

Production of Dwarf Lettuce by Overexpressing a Pumpkin Gibberellin 20-Oxidase Gene

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We investigated the effect of overexpressing a pumpkin gibberellin (GA) 20-oxidase gene encoding an enzyme that forms predominantly biologically inactive products on GA biosynthesis and plant morphology in transgenic lettuce (*Lactuca sativa* cv Vanguard) plants. Lettuce was transformed with the pumpkin GA 20-oxidase gene downstream of a strong constitutive promoter cassette (E12-35S- Ω). The transgenic plants in which the pumpkin gene was detected by polymerase chain reaction were dwarfed in the T₂ generation, whereas transformants with a normal growth phenotype did not contain the transgene. The result of Southern-blot analysis showed that the transgene was integrated as a single copy; the plants segregated three dwarfs to one normal in the T₂ generation, indicating that the transgene was stable and dominant. The endogenous levels of GA₁ and GA₄ were reduced in the dwarfs, whereas large amounts of GA₁₇ and GA₂₅, which are inactive products of the pumpkin GA 20-oxidase, accumulated in these lines. These results indicate that a functional pumpkin GA 20-oxidase is expressed in the transgenic lettuce, resulting in a diversion of the normal pathway of GA biosynthesis to inactive products. Furthermore, this technique may be useful for controlling plant stature in other agricultural and horticultural species.

The gibberellin (GA) plant hormones play important roles in plant development, controlling such processes as seed germination, internode elongation, flower development, and fruit set in higher plants (Crozier, 1983). Control of such processes is achieved, at least in part, by the development-dependent and organ-specific adjustment of the concentrations of biologically active GAs, so that the biosynthetic enzymes are potential regulators of plant development. Furthermore, manipulation of these enzymes by genetic engineering could be used to control plant growth and thereby introduce agronomically useful traits (Hedden and Kamiya, 1997; Lange, 1998).

In higher plants, GA biosynthesis requires the conversion of geranylgeranyl diphosphate to entkaurene via copalyl diphosphate catalyzed by the diterpene cyclases, and further converted to biologically active GAs through a series of oxidative steps catalyzed by cytochrome P450-dependent monooxygenases and 2-oxoglutarate-dependent dioxygenases (Hedden and Kamiya, 1997). One of the dioxygenases, GA 20-oxidase, which catalyzes the sequential oxidation and elimination of C-20, has been shown to be a regulatory enzyme. In recent studies, cDNA clones encoding the GA 20-oxidase gene have been isolated from several higher plants (Lange et al., 1994; Phillips

et al., 1995; Wu et al., 1996; Toyomasu et al., 1997) and the expression of the gene has been shown to be controlled by photoperiod (Wu et al., 1996; Xu et al., 1997), by GA in a type of feedback regulation (Phillips et al., 1995; Xu et al., 1995; Martin et al., 1996; Carrera et al., 1999), and according to organ or developmental stage (Garcia-Martinez et al., 1997; Rebers et al., 1999). Thus, expression of GA 20-oxidase genes controls the production of biologically active GA in response to developmental and environmental stimuli. Furthermore, overexpression of GA 20-oxidase genes in Arabidopsis has been shown to increase GA production and stimulate growth, so confirming that GA 20-oxidase activity is a limiting factor in both processes (Huang et al., 1998; Coles et al., 1999).

Among the GA 20-oxidases known to date, only that from developing pumpkin seeds has been shown to produce biologically inactive GAs as the major products; the pumpkin enzyme converts the aldehyde intermediates GA₂₄ and GA₁₉ to the tricarboxylic acids, GA₂₅ and GA₁₇, respectively (Lange, 1998). Overexpression of the pumpkin GA 20-oxidase gene in transgenic plants could, therefore, result in a reduction of biologically active GAs by diverting the pathway to the tricarboxylic acids. Thus, the gene is a potentially useful tool for controlling plant growth.

In the present study, we tested this hypothesis by overexpressing the pumpkin GA 20-oxidase gene in lettuce (*Lactuca sativa* cv Vanguard) and we demon-

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strated that the approach can result in a reduction in active GA content and growth.

RESULTS

Transformation and Selection of Lettuce Plants Overexpressing the Pumpkin GA 20-Oxidase Gene

In a preliminary study, lettuce plants harboring the pumpkin GA 20-oxidase gene driven by the cauliflower mosaic virus (CaMV) 35S promoter did not show an altered phenotype (data not shown). Therefore, in subsequent experiments a stronger promoter cassette containing a translational enhancer (EI2-35S- Ω) was used to enhance the pumpkin GA 20-oxidase expression (Fig. 1). Sixteen transformants were obtained by kanamycin selection, one of which (SG Ω -4) showed a dwarf phenotype with smaller leaves compared with wild-type plants. In the T₂ generation obtained by selfing SG Ω -4, dwarf plants were obtained in which hypocotyls, internodes, and leaves were reduced in length by 60%, 70%, and 40%, respectively, compared with wild-type plants (Table I). However, no remarkable differences were observed comparing the normal-type T₂ plants from SG Ω -4 with wild-type plants (Table I). The dwarfs segregated 3:1 from plants with a normal phenotype, indicating that the transgene was stable and dominant in transgenic plants.

Molecular Analysis of SG Ω -4 Plants

The presence of the integrated pumpkin GA 20-oxidase gene was tested by PCR, and the product of this was sequenced to ensure that it corresponded to the transgene. The transgene was detected only in genomic DNA from dwarf SG Ω -4 plants; transformants with a normal phenotype did not contain the gene (Table I). The result of Southern-blot analysis is shown in Figure 2. A single band was present in all lanes of genomic DNA from dwarf plants digested with each of three restriction enzymes, suggesting that the transgene is integrated as a single copy. The probe used for the Southern-blot analysis did not cross-hybridize with endogenous GA 20-oxidase genes in lettuce and is assumed to be specific for the transgene.

As shown by northern-blot analysis, large amounts of the transcript for the transgene were detectable only in the dwarf SG Ω -4 plants (Fig. 3). These results,

together with the observed dominance of the dwarfism conferred by the GA 20-oxidase transgene, confirm that the gene is being transcribed and that the observed dwarf phenotype is definitely linked to overexpression of the transgene.

Morphological Characterization of SG Ω -4 Plants

Morphological differences between wild-type and SG Ω -4 plants are shown in Figure 4. In addition to reduced hypocotyl length (Table I), dwarf seedlings contained more lateral roots than the tall plants (Fig. 4A). Leaves of mature dwarf plants maintained the folded morphology of normal-type transformants and wild-type plants, but were shorter and narrower (Fig. 4B). Furthermore, flowering of the dwarf plants that had short, thin floral axes and fewer branches was delayed by about 2 weeks compared with the wild-type plants (data not shown). Seed production was reduced in the dwarfs, although no differences in seed germination between dwarfs and wild-type plants were observed (data not shown).

Inhibitory Effect of Uniconazol (UCZ) on Plant Growth of Lettuce Plants

GA is known to stimulate elongation of floral axes and to accelerate flower production, and, hence, it is likely that the observed morphological changes in the transgenic dwarf-type plants are due to a reduction in the content of biologically active GAs. As shown in Figure 5, seedlings of wild-type plants treated with UCZ were dwarfed to the same degree as transgenic dwarf-type plants with shorter and narrower leaves. In contrast, when 100 ng of GA₃ was applied to the dwarfs, the length and width of newly developed leaves and internode length were restored to the same extent of that of wild-type plants (data not shown). These results indicate that the plants overexpressing the pumpkin GA 20-oxidase gene would have a reduced content of biologically active GAs.

Analysis of Endogenous GA Levels in Dwarf Lettuce Plants

We analyzed endogenous GA levels in the transgenic plants by gas chromatography-mass spectrometry to confirm that GA biosynthesis is altered in the dwarf plants and to determine which steps of the GA

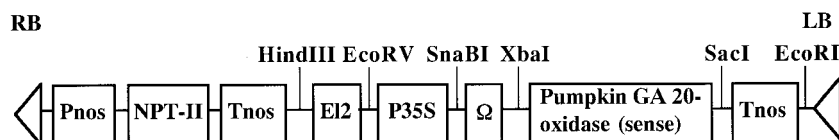


Figure 1. Structure of T-DNA region of pSG Ω . Pnos, 5'-upstream region of nopaline synthase gene. NPT-II, Coding region of nopaline synthase gene. Tnos, Polyadenylation region of nopaline synthase gene. EI2, 5'-upstream region of CaMV 35S promoter (-419 to -90) \times 2. P35S, 5'-upstream region of CaMV promoter (-90 to -1). Ω , 5'-Untranslated region of tobacco mosaic virus.

Table I. Phenotypic characterization of SGΩ-4 plants

Seeds of T₂ generation were germinated on soil in pots and hypocotyl length was measured at 2 weeks after sowing. After growing for 1 month, leaf and internode length were measured. Genomic DNA was extracted from a leaf of the seedlings and the integrated pumpkin GA 20-oxidase gene was detected by PCR using a specific primer.

Phenotype	No. of Plants	Segregation Ratio	Hypocotyl Length ^a	Internode Length ^{a,b}	Leaf Length ^a	PCR Band
<i>cm</i>						
SGΩ-4 plants						
Dwarf type	67	3	0.99 ± 0.30	0.10 ± 0.01	6.66 ± 1.41	+
Normal type	24	1	2.72 ± 0.31	0.32 ± 0.05	12.88 ± 1.50	-
Wild-type plants	24	-	2.44 ± 0.61	0.32 ± 0.03	11.37 ± 2.12	-

^a Values are means ± SE.

^b Average values of primary to fourth leaf.

biosynthetic pathway are affected. The pumpkin GA 20-oxidase is known to produce inactive tricarboxylic acid GAs of no known physiological function (Lange, 1998). As shown in Table II, for early-13-hydroxylated GAs as the major products in lettuce plants, the level of endogenous GA₁ (a biologically active GA that is formed via GA₁₉ and GA₂₀) in the dwarfs was reduced to 15% to 25% of that in wild-type plants. In addition, the contents of GA₂₀ and GA₁₉ were also reduced in the dwarfs. However, large amounts of GA₁₇, which is a biologically inactive GA formed as the major product from GA₁₉ by the pumpkin GA 20-oxidase (Lange et al., 1994), accumulated in the dwarfs to levels that are >50-fold higher than in the wild-type plants (Table II). Furthermore, in the case of non-13-hydroxylated GAs that were massively minor products, it was the same tendency as early-13-hydroxylated GAs that a biologically active GA₄ and its precursor GA₂₄ were reduced and an inactive tricarboxylic acid GA₂₅ was accumulated in the dwarfs. Thus, there is a massive increase in the ratios of inactive GA₁₇ to active GA₁ and GA₂₅ to GA₄ in the dwarfs, indicating that overexpression of the pumpkin GA 20-oxidase results in a switch of GA biosynthesis from GA₁₉ to GA₁₇ and GA₂₄ to GA₂₅ instead of GA₁₉ to GA₂₀ and GA₂₄ to GA₉ and following GA₁ and GA₄, respectively (Fig. 6).

DISCUSSION

Mutations in genes encoding GA-biosynthetic enzymes have been shown to cause dwarfism in many plant species (Martin et al., 1997; Hedden and Proebsting, 1999). In GA biosynthesis, GA 20-oxidase is a regulatory enzyme being controlled by developmental and environmental stimuli (Phillips et al., 1995; Xu et al., 1995; Garcia-Martinez et al., 1997; Tanaka-Ueguchi et al., 1998; Rebers et al., 1999), and as such, is a prime target for genetic manipulation. In Arabidopsis, overexpression of GA 20-oxidase genes produced a GA-overproduction phenotype with elongated hypocotyls and stem and early flowering (Huang et al., 1998; Coles et al., 1999). In a converse manner, antisense expression of a stem-specific 20-oxidase gene caused reduced stem elongation and

delayed flowering in short days (Coles et al., 1999). In the present study we have used a different strategy to reduce GA content: we have introduced an enzyme, a GA 20-oxidase from developing pumpkin seed (Lange et al., 1994), that diverts the biosynthetic flux to an inactive by-product, thereby reducing the precursors available for biologically active GAs.

In transgenic lettuce plants, expression of the transgene, in terms of the presence of transcript, was associated with a dwarf phenotype. Furthermore, the dwarf plants had reduced levels of the biologically active GA₁ and GA₄, and their growth corresponded to UCZ-treated wild-type plants, which would be inhibited in GA biosynthesis. Thus, the introduced pumpkin GA 20-oxidase could have the expected

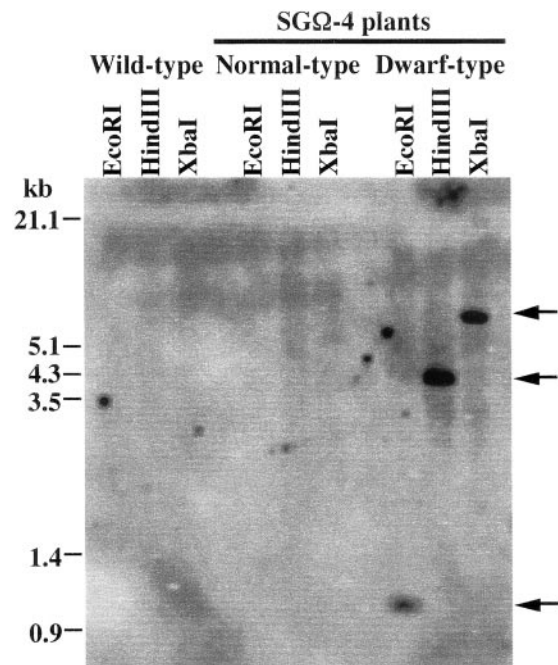


Figure 2. Southern-blot analysis of transgene in SGΩ-4 plants. Genomic DNA extracted from wild-type or SGΩ-4 plants was digested with *EcoRI*, *HindIII*, or *XbaI*, respectively, and 10 μg of digested DNA was loaded per lane. An *EcoRI*-digested PCR fragment of the pumpkin GA 20-oxidase gene, including a 3'-specific region (368 bp), was used as a probe.

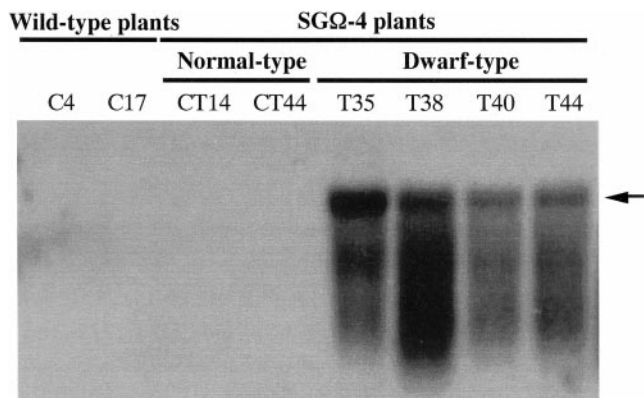


Figure 3. Northern-blot analysis of transgene expression. Total RNA was extracted from mature leaves of wild-type and SGΩ-4 plants, and 15 μg of total RNA per lane was blotted onto a membrane. Probe for hybridization was as described in Figure 2.

function in the transgenic lettuce; the abnormally low levels of GA₁ and its biosynthetic precursors, GA₁₉ and GA₂₀, were accompanied by elevated levels of the inactive tricarboxylic acid, GA₁₇, which is the major product formed from GA₁₉ in vitro by this enzyme (Lange et al., 1994).

It is well known that GA 20-oxidases can participate in two pathways to biologically active GAs. In the non-13-hydroxylation pathway, GA₁₂ is converted by GA 20-oxidase to GA₉, which is then 3β-hydroxylated to the biologically active GA₄. GA₁ is produced by the equivalent early-13-hydroxylation pathway (Fig. 6), which appears to predominate in lettuce plants (Waycott et al., 1991; Toyomasu et al., 1993). The pumpkin GA 20-oxidase converts non-hydroxylated substrates more efficiently than 13-hydroxylated GAs (Lange et al., 1994) and would, therefore, be expected to also influence the formation of GA₄. The minor contents of non-13-hydroxylated GAs, including GA₄, could not be detected, whereas GA₂₅, which could not be detected in wild-type plants, accumulated in dwarf-type plants (Table II). However, in *Arabidopsis*, in which GA₄ is the major biologically active GA, overexpression of the pumpkin GA 20-oxidase also resulted in reduced amounts of GA₄, although GA₁ levels were apparently not affected (Xu et al., 1999). It is surprising that there was only a slight height reduction in the transgenic *Arabidopsis* plants, prompting the authors to suggest that GA₄ content was not limiting. Furthermore, in a recent study with *Solanum dulcamara* (Curtis et al., 2000), in which GA₁ is the major biologically active GA as in lettuce, the plants overexpressing the pumpkin 20-oxidase gene were semi-dwarfed. In the case of *S. dulcamara*, the levels of the major biologically active GA₁ were reduced, whereas GA₄ was the same in stems or increased in the leaves.

It was suggested that the effects of overexpressing the pumpkin GA 20-oxidase gene were compromised by up-regulation of the endogenous 20-oxidase gene due to operation of the feedback control mechanism

in GA biosynthesis (Xu et al., 1999; Curtis et al., 2000). In our experiments, expression of the transgene must override the feedback mechanism, possibly because of the use of a very strong promoter cassette (E12–35S–Ω) by which introduced pumpkin GA 20-oxidase could compete with the endogenous GA 20-oxidase and converts most of the available GA-precursors into inactive forms (Fig. 6). In preliminary experiments with a weaker promoter, no dwarf plants were obtained, suggesting that very high levels of the pumpkin enzyme may be needed to perturb the mechanism that maintains GA homeostasis.

Morphological changes observed in dwarf-type plants corresponded to the growth of wild-type plants that was inhibited in GA biosynthesis by treatment with UCZ (Fig. 5). In addition to reduced leaf size and stem height, the dwarf seedlings also contained thinner roots and more lateral roots as compared with wild-type plants (Fig. 4). Although GAs are not known to affect lateral root development, it is possible that the effect of reduced GA₁ content on lateral root numbers is due to a change in hormone balance as a result of altered shoot morphology. Furthermore, we observed that flowering was delayed and seed production was reduced in the dwarf lettuce plants (data not shown), whereas in the case of *S. dulcamara*, semi-dwarfed plants overexpressing the pumpkin GA 20-oxidase gene flowered earlier and produced more fruit and seeds (Curtis et al., 2000).

In conclusion, we demonstrated that overexpression of the pumpkin GA 20-oxidase gene could result in a reduction of biologically active GAs causing pro-



Figure 4. Morphological changes in SGΩ-4 plants. A, Seedlings (3 weeks after sowing) of wild-type (right) and SGΩ-4 plants (normal, center and dwarf type, left). B, Mature plants (wild type, right. SGΩ-4 plants: normal, center and dwarf type, left).



Figure 5. Inhibitory effect of UCZ treatment on plant growth of wild-type lettuce plants. Seedlings of lettuce plants (dwarf type of SGΩ-4, left and wild type, center-left, center-right, and right) were treated with 0.001% (w/v, center-right) and 0.01% (w/v, right) or without (left and center-left) UCZ. Each concentration of 10 mL of UCZ solution was added to the soil. The plants were photographed at 1 month after the treatment.

duction of dwarf lettuce plants. Furthermore, this method may be useful for controlling plant stature in other agricultural and horticultural species.

MATERIALS AND METHODS

Plant Material

Seeds of lettuce (*Lactuca sativa* cv Vanguard) and its transformants were germinated on soil in pots. The seedlings were grown in the greenhouse under natural daylight at 23°C during the day (10 h of light) and at 18°C at night (14 h of darkness).

Transformation of Lettuce Plants

Lettuce seeds were surface sterilized and germinated on Murashige and Skoog medium with 1% (w/v) Suc and 0.2% (w/v) gellan gum under continuous light at 25°C. Leaves of the seedlings were cut and pre-cultured on the medium for 1 d. Transformation of lettuce plants was performed by infection with *Agrobacterium tumefaciens* LBA4404 harboring a construct prepared by replacing the β -glucuronidase gene of pBI121 with the pumpkin GA 20-oxidase sequence in sense orientation as a *Xba*I-*Sac*I fragment, and the CaMV 35S promoter sequence with the strong constitutive promoter cassette El2-35S- Ω from pBE2113 as a *Hind*III-*Xba*I fragment (Mitsuhara et al., 1996). After incubation in the conditioned medium, transformants were selected on the medium containing kanamycin.

Extraction of Genomic DNA and Analysis of Integrated Pumpkin GA 20-Oxidase Gene

Genomic DNA was extracted from leaves of lettuce seedlings using a DNA extraction kit (ISOPANT, Wako Life Science, Japan). Extracted DNA was purified through a spin-column (Chroma spin-1000, CLONTECH Laboratories, Palo Alto, CA), and the integrated pumpkin GA 20-oxidase gene was detected by PCR using specific primers, including a 3'-specific region and an additional *Eco*RI site (forward: 5'-CGAGAATTCATAGAAATGATGGGC-3' and reverse: 5'-GACGAATTCAGCAACACATAAGA-3'). After agarose gel electrophoresis, the PCR fragment was recovered from the gel using a DNA purification kit (Toyobo, Japan). This fragment was inserted into *Eco*RV-digested pBluescript SK (-) and the DNA sequence was analyzed with a Dye terminator cycle sequencing kit and a DNA sequencer (PE-Applied Biosystems, Foster City, CA).

Southern-Blot Analysis

Genomic DNA was extracted in the same way as described above. For Southern-blot analysis, 10 μ g of genomic DNA was digested with *Eco*RI, *Hind*III, or *Xba*I, respectively, and, after electrophoresis, it was blotted onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Buckinghamshire, UK). The purified PCR fragment described above was digested with *Eco*RI, giving a pumpkin GA 20-oxidase gene-specific probe (368 bp), which was labeled with digoxigenin using digoxigenin-high prime DNA labeling kit (Boehringer Mannheim, Germany). Procedures for hybridization and detection were according to the manufacturer's instructions.

Extraction of Total RNA and Northern-Blot Analysis

Total RNA was extracted by the aurin tricarboxylic acid method (Gonzalez et al., 1980). For northern-blot analysis, 15 μ g of total RNA per lane was subjected to electrophoresis and was blotted onto a nylon membrane (Hybond-N+, Amersham). The probe and the procedures for hybridization and detection were as for the Southern-blot analysis.

Treatment of Lettuce Plants with Uniconazol

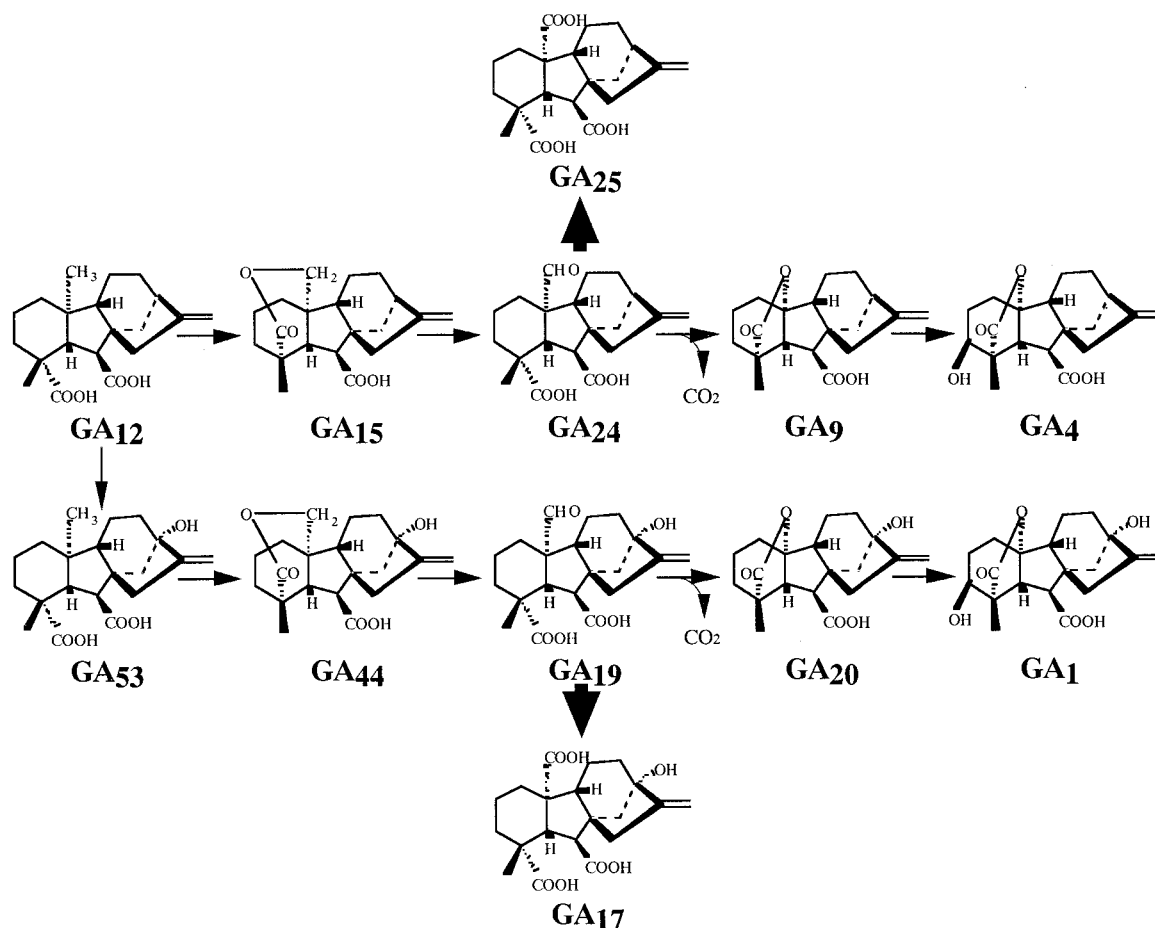
Seedlings of wild-type lettuce plants were treated with UCZ by adding 10 mL of solution to the soil. The length of leaves was measured at 1 month after the treatment.

Table II. Levels of endogenous GAs in dwarf-type of SGΩ-4 plants and in wild-type plants

ND, Not detected. All values are the means \pm SE of three independent experiments.

Phenotype	13-Hydroxylated GAs				Non-13-Hydroxylated GAs			
	GA ₁₉	GA ₂₀	GA ₁	GA ₁₇	GA ₂₄	GA ₉	GA ₄	GA ₂₅
	<i>pg g⁻¹ fresh wt</i>							
SGΩ-4 plants								
Dwarf type	87.2 \pm 10.0	14.3 \pm 7.1	30.9 \pm 4.1	11878.0 \pm 718.0 ^a	ND	ND	ND	36.6 \pm 10.4
Wild-type plants	175.0 \pm 34.0	207.0 \pm 26.0	177.0 \pm 35.0	230.0 \pm 54.0 ^a	1.9 \pm 1.8	ND	7.7 \pm 7.2	ND

^a Values of the concentration of GA₁₇ are relative to [²H₂]GA₁₉.



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Figure 6. GA biosynthesis pathway showing reactions catalyzed by GA 20-oxidase (GA₅₃ to GA₂₀ and GA₁₂ to GA₉) and 3 β -hydroxylase (GA₂₀ to GA₁ and GA₉ to GA₄). Conversion of GA₁₇ from GA₁₉ and GA₂₅ from GA₂₄, catalyzed by the pumpkin GA 20-oxidase, are shown with heavy arrows.

Analysis of Endogenous GA Levels

Leaves of 2-month-old lettuce plants (approximately 100 g of fresh weight) were extracted with 80% (v/v) methanol (MeOH). After addition of internal standards (100 ng of [²H₂]GA₁, 100 ng of [²H₂]GA₄, 100 ng of [²H₂]GA₉, 200 ng of [²H₂]GA₁₉, 100 ng of [²H₂]GA₂₀, 200 ng of [²H₂]GA₂₄, and 200 ng of [²H₂]GA₂₅), the extract was concentrated in vacuo and the aqueous residue was adjusted to pH 3 with HCl and was then extracted with ethyl acetate (EtOAc). The EtOAc fraction was extracted with 0.5 M phosphate buffer (pH 8.3), and 5% (w/v) of polyvinylpyrrolidone was added to the buffer fraction. After filtration, the fraction was adjusted to pH 3 and was extracted with EtOAc again. The EtOAc fraction was dried over anhydrous Na₂SO₄ and was evaporated in vacuo. The resulting residue was dissolved in 80% (v/v) MeOH and was passed through a Sep-Pak (ODS) cartridge (Waters, Milford, MA). The eluate was dissolved in MeOH and charged onto a Bondesil DEA column. The column was washed with MeOH and was then eluted with 0.5% (v/v)

acetic acid (HOAc) in MeOH. The eluate was subjected to HPLC on an Inertsil-2 ODS column (25 × 0.46 cm i.d., Gasukuro Kogyo, Japan). Samples were eluted by linear gradient as follows: solvent, 0 to 2 min, 30% (v/v) MeOH (0.1% [v/v] HOAc); 2 to 10 min, 30% to 50% (v/v) MeOH (0.1% [v/v] HOAc); 10 to 50 min, 50% to 70% (v/v) MeOH (0.1% [v/v] HOAc); and 50 to 51 min, 70% to 100% (v/v) MeOH (0.1% [v/v] HOAc); flow rate, 0.6 mL min⁻¹; column temperature, 40°C. The eluates were collected every 1 min and the fractions of retention time (Rt; 18 to 20 min for GA₁, 27 to 29 min for GA₂₀, and 32 to 35 min for GA₁₇ and GA₁₉) were used for early-13-hydroxylated GAs. For non-13-hydroxylated GAs, the fractions (Rt of 42–52 min) were combined and dissolved in MeOH containing 0.1% (v/v) HOAc and were then loaded onto a Senshu-Pak N(CH₃)₂ 4151 N column (15 × 1.0 i.d. cm), which was eluted with the same solvent at a flow rate of 2 mL min⁻¹ at 50°C. The elutes were collected every 2 min and the fractions of Rt 19 to 24 min (for GA₄ and GA₂₅), Rt 25 to 30 min (for GA₉), and Rt 31 to 36 min (for GA₂₄). All of those fractions were

methylated with ethereal diazomethane, and were subsequently trimethylsilylated with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide at 80°C for 30 min. Gas chromatography-selected ion monitoring analysis was performed as described in Tanaka-Ueguchi et al. (1998) except that we used a capillary column (30 m × 0.25 mm i.d., HP-5, Hewlett-Packard, Palo Alto, CA), and ions were selected as follows: for early-13-hydroxylated GAs, *m/z* 506, 448, and 375 for GA₁ MeTMSi; *m/z* 508, 450, and 377 for [²H₂]GA₁ MeTMSi; *m/z* 492, 432, 402, 373 for GA₁₇ MeTMSi; *m/z* 434, 402, 374 for GA₁₉ MeTMSi; *m/z* 436, 404, 376 for [²H₂]GA₁₉ MeTMSi; *m/z* 418, 403, and 375 for GA₂₀ MeTMSi; *m/z* 420, 405, and 377 for [²H₂]GA₂₀ MeTMSi; for non-13-hydroxylated GAs, *m/z* 418, 386, 328, and 284 for GA₄ MeTMSi; *m/z* 420, 338, 330, and 286 for [²H₂]GA₄ MeTMSi; *m/z* 330, 298, 270, and 243 for GA₉; *m/z* 332, 300, 272, and 245 for [²H₂]GA₉ MeTMSi; *m/z* 374, 342, 314, and 286 for GA₂₄; *m/z* 376, 344, 316, and 288 for [²H₂]GA₂₄ MeTMSi; *m/z* 404, 372, 312, and 286 for GA₂₅; and *m/z* 406, 374, 314, and 288 for [²H₂]GA₂₅ MeTMSi. The levels of endogenous GA₁, GA₄, GA₉, GA₁₇, GA₁₉, GA₂₀, GA₂₄, and GA₂₅ were determined from the ratios of peak areas at *m/z* 506/508, 284/286, 298/300, 432/436, 434/436, 418/420, 314/316, and 312/314, respectively. Only GA₁₇, whose existence was confirmed in the transgenic plants by full-mass scanning, was quantified relative to [²H₂]GA₁₉ because [²H₂]GA₁₇ has not been synthesized. The behavior of GA₁₇ was very similar to that of GA₁₉ on HPLC; however, GA₁₇ and GA₁₉ were fully separated on gas chromatography-mass spectrometry.

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LITERATURE CITED

- Carrera E, Jackson SD, Prat S (1999) Feedback control and diurnal regulation of gibberellin 20-oxidase transcript levels in potato. *Plant Physiol* **119**: 765–773
- Coles JP, Phillips AL, Croker SJ, Garcia-Lepe R, Lewis MJ, Hedden P (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. *Plant J* **17**: 547–556
- Crozier A (1983) *The Biochemistry and Physiology of Gibberellins*, Vol 2. Praeger, New York
- Curtis IS, Ward DA, Thomas SG, Phillips AL, Davey MR, Power JB, Lowe KC, Croker SJ, Lewis MJ, Magness SL et al. (2000) Induction of dwarfism in transgenic *Solanum dulcamara* by overexpression of a gibberellin 20-oxidase cDNA from pumpkin. *Plant J* **23**: 329–338
- Garcia-Martinez JL, Lopez-Diaz I, Sanchez-Beltran MJ, Phillips AL, Ward DA, Gaskin P, Hedden P et al. (1997) Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. *Plant Mol Biol* **33**: 1073–1084
- Gonzalez RG, Haxo RS, Schleich T (1980) Mechanism of action of polymeric aurintricarboxylic acid, a potent inhibitor of protein-nucleic acid interactions. *Biochemistry* **19**: 4299–4303
- Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 431–460
- Hedden P, Proebsting WM (1999) Genetic analysis of gibberellin biosynthesis. *Plant Physiol* **119**: 365–370
- Huang S, Raman AS, Ream JE, Fujiwara H, Cerny RE, Brown SM (1998) Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in *Arabidopsis*. *Plant Physiol* **118**: 773–781
- Lange T (1998) Molecular biology of gibberellin synthesis. *Planta* **204**: 409–419
- Lange T, Hedden P, Graebe JE (1994) Expression and cloning of a gibberellin 20-oxidase, a multifunctional enzyme involved in gibberellin biosynthesis. *Proc Natl Acad Sci USA* **91**: 8552–8556
- Martin DN, Proebsting WM, Hedden P (1997) Mendel's dwarfing gene: cDNAs from the *Le* alleles and function of the expressed proteins. *Proc Natl Acad Sci USA* **94**: 8907–8911
- Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, Lewis MJ, Gaskin P, Hedden P (1996) Feedback regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. *Planta* **200**: 159–166
- Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S et al. (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* **37**: 49–59
- Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P (1995) Isolation and expression of three gibberellin 20-oxidase cDNA clones from *Arabidopsis*. *Plant Physiol* **108**: 1049–1057
- Rebers M, Kaneta T, Kawaide H, Yamaguchi S, Yang YY, Imai R, Sekimoto H, Kamiya Y (1999) Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. *Plant J* **17**: 241–250
- Tanaka-Ueguchi M, Itoh H, Oyama N, Koshioka M, Matsuoka M (1998) Overexpression of a tobacco homeobox gene, *NTH15*, decreases the expression of a gibberellin biosynthetic gene encoding GA 20-oxidase. *Plant J* **15**: 391–400
- Toyomasu T, Kawaide H, Sekimoto C, von Numers C, Phillips AL, Hedden P, Kamiya Y (1997) Cloning and characterization of a cDNA encoding gibberellin 20-oxidase from rice (*Oryza sativa* L.) seedlings. *Plant Physiol* **99**: 111–118
- Toyomasu T, Tsuji H, Yamane H, Nakayama M, Yamaguchi I, Murofushi N, Nakahashi N, Inoue Y (1993) Light

- effects on endogenous levels of gibberellins in photoblastic lettuce seeds. *J Plant Growth Regul* **12**: 85–90
- Waycott W, Smith VA, Gaskin P, MacMillan J, Taiz L** (1991) The endogenous gibberellin of dwarf mutants of lettuce. *Plant Physiol* **95**: 1169–1173
- Wu K, Li L, Gage DA, Zeevaart JAD** (1996) Molecular cloning and photoperiod-regulated expression of gibberellin 20-oxidase from the long-day plant spinach. *Plant Physiol* **110**: 547–554
- Xu Y-L, Gage DA, Zeevaart JAD** (1997) Gibberellins and stem growth in *Arabidopsis thaliana*: effects of photoperiod on expression of the *GA4* and *GA5* loci. *Plant Physiol* **114**: 1471–1476
- Xu Y-L, Li L, Gage DA, Zeevaart JAD** (1999) Feedback regulation of *GA5* expression and metabolic engineering of gibberellin levels in *Arabidopsis*. *Plant Cell* **11**: 927–935
- Xu Y-L, Li L, Wu K, Peeters AJM, Gage DA, Zeevaart JAD** (1995) The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. *Proc Natl Acad Sci USA* **92**: 6640–6644