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Characterization of New Gibberellin-Responsive Semidwarf Mutants of Arabidopsis[1]

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Chemical mutagenesis of *Arabidopsis thaliana* (L.) Heynh. yielded four semidwarf mutants, all of which appeared to be gibberellin (GA)-biosynthesis mutants. All four had atypical response profiles to C_{20}-GAs, suggesting that each had impaired 20-oxidation. One mutant, *11.2*, was shown to be allelic to *ga5* and has been named *ga5-2*. It had altered metabolism of [^{14}C]GA_{15} relative to that in wild-type plants and undetectable levels of C_{19}-GAs in young stems, consistent with the known function of GA5 as a stem-expressed GA 20-oxidase. Two mutants (*2.1* and *10.3*), which had very short inflorescences and siliques, were allelic to each other but not to the known GA-responding mutants, *ga1* to *ga5*. The locus defined by these two mutations is provisionally named *GA6* and is purported to encode an inflorescence- and silique-expressed GA 20-oxidase. A double mutant, *ga5-2 ga6-2*, had an extreme dwarf phenotype with very short siliques. The fourth mutation, *1.1*, gave a phenotype like *ga5*, but was not allelic to any of the known *ga* mutations. It has not yet been given a gene symbol pending further studies.

Stem-length mutants of *Arabidopsis thaliana* (L.) Heynh. are providing excellent material for the analysis of GA biosynthesis and for the study of mechanisms of GA action. Five recessive mutations, *ga1* to *ga5* (Koornneef and van der Veen, 1980), have been shown to cause a dwarf or semidwarf phenotype as a consequence of impaired GA biosynthesis. Detailed analyses of endogenous GAs in these mutants, coupled with measurements of the bioactivity of applied GA precursors and GAs, have been conducted (Talon et al., 1990). Each mutation appears to block one metabolic event in the GA biosynthetic pathway (Zeevaart and Talon, 1992). This work has led to the successful cloning of genes for several enzymes in the GA pathway in Arabidopsis, namely *GA1*, which encodes *ent*-kaurene synthase A (Sun et al., 1992; Sun and Kamiya, 1994), *GA2*, which encodes *ent*-kaurene synthase B (Yamaguchi et al., 1997), *GA4*, which codes a 3β-hydroxylase (Chiang et al., 1995), and *GA5*, which encodes a GA 20-oxidase (Xu et al., 1995).

The production of additional stem-length mutants of Arabidopsis has been undertaken to identify genes that might encode different enzymes or regulatory factors in the GA biosynthetic pathway or other elements in the signal transduction chain. There is a precedent for expecting stem growth to be controlled by many genes. It is known, for example, that in garden pea (*Pisum sativum* L.) there are at least 15 loci that alter stem growth, 9 of which exist as multiple alleles (Reid and Ross, 1993). Not unexpectedly, the cloning of genes in pea has proceeded less rapidly than that of the Arabidopsis genes, and, to date, only *ent*-kaurene synthase B (*LS*) (Ait-Ali et al., 1997) and 20-oxidase (Martin et al., 1996) have been cloned. The identification of additional loci in Arabidopsis that control stem growth would therefore help in our understanding of this process in Arabidopsis and provide molecular genetic information applicable to other plants.

GAs not only regulate stem growth in Arabidopsis but are essential for overcoming ABA-imposed seed dormancy (Koornneef et al., 1982). We therefore sought to produce novel stem-length mutants of Arabidopsis by mutagenesis of an ABA-insensitive (*abi3*) line (Koornneef et al., 1984; Finkelstein and Somerville, 1990). In the *abi3* genotype, ABA insensitivity prevents seed dormancy and negates the requirement for GAs (Nambara et al., 1991). Therefore, using *abi3* seeds for mutagenesis might facilitate the isolation of GA mutants that would not germinate in a WT background. We describe here the isolation of several new stem-length mutants of Arabidopsis and their preliminary characterization.

MATERIALS AND METHODS

Arabidopsis thaliana (L.) Heynh. ecotype L*er* plants were grown in 13-cm clay pots in a growing medium such as Metro Mix or Redi Earth (Hummert International, Earth City, MO). Plants were maintained in greenhouses under natural daylight with either supplemental incandescent and cool-white fluorescent lights or supplemental cool-white fluorescent lights only, to give an 18- or 24-h photoperiod. Total light intensity was variable within the range of 300 to 1500 μmol m^{-2} s^{-1}, depending on the weather.

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Abbreviations: DEA, diethylaminopropyl; L*er*, Landsberg *erecta*; WT, wild type.

Alternatively, plants were maintained in controlled environments under continuous illumination provided either by incandescent and cool-white fluorescent lights giving an intensity of 400 μmol m^{-2} s^{-1}, or by fluorescent lights only (equal numbers of cool-white and warm-white bulbs giving 120 μmol m^{-2} s^{-1}).

Seeds were either sown directly into pots as a suspension in 0.1% agar or were sterilized and plated onto Petri dishes containing nutrient agar as described by Lincoln et al. (1990). Seeds of *ga1*, *ga2*, and *ga3*, which required GA for germination, were plated onto nutrient agar to which GA_4/GA_7 (Sigma) had been added in a small volume of methanol before gelling, giving a final concentration of 10^{-5} M. Petri dishes were kept at 25°C in continuous light at an intensity of 120 μmol m^{-2} s^{-1}, and seedlings were transplanted into pots after 7 to 10 d. Plants were fertilized approximately once every 14 d using the mineral nutrient solution described by Lincoln et al. (1990).

Mutagenesis

Seeds of *A. thaliana* ecotype L*er* with the *ABA-insensitive 3* (*abi3*) mutation and the *glabrous 1* (*gl1*) mutation as a marker, were originally obtained as a gift from R. Finkelstein (University of California, Santa Barbara). Seeds were bulked to obtain approximately 50,000 for mutagenesis. The mutagenesis was performed by soaking the seeds in 0.3% ethylmethane sulfonate for 20 h, followed by thorough rinsing overnight. One-half of these M_1 seeds were densely planted in 30 trays, each containing eight pots, as described above, and plants were allowed to self-pollinate. Seeds from each tray were harvested separately, giving 30 M_2 populations. Seeds from all 30 populations were sparingly sown into 30 trays, allowing for inspection and selection of individual plants. Seeds were harvested from M_2 plants that exhibited a dwarf or semidwarf phenotype or were excessively tall. Each line was named according to the M_2 population and the individual; for example, *10.3* came from population 10 and was the third individual selected from that population. Seeds were immediately resown and plants were allowed to self-pollinate, thereby generating enough seeds for subsequent work. Although initial GA treatments were performed on the original mutant lines, lines that had been backcrossed at least once were used for subsequent work.

Chemicals

GA_{36} 7-methyl ester 3-methoxymethyl ether (Fig. 1, structure **1**) (11.5 mg), prepared by the method of Dawe et al. (1985), was dissolved in methanol (0.6 mL), 2 N NaOH (2.8 mL, aqueous) was added, and the resulting solution was heated at reflux for 48 h under an atmosphere of N_2. The mixture was cooled, acidified to pH 4, and extracted three times with 10 mL of ethyl acetate:*n*-butanol (4:1). Toluene (10 mL) was added to the combined extracts, which were then dried (Na_2SO_4), and the solvent removed to afford the 7,19-dicarboxylic acid (Fig. 1, structure **2**) as a colorless, glassy residue (11.0 mg): ^{1}H NMR (300 MHz, d_4-MeOH) δ 1.30 (*s*, 3H, 4-Me), 1.32–2.15 (*m*, 16H), 2.66 (*m*, 1H, H13), 2.74 (*d*, 1H, J = 13.1 Hz, H5), 2.9 (broad envelope, 1H, H6), 3.40 (*s*, 3H, OCH_2OMe), 3.76 (broad *s*, 1H, H3), 4.63, 4.75 (ABd, 2x1H, J = 7.0 Hz, OCH_2OMe), 4.82 (broad *s*, 1H, H17), 4.93 (broad *s*, 1H, H'17), 4.98 (broad *s*, 1H, H20). Some NMR resonances were broadened because of slow exchange between aldehyde and hydroxylactone tautomers; chemical shift was also variable. H6 is observed as a sharp doublet (J = 13 Hz) at δ 3.91 and H_2O as a sharp singlet at δ 9.96 in the spectrum of GA_{36} dimethyl ester. Electron-impact MS consisted of the following: *m/z* 374 (M^+ −32, 12%), 356 (22), 328 (27), 312 (17), 298 (28), 270 (100), 241 (22), 225 (38), 217 (24), 202 (19), 173 (23), 149 (40), 129 (28), 105 (42), 91 (74).

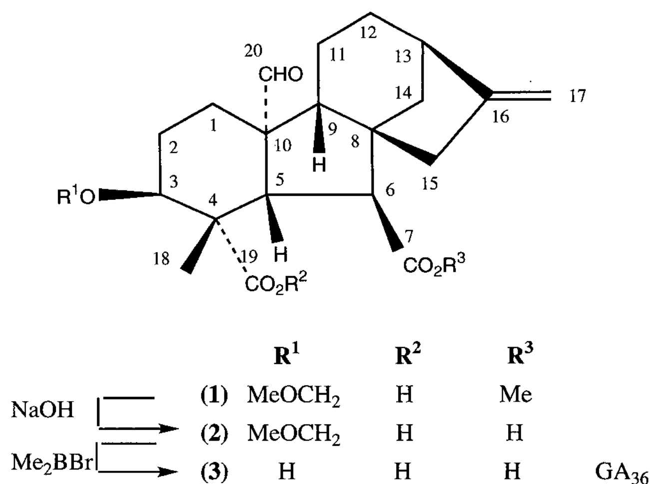

Figure 1. Steps in the preparation of GA_{36}.

To a rapidly stirred solution of the dicarboxylic acid (Fig. 1, structure **2**) (5 mg) prepared in dry dichloromethane (2.0 mL) at −78°C under a N_2 atmosphere was added dimethylboron bromide (3 drops). After 5 min the reaction mixture was added via pipette to a vigorously stirred mixture of saturated $NaHCO_3$ solution (4 mL) and dichloromethane (3 mL). After another 5 min the organic phase was separated and the aqueous phase extracted with dichloromethane (2 × 5 mL) and then acidified with 10% phosphoric acid. Extraction with ethyl acetate (3 × 10 mL), drying (Na_2SO_4), and removal of solvent afforded GA_{36} (Fig. 1, structure **3**), which was purified by HPLC using a 6-μm C_{18} column (NovaPak HR, Waters; 7.8 × 300 mm) and isocratic elution with methanol:water (51:49) (retention time 19.66 min): ^{1}H NMR (300 MHz, d_4-MeOH) δ 1.27 (*s*, 3H, 4-Me), 2.65 (*m*, 1H, H13), 2.77 (*d*, 1H, J = 13 Hz, H5) 2.90 (broad envelope, 1H, H6)[2] 3.72 (broad *s*, 1H, H3), 4.82 (broad *s*, 1H, H17), 4.97 (broad *s*, 1H, H'17) 4.97 (broad *s*, 1H, H20). Electron-impact MS was: *m/z* 344 (M^+ −18, 26%) 318 (28), 298 (12), 272 (15), 254 (24), 229 (21), 199 (17), 183 (13), 171 (15), 155 (13), 127 (30), 105 (31), 91 (100). High-resolution MS: $C_{20}H_{24}O_5$ (M^+ −18) requires 344.1624; found 344.1622.

GA_{12} was prepared according to the method of Cross et al. (1968). GA_1, GA_4, GA_9, and GA_{20} were gifts from M.H. Beale (University of Bristol, UK). GA_{15}, *ent*-kaurene, and *ent*-kaurenoic acid were gifts from L.N. Mander (Australian National University, Canberra, Australia). Paclobutrazol was a gift from J.R. Lenton (University of Bristol, UK).

GA Treatments

ent-Kaurenoids and GAs were applied to seedlings that were just beginning to bolt. Each test compound was applied to a rosette leaf as a 2-μL droplet of methanol or ethanol containing either 20 μg of *ent*-kaurenoid or 10 μg of GA. The *ent*-kaurenoids used were *ent*-kaurene (Fig. 2, structure **4**) and *ent*-kaurenoic acid (**5**) and the GAs were GA_1(**17**), GA_4 (**14**), GA_9 (**13**), GA_{12} (**6**), GA_{15} (**10**), GA_{20} (**15**), and GA_{36} (**3**). There were 8 to 10 plants per treatment. Some of the test compounds left a white residue on the treated leaves, so to facilitate uptake an additional droplet of solvent was applied after 24 h. Seedlings of *abi3* were chemically dwarfed by applying paclobutrazol just before the onset of bolting. Paclobutrazol inhibits *ent*-kaurene oxidation, and thus blocks the biosynthetic pathway before *ent*-kaurenoic acid. Approximately 40 mL of a 50 μM solution of paclobutrazol was applied to each pot as a soil drench. *ent*-Kaurenoids and GAs were applied to paclobutrazol-treated seedlings 3 d later. Lengths of the main stem, including the primary inflorescence of all seedlings, were measured 14 d after *ent*-kaurenoid/GA application.

GA Extraction

Plants for extraction were grown in controlled environments with a 24-h photoperiod provided by incandescent and fluorescent lights, or by fluorescent light only, as specified above. Several different types of plant material were used. For shoot extracts *abi3* (WT for stem length), *11.2*, and *10.3* plants, grown under mixed incandescent and fluorescent light, were harvested when they had bolted, but before any siliques were visible. All aerial portions of the plant (rosette leaves, stems, cauline leaves, buds, and flowers) were harvested en masse, and were frozen in liquid N_2 before storing at $-80°$. For stem extracts *abi3* and *11.2* plants grown under only fluorescent light were harvested

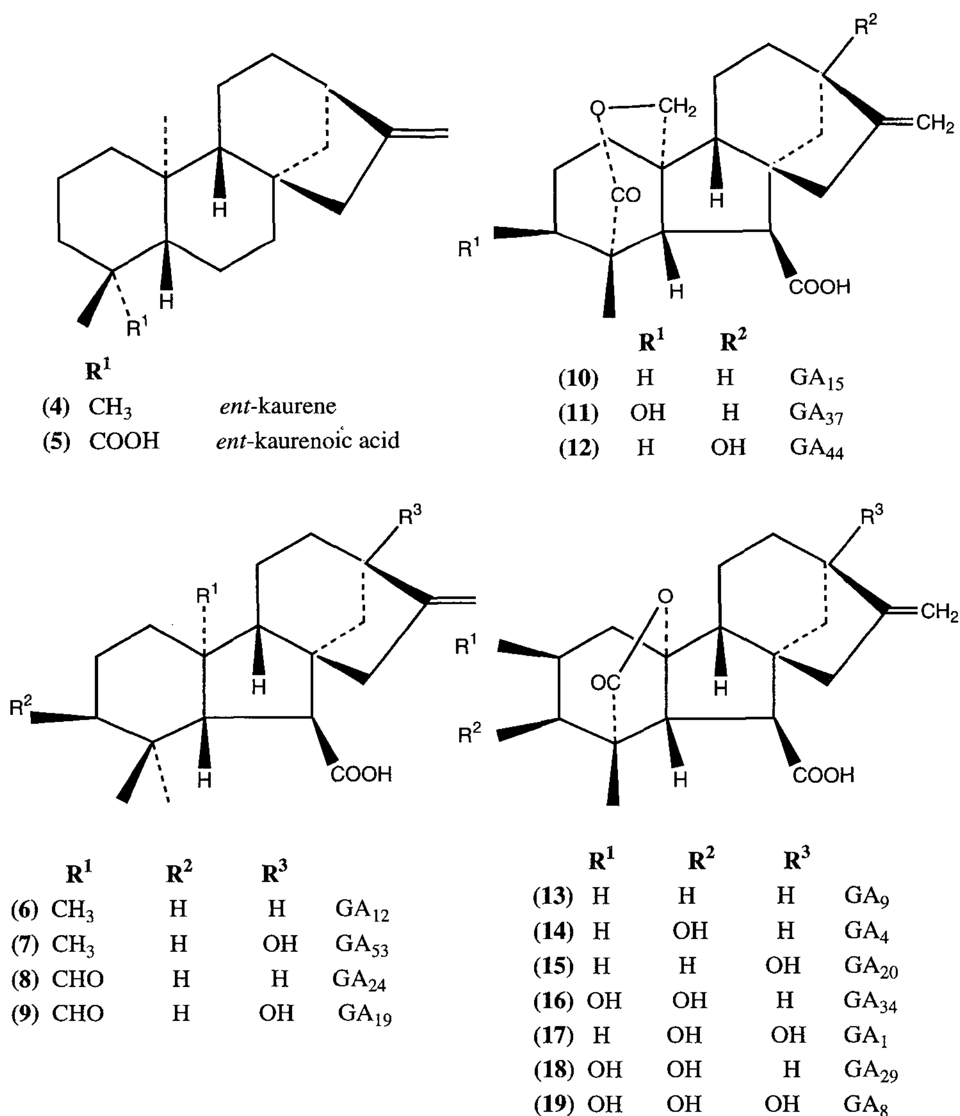

Figure 2. Additional structures of compounds discussed in the text.

either just as the first flower buds were opening (referred to as "young" stems) or when they had maturing siliques ("older" stems). In either case stems were excised above the rosette and below the flowers/siliques, and cauline leaves were removed before the stems were frozen as described above.

Frozen plant material was ground in ice-cold 80% methanol. Stable, isotope-labeled internal standards were added to methanolic extracts for quantification of endogenous GAs by MS. Internal standards, purchased from L.N. Mander, were [17-$^2H_2$]GA_1, [17-$^2H_2$]GA_4, [17-$^2H_2$]GA_8, [17-$^2H_2$]GA_9, [17-$^2H_2$]GA_{15}, [17-$^2H_2$]GA_{19}, [17-$^2H_2$]GA_{20}, [17-$^2H_2$]GA_{24}, [17-$^2H_2$]GA_{29}, and [17-$^2H_2$]GA_{34}. Also added was [^{14}C]GA_{12}, for use both as an internal standard and as a radioactive tracer. [^{3}H]GA_1 (19.6 Ci $mmol^{-1}$) from P.J. Davies (Cornell University, Ithaca, NY) was also added as a tracer.

Plant material was extracted three times, and extracts were partitioned and partially purified with polyvinylpolypyrrolidone using procedure A described by Sponsel et al. (1996). Acidic ethyl acetate extracts were further purified using C_{18} cartridges (Sep-Pak, Waters). For shoot extracts, an elution method (not described) was used that was later found to have resulted in unacceptable losses of the less polar GAs. For stem extracts the Sep-Pak procedure was as follows. Extracts were loaded in 0.4% acetic acid and cartridges were washed first with 0.4% acetic acid, then with 5% methanol in 0.4% acetic acid. GAs were eluted with 80% methanol. This GA-containing fraction was loaded onto a DEA cartridge (Varian, San Fernando, CA) in methanol, and after further methanol rinsing, the GAs were eluted from the DEA cartridge in 0.5% acetic acid in methanol. HPLC was performed using a semipreparative C_{18} column (25 cm × 4.5 mm) fitted with a precolumn and preequilibrated in 30% methanol containing 50 $\mu L\ L^{-1}$ acetic acid. The gradient was triphasic, with the methanol proportion increasing to 60% by 27 min, being held almost constant until 55 min, and then increasing to 90% by 60 min. The flow rate was 2.5 mL min^{-1}; 1-min fractions were collected for 80 min.

Aliquots of fractions were counted to locate the [^{3}H]GA_1 and [^{14}C]GA_{12} tracers, and fractions were pooled according to the expected retention times of the GAs (GA_8 and GA_{29} 9–13 min, GA_1 18–21 min, GA_{20} 35 and 36 min, GA_{19} and GA_{34} 38–41 min, GA_4 44–46 min, GA_9 and GA_{24} 51–55 min, GA_{15} 57 and 58 min, and GA_{12} 68 min). Pooled fractions were evaporated to dryness and then methylated, silylated, and analyzed by GC-MS using methods described previously (Sponsel et al., 1996, procedure A). Samples were analyzed by selected ion monitoring. Identifications were based on the presence of at least four diagnostic ions at the correct Kovats retention index.

GA Metabolism

[^{14}C]GA_{15} (specific activity 55 μCi μmol^{-1}), which was a gift from L.N. Mander, was fed to *abi3* and *11.2* seedlings as they were about to bolt. The substrate was applied to a rosette leaf of each of 50 plants of each genotype as a 2-μL droplet containing 6 nCi of [^{14}C]GA_{15}. Complete shoots were harvested 48 h later. Extraction was conducted as described above using the Sep-Pak elution method as described, but extracts were not subjected to DEA purification. Distribution of radioactivity in HPLC fractions was determined by liquid scintillation counting aliquots of fractions 8 to 65.

RESULTS

Endogenous GAs of *abi3*

There is no reason to think that the *abi3* mutation, which confers ABA insensitivity to seeds, would affect the GA content of seedlings. However, stems of *abi3* seedlings were extracted to show that the GA content is qualitatively and quantitatively similar to that reported for WT L*er* seedlings (Talon et al., 1990). Results (Table I) show that at least nine of the GAs known to be present in L*er* (*ABI3*) are also present in *abi3* stems, at two developmental stages. Although it is not possible to make direct quantitative comparisons with the data of Talon et al. (1990), the presence in *abi3* extracts of C_{20}- and C_{19}-GAs with hydroxylation(s) at C-2, C-3, and/or C-13 (see structures in Fig. 2) provide evidence that the expected GA metabolic pathways (see Fig. 3) are operating in *abi3* plants. The *abi3* genotype is therefore referred to throughout this paper as being WT with respect to stem length and GA content.

Selection of Mutants and Description of Phenotypes

The mutagenesis of *abi3* seeds and the growth of M_1 and M_2 generations is described in "Materials and Methods." More than 20 individual dwarf, semidwarf, or excessively tall plants were selected from the M_2 populations. Four semidwarf lines were initially chosen for characterization because they produced uniform progeny with a distinctive phenotype and copious amounts of seed. These four lines, each of which came from a different M_2 population, were designated *1.1*, *2.1*, *10.3*, and *11.2*. Their stem heights, measured after 28 and 42 d of growth in continuous illumination (greenhouse with supplemental fluorescent and incandescent lighting), are shown in Table II, together with heights of WT and two known semidwarf mutants, *ga4* and *ga5*. Figure 4 shows additional information for 36-d-old WT, *2.1*, *10.3*, and *11.2* seedlings, and of a double mutant (48-d-old), which is discussed below. These plants had all been grown with only fluorescent light in an 18-h photoperiod. (In Fig. 4 unexpanded internodes within the rosette are disregarded. The positions of cauline leaves were used to measure expanded internodes above the youngest rosette leaf. The inflorescence measurement is defined here as the distance between the lowest flower and the stem apex.)

Expanded internodes of *2.1* and *10.3* are comparable or longer than those of WT plants (Fig. 4). Both *2.1* and *10.3* have extremely short inflorescences consisting of compact clusters of flowers and buds. In comparison, *11.2* has a more proportionate reduction in the size of all internodes (Fig. 4). All of the lines were fertile, although *2.1* and *10.3* were less fertile and tended to produce siliques only after an extended period of flowering. Also shown in Table II are

Table I. *GA levels in young and old stems of abi3 seedlings*

GA	Young Stems	Older Stems
	ng 100 g^{-1} fresh wt	
GA_1	7.4	10.0
GA_4	48.8	18.3
GA_9	70.0	93.5
GA_{12}	39.0	62.6
GA_{15}	15.1	19.3
GA_{19}	11.3	14.2
GA_{20}	3.6	3.3
GA_{24}	51.3	46.2
GA_{29}	8.1	nd[a]
GA_{34}	69.2	40.0

[a] nd, Not detectable.

the lengths of fully grown siliques for each mutant, which tended to be shorter than those of WT plants.

Initial Genetic Characterization

All four lines, *1.1*, *2.1*, *10.3*, and *11.2*, were crossed to both *ABI3* and *abi3*. Data for the *ABI3* crosses are presented in Table III. Stem heights of F_1 plants were measured at 42 d. All F_1 plants were WT with respect to stem length. F_1 plants were allowed to self-pollinate. Segregation of WT and semidwarf plants was observed in each F_2 generation, and frequencies of each genotype are also recorded in Table III. Each mutant segregated in a manner consistent with a 3:1 (WT-to-semidwarf) ratio. It was therefore concluded that each line resulted from a single, recessive mutation and that *abi3* was not necessary for expression of the semidwarf phenotypes. Further genetic analysis showed that when *2.1* and *10.3* lines were crossed all progeny in the F_1 and F_2 generations were semidwarf. Therefore, *2.1* and *10.3* are allelic.

The new mutants were then crossed to each of the known GA-deficient mutants, *ga1*, *ga2*, *ga3*, *ga4*, and *ga5* (Table IV). All F_1 plants had WT stem phenotypes, with the exception of the progeny of the cross *11.2* × *ga5*, which had a semidwarf phenotype.

These results (Table IV) provide evidence that *1.1*, *2.1*, and *10.3* are single, recessive mutations that can be complemented by each of the known *ga* mutations. *1.1* has not yet been assigned a name. The locus defined by the *2.1* and *10.3* mutations is provisionally named *GA6*, on the strength of genetic data and physiological data presented in the next section showing these mutants to be GA responders. *2.1* and *10.3* are named *ga6-1* and *ga6-2*, respectively. Complementation does not occur in the progeny of the cross *11.2* × *ga5* (Table IV). Therefore, the mutation designated *11.2* occurs at the *GA5* locus and is named *ga5-2*.

Response to GA Precursors and GAs

Each of the four lines, *1.1*, *ga5-2* (*11.2*), *ga6-1* (*2.1*), and *ga6-2* (*10.3*), were treated with GA precursors and GAs to determine whether the mutant phenotypes could be reversed. The structures of these test compounds are shown in Figures 1 and 2, and the probable GA biosynthetic pathways in Arabidopsis are shown in Figure 3. C-20 is successively oxidized in three stages, namely $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow CO_2$ or COOH. The removal of C-20 as CO_2 from a GA leads to the important production of C_{19}-GAs and is thought to be favored in Arabidopsis over the production of the inactive 20-COOH. In Arabidopsis these successive oxidations probably occur in the sequence GA_{12} (6) → GA_{15} (10) → GA_{24} (8) → GA_9 (13), although parallel sequences in which the GA is also hydroxylated at C-3 or C-13 may occur as well (Fig. 3).

The protein encoded by the *GA5* locus in Arabidopsis is a multifunctional enzyme that can catalyze each of the sequential oxidations at C-20, thus converting GA_{12} right

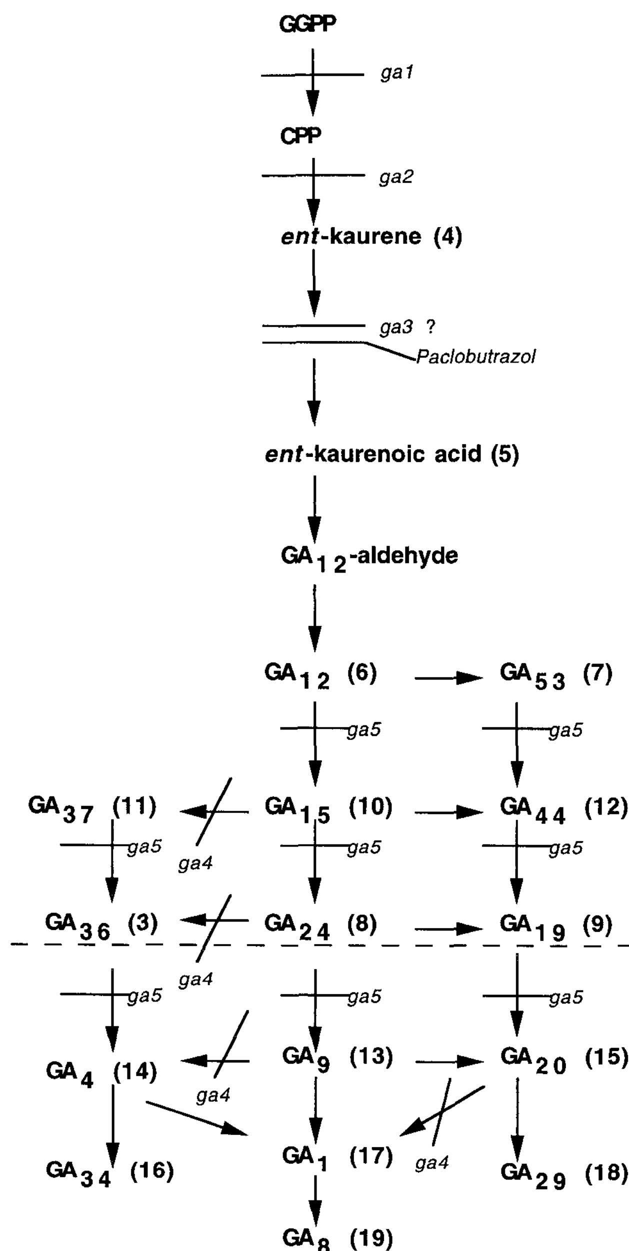

Figure 3. Metabolic pathways thought to operate in Arabidopsis shoots, and the location of steps blocked in the *ga1* to *ga5* mutants.

Table II. *Stem heights and silique lengths of four Arabidopsis lines produced by mutagenesis of abi3*

Values are means ± 1 SE (n = 10).

Line	Stem Length		Silique Length
	28 d	42 d	
		mm	
1.1	12.6 ± 3.1	77.9 ± 4.8	5.9 ± 0.2
2.1	52.6 ± 3.8	126.6 ± 5.6	5.7 ± 0.3
10.3	52.3 ± 4.1	123.5 ± 5.9	5.7 ± 0.3
11.2	61.6 ± 3.7	100.5 ± 4.9	9.0 ± 0.3
abi3	174.0 ± 6.1	226.3 ± 7.4	10.6 ± 0.3
ga4	34.2 ± 4.5	91.3 ± 6.3	9.4 ± 0.3
ga5	50.0 ± 10.4	115.5 ± 4.3	8.3 ± 0.3

through to GA_9 (Phillips et al., 1995; Xu et al., 1995). The enzyme can also utilize 13-hydroxylated GAs as substrates (Phillips et al., 1995), and probably also 3β-hydroxylated GAs. GA_{24} (**8**) was not available for use in the current experiments, so its 3-hydroxylated counterpart, GA_{36} (**3**), which is the immediate precursor of GA_4 (**14**), was used instead. GA_9 and GA_4 are both C_{19}-GAs. However, the 3β-hydroxylated GA, GA_4, is purported to be the primary active GA for stem growth in Arabidopsis (Talon et al., 1990; Zeevaart and Talon, 1992).

Response profiles are shown in Figure 5, which orders the *ent*-kaurenoids and GAs in the probable biosynthetic sequence (Fig. 3). Because of its limited availability, *ent*-kaurene was not tested on *1.1*, and *ent*-kaurenoic acid was not tested on *abi3*. The *1.1* mutant did not respond to *ent*-kaurenoic acid (**5**) or to GA_{12} (**6**), but responded to GA_{15} (**10**), GA_{36} (**3**), GA_9 (**13**), GA_4 (**14**), and GA_1 (**17**). The *ga5-2* mutant showed no response to the *ent*-kaurenoids or GA_{12}, but gave a good response to GA_{15} and all GAs except GA_{20} later in the pathway. The *ga6-1* mutant responded to neither *ent*-kaurenoid and showed only a small response to GA_{12} and GA_{15}, but gave a good response to GA_{36} and a moderate response to GAs later in the pathway, with the exception of GA_{20}. In contrast, *ga6-2* showed a response profile that was very similar to that of *ga5-2* seedlings. Seedlings of *abi3*, which were treated with paclobutrazol to inhibit GA biosynthesis at *ent*-kaurene oxidation before GA application, responded to GA_{12} and all GAs later in the pathway.

In Arabidopsis GAs that are 13-hydroxylated have less activity than their 13-deoxy counterparts. GA_{20} (**15**) showed less activity than GA_9 (**13**), and GA_1 (**17**) showed less activity than GA_4 (**14**) (Fig. 5). This was true for WT and all mutant lines, and further supports the contention that GA_4, rather than GA_1, is the main active hormone for stem growth in Arabidopsis. GA_{20} and GA_1 were not used in subsequent assays (Figs. 6, 7, and 10). All of the mutant lines responded to GA_9 (Fig. 5), suggesting that each had the 3β-hydroxylating capability to convert GA_9 (**13**) to GA_4 (**14**) (see Fig. 3).

GA_{12} (**6**) showed no or low activity on each of the new mutants (Fig. 5), indicating that metabolism of GA_{12} was impaired in all of these lines. Knowing that the 20-oxidase is a multifunctional enzyme that can convert GA_{12} to C_{19}-GAs, it is difficult to rationalize the occurrence of mutants that did not respond to GA_{12} but did respond to GA_{15} (**10**) and GA_{36} (**3**).

To verify these results the same test compounds were assayed on *ga1* seedlings in which the pathway is blocked before *ent*-kaurene (Fig. 3). *ga1* showed a significant, reproducible response to GA_{12} in addition to GA_{15}, GA_{36}, GA_9, and GA_4 (Fig. 6). This result, coupled with data showing that GA_{12} is active in paclobutrazol-treated *abi3* seedlings (Fig. 5), is evidence that the failure of *1.1*, *ga5-2*, and *ga6-2* seedlings to respond to applied GA_{12} is unlikely to be the result of problems with uptake, and is likely to be a valid result.

Further Studies of GA Bioactivity and GA Metabolism in *ga5*

The *ga5-1* mutant (Koornneef and van der Veen, 1982) is known to lack a functional 20-oxidase that is encoded by the *GA5* locus and is normally expressed in stems (Phillips et al., 1995; Xu et al., 1995). Comparative assays of C_{20}- and C_{19}-GAs on *ga5-1* and *ga5-2* were therefore conducted, because information on the response of *ga5-1* to GA_{12} and GA_{15} does not appear to have been published. GA_{36} has activity in *ga5-1* comparable to that of GA_1 (Zeevaart and Talon, 1992). Like *ga5-2*, the *ga5-1* mutant showed no response to GA_{12}, but showed a significant growth response to GA_{15} and GA_{36} (Fig. 7). The difference in activity between GA_{12} and GA_{15} was profound: GA_{12} had no activity, whereas GA_{15} was almost able to restore normal stem height. Because GA_{15} is unlikely to be active per se, *ga5-1* and *ga5-2* plants must metabolize applied GA_{15} to an active GA using enzyme(s) other than the GA5 protein.

To compare the metabolic fates of [^{14}C]GA_{15} in *ga5-2* and WT plants, seedlings were treated with [^{14}C]GA_{15} at the beginning of the bolting stage. It is known that recombinant Arabidopsis 20-oxidase accepts only the 20-hydroxy-19-carboxylic acid ("GA_{15}-open-lactone") as a

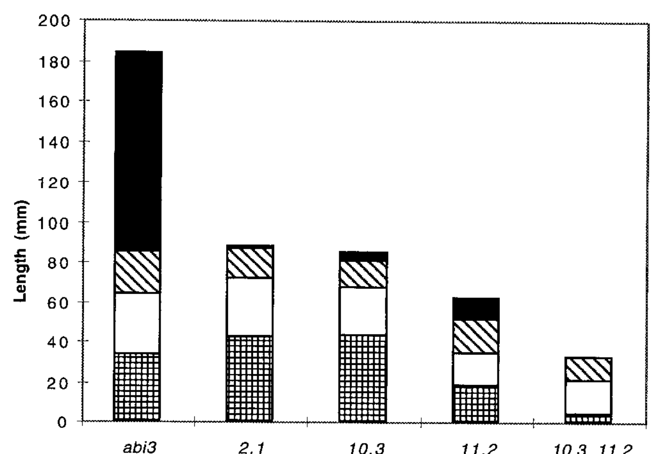

Figure 4. Total stem and individual internode and inflorescence lengths for *abi3*, *2.1*, *10.3*, and *11.2* mutant lines, and a *10.3 11.2* double mutant. Each column represents total seedling height and is subdivided to show individual internodes (oldest at the bottom) and the inflorescence (top). Only internodes above the rosette are included. Values are means ± SE (n = 10).

Table III. *Stem heights of F_1 plants, and segregation of F_2 plants after crossing four mutant lines to Ler (WT)*

Stem lengths are means ± 1 SE (n = 16 for all except *1.1* × WT, where n = 6).

Cross	F_1	F_2		
		Frequency of phenotypes		χ^{2a}
	Stem height at 42 d	WT	Dwarf	
1.1 × WT	212.4 ± 17.7	108	35	0.02^b
2.1 × WT	232.2 ± 18.1	84	30	0.11^b
10.3 × WT	235.2 ± 13.5	69	23	0^b
11.2 × WT	205.7 ± 9.2	73	30	0.9^b

[a] Calculated based on an expected ratio of 3 WT to 1 dwarf. [b] $P > 0.5$.

substrate (Ward et al., 1997). However, there is some evidence (e.g. Gilmour et al., 1986; Graebe, 1987) that other enzyme(s) present in intact plants or in cell-free homogenates from vegetative tissues may be able to convert applied GA_{15} or GA_{44} (**12**) to their open-lactone forms so that they can subsequently be oxidized at C-20. Radioactive metabolites were separated by reverse-phase HPLC and are shown in Figure 8. WT plants were able to metabolize $[^{14}C]GA_{15}$ to several metabolites. At least one of these metabolites (retention time 46 min) was not present in feeds to *ga5-2* plants. The *ga5-2* plants, however, contained a metabolite at 40 min retention time with a production that vastly exceeded that of any metabolite in WT plants. The identities of this and other metabolites are currently being investigated.

Phenotype of *ga6-2* (*10.3*) and Production of a *ga5-2 ga6-2* Double Mutant

The response of the *ga6-2* (*10.3*) mutant to applied GAs was very similar to that of *ga5* mutants (Figs. 5 and 7). However, *ga6-2* and *ga5-2* have quite different phenotypes, which are evident from Figures 4 and 9. The semidwarfism of *ga6-2* arises from a reduction of stem growth once the flower buds begin to swell. In addition, *ga6-2* has very short siliques. The possibility that *ga6-2* has a mutated form of an inflorescence-expressed 20-oxidase is discussed below.

To determine whether *ga5-2* and *ga6-2* had additive phenotypes, a double mutant was prepared by crossing *ga5-2* to *ga6-2*. F_1 plants were WT with respect to stem length. Segregation in the F_2 generation is shown in Table V. The frequencies of phenotypes were consistent with a 9:3:3:1 (WT:*ga5-2*:*ga6-2*:*ga5-2 ga6-2*) segregation. The double mutant *ga5-2 ga6-2* had an extreme dwarf phenotype (Figs. 4 and 9) comparable to that of *ga1* or *ga2*. Treatment of *ga5-2 ga6-2* plants with GAs resulted in stem growth (Fig. 10), although a single application of any GA was not sufficient to restore WT stem growth. The efficacy of repeated applications of GA_4 must now be determined. Although untreated *ga5-2 ga6-2* plants were extremely slow to bolt and flower (the plant in Fig. 9 is 42 d old; the others are 28 d old), the double mutant did produce fertile flowers and seeded siliques without requiring GA application.

Endogenous GAs in *ga5-2* and *ga6-2* Seedlings

Preliminary extractions of seedlings and stems of *ga5-2* have shown that levels of all GAs are below the limits of our detection. Although further extraction and GC-MS with a more sensitive MS system are now required, this preliminary result does provide evidence that *ga5-2* contains less GA than WT plants.

Preliminary extractions of *ga6-2* seedlings were performed before the methods for Arabidopsis extraction described in "Materials and Methods" were developed fully. Some losses of less polar GAs occurred, and only four GAs could be detected. However, the data showed that when seedlings were extracted at the early bolting stage, *ga6-2* and *abi3* seedlings contained comparable levels of these four GAs (Table VI). Clearly, the extraction of inflorescences of *ga6-2*, using the preferred Sep Pak and DEA purification procedures, are now required.

DISCUSSION

Chemical mutagenesis of Arabidopsis seeds was conducted to obtain new dwarf or semidwarf mutants that were either GA-deficient or had altered responsiveness to GAs. An ABA-insensitive (*abi3*) line was used to facilitate

Table IV. *Complementation analysis of stem length mutants*

New mutants were crossed with known GA mutants, and stem heights of 42-d-old F_1 plants were measured. Values are mean stem heights, with SE values in parentheses ($n \geq 10$).

New Mutant	Self-Pollinated	× *ga1*	× *ga2*	× *ga3*	× *ga4*	× *ga5*
			mm			
1.1	80.3 (2.5)	234.1 (10.4)	291.2 (3.9)	198.0 (7.1)	212.8 (13.6)	213.0 (9.7)
2.1	122.6 (5.6)	175.7 (4.8)	169.3 (4.5)	238.5 (6.6)	164.0 (10.2)	212.3 (4.4)
10.3	121.6 (3.7)	221.8 (4.4)	256.1 (7.4)	148.5 (3.7)	170.3 (5.5)	214.1 (5.6)
11.2	80.1 (2.4)	125.5 (4.6)	169.7 (4.8)	147.6 (8.2)	149.9 (4.5)	85.9 (3.2)

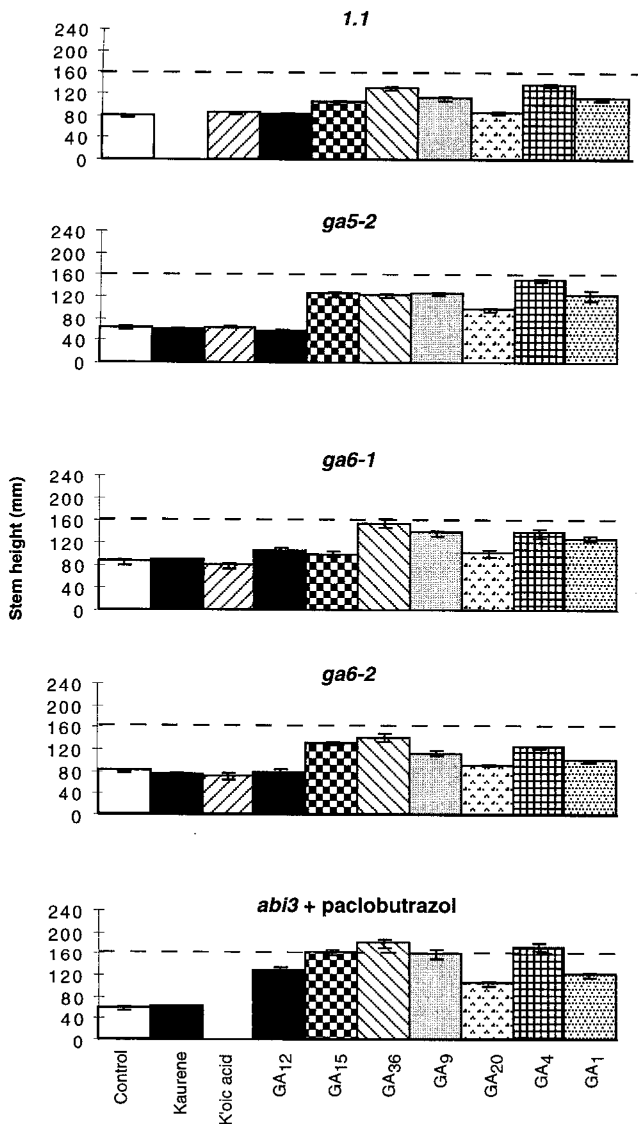

Figure 5. Responses of the mutant lines *1.1*, *ga5-2* (*11.2*), *ga6-1* (*2.1*), *ga6-2* (*10.3*), and of paclobutrazol-treated *abi3* seedlings to *ent*-kaurenoids (20 μg plant^{-1}) and seven GAs (10 μg plant^{-1}). Plants were grown in the greenhouse with a 24-h photoperiod and stem height was measured 2 weeks after treatment. The dotted line on each bar diagram represents the height of untreated *abi3* plants grown under the same environmental conditions.

the isolation of mutants that might not germinate in a WT (*ABI3*) background, although *abi3* was later shown to have no role in the phenotypes of the four semidwarf mutants described in this paper. Nevertheless, as a preliminary to this study, GAs were identified and quantified in *abi3* stems (Table I), and were shown to be consistent with those present in L*er* (*ABI3*) seedlings (Talon et al., 1990). The *abi3* line is therefore considered to be WT with respect to stem length and GA content.

Mutagenesis yielded a number of mutants with altered stem length, and characterization of four of these, *1.1*, *2.1*, *10.3*, and *11.2*, each of which was from a different M_2 population, was undertaken. Each mutant had a semidwarf phenotype and shorter siliques than WT plants (Table II; Fig. 4), suggesting that they were either deficient in or nonresponsive to endogenous GAs. In Arabidopsis GAs are known to be necessary for the normal growth of both stems (Koornneef and van der Veen, 1980) and siliques (Barendse et al., 1986).

Each mutant, when crossed to *ABI3* or *abi3*, gave F_1 progeny that were WT with respect to stem length. Segregation in the F_2 generation was consistent with a ratio of 3 WT to 1 semidwarf (Table III). Therefore, each mutant is the consequence of a single recessive mutation.

Crossing each of the known GA-deficient mutants, *ga1* to *ga5* (Koornneef and van der Veen, 1980), to each of the new mutants showed that complementation occurred with the *1.1*, *2.1*, and *10.3* mutants (Table IV), but that *ga5* could not complement *11.2* (Table IV). Therefore, *11.2* and *ga5* are allelic. Subsequent crosses of *2.1* to *10.3* gave semidwarf F_1 and F_2 progeny, indicating that they are allelic. The *1.1* mutation has not yet been given a gene symbol. Mutants *2.1* and *10.3* are assigned the names *ga6-1* and *ga6-2*, respectively, and *11.2* is named *ga5-2*.

Treatment of mutant seedlings with *ent*-kaurene, *ent*-kaurenoic acid, and seven GAs showed that they were all GA responders (Fig. 5). It was therefore assumed that they were all GA-deficient mutants in which GA biosynthesis is impaired. Results (Fig. 5) showed atypical responses to C_{20}-GAs for all of the mutants compared with either the response of paclobutrazol-treated WT seedlings (Fig. 5) or of *ga1* (Fig. 6) to the C_{20}-GAs. Therefore, the mutants all seemed to have impaired 20-oxidation.

An altered response of *ga5-2* seedlings to C_{20}-GAs (Fig. 5) corroborated our earlier complementation analysis (Table

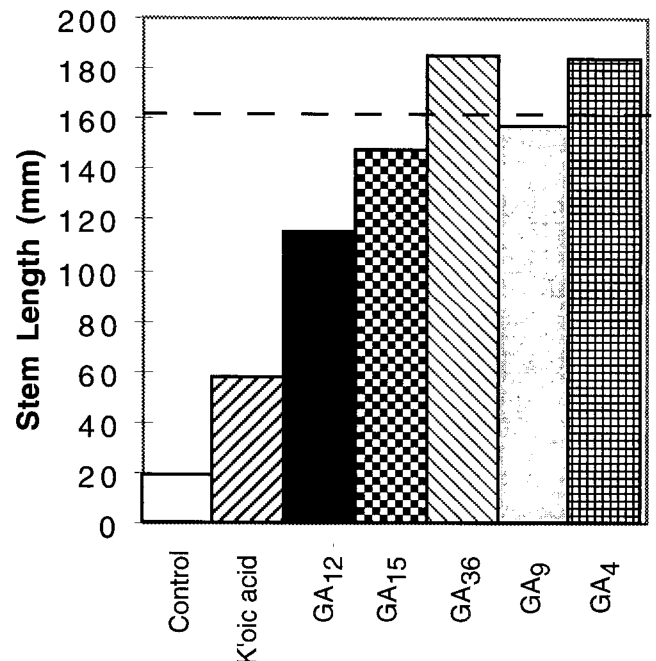

Figure 6. Response of *ga1* plants to five GAs (applied at 10 μg plant^{-1}). Plants were grown in the greenhouse with a 24-h photoperiod and stem heights were measured after 2 weeks.

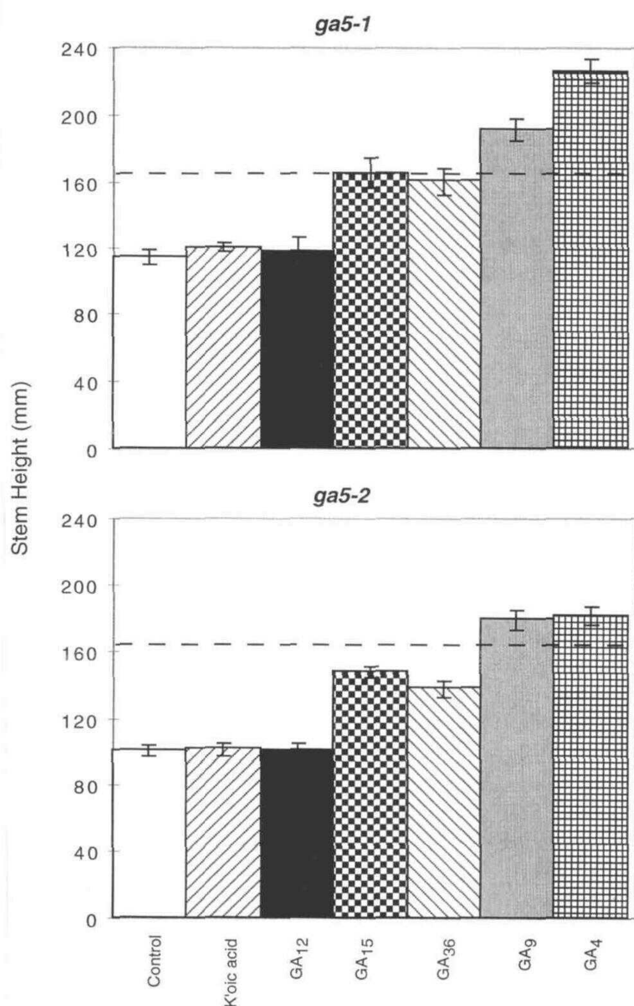

Figure 7. Responses of *ga5-1* and *ga5-2* (*11.2*) plants to five GAs (applied at 10 μg plant^{-1}). Plants were grown in the greenhouse with a 24-h photoperiod and stem heights were measured after 2 weeks.

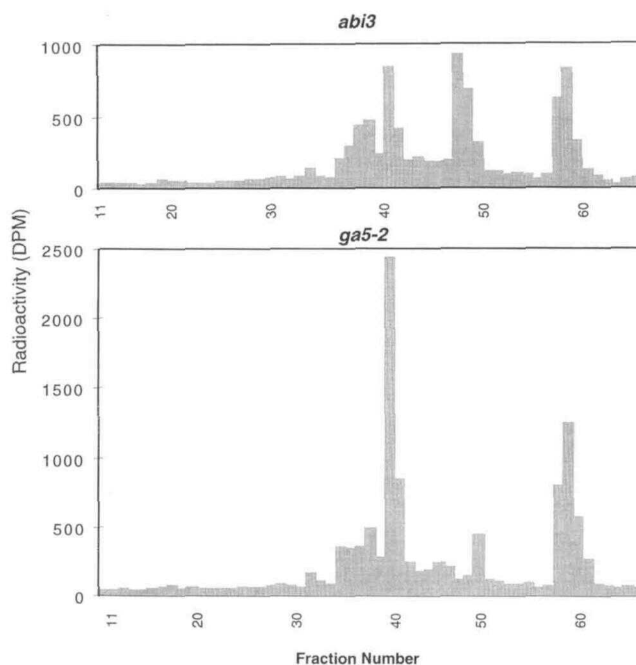

Figure 8. Radioactivity in aliquots of reverse-phase HPLC fractions from *abi* and *ga5-2* (*11.2*) seedlings after treatment with [^{14}C]GA_{15}. Fractions 11 to 65 were counted. [^{14}C]GA_{15} elutes in fractions 57 and 58. DPM, Disintegrations per minute.

IV). However, the strong bioactivity of GA_{15} (**10**) in both *ga5-1* and *ga5-2* seedlings (Figs. 5 and 7) was remarkable. This result implied that only the first oxidation step, i.e. that from GA_{12} (**6**) to GA_{15} (**10**) (Fig. 3), was blocked in these mutants, and was quite unexpected because the 20-oxidase encoded by the *GA5* locus catalyzes all the oxidative steps from GA_{12} to GA_9 (**13**) (Phillips et al., 1995; Xu et al., 1995). Although it is conceivable that a mutation could knock out only one of several functions in a multifunctional enzyme, this explanation is untenable in the case of the *ga5-1* mutant, which is known to have a truncated GA5 protein (Xu et al., 1995). Therefore, a more probable explanation for the activity of GA_{15} in *ga5* seedlings is that Arabidopsis stems contain an additional enzyme that can oxidize GA_{15} but not GA_{12}. This possibility has already been raised by Phillips et al. (1995) as an explanation for why the *ga5* mutant accumulates GA_{12} and GA_{24} but not GA_{15} (Talon et al., 1990).

Small-scale feeds of [^{14}C]GA_{15} to *abi3* and *ga5-2* seedlings were conducted to compare its metabolic fate in the two genotypes. Results based on the retention times of metabolites on HPLC (Fig. 8) showed that there were quantitative and qualitative differences in the metabolism of [^{14}C]GA_{15} in *ga5-2* and WT seedlings. Determination of the identity of metabolites in both genotypes will require additional larger-scale feeds.

The observation that the *ga6-2* mutant had an almost identical pattern of response to the C_{20}-GAs as the *ga5*

Figure 9. Left to right, *abi3*, *ga6.2* (*10.3*), *ga5-2* (*11.2*) (all 28 d old), and the *ga5-2*, *ga6-2* double mutant (42 d old). All plants were grown in the greenhouse with supplemental fluorescent light giving a 24-h photoperiod.

Table V. *Segregation of F_2 plants after crossing ga5-2 and ga6-2*

Experiment	Frequency of Phenotypes				χ^{2a}
	WT	*ga5-2*	*ga6-2*	*ga5-2 ga6-2*	
1	166	47	42	15	3.4
2	143	42	36	14	2.4[b]

[a] Calculated based on an expected ratio of 9 wild type to 3 *ga5-2*, to 3 *ga6-2*, to 1 *ga5-2* ga6-2. [b] $P > 0.5$.

mutants (Figs. 5 and 7) indicated that *ga6-2* might also be missing a functional 20-oxidase, but a different one from that encoded by the *GA5* locus. In *ga6-2* seedlings a reduction in stem growth is not apparent until the inflorescence begins to mature (Figs. 4 and 9). This phenotype is consistent with the stem-expressed 20-oxidase being present and functional, but with the 20-oxidase that is normally expressed in inflorescences and reproductive tissue being impaired. Data showing that vegetative *ga6-2* seedlings contain similar or even more GAs than WT seedlings (Table VI) would appear to confirm the presence of a functional, stem-expressed enzyme. Information on the GA content and metabolic capabilities of inflorescences and siliques of the *ga6-2* mutant is now required.

An alternative approach to characterizing the *ga6-2* mutant is also available, because Phillips et al. (1995) have obtained two full-length cDNA clones encoding two different 20-oxidases from Arabidopsis shoots. One (At2301) is identical to the transcribed region of the genomic clone matched to the *GA5* locus by Xu et al. (1995). Phillips et al. (1995) have demonstrated that At2301 shows very high levels of expression in stems, low expression in flowers, and none in leaves, siliques, and roots (Phillips et al., 1995). This pattern is consistent with the known expression pattern of *GA5*. The second clone (At2353), which has 76% amino acid identity with At2301, shows high levels of expression in flowers and siliques, but none in stems, leaves, or roots. Heterologous expression of At2353 in *Escherichia coli* provided evidence that this is another multifunctional 20-oxidase that can convert GA_{12} to GA_9. The patterns of expression and known activity of At2353 are entirely consistent with the hypothesis that At2353 is the

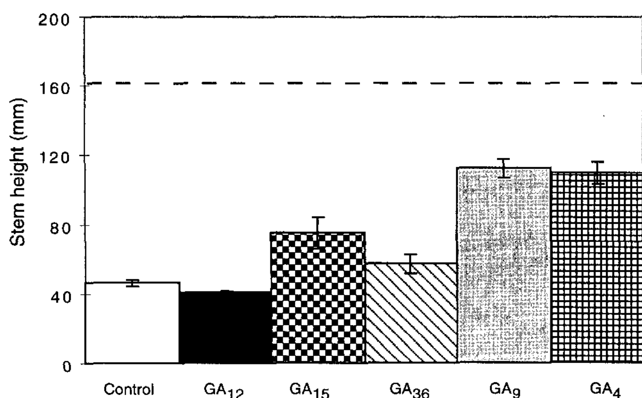

Figure 10. Response of *ga5-2 ga6-2* plants to GAs. Plants were grown in a controlled environment at 21°C with mixed incandescent and fluorescent light giving a 24-h photoperiod.

20-oxidase that is missing or mutated in *ga6-2*. Successful rescue of *ga6-2* with a genomic clone of At2353 would confirm our hypothesis. This rescue is to be attempted in collaboration with A. Phillips and P. Hedden.

The *ga5* and *ga6* mutants have additive phenotypes, as shown by the isolation of a double mutant (Table V; Fig. 3). The *ga5-2 ga6-2* mutant is an extreme dwarf with very short siliques. This phenotype is consistent with a plant that is missing both stem- and inflorescence-/silique-expressed 20-oxidases. *ga5-2 ga6-2*, despite having a growth habit almost identical to *ga1*, is different from *ga1* in that it does not require GA for germination or fertility. The lack of seed dormancy may be a consequence of the *abi3* background. *ga5-2* and *ga6-2* each germinate in an *ABI3* background, but no information is available yet for the double mutant. It is unlikely that the fertility of the double mutant is related to its ABA insensitivity, because the *abi3* allele is expressed only in seeds (Finkelstein and Somerville, 1990). The possibility that some bioactive GAs are produced even in the double mutant cannot be discounted.

The *ga6-1* and *ga6-2* mutants have indistinguishable phenotypes (Table II; Fig. 4) but respond differently to GA_{12} and GA_{15} (Fig. 5). Therefore, the interesting possibility exists that the two mutations affect different catalytic activities of the multifunctional 20-oxidase. Mapping the mutation and sequencing the corresponding gene may serve to shed light on the mechanism of this enzyme.

The *1.1* mutant also has altered response to C_{20}-GAs (Fig. 5) and could potentially be another 20-oxidase mutant. *1.1* has a phenotype like that of *ga5*, but is not allelic to *ga5*, suggesting the surprising possibility that there may be another stem-expressed 20-oxidase, which when mutated or missing will give a semidwarf phenotype, even in the presence of a functional GA5 protein. Although Phillips et al. (1995) have obtained a third 20-oxidase cDNA (YAP169) from a database of expressed sequence tags, it is expressed only in Arabidopsis siliques, and so would not be a candidate for an additional stem-expressed 20-oxidase. Whether the additional enzyme responsible for metabolizing GA_{15} in *ga5* seedlings could be the enzyme impaired in *1.1* plants

Table VI. *GA levels in whole shoots of abi3 (WT) and ga6-2 at an early bolting stage*

GA	WT	*ga6-2*
	ng 100 g^{-1} fresh wt	
GA_1	3.8	6.5
GA_{19}	54.4	69.8
GA_{20}	5.0	6.4
GA_{34}	59.0	97.2

is debatable. An alternative explanation, namely that *1.1* could be a mutation that regulates *GA5* expression, must also be examined. Additional work on *1.1*, specifically on $[^{14}C]GA_{12}$ and $[^{14}C]GA_{15}$ metabolism in *1.1* seedlings, and on the production of a *1.1 ga5* double mutant, is planned. Until this is accomplished the *1.1* mutation will not be assigned a gene symbol.

In conclusion, to our knowledge, *ga5-1* isolated by Koornneef and van der Veen (1980) was the only 20-oxidase mutant of Arabidopsis described until now. The *GA5* locus encodes a stem-expressed 20-oxidase (Philips et al., 1995; Xu et al., 1995). Several additional mutant lines with altered GA_{20} oxidation have now been obtained by mutagenesis. *11.2*, which is allelic to *ga5*, is named *ga5.2*. The locus defined by the *2.1* and *10.3* mutation is provisionally named *GA6*, with *2.1* being *ga6.1* and *10.3* being *ga6.2*. The *GA6* locus is purported to encode an inflorescence- and silique-expressed 20-oxidase, for which the clone At2353 isolated by Phillips et al. (1995) is a candidate. The 20-oxidation of GAs in stems and reproductive tissues by two different enzymes would facilitate both tissue- and development-specific regulation of GA biosynthesis. Whether the facile isolation of 20-oxidase mutants from the current mutagenesis was aided by use of an ABA-insensitive parent is not known, but it is known that an *abi3* background is not required for these mutant phenotypes to be expressed.

It is interesting to note that although mutations that inhibit *ent*-kaurene synthesis and 3β-hydroxylation have been isolated from many species, for example, pea, sweet pea, maize, rice, and tomato (for review, see Reid, 1993), 20-oxidase mutants are known only in Arabidopsis. 20-Oxidation is necessary for the production of C_{19}-GAs in all plants, and evidence is accumulating that up- and down-regulation of the expression of 20-oxidase genes can regulate the levels of bioactive GAs in, for example, spinach (Wu et al., 1996), pea (Martin et al., 1996), and rice (Toyomasu et al., 1997).

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