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 through 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
with reduced GA biosynthesis. To date, three mutants belonging to this class have been identified in Arabidopsis: gai (GA insensitive), sly1 (sleepy1), and pk1 (pickle) (Koomneef 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 GA-1 MUTANT; Silverstone et al., 1998; or for RESTITUTION 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-pho-toaffinity 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 pk1 mutant is only partially suppressed by GA, whereas GA strongly suppresses the embryonic differentiation characteristics in the primary roots of the pk1 mutant (J. Ogas, J. C. Cheng, Z. R. Sung, and C. Somerville, unpublished data). Despite the GA responsiveness of the pk1 mutant, PKL has been suggested to be involved in GA signaling because pk1 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 F2 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 F2 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 (Ler) background. As expected, the F2 progeny were all semidwarfs, and the F2 population segregated 1:2:1 for the dwarf, semidwarf, and wild-type phenotypes. The homozygous dwarf mutant was designated shi.

Linkage analysis of 111 F3 families segregating for the shi
Reduced GA Responsiveness in shi 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 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.

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.
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 GA4 plus GA7 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 (GA3 or GA4 plus GA7) 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 (Ler) 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-
Reduced GA Responsiveness in shi

Wild-type, gai, and ga1-5 plants grown under SD conditions were repeatedly sprayed with 100 μM GA3. 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 GA3 or GA4) 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 inflorescence 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,
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 C19-GAs, including the bioactive GA4, at a level between two and four times higher than wild-type levels. We also observed a decrease of precursor C20-GAs to between one-third and one-tenth that of the wild-type level (Figure 7). In **shi**, the level of GA34, 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 in **shi**, which 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-X2-C-X7-C-X4-C-X2-C-X7-C arrangement (where X is a variable amino acid) is suggested to make up a zinc finger domain similar to the Zn2Cys6 cluster found in the...
Reduced GA Responsiveness in *shi* 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 AT2F23E13; 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 terminator. 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.
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 C19-GAs to a level three times that occurring in the wild type and a corresponding decrease of the precursor C20-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

**DISCUSSION**

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

Comparison of the putative zinc finger domains of SHI (amino acids 111 to 165), LRPI, 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.

**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, stems, cauleine leaves, rosette leaves, roots, and seedlings 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 F1 progeny were allowed to self-fertilize, and the F2 populations were scored for seedlings carrying transposed elements, indicated by resistance to both hygromycin and
and dissolved in 50 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/isomyl alcohol (24:1). DNA was precipitated in 96% ethanol and pelletted 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). Competent XL1-Blue MRF cells of Escherichia coli (Stratagene) were used for transformations, which were performed as described by Sambrook et al. (1989).

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 ~6 × 10^6 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 jetsetor 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 (~7 × 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 Prep 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 miniprep 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.
A restriction fragment length polymorphism (RFLP) was found between the ecotypes Ler 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 independently inbred lines (Lister and Dean, 1993) as having the SHI-hybridizing XbaI fragment from Ler or Columbia. The data were used to give the SHI position relative to the known RFLP markers.

**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:5 (-3'ATGATCTCTACTTACAGAGAGGACATC-3') and SHI:O (-5'GGATCCACATCTTACGGAACTCACC-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 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 32P-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).

**Hormone Treatments**

For testing of hypocotyl elongation response, we germinated seeds on GM medium only or on GM medium supplemented either with 10⁻³ M GA₄ plus GA₇ (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 GA₄ (Duchefa, Haarlem, The Netherlands) or 10⁻⁵ M GA₄ (Sigma) containing 0.02% Tween 20 for analyses of flowering time for analyses of stem elongation, 10⁻⁴ M GA₄, or GA₄ plus GA₇ containing 0.02% Tween 20 was used. Control plants were treated with a solution containing only 0.02% Tween 20.

**Mapping the SHI Locus**

A restriction fragment length polymorphism (RFLP) was found between the ecotypes Ler 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 independently inbred lines (Lester and Dean, 1993) as having the SHI-hybridizing XbaI fragment from Ler 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 Ler plants were measured. Samples were extracted overnight in 20 mL of 80% methanol with 17.17-(P₂H₂)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 pre-equilibrated 2-g aminnopropyl column (Sorbent AB, V. Fröluanda, 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 pre-equilibrated 500-mg C₁₈ cartridge (Sorbent AB). Thereafter, the GAs were eluted from the C₁₈ 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, Milford, MA) and a 717-AutoSampler (Waters) connected to a 4.6 × 150-mm reversed-phase HPLC Nova-Pak C₁₈ 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).

**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 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 pre-equilibrated 2-g aminnopropyl column (Sorbent AB, V. Fröluanda, 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 pre-equilibrated 500-mg C₁₈ cartridge (Sorbent AB). Thereafter, the GAs were eluted from the C₁₈ 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, Milford, MA) and a 717-AutoSampler (Waters) connected to a 4.6 × 150-mm reversed-phase HPLC Nova-Pak C₁₈ 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).
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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The Arabidopsis Dwarf Mutant shi Exhibits Reduced Gibberellin Responses Conferred by Overexpression of a New Putative Zinc Finger Protein

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