Isolation of the GA-Response Mutant *sly1* as a Suppressor of *ABI1-1* in *Arabidopsis thaliana*

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ABSTRACT

Seed dormancy and germination in higher plants are partially controlled by the plant hormones abscisic acid (ABA) and gibberellic acid (GA). ABA establishes dormancy during embryo maturation, whereas GA breaks dormancy and induces germination. Previous attempts to identify GA response genes were confounded because GA mutants are not expected to germinate and, unlike GA auxotrophs, should fail to be rescued by exogenous GA. Here, we describe a screen for suppressors of the ABA-insensitive mutant *ABI1-1* that enriches for GA auxotrophs and GA-insensitive mutants. The vast majority (76%) of the suppressors of *ABI1-1* strongly resemble GA auxotrophs in that they are severely dwarfed and have dark green foliage and flowers with underdeveloped petals and stamen. Three isolates were alleles of the GA auxotroph *ga1*. The remaining severe dwarves were not rescued by GA and belong to a single complementation group that we designate *sly1* (Sleepy 1). The alleles of *sly1* identified are the first recessive GA-insensitive mutations to reflect the full spectrum of GA-associated phenotypes, including the failure to germinate in the absence of the *ABI1-1* lesion. Thus, we postulate that *SLY1* is a key factor in GA reception.

THE choice of dormancy over growth allows many organisms to withstand unfavorable conditions. Well-studied genetic systems such as yeast sporulation (Kupiec *et al.* 1997) and *Caenorhabditis elegans* dauer larva formation (Malone *et al.* 1996) have elucidated many of the mechanisms regulating these developmental responses to environmental stimuli. Seed dormancy and germination in higher plants resemble these systems as germination is responsive to environmental cues, but they differ in that seed dormancy is an integral stage of embryo development. Dormancy is established during late embryo maturation and is associated with expression of specific genes as the embryo accumulates nutrient reserves and acquires desiccation tolerance (Goldberg *et al.* 1989; Galau *et al.* 1991). Dormancy ensures that the seed will survive the interval between dissemination from the mother plant and germination. Seeds germinate in response to dormancy-breaking conditions that are beneficial to growth, including light quality, moisture, and transient exposure to cold. The mechanisms governing the establishment and reversal of seed dormancy are of agricultural interest because cereal seeds are often not fully dormant. As a consequence, preharvest germination under cool moist conditions can cause considerable economic losses (Walker-Simmons and Ried 1993; Bewley and Black 1994). *Arabidopsis thaliana* is ideal for the study of seed dormancy because it produces fully dormant seeds in a genetically tractable system. Studies of biosynthetic mutants in Arabidopsis and other seed plants showed that abscisic acid (ABA) is required to establish seed dormancy, while gibberellic acid (GA) is required to break dormancy and trigger germination (reviewed in Koornneef and Karssen 1994; Bewley 1997).

The role of ABA in establishing seed dormancy has been investigated by analysis of mutants with increased or decreased sensitivity to exogenous ABA. Mutants with an enhanced response to ABA (*era1*) were identified based on their inability to germinate in the presence of exogenous ABA at concentrations that fail to inhibit wild-type germination (Cutler *et al.* 1996). This phenotype is accompanied by hyperdormancy of the seed, indicating that increased ABA sensitivity is associated with increased dormancy. Conversely, ABA-insensitive mutants (*ABI*) allow germination at ABA concentrations that are inhibitory to wild-type germination and result in reduced seed dormancy (Koornneef *et al.* 1984; Finkelstein 1994). Two of the ABA-insensitive mutants, *ABI1-1* and *ABI2-1*, also have a vegetative "wilty" phenotype similar to that seen in the ABA auxotrophs (Koornneef *et al.* 1982; Léon-Kloosterziel *et al.* 1996b). The wilty phenotype results from the role of ABA in stomatal closure in response to environmental cues, including drought. The phenotypes of the remaining ABA-insensitive alleles appear to be seed specific (Finkelstein and Somerville 1990; Finkelstein 1994; Parcy *et al.* 1994). The molecular identities of *ERA1*, *ABI1*, *ABI2*, and *ABI3* suggest that a signaling cascade modulates the cellular response to ABA. The *ERA1* gene encodes a protein

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farnesyl transferase and is postulated to negatively regulate ABA signaling by posttranslational modification of ABA-response gene(s) (Cutler *et al.* 1996). *ABI1* and *ABI2* encode protein phosphatase 2C homologs, suggesting that phosphorylation status is important in ABA signaling (Leung *et al.* 1994, 1997; Meyer *et al.* 1994). Finally, the *ABI3* gene is homologous to the maize transcription factor *VP1* (viviparous) and appears to function as a seed-specific transcriptional activator (McCarty *et al.* 1991; Giraudat *et al.* 1992; Parcy *et al.* 1994; Suzuki *et al.* 1997). Hence, *ABI3* is thought to act at the level of gene regulation, whereas *ERA1*, *ABI1*, and *ABI2* appear to act in ABA signal transduction. The original purpose of our screen for extragenic suppressors of *ABI1-1* was to identify additional mutants with enhanced response to ABA. Surprisingly, the majority of extragenic suppressors resulted from defects in GA biosynthesis or response.

Although many genes involved in GA biosynthesis have been characterized, comparatively few GA-response mutants have been identified (Finkelstein and Zeevaart 1994). Severe mutations in genes acting early in GA biosynthesis (*GA1*, *GA2*, and *GA3*) display a number of GA-rescued phenotypes, including failure to germinate, growth of the plant as a dark green dwarf, underdeveloped petals and stamen accompanied by reduced fertility, an increased number of buds per inflorescence, delayed flowering, reduced apical dominance, and delayed senescence (Koornneef and van der Veen 1980; Wilson *et al.* 1992). In principle, a severe GA-insensitive mutant should reflect the full spectrum of phenotypes seen in severe GA biosynthetic mutants but fail to be rescued by GA. Hence, a severe GA-insensitive mutant should be unable to germinate and, thus, difficult to recover. A single Arabidopsis mutant with decreased GA signal transduction has been described, *gai1-1* (GA-insensitive; Koornneef *et al.* 1985). The recent cloning of *GAI1* has led to several new insights into its role in GA signal transduction (Peng *et al.* 1997). The original allele, *gai1-1*, is a 51-bp deletion resulting in a semidominant, GA-insensitive phenotype similar to a weak GA auxotroph in that it grows as a dark green semidwarf with slightly reduced germination and fertility. In contrast, the disruption *gai1-t6* results in a slight increase in GA signal transduction, evidenced by weak resistance to inhibition of stem elongation by the GA biosynthetic inhibitor paclobutrazol. One interpretation of these results is that *GAI1* is a redundant, negative regulator of GA response (Peng *et al.* 1997). This view is supported by identification of *GRS*, a gene with 83% amino acid identity to *GAI1* (Peng *et al.* 1997). Thus, *gai1-1* may be a dominant negative mutation that interferes with both *GAI1* and *GRS* function, while *GRS* may be able to compensate for loss of *GAI1* function, resulting in a weak phenotype. Because of the significant homology between the *GAI1* gene and the putative transcription factor SCARECROW (SCR), Peng *et al.* (1997) suggest that *GAI1* acts late in GA signal transduction, at the level of transcriptional regulation.

Genetic studies of GA response in Arabidopsis have also used mutations causing increased GA signal transduction (reviewed in Swain and Olszewski 1996), including the *spy1* (spindly) and *rga1* (repressor of *ga1-3*) mutants (Jacobsen and Olszewski 1993; Jacobsen *et al.* 1996; Silverstone *et al.* 1997). The disruption allele *spy1-4* is semidominant. Phenotypes of *spy1* alleles reflect all GA responses and include reduced requirement for GA in germination, increased internode length, increased parthenocarpy, and early flowering (Jacobsen and Olszewski 1993; Wilson and Somerville 1995; Silverstone *et al.* 1997). In double mutant studies, *spy1* partly suppresses the GA auxotroph *ga1* for all phenotypes, including germination. In contrast, the recessive *rga1* mutation suppresses only the dwarf phenotype of *ga1* mutants, suggesting the existence of a branch point in the GA signal transduction pathway (Silverstone *et al.* 1997).

Here, we describe GA-insensitive mutations in *Sleepy 1* (*SLY1*) which, unlike the GA-response mutants characterized thus far, is a recessive mutation displaying the full spectrum of phenotypes seen in severe GA auxotrophs, including the failure to germinate. The germination of *sly1* is dependent on the presence of the *ABI1-1* lesion; the name *sleepy1* refers to the dwarf Sleepy of *Snow White.* That GA-insensitive mutations can be recovered in an ABA-deficient background was first suggested by the fact that ABA auxotroph and -insensitive mutants suppress the germination phenotype of GA biosynthetic mutants (Koornneef *et al.* 1982; Nambara *et al.* 1992; Léon-Kloosterziel *et al.* 1996b). The interpretation of this result was that absence of ABA biosynthesis or sensitivity bypasses the requirement for GA in germination because the seeds do not become fully dormant during embryo maturation (Karssen and Lacka 1986). However, the isolation of alleles of *ga1* and *sly1* as suppressors of *ABI1-1* reveals that *ABI* require GA to germinate in the presence of exogenous ABA. We demonstrate that the influence of both hormones determines the level of sensitivity to ABA in the seed, and we discuss some of the developmental implications of these findings.

MATERIALS AND METHODS

Plant material: The *Arabidopsis thaliana* (L.) Heynh ecotype Landsburg *erecta* (Ler) was used as the background for all of these experiments except for those using *spy1-3*, in which case ecotype Columbia (Col) was used. The *ABI1-1* and *abi3-1* mutants were a gift from M. Koornneef. The *spy1-3* mutant and GA auxotrophs were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH).

Growth conditions and germination experiments: Plants were grown under continuous fluorescent light ($\sim$150 μEinstein $m^{-2} s^{-1}$) at 22°. Seeds used in germination assays were allowed to dry in open tubes at room temperature for at least

2 wk after harvest. Seeds used for comparisons were as close as possible to one another in age (within 1 or 2 wk of the same day of harvest). Germination experiments used seeds surface sterilized in 20% bleach / 0.1% SDS for 15 min followed by four to six washes with sterile water. Although seeds were plated in 0.4% top agar in the initial screen and retest, seeds in later experiments were plated simply with water to avoid variability in percent germination in the *sly1* background. This variation may be caused by either altered oxygen diffusion or pressure exerted by different depths of top agar on the seed coat. All germination experiments used 0.8% agar plates containing 0.5× Murashige and Skoog basal salt mixture (MS; Sigma, St. Louis) and buffered to pH 5.5 with 50 mm 2-[*N*-morpholino]ethane sulfonic acid (MES). All plant hormones were obtained from Sigma, and all hormone stock solutions were in absolute ethanol, except for ABA, which was in methanol. Uniconazol was obtained as a gift from Dr. K. Izumi (Sumitomo Heavy Chemical Company, Ltd., Takatsu Kasa, Japan) and was also solubilized in ethanol. Plant hormones were added to autoclaved media cooled to ~55°. The gibberellin GA_3 was used at 10 μm while the gibberellin GA_4 was used at 1 μm because the latter has roughly 10-fold greater activity (Reed *et al.* 1996). Seeds were imbibed on plates at 4° for 4 days to encourage synchronous germination and then moved to lights at 22°. Percent germination was determined after 5 days under lights unless stated otherwise. Seeds were scored as germinated when green expanded cotyledons were observed (Figure 6; see also Leung *et al.* 1997).

The screen for suppressors of *ABI1-1*: Approximately 50,000 *ABI1-1* homozygous seeds were mutagenized in 100 ml of 0.4% (v/v) EMS (Sigma) for 24 hr at 4°. Seeds were washed 15 times with water over a 6-hr period to remove residual EMS. The mutagenized seeds (M_1) were sown into 40 6-inch pots and grown under standard conditions. The M_2 seeds resulting from self-fertilization of the M_1 plants were harvested as 40 independent pools to maximize the number of alleles known to be independent.

To screen for suppressors of *ABI1-1*, 1000 seeds from each M_2 pool were sterilized and plated in 0.4% top agar containing 3 μm ABA onto 0.8% agar plates containing 3 μm ABA. Plates were imbibed for 4 days at 4° to stimulate uniform germination and then transferred to lights at 22°. After 5 days at 22°, ungerminated seeds were transferred to MS plates or soil. The resulting germinated plants were grown to maturity and allowed to self-fertilize. M_3 seeds were retested for suppression of *ABI1-1* by plating on MS, 0.3, 1.2, and 3.0 μm ABA to determine the degree of ABA sensitivity after 5–7 days. Between 16 and 48 seeds were examined per time point, depending on M_2 seed set.

Hypocotyl elongation assay: Surface-sterilized seeds were plated on agar containing MES-buffered MS with or without 10 μm GA_3. Plates were kept in the dark during 4 days imbibition at 4°, followed by 7 days at 22°. Afterward, the hypocotyl length of 10 seedlings was measured for each genotype.

Genetic analysis: A single backcross to *ABI1-1* was performed for each of the suppressors of *ABI1-1* chosen for detailed analysis. F_2 seeds showing suppression of the *ABI1-1* germination phenotype were selected, grown to maturity, and used as a source of F_3 seed for germination studies. Crosses for complementation tests were performed using M_3 or M_4 plants before backcross. Germination on 3 μm ABA of 12–37 F_1 seeds was analyzed for each complementation cross. Results of complementation tests were confirmed by examining segregation of germination and dwarf phenotypes in the F_2. Between 30 and 100 F_2 seeds were examined for each F_1 plant.

Molecular determination of the *ABI1-1* genotype: The *ABI1-1* lesion destroys an *Nco*I restriction site at nucleotide 970 of the *ABI1* sequence (Leung *et al.* 1994; Meyer *et al.* 1994). This RFLP allowed us to examine the *ABI1* genotype as a codominant trait by *Nco*I digestion of a PCR fragment spanning this site. PCR template was prepared as described in McKinney *et al.* (1995). The PCR primers and conditions used were as described by Leung *et al.* (1997).

RESULTS

Isolation of Suppressors of *ABI1-1*: To identify genes required for the germination of *ABI1-1* seeds in the presence of exogenous ABA, a screen for extragenic suppressors of *ABI1-1* was conducted. *ABI1-1* is a semidominant, pleiotropic mutation displaying a vegetative wilty phenotype in addition to reduced dormancy and ABA sensitivity in the seed. Unlike wild-type, *ABI1-1* seeds germinate in the presence of 3 μm ABA. Exploiting this difference in germination, we screened for EMS-induced mutations that suppressed germination of *ABI1-1* on 3 μm ABA. Germination was scored based on the presence of expanded cotyledons (see materials and methods). A total of 62 candidates (21 pools) from a population of 40,000 M2 EMS-mutagenized *ABI1-1* seed (total 40 pools) were identified in this way. Of these, 36 (17 pools) lines exhibiting good germination on minimal media, but less than 15% germination on 3 μm ABA in the M_3 generation, were advanced for further analysis. Intragenic mutations in *ABI1* were expected to revert both the germination and the vegetative wilty phenotypes. Seven nonwilty suppressors were recovered, of which four (SC4-1, SC4-2, SC14-9, and SC22-13) were backcrossed to wild-type. Segregation analysis of F_2 seed from these backcrosses failed to recover the *ABI1-1* phenotype, further suggesting that a lesion within the *ABI1* locus resulted in reversion of both the germination and wilty phenotypes.

Of the remaining 29 suppressors exhibiting the *ABI1-1* vegetative wilty phenotype, 19 lines (10 pools) produced severely dwarfed plants with dark green leaves (Figure 1). The phenotypes of these plants are reminiscent of GA auxotrophs (Figure 1, C, E, G–K), including extreme dwarfism, dark green leaves, underdeveloped petals and stamen accompanied by reduced fertility, an increased number of buds per inflorescence, reduced apical dominance, and delayed senescence. A further three lines (one pool) were phenotypically less severe semidwarves (Figure 1, D and F). The hypothesis that these 22 dwarves were GA auxotrophs was tested by spraying with 10 μm GA_3 or 1 μm GA_4 at weekly intervals for 7 wk. Three lines, SC19-5 (Figure 1D), SC36-1 (Figure 1E), and SC36-5 were rescued by GA treatment, indicating that they are indeed GA auxotrophs. This conclusion was verified when crosses to each of the five known GA auxotrophs showed that all three GA-rescued mutants fail to complement *ga1-3* and, therefore, represent new alleles in this locus (see Table 1). Crosses of SC19-5 to the severe *ga1-3* dwarf in both directions yielded F_1 hybrids resembling the SC19-5 semidwarf, indicating

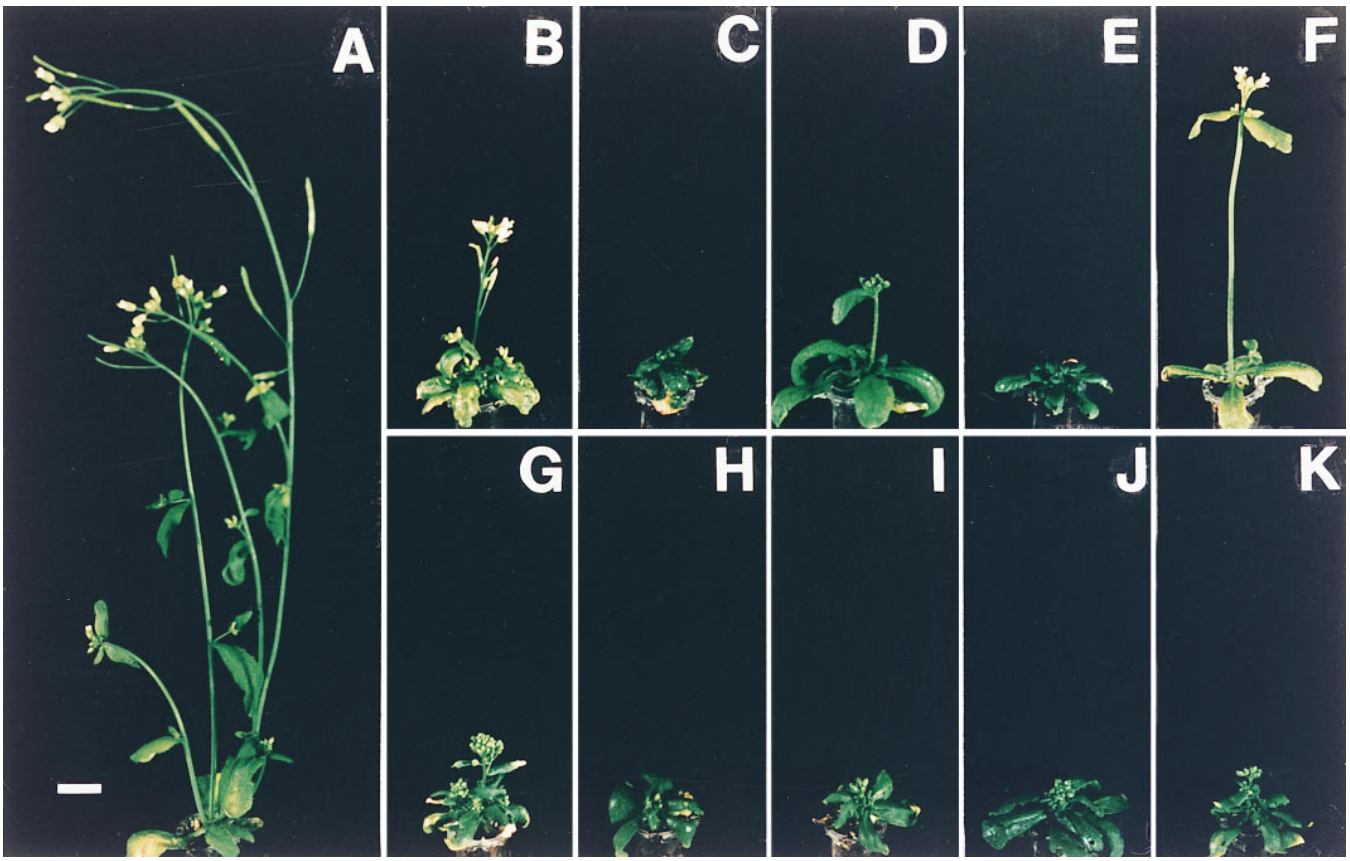

Figure 1.—Comparison of suppressors of *ABI1-1* to *gai1* and to the *ga1-3*. The *ABI1-1* parent strain (A), *gai1* (B), *ga1-3* (C), *ga1-11 ABI1-1* or SC19-5 (D), *ga1-12 ABI1-1* or SC36-1 (E), SC19-6 (F), *sly1-1 ABI1-1* or SC5-6 (G), *sly1-2 ABI1-1* or SC6-6 (H), *sly1-3 ABI1-1* or SC11-6 (I), *sly1-4 ABI1-1* or SC14-10 (J), and *sly1-5 ABI1-1* or SC39-1 (K) shown at same age after ~4 wk of growth under constant light. The *sly1* alleles result in dwarfism and underdeveloped flowers that more closely resemble the severe auxotroph *ga1-3* (C) than *gai1* (B). Bar, 1.1 cm.

that SC19-5 is a weak allele of *ga1*, designated *ga1-11*. SC36-1 and SC36-5 are two isolates from the same pool, and both displayed severe GA-rescued phenotypes and, therefore, likely represent sibling isolates of the allele designated *ga1-12*.

The GA-insensitivity of the remaining dwarves was confirmed using a quantitative assay for hypocotyl elongation in dark-grown seedlings (see materials and methods). Seeds of the parent *ABI1-1* and of several suppressors, including the severe dwarves SC6-6 and

TABLE 1

Complementation analysis of GA-sensitive suppressors of *ABI1-1*

Pollen recipient	Pollen donor							
	SC19-5	SC36-1	SC36-5	*ga1-3*	*ga2-1*	*ga3-1*	*ga4-1*	*ga5-1*
SC19-5 = *ga1-11*	SD			SD	*	*	+	+
SC36-1 = *ga1-12*		D	—	—	*	*	+	+
SC36-5 = *ga1-12*		D	D	D	+	+	+	+
ga1-3	SD	D	—	D				
ga2-1	+	+	*		D			
ga3-1	+	+	*			D		
ga4-1	+	*	+				SD	
ga5-1	+	*	*					SD

Complementation scored based on stature: D, dwarf; SD, semidwarf; +, wild-type stature. D and SD result from noncomplementation. Where the cross was not performed, — indicates that the reciprocal cross is noncomplementing, and * indicates that the reciprocal cross is complementing.

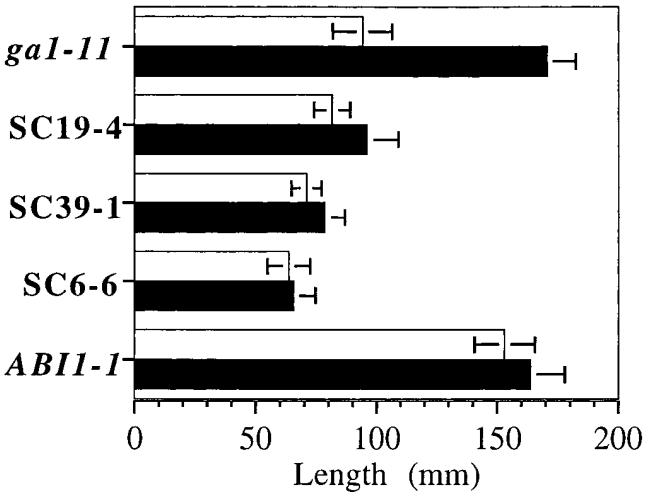

Figure 2.—GA-responsiveness of suppressors of *ABI1-1*. The elongation of dark grown hypocotyls was used as a bioassay for GA response. The average hypocotyl length was determined after 7 days incubation in the dark in the presence (black) or absence (white) of 10 μm GA_3.

SC39-1, the semi-dwarf suppressor SC19-4, and *ga1-11*, were germinated in the dark both in the presence and absence of 10 μm GA_3 and their hypocotyl lengths were measured. Only *ga1-11* showed increased hypocotyl elongation in response to GA (Figure 2).

Recent characterization of auxotrophs for the plant hormone brassinosteroid (BR) described these mutants as dark green dwarves (reviewed in Clouse 1996). In Arabidopsis, such mutants are cabbage-like in appearance and not as dark green as GA auxotrophs. Although suppressors of *ABI1-1* more closely resembled GA than BR auxotrophs, we chose to test whether the dwarfism of these plants was BR-rescued as two GA-insensitive mutants in pea later proved to be BR mutants (Nomura *et al.* 1997). Dwarf plants were sprayed with 1 μm brassinolide at weekly intervals for 6 wk. Neither the dwarf phenotype nor the fertility of these plants was rescued by BR application. We also observed that, unlike known BR mutants, the dwarf suppressors of *ABI1-1* form an apical hook when germinated in the dark, indicating that the etiolation response is intact.

Genetic analysis of the suppressors of *ABI1-1*: To simplify genetic analysis, each of the three GA auxotrophs and representative severe (SC5-6, SC6-6, SC11-6, SC14-10, SC39-1) and weak (SC19-6) GA-insensitive mutants from independent pools were chosen for detailed characterization. Because of the severe male infertility of the suppressor lines, each allele was backcrossed using *ABI1-1* as the pollen donor. In all cases, the resulting F_1 plants were wild type in stature and fertility, indicating that this phenotype is recessive (see Table 2). In addition, the F_2 seed from self-fertilized F_1 plants showed 3:1 segregation for both the germination phenotype (failure to germinate on 3 μm ABA) and for the vegetative phenotypes (dwarfism and reduced fertility). In all cases, failure to germinate cosegregated with dwarfism, indicating that these phenotypes are likely to result from the same genetic lesion.

TABLE 2

Segregation of suppressors of *ABI1-1* germination phenotype in F_2 progeny from backcross to *ABI1-1*

Cross	G^{+a}	G^{-a}	χ^{2b}	*P*
SC5-6	88	35	0.78	>0.25
SC6-6	51	18	0.04	>0.25
SC11-6	132	61	0.36	>0.25
SC14-10	293	78	3.13	0.1–0.05
SC19-5	281	115	3.45	0.1–0.05
SC19-6	66	23	0.05	>0.25
SC36-1	78	33	1.32	0.25
SC39-1	59	19	0.02	>0.25

[a] G^+, number of F_2 seeds germinated; G^-, number ungerminated after 5 days on 3 μm ABA.
[b] Test for 3:1 segregation of the germination phenotype.

All of the severe GA-insensitive dwarves are alleles of SLY1: To determine the number of GA-insensitive complementation groups, the germination phenotype was used to score complementation in crosses between the five severe dwarf suppressors of *ABI1-1* under analysis (see Table 3). For the most part, suppressors SC5-6 and SC11-6 were used as pollen donors because these lines are the most male fertile. All five severe GA-insensitive dwarves fell into a single complementation group that we designate *sly1* (sleepy1). F_1 hybrids were allowed to self-fertilize to obtain F_2 progeny for segregation analysis. All of the F_2 progeny from crosses between GA-insensitive severe dwarves displayed both the suppression of germination on 3 μm ABA and the dwarf phenotype (minimum of 30 F_2s examined). The failure to generate wild-type segregants gives additional proof that these lines are mutations in the same gene. Additional crosses indicate that at least nine independent alleles of *sly1* were recovered in this screen, including the five alleles currently under study. We designate suppressor SC5-6 as *sly1-1* (Figure 1G), SC6-6 as *sly1-2* (Figure 1H), SC11-6 as *sly1-3* (Figure 1I), SC14-10 as *sly1-4* (Figure 1J), and SC39-1 as *sly1-5* (Figure 1K).

Two of the semidwarf suppressors, SC19-4 and SC19-6 (Figure 1F), are GA insensitive and belong to a separate complementation group from *sly1* (see Table 3). Because suppressors SC19-4 and SC19-6 come from a single pool, they are likely to be repeat isolates of the same lesion. This suppressor mutation resembles GA auxotrophs in the following phenotypes: semidwarfism, short, club-shaped siliques, an increased number of buds per inflorescence, and delayed senescence. However, this suppressor mutation differs from GA auxotrophs in the following phenotypes: leaves are not dark green, apical dominance is not decreased, and flowers have petals and anthers of normal length. In light of these differ-

TABLE 3

Complementation analysis of suppressors of *ABI1-1*

Pollen recipient	Pollen donor							
	SC5-6	SC6-6	SC11-6	SC14-10	SC39-1	SC19-4	SC19-5	SC19-6
SC5-6 = *sly1-1*	0	—	0	—	—			50
SC6-6 = *sly1-2*	0	0	0	0	—	100		
SC11-6 = *sly1-3*	—	—	0	—	—			100
AC14-10 = *sly1-4*	0	—	0	0	14			
SC39-1 = *sly1-5*	0	0	0	14	0	100	71	75
SC19-4						0		0
SC19-5 = *ga1-11*							0	88
SC19-6						0		0

Numbers indicate the percent germination after 3 days on 3 μm ABA where noncomplementation results in 0–15% germination. —, cross was not performed, but reciprocal cross fails to complement.

ences, we choose to interpret this GA-insensitive mutation with caution, and we will not assert that the gene mutated plays a direct role in GA signal transduction. This mutant will be referred to by isolation number until sufficient information is available to choose a meaningful name.

sly1 is not an allele of gai1: The *sly1* alleles recovered in this screen are recessive mutations causing severe dwarfism and loss of fertility (Figure 1, G–K), while *gai1* is a semidominant mutation causing semidwarfism and little loss of fertility (Figure 1B) (Koornneef *et al.* 1985; Wilson and Somerville 1995). In spite of these differences, the possibility that the *sly1* mutants are new alleles of *gai1* was investigated by segregation analysis. Towards this end, pollen from a *gai1-1* plant was used to fertilize *sly1-5 ABI1-1*. The F_1 progeny of this cross were semidwarves resembling *gai1-1*. Segregation analysis of 25 F_2 plants found five nondwarves, 14 semidwarves, and six severe dwarves. Assuming that all *sly1-5* homozygotes are severe dwarves regardless of the *gai1* genotype, these frequencies are a good fit for the hypothesis of two unlinked genes ($\chi^2 = 0.029$, $P > 0.97$). Moreover, a significant proportion of wild-type plants segregated in the F_2, further indicating that *sly1-5* is not an allele of *gai1*.

The failure to isolate *gai1* mutants as suppressors of *ABI1-1* suggests that either *gai1* cannot suppress *ABI1-1* under these conditions, or that the *gai1-1* allele is a rare event and difficult to recover. To determine whether the *gai1-1* mutation can suppress germination of *ABI1-1* on 3 μm ABA, we constructed a *gai1-1*/*gai1-1 ABI1-1*/*ABI1-1* double-mutant plant. Seeds of this genotype give 90–100% germination on 3 μm ABA, indicating that *gai1-1* cannot suppress the germination of *ABI1-1* on 3 μm ABA. Thus, we conclude that mutations in *gai1* were not recovered because they did not meet the criteria of the screen.

Reduced GA biosynthesis reduces the germinability of ABA-insensitive seed: While previous data suggested that GA-insensitive mutants could be recovered in an ABA-insensitive background, the recovery of GA-defective mutations as suppressors of *ABI1-1* was unexpected. This result suggests that GA biosynthesis or response is required for germination of ABA-insensitive alleles on exogenous ABA. This GA requirement could be general for ABA-insensitive alleles, or it could be specific to *ABI1-1*, reflecting a unique interaction of this gene product with GA action. To differentiate between these possibilities, the GA biosynthetic inhibitor uniconazol was used to determine if reduced GA biosynthesis in general increases the sensitivity of *ABI1-1* and *abi3-1* germination to ABA. Lesions in the *ABI3* locus differ fundamentally from *ABI1-1* in that they act at the level of transcription and share the germination, not the vegetative, phenotypes of *ABI1-1* (Nambara *et al.* 1992; Ooms *et al.* 1993;

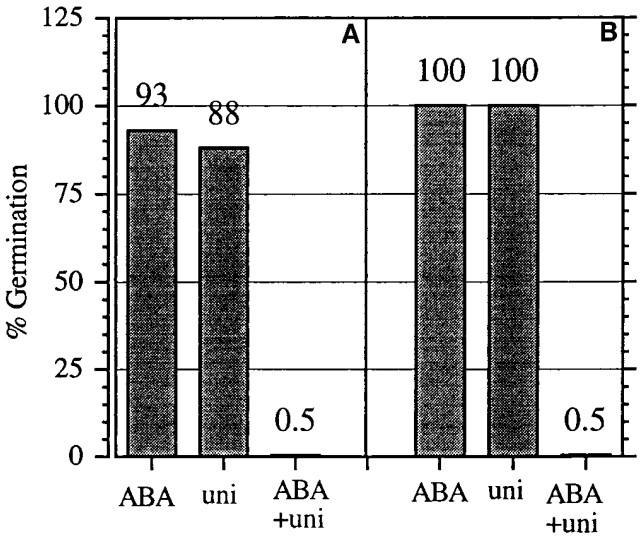

Figure 3.—The GA biosynthesis inhibitor uniconazol suppresses germination of *ABI1-1* and *abi3-1* on exogenous ABA. Percent germination of *ABI1-1* (A) and *abi3-1* (B) on 3 μm ABA (ABA), 10 μm uniconazol (uni), and 3 μm ABA + 10 μm uniconazol. Seeds were imbibed for 4 days at 4° and then germinated under light at 22° for 5 days. Sample size is 30–90 seeds.

Parcy *et al.* 1994). The data shown in Figure 3 demonstrate that 10 μm uniconazol can phenocopy the GA auxotroph suppressors by inhibiting the germination of both *ABI1-1* and *abi3-1* in the presence of 3 μm ABA. This result supports the hypothesis that reduced GA biosynthesis results in reduced germinability of ABA-insensitive mutants in the presence of exogenous ABA.

Decreased GA biosynthesis or sensitivity causes increased ABA sensitivity: The screen for suppressors of *ABI1-1* was intended to identify mutations in ABA signaling that bypass the requirement for *ABI1* to respond to exogenous ABA. Such suppressors would be expected to result in either wild-type or greater than wild-type ABA sensitivity. To quantify the degree to which the GA-sensitive and GA-insensitive suppressors increased the ABA sensitivity of *ABI1-1*, the germination of each suppressor was compared to that of wild-type (Ler) and *ABI1-1* over a range of ABA concentrations. The results shown in Figure 4 indicate that none of the suppressors recovered fully restore ABA sensitivity to that of wild-type Ler. Thus, strictly speaking, none of these suppressors fully bypasses *ABI1-1*. Rather, it appears that the effects of ABA and GA mutants are additive. The curve shown for *sly1-1* demonstrates that it is the least ABA-sensitive allele examined (Figure 4A). This, together with the observation that *sly1-1* is more fertile than the other alleles, indicates that it is the weakest allele recovered. The SC19-6 mutant (Figure 4F) results in a higher degree of ABA sensitivity than any of the *sly1* alleles, as does *ga1-11* (Figure 4E).

In addition to germination over a range of ABA concentrations, the germination of each allele on 3 μm ABA was determined as a function of time (Figure 5). Once again, the *sly1-1* allele allowed germination earlier than the other alleles recovered, with partial germination occurring by day 3 and full germination by day 5. The other alleles allowed partial germination by day 4 and did not fully germinate within the 7 days of the experiment. It is interesting to note that in many cases, *sly1 ABI1-1* and *ga1-11 ABI1-1* seedlings emerged from the seed coat and displayed expanded cotyledons (and thus were germinated by our criteria) but had retarded root development (Figure 6C). In the absence of the *sly1* and *ga1-11* lesion, *ABI1-1* allows root elongation at ABA concentrations greater than threefold higher than those used in this study (Leung *et al.* 1994). On day 8 of the experiment in Figure 5, only five of 28 germinated *sly1-1 ABI1-1* and six of 51 germinated *ga1-11 ABI1-1* seedlings showed root elongation. Wild-type Ler seedlings also fall free of the seed coat if left on 3 μm ABA for 7 days (Figure 6A) or longer. By contrast, these seedlings remain undeveloped with yellow unexpanded cotyledons, so they are scored as ungerminated. Figure 6 compares such a wild-type seedling (Figure 6A) to a *sly1-1 ABI1-1* seedling with green but folded unexpanded cotyledons (Figure 6B), scored as ungerminated, and to a *sly1-1 ABI1-1* seedling with green unfolded expanded cotyledons, scored as germinated (Figure 6C). If these seedlings with undeveloped roots are transferred to medium without ABA, the seedlings grow roots and develop normally. Thus, it appears that *ga1-11* and *sly1* cause a greater increase in the ABA sensitivity of root development than in cotyledon greening and expansion.

***sly1* requires the *ABI1-1* lesion to germinate:** The fact that *sly1* mutants reduce the germinability of *ABI1-1* raised the question of whether *sly1* requires the *ABI1-1* lesion to germinate. If true, this would suggest that the reduced dormancy of the *ABI1-1* background allows germination of these severely GA-insensitive mutants. To determine the phenotype of *sly1-2* in the absence of the *ABI1-1* lesion, *sly1-2*/*sly1-2 ABI1-1*/*ABI1-1* was outcrossed using Ler wild-type pollen. In the F_2 generation, dwarf plants were screened for loss of the wilty phenotype to identify *sly1-2 ABI1*$^+$ candidates. One apparently nonwilty F_2 plant was identified; however, subsequent RFLP analysis revealed that this F_2 plant was heterozygous for the *ABI1-1* lesion (see materials and methods; Leung *et al.* 1997). When the F_3 seeds from the *sly1*/*sly1 ABI1*/$^+$ F_2 plant were plated on MS medium, 56 of 76 seeds germinated, indicating 3:1 segregation of a germination defect ($\chi^2 = 0.07, P > 0.95$). We would predict that the 74% that germinated were either *ABI1-1* homozygous or heterozygous, while the 26% ungerminated seed are homozygous *ABI1*$^+$. If true, this would indicate that *sly1-2* is unable to germinate in the absence of the *ABI1-1* lesion. To test this hypothesis, the *Nco*I restriction site polymorphism associated with *ABI1-1* (Leung *et al.* 1997) was used to molecularly ascertain the genotype of germinated seedlings. Of the 12 seedlings screened, five were *ABI1-1* homozygous and seven were *ABI1-1* heterozygous. Thus, as predicted, all the seeds that germinated contained the *ABI1-1* lesion, allowing us to deduce that the ungerminated seeds were *ABI1* wild type.

The increased GA signal transduction of *spy1-3* causes apparent ABA insensitivity: That reduced GA biosynthesis and sensitivity causes an apparent increase in ABA sensitivity implies that the sensitivity of the seed to exogenous ABA during germination is determined by the cumulative effects of ABA and GA mutations. This hypothesis suggests that mutations causing increased GA signal transduction during germination, such as *spy1*, should cause a decrease in ABA sensitivity in the seed. To test this hypothesis, the ABA sensitivity of *spy1-3* seed was compared to that of the Columbia wild-type parent strain (Col) over a range of ABA concentrations. Wild-type germination was completely inhibited at 2.4 μm ABA and partly inhibited at 1.2 μm after 5 days incubation. Although *spy1-3* germination was inhibited at 5 μm ABA, the mutant was able to germinate at 3 μm ABA, a concentration that completely inhibits wild-type germination (Figure 7). While *spy1-3* is only mildly ABA insensitive compared to *ABI1-1* (Figure 4), it does ap-

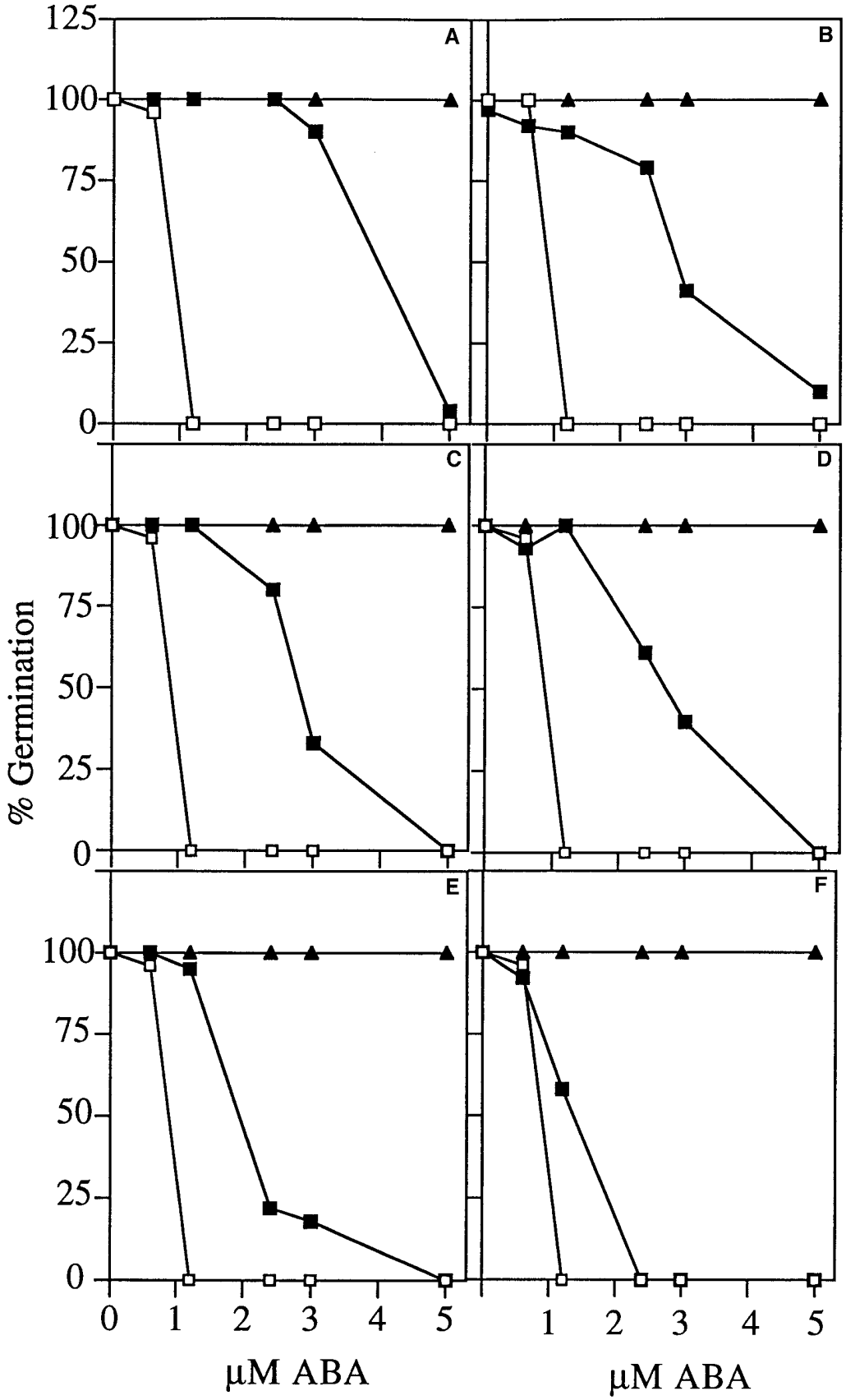

Figure 4.—Effect of suppressors of *ABI1-1* on ABA dose–response curves. Percent germination of *ABI1-1* (▲), Ler wild-type (□), and suppressors in the *ABI1-1* background (■) on increasing concentrations of ABA. Graphs shown are for *sly1-1 ABI1-1* (A), *sly1-2 ABI1-1* (B), *sly1-3 ABI1-1* (C), *sly1-5 ABI1-1* (D), *ga1-11 ABI1-1* (E), and SC19-6 *ABI1-1* (F). Germination was scored for 30–60 seeds per data point after 4 days at 4° and 5 days at 22° under lights.

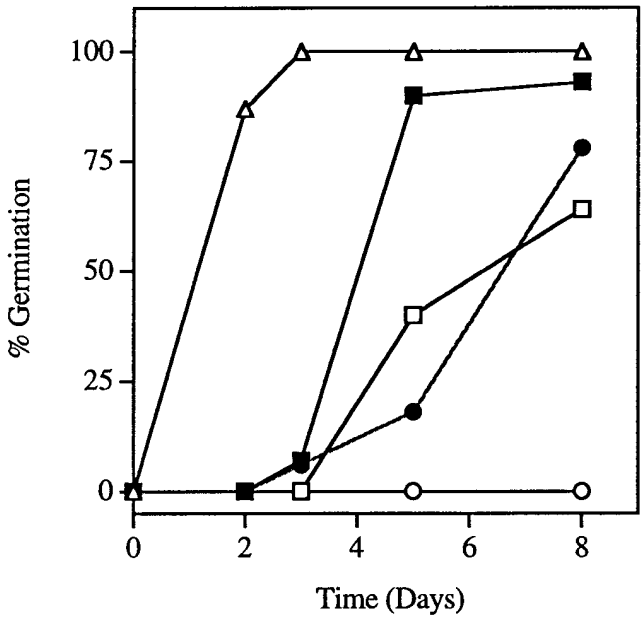

Figure 5.—Effect of suppressors of *ABI1-1* on the timing of germination on ABA. Percent germination on 3 μm ABA is given as a function of time for *ABI1-1* (△), *sly1-1 ABI1-1* (■), *sly1-5 ABI1-1* (□), *ga1-11 ABI1-1* (●), and SC19-6 *ABI1-1* (○).

pear that increased GA signal transduction causes decreased ABA sensitivity.

DISCUSSION

This paper describes the isolation of GA biosynthesis and GA response mutants as suppressors of the ability of *ABI1-1* seed to germinate at exogenous ABA levels inhibitory to wild-type germination. The *ABI1-1* mutation of Arabidopsis was originally identified in a screen for ABA-insensitive mutants (Koornneef *et al.* 1984). This mutant shows defects in ABA-regulated stomatal closing and, more importantly, reduced dormancy in the seed. The reduction in dormancy alleviates the normal requirement for GA to break dormancy and germinate (Koornneef and Karssen 1994). The results presented here demonstrate that removal of the GA requirement for germination in *ABI1-1* allows recovery of GA-insensitive mutations, resulting in failure to germinate. This is in agreement with other seed dormancy studies, demonstrating that reduced ABA biosynthesis or response rescues the germination of mutations in GA biosynthesis (Koornneef *et al.* 1982; Nambara *et al.* 1992; Léon-Kloosterziel *et al.* 1996b). Moreover, the suppression of germination on ABA by these mutants gives some insights into the antagonism between ABA and GA in the control of germination. It appears that decreased GA signal transduction causes increased ABA sensitivity, while increased GA signal transduction causes reduced ABA sensitivity in germination. Thus, it appears that ABA and GA regulate opposing forces in a tug-of-war over the decision to germinate. This does not imply that ABA and GA are the only forces acting on germination. On the contrary, the existence of mutants with reduced seed dormancy in the absence of altered ABA sensitivity suggests that other factors are involved (Keith *et al.* 1994; Léon-Kloosterziel *et al.* 1996a). Nevertheless, the ABA/GA antagonism provides a useful framework for evaluating our results.

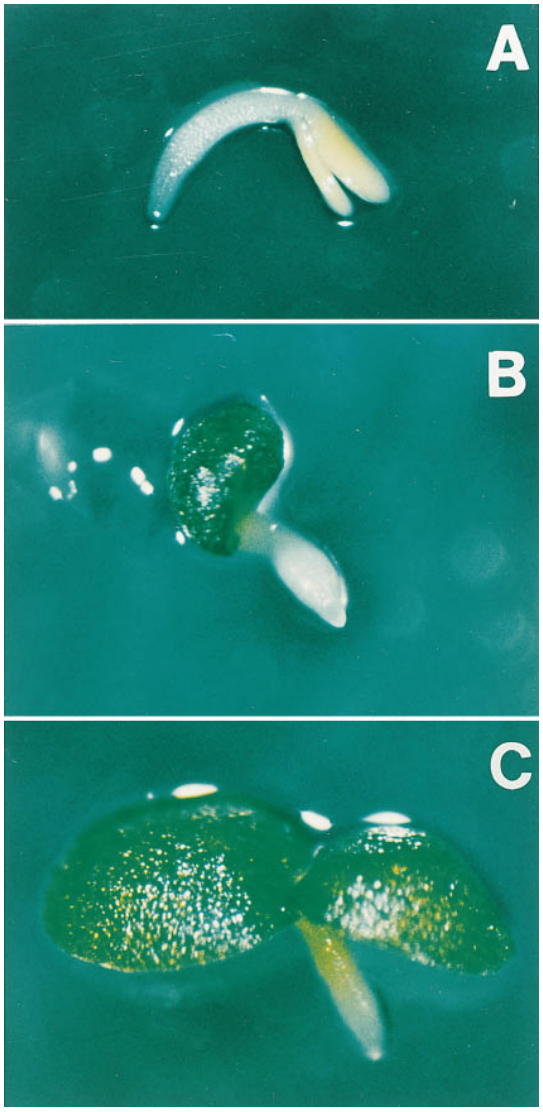

Figure 6.—Ler and *sly1-1 ABI1-1* seedlings germinated on ABA. Wild-type (A) and *sly1-1 ABI1-1* seedlings (B and C) after 7 days on 3 μm ABA. Seedlings in A and B were scored as ungerminated, while the seedling in C was scored as germinated based on green expanded cotyledons. The seedling in C shows retarded root development. Magnification ×10.

The antagonism between ABA and GA in dormancy and germination: The current theory of ABA/GA antagonism in seed dormancy is based on previous work showing that ABA auxotrophic and ABA-insensitive mutants rescue the germination of the *ga1* auxotroph and of seeds treated with GA biosynthetic inhibitor (Koornneef *et al.* 1982; Nambara *et al.* 1991, 1992; Léon-

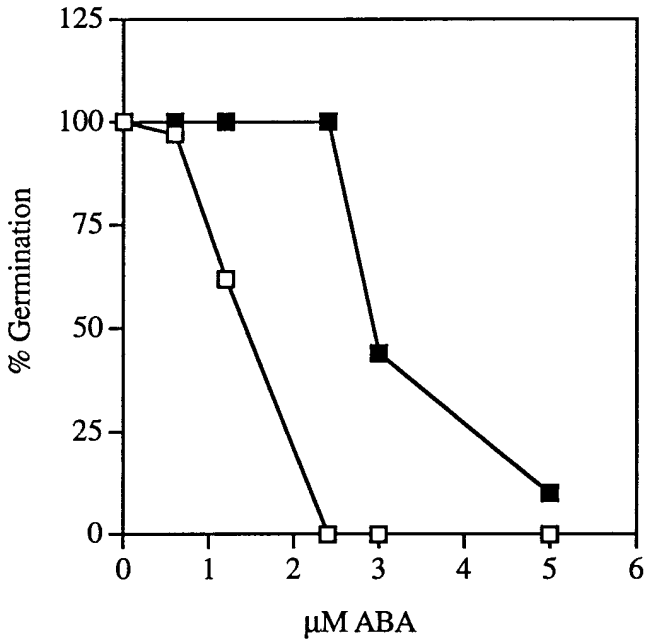

Figure 7.—Reduced ABA sensitivity in *spy1-3*. Percentage germination is given as a function of exogenous ABA concentration for Col (□) and *spy1-3* (■). Approximately 50–80 seeds per point were imbibed 4 days at 4° and germinated 5 days under lights at 22°.

Kloosterziel *et al.* 1996b). These results suggest that the degree of seed dormancy established by ABA during embryo maturation determines the level of GA required at the time of germination to break seed dormancy (Karssen and Lacka 1986). Therefore, less GA is required to germinate seeds in an ABA-defective background because primary dormancy was never fully established during embryo maturation. The observation that embryonic ABA levels reach their peak at 15 days after pollination and then steadily decline to the level in dry seed that is considered insufficient to inhibit germination supports this theory (Karssen *et al.* 1983). Although rescue of germination in the GA-insensitive *sly1* mutants by ABA-insensitive mutants is expected, it is not intuitively obvious that the screen for suppressors of *ABI1-1* germination on exogenous ABA should enrich for GA biosynthetic and GA-insensitive mutants. This result and others presented here indicate that increasing concentrations of exogenous ABA cause a proportional increase in the requirement for GA biosynthesis and sensitivity in the germination of ABA-insensitive seeds (see Figure 4).

The isolation of *ga1* and *sly1* mutants as suppressors of *ABI1-1* does not contradict the notion that the effects of ABA on germination are completed with embryo maturation and are therefore temporally separated from the effects of GA at germination. The transient exposure of wild-type embryos to endogenous ABA during seed maturation results in a state of dormancy that persists after ABA levels decline. In contrast, the apparent dormancy caused by exposure of mature wild-type embryos to exogenous ABA does not persist because seeds germinate soon after they are shifted from ABA plates to minimal plates. In fact, this germination occurs rapidly, suggesting that exogenous ABA does not fully inhibit the dormancy-breaking process, and that some ABA-independent component of dormancy is missing in these reconstruction experiments (C. Steber and P. McCourt, unpublished results). Perhaps not surprisingly, exogenous ABA is an artificial condition that detects alterations in ABA and GA sensitivity after the time for establishment of embryo dormancy has passed. This study uses measurement of sensitivity to exogenous ABA as a tool to detect the effects of altered GA signal transduction. Our results are consistent with the notion that *ABI1-1* and *abi3-1* mutants do not completely alleviate the GA requirement in germination; rather, they reduce the threshold of GA required to the point where residual GA biosynthesis in *ga1* and sensitivity in *sly1* are sufficient to allow germination. Plating the seed on exogenous ABA raises this threshold sufficiently that mutations in GA biosynthesis or sensitivity block germination in an ABA-insensitive background.

Our results also indicate that altered GA sensitivity causes an inverse effect on ABA sensitivity. First, the GA-insensitive *sly1* mutations increase the ABA sensitivity of *ABI1-1* to a level that is intermediate between *ABI1-1* and wild type (Figure 4, A–D). Additionally, the *sly1* mutant displays *ABI1-1*–dependent germination, indicating that *ABI1-1* reduces the requirement for GA responsiveness during germination. Second, the *spy1-3* mutation, which is thought to increase GA signal transduction, results in reduced ABA sensitivity during germination (Figure 7). The *spy1-3* mutant was isolated based on a reduced requirement for GA in germination, and it causes a number of vegetative phenotypes that are considered indicative of increased GA response (Wilson *et al.* 1992; Jacobsen and Olszewski 1993; Silverstone *et al.* 1997). This study shows that *spy1-3* germinates on a concentration of exogenous ABA that fully inhibits wild-type germination.

Differential effects of ABA and GA on aspects of seed germination: The antagonistic effects of ABA and GA on dormancy and germination may result from opposing effects on the same aspects of seed dormancy. For example, in barley, ABA activates transcription of the ABA-induced genes while it inhibits expression of the GA-induced gene α-amylase (Jacobsen *et al.* 1995). Thus, ABA and GA may have opposite effects on transcription of some of the same genes. However, our results detect some differential effects by ABA and GA on some facets of seed germination in Arabidopsis, suggesting that ABA and GA may affect these processes to different degrees. Seedling emergence results from a combination of two forces: (1) the enzymatic degradation of the seed coat releasing pressure from without and (2) the expansion of the embryo resulting in in-

creased pressure from within caused in part by water uptake. After emergence from the seed coat, the seedling must utilize storage reserves and begin growth. If left on ABA for 7 days, wild-type seed will eventually emerge from the seed coat (see Figure 6A). However, these seedlings are still growth arrested. They have germinated if we define germination as emergence, but their cotyledons are neither expanded nor green. This suggests that ABA more strongly inhibits greening and cell expansion than breakdown of the seed coat. Consistent with this interpretation is the recent observation that inhibition of seed germination by exogenous ABA in Arabidopsis is correlated with a block in the utilization of seed storage reserves (Garciarrubio *et al.* 1997). On the other hand, the requirement of *ga1-3* for exogenous GA in germination can be bypassed by dissecting the embryo out of the seed coat (Groot and Karssen 1987; Telfer *et al.* 1997). This raises the possibility that the main reason *ga1-3* seed do not germinate is because GA is needed to stimulate the emergence from the seed coat, either by causing seed coat degradation or by stimulating cell expansion in the embryo. This contention is supported by our observation that on ABA both *ga1 ABI1-1* and *sly1 ABI1-1* embryos often turn green before emerging from the seed coat. Possibly, mutants in the GA biosynthesis and response suppress *ABI1-1* more by slowing the breakdown of the seed coat than the greening of the embryo on ABA. After 7 days on ABA, *sly1 ABI1-1* and *ga1 ABI1-1* seedlings sometimes emerge from the seed coat. In these cases, cotyledon expansion often occurs in the absence of root elongation, suggesting that the *sly1* mutation causes a greater increase in the ABA sensitivity of root elongation than cotyledon greening and expansion (Figure 6C).

Spectrum of mutations recovered as suppressors of *ABI1-1*: The screen for suppressors of *ABI1-1* was originally intended to detect bypass mutations in genes required for germination in the *ABI1-1* background. A bypass mutation would increase the ABA sensitivity of *ABI1-1* to the level in wild-type or enhanced response to ABA (*era*) mutants. Such mutations should include negative regulators of ABA response acting downstream of *ABI1* to reverse dormancy, such as *era1* (Cooney 1996). A total of seven of the extragenic suppressors were not dwarfed and may include the expected class of enhanced response to ABA mutations. This study, however, has focused only on those extragenic suppressors that were dwarfed in stature, particularly on five alleles of the severe GA-insensitive mutant *sly1*.

Recovery of ga1 mutations as a suppressor of ABI1-1: All of the GA auxotrophs identified in this study were alleles of *GA1* (Figure 1, C–E). That the *GA1* gene encodes entkaurene synthetase, which acts early in GA biosynthesis (reviewed in Finkelstein and Zeevaart 1994), suggests that mutations in this gene may be preferentially selected in the *ABI1-1* suppressor screen. Only severe mutations in genes that act early in GA biosynthesis (*GA1*, *GA2*, and *GA3*) have been found to result in strict dependency on exogenous GA for germination. It is also possible that only severe mutations in genes acting early in the GA biosynthetic pathway may result in suppression of the ability of *ABI1-1* to germinate on ABA. However, the *ga1-11* allele is a semidwarf and is quite fertile, indicating that it is a weak allele of *GA1* (Figure 1D). Therefore, this screen is sensitive enough to detect the effect of weak GA auxotrophy. Perhaps, if the screen were carried out to saturation, it might detect mutations in other GA biosynthetic genes.

Recovery of GA-insensitive mutants as suppressors of ABI1-1: The alleles of *SLY1* reported here are the first recessive GA-response mutants to display the full spectrum of phenotypes associated with severe GA auxotrophs (Figure 1, C and G–K), including the failure to germinate in the absence of the *ABI1-1* allele. It is interesting that alleles of *SLY1* were the major class of suppressors of *ABI1-1* recovered. Not only was *gai1* not recovered as a suppressor of *ABI1-1*, but double-mutant analysis also showed that the current *gai1-1* allele is not sufficiently GA-insensitive to suppress the germination phenotype of *ABI1-1* on 3 μmol ABA. A full 59% of the suppressors of *ABI1-1* are mutations in *SLY1*, representing a minimum of nine independent alleles. This raises the question of why no other clear GA-response mutants were identified in this screen. The first possibility is that *SLY1* is the only element in the GA signal transduction pathway. For example, the glucocorticoid receptor is both a receptor and transcriptional regulator in this mammalian hormone signal transduction pathway (reviewed in McEwan *et al.* 1997). The second possibility is that other elements of the GA signal transduction pathway are recalcitrant to detection by mutant screen because they are (1) redundant functions, (2) essential for growth and fertility, or (3) not susceptible to EMS mutagenesis. For example, only dominant mutations have identified ethylene receptors because there are at least four independent receptor genes (Chao *et al.* 1997). The third possibility is that the conditions of the screen somehow enriched for mutations in *SLY1*, either through choice of the *ABI1-1* background or through the choice of ABA concentration used to screen for suppressors. The suppression of *ABI1-1* may be dosage dependent, so that only stronger GA mutants can suppress *ABI1-1* at the low ABA concentration used in this study. Finally, there is the possibility that more GA-insensitive mutants were recovered but are difficult to identify based on vegetative phenotypes. For example, if seed-specific GA-response factors exists, mutations in these genes could exhibit a germination phenotype in the absence of vegetative defects. Further characterization of those suppressors that did not cause dwarfism may address this possibility.

The plant hormone gibberellin was first identified as a plant growth regulator in the 1930s (Phinney 1983). Decades later, little is known about the mechanism of

GA perception and the GA receptor remains unidentified. The fact that *SLY1* mutations show the full spectrum of phenotypes associated with severe GA biosynthetic mutants suggests that this gene acts relatively early in GA perception. Moreover, all of the *sly1* alleles recovered are recessive, raising the possibility that it is a loss-of-function mutation in a GA receptor or other key GA response gene. The fact that *sly1* mutants were the major class of GA-insensitive mutations recovered in this screen and that *sly1* was the only severe GA-insensitive allele identified argue persuasively for the hypothesis that *SLY1* plays a key role in GA response. Future studies will attempt to more accurately place *SLY1* in the GA signal transduction pathway by epistasis analysis and by mapping and cloning the *SLY1* gene.

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