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SPYing on GA Signaling and Plant Development

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Abstract

It has been ten years since the *SPINDLY* (*SPY*) locus was first identified from a screen of mutagenized wild type *Arabidopsis* seeds by selecting for germination in the presence of a gibberellin (GA) biosynthesis inhibitor (Jacobsen and Olszewski 1993). Since then research into this novel protein, an *O*-GlcNAc transferase (OGT), has revealed some fascinating and surprising results. SPY was originally described as a negative regulator specific to the GA signal transduction pathway, but recent research suggests that SPY is involved in additional aspects of plant development. SPY is also being investigated in barley, petunia and rice, adding to the complex story that is SPY.

Key words: *Spindy* (*spy*); Gibberellin signaling; *Arabidopsis*; Signal transduction; GlcNAc

Introduction

Gibberellins (GAs) are important plant hormones that have been shown to participate in most, if not all, stages of plant development. They also mediate between environmental signals such as light and photoperiod and induced physiological responses such as stem elongation and flowering (Hedden and Phillips 2000). Over the last couple of years there have been a number of extensive reviews covering the topic of GA signal transduction (Sun 2000; Richards and others 2001; Olszewski and others 2002). This review focuses on SPINDLY (SPY), an *O*-GlcNAc transferase (OGT), and discusses recent research that attempts to elucidate the physiological roles of this protein in GA signaling and plant development.

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O-GlcNAc Transferases and TPR Proteins

The *SPY* gene encodes a protein that is related to cytosolic *O*-GlcNAc transferase (OGT) of animals (Thornton and others 1999b). SPY has an amino-terminal tetratricopeptide repeat (TPR) domain (containing 10 TPR repeats) and a carboxyl-terminal catalytic domain. Amino acid sequence comparisons between *Arabidopsis* SPY and rat OGT revealed a 40% similarity in the TPR domain and 46% similarity in the catalytic domain (Thornton and others 1999b). The OGT activity of SPY was initially confirmed *in vitro* when AtSPY was expressed in insect cells and protein from *spy* mutants exhibited allele-specific alterations in their OGT activity (Thornton and others 1999a). In addition, it has recently been shown that *spy* mutants exhibit a reduction in *O*-GlcNAcylated proteins (Thornton 2001 quoted in Hartweck and others 2002).

SPY-like genes have now been cloned and characterized from a number of other plant species including barley, petunia, tomato, and rice. Sequence comparisons show that SPY is highly conserved among these different species, including monocots and dicots (Robertson and others 1998; Hartweck and others 2002). Recently, a new OGT gene, *SECRET AGENT* (*SEC*), has been characterized in *Arabidopsis*. SEC shows high sequence similarity to both AtSPY and animal OGTs (Hartweck and others 2002), although SEC is more similar to animal OGTs than is SPY, which shares an equal level of similarity with SEC and animal OGTs. In addition, it has been shown that both SPY and SEC are able to *O*-GlcNAc modify themselves, a property that is also exhibited by human OGT (Thornton and others 1999a; Hartweck and others 2002; Lubas and Hanover 2000).

There is a paucity of information regarding *O*-GlcNAc modification and its activity in plants. However, there has been considerable progress in non-plant systems demonstrating that *O*-GlcNAc modification (termed ''*O*-GlcNAcylation'') involves the addition of single β-*N*-acetylglucosamine moieties (GlcNAc) to specific serine and/or threonine residues via an *O*-linkage (Figure 1) (Comer and Hart 1999). Since it was first described (Torres and Hart 1984), *O*-GlcNAc modification has been found to be common to nearly all eukaryotes including plants, filamentous fungi, animals (including humans) and viruses that infect eukaryotes (Comer and Hart 2000). Therefore, to understand the potential of this regulatory system in plants, it is important to discuss what is known about *O*-GlcNAc modification in non-plant systems.

In animals, nuclear pore proteins were among the first *O*-GlcNAc modified proteins to be structurally characterized. Many other proteins have since been found in both the nucleus and cytoplasm that are modified with *O*-GlcNAc (Comer and Hart 2000). These proteins span a broad spectrum of biological functions including transcription factors, oncogenes, RNA polymerase II, nuclear pore proteins, viral proteins and tumor repressors.

O-GlcNAcylation appears to be highly dynamic with a rapid cycle of addition and removal analogous to protein phosphorylation/dephosphorylation catalyzed by kinases and phosphatases (Figure 1) (Comer and Hart 1999, 2000; Lubas and others 1997). In several documented cases, phosphorylation and *O*-GlcNAc modification are reciprocal and occurring at the same or adjacent moieties (Kelly and others 1993; Comer and Hart 2000). Furthermore, a number of other *O*-GlcNAc modified proteins can occur as phosphorylated proteins, suggesting that *O*-GlcNAc may regulate the target protein by competing with protein kinases (Lubas and others 1997; Chou and others 1995; Gao and others 2001; Wells and others 2001). In support of this, studies using kinase and phosphatase inhibitors have provided direct evidence that changes in phosphorylation can directly affect the level of *O*-GlcNAc in some nuclear and cytoplasmic proteins (Lefebvre and others 1999; Griffith and Schmitz 1999), highlighting the complex relationship between *O*-GlcNAc modification and *O*-phosphorylation.

As expected from the wide range of *O*-GlcNAc-modified proteins described above, *O*-GlcNAcylation has been implicated in the regulation of a number of cellular functions including neurofilament assembly, protein synthesis, and gene transcription (Comer and Hart 1999). Some cytoplasmic and nuclear proteins regulating transcription that are *O*-GlcNAcylated include Sp1, Ap1, Ap2, Ap4, serum response factor (SRF), the estrogen receptor (ER), insulin promoter factor-1 as well as RNA polymerase II and some chromatin proteins (Comer and Hart 1999; Gao and others 2001). Sp1 is multiply *O*-glycosylated, which enhances its activity in transcription. Blocking the addition of GlcNAc residues, which leads to Sp1 becoming hypoglycosylated, subjects the protein to proteasome degradation (Han and Kudlow 1997).

The *O*-GlcNAcase enzyme is responsible for removal of the *O*-GlcNAc molecule from proteins and has been purified from rat spleen (Dong and Hart 1994) and more recently from bovine brain (Gao and others 2001). *O*-GlcNAcase is present in both the cytosol and nucleus, where *O*-GlcNAc modification is known to occur. However, Gao and others (2001) found that *O*-GlcNAcase overexpressed in cos-7 cells is almost exclusively localized to the cytoplasm. This suggests that overexpressing the *O*-GlcNAcase enzyme causes it to form aggregates in the cytoplasm that cannot be correctly translocated.

OGTs are highly conserved members of the tetratricopeptide repeat (TPR) family of proteins and all have TPR motifs in the N-terminal part of the protein. There are 13 TPR tandem repeats in the *C. elegans* protein compared with 9 in humans, 11 in rat, 10 for SPY and 12 for SEC (Lubas and Hanover 2000; Jacobsen and others 1996; Hartweck and others 2002). It has been proposed that the N-terminal TPR domain of the OGT protein is required for optimal recognition of particular substrates. When the first 3 TPR domains of the Human OGT were removed, the addition of *O*-GlcNAc to proteins was greatly reduced (Lubas and Hanover 2000). This was not due to a general reduction in enzyme activity as removal of the first six TPR repeats

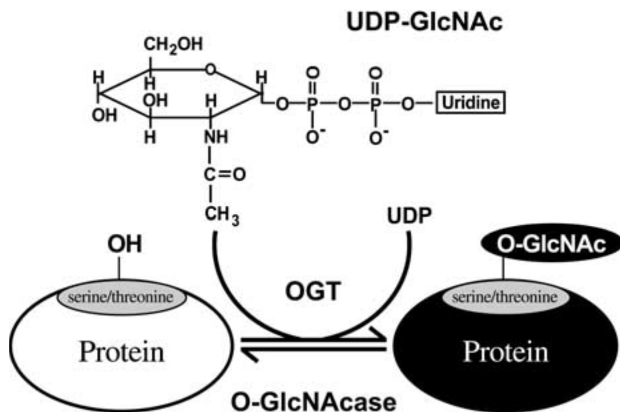

Figure 1. *O*-GlcNAc addition to specific serine/threonine residues can modify protein activity. Addition of a single *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) moiety, from uridine diphospate (UDP)-GlcNAc, to target proteins by *O*-GlcNAc transferase (OGT) can lead to altered protein activity or stability, in a manner analogous to protein phosphorylation by kinases. *O*-GlcNAc residues can be removed by *O*-GlcNAcase, similar to the removal of phosphate groups by phosphatases. For some proteins, addition of a phosphate or GlcNAc residue at a particular serine/threonine can be mutually exclusive.

increased autoglycolsylation of OGT, showing that these repeats are not required for catalysis.

The TPR motif is a protein-protein interaction module that is found in multiple copies in a number of functionally different proteins and facilitates specific interactions with a partner protein(s) (Blatch and Lassle 1999). The motif is widespread in evolution and is found in organisms as diverse as bacteria, cyanobacteria, yeast and other fungi, plants and animals including insects and humans. TPR proteins are also found in a number of subcellular locations such as the cytosol, nucleus, mitochondria and peroxisomes. The number of TPR repeats can vary between different proteins and there appears to be no preferential positioning along the primary sequence of the protein. Although TPRs are tandemly arrayed at the N-terminal for OGT proteins, in other TPR proteins it is not uncommon for single or double TPRs to be found separated from the array (Blatch and Lassle 1999; Lamb and others 1995). Although not all potential TPR's within a particular protein are necessarily functional, the evolutionary conservation of the TPR motif in general suggests that its structure is functionally important (Blatch and Lassle 1999).

The TPR domain is a degenerate 34 amino acid repeat, although the pattern of amino acid similarity is largely conserved with respect to size, hydrophobicity and spacing. Comparing TPRs from a variety of different proteins reveals eight loosely conserved consensus residues at positions 4(W/L/Y), 7(L/I/M), 8(G/A/S), 11(Y/L/F), 20(A/S/E), 24(F/Y/L), 27(A/S/L) and 32(P/K/E) (Figure 2) (Sikorski and others 1990; Hirano and others 1990). Sequence conservation of adjacent TPRs from the same protein is typically only related by the eight consensus residues. However, functionally similar TPRs from different proteins can share sequence similarity beyond the consensus residues, suggesting that individual TPRs are evolutionary conserved (Lamb and others 1995).

The crystal structure of the human protein phosphatase 5 (PP5) has revealed the secondary structure of the TPR motif. Each of the three TPR motifs of PP5 are structurally related and consist of a pair of antiparallel α-helices labeled A and B (Das and others 1998). Adjacent TPR motifs are organized in a parallel manner, so that sequential TPR motifs form a series of antiparallel α-helices. Helices of adjacent TPR repeats are arranged in a right-handed super helical conformation that yields the formation of a channel, an amphipathic groove, which is thought to be responsible for the accommodation of the non-TPR counterparts in TPR-mediated protein complexes (ribbon representations of the TPR domain are shown in Blatch and Lassle (1999), Das and others (1998), Gounalaki and others (2000), and Scheufler and others (2000). The eight consensus residues appear to be important for the structural integrity of the TPR domain. α-helix A spans residues 4, 7, 8, 10 and 11, and the B domain spans residues 20, 24 and 27 (Figure 2) (Das and others 1998; Blatch and Lassle 1999).

The crystal structures of two of the TPR domains of the human HOP protein (Hsp70 and Hsp90 organizing protein), TPR1 and TPR2A, have recently been resolved (Scheufler and others 2000). HOP is an adapter protein that mediates the association of the molecular chaperones Hsp70 and Hsp90 (Scheufler and others 2000). It was found that the crystal conformation of the HOP TPR-peptide is similar to the TPR domains of the PP5 protein, suggesting that secondary structure and folding of TPR proteins is conserved. Thus, from these crystalline structures, 3D models of TPR domains can be constructed to further understand the binding of proteins to the TPR domain. A 3D model of the yeast TPR protein Ssn6 was used to analyze the role of selected mutations in the Ssn6 TPR domain on the binding of the Tup1 protein, known to bind to TPR's 1, 2 and 3 (Gounalaki and others 2000). According to the model, selected mutations in the first TPR domain reduced the ability of Ssn6 to interact with Tup1 by affecting the structural integrity of TPR1

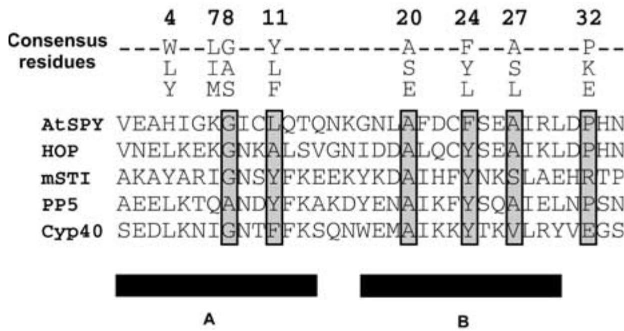

Figure 2. Tetratricopeptide repeats (TPRs) are a degenerate 34 amino acid motif involved in protein interactions. Sequence alignment of the first TPR motif of *Arabidopsis* SPINDLY (SPY) with Hsp70 and Hsp90 organizing protein (HOP), Hsp90-binding regions of murine stress-inducible protein (mSTI1), human phosphatase 5 (PP5) and bovine cyclophilin-40 (Cyp40) are shown. The TPR consensus residues are shown above the alignment with the amino acid position indicated. Grey boxes represent consensus residues present in AtSPY TPR 1. The black horizontal bars represent the extent of helices A and B of the human protein phosphatase 5 (modified from Blatch and Lassle 1999).

and/or the correct spatial arrangement of TPR1 relative to TPR2 and TPR3 (Gounalaki and others 2000). These findings suggest that the structural integrity of TPR proteins is essential for maintaining protein-protein interactions.

The importance of TPR protein complexes and the diverse cellular functions that they perform have been demonstrated in a number of different biological systems. Nevertheless, with the exception of SPY (see below), the functional importance and interactions of TPR proteins within plants has not yet been thoroughly explored. With the completion of the genomic sequence of *Arabidopsis* it is predicted that about 75 proteins contain TPR domains (Chory and Wu 2001).

SPY in *Arabidopsis*; GA Signaling and Plant Development

The ability of *spy* mutant seeds to germinate in the presence of a GA biosynthesis inhibitor, which normally blocks seed germination in *Arabidopsis*, led to the isolation of the first *spy* alleles (Figure 3A) (Jacobsen and Olszewski 1993). A number of other *spy* alleles have since been isolated (Jacobsen and others 1996; Carol and others 1995; Wilson and Somerville 1995; Silverstone and others 1997). Sequencing of these alleles revealed that the majority of mutations are in TPRs 6, 8 and 9 of the TPR domain, or in the catalytic domain (Figure 4) (Tseng and Olszewski unpublished data). Nevertheless, the *spy* allele that is thought to be the closest to a null is *spy-4*, which is tagged by a T-DNA insertion 14 base pairs upstream of the start of transcription (Jacobsen and others 1996).

Analysis of double mutants demonstrated that *spy* can partially suppress the dwarf phenotypes associated with the *ga1* mutant, which lacks an early GA biosynthesis enzyme. Phenotypes suppressed include failure of seed germination, reduced hypocotyl, stem and leaf growth, delayed flowering and male sterility (Jacobsen and others 1996; Swain and others 2001). However, plant height and rosette size of the *spy-4 ga1-2* double mutant is less than that of *spy-4* mutants and they are still able to respond to exogenous GA treatment with an increase in rosette size and plant height. This implies that *spy* mutants are not completely saturated for GA response, as they remain capable of responding to exogenous GA (Jacobsen and others 1996). Similar results were also obtained for plants grown on paclobutrazol, an inhibitor of GA biosynthesis (Izumi and others 1985), and for double mutants between *spy* and the semi-dominant *GA insensitive* (*gai*) mutant (Jacobsen and others 1996; Swain and others 2001). From these results, and the recessive nature of *spy* mutations, *SPY* was proposed to be a negative regulator of the GA signal transduction pathway in *Arabidopsis* (Figure 3).

Further evidence supporting this hypothesis came from analysis of endogenous GA levels in the semi-dwarf *spy ga1-3* mutant, where it was found that the *spy* mutation did not alter the GA content of the dwarf *ga1-3* mutant (Silverstone and others 2001). The partial rescue of *ga1-3* by *spy* is therefore due to alterations in the GA response pathway. Nonetheless, the same study showed that expression of the *GA4* gene, which encodes a GA 3-oxidase enzyme, was increased in *spy-8 ga1-3* mutants compared with *ga1-3* (Silverstone and others 2001). This is the opposite of what would be predicted based on the "feedback" model of GA biosynthesis (Hedden 1999), and is in contrast to results obtained for *rga ga1-3* mutants. RGA (*Repressor of ga1-3*) is another repressor of GA signaling, and loss-of-function *rga* mutations are also able to partially rescue the dwarf *ga1-3* phenotype. Like *spy*, *rga* did not cause any detectable changes in GA content, but caused reductions in *GA4* expression in a *ga1-3* background (Silverstone and others 2001). A contrasting study showed that *spy-5* grown on paclobutrazol, which like *ga1* decreases GA levels, contained lower levels of *GA4* mRNA than untreated *spy-5* plants (Cowling and others 1998).

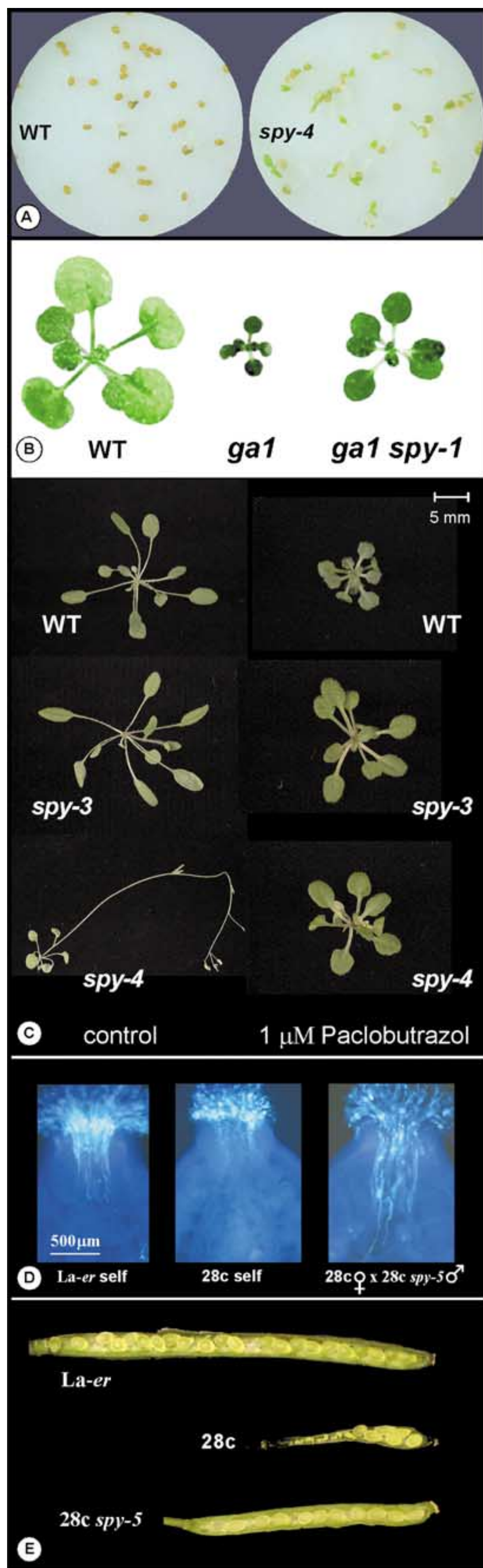

Figure 3. spy Mutant Phenotypes. **A.** Seed germination in the presence of paclobutrazol. *De novo* GA biosynthesis is required for seed germination in *Arabidopsis*, and 10 μM paclobutrazol, a chemical inhibitor of an early GA biosynthesis enzyme, greatly reduces germination of wild-type seeds (7% germination). By contrast, mutant *spy-4* seeds are able to germinate (100% germination) due to an increase in GA response. This differing response to paclobutrazol was used to isolate the original *spy* mutants. **B.** SPY is genetically defined as a negative regulator of GA response. Reduced SPY activity partially suppresses all phenotypes of the GA-deficient *ga1* mutant, including the extreme dwarf phenotype. The *spy-1* allele shown here (without the *hy2* mutation originally present; Jacobsen and others 1996) alters mRNA splicing resulting in SPY protein lacking part of TPRs 8 and 9. **C.** Vegetative growth in the presence of paclobutrazol. Plants grown with or without paclobutrazol in an artificial long day consisting of 8 h of metal halide light followed by 8 h of weak incandescent light. All plants are 30 days old. Control Plants: mild *spy* mutants, such as *spy-3*, appear similar to wild-type, although they flower slightly earlier. By contrast, the severe *spy-4* mutant has a greatly reduced rosette size, flowers extremely early, and is almost completely male sterile. Paclobutrazol-treated plants: If 1 μM paclobutrazol is added at day 10 after germination to reduce endogenous GA levels, wild-type plants are smaller because of reduced leaf growth. Consistent with increased GA response, *spy-3* and *spy-4* plants are larger than wild-type plants under these conditions. Despite the marked differences in growth shown in the control, *spy-3* and *spy-4* are both able to suppress the dwarfing effect of paclobutrazol to a similar extent, suggesting that the two alleles have similar effects on GA signaling. **D.** SPY acts in elongating pollen tubes. Aniline blue staining of pollen tubes 5 h after pollen grain germination reveals that pollen tubes carrying the 35S:2ox2/28c transgene, designed to reduce endogenous GA levels, elongate less than wild-type pollen tubes, and this defect is rescued by the *spy-5* mutation. **E.** *spy-5* rescues the 35S:2ox2/28C small fruit phenotype. Compared with wild-type La-*er*, 35S:2ox2/28c siliques have seeds only at the end nearest the stigma because impaired pollen tube growth reduces fertilization. This phenotype is partially rescued by the *spy-5* mutation, suggesting that increased GA response can largely substitute for reduced GA levels in elongating pollen tubes. Parts D and E modified from Singh and others (2002), copyright the American Society of Plant Biologists, and is reprinted with permission.

Thus, while feedback control mechanisms have illustrated the important role of the GA response pathway in the regulation of GA biosynthesis (for review see Hedden 1999), the role of SPY in this feedback mechanism has not yet been clarified.

To further explore the role of SPY in plant development, *SPY* was overexpressed in wild-type and

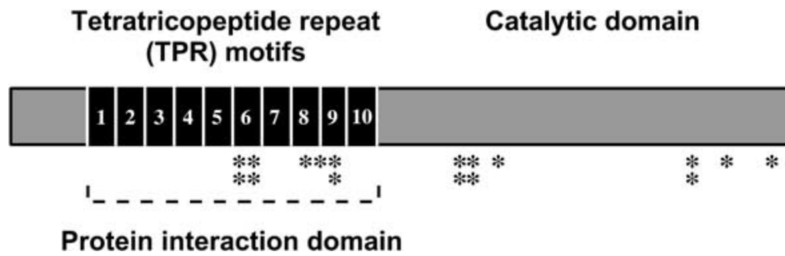

Figure 4. Schematic representation of the SPY protein. SPY is composed of an N-terminal TPR domain, thought to be involved in protein-protein interactions, and a C-terminal putative catalytic domain. A number of *spy* alleles have been isolated (indicated by *) from both the TPR domain and C-terminus, revealing that both regions are essential for normal GA signaling. All of the N-terminal mutations identified to date alter TPRs 6, 8 and/or 9. The *spy-4* mutation is caused by a T-DNA insertion upstream of the start of *SPY* transcription, and greatly reduces *SPY* expression.

a *spy-3* mutant background using the Cauliflower mosaic virus 35S promoter. Germination assays show that *spy-3* is able to germinate on paclobutrazol and this is thought to be due to the increase in GA signaling in *spy* mutants. The 35S:SPY construct containing a genomic *SPY* clone in both a wild-type and *spy-3* background was able to increase the sensitivity of seeds to paclobutrazol compared with wild-type. These results suggest that the 35S:SPY construct is suppressing GA signaling in germinating seeds, as would be expected if SPY was negatively regulating GA signal transduction (Swain and others 2001).

Investigation of the vegetative and reproductive phases of wild-type plants containing 35S:SPY revealed that the relationship between SPY and its effect on GA signaling is much less straightforward. 35S:SPY plants have longer hypocotyls and larger rosettes than wild-type plants (Swain and others 2001), which is not what would be expected if SPY was negatively regulating GA signaling because GAs promote hypocotyl and leaf growth. In addition, 35S:SPY plants are able to partially suppress the vegetative dwarf phenotype of both the *ga1* and *gai* mutants, in a manner similar but not identical to the suppression caused by *spy-3* and *spy-4* (Swain and others 2001). The same result was observed for flowering time, in that 35S:SPY plants flowered earlier than wild-type and could suppress the delayed flowering phenotype of *ga1* and *gai*, but to a lesser extent than strong *spy* alleles (Swain and others 2001). The simplest explanation for these results is that 35S:SPY is increasing GA response in *Arabidopsis* plants after seed germination.

There is evidence suggesting that the effects of 35S:SPY on vegetative growth are unlikely to be caused by co-suppression (Swain and others 2001). Investigation of mRNA in these plants showed that *SPY* mRNA levels were greatly elevated compared with wild-type. This, and the ability of 35S:SPY to rescue the seed germination phenotype of *spy-3*, suggests that the construct does encode a functional protein (Swain and others 2001). These results led to the hypothesis that because SPY is likely to interact with itself and with other proteins through the TPR domain *in vivo*, over-expression of the protein may cause dominant-negative phenotypes, such as an increase in GA response, by disrupting the normal function of SPY complexes.

To test this hypothesis further, Tseng and others (2001) overexpressed the SPY TPR domain without the C-terminus in a wild-type, *spy* and *gai* background. 35S:TPR transgenic plants in a *spy* background were still able to germinate on high concentrations of paclobutrazol, demonstrating that the TPR domain of SPY is not able to rescue the *spy* mutant germination phenotype. In addition, seed germination for 35S:TPR in a wild-type background showed reduced sensitivity to paclobutrazol but was not as resistant as *spy* mutant seeds (Tseng and others 2001). This is in contrast to the increased sensitivity to paclobutrazol exhibited by the germination of wild-type seeds containing 35S:SPY. The 35S:TPR construct also caused slightly earlier flowering in wild-type plants and was able to partially rescue the *gai* mutant phenotype, but to a lesser extent than *spy* mutants (Tseng and others 2001). These results indicate that plants containing the 35S:TPR construct are weak phenocopies of *spy* mutants.

Immunoprecipitation and yeast-2-hybrid experiments confirmed that the full-length SPY protein and the TPR domain could interact (Tseng and others 2001). SPY was also found to be present in an 850-kDa complex suggesting that SPY may be associated with other plant proteins *in vivo*. These results are consistent with the hypothesis that the 35S:TPR and at least some of the 35S:SPY phenotypes are due to dominant-negative effects of excessive levels of the TPR domain with or without the C-terminus (Swain and others 2001; Tseng and others 2001).

During the overexpression studies it was observed that severe *spy* mutants had phenotypes that were apparently not characteristic of an increase in GA signaling, at least based on our present understanding of GA physiology (Figure 3). On closer inspection of the severe *spy* alleles *spy-2* and *spy-4* in

a wild-type La-*er* background and *spy-4* in the Columbia background, it was revealed that these *spy* mutants were not, as previously reported, exact phenocopies of wild-type plants sprayed with GA (Swain and others 2001). Characteristics of *spy* mutants that resemble GA-treated wild-type plants are reduced male fertility, early flowering, pale green foliage and fewer leaves (Jacobsen and others 1996). These characteristics are consistent with an increase in GA signaling. However, wild-type plants sprayed with GA, in addition to the characteristics described above, also exhibited longer hypocotyls, larger rosettes, longer leaves and are larger plants. Yet severe *spy* mutants have short hypocotyls (in white light and in the dark), are smaller plants, have shorter internodes, smaller leaves and rosettes, unserrated leaf margins, extra trichome branches, phyllotaxy defects in the inflorescence, deformed flowers and abnormal root growth (Swain and others 2001, 2002). As far as is known, none of these phenotypes can be observed in wild-type plants treated with GA, and some cannot be explained based on our current understanding of GA action. By contrast, for other GA signaling mutants, such as *rga*, *rgl2*, *gai* (both the gain- and loss-of-function alleles) and *sly*, all of the observed phenotypes are consistent with altered GA response. These observations led to the proposal that SPY may play a role in plant development beyond its role in GA signaling (Swain and others 2001).

On investigation of hypocotyl lengths in *spy* mutants and in transgenic plants, some interesting results were obtained. As previously discussed, *spy* mutations are able to partially suppress the short hypocotyl phenotype of *ga1* (Silverstone and others 1997). Yet, when *spy ga1* plants were grown on a saturating dose of GA_3, they possessed shorter hypocotyls than similarly treated *ga1* plants (Silverstone and others 1997; Swain and others 2001). 35S:SPY in both a wild-type and *spy-3* background had longer hypocotyls, showing that this construct was able to rescue the short hypocotyl phenotype of *spy-3* and confer a phenotype opposite to reduced SPY activity (Swain and others 2001). In contrast, 35S:TPR expressing plants have significantly shorter hypocotyls, but not as short as those of *spy-4* (Tseng and others 2001). Similar contrasting effects of strong *spy* alleles and 35S:SPY were also observed for rosette size. Consequently, although some 35S:SPY phenotypes are probably due to dominant-negative effects, others appear to represent the effect of increased SPY activity.

Another unusual phenotype of *spy* mutants is a reduction in the size and number of leaf serrations (Swain and others 2001). Wild-type, *ga1* and *gai* plants all possess serrated leaves, with or without GA treatments. Yet *spy* and *spy gai* plants have essentially unserrated smooth leaves. This is a phenotype that has not generally been associated with GA action in *Arabidopsis*, although in tomato, the increased GA response mutant, *procera* (*pro*), and wild-type plants treated with GA, have rounder leaves than untreated wild-type plants (Jones 1987).

Recently, the role of GAs in leaf morphology has become clearer, as it has been demonstrated that one function of *KNOX* (*KNOTTED1*-like homeobox) genes is to prevent high GA levels in the shoot apical meristem. *KNOX* proteins are transcription factors central to the acquisition of leaf versus meristem identity, and were first identified by their role in meristem development, and later implicated in the control of GA biosynthesis (Sakamoto and others 2001). Ectopic *KNOX* expression causes increased meristem formation and can have dramatic effects on leaf morphology, depending on the species examined. For example, when *BP* (*BREVIPEDICELLUS*), also known as *KNAT1* (*KNOTTED1-LIKE in ARABIDOPSIS THALIANA 1*), is overexpressed, plants exhibit highly lobed leaves (Hay and others 2002). Significantly, the 35S:KNAT1 leaf phenotype can be reversed by GA application. Furthermore, crosses between 35S:KNAT1 and *spy-1* show that the *spy* mutation decreases the number of lobes per leaf, similar to applied GA (Hay and others 2002). This result can be explained if the 35S:KNAT1 phenotype is largely dependent on localized reductions in GA levels in developing leaf primordia, as normally occurs in wild-type shoot apical meristems.

SPY has also been implicated in meristem behavior in a study examining the effects of phytochrome and gibberellins on the control of meristem identity (Okamuro and others 1997). The *Arabidopsis* mutant *apetala1* (*ap1*) is partially defective in the establishment of flower meristem identity and is characterized by the production of secondary (auxiliary) flowers from axiliary meristems in the primary flower. *apetala2* (*ap2*), in short-day photoperiods, also shows auxiliary flower production. The formation of auxiliary flowers is an indication that the flower meristem has an inflorescence-like character because axillary meristems are suppressed in wild-type flowers. Both *spy-2* and exogenous GA were found to suppress leaf development and auxiliary flower production in *ap2-1* flowers under a short day photoperiod. Similarly, *spy-3* suppressed auxiliary flower development, floral branching and meristem indeterminacy in *ap1-1* flowers under both long and short days. Okamuro and others (1997) therefore concluded that SPY is required to

promote meristem indeterminacy in both *ap2-1* and *ap1-1* and that gibberellins promote flower meristem identity.

Although SPY may play a role in plant development beyond its role in GA signaling, the studies described above suggest that some of the more unexpected *spy* phenotypes could also reflect aspects of GA action that are not presently fully understood. A corollary of this hypothesis is that wild-type plants treated with exogenous GA, and conversely the phenotypes of the ''classic'' mutants with decreased GA levels or response, may not reveal all aspects of GA physiology. This appears to be the case for GAs and *KNOX* gene activity, and suggests that *spy* mutant phenotypes involving meristem behavior, such as defects in cotyledon formation and inflorescence phylotaxy, may reflect novel aspects of GA physiology rather than non-GA roles of SPY.

It is certainly clear that GAs are involved in more aspects of plant development than was originally appreciated from the extensive analysis of GA-related mutants over the last two decades. In addition to the role in meristem development described above, GAs are also involved in seed development and pollen tube growth. The pea *GA 2-oxidase2* (*2ox2*) gene encodes a GA biosynthesis enzyme involved in the irreversible conversion of active C_{19} GAs to inactive forms (Lester and others 1999). When the 35S promoter was used to overexpress *2ox2* in *Arabidopsis*, seed abortion was observed, confirming the role for GAs in seed development. This is in agreement with the defective seed development phenotype observed in GA-deficient *lh* mutants of pea (Swain and others 1997). More interestingly, defects in pollen tube growth were also observed (Figure 3D,E). A range of experiments was used to confirm the requirement for GAs in pollen tube elongation (Singh and others 2002). When 35S:2ox2 plants were crossed with a *spy* mutant, the 35S:2ox2 pollen tube phenotype was partially suppressed. The partial rescue by *spy-5* of the pollen tube defect in 35S:2ox2 plants was also mimicked by GA application *in vitro* and by combining with other mutants with increased GA response (S. Swain unpublished data), thereby extending the known physiological roles of both GAs and SPY in *Arabidopsis*.

Because SPY contains a TPR domain, thought to be involved in protein-protein interactions, yeast-2-hybrid analysis has been used as an additional approach to understand SPY function. One protein that was found to physically interact with SPY, at least *in vitro*, is GIGANTEA (GI) (Tseng and others 2002). GI is a novel nuclear-localized protein required for maintaining the circadian clock and the control of flowering time by photoperiod (Fowler and others 1999; Park and others 1999; Huq and others 2000). Consistent with GI and SPY interacting *in vivo* in a physiologically relevant manner, analysis of the severe *spy-4* mutant reveals that it causes defects in circadian clock function (Sothern and others 2003). This is a particularly exciting (if unexpected) result and may explain some of the *spy* mutant phenotypes that are not obviously GA related, and the altered hypocotyl length and rosette size in 35S:SPY plants. If SPY is involved in regulating flowering time through GA and the circadian clock, then this may also explain the extremely early flowering time of *spy-4* compared with wild-type plants treated repeatedly with a saturating dose of GA (Swain and others 2001).

Research into the newly characterized *Arabidopsis* OGT gene, *SECRET AGENT* (*SEC*), suggests that SPY still has additional functions in plant development that have not been revealed in detail. Although *sec* mutants do not exhibit any obvious phenotypes, *sec* and *spy* double mutants exhibit a synthetic lethal interaction (Hartweck and others 2002). Both male and female gametes carrying *sec* and *spy* are defective, and double mutant embryos, which form at low frequency, abort at various stages of development, with no viable seeds completing embryogenesis. Therefore, the interaction between these two mutants suggests that protein *O*-GlcNAcylation and OGT activity are essential for gamete and seed development (Hartweck and others 2002). Whether the *sec spy* male gamete defect is related to the role of SPY in pollen tube growth (see above) is not yet know, but excessive GA concentrations are known to inhibit pollen tube growth *in vitro* (for example, see Singh and others 2002). Although *sec* mutants have not been shown to have a role in GA signaling, additional characterization of SEC is currently under way to determine if it plays a role in GA signaling redundantly with SPY.

To fully understand the function and action of SPY within plants, it is necessary to characterize its expression patterns and intracellular localization throughout development. Initial experiments examined the localization of *SPY* mRNA by *in situ* hybridization, and showed that *SPY* is present in seedlings and developing flowers (Jacobsen and others 1998). More recently, a SPY:GUS reporter construct has been used to examine *SPY* expression throughout development. It revealed that, in general, *SPY* is expressed in all organs of the plant and at all stages of the life cycle, although expression was highest in young seedlings (Swain and others 2002). Thus, the expression patterns observed from

the SPY:GUS construct are consistent with the *in situ* data and with the observed phenotypes of *spy* mutants.

A SPY:SPY-GFP reporter construct was used to determine the cellular localization of the SPY protein. This construct revealed that in root tips, the majority of the GFP fluorescence is from the nucleus, though some fluorescence is also found in the cytoplasm (Swain and others 2002). The intracellular localization was confirmed by detecting SPY on protein blots containing proteins from different subcellular fractions (Swain and others 2002). Nonetheless, the SPY protein contains no obvious nuclear localization signals, which raises the question of how SPY becomes localized to the nucleus. One possibility is that nuclear localization may occur through the interaction of SPY'S TPR domain with other proteins containing a nuclear localization signal.

SPY in Barley

The *Arabidopsis SPY* gene was used to clone and investigate the role of the barley (*Hordeum vulgare* L.) HvSPY in regulating the GA response of barley aleurone cells. The cereal aleurone is an established model system used for studying the regulation of plant cell function by the hormones GA and abscisic acid (ABA). Both hormones elicit distinct responses, including well-characterized changes in gene expression, in individual aleurone cells. The aleurone layer is a specialized tissue that responds to GA produced during seed germination by synthesizing hydrolyses (including α-amylase) that mobilize seed reserves stored in the starchy endosperm (Jones and Jacobsen 1991).

Analysis involved co-bombarding HvSPY, driven by the constitutive ubiquitin promoter, into aleurone cells with a GUS reporter gene under the control of a high-pl α-amylase promoter. HvSPY largely prevented GA activation of α-amylase gene expression, as measured by GUS activity (Robertson and others 1998), confirming SPY's role as a negative regulator of GA signaling.

In a further study, the effect of HvSPY on the expression of an ABA-regulated gene, *Dehydrin* (*Dhn*), was examined. Dehydrins are proteins that are expressed in response to dehydration stress and ABA and are proposed to be key components for dehydration stress tolerance (Chandler and Robertson 1994; Close 1996). The increase in *Dhn* mRNA levels in response to ABA is at the level of transcription, and is antagonized by GA via post-transcriptional control (Robertson and others 1995). Recent co-bombardment experiments, with the *Dhn1-2* promoter fused to a GUS reporter gene, demonstrated that both ABA and HvSPY increased *Dhn* promoter activity (Robertson 2003).

ABA-responsive elements within the *Dhn* promoter have been identified and include a G-box motif, a GC-rich element and a Sph element (Robertson and others 1995; Leung and Giraudat 1998). To investigate whether HvSPY uses the ABA signaling pathway, deleted or mutagenized forms of the *Dhn* promoter were used in further co-bombardment experiments. When the ABA-responsive elements were deleted or mutagenized, ABA-induced activity of *Dhn* was decreased, but when HvSPY was overexpressed, *Dhn* activity was still induced (Robertson 2003). Consistent with this result, further analysis of the *Dhn* promoter revealed that HvSPY acts through a region downstream of the ABA responsive elements. Therefore HvSPY is unlikely to be operating through the ABA signaling pathway. Additional evidence for this conclusion comes from experiments using two other ABA-induced genes, wheat *Em* and *EmH5*. These constructs showed a very low activity in the control in the absence of ABA, but when ABA was present there was a 10-fold increase in activity. In contrast, when HvSPY was overexpressed there was no change in the activity of these genes, suggesting that the effect of HvSPY on *Dhn* is not universal to ABA response (Robertson 2003).

Investigation of HvSPY function using barley aleurone layers has provided some interesting results and insights into the activity and function of this protein. These observations further support the hypothesis that SPY may be involved in activities beyond GA signaling.

SPY in Petunia

To investigate the GA signaling pathway in petunia (*Petunia hybrida*), plants were transformed with the SPY construct (35S:AtSPY) that was previously used for over-expression studies in *Arabidopsis* (construct A in Swain and others 2001). In contrast to results obtained in *Arabidopsis*, a number of homozygous petunia 35S:AtSPY plants exhibited a dwarf phenotype with short internodes, reduced apical dominance and rounded leaves (leaves of petunia are normally oval) (Izhaki and others 2001). These plants flowered late and young buds aborted at a stage at which GA has been shown to be crucial for flower development. Seeds obtained from transgenic plants also showed reduced germination, which could be restored with application of GA to

the media (Izhaki and others 2001). These phenotypes were also observed when wild-type plants are treated with paclobutrazol, suggesting that the 35S:SPY transgene can negatively regulate GA signaling in petunia. It is not clear why the same 35S:AtSPY construct has such contrasting effects in *Arabidopsis* and petunia, although this phenomenon is presumably due to differences between SPY'S partners and/or substrates in the two species.

To confirm the down-regulation of GA signaling by the *AtSPY* transgene, the expression of an exclusively GA-regulated gene in petunia, GA-induced gene (*GIP*), was investigated. The expression of *GIP* was inhibited in both wild-type plants treated with paclobutrazol and in the 35S:SPY transgenic plants (Izhaki and others 2001). Although the expression of *GIP* could be restored with the application of GA, a higher concentration of GA was needed to restore *GIP* expression in the transgenic lines compared with paclobutrazol-treated wild-type plants. These results support the hypothesis that GA sensitivity is reduced in petunia plants overexpressing the 35S:SPY construct (Izhaki and others 2001).

Petunia plants were also transformed with the same *Arabidopsis* 35S:TPR construct that gave rise to the dominant negative phenotypes observed in *Arabidopsis* (Tseng and others 2001). RT-PCR (Reverse Transcriptase-PCR) was used to confirm that overexpressing the 35S:TPR construct did not cause co-suppression or silencing of the endogenous petunia PhSPY gene (Izhaki and others 2001). The petunia 35S:TPR transgenic plants exhibited dominant-negative phenotypes including reduced sensitivity to paclobutrazol at seed germination, extensive vegetative growth, lanceolate leaves and long petioles (Izhaki and others 2001). All these phenotypes are observed when wild-type petunia plants are treated with GA. However, in contrast to the results obtained in *Arabidopsis*, other GA-regulated processes, such as flowering time and flower development, were not affected in the transgenic petunia plants (Izhaki and others 2001). Based on these results, it was proposed that the observed dominant-negative phenotypes are due to high levels of the truncated AtSPY interacting with, and disrupting the activity of, the petunia SPY, leading to increased GA signaling.

The *Arabidopsis SPY* gene was used to isolate the petunia homolog (*PhSPY*) (Izhaki and others 2001). Analysis revealed that *PhSPY*, like *AtSPY*, is expressed constitutively throughout the plant, with a slightly higher level of expression at the early stages of development. Furthermore, *PhSPY* expression was not affected by application of GA_3, suggesting that *PhSPY* is not regulated at the transcriptional level by this hormone (Izhaki and others 2001).

Overall, the examination of SPY in petunia has added further evidence of SPY's role as a negative regulator of GA signaling and demonstrates that different plant species use similar mechanisms to regulate the GA signal (Izhaki and others 2001).

SPY in Other Plant Species

Recently, *SPY* was isolated and characterized from tomato (*Lycopersicon esculentum* Mill.) (*LeSPY*), and was used to investigate the relationship between *LeSpy* and the tomato *procera* mutant (Greb and others 2002). The *procera* mutant, like *spy*, is a recessive mutation that causes plants to have an excessive overgrowth phenotype that resembles wild-type plants treated with exogenous GA. Gene mapping revealed that *LeSpy* and *Procera* are not the same gene (Greb and others 2002).

In rice, the *OsSPY* gene has been identified and work is currently underway to investigate the function of *OsSPY* using transgenic rice plants containing an antisense or RNAi *OsSPY* construct (Ueguchi-Tanaka and others 2002). Preliminary results suggest that reduced *SPY* expression has broadly similar effects on plant development in both *Arabidopsis* and rice. More interestingly, initial data suggest that *OsSPY* may play a role in the brassinosteroid pathway. However, this role may be indirect because Ogawa and others (2002) have recently suggested that, at least in germinating *Arabidopsis* seeds, GAs promote brassinosteroid biosynthesis.

Models of SPY and GA Signaling

Genetic, biochemical and physiological research into SPY over the last few years has revealed a complex array of results. SPY appears to be involved in many aspects of plant development, and it is hypothesized that it is involved in both GA and non-GA-related aspects of plant development. Based on the current information, predictive models can be constructed to describe the roles of SPY. In the barley aleurone system and in germinating seeds, where the circadian clock appears to play a minor role, a simple model (Figure 5A) can be envisioned in which SPY acts as a negative regulator of GA response. By contrast, in the later vegetative and reproductive stages of development, two models can be constructed. In the first (Figure 5B), some aspects of SPY function, such as its role in GA signaling and

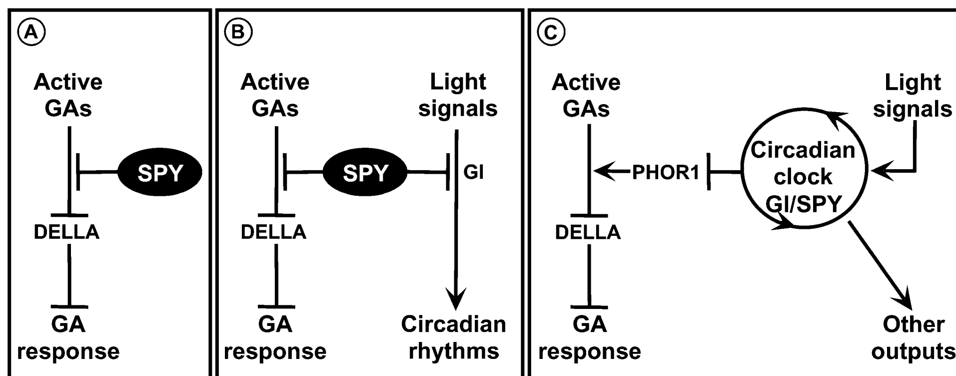

Figure 5. Models of SPY action in *Arabidopsis*. More complex models may be required to explain other *spy* mutant phenotypes and the regulation of *Dehydrin* gene expression by SPY in barley. ''DELLA'' refers to GAI/RGA/RGL2 family members involved in GA response. Note that it is also possible that SPY acts downstream of the ''DELLA'' proteins. **A**. SPY is a negative regulator of GA response during seed germination and in reserve mobilization in aleurone cells. **B**. SPY functions in two unrelated pathways (GA response and the circadian clock). **C**. SPY functions in the circadian clock, which in turn regulates GA signaling via PHOR1.

the circadian clock, reflect two distinct pathways. In this model the two pathways have no connection other than the fact that SPY *O*-GlcNAc modifies different components in each. In the GA response pathway, potential substrates include the ''DELLA'' family members RGA, RGL2 and GAI, whereas in the circadian clock pathway GI may be modified by SPY.

In the second model (Figure 5C), SPY, in conjunction with GI, is required for correct functioning of the circadian clock. Thus, the negative regulation of GA response by SPY reflects a role for the clock in modifying GA response. A significant advantage of this model is that it can explain the role of PHOR1, a protein related to *Drosophila* Armadillo, in GA response. PHOR1 is a positive regulator of GA signaling and its expression varies during the day and in response to photoperiod (Amador and others 2001), consistent with regulation by the circadian clock. This model therefore predicts that one of SPY's functions is to inhibit PHOR1 activity in a clock-dependent manner, and hence regulate GA response. This leads to the following testable hypothesis: if GA response is regulated by the clock, it should exhibit clock-dependent changes in activity. Tools such as GFP-RGA (Silverstone and others 2001) may allow the effect on GA signaling of photoperiod and altered clock function to be explored.

The model in Figure 5A implies that SPY's function is less complex during and immediately after seed germination because the circadian clock may not have a major role in the early events of radicle emergence or in reserve mobilization from the barley aleurone layer. If this hypothesis is correct, it may help to explain why analysis of SPY'S function in these processes has yielded apparently simpler results compared with other stages of plant development. For example, during seed germination in petunia and *Arabidopsis*, and in GA induction of α-amylase in barley aleurones, overexpression of SPY has the predicted effect of reducing GA response. By contrast, in later development, when the clock has major roles in plant development, SPY's function appears to be more complex, and the phenotypes of *spy* mutants and of *Arabidopsis* plants overexpressing SPY are more difficult to interpret.

In conclusion, continuing research into this novel protein in *Arabidopsis*, petunia, barley, tomato and rice seems certain to lead to both a greater understanding of gibberellin action and new insights into the fundamental controls of plant growth and development.

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