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Derivative Alleles of the Arabidopsis Gibberellin-Insensitive (*gai*) Mutation Confer a Wild-Type Phenotype

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The *gai* mutation of Arabidopsis confers a dwarf phenotype resembling that of mutants defective in gibberellin (GA) biosynthesis. However, *gai* mutant plants differ from GA biosynthesis mutants because they fail to respond to exogenous GAs and accumulate endogenous GA species to higher (rather than lower) levels than found in wild-type controls. The *gai* mutation, therefore, identifies a gene that modulates the response of plant cells to GA. We have mapped *gai* with respect to visible and restriction fragment length polymorphism (RFLP) markers from chromosome 1. To observe the phenotype exhibited by individuals potentially lacking wild-type (*GAI*) function, we have also isolated novel irradiation-induced derivative alleles of *gai*. When homozygous, these alleles confer a revertant phenotype that is indistinguishable from the wild type. *gai* is a semidominant mutation that exerts its effects either because it is a gain-of-function mutation or because it is a loss-of-function or reduced-function mutation. The genetic and physiological properties of the derivative alleles are considered with reference to these alternative modes of dominance of *gai*. Because these alleles are potential deletion or rearrangement mutations, together with the closely linked RFLP markers identified in the linkage mapping experiments, they provide useful resources for the isolation of the *gai* locus via a map-based cloning approach.

INTRODUCTION

The gibberellins (GAs) are associated with a number of plant developmental processes. Specific examples include seed germination (Karssen et al., 1989), stem elongation (Spray et al., 1984; MacMillan and Phinney, 1987; Talon and Zeevaart, 1990), regulation of gene expression in the cereal aleurone layer (Baulcombe and Buffard, 1983; Nolan and Ho, 1988; Fincher, 1989), and juvenile-to-adult phase transition and phyllotaxis (Maksymowych and Erickson, 1977; Marc and Hackett, 1991, 1992). GA-related mutants have been identified in several plant species (Reid, 1986). These mutants are classified according to their sensitivity to exogenously applied GA. In Arabidopsis, plants homozygous for recessive GA-sensitive dwarf mutations (at the *GA1*, *GA2*, *GA3*, *GA4*, and *GA5* loci) have been identified. These mutants are reduced in height, display reduced apical dominance, and are darker green than normal. In addition, some alleles confer a requirement for supplied GA during germination, and partial or complete male sterility. The phenotype of GA-sensitive dwarf mutants can be restored to normal by application of exogenous GAs (Koornneef and van der Veen, 1980; Talon et al., 1990a). The *ga4* and *ga5* mutants contain reduced levels of biologically active GA species, and the *GA4* and *GA5* loci appear to control enzymatic steps in the synthesis of these GAs (Talon et al., 1990a). Short-day floral initiation is significantly delayed, or even abolished, in *ga1* mutants. This effect is remedied by GA application and suggests that GA has an important role in floral initiation in short photoperiods (Wilson et al., 1992).

gai is a mutation conferring insensitivity to GA (Koornneef et al., 1985a). Plants containing *gai* resemble plants homozygous for mutations at the *GA1* to *GA5* loci; they are dwarfed, darker green, and display reduced apical dominance with respect to wild-type controls. Floral induction of *gai* can be delayed with respect to the wild type in short days (Wilson et al., 1992). Despite these phenotypic similarities to the GA-sensitive dwarf mutants, *gai* mutant plants are unresponsive to the application of exogenous GAs (Koornneef et al., 1985a; Wilson et al., 1992). Interestingly, plants containing *gai* accumulate endogenous biologically active GAs to significantly higher levels than found in wild-type controls (Talon et al., 1990b). The fact that plants containing *gai* do not respond to these elevated levels of endogenous GAs or to exogenous GA suggests that the locus (*GAI*) identified by the *gai* mutation may be involved with GA reception or signal transduction, or with some other process involved in the modulation of tissue sensitivity to GA. Mutants that display semidominant gibberellin-insensitive dwarfism associated with the accumulation of bioactive GAs have been described in several other species, including maize (*Dwarf-8* and *Miniplant* [*D8* and *Mpl1*], Fujioka et al., 1988; Harberd and Freeling, 1989), wheat (*Reduced height* [*Rht*] homeoallelic series, Stoddart, 1984; Lenton et al., 1987), and oilseed rape (*dwarf1* [*dwf1*], Zanewich et al., 1991). In addition to their intrinsic biological interest, these mutants are of significance to plant breeding and agriculture. For

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example, the use of the *Rht* homeoalleles has had a major impact on wheat breeding, resulting in the semidwarf bread wheat varieties now in widespread use (Gale and Youssefian, 1985).

This paper describes a genetic analysis of the Arabidopsis *GAI* and *GA4* loci. These loci are tightly linked within a small region of chromosome 1 (Koornneef et al., 1985b). Visible marker and restriction fragment length polymorphism (RFLP) linkage maps of the *GAI-GA4* region are presented. The goal of this work is to isolate the *GAI* and *GA4* loci. We have also initiated a mutational analysis of *gai*. The isolation of phenotypic revertant derivative lines (displaying wild-type phenotype) from *gai* homozygous material treated with ionizing radiation is described. Our purpose in isolating these lines was twofold. First, investigations of their genetic, molecular, and physiological properties enhance our understanding of how *gai* (and its presumed wild-type allele) modulates GA sensitivity. Second, such irradiation-induced derivative lines may contain deletion (Wilkinson and Crawford, 1991) or rearrangement (Shirley et al., 1992) mutant alleles of *GAI*, which will facilitate the identification of this locus in gene cloning experiments (Gibson and Somerville, 1992; Sun et al., 1992). Here we describe the genetic behavior and phenotype of four independently isolated *gai* derivative lines. Genetic analysis suggests that these lines contain secondary mutations at the *GAI* locus rather than extragenic suppressor mutations. Plants homozygous for these *gai* derivative alleles are indistinguishable from wild type, suggesting that wild-type (*GAI*) gene function may be dispensable. The significance of these observations is discussed with respect to the possible modes of dominance of the *gai* allele and the possible functions of the *GAI* gene product.

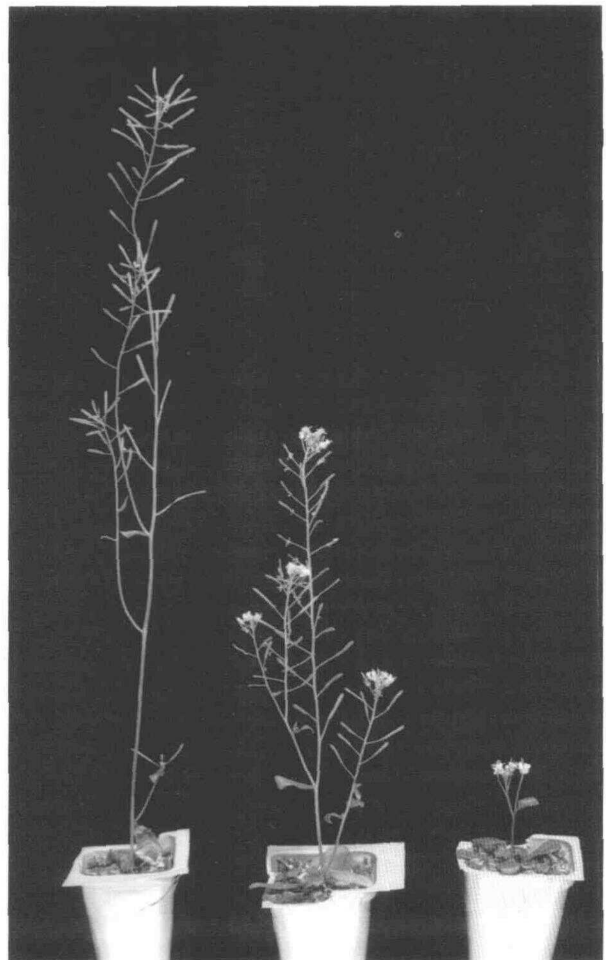

Figure 1. Plants Heterozygous and Homozygous for *gai*.

Shown from left to right are *GAI/GAI*, *gai/GAI*, and *gai/gai* plants segregating in the progeny of a self-pollinated *gai/GAI* plant.

RESULTS

gai Is a Semidominant Dwarfing Mutation

When *gai/gai* homozygotes are backcrossed to the progenitor Landsberg *erecta* strain, the resulting (F_1) *gai/GAI* heterozygotes are dwarfed and have increased numbers of axillary shoots. The severity of the heterozygous phenotype is, however, intermediate between that displayed by *gai/gai* homozygotes and *GAI/GAI* wild-type homozygotes. Upon self-pollination of *gai/GAI* heterozygotes, an F_2 population segregating individuals displaying severe (*gai/gai*), intermediate (*gai/GAI*), and wild-type (*GAI/GAI*) phenotypes is obtained (Koornneef et al., 1985a). Plants heterozygous and homozygous for *gai* are shown in Figure 1. Thus, *gai* is a semidominant mutation whose effect on phenotype is conditioned by gene dosage.

GAI Maps to the Top Arm of Chromosome 1

GAI and *GA4* map to the top arm of chromosome 1 at positions 21.8 and 21.9, respectively (Koornneef, 1987). Previous experiments yielded no detectable recombinant chromosomes with cross-over points between *GAI* and *GA4* (Koornneef et al., 1985b). In further experiments, we have identified such recombinant chromosomes, using the flanking markers *distorted trichomes* (*dis1*) and *thiamine requiring* (*th1*; see Methods). The results of these experiments confirm that the gene order published for the top arm of chromosome 1 (*dis1-gai-ga4-th1*; Koornneef, 1987) is correct and give an approximate distance of 0.6 centimorgans (cM) between *GAI* and *GA4* (N. P. Harberd, unpublished data).

We have identified an Arabidopsis (Landsberg *erecta*) transformant line (A264) in which a T-DNA construct conferring kanamycin resistance (*K*) is inserted into a region of chromosome 1 spanned by yeast artificial chromosome (YAC) clones EG11A5 and EG12E7 (from the Erwin Grill [EG] YAC library, Grill and Somerville, 1991; C. Recknagel and G. Coupland, unpublished data). These clones hybridize to RFLP marker

m322 (Hwang et al., 1991; J. Peng and N. P. Harberd, unpublished data). To improve the integration of the classic genetic and RFLP linkage maps of this region, we performed the crosses shown in Figure 2. Nineteen *K-gai* recombinant plants were identified, giving a genetic map distance of ~11 cM between these two loci. The data from this experiment are summarized in the linkage map shown in Figure 3A.

RFLP Mapping of the *GAI-GA4* Region

Although the top arm of chromosome 1 is well defined by RFLP markers (Chang et al., 1988; Nam et al., 1989), the RFLP and classic genetic maps have few markers in common, making accurate cross-reference between these maps difficult. Accordingly, we attempted to determine the position of *GAI* with respect

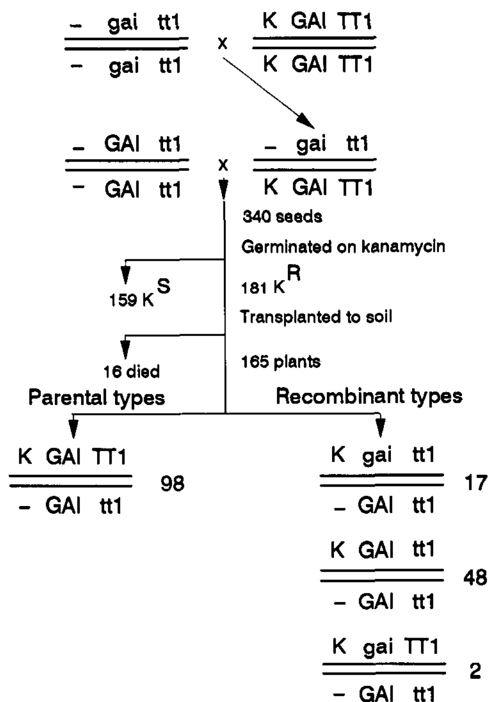

Figure 2. Linkage Analysis between the Kanamycin Resistance Marker (*K*) of the T-DNA Insertion in Transformant Line A264 and the *gai* and *tt1* Loci.

Plants homozygous for *gai* and for *tt1* were crossed as females with plants homozygous for the T-DNA insertion (*K/K*). The F_1 plants were then used to pollinate plants homozygous for *tt1*. The progeny (340 seeds) were germinated on medium containing kanamycin. Kanamycin-sensitive seedlings (159 K^S) bleached out and died, and kanamycin-resistant seedlings (181 K^R) were transplanted to soil. The surviving 165 plants were scored for *gai* and *tt1* phenotypes. The 16 plants that died following transplantation were all *GAI/GAI* homozygotes; *tt1* phenotypes of these plants could not be scored. The numbers of plants in each of the parental and recombinant genotype classes were as shown to the right of each genotype.

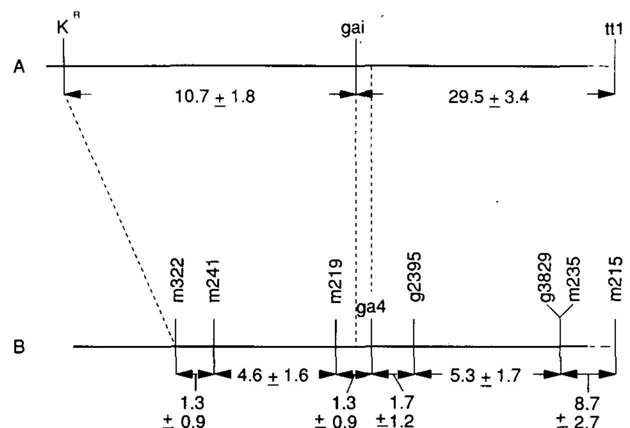

Figure 3. Comparison of Linkage Maps Containing the *gai* and *ga4* Loci.

(A) *gai* and visible markers. The linkage map was calculated from data given in Figure 2.

(B) *ga4* and RFLP markers. The linkage map was calculated from data given in Table 1.

All distances are in centimorgans plus/minus standard error. The T-DNA (A264) is inserted within ~150 kb of RFLP marker m322 (J. Peng and N.P. Harberd, unpublished data). *gai* is ~0.6 cM distal of *ga4* (see Methods). It is therefore likely that *gai* maps between m219 and g2395, although the confidence intervals do not allow us to exclude the possibility that *gai* may be distal of m219. Dotted lines indicate these approximate equivalent positions on the two maps. The right-hand end of each map (region of chromosome represented with broken lines) is not to scale. K^R, kanamycin resistance.

to RFLP markers. *gai* (Landsberg *erecta* background) was crossed to the Columbia and Niederzenz ecotypes with the intention of observing *gai* segregation in subsequent generations. However, we encountered great difficulty in scoring *gai* segregation in these populations, making them useless for the RFLP mapping of *gai*. Our experiments to RFLP map *GA4* were more successful than those with *gai*. Plants homozygous for *ga4* (Landsberg *erecta* background) were crossed with the Niederzenz and Columbia ecotypes. *ga4/ga4* homozygotes were identified in the F_2 generation and confirmed by checking that their (F_3) progeny (obtained through self-pollination) were uniformly dwarfed and did not segregate for tall (wild-type) individuals. *ga4/ga4* homozygotes were observed to segregate with approximate frequencies of one-twelfth in the F_2 populations, a frequency considerably lower than the theoretically expected one-fourth. This could be due either to a lower transmission frequency of the chromosome carrying *ga4* with respect to the transmission frequencies of the Niederzenz and Columbia wild-type chromosomes or to the effects of segregating modifier loci derived from the Niederzenz and Columbia genomes.

Genomic DNA was prepared from F_3 families (*ga4/ga4* homozygotes) obtained from the Niederzenz (68 families) and Columbia (22 families) crosses. Digests of each DNA preparation were tested for the presence of RFLP alleles associated

with the Landsberg *erecta* and Niederzenz or Columbia genomes. The markers used were m322, m241, m219, m235, and m215 (Chang et al., 1988), and g2395 and g3829 (Nam et al., 1989), all of which map in the vicinity of *GA4*. The results are shown in Table 1 and Figure 3B. *GA4* is located between markers m219 and g2395. Due to the proximity of *GAI* and *GA4*, it is likely that *GAI* also maps to the region spanned by these two markers. However, because *gai* was not mapped directly to m219 and g2395, their relative positions remain uncertain.

Identification of Irradiation-Induced *gai* Derivative Mutations

We have devised a method for the induction and recovery of secondary mutations causing loss of the *gai* mutant phenotype from material homozygous for *gai*. *gai/gai* homozygous seeds are treated with γ rays, thus inducing mutations in the cells of the embryo. Among the mutations induced are novel derivative alleles of *gai* (*gai-d*). Cells containing these novel alleles are heterozygous (*gai/gai-d*). Clonal analysis of the shoot apical meristem of the Arabidopsis embryo has shown that sectors containing the mitotic descendants of single meristematic cells can cover a substantial proportion of the mature plant body (Furner and Pumfrey, 1992; Irish and Sussex, 1992). Accordingly, the descendants of a meristematic cell of *gai/gai-d* genotype have the capacity to encompass one or more inflorescence meristems. The bolt stems elaborated from such meristems might display the less severely dwarfed phenotype of a *gai* heterozygote (*gai/GAI*), rather than the severely dwarfed phenotype of a *gai* homozygote (*gai/gai*). Thus, it might be possible to identify secondary mutations causing loss of *gai* phenotype because they result in sectors displaying increased shoot elongation and internode length. A potential advantage of this M1 screen is that it permits the identification and recovery of secondary mutations that are homozygous lethal or that cause impaired transmission of the chromosome carrying them.

Approximately 60,000 seeds homozygous for *gai* and for *transparent testa 1* (*tt1*) were treated with γ rays (for details see Methods). Following germination, the seedlings displayed obvious radiation damage. The emergence of the first pair of true leaves was considerably delayed and many seedlings died at this stage. The surviving seedlings grew on to produce adult plants that were, on the whole, relatively normal in their appearance (normal for *gai/gai*). However, 13 plants bearing bolt stems that were taller and had longer internodes than expected for a *gai/gai* homozygote were observed. Examples are shown in Figures 4A and 4B. These 13 stems displayed a phenotype similar to what might be expected for a *GAI/gai* heterozygote.

Table 1. Linkage Analysis between *ga4* and RFLP Markers on Chromosome 1

Markers	Recombination Frequency[a]	Number Scored[b]
m322	12	154
m241	10	180
m219	2	154
g2395	2	120
m235	11	180
g3829	6	102
m215	17	138

[a] Number of recombinant chromosomes identified.
[b] Number of chromosomes scored.

Genetic Analysis of Irradiation-Induced *gai* Derivative Mutations

Each of the 13 elongated bolt stems identified in the irradiated (M1) *gai tt1/gai tt1* material was allowed to self-pollinate. All seeds obtained were yellow (*tt1/tt1*), thus excluding the possibility that they may be wild-type seed contaminants. Seeds from each bolt stem were planted to determine if they had inherited any potential *gai-d* mutations.

The 13 bolt stems fell into three classes on the basis of their progeny tests, as shown in Table 2. First, seeds from nine of them (NA342A-1, NA342A-3, NA342A-4, NA342B-1, NA342B-2, NA342B-3, NA342C-1, NA342D-1, and NA342D-2) appeared to be uniformly homozygous for *gai*, and did not appear to segregate for taller individuals. Thus, the putative derivative mutations in these stems were not transmitted to the progeny. In the second class, seeds from another three stems (NA273A-1, NA342A-2, and NA342D-3) were found to segregate for plants homozygous for *gai*, plants resembling *gai/GAI* heterozygotes, and plants resembling wild-type (*GAI/GAI*) homozygotes, showing that in these cases, the putative derivative mutations are heritable. The plants resembling wild-type homozygotes are presumably homozygous for the novel derivative mutations. These derivative alleles are designated *gai-d1* (from NA273A-1), *gai-d2* (from NA342A-2), and *gai-d4* (from NA342D-3). They are likely to be secondary mutant alleles of *gai* rather than extragenic suppressor mutations (see below). Plants homozygous for these derivative alleles are indistinguishable from wild-type (*GAI/GAI*) homozygotes, and pure-breeding (homozygous) derivative lines have been maintained over several generations. In the third class, seeds from the final stem (NA342A-5) segregated *gai* homozygotes and plants resembling *gai* heterozygotes, but not plants resembling wild-type (*GAI/GAI*) homozygotes. The possible causes underlying the abnormal inheritance of this derivative (*gai-d3*) were investigated further (see below).

Are the Derivative Mutations Extra- or Intragenic Suppressors?

Derivative mutations *gai-d1*, *gai-d2*, and *gai-d4* may each have arisen in one of two ways. First, they may be secondary mutant alleles of *gai* that no longer specify the *gai* phenotype.

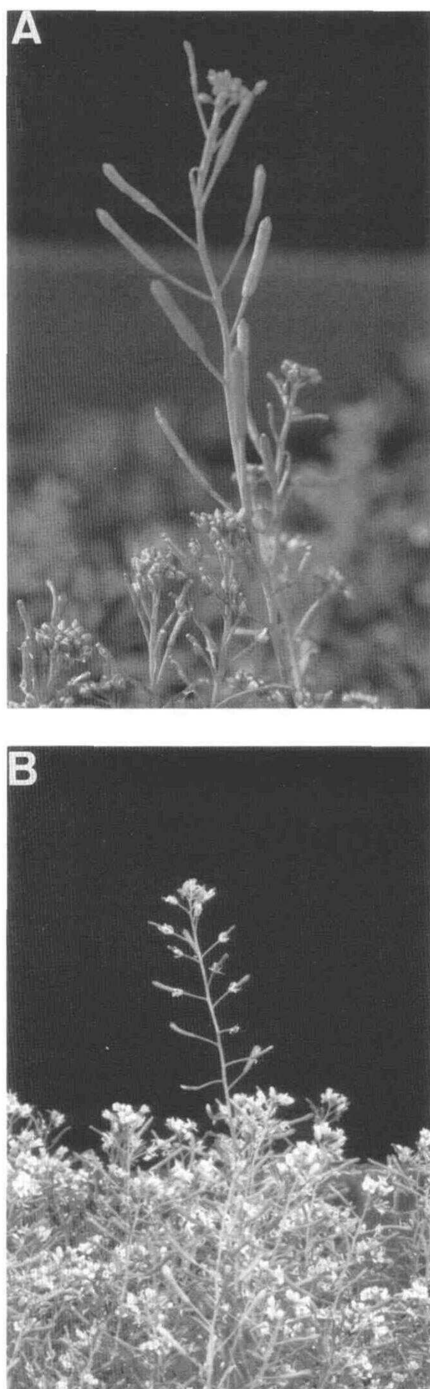

Figure 4. Identification of Elongated Bolt Stems Containing Novel Irradiation-Induced *gai* Derivative Mutations from Material Homozygous for *gai*.

(A) Elongated bolt stem NA273A-1 (from which *gai-d1* is derived).
(B) Elongated bolt stem NA342D-2.
These elongated stems can be seen against a background of bolt stems that are uniformly homozygous for *gai*.

Second, they may be mutations at loci (distinct from *gai*) that cause suppression of the phenotype conferred by *gai*. These two cases can be distinguished by crossing the (homozygous) derivative lines to Landsberg *erecta* and screening the F_2 generation for segregation of *gai*. Crosses involving secondary mutant alleles of *gai* would not be expected to segregate plants displaying the *gai* phenotype in the F_2 generation. Crosses involving unlinked extragenic suppressors of *gai* would be predicted to segregate plants containing *gai* but lacking the suppressor mutation in F_2. These plants would be expected to display the *gai* phenotype (see Methods). Plants from the *gai-d1*, *gai-d2*, and *gai-d4* homozygous lines (also homozygous for *tt1*) were crossed with Landsberg *erecta* (*TT1/TT1*). The F_1 plants looked completely normal. *gai* segregation was not observed in any of the F_2 populations obtained from these crosses (63 F_2 progeny from the *gai-d1* cross, 77 from the *gai-d2* cross, and 70 from the *gai-d4* cross). As expected, these F_2 populations segregated one-fourth of the plants bearing yellow seeds (*tt1/tt1* homozygotes). Note that because the mutations were originally identified as bolt stems resembling *gai/GAI* heterozygotes, any second-site suppressors would be predicted to be semidominant in their effects. The absence of individuals displaying the *gai* heterozygous or homozygous phenotype in the F_2 populations demonstrates that the derivative mutant lines do not contain unlinked semidominant second-site suppressor mutations. Thus, *gai-d1*, *gai-d2*, and *gai-d4* are likely to be allelic to *gai*. However, we cannot exclude the possibility that they may be second-site mutations closely linked to *gai* because the results of our experiments

Table 2. Segregation of Phenotypic Classes of Plants Obtained Following Self-Pollination of Elongated Bolt Stems Identified Following γ-Irradiation of *gai tt1/gai tt1* Homozygotes

Elongated Bolt Stems	*gai* Homozygote	*gai* Heterozygote[a]	Wild-Type Homozygote[b]
NA273A-1[c]	8	11	6
NA342A-1	24	0	0
NA342A-2[d]	3	16	4
NA342A-3	27	0	0
NA342A-4	10	0	0
NA342A-5[e]	13	10	0
NA342B-1	5	0	0
NA342B-2	13	0	0
NA342B-3	24	0	0
NA342C-1	14	0	0
NA342D-1	7	0	0
NA342D-2	14	0	0
NA342D-3[f]	5	11	5

[a] *gai/gai-d* genotype.
[b] *gai-d/gai-d* genotype.
[c] Progeny segregating *gai-d1*.
[d] Progeny segregating *gai-d2*.
[e] Progeny segregating *gai-d3*.
[f] Progeny segregating *gai-d4*.

do not distinguish between linked second-site suppressors and intragenic derivative alleles.

Further Analysis of *gai-d3*

As described above, self-pollination of the elongated stem on plant NA342A-5 resulted in progeny homozygous for *gai* and progeny resembling *gai* heterozygotes (*gai/gai-d3*), but no progeny resembling the wild type (*gai-d3/gai-d3*). This could have occurred for one or several of the following reasons. The chromosome bearing the *gai-d3* allele could be poorly transmitted through either male or female gametophytes (egg or pollen). Alternatively, the *gai-d3* allele could cause inviability when homozygous. Finally, the elongated stem on plant NA342A-5 may have been chimeric, allowing transmission of the *gai-d3* allele through only one of the male and female gametophytes. The inheritance of *gai-d3* was further investigated as follows.

Plant NA407-11, one of the progeny of NA342A-5 (elongated stem) that was scored as resembling a *gai* heterozygote (*gai/gai-d3* genotype), was allowed to self-pollinate. Approximately 200 of the progeny were planted and found to segregate for plants resembling *gai* heterozygotes (*gai/gai-d3*) and homozygotes (*gai/gai*). None of the progeny displayed the wild-type phenotype. Because plant NA407-11 should not have been chimeric, this result suggests that abnormal inheritance of *gai-d3* was not due to chimerism of the original elongating stem from which it was obtained. The inheritance of *gai-d3* in reciprocal crosses (in which the effects of using plants containing the derivative mutation as male or female are compared) was investigated. The results are presented in Table 3. When a *gai/gai-d3* plant was used as female in a cross with Landsberg *erecta* (*GAI/GAI*), 12 plants displaying the *gai* phenotype (*gai/GAI*) and six plants displaying the wild-type phenotype (*gai-d3/GAI*) were obtained. When the cross was performed in the other direction (*GAI/GAI* as female; *gai-d3/gai* as male), only one of 18 plants displayed the wild-type phenotype; the rest displayed the *gai* phenotype. The plants obtained from these crosses showed that *gai-d3*, when heterozygous over a wild-type (*GAI*) allele, confers a wild-type phenotype. In addition, transmission of *gai-d3* through the pollen appears to be impaired, a phenomenon that is frequently correlated with the presence of a deletion mutation (McClintock, 1942; Hake et al., 1989). At present, we cannot exclude the possibility that *gai-d3* may also be homozygous lethal.

Table 3. Reciprocal Cross Analysis of the Inheritance of *gai-d3*[a]

	Phenotype	
Cross	*gai* Heterozygote[b]	Wild Type[c]
gai/gai-d3 × *GAI/GAI*	12	6
GAI/GAI × *gai/gai-d3*	17	1

[a] Number of progeny displaying phenotypes shown following reciprocal crosses of a plant heterozygous for *gai-d3* (*gai/gai-d3*) with wild type (*GAI/GAI*). For each cross the female genotype is written first.
[b] *gai/GAI* genotype.
[c] *gai-d3/GAI* genotype.

Table 4. Test for Complementation of *ga4* by *gai-d3*[a]

	Phenotype	
Cross	*gai* Heterozygote[b]	Wild Type[c]
gai GA4/gai-d3 GA4 × *GAI ga4/GAI ga4*	10	9
GAI ga4/GAI ga4 × *gai GA4/gai-d3 GA4*	17	0

[a] Number of progeny displaying phenotypes shown following reciprocal crosses of a plant heterozygous for *gai-d3* (*gai GA4/gai-d3 GA4*) with a plant homozygous for *ga4* (*GAI ga4/GAI ga4*). For each cross the female genotype is written first.
[b] *gai GA4/GAI ga4* genotype.
[c] *gai-d3 GA4/GAI ga4* genotype.

In a second experiment, reciprocal crosses involving *gai-d3* heterozygotes (*gai-d3/gai*) and *ga4* homozygotes (*ga4/ga4*) were performed. The purpose of this experiment was to determine if *gai-d3* could complement *ga4*. The results are presented in Table 4. When a *gai-d3/gai* plant was used as female in crosses with a *ga4/ga4* homozygote, an approximate segregation of 1 plant displaying the wild-type phenotype (*gai-d3 GA4/GAI ga4*) to 1 plant displaying the *gai* phenotype (*gai GA4/GAI ga4*) was obtained in the F_1 generation. When the cross was done in the opposite direction (*GAI ga4/GAI ga4* as female; *gai-d3 GA4/gai GA4* as male), no wild-type plants were obtained, confirming the impaired pollen transmission of *gai-d3* observed in the preceding experiment. These results show that *gai-d3* complements *ga4*. Thus, if *gai-d3* is a deletion mutation, this deletion does not extend over *ga4*.

Further Analysis of the Phenotype Conferred by the *gai* Derivative Alleles

As described above, the visible phenotype of plants homozygous for *gai-d1*, *gai-d2*, or *gai-d4* is indistinguishable from that of wild-type (Landsberg *erecta*) plants. However, it was still possible that these plants might display altered responses to changes in endogenous GA levels. Accordingly, the effects of paclobutrazol, an inhibitor of GA biosynthesis, on growth of seedlings homozygous for *gai-d1*, *gai-d2*, and *gai-d4* were investigated. Paclobutrazol is a triazole derivative that specifically inhibits GA biosynthesis at the kaurene oxidase reaction (Hedden and Graebe, 1985). Seedlings were grown on GM medium (Valvekens et al., 1988) containing 10^{-5} M paclobutrazol. In these growth conditions, wild-type (Landsberg *erecta*) seeds display reduced germination, and the resultant seedlings are small, dark green, and compact (as would be expected for plants with reduced endogenous GA levels). Plants homozygous for *gai-d1*, *gai-d2*, or *gai-d4* are indistinguishable

from the wild type (Landsberg *erecta*) when grown in these conditions (data not shown). Thus, these mutants do not correspond to the *slender* genotypes described in other species. *slender* genotypes display GA-independent extension growth that is insensitive to reductions in endogenous GA levels (Potts et al., 1985; Chandler, 1988; Lanahan and Ho, 1988).

DISCUSSION

gai is a semidominant mutation of Arabidopsis conferring GA-insensitive dwarfism. The phenotype conferred by this mutation has several features in common with the *D8/Mpl1* mutants of maize and the *Rht* mutants of wheat. Genetic analyses of the *D8/Mpl1* and *Rht* alleles have indicated that the dominant effects exerted are due to these mutations being gain-of-function alleles (Gale and Marshall, 1975; Harberd and Freeling, 1989). In addition, clonal analysis of the phenotype conferred by *D8/Mpl1* (via x-ray–induced chromosome breakage) demonstrated that in plants heterozygous for *D8* or *Mpl1*, clones of cells that have lost these dominant alleles can display the wild-type phenotype. These experiments indicate that in chimeric plants the effects of *D8* and *Mpl1* can be limited to the tissues containing them and that sectors of tissue containing cells of altered genotype (with respect to *D8/Mpl1*) can be identified on the basis of an alteration in phenotype (Harberd and Freeling, 1989). On the assumption that *gai*-loss sectors in Arabidopsis would display similar properties, we devised a method for the isolation of mutant lines containing allelic derivatives of *gai*. This M1 screening technique is novel and may be useful for the analysis of other genes in Arabidopsis. Segregation analysis suggests that three of the mutations isolated (*gai-d1*, *gai-d2*, and *gai-d4*) are indeed apparent intragenic derivative alleles of *gai* rather than unlinked second-site suppressor mutations. The fourth mutation (*gai-d3*) displays inheritance properties consistent with the presence of a large-scale deletion or chromosomal rearrangement.

What might be the genetic basis for the generation of these derivative alleles? This depends on whether *gai* itself is a gain-of-function or a loss-of-function mutation. If *gai* is a gain-of-function mutation, then the *gai-d* alleles are most likely to be loss-of-function alleles (rather than revertant alleles with restored wild-type gene function). This is because there are likely to be many ways in which a gain-of-function allele can be inactivated, but relatively few ways in which wild-type function can be restored. Conversely, if *gai* is actually a loss-of-function (or reduced-function) mutation, then the *gai-d* alleles must have restored wild-type function. The following points favor the former hypothesis (*gai* gain-of-function; *gai-d* loss-of-function). First, the *D8/Mpl1* and *Rht* mutants of maize and wheat, which share many properties in common with *gai*, appear to be gain-of-function mutations. Second, because the generation of loss-of-function derivative mutations is more likely than the generation of restored wild-type function derivative mutations, the fact that we were able to isolate the *gai-d* alleles itself suggests that *gai* is a gain-of-function mutation. Third, if *gai-d3* is genuinely a deletion mutation, it seems unlikely that this would result in a restoration of wild-type gene function. Whereas these arguments make it more likely that *gai* is a gain-of-function mutation, none of them are conclusive, and the possibility that *gai* is actually a loss-of-function mutation remains. This question will finally be resolved when molecular analysis of *GAI*, *gai*, and *gai-d* alleles becomes possible.

Plants homozygous for the *gai-d1*, *gai-d2*, or *gai-d4* alleles are indistinguishable from wild-type (Landsberg *erecta*) Arabidopsis plants. If these are loss-of-function alleles of *gai*, then the phenotypes displayed show that loss-of-function alleles have no effect on phenotype and that wild-type gene function is dispensable. Mutational analyses of genes identified by dominant gain-of-function alleles in other organisms have demonstrated that it is not uncommon for their (presumed) loss-of-function alleles to confer wild-type phenotypes (Park and Horwitz, 1986). This can occur if the gene is a member of a family of two or more genes that are able to substitute for each other's functions. Interestingly, the GA-insensitive slender phenotype of pea provides a precedent for the involvement of duplicate gene functions in the control of GA sensitivity. Homozygosity for recessive alleles (*la* and cry^s) at both of the unlinked *La* and *Cry* loci is required to obtain the GA-insensitive slender phenotype (Reid et al., 1983), suggesting that each of the dominant alleles is able to substitute for the other in its absence. Thus, the fact that the Arabidopsis *GAI* (wild-type) gene product may be dispensable does not necessarily preclude it from involvement with the mediation of GA signal transduction. However, it is also possible that the *GAI* gene product is unique but not essential. It is difficult to imagine that a unique gene product playing an important role in GA signal transduction would be dispensable. Gain-of-function mutations are poor indicators of gene function, and it may be that *GAI* actually has a function unrelated to the action of GA.

The mapping data presented above show that *ga4* lies between RFLP markers m219 and g2395 on the top arm of chromosome 1. We have isolated YAC clones that contain DNA hybridizing to these markers, and we are bridging the distance between them by the isolation of further contiguous YAC clones. The proximity of *GAI* and *GA4* should enable us to isolate and study both loci. *GA4* is of particular interest because it probably encodes a protein with 3β-hydroxylase activity. This activity plays a crucial role in the conversion of GA molecules from a bioinactive to a bioactive form (Talon et al., 1990a). Due to its apparent involvement with the GA response, the molecular cloning of *gai* is clearly an important goal. Because at least one of the *gai* derivative alleles (*gai-d3*) has transmission properties suggestive of a deletion mutation, we intend to make use of these lines in our efforts to isolate the *gai* locus. Several possible strategies are available to us, including pulse field gel analysis of DNA hybridizing to linked markers (Joslyn et al., 1991) and genomic subtraction (Sun et al., 1992). In addition, we have shown that the T-DNA insertion in transformant A264 is located ∼11 cM distal of *gai*. Because this T-DNA carries a functional *Dissociation* (*Ds*) element (Swinburne et al.,

1992), it may be possible to use this element to isolate "knockout" alleles of *gai* and to use these for molecular isolation of the locus via transposon tagging. Molecular characterization of *gai* and of its wild-type allele will permit us to assess the role played by this locus in the modulation of the GA response and to determine if *gai* is indeed a gain-of-function mutation.

METHODS

Plant Mutant Lines

The *gibberellin-insensitive* (*gai*) mutant was isolated from the *Arabidopsis thaliana* Landsberg *erecta* progenitor strain following x-ray mutagenesis (Koornneef et al., 1985a). We obtained this mutant line from Maarten Koornneef (Wageningen Agricultural University, The Netherlands), who also supplied us with Landsberg *erecta*, *ga4*, *tt1*, and a line homozygous for several markers from the top arm of chromosome 1, including *dis1*, *ga4*, and *th1*. We also obtained *gai* (this material also originates from Maarten Koornneef) from Ruth Wilson (Michigan State University, Department of Energy Plant Research Laboratory, East Lansing, MI). The Columbia ecotype was provided by Chris Somerville (Michigan State University, Department of Energy Plant Research Laboratory, East Lansing, MI), and the Niederzenz ecotype was obtained from the Arabidopsis Information Service seed bank (Johann Wolfgang Goethe University, Frankfurt am Main, Germany). The T-DNA transformant line A264 was obtained from George Coupland (Cambridge Laboratory, John Innes Centre, Norwich, UK). *gai* is a semidominant mutation conferring GA-insensitive dwarfism; *ga4* is a recessive mutation conferring GA-sensitive dwarfism; *tt1* is a recessive mutation conferring a transparent testa (yellow seeds); *dis1* is a recessive mutation conferring distorted trichomes; *th1* is a recessive mutation conferring a requirement for thiamine. The genetic nomenclature used in this paper follows the conventions recommended at the Third International Arabidopsis Meeting (East Lansing, MI, April 1987; Koornneef and Stam, 1992). Genotypes are italicized; the wild-type genotype is capitalized (e.g., *GAI*), and the mutant genotype is represented in lowercase letters (e.g., *gai*).

Plant Maintenance

For plants grown in nonsterile conditions, seeds were imbibed on moistened filter paper at 4°C for 4 days (to break dormancy) and then planted on "Arabidopsis mix" (2 parts Levington's M3 potting compost: 1 part grit/sand). Plants were then grown in standard glasshouse conditions, in a short-day glasshouse in which the photoperiod was artificially reduced to 10 hr, or in controlled environment chambers (long-day 18-hr photoperiod; short-day 10-hr photoperiod).

For plants grown in sterile conditions, seeds were first surface sterilized (Swinburne et al., 1992) and then sown on GM medium (Valvekens et al., 1988) supplemented (where appropriate) with 50 mg/L kanamycin. Kanamycin-resistant seedlings were subsequently transplanted into Arabidopsis mix and grown in the glasshouse. In other experiments, sterilized seeds were sown on Petri plates containing GM supplemented with 10^{-5} M paclobutrazol (obtained from Peter Hedden, Long Ashton Research Station, Bristol, UK). These plants were grown in continuous (24-hr) light, and the responses of the various genotypes were compared by visual estimation. Transgenic plants were grown according to United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF) regulations (License Number PHF 1418/8/22).

Detection of Recombination between *gai* and *ga4*

DIS1 GAI ga4 TH1/DIS1 GAI ga4 TH1 homozygous plants were pollinated with *DIS1 gai GA4 TH1/dis1 GAI ga4 th1* heterozygotes. The F_1 seeds obtained from this cross were planted out. All plants should have displayed the dwarf phenotype (*GAI ga4/gai GA4* and *GAI ga4/GAI ga4*), except for those carrying a wild-type recombinant chromosome (*GAI ga4/GAI GA4*), which would be tall. (Note that plants containing the reciprocal recombinant chromosome, *GAI ga4/gai ga4*, will display the dwarf phenotype and will not be detectable.) Three tall and 943 dwarf individuals were obtained, giving an approximate distance of 0.6 centimorgans (cM) between *GAI* and *GA4*. The three tall individuals were allowed to self-pollinate and their (F_2) seeds were planted. All three F_2 families segregated for *dis1*. This marker is relatively close to *gai* and *ga4* (~4 cM distal of this region). The presence of *dis1* in all three families suggests that the gene order published for the top arm of chromosome 1 (*dis1-gai-ga4-th1*; Koornneef, 1987) is correct. In addition to segregating for *dis1*, one of the F_2 families also segregated for *th1*. Because *th1* maps 11 cM proximal of *ga4*, this segregation probably represents a cross-over event independent of recombination between *gai* and *ga4*.

Restriction Fragment Length Polymorphism Mapping

Restriction fragment length polymorphism (RFLP) markers are designated as described in the AAtDB *A. thaliana* data base (Hauge and Goodman, 1992). The λ clone RFLP markers m322, m241, m219, m235, and m215 (Chang et al., 1988) were obtained from Elliot Meyerowitz (California Institute of Technology, Pasadena, CA). Phage DNA (prepared as described in Sambrook et al., 1989) was used directly as a probe in hybridization experiments. Cosmid clone RFLP markers g2395 and g3829 (Nam et al., 1989) were provided by Brian Hauge and Howard Goodman (both Harvard Medical School and Massachusetts General Hospital, Boston, MA). Cosmid DNA was prepared as described by Sambrook et al. (1989). This DNA was digested with EcoRI and passed through Sepharose CL-6B before being used as a hybridization probe.

Plant genomic DNA was prepared using a CTAB extraction method (Murray and Thompson, 1980) and digested with excess restriction enzymes (EcoRI, BglII, PvuII, and HindIII). The digested DNA was size fractionated via electrophoresis on a 0.8% agarose gel (TAE, 70 mA, run overnight; Sambrook et al., 1989). After electrophoresis, the gels were treated with 0.2 M HCl for 10 min, denatured twice with 0.5 M NaOH, 1.5 M NaCl for 15 min each, and finally neutralized with two washes in 3 M NaCl, 1 M Tris-HCl, pH 5.5, for 15 min each. DNA was transferred from the gel to Hybond-N (Amersham International). The methods of electrophoresis, depurination, denaturation, neutralization, and blotting were all essentially as described by Sambrook et al. (1989). After transfer, the DNA was cross-linked to the filter by UV treatment and subsequent baking at 80°C for 2 hr.

The ^{32}P-labeled DNA probes were prepared by random primer extension (Feinberg and Vogelstein, 1983). Filters were prehybridized and hybridized without formamide (Sambrook et al., 1989). Hybridization took place for 18 hr at 65°C. Filters were washed once with 3 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS for 25 min at 65°C, once with 0.1 × SSC, 0.1% SDS for 25 min at 65°C, and then exposed to Kodak XAR film. Recombinant individuals were

identified by comparison of band positions on the autoradiographs with those obtained from the *ga4/ga4* (Landsberg *erecta*), Niederzenz, and Columbia parents. Before use in further hybridization experiments, the filters were stripped by submerging them in boiling 0.1% SDS and then allowing them to cool to 60°C. The filters were then stored at −20°C until further use.

Genetic linkage distances were calculated as described by Koornneef and Stam (1992).

γ-Irradiation Mutagenesis

γ-Irradiation mutagenesis was performed using the cesium-137 source at the University of Nottingham, United Kingdom. Approximately 10,000 dry *gai tt1/gai tt1* seeds were exposed to 90 kR γ rays; another group of ∼50,000 dry seeds of the same genotype were treated with 70 kR γ rays. Seeds were then chilled and planted as described above. *gai-d1* was obtained from the 90 kR-treated material. *gai-d2*, *gai-d3*, and *gai-d4* were all obtained from the 70 kR–treated material.

Second-Site Suppressor Analysis

Plants homozygous for *gai-d1*, *gai-d2*, or *gai-d4* display the wild-type phenotype. Experiments to determine if this phenotype results from the action of an unlinked secondary mutation that suppresses the phenotype conferred by *gai* were performed. Because the mutational events were originally identified as bolt stems resembling *gai/GAI* heterozygotes, any such second-site suppressors would be predicted to be semidominant in their effects. On the asssumption that the *gai-d1*, *gai-d2*, and *gai-d4* lines had indeed carried unlinked semidominant suppressors of *gai*, the segregations expected in the F_2 generation of the backcross experiments (described in the text) can be calculated using a Punnett square (data not shown). Under such circumstances, plants displaying the *gai* heterozygous or homozygous phenotype would have been expected to have segregated with a minimum frequency of 5 *gai* phenotype to 11 wild-type phenotype. This is a minimum estimate because genotypes of unknown phenotype would also segregate in these F_2 families. Because no individual displaying the *gai* phenotype was observed in these F_2 families, the hypothesis that the *gai-d1*, *gai-d2*, and *gai-d4* lines contain unlinked second-site suppressor mutations can be excluded.

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