The Plant Cell, Vol. 10, 995–1007, June 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

Identification of a Negative Regulator of Gibberellin Action, HvSPY, in Barley

Masumi Robertson,[a,b,1] Stephen M. Swain,[c] Peter M. Chandler,[a] and Neil E. Olszewski[c]

[a] CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia
[b] Cooperative Research Centre for Plant Science, Australian National University, GPO Box 4, Canberra, ACT 2601, Australia
[c] Department of Plant Biology and Plant Molecular Genetics Institute, University of Minnesota, St. Paul, Minnesota 55108

To broaden our understanding of the molecular mechanisms of gibberellin (GA) action, we isolated a spindly clone (HvSPY) from barley cultivar Himalaya and tested whether the HvSPY protein would modulate GA action in barley aleurone. The HvSPY cDNA showed high sequence identity to Arabidopsis SPY along its entire length, and the barley protein functionally complemented the *spy-3* mutation. HvSPY and SPY proteins showed sequence relatedness with animal O-linked *N*-acetylglucosamine transferases (OGTs), suggesting that they may also have OGT activity. *HvSPY* has a locus distinct from that of *Sln*, a mutation that causes the constitutive GA responses of *slender* barley, which phenotypically resembles Arabidopsis *spy* mutants. The possibility that the *HvSPY* gene encodes a negative regulator of GA action was tested by expressing HvSPY in a barley aleurone transient assay system. HvSPY coexpression largely abolished GA_3-induced activity of an α-amylase promoter. Surprisingly, HvSPY coexpression increased reporter gene activity from an abscisic acid (ABA)–inducible gene promoter (dehydrin), even in the absence of exogenous ABA. These results show that HvSPY modulates the transcriptional activities of two hormonally regulated promoters: negatively for a GA-induced promoter and positively for an ABA-induced promoter.

INTRODUCTION

The plant hormone gibberellin (GA) is involved in regulating a number of processes during plant growth and development, including cereal grain germination, elongation, flowering, and fruit development (Pharis and King, 1985; Klee and Estelle, 1991; Okamuro et al., 1996). Such regulation depends on the presence of active GAs and the ability of cells to perceive and respond to the hormone. Considerable progress has been made in understanding the regulation of GA levels, both by elucidating GA biosynthetic pathways and, more recently, by cloning GA biosynthetic enzymes (Hedden and Kamiya, 1997). Less progress has been made in understanding how cells perceive GA and transmit this signal. Cereal aleurone layers are highly suited to studying the action of GA because they lack endogenous active GAs and have a well-characterized GA response involving the synthesis of hydrolytic enzymes, in particular, α-amylase (Jones and Jacobsen, 1991). Much of the progress in understanding GA action has been made using this model system. In aleurone, GA perception is proposed to be at the external face of the plasma membrane (Hooley et al., 1991; Gilroy and Jones, 1994). One component of GA signal transduction is the transcriptional activator GAMyb, which can transactivate a high-pI α-amylase gene promoter (Gubler et al., 1995). Other components of the signal transduction pathway are largely unknown, although a number of second messengers implicated in GA signaling have been described (Bethke et al., 1997).

GA response mutants offer the potential for further advances to be made by using molecular genetic approaches. There are two categories of response mutants: GA-insensitive dwarfs and constitutive GA response slenders. The dwarf mutants include wheat *Rht3* (Gale and Marshall, 1973), maize *D8* and *D9* (Phinney, 1956; Harberd and Freeling, 1989), and Arabidopsis *gai* (Koornneef et al., 1985). These mutants are all dominant or semidominant and have been proposed to be gain-of-function mutants of a negative regulator or dominant-negative mutants (Hooley, 1994; Swain and Olszewski, 1996). It is interesting that *Rht3* and *D8* have been mapped to corresponding loci on cereal chromosome 4 (Moore et al., 1995), suggesting that they may encode homologous genes. The slender-type mutants include barley *sln* (Foster, 1977), Arabidopsis *spindly* (*spy)* (Jacobsen and Olszewski, 1993), pea *cry^{s}la* (de Haan, 1930), and tomato *procera* (Jones, 1987). These mutants are recessive and are thought to result from the loss of function of a negative regulator (Hooley, 1994; Swain and Olszewski, 1996). Recently, extragenic suppressors of GA mutants, such as *rga* mutants, have been identified (Silverstone et al., 1997). Interestingly,

[1] To whom correspondence should be addressed. E-mail masumi.robertson@pi.csiro.au; fax 61-2-6246-5000.

rga mutants in the wild-type background display only a subtle phenotype.

The *sln* mutant of barley (cv Herta) (*sln-1*) is characterized by nondormant grain, rapid elongation of stems and leaves, sterility, and high α-amylase activity in aleurone layers in the absence of applied GA (Foster, 1977; Chandler, 1988; Lanahan and Ho, 1988). These characteristics can be phenocopied by the application of active GAs to wild-type barley. Because most, if not all, GA-regulated responses are affected in the mutant, despite low levels of endogenous active GAs (Croker et al., 1990), the *Sln* gene product is thought to function early in the GA signal transduction pathway (Hooley, 1994).

A similar mutant of Arabidopsis has been described recently (Jacobsen and Olszewski, 1993). The *spy* mutants are characterized by pale green to yellow leaves, early flowering, parthenocarpy, and partial male sterility. Although *spy* mutants exhibit many exaggerated GA responses, *spy-4* is only a partial suppressor of a GA-deficient mutation, *ga1-2*, and retains responsiveness to applied GA_3 and to the GA biosynthesis inhibitor paclobutrazol (Jacobsen et al., 1996). Epistatic analysis of *spy* and *gai* suggests that SPY functions downstream of GAI. The cloned *SPY* gene encodes a protein that belongs to the tetratricopeptide repeat (TPR) group of proteins, and the presence of a similar gene in *Caenorhabditis elegans* suggests common mechanisms of signaling among eukaryotes (Jacobsen et al., 1996). However, the biochemical function of SPY has yet to be determined, and its involvement in GA signaling has not been demonstrated unequivocally.

Our interest in GA signal transduction in barley, and the similarity between Arabidopsis *spy* and barley *sln* mutants, led us to initiate cloning of SPY cDNA-related sequences in barley. The isolation of a functionally equivalent sequence in barley, HvSPY, allowed us to utilize the advantages of barley aleurone to test the function of the HvSPY protein in regulating GA action and to establish the relationship between *Sln* and *HvSPY.*

RESULTS

SPY Sequences Are Highly Conserved between Monocot and Dicot Species

A combined sequence of HvSPY cDNA was obtained by screening a barley cultivar Himalaya leaf cDNA library and 5′ rapid amplification of cDNA end (RACE) reactions (see Methods). The cDNA was 3290 bases in length, including 18 alanine residues at the 3′ end; the GenBank accession number is AF035820. The HvSPY cDNA contained a 2835-base open reading frame capable of encoding a polypeptide of 944 amino acid residues, as shown in Figure 1A. Its nucleotide sequence showed 65% identity to the Arabidopsis SPY sequence, which is high considering that they represent monocot and dicot sequences. For example, maize viviparous1 (Vp1) and Arabidopsis homolog abscisic acid–insensitive3 (ABI3) have only 46% identical nucleotides (McCarty et al., 1991; Giraudat et al., 1992). The HvSPY and SPY nucleotide sequences are similar along their entire length, except near the 5′ and 3′ termini (including the 5′ and 3′ untranslated regions). The deduced amino acid sequence showed 76% identity and 87% similarity to the SPY sequence. The two amino acid residues that were mutated in the *spy-3* and *spy-5* alleles in Arabidopsis are conserved in HvSPY.

The deduced amino acid sequence of HvSPY contained 10 TPR motifs (Figure 1B) in the amino half of the polypeptide (Figure 1C). The TPR motifs were direct repeats, with additional amino acid residues present between repeats 2 and 3 and repeats 5 and 6 (Figure 1B). The positions as well as the numbers of intervening amino acid residues were identical for the barley and the Arabidopsis sequences. The non-TPR region was as highly conserved as the TPR region. The only notable difference was found near the C terminus. The last 60 amino acid residues of the Arabidopsis sequence showed much less identity with HvSPY, and the barley sequence contained 34 additional residues (Figure 1A).

A database search revealed that HvSPY is related to animal cDNAs encoding O-linked *N*-acetylglucosamine transferase (OGT). The deduced amino acid sequence of the rat OGT cDNA shows 32% identity and 54% similarity to the HvSPY sequence, which is considerably lower than the 94% identity between mammalian OGTs (Kreppel et al., 1997; Lubas et al., 1997). A previously identified gene on chromosome III of *C. elegans*, K04g7.3 (Wilson et al., 1994), whose product has amino acid sequence relatedness to SPY (29% identity and 50% similarity), shows 68% sequence identity and 80% similarity to human OGT and is likely to encode a polypeptide with OGT activity (Lubas et al., 1997). One notable difference between plant SPY sequences and animal OGT sequences is the absence of the putative nuclear localization signal in the plant sequences. Another gene product with a significant BLAST score (Altschul et al., 1990) identified a gene, D90908, of a cyanobacterium, *Synechocystis* sp, which was the only one identified within the genome (Kaneko et al., 1996).

In addition, two rice expressed sequence tag (EST) sequences, C26602 and C73901 (K. Yamamoto and T. Sasaki, unpublished data), were identified with very high sequence relatedness to HvSPY. The combined sequence of the ESTs showed 91% nucleotide identity, 97% amino acid similarity, and 93% amino acid identity over 189 amino acid residues deduced from a 569-base internal sequence. The combined amino acid sequence of the rice ESTs showed $<$40% similarity to another rice EST, D24403 (Y. Minobe and T. Sasaki, unpublished data), which was found to be related to animal OGT sequences (Kreppel et al., 1997; Lubas et al., 1997). D24403 shared higher amino acid sequence identity with the rat OGT (48%) than with the barley HvSPY (22%) over 108 amino acid residues, which were deduced from a 326-base internal sequence.

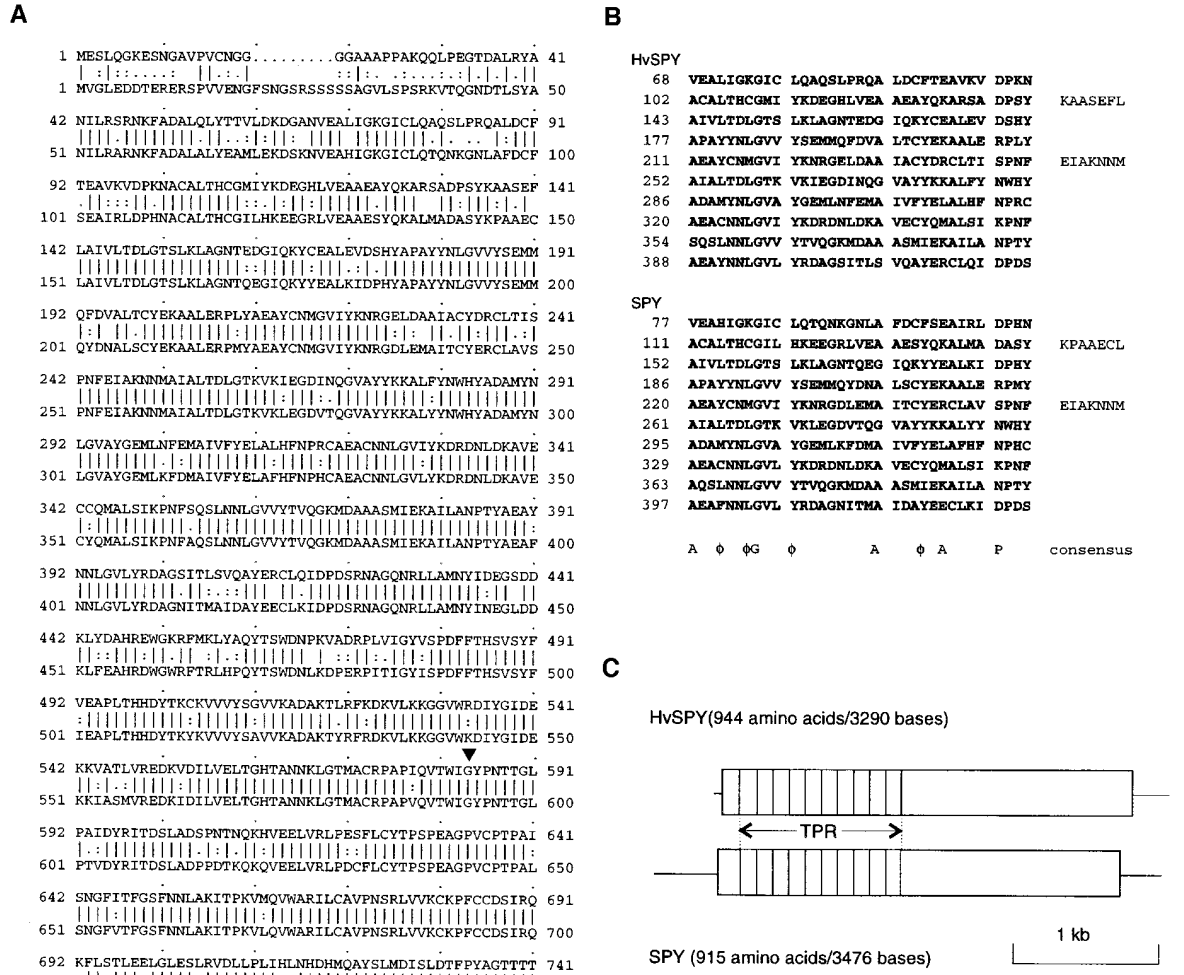

Figure 1. Structure of the HvSPY cDNA.

(A) Amino acid comparison between barley HvSPY (top) and Arabidopsis SPY (bottom). Exact matches are indicated by a vertical bar; the periods and colons indicate chemically less related residues. Gaps were also introduced for alignment. Two single amino acid residues mutated in *spy-3* and *spy-5* are indicated by an arrowhead and a diamond, respectively. Asterisks indicate the termination codon.

(B) TPR motifs in HvSPY and SPY. The 34–amino acid repeats are shown in boldface type; intervening residues (between repeats 2 and 3 and 5 and 6) are shown in normal type to the right of the TPR. Consensus of the TPR residues is shown at the bottom, where Φ indicates amino acids with a large hydrophobic side chain.

(C) Structures of the HvSPY and SPY cDNAs. Open reading frames are boxed, with the 5′ and 3′ untranslated regions shown as line extensions. The TPR regions are 10 boxes indicating 10 TPRs. The bar at the bottom corresponds to 1 kb.

HvSPY Functionally Complements the Arabidopsis *spy-3* Mutation

To determine whether HvSPY was able to function in Arabidopsis, we transformed *spy-3* plants with a construct expressing either the Arabidopsis SPY cDNA or the HvSPY cDNA under the control of the 35S promoter of cauliflower mosaic virus. The progeny homozygous for a single transgene were compared with *spy-3.* The constructs contained partial 5′ untranslated sequence of similar length from either the SPY or HvSPY cDNAs. The results of germination studies in the presence of the GA biosynthesis inhibitor paclobutrazol are shown in Table 1. After 4 days in the light, the wild-type Columbia seeds had not germinated, whereas 64% of the *spy-3* seeds had. Two transgenic lines with the SPY cDNA and four of the five lines with the HvSPY cDNA showed reduced germination compared with *spy-3*, whereas the remaining HvSPY line germinated as well as *spy-3.* By day 11, *spy-3* and all of the transgenic lines germinated well, in contrast to the wild type. Thus, both Arabidopsis SPY and HvSPY delayed germination of *spy-3* equally well but were not able to fully restore the wild-type phenotype. Germination was also compared in the dark because light promotes germination of Arabidopsis seeds. Under these conditions, the germination of all genotypes was reduced, but both the SPY cDNA lines and two of the HvSPY cDNA lines completely prevented the germination of *spy-3.*

In contrast, the phenotype of *spy* mutants was fully restored to the wild-type phenotype when mutants were transformed with an Arabidopsis genomic clone containing the entire *SPY* gene (Jacobsen et al., 1996; S.M. Swain and N.E. Olszewski, unpublished data). Therefore, it appears that weak suppression of the *spy-3* phenotype by Arabidopsis SPY and HvSPY cDNAs reported here for germination in the light results from the absence of 5′ untranslated or intronic sequences.

Table 1. Germination on Paclobutrazol

	Percentage of Germination[a]		
	Light		Dark
Genotype	Day 4	Day 11	Day 7
WT[b]	0	5	0
spy-3[c]	64	100	40
35S:SPY No. 1[d]	1	70	0
35S:SPY No. 2	1	72	0
35S:HvSPY No. 1	0	92	0
35S:HvSPY No. 2	0	90	11
35S:HvSPY No. 3	8	89	2
35S:HvSPY No. 4	78	100	47
35S:HvSPY No. 5	1	99	0

[a] Seeds were germinated on paclobutrazol for the number of days shown after a 3-day cold treatment.
[b] WT, wild type in the Columbia background.
[c] *spy-3*, in the Columbia background.
[d] Transgenic lines 1 to 5 are T_3 homozygous.

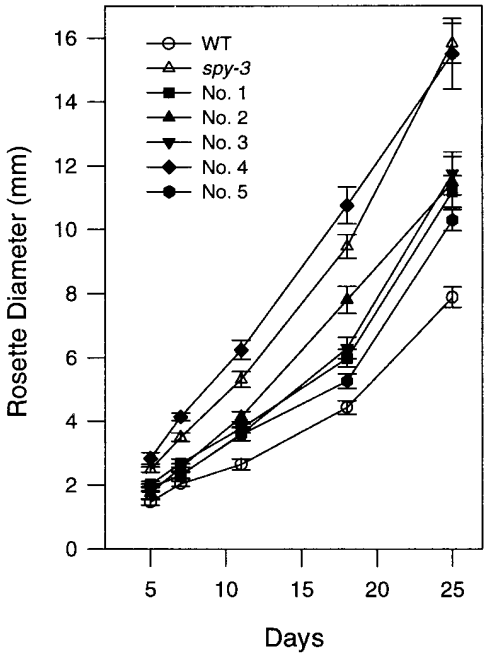

Figure 2. Functional Complementation of the Arabidopsis *spy-3* Mutation by HvSPY.

Rosette diameters of wild type (WT), *spy-3*, and HvSPY transgenic lines 1 to 5 are given. Plants were grown on 10^{-7} M uniconazole.

The same five HvSPY lines were tested for their ability to suppress the growth of *spy-3* on uniconazole, an inhibitor of GA biosynthesis with a mode of action similar to paclobutrazol (Hedden and Kamiya, 1997). Figure 2 shows that the rosette diameter of wild-type plants was <8 mm at 25 days, whereas that of *spy-3* was almost 16 mm. The diameter of the HvSPY lines was less than that of *spy-3*, except for one line (No. 4), showing that HvSPY also partially suppressed the resistance of *spy-3* to the dwarfing effects of uniconazole on vegetative growth. In both germination and growth tests, there was concordance in the different degrees of suppression of the *spy-3* phenotype: lines 1 and 5 were most effective, whereas line 4 had little effect.

Genomic DNA Gel Blot Analyses

Genomic DNA gel blot analysis was performed to estimate the copy number of genes related to the HvSPY cDNA. Barley genomic DNA was digested with EcoRI, HindIII, XbaI,

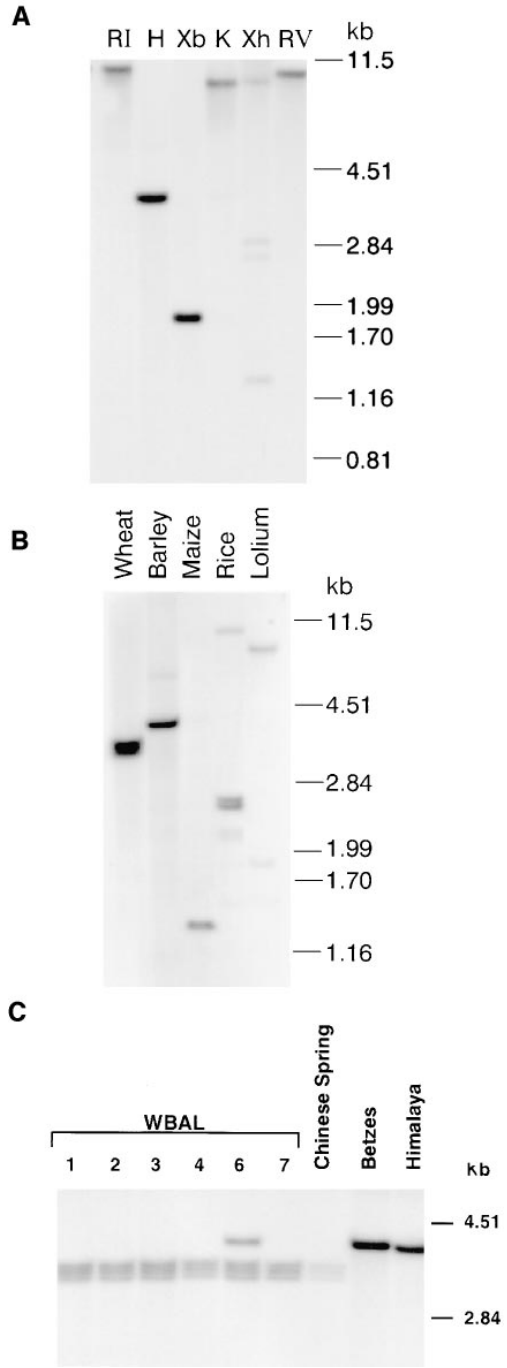

Figure 3. Genomic DNA Gel Blot Analyses of HvSPY.

(A) HvSPY is a single-copy gene. The hybridization pattern of the 3′ half of HvSPY cDNA with Himalaya genomic DNA (10 μg) digested with EcoRI (RI), HindIII (H), XbaI (Xb), KpnI (K), XhoI (Xh), and EcoRV (RV) is shown. The numbers at right indicate lengths in kilobases.

(B) Sequences similar to HvSPY are conserved in DNA of several grass species. HvSPY hybridization patterns with DNA from wheat (20 μg), barley (10 μg), maize (10 μg), rice (5 μg), and *L. temulentum* (Lolium; 10 μg) digested with HindIII are shown. The numbers at right indicate lengths in kilobases.

(C) HvSPY is located on chromosome 6. The HvSPY hybridization pattern with DNA (10 μg) from wheat–barley addition lines (WBAL) 1, 2, 3, 4, 6, and 7, Chinese Spring, Betzes, and Himalaya digested with HindIII is shown. The numbers at right indicate lengths in kilobases.

KpnI, XhoI, and EcoRV. The blotted membrane was hybridized with the 5′ half (data not shown) or the 3′ half (Figure 3A) of the cDNA. There were strong single bands in all lanes, except for the lane with XhoI-digested DNA, which had four bands weakly hybridizing with the 3′ probe. The number of bands was as expected from the cDNA sequence for five of the restriction enzymes; there was no internal site in the 3′ sequence for EcoRI, HindIII, XbaI, KpnI, and EcoRV. Because there were two XhoI sites, the fourth band in the lane with XhoI-digested DNA may indicate a XhoI site in an intron. Alternatively, it may represent a distantly related sequence, because single weak bands are present in other lanes. The *HvSPY* gene is most likely to be present as a single-copy gene in the Himalaya barley genome. The same conclusion was obtained using the 5′ half of the cDNA as a probe.

To examine related sequences in other grass species, we performed DNA gel blot analysis by using genomic DNA from wheat (cv Lawson), maize, *Lolium temulentum* (selection CERES), and rice (cv Taipei). Figure 3B shows results of autoradiography with HindIII-digested DNA probed with the 3′ half fragment of the HvSPY cDNA. One or more hybridizing bands were detected in all species tested even when normal hybridizing and washing stringencies were used, suggesting that *SPY* sequences are highly conserved among the grasses.

To determine the chromosomal location of the *HvSPY* gene, genomic DNAs of wheat–barley addition lines, the wheat parental cultivar Chinese Spring, and the barley chromosomal donor cultivar Betzes were digested with HindIII and probed with the 3′ half fragment of the HvSPY cDNA (Figure 3C). The length of a single hybridizing band in Betzes (4 kb) was the same as in Himalaya. Chinese Spring contained three slightly smaller (3, 3.2, and 3.5 kb) hybridizing bands, which probably reflects the hexaploid nature of this species. The 4-kb band identified in Betzes was present in the addition line 6, providing evidence that *HvSPY* is located on chromosome 6 (6H).

HvSPY mRNA Expression

HvSPY mRNA was not detectable in RNA gel blots using total RNA, even after a 2-week exposure (data not shown). However, it was detected by reverse transcription–polymerase

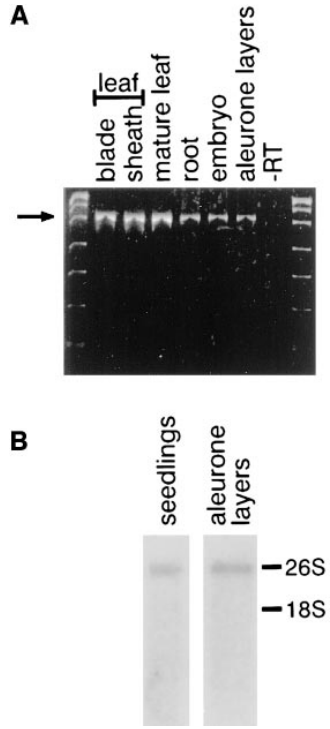

Figure 4. HvSPY mRNA Expression.

(A) HvSPY mRNA expression examined by RT–PCR. Expression in the blade and sheath of young leaves, mature leaves, roots, embryos, and aleurone layers is shown. An RT-PCR control reaction without reverse transcriptase (−RT) is also shown. The arrow indicates the position of the product of the expected size.

(B) HvSPY mRNA expression examined by RNA gel blot analysis. Lanes contain 1 μg of poly(A)$^+$ RNA from seedlings or aleurone layers of Himalaya. The positions of 26S and 18S rRNA are indicated at right.

chain reaction (RT-PCR) and in RNA gel blots using poly(A)$^+$ RNA (Figure 4). Figure 4A shows HvSPY mRNA expression analyzed by RT-PCR. It is expressed in all parts of barley plants that we examined, including immature leaf blade, leaf sheath, mature leaf blade, roots, germinating embryos, and aleurone layers. PCR reaction controls using the same amount of total RNA but without RT did not produce the product of the expected length; therefore, we know that the total RNA preparation was free from contaminating DNA.

RNA gel blot analysis using poly(A)$^+$ RNA was performed to detect and estimate the size of the mRNA. Figure 4B shows a single band in poly(A)$^+$ RNA from young seedlings and aleurone layers after a 2-week exposure. The size of the mRNA was estimated to be ~3.5 kb because the band comigrated with the 26S rRNA. Hybridization was not due to the 26S rRNA, because a lane containing total RNA, or flowthrough poly(A)$^-$ RNA, did not show any hybridization signal (data not shown).

Relationship between *Sln* and *HvSPY*

The similarities between the barley *sln* and Arabidopsis *spy* mutants suggested that *Sln* and *SPY* may be homologous genes. The cloning of the HvSPY cDNA allowed us to test this possibility. Restriction fragment length polymorphism analysis using 35 restriction enzymes showed identical patterns in Himalaya, *sln-1* in Himalaya, *sln-2* in Himalaya, Herta (the genetic background of *sln-1*), and a breeding line (the genetic background of *sln-2*). These results indicate that the *HvSPY* gene and its surrounding DNA are well conserved in these genotypes. With only a single enzyme (BstNI) was there evidence for polymorphism: Himalaya and *sln-1* and *sln-2* backcross lines in Himalaya showed the same pattern, whereas Herta and the breeding line showed a different pattern, which suggested that *HvSPY* and *Sln* are distinct loci (data not shown).

To obtain stronger evidence, HvSPY cDNA sequences were compared in the five genotypes used in restriction fragment length polymorphism analysis. If HvSPY were the product of the *Sln* locus, then we expected to detect sequence variation due to (1) differences in genetic background among Himalaya, Herta, and the breeding line but not between Herta and *sln-1* or the breeding line and *sln-2* and/or (2) mutations responsible for the *sln-1* and *sln-2* phenotypes. A single base substitution was detected. Position 2461 was a T residue in Himalaya, whereas it was a C residue in Herta and the breeding line, indicating that the codon containing this nucleotide encodes valine and alanine, respectively. In the two *sln* mutants in a Himalaya background (*sln-1* and *sln-2*), position 2461 was a T residue. If *HvSPY* were *Sln*, then the HvSPY cDNA in the *sln* mutants should have retained the original C residue at position 2461. In addition, the cDNA sequences of HvSPY in Himalaya, *sln-1*, and *sln-2* were identical. Therefore, we conclude that the *Sln* locus does not encode HvSPY.

HvSPY Modifies the Promoter Activities of GA- and Abscisic Acid–Regulated Genes

The effect of HvSPY on GA action was examined in barley aleurone by using cobombardment experiments, as shown in Figure 5. The effect of HvSPY coexpression on GA upregulated promoter activity was studied using a barley high-pI α-amylase promoter–β-glucuronidase (*GUS*) reporter gene construct, Amy(−877)–IGN, and a blank ubiquitin effector cassette (UbiCass) or a HvSPY overexpression effector construct (Ubi–HvSPY). The structures of these constructs are shown in Figure 5A. Figure 5B shows that GUS activity increased fivefold above the control levels when half-grains cobombarded with the blank effector cassette were treated with 1 μM GA_3. When Ubi–HvSPY was used as the effector, GUS activity in the grains treated with GA_3 was reduced significantly. The results demonstrate that coexpression of HvSPY blocks most, if not all, of the GA_3-induced, high-pI

α-amylase gene promoter activity. This provides strong evidence for the predicted function of the *SPY* gene product as a negative regulator of GA signal transduction.

The possibility that HvSPY acts as a general negative regulator of transcription, not limited to GA action, is unlikely because the promoter activity of the grains in the control medium was not decreased (Figure 5B). In addition, promoter activities of a rice actin (McElroy et al., 1990) or a maize ubiquitin (Christensen et al., 1992) gene were not reduced by HvSPY coexpression (data not shown).

Abscisic acid (ABA) antagonizes many GA-induced responses, including α-amylase expression. To test further whether the decrease in α-amylase promoter activity in the presence of HvSPY was a general response or a response specific to GA action and to test whether negative regulation by HvSPY involved ABA, we used a dehydrin promoter–GUS construct, Dhn(−935)–IGN, as a reporter in cobombardment experiments (Figure 6A). GUS activity increased 14-fold over the control in aleurone treated with ABA when the blank effector cassette (UbiCass) was used (Figure 6B). In half-grains cobombarded with Ubi–HvSPY, GUS activity increased even in the absence of ABA. This surprising result indicates that expression of HvSPY substitutes for ABA treatment in activating the dehydrin promoter. GUS activity was further increased in aleurone cobombarded with Ubi–HvSPY and treated with ABA, with

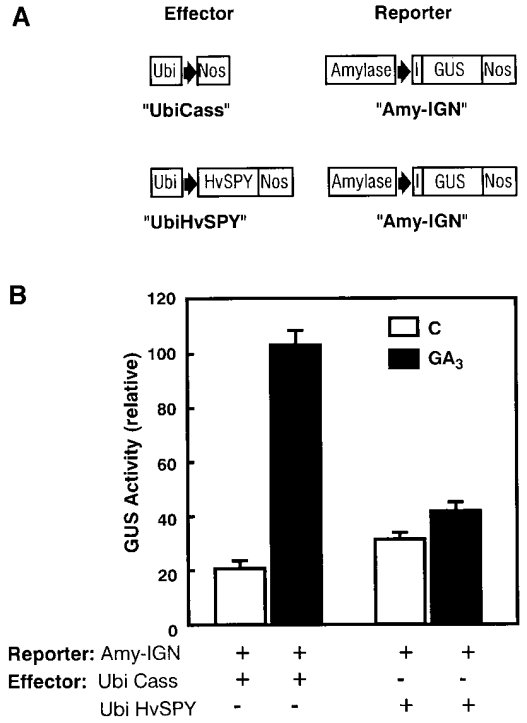

Figure 5. Functional Analysis of the HvSPY Protein: Negative Regulation of GA_3 Induction of High-pI α-Amylase Promoter Activity.

(A) Structure of effector and reporter constructs. UbiCass is a blank effector cassette with a ubiquitin promoter but lacking a coding region, and Ubi–HvSPY is an effector construct with HvSPY cDNA inserted downstream of the ubiquitin promoter. Amy–IGN is a reporter construct with a high-pI α-amylase promoter driving the intron–GUS–Nos (IGN) reporter cassette. Nos, nopaline synthase.
(B) Effect of HvSPY on amylase promoter activity. Shown is relative GUS activity, where GUS activity of aleurone layers cobombarded with Amy–IGN and UbiCass in GA_3 is expressed as 100%. Reporter and effector constructs used in cobombardment experiments are indicated below the graph: (+) indicates the presence and (−) indicates the absence of the construct at left. GUS activity of layers in solution without added GA_3 is shown by open bars (C); those treated with GA_3 are shown by black bars. The means and standard error of 24 replicated bombardments from four independent experiments are shown.

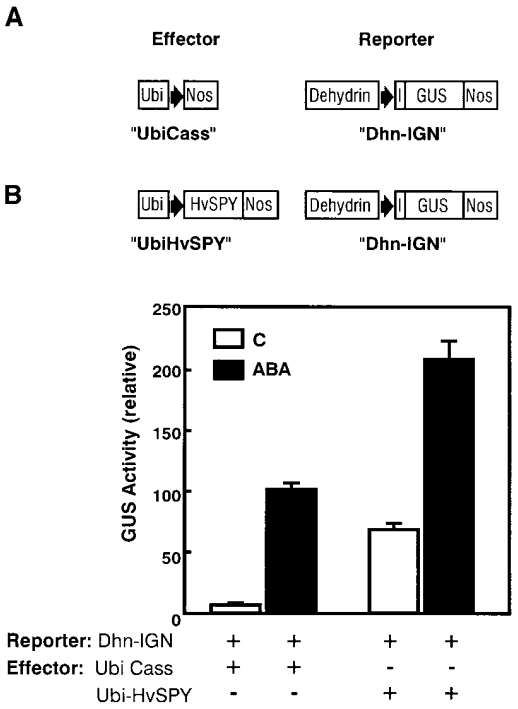

Figure 6. Functional Analysis of the HvSPY Protein: Positive Regulation of Dehydrin Promoter Activity.

(A) Structure of effector and reporter constructs. Effector constructs are as given in Figure 5. The reporter construct is a dehydrin promoter driving IGN and is designated Dhn–IGN. Nos, nopaline synthase.
(B) Effect of HvSPY on dehydrin promoter activity. Shown is relative GUS activity, where GUS activity of aleurone layers cobombarded with Dhn–IGN and UbiCass in ABA is expressed as 100%. Reporter and effector constructs used in cobombardment experiments are indicated below the graph: (+) indicates the presence and (−) indicates the absence of the construct at left. GUS activity of layers in solution without added ABA is shown by open bars (C); those treated with ABA are shown by black bars. The means and standard error of 24 replicated bombardments from four independent experiments are shown.

a 3.3-fold increase over control values with Ubi–HvSPY and a 33-fold increase over control values with UbiCass. This result suggests that HvSPY acts as a positive regulator of ABA responses, at least for the dehydrin gene promoter activity.

DISCUSSION

In this work, we have demonstrated that HvSPY is a negative regulator of GA action, a function originally proposed for the Arabidopsis *SPY* gene product based on an analysis of *spy* mutants (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996). The barley gene *HvSPY* appears to be a homolog of *SPY*. This conclusion is supported by both the molecular characteristics of *HvSPY* and functional complementation of the Arabidopsis *spy-3* mutation. The high degree of conservation of this sequence between dicot and monocot plant species and the presence of related sequences in a number of grass species indicate that similar GA signaling mechanisms exist in higher plants.

HvSPY Is a Member of a Family of TPR Proteins

The HvSPY deduced amino acid sequence does not contain a DNA binding or activation (acidic) domain; thus, it is unlikely to be a *trans*-acting transcriptional factor. The only motif identified was that of TPR. This motif has 34 amino acid residues and is characterized by degenerate consensus sequences so that amino acid residues with similar chemical characteristics are retained (Goebl and Yanagida, 1991). To date, >30 polypeptides with three to 16 TPR motifs have been identified in databases. They function in diverse areas, including transcriptional regulation, cell cycle regulation, macromolecule translocation, hormone perception, and translational regulation (Goebl and Yanagida, 1991). Protein–protein interactions involving the TPR region have been shown to be essential for their biochemical function (Williams et al., 1991; Smith et al., 1995; Shpungin et al., 1996).

Recently, animal cDNA clones with sequence relatedness to Arabidopsis SPY were isolated (Kreppel et al., 1997; Lubas et al., 1997). They encode a catalytic 110-kD subunit of the heterotrimeric enzyme UDP–*N*-acetylglucosamine: peptide *N*-acetylglucosaminyl transferase (*O*-GlcNAc transferase or OGT), and OGT activity of the expressed polypeptides has been demonstrated. The enzyme catalyzes additions of a single *N*-acetylglucosamine in O-glucosidic linkage to serine or threonine residues. The dynamic modification by O-linked *N*-acetylglucosamine glycosylation (*O*-GlcNAcylation) is similar to phosphorylation reactions and thought to play an analogous regulatory role (Hart, 1997). The site of *O*-GlcNAc residue attachment could be the same site as the serine/threonine kinase site, and a number of proteins modified by phosphorylation are also modified by *O*-GlcNAc. Therefore, it has been proposed that *O*-GlcNAc modification may provide an additional level of control for proteins whose activities are known to be modified by phosphorylation. A diverse array of proteins can be modified by *O*-GlcNAc, and these are distributed in both the cytoplasm and nucleus. They include RNA polymerase II and its transcription factors, the nuclear pore protein, structural proteins, and regulatory proteins (Hart, 1997). In a reverse reaction, the single *O*-GlcNAc residues are removed by *N*-acetyl-β-D-glucosaminidase. The sequence relatedness suggests that plant and *C. elegans* genes encode polypeptides with OGT activity and that similar molecular mechanisms for signal transduction exist among a wide range of eukaryotes.

The identification of two classes of rice EST sequences in the database raises the possibility that HvSPY and SPY may still encode OGTs but that they may belong to a different subclass from the animal OGTs. It seems unlikely that a single enzyme would be responsible for the specific addition of *O*-GlcNAc to all of the proteins that become modified (Kreppel et al., 1997). Therefore, there may be several subclasses of enzymes with OGT activity, and the rice ESTs and HvSPY may define two of the subclasses in cereals.

The identification of OGT-like sequences in a number of eukaryotic systems suggests that a similar sequence could be present in yeast. The yeast (*Saccharomyces cerevisiae*) genome is now completely sequenced (Goffeau et al., 1997), and the yeast genome database was searched. Ssn6 (or Cyc8) showed the highest overall BLAST scores when compared with HvSPY, SPY, RnOGT, and HsOGT. Ssn6 is structurally very similar to SPY and HvSPY; it contains 10 TPR motifs in the N-terminal half of the polypeptide, and the C-terminal half does not contain any defined motifs (Schultz and Carlson, 1987). Although its sequence relatedness to animal OGTs and plant SPY sequences is lower (38 to 41% similarity) than those among the higher eukaryotes (50 to 96% similarity), it has been suggested that a similar substructure could be obtained with a degenerate sequence conservation with 42% sequence similarity (Xie et al., 1996). Ssn6 negatively regulates the transcription of several genes by interacting with TUP1 and a pathway-specific DNA-binding homeodomain protein (Williams et al., 1991; Keleher et al., 1992; Smith et al., 1995). If similar signaling mechanisms exist in higher and lower eukaryotes, it is possible that the molecular mechanism of gene regulation by HvSPY and OGT would involve protein–protein interactions similar to those of Ssn6.

Negative Regulators of GA Action

Many proposed models for GA signal transduction are based on GA activation of the signal pathway (Hooley, 1994;

Swain and Olszewski, 1996; Silverstone et al., 1997). GA receptors in aleurone cells have been suggested to be on the external face of the plasma membrane (Hooley et al., 1991; Gilroy and Jones, 1994), and the perception of active GAs is thought to activate a positive signaling cascade resulting in, for example, induction of α-amylase gene expression. The product of the *SPY* gene has been proposed to function as a negative regulator of GA action (Jacobsen and Olszewski, 1993), and this has now been confirmed for the product of the *HvSPY* gene in barley aleurone. When HvSPY was expressed, it prevented GA activation of α-amylase gene expression, as measured by promoter activity. This demonstrates that a proposed signal transduction component functions as a negative regulator of GA action for a well-defined GA response. The mechanism by which HvSPY activity is modified when plants are responding to GA remains to be determined.

The similarities between barley *sln* and Arabidopsis *spy* mutants suggest that *HvSPY* might be the *Sln* gene. However, we have shown that *HvSPY* and *Sln* are distinct loci. This finding was surprising, because the two "slender" mutants isolated in barley are not *hvspy* mutants and are likely to be allelic mutants of *sln*, whereas in Arabidopsis, "slender-type" mutants are alleles of *spy* (there are now at least 17). If there is a *Sln* gene homolog in Arabidopsis, then these observations raise three possibilities. First, in Arabidopsis, the absence of any slender-type mutants that are not allelic to *spy* might suggest that *SLN* is a redundant or duplicate gene. A redundancy in GA signaling genes has been suggested in pea (Potts et al., 1985). In contrast, both *Sln* and *HvSPY* in barley are most likely single genes, as shown by *sln* mutant isolation and HvSPY genomic DNA gel blot analysis, and we may be able to identify mutants of HvSPY when additional slender-type mutants are isolated in barley. A second possibility is that Arabidopsis slender mutants are not easily detected in a wild-type background. For example, *rga* mutants in Arabidopsis can partly restore tall growth in a GA-deficient background (*ga1-3*), suggesting that *RGA* could potentially define a *Sln* homolog. However, *rga* mutants in the wild-type background had only a subtle phenotype (Silverstone et al., 1997). Either of these two possibilities would lead to *SLN* in Arabidopsis exerting a different degree of regulation on GA action compared with *Sln* in barley. A third possibility is that *Sln* in Arabidopsis is a single gene, not allelic to RGA, and has yet to be defined by a mutant phenotype or perhaps will never be defined because of a lethal phenotype.

Taken together, at least three, and possibly four, loci that encode negative regulators of GA responses (*GAI*, *SPY*, *RGA*, and *Sln*) have been defined by mutant isolation and characterization in barley and Arabidopsis. The relationship of *Sln* to *GAI* and/or *RGA* remains unknown, but the cloning of *GAI* and *RGA* in barley would enable this issue to be addressed using *sln* mutants. GAMyb is a positive regulator affecting transcription of the α-amylase gene, and its relationship to these negative regulators, in particular to HvSPY, remains undetermined. HvSPY and GAMyb may operate through a common pathway or in separate pathways.

Dual Function of HvSPY in Modulating GA and ABA Responses

In addition to being a negative regulator of GA responses, HvSPY positively regulated dehydrin promoter activity. A transcriptional regulator with broadly similar activities has been characterized in maize. Vp1 is a seed-specific transcriptional activator that acts synergistically with ABA (McCarty et al., 1991). It contains an acidic activator domain and activates transcription of the *C1* gene in vivo (Hattori et al., 1992). When tested in cobombardment experiments, overexpression of Vp1 increased promoter activity of another ABA-induced gene, *Em* (McCarty et al., 1991; Hattori et al., 1992). In addition, it blocked GA_3-induced high-pI α-amylase gene promoter activity (Hoecher et al., 1995). Thus, it activates an ABA-upregulated gene (*Em*) and negatively regulates a GA-upregulated gene (α-amylase).

This simple analogy might suggest that HvSPY and Vp1 function similarly; however, their sequences are unrelated, and at least three other differences can be recognized. First, Vp1 and ABI3 (an Arabidopsis Vp1 homolog; Giraudat et al., 1992) are expressed only in seeds, whereas HvSPY and SPY expression has been detected in many parts of the plant. Second, the *spy* mutation affected GA responses in both seeds and vegetative parts (Jacobsen and Olszewski, 1993) and HvSPY functionally complemented the uniconazole-resistant vegetative growth of *spy-3*, whereas only seeds are affected in *vp1* and *abi3* mutants. Third, expression levels of Vp1 are high, whereas those of HvSPY and SPY are very low. It is still possible that the SPY pathway may interact or be shared with the Vp1 pathway in seeds. Phenotypic characterization of the *abi3 spy* double mutant may provide a better understanding of the relationship between ABI3 and SPY and possibly between Vp1 and HvSPY.

The apparent dual function of HvSPY in transient assays might indicate that HvSPY has two discrete activities, but it is also possible that both effects result from enhanced ABA signaling activity. The latter possibility would imply that *spy* mutants have reduced ABA responses; however, this has not been reported. In barley, the positive regulation of the dehydrin promoter activity by HvSPY could be due to mechanisms other than through ABA signaling. This will be tested with mutant dehydrin promoters that do not respond to ABA in cobombardment experiments. Functional analysis of HvSPY domains might be the most direct way to determine whether HvSPY has two distinct activities; for instance, the two discrete activities of HvSPY might be uncoupled using mutant HvSPY proteins.

METHODS

Plant Material

Barley (*Hordeum vulgare* cv Himalaya) was grown in a greenhouse at 22°C/16°C day/night temperatures under natural lighting. Grains of Himalaya from Washington State University at Pullman (1985 harvest) were also used. Stocks of two *slender* (*sln*) mutants were originally obtained from C.A. Foster (Welsh Plant Breeding Station, Plas Goggerdan, Aberystwyth, Wales, UK; Foster, 1977). The *sln-1* mutant was isolated from cultivar Herta, and *sln-2* was isolated from a breeding line. Near-isogenic lines of *sln-1* (BC6) and *sln-2* (BC4) were established in a Himalaya background. Wheat–barley addition lines (Islam and Shephard, 1981) and their parental lines, Chinese Spring and Betzes, were obtained from A.H.D. Brown (CSIRO Plant Industry). The *Arabidopsis thaliana* lines are in the Columbia ecotype.

Construction of a cDNA Library and Identification of the HvSPY cDNA Sequence

Total RNA was isolated from elongating leaves of Himalaya seedlings, as described previously (Close et al., 1989), and poly(A)$^+$ RNA was isolated by using oligo(dT)–cellulose (Boehringer Mannheim) or Quick Prep (Pharmacia Biotechnology, Melbourne, Australia). A cDNA library was made in λZAPII (Stratagene, La Jolla, CA), according to the manufacturer's instructions, using size-fractionated cDNA. Plaques (750,000) were screened at low stringency with 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 1% [w/v] Ficoll, 1% [w/v] PVP, 1% [w/v] BSA Pentax Fraction V), 0.5% [w/v] SDS, 10 μg/mL poly(A), and 100 μg/mL sheared salmon sperm DNA at 55°C, using the 3.5-kb insert of the Arabidopsis SPY cDNA clone as probe (Jacobsen et al., 1996). Filters were washed three times in 2 × SSC and 0.5% [w/v] SDS at 55°C for 20 min each. Hybridizing plaques were purified, and the inserts were subcloned by in vivo excision. More than 50 independent plaques with varying degrees of hybridization signal strength were purified, and their sequences were determined by using a PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA) with dye terminator fluorescent cycle sequencing reactions (Applied Biosystems/Perkin-Elmer, Scoresby, Australia). In most cases, their sequences were not closely related to SPY: only 35 to 40% of the nucleotides were identical in the sequenced regions. Three clones showed nucleotide sequence identity >60%, and these were characterized further. Their sequences were identical to each other in the large regions of overlap, and the longest sequence of clone HvSPY88 has 65% nucleotide sequence identity to SPY and 72% identity and 81% similarity in amino acid sequence over 1.4 kb of sequence.

Sequence Analysis and 5′ Rapid Amplification of cDNA Ends

The three barley cDNA clones contained identical overlapping sequences, but they were partial cDNA clones. The 5′ sequence was obtained by 5′ rapid amplification of cDNA ends (RACE). An antisense primer was made near the 5′ end of HvSPY88, and 5′ fragments were amplified from an adaptor-ligated cDNA pool (Marathon kit; Clontech, Sydney, Australia), using an Advantage cDNA polymerase chain reaction (PCR) kit (Clontech), except that PCR parameters were slightly modified to include step-down T_m cycles (T_m = 68°C) to increase primer specificity. Among the primary amplification products, three products at 1.9, 1.7, and 1.5 kb hybridized strongly with the 180-bp 5′ fragment of HvSPY88. The two longer products were reamplified with a nested adaptor (AP2) primer and the HvSPY-specific antisense primer and cloned into the pGEM-T vector (Promega). Three 5′ RACE clones were fully sequenced on both strands. The cloned fragments were identical in sequence, except that the 1.7-kb clones were 240 bp shorter. The longest 5′ RACE product contained the overlapping 60 bases of HvSPY88, but the combined sequence and its deduced amino acid sequence appeared to lack 100 bases of open reading frame when compared with SPY. Therefore, another 5′ RACE reaction was performed using the same cDNA pool, and an additional 180 bases were obtained. The combined cDNA and 5′ RACE sequences were analyzed using the Genetics Computer Group (Madison, WI) software package (Devereux et al., 1984).

Genomic DNA and RNA Gel Blot Analyses

DNA was extracted from young leaves, according to the method of Dellaporta et al. (1983). Digested DNA was fractionated in 0.8% [w/v] agarose gels in 0.5 × Tris–borate–EDTA and transferred to a Hybond-N (Amersham International) membrane by using 20 × SSC, and the membrane was hybridized in 6 × SSC, 5 × Denhardt's solution, 0.5% [w/v] SDS, 10 μg/mL poly(A), and 100 μg/mL sheared salmon sperm DNA at 65°C with a 5′ half (the 1.9-kb 5′ RACE product) or 3′ half (HvSPY88 cDNA) cDNA fragment as a probe. The membrane was washed three times in 2 × SSC and 0.5% [w/v] SDS at 65°C for 20 min each, followed by a 50°C wash in 0.1 × SSC and 0.5% [w/v] SDS for 20 min. The membrane was exposed to Fuji (Tokyo, Japan) x-ray film with two intensifying screens at −80°C.

For RNA gel blot analysis, poly(A)$^+$ RNA was isolated from total RNA (PolyATract; Promega) and fractionated in 1% agarose gels containing formaldehyde. RNA was transferred, hybridized, and exposed to x-ray films, as described for genomic DNA gel blot analysis.

Reverse Transcription–PCR

Reverse transcription (RT) followed by PCR amplification was used to examine HvSPY mRNA expression and to clone and analyze HvSPY in various barley genotypes. Total RNA was extracted from shoots of young seedlings (Close et al., 1989), and 5 μg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The single-stranded cDNA was the template for 10-μL PCR reactions, using a forward primer (5′-CGGAGGATGAATATGTTAG-3′) and a reverse primer (5′-CTGCTCACCTCCCTTG-3′) for expression analysis. The PCR product was fractionated in 5% total acrylamide and *bis*-acrylamide in Tris–borate–EDTA and stained with ethidium bromide. For cloning, PCR reactions were performed in 50- to 100-μL reactions, using a number of primer pairs, and the PCR products were fractionated in 1.2 % [w/v] agarose gels. Bands of the correct size were excised, and DNA was purified using QIAEXII resin (Qiagen, Melbourne, Australia). The purified DNA was cloned into the pGEM-T vector, and the sequence was analyzed as described above.

Functional Complementation of the Arabidopsis *spy-3* Mutation

The vector used for complementation experiments was pOCA121, which contains the HindIII-EcoRI fragment (cauliflower mosaic virus

35S promoter:β-glucuronidase [*GUS*]:nopaline synthetase [*NOS*] 3′) from pBI121 cloned into the HindIII and EcoRI sites of pOCA28, a derivative of pOCA18 (Olszewski et al., 1988). A construct was made using the Arabidopsis SPY cDNA subcloned into pBluescript II KS+. A primer was used to introduce a XbaI site 51 bp upstream of the presumed start codon by PCR to amplify several hundred base pairs of the SPY cDNA. The resulting PCR product was digested with XbaI and BstEII (35 bp downstream of the start codon) and ligated into pOCA121 with the BstEII-SacI fragment of the pBluescipt II KS+ SPY cDNA clone. This generated the entire SPY cDNA coding region with 51 bp of the 5′ untranslated leader driven by the 35S promoter.

A HvSPY cDNA with a complete open reading frame and 32 bp of 5′ untranslated leader was obtained by RT-PCR because it was not possible to construct a continuous cDNA with the cDNA clone and the 5′ RACE products because of limitations in restriction enzyme sites. From poly(A)$^+$ RNA, cDNA was synthesized using oligo(dT)$_{18}$ priming and then amplified using a mixture of Taq and Pfu DNA polymerases. The PCR product was cloned into a SmaI-digested, calf intestine phosphatase–treated pGEM7 (Promega) vector. Three clones were sequenced in full to test for errors in PCR. Each of the three clones contained one or two errors, but the deduced amino acid sequence of one of the clones was identical to those of the cDNA and the 5′ RACE sequences, so this clone was used in a binary vector construct. The XbaI-SacI fragment of the full-length HvSPY cDNA in pGEM7 was ligated into the XbaI and SacI sites of the above vector to replace the Arabidopsis SPY cDNA.

Both constructs were transformed into *Agrobacterium tumefaciens* C58C1 by electroporation. The binary vectors were then transformed into *spy-3* plants by vacuum infiltration. T_1 seeds (self-pollinated) harvested from the infiltrated *spy-3* plants were screened on kanamycin plates (50 μg/mL) to identify transgenic plants. The kanamycin-resistant putative transgenic seedlings containing a single kanamycin resistance locus were grown to maturity to obtain T_2 seeds. The segregating population of T_2 plants was selfed, and T_3 seeds were germinated on kanamycin plates to identify homozygous T_2 plants. T_3 seeds from homozygous plants were used for analysis. The homozygous lines were obtained from different infiltrated plants and therefore represent independent transformation events.

The ability of both the Arabidopsis SPY and barley HvSPY constructs to suppress the mutant phenotype of *spy-3* was tested by germination on paclobutrazol in both the light and the dark (Jacobsen and Olszewski, 1993). Germination was scored on days 4 and 11 in the light and day 7 in the dark. The 35S:HvSPY construct was also tested for its ability to suppress the vegetative phenotype of *spy-3.* Seeds were sown on 0.6% [w/v] agar plates containing 1 × Murashige and Skoog salts (Murashige and Skoog, 1962), 1% sucrose, and 10^{-7} M uniconazole and grown in an 18-hr day (22°C) and 6-hr night (20°C) photoperiod. The light intensity was 180 μmol m^{-2} sec^{-1}.

Transient Expression Analysis

Himalaya barley aleurone transient assays were performed essentially as described by Gubler et al. (1995), using 1985 Pullman harvest of cultivar Himalaya grains. Reporter plasmid (0.5 μg; either Amy[−877]–IGN [Jacobsen and Close, 1991] or Dhn7[−935]–IGN [Robertson et al., 1995]) and effector plasmid (1.0 μg; either UbiCass or Ubi–HvSPY) were coprecipitated on 3 mg of gold particles. UbiCass is a maize Ubi1 expression cassette (pLZUbicas) consisting of the maize ubiquitin gene promoter (Christensen et al., 1992) fused to a multiple cloning site and a *Nos* 3′ terminator (Z. Li, unpublished data). Ubi–HvSPY was constructed using the full-length open reading frame described above. It was inserted into the KpnI-SalI site of the UbiCass vector. Each transient expression replicate used six half-grains and 15% of the gold preparation with precipitated DNA (i.e., 75 ng of reporter construct and 150 ng of effector construct on 0.45 mg of gold). The transformed half-grains were cut longitudinally along the crease, and six quarter-grains were incubated at 25°C for 24 hr with gentle shaking in 25-mL flasks containing 2 mL of 10 mM $CaCl_2$ (control) supplemented with 10^{-6} M GA_3 or 10^{-6} M abscisic acid (ABA), as appropriate. After treatment and removal of the solutions, the grains were stored at −20°C, and GUS assays were performed as described previously (Jefferson, 1988).

ACKNOWLEDGMENTS

We thank Judy Radik and Eva Chung for valuable technical assistance and Zhongyi Li for providing the UbiCass plasmid. This work was supported by grants to N.E.O. from the National Science Foundation (Nos. IBN 9317524 and MCB 9604126).

Received January 6, 1998; accepted March 19, 1998.

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