Gibberellin Biosynthesis in Developing Pumpkin Seedlings[1,2]

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A gibberellin (GA) biosynthetic pathway was discovered operating in root tips of 7-d-old pumpkin (*Cucurbita maxima*) seedlings. Stepwise analysis of GA metabolism in cell-free systems revealed the conversion of GA_{12}-aldehyde to bioactive GA_4 and inactive GA_{34}. Highest levels of endogenous GA_4 and GA_{34} were found in hypocotyls and root tips of 3-d-old seedlings. cDNA molecules encoding two GA oxidases, *CmGA20ox3* and *CmGA3ox3*, were isolated from root tips of 7-d-old LAB150978-treated seedlings. Recombinant CmGA20ox3 fusion protein converted GA_{12} to GA_9, GA_{24} to GA_9, GA_{14} to GA_4, and, less efficiently, GA_{53} to GA_{20}, and recombinant CmGA3ox3 protein oxidized GA_9 to GA_4. Transcript profiles were determined for four GA oxidase genes from pumpkin revealing relatively high transcript levels for *CmGA7ox* in shoot tips and cotyledons, for *CmGA20ox3* in shoot tips and hypocotyls, and for *CmGA3ox3* in hypocotyls and roots of 3-d-old seedlings. Transcripts of *CmGA2ox1* were mainly found in roots of 7-d-old seedlings. In roots of 7-d-old seedlings, transcripts of *CmGA7ox*, *CmGA20ox3*, and *CmGA3ox3* were localized in the cap and the rhizodermis by in situ hybridization. We conclude that hypocotyls and root tips are important sites of GA biosynthesis in the developing pumpkin seedling.

Gibberellins (GAs) are signaling molecules that regulate and integrate developmental processes during the entire life cycle of higher plants, including shoot elongation and root development (Richards et al., 2001; Olszewski et al., 2002; Fu and Harberd, 2003; Reid et al., 2004; Sun, 2004).

GA biosynthetic pathways are of considerable complexity (for review, see Hedden and Kamiya, 1997; Sponsel and Hedden, 2004). They are divided into nonhydroxylated, 3β-hydroxylated, and 13-hydroxylated pathways (Fig. 1, A–C, respectively; Graebe, 1987). A principal pathway to GA plant hormones can be drawn from GA_{12}-aldehyde. First, 7-oxidation of GA_{12}-aldehyde results in the formation of GA_{12}. GA_{53} is often formed by 13-hydroxylation of GA_{12}. The following three oxidation steps at carbon (C)-20 are catalyzed by one enzyme, the GA 20-oxidase, which, in general, leads to the formation of a C_{19}-GA (e.g. GA_9 and GA_{20}; Fig. 1). Alternatively, C-20 oxidation leads to the formation of a carboxylic acid (e.g. GA_{25} and GA_{17}). The resulting C_{20}-GAs usually are minor products of GA 20-oxidase activity. Finally, 3-oxidation produces GA plant hormones (GA_4 and GA_1), which are subsequently inactivated by 2-oxidation (GA_{34} and GA_8; Fig. 1). In many plant species, 7-oxidation and 13-hydroxylation are catalyzed by NADPH-dependent cytochrome P450 mono-oxygenases. In Arabidopsis (*Arabidopsis thaliana*), a multifunctional *ent*-kaurenoic acid oxidase with 7-oxidation catalytic properties has been characterized (Helliwell et al., 2001). In pumpkin (*Cucurbita maxima*), however, 7-oxidation is catalyzed by an additional multifunctional GA 7-oxidase that belongs to the class of 2-oxoglutarate-dependent dioxygenases (Lange, 1997). Recently, recombinant pumpkin GA 7-oxidase has been shown to catalyze 3-oxidation of GA_{12} (Frisse et al., 2003), which might initiate an early 3β-hydroxylated pathway via GA_{14} to GA_4 (Fig. 1).

Most of the genes coding for enzymes of the GA biosynthetic pathway have been isolated and characterized and our understanding is increasing about their tissue-cell specificity during plant development (for review, see Sponsel and Hedden, 2004). Genes encoding GA oxidases from pumpkin are summarized in Table I. Localization of the transcripts of GA biosynthetic genes indicates complex tissue- and developmental-specific expression patterns in many species (Yamaguchi et al., 2001). Endogenous GAs have frequently been identified in shoots and occasionally in roots of higher plants (for review, see MacMillan, 2002). In seedlings, GA oxidase genes are mainly expressed in rapidly developing tissues, including shoot and root tips (for review, see Olszewski et al., 2002). Recently, a growing body of evidence supports the hypothesis that GAs are produced close to or at their site of action (for review, see Sponsel and Hedden, 2004), which might still include unknown transport mechanisms (Fleet and Sun, 2005). There is some indication that GAs derived from cotyledons

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant nos. La880/4–2 and La880/4–3).

[2] This paper is dedicated to Jan E. Graebe on the occasion of his 75th birthday.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.064162.

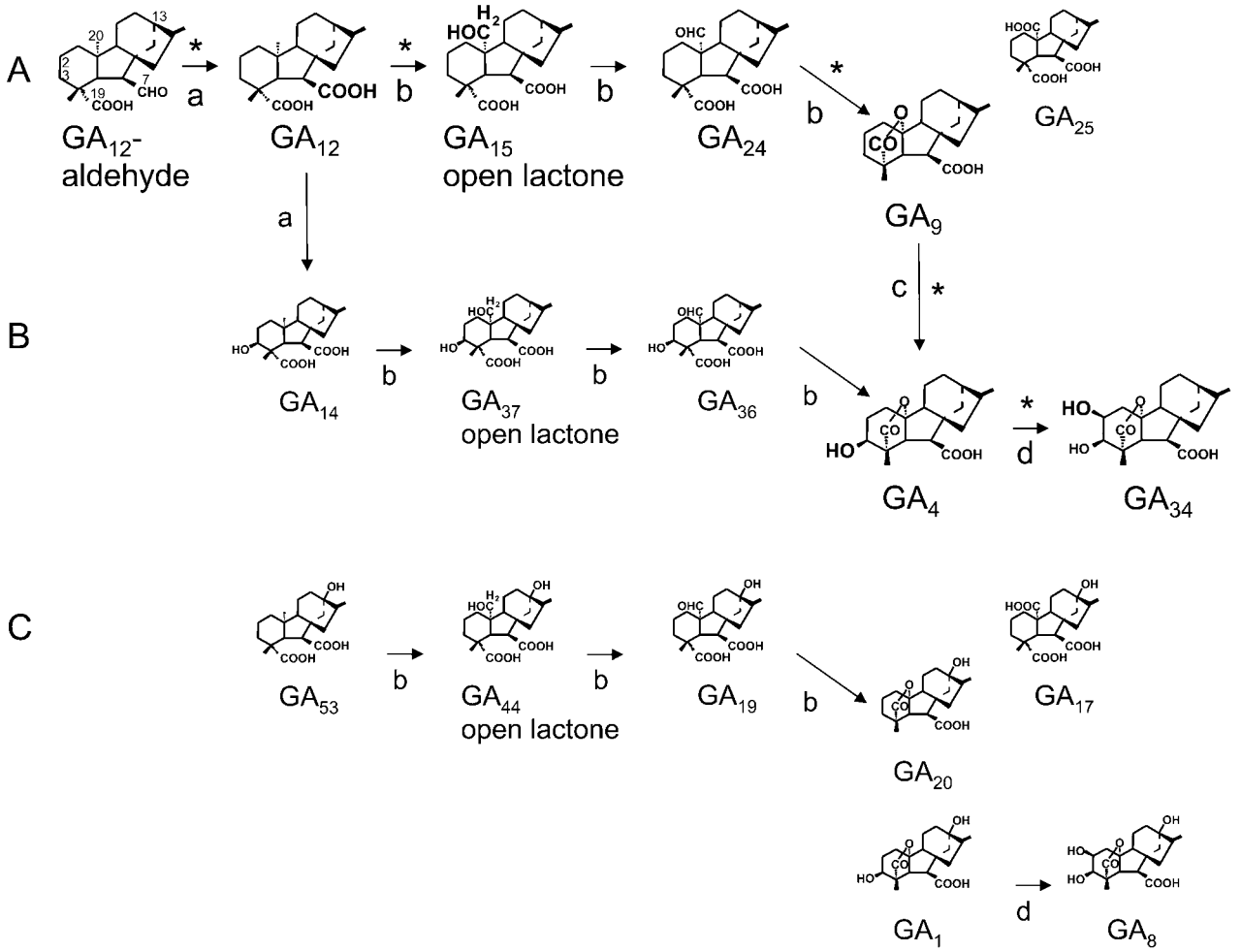

Figure 1. GA biosynthetic pathways from GA_{12}-aldehyde in pumpkin seedlings: nonhydroxylated pathway (A), 3β-hydroxylated pathway (B), and 13-hydroxylated pathway (C; Graebe, 1987). GA biosynthetic steps identified in cell-free systems are labeled with asterisks. Reactions are catalyzed by recombinant GA7ox (a), GA20ox2 (b), GA3ox3 (c), and GA2ox1 (d; Frisse et al., 2003). Structures and metabolic relationships are discussed in the text.

play a role in cell division during tissue reunion in the cortex of hypocotyls, as has been shown for cucumber and tomato (Asahina et al., 2002). It has been shown that GAs promote root growth in Arabidopsis by inducing the degradation of the DELLA proteins RGA (*repressor of gal-3*) and GAI (GA insensitive) and that other hormones are involved in regulating this action (Achard et al., 2003; Fu and Harberd, 2003; Fleet and Sun, 2005). However, direct evidence for GA biosynthesis occurring in roots is missing.

To address this question and investigate potential sites of GA biosynthesis in pumpkin seedlings, endogenous GA levels were analyzed and a GA biosynthetic pathway in root tips of 7-d-old pumpkin seedlings was identified. Characteristic genes of this pathway were isolated, including those coding for GA 20-oxidase (*CmGA20ox3*) and GA 3-oxidase (*CmGA3ox3*; Table I). GA oxidase transcripts were quantified in different parts of developing pumpkin seedlings by competitive reverse transcription (RT)-PCR and localized in roots and, more specifically, in root tips by in situ hybridization.

RESULTS

Endogenous GAs of Developing Pumpkin Seedlings

In preliminary experiments, seedlings were treated for 7 d with inhibitor of GA biosynthesis 1-(4-trifluormethyl)-2-(1,2,4-triazolyl-(1))-3-(5-methyl-1,3-dioxan-5-yl)-propen-3-ol (LAB150978), with GA_4, and with both LAB150978 and GA_4 (Table II). LAB150978 serves as an inhibitor of *ent*-kaurene oxidase, an enzyme that catalyzes early steps of the GA biosynthetic pathway (Jung et al., 1986). Primary root elongation was inhibited significantly in the presence of the GA inhibitor, while GA_4 or GA_4 plus LAB150978 treatment had no significant effect. In contrast, radial growth of primary root tips was elevated in

Table I. *Pumpkin GA oxidases, coding genes, expression sites, and accession numbers*

GA Oxidases	Coding Gene	Previously Described as	Expression Sites	Accession Nos.
7-Oxidase	*CmGA7ox*	GA 7-oxidase, Cm 7-ox	Developing seed, seedling	U61386
20-Oxidases	*CmGA20ox1*	GA 20-oxidase, Cm 20-ox	Embryo of developing seed	X73314
	CmGA20ox2	GA 20-oxidase, Cm 20-ox	Endosperm of developing seed	U61385
	CmGA20ox3	Cm 20-ox-RT	Developing seed, seedling	AJ308480
3-Oxidases	*CmGA3ox1*	GA 2β-3β-hydroxylase; Cm 2, 3-ox	Endosperm of developing seed	U63650
	CmGA3ox2	Cm 3-ox; GA 3-oxidase	Embryo of developing seed	AJ006453
	CmGA3ox3	Cm 3-ox-RT	Seedling	AJ302040
2-Oxidases	*CmGA2ox1*	Cm 2-ox, GA 2-oxidase	Developing seed, seedling	AJ302041, AJ315663
	CmGA2ox2	Truncated GA 2-oxidase	Seedling	AJ315662

LAB150978-treated plants, while GA_4 treatment resulted in significantly thinner root tips. Our results, summarized in Table II, indicate that GAs are important for normal hypocotyl and root growth of pumpkin seedlings.

Endogenous GAs were quantified from embryos of dry pumpkin seeds and from different parts of 3-, 5-, and 7-d-old pumpkin seedlings by gas chromatography-mass spectrometry (GC-MS) single ion monitoring (Fig. 2). Levels of precursors of the early 3β-hydroxylated pathway (GA_{14}, GA_{37}, and GA_{36}) were low in all tissues (<0.1 ng g^{-1} fresh weight; data not shown) with the exception of embryos from dry seeds where GA_{14} and GA_{36} levels were 1.9 and 0.6 ng g^{-1} fresh weight, respectively. Embryos of pumpkin seeds contain very high levels of the tricarboxylic acids GA_{25} and GA_{17}, as well as catabolic GA_8 (Fig. 2, A and B). High levels of the latter two GAs persist during the first 7 d of seedling development (Fig. 2, D, F, and H). Levels of 13-hydroxylated GAs decreased during the first 7 d of seedling development, with the exception of the plant hormone GA_1, which was found at low levels in roots and root tips of 7-d-old seedlings (Fig. 2, D, F, and H).

Precursors of the nonhydroxylated pathway were mainly found in shoot and root tips and in the hypocotyls, with decreasing levels in seedlings getting older (Fig. 2, C, E, and G). Highest levels of the plant hormone GA_4 were identified in root tips and hypocotyls of 3-d-old seedlings, which decrease at later stages of development (Fig. 2, C, E, and G). Catabolic GA_{34} was found in the embryo and in early stages of seedling development, particularly in hypocotyls and root tips. These results indicate that mainly hypocotyls and root tips of developing seedlings contain relatively high concentrations of bioactive GAs compared to the other tissues. In addition, hypocotyl elongation was maximal between days 3 and 5, while the primary root length doubled approximately every second day between days 3 and 7 of seedling development (Fig. 2, left).

GA Metabolism in Cell-Free Systems Prepared from Root Tips of 7-d-Old Pumpkin Seedlings

GA biosynthetic pathways were investigated in cell-free systems prepared from root tips of untreated seedlings and of seedlings that have been treated with LAB150978. The identities of the products were confirmed by full-scan GC-MS (Table III). From ^{14}C-GA_{12}-aldehyde ^{14}C-GA_{12} was formed, indicating the presence of GA 7-oxidase activity. Conversion was higher in root tips of untreated seedlings than in LAB150978-treated seedlings. ^{14}C-GA_{12} was converted to ^{14}C-GA_{15}, and ^{14}C-GA_{24} was metabolized to ^{14}C-GA_9, indicating the presence of GA 20-oxidase activity different from the one previously identified in developing pumpkin seeds (Table III; Lange et al., 1994; Lange, 1997). No difference of GA 20-oxidation was observed between untreated and LAB150978-treated seedlings. Incubation with ^{14}C-GA_9 gave two products, ^{14}C-GA_4 and ^{14}C-GA_{34}, indicating GA 3-oxidase and 2-oxidase activity, respectively (Table III). GA 3-oxidation was

Table II. *Effect of LAB150978 (10^{-6} M), of GA_4 (10^{-6} M), and of LAB150978 plus GA_4 (both 10^{-6} M) treatment on hypocotyl and root growth of 7-d-old pumpkin seedlings*

Data are shown as mean ± SE (n=10).

Treatment	Hypocotyl Length	Primary Root Length	ϕ of Primary Root Tip [a]
	cm	*cm*	*mm*
Control	2.9 ± 0.3	15.6 ± 1.7	0.70 ± 0.07
LAB150978	1.7 ± 0.2[b]	11.1 ± 2.0[b]	0.91 ± 0.05[b]
GA_4	5.4 ± 0.3[b]	15.7 ± 2.1	0.57 ± 0.04[b]
LAB150978 + GA_4	5.0 ± 0.7[b]	14.7 ± 8.5	0.76 ± 0.12

[a]Measured at 2 mm from the tip of the primary roots. [b]Statistically significant differences between control plants and treated plants at the 5% level; Student's t test.

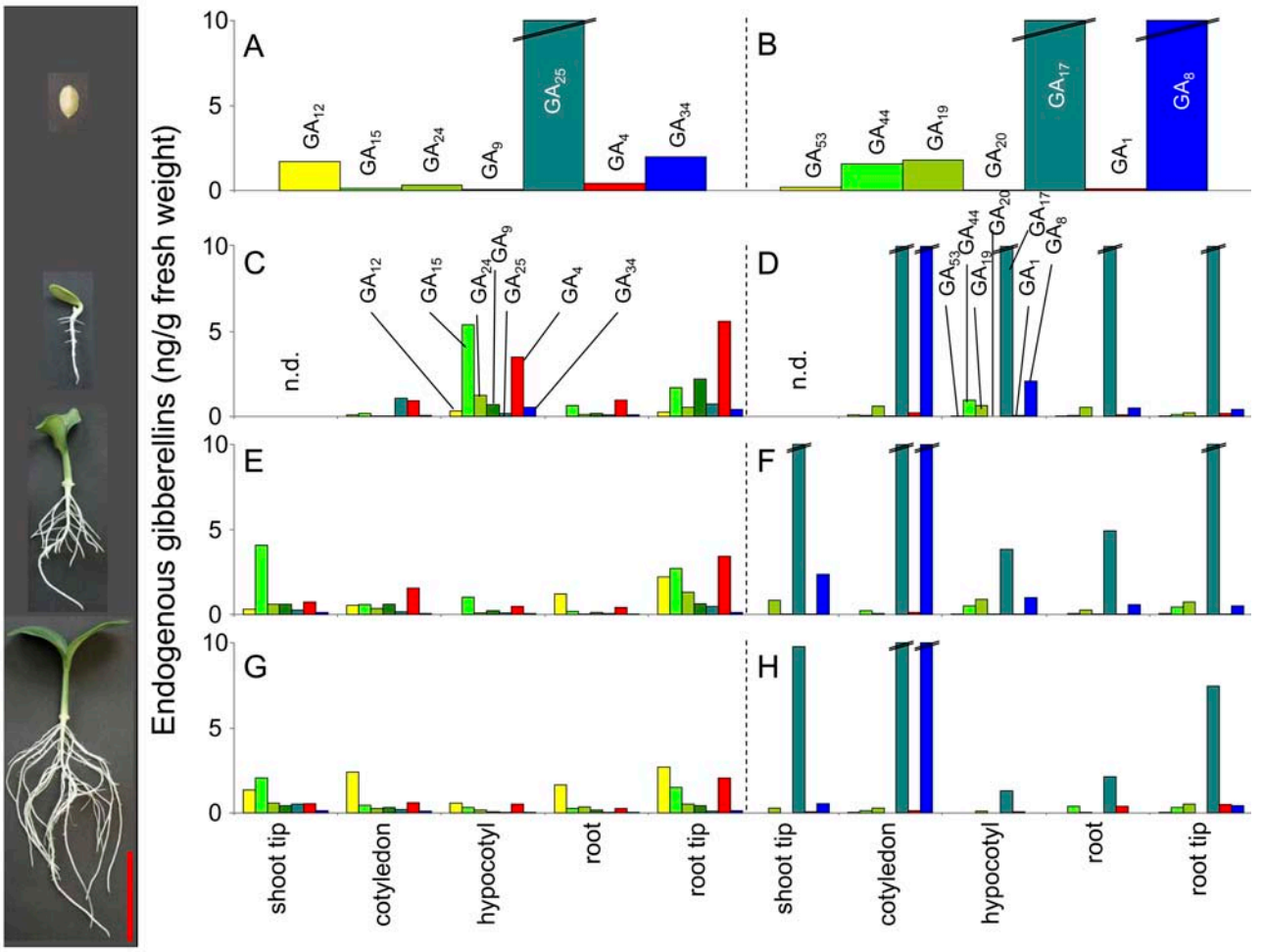

Figure 2. Endogenous GAs in embryos of dry seeds (A and B) and in different tissues of 3-d-old (C and D), 5-d-old (E and F), and 7-d-old (G and H) pumpkin seedlings. Images at the left illustrate pumpkin seed and seedlings at respective developmental stages (bar = 5 cm). GAs of the non- and 3β-hydroxylated pathway are depicted in A, C, E, and G, and GAs of the 13-hydroxylated pathway are depicted in B, D, F, and H. Double lines across the top of a bar indicate values >10 ng GAs/g fresh weight. Measurements have been repeated at least once with similar results. Shoot tips were analyzed together with cotyledons of 3-d-old seedlings. n.d., Not determined.

higher in LAB150978-treated than in untreated plants. Finally, ^{14}C-GA$_4$ was metabolized to ^{14}C-GA$_{34}$, a product of GA 2-oxidase activity (Table III). These results confirm the presence of a specific GA biosynthetic pathway in root tips distinct from the pathways operating in developing pumpkin seeds; in root tips, C_{19}-GAs are the major end products and in developing seeds C_{20}-GAs are mainly formed (Lange et al., 1993a, 1993b).

Cloning and Expression of GA Oxidase Genes from Root Tips of 7-d-Old Pumpkin Seedlings

The GA biosynthetic pathway discovered in root tips requires 20-oxidases and 3-oxidases with different catalytic properties to the ones identified previously from developing pumpkin seeds (Lange, 1998; Frisse et al., 2003). To further characterize these enzymes, first their encoding cDNA molecules were isolated from an amplified pBluescript SK+ cDNA library derived from root-tip poly(A)$^+$ RNA of LAB150978-treated pumpkin seedlings. The cDNA library was screened for cDNA molecules encoding GA 20-oxidases and 3-oxidases by a PCR-based strategy using respective degenerated oligonucleotide primers (Frisse et al., 2003). Two clones, designated *CmGA20ox3* and *CmGA3ox3*, were isolated that share high homology to known sequences of other GA 20- and GA 3-oxidases, respectively.

Catalytic properties of recombinant CmGA20ox3 (GA 20-oxidase) and CmGA3ox3 (GA 3-oxidase) proteins were investigated by expression of the respective cDNA molecules in pUC18 in *Escherichia coli* NM522 (Table IV). Cell lysates of recombinant CmGA20ox3 protein, prepared from the full-length clone, oxidized

Table III. *Metabolism of ^{14}C-labeled GAs by cell-free systems prepared from root tips of 7-d-old pumpkin seedlings*[a]

Substrate[c]	Products[b]				
	Compounds Formed	Untreated Plants	LAB150978-Treated Plants	KRI	Characteristic Ions at *m/z* (% Relative of Base Peak)[d]
		pmol	*pmol*		
GA_{12}-ald.	GA_{12}	78.3	29.4	2422	368(0.01), 360(0.01), 308(100), 300(60), 291(27), 285(13), 247(37), 246(28), 241(25), 240(19)
GA_{12}	GA_{15}	62.8	61.5	2746	352(15), 344(1), 320(8), 312(14), 290(44), 284(18), 245(100), 239(72), 197(57), 195(30)
GA_{24}[e]	GA_9	28.4	34.0	2418	332(4), 330(0), 300(96), 298 (30), 272(100), 270(9), 229(56), 228(75), 227(46), 226(35)
GA_9[f]	GA_4	24.1	58.8	2563	420(17), 418(4), 402(14), 400(4), 330(48), 328(17), 291(94), 289(38), 286(100), 284(83)
	GA_{34}	13.8	20.2	2672[g]	
GA_4[f]	GA_{34}	n.d.[h]	40.4	2672	508(100), 506(0), 418(9), 416(2), 358(13), 356(2), 290(32), 288(22), 219(84), 217(40)

[a]Protein concentrations of root tips of untreated plants and of LAB150978-treated plants were 10.8 and 11.5 mg/mL, respectively. [b]Identification of ^{14}C-GA metabolic products by GC-MS on the basis of mass spectra (Gaskin and MacMillan, 1992) and Kovats retention indices (KRI) of the methyl ester trimethylsilylether derivatives. [c]GA_{12}-aldehyde and GA_{12} were (1-, 7-, 12-, 18-$^{14}C_4$)-labeled; GA_{24}, GA_9, and GA_4 were (17-^{14}C)-labeled. [d]Based on ions above a mass-to-charge ratio (*m/z*) of 50. [e]Incubation volumes 6 times the standard assay. [f]Incubation volumes 2 times the standard assay. [g]Spectrum was seriously contaminated with extraneous ions. The identification was made on the basis of co-occurence of characteristic ions (Gaskin and MacMillan, 1992) at the appropriate KRI. [h]Not determined.

^{14}C-GA_{12} to -GA_{15} and ^{14}C-GA_{24} to -GA_9 in standard enzyme assays (data not shown). GA 20-oxidation activity highly increased in cell lysates prepared from the predicted open reading frame (ORF) of the *CmGA20ox3* clone (designated *CmGA20ox3* ORF) and, therefore, was used for further characterization (Table IV). Recombinant CmGA20ox3 ORF protein is capable of oxidizing and subsequently removing the C-20 position of ^{14}C-labeled substrates GA_{12}, GA_{24}, GA_{14}, and, less efficiently, GA_{53}. Of all GAs tested, recombinant CmGA3ox3 protein oxidizes only ^{14}C-GA_9 at the C-3β position (Table IV). No conversion of the ^{14}C-labeled substrates GA_{12}-aldehyde, GA_{12}, GA_{15}, GA_{24}, GA_{25}, GA_{13}, and GA_4 was observed with recombinant CmGA3ox3 protein (data not shown). No conversion of the ^{14}C-labeled substrates GA_{12}-aldehyde, GA_{12}, GA_{15}, GA_{24}, GA_{25}, GA_{13}, GA_9, GA_4, GA_{14}, and GA_{53} was obtained in standard incubation assays, with cell lysates of *E. coli* harboring the pUC18 plasmid without the cDNA insert (data not shown).

Transcript Analysis of GA Oxidases in Developing Pumpkin Seedlings

Expression levels of mRNA were quantified for GA oxidase-encoding genes in different tissues from 3-, 5-, and 7-d-old pumpkin seedlings by competitive RT-PCR (Fig. 3). The primers used were based on the sequence of *CmGA7ox* and *CmGA2ox1* cDNAs that were originally isolated from pumpkin endosperm and embryo, respectively (Lange, 1997; Frisse et al., 2003), and on the sequence of *CmGA20ox3* and *CmGA3ox3* clones from root tips of the pumpkin seedling.

Highest transcript levels of *CmGA7ox*, *CmGA20ox3*, and *CmGA3ox3* were identified in 3-d-old seedlings (Fig. 3, A–C, E–G, and I–K): *CmGA7ox* transcripts were found mainly in shoot tips and cotyledons of 3-d-old seedlings, with moderate levels in hypocotyls and root tips (Fig. 3A), *CmGA20ox3* transcript levels were highest in shoot tips and hypocotyls (Fig. 3B), and *CmGA3ox3* transcripts were mostly detectable in hypocotyls (Fig. 3C). Moderate transcript levels of *CmGA7ox*, *CmGA20ox3*, and *CmGA3ox3* were visible in root tips of seedlings 5 d after imbibition (Fig. 3, E–G). In contrast, *CmGA2ox1* transcript levels increased in roots from day 3 to day 7 after imbibition of the seedling (Fig. 3, D, H, L). Highest *CmGA2ox1* transcript levels were found in roots, together with moderate levels in shoot and root tips. Additionally, transcript levels of three previously cloned GA oxidase genes from developing pumpkin seeds (Lange, 1997; Lange et al., 1997; Frisse et al., 2003) were

Table IV. *Metabolism of ^{14}C-GAs by cell lysates from E. coli transformed with pUC18 clones Cm20ox3-ORF and CmGA3ox3*

Clone	Substrate[b]	Products[a]			
		Compounds Formed	% by HPLC	KRI	Characteristic Ions at *m/z* (% Relative of Base Peak)[c]
Cm20ox3-ORF	GA_{12}[d]	GA_9	100	2418	338(12), 330(8), 306(53), 298(61), 276(58), 270(100), 251(89), 243(99), 232(78), 226(91)
	GA_{24}	GA_9	100	2420	332(0), 330(0), 300(40), 298(1), 272(89), 270(5), 243(100), 229(57), 228(77), 227(41), 226(17)
	GA_{14}	GA_4	100	2561	426(1), 418(0), 336(4), 328(2), 292(56), 284(32), 231(99), 230(100), 225(46), 224(53)
	GA_{53}	$GA_{44/19}$[e]	12		
		GA_{20}	88	2544	426(42), 418(100), 409(1), 403(10), 381(29), 375(68), 365(9), 359(19), 307(10), 301(27)
CmGA3ox3	GA_9	GA_4	48	2563	420(1), 418(1), 330(7), 328(6), 286(55), 284(20), 227(99), 226(100), 225(58), 224(37)

[a]Identification of ^{14}C-GA metabolic products by GC-MS on the basis of mass spectra (Gaskin and MacMillan, 1992) and Kovats retention indices (KRI) of the methyl ester trimethylsilylether derivatives. [b]GA_{12}, GA_{14}, and GA_{53} were (1-, 7-, 12-, 18-$^{14}C_4$)-labeled; GA_{24} and GA_9 were (17-^{14}C)-labeled. [c]Based on ions above a mass-to-charge ratio (*m/z*) of 50. [d]Incubation volumes 0.6 times the standard assay. [e]Preliminary identification according to HPLC retention time.

analyzed in untreated 5-d-old seedlings and either were not detectable (*CmGA20ox1*, *CmGA20ox2*, and *CmGA3ox1*) or were identified at very low levels in root tips (*CmGA3ox2*, 0.02 μg transcript per gram of total RNA; data not shown).

The impact of LAB150978 treatment on transcript levels was analyzed for four GA oxidase genes in 7-d-old seedlings. Transcript levels of *CmGA7ox* and *CmGA2ox1* did not alter significantly between treated and untreated plants (Fig. 4, A and D). However, transcript levels of *CmGA20ox3* and of *CmGA3ox3* genes were low in general and, after LAB150978 treatment, increased dramatically in all parts of the seedling (Fig. 4, B and C).

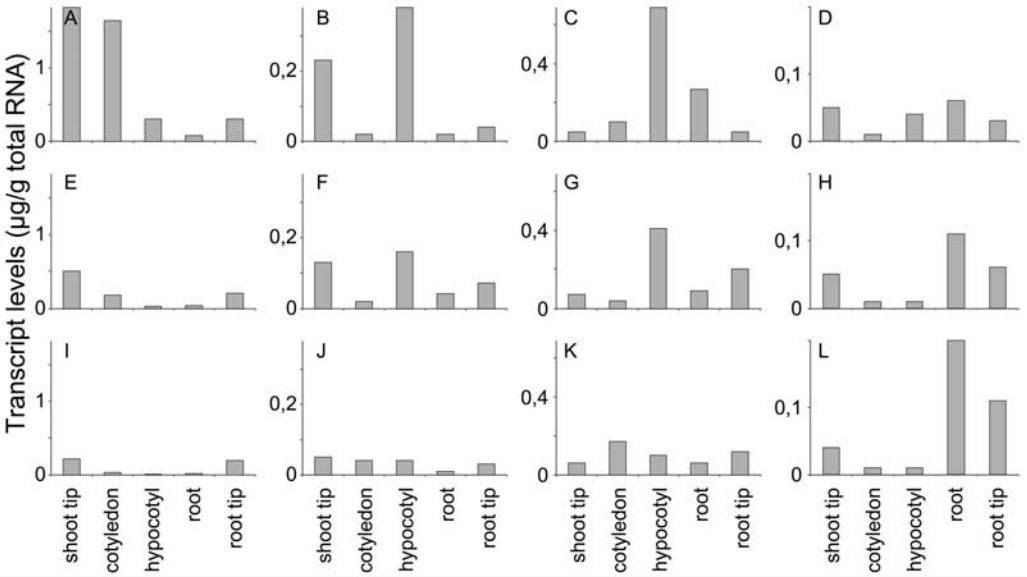

Figure 3. Transcript levels of *CmGA7ox* (A, E, and I), *CmGA20ox3* (B, F, and J), *CmGA3ox3* (C, G, and K), and *CmGA2ox1* (D, H, and L) genes in different parts of 3- (A–D), 5- (E –H), and 7-d-old (I–L) pumpkin seedlings as determined by quantitative RT-PCR.

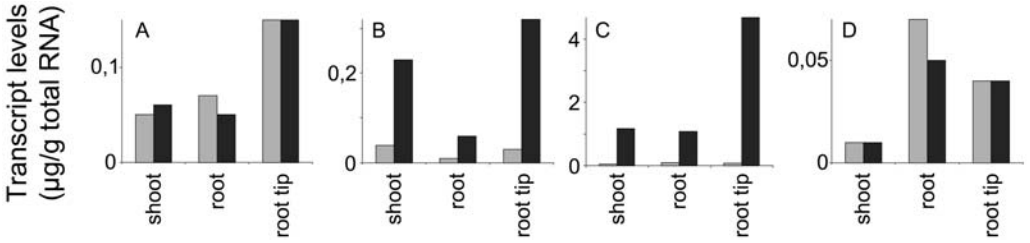

Figure 4. Transcript levels of *CmGA7ox* (A), *CmGA20ox3* (B), *CmGA3ox3* (C), and *CmGA2ox1* (D) genes as determined by competitive RT-PCR in tissues from shoots, roots, and root tips of 7-d-old seedlings. Seedlings were treated with water (gray bars) or with LAB150978 (10^{-6} M; black bars).

Expression patterns for transcripts of four GA oxidases were investigated by in situ hybridization in root tips of 7-d-old pumpkin seedlings. *CmGA7ox*, *CmGA20ox3*, and *CmGA3ox3* transcripts were localized in the cap of the root and the rhizodermis (Fig. 5, A–C and E–G). However, only weak signals for *CmGA2ox1*-encoding transcripts were detectable in root tips by in situ hybridization (Fig. 5, D and H). No differences in expression pattern of these transcripts were detectable between 3- and 7-d-old plants and between untreated and LAB150978-treated plants by in situ hybridization. However, the intensity of the signals obtained for *CmGA20ox3* and *CmGA3ox3* were stronger in LAB150978-treated plants (data not shown).

DISCUSSION

In pumpkin, GA biosynthetic pathways have been elucidated mainly in cell-free systems prepared from developing seeds (Lange et al., 1993a, 1993b; for review, see Graebe, 1987). These pathways are still active in mature pumpkin seeds even 36 h after imbibition. However, they disappear rapidly in later stages of the developing seedling (Lange et al., 1997). The main products formed via those seed-specific pathways in pumpkin are GAs containing C-20 carboxylic acids (e.g. GA_{25} and GA_{17}; Fig. 1). In fact, the mature embryo (as well as the testa; data not shown) contains large quantities of those tricarboxylic GAs that have no known physiological function (Fig. 2).

There is growing evidence for the presence of root-based GA biosynthesis from many plant species, including pumpkin (Yamaguchi et al., 1996; Smith et al., 1998), pea (*Pisum sativum*; Lester et al., 1999; Martin et al., 1999; Elliott et al., 2001; Davidson et al., 2003, 2004), Arabidopsis (Chiang et al., 1995; Silverstone et al., 1997; Thomas et al., 1999), and rice (*Oryza sativa*; Sakamoto et al., 2001, 2004; Monna et al., 2002; Sasaki et al., 2002; Kaneko et al., 2003; Itoh et al., 2004). In this study, we present direct evidence for GA biosynthesis in root tips of young pumpkin seedlings. The pathway involves oxidations at four different positions of the

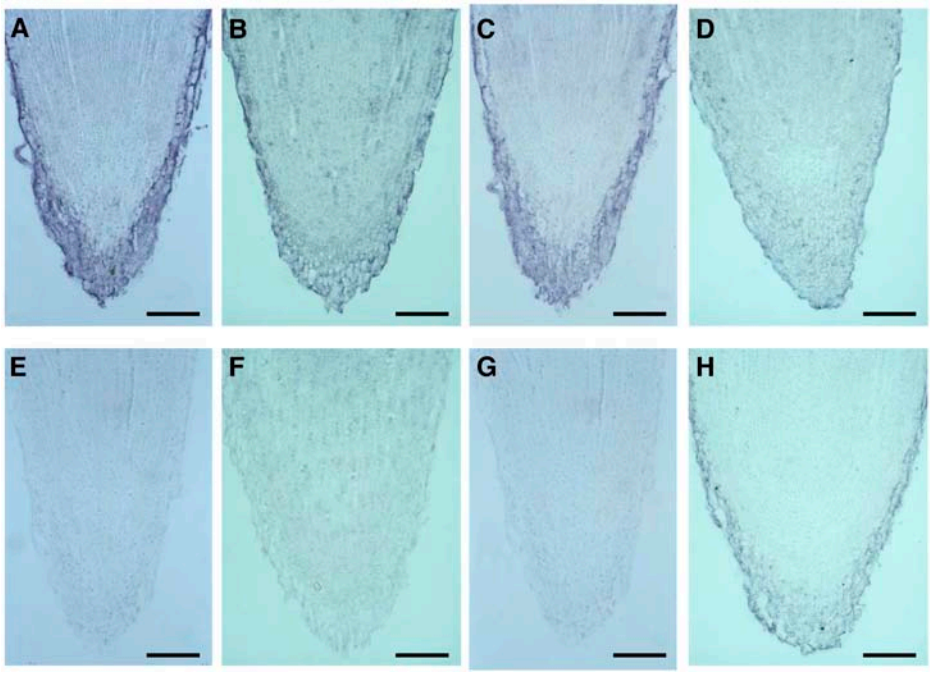

Figure 5. Localization of *CmGA7ox* (A and E), *CmGA20ox3* (B and F), *CmGA3ox3* (C and G), and *CmGA2ox1* (D and H) mRNA in longitudinal sections from root tips of 7-d-old pumpkin seedlings by in situ hybridization. The sections were hybridized to either antisense (A–D) or sense (E–H) RNA probes of the entire respective cDNA (A, C, E, and G) or the predicted ORF (B, D, F, and H) that was labeled with digoxygenin. Bar = 0.1 mm.

GA molecule, C-7, C-20, C-3β, and C-2β (Fig. 1) and differs from the one previously identified in developing pumpkin seeds. The main products of GA 20-oxidase activity are C_{19}-GAs, which are different from developing pumpkin seeds where the main products are C_{20}-GAs (Lange et al., 1994; Lange, 1997). No metabolism of GA_{15} was observed, indicating a different GA 3-oxidase activity to the one that is present in developing seeds where GA 3-oxidases efficiently convert C_{20}-GAs, including GA_{15} (Lange et al., 1997; Frisse et al., 2003). All GAs obtained in the metabolic studies were identified as endogenous compounds of the seedling. Investigations based on studies of endogenous GA levels led to the presumption that in vegetative tissues bioactive GAs are synthesized via a late 3β-hydroxylation step that occurs after C_{19}-GAs are formed (Graebe, 1987; Hedden, 1999). Our metabolic studies confirm this supposition for GA biosynthesis in pumpkin seedlings. Moreover, none of the early 3β-hydroxylated precursors (GA_{14}, GA_{37}, and GA_{36}) accumulated during seedling development. However, the presence of an early 3-oxidation step in the GA biosynthetic pathway of root tips cannot be completely excluded. Recently, we demonstrated that recombinant pumpkin GA 7-oxidase hydroxylated, at low efficiency, GA_{12} at the C-3β position, leading to the formation of GA_{14} (Frisse et al., 2003), which can be further metabolized to GA_4 by recombinant CmGA20ox3 (Fig. 1; Table IV).

GA metabolic studies demonstrate that the products resulting from GA 20-oxidase and GA 3-oxidase activities are different in developing pumpkin seeds and seedlings. We isolated two cDNA molecules, *CmGA20ox3* and *CmGA3ox3*, from root tip poly(A)$^+$ RNA of 7-d-old LAB150978-treated pumpkin seedlings, encoding GA 20-oxidase and 3-oxidase, respectively. The broad specificity of the recombinant CmGA20ox3 protein indicates that a single enzyme is capable of catalyzing alternative GA biosynthetic pathways (Fig. 1). Unlike CmGA20ox1 and CmGA20ox2 proteins from developing seeds, recombinant CmGA20ox3 protein exhibits strong decarboxylation activities and converts GA precursors GA_{12}, GA_{14}, and GA_{53} to their respective C_{19}-GAs. Recombinant CmGA3ox3 protein, however, reveals more stringent substrate specificity than CmGA3ox1 or CmGA3ox2 protein from developing pumpkin seeds (Lange et al., 1997, Frisse et al., 2003). It catalyzes 3-oxidation, after C_{19}-GA is formed, but not of intermediates of the pathway, which might have some importance for its activity in situ, where huge quantities of endogenous tricarboxylic GAs might otherwise inhibit its activity. Such enzymatic properties are typical for GA 20-oxidases and GA 3-oxidases from most plant species, but up to now were not known in pumpkin (Lange, 1998). However, it is likely that the cloning of the two genes from root-tip tissues was facilitated by a negative type of feedback regulation that occurred in LAB150978-treated pumpkin plants (Fig. 4) and was observed in several other plant species (for review, see Hedden and Phillips, 2000; Fleet and Sun, 2005). A positive feed-forward regulation as reported for GA 2-oxidase transcript levels from some other plant species (Martin et al., 1999; Thomas et al., 1999; Elliott et al., 2001) was not observed for *CmGA2ox1* gene expression, similar to observations obtained with rice GA 2-oxidase (Sakamoto et al., 2001).

Sites of bioactive GA synthesis seem to be tissues containing rapidly expanding cells (Silverstone et al., 1997, Yamaguchi et al., 2001; for review, see Olszewski et al., 2002). Our results show that transcript and endogenous GA profiles correlate well for most of the tissues analyzed (Figs. 2 and 3). For example, in rapidly growing hypocotyl tissues, this site of GA action was paralleled by the transcript levels of the GA oxidases as well as endogenous GA levels, including the plant hormone GA_4. As hypothesized recently by Sponsel and Hedden (2004), our investigations support the view that the site of GA biosynthesis and GA action might be similar, if not identical, in hypocotyls. Additional evidence comes from expression studies on *GA1*, *GA4*, and *GA5* genes, all expressed in the hypocotyl of young Arabidopsis seedlings (Silverstone et al., 1997; Bouquin et al., 2001; Meier et al., 2001).

In root tips, however, transcript levels determined for pumpkin GA 20-oxidase (*CmGA20ox1*, *CmGA20ox2*, and *CmGA20ox3*) and GA 3-oxidase (*CmGA3ox1*, *CmGA3ox2*, and *CmGA3ox3*) genes do not correlate well with endogenous GA levels. GA oxidases form multigene families (Sponsel and Hedden, 2004) and it is likely that additional GA oxidases are expressed that might account for further enzyme activities and for the high endogenous GA levels determined in this tissue. It is also possible that other GA oxidases present in this tissue do not show a negative type of feedback regulation and were, therefore, not favored in the cloning process from LAB150978-treated pumpkin seedlings. However, transcripts of *CmGA7ox* and *CmGA20ox3*, together with transcripts of *CmGA3ox3*, are localized in root caps and rhizodermis of the root tip by in situ hybridization, indicating the contribution of their respective GA oxidases for the GA biosynthesis in this tissue. GA synthesis in root tips might have a function for root development of the young seedling. For instance, optimal GA levels are necessary to achieve stability of principal directions of root growth (Nakielski and Barlow, 1995) and regulate root elongation as well as radial root growth (see Table II; Tanimoto, 1990; Barlow et al., 1991; Yaxley et al., 2001). Recently, DELLA proteins, components of the GA-signaling cascade, have been identified to regulate root growth in Arabidopsis seedlings and this action seems to be modulated by other hormones (Fu and Harberd, 2003).

MATERIALS AND METHODS

Plant Material, Enzyme, and Total RNA Preparations

Seeds of pumpkin (*Cucurbita maxima* L. cv Riesenmelone, gelb genetzt) were sown in 100-mL pots containing 10 g of vermiculite moistened with 50 mL of 0.01% methanol, 0.01% methanol containing GA_4 (10^{-6} M), or 0.01%

methanol containing the plant growth retardant 1-(4-trifluormethyl)-2-(1,2,4-triazolyl-(1))-3-(5-methyl-1,3-dioxan-5-yl)-propen-3-ol (10^{-6} M; LAB150978; a gift from Dr. Rademacher, BASF Agricultural Center, Limburgerhof, Germany). Water and growth regulators were replenished 10 mL/d. Germination and growth of the seedlings occurred under an 18-h photoperiod at 24°C during the day and 18°C during the night. Light was supplied by Osram Powerstar HQI-T 400W/D daylight lamps, located 1 m above the plants, giving a photon fluence rate of 200 μE m^{-2} s^{-1}. At the time of sampling, the plants were rinsed, dissected into shoot tips (the upper 5 mm of the stem), cotyledons, hypocotyl, roots, and root tips (the lower 2 mm of the primary and lateral roots) and immediately frozen in liquid N_2 and stored at −80°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 (25 mg) with a NucleoSpin RNA plant kit, according to the manufacturer's instructions (Machery-Nagel), and treated with DNase I (Sigma; 10 units/μg total RNA) for 20 min at 37°C, followed by phenol-chloroform extraction. Total RNA was stored at −80°C and used for quantification of specific GA oxidase transcripts as described below. Cell-free enzyme preparations were obtained from the frozen powder (1 g) to which 200 mM Tris-HCl buffer (pH 7.9, as measured at 4°C) containing 10 mM dithiothreitol was added 1:1 (w/v). After thawing, the extract was centrifuged for 30 min at 40,000*g* and the supernatant was stored at −80°C.

Construction of a pBluescript SK+ cDNA Plasmid Library

Poly(A)$^+$ RNA (5 μg) was extracted from root tips of 7-d-old pumpkin seedlings treated with the growth retardant LAB150978, using a mRNA isolation kit, according to the manufacturer's instructions (Fast Track 2.0; Invitrogen), and used for the preparation of an oligo(dT)-primed cDNA library in pBluescript SK+ using commercial kits (pBluescript II XR cDNA library construction kit; Stratagene). A cDNA plasmid library in *Escherichia coli* XL 10 GOLD of 3.4×10^5 independent cell-forming units was obtained and amplified, 95% of which contained inserts of a length between 800 to 2,000 bp, as shown by agarose gel electrophoresis of PCR products using pBluescript specific M13 primers.

Screening of pBluescript SK+ cDNA Library by PCR

Screening of the pBluescript SK+ cDNA library for GA 20-oxidase, 3-oxidase, and 2-oxidase genes was performed by a PCR-based cloning strategy (Frisse et al., 2003) with the following modifications: For GA 20-oxidase screening, degenerate primer pairs (sense primer 5′-TNCCNTGGAA(AG)-GA(AG)AC-3′ and antisense primer 5′-GG(AG)CANA(AG)(AG)AA-(AG)AANGC-3′, where N is a mixture of A, C, G, and T) were used in a PCR reaction at an annealing temperature of 50°C. One clone (designated *CmGA20ox3*) was isolated that gave an approximately 530-bp band, which is the expected size for a putative GA 20-oxidase. As described by Frisse et al. (2003), using degenerate GA 3-oxidase primer pairs, one clone (*CmGA3ox3*) was identified harboring a putative GA 3-oxidase cDNA, and using degenerate GA 2-oxidase primer pairs, one clone was isolated harboring putative GA 2-oxidase cDNAs. The pBluescript SK+ plasmids containing the cDNA inserts of the three clones, *CmGA7ox*, *CmGA3ox3*, and the putative 2-oxidase clone, were custom sequenced on both strands (AGOWA). The 2-oxidase sequence corresponded to the previously cloned *CmGA2ox1* from pumpkin embryos (Frisse et al., 2003).

Heterologous Expression of Recombinant GA Oxidases

DNA sequence analysis revealed that the cDNA inserts of the three clones were not in frame to the *lac*-promotor of the pBluescript vector. The pBluescript SK+ vector containing *CmGA20ox3* cDNA was digested with *Xba*I and *Eco*RI, filling in recessed 3′-termini with Klenow fragment and then religated. The predicted ORF of the *CmGA20ox3* clone was amplified by PCR (at an annealing temperature of 60°C), using sense primer 5′-NGAATTCAAAC-CAAACCATGCATGTCGTGAC-3′ and antisense primer 5′-NGGATCCTT-TTCCTCAGGCGAGGAAAAGTG-3′, cut by *Eco*RI and *Bam*HI digest and cloned into the appropriate cloning sites of pUC18 vector (and pBluescript SK− vector for preparation of riboprobes for in situ hybridization; see below). The cDNA of clone *CmGA3ox3* was excised by *Xho*I and *Xba*I and recessed 3′-termini were filled in by Klenow fragment and subcloned into the *Sma*I site of pUC18. The plasmid vectors containing respective inserts were used to transform *E. coli* NM 522 cells. Protein induction and cell lysis were carried out as described by Lange (1997).

Standard Enzyme Assay and Analysis of Incubation Products

All ^{14}C-labeled GAs were prepared as described elsewhere (Lange and Graebe, 1993; Frisse et al., 2003). Specific radioactivity for (1, 7, 12, and 18-$^{14}C_4$)-labeled GA_{12}-aldehyde, GA_{12}, GA_{15}, GA_9, GA_{14}, and GA_{53}, were determined to be 5.80×10^{12}, 5.93×10^{12}, 5.05×10^{12}, 5.80×10^{12}, 5.80×10^{12}, and 5.4×10^{12} Bq mol^{-1}, respectively. Specific radioactivity for (17-^{14}C)-labeled substrates was 1.95×10^{12} Bq/mol. Cell-free enzyme preparations from different parts of 7-d-old pumpkin seedlings (450 μL each) were incubated with 2-oxoglutarate and ascorbate (4 mM each, final concentrations), and preparations of *E. coli* cell lysates (350 μL) were incubated with 2-oxoglutarate and ascorbate (100 mM each, final concentrations). $FeSO_4$ (0.5 mM), catalase (1 mg/mL), and the ^{14}C-labeled substrate (10 μL in 50% methanol; 0.33 nmol for (1, 7, 12, and 18-$^{14}C_4$)-labeled GAs and 1 nmol for (17-^{14}C)-labeled GAs) were added in a total volume of 500 μL and incubated for 16 h at 37°C. Incubation products were extracted and analyzed by reverse-phase HPLC with online radiocounting, using gradients of increasing methanol in acidic water, as described by Lange and Graebe (1993). HPLC fractions were analyzed by combined GC-MS (Xu et al., 2002). Variations in incubation conditions are specified for particular experiments.

Quantitative Analysis of GA

For quantitative determination of endogenous GAs, 2 g fresh weight of frozen plant tissue were spiked with 17, 17-d2-GA standards (2 ng each; from Professor L. Mander, Canberra, Australia) and pulverized under liquid nitrogen. Eighty percent methanol-water (8 mL) was added and the extract was stirred for 1 h at 4°C. After centrifugation, the pellet was re-extracted with methanol (4 mL) for 30 min and recentrifuged. The re-extraction procedure was repeated two times. The combined methanol extracts were evaporated to dryness and resuspended in water (3 mL), adjusted to pH 8.0 (1 M KOH). Solvent partition was performed using ethyl acetate (four times, 1 mL). The aqueous phase was adjusted to pH 3 (acetic acid) followed by solvent partition with ethyl acetate (four times, 1 mL). The combined ethyl acetate fractions were evaporated to dryness, redissolved in methanol (100 μL), and methylated with ethereal diazomethane (two times, 200 μL). The methyated samples were dried and redissolved in methanol (100 μL) and water (5 mL), adjusted to pH 3.2 (acetic acid). After loading onto a C18 cartridge (Waters), the cartridge was washed with water (10 mL), pH 3.2. Methylated GAs were eluted with methanol (6 mL), which was then dried. For purification by HPLC, the residues were redissolved in 200 μL methanol-water, pH 3.2 (1:1), and applied to a C18 reverse-phase column (10 cm long, 8 mm i.d., 4 μm particle size, Novapack liquid chromatography cartridge in a RCM100 radial compression system; Waters), which was eluted with a gradient from 25% methanol in water to 100% methanol in 40 min delivered by a two-pump HPLC system (models 501 and 510; Waters) at a flow rate of 1 mL min^{-1}. Starting from 13.5 mL, 18 fractions were collected per run, each containing 2 mL eluate and dried. Dried HPLC fractions were redissolved in 2 μL *N*-methyl-*N*-trimethylsilyltrifluoracetamide (Macherey-Nagel). The derivatized samples were analyzed using a Turbo-Mass MS system (Perkin-Elmer) equipped with a Perkin-Elmer AutoSystem XL gas chromatograph. Samples (1–2 μL) injected into a SGE BPX5 capillary column (30 m long, 0.25 mm i.d., 0.25-μm film thickness; SGE) at an oven temperature of 60°C. The split value (30:1) was open after 1 min, after which the temperature was increased by 45°C min^{-1} to 220°C and then with 4°C min^{-1} to 300°C. The He inlet was pneumatic pressure controlled at a constant flow rate of 1.5 mL min^{-1} and the injector, transfer line, and source temperatures were 220°C, 280°C, and 240°C, respectively. Data were acquired in the selected ion monitoring mode after 5 min. The ions monitored for quantification of endogenous GAs were 270 and 272 (GA_{12}-aldehyde), 300 and 302 (GA_{12}), 239 and 241 (GA_{15}), 314 and 316 (GA_{24}), 270 and 272 (GA_9), 284 and 286 (GA_{25}), 284 and 286 (GA_4), 506 and 508 (GA_{34}), 207 and 209 (GA_{53}), 207 and 209 (GA_{44}), 374 and 376 (GA_{19}), 418 and 420 (GA_{20}), 492 and 494 (GA_{17}), 506 and 508 (GA_1), 594 and 596 (GA_8), 416 and 418 (GA_{14}), 432 and 434 (GA_{37}), and 284 and 286 (GA_{36}). Identification was confirmed on the basis of retention time and the co-occurrence of additional ions. Endogenous levels were calculated on the basis of peak areas, after corrections were made for the contribution of naturally occurring isotopes and for the presence of unlabeled GAs in the internal standards, where necessary.

Quantitative RT-PCR

Quantification of *CmGA7ox* transcripts was performed as described by Lange et al. (1997). For each of the *CmGA20ox3*, *CmGA3ox3*, and *CmGA2ox1* (Frisse et al., 2003) genes, three specific oligonucleotides were synthesized based on their cDNA sequence: clone *CmGA20ox3* F (5′-AAA CCA AAC CAT GCA TGT CGT GAC-3′), R (5′-TTT TCC TCA GGC GAG GAA AAG TG-3′), and RT (5′-TTT AAA TGG AAG GGT TC-3′); clone *CmGA3ox3* F (5′-CAA CAT GGC CAC CAC AAT AGC-3′), R (5′-GGG ATT AGC CTA CTT TGA CCC GGC-3′), and RT (5′-TAG GGT GGG ATT AG-3′); and *CmGA2ox1* F (5′-AAT GAG AAG CTC CAC GTC CAT G-3′), R (5′-GAT GTT CGA ATC CTG TCA CCT C-3′), and RT (5′-AGA TGT TCG AAT CC-3). For the preparation of internal RNA standards, pBluescript SK plasmids containing the *CmGA20ox3* clone were digested with *Sal*I and *Hin*dIII, the *CmGA3ox3* clone was digested with *Hin*cII, and the ORF of *CmGA2ox1* was digested with *Hin*dIII that released a 299-, 379-, and 448-bp fragment, respectively. The vectors containing the remaining cDNAs were religated and used for standard RNA synthesis. For stability reasons, dilution of standard RNA was performed in the presence of *lambda* RNA (100 ng/μL). Quantification of *CmGA20ox3*, *CmGA3ox3*, and *CmGA2ox1* transcripts were performed as described by Lange et al. (1997), except that total RNA (50–100 ng) was reverse transcribed using the RevertAid H Minus first-strand cDNA synthesis kit (MBI Fermentas), and PCR was performed at an annealing temperature of 60°C for *CmGA20ox3* and *CmGA3ox3* genes and 65°C for the *CmGA2ox1* gene.

In Situ Hybridization

Sense and antisense riboprobes of full-length cDNAs of *CmGA7ox* (Lange, 1997) and *CmGA3ox3* and of the predicted ORF encoding *CmGA2ox1* (Frisse et al., 2003) and *CmGA20ox3* were synthesized using the dioxygenin nucleic acid labeling kit, according to the manufacturer's protocol with T7 and T3 RNA polymerases (Roche Molecular Biochemicals) and used for in situ hybridization as described by Frisse et al. (2003).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ315663 and AJ302040.

ACKNOWLEDGMENTS

We thank Anja Liebrandt for technical assistance and Julia Niemeyer for help with RT-PCR.

Received April 25, 2005; revised June 13, 2005; accepted July 10, 2005; published August 26, 2005.

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