carboxylic acids produced from mevalonic acid via geranylgeranyl diphosphate, the cyclization of which is the first committed step in the pathway. This reaction is catalyzed by the A activity of *ent*-kaurene synthetase, an enzyme that was recently cloned from Arabidopsis by genomic subtraction (Sun et al., 1992; Sun and Kamiya, 1994). *ent*-Kaurene is metabolized to GAs by membrane-associated monooxygenases and soluble, 2-oxoglutarate-dependent dioxygenases (Graebe, 1987). The latter group includes enzymes responsible for successive oxidations at C-20, leading to its loss as CO_2 and hence to the formation of the biologically active C_{19} GAs (Fig. 1). Oxidation at C-20 is thought to be a site of regulation. In spinach (*Spinacia oleracea*), an LD rosette plant, bolting in response to long days is associated with increased rates of GA_{53} and GA_{19} 20-oxidase activities (Gilmour et al., 1986). Furthermore, there is evidence to suggest that these enzyme activities are down-regulated in maize (*Zea mays*) seedlings as a result of GA action in a type of feedback control (Hedden and Croker, 1992).

We have recently cloned a GA 20-oxidase from developing cotyledons of pumpkin (*Cucurbita maxima*) by screening a cDNA expression library with antibodies raised against a synthetic peptide corresponding to the sequence of a proteolytic fragment of purified GA 20-oxidase from pumpkin endosperm (Lange et al., 1994). Heterologous expression of this cDNA clone in *Escherichia coli* gave a fusion protein that catalyzed three successive oxidations of GA_{12} at C-20. However, 99% of the final product was the tricarboxylic acid GA_{25} with only 1% of the C_{19} product GA_9, which can be converted by 3β-hydroxylation to the biologically active GA_4. It is not yet clear whether the 20-oxidase present in vegetative tissues of plants, in which C_{19} GAs, rather than the tricarboxylic acid C_{20} GAs, are the predominant forms, has a similar catalytic activity or, indeed, how many enzymes are involved in 20-oxidation in such tissues. To address these points and to examine the regulation of 20-oxidase activity, we set out to clone GA 20-oxidases from *Arabidopsis thaliana*.

In this paper we report the isolation of two cDNA clones encoding GA 20-oxidases from shoots of Arabidopsis by PCR amplification of internal fragments using primers based on the pumpkin 20-oxidase cDNA, followed by screening of a cDNA library. A third cDNA clone was identified in the Database of Expressed Sequence Tags (Höfte et al., 1993). The three cDNAs show different pat-

* Corresponding author; e-mail andy.phillips@bbsrc.ac.uk; fax 44–1275–394281.

Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; *Ler*, Landsberg *erecta*.

terns of tissue-specific expression. In a GA-deficient mutant of Arabidopsis, *ga1–2*, transcript levels corresponding to each gene are dramatically reduced after application of biologically active GA_3, suggesting that the biosynthesis of these enzymes might be under feedback control.

MATERIALS AND METHODS

Plant Material

Seeds of the *ga1–2* (isolate 6.59) mutant of *Arabidopsis thaliana* Heyhn. (Koornneef et al., 1983) were obtained from Dr. M. Koornneef. Seeds were germinated by overnight treatment with 10 μM GA_3, then washed in water, suspended in 0.15% agar, and distributed over the surface of potting compost in seed trays. Plants were grown under a 16-h photoperiod in a glasshouse. Wild-type (*Ler*) Arabidopsis plants grown for RNA extraction were sown directly onto compost and grown as above. For production of plants for genomic DNA extraction, seeds were surface sterilized, suspended in 0.15% agar, and distributed onto the surface of sterile 1% agar containing Murashige and Skoog medium with B5 salts, supplemented with 3% Suc.

Isolation of Genomic DNA

Genomic DNA was isolated from shoots of sterile-grown plants of *A. thaliana* Ler essentially as described by Lichtenstein and Draper (1985), except that the plant material was frozen in liquid nitrogen before being ground to a slurry in extraction buffer with the aid of a little acid-washed sand. The DNA was further purified by ethidium bromide-CsCl density gradient centrifugation.

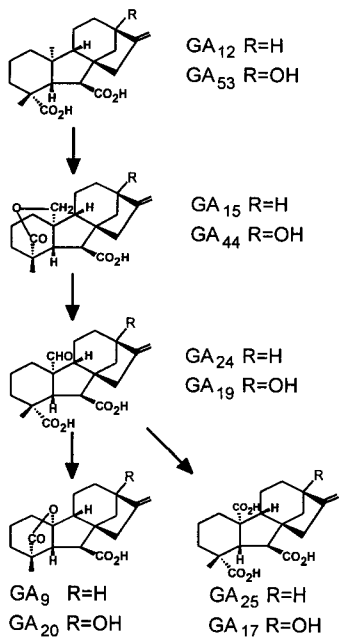

Figure 1. Steps in GA biosynthesis catalyzed by 20-oxidases, showing both the 13-deoxy and 13-hydroxylated intermediates. The C_{19} GAs (GA_9 and GA_{20}) are subsequently converted to bioactive GAs (GA_4 and GA_1, respectively) by 3β-hydroxylation.

PCR with Degenerate Primers

Degenerate oligonucleotides were based on six regions of conserved amino acid sequence in the dioxygenase superfamily (Lange et al., 1994). A total of eight oligonucleotides (five sense and three antisense primers) were synthesized, each incorporating an *Eco*RI or *Hin*dIII site, respectively, at its 5′ end to facilitate cloning: primer A, 5′-GCAAGCTTGTXCCXACXAT(ACT)GA(CT)(CT)T-3′; primer B, 5′-GCAAGCTT(CT)TT(CT)GTXAA(CT)CA(CT)-GG-3′; primer C, 5′-GCAAGCTTAA(CT)TA(CT)TA(CT)-CCXACXTG-3′; primer D, 5′-GCGAATTC(GA)CAXGTXG-G(AG)TA(AG)TA(AG)TT-3′; primer E, 5′- GCAAGCTTG-GXACXGGXCCXCA(CT)ACXGA-3′; primer F, 5′-GCAA-GCTT(CT)GTXAT(ACT)AA(CT)AT(ACT)GGXGA-3′; primer G, 5′-GCGAATTCXCC(AGT)AT(AG)TT(AGT)ATX-AC(AG)AA-3′; primer H, 5′-CGCAATTCTTXACXACXG-CXC(GT)(AG)TG-3′.

Pairs of sense and antisense oligonucleotides were used as primers in PCR reactions with Arabidopsis genomic DNA as substrate. Each 25-μL reaction contained 50 ng of genomic DNA, 2.5 μL of 10× PCR buffer, 2.5 μL of 25 mM deoxynucleoside triphosphates, 2.5 μg of each primer, and 1 unit of *Taq* polymerase (Amplitaq, Applied Biosystems); the final concentration of $MgCl_2$ was 1.5 mM. Samples were heated to 94°C for 5 min, then subjected to 40 cycles of 94°C 1 for min, 35°C for 2 min, and 72°C for 3 min; the polymerization step was extended by 5 s each cycle and the reaction was completed by a 10-min incubation at 72°C. The products were purified by agarose gel electrophoresis and 1/100 of each was used as substrate in a second round of PCR under the conditions described above. Amplified products were again isolated from an agarose gel, digested with *Eco*RI and *Hin*dIII, and ligated to pUC19 that had been digested with the same enzymes and dephosphorylated. Ligation products were introduced into *Escherichia coli* XL1-Blue by transformation, and recombinant clones were identified.

Hybridization to Northern and Southern Blots

Plants of the GA-deficient dwarf mutant of Arabidopsis, *ga1–2*, were grown for 4 to 6 weeks until flower buds had formed within the rosette. GA was applied to plants as a spray of approximately 20 mL of 100 μM GA_3 in water; control plants were sprayed with water. Shoot tissue was harvested after 16 h, frozen in liquid nitrogen, and stored at −75°C. Roots, stems, leaves, flowers, and developing siliques of the *Ler* ecotype were collected in a similar manner. Poly(A)$^+$ RNA was isolated from homogenized, frozen tissue by proteinase K extraction followed by phenol-chloroform extraction, as described by Bartels and Thompson (1983), followed by two rounds of chromatography on oligo(dT) cellulose.

Northern blots were prepared by electrophoresis of 1-μg samples of poly(A)$^+$ RNA through agarose gels in the presence of formaldehyde (Sambrook et al., 1989), followed by transfer to nitrocellulose as described by Thomas (1980). For Southern blots, Arabidopsis genomic DNA was digested with restriction enzymes, separated by agarose gel

electrophoresis, and transferred to nitrocellulose (Sambrook et al., 1989). PCR products, cloned in pUC19, were labeled with ^{32}P by primer extension with Klenow DNA polymerase from the universal or reverse sequencing primer, as appropriate, to produce a labeled, antisense probe (Huttly et al., 1988). Larger fragments were labeled by random-primed labeling (Feinberg and Vogelstein, 1983). Hybridization was carried out at 42°C under the conditions described by Phillips and Huttly (1994). Blots were hybridized for 16 h and then washed for 10 min in 2× SSC, 0.1% SDS at 25°C. Additional 10-min washes were carried out at low (1× SSC, 0.1% SDS; 40°C), medium (0.1× SSC, 0.1% SDS; 40°C), or high (0.1× SSC, 0.1% SDS; 65°C) stringency. Blots were sealed into polyethylene bags and exposed to Kodak XAR film at −75°C with intensifying screens.

Isolation of Full-Length cDNA Clones

A cDNA library was constructed from poly(A)$^+$ RNA isolated as above from floral shoots of *A. thaliana ga1–2*. Double-stranded cDNA was synthesized using a cDNA Synthesis Kit (Amersham), *Eco*RI adapters were added (λgt11 cDNA Cloning Kit, Amersham), and the products were ligated into λZapII (Stratagene) that had been digested with *Eco*RI and dephosphorylated. Ligation products were packaged using Gigapack Gold (Stratagene), and the resulting cDNA library of 2× 10^5 recombinants was amplified by passage through *E. coli* XL1-Blue.

A total of 4× 10^5 phage was plated in *E. coli* XL1-Blue on eight 10-cm-square Petri dishes. Plates were grown for 5 to 6 h until plaques were approximately 0.5 mm in size. Duplicate lifts on nitrocellulose filters (0.45 μm; Schleicher & Schuell) were made as described by Sambrook et al. (1989). Single-stranded, labeled probes were produced from PCR products cloned in pUC19 by primer extension from the universal sequencing primer in the presence of [^{32}P]dCTP, followed by digestion with *Hin*dIII and isolation of the product on a denaturing polyacrylamide gel (Huttly et al., 1988). Hybridization to filters was carried out as described above. Positive signals were identified by autoradiography, cored from the plates, and rescreened as above until plaque pure. Inserts were subcloned by plasmid rescue as described by Stratagene.

DNA Sequence Analysis

PCR-generated fragments cloned into pUC19 were sequenced by the dideoxynucleotide chain termination method from the universal and reverse sequencing primers, using the T7 Sequencing Kit (Pharmacia), except that after denaturation with NaOH, plasmids were purified by spin-desalting (Murphy and Kavanagh, 1988). The inserts of full-length cDNA clones At2301 and YAP169 were sequenced through the construction of nested sets by TN1000 transposon insertion (Strathmann et al., 1991). DNA sequencing of At2353 was performed using chain-termination fluorescent-dideoxynucleotide triphosphates and an Applied Biosystems 373A automated DNA sequencer; analysis was performed using the program Inherit from Applied Biosystems.

General nucleotide and protein sequence analysis was carried out using the University of Wisconsin Genetics Computer Group suite of programs. Homology searches of the EMBL, GenBank, PIR, and SwissProt data bases were performed using the FASTA program; searches of the Database of Expressed Sequence Tags were carried out via the e-mail server at blast@ncbi.nlm.nih.gov.

Heterologous Expression of cDNA Clones in *E. coli*

Full-length cDNA clones were expressed as fusion proteins in *E. coli* using the Invitrogen (San Diego, CA) Xpress Kit. The inserts from At2301 and At2353 were excised as *Eco*RI fragments and inserted into pTrcHisA, and the insert from YAP169 was excised as a *NsiI-PstI* fragment and inserted into pTrcHisC. Recombinant clones in *E. coli* TOP10 were grown shaking at 30°C in 1 L of 2× YT broth with ampicillin (50 μg/mL) to mid-logarithmic phase. Expression was induced by the addition of IPTG to 5 mM; cultures were grown for a further 2 h at 30°C and harvested by centrifugation (4,000 rpm for 30 min at 4°C). The pellets were resuspended twice in 2× YT broth (25 mL), transferred to 50-mL tubes, and recentrifuged as above. After resuspension of the approximately 3-mL pellets in 1.6 mL of 200 mM Tris, pH 7.8, containing 20 mM DTT, the cells were disrupted by the addition of 200 μL of lysozyme solution (20 mg/mL in 200 mM Tris, pH 7.8), incubating on ice for 45 min, and freeze-thawing. The lysates were centrifuged at 50,000*g* for 30 min at 4°C to give approximately 800 μL of supernatant, which was used for enzyme assays.

Enzyme Activities

Cell lysates (different amounts) were incubated at 30°C for 25 h with [^{14}C]GA_{12} or [^{14}C]GA_{53} (2500 Bq, 450 pmol), prepared as described elsewhere (Lange and Graebe, 1993), in the presence of 50 mM Tris, pH 7.8, and a cofactor mixture (24 μL, containing 133 mM 2-oxoglutarate, 133 mM ascorbate, 16.7 mM $FeSO_4$, and 33.3 mg/mL catalase) in a total volume of 224 μL. Fresh cofactors were added after 2, 4, 6, and 8 h (24, 24, 24, and 104 μL, respectively). Products were extracted and separated by HPLC with on-line radiocounting as described previously (Lange, 1994). Fractions containing radioactivity were converted to methyl esters with ethereal diazomethane and then to trimethylsilyl ethers by reaction with *N*-methyl-*N*-trimethylsilyltrifluoracetamide, after which they were analyzed by GC-MS (Lange et al., 1994).

RESULTS

PCR from Arabidopsis Genomic DNA with Degenerate Primers

The degenerate primers used to amplify GA 20-oxidase sequences from *A. thaliana* were designed after comparison of the amino acid sequence of the *Cucurbita maxima* 20-oxidase with that of other plant dioxygenases, which revealed a number of conserved regions (Lange et al., 1994).

Where an amino acid was not conserved in all the dioxygenases, the sequence of the oligonucleotide was based on the amino acid present in the *C. maxima* 20-oxidase. A total of five sense (A–E) and three antisense (F–H) primers were synthesized and were used in all combinations in PCR reactions using genomic DNA from *A. thaliana* Ler as substrate. After 40 cycles of PCR, only two primer combinations, C+G and E+G, yielded products. These fragments were excised from an agarose gel and reamplified with the same primers. The resulting products were cloned into pUC19, and six clones from each class were characterized by restriction endonuclease digestion and DNA sequencing.

None of the products generated by primer pair E+G had significant nucleotide or amino acid sequence homology with the dioxygenase superfamily. In contrast, all of those generated by primers C+G encoded amino acid motifs characteristic of these enzymes. An additional 24 clones derived from primers C+G were chosen at random and sequenced. This yielded a total of six different PCR products (Fig. 2) with between 29 and 66% amino acid identity with the GA 20-oxidase from *C. maxima*. The PCR product most closely related to the *C. maxima* sequence, At2204, was selected for further study.

Hybridization of the ^{32}P-labeled insert of At2204 to a northern blot containing poly(A)$^+$ RNA from the GA-deficient mutant of Arabidopsis, *ga1–2*, identified an mRNA of approximately 1.5 kb that was severely reduced in abundance by treatment of the dwarf plants with GA_3 (not shown). Hybridization of At2204 to a Southern blot of Arabidopsis genomic DNA at low stringency identified two genomic fragments in each enzyme digest (Fig. 3); in contrast, hybridization at high stringency identified only one fragment in each track. This indicates that At2204 is probably represented by a single locus in the Arabidopsis genome, but also suggests the presence of a second, closely related gene. Therefore, we set out to isolate full-length cDNA clones corresponding to both At2204 and this related gene.

Isolation of Full-Length cDNA Clones

Because we had evidence that suggested that mRNA identified by At2204 was more abundant in the absence of GA, we constructed a cDNA library in λZapII from mRNA isolated from shoots of the GA-deficient Arabidopsis mutant *ga1–2*. This library was screened with the ^{32}P-labeled insert of At2204; 20 replicated positive signals were identified in 4×10^5 plaques from the amplified library. Ten of these positives were rescreened until plaque pure and converted into plasmid clones by in vivo rescue. Restriction endonuclease digestion showed that eight of these clones possessed inserts of 1.3 to 1.5 kb, the expected size for a full-length dioxygenase cDNA clone. Limited 5′ and 3′ sequencing of these clones confirmed that they were identical at the nucleotide level and differed only in the length of the 5′ untranslated region; the clone with the shortest 5′ extension, At2301, was used for all subsequent experiments. The preliminary sequence data also confirmed that these clones encoded an enzyme of the dioxygenase class.

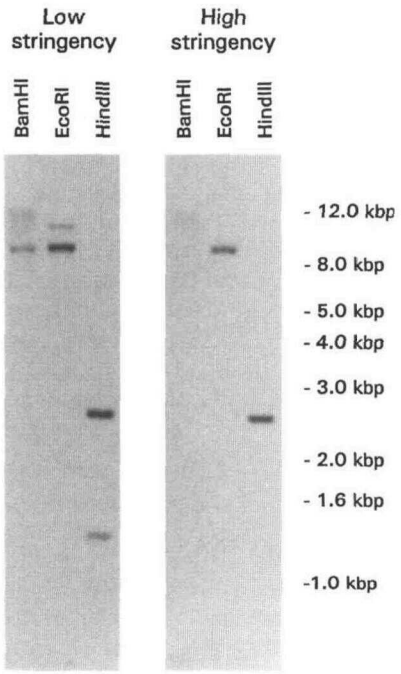

Figure 3. Hybridization at low and high stringency of PCR fragment At2204 to a Southern blot containing Arabidopsis genomic DNA digested with *Bam*HI, *Eco*RI, or *Hin*dIII.

Hybridization of At2204 to a Southern blot of *A. thaliana* genomic DNA had suggested the presence of two closely related genes (Fig. 3). Therefore, we attempted to isolate a cDNA clone corresponding to this second gene by screening the cDNA library with both the PCR product At2204 and its full-length clone (At2301) at low stringency. Approximately 40 positive clones were identified; both probes hybridized to the same plaques. Ten clones were replated and screened until plaque pure, and rescued into plasmid clones. Restriction endonuclease digestion showed that 9 of the 10 clones contained inserts of the expected size for full-length clones. Limited DNA sequencing revealed that

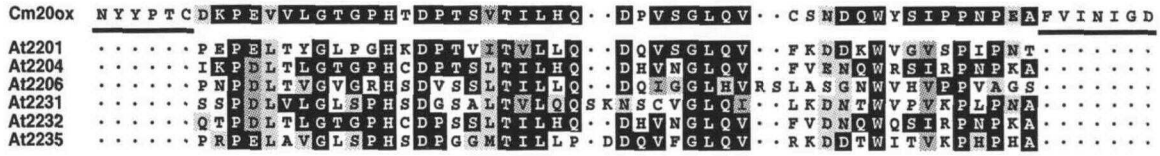

Figure 2. Predicted amino acid sequence of PCR products (At22xx) amplified from Arabidopsis genomic DNA using degenerate oligonucleotide primers C and G (underlined). Residues conserved in the GA 20-oxidase from *C. maxima* (Cm20ox) are boxed in black; similar residues are shaded in gray.

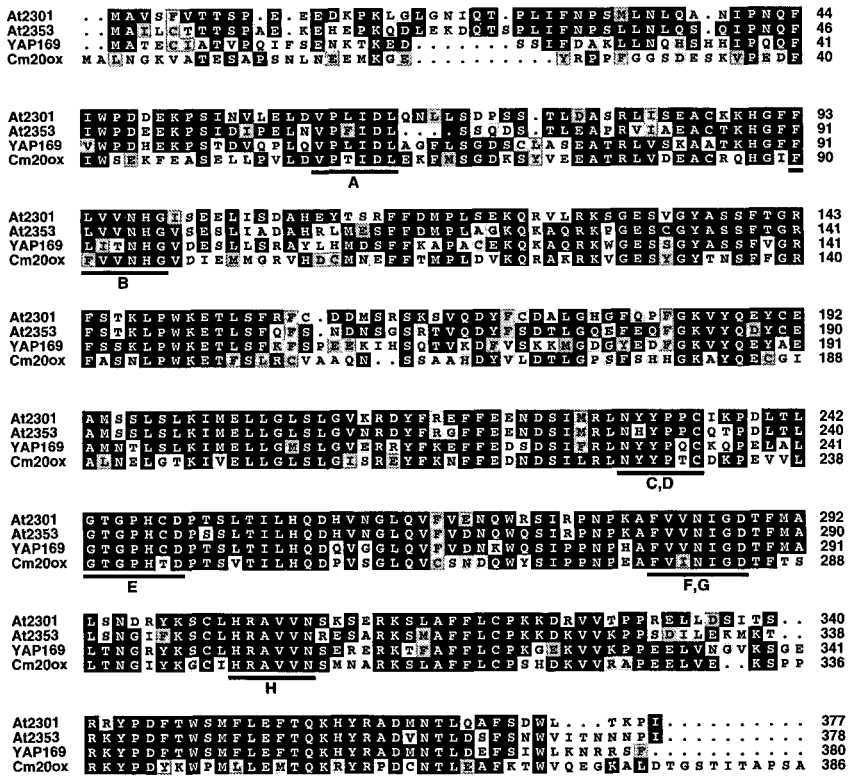

Figure 4. Predicted amino acid sequences of full-length cDNA clones At2301, At2353, and YAP169, with the sequence of a GA 20-oxidase from *C. maxima* (Cm20ox; Lange et al., 1994). Identical residues are boxed in black; similar residues are shaded in gray. The amino acids underlined in Cm20ox indicate the regions used in the design of the degenerate oligonucleotide primers for PCR amplification from Arabidopsis genomic DNA. Sense primers: A, B, C, E, and F; anti-sense primers: D, G, and H.

5 of these clones were identical to the previously isolated cDNA clone, At2301, that was used to probe the library. The remaining 4 clones, although clearly related to At2301, represented a second novel dioxygenase cDNA from Arabidopsis. One such clone, At2353, with an insert of approximately 1.5 kb, was chosen for further study.

To identify any related cDNA clones already isolated from Arabidopsis, we searched the Database of Expressed Sequence Tags with the predicted N-terminal amino acid sequences of At2301 and At2353. This revealed that a third cDNA clone with significant amino acid sequence homology to both cDNAs had been isolated as a random clone from a library constructed from immature siliques of Arabidopsis (Höfte et al., 1993). This clone, YAP169, was obtained from Dr. M. Delseny (University of Perpignan, France).

DNA Sequence Analysis of cDNA Clones

DNA sequencing of the inserts of the cDNA clones At2301, At2353, and YAP169 identified open reading frames of 377, 378, and 380 amino acids, respectively (Fig. 4). The sequence data indicated that At2301 was a full-length clone corresponding to the PCR product At2204, with 100% nucleotide identity; it also revealed that clone At2353 was a full-length clone of PCR product At2232, the PCR product most closely related to At2204. Clone YAP169 was not represented in the PCR products.

The predicted amino acid sequences of the three clones confirmed that At2301 and At2353 were more closely related to each other than to either YAP169 or to the GA 20-oxidase from *C. maxima* (Table I). All three clones were closer to the *C. maxima* enzyme than to any other member of the dioxygenase family. However, it was not possible to conclude simply from the sequence data that any or all of the three Arabidopsis clones encoded GA 20-oxidases.

Heterologous Expression in *E. coli*

Since we had shown previously that the GA 20-oxidase cDNA clone from *C. maxima* produces catalytically active enzyme in *E. coli* when expressed as a fusion with β-galactosidase in λgt11 (Lange et al., 1994), we decided to investigate the function of the proteins encoded by the three Arabidopsis dioxygenase clones by expressing them in *E. coli*. Each of the three cDNAs was inserted, in sense orientation and in frame with the short N-terminal tag, in the pTrcHis series of expression vectors (Invitrogen). Logarithmic-phase, liquid cultures were induced with 5 mM IPTG and grown at 30°C. Soluble extracts of these cells were assayed for GA 20-oxidase activity by incubation with [^{14}C]GA_{12} and [^{14}C]GA_{53} under the conditions described in "Materials and Methods." Products were separated by HPLC (Fig. 5) and identified by GC-MS.

Table I. *Amino acid sequence identity (%) between full-length dioxygenase cDNA clones from Arabidopsis and GA 20-oxidase from developing cotyledons of C. maxima (Cm20ox)*

	At2353	YAP169	Cm20ox
At2301	76	64	53
At2353		66	53
YAP169			52

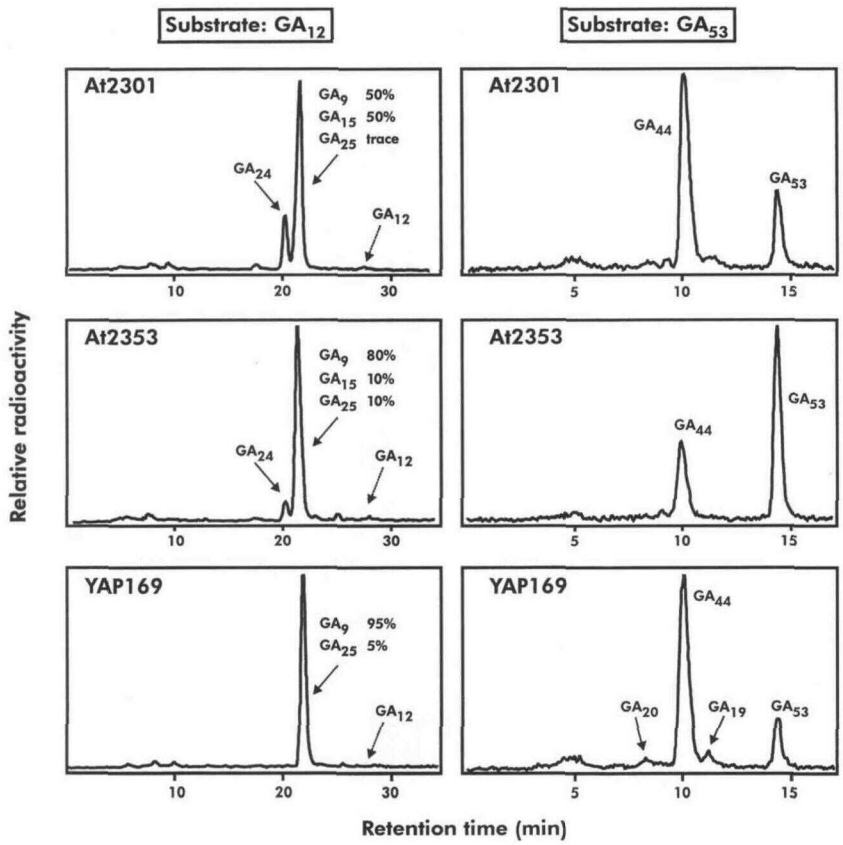

Figure 5. HPLC separation of radiolabeled products after incubation of extracts of *E. coli* expressing fusion proteins derived from cDNA clones At2301, At2353, and YAP169 with [^{14}C]GA_{12} and [^{14}C]GA_{53}. The traces shown derive from assays containing 50 μL of bacterial extract for At2301 and YAP169 and 3 μL of extract for At2353. The products were detected by online radio-monitoring and the contents of each peak were identified by GC-MS. All the GA_{12} was converted in the enzyme assay; the time at which this would be expected to elute is indicated on the HPLC traces.

The expression products of At2301, At2353, and YAP169 were each capable of using GA_{12} as substrate. The products of the reaction were, in each case, mixtures containing combinations of GA_{15}, GA_{24}, GA_{25}, and GA_9 (see Fig. 1), the composition depending on the construct and on the volume of cell lysate used. Incubations with products from At2353 (3 μL of extract) and YAP169 (50 μL of extract) resulted in the formation of GA_9 as the major product, with small amounts of the tricarboxylic acid GA_{25}, whereas incubations with At2301 (50 μL of extract) produced substantial quantities of GA_{15} and GA_{24}. GA_9 and GA_{25} result from three oxidations at C-20, GA_9 formation involving loss of this carbon atom as CO_2 and formation of the γ-19,10-lactone (Kamiya et al., 1986). GA_{15} and GA_{24} are intermediates in the formation of GA_9 and GA_{25}, requiring, respectively, one and two oxidations on C-20 of GA_{12}. It is not yet known whether the differences in activity from the different cDNA clones are due to the relative efficiencies of expression in *E. coli* or to differences in the properties of the enzymes. GA_{53} was also a substrate for the expression products, although, in each case it was utilized less effectively than was GA_{12} (Fig. 5), and the major product was GA_{44} (see Fig. 1). In addition, GA_{19} and GA_{20} were formed in low yield in incubations with YAP169. As a control, extracts of IPTG-treated, transformed *E. coli* cells containing the pTrcHis vector with no insert did not metabolize

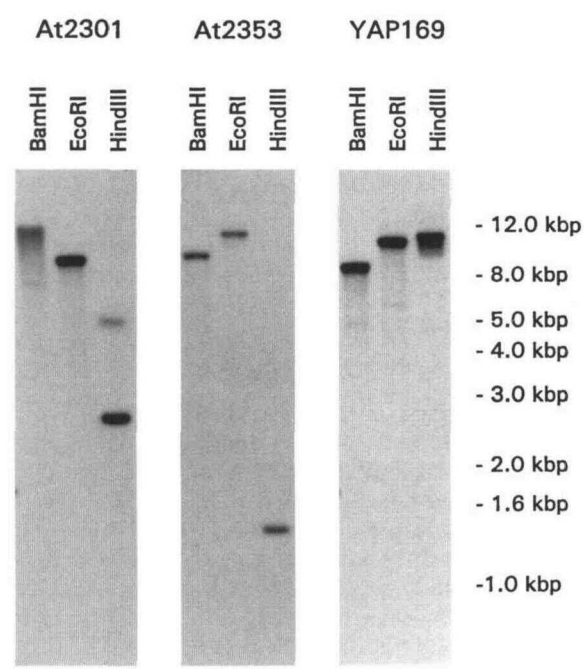

Figure 6. Hybridization of cDNA clones At2301, At2353, and YAP169 at high stringency to Southern blots containing Arabidopsis genomic DNA digested with *Bam*HI, *Eco*RI, or *Hin*dIII.

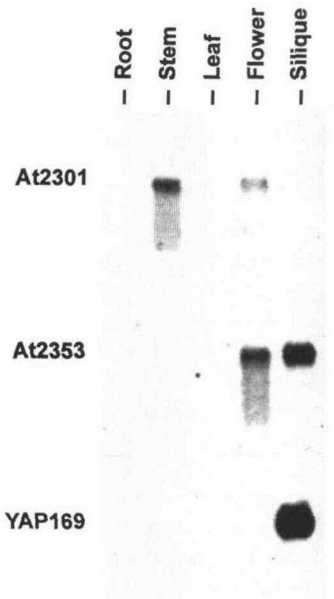

Figure 7. Expression patterns of GA 20-oxidases in wild-type (L*er*) *A. thaliana* plants. Hybridization of 20-oxidase cDNA clones At2301, At2353, and YAP169 to northern blots containing 1 μg of poly(A)$^+$ RNA extracted from roots, stems, expanded leaves, inflorescences, and developing siliques.

GA_{12}, GA_{15}, GA_{53}, GA_{44}, or GA_{19}; [^{14}C]GA_{24} was not available for testing.

Pattern of Expression in Arabidopsis

The pattern of mRNA accumulation of each cDNA clone was investigated by hybridization to northern blots of poly(A)$^+$ RNA extracted from various organs of *A. thaliana* L*er*. To be confident that each cDNA probe was specific for a single mRNA species, we first performed genomic Southern analysis with each clone. When hybridization and washing were carried out at high stringency, each cDNA probe hybridized strongly to one or two *Bam*HI, *Eco*RI, or *Hin*dIII fragments of Arabidopsis L*er* genomic DNA (Fig. 6), suggesting that each cDNA clone was represented by a single locus in the haploid genome. No cross-hybridization between probes was evident. As expected, clone At2301 hybridized to the same genomic fragments as did the PCR-generated clone At2204 at high stringency, with the exception that the full-length clone hybridized to a new 5-kb fragment not recognized by the PCR product; this is explained by the presence of a *Hin*dIII site in the cDNA sequence at 450 bp from the 5′ end, in a region not included within the PCR product. Clone At2353 produced the same pattern as those additional bands identified by At2204 at low stringency (cf. Figs. 3 and 6). Although At2301 and At2353 are fairly closely related in DNA sequence (76% identity), we found no evidence that they are very closely linked in the genome; each probe hybridized to a different genomic fragment, and genomic λ clones corresponding to the two clones show no overlapping fragments (J. Coles, A. Phillips, and P. Hedden, unpublished data). Clone YAP169 also hybridized to a faint band in each digest, possibly suggesting that a fourth, distantly related gene is present in the Arabidopsis genome.

Northern blots of mRNA extracted from roots, stems, mature leaves, inflorescences, and developing siliques of L*er* were probed with ^{32}P-labeled inserts of the three GA 20-oxidase clones (Fig. 7). The inserts of At2301 and At2353 each identified an mRNA species of approximately 1.5 kb expressed at very low levels; accumulation of mRNA hybridizing to At2301 was confined to stems and inflorescence tissues, whereas that for At2353 appeared to be present in inflorescence and developing siliques. In contrast, an approximately 1.5-kb mRNA corresponding to YAP169 was found at high levels in developing siliques, correlating with its isolation as a random clone from this tissue, but was absent in all vegetative and floral tissues.

The *ga1–2* mutant of Arabidopsis used to isolate two of the three cDNA clones is deficient in *ent*-kaurene synthetase A activity (Sun and Kamiya, 1994) and thus has low levels of GAs (Zeevaart and Talon, 1992). These plants form compact rosettes, which finally develop flower buds that arrest before maturity. Expression of the three 20-oxidase clones was investigated in the arrested flower buds and pedicels of the GA-deficient plants (Fig. 8). At2301 and At2353 were both expressed at high levels in these tissues; YAP169 was expressed at a significantly lower level. Treatment of *ga1–2* plants with GA_3 16 h before RNA extraction reduced substantially the abundance of mRNA corresponding to each of the three clones.

DISCUSSION

We recently isolated a cDNA clone encoding a GA 20-oxidase from developing cotyledons of pumpkin seeds,

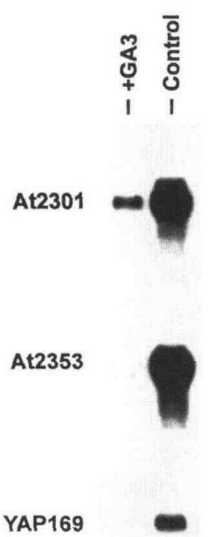

Figure 8. Expression of GA 20-oxidases in floral shoots of the GA-deficient mutant *ga1–2*. Hybridization of 20-oxidase cDNA clones At2301, At2353, and YAP169 to northern blots containing 1 μg of poly(A)$^+$ RNA extracted 16 h after spraying the plants with 100 μM GA_3 or with water.

which are highly active in GA biosynthesis (Lange et al., 1994). The products of heterologous expression of this cDNA were capable of catalyzing three successive oxidations at C-20 of GA_{12}, but the major product was the tricarboxylic acid GA_{25}, with only a small (1%) yield of the C_{19} metabolite, GA_9. This gene is not expressed at detectable levels in vegetative tissues of pumpkin (P. Krubasik, A.L. Phillips, and P. Hedden, unpublished data), suggesting that the enzyme may be unique to the developing seed, and that a different enzyme (or enzymes) is involved in the production of biologically active GAs in vegetative tissues. On the assumption that there would be conserved amino acid sequences in the Arabidopsis and pumpkin GA 20-oxidase genes, we have used degenerate PCR, with oligonucleotide primers based on consensus regions with other dioxygenases, to amplify internal fragments of related genes from Arabidopsis DNA. These were used, in turn, to isolate two closely related, full-length clones (At2301 and At2353) from a cDNA library; a third, more distantly related clone (YAP169) was identified in a data base of randomly isolated cDNAs from developing siliques of Arabidopsis. After this manuscript was submitted, we learned that J.A.D. Zeevaart and colleagues had independently isolated a genomic clone encoding a 20-oxidase from Arabidopsis by using a pumpkin endosperm GA 20-oxidase cDNA clone as a heterologous probe (Xu et al., 1995). The transcribed region of this genomic clone is essentially identical to cDNA clone At2301, and restriction fragment length polymorphism mapping revealed that it is very closely linked to the *ga5* semi-dwarf locus, which is impaired in GA 20-oxidase activity (Talon et al., 1990; Xu et al., 1995).

Heterologous expression products of all three Arabidopsis cDNA clones catalyzed three successive oxidations at C-20 of GA_{12}, resulting in formation of the C_{19} GA_9 as well as the alcohol and aldehyde intermediates GA_{15} and GA_{24}. In plants, GA_9 may be converted by 3β-hydroxylation to GA_4 (Takahashi et al., 1986; Albone et al., 1990; Junttila et al., 1992; Rood and Hedden, 1994), which is probably the major biologically active GA in Arabidopsis (Talon et al., 1990), or by 13-hydroxylation to GA_{20} (Takahashi et al., 1986; Rood and Hedden, 1994), which is the immediate precursor of GA_1. Thus, the enzymes encoded by these three cDNAs all perform three consecutive steps in the biosynthesis of active GAs. In addition to GA_9, the expression products of each clone produced a small (<5%) proportion of the tricarboxylic acid GA_{25}. This is a biologically inactive side product of the pathway to C_{19} GAs resulting from oxidation of the C-20 of GA_{24} to a carboxylic acid instead of this C atom being lost as CO_2 in GA_9 formation. The Arabidopsis GA 20-oxidases are therefore different from the enzyme in pumpkin seed that converts GA_{12} predominantly to GA_{25} (Lange et al., 1994). The availability of these clones and the potential to prepare large amounts of enzymes by heterologous expression will allow us to investigate the intriguing, and largely unknown, reaction by which C-20 is lost. Furthermore, comparison of the Arabidopsis and pumpkin enzymes may enable us to identify features of the enzyme active sites that determine the fate of C-20 after oxidation.

In common with the pumpkin GA 20-oxidase, the Arabidopsis enzymes oxidize GA_{53} less efficiently than GA_{12}. This may explain the relatively high abundance of 13-deoxy GAs in Arabidopsis shoots (Talon et al., 1990). However, it is possible that the preference for GA_{12} is a general feature of GA 20-oxidases and that 13-hydroxylation occurs preferentially after C_{19} GA formation. The accumulation of 13-hydroxy C_{20} GAs often found in plant tissues may reflect the slow turnover of these metabolites. These questions may be resolved when 20-oxidases are cloned from tissues in which 13-hydroxy GAs are the predominant forms.

As described previously, in spinach, an LD rosette plant, bolting in LD conditions correlates with an increase in the activities of GA_{53} and GA_{19} 20-oxidases, catalyzing the first and third oxidations at C-20 (Gilmour et al., 1986). GA_{44} 20-oxidase, which catalyzes the second 20-oxidative step, is present at high levels in SD plants and does not increase in activity after induction of bolting. GA_{44} 20-oxidase activity can also be separated from the other 20-oxidase activities by ion-exchange chromatography (Gilmour et al., 1987). These observations suggest that, in spinach leaves, at least two different enzymes catalyze the three oxidations at C-20, in contrast to the Arabidopsis enzymes described above. The semi-dwarf *ga5* mutant of Arabidopsis has a point mutation, leading to a truncated protein product, in a GA 20-oxidase gene corresponding to cDNA clone At2301 (Xu et al., 1995). This mutant accumulates GA_{12} and GA_{24}, but not GA_{15} (Talon et al., 1990), suggesting that it is deficient in the activities that catalyze the first and third oxidations at C-20; yet our heterologous expression results demonstrate that the enzyme encoded by the wild-type locus, At2301, will catalyze all the steps between GA_{12} and GA_9. This suggests the presence of another enzyme activity in spinach and Arabidopsis that converts the C-20 alcohol of GA_{44} and GA_{15} to the aldehydes GA_{24} and GA_{19}.

There is evidence from maize seedlings that bioactive GAs regulate their own biosynthesis through control of 20-oxidase activity (Hedden and Croker 1992). The *ga1–2* mutant of Arabidopsis, which has low levels of endogenous GAs (Zeevaart and Talon, 1992), accumulates high levels of mRNA corresponding to At2301 and At2353 in the floral shoots, whereas mRNA for YAP169 is present at a lower level. In all cases the mRNA level was considerably reduced by application of the biologically active GA_3. This down-regulation of transcript levels is rapid, being observed within 3 h of GA_3 application (A.L. Phillips and P. Hedden, unpublished data), and supports the view that GA biosynthesis may be, at least in part, regulated by feedback suppression of expression of the 20-oxidase genes. We are currently investigating transgenic Arabidopsis plants containing sense and anti-sense copies of the three 20-oxidase cDNA clones, which should help to assess the extent to which these enzymes control the overall rate of GA biosynthesis.

Analysis of mRNA accumulation of the 20-oxidase cDNAs in tissues of wild-type (*Ler*) plants suggests a tissue-specific pattern of expression. YAP169 is expressed at a high level, exclusively in siliques; a high rate of GA bio-

synthesis is a common feature of fruit tissue of many species. The distribution of mRNA hybridizing to At2301 and At2353 suggests that the former may be expressed in green vegetative tissues and the latter in floral tissues. No mRNA hybridizing to any of the three cDNAs was detected in roots: it is possible that more 20-oxidases remain to be identified, or that expression in roots is at a lower level. Further work is in progress to localize the expression of these three cDNAs more precisely, but the expression patterns described above present the possibility that the three cDNA clones, At2301, At2353, and YAP169, are responsible for GA biosynthesis associated with different aspects of plant development. There is genetic evidence that other GA-biosynthetic enzymes may also be encoded by more than one gene with tissue-specific expression. For example, the *Na* gene of pea (*Pisum sativum*) that encodes an enzyme responsible for an early step, possible GA_{12}-aldehyde synthetase (Ingram and Reid, 1987), is expressed in vegetative tissues but not in developing seeds (Potts and Reid, 1983).

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