The Role of Two F-Box Proteins, SLEEPY1 and SNEEZY, in Arabidopsis Gibberellin Signaling[1][C][W][OA]

Tohru Ariizumi[2], Paulraj K. Lawrence, and Camille M. Steber*

United States Department of Agriculture-Agricultural Research Service, Wheat Genetics, Quality Physiology and Disease Research Unit, Pullman, Washington 99164–6420 (C.M.S.); and Department of Crop and Soil Science, Washington State University, Pullman, Washington 99164–6420 (T.A., P.K.L., C.M.S.)

The *SLEEPY1* (*SLY1*) F-box gene is a positive regulator of gibberellin (GA) signaling in Arabidopsis (*Arabidopsis thaliana*). Loss of *SLY1* results in GA-insensitive phenotypes including dwarfism, reduced fertility, delayed flowering, and increased seed dormancy. These *sly1* phenotypes are partially rescued by overexpression of the *SLY1* homolog *SNEEZY* (*SNE*)/*SLY2*, suggesting that *SNE* can functionally replace *SLY1*. GA responses are repressed by DELLA family proteins. GA relieves DELLA repression when the SCF^{SLY1} (for Skp1, Cullin, F-box) E3 ubiquitin ligase ubiquitinates DELLA protein, thereby targeting it for proteolysis. Coimmunoprecipitation experiments using constitutively expressed 35S:hemagglutinin (HA)-SLY1 and 35S:HA-SNE translational fusions in the *sly1-10* background suggest that SNE can function similarly to SLY1 in GA signaling. Like HA-SLY1, HA-SNE interacted with the CULLIN1 subunit of the SCF complex, and this interaction required the F-box domain. Like HA-SLY1, HA-SNE coimmunoprecipitated with the DELLA REPRESSOR OF GA1-3 (RGA), and this interaction required the SLY1 or SNE carboxyl-terminal domain. Whereas *HA-SLY1* overexpression resulted in a decrease in both DELLA RGA and RGA-LIKE2 (RGL2) protein levels, *HA-SNE* caused a decrease in DELLA RGA but not in RGL2 levels. This suggests that one reason *HA-SLY1* is able to effect a stronger rescue of *sly1-10* phenotypes than *HA-SNE* is because SLY1 regulates a broader spectrum of DELLA proteins. The FLAG-SLY1 fusion protein was found to coimmunoprecipitate with the GA receptor HA-GA-INSENSITIVE DWARF1b (GID1b), supporting the model that SLY1 regulates DELLA through interaction with the DELLA-GA-GID1 complex.

This study examines the roles of the *SLEEPY1* (*SLY1*) F-box gene and its homolog *SNEEZY* (*SNE*)/*SLY2* in GA hormone signaling. GA is required for several important transitions in Arabidopsis (*Arabidopsis thaliana*) development, including seed germination, stem elongation, fertility, and the transition to flowering (for review, see Sun and Gubler, 2004; Ueguchi-Tanaka et al., 2007; Aya et al., 2009). GA is also required for normal fertility and flower development and plays an important role in adaptations to cold, drought, and anoxia (Achard et al., 2006, 2008; Fukao and Bailey-Serres, 2008). During the green revolution, GA-insensitive semidwarf mutations in the DELLA genes provided resistance to lodging as well as increased yield for biomass (Allan, 1986). The mechanisms of GA signaling are highly conserved between Arabidopsis, rice (*Oryza sativa*), barley (*Hordeum vulgare*), and tomato (*Solanum lycopersicum*; for review, see Sun and Gubler, 2004; Jasinski et al., 2008). Thus, elucidating the fundamental mechanisms of GA signaling will be important in developing future strategies for crop improvement.

Previous research has shown that GA stimulates GA responses through destruction or deactivation of DELLA repressors of GA responses (McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004). The DELLA gene family is a subset of the GRAS family of putative transcription factors defined by the presence of a conserved N-terminal DELLA regulatory domain and a C-terminal GRAS functional domain. The term DELLA refers to the signature conserved amino acid sequence Asp-Glu-Leu-Leu-Ala (D-E-L-L-A). There are five DELLA genes in Arabidopsis with partly overlapping functions defined based on the capacity of each DELLA mutation to suppress the phenotypes of the severe GA biosynthesis mutant *ga1-3*. DELLAs *REPRESSOR OF GA1-3* (*RGA*) and *GA-INSENSITIVE* (*GAI*) are the main DELLA genes repressing stem elongation (Dill and Sun, 2001), but the DELLA *RGA-LIKE1* (*RGL1*) also contributes (Wen and Chang, 2002). DELLAs *RGA*, *RGL2*, and *RGL1* repress flowering and fertility (Cheng et al., 2004).

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[2] Present address: University of Tsukuba, Gene Research Center, 1–1–1 Tenno-dai, Tsukuba 305–8572, Japan.

* Corresponding author; e-mail csteber@wsu.edu.

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Finally, the DELLA RGL2 is the main repressor of seed germination (Lee et al., 2002; Peng and Harberd, 2002; Tyler et al., 2004; Ariizumi and Steber, 2007), although DELLAs RGA, GAI, and RGL3 also function in seed germination (Cao et al., 2005; Piskurewicz and Lopez-Molina, 2009). The resemblance of the GRAS domain to STAT transcription factors and the fact that DELLA proteins localize to the nucleus initially suggested that DELLAs might function in transcriptional control. Chromatin immunoprecipitation experiments showed that DELLA RGA localizes to promoter elements and appears to activate the expression of downstream negative regulators of GA responses (Zentella et al., 2007). Nevertheless, Zentella et al. (2007) suggested that the enrichment for DELLA RGA at these promoters is weak (2- to 3.5-fold), because DELLA appears to interact indirectly with these promoters rather than through protein-protein interaction. DELLA also appears to repress hypocotyl elongation in the dark by direct protein-protein interaction with the phytochrome-interacting factors PIF3 and PIF4 (de Lucas et al., 2008; Feng et al., 2008). DELLA binding prevents PIF3 and PIF4 transcriptional activators from binding to their promoter elements, thus blocking their transcription. Thus, it appears that DELLA may repress GA responses both with and without association with promoter elements.

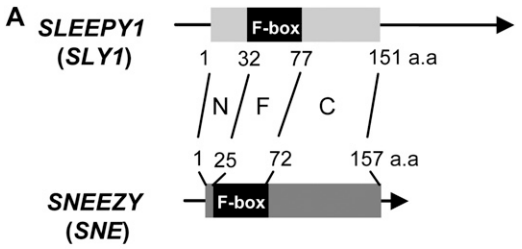
The SCF^{SLY1} E3 ubiquitin ligase complex lifts DELLA repression by targeting DELLA for destruction by the ubiquitin-proteasome pathway. The SCF complex is composed of a Skp1 homolog termed ASK (for Arabidopsis Skp1), a Cullin homolog, an Rbx1 homolog, and an F-box protein that binds a specific target. There are 23 ASK genes, five Cullins (CULs), two Rbx1 homologs, and 694 F-box proteins in Arabidopsis (Gagne et al., 2002; Gray et al., 2002; Risseeuw et al., 2003). The F-box protein binds to the Skp1 homolog of the complex through direct protein-protein interaction via the F-box domain. The F-box protein generally binds the substrate protein via the C-terminal domain. Yeast two-hybrid data indicate that the SLY1 protein can bind to DELLA protein via the C-terminal domain (Dill et al., 2004; Fu et al., 2004). GA binding stimulates the ability of the GA receptor GA-INSENSITIVE DWARF1 (GID1) to bind DELLA proteins. Based on yeast three-hybrid data, Arabidopsis GID1-GA binding to DELLA appears to increase the affinity of the F-box protein SLY1 for DELLA (Griffiths et al., 2006). Thus, GA stimulates SCF^{SLY1} binding to DELLA proteins, thereby allowing SCF^{SLY1} to catalyze the polyubiquitination of DELLA protein. Addition of four ubiquitin moieties to a target protein triggers its recognition and proteolysis by the 26S proteasome (Smalle and Vierstra, 2004). It appears that DELLA is destroyed via the ubiquitin-proteasome pathway, because both mutations in *SLY1* and 26S proteasome inhibitors result in stabilization and increased accumulation of DELLA protein in the presence of GA (McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004).

We first identified MIF21.6 as a homolog of *SLY1* in Arabidopsis by BLAST database search (At5g48170; McGinnis et al., 2003), and this predicted protein was subsequently named SNE (Strader et al., 2004) or SLY2 (Fu et al., 2004). Functional analysis showed that *SNE/SLY2* overexpression can rescue the *sly1* mutant phenotype (Fu et al., 2004; Strader et al., 2004). We chose to refer to this gene as *SNE* rather than as *SLY2* because it is not yet clear whether *SNE/SLY2* normally shows functional overlap with *SLY1*. Moreover, it is still possible that *SNE/SLY2* may have some functions that do not overlap with those of *SLY1*. This study examines the relative roles of the two F-box proteins, SLY1 and SNE/SLY2, first through a careful analysis of their ability to complement the *sly1-10* mutation, then by determining which domains are required for function, and finally by examining the ability of these proteins to bind to other proteins involved in GA signaling, including the CUL1 subunit of the SCF complex and the DELLA protein RGA in Arabidopsis. The ability of SNE to regulate the accumulation of DELLAs RGA and RGL2 is examined. Finally, we established the interaction of the SLY1 protein with the GA receptor GID1 through coimmunoprecipitation.

RESULTS

Sequence Homology of SLY1 and SNE/SLY2

The homology of *SLY1* to *SNE* is diagrammed in Figure 1 at both the level of mRNA and predicted protein sequence. The 453-bp *SLY1* gene encodes a



B

Domain	DNA	Amino acid
N terminal (N)	55.4%	16.7%
F-box (F)	55.0%	26.8%
C terminal (C)	64.2%	45.1%
Total	55.1%	33.1%

Figure 1. Domain structure and homology of the *SLY1* and *SNE* genes and predicted protein sequences. A, Gene structure of two F-box proteins, SLY1 and SNE, consisting of N-terminal (N), F-box (F), and C-terminal (C) domains. The number below the SLY1 and above the SNE gene shows the amino acid (a.a.) number, where the first amino acid is +1. B, DNA and amino acid homology between SLY1 and SNE in the N-terminal, F-box, and C-terminal domains.

predicted 151-amino acid protein, whereas the 471-bp *SLY2/SNE* open reading frame (ORF) encodes a predicted protein of 157 amino acids. Both gene sequences contain no introns (McGinnis et al., 2003). Based on InterPro motif analysis (http://www.ebi.ac.uk/Tools/InterProScan/; Hunter et al., 2009), the predicted SNE protein contains an F-box motif composed of amino acids 25 to 72. The presence of this functional domain suggests that *SNE* encodes an F-box subunit protein of an SCF E3 ubiquitin ligase (Fig. 1; Supplemental Fig. S1). The homology of SLY1 and SNE was considered by dividing the proteins into three domains: the F-box domain, the N-terminal domain, and the C-terminal domain. Previously published yeast two-hybrid studies indicated that the C-terminal domain of SLY1 is required for interaction with DELLA proteins (Dill et al., 2004; Fu et al., 2004). The full-length *SLY1* and *SNE* genes have 55.1% DNA and 33.1% amino acid sequence homology. The N-terminal domains before the F-box domain (SLY1, amino acids 1–32; SNE, 1–24) have 55.4% DNA/16.7% amino acid homology; the F-box domain itself (SLY1, amino acids 32–77; SNE, 25–72) has 55.0% DNA/26.8% amino acid homology; and the C-terminal domain (SLY, amino acids 78–151; SNE, 73–157) has 64.2% DNA/45.1% amino acid homology. Thus, the C-terminal region, which is believed to be involved in SLY1 interaction with its DELLA target protein, contains the highest degree of sequence homology with SNE (Fig. 1B).

Functional Analysis of *SLY1* and Its Homolog *SNE* in Arabidopsis

Previous research showed that the *SNE* gene sequence was able to partly suppress the *sly1* mutant phenotypes when expressed under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Fu et al., 2004; Strader et al., 2004). This study used deletion analysis to further explore the functional importance of each of the three domains in controlling plant height, fertility, and DELLA destruction. An N-terminal hemagglutinin (HA) epitope tag was fused in-frame to *SLY1* and *SNE* full-length ORFs and ORFs containing deletions of each of the three domains defined in Figure 1 and placed under the control of the 35S promoter. The full-length constructs are referred to as *HA-SLY1* and *HA-SNE*, whereas the constructs lacking the N-terminal, F-box, and C-terminal domains are referred to as *ΔN*, *ΔF*, and *ΔC* alleles, respectively (Supplemental Table S1). These chimeric constructs and an HA vector-only control were then transformed into the *sly1-10* mutant to determine whether they were able to rescue the *sly1-10* dwarfism and fertility phenotypes. Supplemental Table S1 shows the number of transgenic plants obtained for each construct and the number of transformed plants that appeared to rescue the *sly1-10* dwarf phenotype in the T2 generation. Based on initial observations, the *HA-SLY1* construct fully complemented the *sly1-10* dwarfism, whereas *HA-SNE*, *HA-sly1ΔN*, and *HA-sneΔN* partially complemented the dwarfism. The remaining constructs failed to complement.

When the expression of each chimeric protein was examined by protein-blot analysis using the HA antibody, bands corresponding to the predicted full-length and truncated HA fusion proteins were observed (Supplemental Fig. S2). For each construct, two lines showing similar levels of protein expression were used for further analysis. However, it was noted that HA-SNE protein levels were on the whole lower than those of the HA-SLY1 constructs and that loss of the C-terminal domain led to some decrease in protein accumulation.

The degree to which each *HA-SLY1* and *HA-SNE* construct was able to complement the *sly1-10* mutation was determined by observing 30-d-old plants (Fig. 2A) and 45-d-old plants (Supplemental Fig. S3) and by measuring final plant height and the number of seeds per silique (Fig. 3). The full-length *HA-SLY1* construct fully complemented the *sly1-10* mutant phenotypes, including dwarfism, infertility, and delayed flowering (Fig. 2A; Supplemental Table S1). The *HA-sly1ΔN* construct appeared to fully rescue the final plant height and fertility phenotypes; however, these plants were shorter than the wild-type and the *sly1-10 HA-SLY1* lines at 30 d due to slower plant growth (Figs. 2A and 3). *HA-sly1ΔF* and *HA-sly1ΔC* resulted in no apparent rescue of *sly1-10* phenotypes compared with the untransformed and the vector-only controls. These results indicated that the F-box and C-terminal domains of SLY1 are required for SLY1 function, whereas the N-terminal region of SLY1 is required for full functionality of the SLY1 protein. The *HA-SNE* construct strongly complemented the *sly1-10* mutation, resulting in a final plant height approximately 89% of the wild-type plant height and in fertility approximately 84% of the wild type (Figs. 2B and 3; Supplemental Fig. S3B). The *sly1-10 HA-sneΔN* did not show significant restoration of plant height in 30-d-old plants but appeared to result in some restoration of plant height and fertility in 45-d-old plants (Figs. 2A and 3; Supplemental Fig. S3C). The *sly1-10 HA-sneΔF* and the *sly1-10 HA-sneΔC* showed no suppression of *sly1-10* phenotypes (Figs. 2A and 3). Thus, the F-box and C-terminal domains are required for *SLY1* function and for *SNE* rescue of the *sly1-10* plant height and fertility phenotypes. These results are consistent with previous reports indicating that *SNE/SLY2* overexpression rescued *sly1* mutant phenotypes (Fu et al., 2004; Strader et al., 2004).

The Effect of *HA-SLY1* and *HA-SNE* Constructs on DELLA Protein Accumulation

To determine if the restoration of *sly1-10* plant height was associated with a decrease in the accumulation of DELLA repressors of stem elongation, protein-blot analysis was performed to detect DELLAs RGA and GAI (Fig. 2C). Expression of *HA-SLY1* and *HA-sly1ΔN* was associated with a considerable de-

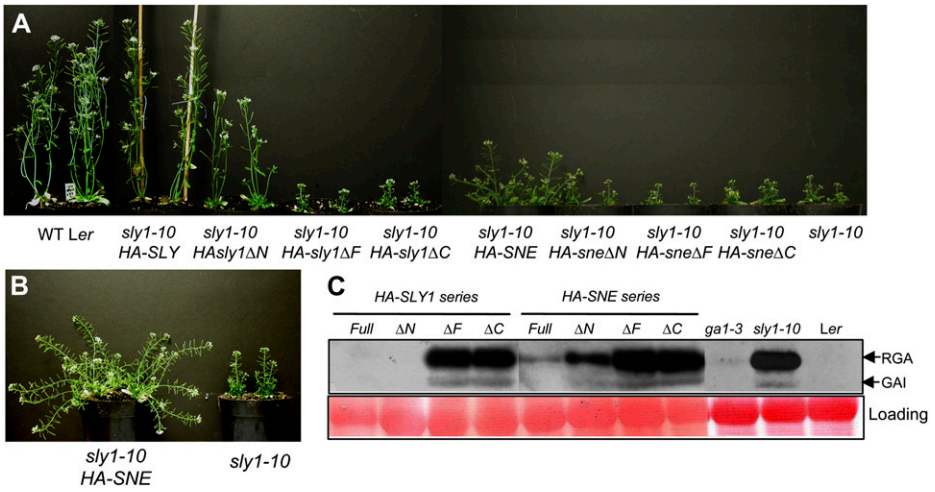


Figure 2. The effect of N-terminal, C-terminal, and F-box domain deletions on the ability of *HA-SLY1* and *HA-SNE* to rescue *sly1-10*. A, Shown are 30-d-old wild-type (WT) L*er*, *sly1-10*, and *sly1-10* transformed with the indicated HA fusion constructs. B, Shown are 45-d-old *sly1-10* and *sly1-10 HA-SNE*. The *HA-SNE* construct partially rescued the dwarfism. C, DELLA RGA and GAI protein accumulation in wild-type L*er*, *ga1-3*, *sly1-10*, and *sly1-10* transformed with constructs described above was determined by anti-RGA immunoblot analysis of 40 μg of total protein extracted from 21-d-old rosette leaves.

crease in DELLA protein accumulation in 21-d-old *sly1-10* seedlings, whereas expression of *HA-sly1ΔF* and *HA-sly1ΔC* did not result in decreased DELLA protein accumulation. This suggests that the *HA-SLY1* and *HA-sly1ΔN* constructs relieve DELLA repression through ubiquitination and proteolysis and that the F-box and C-terminal regions are required for this E3 ubiquitin ligase activity. Expression of *HA-SNE* also resulted in decreased DELLA protein accumulation in *sly1-10* seedlings, suggesting that the *HA-SNE* fusion protein can partly replace SLY1 function in DELLA protein ubiquitination and destruction during stem elongation (Fig. 2C). Only a slight decrease in DELLA protein accumulation was observed in *sly1-10* seedlings transformed with *HA-sneΔN*, suggesting that the N terminus may be needed for full function. Alternatively, the fact that HA-SLY1ΔN and HA-SNEΔN proteins accumulate at lower levels than HA-SLY1 and HA-SNE may explain their lesser effect on DELLA protein accumulation (Supplemental Fig. S2). The *HA-sneΔF* and *HA-sneΔC* constructs resulted in no significant decrease in DELLA protein accumulation in *sly1-10* seedlings, suggesting that the F-box and C-terminal domains are required for SNE/SLY2 to regulate DELLA protein accumulation.

Because the infertility phenotype of the *sly1-10* mutant was also fully suppressed by the *HA-SLY1* construct and partially suppressed by the *HA-sly1ΔN*, *HA-SNE*, and *HA-sneΔN* constructs (Fig. 3B), we examined whether this suppression is associated with decreased DELLA protein accumulation. Protein-blot analysis was performed to examine the accumulation of DELLA proteins RGA, GAI, and RGL2 in flower bud tissue from 30-d-old plants. Transformation of *sly1-10* with the *HA-SLY1* and *HA-SNE* constructs resulted in a dramatic decrease in DELLA RGA and GAI accumulation compared with untransformed *sly1-10* (Supplemental Fig. S4); transformation with *HA-sly1ΔN* resulted in a large decrease; transformation with *HA-sneΔN* resulted in a slight decrease; and transformation with *HA-sly1ΔF*, *HA-sly1ΔC*, *HA-sneΔF*, and *HA-sneΔC* resulted in no decrease in DELLA RGA and GAI protein accumulation. These results suggested that the suppression of infertility by *SNE* gene overexpression was, at least in part, the result of decreased RGA and GAI protein accumulation. Interestingly, a decrease in RGL2 protein levels was observed when *sly1-10* was transformed with *HA-SLY1* and *HA-sly1ΔN* (Supplemental Fig. S4A), but no decrease in RGL2 was observed when *sly1-10* was transformed with *HA-SNE* (Supplemental Fig. S4B). This suggests that SNE is able to direct the degradation of DELLAs RGA and GAI but not of the DELLA RGL2.

Because RGL2 is the major DELLA repressing seed germination, we next examined the effect of *HA-SNE* and *HA-SLY1* overexpression on DELLA accumulation and on seed germination efficiency in *sly1-10*. The *sly1-10* mutant has increased seed dormancy and when after-ripened germinates more slowly than the wild type (Ariizumi and Steber, 2007). The *sly1-10* seed germination phenotype was fully rescued by *HA-SLY1* and partially rescued by *HA-SNE* overexpression after 4 d of incubation (Fig. 4A). Protein-blot analysis showed that *HA-SLY1* overexpression was associated with the disappearance of both RGA and RGL2. In contrast, the rescue of seed germination by *HA-SNE* overexpression was associated with a decrease in RGA protein but not in RGL2 protein levels (Fig. 4B). This suggests that an SCF^{SNE} E3 complex may be able to ubiquitinate and target RGA but not RGL2 for destruction.

Evidence for the Formation of SCF^{SLY1} and SCF^{SNE} Complexes and for Protein Interaction with DELLA RGA

The fact that *sly1-10* rescue by overexpression of full-length and N-terminal deletions of *SLY1* and *SNE* is associated with a decrease in DELLA protein accumulation suggested that these two F-box proteins form SCF complexes that interact with and regulate DELLA proteins by ubiquitination. If this is true, we would expect SLY1 and SNE to interact with DELLA RGA

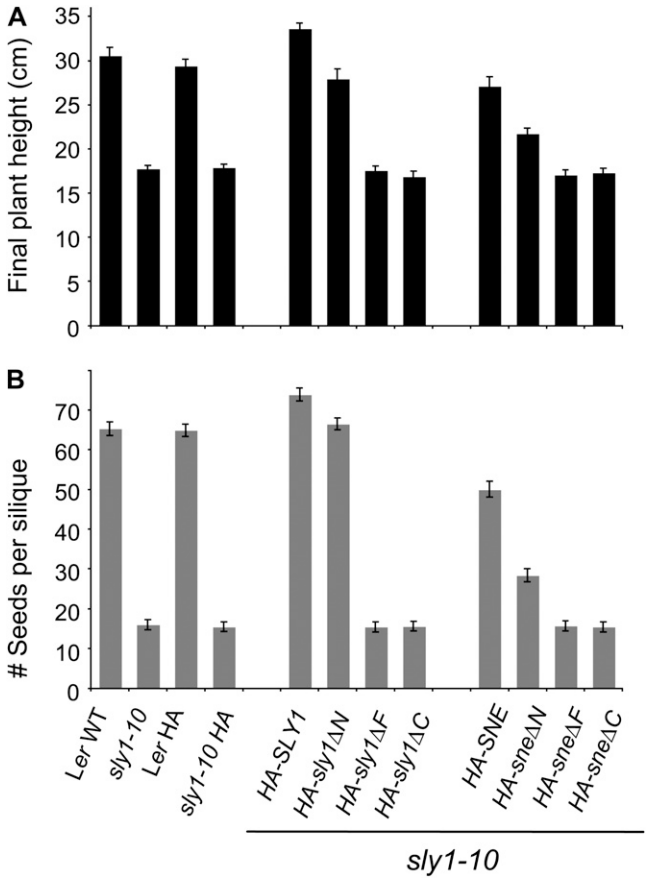


Figure 3. Final plant height and fertility in the wild-type (WT) L*er*, *sly1-10,* and *sly1-10* transformed with the indicated constructs. A, Final plant height (cm) was determined after 120 d of incubation in a growth chamber. B, Fertility was determined based on the number of seeds per silique. Wild-type L*er* and *sly1-10* plants transformed with the HA vector were used as controls.

protein and with the SCF CUL1 subunit via the F-box domain. To examine in planta protein-protein interactions, we performed coimmunoprecipitation experiments using an HA antibody matrix to affinity purify full-length and deletion alleles of HA-SLY1 and HA-SNE proteins from seedling extracts. Protein-blot analysis of these extracts showed that CUL1 coimmunoprecipitated with the HA-SLY1, HA-sly1ΔN, HA-sly1ΔC, HA-SNE, HA-sneΔN, and HA-sneΔC fusion proteins but not with the HA-vector control or with the HA-sly1ΔF and HA-sneΔF proteins (Fig. 5; Supplemental Fig. S5). This result suggests that SLY1 and SNE proteins form SCF complexes that include CUL1 and that complex formation requires the F-box domain. Protein-blot analysis also showed that the DELLA protein RGA coimmunoprecipitated with the HA-SLY1, HA-sly1ΔN, HA-sly1ΔF, HA-SNE, HA-sneΔN, and HA-sneΔF fusion proteins but not with the HA-sly1ΔC and HA-sneΔC proteins and not with the HA-vector control. The addition of GA to the protein extract increased the quantity of RGA coimmunoprecipitated with HA-SLY1, but the interaction was still observed without GA addition due to the presence of endogenous GA in seedling extracts. This result indicates that both SLY1 and SNE can interact with RGA protein in planta and that the C-terminal domain of SLY1 and SNE are required. Taken together, these data indicate that SCF^{SNE} and SCF^{SLY1} E3 ubiquitin ligases form in vivo and regulate DELLA RGA by direct protein interaction.

The Effect of SNE Overexpression on Plant Growth Habit

In addition to partly suppressing the *sly1-10* dwarf and infertility phenotypes, we observed that *HA-SNE* plants show an aberrant plant growth habit consisting of decreased apical dominance and a prone growth habit (Fig. 2, A and B). All 17 *sly1-10 HA-SNE* plants showed a similar phenotype where secondary lateral shoots formed at a wide angle to the primary shoot (Supplemental Fig. S3). To determine whether this *HA-SNE* overexpression phenotype was dependent on the *sly1-10* background, wild-type ecotype Landsberg *erecta* (L*er*) and the GA biosynthesis mutant *ga1-3* were transformed with the *HA-SNE* overexpression construct. It appeared that wild-type L*er HA-SNE* plants also showed the decreased apical dominance

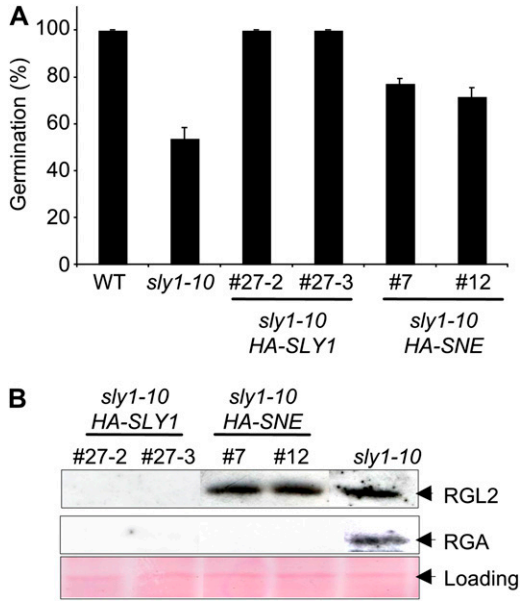


Figure 4. *SNE* overexpression partial rescue of the *sly1-10* seed germination phenotype is associated with decreased DELLA RGA but not RGL2 protein accumulation. A, Germination of *sly1-10* and two independent *sly1-10* lines transformed with *HA-SLY1* (#27-2 and #27-3) and *HA-SNE* (#7 and #12). Seeds were incubated on MS agar plates at 4°C for 3 d, followed by 22°C for 4 d. WT, Wild type. B, Protein-blot analysis of RGA and RGL2 in *sly1-10 HA-SLY1* and *sly1-10 HA-SNE* seeds imbibed on MS agar plates at 4°C for 3 d, followed by 22°C for 24 h. Protein was detected with anti-RGA and anti-RGL2. Sixty micrograms of total protein was loaded. [See online article for color version of this figure.]

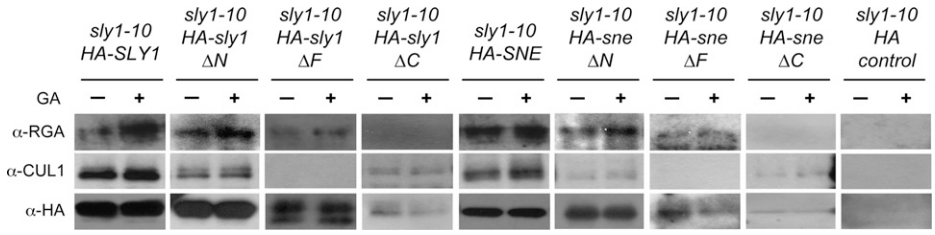


Figure 5. HA-SLY1 and HA-SNE fusion proteins interact with DELLA RGA and CUL1 protein in planta. Protein was extracted from 12-d-old *sly1-10* plants transformed with the indicated constructs and incubated with HA matrix agarose in the presence of 0.1% ethanol (mock) or 100 μM GA_3. Immunoprecipitated protein was loaded on an SDS-PAGE gel and detected with anti-HA antibody. Coimmunoprecipitated protein was detected with anti-RGA and anti-CUL1.

and prone growth habit (Supplemental Fig. S6A). Thus, the aberrant growth phenotype is not dependent on the *sly1-10* background. The *HA-SNE* overexpression construct did not rescue the dwarfism of the *ga1-3* plants. GA treatment of the *ga1-3 HA-SNE* plants rescued the *ga1-3* dwarf and fertility phenotypes but resulted in the same aberrant prone growth habit seen in the wild-type L*er* transformants (Supplemental Fig. S6B). These results suggest that the aberrant growth phenotype is a direct result of *SNE* overexpression and does not result from an interaction between *SNE* overexpression and the *sly1* mutant background.

Expression Analysis of *SNE/SLY2*

Previous reverse transcription-PCR and northern-blot analyses showed that the *SLY1* mRNA is present throughout the plant, whereas the *SNE* mRNA is mainly present in flowers and to a lesser extent in stems (Strader et al., 2004). The meta-analysis tool Genevestigator (https://www.genevestigator.com/gv/index.jsp) and the Arabidopsis microarray database were used to compare the spatiotemporal pattern of *SLY1* and *SNE* gene expression (Hruz et al., 2008). The level of *SLY1* mRNA accumulation appears to be 3- to 5-fold higher than that of *SNE* in most tissues, with the two genes showing similar expression trends in a developmental analysis (Supplemental Fig. S7A). *SNE* transcript appeared highest in callus, shoot apex, and root endodermis (Supplemental Fig. S8) and lowest in germinating seeds and seed tissues (endosperm, seed coat). *SLY1* expression appeared highest in the abscission zone, hypocotyl, shoot apex, and petals and lowest in pollen and stigma.

GUS transcriptional fusions were used to further compare the expression patterns of these F-box genes. A construct containing the *SLY1* promoter region fused to the GUS reporter gene was transformed into wild-type L*er* and the expression pattern analyzed by histochemical staining for GUS activity (Ariizumi et al., 2002a). A total of six independent *SLY1p*-GUS lines were examined; representative GUS expression patterns are shown in Supplemental Figure S9A. Consistent with the previous reverse transcription-PCR analysis (McGinnis et al., 2003), *SLY1p*-GUS expression was found in most parts of the plant. Strong expression was seen in the cotyledons and hypocotyls of 6-d-old seedlings, in the vasculature of seedlings, leaves, and roots, in the primary root tip, and in the anthers, filaments, petals of flowers, and the receptacle of siliques. In contrast, the expression of *SNE*-GUS appears to be much less widespread. An enhancer trap line in which a T-DNA containing the GUS reporter was inserted just before the *SNE* translational start site was used to examine expression in germinating seeds, seedlings and seedling roots, mature leaves, and flowers. Low-level *SNE-GUS* expression was evident only in flower anthers (Supplemental Fig. S9B).

Protein Interaction between SLY1 and the GID1b GA Receptor

Based on our results, *SLY1* most likely is the predominant F-box protein functioning in GA signaling because it is expressed at higher levels and because it can target more DELLA proteins for destruction. In the current model of GA signaling, the formation of the GID1-GA-DELLA complex allows SCF^{SLY1} to bind to and ubiquitinate DELLA protein (Hirano et al., 2008). This model predicts that SLY1 should form a complex that includes not only DELLA but also GID1 protein. Coimmunoprecipitation was used to examine whether or not the SLY1 protein interacts with GID1b in planta using *sly1-10* plants transformed with an *HA-GID1b* and *FLAG-SLY1* epitope-tagged fusion proteins expressed on the 35S promoter. When the FLAG-SLY1 protein was affinity purified from 12-d-old seedling extracts using a FLAG antibody matrix, protein-blot analysis showed that HA-GID1b coimmunoprecipitated with FLAG-SLY1 and that this interaction increased with the addition of GA to the plant protein extract (Fig. 6). This indicates that SCF^{SLY1} interacts with a complex that includes GID1b.

DISCUSSION

Previous work suggested that the *SLY1* homolog *SNE/SLY2* may encode a second F-box protein functioning in GA signaling, since overexpression of *SNE*

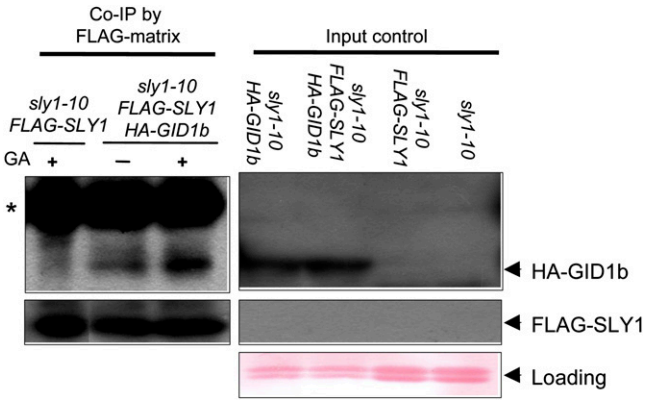


Figure 6. Interaction of SLY1 and GID1 in coimmunoprecipitation (Co-IP) assays. The coimmunoprecipitation experiment was performed using protein extracted from 12-d-old *sly1-10* plants transformed with *HA-GID1a* and/or *FLAG-SLY1*. The protein extract was incubated with FLAG matrix agarose in the presence of 0.1% ethanol (mock) or 10 μM GA_3 and loaded on an SDS-PAGE gel. Protein-blot analysis was performed using anti-FLAG and anti-HA antibodies. The asterisk indicates a background band. Forty micrograms of total protein was loaded (input). [See online article for color version of this figure.]

on the 35S promoter partly rescued the *sly1-10* dwarf phenotype and resulted in reduced DELLA RGA accumulation (Fu et al., 2004; Strader et al., 2004). This paper examined the mechanism by which SNE substitutes for SLY1 in the regulation of GA signaling, whether the SNE protein could form an SCF E3 ubiquitin ligase complex in planta, and the relative roles of SLY1 and SNE/SLY2 in GA signaling. Finally, we examined the interaction of SLY1 with the GA receptor GID1.

Functional Analysis of SLY1 and SNE F-Box Proteins in GA Signaling

A side-by-side comparison was conducted to determine the domains required for HA-SLY1 and HA-SNE to rescue *sly1-10* and to interact with the SCF subunit CUL1 and the DELLA RGA proteins in planta (Fig. 5). Consistent with previous results, HA-SLY1 gave complete rescue of the germination, fertility, and plant height phenotypes of *sly1-10*, whereas HA-SNE only partly rescued these phenotypes (Figs. 2–4; Supplemental Fig. S3). In both cases, the F-box and C-terminal domains were required for function. Deletion of the N-terminal domains of HA-SLY1 and HA-SNE decreased the capacity of these proteins to complement the *sly1-10* phenotype (Figs. 2 and 3; Supplemental Fig. S3). Thus, it appears that although the N terminus is not essential, it does play some role. Consistent with the functional data, it was observed that the C terminus was required for HA-SLY1 and HA-SNE to interact with the DELLA RGA protein in planta in coimmunoprecipitation experiments (Fig. 5). Previous work demonstrated the interaction of SLY1 with DELLA protein in vitro, by yeast two-hybrid assay, and by pull-down assay (Dill et al., 2004; Fu et al., 2004; Wang et al., 2009) as well as the association of SLY1 with the SCF complex protein CUL1 in vitro (Wang et al., 2009). In this study, both HA-SLY1 and HA-SNE coimmunoprecipitated with CUL1 in planta, and this interaction required the F-box domain (Fig. 5), indicating that it is required for SCF complex formation. The F-box domain is required for the F-box protein to interact with the Skp1 subunit during SCF complex formation (Gagne et al., 2002, 2004). Both DELLA RGA and CUL1 proteins coimmunoprecipitate with the HA-SLY1ΔN and HA-SNEΔN proteins, indicating that the N-terminal domain is not absolutely essential for these protein-protein interactions (Fig. 5). It is possible that less RGA coimmunoprecipitates with HA-SLY1ΔN than with HA-SLY1 protein because the N-terminal deletion accumulates at lower levels (Supplemental Fig. S2). Taken together, these results indicate that SNE can function in a manner very similar to SLY1, forming an SCF complex in planta that can function in GA signaling through interaction with the DELLA RGA.

SNE Function Partly Overlaps with SLY1 Function in GA Signaling

Overexpression of *HA-SNE* partially rescued the dwarfism, infertility, and germination phenotypes of the *sly1-10* mutant (Figs. 2–4; Supplemental Fig. S3). This rescue was correlated with decreased levels of the DELLA proteins RGA and GAI, suggesting that an SCF^{SNE} E3 ubiquitin ligase can regulate these DELLA proteins via ubiquitination and destruction by the 26S proteasome (Figs. 2 and 4; Supplemental Fig. S4). Several observations support this theory, including that HA-SNE interacts with DELLA RGA and forms an SCF complex in planta (Fig. 5). Both interaction with CUL1 and rescue of the *sly1-10* phenotypes required the F-box domain, indicating that formation of an SCF^{SNE} complex is required for its function in GA signaling (Figs. 2, 3, and 5). This is consistent with previously published data showing that SNE protein can interact with Arabidopsis Skp1 homologs in the yeast two-hybrid system (Fu et al., 2004). Finally, *HA-SNE* overexpression does not rescue the dwarfism or flowering phenotypes of the GA biosynthesis mutant *ga1-3* in the absence of GA (Supplemental Fig. S6C). This suggests that *SNE* acts through GA signaling rather than via a parallel pathway and that the SCF^{SNE} E3 ubiquitin ligase targets DELLA for destruction via a GA-dependent mechanism similar to the SCF^{SLY1} (McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004).

The function of *SNE/SLY2* in GA signaling appears to only partly overlap with that of *SLY1*. Whereas overexpression of *HA-SLY1* caused a decrease in DELLA RGA, GAI, and RGL2 protein accumulation in seeds and flowers of the *sly1-10* mutant, overexpression of *HA-SNE* caused a decrease in DELLA RGA and GAI but not in DELLA RGL2 protein accumulation (Fig. 4; Supplemental Fig. S4). This suggests that SNE can only regulate a subset of DELLA proteins.

Future work will need to examine whether SNE protein shows stronger binding to RGA and GAI than to RGL2 or whether SNE binds to RGL2 but fails to ubiquitinate RGL2. RGL2 is a key DELLA protein controlling seed germination as well as flowering and fertility (Lee et al., 2002; Cheng et al., 2004; Tyler et al., 2004; Cao et al., 2005, 2006). Thus, failure to regulate RGL2 may partly explain why *HA-SNE* overexpression only partly rescues the fertility and germination of *sly1-10* (Figs. 3 and 4). This, taken together with the facts that the *SNE* mRNA accumulates at lower levels than the *SLY1* mRNA (Supplemental Figs. S7–S9) and that the *sly1* mutant shows strong dwarfism and infertility phenotypes in the presence of the normal *SNE* gene, supports the conclusion that *SLY1* is the major F-box protein contributing to GA signaling in Arabidopsis.

The *SNE* gene can regulate DELLA proteins, but might it also regulate genes that are not part of GA signaling? The overexpression of *HA-SNE* not only rescued the GA-insensitive phenotypes of *sly1-10* but also resulted in changes in growth habit, including loss of apical dominance and a prone growth habit where stems were angled downward (Fig. 2, A and B; Supplemental Fig. S3). The phenotype was not dependent on the *sly1-10* background, as these phenotypes were also observed when *HA-SNE* was transformed into wild-type L*er* and *ga1-3* (Supplemental Fig. S6). Digital northern analysis shows that the *SNE* mRNA is expressed in the shoot apex and in callus cells (Supplemental Fig. S8). It is not clear whether the prone phenotype does or does not result from changes in GA signaling. Thus, future research will need to examine whether *SNE* regulates an alternative target involved in meristem function and/or GA signaling. Previous work demonstrated that *SNE* mRNA is expressed in the endodermis and the quiescent center of the root, whereas *SLY1* is only expressed in the stele (Cui and Benfey, 2009). Cui and Benfey (2009) hypothesized that *SNE* may be important for DELLA regulation in the root cells that do not show *SLY1* expression.

SLY1 Physical Interactions with the GID1 GA Receptor

This study next examined the interaction of the SCF^{SLY1} E3 ubiquitin ligase with the GA receptor, GID1. The current model of GA signaling proposes that the F-box protein SLY1 of Arabidopsis and the orthologous F-box GID2 of rice trigger DELLA destruction only when DELLA protein is bound by the GA receptor GID1. GID1 protein affinity for DELLA protein increases when GID1 binds GA hormone (Griffiths et al., 2006; Nakajima et al., 2006; Willige et al., 2007). Yeast three-hybrid analysis has demonstrated that the rice F-box GID2 only binds to DELLA protein when it is in the GID1-GA-DELLA complex (Hirano et al., 2010). It appears that GA may also stimulate SLY1-GID1 interaction in Arabidopsis, as coimmunoprecipitation demonstrated that FLAG-SLY1 forms a complex with HA-GID1b (Fig. 6). Based on the work of Hirano et al. (2010), the GID1-SLY1 complex likely includes a DELLA protein. This is, to our knowledge, the first in planta demonstration that the F-box protein forms a complex that includes the GA receptor, thus supporting the rice model derived from yeast three-hybrid data.

Implications for GA Signaling

The research presented here has several broader implications for GA signaling. This study and others indicate that SLY1 is the major F-box protein regulating DELLA protein in GA signaling (Dill et al., 2004; Fu et al., 2004; Ariizumi and Steber, 2007). It appears that SNE can regulate DELLAs RGA and GAI but not RGL2 (Fig. 4; Supplemental Fig. S4). However, based on the unusual growth phenotype of *SNE* overexpression lines and the apparently unique role of *SNE* in the control of root elongation, it appears that SCF^{SNE} may play a specialized role that will need to be considered in future studies (Supplemental Fig. S3; Cui and Benfey, 2009). This study demonstrated that the SLY1 protein exists in complex with the GID1b receptor protein in planta. This result supports the model that SCF^{SLY1} binding to and ubiquitination of DELLA protein is stimulated when DELLA is in complex with GID1 and GA hormone (Fig. 6; Hirano et al., 2010), resulting in GA-stimulated ubiquitination and destruction of DELLA (McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana*) L*er*, as well as *sly1-10* and *ga1-3* mutations in the L*er* background, were obtained as described previously (Koornneef and van der Veen, 1980; Ariizumi et al., 2008). The germination of *ga1-3* seed was stimulated by imbibing in 10 μM GA_4 for 3 d at 4°C and then washing with sterile water. Seeds were sterilized and imbibed in sterile water for 3 d at 4°C to break dormancy, and then all seeds were transferred to half-strength Murashige and Skoog (MS) salts (Sigma-Aldrich)/0.8% agar (MS agar) and incubated at 22°C under constant fluorescent light for 10 to 14 d. Seedlings were transferred to soil and grown at 22°C under fluorescent light (16-h day; McGinnis et al., 2003) for growth rate and fertility comparisons. To determine the effect of GA treatment on plant growth, plants grown in soil were sprayed every 3 d with 10 μM GA_4.

A *sly1-10* line transformed with the *FLAG-SLY1* and *HA-GID1b* translation fusions was constructed by crossing the *sly1-10 FLAG-SLY1* line (described below) to the previously constructed *sly1-10 HA-GID1b* line (Ariizumi et al., 2008). F1 and F2 seeds were sown on MS agar containing 20 mg L^{-1} hygromycin. F3 seeds from each F2 individual were harvested. Among several independent F3 plants, a *FLAG-SLY1* F3 individual expressing HA-GID1b protein at a level similar to the original *sly1-10 HA-GID1b* line was selected based on protein-blot analysis (Fig. 5).

Plasmid Construction and Plant Transformation

In-frame N-terminal fusions of the full-length or truncated *SLY1* (At4g24210; GenBank accession no. NM_118554) and *SNE* (At5g48170; NM_124191) coding regions to the HA epitope tag were constructed under the control of the constitutive CaMV 35S promoter. Fragments containing the full-length, N-terminal, and C-terminal deletions of the *SLY1* and *SNE* genes were obtained by PCR using gene-specific primer pairs (Supplemental Table S2). N-terminal deletions of the *SLY1* and *SNE* genes were generated using

the SLY-Ndel-F/SLYorf-R and SNE-Ndel-F/SNEorf-R primers, respectively. C-terminal deletions were generated using SLYorf-F/SLY-Cdel-R and SNEorf-F/SNE-Cdel-R primers. Internal deletions of the *SLY1* and *SNE* F-box domains were created using the following strategy. (1) The SLYorf-F and SLY-Fbox-R primers were used to amplify fragment A, while the SLY-Fbox-Fr and SLYorf-R primers were used to amplify fragment B. (2) These PCR products were gel purified and then diluted in water (1:1,000). (3) A reaction was set up using 0.5 μL of the A and B fragments as a PCR template, generating a deletion of the F-box by amplification with the SLY1orf-F and SLYorf-R primers (SNEorf-F and SNEorf-R). The proofreading enzyme KOD Hotstart DNA polymerase (Novagen) was used, and all constructs were confirmed by sequencing. These amplified PCR fragments were then phosphorylated and directly cloned as blunt-end fragments into the *Sma*I site of HA/pBluescript (Ariizumi et al., 2008) to obtain the *HA-SLY1*, *HA-SLY1ΔN*, *HA-SLY1ΔF*, *HA-SLY1ΔC*, *HA-SNE*, *HA-SNEΔN*, *HA-SNEΔF*, and *HA-SNEΔC* translational fusions. The *Hin*dIII-*Sac*I fragments from these HA-fused full-length and truncated SLY1/pBluescript plasmids were excised and cloned into the *Hin*dIII-*Sac*I site of T-DNA binary vector pBI101, while the *Hin*dIII-*Sac*I fragments from *HA-SNE*/pBluescript and *HA-SNEΔC*/pBluescript plasmids were excised and cloned into the *Hin*dIII-*Sac*I site of T-DNA binary vector pBI101H (Ariizumi et al., 2002b).

To construct the N-terminal FLAG-tagged SLY1 construct, the DNA sequence for three repeats of the FLAG epitope was amplified using FLAG-F and FLAG-R primers (Supplemental Table S2). The PCR fragment was phosphorylated with T4 polynucleotide kinase (Fermentas) and then blunt ligated into the *Eco*RV site of pBluescript II KS− vector to generate FLAG/pBluescript. PCR fragments containing the full-length *SLY1* ORF were directly cloned as a blunt-end fragment into the *Sma*I site of FLAG/pBluescript to obtain FLAG-SLY1/pBluescript. The *Hin*dIII-*Sac*I fragment from the FLAG-SLY1/pBluescript plasmids was cloned into the *Hin*dIII-*Sac*I site of T-DNA binary vector pGTV-HPT.

The constructs were transformed into *Agrobacterium tumefaciens* GV3101 by the freeze-thaw method (An et al., 1988). These constructs were transformed into *sly1-10*, wild-type L*er*, or *ga1-3* and selected on MS agar with 20 mg L^{-1} hygromycin (Clough and Bent, 1998). The expression of each chimeric protein of appropriate size was confirmed by protein-blot analysis using HA antibody (Supplemental Fig. S2). Two independent lines showing similar expression of each fusion protein were used for further analysis.

To create the GUS transcriptional fusion to the *SLY1* promoter, the 2.0-kb region upstream of the *SLY1* ORF was amplified with KOD Hotstart DNA polymerase using the SLY1pro-F and SLY1pro-R primers, phosphorylated, and cloned as a blunt-end fragment into the *Sma*I site of pBI121 (Clontech). These constructs were transformed into L*er* and selected on MS agar plus kanamycin (30 mg L^{-1}). Expression of the *SNE* gene was examined using the *sne-t2* line, which contains an enhancer trap T-DNA insertion 1 bp before the translational start site. The *sne-t2* line was isolated by PCR screening of the Sussman Basta line pools using the T-DNA left border primer JL202 (5′-CATTTTATAATAACGCTGCGGACATCTAC-3′) and the SNE-specific primer T-sly2F (5′-AAGAAACAGGAGTGGGAAAAAATCACG-3′) to obtain a 1.2-kb product (Krysan et al., 1999; Sussman et al., 2000). The pD991 T-DNA binary vector contains the −60 CaMV minimal promoter fused to the *uidA* GUS gene (http://www.dartmouth.edu/~tjack/et.html#pD991). This construct shows no expression until in the vicinity of an enhancer, allowing histochemical detection of *SNE-GUS* expression.

Gene Expression Analysis

GUS activity was examined according to Ariizumi et al. (2002a). Two-day-old, 6-d-old, 14-d-old, and 30-d-old plants were soaked in GUS solution and incubated for 2 to 24 h at 37°C. After incubation, plants were incubated in 70% ethanol for 24 h to bleach the pigments. In silico expression analysis was performed using the online tool Genevestigator (Hruz et al., 2008).

Protein Expression Analysis

Protein-blot analysis was used to examine DELLA protein (RGA, GAI, and RGL2) accumulation in nonsegregating T3 seedlings, flower buds, and seeds. Seeds were germinated under kanamycin selection and imbibed for 3 d at 4°C, followed by incubation at 22°C for 10 to 14 d prior to tissue collection. Flower buds were collected from 44-d-old plants. Total plant protein was extracted according to Silverstone et al. (2001) with a modified extraction buffer X (50 mM Tris, pH 6.8, 10% glycerol, and 1% SDS). Total protein was extracted from seed tissue as described by Ariizumi and Steber (2007). Forty micrograms of total protein from seedlings and flower buds, or 60 μg of total protein from seeds, was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Immobulon). Protein concentration was determined using the Bio-Rad protein assay, and even loading was confirmed by Ponceau membrane staining. Protein detection was performed using an enhanced chemiluminescence system (GE Healthcare) according to the manufacturer's protocol except that primary antibody incubations were conducted overnight. RGA and GAI proteins were detected using the RGA polyclonal antibody (1:25,000; Silverstone et al., 2001) and RGL2 using an RGL2 polyclonal antibody (1:25,000; Hussain et al., 2005) using controls described previously (Ariizumi and Steber, 2007). HA fusion proteins were detected using monoclonal HA antibody (1:25,000; Sigma-Aldrich). CUL1 protein was detected using CUL1 antibody (1:25,000; Chen et al., 2006). The anti-rabbit IgG-horseradish peroxidase (GE Healthcare) was used as a secondary antibody (1:250,000).

Germination Experiments

For germination experiments, 30 to 60 seeds from each genotype grown in the same incubator were sterilized with 10% bleach for 15 to 20 min and plated on MS agar or MS agar including 0 to 1.2 μM (+)-abscisic acid (PBI58; gift of S. Abrams). Percentage germination based on radicle emergence was determined following 3 d of incubation at 4°C followed by incubation under constant fluorescent light at 22°C. The average germination rate was calculated using three independent replicates.

Coimmunoprecipitation Experiment

For coimmunoprecipitation experiments, 10-d-old *sly1-10* plants transformed with the indicated constructs were incubated as a suspension of 100 μM MG132 in half-strength MS buffered with 5 mM MES, pH 5.5, for 2 h on ice. The cross-linking reagent dithiobis-succinimidyl propionate (Pierce) was added to a final concentration of 1 mM and further incubated for 30 min on ice. The cross-linking reaction was stopped by adding 2 mM Gly for 15 min on ice. Seedlings were washed twice with chilled phosphate-buffered saline (pH 7.6), blotted dry on Kimwipes, and ground under liquid N_2. Ground tissue was transferred to buffer A (100 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitor cocktail [Roche]) on ice, then centrifuged at 21,000*g* for 15 min. Protein concentration of the supernatant was determined, and 5 mg of protein extract was incubated with 40 μL of anti-HA matrix (Roche) in the presence of 100 μM GA_3 or no GA_3 (mock; 0.1% ethanol) for 16 h at 4°C. After the anti-HA matrix was washed three times with buffer A, it was resuspended in 1× SDS sample buffer, boiled for 3 min, and pelleted for 2 min at 14,000 rpm, and 20 μL of the supernatant was loaded for SDS-PAGE separation.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_118554 (SLY1), NM_124191 (SNE/SLY2), NM_126218 (RGA), NM_101361 (GAI), NM_111216 (RGL2), and NM_116166 (GID1b).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of predicted SLY1 and SNE proteins.

Supplemental Figure S2. Expression of HA fusion proteins in *sly1-10*.

Supplemental Figure S3. Vegetative phenotype of *sly1-10 HA-SNE* lines.

Supplemental Figure S4. DELLA protein expression in flower buds.

Supplemental Figure S5. Input control for Figure 5.

Supplemental Figure S6. Vegetative phenotype of L*er* and *ga1-3 HA-SNE* lines.

Supplemental Figure S7. Digital northern of *SNE* and *SLY1*.

Supplemental Figure S8. Developmental pattern of *SNE* and *SLY1* mRNA expression.

Supplemental Figure S9. GUS histochemical staining showing the pattern of expression from the *SLY1* and *SNE* promoters.

Supplemental Table S1. Complementation of *sly1-10*.

Supplemental Table S2. Primers used.

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