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The Arabidopsis Dwarf Mutant *shi* Exhibits Reduced Gibberellin Responses Conferred by Overexpression of a New Putative Zinc Finger Protein

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***shi* (for *short internodes*), a semidominant dwarfing mutation of Arabidopsis caused by a transposon insertion, confers a phenotype typical of mutants defective in the biosynthesis of gibberellin (GA). However, the application of GA does not correct the dwarf phenotype of *shi* plants, suggesting that *shi* is defective in the perception of or in the response to GA. In agreement with this observation, the level of active GAs was elevated in *shi* plants, which is the result expected when feedback control of GA biosynthesis is reduced. Cloning of the *SHI* gene revealed that in *shi*, the transposon is inserted into the untranslated leader so that a cauliflower mosaic virus 35S promoter in the transposon reads out toward the *SHI* open reading frame. This result, together with mRNA analysis, suggests that the phenotype of the *shi* mutant is a result of overexpression of the *SHI* open reading frame. The predicted amino acid sequence of SHI has acidic and glutamine-rich stretches and shows sequence similarity over a putative zinc finger region to three presumptive Arabidopsis proteins. This suggests that *SHI* may act as a negative regulator of GA responses through transcriptional control.**

INTRODUCTION

Gibberellins (GAs) constitute a large family of tetracyclic diterpenoid plant hormones of which most are believed to be precursors, deactivated forms, or secondary metabolites (Swain and Olszewski, 1996). Bioactive GAs are associated with many growth and developmental processes in higher plants, such as leaf expansion and stimulation of epidermal cell elongation of the inflorescence stem as well as induction of seed germination and flower development (Hooley, 1994; Swain and Olszewski, 1996). Thus, GA is a key regulator of plant growth and phase transition throughout plant development. For us to fully understand the physiological roles of GA in the control of these diverse processes, characterization of the GA biosynthesis and signaling pathways is important.

Our knowledge of GA biosynthesis has increased during the past decade by biochemically analyzing and studying GA biosynthesis mutants of several species (Koornneef et al., 1990; reviewed in Hedden and Kamiya, 1997; Phillips, 1998). In Arabidopsis, a number of GA biosynthesis mutants have been well characterized, and the recent cloning of several of the corresponding genes has provided information about how GA biosynthesis is regulated (reviewed in Phillips, 1998). Much less is known about GA perception and signal transduction. Thus far, GA signaling has been well defined only in cereal aleurone layers. In these tissues, GAMyb has been shown to be a positive regulator of GA-induced α-amylase gene transcription (Gubler et al., 1995). However, research on GA signal transduction during plant growth and development is rapidly progressing, because new GA response mutants have been isolated and the corresponding genes have been cloned.

Mutants with altered GA responsiveness fall into two phenotypic categories and exhibit either increased or decreased GA signal transduction (Hooley, 1994). Members of the first class carry recessive alleles and are elongated, slender mutants that resemble GA-treated wild-type plants. In Arabidopsis, the *spindly* (*spy*) mutation (Jacobsen and Olszewski, 1993) belongs to this class. The *SPY* gene has been cloned (Jacobsen et al., 1996), and sequence comparison with mammalian GlcNAc transferase genes suggests that SPY acts through glycosylation of serine and threonine residues of target proteins (Lubas et al., 1997; Robertson et al., 1998). SPY is thought to function as a repressor of the GA response, because the *spy* mutation partially suppresses GA-deficient phenotypes of severe GA biosynthesis mutants. Furthermore, in the aleurone layer of germinating barley seeds transiently expressing a *SPY*-like cDNA (*HvSPY*), GA-induced activity of an α-amylase promoter is repressed (Robertson et al., 1998).

The second class of GA response mutants comprises dwarf and semidwarf mutants that are phenocopies of plants

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with reduced GA biosynthesis. To date, three mutants belonging to this class have been identified in Arabidopsis: *gai* (*GA insensitive*), *sly1* (*sleepy1*), and *pkl* (*pickle*) (Koornneef et al., 1985; Peng and Harberd, 1993; Ogas et al., 1997; Steber et al., 1998). The original *gai* mutation is a semidominant gain-of-function mutation, and the mutant allele carries an in-frame deletion that removes 17 amino acids from the encoded protein (Peng et al., 1997). The phenotype of the original *gai* mutation resembles weak GA biosynthesis mutants, whereas all other subsequently identified disruptions of the *GAI* gene (e.g., *gai-t6*) lead to a slight increase in GA signal transduction. The *gai-t6* mutation is recessive and probably represents a null allele. This indicates that GAI acts as a repressor of the GA response pathway, and analysis of the *GAI* gene product suggests that it functions as a transcriptional regulator (Peng et al., 1997).

Recently, a gene showing sequence similarity to *GAI* has been cloned. This gene has been designated both *RGA* (for *REPRESSOR OF THE GA1-3 MUTANT*; Silverstone et al., 1998; or for *RESTORATION OF GROWTH ON AMMONIA*; Truong et al., 1997) and *GRS* (for *GAI-RELATED SEQUENCE*; Peng et al., 1997). Recessive mutations in this gene (*rga*) partially suppress phenotypic defects of the severe GA biosynthesis mutant *ga1-3* (Silverstone et al., 1997). In the wild-type background, *rga* resembles mutants carrying a disruption in the *GAI* locus, because it shows a slight increase in GA signal transduction. This suggests that RGA and GAI may have overlapping functions as repressors of GA signal transduction (Harberd et al., 1998; Silverstone et al., 1998). In screens for extragenic suppressors of the dwarf phenotype conferred by the original *gai* mutation, a new locus, *GAR2* (for *GAI REPRESSOR2*; Wilson and Somerville, 1995), has been identified. Hence, it has been suggested that GAR2 acts as a component in GA signal transduction (Peng et al., 1997).

The *sly1* mutant is the first recessive GA-insensitive dwarfing mutation isolated and the first that shows the full spectrum of phenotypes associated with severe GA biosynthetic mutants (Steber et al., 1998). Consequently, it has been proposed that SLY1 could play a central role in GA response, acting as a putative receptor or as a key positive regulator. Cloning of *SLY1* may provide clues to the role that SLY1 plays in GA signaling. No GA receptor has yet been identified, although GA binding polypeptides have recently been found in the plasma membrane of oat aleurone cells and in stem and leaf cells of Arabidopsis by using GA-photoaffinity labeling (Lovegrove et al., 1998). Cloning and analysis of the genes encoding these polypeptides should help to reveal whether they are involved in GA perception.

The dwarf phenotype of the recessive *pkl* mutant is only partially suppressed by GA, whereas GA strongly suppresses the embryonic differentiation characteristics in the primary roots of the *pkl* mutant (J. Ogas, J.-C. Cheng, Z.R. Sung, and C. Somerville, unpublished data). Despite the GA responsiveness of the *pkl* mutant, PKL has been suggested to be involved in GA signaling because *pkl gai* double mutants exhibit a much stronger dwarf and late-flowering phenotype than do any of the progeny from the parental lines (Ogas et al., 1997). If so, PKL is most likely active in a novel pathway affecting both shoot and root development.

Despite the recent progress in the recovery of GA response mutants, our understanding of the GA signaling pathways is still rudimentary. To elucidate these pathways, the isolation of additional response mutants (in particular, mutants affected in specific rather than all responses to GA) and further studies of the interaction between the isolated genes are needed. Here, we report the identification and characterization of a new semidominant dwarfing mutation in Arabidopsis, designated *shi* (for *short internodes*).

We propose that the *SHI* gene product might be a new negative regulator of GA signaling pathways, specifically affecting cell elongation in the inflorescence stem and the transition to flowering. We also suggest that the *shi* mutant might be of importance in crop improvement because of its reduced stem growth and high flower production capability and because early flowering of *shi* can be restored by the application of GA.

RESULTS

The *shi* Mutation Is Caused by Insertion of a Transposable Element

The *shi* mutant was isolated in a mutant screen by using a two-component *Activator*/*Dissociation* (*Ac*/*Ds*) transposon tagging strategy specifically designed to identify dominant mutations (Long et al., 1993; Wilson et al., 1996). Four individuals from the same F_2 family (see Methods) produced offspring that segregated for a phenotype with short hypocotyl, epinastic cotyledons, and leaves with a narrow base and short petiole, particularly the first leaves of the rosette, as shown in Figures 1A and 1B. The individual ES724a was shown to carry a stable *Ds* insertion and was chosen for further analysis. Its progeny were shown to comprise three phenotypic classes, as seen in Figure 1C: four dwarfed plants, 11 semidwarfed plants, and three wild-type plants. The segregation into three different phenotypes in a ratio of approximately 1:2:1 suggested that the initial F_2 individual ES724a carried one copy of a semidominant dwarfing mutation and that the dwarf, semidwarf, and wild-type phenotypes correspond to plants homozygous for, heterozygous for, or lacking the mutation, respectively. To confirm that the dwarf phenotype was the result of a mutation at a single, semidominant locus, an individual showing the dwarf phenotype was crossed to a wild-type plant in the Landsberg *erecta* (L*er*) background. As expected, the F_1 progeny were all semidwarfs, and the F_2 population segregated 1:2:1 for the dwarf, semidwarf, and wild-type phenotypes. The homozygous dwarf mutant was designated *shi.*

Linkage analysis of 111 F_3 families segregating for the *shi*

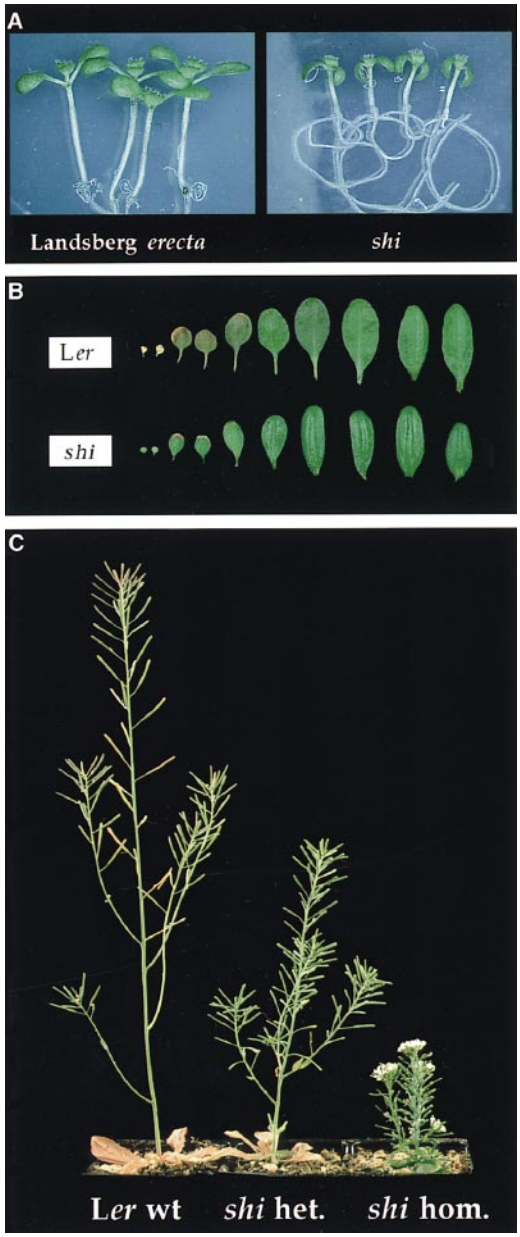

Figure 1. Comparison between the *shi* Mutant and the Wild Type.

(A) Nine-day-old wild-type and *shi* seedlings. The seedlings shown were grown on horizontal plates and tipped over for photography.
(B) Wild-type and *shi* rosette leaves from plants grown under long-day conditions.
(C) A wild-type (wt) plant, a *shi* heterozygote (het.), and a *shi* homozygote (hom.) grown under long-day conditions for 5 weeks.

mutation revealed that no recombination had occurred between the *Ds* hygromycin (*Ds*[*Hyg*]) element and the mutation, indicating that they are separated by a maximum of 1.5 centimorgans (cM; calculated by the Haldane [1919] mapping function). Furthermore, the mutation was unstable in the presence of the transposase gene, resulting in reversion to the wild-type phenotype. To verify that the identified revertant plants were true revertants, we sequenced *Ds* excision sites. *Ac*/*Ds* elements characteristically generate an 8-bp duplication of target sequence when inserted into the plant genome. When the elements are excised, this 8-bp duplication is retained as a footprint, although often slightly rearranged (Pohlman et al., 1984; Baker et al., 1986). All revertants carried the 8-bp duplication at the *Ds* excision site, but the two bases adjacent to *Ds* were altered from AC to TG (Figure 2). The fact that they all carry an identical base rearrangement indicates that all of the isolated revertants originated from the same excision event.

The Phenotype of the *shi* Mutant Is a Phenocopy of Weak GA Deficiency

The weak GA biosynthesis mutants as well as the GA-insensitive mutant *gai* all have a phenotype that, compared with the wild type, includes dwarfism, darker green color, narrow leaves, reduced apical dominance, and late flowering. The *shi* mutant is very similar to these mutants, although the coloring of its leaves is not as dark green as that of *gai* leaves (Figure 3).

We measured the hypocotyl length of 10-day-old light-grown seedlings derived from a wild-type plant and plants heterozygous or homozygous for *shi*, respectively. As shown in Figure 4A, the progeny of a heterozygous *shi* plant segregated for short, medium, or long hypocotyls ranging from 1 to 6 mm. Of the wild-type hypocotyls, the majority measured >4 mm, whereas the offspring from the homozygous *shi* parent had hypocotyls that measured ⩽4 mm. These data confirm that the short hypocotyl of *shi* is a mutant-specific trait.

Figure 2. Analysis of the *Ds* Insertion Site in the *SHI* DNA Sequence.

Sequences at the *Ds* insertion site are shown from the wild type, the *shi* mutant, and a revertant plant. The *Ds* element is represented as a triangle. In *shi*, there is a characteristic 8-bp duplication of the *Ds* target sequence; in the revertant, this sequence is retained as a "footprint," although slightly rearranged. The altered bases in the revertant are marked with asterisks.

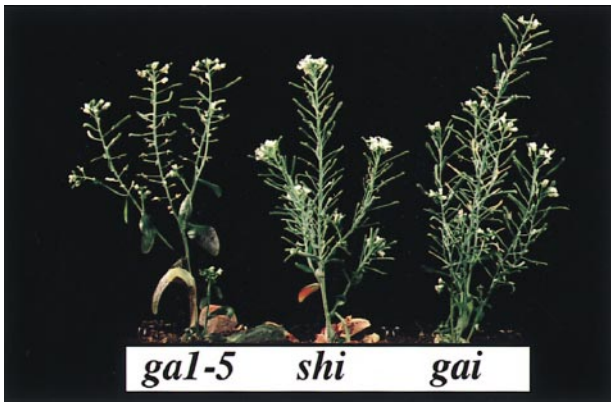

Figure 3. Phenotypic Comparison of *shi* to Two GA Mutants.

The *shi* mutant, the weak GA biosynthesis mutant *ga1-5*, and the GA-insensitive mutant *gai* all have a similar dwarfed phenotype. The individuals shown are homozygous for the mutated alleles.

The plant heights of *shi*, *gai*, and *ga1-5* are significantly shorter than those of the wild type, but they are similar in height to each other (Figure 4B). Scanning electron microscopy showed that the epidermal cells of the inflorescence stem of *shi*, *gai*, and *ga1-5* are significantly less elongated than are the epidermal cells of the wild-type stem ($P < 0.0001$; Student's *t* test) (Figures 5A to 5D). Taken together, these data confirm that the *shi* mutant has a dwarf phenotype due to reduced cell elongation in the bolting stem, which is similar to the GA biosynthesis mutant *ga1-5* and the GA-insensitive mutant *gai.*

An interesting observation is that the *shi* mutant produces significantly more flowers per plant than do both wild-type and *gai* plants, as shown in Figure 6A. The morphology of the *shi* flowers is indistinguishable from that of the wild type.

GA Application Has Different Effects on *shi* Stem Elongation and Flowering Time

A well-known effect of GA is to stimulate elongation of hypocotyls and bolting inflorescence stems. To study hypocotyl elongation, we germinated wild-type and *shi* seeds either on GM medium only (Valvekens et al., 1988) or on GM medium containing 10 μM GA_4 plus GA_7 and studied hypocotyl elongation after 7 days. GA treatment of germinating *shi* seeds did not restore wild-type elongation of the *shi* hypocotyl (data not shown). To study elongation of the bolting stem, we repeatedly sprayed soil-grown *shi* plants with 100 μM GA (GA_3 or GA_4 plus GA_7) starting before flower induction. Applied GA did not lead to additional elongation of the *shi* inflorescence, indicating that the dwarf phenotype of *shi* is not a result of impaired GA biosynthesis (data not shown).

GAs have been shown to be important for flower induction in Arabidopsis, especially under short-day (SD) conditions (Wilson et al., 1992). A common trait of GA-deficient and response dwarf mutants grown under SD conditions is that they are late flowering as a result of reduced GA content and GA signal transduction, respectively. As occurs in these GA mutants, flower induction in SD-grown *shi* mutants was shown to be delayed (Figure 6B). The wild type (L*er*) flowered after 58 days, whereas *shi* plants flowered after 78 days, *ga1-5* after 63 days, and *gai* after 80 days.

GA-deficient mutants can be induced to flower at the same time as wild-type plants if they are treated exoge-

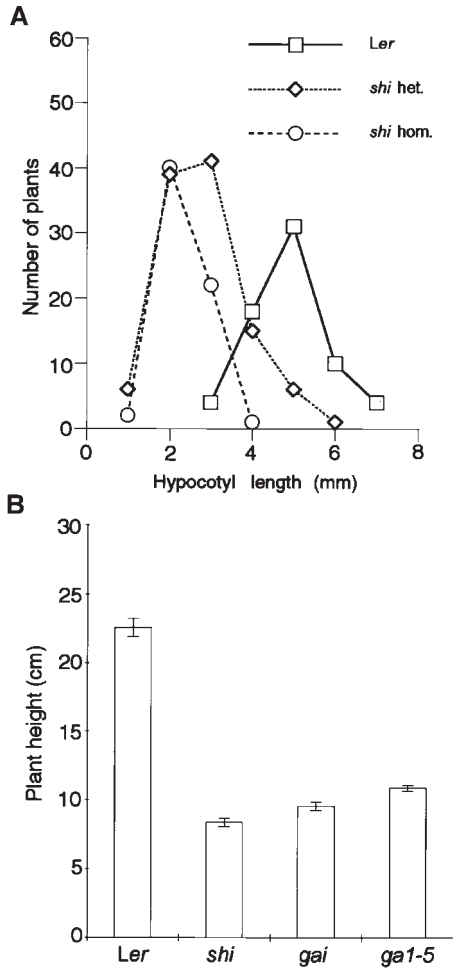

Figure 4. Lengths of Hypocotyls and Stems.

(A) Hypocotyl lengths of 10-day-old light-grown seedlings from a wild-type L*er* population, a population descended from a plant heterozygous for the *shi* mutation (het.), and a population homozygous (hom.) for the *shi* mutation.

(B) Adult plant heights of wild-type L*er*, homozygous *shi*, and plants homozygous for the different GA mutations *gai* and *ga1-5.* The values represent the mean of >12 plants per line; bars represent standard errors.

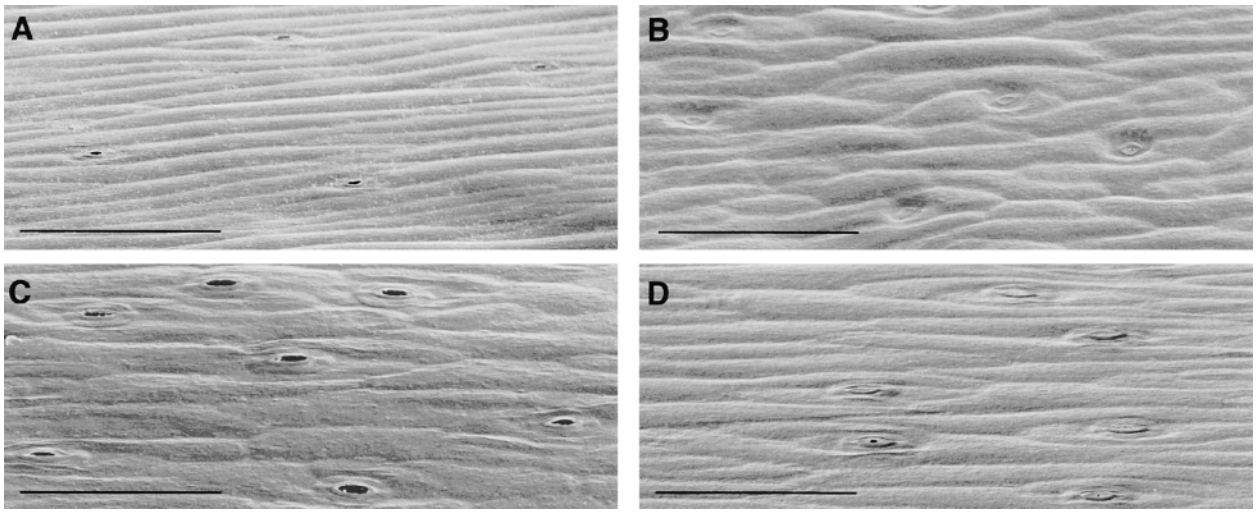

Figure 5. Scanning Electron Micrographs of Epidermal Cells of the Inflorescence Stem.

(A) Wild-type L*er*.
(B) *shi*.
(C) *gai*.
(D) *ga1-5*.
The cells of *shi*, *gai*, and *ga1-5* mutants are significantly shorter than are the cells of wild-type plants ($P < 0.0001$; *t* test). Samples were taken from the first internode of adult plants. Bars = 100 μm.

nously with GA (Wilson et al., 1992). *shi*, wild-type, *gai*, and *ga1-5* plants grown under SD conditions were repeatedly sprayed with 100 μM GA_3. When treated with GA, wild-type plants flowered after 43 days and *shi* plants flowered after 44 days. The GA-responsive mutant *ga1-5* flowered after 43 days with the same treatment, whereas *gai* took 74 days to flower (Figure 6B). These data show that with respect to flowering time, the *shi* mutant is as responsive to GA applied at high concentrations as are the GA-deficient mutant *ga1-5* and the wild type, whereas *gai* is only slightly affected. This implies that *shi* affects flowering time as severely as does *gai* but that the response to applied GA differs between the two mutants in this aspect. Furthermore, the application of GA at a lower concentration (10 μM GA_3 or GA_4) does not fully restore the flowering time of *shi* mutants, suggesting that flowering time response is GA dose dependent in *shi* (data not shown). In these experiments, *shi* flowers several days later than does *ga1-5.*

shi Is Not a New Brassinosteroid Mutant

Mutants defective in the biosynthesis of or in the response to brassinosteroids (BRs) are known to elicit a dwarf phenotype similar to that of the severe GA-deficient mutants. This phenotype is characterized by reduced hypocotyl and inflorescense stem cell elongation, dark green leaf foliage, and reduced apical dominance (reviewed in Clouse, 1996; Szekeres and Koncz, 1998). Seeds of wild-type and *shi* plants were germinated on GM medium containing 10^{-6}, 10^{-7}, or 10^{-8} M epibrassinolide. *shi* mutants did respond to the treatment, although hypocotyl length was not restored to that of the wild-type plants germinated on BR-containing media (data not shown). The same result was previously observed for the GA mutants *ga1* to *ga5* as well as for *gai* when treated with BR (Kauschmann et al., 1996) and indicates that the short hypocotyl phenotype of *shi* is not the result of either reduced BR biosynthesis or insensitivity to BR.

When germinated in the dark, GA mutants respond to darkness in a manner similar to the wild type, whereas the Arabidopsis BR mutants display a deetiolated growth habit with a short hypocotyl, opened cotyledons, and no hook (Kauschmann et al., 1996; Li et al., 1996). *shi* seedlings grown in the dark display an intact etiolated response identical to that of the wild type, suggesting that *shi* does not affect the BR signaling pathway (data not shown).

Levels of Active GAs Are Elevated in the *shi* Mutant

Several studies suggest that GA biosynthesis in Arabidopsis is controlled by a variety of feedback mechanisms (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995). In several known GA signal transduction mutants, the level of endogenous GAs is consistent with this hypothesis. In mutants with increased signal transduction, the GA levels are decreased,

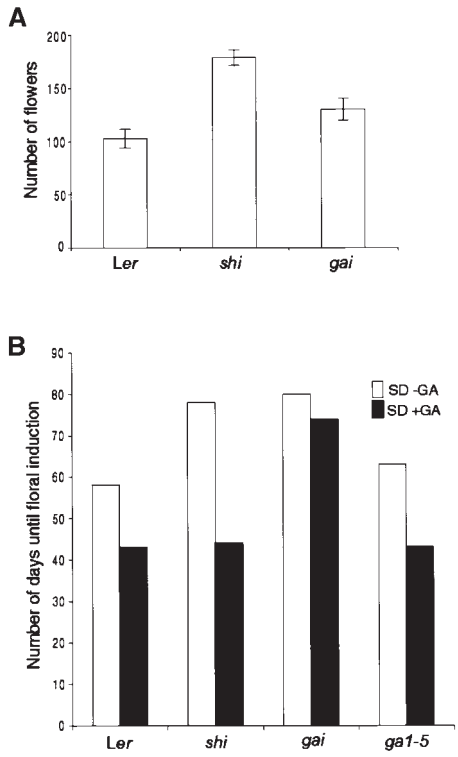

Figure 6. Flower Production and SD Flower Induction.

Comparison between *shi* and other GA-related mutants with respect to flower production and SD flower induction.
(A) The *shi* mutant produces more flowers compared with wild-type and *gai* plants. The values represent the mean of 10 plants; bars represent standard errors.
(B) The *shi* mutant can restore time for flower induction under SD conditions when treated with high concentrations of GA. Plants sprayed with GA_3 (100 μM) are shown by black bars. Untreated plants are shown by white bars. Time for flower induction is defined as the day when flower buds could be detected with a stereomicroscope in 50% of the individuals in each test population.

whereas in those with decreased signal transduction, there is an accumulation of bioactive GAs to a level higher than that in the wild type (Ross, 1994). This feedback regulation of GA levels seems to be specific for responses in GA signal transduction. For *shi* mutants, we observed an increase of C_{19}-GAs, including the bioactive GA_4, at a level between two and four times higher than wild-type levels. We also observed a decrease of precursor C_{20}-GAs to between one-third and one-tenth that of the wild-type level (Figure 7). In *shi*, the level of GA_{34}, a 2β-hydroxylated inactivated end product, was found to be dramatically increased. Similar results were previously found in the *gai* mutant (Talon et al., 1990), suggesting that 2β-hydroxylation is stimulated by increased GA biosynthesis.

SHI Encodes a New Putative Zinc Finger Protein

Using inverse polymerase chain reaction (IPCR), we determined the flanking sites of the *Ds* insertion in *shi*, which allowed us to isolate a *SHI* genomic clone from the wild type (Figure 8). A *SHI* cDNA was isolated by reverse transcription–PCR (RT-PCR). Comparison of *SHI* cDNA sequence from rapid amplification of 5′ cDNA ends with that of the two IPCR fragments obtained from each side of the *Ds*(*Hyg*) element revealed that the transposon is inserted in the untranslated leader 363 bp upstream of the translational start site and that 8 bp of genomic DNA is duplicated at the *Ds*(*Hyg*) insertion site (Figure 8).

The predicted SHI protein consists of 331 amino acids. A region containing six cysteine residues (amino acids 120 to 147) in a $C\text{-}X_2\text{-}C\text{-}X_7\text{-}C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_7\text{-}C$ arrangement (where X is a variable amino acid) is suggested to make up a zinc finger domain similar to the Zn_2Cys_6 cluster found in the

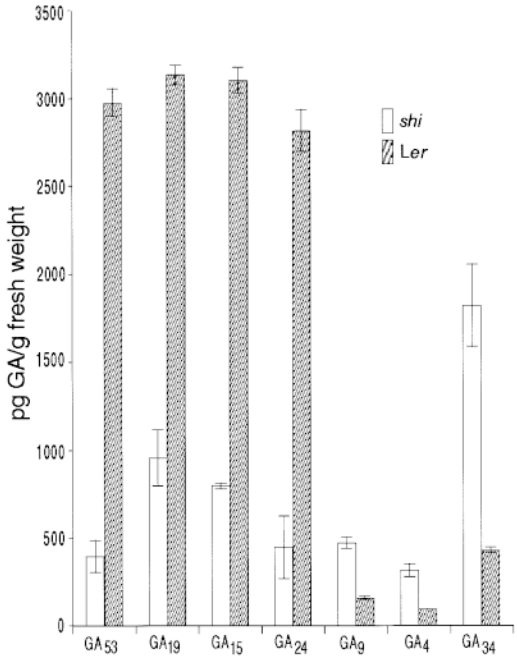

Figure 7. Level of Active GA Is Increased in the *shi* Mutant.

The levels of different GAs in stem segments of 5-week-old *shi* and L*er* plants were measured in picograms per gram fresh weight. Two separate experiments were performed, and the sum of the two is presented (no significant differences were found between the experiments; $F_{1,4} = 0.06$; $P > 0.8$). L*er* values represent the mean of six samples and *shi* values the mean of four samples. Bars represent standard errors. The level of C_{20}-GAs (GA_{53}, GA_{19}, GA_{15}, and GA_{24}) is significantly reduced in *shi*, whereas the amount of C_{19}-GAs (GA_9, GA_4, and GA_{34}) is significantly increased ($P < 0.001$ for all GAs measured).

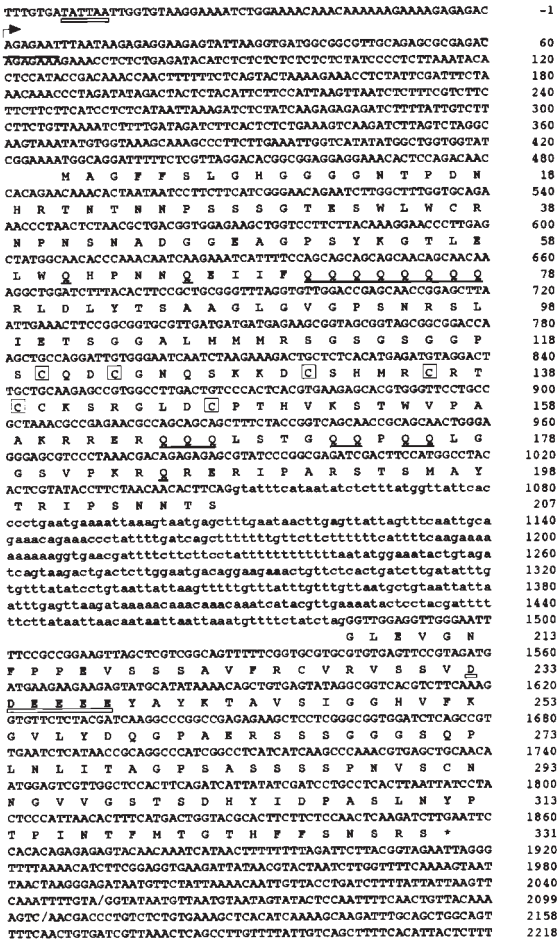

Figure 8. *SHI* Genomic Sequence.

The sequence of genomic DNA at the *SHI* locus in wild-type Columbia is shown. The putative transcriptional start site is marked with an arrow, and a possible TATA box is underlined with a bar. The *Ds* insertion site with the 8-bp sequence duplicated upon insertion is overlined. The intron is presented in lowercase letters. The predicted amino acid sequence is shown below the *SHI* open reading frame; the asterisk indicates the termination codon. The cysteine residues comprising the putative zinc finger are boxed. The glutamines in the two glutamine-rich regions are underlined, and the amino acids of the acidic stretch are underlined with an open bar. The possible polyadenylation sites as identified from RT-PCR products are indicated by slashes. The GenBank accession number is AF152555.

DNA binding region of the yeast transcriptional activator GAL4 (Kraulis et al., 1992). Two regions rich in glutamine residues were found adjacent to the zinc finger domain, as well as a short stretch of acidic residues in the C-terminal third of the SHI protein. These features have been found in transcription factor proteins and have been demonstrated to be important for their function as transcriptional activators (Mitchell and Tjian, 1989). Furthermore, two putative nuclear localization signals (NLSs) were found in the SHI sequence. These are two basic stretches (amino acids 160 to 165 and 183 to 188) that conform to the consensus of the simian virus 40–like "typical NLS" defined as four arginines and lysines within a region of six amino acids (Boulikas, 1994; LaCasse and Lefebvre, 1995).

As shown in Figure 9, the SHI amino acid sequence shows extensive sequence similarity over the putative zinc finger region to Lateral Root Primordium1 (LRP1; Smith and Fedoroff, 1995), to an expressed sequence tag (GenBank accession number T88542), and to a chromosome 4 bacterial artificial chromosome clone sequence identified in the European Union Arabidopsis sequencing project (locus ATF23E13; accession number AL022141).

Restriction fragment length polymorphism (RFLP) mapping using recombinant inbred lines (Lister and Dean, 1993) showed that *SHI* is located at the bottom of chromosome 5 (denoted ES724A) ~2.5 cM below the pHJ5 locus (Lister and Dean recombinant inbred map; http://nasc.nott.ac.uk/new ri map.html) and ~2.9 cM above the RFLP marker m555 (Chang et al., 1988).

In the parental line Tn108 used to generate the *shi* mutant, the position of the *Ds*(*Hyg*) element is on chromosome 3 (Long et al., 1997). This implies that *Ds* has transposed between chromosomes and not to a linked site, which would be a more common event (Bancroft and Dean, 1993; Keller et al., 1993).

The *SHI* Gene Is Overexpressed in the *shi* Mutant

The *shi* mutation is caused by insertion of a *Ds*(*Hyg*) transposable element, which carries a cauliflower mosaic virus 35S promoter transcribing out of the element over one terminus. The position and orientation of the *Ds* element in the *SHI* gene suggest that transcription from the cauliflower mosaic virus 35S promoter within *Ds* would lead to overexpression of the *SHI* open reading frame rather than a disruption of the *SHI* gene. RNA gel blot analysis was used to estimate the level of *SHI* transcript in the mutant and the wild type. Figure 10A shows the presence of high levels of *SHI* mRNA in the homozygous 4-week-old *shi* plants, whereas a transcript could not be detected in the 4-week-old wild-type plant. We also performed RNA gel blot analysis using total RNA isolated from different tissues of a wild-type plant; however, no *SHI* transcript could be detected.

Using RT-PCR analysis, we detected low levels of *SHI* mRNA in wild-type flowers, siliques, inflorescence stems, cauline and rosette leaves, and roots and in young seedlings (Figure 10B). Taken together, these data suggest that overexpression of the *SHI* open reading frame confers the phenotype of the *shi* mutant and that the transcript in the wild type is present at low levels in tissues subjected to GA signaling.

Figure 9. Amino Acid Sequence Alignment of Zinc Finger Domains.

Comparison of the putative zinc finger domains of SHI (amino acids 111 to 165), LRP1, an expressed sequence tag (T88542), and a bacterial artificial chromosome clone (ATF23E13). Identical and similar residues are displayed in black and gray boxes, respectively. Cysteine residues comprising the putative zinc finger are denoted with asterisks.

DISCUSSION

We report the isolation and characterization of *shi*, a new semidominant mutation of Arabidopsis conferring GA-insensitive dwarfism to plants overexpressing the *SHI* gene. The phenotype of the *shi* mutant is similar to the phenotypes of the weak GA biosynthesis mutants *ga1* to *ga6* (Koornneef and van der Veen, 1980; Sponsel et al., 1997) and to the GA-insensitive mutant *gai* (Koornneef et al., 1985). This phenotype includes dwarfism, slightly narrower and darker green leaves, reduced apical dominance, and delayed flowering time under SD conditions. Dwarfism of the *shi* mutant is due to reduced elongation of epidermal cells in the inflorescence stem, as occurs in *gai* and the weak GA biosynthesis mutant *ga1-5.*

The dwarf phenotype of the *shi* mutant cannot be restored by treatment with GA, suggesting that *shi* is not defective in the GA biosynthesis pathway. In the *shi* mutant, we found an accumulation of active C_{19}-GAs to a level three times that occurring in the wild type and a corresponding decrease of the precursor C_{20}-GAs. Huang et al. (1998) have shown that a three-fold increase in active GA is of biological significance. Arabidopsis plants overexpressing a gene encoding the GA biosynthetic enzyme 20-oxidase were found to accumulate active GA to a level three times that of the wild-type level, which resulted in elongated growth similar to *spy-3* and plants sprayed with high levels of active GA. GA biosynthesis genes are highly regulated during growth and development, and several studies suggest that GA biosynthesis is controlled by a variety of negative feedback mechanisms (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995). In several known mutants with decreased GA signal transduction, including *gai*, there is an accumulation of the bioactive GAs to a level higher than that in the wild type (Ross, 1994). These data together with the phenotypic resemblance between *shi* and GA biosynthesis mutants suggest that the *shi* mutant is defective in its response to GA.

However, the *shi* mutant is not completely insensitive to GA. Application of high concentrations of GA can restore the delayed transition to floral development in *shi*, whereas the dwarfing phenotype of a *shi* mutant is not affected by this treatment. This difference in GA responsiveness has not been detected in *gai* mutants, in which neither the dwarfing nor the late-flowering phenotype responds to the application of GA. We propose three models that could explain the difference in GA responsiveness of *shi* and *gai* mutants. The first model suggests that *shi* and *gai* are defective in the same GA signal transduction pathway leading to both cell elongation and flower induction. In this case, signal transduction in the *gai* mutant must be more severely repressed. This would also imply that flowering-time control responds

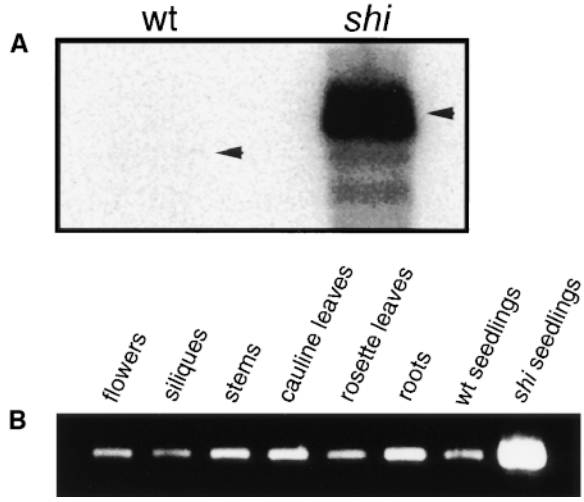

Figure 10. Overexpression of *SHI* in the Mutant.

SHI is expressed at low levels in the wild type (wt) and overexpressed in the *shi* mutant.

(A) RNA gel blot analysis of *SHI* transcript abundance in wild-type plants and plants homozygous for the *shi* mutation. Total RNA was extracted from 4-week-old wild-type and *shi* plants, and 5 μg was loaded in each lane. To detect the *SHI* transcript, we used a probe corresponding to a part of the 5′ untranslated leader of the *SHI* cDNA. The length of the *SHI* transcript in the mutant is ~1.9 kb (arrowhead). The arrowhead in the wild-type lane indicates the expected position of a wild-type *SHI* transcript (~1.6 kb).

(B) RT-PCR analysis using total RNA isolated from wild-type flowers, siliques, inflorescence stems, cauline leaves, rosette leaves, and roots and from wild-type and *shi* 12-day-old seedlings. Data are from one representative experiment.

to lower levels of GA than does stem elongation. A slightly stronger response in *shi* would then be sufficient to induce flowering if GA levels are increased artificially. Alternatively, *shi* and *gai* might be defective in different GA signaling pathways. In this model, the pathway defective in *gai* influences both cell elongation and flower induction, whereas the *shi* mutation affects a pathway with stronger influence on cell elongation than on phase transition to flowering.

In keeping with the first model, it could be argued that a firmer repression of GA signaling in *gai* mutants would lead to a more severe dwarf appearance and delay in flowering time than occurs in *shi* mutants. Because we could not detect any difference between *gai* and *shi* mutants in these respects, we currently favor the second model. In the third model, we suggest that because the *shi* mutation is due to overexpression of *SHI* by the 35S promoter, it is also possible that different expression levels of *SHI* by this promoter in different tissues cause the difference in GA response to flower induction versus stem growth. It may be argued that the effect of *shi* on flower induction could be unrelated to GA, thus explaining the difference in responsiveness to GA in floral transition between *shi* and *gai.* However, this does not seem likely because the increase in GA levels in the *shi* mutant, which corresponds to a significant external dose (Huang et al., 1998), was not sufficient to restore flowering time. Furthermore, application of smaller external doses that do correct the *ga1-5* flowering time do not fully restore the early flowering time of *shi*.

The predicted amino acid sequence of SHI suggests that the gene encodes a new putative zinc finger protein. Zinc finger domains are known to be involved in DNA binding but also have been shown to interact with both RNA and other proteins (reviewed in Mackay and Crossley, 1998). The amino acid sequence of SHI shows extensive sequence similarity over the putative zinc finger region to the predicted gene products of three identified Arabidopsis genes, suggesting that this region is important for the function of the encoded proteins in this new gene family. The SHI amino acid sequence also contains acidic and glutamine-rich stretches. These motifs are commonly found in proteins that are known to function as transcriptional regulators (Mitchell and Tjian, 1989). Furthermore, we have identified two possible NLS sequences, which might suggest a nuclear localization of SHI. Taken together, these data suggest that SHI might function in transcriptional regulation.

Overexpression of the *SHI* gene leads to dwarfism due to reduced elongation of cells in the bolting stem, as well as other features typical for reduced levels of active GA, and this implies that the wild-type SHI protein might be a repressor of GA responses, in particular GA-induced cell elongation in bolting stems. mRNA accumulation analysis suggests that *SHI* in the wild type is active in elongating stems, developing flowers and pods, leaves, and roots, thus supporting this hypothesis. However, we cannot exclude the possibility that some or all of the mutant phenotypic traits seen in *shi* are the result of ectopic expression of *SHI*, causing SHI to interfere with the GA response pathway in cells in which *SHI* normally is not active.

To date, only three cloned Arabidopsis genes suggested to be involved in GA response have been reported. These genes are *SPY* (Jacobsen et al., 1996) and the two homologous genes *GAI* and *RGA* (Peng et al., 1997; Silverstone et al., 1998), and they are all thought to be negative regulators of the GA response. It has therefore been suggested that GA signaling, at least in one pathway, could be regulated by a negative derepressible system, with *SPY*, *GAI*, and *RGA* as components or modulators (Peng et al., 1997; Harberd et al., 1998). The cloning of *SHI*, which also appears to encode a repressor of GA signaling, lends further support to this hypothesis. From genetic and phenotypic analyses of the *pkl* and *sly1* mutants (Ogas et al., 1997; Steber et al., 1998), both PKL and SLY1 have been suggested to be activators of GA signaling. If so, they may be involved in controlling derepression of GA signaling.

Recent progress in our understanding of GA signal transduction has indicated the importance of negative regulators in GA signaling. One central question that remains to be answered is how GA can influence a number of different developmental processes. Are there separate GA signaling pathways leading to stem elongation, flower induction, flower development, and seed germination? Our current data indicate that SHI might be involved in a pathway acting primarily on stem growth and the transition to flowering but that the SHI-mediated control is stronger for stem elongation than it is for flower induction. Further studies on the function of the *SHI* gene are important to attain a more comprehensive model of the GA signaling pathways. The cloned *SHI* gene may also have a direct application to crop improvement. Mutants defective in the GA response have had a major impact on wheat breeding. Reduced plant height in *Rht* mutant dwarf wheat varieties has resulted in reduced lodging and, as a consequence, increased yield (Stoddart, 1984; Lenton et al., 1987). Construction of transgenic plants carrying the *SHI* open reading frame behind a constitutive promoter represents a potential strategy to reduce the height of crop plants without reducing flower production.

METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana shi* (for *short internodes*) mutant was identified in a transposon-tagging mutant screen by using a two-component *Activator*/*Dissociation* (*Ac*/*Ds*) strategy described previously (Long et al., 1993; Wilson et al., 1996). To induce transposition, we crossed plants homozygous for the transposase source *Ac* 35S::TPase (line Tn25, transformant A; Swinburne et al., 1992) to plants homozygous for the *Ds* hygromycin (*Ds*[*Hyg*]) element (line Tn108; Long et al., 1997). The F_1 progeny were allowed to self-fertilize, and the F_2 populations were scored for seedlings carrying transposed elements, indicated by resistance to both hygromycin and

streptomycin. The stability of the *Ds* element in the F_2 individuals was investigated by testing for the presence of β-glucuronidase reporter gene activity linked to the *Ac* 35S::TPase gene. Seeds were surface sterilized (washed in 70% ethanol for 2 min and in 15% chlorine and 0.5% SDS for 10 min followed by at least four washes in sterile distilled water) and sown on GM medium (Valvekens et al., 1988). Before cultivation, seed dormancy was broken by 3 to 4 days of cold treatment (4°C). For antibiotic testing, the medium was supplemented with streptomycin (200 mg/L) and hygromycin (40 mg/L). To test for expression of β-glucuronidase, we placed seedlings in a Petri dish with the roots immersed in 5-chloro-4-bromo-3-indolyl β-D-glucuronide (X-Gluc) substrate (0.5 mg/L X-Gluc cyclohexylammonium salt in 50 mM sodium phosphate, pH 7.0, and 0.05% Triton X-100) and incubated at 37°C for 15 min. Expression of the gene was detected by the appearance of a blue precipitate in the roots.

Plants grown under nonsterile conditions were planted on soil mixed with vermiculite (2:1). All plants were cultivated in controlled environmental chambers at 20 to 22°C, soil-grown material was cultivated under long-day (18 hr of light and 6 hr of darkness) or short-day (SD) conditions (9 hr of light and 15 hr of darkness), and in vitro–grown plants were cultivated in 12 hr of light and 12 hr of darkness. The *Ac* and *Ds* lines used are in the Landsberg *erecta* (L*er*) background and were kindly provided by G. Coupland (John Innes Centre, Norwich, UK). The wild-type plants used in all experiments are of the L*er* ecotype. Seeds from the gibberellin (GA) mutants *ga1-5* and *gai* were kindly provided by the Arabidopsis Biological Resource Center (Ohio State University, Columbus).

Isolation of Plant DNA

DNA from the *shi* mutant was isolated according to the protocol of Dellaporta et al. (1983), with the following modifications. In step 7, the pellet was dissolved in 480 μL of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0). Rather than step 8, 30 μL of 5 M NaCl and 20 μg of RNase A were added, and the tube was incubated for 30 min at 37°C. The solution was extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated in 96% ethanol and pelleted in a microcentrifuge, washed with 70% ethanol, dried, and dissolved in TE buffer. DNA from revertant plants was isolated using the FastDNA Kit (Bio-101, La Jolla, CA), according to the manufacturer's protocol.

Polymerase Chain Reactions

Inverse polymerase chain reaction (IPCR) was used to isolate DNA adjacent to the *Ds*(*Hyg*) element. *shi* mutant DNA was cleaved with BstYI and then precipitated and religated according to Long et al. (1993). The circularized DNA fragments were ethanol precipitated and dissolved in 50 μL of TE buffer. For PCR, 0.5 and 5 μL of this solution was used in a total reaction volume of 50 μL. The primers used to amplify DNA adjacent to the termini of *Ds*(*Hyg*) were for the 5′ ends E4 (5′-AAACGGTAAACGGAAACGGAAACGG-3′) and I1 (5′-TATACGATAACGGTCGGTACGGG-3′) and for the 3′ ends DL3 (5′-CACCGGTACCGACCGTTACCGACCG-3′) and DL6 (5′-TTGCTGCAGCAATAACAGAGTCTAGC-3′). Dynazyme Taq polymerase (Finnzymes, Espoo, Finland) was used. The reaction was heated to 94°C for 4 min and then exposed to 34 cycles of 94°C for 1 min, 60°C (I1 and E4) or 57°C (DL3 and DL6) for 2 min, and 72°C for 3 min, and then finally incubated at 72°C for 7 min.

One microliter of the IPCR products was used in a second PCR with nested primers for the 5′ end fragments D73 (5′-TTCCCATCCTACTTTCATCCCTG-3′) and DL1 (5′-TAGAGCTAGTTACCCGACCG-3′) and for the 3′ end fragments D71 (5′-CGTTACCGACCGTTTTCATCCCTA-3′) and DL6. The PCR program used was the same as for the IPCR reactions but with the annealing temperatures 57°C (D73 and DL1) and 65°C (D71 and DL6), respectively.

To amplify the *Ds* donor site in the revertant plants, we ran the PCR using AmpliTaq Gold polymerase (Perkin-Elmer) and two *SHI*-specific primers, rev5′ (5′-GAGAGGATGAAGAAGAAGAAG-3′) and rev3′ (5′-CACGTGATGATCAACGGTAAG-3′), with the following cycling conditions: 94°C for 10 min; 34 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min.

The probe fragment corresponding to a part of the 5′ untranslated leader of *SHI* was amplified using AmpliTaq Gold polymerase and the primers 5′SHI (5′-CTTCTTCCTCTTCATCCTCTC-3′) and SHI:F (5′-TGTTTCCTCCTCCGCCGTGTCCTAACG-3′) under the following cycling conditions: 94°C for 10 min; 34 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and 72°C for 10 min.

DNA Cloning and Sequencing and Sequence Analysis

A genomic library of the Arabidopsis Columbia ecotype (EMBL, Heidelberg, Germany) was screened with a 210-bp fragment obtained by IPCR using the 3′ end of the *Ds* element. Three clones were isolated from $\sim 6 \times 10^4$ plaque-forming units (pfu). Phage DNA was isolated and analyzed by restriction enzyme digestion and DNA gel blotting, according to standard procedures. A 4.2-kb BamHI-EcoRI genomic fragment hybridizing with the IPCR fragment was gel purified using a Jetsorb gel extraction kit (Genomed, Bad Oeynhausen, Germany) and subcloned into the pBluescript II SK+ vector (Stratagene, La Jolla, CA). Competent XL1-Blue MRF cells of *Escherichia coli* (Stratagene) were used for transformations, which were performed as described by Sambrook et al. (1989).

A cDNA library of the Arabidopsis Columbia ecotype (λ PRL2; Arabidopsis Biological Resource Center) was screened with the 4.2-kb *SHI* genomic fragment ($\sim 7 \times 10^5$ pfu) and with the PCR fragment corresponding to the untranslated leader of *SHI* (another 4×10^5 pfu); however, no positive signals could be detected.

The IPCR and PCR fragments were directly purified using the Wizard PCR Preps DNA purification system (Promega) and cloned into the pCR-script Amp SK+ vector (Stratagene) or the pCR 2.1-TOPO vector (Invitrogen, Leek, The Netherlands). All plasmid DNA was extracted and purified using the Wizard minipreps DNA purification system (Promega). Sequencing was performed using an ABI automated sequencer (Perkin-Elmer, Norwalk, CT). Primary sequence analysis was performed using MacVector version 3.0 (Oxford Molecular, Campbell, CA) and auto assembler (ABI PRISM; Perkin-Elmer) computer programs.

RNA Isolation and RNA Gel Blot Analysis

Arabidopsis plant material for expression analyses was harvested, frozen in liquid nitrogen, and stored at −70°C until used. The material used was in vitro–grown 4-week-old wild-type and homozygous *shi* plants and in vitro–grown 12-day-old wild-type and homozygous *shi* seedlings, as well as flowers, siliques, inflorescence stems, cauline and rosette leaves, and roots from soil-grown adult wild-type plants. Total RNA was extracted using a protocol from Verwoerd et al.

(1989). The RNA preparations were DNase treated using the removal of DNA procedure from the S.N.A.P. total RNA isolation kit (Invitrogen). For RNA gel blot analysis, 5 μg of each RNA sample was electrophoresed in 1% agarose gels with 17% formaldehyde in 1 × running buffer (10 × running buffer is 0.2 M Mops, pH 7.0, 80 mM NaOAc, and 5 mM EDTA, pH 8.0) and transferred to Hybond N^+ nylon membranes (Amersham), according to the manufacturer's instructions. Prehybridization, hybridization, and washing were conducted according to the manufacturer's directions. The final wash was conducted in 0.5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 8.0) and 0.1% SDS for 10 min at 65°C. The hybridization probe was radiolabeled with ^{32}P-dCTP by random priming using the Rediprime kit (Amersham). Blots were exposed on a PhosphorImager plate and analyzed using a PhosphorImager BAS 2000II Bio-Analyzer (Fuji, Tokyo, Japan).

Reverse Transcription–PCR and Rapid Amplification of 5′ cDNA Ends

Reverse transcription–PCR (RT-PCR) was performed using the Access RT-PCR system (Promega). For expression analysis, 500 ng of DNase-treated total RNA was used in each RT-PCR reaction. The primers used were SHI:S (5′-AAGATCTCTATCAAGAGAGAGATC-3′) and SHI:O (5′-GGATCCATCATCTACGGAACTCACAC-3′). The identity of the products was confirmed by DNA gel blot analysis according to standard procedures, using a probe fragment corresponding to a part of the 5′ untranslated leader of *SHI.* The amplification of DNA from extracted RNA was verified by repeating the RT-PCR experiment four times.

To isolate a *SHI* cDNA, we performed first-strand cDNA synthesis using the standard oligo$(dT)_{17}$ adapter primer (Frohman et al., 1988). PCR reactions were performed using the adapter primer (Frohman et al., 1988) and the upstream primer rev5′ (described earlier). One hundred nanograms of total RNA from 4-week-old wild-type plants was used in the reactions. One microliter of a 10-fold dilution of a reaction was used in a second PCR with the nesting primers SHI:V (5′-ATGATGATGAGAAGCGGTAGC-3′) and SHI:H (5′-ATGATCTGAAGTGGAGCCAACG-3′).

To identify transcriptional start sites, we performed rapid amplification of 5′ cDNA ends with adapter-ligated cDNA, using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). The gene-specific primer used in the initial PCR reaction was SHI:F (5′-TGTTTCCTCCTCCGCCGTGTCCTAACG-3′), and the gene-specific nesting primer was SHI:E (5′-ATCCTGCCATTTTCCGATACCACCAGCC-3′). The PCR reactions were performed with AmpliTaq Gold polymerase with the following cycling conditions: 94°C for 10 min; 34 cycles at 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min; 72°C for 10 min and 94°C for 10 min; 25 cycles at 94°C for 1 min, 68°C for 2 min, and 72°C for 3 min; and 72°C for 10 min, respectively.

Mapping the *SHI* Locus

A restriction fragment length polymorphism (RFLP) was found between the ecotypes L*er* and Columbia when genomic DNA was cleaved with XbaI, separated on a 0.8% agarose gel, transferred to a Hybond N^+ membrane according to the manufacturer's instructions, and probed with the *SHI* 210-bp IPCR fragment. Radiolabeling of the probe, hybridization, and washing were performed as described for RNA gel blot analysis. The XbaI RFLP was used to score 46 independent recombinant inbred lines (Lister and Dean, 1993) as having the *SHI*-hybridizing XbaI fragment from L*er* or Columbia. The data were used to give the *SHI* position relative to the known RFLP markers.

GA Analysis

The levels of different GAs in whole inflorescence stem segments of 5-week-old *shi* and L*er* plants were measured. Samples were extracted overnight in 20 mL of 80% methanol with 17,17-($^2H_2$)GAs added as internal standards (purchased from Prof. L.N. Mander, Australian National University, Canberra, Australia). After concentration in vacuo, the volume of the aqueous phase was adjusted to 20 mL, and the pH was adjusted to 2.8 with 1 M HCl. The aqueous phase was partitioned three times against ethylacetate. After evaporation of the ethylacetate phase, the sample was dissolved in 2 mL of ethylacetate and applied to a preequilibrated 2-g aminopropyl column (Sorbent AB, V. Frölunda, Sweden). The column was washed with 10 mL of ethylacetate, and the GAs were eluted with 25 mL of 0.2 M formic acid directly on to a preequilibrated 500-mg C_{18} cartridge (Sorbent AB). Thereafter, the GAs were eluted from the C_{18} cartridge with 4 mL of methanol. The samples were further purified, and the respective GA fractions were separated by reversed-phase HPLC. The HPLC system consisted of a 600 pump (Waters Associates, Millford, MA) and a 717-AutoSampler (Waters) connected to a 4.6 × 150-mm reversed-phase HPLC Nova-Pak C_{18} column (Waters). The flow was 1 mL/min, and the GAs were separated by means of gradient elution. The gradient was from 20% methanol (1% acetic acid [v/v]) to 100% methanol (1% acetic acid [v/v]) for 20 min. The HPLC fractions were methylated with ethereal diazomethane and analyzed by using combined gas chromatography/high-resolution selected ion monitoring mass spectrometry, as described previously (Moritz and Olsen, 1995).

Hormone Treatments

For testing of hypocotyl elongation response, we germinated seeds on GM medium only or on GM medium supplemented either with 10^{-5} M GA_4 plus GA_7 (Sigma) or with epibrassinolide (Sigma) at a concentration of 10^{-6}, 10^{-7}, or 10^{-8} M. Hypocotyl elongation was documented after 4 and 10 days.

For the stem elongation and flowering time experiments, seeds were sown on soil under SD conditions. Beginning at ~25 days after sowing, plants were sprayed generously once a week with 10^{-4} or 10^{-5} M GA_3 (Duchefa, Haarlem, The Netherlands) or 10^{-5} M GA_4 (Sigma) containing 0.02% Tween 20 for analyses of flowering time; for analyses of stem elongation, 10^{-4} M GA_3 or GA_4 plus GA_7 containing 0.02% Tween 20 was used. Control plants were treated with a solution containing only 0.02% Tween 20.

Scanning Electron Microscopy

From adult plants, 1-cm segments from the first internode were harvested. Tissues were fixed in 50% ethanol, 5.0% acetic acid, and 3.7% formaldehyde and exposed to a 30-min vacuum. After dehydration through an ethanol series (50% ethanol for 2 × 30 min, 60% ethanol for 30 min, and 70% ethanol until no chlorophyll remained), samples were stored in 70% ethanol until exposure to 85 and 95% ethanol and critical point drying. Samples were coated with gold and analyzed in a scanning electron microscope (model XL 30; Philips

Technologies, Cheshire, CT), and micrographs were taken. Five samples from each genetic line were analyzed, and from each sample, at least 10 cells were measured with respect to length by using National Institutes of Health Image version 1.61 software. Data were statistically analyzed using a nested analysis of variance and the Student's *t* test.

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