Mutations at the SPINDLY Locus of Arabidopsis Alter Gibberellin Signal Transduction

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Three independent recessive mutations at the SPINDLY (SPY) locus of Arabidopsis confer resistance to the gibberellin (GA) biosynthesis inhibitor paclobutrazol. Relative to wild type, spy mutants exhibit longer hypocotyls, leaves that are a lighter green color, increased stem elongation, early flowering, parthenocarpy, and partial male sterility. All of these phenotypes are also observed when wild-type Arabidopsis plants are repeatedly treated with gibberellin A₃ (GA₃). The spy-1 allele is partially epistatic to the ga1-2 mutation, which causes GA deficiency. In addition, the spy-1 mutation can simultaneously suppress the effects of the ga1-2 mutation and paclobutrazol treatment, which inhibit different steps in the GA biosynthesis pathway. This observation suggests that spy-1 activates a basal level of GA signal transduction that is independent of GA. Furthermore, results from GA₃ dose-response experiments suggest that GA₃ and spy-1 interact in an additive manner. These results are consistent with models in which the SPY gene product regulates a portion of the GA signal transduction pathway.

INTRODUCTION

Gibberellins (GAs) have long been known to play major roles in plant growth and development (for reviews, see Jones, 1973; Phariss and King, 1985; Graebe, 1987; Klee and Estelle, 1991). GA-deficient mutants have been particularly useful in determining which stages of plant development involve GAs. Mutations at the GA1 locus of Arabidopsis block GA biosynthesis prior to the formation of ent-kaurene (Barendse et al., 1986; Zeevaart and Talon, 1992). The phenotypes of ga1 mutants include dwarfism, reduced apical dominance, failure to germinate, male sterility, and incomplete petal development. All of these phenotypes are reversed by applied GAs (Koornneef and van der Veen, 1980).

Although much is known about the GA biosynthesis pathway in higher plants (Graebe, 1987; Rademacher, 1989), very little is known about GA perception or GA signal transduction. Genetic analysis, however, has identified several classes of mutants that may be affected in their response to GAs (reviewed in Scott, 1990). In a number of plant species, dominant or semi-dominant mutations result in reduced sensitivity to GA and produce a phenotype similar to that of GA-deficient mutants. Mutants of this type include the ga1 mutant of Arabidopsis (Koornneef et al., 1985), the Miniplant and Dwarf-8 mutants of maize (Phinney, 1956; Harberd and Freeling, 1989), the Rht mutants of wheat (Stoddart, 1984), and the dwarf1 mutant of oilseed rape (Zanewich et al., 1991). Interestingly, for each case in which GA levels have been measured, these "GA-insensitive" mutants contain higher levels of biologically active GAs than do isogenic wild-type plants (Lenton et al., 1987; Fujioka et al., 1988; Talon et al., 1990; Zanewich et al., 1991).

A different class of mutants putatively affected in GA perception or signal transduction is the "slender" mutants. The phenotype of these mutants is similar to that of wild-type plants that have been repeatedly treated with GA. The most extensively characterized slender mutants are in pea (de Haan, 1927), barley (Foster, 1977), and tomato (Jones, 1987). Interestingly, slender mutants in all three of these species contain lower endogenous levels of GAs than wild-type plants (Potts et al., 1985; Jones, 1987; Croker et al., 1990).

Slender pea is the result of recessive mutations at both the CRY and LA loci (de Haan, 1927). crya la plants exhibit long, thin internodes, pale green foliage, and parthenocarpic fruit development (de Haan, 1930). These phenotypes are also observed when wild-type plants are treated with gibberellin A₃ (GA₃) (Dalton and Murfet, 1975; Potts et al., 1985). crya la plants appear to be insensitive to changes in endogenous GA levels, because crya la na triple mutant plants, which have reduced endogenous GA due to the na mutation, exhibit a phenotype that is indistinguishable from that of crya la plants (Potts et al., 1985). Similarly, crya la plants are nearly insensitive to chemical inhibitors of GA biosynthesis and unresponsive to exogenous GA treatment (Potts et al., 1985).

In barley, a single recessive mutation, slender (sin), results in plants that appear as if they have been treated with high doses of GA (Foster, 1977). Phenotypes of this mutant include long internodes, narrow leaves, and complete male and female sterility. Similar to crya la pea plants, sin barley plants...
appear to be insensitive to GA, because their phenotype is not affected by GA biosynthesis inhibitors (Lanahan and Ho, 1988; Croker et al., 1990). In addition, synthesis of hydrolytic enzymes such as protease, ribonuclease, and α-amylase is GA independent in aleurone cells but GA dependent in wild-type aleurone cells (Chandler, 1987; Lanahan and Ho, 1988).

In tomato, the recessive procera (pro) mutation results in plants with elongated stems, pale green foliage, a distinctive change in leaf shape, and other characteristics that are observed in GA-treated wild-type tomato plants (Jones, 1987). The pro mutation is almost completely epistatic to a mutation causing GA deficiency, gib1 (Koornneef et al., 1993). In contrast to mutants in pea and barley, pro plants are still responsive to applied GA and to GA biosynthesis inhibitors (Jones, 1987; Hedden and Lenton, 1988).

We have attempted to isolate slender-type mutants in Arabidopsis by selecting for mutants that are resistant to the GA biosynthesis inhibitor paclobutrazol. This work has resulted in the isolation of three recessive alleles at the SPINDLY (SPY) locus that result in a "GA overdose" phenotype similar to that of slender mutants in other species. We present an analysis of the phenotype of spy mutants and the phenotype of plants that are doubly mutant for spy-1 and ga1-2. We also describe the response of spy mutants to exogenous GA$_3$ treatments and discuss SPY's putative role in GA perception or signal transduction.

**RESULTS**

**Isolation of spindly Mutants**

A two-step screen was used to isolate mutants of Arabidopsis that were resistant to the plant growth regulator paclobutrazol. Paclobutrazol inhibits the monooxygenases involved in the oxidation of ent-kaurene to ent-kaurenoic acid and therefore reduces the plant's ability to synthesize active GAs (Rademacher, 1989). Similar to what has been reported for other GA biosynthesis inhibitors (Karssen et al., 1989; Nambara et al., 1991), Table 1 shows that paclobutrazol inhibited the germination of wild-type Arabidopsis seeds but that this inhibition was reversed by GA$_3$. We reasoned that mutants that germinate in the presence of paclobutrazol may be affected in GA perception or GA signal transduction. Thus, the first step of our screen was to select for mutants that germinated in a medium containing paclobutrazol (see Methods). Screening of 440,000 M$_2$ seed derived from Arabidopsis seed mutagenized with ethylmethyl sulfonate (EMS) identified 69 lines that exhibited paclobutrazol-resistant germination. Because screens similar to this have identified a number of mutants involved in abscisic acid (ABA) metabolism (Koornneef et al., 1982) and ABA response (Nambara et al., 1992), we expected that some of the mutants isolated by the first step of our screen would be of this type. Indeed, of the 69 lines that germinated in the presence of paclobutrazol, 27 lines exhibited a wilty phenotype that is characteristic of ABA-deficient (aba) and some ABA-insensitive (abi) mutants (Koornneef et al., 1982, 1984).

The second step of our screen was to examine the lines that germinated in the presence of paclobutrazol to identify mutants that were also resistant to the dwarfing effects of paclobutrazol. Seeds from each of the 69 lines were grown on soil and at the two- to four-leaf stage plants were treated with a paclobutrazol solution (see Methods). After 4 weeks of treatment, most of the mutants, including all of those with a wilty phenotype, exhibited an extreme dwarf growth habit. However, plants from two lines exhibited stature and leaf size that were intermediate between that of paclobutrazol-treated wild-type and control wild-type plants. For reasons described below, these mutants were designated spindly-1 (spy-1) and spy-2. An additional screen was performed with an independent lot of EMS-mutagenized M$_2$ seed in which the order of the screening steps was reversed. This screen identified one additional mutant (spy-3) that exhibited paclobutrazol-resistant germination and vegetative growth.

**spy Mutations Are Recessive and Allelic**

Table 1 shows that in the presence of paclobutrazol, the germination frequency of seeds resulting from self-pollination of spy/spy plants was between 61 and 86%, and the germination frequency of seeds from SPY/spy plants was between 11 and 22%. These frequencies are most consistent with the hypothesis that, with respect to germination in the presence of paclobutrazol, 27 lines exhibited a wilty phenotype that is characteristic of ABA-deficient (aba) and some ABA-insensitive (abi) mutants (Koornneef et al., 1982, 1984).

The second step of our screen was to examine the lines that germinated in the presence of paclobutrazol to identify mutants that were also resistant to the dwarfing effects of paclobutrazol. Seeds from each of the 69 lines were grown on soil and at the two- to four-leaf stage plants were treated with a paclobutrazol solution (see Methods). After 4 weeks of treatment, most of the mutants, including all of those with a wilty phenotype, exhibited an extreme dwarf growth habit. However, plants from two lines exhibited stature and leaf size that were intermediate between that of paclobutrazol-treated wild-type and control wild-type plants. For reasons described below, these mutants were designated spindly-1 (spy-1) and spy-2. An additional screen was performed with an independent lot of EMS-mutagenized M$_2$ seed in which the order of the screening steps was reversed. This screen identified one additional mutant (spy-3) that exhibited paclobutrazol-resistant germination and vegetative growth.

**Table 1. Germination Frequency of spy Mutants in the Presence of Paclobutrazol**

<table>
<thead>
<tr>
<th>Genotype of Parent(s)</th>
<th>Seeds Tested</th>
<th>Treatment</th>
<th>Percent Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (SPY/SPY)</td>
<td>Self</td>
<td>Pac$^b$</td>
<td>0 (162$^c$)</td>
</tr>
<tr>
<td>Wild type$^a$</td>
<td>Self</td>
<td>Pac + GA$_3$</td>
<td>96 (137)</td>
</tr>
<tr>
<td>spy-1/spy-1$^b$</td>
<td>Self</td>
<td>Pac</td>
<td>86 (165)</td>
</tr>
<tr>
<td>spy-2/spy-2$^b$</td>
<td>Self</td>
<td>Pac</td>
<td>62 (29)</td>
</tr>
<tr>
<td>spy-3/spy-3$^b$</td>
<td>Self</td>
<td>Pac</td>
<td>61 (82)</td>
</tr>
<tr>
<td>Spy1/spy-1$^c$</td>
<td>Self</td>
<td>Pac</td>
<td>22 (95)</td>
</tr>
<tr>
<td>SPY/spy-2$^c$</td>
<td>Self</td>
<td>Pac</td>
<td>15 (31)</td>
</tr>
<tr>
<td>SPY/spy-3$^c$</td>
<td>Self</td>
<td>Pac</td>
<td>11 (115)</td>
</tr>
<tr>
<td>Spy1/spy-1 x SPY/spy-2</td>
<td>F$_1$</td>
<td>Pac</td>
<td>9 (53)</td>
</tr>
<tr>
<td>SPY/spy-1 x SPY/spy-3</td>
<td>F$_1$</td>
<td>Pac</td>
<td>7 (63)</td>
</tr>
<tr>
<td>SPY/spy-2 x SPY/spy-3</td>
<td>F$_1$</td>
<td>Pac</td>
<td>13 (131)</td>
</tr>
</tbody>
</table>

$^a$ Seeds were sown on Petri plates on filter papers saturated with 35 mg/L paclobutrazol (Pac), as described in Methods. Seeds were incubated at 4°C for 3 days and then 23°C for 4 days before scoring for germination (radical protrusion).

$^b$ Number in parentheses indicates the number of seeds tested.

$^c$ Seeds were assayed for germination in the presence of 35 mg/L paclobutrazol plus 5 x 10$^{-5}$ M GA$_3$. 

...
paclorbutrazol, spy mutations are recessive and do not exhibit complete penetrance. With respect to other phenotypes, including increased stem elongation (see below), spy mutations are also recessive. In an F₂ population resulting from a backcross of spy-1, 19 of 79 plants exhibited an elongated phenotype (χ² (3:1) = 0.038; P > .5).

spy-1, spy-2, and spy-3 were tested for alleleism by intercrossing plants heterozygous for each mutation. Table 1 shows that the F₁ seed resulting from these crosses germinated in the presence of paclobutrazol at approximately the same frequency as that of seed resulting from self-pollination of SPLY spy plants. Plants grown from F₁ seeds that had germinated in the presence of paclobutrazol exhibited vegetative and reproductive phenotypes characteristic of spy/spy plants (data not shown). Thus, spy-1, spy-2, and spy-3 are alleles of a single locus, SPINDLY (SPY).

**spy-1 Is Partially Epistatic to ga1-2**

To confirm that spy mutations confer resistance to paclorbutrazol by suppressing the effects of GA deficiency rather than by inhibiting the action of paclorbutrazol, the double mutant spy-1 ga1-2 was constructed (see Methods) and analyzed phenotypically. ga1-2 plants have reduced endogenous GA levels due to chromosomal rearrangement within the GA1 gene (Sun et al., 1992). Table 2 shows that ga1-2 seeds required exogenous GA for germination (see also Koornneef and van der Veen, 1980) but that spy-1 ga1-2 double mutant seeds germinated in the absence of exogenous GA. Thus, spy-1 suppresses the germination phenotype of ga1-2. Table 2 also shows that seeds resulting from self-pollination of a plant homozygous for ga1-2 but heterozygous for spy-1 germinated at a frequency of 24% (χ² (3:1) = 0.023; P > .5), indicating that spy-1 is recessive with respect to its ability to suppress the germination phenotype of ga1-2. Interestingly, spy-1 ga1-2 seeds germinate at approximately the same frequency both in the presence or absence of paclorbutrazol (Table 2). This indicates that spy-1 can simultaneously suppress the effects of the ga1-2 mutation and paclorbutrazol, which act at different steps in the GA biosynthesis pathway (Barendse et al., 1986; Rademacher, 1989; Zeevaart and Talon, 1992).

The phenotype of spy-1 ga1-2 plants indicates that spy-1 partially suppresses all of the vegetative and reproductive phenotypes associated with GA deficiency. Figure 1A shows that whereas ga1-2 plants exhibited an extreme dwarf growth habit, very limited stem elongation, complete male sterility, and incomplete petal development (see also Koornneef and van der Veen, 1980), spy-1 ga1-2 plants developed an expanded rosette, displayed significant stem elongation, exhibited only partial male sterility, and exhibited normal petal development.

**spy Mutants Resemble Wild-Type Plants That Have Been Repeatedly Sprayed with GA₃**

Relative to wild-type plants, spy-1, spy-2, and spy-3 plants had longer hypocotyls, more erect rosette leaves, and a paler green color (Figure 1B; data not shown). Wild-type plants that were sprayed daily with 5 × 10⁻⁵ M GA₃ also exhibited these phenotypes (Figure 1C; data not shown).

Table 3 presents quantitation of several additional phenotypes exhibited by both spy mutants and wild-type plants that have been sprayed daily with GA₃. As measured either by the number of rosette leaves present at floral initiation or by the number of days required to develop a flowering stem, spy plants and GA₃-treated wild-type plants flowered earlier than control wild-type plants (Table 3; Figures 1B and 1C). The spy-1 plants shown in Figures 1A and 1B demonstrate that spy mutants have the ability to flower after producing only two rosette leaves. spy mutants also exhibited more extensive elongation of the main stem than did wild-type plants (Table 3; Figure 1A). Interestingly, the increased stem elongation exhibited by spy-1 was caused primarily by an increase in internode length, whereas the increase found in spy-2 and spy-3 was caused primarily by an increase in the number of nodes in the inflorescence (Table 3). This difference between spy alleles was observed in three independent experiments (data not shown). Because wild-type plants treated with GA₃ exhibited an increase in stem length that was caused only by an increase in the number of nodes (Table 3), it is unclear why spy-1 displayed increased internode elongation.

Figure 1D shows that siliques of spy plants developed parthenocarpically. When spy-2 and wild-type flowers were emasculated prior to anthesis, spy-2 siliques elongated to a greater extent than did wild-type siliques. Nine days after emasculation, the length of wild-type siliques was 2.3 ± 0.2 mm (mean ± se), whereas the length of spy-2 siliques was 3.8 ± 0.2 mm. Siliques of spy-1, spy-3, and GA₃-treated wild-type plants also developed parthenocarpically (data not shown). spy mutants exhibited partial to full male sterility. Anthers from spy plants shed less pollen than wild-type plants (data not shown). In addition, Table 4 shows that when spy plants were allowed to self-pollinate, they produced fewer seeds than wild-type plants. When pollinated with wild-type pollen,

**Table 2. Germination Frequency of ga1-2 and spy-1 ga1-2 Double Mutant Seed**

<table>
<thead>
<tr>
<th>Genotype of Parent</th>
<th>Treatment</th>
<th>Percent Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPLY/spy ga1-2ga1-2</td>
<td>Water</td>
<td>0 (60)</td>
</tr>
<tr>
<td>SPLY/spy ga1-2ga1-2</td>
<td>Water</td>
<td>24 (58)</td>
</tr>
<tr>
<td>spy-1/spy ga1-2ga1-2</td>
<td>Water</td>
<td>100 (58)</td>
</tr>
<tr>
<td>spy-1/spy ga1-2ga1-2</td>
<td>Pac</td>
<td>94 (31)</td>
</tr>
</tbody>
</table>

* Seeds resulting from self-pollination of the above genotypes were assayed for germination in sterile water (Water) or paclorbutrazol (Pac), as described in Table 1. Seed viability for all genotypes was tested by germinating seeds in the presence of 5 × 10⁻⁵ M GA₃ and was found to be between 94 and 100%.

* Number in parentheses indicates the number of seeds tested.
Figure 1. Phenotypes of spy Mutants and spy ga1 Double Mutants.

(A) Wild-type, spy-1, spy-1 ga1-2, and ga1-2 plants shown 4 weeks after germination. Col, wild-type Columbia; Ler, wild-type Landsberg erecta.

(B) spy-1 and wild-type Columbia (Col) plants photographed 18 days after germination.

(C) Wild-type Columbia plants sprayed daily with either GA$_3$ or water (H$_2$O) and photographed 18 days after germination.

(D) Siliques 9 days after emasculation of wild-type Columbia (Col) and spy-2 flowers.

(E) spy-1 ga1-2 plants sprayed daily with either GA$_3$ or water (H$_2$O) and photographed 12 days after germination.
Table 3. Phenotype of spy Mutants and of Wild-Type Plants Sprayed with GA3a

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Rosette Leaves</th>
<th>Number of Days from Germination to Bolting b</th>
<th>Final Length of Main Stem (cm)</th>
<th>Number of Nodes in Inflorescence c</th>
<th>Average Internode Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.1 ± 0.5 a</td>
<td>21.0 ± 0.7 a</td>
<td>29.2 ± 1.2 a</td>
<td>24.3 ± 1.6 a</td>
<td>1.2 ± 0.04 a,b</td>
</tr>
<tr>
<td>spy-1</td>
<td>4.0 ± 0.1 b</td>
<td>15.2 ± 0.5 b</td>
<td>40.4 ± 1.9 b</td>
<td>25.7 ± 1.9 a</td>
<td>1.6 ± 0.07 c</td>
</tr>
<tr>
<td>spy-2</td>
<td>7.9 ± 0.7 c</td>
<td>20.0 ± 0.7 a,c</td>
<td>36.4 ± 2.0 a,b</td>
<td>34.2 ± 2.5 b</td>
<td>1.1 ± 0.05 a</td>
</tr>
<tr>
<td>spy-3</td>
<td>7.0 ± 0.6 c</td>
<td>18.0 ± 0.9 c</td>
<td>39.5 ± 2.6 b</td>
<td>33.1 ± 3.7 b</td>
<td>1.3 ± 0.09 b</td>
</tr>
</tbody>
</table>

Wild-type plants treated with a

<table>
<thead>
<tr>
<th>Water</th>
<th>9.8 ± 0.5 a</th>
<th>22.8 ± 0.3 a</th>
<th>37.6 ± 0.2 a</th>
<th>30.8 ± 0.3 a</th>
<th>1.2 ± 0.01 a</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>6.2 ± 0.2 b</td>
<td>15.8 ± 0.4 b</td>
<td>49.2 ± 1.0 b</td>
<td>41.4 ± 1.9 b</td>
<td>1.2 ± 0.03 a</td>
</tr>
</tbody>
</table>

a Numbers are means of measurements of 7 to 10 plants followed by the se. Values that are followed by the same letter are not significantly different from each other at P = .05, as determined by analysis of variance and the Fisher protected least significant difference test.
b Bolting was defined as the day that the main stem had reached a height of 1 cm.
c The number of nodes in the inflorescence was determined by counting the number of cauline leaves and flowers arising from the main stem.
d Average internode length on the main stem was determined by dividing the final length of the main stem by the number of nodes in the inflorescence.

In a separate experiment, wild-type Arabidopsis ecotype Columbia seeds were germinated in either water or 5 x 10^-5 M GA3, and then plants were sprayed daily with water or GA3 until the main stem had ceased flowering.

Table 4. Effect of spy Mutations and GA3 Treatment on Plant Fertilitya

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Seeds/ Silique at 26°C</th>
<th>Number of Seeds/ Silique at 18°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>38.2</td>
<td>41.2</td>
</tr>
<tr>
<td>spy-1</td>
<td>0.23</td>
<td>22.0</td>
</tr>
<tr>
<td>spy-2</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>spy-3</td>
<td>2.6</td>
<td>39.0</td>
</tr>
</tbody>
</table>

Wild-type plants treated with b

<table>
<thead>
<tr>
<th>Water</th>
<th>38.6</th>
<th>ND c</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA3</td>
<td>15.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

a For each genotype, 50 to 100 siliques were analyzed, except spy-2 in which 590 siliques were analyzed at 26°C and 706 siliques were analyzed at 18°C. Seed number was either counted directly or estimated by seed weight. Each value represents the total number of seeds divided by the total number of siliques analyzed.
b In a separate experiment, wild-type Arabidopsis ecotype Columbia seeds were germinated in either water or 5 x 10^-5 M GA3, and then plants were sprayed daily with water or GA3 until the main stem had ceased flowering.
c ND, not determined.
seedlings exhibited defects in cotyledon number and phyllotaxy (data not shown).

It is unclear why spy-2 displays these unique floral and seedling phenotypes, because in all other respects spy-2 exhibits the same phenotypes as spy-1 but to a lesser degree. One possible explanation is that the extreme sterility and the ovary and seed development phenotypes are caused by a second mutation in the spy-2 background. However, all of these phenotypes cosegregated with paclobutrazol resistance in an F2 population (21 plants) resulting from backcrossing spy-2 to wild type. Therefore, if a second mutation is present, it is most likely linked to spy-2. A second possibility is that spy-2 is a gain-of-function mutation that affects reproductive processes in addition to being a loss-of-function mutation that reduces the normal function of the SPY gene product. This second possibility is supported by the observation that the F1 plants from the cross spy-1/spy-1 × spy-2/spy-2 exhibited phenotypes that were most similar to those of spy-2, including extreme sterility and extra carpel development. Further analysis is needed to clarify the genetic basis of these extra phenotypes of spy-2.

The long hypocotyl phenotype of spy mutants prompted us to compare the phenotypes of spy mutants with those of the long hypocotyl (hy) mutants of Arabidopsis, which are affected in photomorphogenesis. hy mutants exhibit some of the phenotypes observed in spy mutants, including early flowering, elongated hypocotyls, yellow-green leaves, and elongated stems (Koombe et al., 1980; Kendrick and Nagatani, 1991; Reed et al., 1993). In fact, hy3 and hy6 exhibited some of these phenotypes to a greater extent than did spy-3 (Table 5; data not shown). However, hy1, hy2, hy3, hy4, hy5, and hy6 are clearly distinguishable from spy mutants because they did not exhibit male sterility and did not germinate in the presence of paclobutrazol (data not shown).

**Response of spy Mutants to Exogenous GA**

Table 5 shows that the number of rosette leaves and the stem length of spy-1 ga1-2 plants was intermediate between that of

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Rosette Leaves</th>
<th>Final Length of Main Stem (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Columbia</td>
<td>12.2 ± 0.5 a</td>
<td>30.7 ± 2.4 a,b</td>
</tr>
<tr>
<td>spy-1</td>
<td>4.6 ± 0.2 b</td>
<td>39.7 ± 2.8 c</td>
</tr>
<tr>
<td>spy-3</td>
<td>8.4 ± 0.8 c</td>
<td>39.6 ± 3.6 c</td>
</tr>
<tr>
<td>spy-1 ga1-2</td>
<td>5.2 ± 0.2 b</td>
<td>17.7 ± 1.2 d</td>
</tr>
<tr>
<td>ga1-2</td>
<td>10.8 ± 0.4 d</td>
<td>0.6 ± 0.1 e</td>
</tr>
<tr>
<td>Wild-type Ler a</td>
<td>8.8 ± 0.3 c</td>
<td>20.8 ± 1.1 d</td>
</tr>
<tr>
<td>hy3</td>
<td>5.4 ± 0.3 b</td>
<td>32.7 ± 1.5 a</td>
</tr>
<tr>
<td>hy6</td>
<td>7.1 ± 0.6 e</td>
<td>23.5 ± 1.5 b,d</td>
</tr>
</tbody>
</table>

* Wild-type A. thaliana ecotype Landsberg erecta. All other symbols are defined in Table 3.

**DISCUSSION**

Except for the unique floral and seedling development phenotypes of the spy-2 mutant, all of the spy phenotypes can be reproduced by repeatedly treating wild-type plants with GA3. spy mutations could cause this "GA overdose" phenotype by at least three mechanisms: increasing the plant's ability to synthesize GA, reducing the plant's ability to catabolize GA, or constitutively activating GA perception or GA signal transduction.

It is unlikely that spy mutants act by increasing the plant's ability to synthesize GA because spy mutations suppress the effects of GA deficiency whether caused by paclobutrazol or

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**Table 5. Comparison of the Phenotypes of spy-1 Plants with the Phenotypes of spy-1 ga1-2 and hy Plants**

**Table 6. Response of the spy-1 ga1-2 Double Mutant to GA3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Rosette Leaves</th>
<th>Final Length of Main Stem (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>6.3 ± 0.2 a</td>
<td>16.3 ± 1.3 a</td>
</tr>
<tr>
<td>GA3</td>
<td>2.4 ± 0.2 b</td>
<td>37.7 ± 1.3 b</td>
</tr>
</tbody>
</table>

* spy-1 ga1-2 seeds were germinated in either water or 5 × 10⁻⁵ M GA3, and plants were sprayed daily with water or GA3. Symbols are defined in Table 3.
by the *gat-2* mutation. Paclobutrazol and *gat* act on different steps in the GA biosynthesis pathway. Paclobutrazol inhibits the monooxygenases involved in the oxidation of *ent*-kaurenoic acid (Rademacher, 1989), whereas *gat* blocks GA biosynthesis before the formation of *ent*-kaurene (Barendse et al., 1986; Zeevaart and Talon, 1992). Furthermore, a mutant that increased GA biosynthesis would be expected to have longer hypocotyls than the wild type in the absence of exogenous GA, but, at saturating concentrations of applied GA, it would be expected to exhibit the same hypocotyl length as the wild type. However, the GA3 dose–response curves for hypocotyl elongation shown in Figure 2 demonstrate that at saturating concentrations of GA3, *spy-1 gat-2* hypocotyls are longer than *gat-2* hypocotyls.

It is also unlikely that *spy* acts by decreasing GA catabolism because the *spy-1* mutation can simultaneously suppress the effects of paclobutrazol and the *gat-2* mutation. If *spy-1 gat-2* seeds germinate because of reduced breakdown of any endogenous GA in the *gat* background, one would expect that paclobutrazol treatment would further reduce GA biosynthesis and therefore reduce the frequency of germination of *spy-1 gat-2* seeds. As indicated in Table 2, however, *spy-1 gat-2* seeds germinate at approximately the same frequency in the presence or absence of paclobutrazol. Furthermore, the GA3 dose–response curves (Figure 2) strongly suggest that *spy* mutations do not act through changes in GA catabolism. If GA3 is perceived at the external face of the plasma membrane, as suggested by Hooley et al. (1991), then a catabolism mutant would be expected to exhibit the same maximum hypocotyl length at saturating GA3 concentrations as the wild type. However, as mentioned above, Figure 2 shows that at saturation, *spy-1 gat-2* hypocotyls are longer than *gat-2* hypocotyls. Alternatively, if GA3 is passively taken into plant cells (as indicated by the work of O'Neill et al., 1986) and GA3 is active inside the cell or is converted to an active form inside the cell, then a mutant that displayed reduced GA catabolism would be expected to be more sensitive to exogenous GA treatment because it would accumulate more intracellular GA at any given extracellular GA concentration than the wild type. In this case, one would expect *spy* hypocotyls to be sensitive to a lower concentration of applied GA3 than wild-type hypocotyls and *spy* hypocotyls to become saturated for GA response at a lower concentration of applied GA3 than wild-type hypocotyls (Finn, 1986). However, Figure 2 shows that with regard to these aspects of GA sensitivity, *gat-2* plants and *spy-1 gat-2* plants are quite similar. Therefore, *spy* mutations do not seem to act through changes in GA catabolism.

The GA3 dose–response curves shown in Figure 2 also suggest that *spy* mutations do not significantly affect the plant's ability to respond to GA. Finn (1986) describes the characteristics of hormone dose–response curves that would indicate that a particular mutation changed the plant's "sensitivity" to a hormone. By the criteria described by Finn (1986), *spy* mutations do not affect GA receptivity (the concentration of GA receptors in a cell), the affinity of a GA receptor for GA, or the plant's capacity to respond to GA. Even though *spy-1 gat-2*

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**Figure 2.** Response of *gat-2* and *spy-1 gat-2* Seedlings to GA3.

(A) Hypocotyl length of seedlings incubated in concentrations of GA3 ranging from $10^{-10}$ to $3 \times 10^{-3}$ M or incubated without GA3. No *gat-2* seeds germinated in the $10^{-8}$, $10^{-9}$, $3 \times 10^{-4}$, and no GA3 treatments. Dotted squares, *spy-1 gat-2*; closed diamonds, *gat-2*. Error bars illustrate the SE of each mean.

(B) Linear regression analysis of the correlation between hypocotyl length and GA3 concentration for the range $10^{-9}$ to $3 \times 10^{-9}$ M GA3. $r^2$ = the coefficient of determination.
hypocotyls are longer than ga1-2 hypocotyls at any given GA3 concentration, the response of these two genotypes to GA3 is nearly identical. Thus, our working hypothesis is that spy mutations increase the level of GA signal transduction without significantly altering GA responsiveness. Because spy mutations suppress all of the phenotypes associated with GA deficiency, spy must act at an early portion of the GA signal transduction pathway that is common to all GA responses. The recessive nature of the three known spy alleles also suggests that the function of the wild-type SPY gene product is to negatively regulate the flux of signal through the pathway.

The observation that spy-1 ga1-2 seeds germinate even in the presence of paclobutrazol suggests that spy-1 affects GA signal transduction in a GA-independent manner. Furthermore, throughout the range of GA concentrations used in the GA3 dose-response experiments shown in Figure 2, spy-1 ga1-2 hypocotyls were longer than ga1-2 hypocotyls to approximately the same extent, indicating that the spy-1 mutation and applied GA interact in an additive manner. At least two models can be proposed to explain these properties of spy mutants. First, as shown in Figure 3A, the GA signal transduction pathway could be functionally redundant and SPY could regulate only a portion of the pathway. spy mutations would constitutively activate one branch of the pathway, while GA perception and signal transduction from the other branch of the pathway would still function normally. This would explain why spy mutants still exhibit a significant response to applied GA. Redundancy of a component of the GA signal transduction pathway is suggested by the la and crya mutations in pea, which are both required to cause the GA-insensitive slender phenotype (Reid et al., 1983). Another possible example of redundancy in the GA signal transduction pathway is the GA-insensitive (GAI) gene of Arabidopsis, which appears to be dispensable, because apparent loss-of-function mutations at this locus have a phenotype indistinguishable from wild type (Peng and Harberd, 1993). This may suggest that other genes can substitute for the function of the GAI gene (Peng and Harberd, 1993).

A second model that is consistent with the properties of spy mutants is that the primary function of the SPY gene product is to negatively regulate cross-talk from a non-GA–regulated signal transduction pathway to the GA signal transduction pathway (Figure 3B). Because all GA responses are affected in spy mutants, this model would predict that signal from the non-GA pathway feeds into a very early portion of the GA signal transduction pathway.

Slender mutants including the crya la double mutant of pea, the sin mutant of barley, the pro mutant of tomato, and the spy mutants of Arabidopsis are similar in that they can be phenocopied by treating wild-type plants with GA. However, with respect to GA responsiveness, slender mutants can be divided into a GA-responsive class, consisting of the spy and pro mutants that respond to both applied GA and GA biosynthesis inhibitors (Jones, 1987; Hedden and Lenton, 1988), and a GA-unresponsive class, which includes the crya la pea and sin barley mutants whose phenotypes are unaffected by changing GA levels (Potts et al., 1985; Lanahan and Ho, 1988; Croker et al., 1990). One possible interpretation of these two classes of slender phenotypes is that they are all caused by recessive mutation of genes encoding the same component of the GA signal transduction pathway but that the GA-responsive mutants are weaker alleles than the GA-unresponsive mutants (Jupe et al., 1988). However, the observation that the spy-1 mutation does not have a significant effect on the response to GA3 (Figure 2) suggests that spy mutants are affected in a different component of the GA signal transduction pathway than are the GA-unresponsive mutants. Thus, future research may uncover mutations at additional SPY genes in Arabidopsis that are more similar to the GA-unresponsive slender mutations in pea and barley.

**METHODS**

**Mutant Stocks**

Seeds of the long hypocotyl (hy1, hy2, hy3, hy4, and hy5), and gibberellic (GA)-deficient (ga1-2) mutants of Arabidopsis thaliana (ecotype Landsberg erecta) and the hy6 mutant (in the ecotype Columbia) were obtained from J. Chory (Salk Institute, La Jolla, CA). All hy alleles are
those described by Chory et al. (1989). The gal-2 plants described in Table 5 were obtained by imbibing gal-2 seeds in water for 24 hr at 23°C, removing the seed coats, incubating the embryos in sterile water under fluorescent lights for 48 hr, and then transferring the green seedlings to soil. Unless otherwise noted, plants were grown in potting soil (Gardener's Supply Company, Burlington, VT) in a growth chamber at 26°C and a 20-hr photoperiod (75 μE m⁻² sec⁻¹).

**Mutagenesis and Screening**

Arabidopsis ecotype Columbia seeds (20,000) were imbibed in 0.3% ethylmethane sulfonate (EMS) for 16 hr. The mutagenized seed were then sown on soil, and M₁ plants were allowed to self-pollinate and male sterility were pollinated with wild-type pollen, and the resulting F₂ plants were allowed to self-pollinate and M₂ plants were allowed to self-pollinate and M₂ seeds were then retested for germination on paclobutrazol. M₂ plants exhibiting male sterility were pollinated with wild-type pollen, and the resulting F₂ plants were then retested for germination on paclobutrazol. This screen identified 69 lines that exhibited significant germination on paclobutrazol. To screen for mutants that exhibited resistance to the dwarfing effects of paclobutrazol, plants were grown on soil, and at the two- to four-leaf stage, plants were sprayed to run off and the soil was drenched with 35 mg/L paclobutrazol. At weekly intervals, plants were retreated to synchronize seed germination. Hypocotyl length was determined 6 days after transferring plates to a growth chamber at 26°C.

**Dose-Response Experiments**

Seeds were surface sterilized as described by Parks and Quail (1993) and sown in Petri plates containing two filter paper discs saturated with a solution of 0.5 × Murashige and Skoog (MS) salts (Sigma), pH 5.8, and various concentrations of GA₃ (diluted from a GA₃ stock in 1M KOH). Plates were placed at 4°C for 3 days to break dormancy and synchronize seed germination. Hypocotyl length was determined 6 days after transferring plates to a growth chamber at 26°C.

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