The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses

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The *Arabidopsis gai* mutant allele confers a reduction in gibberellin (GA) responsiveness. Here we report the molecular cloning of *GAI* and a closely related gene *GRS*. The predicted GAI (wild-type) and gai (mutant) proteins differ only by the deletion of a 17-amino-acid segment from within the amino-terminal region. GAI and GRS contain nuclear localization signals, a region of homology to a putative transcription factor, and motifs characteristic of transcriptional coactivators. Genetic analysis indicates that GAI is a repressor of GA responses, that GA can release this repression, and that gai is a mutant repressor that is relatively resistant to the effects of GA. Mutations at *SPY* and *GAR2* suppress the *gai* phenotype, indicating the involvement of GAI, SPY, and GAR2 in a signaling pathway that regulates GA responses negatively. The existence of this pathway suggests that GA modulates plant growth through derepression rather than through simple stimulation.

[*Key Words: Arabidopsis thaliana; gai;* gibberellin; mutant; signal transduction]

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Gibberellins (GAs) are tetracyclic diterpenoid growth factors that are essential regulators of stem elongation and other plant developmental processes (Hooley 1994; Swain and Olszewski 1996). GA-related mutants have been identified in several plant species, including *Arabidopsis* (Ross 1994). GA-deficient *Arabidopsis* mutants display characteristic phenotypes, including dark green leaves and a dwarf growth habit attributable to reduced stem elongation (Koornneef and van der Veen 1980; Talon et al. 1990a; Sun and Kamiya 1994; Peng and Harberd 1997). *gai* is a semidominant mutation of *Arabidopsis*, which also confers a dark green, dwarf phenotype (Koornneef et al. 1985; Peng and Harberd 1993, 1997; Wilson and Somerville 1995). The *gai* mutation affects GA reception or subsequent signal transduction, and does not result in GA deficiency (Koornneef et al. 1985; Talon et al. 1990b; Wilson et al. 1992; Peng and Harberd 1993; Wilson and Somerville 1995).

Dominant mutations conferring visible phenotypes resembling those attributable to GA deficiency are also known in other plants, including maize (*D8* allelic series; Harberd and Freeling 1989; Winkler and Freeling 1994) and wheat (*Rht* homeoallelic series; Gale et al. 1975). Previous genetic and physiological analyses of *gai*, *D8*, and *Rht* indicate that all are gain-of-function mutations (Gale et al. 1975; Harberd and Freeling 1989; Peng and Harberd 1993; Winkler and Freeling 1994; Wilson and Somerville 1995) conferring reduced GA responses and increased endogenous GA levels (Lenton et al. 1987; Fujioka et al. 1988; Talon et al. 1990b). The increased endogenous GA levels found in *gai*, *D8*, and *Rht* mutants are likely to arise through perturbation of the feedback control mechanisms by which GAs regulate *in planta* GA levels negatively (Croker et al. 1990; Chiang et al. 1995; Phillips et al. 1995; Xu et al. 1995). These dominant GA-response mutations are of considerable agricultural significance. The *Rht* mutations are especially important because they are the genetic basis of the high-yielding, semi-dwarf wheat varieties of the ''green revolution'' (Gale and Youssefian 1985). We cloned *GAI* to enhance our understanding of the mechanisms of GA signal transduction, and because of the potential use for *gai* in crop improvement.

Previous experiments had identified a T-DNA insertion, genetically linked to *GAI*, which contained a *Ds* transposable element (Peng and Harberd 1993). This *Ds* was used to clone *GAI* through targeted insertional mutagenesis. Comparison of *GAI* and *gai* DNA sequences shows that the predicted mutant protein (gai) lacks a short (17-amino-acid) segment of the GAI protein sequence. We propose that this structural alteration is re-

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sponsible for the dominant, gain-of-function properties of *gai*. In addition, presumed null alleles of *GAI* confer increased resistance to the growth-retarding effects of paclobutrazol (PAC), an inhibitor of GA biosynthesis. These observations suggest the following hypotheses to explain the role of GAI in GA signaling. First, GAI is proposed to be a negative regulator that represses GA responses but whose activity is opposed by GA. Second, gai is proposed to be a mutant repressor that is relatively resistant to the effects of GA and, therefore, maintains repression irrespective of the presence of GA.

Several recent publications have described extragenic mutations that suppress the phenotype conferred by *gai* (Carol et al. 1995; Wilson and Somerville 1995; Jacobsen et al. 1996) or by GA deficiency mutations (Jacobsen and Olszewski 1993; Silverstone et al. 1997). Here we extend the analysis of the phenotypes conferred by two of these suppressors (*spy-7* and *gar2-1*). First, we compare the effects of *spy-7* and *gar2-1* (alone and in combination) on the growth of and PAC resistance of plants containing *gai*. We have also investigated the effects of *spy-7* and *gar2-1* on the regulation of GA biosynthesis, by comparing the steady-state levels of gene transcripts encoding GA C-20 oxidase, the enzyme that catalyzes the penultimate step in the synthesis of biologically active GAs (Phillips et al. 1995; Xu et al. 1995). Finally, we have investigated the effects of *spy-7* and *gar2-1* on steady-state levels of *gai* transcripts.

The results of the above experiments indicate that GAI, SPY, and GAR2 operate within, or modulate, a signal-transduction pathway that represses growth and whose activity is opposed by GA. Because of the existence of mutations having comparable effects to *gai* and *spy* in other plant species (Swain and Olszewski 1996), and because GA is an essential growth regulator in a wide variety of plant species (Hooley 1994), it seems likely that the *Arabidopsis GAI, SPY,* and *GAR2* genes define a system for GA-mediated growth regulation that is common to all higher plants.

Results

Cloning of gai *through insertional mutagenesis*

gai maps to chromosome 1 of *Arabidopsis* (Koornneef et al. 1985; Peng and Harberd 1993), ~11 cM from a T-DNA insertion (A264) carrying a *Ds* transposon (Peng and Harberd 1993; Balcells et al. 1991). Genetic analyses suggested that loss-of-function alleles of *gai* confer a tall plant phenotype, similar to that conferred by the wild-type allele (*GAI;* Peng and Harberd 1993; Wilson and Somerville 1995). Therefore, we isolated *Ds* insertion loss-of-function alleles of *gai*, exploiting the tendency of *Ds* (in the presence of the *Ac* transposase) to transpose preferentially to linked sites (Bancroft and Dean 1993; Jones et al. 1994). We constructed plant lines homozygous for both *Ds*-bearing T-DNA insertion A264 (Peng and Harberd 1993) and *gai*, and also containing a transgene expressing *Ac* transposase (Δ*Nae*I–s*Ac*(GUS)-1; Bancroft and Dean 1993). Potential *Ds* insertion *gai* alleles were isolated from F_1 to F_4 generations of this material. The plants were screened for reduction or loss of the dark green, dwarf phenotype conferred by *gai*, by searching for rare individuals that were paler green and taller than expected for a *gai* homozygote (Peng and Harberd 1993). NA735B-1 was one such plant, being taller and paler green than a *gai* homozygote (*gai*/*gai*), but not as tall or pale as a wild-type (*GAI*/*GAI*) homozygote. In accord with previous observations (Peng and Harberd 1993), NA735B-1 was identified provisionally as a *gai* heterozygote of genotype *gai*/*gai-t6*, where *gai-t6* was a new allele possibly carrying a *Ds* insertion. Self-pollination of NA735B-1 resulted in a progeny population that segregated for *gai* homozygotes, *gai* heterozygotes (*gai*/*gai-t6*), and a new class of plants (*gai-t6*/*gai-t6*) displaying a tall phenotype similar to that of wild type. Plants homozygous for *GAI*, *gai*, and *gai-t6* are shown in Figure 1A.

DNA gel-blot experiments (using a *Ds* hybridization probe; see Materials and Methods) revealed that *gai-t6* contains two *Ds* elements, one in the original position (as in A264), the other (transposed *Ds*) in a new position (Fig. 1B). Using map-based cloning methods we had previously isolated *Arabidopsis* genomic DNA fragments spanning an ~200-kb segment of chromosome 1 known to contain *GAI* (see Materials and Methods; P. Carol, D.E. Richards, R. Cowling, J. Peng, and N.P. Harberd, unpubl.). An IPCR fragment (JP95) containing genomic DNA adjacent to the 3′ end of the transposed *Ds* in *gai-t6* (see Materials and Methods) hybridized specifically to DNA from within this segment, suggesting that this *Ds* is inserted into, or in the vicinity of, *gai* (P. Carol, J. Peng, D.E. Richards, and N.P. Harberd, unpubl.). A cDNA (insert of pPC1) was identified through hybridization to cosmid JP2 (see Materials and Methods) and JP95. DNA gel-blot experiments showed that the transposed *Ds* in *gai-t6* interrupts the gene (*GAI*) encoding the mRNA represented in pPC1 (Fig. 1C).

In addition to *GAI,* the pPC1 cDNA also hybridizes with a genomic fragment containing a second gene *GRS* (for *GAI*-related sequence) (Fig. 1C). A cDNA clone containing *GRS* sequence was identified through hybridization with probes containing the *GAI* sequence. Although *GAI* maps to chromosome 1 (see above), *GRS* is located close to the top of chromosome 2 (K. King, P. Carol, and N.P. Harberd, unpubl.).

The gai *mutant allele encodes an altered product*

The DNA sequences of two overlapping *GAI* cDNAs revealed an open reading frame (ORF) encoding a protein (GAI) of 532 amino-acid residues. DNA fragments containing this ORF were amplified from *GAI* and *gai* genomic DNA; their sequences showed that the *GAI* ORF is not interrupted by introns. The predicted primary sequence of the GAI and *gai* proteins is shown in Figure 2A. The gai allele contains a deletion of 51-bp from within the *GAI* ORF. This in-frame deletion results in the absence of a 17-amino-acid residue segment situated close to the amino terminus of the predicted protein se-

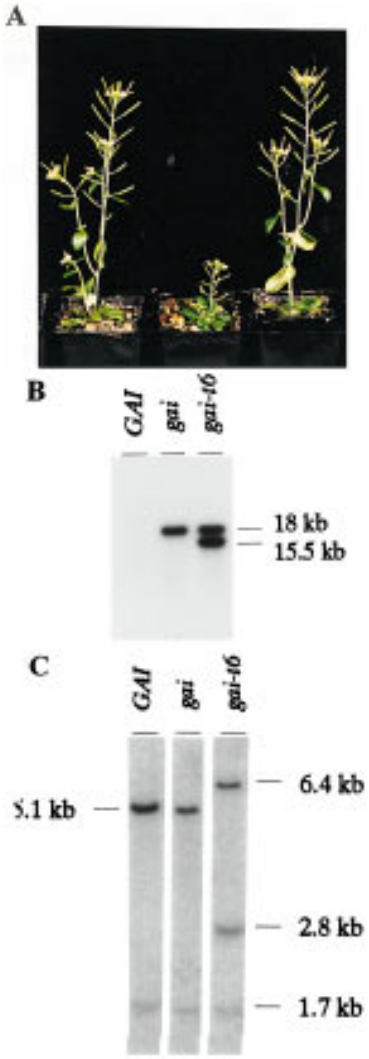

Figure 1. The *gai-t6* line contains a transposed *Ds* that interrupts a transcribed gene. (*A*) Plants shown are (*left* to *right*) homozygous for *GAI*, *gai*, and *gai-t6*. *GAI* and *gai-t6* plants display a tall phenotype. (*B*) DNA gel-blot hybridization using a radiolabeled *Ds* probe. DNA in the *GAI* lane lacks *Ds*. The *gai* lane contains DNA from plants homozygous for *gai* and for T-DNA A264, which contains *Ds* (18.0-kb *Eco*RI fragment). The *gai-t6* lane contains DNA from plants homozygous for A264 and for a transposed *Ds* (15.5-kb fragment). *gai-t6* has lost Δ*Nae*I–s*Ac*(GUS)-1 through genetic segregation. (*C*) DNA gel-blot hybridization using a radiolabeled *GAI* cDNA probe. The cDNA (insert of pPC1) hybridizes with a 5.1-kb *Bcl*I fragment in DNA from *GAI* and *gai*, replaced in *gai-t6* by 6.4- and 2.8-kb fragments. Because *Bcl*I cuts once within *Ds*, the *Ds* insertion is flanked on either side by the gene (*GAI*) encoding the cDNA. The fainter hybridization at 1.7 kb identifies *GRS*.

quence (Fig. 2A). There are no other differences between the proteins encoded by *GAI* and *gai*.

Figure 2A also shows the predicted primary sequence of the *GRS* gene product. *GRS* encodes a protein (GRS) of 587 amino acids, somewhat larger than *GAI*. GRS shares a high degree of sequence similarity with GAI (83% amino acid identity) and contains a region of identical amino acid sequence to the segment that is deleted in the gai mutant protein.

The gai-derivative *alleles contain mutations that disrupt the* GAI *ORF*

A series of presumed *gai-derivative* alleles conferring tall phenotypes similar to that conferred by *GAI* were isolated after γ-irradiation mutagenesis of *gai* (Peng and Harberd 1993). These alleles (*gai-d1*, *gai-d2*, *gai-d5*, and *gai-d7*) contain the 51-bp deletion characteristic of *gai* (thus confirming that they are derived from *gai*), together with additional mutations that disrupt the *GAI* ORF (Table 1). Thus, loss of *gai* mutant phenotype is associated with the occurrence of mutations that may result in a nonfunctional gene product. Furthermore, in reversion experiments, excision of *Ds* from *gai-t6* was associated with restoration of a genetically dominant, dwarf phenotype (J. Peng, P. Carol, D.E. Richards, and N.P. Harberd, unpubl.). These observations confirm that the transposed *Ds* in *gai-t6* is inserted within *GAI*, and that *GAI* has been cloned. They are also consistent with predictions that the *gai-d* alleles would be null alleles (Peng and Harberd 1993; Wilson and Somerville 1995).

GAI contains a consensus nuclear localization signal, a LXXLL motif, and is a new member of the VHIID domain family

Searches of the DNA and protein sequence databases using the BLAST program (Altschul et al. 1990) revealed that GAI is closely related in sequence to the predicted product (SCR) of a recently cloned *Arabidopsis* gene *SCARECROW* (*SCR;* Di Laurenzio et al. 1996), a member of a novel family of candidate transcription factors (Fig. 2B). GAI has homology to the carboxyl terminus of SCR, especially to the VHIID domain that characterizes the new family. GAI contains two heptad repeat regions similar to leucine zippers (GAI amino acid residues 169–203 and 316–336), as described previously for SCR (Fig. 2B; Di Laurenzio et al. 1996). GAI contains the leucine zipper region of the proposed basic leucine zipper (bZIP) domain in SCR, but lacks the basic domain (Di Laurenzio et al. 1996). There is no significant homology between GAI and SCR in regions amino terminal to the area shown in Figure 2B. A short segment of GAI (amino acid residues 403–427), and also of SCR, shows homology with cdr 29 (BLAST Poisson probability score for GAI: $1.2 \times e^{-5}$), a barley homolog of peroxisomal acyl CoA oxidase genes (Grossi et al. 1995). The significance of this finding is unknown.

Using PSORT, a program for the prediction of protein localization in cells (Nakai and Kanehisa 1992), the highest score assigned to GAI was for nuclear localization (certainty value = 0.760). The GAI protein contains two basic regions that are characteristic of nuclear localization signals (NLSs). The first region (^{206}RKVATYFAEA-

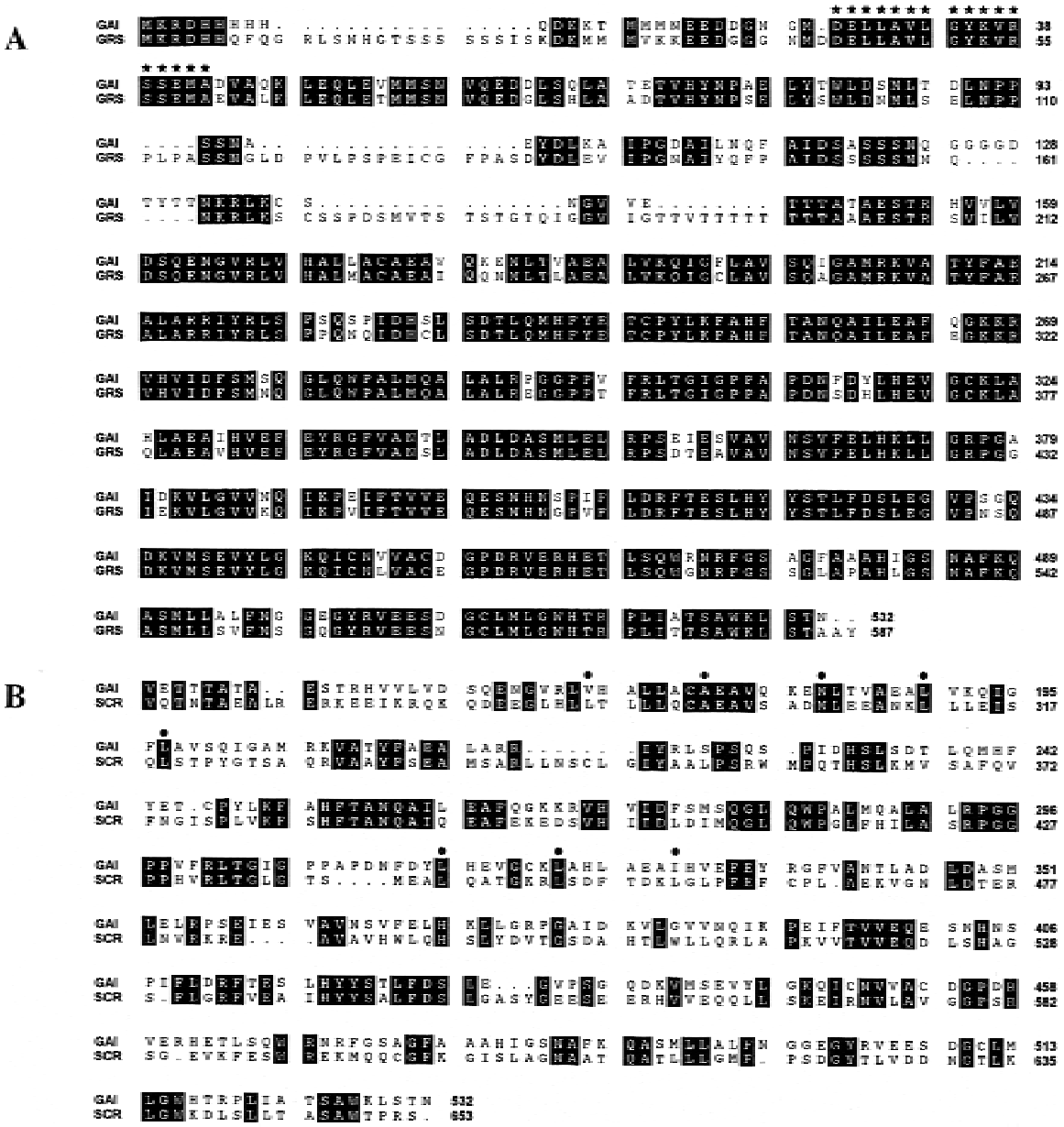

Figure 2. Analysis of GAI, gai and GRS amino acid sequences. (*A*) Alignment of the amino acid sequences (single-letter code) of GAI (predicted from the genomic DNA sequence of *GAI*) and GRS (from cDNA sequence) is shown. Gaps are introduced to maximize alignment, and identical amino acid residues are highlighted in black. The 17 amino acids missing in the gai mutant protein (D^{27}–A^{43}) have black stars above them. The *Ds* in *gai-t6* is inserted between the E^{182} and N^{183} codons. (*B*) Alignment of the carboxyl termini of GAI (from V^{143}) and SCR (from V^{263}) is shown. The third row of the comparison shows the VHIID domain described in Di Laurenzio et al. (1996). Residues defining leucine heptad repeats are identified by closed circles. (*C*) Comparison of bipartite nuclear localization signals in GAI, GRS, and other proteins. Basic amino acids are shown in bold uppercase letters. Sequence information is from the following: for TGA-1A, Opaque-2 (NLS-B; O2) and VirD2, see Raikhel (1992); for TSL, see Roe et al. (1993, 1997); for Ac see Boehm et al. (1995); for p53, see Dingwall and Laskey (1991); for IL-5, see Jans et al. (1997); for Nucleoplasmin and N1, see Robbins et al. (1991).

Table 1. *Mutations in* GAI *alleles*

Allele	Nature of mutation[a]	Position in coding sequence	Consequence of mutation
gai-d1	CAG to TAG	Gln^{239}	stop codon, truncated polypeptide
gai-d2	GAT to GA, 1-base deletion	Asp^{274}	frameshift, addition of two novel amino acids, truncated polypeptide
gai-d5	7-base deletion, also C to G	follows Leu^{281}	frameshift, addition of 18 novel amino acids, truncated polypeptide
gai-d7	GTT to GT, 1-base deletion	Val^{156}	frameshift, addition of 27 novel amino acids, truncated polypeptide

[a]Underlining denotes nucleotide substitution in each allele.

LARRIYR^{222}) exactly fits the consensus for bipartite NLSs, which has been defined as two basic amino acid residues, a spacer region of ~10 residues, and at least three basic residues out of the next five (Fig. 2C; Dingwall and Laskey 1991; Robbins et al. 1991; Raikhel 1992). In addition, GAI contains a second basic region (^{134}KRLK^{137}) that conforms to the consensus (K-R/K-X-R/K) proposed for nontypical SV40-like NLSs (Boulikas 1994; LaCasse and Lefebvre 1995). The presence of these sequences suggests that GAI may be targeted to the nucleus. Interestingly, GAI also contains two motifs, ^{169}VHALL^{173} and ^{370}LHKLL^{374}, which are, respectively, closely related and identical to a consensus motif (LXXLL) that has been shown recently to mediate binding of transcriptional coactivators to nuclear receptors (Heery et al. 1997).

All of the features described above for GAI (SCR homology, cdr 29 homology, NLSs, LXXLL motifs), are also found in GRS, suggesting that GAI and GRS have similar functions.

GAI *null alleles confer increased resistance to PAC*

PAC is a triazole derivative that inhibits GA biosynthesis at the kaurene oxidase reaction (Hedden and Graebe 1985; Davis and Curry 1991). Wild-type *Arabidopsis* plants require GA for seed germination and stem elongation (Koornneef and van der Veen 1980), and depletion of endogenous GA levels by PAC inhibits these processes (Jacobsen and Olszewski 1993). There are several plant mutants that display increased resistance to the effects of PAC. Among these are the *la cry*^{s} and *slender* mutants of pea and barley (Brian 1957; Potts et al. 1985; Chandler 1988; Lanahan and Ho 1988; Croker et al. 1990), and the *spy* mutants of *Arabidopsis* (Jacobsen and Olszewski 1993; Jacobsen et al. 1996). These mutants exhibit growth that, to varying degrees, is less dependent on GA than is the growth of wild-type plants. Thus, in the *la cry*^{s}, *slender*, and *spy* mutants, stem elongation is at least partially uncoupled from the GA-mediated control characteristic of normal plants.

Experiments to determine whether the *gai-t6* allele confers greater PAC resistance than the *GAI* allele were performed. The purpose of these experiments was to determine whether a loss-of-function allele of *GAI* confers a reduction in the GA dependency of growth. We chose *gai-t6* for these experiments because it is a *Ds* insertion mutation, and a likely null allele. Initial experiments showed that *gai-t6* does not germinate on 10^{-4} M PAC, a PAC concentration that permits germination of *spy* mutants (data not shown; Jacobsen and Olszewski 1993). However, as shown in Figure 3, *gai-t6* does confer increased PAC resistance; when grown on medium containing 10^{-6} M PAC, *gai-t6* mutants display longer floral bolt stems than *GAI* control plants. This result suggests that loss of *GAI* function causes a reduction in the GA dependency of stem elongation, that plants lacking GAI require less GA than normal plants for equivalent growth, and that GAI is a negative regulator of GA responses. However, the degree of PAC resistance conferred by *gai-t6* is less than that conferred by the currently available *spy* alleles. One explanation for the fact that *gai-t6* does not confer strong PAC resistance is that GRS can compensate substantially for loss of GAI.

gai-suppressor *mutations have additive effects on stem elongation and PAC resistance*

Screens for extragenic suppressors of the dwarf phenotype conferred by *gai* identified mutations at the *GAS1* and *GAR2* loci (Carol et al. 1995; Wilson and Somerville 1995). Complementation analysis showed that *gas1-1* is a *spy* allele (now renamed *spy-7;* data not shown). This result is consistent with the observation that the *spy-4* and *spy-5* alleles suppress the dwarf phenotype conferred by *gai* (Wilson and Somerville 1995; Jacobsen et al. 1996). *gar2-1*, however, identifies a distinct genetic locus that segregates independently of *SPY*. A recent report describes the isolation of multiple alleles at the *Arabidopsis RGA* locus, which partially suppress the phenotype conferred by the GA deficiency mutation *ga1-3* (Silverstone et al. 1997). Although complementation tests have not been performed, it is unlikely that *RGA* and *GAR2* are allelic, because all known *rga* alleles are recessive (Silverstone et al. 1997), whereas *gar2-1* is dominant (Wilson and Somerville 1995).

gai mutants exhibit reduced GA responses (Koornneef et al. 1985; Wilson and Somerville 1995). One possible explanation for the suppression of the *gai* phenotype by *spy-7* and *gar2-1* is that they restore partially the GA responses of *gai*. However, previous experiments showed that *spy-7* does not increase the response of *gai* mutants to exogenous GA (Carol et al. 1995). Similarly, we have

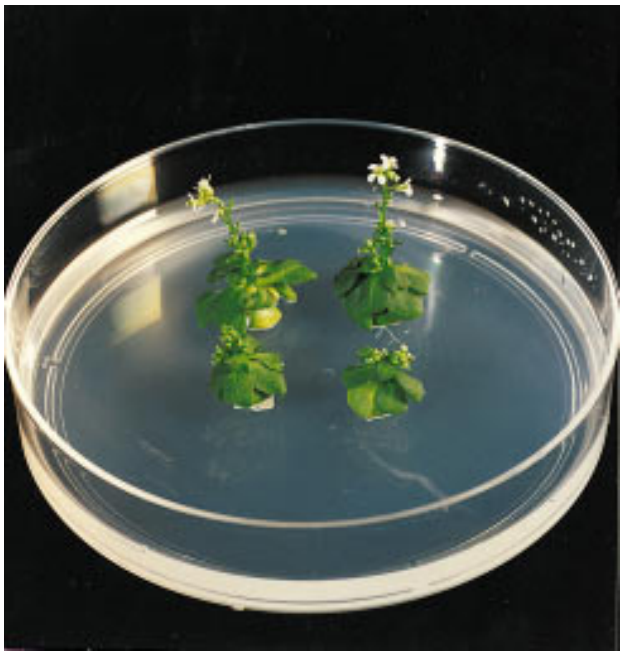

Figure 3. *gai-t6* confers increased resistance to PAC. Bolt stem elongation of *gai-t6* (rear two plants) and *GAI* (front two plants) plants grown on 10^{-6} M PAC is shown. The plants were photographed 57 days after sowing. Because PAC inhibits GA biosynthesis both classes of plant are dwarfed and darker green than when grown on medium lacking PAC. However, *gai-t6* plants grow taller than the *GAI* plants and, therefore, are more resistant to the effects of 10^{-6} M PAC. As shown, the *gai-t6* plants have open flowers, with the petals clearly visible, whereas the flowers on the *GAI* plants are not open (retarded petal and stamen elongation is characteristic of severe GA deficiency; Koornneef and van der Veen 1980). *gai-d1* and *gai-d5* mutants behave like *gai-t6* under these conditions, and not like *GAI*. When grown on medium lacking PAC, *gai-t6*, *gai-d1*, and *gai-d5* are indistinguishable from *GAI*.

shown that the *gai gar2-1* double mutant also does not exhibit a significant growth response to exogenous GA (data not shown). Thus, *gar2-1*, like *spy-7*, does not restore GA responses to *gai* mutant plants.

On their own, *spy-7* and *gar2-1* cause partial suppression of the phenotype conferred by *gai*. As shown in Figure 4A, *gai spy-7* homozygotes are taller and paler than *gai* homozygotes, but not as tall as wild-type plants (Carol et al. 1995). *gai gar2-1* homozygotes are taller, although not paler, than *gai* homozygotes, and not as tall as wild-type plants (Fig. 4A; Wilson and Somerville 1995). When combined, in a *gai spy-7 gar2-1* homozygote, the two *gai-suppressor* mutations confer complete suppression of the *gai* phenotype (Fig. 4A). *gai spy-7 gar2-1* plants exhibit increased internode length and apical dominance, are paler than, and at least as tall as, wild-type plants.

The *SPY* locus was identified originally by mutations that confer PAC-resistant seed germination (Jacobsen and Olszewski 1993). *gar2-1* (on a *GAI*/*GAI* rather than a *gai*/*gai* background) also confers PAC-resistant seed germination (data not shown). Accordingly, we investigated the effects of PAC on the growth of plants carrying the *gai-suppressor* mutations. As shown in Figure 4B, growth of *gai spy-7 gar2-1* plants is more resistant to PAC than is that of *gai gar2-1* plants or *gai spy-7* plants (which are themselves more resistant than *gai* or *GAI*). *gai spy-7 gar2-1*, *gai gar2-1*, and *gai spy-7* all grow taller

Figure 4. Additive suppression of *gai* phenotype by *spy-7* and *gar2-1* alleles. (*A*) Final adult growth of *GAI*, *gai*, *gai spy-7*, *gai gar2-1*, and *gai spy-7 gar2-1* plants in standard glasshouse conditions is shown. As previously described, *gai spy-7* and *gai gar2-1* plants are taller than *gai*, but less tall than *GAI* (Carol et al. 1995; Wilson and Somerville 1995). In *gai spy-7 gar2-1* plants the effects of *gai* are suppressed completely, resulting in plants at least as tall as *GAI*. (*B*) Growth of plants grown on 10^{-6} M PAC. Plants were grown as in Fig. 3, and photographed 30 days after sowing. *gai spy-7 gar2-1* displays the greatest resistance to PAC, followed by *gai gar2-1* and *gai spy-7*, which are themselves more resistant than *GAI* or *gai*.

than *gai-t6* on 10^{-6} M PAC (plants shown in Fig. 3 are more mature than those shown in Fig. 4B).

GA C-20 oxidase transcript abundance is affected by gai *and by* gai-suppressor *mutations*

The GA C-20 oxidase gene family encodes enzymes that catalyze the penultimate step in the biosynthesis of biologically active GAs. Steady-state levels of C-20 oxidase transcripts are regulated negatively by GA (or the GA signal), and are elevated in *gai* (Phillips et al. 1995; Xu et al. 1995). RNA gel-blot analysis was used to visualize C-20 oxidase transcripts in *gai* and in the *gai-suppressor* mutants (Fig. 5A,C). As previously reported, C-20 oxidase transcript levels are greater in *gai* than in the *GAI* control. Interestingly, the abundance of the C-20 oxidase transcripts is restored to approximately wild-type levels in *gai spy-7*, *gai gar2-1*, and *gai spy-7 gar2-1*. Thus, in addition to suppressing the dwarf phenotype conferred by *gai*, *spy-7* and *gar2-1* also suppress the elevated C-20 oxidase transcript levels conferred by *gai*.

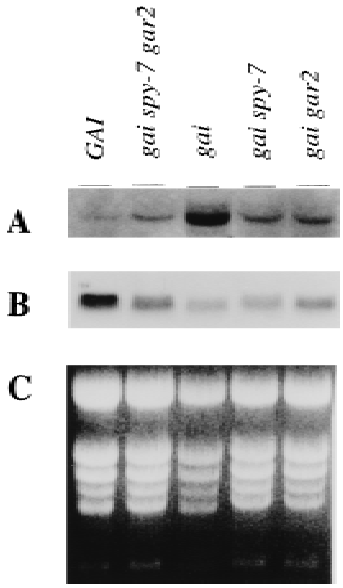

Figure 5. Effect of *gai*, *gai spy-7*, *gai gar2-1*, and *gai spy-7 gar2-1* on GA C-20 oxidase and *GAI*/*gai* transcript levels. (*A*) A radiolabeled GA C-20 oxidase probe detects the C-20 oxidase message in total RNA isolated from seedlings of the indicated genotypes. As previously described, *gai* accumulates higher levels of C-20 oxidase transcript than does *GAI* (Xu et al. 1995). In *gai spy-7*, *gai gar2-1*, and *gai spy-7 gar2-1* mutants, C-20 oxidase levels are similar to those found in *GAI*. (*B*) A radiolabeled *GAI* probe detects *GAI* or *gai* messages in total RNA from seedlings of the indicated genotypes. Levels of *gai* message in *gai* are similar to, and may be slightly lower than, levels of *GAI* message in *GAI*. There are no large differences between the *gai* message levels found in the *gai*, *gai spy-7*, *gai gar2-1*, and *gai spy-7 gar2-1* samples. (*C*) UV fluorescence of ethidium bromide-stained gel blotted for use in the hybridizations shown in *A* and *B*. Note that there was less RNA in the *gai* lane.

GAI *transcript abundance is not significantly affected by* gai *or* gai-suppressor *mutations*

RNA gel-blot analysis was also used to visualize *GAI* (or *gai*) transcripts in wild-type, *gai*, and *gai-suppressor* mutants (Fig. 5B,C). Comparison of wild-type and *gai* samples shows that *gai* transcript levels in the *gai* mutant are similar to *GAI* transcript levels in the wild type, indicating that overexpression is an unlikely explanation for the genetic dominance of *gai*. If anything, *gai* transcript levels are actually slightly lower in *gai* than are *GAI* transcript levels in the wild type.

As shown above (see Fig. 3), apparent null alleles of *gai* confer a tall plant phenotype. It could be thought that *spy-7* and *gar2-1* might cause suppression of the *gai* phenotype through a reduction in *gai* transcript levels. However, this is unlikely, because *gai* transcript levels are not detectably different in *gai*, *gai spy-7*, *gai gar2-1*, or *gai spy-7 gar2-1*, although the latter genotype confers a phenotype that is at least as tall as wild type. Thus, *spy-7* and *gar2-1* are unlikely to suppress *gai* phenotype by an effect on *gai* transcript levels.

Discussion

The cloning of *GAI* through insertional mutagenesis of the *gai* allele demonstrates that *gai* is a gain-of-function, rather than a dominant loss-of-function mutation. Gain-of-function mutations can have dominant effects for a variety of reasons, including ectopic or increased expression of a normal gene product, or altered function of a mutant gene product. Here we show that *gai* does not confer a detectable increase in *gai* transcript abundance, suggesting that increased expression is not the explanation for the dominance of *gai*. We also show that *gai* encodes an altered product, suggesting that *gai* is dominant because this alteration in structure results in an altered function. Thus, deletion of a 17-amino-acid segment from GAI results in a mutant protein (gai) that, in a genetically dominant fashion, causes a reduction in GA responses. We also show that loss of *GAI* function results in increased resistance to the growth-retarding effects of the GA biosynthesis inhibitor PAC. This observation is significant, because it demonstrates that the wild-type gene product GAI is a GA signal-transduction component. To explain these observations we propose that GAI is a repressor of stem elongation, and that GA derepresses stem elongation by opposing GAI action (Fig. 6). The segment missing in the mutant gai protein could be responsible for interacting with the GA signal (or with GA itself); gai would then constitutively repress stem elongation because it can no longer interact with GA or with the GA signal. Alternatively, the segment deleted in gai may have some other function, and the gai mutant protein may be locked into a repressive conformation for reasons other than the loss of a segment that interacts with the GA signal.

An alternative explanation for the dominance of *gai* is that gai interferes with the activity of a signal-transduction pathway that activates stem elongation in response to GA. We prefer the GAI repression explanation because

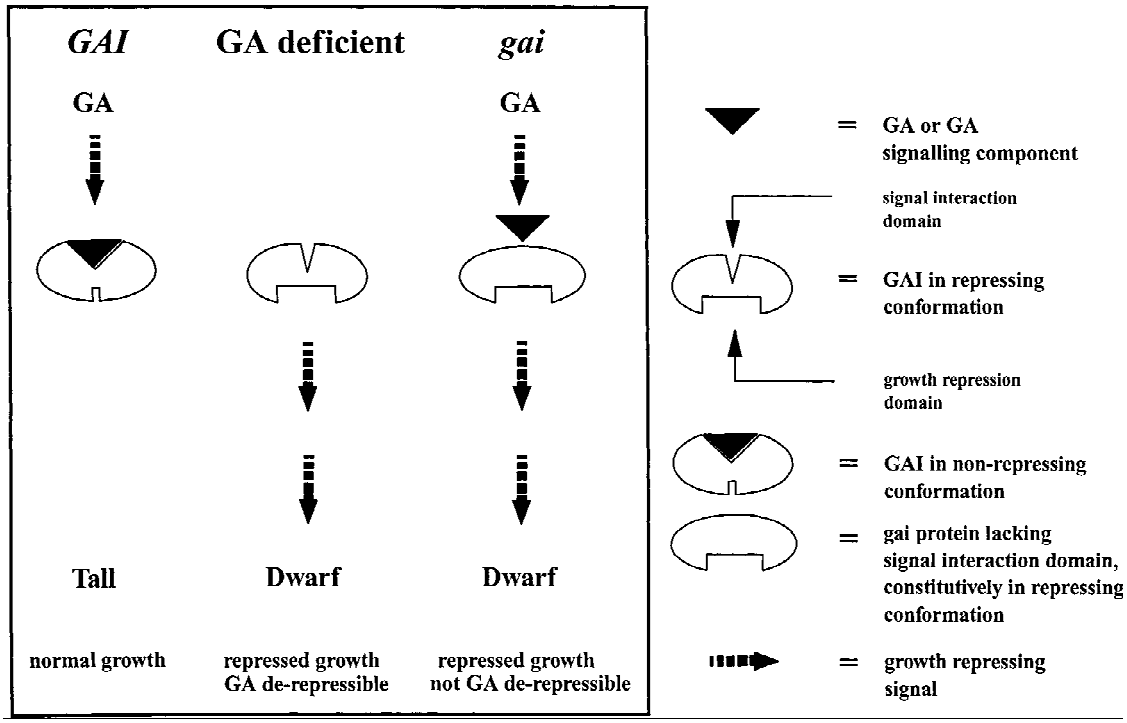

Figure 6. Derepression model for regulation of plant stem elongation by GA. GA derepresses stem elongation because it (or a GA signaling component) opposes the activity of GAI, a protein that represses stem elongation. GAI contains signal interaction and growth-repressing domains, and exists in one of two distinct conformations. Interaction between GA (or the GA signal) and GAI transforms GAI into the nonrepressing conformation. Normal plants (*GAI*) grow tall because the level of endogenous GA is sufficiently high to oppose the activity of the GAI repressor. GA-deficient plants contain insufficient GA to oppose GAI repression to the same degree and, therefore, are thus dwarfed. *gai* mutant plants are dwarfed because the mutant gai protein is relatively resistant to the effects of GA, and represses growth in a dominant fashion. Null alleles at *GAI* (eg., *gai-t6*) confer a tall, PAC-resistant phenotype, because absence of GAI results in loss of its growth repression function. *gai-t6* mutant plants are not totally PAC resistant because of the probable activity of GRS. This model provides a general explanation for the regulation of plant stem elongation by GA.

it predicts that loss-of-function alleles of *GAI* should confer increased resistance to GA biosynthesis inhibitors, whereas the gai interference explanation does not. As shown above, the *gai-t6* allele does indeed confer increased resistance to PAC. Previous studies have suggested that a repressor function is involved in GA signal transduction (Brian 1957; Potts et al. 1985; Chandler 1988; Lanahan and Ho 1988; Harberd and Freeling 1989; Croker et al. 1990). Our work provides direct evidence that such a repressor exists, and that it is GAI. A further consequence of these findings is that GA regulates stem elongation not through activation but by derepression.

GAI contains leucine heptad repeats, NLSs, and the LXXLL motif characteristic of transcriptional coactivators. All of these features are found in proteins that modify transcription (Montminy 1997; Torchia et al. 1997). Perhaps GAI acts as a transcriptional regulator, repressing transcription of genes that promote stem elongation. GAI lacks any obvious membrane-spanning domain and, therefore, is unlikely to be the plasma membrane-associated GA receptor implicated in the cereal aleurone GA response (Hooley et al. 1991; Smith et al. 1993; Gilroy and Jones 1994; Jacobsen et al. 1995).

Here we show that the *spy-7* and *gar2-1* mutations cause partial, and, when combined, total suppression of the dwarf phenotype conferred by *gai*, and also suppress the effect of *gai* on the accumulation of C-20 oxidase transcripts. Suppressed *gai* mutants (*gai spy-7*, *gai gar2-1*, *gai spy-7 gar2-1*) accumulate *gai* transcripts to levels similar to that found in plants carrying the *gai* mutation alone. These observations suggest that the *SPY* and *GAR2* gene products do not modify *GAI* expression, but rather act as GA signal transduction components upstream or downstream of the *GAI* gene product. This idea may be an oversimplification with respect to GAR2 because the *gar2-1* allele is a dominant, potential gain-of-function mutation (Wilson and Somerville 1995). Perhaps a mutant *gar2-1* gene product interferes with the function of an unidentified GA signal transduction component.

The additive effects of *spy-7* and *gar2-1*, together with their differing effects on paleness, might suggest that they identify different branches of the GA signaling pathway. Alternatively, because the *spy-7* allele is weaker in its effects than *spy-5* (*spy-5* itself is not a strong *spy* allele; J. Peng and N.P. Harberd, unpubl.; Wilson and Somerville 1995; Jacobsen et al. 1996), *spy-7* is unlikely to be a null allele. The combination of two par-

tial blocks in a single (unbranched) pathway may be the equivalent of a complete block in that pathway.

The observation that *spy* mutations suppress the *gai* phenotype has led to suggestions that SPY acts downstream of GAI (Jacobsen et al. 1996; Swain and Olszewski 1996). However, interpretation of epistasis relationships in terms of the ordering of gene functions in pathways is not always a simple matter (Avery and Wasserman 1992). Recent evidence suggests that *SPY* encodes an *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase (OGT; Kreppel et al. 1997), an activity that is involved in the dynamic modification of regulatory proteins in a manner analogous to that of protein phosphorylation (Kreppel et al. 1997; Lubas et al. 1997). SPY may modify GAI, rather than being a component of the GA signaling pathway downstream of GAI.

The resistance to PAC conferred by *gai-t6* is not as strong as that observed in the *gai spy-7 gar2-1* mutant. This observation is consistent with the hypothesis that at least one other *Arabidopsis* gene product has a function that substantially duplicates that of GAI. As described above, the primary sequence of GRS is very similar to that of GAI, and is identical to GAI in the region deleted in gai. Perhaps GRS shares functional properties in common with GAI. If so, mutants lacking GRS, like those lacking GAI, might be predicted to exhibit PAC resistance. Also, because *gai spy-7 gar2-1* is more PAC resistant than *gai-t6*, it seems that GA signaling from both GAI and GRS (assuming that GAI and GRS do have overlapping functions) is mediated or modulated by GAR2 and SPY.

The GA signaling system outlined in this paper has several intriguing properties. First, although GA is an essential regulator of various stages of the life cycle of normal plants (e.g., seed germination in *Arabidopsis;* Koorneef and van der Veen 1980), it is no longer essential if the GA signaling system is compromised. Second, the different degrees of PAC resistance exhibited by the various mutants described in this paper show that the GA signaling system is capable of eliciting a graduated, rather than an all-or-nothing response, such that a partially compromised system yields a partial reduction in GA dependency. Ethylene is another factor that plays an important part in the control of plant growth and development. Genetic dissection of ethylene signal transduction in *Arabidopsis* suggests that the *ETR* and *ERS* genes encode redundant ethylene receptors, that *CTR1* acts downstream of *ETR* and *ERS*, and that the *ETR*, *ERS*, and *CTR* gene products define a pathway that operates as a negative regulator of ethylene responses (Bleeker et al. 1988; Chang et al. 1993; Kieber et al. 1993; Hua et al. 1995; Schaller and Bleeker 1995). Genetic analysis of GA signal transduction in *Arabidopsis* has identified the involvement of *GAI*, *SPY*, and *GAR2*. It appears that *GAI* encodes largely redundant functions, and that GAI, SPY, and GAR2 are components (or modulators) of a pathway that acts as a negative regulator of GA responses. Negative, derepressible regulatory systems may be common features of the mechanisms by which plant growth factors regulate plant development.

Materials and methods

Genetic nomenclature

In this paper genotypes are written in italics; the wild-type genotype is in capitals (e.g., *GAI*), and the mutant genotype is in lowercase letters (e.g., *gai*). The polypeptide product of the *GAI* gene is written as GAI, and of the *gai* gene as gai.

Plant materials

Mutant plant lines were obtained as previously described (Peng and Harberd 1993; Carol et al. 1995). The *gar2-1* mutant was obtained from Ruth Wilson. Seeds were chilled on moistened filter paper at 4°C for 4 days (to break dormancy) and then planted on two parts Levington's M3 potting compost to one part grit/sand. Plants were then grown in standard greenhouse conditions or in controlled environment chambers. Transgenic plants were grown according to United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF) regulations (License no. PHF 1418/8/22).

Growth of plants on medium containing PAC

Seeds were surface-sterilized and germinated on GM medium, with or without supplementary PAC (obtained from Zeneca Agrochemicals), and seedlings were maintained on this medium, under the same conditions as previously described (Peng and Harberd 1993).

DNA and RNA gel-blot hybridizations, DNA sequencing

Genomic DNA preparation and gel-blot hybridizations were performed as described (Peng and Harberd 1993). The *Ds* probe was a radiolabeled 3.4-kb *Xho*I–*Bam*HI subfragment of *Ac*. RNA was extracted from 27-day-old seedlings (grown under natural photoperiod), and RNA gel-blot transfers were performed as described (Whitelam et al. 1993). DNA–RNA hybridizations were in 0.3 M Na phosphate (pH 7.2), 7% SDS, 1% BSA, and 1 mM EDTA, at 65°C, with two subsequent washes for 30 min each in 0.5 M Na phosphate (pH 7.2), 5% SDS, 50 mM EDTA at 65°C. For DNA–RNA hybridizations, the *GAI* (gene specific) probe was a 369-bp PCR amplified fragment containing a 150-bp 5′ noncoding sequence and a portion encoding the amino terminus of GAI. The C-20 oxidase probe was an ~800-bp PCR amplified fragment from *GA5* (Xu et al. 1995). DNA sequences were determined using the dideoxynucleotide chain termination method.

Isolation of genomic DNA flanking the transposon insertion in gai-t6

JP95 is an ~2.5-kb IPCR fragment containing DNA flanking the transposed *Ds* in *gai-t6*. This fragment extends from the 3′ end of *Ds* into the adjacent *Arabidopsis* chromosomal DNA and terminates at the next *Bcl*I site. To make JP95, genomic DNA from *gai-t6* was digested with *Bcl*I, recircularized, and then amplified using primers DL5 and B39 for the first round and DL6 and D71 for the second, nested, round of amplification (Long et al. 1993).

Identification of cosmid clones containing GAI

Using methods described elsewhere (Putterill et al. 1993, 1995; Macknight et al. 1997) we established a contig of yeast artificial chromosomes (YACs; supplied by J. Ecker, University of Pennsylvania, Philadelphia) that contained *GAI*. This contig was

based in part on unpublished hybridization data from the laboratories of J. Ecker and G. Jürgens (University of Tübingen, Germany). Cosmids containing DNA from a subregion of this contig [shown by restriction fragment length polymorphism (RFLP)-marked recombinant analysis to contain *GAI;* P. Carol, J. Peng, D.E. Richards, R. Cowling, and N.P. Harberd, unpubl.] were isolated from a Landsberg *erecta* DNA cosmid library (gift of C. Lister and C. Dean, John Innes Centre, Norwich, UK).

Identification and characterization of GAI *and* GRS *cDNAs and genomic DNAs from* GAI, gai, *and* gai-derivative *alleles*

A 10-kb subfragment of a cosmid (JP2) containing *GAI*, previously shown to hybridize with the IPCR fragment JP95, was used to screen a cDNA library made from young seedling aerial parts (Columbia ecotype). We identified cDNA clones pPC1 (*GAI*) and pPC2 (*GRS*). Part of the DNA sequence of pPC1 was identical with that of an ~150-bp region of genomic DNA flanking the *Ds* insertion in *gai-t6* (from JP95; J. Peng, P. Carol, D.E. Richards, and N.P. Harberd, unpubl.). In addition, searches of the dbEST database (Boguski et al. 1993) with the BLAST programme (Altschul et al. 1990) revealed an *Arabidopsis*-expressed sequence tag (EST; GenBank Identifier ATTS3217) containing sequence identical to that of JP95. cDNA ATTS3217 was obtained from the *Arabidopsis* Biological Resources Centre, and the complete DNA sequences of the pPC1 insert and of ATTS3217 were determined. These overlapping sequences revealed an ORF, together with 5′ and 3′ noncoding regions, for *GAI*. Oligonucleotide primers derived from 5′ and 3′ noncoding sequence of *GAI* were used to amplify, with PCR, 1.7-kb fragments from *GAI, gai, gai-d1, gai-d2, gai-d5,* and *gai-d7* genomic DNA. The DNA sequences of these fragments were determined from duplicate amplifications, thus avoiding potential errors introduced by PCR. The *GAI* genomic sequence was almost identical with that of the overlapping cDNAs. Three nucleotide substitutions were detected, which could be attributable to differences between ecotypes (the *GAI* genomic sequence is from Landsberg *erecta,* the cDNAs from Columbia) and do not alter the predicted amino acid sequence of GAI. Amino-acid sequence alignments in Figure 2 were performed using the PILEUP and PRETTYBOX programs (Wisconsin Package, Genetics Computer Group, Madison, WI), using default parameters.

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Note added in proof

Nucleotide sequences corresponding to the amino acid sequences described in this paper have been submitted to the EMBL data library under accession nos. Y15193 and Y15194.

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