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Molecular Cloning and Photoperiod-Regulated Expression of Gibberellin 20-Oxidase from the Long-Day Plant Spinach[1]

Keqiang Wu[2], Li Li[3], Douglas A. Gage, and Jan A. D. Zeevaart*

Michigan State University-Department of Energy Plant Research Laboratory (K.W., L.L., J.A.D.Z.) and Department of Biochemistry (D.A.G.), Michigan State University, East Lansing, Michigan 48824

Spinach (*Spinacia oleracea* L.) is a long-day (LD) rosette plant in which stem growth under LD conditions is mediated by gibberellins (GAs). Major control points in spinach are the later steps of sequential oxidation and elimination of C-20 of C_{20}-GAs. Degenerate oligonucleotide primers were used to obtain a polymerase chain reaction product from spinach genomic DNA that has a high homology with GA 20-oxidase cDNAs from *Cucurbita maxima* L. and *Arabidopsis thaliana* Heynh. This polymerase chain reaction product was used as a probe to isolate a full-length cDNA clone with an open reading frame encoding a putative 43-kD protein of 374 amino acid residues. When this cDNA clone was expressed in *Escherichia coli*, the fusion protein catalyzed the biosynthetic sequence $GA_{53}\rightarrow GA_{44}\rightarrow GA_{19}\rightarrow GA_{20}$ and $GA_{19}\rightarrow GA_{17}$. This establishes that in spinach a single protein catalyzes the oxidation and elimination of C-20. Transfer of spinach plants from short day (SD) to LD conditions caused an increase in the level of all GAs of the early-13-hydroxylation pathway, except GA_{53}, with GA_{20}, GA_1, and GA_8 showing the largest increases. Northern blot analysis indicated that the level of GA 20-oxidase mRNA was higher in plants in LD than in SD conditions, with highest level of expression in the shoot tips and elongating stems. This expression pattern of GA 20-oxidase is consistent with the different levels of GA_{20}, GA_1, and GA_8 found in spinach plants grown in SD and LD conditions.

Spinach (*Spinacia oleracea* L.) is an LD rosette plant in which exposure to LD conditions results in stem elongation and subsequent floral development. There is considerable evidence that photoperiodic control of stem elongation in rosette plants is mediated by GAs. Application of GA to rosette plants under SD conditions promotes stem growth, whereas treatment with inhibitors of GA biosynthesis suppresses stem elongation under LD conditions (Zeevaart, 1971; Zeevaart et al., 1993). In another LDP, *Silene armeria*, the GA content increases severalfold after the transfer of plants from SD to LD conditions, particularly in the subapical region (Talon et al., 1991a; Talon and Zeevaart, 1992). In spinach, several steps of GA biosynthesis were shown to be regulated by photoperiod, *ent*-kaurene biosynthesis, an early step of the GA biosynthetic pathway, being enhanced by long photoperiods (Zeevaart and Gage, 1993). In addition, $[^3H]GA_{20}$ was metabolized more rapidly in spinach plants in LD than in SD conditions (Metzger and Zeevaart, 1982). Photoperiod was also shown to regulate at least another two steps in GA metabolism, namely the conversion of GA_{53} to GA_{44} and of GA_{19} to GA_{20}. Here, the levels of GA_{53} and GA_{19} decreased, whereas GA_{20} increased when spinach plants were transferred from SD to LD conditions (Talon et al., 1991b). This coincided with increases in GA_{53}-oxidase and GA_{19}-oxidase activities (Gilmour et al., 1986; Zeevaart et al., 1990).

GA 20-oxidase (GA, 2-oxoglutarate:oxygen oxidoreductase [20-hydroxylating, oxidizing], EC 1.14.11-) catalyzes the sequential oxidation at C-20, ultimately leading to its loss as CO_2 (Fig. 1). Previously, cDNA clones encoding GA 20-oxidases from pumpkin (Lange et al., 1994; Zeevaart et al., 1994) and *A. thaliana* (Phillips et al., 1995; Xu et al., 1995) were isolated. Heterologous expression of these cDNA clones in *Escherichia coli* has shown that their fusion proteins can catalyze the biosynthetic sequence $GA_{53}\rightarrow GA_{44}\rightarrow GA_{19}\rightarrow GA_{20}$ and $GA_{19}\rightarrow GA_{17}$, suggesting that a single protein is responsible for the oxidation and elimination of C-20. To further understand how the photoperiod regulates these GA biosynthetic steps in spinach, we have cloned a GA 20-oxidase gene from spinach. Our results indicate that expression of GA 20-oxidase in spinach is regulated by photoperiod, with a higher level of transcription in plants grown in LD conditions than in those grown under SD conditions.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* L., Savoy Hybrid 612; Harris Seed Co., Rochester, NY) was grown as described earlier (Zeevaart and Gage, 1993). The SD conditions consisted of 8 h of light from fluorescent tubes and incandescent bulbs (450 μmol m^{-2} s^{-1}) at 23°C, followed by 16 h of darkness at 20°C. For the LD condition, the 8-h main light period was followed by 16 h of weak light from incandescent bulbs (10 μmol m^{-2} s^{-1}) at 20°C.

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[2] Present address: Department of Physiology, Medical School, University of Minnesota, Minneapolis, MN 55455.

[3] Present address: Department of Plant Breeding, Cornell University, Ithaca, NY 14850.

* Corresponding author; e-mail 22900mgr@msu.edu; fax 1-517-353-9168.

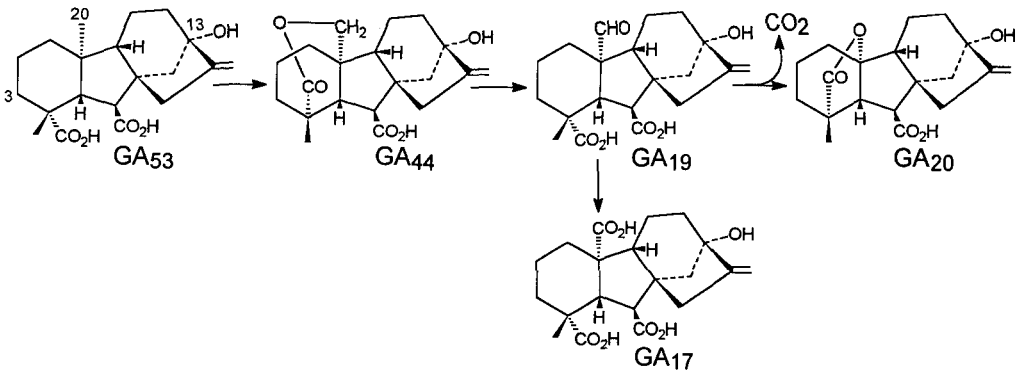

Figure 1. Reactions catalyzed by GA 20-oxidase in the early-13-hydroxylation GA biosynthetic pathway.

Genomic DNA Isolation and PCR with Degenerate Primers

Total genomic DNA from spinach was extracted as described by Dellaporta et al. (1983). The degenerate primers used for PCR to amplify a GA 20-oxidase sequence from spinach were designed after comparison of the amino acid sequences of the pumpkin and *Arabidopsis thaliana* GA 20-oxidases with those of other plant dioxygenases. A total of three antisense primers (JZ14, JZ16, and JZ20) and three sense primers (JZ15, JZ17, and JZ19) were synthesized, based on six regions of conserved amino acid sequences:

JZ14: 5′-GG(AG)CACA(AG)(AG)AAGAAIGCIA(AG)IG-3′;
JZ15: 5′-GA(AG)CTT(CT)TIGGICT(AT)AG-3′;
JZ16: 5′-GAAIGT(AG)TCICCGAT(AC)TT-3′;
JZ17: 5′-AGGCTIAA(CT)TA(CT)TA(CT)CC-3′;
JZ19: 5′-AA(AGCT)CT(AGCT)CC(AGCT)TGGAA(AG)-GA(AG)AC-3′;
JZ20: 5′-GTC(CT)TG(AG)TG(AGCT)AG(AGT)AT(AGCT)-GT-3′.

These primers were used in PCR reactions to amplify spinach genomic DNA fragments. Each 50-μL reaction mixture contained 0.5 μg of genomic DNA, 5 μL of PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of all four deoxyribonucleoside triphosphates, 0.5 μM each primer, and 1 unit of *Taq* polymerase (GIBCO-BRL). The reaction mixtures were heated to 94°C for 4 min and then subjected to 30 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min. A final extension was performed at 72°C for 7 min. The products were analyzed by agarose gel electrophoresis and purified with a Wizard PCR purification system (Promega). The purified PCR products were cloned into the PCRII vectors (Invitrogen, San Diego, CA).

cDNA Library Screening

A cDNA library was constructed in λZAPII (Stratagene) starting with poly(A)$^+$ RNA isolated from young leaves of spinach grown in the LD condition. The ^{32}P-labeled PCR product, SP26, was used to screen the cDNA library. Prehybridization and hybridization were performed at 60°C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA. Filters were washed once for 15 min in 2× SSC with 0.1% SDS at room temperature and then twice for 20 min in 0.1× SSC, 0.1% SDS at 60°C (high stringency) or 37°C (low stringency). The damp filters were autoradiographed at −80°C using two intensifying screens. Filters were stripped in 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS at 100°C for 2 min when reprobing was required.

DNA Sequence Analysis

Dye primer sequencing of cDNA clone inserts in pBluescript SK$^+$ and dye terminator sequencing of PCR products were performed using an automated sequencing system (Applied Biosystems). The DNA sequence analysis was carried out using the DNASIS program. Protein sequence analysis was performed using PROSIS (Hitachi Software Engineering Co., Ltd., Tokyo, Japan).

Heterologous Expression of a cDNA Clone in *Escherichia coli*

A full-length cDNA pSPC261 cloned in pBluescript SK$^+$ was transformed into *Escherichia coli* strain DH5α. A fresh overnight culture of 10 mL was added to 1 L of Luria-Bertani broth (Sambrook et al., 1989) with 50 mg/L ampicillin and incubated at 37°C with vigorous shaking. When the optical density at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 5 mM and the culture was incubated for another 2 h. The cells were pelleted by centrifugation, washed with 25 mL of Luria-Bertani broth, and then recentrifuged. The resulting pellets were resuspended in 2 mL of lysis buffer (100 mM Tris-HCl, pH 8.0, 3 mM DTT, and 2.5 mg/mL lysozyme) and incubated at room temperature for 10 min. Samples in tubes were submerged in liquid N_2 for 5 min and then thawed in an ice bath for 15 min (Johnson and Hecht, 1994). The lysates were centrifuged in a microcentrifuge at 14,000 rpm for 15 min. The supernatant was used for enzyme assays.

GA 20-Oxidase Activity Assay

The assay for GA 20-oxidase activity was performed according to the method of Gilmour et al. (1986) with some modifications. The reaction mixture contained 100 mM Tris buffer (pH 7.5), 1 mM $FeSO_4$, 10 mM 2-oxoglutarate, 10 mM ascorbate, 100 μL of enzyme extract, and [^{14}C]GA_{53} (30,750 dpm, 125 pmol) or [^{14}C]GA_{19} (31,400 dpm, 107 pmol) in a total volume of 250 μL. Cofactors were replenished after 1

and 3 h. The mixture was incubated for up to 6 h at 30°C with gentle shaking. Products were separated by HPLC with on-line radiocounting (Zeevaart et al., 1993). For product identification by GC-MS (Zeevaart et al., 1993), the reaction mixtures were scaled up to a volume of 2 mL with 10^6 dpm (4.2 nmol) [^{14}C]GA_{53} or 10^6 dpm (3.4 nmol) [^{14}C]GA_{19} as substrates.

Southern and Northern Blot Analysis

For Southern blots, spinach genomic DNA was digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to nylon membranes (Sambrook et al., 1989). For northern blot analysis, total RNA was isolated from 1 to 2 g of spinach tissues using an Extract-A-Plant RNA isolation kit (Clontech, Palo Alto, CA). The poly(A)$^+$ RNA was then isolated by chromatography on oligo(dT)-cellulose (GIBCO-BRL). Northern blots were prepared by electrophoresis of 2- to 3-μg samples of poly(A)$^+$ RNA through agarose gels in the presence of formaldehyde (Strommer et al., 1993), followed by transfer to Duralon-UV membranes (Stratagene). Southern and northern blots were probed with ^{32}P-labeled spinach GA 20-oxidase cDNA clone pSPC261. Hybridization was carried out at 42°C using the same hybridization solution as used for screening of the cDNA library, except that 50% formamide was included in the solution. Blots were washed and stripped as described for cDNA library screening. As a control, all northern blots were also probed with an *A. thaliana* actin (clone 40F11 from T. Newman, Michigan State University, East Lansing) probe. The blots were then exposed to phosphor screens, and relative amounts of mRNA were determined with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Quantification of GAs

The GAs were extracted, purified, and quantified by the methods described earlier (Zeevaart et al., 1993), except that for identification of radiolabeled GAs by GC-MS the gas chromatograph was equipped with a DB-5MS capillary column (30-m × 0.32-mm × 0.25-μm film; J&W Scientific, Folsom, CA). Experiments on quantification of GAs in different plant parts were repeated at least once with similar results.

RESULTS

PCR Amplification of GA 20-Oxidase Sequence using Degenerated Primers

The degenerate primers used for PCR to amplify GA 20-oxidase sequences from spinach were designed by comparison of the amino acid sequences of the pumpkin and *A. thaliana* GA 20-oxidases (Lange et al., 1994; Phillips et al., 1995; Xu et al., 1995) with those of other plant dioxygenases (Prescott, 1993; De Carolis and De Luca, 1994). These primers were used in all combinations in PCR reactions to amplify spinach genomic DNA fragments. Only one pair of primers, JZ16 and JZ17, yielded products of expected size (about 160 bp). The PCR-amplified products were cloned into PCRII vectors (Invitrogen) and sequenced. Sequence analyses identified three different PCR products, SP167, SP149, and SP26, with 39, 54, and 69 amino acid identity, respectively, with the GA 20-oxidase from pumpkin (Fig. 2).

Isolation of a Full-Length GA-20 Oxidase cDNA Clone from Spinach

Since the PCR product SP26 shows highest homology to the GA 20-oxidase genes of pumpkin and *A. thaliana*, it was used for screening a λZAPII cDNA library of spinach. A total of 5×10^5 recombinant phage plaques from the cDNA library were screened with ^{32}P-labeled SP26 by hybridization at high stringency. Five positive clones were recovered from this screening. Restriction endonuclease digestion showed that these clones contained inserts of about 1.5 kb. Sequence analysis indicated that two of them were identical and were designated pSPC261. Complete sequence analysis of pSPC261 confirmed it to be a full-length cDNA (Fig. 3). The other three clones were found to be chimeric, with 770 bp of the 3′ region of pSPC261 being fused to an unrelated cDNA fragment.

To investigate the copy number of pSPC261 genes in the spinach genome, a ^{32}P-labeled pSPC261 DNA probe was hybridized at high stringency to total spinach genomic DNA that had been digested with *Eco*RI, *Hin*dIII, *Nhe*I, and *Spe*I restriction enzymes (Fig. 4). A single band was observed in each lane, indicating that the pSPC261 gene is probably present as a single locus in the spinach genome.

The deduced amino acid sequence of pSPC261 shares 52 and 65% identity with the pumpkin GA 20-oxidase and *GA5* of *A. thaliana*, respectively (Fig. 5). Thus, pSPC261 may encode a GA 20-oxidase. The open reading frame of pSPC261 encodes a putative 43-kD protein of 374 amino acid residues. This agrees with the estimated molecular masses of partially purified GA_{53}-oxidase and GA_{19}-oxidase from spinach leaves by gel-filtration HPLC, which are in the range 38 to 43 kD (Gilmour et al., 1987). GA 20-oxidase has been classified as a member of the family of 2-oxoglutarate-dependent dioxygenases (Prescott, 1993; De Carolis and De Luca, 1994). The highly conserved consensus sequence N-Y-Y-P-X-C-X-X-P (residues 223–231 of pSPC261, Fig. 3), which is proposed to be involved in the

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p16    RLNYYPTCDKPEVVLGTGPHTDPTSVTILHQDPVSG...LQVCSNDQ.WYSIPPNPEAFVINIGDTF
SP149          MSKDRFVLGTGPHSDRSALTILHQEE.GG..CFQVVV.DQ.RSSIPLIPVAGVI
SP167          CPRPDLVLGLSPHSDGSALTILQQKE.GGSVGLQLLK.DNNXVSIIPIPGALVV
SP26           CQKPELTLGTGPHCDPTSLTILHQDHVGG...LEVFV.DQKWYSIRPNQKAFVV
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Figure 2. Predicted amino acid sequence of PCR products (SP149, SP167, and SP26) amplified from spinach genomic DNA, using degenerate primers JZ16 and JZ17. Amino acids of the GA 20-oxidase from pumpkin (p16) that are conserved in the PCR products are shaded.

```
     ACCTCCATTTTCCTAACAAAGCTAGCTTAATTGGCTTCAAACCAACAAAAA
1    ATGATGCAACCACTTCTTACCATTCCACCACCACTTCCAACAACCTCATTG
1      M  M  Q  P  L  L  T  I  P  P  P  L  P  T  T  S  L

52   TTTGATACATCATTTCTTAAACATGAAGATAACATACCAAGCCAATTTATA
18     F  D  T  S  F  L  K  H  E  D  N  I  P  S  Q  F  I

103  TGGCCAGATGATGAAAAACCATGCTCGGAAACGCCTCCAGAGCTCGAGGTA
35     W  P  D  D  E  K  P  C  S  E  T  P  P  E  L  E  V

154  CCTCCCATTGATCTAGGGGGGTTCCTCTCAGGAGACCCAGTTGCAGTGTCA
52     P  P  I  D  L  G  G  F  L  S  G  D  P  V  A  V  S

205  AAGGCAACTACACTTGCCAATGAAGCATGCAAGTGGCATGGGTTCTTCTTG
69     K  A  T  T  L  A  N  E  A  C  K  W  H  G  F  F  L

256  ATTGTCAACCATGATATCTATTTCGAGCTCTTAGTTAAAGCTCATGAAGCT
86     I  V  N  H  D  I  Y  F  E  L  L  V  K  A  H  E  A

307  ATGGATTACTTTTTTAGTCAGCCGTTTTCCCAAAAACAAAAAGCTCTCAGG
103    M  D  Y  F  F  S  Q  P  F  S  Q  K  Q  K  A  L  R

358  AAACAAGGTGATCATTGTGGCTATGCTAGTAGCTTTCTTGGAAGATTTGCC
120    K  Q  G  D  H  C  G  Y  A  S  S  F  L  G  R  F  A

409  ACAAAACTTCCTTGGAAAGAGACTCTTTCCTTTCGATATTATGATGATGAT
137    T  K  L  P  W  K  E  T  L  S  F  R  Y  Y  D  D  D

460  GATGATAAGTCCTCAAAAATGGTACAAAACTACATCTCCAACTTAATGGGG
154    D  D  K  S  S  K  M  V  Q  N  Y  I  S  N  L  M  G

511  ACTGACTTTCAAGAATTTGGGAGGGTGTACCAAGAATATTGTAAGGCTATG
171    T  D  F  Q  E  F  G  R  V  Y  Q  E  Y  C  K  A  M

562  AGCAAGTTGTCCCTTGGTATCATGGAGCTTTTGGGAATGAGCCTAGGAGTT
188    S  K  L  S  L  G  I  M  E  L  L  G  M  S  L  G  V

613  GGAAGAAACTATTTCAGGGAATTTTTCAAAGGGAATGACTCAATAATCAGA
205    G  R  N  Y  F  R  E  F  F  K  G  N  D  S  I  I  R

664  CTAAACTACTACCCGCCTTGCCAAAAACCCGAATTAACTCTTGGGACGGGG
222    L  N  Y  Y  P  P  C  Q  K  P  E  L  T  L  G  T  G

715  CCTCACTGTGATCCCACGTCGCTGACGATTCTTCATCAAGATCATGTTGGT
239    P  H  C  D  P  T  S  L  T  I  L  H  Q  D  H  V  G

766  GGCCTTGAAGTCTTCGTCGACCAAAAATGGTACTCCATCCGTCCCAACCAG
256    G  L  E  V  F  V  D  Q  K  W  Y  S  I  R  P  N  Q

817  AAAGCATTTGTCGTCAACATTGGAGATACCTTCATGGCTTTGTCAAATGGG
273    K  A  F  V  V  N  I  G  D  T  F  M  A  L  S  N  G

868  AAATACAAGAGTTGCTTGCACAGGGCAGTGGTGAATAGCAAAACTCCTAGA
290    K  Y  K  S  C  L  H  R  A  V  V  N  S  K  T  P  R

919  AAATCAGTGGCTTTCTTTCTGTGTCCAAGGGGAAACAAAGTGATTCGTCCA
307    K  S  V  A  F  F  L  C  P  R  G  N  K  V  I  R  P

970  CCAATTGAGTTAGGGCATCCAAGGGTATACCCGGATTTTACATGGCCGCTT
324    P  I  E  L  G  H  P  R  V  Y  P  D  F  T  W  P  L

1021 CTTTTGGAGTTTACACAGAAACATTATAGGGCCGACACAAAAACTCTAGAT
341    L  L  E  F  T  Q  K  H  Y  R  A  D  T  K  T  L  D

1072 TCTTTTACAAAGTGGCTTCAAAAGAGATCAACTGAAGACGAGCGAGTAAAG
358    S  F  T  K  W  L  Q  K  R  S  T  E  D  E  R  V  K

1123 TAAAACCATGCAACAGCAACAGCAACAGCAACAGCAACAGCAGCACGCAAA
       *
     GGACATACATGTAAAAACATGATGCACTCGTTATAGAAAAGCATTGTACTC
     CGTATATGAATTGAAGAGACAAAAGGGTGCCAATTGAAGTTAGCTAAAGGA
     TCGATCTGATTAAATTACAACTGAAGCACCGCTGTTGG
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Figure 3. Nucleotide sequence of GA 20-oxidase cDNA pSPC261 and predicted amino acid sequence. Shaded residues are highly conserved in 2-oxoglutarate-dependent dioxygenases, except for the sequence L-P-W-K-E-T (residues 139–144), which is conserved only in GA 20-oxidases (see text).

binding of the co-substrate, 2-oxoglutarate, is conserved in the spinach putative GA 20-oxidase. The three His residues (His^{89}, His^{240}, and His^{296} of pSPC261), which may be involved in the binding of Fe^{2+}, are also conserved. In addition, the sequence L-P-W-K-E-T (residues 139–144 of pSPC261, Fig. 5) in the putative spinach GA 20-oxidase is also conserved in the GA 20-oxidases of pumpkin and *A. thaliana* (Lange et al., 1994; Phillips et al., 1995; Xu et al., 1995). Since this motif is highly conserved in all of the GA 20-oxidases reported but diversified in other 2-oxoglutarate-dependent dioxygenases, it was proposed that this motif may be important for the GA substrate binding (Xu et al., 1995).

Heterologous Expression in *E. coli*

To confirm that the pSPC261 clone encodes a GA 20-oxidase, the function of its encoding protein was analyzed by expression in *E. coli*. The pSPC261 clone was inserted, in sense orientation and in frame, in the pBluescript SK^+ vector (Stratagene) and maintained in a bacterial host, *E. coli* strain DH5α. Protein extracts containing the pSPC261 insert were assayed for GA 20-oxidase activity by using [^{14}C]GA_{53} and [^{14}C]GA_{19} as substrates, and the reaction products were separated by HPLC (Fig. 6). The expression product of pSPC261 was able to convert [^{14}C]GA_{53} to [^{14}C]GA_{44}, [^{14}]GA_{19}, and [^{14}C]GA_{20} (Fig. 6B). When [^{14}C]GA_{19} was used as a substrate, the major product was [^{14}C]GA_{20}, and [^{14}C]GA_{17} was a minor product, never amounting to more than 5% of the total radioactivity recovered (Fig. 6D). All products were identified by their full-scan mass spectra and retention times from GC-MS analysis. No enzyme activity was detected in protein extracts from *E. coli* DH5α containing pBluescript SK^+ with the pSPC261 insert in antisense orientation (Fig. 6, A and C).

Effect of Daylength on GA Levels in Spinach

The major GAs of spinach belong to the early-13-hydroxylation pathway (Talon et al., 1991b). It was shown earlier that of these GAs only GA_1 is active per se (Zeevaart et al., 1993). No evidence has been obtained for qualitative differences in GA composition between spinach plants growing as rosettes in SD and bolting plants in LD condi-

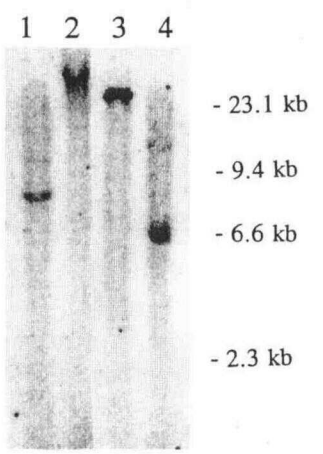

Figure 4. Genomic Southern blot analysis of pSPC261. Spinach genomic DNA (approximately 10 μg) was digested with *Nhe*I (lane 1), *Spe*I (lane 2), *Hin*dIII (lane 3), or *Eco*RI (lane 4) and then fractionated by agarose gel electrophoresis and transferred to a nylon membrane, where it was hybridized with the ^{32}P-labeled spinach GA 20-oxidase cDNA insert of pSPC261.

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p16        ....MALNGK VATESAPSNL NEEMKGEYRP PFGGSDESKV PEDFIWSEKF 46
GA5        MAVSFVRTSP EEEDKPKLGL GNIQTPLIFN PSMLDLQANM ANQFHWPDDE 50
pSPC261    .......... .MMQPLLTIP PPLPTTSLFD TSFLKHEDNI PSQFIWPDDE 39

p16        EA.SELLPVL DVPTIDLEKF MSGDKSYVEE PTRLVDEACR QHGIFFVVNH 95
GA5        KP.STLQLEL DVPLIDLQNL LS.DPSSTLD ASRLISEACK KHGFFLVVNH 98
pSPC261    KPCSETPPEL EVPPIDLGGF LSGDPVAVSK ATTLANEACK WHGFFLIVNH 89

p16        GVDIEMMGRV HDCMNEFFTM PLDVKQRAKR KVGESYGYTN SFFGRFASNL 145
GA5        GISEELISDA HEYTSRFFDM PLSEKQRVLR KSGESVGYAS SFTGRFSTKL 148
pSPC261    DIYFELLVKA HEAMDYFFSQ PFSQKQKALR KQGDHCGYAS SFLGRFATKL 139

p16        PWKETFSLRC VAAQNSSAAH ...DYVLDTL GPSFSHHGKA YQECGIALNE 192
GA5        PWKETLSFRF C..DDMSRSK SVQDYFCDAL GHGFQPFGKV YQEYCEAMSS 196
pSPC261    PWKETLSFRY YDDDDDKSSK MVQNYISNLM GTDFQEFGRV YQEYCKAMSK 189
           JZ19

p16        LGTKIVELLG LSLGISREYF KNFFEDNDSI LRLNYYPTCD KPEVVLGTGP 242
GA5        LSLKIMELLG LSLGVKRDYF REFFEENDSI MRLNYYPPCI KPDLTLGTGP 246
pSPC261    LSLGIMELLG MSLGVGRNYF REFFKGNDSI IRLNYYPPCQ KPELTLGTGP 239
                  JZ15                        JZ17

p16        HTDPTSVTIL HQDPVSGLQV CSNDQWYSIP PNPEAFVINI GDTFTSLTNG 292
GA5        HCDPTSLTIL HQDHVNGLQV FVENQWRSIR PNPKAFVVNI GDTFMALSND 296
pSPC261    HCDPTSLTIL HQDHVGGLEV FVDQKWYSIR PNQKAFVVNI GDTFMALSNG 289
                 JZ20                                  JZ16

p16        IYKGCIHRAV VNSMNARKSL AFFLCPSHDK VVRAPEELVE KSPPRKYPDY 342
GA5        RYKSCLHRAV VNSERMRKSL AFFLCPKKDR VVTPPRELLD SITSRRYPDF 346
pSPC261    KYKSCLHRAV VNSKTPRKSV AFFLCPRGNK VIRPPIEL.G H..PRVYPDF 336
                                  JZ14

p16        KWPMLLEM.Q KRYRPDCNTL EAFKTWVQEG KALDTGSTIT APSA 386
GA5        TWSMFLEFTQ KHYRADMNTL QAFSDWLTKP I..............  377
pSPC261    TWPLLLEFTQ KHYRADTKTL DSFTKWLQKR STEDERVK.......  374
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Figure 5. Sequence alignment of the deduced amino acid sequence of the cDNA clone pSPC261 with amino acids from the GA 20-oxidases from pumpkin (p16) and *A. thaliana* (GA5). Identical amino acids within the sequences are shaded. The amino acids underlined indicate the regions used in the design of the degenerate primers JZ14, JZ15, JZ16, JZ17, JZ19, and JZ20.

tions. However, levels of most of the GAs are greatly affected by the photoperiod. Transfer of spinach plants from SD to LD conditions caused an increase in GAs of the early-13-hydroxylation pathway, with GA_{20}, GA_1, and GA_8 showing the largest increases (see table 6 in Zeevaart et al., 1993).

Previous studies have established that more *ent*-kaurene is produced in petioles of spinach leaves than in the blades (Zeevaart and Gage, 1993). In the present study, we assessed the distribution of GAs in various organs of spinach (Table I). However, these data do not necessarily represent in situ GA biosynthesis, since transport from other sites of biosynthesis may have taken place. Nevertheless, on a dry weight basis, petioles had higher GA levels than their corresponding blades, and GA_{53}, a substrate for GA 20-oxidase, decreased in all parts, except in the shoot tips, after plants were transferred from SD to LD conditions. There was also a striking increase in the GA_{20} content following LD treatment and to a lesser extent in the levels of GA_1 and GA_8. The highest GA contents were present in the shoot tips, the site where GA causes cell division prior to stem elongation (Talon et al., 1991a). Thus, the GA levels in spinach are highest in organs that undergo rapid elongation (petioles) and cell division (apex plus subapical meristems) in LD conditions. The increase in GA_1 under LD conditions, particularly in the subapical meristem, is most likely the primary cause of subsequent stem elongation in spinach.

Effect of Daylength on GA 20-Oxidase Gene Expression

The expression patterns of GA 20-oxidase mRNA in different plant parts and their relationship to the photoperiod were investigated. Plants grown in the SD condition were placed in the LD condition for 14 d. Northern blots of poly(A)$^+$ RNA extracted from shoot tips, stems, and blades and petioles of young leaves of spinach grown in SD and LD conditions were probed with ^{32}P-labeled spinach GA 20-oxidase cDNA pSPC261 (Fig. 7). An mRNA species of about 1.5 kb was identified that was of low abundance relative to actin mRNA, except in stems, where its abundance was similar to that of actin. Expression of GA 20-oxidase in leaf blades was too low for a reliable comparison of mRNA levels between SD- and LD-grown plants. The highest level of expression was in stems, and moderate levels of expression were also observed in shoot tips and petioles of plants grown in LD conditions. The GA 20-oxidase was expressed at significantly lower levels in shoot tips and petioles of plants grown in SD than in those in LD conditions (Fig. 7). Thus, the accumulation of GA 20-oxidase mRNA in the organs that undergo rapid elongation (petioles) and cell division (shoot tips) in LD conditions coincides with their high GA levels.

When plants grown in the SD condition were placed in the LD condition for increasing periods, a substantial increase in the levels of GA 20-oxidase mRNA in the shoot tips was detected (Fig. 8A). Conversely, when plants were

transferred to the SD condition after exposure to 8 long days, the levels of GA 20-oxidase mRNA decreased significantly (Fig. 8B). These results are consistent with the observation that the activities of the enzymes oxidizing GA_{53} and GA_{19} are increased in LD and decreased in SD conditions (Gilmour et al., 1986).

Plants exposed to 16 long days were placed in darkness and harvested 24 h later. Levels of GA 20-oxidase mRNA were determined in shoot tips, stems, and young leaves. A decrease of about 2-fold in the level of GA 20-oxidase mRNA in stems and shoot tips was observed after this transfer (Fig. 9). This agrees with the previous findings that the activities of GA_{53}- and GA_{19}-oxidizing enzymes decreased when plants were transferred from LD conditions to darkness (Gilmour et al., 1986).

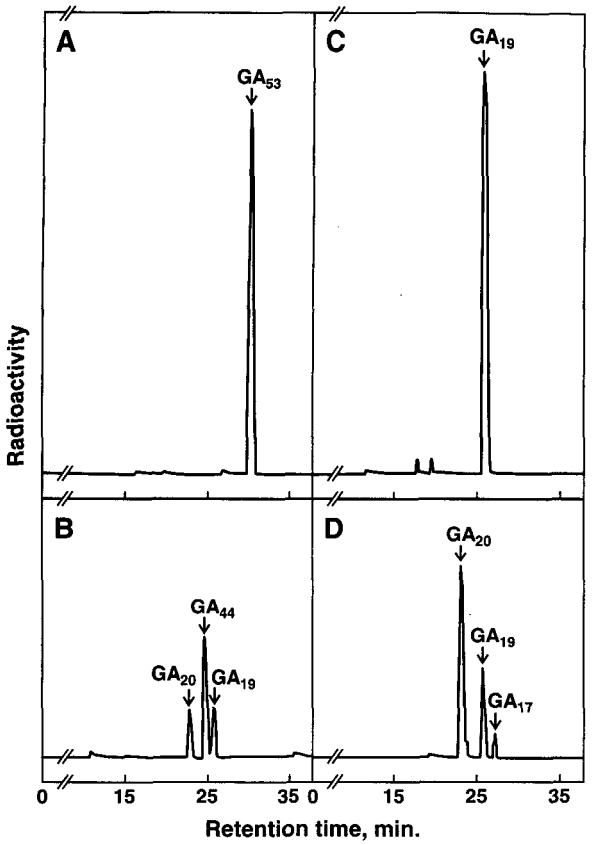

Figure 6. HPLC profiles of radiolabeled products from incubations of [^{14}C]GA_{53} and [^{14}C]GA_{19} with protein extracts from transformed *E. coli* that express pSPC261 as a fusion protein, either in sense (B and D) or in antisense (A and C) orientation. Substrates and products were identified by full-scan GC-MS as the methyl ester trimethylsilyl ethers. With GA_{53} as substrate (A and B), characteristic m/z ions (percentage of relative abundance) for GA_{53}: 456 (31), 448 (49), 424 (13), 416 (16), 395 (20), 389 (31), 209 (100), 207 (89); for GA_{44}: 440 (24), 432 (56), 379 (7), 373 (16), 240 (21), 238 (34), 209 (61), 207 (100); for GA_{19}: 442 (23), 434 (36), 410 (8), 402 (14), 381 (13), 380 (12), 375 (19), 374 (22); for GA_{20}: 426 (34), 418 (100), 381 (19), 375 (53), 365 (6), 359 (16), 307 (6), 301 (13), 209 (17), 207 (32). With GA_{19} as substrate (C and D), characteristic m/z ions (percentage of relative abundance) for GA_{19}: 442 (100), 434 (99), 410 (35), 402 (33), 381 (50), 380 (52), 375 (51), 374 (55); for GA_{20}: 426 (92), 418 (100), 381 (66), 375 (60), 365 (20), 359 (18), 307 (19), 301 (16), 209 (46), 207 (41); for GA_{17}: 500 (40), 492 (39), 468 (30), 460 (26), 441 (18), 433 (24), 409 (18), 401 (14), 379 (18), 373 (21), 253 (21), 251 (20), 210 (100), 208 (77).

Table I. *Comparison of GA levels in different organs of spinach in the SD condition and after 8 long days*

Spinach plants were grown in the continuous SD condition or had received 8 long days at the time of harvest. The different parts were harvested as described earlier (Zeevaart and Gage, 1993), and levels of GAs were determined by GC-MS-selected ion monitoring, using stable isotope internal standards (Talon et al., 1991b).

Plant Part	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_1	GA_8
	ng g^{-1} dry wt					
Mature leaves						
Blades						
SD	5	2	18	3	2	36
LD	1	1	9	11	2	167
Petioles						
SD	42	9	76	3	3	70
LD	14	18	148	47	3	712
Immature leaves						
Blades						
SD	7	2	13	4	3	25
LD	1	2	18	22	4	126
Petioles						
SD	44	12	49	4	4	119
LD	13	32	104	40	6	860
Young leaves[a] (1.5–10 cm)						
SD	28	8	101	5	10	46
LD	12	30	98	86	14	655
Shoot tips[b]						
SD	39	17	108	4	12	188
LD	53	80	213	62	35	1266

[a] Blades and petioles not separated. [b] Upper part of roots included.

DISCUSSION

The deduced amino acid sequence of the full-length cDNA pSPC261 shares high homology with GA 20-oxidases from pumpkin and *A. thaliana*. This strongly suggests that pSPC261 is a GA 20-oxidase cDNA from spinach. More direct evidence comes from the heterologous expression of this cDNA in *E. coli*. The fusion protein of spinach GA 20-oxidase was able to catalyze the biosynthetic sequence $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$ and $GA_{19} \rightarrow GA_{17}$. This establishes that in spinach, as in pumpkin (Lange et al., 1994; Zeevaart et al., 1994) and *A. thaliana* (Phillips et al., 1995; Xu et al., 1995), a single protein catalyzes the multiple steps of oxidation and elimination of C-20 (Fig. 1).

The reaction catalyzed by the heterologous expression product of pSPC261 produced the major end product GA_{20}, as well as a small amount of GA_{17}. The expression products of *A. thaliana* GA 20-oxidase cDNAs also produced mainly GA_{20} (Phillips et al., 1995; Xu et al., 1995). In plants, GA_{20} is readily converted by 3β-hydroxylation to the bioactive GA_1, whereas GA_{17} is biologically inactive. The production of GA_{20} as the major product thus ensures the synthesis of GA_1 that is necessary for petiole and stem elongation in these two rosette plant species. In contrast, the recombi-

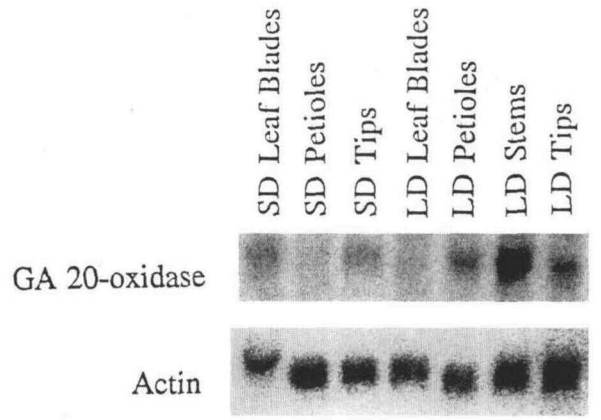

Figure 7. Relative abundance of GA 20-oxidase mRNA in different parts of spinach plants grown in SD and LD conditions. Poly(A)$^+$ RNA was probed with ^{32}P-labeled spinach GA 20-oxidase cDNA insert of pSPC261. The RNA was isolated from various parts of plants grown in the SD condition or from plants that were exposed to 14 long days. In the case of plants in the LD condition, "Tips" included the upper 1 cm and "Stems" the next 1 to 2 cm of the elongating stems.

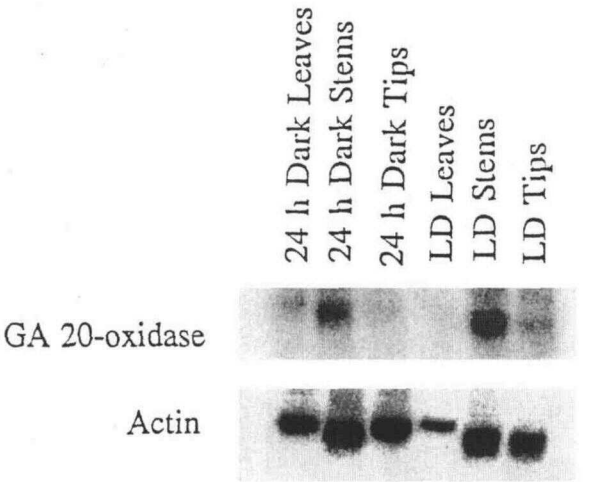

Figure 9. Effect of 24 h of darkness on GA 20-oxidase gene expression in spinach. Poly(A)$^+$ RNA was isolated from young leaves, stems, and shoot tips of plants grown in the LD condition for 16 d and from plants that were transferred from the LD condition to darkness after 24 h in darkness.

nant enzyme of pumpkin yielded GA_{17} as the major product (Lange et al., 1994; Xu et al., 1995). The spinach GA 20-oxidase is, therefore, similar to the enzymes of *A. thaliana* but different from the enzyme of pumpkin. Since the cDNA clone of the pumpkin GA 20-oxidase was isolated from immature seeds, and there is evidence that it is expressed only in the developing seeds and not in vegetative tissues, it was suggested that a different enzyme (or enzymes) may be involved in the production of biologically active GAs in vegetative tissues of pumpkin (Phillips et al., 1995).

Previous studies in this laboratory have established that the activities of GA_{53}- and GA_{19}-oxidase, catalyzing the first and the third oxidation at C-20 (Fig. 1), are light dependent, whereas GA_{44}-oxidase, which catalyzes the second step, is not (Gilmour et al., 1986). Likewise, the semi-

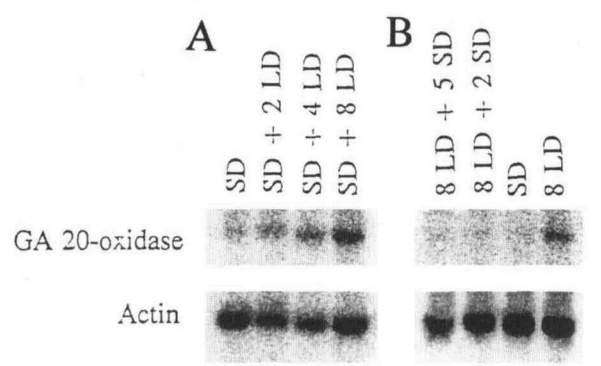

Figure 8. Effect of daylength on GA 20-oxidase gene expression in spinach. A, Poly(A)$^+$ RNA was isolated from shoot tips of plants grown in the SD condition and from plants that had received 2, 4, and 8 LD treatments. B, Poly(A)$^+$ RNA was isolated from shoot tips of plants grown in the SD or 8 LD conditions and from plants that were exposed to 8 long days and then transferred to the SD condition, after 2 and 5 short days.

dwarf *ga5* mutant of *A. thaliana*, which has a point mutation resulting in a truncated GA 20-oxidase protein (Xu et al., 1995), appears to be blocked only at the first and the third steps of GA 20-oxidase activity (Talon et al., 1990). Furthermore, GA_{53}-oxidase and GA_{19}-oxidase activities could be separated from GA_{44}-oxidase activity by anion-exchange HPLC (Gilmour et al., 1987). Hence, these earlier findings appear to be in conflict with the current results that all three reactions are catalyzed by one enzyme with a single active site. Therefore, we have to consider the possibility that there are two enzymes that can convert GA_{44} to GA_{19}: the multifunctional GA 20-oxidase that catalyzes the sequential oxidation at C-20 and a second enzyme catalyzing a single step, namely the conversion of GA_{44} to GA_{19}. A similar case was reported for *Neurospora crassa* in which the methyl group of thymine is successively oxidized to hydroxymethyl, formyl, and carboxyl by a single 2-oxoglutarate-dependent dioxygenase. However, for the last step, there is an additional enzyme that is not dependent on 2-oxoglutarate (Liu et al., 1973).

There is considerable evidence indicating that the steps catalyzed by GA 20-oxidase are important, regulatory steps in GA biosynthesis. In maize seedlings, it was found that bioactive GAs regulate their own biosynthesis through control of GA 20-oxidase activity (Hedden and Croker, 1992). The *ga1* and *ga5* mutants of *A. thaliana*, which have low levels of endogenous GAs (Zeevaart and Talon, 1992), accumulate high levels of GA 20-oxidase mRNA compared with wild-type plants (Phillips et al., 1995; Xu et al., 1995). In addition, expression of three GA 20-oxidase genes in flower buds and pedicels of the *ga1–2* mutant of *A. thaliana* was considerably reduced after application of GA_3 (Phillips et al., 1995). These findings support the view that bioactive GAs may control their own synthesis through a negative feedback mechanism on the mRNA level of the GA 20-

As described previously, the conversions of GA_{53}→ GA_{44} and of GA_{19}→ GA_{20} are regulated by the photoperiod (Gilmour et al., 1986; Zeevaart et al., 1990). Measurement of GA levels indicated that transfer of spinach plants from SD to LD conditions caused an increase in all GAs of the early-13-hydroxylation pathway except GA_{53}, with GA_{20}, GA_1, and GA_8 showing the largest increases. This correlates well with the increases of GA_{53}-oxidase and GA_{19}-oxidase activities (Gilmour et al., 1986; Zeevaart et al., 1990) and is compatible with the idea that in spinach the flow through the GA biosynthetic pathway is much enhanced by long photoperiods. An increase in the later GAs of the early-13-hydroxylation pathway in long photoperiods also suggests that light may regulate other step(s) in the GA biosynthetic pathway in addition to the steps catalyzed by GA 20-oxidase (Metzger and Zeevaart, 1982; Talon et al., 1991b; Zeevaart and Gage, 1993).

Studies of the expression of GA 20-oxidase in spinach plants indicate that there is a significantly higher level of GA 20-oxidase mRNA in plants grown in LD conditions than in those in SD conditions (Figs. 7 and 8). When spinach plants were transferred from the SD to the LD condition for up to 8 d, an increase in GA 20-oxidase mRNA abundance was observed, which parallels the increase in GA_{20} and GA_1 levels. If the steps catalyzed by GA 20-oxidase are rate limiting for GA biosynthesis, then increases in GA 20-oxidase transcription and/or mRNA stability, as well as in enzyme activity, will lead to an increase in the GA_{20} content and subsequently in the GA_1 content (assuming GA_{20}→ GA_1 is not a rate-limiting step), with GA_1 being the effector of stem elongation. Conversely, when spinach plants grown in the LD condition were transferred to the SD condition or darkness for 24 h, there was a decrease in GA 20-oxidase mRNA (Figs. 8B, 9). In addition, posttranscriptional regulation is likely, since GA 20-oxidase mRNA was still detectable after plants had been in darkness for 24 h (Fig. 9), whereas enzyme activity decreased rapidly during the first 8 h in darkness (Gilmour et al., 1986).

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