The Plant Cell, Vol. 5, 887–896, August 1993

Mutations at the *SPINDLY* Locus of Arabidopsis Alter Gibberellin Signal Transduction

Steven E. Jacobsen and Neil E. Olszewski[1]

Department of Plant Biology and Plant Molecular Genetics Institute, University of Minnesota, St. Paul, Minnesota 55108

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 A_3 (GA_3). 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 GA_3 dose–response experiments suggest that GA_3 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; Pharis 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 semidominant mutations result in reduced sensitivity to GA and produce a phenotype similar to that of GA-deficient mutants. Mutants of this type include the *gai* 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). *cry*^{s} *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 A_3 (GA_3) (Dalton and Murfet, 1975; Potts et al., 1985). *cry*^{s} *la* plants appear to be insensitive to changes in endogenous GA levels, because *cry*^{s} *la na* triple mutant plants, which have reduced endogenous GA due to the *na* mutation, exhibit a phenotype that is indistinguishable from that of *cry*^{s} *la* plants (Potts et al., 1985). Similarly, *cry*^{s} *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* (*sln*), 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 *cry*^{s} *la* pea plants, *sln* barley plants

[1] To whom correspondence should be addressed at: Department of Plant Biology, 220 Bioscience Center, 1445 Gortner Ave., University of Minnesota, St. Paul, MN 55108.

appear to be insensitive to GA, because their phenotype is unaffected 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 *sln* aleurone cells but GA dependent in wild-type aleurone cells (Chandler, 1988; 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 ethylmethane 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).

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

Genotype of Parent(s)	Seeds Tested	Treatment	Percent Germination
Wild type (*SPY/SPY*)[a]	Self	Pac[b]	0 (162)[c]
Wild type[a]	Self	Pac + GA_3[d]	96 (137)
spy-1/spy-1[a]	Self	Pac	86 (185)
spy-2/spy-2[a]	Self	Pac	62 (29)
spy-3/spy-3[a]	Self	Pac	61 (82)
SPY/spy-1[a]	Self	Pac	22 (95)
SPY/spy-2[a]	Self	Pac	15 (31)
SPY/spy-3[a]	Self	Pac	11 (115)
SPY/spy-1 × *SPY/spy-2*	F_1	Pac	9 (53)
SPY/spy-1 × *SPY/spy-3*	F_1	Pac	7 (83)
SPY/spy-2 × *SPY/spy-3*	F_1	Pac	13 (131)

[a] Seed viability was tested by germinating seeds in sterile water and was found to be between 96 and 100%.
[b] Seeds were sown in 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).
[c] Number in parentheses indicates the number of seeds tested.
[d] Seeds were assayed for germination in the presence of 35 mg/L paclobutrazol plus 5×10^{-5} M GA_3.

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, *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 F_2 population resulting from a backcross of *spy-1*, 19 of 79 plants exhibited an elongated phenotype [χ^2 (3:1) = 0.038; $P > .5$].

spy-1, *spy-2*, and *spy-3* were tested for allelism by intercrossing plants heterozygous for each mutation. Table 1 shows that the F_1 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 *SPY/spy* plants. Plants grown from F_1 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 paclobutrazol by suppressing the effects of GA deficiency rather than by inhibiting the action of paclobutrazol, 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% [χ^2 (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 paclobutrazol (Table 2). This indicates that *spy-1* can simultaneously suppress the effects of the *ga1-2* mutation and paclobutrazol, 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.

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

Genotype of Parent	Treatment[a]	Percent Germination
SPY/SPY ga1-2/ga1-2	Water	0 (60)[b]
SPY/spy-1 ga1-2/ga1-2	Water	24 (58)
spy-1/spy-1 ga1-2/ga1-2	Water	100 (58)
spy-1/spy-1 ga1-2/ga1-2	Pac	94 (31)

[a] Seeds resulting from self-pollination of the above genotypes were assayed for germination in sterile water (Water) or paclobutrazol (Pac), as described in Table 1. Seed viability for all genotypes was tested by germinating seeds in the presence of 5×10^{-5} M GA_3 and was found to be between 94 and 100%.

[b] Number in parentheses indicates the number of seeds tested.

spy Mutants Resemble Wild-Type Plants That Have Been Repeatedly Sprayed with GA_3

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 GA_3 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 GA_3. 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 GA_3-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, while 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 GA_3 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 GA_3-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,

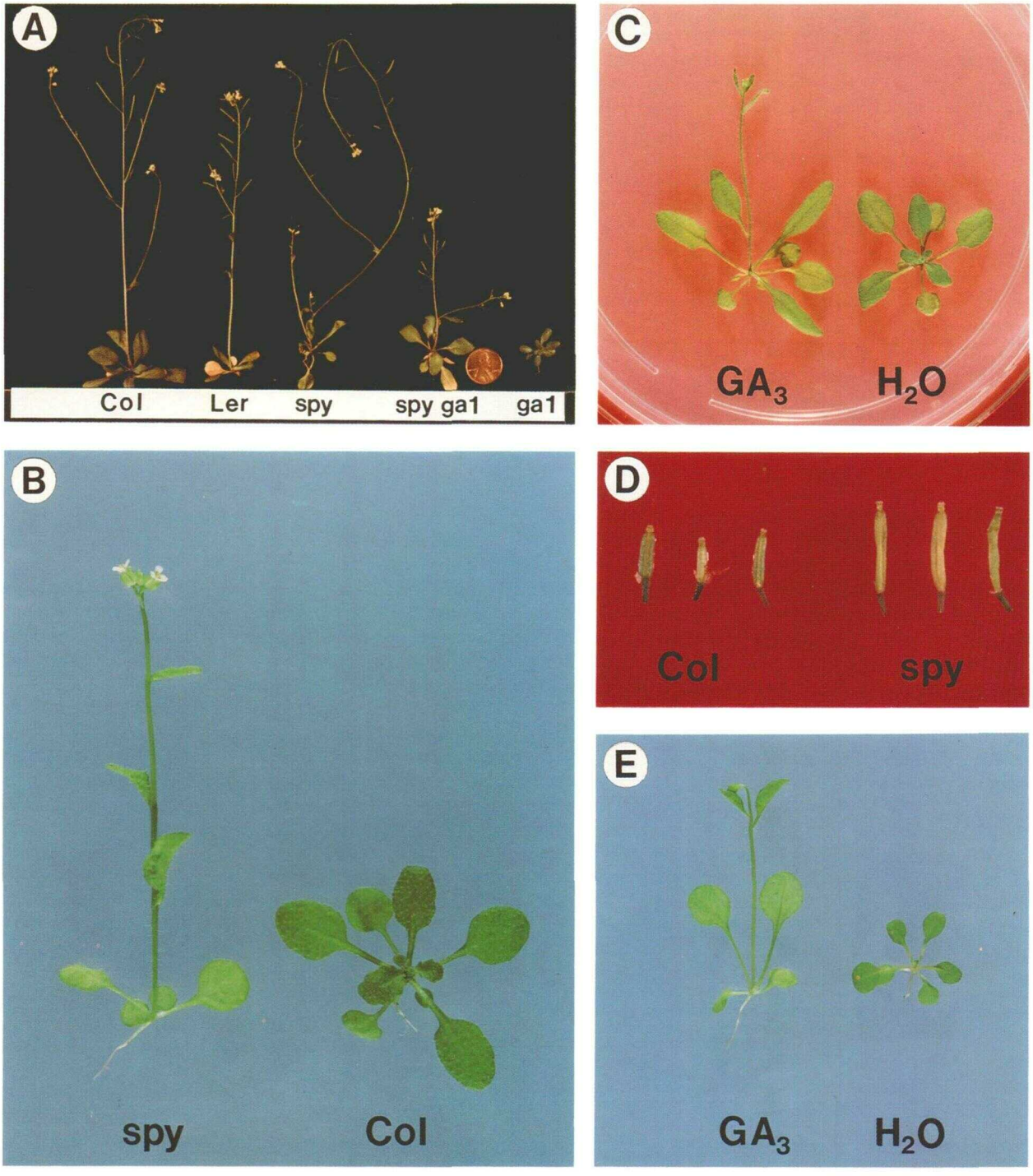

Figure 1. Phenotypes of *spy* Mutants and *spy ga1* Double Mutants.

(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_2O) 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_2O) and photographed 12 days after germination.

Table 3. Phenotype of *spy* Mutants and of Wild-Type Plants Sprayed with GA_3[a]

Genotype	Number of Rosette Leaves	Number of Days from Germination to Bolting[b]	Final Length of Main Stem (cm)	Number of Nodes in Inflorescence[c]	Average Internode Length (cm)[d]
Wild type	10.1 ± 0.5 a	21.0 ± 0.7 a	29.2 ± 1.2 a	24.3 ± 1.6 a	1.2 ± 0.04 a,b
spy-1	4.0 ± 0.1 b	15.2 ± 0.5 b	40.4 ± 1.9 b	25.7 ± 1.9 a	1.6 ± 0.07 c
spy-2	7.9 ± 0.7 c	20.0 ± 0.7 a,c	36.4 ± 2.0 a,b	34.2 ± 2.5 b	1.1 ± 0.05 a
spy-3	7.0 ± 0.6 c	18.0 ± 0.9 c	39.5 ± 2.6 b	33.1 ± 3.7 b	1.3 ± 0.09 b
Wild-type plants treated with[e]					
Water	9.8 ± 0.5 a	22.8 ± 0.3 a	37.6 ± 0.2 a	30.8 ± 0.3 a	1.2 ± 0.01 a
GA	6.2 ± 0.2 b	15.8 ± 0.4 b	49.2 ± 1.0 b	41.4 ± 1.9 b	1.2 ± 0.03 a

[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.
[e] In a separate experiment, wild-type Arabidopsis ecotype Columbia seeds were germinated in either water or 5×10^{-5} M GA_3, and then plants were sprayed daily with water or GA_3 until the main stem had ceased flowering.

however, *spy* mutants produced relatively normal amounts of seed, indicating that they were female fertile (data not shown). Wild-type plants that were sprayed with GA_3 also exhibited reduced fertility (Table 4).

The severity of the male sterility phenotype of *spy* mutants was greatly influenced by the temperature at which the plants were grown. Table 4 shows that the male sterility of *spy-1* and *spy-3* was greatly reduced when plants were grown at 18°C. Furthermore, *spy-2* plants, which were completely sterile when grown at 26°C, were only partially sterile when grown at 18°C. However, relative to wild type, *spy* mutations influenced flowering time and stem length to approximately the same extent whether the plants were grown at 26°C or 18°C (data not shown). In addition, seed from all three *spy* mutants germinated in the presence of paclobutrazol at approximately the same frequency at both 26 and 18°C (data not shown). Thus, it appears that the *spy-1*, *spy-2*, and *spy-3* mutations are not temperature sensitive but that temperature can modulate the severity of the sterility phenotype of all three *spy* mutants.

The three *spy* mutants differ in the extent to which they express the various phenotypes. In general, *spy-1* is the most extreme allele because it showed the most dramatic phenotype with respect to germination in the presence of paclobutrazol, hypocotyl length, leaf erectness, leaf color, time to flowering, and parthenocarpy. In fact, *spy-1* has the additional phenotype that early stem elongation is so rapid that flower buds occasionally remained wrapped in the yet unfolded cauline leaves, resulting in bending of the main stem (Figure 1E shows an example in a *spy-1 ga1-2* plant). In contrast, *spy-2* exhibited the most dramatic male sterility (Table 4). Thus far, we have not recovered seed resulting from self-pollination of *spy-2* plants grown at temperatures higher than or equal to 25°C (Table 4; data not shown). In addition, *spy-2* has unique phenotypes relating to ovary and seed development. *spy-2* ovaries often developed three and occasionally four carpels (data not shown). Within some of these multicarpelate ovaries were structures that appeared to develop from unfertilized ovules and that may have been additional carpels because they often had stigmatic papillae on their surfaces (data not shown). Finally, *spy-2*

Table 4. Effect of *spy* Mutations and GA_3 Treatment on Plant Fertility[a]

Genotype	Number of Seeds/ Silique at 26°C	Number of Seeds/ Silique at 18°C
Wild type	38.2	41.2
spy-1	0.23	22.0
spy-2	0	0.08
spy-3	2.6	39
Wild-type plants treated with[b]		
Water	38.6	ND[c]
GA_3	15.4	ND

[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 5×10^{-5} M GA_3, and then plants were sprayed daily with water or GA_3 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 F_2 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 F_1 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 (Koornneef 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 *spy-1* plants and that of *ga1-2* plants (see also Figure 1A). This intermediate phenotype could be attributable to genetic background effects (because *spy-1* is in the Columbia background, whereas *ga1-2* is in the Landsberg *erecta* background) or could suggest that *spy* mutants are still responsive to changes in endogenous GA levels.

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

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

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

Table 6. Response of the *spy-1 ga1-2* Double Mutant to GA_3[a]

Treatment	Number of Rosette Leaves	Final Length of Main Stem (cm)
Water	6.3 ± 0.2 a	16.3 ± 1.3 a
GA_3	2.4 ± 0.2 b	37.7 ± 1.3 b

[a] *spy-1 ga1-2* seeds were germinated in either water or 5×10^{-5} M GA_3, and plants were sprayed daily with water or GA_3. Symbols are defined in Table 3.

To test whether *spy-1* plants respond to exogenous GA treatment, *spy-1 ga1-2* double mutant plants were sprayed daily with either water or 5×10^{-5} M GA_3. Table 6 shows that GA treatment decreased the number of rosette leaves and increased the stem length of *spy-1 ga1-2* plants (see also Figure 1E). Thus, the *spy-1* mutation did not eliminate the plant's ability to respond to applied GA.

We have further investigated the influence of *spy-1* on GA response by examining the effect of GA_3 concentration on the elongation of *spy-1 ga1-2* and *ga1-2* hypocotyls. These GA_3 dose–response experiments, shown in Figure 2A, demonstrate that at any given GA_3 concentration *spy-1 ga1-2* hypocotyls were longer than *ga1-2* hypocotyls. However, the shape of the GA_3 dose–response curves for both *spy-1 ga1-2* and *ga1-2* hypocotyls was quite similar. Both genotypes began to exhibit a significant increase in hypocotyl elongation at a concentration of 10^{-7} M GA_3, and both genotypes exhibited saturation of GA_3 response at $\sim 3 \times 10^{-5}$ M GA_3. In addition, the slopes of the linear portion of the GA_3 response curves (concentrations from 10^{-7} to 3×10^{-5} M GA_3) were very similar (Figure 2B). These results suggest that, with respect to promoting hypocotyl elongation, *spy-1* exhibits a simple additive interaction with GA_3 treatment.

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 GA_3. *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

by the *ga1-2* mutation. Paclobutrazol and *ga1* act on different steps in the GA biosynthesis pathway. Paclobutrazol inhibits the monooxygenases involved in the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher, 1989), whereas *ga1* 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 GA_3 dose–response curves for hypocotyl elongation shown in Figure 2 demonstrate that at saturating concentrations of GA_3, *spy-1 ga1-2* hypocotyls are longer than *ga1-2* 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 *ga1-2* mutation. If *spy-1 ga1-2* seeds germinate because of reduced breakdown of any endogenous GA in the *ga1* background, one would expect that paclobutrazol treatment would further reduce GA biosynthesis and therefore reduce the frequency of germination of *spy-1 ga1-2* seeds. As indicated in Table 2, however, *spy-1 ga1-2* seeds germinate at approximately the same frequency in the presence or absence of paclobutrazol. Furthermore, the GA_3 dose–response curves (Figure 2) strongly suggest that *spy* mutations do not act through changes in GA catabolism. If GA_3 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 GA_3 concentrations as the wild type. However, as mentioned above, Figure 2 shows that at saturation, *spy-1 ga1-2* hypocotyls are longer than *ga1-2* hypocotyls. Alternatively, if GA_3 is passively taken into plant cells (as indicated by the work of O'Neill et al., 1986) and GA_3 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 GA_3 than wild-type hypocotyls and *spy* hypocotyls to become saturated for GA response at a lower concentration of applied GA_3 than wild-type hypocotyls (Firn, 1986). However, Figure 2 shows that with regard to these aspects of GA sensitivity, *ga1-2* plants and *spy-1 ga1-2* plants are quite similar. Therefore, *spy* mutations do not seem to act through changes in GA catabolism.

The GA_3 dose–response curves shown in Figure 2 also suggest that *spy* mutations do not significantly affect the plant's ability to respond to GA. Firn (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 Firn (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 ga1-2*

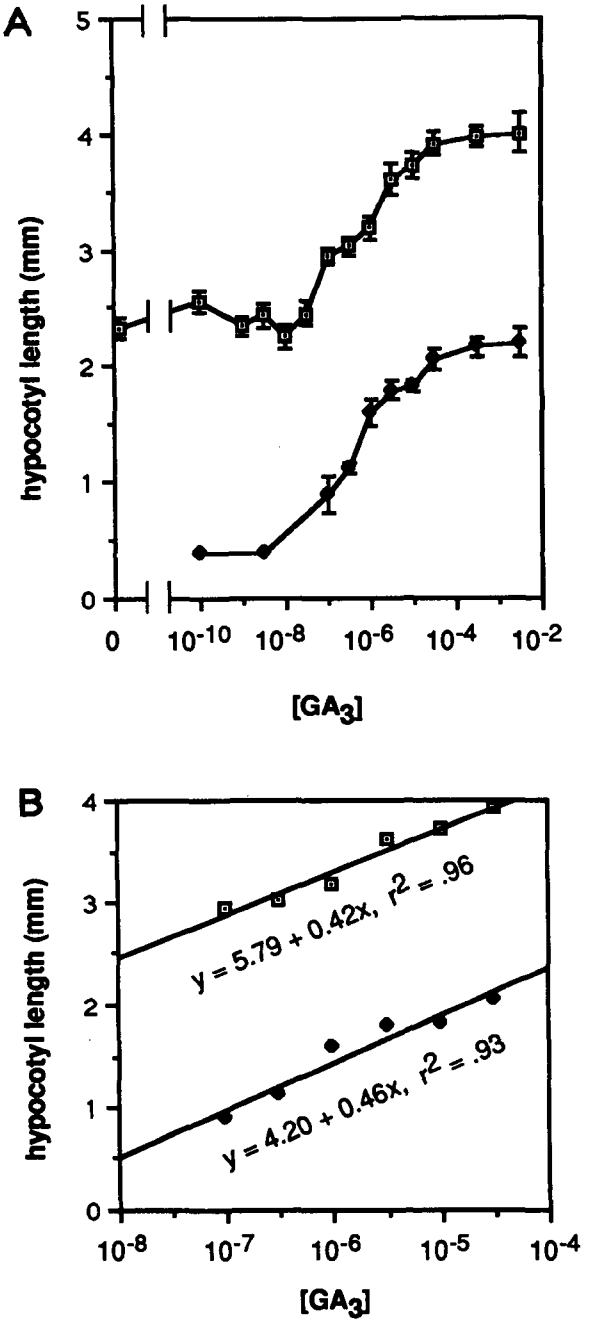

Figure 2. Response of *ga1-2* and *spy-1 ga1-2* Seedlings to GA_3.

(A) Hypocotyl length of seedlings incubated in concentrations of GA_3 ranging from 10^{-10} to 3×10^{-3} M or incubated without GA_3. No *ga1-2* seeds germinated in the 10^{-9}, 10^{-8}, 3×10^{-8}, and no GA_3 treatments. Dotted squares, *spy-1 ga1-2*; closed diamonds, *ga1-2*. Error bars illustrate the SE of each mean.

(B) Linear regression analysis of the correlation between hypocotyl length and GA_3 concentration for the range 10^{-7} to 3×10^{-5} M GA_3. r^2 = the coefficient of determination.

hypocotyls are longer than *ga1-2* hypocotyls at any given GA_3 concentration, the response of these two genotypes to GA_3 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 GA_3 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 *cry*^{s} 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).

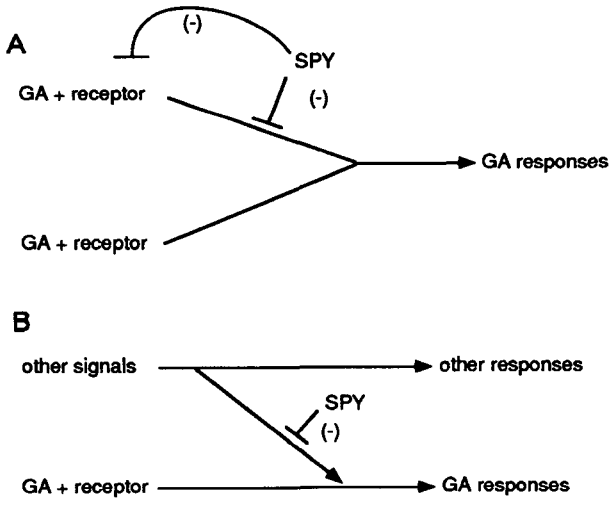

Figure 3. Alternative Models for the Action of the *SPY* Gene in GA Perception and Signal Transduction.

(A) A model for a GA perception and signal transduction pathway that is redundant, with SPY as a negative acting component of one of the duplicate pathways. The two lines originating from "SPY" indicate that the SPY gene product could act at GA perception or at GA signal transduction.

(B) A model in which SPY negatively regulates the cross-talk between a separate pathway that is not influenced by GAs and the GA perception and signal transduction pathway.

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 *cry*^{s} *la* double mutant of pea, the *sln* 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 *cry*^{s} *la* pea and *sln* 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 GA_3 (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 gibberellin (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 *ga1-2* plants described in Table 5 were obtained by imbibing *ga1-2* 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 $\mu E\ m^{-2}\ sec^{-1}$).

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 M_1 plants were allowed to self-pollinate and produce M_2 seeds at 18°C. M_2 seeds (440,000) were sown in Petri plates containing two filter paper discs (Grade 363; Baxter Healthcare Corporation, McGaw Park, IL) saturated with a solution of 35 mg/L (1.2×10^{-4} M) paclobutrazol (experimental plant growth regulator PP333; ICI Americas Inc., Goldsboro, NC) and 0.01% (v/v) Tween-20 (polyoxyethylene-sorbitan monolaurate). Petri dishes were incubated at 23°C under cool white fluorescent lights (35 $\mu E\ m^{-2}\ sec^{-1}$). Seedlings that germinated in the presence of paclobutrazol (571 total) were transferred to plates containing 5×10^{-5} M gibberellin A_3 (GA_3) (Sigma Chemical Co.), incubated at 23°C for 24 hr, and then transferred to soil. M_2 plants were allowed to self-pollinate, and M_3 seeds were retested for germination on paclobutrazol. M_2 plants exhibiting male sterility were pollinated with wild-type pollen, and the resulting F_2 seeds 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 with paclobutrazol in the same manner. After ~4 weeks, mutants that exhibited increased stature and leaf size relative to wild-type controls were identified. These screens identified three recessive mutations at the *SPINDLY* locus. These mutants, designated *spy-1*, *spy-2*, and *spy-3*, were backcrossed twice to wild-type Columbia, and homozygous *spy* plants were selected by germinating the F_2 seed resulting from these crosses in the presence of paclobutrazol.

Construction of the *spy-1 ga1-2* Double Mutant

SPY/spy-1 GA1/GA1 and *SPY/SPY ga1-2/ga1-2* plants were crossed, and an F_1 plant with the genotype *SPY/spy-1 GA1/ga1-2* was identified by the ability of 25% of its progeny to germinate in the presence of paclobutrazol. F_2 seeds from this plant were then imbibed in 5×10^{-5} M GA_3 for 24 hr and planted on soil. Plants homozygous for *ga1-2* were identified by their dwarf growth habit and lack of flowering. These *ga1-2/ga1-2* plants were then sprayed with 5×10^{-5} M GA_3, and the plants were allowed to self-pollinate. Seeds from a number of these *ga1-2/ga1-2* lines germinated on water (*ga1-2/ga1-2* seeds normally do not germinate without exogenous GA). A germinating seed from one such line was grown on soil and test crossed to both a *spy-1/spy-1 GA1/GA1* plant and a *SPY/SPY ga1-2/ga1-2* plant to confirm that its genotype was *spy-1/spy-1 ga1-2/ga1-2*. This line (referred to simply as the *spy-1 ga1-2* double mutant) was then used in all of the experiments described.

GA_3 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 GA_3 (diluted from a GA_3 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.

ACKNOWLEDGMENTS

We thank Pete Lefebvre and Gary Gardner for critically reviewing the manuscript. This work was supported by a grant to N.E.O. from the National Institutes of Health (No. GM40553) and by a predoctoral fellowship to S.E.J. from the National Institutes of Health (No. GM07323).

Received May 17, 1993; accepted June 25, 1993.

REFERENCES

Barendse, G.W.M., Kepczynski, J., Karssen, C.M., and Koornneef, M. (1986). The role of endogenous gibberellins during fruit and seed development: Studies on gibberellin-deficient genotypes of *Arabidopsis thaliana.* Physiol. Plant. **67,** 315–319.

Chandler, P.M. (1988). Hormonal regulation of gene expression in the "*slender*" mutant of barley (*Hordeum vulgare* L.). Planta **175,** 115–120.

Chory, J., Peto, C.A., Ashbaugh, M., Saganich, R., Pratt, L., and Ausubel, F. (1989). Different roles for phytochrome in etiolated and green plants deduced from characterization of *Arabidopsis thaliana* mutants. Plant Cell **1,** 867–880.

Croker, S.J., Hedden, P., Lenton J.R., and Stoddart, J.L. (1990). Comparison of gibberellins in normal and slender barley seedlings. Plant Physiol. **94,** 194–200.

Dalton, P.J., and Murfet, I.C. (1975). The effect of gibberellic acid and genotype *le la cry*^{c} on flowering in peas. *Pisum* Newslett. **7,** 5–7.

de Haan, H. (1927). Length factors in *Pisum.* Genetica **9,** 481–497.

de Haan, H. (1930). Contributions to the genetics of *Pisum.* Genetica **12,** 321–439.

Firn, R.D. (1986). Growth substance sensitivity: The need for clearer ideas, precise terms and purposeful experiments. Physiol. Plant. **67,** 267–272.

Foster, C.A. (1977). Slender: An accelerated extension growth mutant of barley. Barley Genet. Newslett. **7,** 24–27.

Fujioka, S., Hisakazu, Y., Spray, C.R., Katsumi, M., and Phinney, B.O. (1988). The dominant non-gibberellin-responding dwarf mutant (D8) of maize accumulates native gibberellins. Proc. Natl. Acad. Sci. USA **85,** 9031–9035.

Graebe J.E. (1987). Gibberellin biosynthesis and control. Annu. Rev. Plant Physiol. **38,** 419–465.

Harberd, N.P., and Freeling, M. (1989). Genetics of dominant gibberellin-insensitive dwarfism in maize. Genetics **121,** 827–838.

Hedden, P., and Lenton, J.R. (1988). Genetical and chemical approaches to the metabolic regulation and mode of action of gibberellins in plants. In Biomechanisms Regulating Growth and Development, Beltsville Symposia in Agricultural Research 12, G.L. Steffens and T.S. Rumsey, eds (Dordrecht: Kluwer Academic), pp. 175–204.

Hooley, R., Beale, M.H., and Smith S.J. (1991). Gibberellin perception at the plasma membrane of *Avena fatua* aleurone protoplasts. Planta **183,** 274–280.

Jones, M.G. (1987). Gibberellins and the *procera* mutant of tomato. Planta **172,** 280–284.

Jones R.L. (1973). Gibberellins: Their physiological role. Annu. Rev. Plant Physiol. **24,** 571–598.

Jupe, S.C., Causton, D.R., and Scott, I.M. (1988). Cellular basis of the effects of gibberellin and the *pro* gene on stem growth in tomato. Planta **174,** 106–111.

Karssen, C.M., Zagorski, S., Kepczynski, J., and Groot, S.P.C. (1989). Key role for endogenous gibberellins in the control of seed germination. Ann. Bot. **63,** 71–80.

Kendrick, R.E., and Nagatani, A. (1991). Phytochrome mutants. Plant J. **1,** 133–139.

Klee, H., and Estelle, M. (1991). Molecular genetic approaches to plant hormone biology. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 529–551.

Koornneef M., and van der Veen, J.H. (1980). Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. **58,** 257–263.

Koornneef, M., Rolff, E., and Spruit, C.J.P. (1980). Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L.) Heynh. Z. Pflanzenphysiol. **100,** 147–160.

Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D L.C., and Karssen, C.M. (1982). The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. **61,** 385–393.

Koornneef, M., Reuling, G., and Karssen, C.M. (1984). The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana.* Physiol. Plant. **61,** 377–383.

Koornneef, M., Elgersma, A., Hanhart, C.J., van Loenen-Martinet, E.P., Rijn L., and Zeevaart, J.A.D. (1985). A gibberellin insensitive mutant of *Arabidopsis thaliana.* Physiol. Plant. **65,** 33–39.

Koornneef, M., Karssen, C.M., Kendrick, R.E., and Zeevaart, J.A.D. (1993). Plant hormone and photomorphogenic mutants of tomato. In Molecular Biology of Tomato, J.I. Yoder, ed (Lancaster, PA: Technomic Publishing Co.), pp. 119–128.

Lanahan, M.B., and Ho, T.D. (1988). Slender barley: A constitutive gibberellin-response mutant. Planta **175,** 107–114.

Lenton, J.R., Hedden, P., and Gale, M.D. (1987). Gibberellin insensitivity and depletion in wheat—consequences for development. In Hormone Action in Development—A Critical Appraisal, G.V. Hoad, J.R. Lenton, M.B. Jackson, and R.K. Atkin, eds (London: Butterworths), pp. 145–160.

Nambara, E., Akazawa, T., and McCourt, P. (1991). Effects of the gibberellin biosynthetic inhibitor uniconazol on mutants of *Arabidopsis.* Plant Physiol. **97,** 736–738.

Nambara, E., Naito, S., and McCourt, P. (1992). A mutant of *Arabidopsis* which is defective in seed development and storage protein accumulation is a new *abi3* allele. Plant J. **2,** 435–441.

O'Neill S.D., Keith, B., and Rappaport, L. (1986). Transport of gibberellin A_1 in cowpea membrane vesicles. Plant Physiol. **80,** 812–817.

Parks, B.M., and Quail, P.H. (1993). *hy8,* a new class of Arabidopsis long hypocotyl mutants deficient in functional phytochrome A. Plant Cell **5,** 39–48.

Peng, J., and Harberd, N.P. (1993). Derivative alleles of the Arabidopsis gibberellin-insensitive (*gai*) mutation confer a wild-type phenotype. Plant Cell **5,** 351–360.

Pharis R.P., and King R.W. (1985). Gibberellins and reproductive development in seed plants. Annu. Rev. Plant. Physiol. **36,** 517–568.

Phinney, B.O. (1956). Growth response of single-gene dwarf mutants in maize to gibberellic acid. Proc. Natl. Acad. Sci. USA **42,** 185–189.

Potts, W.C., Reid, J.B., and Murfet, I.C. (1985). Internode length in *Pisum.* Gibberellins and the slender phenotype. Physiol. Plant. **63,** 357–364.

Rademacher, W. (1989). Gibberellins: Metabolic pathways and inhibitors of biosynthesis. In Target Sites of Herbicide Action. P. Boger and G. Sandman, eds (Boca Raton: CRC Press), pp. 128–140.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. Plant Cell **5,** 147–157.

Reid, J.B., Murfet, I.C., and Potts, W.C. (1983). Internode length in Pisum. II. Additional information on the relationship and action of the loci *Le, La, Cry, Na* and *Lm.* J. Exp. Bot. **34,** 349–364.

Scott, I.M. (1990). Plant hormone response mutants. Physiol. Plant. **78,** 147–152.

Stoddart, J.L. (1984). Growth and gibberellin-A_1 metabolism in normal and gibberellin-insensitive (*Rht3*) wheat (*Triticum aestivum* L.) seedlings. Planta **161,** 432–438.

Sun, T.-p., Goodman, H.M., and Ausubel, F.M. (1992). Cloning the Arabidopsis *GA1* locus by genomic subtraction. Plant Cell, **4,** 119–128.

Talon, M., Koornneef, M., and Zeevaart, J.A.D. (1990). Accumulation of C19-gibberellins in the gibberellin-insensitive dwarf mutant *gai* of *Arabidopsis thaliana* (L.) Heynh. Planta **182,** 501–505.

Zanewich, K.P., Rood, S.B., Southworth, C.E., and Williams, P.H. (1991). Dwarf mutants of *Brassica*: Responses to applied gibberellins and gibberellin content. J. Plant Growth Regul. **10,** 121–127.

Zeevaart, J.A.D., and Talon, M. (1992). Gibberellin mutants in *Arabidopsis thaliana.* In Progress in Plant Growth Regulation. C.M. Karssen, L.C. van Loon, and D. Vreugdenhil, eds (Dordrecht: Kluwer Academic), pp. 34–42.