Cloning and Expression of a Gibberellin 2β,3β-Hydroxylase cDNA from Pumpkin Endosperm

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A cDNA expression library in λMOSElox derived from poly(A)+ RNA from pumpkin endosperm was screened immuno-logically with a polyclonal antibody raised against partially purified gibberellin (GA) 2β,3β-hydroxylase from pumpkin endosperm. A recombinant fusion protein encoded by a selected positive clone catalyzed 3β-hydroxylation of GA15, GA24, GA25, and GA4, and as identified putatively, GA34. The fusion protein also catalyzed 2β-hydroxylation of the C20 GAs GA25, GA34, and GA4, and GA34, albeit less efficiently. The full-length clone contains an open reading frame of 1041 nucleotides encoding 346 amino acid residues with a predicted molecular weight of 38,992 and pl of 7.2. Transcript levels of this gene and of the previously cloned GA 7-oxidase and 20-oxidase genes from pumpkin endosperm rose until day 2 after the start of imbibition of the mature seeds, but only at one–two hundredth to one–six thousandth of the level found in the endosperm, as determined by quantitative reverse transcriptase–polymerase chain reaction. In contrast, GA 7-oxidase, 20-oxidase, and 3β-hydroxylase enzyme activities were present in cell-free systems prepared from embryos of mature seeds and decreased after imbibition.

INTRODUCTION

Many important steps in plant growth and development are controlled by gibberellin (GA) hormones. The physiological function of GAs is well established in vegetative plant organs (Crozier, 1983). In contrast, very little is known about their role in developing seeds (Pharis and King, 1985), which generally contain higher levels of GAs than any other tissues and which have been used extensively to study GA biosynthetic pathways (Graebe, 1987). However, there is growing evidence that GAs have an important if not essential role in early stages of embryogenesis (Swain et al., 1993, 1995; Yeung and Meinke, 1995).

GAs, which are physiologically active in growing plants and which possibly play important roles in seed development, are produced in the biosynthetic pathway by oxidation of the GA precursor GA13-aldehyde at three different positions: carbon-7, carbon-20, and carbon-3β (Figure 1). In immature pumpkin seeds, the pathway splits into two routes. The first one, which represents the major pathway, leads to GAs with 20 carbons (C20 GAs, including GA4, and GA5, the other produces the C19 GAs (GA5 or GA4). Some of the 3β-hydroxylated C19 GAs (GA, and GA5) are active in bioassays. Subsequently, such bioactive GAs can be deactivated by 2β-hydroxylation (e.g., GA34). In immature pumpkin seeds, 2β-hydroxylation is tissue specific: in cell-free systems from pumpkin embryos, only C19 GAs are 2β-hydroxylated, whereas in pumpkin endosperm, only 2β-hydroxylations of C20 GAs (e.g., GA4) have been obtained (Lange et al., 1993a, 1993b). The physiological function of the latter reaction is not known. In most plant species studied to date, 20-oxidation, 2β-hydroxylation, and 3β-hydroxylation are catalyzed by 2-oxoglutarate–dependent dioxygenases: in pumpkin, 7-oxidation is also catalyzed by these dioxygenases (Prescott and John, 1996; Hedden and Kamiya, 1997).

The recent cloning of the GA 7-oxidase gene from pumpkin endosperm and the GA 20-oxidase genes from several sources including pumpkin endosperm (Lange et al., 1994b; Lange, 1997) revealed that the encoded enzymes are multifunctional with broad substrate specificities (Figure 1). Also, the cloning of the GA4 gene from Arabidopsis has been reported (Chiang et al., 1995). GA4 has been shown to encode a GA 3β-hydroxylase (J. Williams, A.L. Phillips, and P. Hedden, unpublished data). Recently, maize d1 mutants have been shown to be defective in three GA biosynthetic steps, including 3β-hydroxylation (Spray et al., 1996). A partially purified enzyme preparation from pumpkin endosperm is known to catalyze 3β-hydroxylations of various precursors of the GA biosynthetic pathway, including both C20 and C19 GAs (Graebe, 1987; Lange and Graebe, 1993; Lange et al., 1994a).

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In pumpkin endosperm, reactions are catalyzed by GA 7-oxidase (A), GA 20-oxidase (B), and GA 2β,3β-hydroxylase (C). Structures and metabolic relationships of GAs are discussed in the text.

Isolation of cDNA Clones

The IgG fraction containing anti-2β,3β-hydroxylase antibodies was used for immunological screening of an amplified λMOSEloX cDNA expression library derived from endosperm poly(A)+ RNA of immature pumpkin seeds. Thirteen positive plaques from 200,000 recombinants were identified. Four positive plaques contained inserts of ~1350 bp and were shown to be similar by restriction enzyme digest analysis. One of these λ clones was used for bacteriophage P1 cre recombinase-mediated excision to give pMOSEloX plasmids (designated clone 83). The unamplified library was
shown to contain 27 positives among 300,000 plaque-forming units (pfu).

Substrate Specificity of Recombinant GA 2β,3β-Hydroxylase

The catalytic properties of the recombinant protein were investigated by using cell lysates prepared from Escherichia coli BL21(DE3) harboring pMOSElox clone 83. Recombinant 2β,3β-hydroxylase is capable of oxidizing 14C-labeled substrates GA12-aldehyde, GA12, GA15, GA24, GA9, GA25, GA26, and GA17 at position C-3β. The C20 GAs GA15, GA24, and GA25 were the preferred substrates (Table 2). Compared with the conversion rate of 14C-GA12 (Figure 2A), those of 14C-GA15 (Figure 2B), 14C-GA24 (Figure 2C), 14C-GA25 (Figure 2D), and 14C-GA9 (Figure 2E) were 130, 250, 70, and four times higher, respectively. Only the C20 GAs 14C-GA25, 14C-GA15, and probably 14C-GA17 as well were oxidized at position C-2β (Table 2 and Figure 2). These C20 GAs all contain the C-20 carboxylic acid groups. Specific radioactive activities of substrates and the respective products thereof were identical, as determined by gas chromatography–mass spectrometry (GC-MS) single-ion monitoring (data not shown). No conversion of the 14C-labeled substrates GA12-aldehyde, GA12, GA15, GA24, GA9, GA25, GA13, GA26, GA17, and GA1 was obtained in standard incubation assays, with cell lysates of E. coli harboring the pMOSElox plasmid without the cDNA insert (data not shown).

cDNA Sequence Analysis

The cDNA sequence of the insert of clone 83 contains 1252 nucleotides encoding an open reading frame of 346 amino acids.

Table 1. Immunoinhibition of Partially Purified GA 2β,3β-Hydroxylase from Pumpkin Endosperm

<table>
<thead>
<tr>
<th>IgG Fraction of Rabbit Serum</th>
<th>Products (% by HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before immunization</td>
<td>GA25 GA12</td>
</tr>
<tr>
<td>After immunization</td>
<td>620 38</td>
</tr>
</tbody>
</table>

*aThe IgG fractions were obtained from rabbit sera before or after immunization with partially purified GA 2β,3β-hydroxylase. Partially purified 2β,3β-hydroxylase from pumpkin endosperm was immunoprecipitated by using these IgG fractions, and supernatants were assayed for 2β,3β-hydroxylase activity by incubations with 14C-GA2, as described in Methods, except that incubation time was 90 min.*

*bUnmetabolized substrate.

Table 2. Metabolism of 14C-GAs by Cell Lysates from E. coli Transformed with pMOSElox Clone 83

<table>
<thead>
<tr>
<th>Substrate Formed</th>
<th>Products (a)</th>
<th>% by HPLC</th>
<th>KRI</th>
<th>Characteristic Ions at m/z (% Relative Intensity of Base Peak (b))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA12ald.</td>
<td>GA12ald.</td>
<td>9</td>
<td>2490</td>
<td>SIM</td>
</tr>
<tr>
<td>GA12</td>
<td>GA14</td>
<td>29</td>
<td>2763</td>
<td>440(19), 432(4), 350(38), 342(16), 318(78), 310(40), 292(50), 284(32), 243(58), 237(26)</td>
</tr>
<tr>
<td>GA14</td>
<td>GA12</td>
<td>100</td>
<td>2600</td>
<td>470(3), 462(3), 438(26), 430(31), 410(23), 402(26), 320(47), 312(44), 290(98), 284(100)</td>
</tr>
<tr>
<td>GA24</td>
<td>GA24</td>
<td>25</td>
<td>2511</td>
<td>426(12), 418(15), 394(11), 386(33), 336(22), 328(25), 320(25), 292(25), 284(100), 313(138), 225(214)</td>
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<tr>
<td>GA24</td>
<td>GA24</td>
<td>81</td>
<td>2592</td>
<td>485(5), 477(2), 442(12), 436(4.5), 406(24), 400(8), 350(14), 342(12), 318(38), 310(25)</td>
</tr>
<tr>
<td>GA26</td>
<td>GA26</td>
<td>5</td>
<td>2615</td>
<td>500(7), 492(9), 488(34), 460(14), 406(7), 400(31), 350(68), 342(38), 318(74), 310(39)</td>
</tr>
<tr>
<td>GA26</td>
<td>GA26</td>
<td>4</td>
<td>2730</td>
<td>588(3), 580(2), 498(6), 490(6), 439(100), 431(40), 406(12), 398(10), 379(18), 371(15)</td>
</tr>
<tr>
<td>GA26</td>
<td>GA26</td>
<td>7</td>
<td>2729</td>
<td>588(3), 580(4), 498(9), 490(6), 439(97), 431(100), 406(10), 398(10), 379(14), 371(21)</td>
</tr>
<tr>
<td>GA13</td>
<td>GA13</td>
<td>6</td>
<td>2687</td>
<td>SIM</td>
</tr>
<tr>
<td>GA13</td>
<td>GA13</td>
<td>92</td>
<td>2718</td>
<td>SIM</td>
</tr>
</tbody>
</table>

*Identification of 14C-GA metabolic products by GC-MS on the basis of Kovats retention indices (KRI) and single-ion monitoring (SIM) of characteristic masses or full-scan mass spectra of the methyl ester trimethylsilyl ether derivatives and by comparison with published spectra (Gaskin and MacMillan, 1992).

Based on ions above a mass-to-charge ratio (m/z) of 100.

GA9, GA12-aldehyde. Incubation volumes three times that of the standard assays.

GA9, GA12-aldehyde. Putative identification on the basis of HPLC retention time.

Incubation volumes 10 times that of the standard assays.

Incubation volumes five times that of the standard assays.

Putative 2β-hydroxyGA25 on the basis of HPLC retention time (Lange and Graebe, 1993).

No product formation observed.
Transcript Levels of GA 7-Oxidase, 20-Oxidase, and 2β,3β-Hydroxylase Genes in Immature and Germinating Pumpkin Seeds

mRNA expression levels were determined for three GA dioxygenase genes in the endosperm of immature seeds and in embryos during germination by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Highest transcript levels were found for all three genes in immature seeds. They contained 40, 300, and 20 μg of transcripts per g of total RNA encoding GA 7-oxidase, 20-oxidase, and 2β,3β-hydroxylase, respectively. However, no transcripts of the three GA dioxygenase genes were detected in mature seeds (data not shown) and in seeds 4 hr after the start of imbibition (Figures 4A to 4C). These seeds contained 0.2, 0.05, and 0.005 μg of transcripts per g of total RNA encoding GA 7-oxidase, 20-oxidase, and 2β,3β-hydroxylase, respectively (Figures 4A to 4C, lane 3).

Metabolism of 14C-GA12-Aldehyde, 14C-GA20, and 14C-GA39 in Cell-Free Systems from Mature and Germinating Seeds

Cell-free enzyme systems prepared from the embryos of mature seeds 2, 30, and 72 hr after the start of imbibition metabolize 14C-GA12-aldehyde, 14C-GA12, and 14C-GA25 (Figure 5). GA 7-oxidation and 20-oxidation activities remained nearly constant during the first 30 hr after the start of imbibition, and GA 3β-hydroxylation activity increased slightly. However, conversion of all three substrates was low in cell-free preparations of seeds 72 hr after the start of imbibition (Figure 5). GA metabolism was investigated in cell-free systems of embryos from mature pumpkin seeds and of embryos from seeds 36 hr after the start of imbibition, using 14C-GA20-aldehyde, 14C-GA25, 14C-GA33, and 14C-GA4 as substrates (Table 3). Both systems metabolize 14C-GA12-aldehyde primarily to 14C-GA39. Minor products were the 12α-hydroxy-GA13, putative 12α-hydroxy-GA25, and GA4. Incubations with 14C-GA25 produced primarily 14C-GA39, and incubations with 14C-GA33 gave only 14C-GA39 as a minor product. 14C-GA4 was primarily converted to 14C-GA4, but in addition, 14C-GA40 was produced as identified by full-scan GC-MS. The product pattern did not change if enzyme assays were performed in the presence of DNase I (100 units) or RNase A (1 Kunitz unit; data not shown), indicating that no in vitro transcription or translation had occurred.

DISCUSSION

In this study, we present evidence for the cloning of a GA 2β,3β-hydroxylase cDNA derived from endosperm poly(A)+...
RNA of immature pumpkin seeds. The GA 2β,3β-hydroxylase cDNA (clone 83) was cloned by immunological screening of a cDNA expression library in A. rhizogenes with a specific antibody. The recombinant GA 2β,3β-hydroxylase has a theoretical molecular weight of 38,992, which was confirmed by 5′ RACE. The molecular weight of partially purified GA 2β,3β-hydroxylase from pumpkin endosperm, as determined by SDS-PAGE, was also 40,000 (Lange et al., 1994a). However, the apparent molecular weight, as determined by gel filtration HPLC, was considerably higher (58,000) for the native enzyme (Lange et al., 1994a). Because gel filtration HPLC was performed at the calculated isoelectric point of the 2β,3β-hydroxylase (pI 7.2), the elution profile might have been affected by interactions (e.g., forming aggregates) due to the zero net charge of the molecule.

The broad substrate specificity of the recombinant GA 2β,3β-hydroxylase indicates that a single enzyme is capable of catalyzing all 3β-hydroxylations and 2β-hydroxylations that occur in the GA biosynthetic pathway, as observed in cell-free systems of endosperm from immature pumpkin seeds (Graebe, 1987; Lange et al., 1993a). The recombinant enzyme prefers GA substrates containing C-20 alcohol, aldehyde, or carboxylic acid groups, such as GA15, GA24, and GA25, respectively. It catalyzes, at lower efficiency, the hydroxylation at position 2β of those GA precursors containing a C-20 carboxylic acid group (as shown for GA25, GA13, and probably GA12). All other substrates, including C19 GAs, were only hydroxylated at the 3β position. These catalytic properties correspond exactly to the ones found for the partially purified 2β,3β-hydroxylase from pumpkin endosperm (Lange and Graebe, 1993; Lange et al., 1994a). The native enzyme is also able to 2β-hydroxylate GA precursors containing C-20 carboxylic acid groups (data not shown). By contrast, in cell-free systems from immature and mature pumpkin embryos, no 2β-hydroxylation of C20 GAs was observed. Embryo systems hydroxylate C19 GAs at position 2β.

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Figure 3. Amino Acid Sequence Comparison of the GA 2β,3β-Hydroxylase (Cm3h) from Pumpkin Endosperm with Other Members of the GA Dioxygenase Family.

Enzymes included in the alignment are GA 3β-hydroxylase sequence (At3h) from Arabidopsis (regarding the revised amino acid sequence for GenBank accession number L37126; see Chiang et al., 1995) and GA 7-oxidase (Cm7ox) and GA 20-oxidase (Cm20ox) from pumpkin endosperm (Lange, 1997). Identical residues are boxed in black; similar residues are shaded in gray. Dashes were introduced to optimize alignment. The GenBank accession number for Cm3h is U63650.

Figure 4. GA 7-Oxidase, GA 20-Oxidase, and GA 2β,3β-Hydroxylase mRNA Expression in Germinating Seeds and Young Seedlings of Pumpkin Shown by RT-PCR.

Reverse transcription was conducted with internal RNA standards and was followed by a PCR reaction, as described in Methods.

(A) GA 7-oxidase (25 fg).

(B) GA 20-oxidase (2.5 fg).

(C) GA 2β,3β-hydroxylase (0.25 fg).

Lane 1 contains RNA from seeds 4 hr after imbibition, lanes 2 to 6 contain RNA from seeds 1 to 5 days after imbibition, respectively, and lane 7 contains the 100-bp ladder. The positions of the 800-bp bands are shown at right.
Enzyme preparations were obtained from seeds 2, 30, and 72 hr after the start of imbibition; the preparations contained 13.2, 9.8, and 12.5 mg/mL protein, respectively. Standard incubations were performed for GA 7-oxidase with 14C-GA7-aldehyde, for 20-oxidase with 14C-GA20, and for 3β-hydroxylase with 14C-GA3, except for enzyme preparation volumes, which were 1, 0.25, and 3 μL, respectively; incubation time was 90 min. (Lange et al., 1993b; Table 3) and therefore must require an enzyme(s) with properties distinct from the 2β,3β-hydroxylase of immature pumpkin endosperm. Whether such an enzyme also exhibits 2β-hydroxylation and 3β-hydroxylation activities, as suggested by a partially purified 3β-hydroxylase from developing bean embryos (Smith et al., 1990), or whether it has solely 2β-hydroxylase activity, as suggested by the enzyme from cotyledons of mature bean seeds (Griggs et al., 1991), might be addressed when the gene encoding this enzyme has been cloned.

The composition of the endogenous GAs in pumpkin endosperm (Blechschmidt et al., 1984) perfectly reflects the catalytic properties of the recombinant 2β,3β-hydroxylase. Its major constituents are 3β-hydroxylated C19 GAs (mainly GA1, equivalent to 12α-hydroxy GA4) and 2β,3β-hydroxylated C20 GAs (mainly GA3). The pumpkin endosperm surrounds the developing embryo, apparently gets absorbed during embryogenesis, and might therefore serve the embryo as a source of GAs. For instance, the total endogenous GA19 level of developing pumpkin seeds decreased in the course of embryogenesis, but it was not found in pumpkin embryos of early developmental stages and was present later in developed embryos (Blechschmidt et al., 1984).

The GA levels in seeds from other species are also dependent on developmental stage and tissue examined. For instance, decreasing levels of GA4, as found for several species during early seed development (García-Martínez et al., 1987; Koshioka et al., 1993; Tadeo et al., 1994; Swain et al., 1995; Rodrigo et al., 1997), are consistent with the existence of different GA 2β- and 3β-hydroxylase activities in endosperm and embryo tissues. GAs are important for early embryogenesis and embryo growth (Pharis and King, 1985; Yeung and Meinke, 1993). Swain et al. (1993, 1995) provided clear evidence that GA deficiency during early embryogenesis in pea seeds of the lhir mutant led to abortion unless their endogenous GA levels were restored. In particular, C19 GAs that have been modified by a (multifunctional) GA 3β-hydroxylase(s) are regarded as having physiological functions (Spray et al., 1996); as yet, this has not been shown for plant embryogenesis. In light of these results, GA 2β,3β-hydroxylase in the endosperm might be essential for GA biosynthesis during early embryogenesis and for seed survival.

Transcript levels of the GA 2β,3β-hydroxylase gene were studied in the endosperm of immature seeds and in embryos of mature and germinating pumpkin seeds. In addition, the transcript levels of two other GA dioxygenase genes, encoding 7-oxidase and 20-oxidase (Lange et al., 1994b; Lange, 1997), were monitored. In the endosperm of immature pumpkin seeds, transcript levels, as determined by quantitative RT-PCR, were 200 times higher for 7-oxidase and 4000 to 6000 times higher for 20-oxidase and 2β,3β-hydroxylase, respectively, compared with the maximum yield found in germinating seeds 2 days after imbibition. This indicates that the three GA dioxygenase genes have considerably higher transcript levels in immature pumpkin seeds.

However, relatively high GA 7-oxidation, 20-oxidation, and 3β-hydroxylation activities were detected in cell-free preparations from mature dry seeds. The conversion rates of GA7-aldehyde and GA12 were only ~10 to 15 times lower than rates previously determined in cell-free preparations from embryos of immature pumpkin seeds (Lange et al., 1993b; Table 3).

### Table 3. Metabolism of 14C-GAs by Cell-Free Preparation from Mature Seeds 0 and 36 hr from the Start of Incubation

<table>
<thead>
<tr>
<th>Time from Start of Imbibition (Hr)</th>
<th>Products (% by HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA19-aldehyde</td>
<td>GA20 GA20-OH GA20b GA20c GA3 GA3b</td>
</tr>
<tr>
<td>0</td>
<td>GA19-aldehyde 16 80 2 2 0</td>
</tr>
<tr>
<td></td>
<td>GA9 95 5</td>
</tr>
<tr>
<td></td>
<td>GA20 20b 80 0</td>
</tr>
<tr>
<td></td>
<td>GA13 96b 4</td>
</tr>
<tr>
<td>36</td>
<td>GA19-aldehyde 87 6 3 4</td>
</tr>
<tr>
<td></td>
<td>GA9 93 7</td>
</tr>
<tr>
<td></td>
<td>GA20 20 85 2</td>
</tr>
<tr>
<td></td>
<td>GA13 93b 7</td>
</tr>
</tbody>
</table>

* Incubations were performed as described in Methods, except that fresh cofactors were added in 4 μL after 4 hr; incubation time was 15.5 hr.

* The pooled HPLC fractions contained <10% of putative 14C-12α-hydroxy GA20, as determined by GC-MS.

* Unmetabolized substrate.
In addition, the GA pathways found in cell-free systems from both tissues lead to the same embryo-typical end products (see above). Transcripts encoding the three GA dioxygenases from pumpkin endosperm were not detectable by RT-PCR in embryos of mature seeds up to 4 hr after the start of imbibition. Forty-eight to 72 hr after the start of imbibition, when transcripts peaked at a very low level, GA dioxygenase activities decreased dramatically, indicating that the contribution of de novo synthesis by expression of the three GA dioxygenase genes from endosperm is negligible. Therefore, we propose that the initial GA dioxygenase activities, as found in the embryo of the mature seed, are already produced by gene expression during embryo development. These GA dioxygenase activities might contribute to GA biosynthesis occurring during early seed germination.

Tissue-specific transcription pattern and feedback regulation of transcript levels were found previously for different GA dioxygenase genes in several species (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995; Martin et al., 1996; Wu et al., 1996; MacMillan et al., 1997; Toyomasu et al., 1997). Our report, however, clearly demonstrates that for the three very persistent GA dioxygenase genes in mature pumpkin seeds, transcript levels do not correlate with enzyme activities present and thus with operating GA biosynthetic pathways.

**METHODS**

**Preparation of ¹⁴C-Labeled Substrates**

(1, 7, 12, 18-¹⁴C₂⁶-GA₁₂)-aldehyde (5.93 X 10¹² Bq/mol) and -GA₁₃ (5.80 X 10¹² Bq/mol) were prepared from R-2-¹⁴C-mevalonic acid (1.96 X 10¹² Bq/mol; Amersham), and ¹⁴C-GA₁₆ (5.05 X 10¹² Bq/mol), ¹⁴C-GA₂₅ (4.75 X 10¹² Bq/mol), ¹⁴C-GA₃₃ (5.33 X 10¹² Bq/mol), ¹⁴C-GA₄₇ (5.35 X 10¹² Bq/mol), and ¹⁴C-GA₅₈ (5.88 X 10¹² Bq/mol) were prepared from ¹⁴C-GA₂₅ by using a cell-free system from Cucurbita maxima endosperm (Lange and Graebe, 1993). ¹⁴C-GA₃₉ (7.56 X 10¹² Bq/mol) was a gift from P. Hedden (Long Ashton Research Station, Bristol, UK), and ¹⁴C-GA₉₃ was a gift from L. Mander (Australian National University, Canberra, Australia). ¹⁴C-GA₂₁ (1.05 X 10¹² Bq/mol) was prepared from ¹⁴C-GA₂₀ by using a partially purified 20-oxidase from immature Pisum sativum seeds (Lange and Graebe, 1989). ¹⁴C-GA₅₇ (4.77 X 10¹² Bq/mol) was prepared by incubation of ¹⁴C-GA₁₂ (28,000 Bq) with cell lysates (2.5 mL) from Escherichia coli transformed with clone A24242 (Phillips et al., 1995) and cofactors (100 mM 2-oxoglutarate, 100 mM ascorbate, 0.5 mM FeSO₄, and 1 mg/mL catalase, final concentrations) in a total volume of 4.1 mL for 16 hr at 30°C. ¹⁴C-GA₅₇ was extracted and separated by HPLC, using a methanol-50 mM Tris-HCl gradient at pH 8.3 (Lange et al., 1997).

**Plant, Enzyme, RNA, and DNA Preparations**

Seeds of C. maxima cv Riesemelone, gelb genetzt (van-Waveren, Göttingen, Germany), which were stored for 16 months at room temperature (1995 harvest), were imbibed for 4 hr in water, and were grown for up to 5 days in a growth cabinet under an 18-hr photoperiod at 25°C (light) at 80 μmol m⁻² sec⁻¹ (model L65W/25 white-universal tubes; Osram, Munich, Germany) and at 22°C (dark). The testa were removed, and the embryo or seedling was frozen immediately in liquid nitrogen and stored at −87°C in a freezer. In addition, embryos of dry mature seeds and the endosperm nucellus of immature seeds without developed cotyledons were frozen in liquid nitrogen and stored at −87°C. The plant material was ground to a fine powder in liquid nitrogen with a mortar and pestle. Total RNA was isolated from the frozen powder (100 μg) with an RNaseasy kit (Qiagen, Hilden, Germany) and treated with DNase I (Sigma, Deisenhofen, Germany; 10 units per μg of total RNA) for 20 min at 37°C, followed by phenol-chloroform extraction. Total RNA was stored at −87°C and used for quantification of specific gibberellin (GA) dioxygenase gene transcripts as described below. Enzyme preparations were obtained from the frozen powder (1 g) as described by Lange et al. (1993b), but the gel filtration step was omitted. The endosperm nucellus from immature pumpkin seeds was also used for preparation of partially purified GA 2β,3β-hydroxylase, according to Lange et al. (1994a), and for genomic DNA isolation (Ausubel et al., 1987).

**Antibodies and Immunoinhibition of Native GA 2β,3β-Hydroxylase Activity**

Polyclonal antibodies were developed in rabbit according to the method of Sambrook et al. (1989) against a partially purified 2β,3β-hydroxylase preparation from pumpkin endosperm (Lange et al., 1994a), and the IgG fractions from rabbit sera before and after immunization were purified on a protein A column, lyophilized, and stored at 4°C. Partially purified 2β,3β-hydroxylase (0.5 μL is equivalent to 0.34 μg of protein) was incubated with the purified IgG fractions (10 μL is equivalent to 67.4 μg of protein) in TBS buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl) in a total volume of 200 μL for 150 min at 30°C in a shaking water bath. Protein A-Sepharose CL-4 gel (100 μL is equivalent to 12.5 mg in TBS buffer; Sigma) was added, and the mixture was incubated for another 150 min at 30°C in a shaking water bath. The mixtures were centrifuged at 15,000g for 5 min, and 94 μL of each supernatant was removed and directly incubated with ¹⁴C-GA₅₇, as described below.

**cDNA Library Construction, Antibody Screening, and Heterologous Expression of Recombinant GA 2β,3β-Hydroxylase Activity**

An oligo(dT)-primed and amplified cDNA expression library was prepared in XMOSElox (Amersham) from poly(A)⁺ RNA from the endosperm nucellus of immature pumpkin seeds without developed cotyledons, as described previously (Lange, 1997). Ten 90-mm plates, each containing ~20,000 plaque-forming units (pfu) of the amplified library, were immunologically screened with the polyclonal antibody (Lange et al., 1994b). Thirteen positive plaques were cored from the plates, replated at ~160 pfu per plate, and rescreened until the plaque was pure, and the size of the cDNA inserts was determined by polymerase chain reaction (PCR). For heterologous expression and cDNA sequence analysis, one clone (designated 83) was subcloned in pMOSElox plasmids by bacteriophage P1 cre recombinase-mediated excision in E. coli BM25.8, following the manufacturer’s instructions (Amersham). E. coli BL21 (DE3) were transformed with the pMOSElox plasmids containing clone 83 and used for heterologous expression of GA 2β,3β-hydroxylase activity, as described by Lange (1997).
Standard Enzyme Assay and Analysis and Identification of Incubation Products

Native enzyme preparations (94 µL; 8.7 mg/mL protein) were incubated with 2-oxoglutarate and ascorbate (4 mM each, final concentrations), and preparations of E. coli cell lysates (70 µL) were incubated with 2-oxoglutarate and ascorbate (100 mM each, final concentrations), FeSO₄ (0.5 mM), catalase (1 mg/mL), and the 14C-labeled GA substrate (2 µL in 50% methanol; 40 pmol) were added in a total volume of 100 µL and incubated for 16 hr at 30°C. GA substrates and variations of the incubation conditions are specified for particular experiments. Incubation products were extracted and analyzed by reverse phase HPLC with on-line radiocounting, using gradients of increasing methanol in acidic H₂O₃, as described by Lange and Graebe (1993). More than 95% of the radioactivity originally added as substrate was recovered. Radioactive fractions were dried, derivatized, and analyzed by combined gas chromatography–mass spectrometry (GC-MS) (Lange, 1997).

DNA Sequence Analysis

The cDNA inserts of clone 83 were released with EcoRI and further digested with Eco88.1, HindIII, and with both Eco88.1 and HindIII. 5'-Rapid amplification of cDNA ends (RACE) was performed using mRNA from immature pumpkin seeds and the 5'/3' RACE kit (Boehringer Mannheim). The undigested cDNA insert, fragments after restriction enzyme cleavages, and RACE products were subcloned by using specific primer pairs of sense (F) and antisense (R) oligonucleotides, and preparations of

For the preparation of internal RNA standards, genomic DNA was isolated from the endosperm of immature pumpkin seeds and amplified by using specific primer pairs of sense (F) and antisense (R) oligonucleotides in PCR reactions. Each 50-µL reaction contained 1 µg of genomic DNA, 5 µL of 10 X PCR buffer containing 15 mM MgCl₂, 1 µL of 10 mM deoxynucleotide triphosphates, 10 pmol of each primer, and 1.5 units of BioTherm-polymerase (GenCraft, Münster, Germany). Samples were heated to 94°C for 4 min and then subjected to 35 cycles of 94°C for 30 sec, 45°C for 30 sec, and 72°C for 2 min; the reaction was completed by a 6-min incubation at 72°C. PCR products were purified by agarose gel electrophoresis (Vogelstein and Gillespie, 1979), blunt-ended using the Klenow fragment of DNA polymerase I, and ligated to pBlueScript SK−. The amplified DNAs of each of the three GA genes contain ~200-bp-long introns. Ligation products were introduced into E. coli XL1 Blue, and plasmid DNA was isolated from single transformants by using a plasmid Mici kit (Qiagen). Plasmids containing inserts in sense orientation (1 µg each) were transcribed in vitro by using a T7 transcription kit (MBI-Fermentas, St. Leon-Rot, Germany). RNA molecules were purified by using the RNeasy kit (Qiagen), stored at −87°C, and used as internal RNA standards.

For quantification, total RNA (0.25 µg) supplemented with different amounts of RNA standards and sequence-specific antisense (RT) primers (5 pmol) for each of the three genes was reverse transcribed using first-strand cDNA synthesis reactions (Boehringer Mannheim) in a total volume of 5 µL. One microliter of each of the reverse-transcribed products was amplified by PCR using sequence-specific sense (F) and antisense (R) primers (2 pmol each), 1 µL of 10 X PCR buffer containing 15 mM MgCl₂, 0.2 µL of 10 mM deoxynucleotide triphosphates, and 0.3 units of BioTherm-polymerase in a total volume of 10 µL. Thirty-five cycles were performed consisting of denaturation (94°C for 30 sec) and annealing and extension (72°C for 2 min), and the reaction was completed after 6 min at 72°C. Products were analyzed by electrophoresis on 1% agarose gel followed by ethidium bromide staining and visualized by UV transillumination. Quantification of RNA expression levels was done by comparing the intensity of the bands with those produced by internal RNA standards. Before performing quantitative RT-PCR, total RNA samples (1 µg each) were analyzed by agarose gel electrophoresis as described above to ensure that the RNA was not degraded. In all experiments, control reactions were performed by omitting the reverse transcriptase, the RNA, or the internal standard from the reverse transcriptase reaction. All experiments were repeated at least once, giving identical results.

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