The Arabidopsis Protein SHI Represses Gibberellin Responses in Arabidopsis and Barley[1]

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The current model of gibberellin (GA) signal transduction is based on a derepressible system and a number of candidate negative regulators have been identified in Arabidopsis. We previously have reported the identification of the Arabidopsis gene *SHORT INTERNODES* (*SHI*) that causes suppression of GA responses when constitutively activated. In this paper, we show by using reporter gene analysis that the *SHI* gene is expressed in young organs, e.g. shoot apices and root tips. The model predicts a suppressor of GA responses to be active in these tissues to prevent premature growth or development. To study the effect of SHI on GA signaling, we used a functional assay that measures effects of signaling components on a well-defined GA response; the up-regulation of α-amylase in barley (*Hordeum vulgare*) aleurones in response to GA treatment. We found that SHI was able to specifically block the activity of a high-isoelectric point α-amylase promoter following GA_3 treatment, which further supports that SHI is a suppressor of GA responses. We have identified two putative loss-of-function insertion alleles of *SHI* and lines homozygous for either of the new alleles show no phenotypic deviations from wild type. Because *SHI* belongs to a gene family consisting of nine members, we suggest that *SHI* and the *SHI*-related genes are functionally redundant. We also show that a functional *ERECTA* allele is able to partly suppress the dwarfing effect of the *shi* gain-of-function mutation, suggesting that the *erecta* mutation harbored by the Landsberg *erecta* ecotype is an enhancer of the *shi* dwarf phenotype.

The plant hormone gibberellin (GA) mediates the transition to new developmental phases (e.g. germination and reproductive development) as well as a variety of growth responses. These diverse tasks of GA signaling must rely on a tight regulation. A number of candidate components of the GA signal transduction pathway have been identified by mutation analysis in Arabidopsis. The Arabidopsis proteins GAI (for GA insensitive; Peng et al., 1997) and RGA (for repressor of ga1-3; Silverstone et al., 1998) are members of the GRAS family of putative transcription factors (Pysh et al., 1999), and they are both postulated to function as negative regulators of GA responses in Arabidopsis. Gain-of-function mutations in *GAI* and *RGA* cause dwarf phenotypes that phenocopy GA deficiency, whereas loss-of-function mutations result in reduced sensitivity to reduction in GA levels. The SPY (SPINDLY) protein is believed to act as a negative regulator of GA signaling through GlcNAc modifications of downstream proteins (Jacobsen et al., 1996; Lubas et al., 1997). Mutations in *SPY* cause an elongated growth typical for the response to exogenous application of excessive GA. The identification of an Arabidopsis SPY homolog, SPINDLY2, was reported recently and SPY2 is suggested to function in a partially redundant manner to SPY (Hartweck et al., 2000). Furthermore, PKL (PICKLE), a CHD3 chromatin-remodeling factor, is suggested to be a component of a GA-modulated developmental switch that functions during germination to specifically suppress embryonic differentiation characteristics in Arabidopsis (Ogas et al., 1999). SLY1 (SLEEPY1) is a very interesting putative component of GA signal transduction that still awaits cloning (Steber et al., 1998). The *sly1* mutant displays the full spectrum of phenotypes associated with severe GA deficiency including germination defects, suggesting that the SLY1 protein is a key component of the GA signal transduction pathway, acting as a positive regulator, or possibly as a receptor. We have reported previously on the identification of the Arabidopsis protein SHI (SHORT INTERNODES), which we suggest to be a negative regulator of GA-induced cell elongation (Fridborg et al., 1999). In the *shi* mutant, a transposon insertion confers overexpression of the *SHI* gene resulting in a dwarf phenotype typical of mutants defective in GA biosynthesis or response. Application of high doses of exogenous GA does not correct the dwarf phenotype, suggesting that *shi* is affected in GA responsiveness. Defects in the ability to respond to GA have been shown previously to result in reductions in negative feedback control of GA biosynthesis (Talon et al., 1990). In agreement

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with this, we found an elevated level of active GA in the *shi* mutant (Fridborg et al., 1999). The SHI protein sequence contains a zinc finger motif similar to the previously described RING fingers (Lovering et al., 1993) that are believed to mediate protein-protein interactions involved in ubiquitination for targeted proteolysis (Tyers and Willems, 1999; for review, see Freemont, 2000) or in transcriptional regulation (Peng et al., 2000).

GA responses are easily identifiable phenotypically but are generally poorly characterized at the genetic and molecular level. One exception is the response to GA in germinating cereal grains. The key response is the production of the hydrolytic enzyme α-amylase, which is synthesized in the aleurone cells during germination for breakdown and mobilization of the starch in the endosperm of the seed. This has led to a widespread use of the cereal aleurone as a model system for studying the GA action at the molecular and genetic level. A number of candidate components involved in GA signaling and response has been identified using this system. In aleurone cells, GAs are proposed to be perceived at the plasma membrane, and there is accumulating evidence for a role of heterotrimeric G-proteins, Ca^{2+}, and calmodulin as transducers of the GA signal (for review, see Lovegrove and Hooley, 2000). The coding sequence of a MYB transcription factor gene, *HvGAMYB*, has been isolated from a barley (*Hordeum vulgare*) aleurone cDNA library and the gene product has been demonstrated to transcriptionally upregulate α-amylase and other hydrolytic enzymes in response to GA (Gubler et al., 1995, 1999). In addition, a novel zinc finger transcriptional repressor, HRT (*Hordeum* repressor of transcription) was isolated from barley and shown to bind to the α-amylase promoter, thereby repressing α-amylase expression (Raventós et al., 1998).

Genes encoding proteins similar to HvGAMYB and HRT are present in the Arabidopsis genome. Furthermore, homologs of the Arabidopsis proteins GAI and RGA have been identified in wheat (*Triticum aestivum*; RHT1; Peng et al., 1999), maize (*Zea mays*; D8; Peng et al., 1999), rice (*Oryza sativa*; OsGAI; Ogawa et al., 2000), and barley (SLN; Gubler et al., 2000) and their function as suppressors of GA signaling in these species have been supported by mutant analysis. A barley homolog of the *SPY* gene, *HvSPY*, has been cloned recently, and the gene product is shown to be able to specifically inhibit GA-induced expression of barley α-amylase (Robertson et al., 1998). Furthermore, when the Arabidopsis protein GAI is expressed transiently in aleurone cells, it also represses GA-stimulated induction of α-amylase activity (F. Gubler, personal communication). This indicates that GA signaling components are conserved between distantly related species and different GA responses, and that the well-defined aleurone system could be used for studies of the putative GA response repressors identified in Arabidopsis.

To establish if SHI could act as a negative regulator of GA responses not only when constitutively expressed, but also in wild-type Arabidopsis, we have studied the temporal and spatial distribution of SHI promoter activity in Arabidopsis using promoter-reporter gene constructs. We have also isolated two insertion mutants in *SHI*, and our results are presented in this paper. Furthermore, we have exploited the barley aleurone system to determine if SHI, when transiently expressed, can suppress GA-induced α-amylase promoter activity.

RESULTS

The *SHI* Promoter Is Active Primarily in Shoot and Root Primordia

SHI gene expression in roots, stems, flowers, rosette leaves, cauline leaves, and siliques have been demonstrated previously by reverse transcriptase-PCR analysis (Fridborg et al., 1999). For a more detailed study of the expression of *SHI* in these organs, we introduced constructs with *SHI* upstream regulatory sequences and part of the *SHI* open reading frame (ORF) fused to the reporter gene *uidA* encoding β-glucuronidase (GUS) into Arabidopsis by *Agrobacterium tumefaciens*-mediated transformation. It has been shown previously that a number of Arabidopsis genes require the presence of intron sequences for proper expression (Gidekel et al., 1996; Rose and Last, 1997; Silverstone et al., 1997a). Therefore, we made two different GUS constructs, one with an approximately 1.5-kb sequence upstream of the *SHI* ATG start site plus the first exon of *SHI* fused to the GUS ORF, and the other with the same upstream sequence plus the first exon, the intron, and part of the second exon of *SHI* fused to GUS. For each construct, six independent transformed lines segregating for a single insert were generated, and homozygous offspring of each line were analyzed for GUS activity.

GUS expression was detected in all lines that carry the construct without the *SHI* intron, although the staining was very weak in these lines. In contrast, GUS staining was much stronger in the transformant lines carrying the construct where the *SHI* intron was included, from now on referred to as the SHI-GUS lines. However, the temporal and spatial distribution of GUS staining was the same for the independent lines of both constructs, which implies that the intron is necessary for correct levels of expression of *SHI*, but not for the regulation of temporal and tissue specificity.

Figure 1 shows the GUS staining of a representative SHI-GUS line. In germinating seeds, expression was first detected at about 48 h after imbibition (Fig. 1A). In 4-d-old seedlings grown in continuous light, GUS activity could be detected in the root/shoot

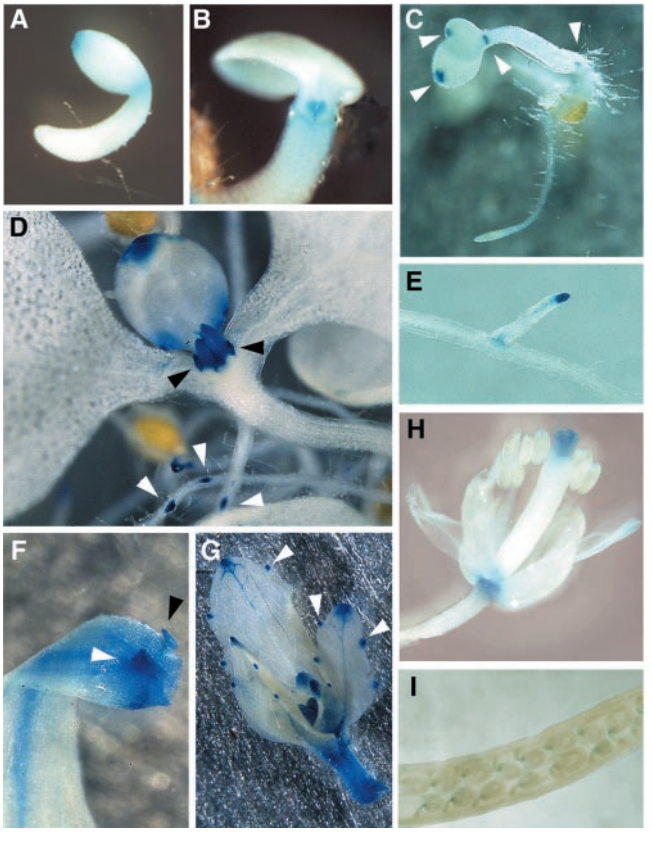

Figure 1. Expression of *SHI* in different tissues. Shown are the results from line 6:1 12-1-1 harboring a construct with an approximately 1.5-kb sequence upstream of the *SHI* ATG start site plus the first exon, the intron, and part of the second exon of *SHI* fused to GUS. A, Two-day-old seedling; B, shoot apex of 4-d-old seedling, front cotyledon is removed; C, 4-d-old seedling. White arrowheads indicate apical hydathodes, apex, and transition zone. D, Shoot apex of 9-d-old seedling, front leaf is removed. Black arrowheads indicate stipules, white arrowheads indicate lateral root primordial. E, Lateral root; F, developing axillary bud (white arrowhead) and stipules (black arrowhead) on the base of a rosette leaf; G, developing secondary shoot from rosette axis. White arrowheads indicate three of the lateral hydathodes. H, Flower; I, mature silique.

transition zone, in the apex, and in the apical region of the cotyledons (Fig. 1, B and C). In 9-d-old seedlings GUS staining was seen in lateral root primordia and in emerging lateral roots, particularly in the root tips (Fig. 1D). No staining was seen in the primary root tip or in the hypocotyl at any time. GUS expression was seen in the shoot apex and in the new leaves, especially in the apical and lateral hydathodes.

In adult plants, GUS expression was seen in lateral root tips (Fig. 1E), lateral root primordia, and axillary shoot primordia (Fig. 1F). Faint expression was detected in stems close to or at branching points (data not shown). Hydathodes of rosette and cauline leaves of primary and secondary shoots stained at all stages of leaf development (Fig. 1G). In the flowers, staining was seen in the style and stigmatic surface of the pistil and in the receptacle (base) of the flower throughout the development of the pistil and the silique (Fig. 1H). No staining was seen in the anthers or filaments of the stamens. In the ovaries, weak staining was detected in the funiculi from just prior to anthesis throughout seed development but no staining was seen in the ovules, in the developing embryo, or in the surrounding maternal tissues (Fig. 1I). No expression of the GUS transgene was detected in dry seeds.

This expression pattern of *SHI* as determined by reporter gene analysis is in agreement with previous data obtained by reverse transcriptase-PCR analysis of the endogenous *SHI* gene activity.

Expression of GUS in SHI-GUS lines was not affected by treatments with GA or the GA biosynthesis inhibitor paclobutrazol (PAC) for 3 d. Similarly, GUS expression was not affected by the addition of the plant hormones epi-Brassinolide, abscisic acid (ABA), benzyladenine, or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, suggesting that the SHI promoter is not regulated by any of these substances (data not shown). However, because GUS in SHI-GUS lines is expressed in lateral root primordia and lateral root tips, auxin treatment (indole-3-acetic acid or 2,4-dichlorophenoxyacetic acid), which causes the development of increased number of lateral roots (Laskowski et al., 1995), indirectly resulted in an increased total expression of GUS in the roots.

Identification of New Insertion Alleles of *SHI*

In the *shi* mutant, the transcriptional regulation of the *SHI* gene is controlled by a 35S promoter located on a *Ds* element inserted in the untranslated leader of the *SHI* gene (Fridborg et al., 1999). To elucidate the function of SHI in the wild type, we aimed at identifying loss-of-function alleles of *SHI*. Several of the available T-DNA and transposon insertion lines were screened for insertions in *SHI*. In the Sainsbury Laboratory Transposants Collection (SLAT; Tissier et al., 1999), we identified one insertion line (denoted *shi-2*) carrying a d*SPM* transposon insertion at a position approximately 100 bp upstream of the predicted TATA box in the *SHI* promoter sequence. In the T-DNA collection from the Arabidopsis Knockout Facility (Krysan et al., 1999), we identified a second insertion line (denoted *shi-3*) harboring a T-DNA insertion in the first part of the second exon (amino acid position 264).

Homozygous plants of these insertion lines were grown in long-day light conditions and screened for a mutant phenotype. The development of shoot apices, lateral root primordia, lateral roots, as well as floral organs were characterized with specific care because these are sites of *SHI* promoter activity and thus expected sites of SHI protein function. We could not detect any phenotypic deviations from wild type of either *shi-2* or *shi-3* at any developmental stage under these growth conditions (data not shown), suggesting that a disruption of the *SHI* gene does not

notably affect the development of the plant. However, the positions of the insertions do not rule out that SHI protein activity in these lines may not have been completely abolished.

Neither the *shi-2* nor the *shi-3* mutant show increased elongation growth, which suggest that none of them are strongly over-responsive to GA signal transduction. Because a weak GA oversensitivity would not be detected during post-germination stages, we studied the germination ability of the insertion lines on the GA biosynthesis inhibitor PAC. We found no increased resistance compared with wild type on any of the concentrations used (5, 10, 25, 50, 75, 120, and 200 μM, data not shown).

We also attempted to silence the expression of *SHI* by making transgenic Landsberg *erecta* (L*er*) plants expressing *SHI* cDNA in antisense orientation behind the constitutive 35S promoter. A high number of stably transformed lines were generated; however, as for the insertion lines, none of the transformants displayed a phenotype different from the wild type (data not shown). These observations further support the notion that a reduction in *SHI* expression has no visible effect on plant growth and development under normal growth conditions.

Identification of Additional Members of the *SHI* Gene Family

The lack of a mutant phenotype of the *SHI* insertion lines and antisense transgenic lines indicate the existence of proteins that are functionally redundant to *SHI*. Many molecules that control genetic regulatory circuits act at extremely low intracellular concentrations. Functional redundancy is often observed between closely related proteins and this may be particularly true for proteins that at low intracellular concentrations control regulatory functions (McAdams and Arkin, 1999). We have shown previously that *SHI* is a member of a small family consisting of at least four genes (Fridborg et al., 1999). Searches in the Arabidopsis Genome Initiative (http://www.Arabidopsis.org/agi.html) databases revealed the existence of five additional, previously unidentified sequences with high similarity to *SHI*, suggesting that the gene family comprises at least nine genes, two of which are *SHI* and *LRP1* (*LATERAL ROOT PRIMORDIUM1*; Smith and Fedoroff, 1995). We have designated the remaining sequences *SRS1* to *SRS7*, for *SHI RELATED SEQUENCE 1–7*, and the gene family was denoted the *SHI* gene family.

The predicted proteins encoded by the genes of the family show particularly high sequence identity over two regions. The first region is positioned in the N-terminal one-half of the proteins, and the sequence identity varies between 64% and 90% (Fig. 2A). This region shows similarity to the consensus sequence of the previously described zinc binding RING finger motif (Freemont, 1993; for review, see Borden, 2000), as seen in Figure 2B. The putative RING domains of SHI, SRS1, SRS2, SRS4, SRS5, and SRS7 comprise 31 amino acid residues in a Cys-X_2-Cys-X_7-Cys-X-His-X_2-Cys-X_2-Cys-X_7-Cys-X_2-His consensus arrangement (X denotes any amino acid). We refer to this motif as a C3HC3H RING domain. This arrangement is consistent with the classical RING consensus se-

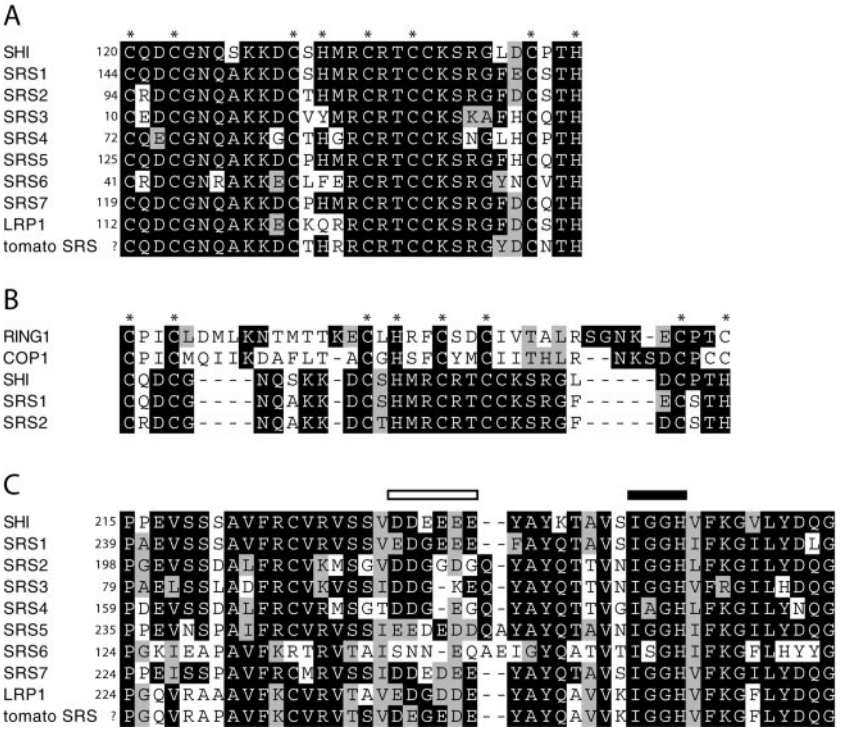

Figure 2. Amino acid sequence similarites between SHI, SHI family members, and other RING proteins. A, Sequence comparison of the RING domains of the SHI related proteins. The amino acid position of the domain in the different proteins is indicated relative to the translational start site. Asterisks indicate the conserved Cys and His zinc ligand residues in the RING finger motif. Identical and similar residues are displayed in black and gray boxes, respectively. B, Alignment of the RING domains of SHI, SRS1, and SRS2 with RING domains from different organisms. RING1, Human; COP1, Arabidopsis. C, Comparison of the IGGH domain of the SHI-related proteins. Acidic stretches (white box) and the four IGGH residues (black box) are indicated.

quence defined as Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys-X_2-Cys-X_{4-48}-Cys-X_2-Cys, or C3HC4 (Fig. 2B; Freemont, 1993), with two exceptions. First, the SHI family proteins have only seven amino acids between the second and third Cys (loop1) where the consensus sequence predicts at least nine. Second, the SHI proteins contain a His residue in the position of the last Cys zinc ligand of the RING consensus (Fig. 2B). As can be observed in Figure 2A, SRS3, SRS6, and LRP1 differ from the other family members in that they lack the first conserved His residue of the restricted C3HC3H RING consensus.

The second conserved region of the SHI family proteins is positioned in the C-terminal part (Fig. 2C). This domain is unique to proteins in the SHI family and does not show similarity to any previously described protein motif. This second conserved region will be referred to as the IGGH domain, named after four highly conserved residues within the region. A short acidic cluster is present in the IGGH domain in several of the SHI-related proteins.

The SHI family is not unique to Arabidopsis. Putative SHI homologs have been found in several other plant species as revealed by database searches, such as tomato (*Lycopersicon esculentum*; Fig. 2, A and C), rice, soybean (*Glycine max*), and *Medicago truncatula*, but not in any organism outside the plant kingdom, suggesting the SHI family to be plant specific.

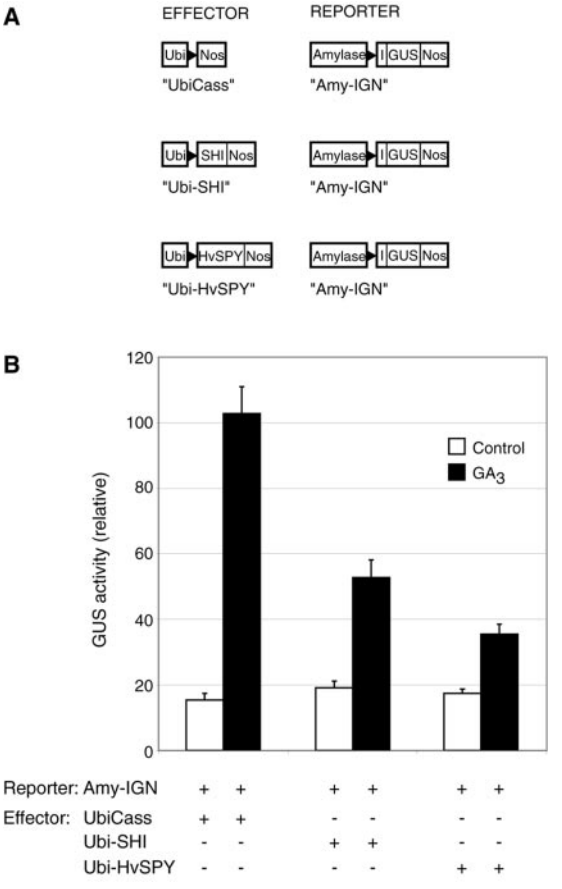

Figure 3. Analysis of SHI function in barley aleurone cells. A, Effector and reporter constructs used in cobombardment experiments. UbiCass is a blank effector cassette with a ubiquitin promoter lacking a coding region. Ubi-SHI and Ubi-HvSPY are effector constructs carrying the *SHI* cDNA and the *HvSPY* cDNA, respectively, fused to the ubiquitin promoter. The reporter construct, Amy-IGN, contains the high-pI α-amylase promoter fused to the IGN (intron-GUS-NOS) reporter cassette. B, Response of the high-pI α-amylase promoter to SHI and HvSPY expression in transient expression analyses. De-embryonated barley half-grains were cobombarded with effector and reporter constructs and incubated with 10 mM $CaCl_2$ (control) or with 10^{-6} M GA_3 in 10 mM $CaCl_2$. Induction of α-amylase promoter activity was measured as relative GUS activity. The results represent two experiments, each with six shootings and three independent GUS measurements of each shooting. Bars represent SES. The difference between the effect of the control effector UbiCass and Ubi-SHI on GUS activity in GA-treated barley half-grains is statistically significant ($P < 0.0001$).

SHI Can Suppress GA Induction of Barley α-Amylase Expression

In the *shi* mutant, overexpression of the *SHI* gene generates a phenotype that suggests an involvement of the SHI protein in the GA signal transduction pathway (Fridborg et al., 1999). To further study the effect of SHI on GA signaling, we used a functional assay that measures the effect of transiently expressed proteins on the GA mediated up-regulation of the hydrolytic enzyme α-amylase in barley aleurones (Gubler et al., 1995; Robertson et al., 1998). A *SHI* overexpression effector construct (Ubi-SHI) was generated by fusing the ubiquitin promoter to the *SHI* cDNA, and this construct was cobombarded into de-embryonated barley half-grains together with a reporter construct (Amy-IGN) consisting of a barley high-pI α-amylase promoter fused to the GUS gene *uidA*. The constructs are shown in Figure 3A. The bombarded grains were treated with GA_3, and the activity of the α-amylase promoter was monitored by measuring GUS activity fluorometrically. As a control, a blank ubiquitin effector cassette (UbiCass) was used instead of Ubi-SHI.

The results show that GA_3 treatment increased GUS activity 7-fold above background levels in barley half-grains transiently transformed with the blank UbiCass together with Amy-IGN (Fig. 3B). When the Ubi-SHI construct was used as the effector, the GA_3-induction of the α-amylase promoter activity was reduced to only three times above background level. As a positive control, the barley *HvSPY* fused to the ubiquitin promoter (Ubi-HvSPY) was used as the effector. HvSPY has been demonstrated to be a strong suppressor of GA_3-induced α-amylase expression (Robertson et al., 1998) and in our experiments, transient expression of HvSPY resulted in a reduction of GUS activity to only 2-fold over the background level (Fig. 3B). The level of α-amylase expression in the absence of GA was similar for all three constructs (Fig. 3B).

To rule out the possibility that SHI, when transiently expressed in the aleurone system, is acting as a general repressor of transcription not limited to GA-associated gene induction, we studied the effect of SHI on the activity of an additional promoter. We used a constitutively active rice actin promoter (from the *rice actin 1 gene*, *Act1*; McElroy et al., 1990) and as expected, transient *Act1* promoter activity in barley aleurones was not significantly altered by GA_3 treatment or by SHI co-expression (data not shown).

The *shi* Overexpressor Mutant Is Sensitive to Reductions in the Endogenous GA Level

The dwarf phenotype of the *shi* gain-of-function mutant cannot be restored to wild type by exogenously applied GA, suggesting that the reduction in stem length of *shi* is not due to impaired GA biosynthesis (Fridborg et al., 1999). Furthermore, the lack of elongation response in *shi* following GA treatment indicates that the *shi* mutant is saturated in GA responses in respect to stem elongation. The endogenous levels of active GA were previously shown to be elevated in *shi*, most likely as a result of reduction in GA biosynthesis feedback regulation, further supporting the hypothesis that GA-regulated elongation response in *shi* are specifically suppressed (Fridborg et al., 1999). If the *shi* mutant is not insensitive to the GA signal per se, we expect the *shi* mutant to be sensitive to reductions in endogenous GA levels. To test this hypothesis, we generated *shi ga1-3* double mutants. Plants homozygous for the *ga1-3* mutation are GA deficient and severely dwarfed unless treated with exogenous GA (Koornneef and van der Veen, 1980). Similar to the *ga1-3* single mutant, the *shi ga1-3* double mutant was unable to germinate without application of exogenous GA. After germination on GA medium but without further supplement of GA, the *shi ga1-3* double mutant developed into a phenocopy of untreated *ga1-3* plants (Fig. 4A) except for the narrow leaves typical of *shi* (Fridborg et al., 1999). Treatment with high doses of GA_3 fully restored the *ga1-3* single mutants to wild-type height, whereas the height of *shi ga1-3* plants was restored to that of *shi*, but no further (Fig. 4A). The height of the *shi* single mutant plants was unaffected by the GA treatment.

To test the degree of responsiveness of *shi ga1-3* to exogenous GA at early stages of development, we measured hypocotyl growth of *ga1-3* and *shi ga1-3* in response to different concentration of GA_3 (Fig. 4B). In both lines, application of doses ranging from 0.01 to 5 μM GA_3 resulted in linear responses. The difference between the slopes indicate that *shi* slightly represses the GA responsiveness of *ga1-3* (Fig. 4B). Also, the response was saturated at lower GA levels in *shi ga1-3* than in *ga1-3*. At 10 μM GA_3, *shi ga1-3* hypocotyl growth was less induced, compared with application of the lower dose 5 μM GA_3. This reduction in response was not detected in *ga1-3* until the dose exceeded 10 μM (data not shown).

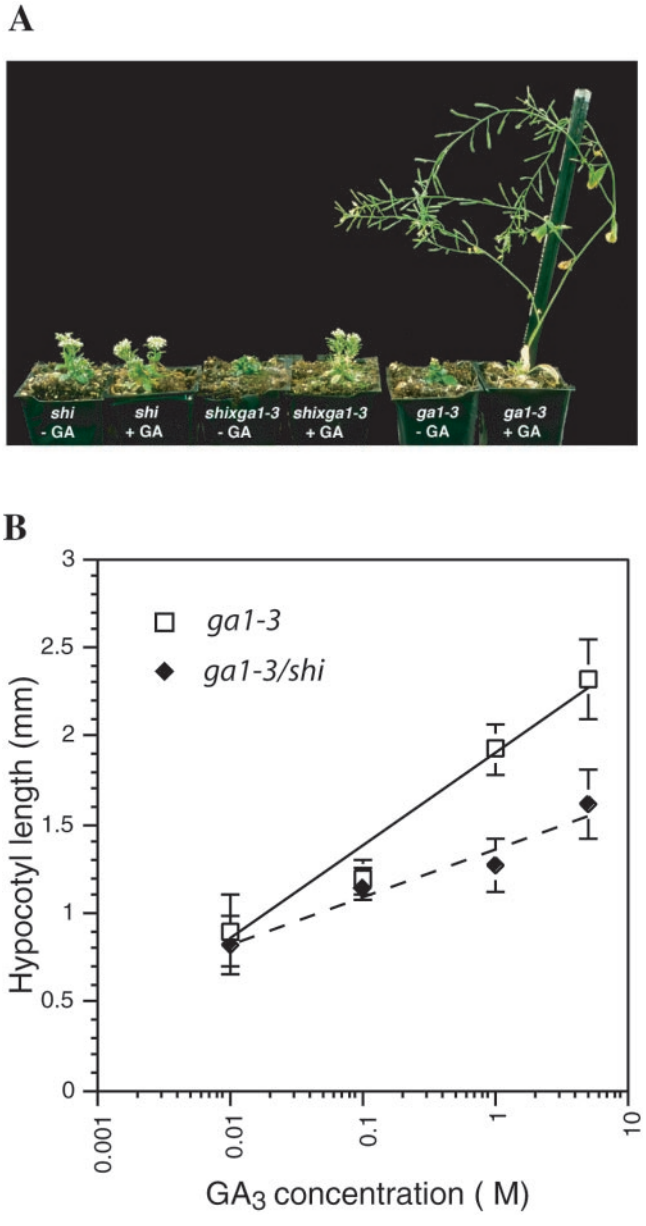

Figure 4. Sensitivity of *shi* to reductions in endogenous GAs. A, *shi ga1-3* double-mutant plants were grown in long-day conditions, repeatedly sprayed with 10^{-4} M GA_3, and compared with GA-treated *shi* and *ga1-3* plants, plus untreated plants of the same genotypes. B, *shi ga1-3* and *ga1-3* seedlings were grown on different concentrations of GA_3 and the hypocotyl length under these conditions are shown as the mean ± SE of 10 to 15 plants measured. The equations for the linear regression are as follows: *shi ga1-3*, $y = 1.37+0.269\log(x)$ and $R = 0.97$; and *ga1-3*, $y = 1.9+0.546\log(x)$ and $R = 0.99$.

In addition, *shi* seeds were unable to germinate on the GA biosynthesis inhibitor PAC at similar concentrations as prevented germination of wild-type seeds (10^{-5} M), and exogenous treatment with PAC further reduced the elongation of the *shi* inflorescence compared with untreated *shi* plants (data not shown). Taken together, these results demonstrate that the *shi*

mutant is not completely insensitive to reductions in the endogenous GA level and that it is fully capable of responding to exogenous GA by seed germination and by some elongation growth.

The *erecta* Mutation Is an Enhancer of the *shi* Dwarf Phenotype

In Arabidopsis, all but one (*pkl*; Ogas et al., 1997) of the GA biosynthesis/response related dwarf mutants have been isolated in the L*er* ecotype. These include the *ga1-ga5* biosynthesis mutants (Koornneef and van der Veen, 1980), the *gai* mutant (Koornneef et al., 1985), the *sly1* mutant (Steber et al., 1998), and the *shi* mutant. The L*er* ecotype carries the *erecta* mutation resulting in a nonfunctional ERECTA protein. ERECTA is believed to function as a transmembrane receptor protein kinase with an extracellular ligand-binding domain, and the protein kinase domain shows a high degree of sequence similarity to other predicted receptor-like protein kinases in higher plants (Torii et al., 1996). In the protein kinase domain, the *erecta* mutation results in a substitution of Ile to Lys at a residue that is highly conserved between the plant receptor-like protein kinases (Torii et al., 1996). Because the *erecta* mutation is in itself a dwarfing mutation, strongly affecting inflorescence elongation and organ shape, and because almost all of the GA mutant dwarfs have been isolated in the *erecta* background, we expected that *erecta* might influence the phenotypic effects of mutations in the reductions in GA levels or responses. To determine the impact of the *erecta* mutation on the *shi* dwarf phenotype, *shi* was crossed with Landsberg$^+$ (Ler$^+$) plants (lacking the *erecta* mutation) and the F_2 individuals were screened by PCR for the presence of the *shi* mutation and the *ERECTA* wild-type allele. The identified *shi ER* homozygotes were allowed to self-pollinate, and the height of the offspring was measured and compared with that of *shi er*, L*er*, and Ler$^+$ plants and the results are presented below as mean value in centimeters ± SE of 12 individuals per line. In these measurements, the *shi* mutant in L*er* background (3.4 ± 0.1) was less than 20% the height of L*er* (20.9 ± 0.65), whereas the *shi ER* individuals displayed a mild dwarf phenotype (19 ± 0.5), reaching 40% to 50% the height of wild-type Ler$^+$ plants (42 ± 1.4) as seen in Figure 5. The super additive effect of the two mutations in the *shi er* plants shows that the *erecta* mutation is a strong enhancer of the *shi* dwarf phenotype.

To investigate if *erecta* influences the phenotype of other GA dwarfs, we made crosses between the GA response mutant *gai* and Ler$^+$ plants. The *gai* mutant in L*er* background were 40% the height of L*er* plants, whereas the *gai ER* individuals were 55% the height of Ler$^+$ plants, suggesting an additive effect of the two mutations in *gai er* plants (data not shown). In conclusion, our results suggest that the *erecta* mutation strongly affect the GA dwarf phenotypes, which should be taken into consideration when characterizing the phenotypic effects of defective GA responses in the L*er* background.

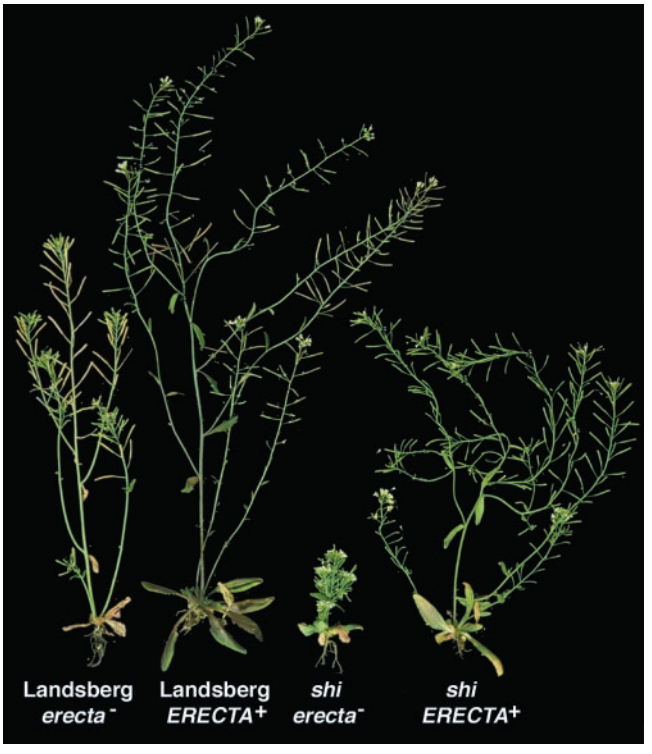

Figure 5. The impact of *ERECTA* on the *shi* phenotype. The *shi* mutant in *erecta* and *ERECTA* background compared with wild-type L*er* and Ler$^+$. Plants were grown on soil under long-day conditions.

DISCUSSION

It has been hypothesized that GA signaling is regulated by a derepressible system, similar to the ethylene signal transduction pathway (Harberd et al., 1998). GAI, RGA, and SPY have all been suggested to act as GA derepressible suppressors of GA responses (Sun, 2000). The hypothesis is based on the finding that when GA biosynthesis is diminished, as in the *ga1-3* mutant, inactivation of the negative regulator genes *SPY* or *RGA* is enough to at least partially restore growth in the GA deficient background (Silverstone et al., 1997b). In these mutants, a signal from active GA is not required for growth as a suppressor gene is turned off. This suggests that, in wild type, GA mediates growth responses by inactivation of a number of negative regulators of growth. If SHI is one of these proteins, we would assume *SHI* to be expressed both in young tissue prior to GA-induced expansion as well as at the time of GA induction. Furthermore, if GA signaling inactivates the SHI protein and not *SHI* transcription, we would assume transcription of the *SHI* gene to be active also some time after initiation of GA-induced expansion. In agreement with this, we have been able to show that

SHI is active in apical shoots, including very young organs and organ primordia, and in lateral root tips and primordia, suggesting that SHI could play a role as a GA-modulated repressor.

Alternatively, SHI could act as a suppressor of unwanted GA responses at the sites of GA biosynthesis. According to studies of reporter gene activity, the wild-type expression pattern of *SHI* is largely similar to that of the GA biosynthesis gene *GA1* encoding copalyl diphosphate synthase (previously known as *ent*-kaurene synthase A), an enzyme that is regulating the first committed step in the GA biosynthesis pathway (Silverstone et al., 1997a). As *SHI*, the *GA1* gene is expressed at high levels in young organs, e.g. shoot apices and root tips, and in the receptacle and funiculi of the flower. As opposed to *SHI*, *GA1* is also expressed in anthers and developing seeds. It is not known exactly where active GAs are produced, but it is generally believed that the three principal sites for GA biosynthesis are developing seeds and fruits, young leaves of apical buds and elongating shoots, and the apical regions of roots. This is in agreement with the expression pattern of *GA1*, and the presence of SHI in several of these tissues could be explained by the need for a negative regulator of GA-induced responses at the sites where GA is produced, to repress premature growth or development.

Independently of whether SHI acts as a derepressible negative regulator to prevent GA responses until GA levels have reached a certain threshold, or prevents GA responses at sites of especially high GA levels, we would expect a knockout allele of *SHI* to generate a slender, elongated mutant phenotype due to increased GA signaling in the apex region. The lack of a mutant phenotype in the presumed loss-of-function *SHI* alleles, generated by insertional mutagenesis or antisense constructs, suggests that the *SHI* gene product is largely dispensable for normal elongation growth under ordinary growth conditions. A likely explanation to the lack of a loss-of-function phenotype of *shi* mutants is functional redundancy between *SHI* and *SHI*-related genes. *SHI* is a member of a gene family, comprising at least nine members in Arabidopsis. The members of the *SHI* gene family show significant sequence similarities over two distinct regions of the predicted proteins; the first region comprises a putative zinc-binding RING finger-like motif, whereas the second region shows no resemblance to any previously identified protein motif. The sequence similarity between the proteins is most pronounced within the RING domain, which suggests that this region has a conserved function. Overexpression of at least three other genes in the family confers a dwarf phenotype identical to the *shi* mutant phenotype, implicating a role of the proteins as negative regulators of cell elongation in a similar manner as SHI (I. Fridborg, S. Kuusk, and E. Sundberg, our unpublished data). We are currently screening for knockout alleles of the *SHI*-related genes, as the most straightforward way to verify redundant gene function within a gene family is to make double, triple, and possible even more combinations of loss-of-function mutations. This has been demonstrated previously for five members of the ethylene receptor gene family. Gain-of-function mutants display dominant ethylene insensitivity, but none of the single knock-out alleles generate a constitutive ethylene response. However, when several loss-of-function mutations are combined, a constitutive ethylene response is progressively activated (Hua and Meyerowitz, 1998).

The role of SHI in GA signaling as a negative regulator was further demonstrated by using the well characterized GA response in cereal aleurone layers. We have shown that the SHI protein can suppress GA-induced expression of barley α-amylase. Robertson et al. (1998) have reported that HvSPY, the barley homolog of Arabidopsis SPY, can efficiently suppress α-amylase expression in GA-treated barley half-grains in the same experimental system and these results have been verified in this investigation. HvSPY is able to partially complement the Arabidopsis *spy-3* mutation, indicating that the function is conserved between the proteins of the two species (Robertson et al., 1998). The SHI-mediated suppression of α-amylase expression is not as strong as that mediated by HvSPY, which could be due to the heterologous origin of the SHI protein. HvSPY has been shown to affect the activity not only of the GA-induced α-amylase promoter but also of an ABA-activated promoter. Robertson et al. (1998) have shown that HvSPY expression can substitute for ABA treatment in activating a dehydrin promoter (from the *dehydrin gene 7*, *Dhn7*; Robertson et al., 1995) in barley aleurone tissue. This is not surprising because in late stages of seed development, GA and ABA act antagonistically. GA stimulates seed germination and degradation of storage reserves, whereas ABA prevents seed germination-related processes but stimulates seed maturation and drought and dehydration tolerance. We have studied the effect of SHI on transient *Dhn7* promoter activity and found that cobombardment with Ubi-SHI resulted in significantly higher *Dhn7* promoter activity compared with the control without ABA application (M. Robertson, unpublished data). In addition, ABA responsiveness of *Dhn7* promoter was significantly increased by SHI co-expression. In summary, our data suggest that transiently expressed SHI in aleurone layers specifically suppress GA-induced responses in a similar manner as HvSPY.

The Arabidopsis protein GAI is also able to suppress the activity of the α-amylase promoter following GA treatment of barley aleurone cells (F. Gubler, personal communication). In dicots, the aleurone layer is present but its role in germination is unknown (Melan et al., 1994). The ability of the two Arabidopsis proteins SHI and GAI to specifically

down-regulate the expression of a monocot aleurone-specific gene when ectopically expressed suggests that the nature of GA signaling, and the components acting in it, are largely conserved between tissues and between distantly related species of higher plants. Thus, SHI and GAI might be tissue-specific components of a general GA signaling pathway that is common for several GA-regulated processes in plants.

By introducing the *shi* mutation into wild-type *ERECTA* background, we have shown that a functional *ERECTA* allele was able to partly suppress the dwarfing effect of *shi*. Conversely, the *erecta* mutation is a strong enhancer of the *shi* dwarf phenotype. The effect of the *erecta* mutation on the *gai* phenotype is less dramatic, but fully additive. These relationships between *erecta* and the GA response mutations indicate that stem elongation in Arabidopsis is regulated through additional pathways that are not GA dependent and that in one of these pathways, the ERECTA protein is a key component. However, because *shi* and *gai* are both gain-of-function mutations, it is not possible to draw any definite conclusions toward the relationships of the affected genes based on the double-mutant phenotypes.

In this report, we have shown that the SHI protein is able to specifically suppress expression of a GA-induced gene in a heterologous system in a manner similar to the established GA signal suppressors HvSPY and GAI. We have also shown that *SHI* is expressed in vegetative tissues believed to be the site of GA biosynthesis, and that SHI-related proteins might be functionally redundant to SHI. In summary, our data support a role of the wild-type SHI protein as a negative regulator of GA-induced cell elongation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The *shi* mutant was identified in a transposon-tagging mutant screen previously described (Fridborg et al., 1999). The wild type used are of the L*er* and Ler^{+} ecotypes as stated in the experiments. Seeds from wild type and from the GA mutants *ga1-3* and *gai* were kindly provided by the Arabidopsis Biological Resource Center (Ohio State University, Columbus).

Seeds were surface sterilized (70% [w/v] ethanol for 2 min, 15% [w/v] chlorine, and 0.5% [w/v] SDS for 10 min followed by at least four washes in sterile distilled water) and sown on Germination Medium (GM; Valvekens et al., 1988). Prior to cultivation, seed dormancy was broken by 3 to 4 d of cold treatment (4°C). Plants grown in nonsterile conditions were planted on soil mixed with vermiculite (2:1, v/v). All plants were cultivated in controlled environmental chambers at 20°C to 22°C, soil-grown material and in vitro grown material for GA and PAC treatment under long-day conditions (18 h light, 6 h darkness), and in vitro-grown plants for GUS staining in continuous light.

Reporter Gene Constructs, Sense/Antisense Constructs, and Plant Transformation

For reporter gene constructs, the *SHI* genomic fragment was PCR amplified using the primer SHI:YY (5′-GTC GAC AAT ACG GTG AAG AGG TTG GAT AG-3′) in combination with either SHI:N (5′-GGA TCC TCT CTG TCG TTT AGG GAC GC-3′) for the 5:1 construct, or SHI:O (5′-GGA TCC ATC ATC TAC GGA ACT CAC AC-3′) for the 6:1 construct. The PCR fragments were cleaved with *Sal*I and *Bam*HI and cloned in frame with the *uidA* ORF in the pBI101.1 binary vector (CLONTECH, Palo Alto, CA). The constructs were introduced into the *Agrobacterium tumefaciens* strain GV3101 containing the helper plasmid pMP90, using standard methods.

For sense and antisense constructs, the *SHI* cDNA was ligated into the *Bam*HI site of pHTT202, the parent vector of pHTT370 (Elomaa et al., 1993) in sense or antisense direction, and introduced into *A. tumefaciens* strain C58::pGV2260 using triparental mating.

The plasmid constructs were *A. tumefaciens* transformed into Arabidopsis plants of ecotype L*er*, essentially as described by Bent and Clough (1998). For antibiotic testing of T_1 seeds, the GM medium was supplemented with kanamycin (50 mg L^{-1}). The resulting transgenic plants were self-fertilized and the T_2 seeds screened for segregation on kanamycin.

Detection of GUS Expression

To test for expression of GUS, plant tissue was immersed in 5-chloro-4-bromo-3-indolyl β-D-glucuronide (X-GlcU) substrate (0.5 mg of X-GlcU cyclohexylammonium salt per liter in 50 mM sodium phosphate, pH 7/0.05% [w/v] Triton X-100), and incubated at 37°C over night. Tissues were destained in 95% (w/v) ethanol. Expression of GUS was detected by the appearance of a blue precipitate in the tissue. Photographs were taken using a stereo microscope (Leica Microsystems AG, Wetzlar, Germany).

Screening of Insertion Lines

Superpools 1 through 8 of the SLAT collection was screened using the gene-specific primers SHI:BB (5′-CAC AGT TGA AAA CTG CCA GCT GC-3′) and SHI:R (5′-TTC CAG CTG CGA ACT TTA GGC AC-3′) in combination with the transposon primers dSPM1 and dSPM11, essentially as described in the SLAT manual (http://nasc.nott.ac.uk/info/slat_info1.html). PCR products were checked by Southern analysis using a gene-specific *SHI* probe, according to standard procedures. A second PCR screen was performed on 48 pools, each of 50 lines, from the positive superpool. From the identified pool of 50 lines, approximately 400 seeds were germinated on soil and sprayed with 100 mg L^{-1} Basta (Hoechst AG, Frankfurt am Main, Germany) for selection. DNA was extracted, following standard procedures, from pools of 10 plants using one rosette leaf per plant. Positive pools and subsequently individual positive plants were identified by PCR.

The Wisconsin T-DNA collection was screened by the Arabidopsis Knockout Facility according to Krysan et al. (1999) using the gene specific primers SHI:VV (5′-ATC TAA AAC ACG TGA TGA TCA ACG GTA AG-3′) and SHI:EE (5′-AAC AAG GCT GAG TTT AAC GAT CAC AGT TG-3′) in combination with the T-DNA primer JL202. PCR products were checked by Southern analysis using a gene-specific *SHI* probe, according to standard procedures. After identification of a positive pool, approximately 250 seeds from 25 subpools (each representing nine lines) were sown on GM, and DNA was prepared from one-third of the seedlings from each subpool. After PCR identification of the positive subpool, the remaining two-thirds of the seedlings were transferred to soil and DNA was prepared from pools of 10 plants. Positive pools and individual positive plants were identified by PCR.

Transient Expression Assays

Preparation of barley (*Hordeum vulgare* cv Himalaya) half-grains particle bombardment and precipitation of plasmid DNA onto gold preparations was performed essentially as described by Gubler et al. (1995). For Ubi-SHI, the *SHI* cDNA was amplified using PCR primers with *Bam*HI sites: SHI:S3 (5′-CGG GAT CCA AGA TCT CTA TCA AGA GAG AGA TC-3′) and SHI:L3 (5′-CGG GAT CCA AAC CCT AAT TCT ACC GTA AGA ATC-3′). The PCR fragment was ligated into the *Bam*HI site of the Ubi-Cass vector behind the ubiquitin promoter (Robertson et al., 1998). Per 3-mg gold particles, 0.5 μg of reporter plasmid (Amy[-877]-IGN [Jacobsen and Close, 1991], Dhn7-[-935]-IGN [Robertson et al., 1995], or Act1-IGN [McElroy et al., 1990]) and 1.0 μg of effector plasmid (UbiCass, Ubi-HvSPY [Robertson et al., 1998] or Ubi-SHI) were used. Each experiment used six half-grains and 15% of the DNA gold preparation, resulting in the molar ratio of reporter to effector of 1:2.1 for SHI and 1:1.6 for HvSPY for all three reporter constructs. The bombarded half-grains were cut longitudinally into two equal quarter-grains and incubated with 10 mM $CaCl_2$ (control) supplemented with 10^{-6} M GA_3 or 10^{-6} M ABA as described by Robertson et al. (1998). Preparation of extracts and assays of GUS activity was performed essentially as described by Jefferson (1987). ANOVA using multiple comparisons (Fisher's PLSD, Scheffe's, and Bonferroni/Dunn) was conducted to test for differences in GUS activity. The calculations were performed using StatView 4.01 software (SAS Institute, Cary, NC).

Double Mutant Analysis

Plant DNA was extracted essentially as described by Edwards et al. (1991). The presence of mutant alleles was confirmed by PCR. The *shi* mutant allele was recognized by the presence of the *Ds* element in the *SHI* gene, using primer pair rev3′/DL3 (Fridborg et al., 1999). The *SHI* wild-type allele was recognized by amplification of a fragment spanning the *Ds* insertion site, using primer pair rev3′/rev5′ (Fridborg et al., 1999). The *ga1-3* and *GA1-3* alleles were identified using primer pairs 25/34 and 9/10, respectively (Silverstone et al., 1997b). The *erecta* and *ERECTA* alleles were distinguished by sequence analysis of a fragment spanning the site of the *erecta* mutation, obtained using primers ER1 (5′-CGA GAT GCT AAG TAG CAT CAA GC-3′) and ER2 (5′-GTA TGT GAC TTT GAC ACA CAC AAG C-3′). The *gai* and *GAI* alleles were distinguished by size using primers GAI1:1 (5′-GAT CCG AGA TTG AAG GAA AAA CC-3′) and GAI1:2 (5′-TTG TAG TAT ACG TAT CTC CTC CT-3′).

Growth Regulator Treatments

For stem elongation experiments, seeds were sown on soil in long-day conditions. Beginning at approximately 25 d after sowing, plants were sprayed generously once a week with 10^{-4} M GA_3 (Duchefa, Haarlem, the Netherlands) or 5×10^{-4} M PAC (Bonzi, ZENECA Agro, Copenhagen), each supplemented with 0.02% (w/v) Tween 20. Control plants were sprayed with a solution containing only 0.02% (w/v) Tween 20.

For hypocotyl elongation experiments, *ga1-3* and *shi/ga1-3* seeds were incubated with 100 μM GA_3 during stratification and rinsed thoroughly with water before plating on GM supplemented with 0.01, 0.1, 1, 5, or 10 μM GA_3. Seedlings were grown in long day conditions and hypocotyl lengths were measured after 8 d.

For germination experiments, seeds were sown in vitro on GM supplemented with 0, 5, 10, 25, 50, 75, 120, or 200 μM PAC. Germination was scored after 5 d in long-day conditions.

For studies of GUS expression, seeds were sown on GM supplemented with 1 or 10 μM of 1-aminocyclopropane-1-carboxylic acid, benzyladenine, indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid, PAC, GA_3, naphthylphthalamic acid, ABA, or epi-Brassinolide. Seedlings were stained for GUS activity after 3 d in continuous light.

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