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Arabidopsis Paired Amphipathic Helix Proteins SNL1 and SNL2 Redundantly Regulate Primary Seed Dormancy via Abscisic Acid–Ethylene Antagonism Mediated by Histone Deacetylation[W]

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Histone (de)acetylation is a highly conserved chromatin modification that is vital for development and growth. In this study, we identified a role in seed dormancy for two members of the histone deacetylation complex in *Arabidopsis thaliana*, SIN3-LIKE1 (SNL1) and SNL2. The double mutant *snl1 snl2* shows reduced dormancy and hypersensitivity to the histone deacetylase inhibitors trichostatin A and diallyl disulfide compared with the wild type. SNL1 interacts with HISTONE DEACETYLASE19 in vitro and in planta, and loss-of-function mutants of SNL1 and SNL2 show increased acetylation levels of histone 3 lysine 9/18 (H3K9/18) and H3K14. Moreover, SNL1 and SNL2 regulate key genes involved in the ethylene and abscisic acid (ABA) pathways by decreasing their histone acetylation levels. Taken together, we showed that SNL1 and SNL2 regulate seed dormancy by mediating the ABA-ethylene antagonism in *Arabidopsis*. SNL1 and SNL2 could represent a cross-link point of the ABA and ethylene pathways in the regulation of seed dormancy.

INTRODUCTION

Adaptation of germination to the growth environment is decisive for fitness and survival of a plant species. Desiccated, dormant seeds display very low metabolic activities and absence of growth and development, ensuring survival in adverse conditions. This is especially important since plants cannot escape environmental threats. Understanding the mechanisms underlying the regulation of seed dormancy not only is of academic interest but also forms a basis for improving crop yield and quality (Gubler et al., 2005).

A role for chromatin remodeling–related genes in seed dormancy has recently been demonstrated. HISTONE MONO-UBIQUITINATION1 (HUB1) protein is required for mono-ubiquitination of histone H2B, and mutations in *HUB1* can cause reduced seed dormancy (Liu et al., 2007). Elimination of *HUB1* causes decreased expression of genes related to abscisic acid (ABA) metabolism and response, such as *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE9* (*NCED9*) and *ABA INSENSITIVE4* in seeds and also results in reduced ABA levels (Peeters et al., 2002), which partly explains the reduced seed dormancy phenotype. Mutations in *KRYPTONITE/SU(VAR)3-9 HOMOLOG4* (*KYP/SUVH4*), encoding a histone methyltransferase that is required for histone 3 lysine 9 (H3K9) dimethylation (Jackson et al., 2002), result in increased seed dormancy. On the other hand, *KYP/SUVH4*-overexpressing *Arabidopsis thaliana* plants show decreased dormancy (Zheng et al., 2012). KYP/SUVH4 is putatively involved in the regulation of balance between ABA and gibberellin (GA) to affect seed dormancy based on the altered response of the *kyp* mutant to ABA and GA biosynthesis inhibitors paclobutrazol (PAC) in seed germination. Mutants in FERTILIZATION-INDEPENDENT ENDOSPERM, an essential component of the Polycomb Repressive Complex 2, display genome-wide abolition of H3K27me3 and exhibit increased seed dormancy and germination defects (Bouyer et al., 2011). H3K27me3 deposition is very important for the transition from seed dormancy to germination (Müller et al., 2012). Moreover, mutation analysis of genes encoding HD2 family histone deacetylase (HDAC) shows that histone acetylation is also involved in seed dormancy and germination. Seed germination is enhanced in the *hd2a* null mutants; by contrast, *hd2c* mutants are restrained in germination relative to the wild-type seeds (Colville et al., 2011). Overexpression of *HD2C* confers an ABA-insensitive phenotype as monitored by enhanced germination and expression of

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the *LATE EMBRYOGENESIS ABUNDANT PROTEIN* class genes (Sridha and Wu, 2006). Taken together, the data indicate that histone modifications function in the regulation of seed dormancy and germination probably through affecting phytohormone metabolism and response.

The phytohormone ABA has been found to play a key role in the regulation of seed dormancy and germination based on genetic and physiological studies in *Arabidopsis* and other species (Finkelstein et al., 2008; Holdsworth et al., 2008; Graeber et al., 2012). Altered expression of genes regulating ABA levels or sensitivity leads to changed seed dormancy levels (Rodríguez-Gacio et al., 2009). Induction of NCED6 during imbibition increases ABA levels and is sufficient to prevent seed germination (Martínez-Andújar et al., 2011). The ABA 8′-hydroxylase mutants *cyp707a2* and *cyp707a1* and the double mutant *cyp707a2 cyp707a1* accumulate ABA in dry and imbibed seeds and exhibit enhanced seed dormancy (Saito et al., 2004; Okamoto et al., 2006). ABA perception by PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL) proteins plays a major role in the regulation of seed dormancy and germination; the enhanced seed germination of plants lacking three to six PYR/PYLs indicates quantitative regulation by this family of ABA receptors (Gonzalez-Guzman et al., 2012).

Ethylene can promote seed germination and repress seed dormancy establishment by antagonizing the ABA pathway (Matilla and Matilla-Vázquez, 2008; Linkies and Leubner-Metzger, 2012). Ethylene-insensitive *ethylene response1* (*etr1*) mutant seeds show enhanced dormancy, and their germination is ABA hypersensitive. This is at least partly caused by higher ABA contents in *etr1* seeds (Beaudoin et al., 2000; Chiwocha et al., 2005). Mutations in *ENHANCED RESPONSE TO ABA3* (*ERA3*)/*ETHYLENE INSENSITIVE2* (*EIN2*) genes lead to an overaccumulation of ABA and increased seed dormancy, suggesting that ERA3/EIN2 is a negative regulator of ABA synthesis (Ghassemian et al., 2000). Ethylene biosynthesis during seed germination is regulated by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) and is involved in counteracting the inhibiting effects of ABA on endosperm cap weakening and endosperm rupture, which is important for germination and dormancy (Linkies et al., 2009). The regulation of the ethylene-ABA antagonism during seed dormancy and germination is still poorly understood.

SNL1 and SNL2 belong to the SWI-INDEPENDENT3 (SIN3)-LIKE (SNL) protein family containing paired amphipathic helix repeats (Bowen et al., 2010). In yeast and mammals, studies have showed that SIN3 acts as a scaffold protein recruiting histone binding proteins RbAp46/RbAp48 and HDAC1/HDAC2, which deacetylate the core histones resulting in a transcriptionally repressed state of the chromatin (Grzenda et al., 2009). In *Arabidopsis*, SNL1 also possesses inherent transcription repression capability that is dependent on functional HDAC activity in vitro (Bowen et al., 2010). However, little is known about the importance of the SNL protein family in growth and development of land plants. In this study, we show strong genetic evidence for a redundant function of SNL1 and SNL2 in the regulation of seed dormancy in *Arabidopsis*. Our results also indicate that SNL1 and SNL2 could act as components of the HDAC-SNL complex to modulate the transcription of genes involved in the ethylene and ABA pathways by modifying their histone acetylation abundance. Enhanced ABA-ethylene antagonism in the *snl* mutants results in altered seed dormancy.

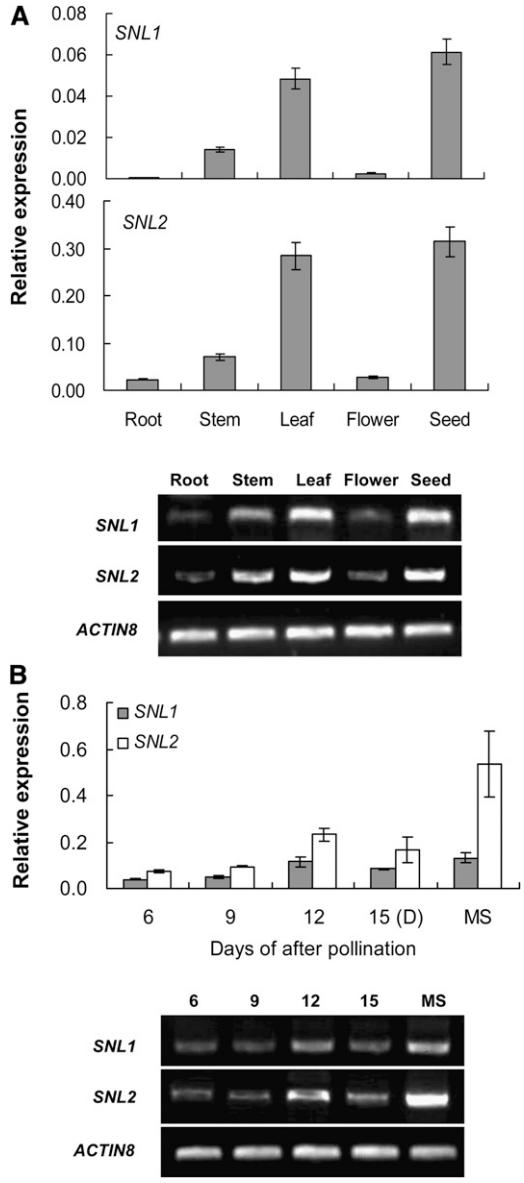

Figure 1. Expression Patterns of *SNL1* and *SNL2*.

(A) qRT-PCR analysis of *SNL1* and *SNL2* expression in *Arabidopsis* tissues. Results were normalized against the expression of *ACTIN8*. The mean values and SE were from three independent experiments. A representative result of gel electrophoresis is displayed at the bottom.
(B) qRT-PCR analysis of *SNL1* (gray) and *SNL2* (white) in developing siliques. The *x* axis presents the developmental stage after pollination. The mean values and SE were from three independent experiments. MS, mature seed. A representative result of gel electrophoresis is displayed at the bottom.

RESULTS

SNL1 and *SNL2* Positively Regulate Seed Dormancy

We analyzed the expression pattern of various genes encoding potential chromatin-regulating proteins using publicly available gene expression data and found that *SNL1* and *SNL2* show their

highest expression level in dry seeds (https://www.genevestigator.com/gv/plant.jsp) (see Supplemental Figure 1 online). This suggested a putative role of these genes in seeds, which was further investigated.

First, we examined the expression pattern of *SNL1* and *SNL2* in *Arabidopsis* using quantitative RT-PCR (qRT-PCR). This analysis confirmed a high expression of *SNL1* and *SNL2* in mature seeds (Figure 1A; see Supplemental Figure 2A online). A more detailed expression analysis showed that the expression of *SNL1* and *SNL2* gradually increased during seed maturation (Figure 1B; see Supplemental Figure 2B online). This pattern is reminiscent of that of dormancy genes such as *DELAY OF GERMINATION1* (*DOG1*) (Bentsink et al., 2006) and suggests a possible role of *SNL1* and *SNL2* in seed dormancy.

To study the role of the *SNL* genes in seeds, we obtained insertion mutants of *SNL1* and *SNL2*. Analysis of these mutants by RT-PCR (Figures 2A and 2B) showed that they are likely to be null mutants. The *SNL1* and *SNL2* genes are highly similar, sharing 71% identity between their protein sequences, and we created the double mutant *snl1 snl2* by crossing. The seed dormancy level of the single and double mutants was

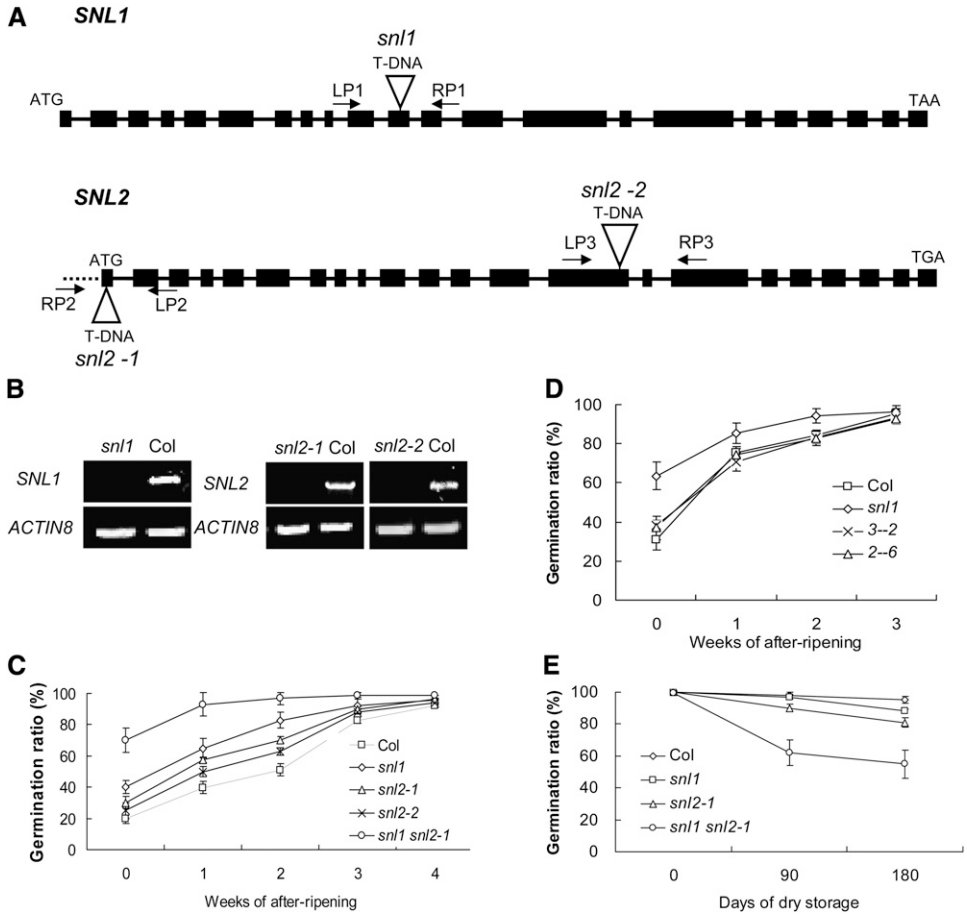

Figure 2. The Seed Dormancy Phenotype of *SNL1* and *SNL2* T-DNA Insertion Lines.

(A) Schematic diagrams of the *SNL1* and *SNL2* gene structures with the positions of the T-DNA insertions. Exons are shown as black boxes and introns and 5′ untranslated region as lines and dashed lines, respectively. The positions of the primers used for RT-PCR analysis in **(B)** are indicated next to the structures.

(B) RT-PCR analysis of the *SNL1* and *SNL2* transcripts in leaves of wild-type and T-DNA insertion mutants. Total RNA was extracted from 10-d-old fresh leaves and reverse transcribed to single-stranded cDNA. The primers for RT-PCR are indicated in **(A)** and listed in Supplemental Table 1 online. *ACTIN8* was used as a loading control.

(C) Seed germination phenotypes of Col (the wild type), *snl1*, *snl2-1*, *snl2-2*, and the *snl1 snl2-1* double mutant on water in the light after different periods of after-ripening. Percentages of seed germination are means (±SE) based on at least eight individual plants for each genotype.

(D) Complementation of *snl1*. Seed germination of Col, *snl1*, and two homozygous transgenic lines (2-6 and 3-2) containing a *P35S*:*SNL1* construct. Percentages of seed germination are means (±SE) based on at least eight individual plants for each genotype.

(E) Seed survival of Col and the *snl1*, *snl2-1*, and *snl1 snl2-1* mutants after dry storage for 90 and 180 d. The seeds were harvested under identical conditions and stored at 25°C in a controlled chamber with 16-h/8-h light/dark. This experiment was repeated three times with independent samples, and a representative result is shown. Percentages of seed germination are means (±SE) based on at least eight individual plants for each genotype.

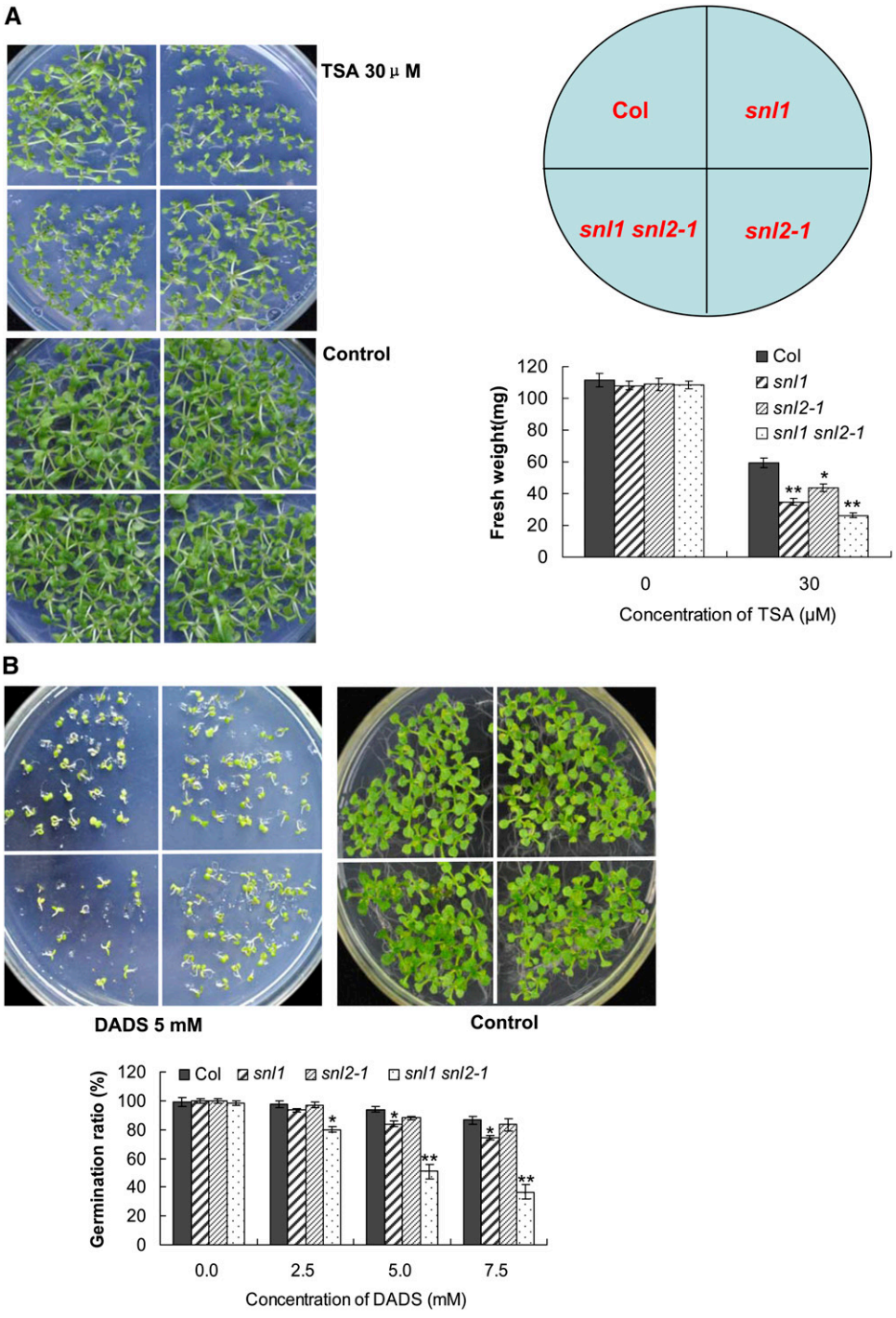

Figure 3. *snl* Mutants Response to the HDAC Inhibitors TSA and DADS.

(A) The postgermination growth of Col and mutants in response to TSA after 17 d growth in a chamber with 16 h light at 22°C. The left photographs show Col and mutants in MS with (top) or without TSA (bottom). The diagram on the right shows the positions of the different lines on the plates. The graph shows fresh weight of Col and mutants growing on the plate. At least three independent experiments were performed, similar results were obtained, and representative data are displayed. Error bars denote SD ($n > 30$), and single asterisk and double asterisk indicate significant differences from Col by Student's *t* test ($P < 0.05$ and $P < 0.01$, respectively).

(B) Seed germination phenotype of Col and mutants in response to DADS after 12 d of growth. The photograph shows the germination of Col and mutants in MS with (left) or without DADS (right); the positions of different lines in the plate are the same as in **(A)**. The graph shows the seed germination percentage of each genotype in the plates. Three independent experiments were performed, similar results were obtained, and representative data are displayed. Error bars denote SD ($n > 6$), and single and double asterisks indicate significant difference between Col and *snl* mutants by Student's *t* test ($P < 0.05$ and $P < 0.01$, respectively).

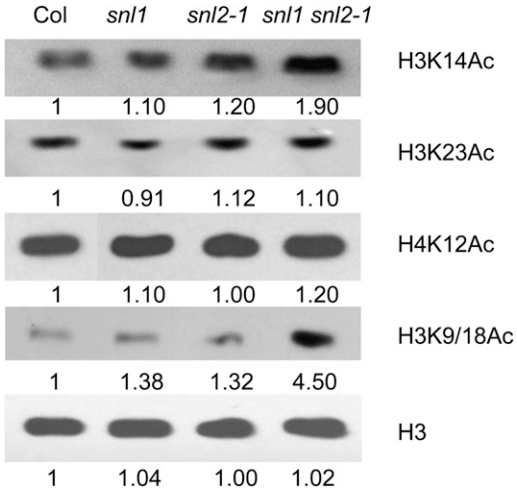

Figure 4. Histone Acetylation Status in *snl* Mutants.

H3 and H4 Lys acetylation levels in Col, *snl1*, *snl2-1*, and *snl1 snl2-1* were determined by immunoblot with the specific antibodies indicated on the right. Immunoblot signals with H3 antibody are shown as loading control. The histone proteins were extracted from 7-d-old seedlings of different lines.

subsequently analyzed by measuring germination during seed storage. All mutants showed reduced dormancy, which was most evident in the double mutant *snl1 snl2-1* (Figure 2C). The single mutants *snl2-1* and *snl2-2* only showed slightly reduced seed dormancy (Figure 2C). This indicates that SNL1 and SNL2 have partially redundant roles in seed dormancy. Transgenic plants containing the *P35S*:*SNL1* construct in a mutant *snl1* background showed similar germination levels as wild-type seeds, independent of the addition of ABA in the medium (Figure 2D; see Supplemental Figure 3 online). We also selected additional independent double mutants from the crosses of *snl1* to *snl2-1* and *snl2-2* to *snl1*, which all showed significantly reduced seed dormancy compared with the single mutants (see Supplemental Figure 4 online).

Several seed dormancy mutants, including *hub1* and *dog1*, show reduced seed longevity (Bentsink et al., 2006; Liu et al., 2007). Therefore, we examined seed longevity of *snl* mutants after 3 and 6 months storage at room temperature. Germination of 6-month-stored seeds of Columbia (Col), *snl1*, *snl2-1*, and *snl1 snl2-1* was 95, 88, 84, and 55%, respectively (Figure 2E). This indicates that the decrease of seed dormancy is associated with reduced seed longevity in these lines.

The *snl* Mutants Show Enhanced Sensitivity to HDAC Inhibitors

SNL1 and SNL2 are predicted to be components of the HDAC complex (Bowen et al., 2010). To confirm a role of SNL1 and SNL2 in histone deacetylation, the mutant growth response to the histone deacetylation inhibitors trichostatin A (TSA) (Tanaka et al., 2008) and diallyl disulfide (DADS) (Zhao et al., 2006) was tested. The *snl1* and *snl2* mutants showed increased sensitivity to TSA in postgermination growth (Figure 3A). In Murashige and Skoog (MS) medium containing 30 μM TSA, the postgermination growth of mutants was severely inhibited, and the *snl1 snl2-1* double mutant showed the highest sensitivity.

The germination of these mutants was also inhibited by DADS. The germination percentage of *snl1 sn2-1* was 49%, which is markedly lower than the 94% germination of wild-type seeds observed in medium containing 5 mM DADS (Figure 3B). These results suggest that SNL1 and SNL2 function partially redundantly in histone deacetylation.

The *snl1 snl2-1* Double Mutant Shows Altered Histone Acetylation Levels

To assess the influence of SNL on histone acetylation in more detail, the acetylation levels of histones H3 and H4 at different positions were analyzed using specific antibodies. Acetylation levels of H3K9/18 and H3K14 were increased in the 7-d-old seedlings of the *snl1 snl2-1* double mutant compared with the Col wild type. However, H3K23 and H4K12 acetylation levels were not affected (Figure 4). The levels of H3K14ac and H3K9/18ac in *snl1 snl2-1* were 1.9- and 4.5-fold higher than in Col, respectively. Slightly increased H3K9/18ac levels were also found in the *snl1* and *snl2-1* single mutants (Figure 4). These alterations of histone acetylation levels in the mutants indicate that SNL1 and SNL2 redundantly regulate this histone modification.

SNL1 Physically Interacts with HDAC

SIN3 protein needs to associate with the HDAC reduced potassium dependency3 (RPD3) to regulate gene transcription in yeast and mammals (Grzenda et al., 2009). Therefore, it is plausible that the two SIN3 orthologous proteins SNL1 and SNL2 interact with RPD3-type HDACs to execute their function in *Arabidopsis*. HISTONE DEACETYLASE19 (HDA19) protein is a good candidate for an RPD3-type HDAC because it was found to physically interact with SNL1 in the yeast two-hybrid screening database on the BAR website (http://bar.utoronto.ca/interactions/cgi-bin/Arabidopsis_interactions_viewer.cgi). Therefore, we first confirmed the interaction between SNL1 and HDA19 by yeast two-hybrid assays (Figure 5A). The SNL1 protein strongly interacts with HDA19 on the high-stringency selection plates. This interaction was also confirmed using the chromogenic marker X-α-Gal by filter lift assay (Figure 5A).

SNL1 and HDA19 were colocalized in the nucleus after cotransformation of CFP-HDA19 and yellow fluorescent protein YFP-SNL1 in tobacco (*Nicotiana benthamiana*) (see Supplemental Figure 5 online), confirming previous observations by Long et al. (2006) and Bowen et al. (2010). For verification of the interaction between SNL1 and HDA19 in planta, we performed bimolecular fluorescence complementation (BiFC) in *Agrobacterium tumefaciens*–infiltrated tobacco leaves (Waadt et al., 2008). The YFP fluorescence signal was observed only in *N. benthamiana* leaves that coexpressed the constructs $YFP_{1\text{-}174\ aa}$-HDA19 and $YFP\text{-}_{175\text{-end}\ aa}$-SNL1 or $YFP\text{-}_{175\text{-end}\ aa}$-HDA19 and $YFP_{1\text{-}174\ aa}$-SNL1 (Figure 5B).

In addition, the *HDA19* loss-of-function mutants *hda19-1* and *hda19-2* (Long et al., 2006) both showed decreased seed dormancy (see Supplemental Figure 6 online). The *hda19-2* mutant showed a stronger reduction of seed dormancy than *hda19-1*.

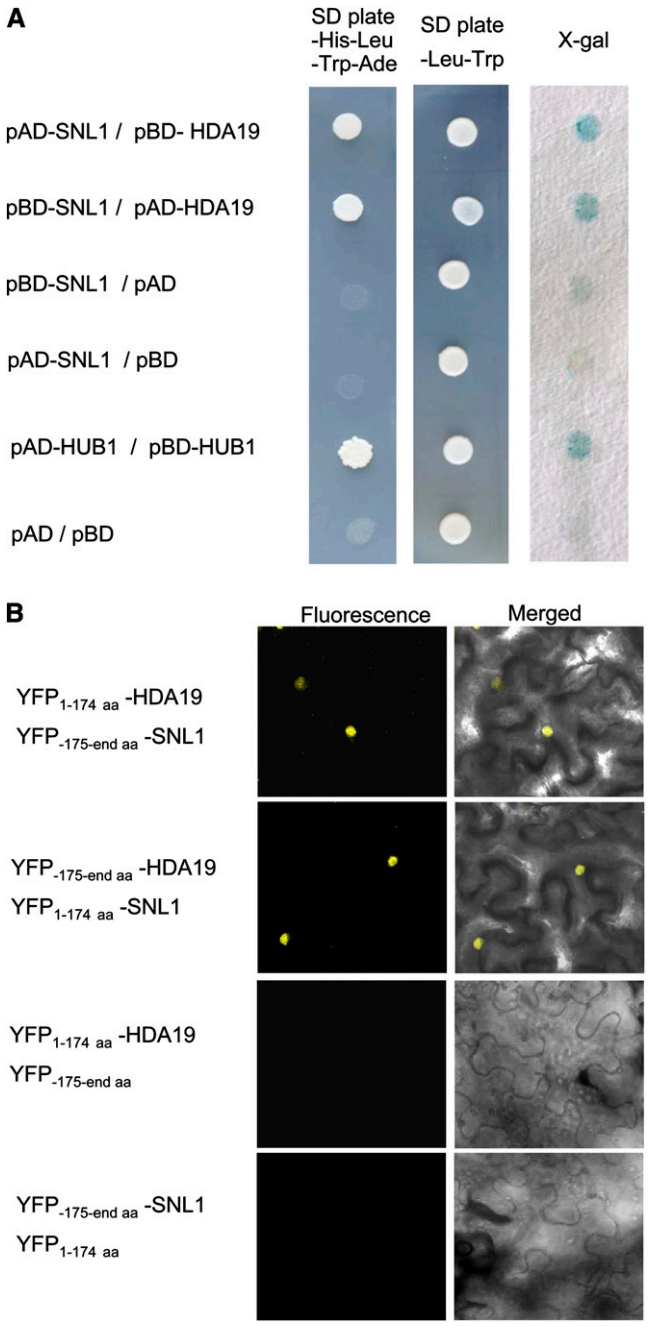

Figure 5. SNL1 Physically Interacts with HDA19.

(A) Yeast two-hybrid analysis of the interaction between SNL1 and HDA19. All transformants were cultured in selective medium lacking Leu and Trp (control) or Leu, Trp, His, and Ade. As an additional indicator of interaction, colonies were monitored for LacZ activity (blue color with X-Gal) using a filter lift assay. Self-interaction of HUB1 (Cao et al., 2008) was used as a positive control (*pAD-HUB1*/ *pBD-HUB1*). Cotransformation of *pAD-SNL1* and *pBD-SNL1* with empty *pAD* or *pBD* vectors is shown as negative control.

(B) BiFC of the interaction between SNL1 and HDA19 in *N. benthamiana* leaf epidermal cells. Top panel shows the signal from enhanced YFP, reconstituted from $YFP_{1\text{-}174\ aa}$-HDA19 and YFP-$_{175\text{-}end\ aa}$-SNL1, and transmitted light detector signals. Second panel includes enhanced YFP, reconstituted from $YFP_{1\text{-}174\ aa}$-SNL1 and YFP-$_{175\text{-}end\ aa}$-HDA19, and transmitted light detector signals. The bottom two panels show that no fluorescence signal was observed with cotransformants of constructs $YFP_{1\text{-}174\ aa}$-HDA19 and YFP-$_{175\text{-}end\ aa}$ or YFP-$_{175\text{-}end\ aa}$-SNL1 and $YFP_{1\text{-}174\ aa}$.

This could be due to the position of the *hda19-2* T-DNA, which inserted five base pairs upstream of the start codon and disrupted the HDA19 function more severely than the *hda19-1* insertion (Long et al., 2006). The reduced dormancy phenotype of *hda19* mutants is consistent with the *snl1* phenotype and indicates a role for the histone deacetylation complex comprising SNL1 and HDA19 in seed dormancy.

Transcriptome Analysis of *snl1 snl2-1* Mutant Seeds

Reduced histone acetylation results in the repression of target genes (Richon and O'Brien, 2002). We showed that the histone acetylation level in the *snl1 snl2-1* double mutant was significantly increased (Figure 4). Therefore, we performed an RNA-sequencing (RNA-seq) analysis of 12-h imbibed freshly harvested seeds of the *snl1 snl2-1* mutant and Col to identify genes with altered expression levels. In *snl1 snl2-1*, we identified 1517 and 278 genes that were upregulated and downregulated, respectively (Figure 6), indicating that a large proportion of genes are negatively regulated by SNL1 and SNL2. Many of the differentially expressed genes were involved in the cell cycle (Figure 6; see Supplemental Figure 7 online) as previously shown in mammals and yeast (Pennetta and Pauli, 1998; Silverstein et al., 2003; Cowley et al., 2005; Dannenberg et al., 2005). Transcription levels of genes regulating the plant hormone pathways were also affected, specifically those for ABA, auxin, and ethylene (see Supplemental Figure 7 online). There is abundant evidence that ABA and ethylene play important roles in the regulation of seed dormancy (Finkelstein et al., 2008). Therefore, we speculate that these hormones could be involved in the reduced dormancy phenotype of the *snl* mutants. Table 1 gives an overview of the expression of several hormone- and dormancy-related genes in the *snl1 snl2* double mutant. We validated the expression patterns of these genes in *snl* mutants by qRT-PCR (Figures 7A and 8C; see Supplemental Figure 8 online).

SNL1 and SNL2 Promote Seed Dormancy by Negatively Regulating Ethylene Biosynthesis and Response

Our transcriptome analysis identified 16 genes involved in the ethylene pathway with significantly increased expression in the *snl1 snl2-1* double mutant (see Supplemental Figure 7 online), including some ethylene biosynthesis genes (Table 1). qRT-PCR confirmed the upregulation of the genes *ACO1*, *ACO4*, *ERF105*, *ERF9*, and *ERF112* (Figure 7A; see Supplemental Figure 8 online). This pointed to the involvement of *SNL1* and *SNL2* in seed dormancy by repressing ethylene synthesis and response. Therefore, we examined the ethylene release of freshly harvested seeds and the triple response in the mutant lines. Seeds of *snl1* and *snl1 snl2-1* released more ethylene than the wild type (Figure 7B), whereas *snl2-1* produced a similar amount of ethylene.

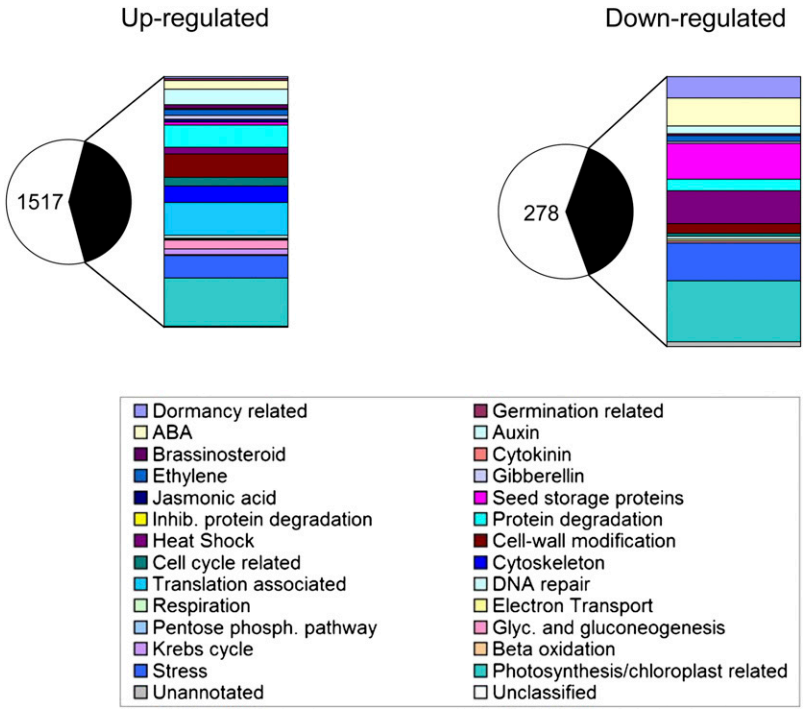

Figure 6. Transcriptome Analysis of *snl1 snl2-1* 12-h Imbibed Freshly Harvested Seeds.

TAGGIT gene ontology classification of up- and downregulated genes in the *snl1 snl2-1* double mutant. The data consist of genes that have a fold change ≥ 1.5 ($\log_2$ ratio ≥ 0.6) and an FDR value ≤ 0.05 from two independent RNA-seq experiments. The complete list is presented in Supplemental Data Set 1 online.

This result was consistent with the increased expression of ethylene biosynthesis genes. Moreover, a significantly increased triple response to 1-aminocyclopropane-1-carboxylate (ACC) was observed in seedlings of *snl1* and *snl1 snl2-1* (Figure 7C). The hypocotyl length of *snl1* and *snl1 snl2-1* was much shorter than Col in medium with 1 μM ACC, whereas *snl2-1* showed a relatively mild decrease in hypocotyl length (Figure 7D). However, ACC treatment had only a minor influence on the expression of *SNL1* and *SNL2* in seed (see Supplemental Figure 9 online).

To better understand the role of SNL1 and SNL2 in the ethylene pathway, the triple mutant *snl1 snl2-1 etr1-2* was created. ETR1 is an ethylene receptor with a negative role in the ethylene pathway; the dominant allele *etr1-2* blocks ethylene signaling and causes increased seed dormancy (Bewley, 1997). The triple mutant *snl1 snl2-1 etr1-2* showed a similar germination pattern and a similar triple response as the wild type (Figure 7E; see Supplemental Figure 10 online). This indicates that *etr1-2* suppresses the effects of *snl1 snl2-1* on seed dormancy and germination by blocking ethylene signaling.

Increased ABA Hydrolysis and Repressed ABA Response in *snl1* and *snl2* Mutants

Seed dormancy is set during seed maturation, and the phytohormone ABA is believed to be a central player in seed dormancy establishment and maintenance (Koornneef et al., 2002). Our transcriptomic analysis identified some ABA-related genes with altered expression levels in the *snl1 snl2* double mutant (Figure 6, Table 1). Interestingly, *snl1*, *snl2-1*, and *snl1 snl2-1* displayed reduced sensitivity to ABA during germination compared with wild-type Col (Figure 8A). The sensitivity to ABA of root growth after germination was similarly affected (see Supplemental Figure 11 online). The reduced sensitivity could be caused by reduced ABA levels in the mutants. Indeed, high performance liquid chromatography (HPLC) analysis showed that the ABA content was markedly reduced in *snl1*, *snl2-1*, and *snl1 snl2-1* fresh seeds (Figure 8B). In agreement with the reduced ABA content in mutant seeds, several genes involved in the hydrolysis of ABA showed altered expression levels between mutants and the wild type (Table 1). The genes *CYP707A1* and *CYP707A2* encoding ABA hydrolases were upregulated, and a *NCED4* gene was downregulated, with different profiles in *snl1*, *snl2-1*, and *snl1 snl2-1* (Figure 8C). *FUSCA3* (*FUS3*) and *DOG1* are two key factors in seed maturation and dormancy (Gazzarrini et al., 2004; Graeber et al., 2010; Kendall et al., 2011). Both genes showed reduced gene expression (Table 1, Figure 8C). Moreover, qRT-PCR showed that the transcripts of *SNL1* and *SNL2* were induced by ABA (Figure 8D). Altogether, we showed that SNL1 and SNL2 are positive regulators of the ABA pathway.

GA is another important factor involved in seed germination (Fleet and Sun, 2005) that is negatively regulated by ABA. However, we could not detect any significant differences in the responses of the *snl* mutants to the GA synthesis inhibitor PAC. Moreover, the GA contents in mature seeds of the wild type and *snl* mutants were identical (see Supplemental Figure 12 online). Therefore, we assume that *SNL1* and *SNL2* do not influence seed germination through the GA pathway.

Table 1. Selected Genes with Altered Expression in the Seeds of *snl1 snl2-1* as Identified by RNA-Seq Analysis

Gene Function Annotation	Gene Name	Locus Identifier	Expression Level	Fold Change (Log_2)	FDR
Ethylene metabolism					
1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE1	*ACO1*	At2g19590	Up	2.2	1.85E-08
1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE4	*ACO4*	At1g05010	Up	2.2	0.000137
1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE5	*ACO5*	At1g77330	Up	9.8	0.00925
Ethylene signaling					
ETHYLENE RESPONSE FACTOR9	*ERF9*	At5g44210	Up	5.1	0.0153679
ETHYLENE RESPONSE FACTOR112	*ERF112*	At2g33710	UP	8.5	0.0088264
ETHYLENE RESPONSE FACTOR105	*ERF105*	At5g51190	UP	2.3	3.41E-12
ETHYLENE RESPONSE FACTOR114	*ERF114*	At5g61890	UP	3.1	0.001728
ETHYLENE RESPONSE FACTOR6	*ERF6*	At4g17490	Up	1.3	0.046749
ETHYLENE RESPONSE FACTOR5	*ERF5*	At5g47230	Up	2.0	0.00435
ETHYLENE RESPONSE FACTOR088	*ERF088*	At1g12890	Up	7.0	8.80E-05
β-*EXPANSIN1*	*EXPB1*	At2g20750	Up	5.9	0.002262
EXPANSIN A6	*EXPA6*	At2g28950	Up	5.8	0.0182439
EXPANSIN A2	*EXPA2*	At5g05290	Up	4.1	2.49E-07
EXPANSIN A8	*EXPA8*	At2g40610	Up	4.9	3.54E-06
ABA pathway					
NINE-cis-EPOXYCAROTENOID DIOXYGENASE4	*NCED4*	At4g19170	Down	2.0	2.63E-29
DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2	*DREB2B*	At3g11020	Down	1.5	4.70E-07
Cytochrome P450, *Family 707*, *Subfamily A*, *Polypeptide1*	*CYP707A1*	At4g19230	Up	7.3	0.00541734
Cytochrome P450, *Family 707*, *Subfamily A*, *Polypeptide2*	*CYP707A2*	At2g29090	Up	1.2	0
Seed dormancy					
DELAY OF GERMINATION1	*DOG1*	At5g45830	Down	2.9	2.32E-12
FUSCA3	*FUS3*	At3g26790	Down	1.2	1.57E-07
Histone deacetylation					
SIN3-LIKE1	*SNL1*	At3g01320	Down	1.2	1.82E-16
SIN3-LIKE2	*SNL2*	At5g15020	Down	4.6	2.46E-114

Fold change $\geq$ 1.5 (log_2 ratio $\geq$ 0.6) and FDR $\leq$ 0.05 were used as the threshold to judge the significance of gene expression difference.

SNL1 and *SNL2* Modify Histone Acetylation of Upregulated Genes

We have shown that loss of function of *SNL1* and *SNL2* increased the histone acetylation level and activated gene expression of several key genes involved in the ethylene and ABA pathways. To verify whether these upregulated genes were directly targeted by histone acetylation, we performed chromatin immunoprecipitation (ChIP) assays with 7-d-old seedlings. As the gene expression levels were studied in imbibed freshly harvest seeds, additional qRT-PCR experiments with 7-d-old seedlings were performed, which yielded similar expression patterns (see Supplemental Figure 13 online). Because of the increased H3K9/18ac level in the *snl1 snl2-1* mutant (Figure 4), we used the specific H3K9/18ac antibody to immunoprecipitate chromatin in Col and *snl* mutants. After ChIP, the relative enrichment of histone H3 acetylation was determined by quantitative PCR (qPCR) using gene-specific primers (see Supplemental Table 1 online). Among the upregulated genes, hyperacetylation of the promoter region was detected for *ACO1*, *ACO4*, *ERF112*, *ERF9*, and *CYP707A1* in the *snl1 snl2-1* double mutant. However, increased acetylation also occurred in the promoter (first) and coding (second) regions in *ERF105* and in the coding (third) region in *CYP707A2* (Figure 9; see Supplemental Figure 14 online). These data suggest that the upregulation of gene expression in *snl1 snl2* was tightly associated with increased histone acetylation.

SNL1 Regulates Seed Dormancy Independently from *HUB1*

Previous research showed that the ubiquitin E3 ligase HUB1 promotes seed dormancy in *Arabidopsis* (Liu et al., 2007). We were interested in the relation between histone acetylation and ubiquitination in the regulation of seed dormancy. Therefore, we obtained the double mutant *snl1 hub1-5* after crossing of the single mutants. The *snl1 hub1-5* double mutant showed a further reduction in dormancy compared with the single mutants (Figure 10). This implied that SNL1 and HUB1 regulate seed dormancy independently.

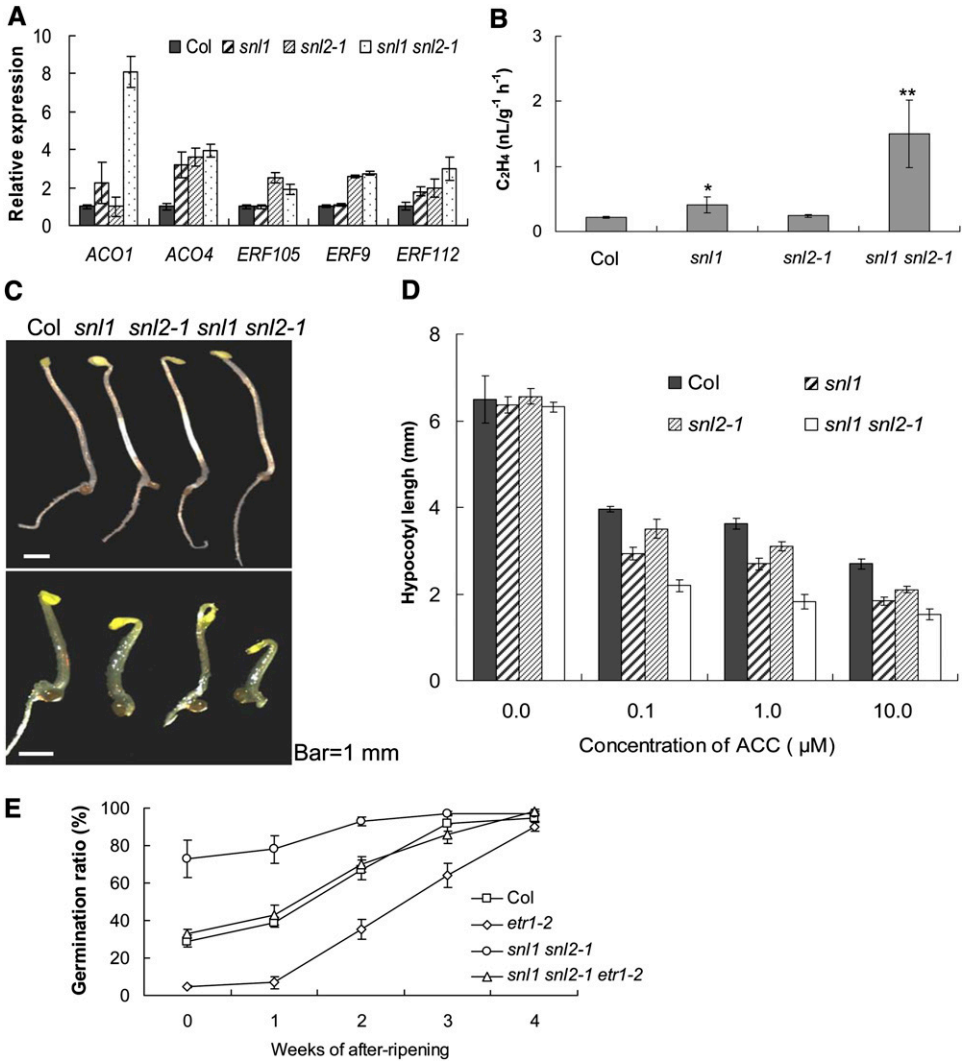

Figure 7. *SNL1* and *SNL2* Negatively Regulate Ethylene Signaling in Seed Germination of *Arabidopsis*.

(A) Expression pattern of genes involved in ethylene synthesis and signal transduction in Col and *snl* mutants. Expression levels were analyzed using qRT-PCR and normalized using the A*CTIN8* as an internal control. Each experiment had three biological replicates, and the average value is shown with SE. The relative level in Col was set at 1. Total RNA was extracted from 12-h imbibed freshly harvested seeds.

(B) The ethylene production of freshly harvested seeds from Col and *snl* mutants was determined by gas chromatography. Three independent experiments were performed, and the average value is shown with SE. The single and double asterisks indicate significant difference from Col by Student's *t* test ($P < 0.05$ and $P < 0.01$, respectively).

(C) Ethylene responses of Col and mutants. The photos show a representative result of 3-d-old seedlings in the dark in MS medium without (top) and with (bottom) 1 μM ACC. The mutants *snl1*, *snl2-1*, and *snl1 snl2-1* are ethylene sensitive to different extents. Bars = 1 mm.

(D) Hypocotyl growth in response to 1 μM ACC. The hypocotyl lengths of different lines were measured after 3 d in dark. The average value is shown with SD ($n = 15$).

(E) Germination rates of Col and mutants *etr1-2*, *snl1 snl2-1*, and *snl1 snl2-1 etr1-2*. The triple mutant *snl1 snl2-1 etr1-2* was obtained and identified by crossing homozygous *etr1-2* and *snl1 snl2-1*. The average value is shown with SE ($n \geq 8$).

DISCUSSION

SNL1 and *SNL2* Are Highly Expressed in Seeds and Involved in Seed Dormancy

SIN3 has an important role in the regulation of centromeres, the cell cycle, and chromatin remodeling in several organisms (Kawaguchi et al., 2003; Sakuma et al., 2006; Wilson et al., 2006). *Drosophila melanogaster sin3* null mutants die during the larval stage of development due to a delay in the G2 phase of the cell cycle (Pennetta and Pauli, 1998). The deletion of mSIN3A in mouse results in death at the postimplantation stage of development (Dannenberg et al., 2005). However, little is known about SIN3-like proteins in plants. *Arabidopsis* contains six *SNL* genes (Bowen

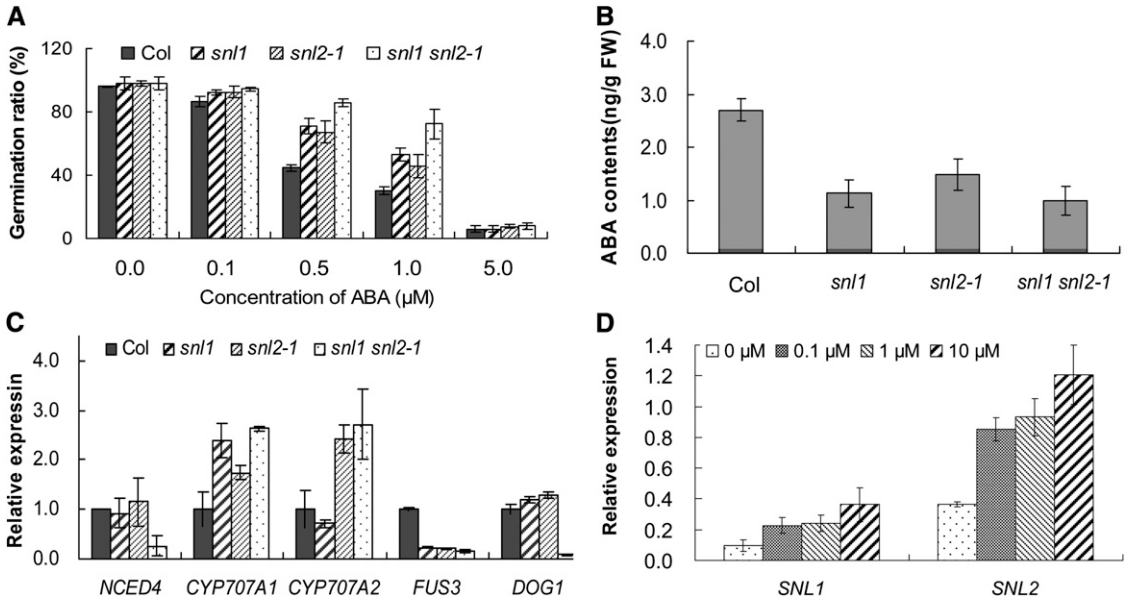

Figure 8. *SNL1* and *SNL2* Positively Regulate ABA Signaling in Seed Dormancy of *Arabidopsis*.

(A) Germination rates of Col, *snl1*, *snl2-1*, and *snl1 snl2-1* in response to ABA. Percentages of seed germination are means (±SD) based on eight individual plants. The seeds were immersed in different solutions (including control) at 4°C for 3 d; then, germination percentages were counted after 7 d in a growth chamber at 22°C with 16 h of light and 8 h of darkness.

(B) ABA contents in freshly harvested seeds of mutants and Col. ABA levels were measured by HPLC. Error bars denote SD from two independent samples. FW, fresh weight.

(C) Expression patterns of genes involved in the ABA pathway. Total RNA was extracted from 12-h imbibed Col and mutant seeds. The mean values and SE were from three biological replicates and normalized using *ACTIN8* as an internal control. The relative expression level in Col was set at one.

(D) Expression patterns of *SNL1* and *SNL2* in response to ABA. Expression levels were measured using qRT-PCR. Total RNA was extracted from Col seeds imbibed for 12 h in different ABA concentrations (0.1, 1, and 10 μM). The mean values and SE were from three biological replicates.

et al., 2010), which is much more than the two SIN3-encoding genes in yeast and mouse (Grzenda et al., 2009). Among the six *SNL* genes, *SNL1* and *SNL2* were highly expressed in dry seeds (see Supplemental Figures 1 and 2 online; Figure 1). This expression pattern suggested a potential role for *SNL1* and *SNL2* in seeds; indeed, we observed that these genes have a partially redundant role in seed dormancy (Figure 2C). Mutations in *SNL1* and *SNL2* resulted in significantly reduced seed dormancy (Figure 2). No visible phenotypes were observed in the mutants under normal growth conditions. This suggests that SNL1 and SNL2 mainly function in seed dormancy. However, we cannot exclude their function in other traits since *SNL1* and *SNL2* show some expression in other tissues (Figure 1) and their homologous gene *SNL3* has been found to function in salt tolerance (Song et al., 2005).

SNL1 and SNL2 Regulate Seed Dormancy by Influencing Ethylene-ABA Antagonism

In our study, mature seeds of the *snl1 snl2-1* double mutant produced more ethylene compared with the wild type (Figure 7B). This correlated with increased expression of the ethylene synthesis–related genes *ACO1*, *ACO4*, and *ACO5* (Figure 7A; see Supplemental Figure 8 online). In addition, some ethylene signaling genes like *ERF6*, *ERF9*, *ERF105*, and *ERF112* were upregulated in *snl1* or *snl2-1* and *snl1 snl2-1* (Figure 7A; see Supplemental Figure 8 online), indicating that ethylene signaling was also enhanced in these mutants. Ethylene has been found to play a key role in the release of coat-imposed seed dormancy and the promotion of seed germination by endosperm cap weakening and endosperm rupture (Kepczynski and Kepczynska, 1997; Linkies et al., 2009). The main role of ethylene could be the promotion of radial cell expansion in the embryonic hypocotyl, decreased seed base water potential, and enhanced seed respiration or activity of cell wall hydrolases in the endosperm cap (Kucera et al., 2005; Matilla and Matilla-Vázquez, 2008). In our work, several downstream ethylene-responsive genes, including the cell wall–modifying proteins β-1,3-glucanase and expansin, have been found to be upregulated in the *snl1 snl2-1* double mutant (Table 1; see Supplemental Figure 8 online), which further indicates that ethylene functions in the regulation of seed dormancy mediated by SNL1 and SNL2.

We also obtained genetic evidence for the involvement of SNL1 and SNL2 in the ethylene pathway. The *Arabidopsis* ethylene receptor ETR1 plays a negative role in the ethylene pathway, and point mutations in the *ETR1* gene can confer dominant ethylene insensitivity, resulting in enhanced seed dormancy (Chiwocha et al., 2005). We constructed a triple mutant *snl1 snl2-1 etr1-2*, which showed that *etr1-2* enhanced seed dormancy and partially reduced the triple response of the *snl1*

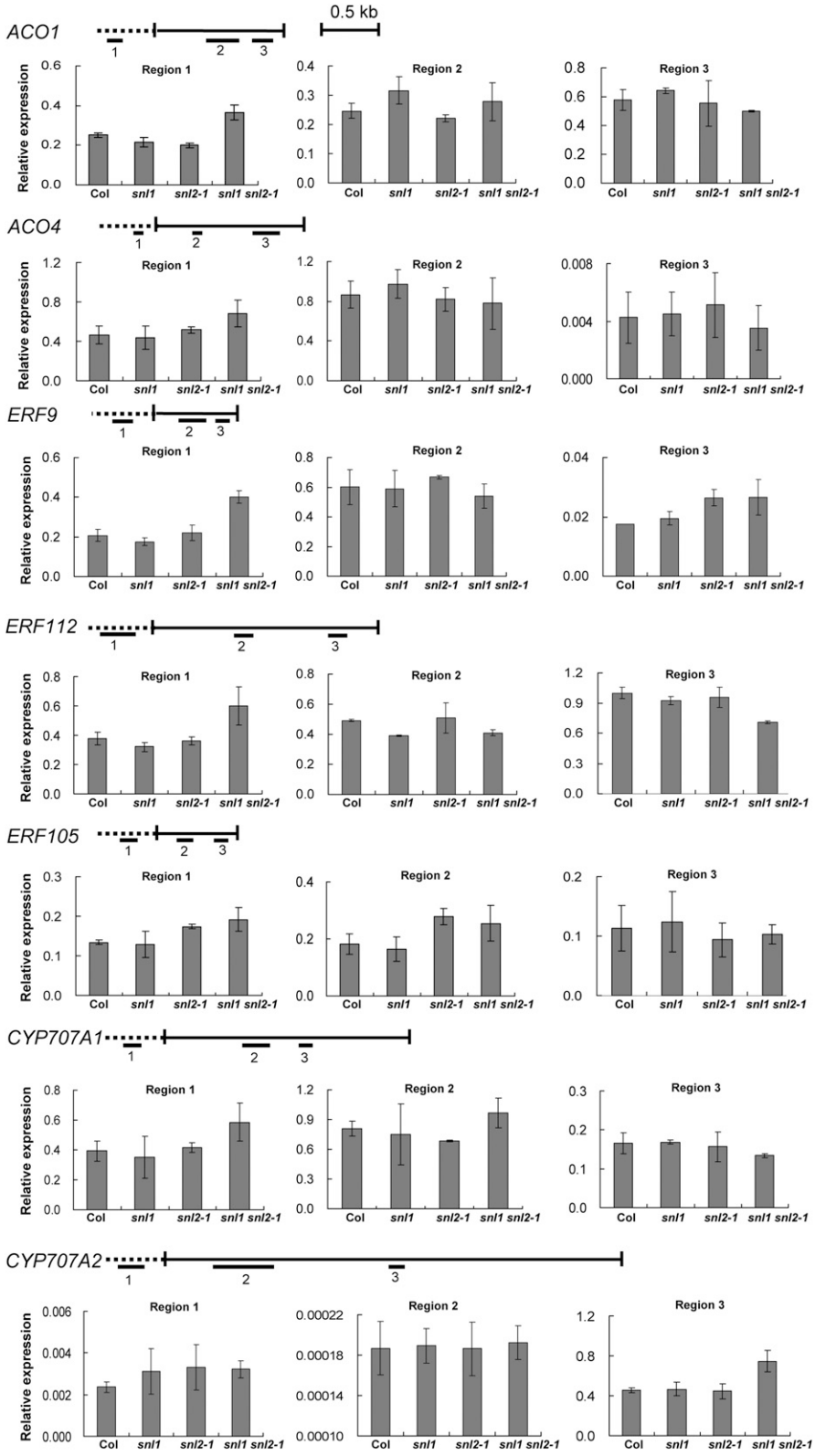

Figure 9. ChIP Assay of Upregulated Key Genes Involved in the Ethylene and ABA Pathways in *snl* Mutants.

A schematic diagram of each gene structure is shown in the top. Dashed lines indicate the ~500-bp promoter sequence. The amplification sites for ChIP analysis are indicated as numbers (1 to 3) with black lines below the draft. Black lines represent the ORF of the gene from start codon to stop codon. The figures show the accumulated abundance of each gene with specific primers for different regions (regions 1, 2, and 3) in the ChIP assay. Immunoprecipitates were obtained from 7-d-old seedlings with an H3K9/18ac-specific antibody (Upstate 07-593). Relative amounts of the PCR products were calculated and normalized to *ACTIN8*. This experiment was repeated three times with independent samples.

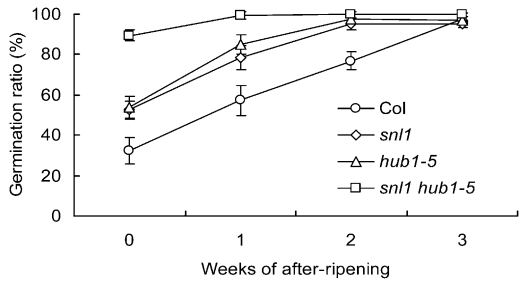

Figure 10. SNL1 and HUB1 Additively Affect Seed Dormancy.

The double mutant *snl1 hub1-5* was obtained by crossing the *snl1* and *hub1-5* mutants. The null mutation of *hub1-5* was identified as before (Liu et al., 2007). Percentages of seed germination (±SE) are based on at least eight individual plants for each line.

snl2-1 double mutant (Figure 7E; see Supplemental Figure 10 online). In the *Arabidopsis* genome, five ethylene receptors, ETR1, ETR2, ERS1, ERS2, and EIN4, were found to negatively regulate ethylene signaling (Hua and Meyerowitz, 1998). The allele *etr1-2* could not completely block ethylene signaling in a *snl1 snl2* background (Figure 7E; see Supplemental Figure 10 online), which implies that other receptors might be also involved in the regulation of seed dormancy and germination.

Impairment of *SNL3* expression showed a role for SNL3 in ABA sensitivity during seed germination through gene repression mediated by HDA19 (Song et al., 2005). Here, we showed that SNL1 and SNL2 were also redundantly involved in the ABA pathway (Figures 8 and 9). The ABA content in *snl* mutants seeds was decreased (Figure 8B), consistent with increased transcript abundance of the ABA hydrolase-encoding genes *CYP707A1* and *CYP707A2* (Figure 8C). Moreover, the expression of *SNL1* and *SNL2* was induced by ABA during seed development (Figure 8D), suggesting a positive feedback mechanism. Together, our work indicates that SNL1 and SNL2 are also involved in seed dormancy via ABA metabolism and signaling.

The antagonistic function of ABA and ethylene in the regulation of seed dormancy and germination has been widely reported (Finkelstein et al., 2008; Linkies and Leubner-Metzger, 2012). In this study, we showed that the reduced seed dormancy phenotype of the *snl1*, *snl2-1*, and *snl1 snl2-1* mutants correlated with decreased ABA signal and increased ethylene signal in seeds. This suggests that SNL1 and SNL2 regulate seed dormancy at least partially by mediating the antagonism between ethylene and ABA. It was recently suggested that ABA and ethylene may regulate each other's biosynthesis, catabolism, or signaling to enhance their antagonistic effects upon seed germination (Cheng et al., 2009; Linkies et al., 2009; Li et al., 2011). Therefore, the debate arises whether SNL1 and SNL2 are directly involved in the regulation of both the ABA and ethylene pathways or regulate one of them indirectly. Our ChIP-qPCR data showed that both ABA and ethylene-related genes like *ACO1*, *ACO4*, *ERF*s, *CYP707A1*, and *CYP707A2* have altered H3K9/18 histone acetylation levels in the *snl1 snl2-1* double mutant (Figure 9). Among them, *ACO4*, *ERF9*, and *ERF105* have been reported to be the direct targets of H3K9ac (Charron et al., 2009). Since SNL1 has been shown to depend on functional HDAC activity for its transcriptional repression (Bowen et al., 2010), we speculate that SNL1 and SNL2 might directly function in the regulation of both ABA and ethylene pathways mediated by histone acetylation. However, this does not exclude the existence of interactions between the ABA and ethylene pathways to enhance their antagonism.

FUS3 and *DOG1*, two key factors that regulate seed maturation and/or seed dormancy, were downregulated in *snl1 snl2-1* (Figure 8C). Loss of FUS3 causes a nondormant phenotype of seed (Keith et al., 1994). FUS3 has been indicated to be involved in ABA metabolism to regulate seed development (Nambara et al., 2000; Gazzarrini et al., 2004). Recently, FUS3 was also found to play a negative role in the ethylene pathway during germination (Lumba et al., 2012), implying that downregulation of *FUS3* could cause enhanced ethylene signaling in the *snl1 snl2* mutant. Expression of *DOG1* is tightly correlated with dormancy levels (Bentsink et al., 2006; Nakabayashi et al., 2012), and DOG1 protein abundance in freshly harvested seeds directly acts as a timer for seed dormancy release. DOG1 functions largely independently from ABA; however, ABA is essential for DOG1 action (Nakabayashi et al., 2012). FUS3 and DOG1 might contribute to the antagonism between ABA and ethylene to regulate seed dormancy downstream of SNL1 and SNL2.

SNL1 and SNL2 Regulate Gene Transcription through Histone Deacetylation

The association of SIN3 with the RPD3-type HDAC protein complex has been shown to play a key role in the regulation of

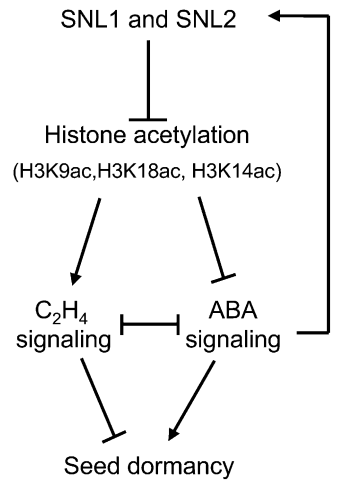

Figure 11. A Model Depicting the Role of SNL1 and SNL2 in Seed Dormancy.

During seed dormancy establishment, SNL1 and SNL2 regulate histone acetylation levels at the genes that are involved in the ABA and ethylene pathways. Histone deacetylation mediated by SNL1 and SNL2 negatively regulates the ethylene pathway and positively regulates the ABA pathway, which leads to an increase in seed dormancy mediated by the antagonism between the ABA and ethylene pathways.

gene expression in a number of eukaryotes, such as budding yeast (*Saccharomyces cerevisiae*), *Drosophila*, and mammals (Silverstein and Ekwall, 2005). Therefore, the SIN3 homologous proteins SNL1 and SNL2 in *Arabidopsis* are also expected to be components of the histone deacetylation complex. Bowen et al. (2010) showed that SNL1 is a nuclear protein that functions as a gene repression factor. Here, we provide four pieces of evidence that support a role for SNL1 and SNL2 in the histone deacetylation complex to repress transcription. First, *SNL1* and *SNL2* mutant seeds showed higher sensitivity to the histone deacetylation inhibitors TSA and DADS compared with the Col wild type (Figure 3). This increased sensitivity is similar to that obtained with *HDA6* RNA interference lines (Tanaka et al., 2008). Secondly, SNL1 was found to interact with HDA19 in vitro and in planta (Figure 5), and the immunoblot analysis showed increased acetylation levels of H3K9/18 and H3K14 in the *snl1 snl2-1* double mutant (Figure 4). Third, a transcriptome analysis of the *snl1 snl2-1* double mutant showed that a majority of the differentially expressed genes were upregulated (Figure 6; see Supplemental Figure 7 online), consistent with increased histone acetylation levels. Finally, we showed that the upregulation of several of these genes in the *snl1 snl2-1* double mutant was indeed associated with increased H3K9/18 acetylation levels at corresponding loci (Figure 9).

The *Arabidopsis* genome contains 12 RPD3-like HDAC-encoding genes (Hollender and Liu, 2008; Bowen et al., 2010). HDA19 is one of the best studied HDACs and is involved in a wide range of developmental stages and environmental responses (Tian and Chen, 2001; Tian et al., 2003; Zhou et al., 2005; Long et al., 2006; Chen and Wu, 2010). *HDA19* mediates deacetylation of H3K9/14, H3K9, H4K12, and H4 (Tian et al., 2005; Chen and Wu, 2010; Jang et al., 2011), which is overlapping with the roles of SNL1 and SNL2 in the deacetylation of H3K9/18 and H3K14 (Figure 4). We showed that SNL1 can interact with HDA19 both in vivo and in planta (Figure 5; see Supplemental Figure 5 online). Interestingly, *hda19* mutants also show decreased seed dormancy (see Supplemental Figure 6 online). However, reduced expression of *HDA19* resulted in various pleiotropic phenotypes (Wu et al., 2000; Tian and Chen, 2001; Tian et al., 2003; Zhou et al., 2005) that were not observed in the *snl* mutants. Therefore, we speculate that the SNL-HDA19 protein complex might be especially important to deacetylate histones of seed dormancy–related genes during seed maturation. Since histone deacetylation complexes can contain different combinations of SIN3 with HDACs (Grzenda et al., 2009), a SIN3 protein can interact with more than one HDAC protein (Laherty et al., 1997; Grzenda et al., 2009). Therefore, besides HDA19, other HDACs might interact with SNL1 or SNL2 to regulate seed dormancy.

SNL1 has been shown to repress transcription in a HDAC-dependent manner (Bowen et al., 2010). Therefore, we presume that gene upregulation in the *snl1 snl2-1* double mutant should be associated with increased histone acetylation abundance. The ChIP assay with anti-H3K9/18ac indeed reflected this positive relationship (Figure 9), which implied that the upregulation of ethylene- and ABA-related genes during seed germination was directly caused by increased histone acetylation mediated by SNL1 and SNL2. Several studies have indicated a good correlation between SIN3 and HDAC direct target genes. Stephan and Koch (2009) found that Sin3 and HDAC Rpd3 were both recruited to promoters of SBF (Swi4/Swi6)-regulated genes (*CLN1*, *CLN2*, and *PCL1*) in yeast. van Oevelen et al. (2008, 2010) showed by ChIP assay that SIN3 directly impacts acetylation levels downstream of the transcriptional start site of target genes in mouse. Although ChIP assays for direct interaction between SNL1 and ethylene/ABA genes did not function in our hands, we speculate that the differentially expressed genes with altered histone acetylation level in the *snl1 snl2* mutant could be the target genes of SNL1 or SNL2.

A Model for the Role of SNL1 in Seed Dormancy

Histone ubiquitination and acetylation are two important modifications for gene transcription activation. An analysis of the double mutant *snl1 hub1-5* indicated that SNL1 and HUB1 regulate seed dormancy additively (Figure 10). A comparison of the *hub1* and *snl1 snl2-1* transcriptomes also showed that there was no significant overlap in differentially expressed genes (see Supplemental Figure 15 online). Overall, these analyses indicated that HUB1 and SNL1 regulate seed dormancy independently, implying that histone acetylation and ubiquitination affect gene transcription independently.

In Figure 11, we present a model of the function of SNL1 and SNL2 during seed dormancy and germination that integrates all presented data. In this model, *SNL1* and *SNL2* can be induced by ABA. Enhanced SNL1 and SNL2 levels inhibit ABA hydrolysis and promote ABA synthesis by histone deacetylation of specific target genes. This mechanism reinforces ABA signaling. In addition, SNL1 and SNL2 reduce ethylene accumulation by altering the transcription of ethylene synthesis or signaling-related genes. Finally, the antagonism between the ABA and ethylene pathways further strengthens seed dormancy. Thus, *SNL1* and *SNL2* could represent a cross-link point for ABA and ethylene antagonism in the regulation of seed dormancy and germination.

METHODS

Plant Materials and Growth Conditions

All experiments were performed with *Arabidopsis thaliana* Col wild-type plants or mutants in the Col background. T-DNA insertion lines Sail_1151_F09 (*snl1*), Salk_097168 (*snl2-1*), and Salk_073549 (*snl2-2*) were ordered from the ABRC, and homozygous individuals were identified by PCR-based screening. The gene-specific primers, designed by the SIGNAL T-DNA verification primer design program, were used in combination with T-DNA left border primers. RT-PCR with RNA isolated from leaves was performed to confirm the homozygous knockout lines. PCR was performed with 25 cycles for *ACTIN8* and 35 cycles for *SNL1* and *SNL2*. The primers for PCR in this article are listed in Supplemental Table 1 online. The mutants *etr1-2*, *hub1-5*, *hda19-1*, and *hda19-2* were described before (Chiwocha et al., 2005; Long et al., 2006; Liu et al., 2007). Double and triple mutants (*snl1 snl2-1*, *snl1 snl2-2*, *snl1 hub1-5*, and *snl1 snl2-1 etr1-2*) were obtained by standard crossing procedures. Seeds were sown in soil and grown in the greenhouse under photoperiodic cycles of 16 h light and 8 h dark at 22°C. The seeds sown on half-strength MS medium were first sterilized with 10% (v/v) NaClO. Plates were kept in the dark at 4°C for 3 d to break dormancy (stratification) before moving into a climate chamber with a photoperiod of 16 h light and 8 h dark at 22°C.

Germination Tests

Germination tests were performed as described by Alonso- Blanco et al. (2003). All germination experiments were performed on filter paper in 6-cm Petri dishes. Each genotype had at least eight replicates (consisting of 80 to 100 seeds from one individual plant per Petri dish). The average germination ratio was determined after 7 d of incubation in a climate room (25°C, 16 h light with 80 to 90 μmol m^{-2} s^{-1} light intensity). DADS and TSA were added into MS medium for the analysis of germination and postgermination growth. For the sensitivity analysis, filter papers were soaked with either control solutions or solutions of the GA biosynthesis inhibitors PAC or ABA. Seeds for each germination assay were collected from plants of different genotypes grown simultaneously and stored under identical conditions. For the triple response assay, ACC was added into the MS medium, and all lines were grown 3 d in the dark at room temperature after 3 d of stratification at 4°C. Each germination or growth test was done with at least three replicates from independent plants.

Constructs and Plant Transformation

Total RNA was isolated from Col young leaves using the Qiagen RNeasy kit (Qiagen). cDNAs were generated by SuperScript II reverse transcriptase (Invitrogen). The open reading frame (ORF) of *SNL1* was amplified with Phusion DNA polymerase (NEB) and primers *SNL1-F*, 5′-AAAAAGCAGGCTTGATGAAGCGGATAAGAGATGAT-3′, and *SNL1-R*, 5′-AGAAAGCTGGGTTTTAAGCTGAGAGAAACCTATGG-3′. *P35S*:*SNL1* was constructed by inserting *SNL1* cDNA into the *pLEELA* vector, which is a derivative of pJawohl3-RNAi (GenBank accession number AF404854) containing a Gateway cassette introduced into the *Hpa*I site, and then transformed in mutant *snl1* plants. The *snl1* plants were transformed by *Agrobacterium tumefaciens* strain GV3101 pm90RK using the floral dip method (Clough and Bent, 1998). Transformants were selected based on their ability to survive after 7 d in MS medium with 10 mg/L DL-phosphinothricin. The 3:1 segregating transformant lines were selected on MS medium with 10 mg/L DL-phosphinothricin. T3 homozygous transgenic plants were used for phenotypic analyses. All of the constructs used in this study were confirmed by sequencing.

Histone Extraction and Protein Gel Blot Analysis

Histone extraction was performed according to Charron et al. (2009) with minor modifications. Four grams of 7-d-old seedlings grown in MS were homogenized in liquid nitrogen and resuspended in extraction buffer 1 (0.4 M Suc, 10 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, and 0.1 mM PMSF), filtered through four layers of Miracloth, and centrifuged for 20 min at 2880*g* at 4°C. Pellets were resuspended in 1 mL of histone extraction buffer 2 (10 mM Tris-HCl, 2 mM EDTA, 0.25 M HCl, 5 mM 2-mercaptoethanol, and 0.2 mM PMSF), and soluble proteins were isolated by centrifugation, precipitated with 25% trichloroacetic acid, and repelleted by centrifugation at 17,000*g* for 30 min. Pellets were washed twice with ice-cold acetone, resuspended in Laemmli buffer (63 mM Tris HCl, 10% glycerol, 2% SDS, and 0.0025% bromophenol blue, pH 6.8), separated electrophoretically on 4 to 12.5% polyacrylamide gels, and transferred to Immobilon polyvinylidene difluoride membranes (Amersham) for immunoblotting. Histone modifications were detected with the H3 (Upstate 05-499) and acetyl-H3 antibodies (Upstate 07-595, 07-593, 06-911, and 07-355) and visualized on x-ray film using a horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG secondary antibody and the Super ECL plus detection system (Applygen). The membrane was stripped before being reprobed with other antibodies in accordance with Sridhar et al. (2007).

RNA Extraction, qRT-PCR, and RNA-Seq

Total RNAs from seedlings or seeds with or without different hormone treatments were extracted using the Qiagen RNeasy kit and RNAqueous small-scale phenol-free total RNA isolation kit (Ambion) according to the manufacturer's instructions and reverse transcribed using the SuperScript RT-PCR system (Invitrogen). qRT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa). The expression value for each gene was quantified using a standard curve with a serial dilution of plasmid of known concentration, and they were normalized to the value of *ACTIN8* and *At2g28390* (Chua et al., 2005; Czechowski et al., 2005; Graeber et al., 2011). At least three biological replicates were analyzed. Primers used are listed in Supplemental Table 1 online. Mean values and standard errors were calculated from the three biological replicates and are shown in the figures.

For the RNA-seq experiment, total RNAs were extracted from freshly harvested Col and *snl1 snl2-1* seeds after 12 h of imbibition. The experiments were repeated to have two biological replicates. The RNA samples were sequenced by BGI at Shenzhen.

mRNA was enriched using oligo(dT) magnetic beads. By adding the fragmentation buffer, the mRNA was interrupted into short fragments; then, first-strand cDNA was synthesized with random hexamer primer using the mRNA fragments as templates. Buffer, deoxynucleotide triphosphates, RNase H, and DNA polymerase I were added to synthesize the second strand. The double-stranded cDNA was purified with the QiaQuick PCR extraction kit and washed with EB buffer for end repair and single nucleotide A addition. Finally, sequencing adaptors were ligated to the fragments. The required fragments were purified by agarose gel electrophoresis and enriched by PCR amplification. The library products were then sequenced on an Illumina HiSeq 2000 platform.

Determination of Ethylene, ABA, and GA Accumulation

The rate of ethylene production by 1 g seeds was measured by enclosing samples in 15-mL airtight containers for 24 h at 20°C, withdrawing 1 mL of the headspace gas, and injecting it into a gas chromatograph (model GC-4CMPF; Shimadzu) fitted with a flame ionization detector and an activated alumina column. The oven temperature was 60°C and the N_2, H_2, and O_2 flow rates were 20, 35, and 300 mL min^{-1}, respectively. Ethylene identification was based on the retention time compared with standard C_2H_4 (purity 99.9%) (Sánchez-Calle et al., 1989). This experiment was repeated thrice for each line.

ABA and GA_4 concentrations in seeds of different mutants and Col were determined by HPLC as described (Mwange et al., 2003) with minor modification. Seeds (0.5 g) were finely ground in liquid nitrogen for extraction and measurement of hormone. Quantification was obtained by comparing the peak areas with those of known amounts of standard ABA and GA_4 (Sigma-Aldrich).

Yeast Two-Hybrid Assay

The ORF cDNAs of *SNL1* and *HDA19* were amplified with *SNL1-F* and *SNL1-R*, previously presented, as well as *HDA19-F*, 5′-AAAAAGCAGGCTTAATGGATACTGG CGGCAATTCG-3′, and *HDA19-R*, 5′-AGAAAGCTGGGTCTTATGTTTTAGGAGGAAACGCC-3′, from Col seeds and in-frame fused with the GAL4 activation domain and the binding domain of the *pAD* (*pACT2-attR*) and *pBD* (*pAS2-attR*) vectors to generate prey and bait plasmids, respectively (modified from Clontech). These pairs of bait and prey plasmids were cotransformed into yeast AH109 cells using a lithium acetate method (Clontech) and analyzed for yeast growth on selective medium lacking His, Leu, Trp, and adenine at 28°C for 2 to 4 d. Positive and negative colonies were transferred to filter paper, permeabilized in liquid nitrogen, and assayed for expression of the *lacZ* reporter gene by detection of β-galactosidase activity with a solution containing an X-Gal substrate. The plates containing the filter papers were incubated at room temperature for 3 h. The color of colonies in filter paper was monitored, and colonies producing β-galactosidase became blue (Agilent).

Interaction between SNL1 and HDA19 by Plant BiFC

To generate the vector system for BiFC analysis, the cDNAs of *HDA19* and *SNL1* were amplified and in-frame fused into vectors *pESPYNE* and *pESPYCE* to produce the N-terminal fusions $YFP_{1\text{-}174aa}$-HDA19 and YFP-$_{175\text{-end aa}}$-SNL1. Additionally, the CFP-HDA19 and YFP-SNL1 fusion vectors were constructed to confirm the fusion protein localization with *pENSG-CFP* and *pENSG-YFP*, respectively (Kwaaitaal et al., 2010). For transient expression, *Agrobacterium* strains (GV3101 pm90RK) carrying the constructs were used (Clough and Bent, 1998) for infiltration of 5- to 6-week-old *Nicotiana benthamiana* leaves. The *Agrobacterium* strains were infiltrated at an OD_{600} of 0.5 for the BiFC constructs. For microscopy analyses, leaf discs were used 2 d after infiltration for BiFC. The multiphoton laser scanning microscope (Olympus FV1000MPE) was used to detect the fluorescence signal.

ChIP Assay

About 1.5 g of 7-d-old seedlings was used for ChIP assay. Chromatin preparation and immunoprecipitation were performed as described (Bowler et al., 2004). Harvested seedlings were fixed in 1% formaldehyde for 10 min in a vacuum. Gly was added to a final concentration of 0.125 M, and the reaction was terminated by incubation for 5 min in a vacuum. Seedlings were rinsed three times with distilled water and frozen in liquid nitrogen. After isolation, chromatin was sheared to 500- to 2000-bp fragments by sonication (Branson Sonifier 250). Immunoprecipitation was performed by adding specific antibody in an extract and protein G agarose/salmon sperm DNA (Millipore). The antibodies used for ChIP assays were anti-H3K9/18ac (Upstate 07-593). After washing, immune complexes were eluted from the protein G beads and reverse cross-linked by incubation overnight at 65°C. Samples were treated with proteinase K for 1 h at 65°C and RNase A for 1 h at 37°C. DNA was extracted in a final volume of 40 μL using the QIAquick PCR purification kit (Qiagen). The ChIP assay was repeated with three biological replicates. DNA (0.5 μL) was used for each qPCR. qPCR with SYBR Premix Ex Taq (TaKaRa) was performed with a real-time system (Eppendorf). Each sample was assayed in triplicate by PCR. Error bars in each graph indicate variance of three technical repetitions. We used *ACTIN8* and *18S* (He et al., 2003; Chua et al., 2005) as internal control for H3K9/18ac; primers used for ChIP assays are listed in Supplemental Table 1 online.

Data Analysis and Statistical Analysis

The original RNA-seq image data were base-called using Illumina GA Pipeline v1.6 software. The raw reads were filtered for adaptors and low-quality reads. Reads were then mapped to reference *Arabidopsis* genome sequences using SOAPaligner/soap2. Mismatches no more than two bases were allowed in the alignment. The gene expression level was calculated as reads per kilobase of transcript per million mapped reads. A statistical method designed for differential expression based on gene sequence tags was applied for the detection of differentially expressed genes (Audic and Claverie, 1997). The method reported both the raw P value and Benjamini-Hochberg procedure adjusted P value (false discovery rate [FDR]) across all detected genes. Only genes with both FDR $\leq$ 0.05 and absolute value of fold change $\geq$ 1.5 were qualified as differentially expressed genes.

We used an annotation tool named TAGGIT, which was specially designed for the seed-specific gene ontology classification (Carrera et al., 2007) to annotate the lists of differentially expressed genes.

We compared the list of differentially expressed genes of *snl1 snl2-1* with those of *hub1*. The list of differentially expressed genes in *hub1* was obtained from Liu et al. (2011). The Affymetrix probe identifiers in the *hub1* transcriptome data were mapped to *Arabidopsis* locus identifiers for comparison. The overlap between the up- and downregulated genes in the *snl1 snl2-1* and *hub1* mutants was counted, and the significances of the overlap were calculated by the phyper function in R. We used the number of all nuclear genes represented on the Affymetrix ATH1 Genome array as the population size in the calculation. For physiological and biochemical data, an analysis of variance was performed to investigate whether there was a significant difference between the samples, and if a significant difference was found, a Tukey's honestly significant difference test was performed to determine which samples were responsible for the significant differences.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *SNL1* (At3g01320), *SNL2* (At5g15020), *HDA19* (At4g38130), *ETR1* (At1g66340), *HUB1* (At2g44950), *ERF5* (At5g47230), *ERF6* (At4g17490), *ERF9* (At5g44210), *ERF105* (At5g51190), *ERF112* (At2g33710), *ERF008* (At1g12890), *ERF114* (At5g61890), *EXPB1* (At2g20750), *EXPA6* (At2g28950), *EXPA2* (At5g05290), *EXPA8* (At2g40610), *ACO1* (At2g19590), *ACO4* (At1g05010), *ACO5* (At1g77330), *CYP707A1* (At4g19230), *CYP707A2* (At2g29090), *NCED4* (At4g19170), *DREB2B* (At3g11020), *DOG1* (At5g45830), *FUS3* (At3g26790), *ACTIN8* (At1g49240), *18S* (At2G01010), and *At2g28390*.

Supplemental Data

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

Supplemental Figure 1. The Expression Patterns of *SNL1* and *SNL2* in Different Tissues of *Arabidopsis* from the Genevestigator Website (https://www.genevestigator.com/gv/index.jsp).

Supplemental Figure 2. Expression Patterns of *SNL1* and *SNL2* Using Gene *At2g28390* as Internal Control.

Supplemental Figure 3. Overexpression of *SNL1* Restores the Seed Germination of *snl1* in Response to ABA.

Supplemental Figure 4. Seed Germination Analysis of Different Double Mutant *snl1 snl2-1* and *snl1 snl2-2* Lines.

Supplemental Figure 5. Nuclear Localizations of SNL1 and HDA19.

Supplemental Figure 6. Seed Dormancy Phenotype of *hda19* Mutants.

Supplemental Figure 7. The Number of Genes that Are Differentially Expressed in *snl1 snl2-1* Mutant Seed.

Supplemental Figure 8. Expression Patterns of Some Genes in *snl* Mutants in Table 1.

Supplemental Figure 9. Gene Expression of *SNL1* and *SNL2* in Response to ACC.

Supplemental Figure 10. The *etr1-2* Mutation Reduced the Triple Response of *snl1 snl2-1* to ACC.

Supplemental Figure 11. The Sensitivity of Root Growth to ABA in the Mutants *snl1*, *snl2-1*, and *snl1 snl2-1*.

Supplemental Figure 12. GA Levels and Responses during Seed Germination in the *snl* Mutants.

Supplemental Figure 13. Expression of Genes Involved in Ethylene and ABA Pathways in 7-d-Old Seedlings of Col and *snl* Mutants.

Supplemental Figure 14. ChIP Assay of Upregulated Key Genes Involved in the Ethylene and ABA Pathways in *snl* Mutants Using *18S* as Internal Control Gene.

Supplemental Figure 15. Analysis of Overlapping Genes between *hub1* and *snl1 snl2-1* that Are Up- and Downregulated in Seeds Compared with Col.

Supplemental Table 1. Primers for Mutant Genotyping, Plasmid Construction, qRT-PCR/qPCR, and ChIP Assay.

Supplemental Data Set 1. RNA-Seq Analysis of *snl1 snl2* versus the Wild Type in the 12 h Imbibed Freshly Harvested Seeds.

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AUTHOR CONTRIBUTIONS

Z.W. and Y.-X. L. designed the research. Z.W., H.C., Y.S., X.L., and F.C. performed the experiments. Z.W., Y.L., A.C., and Y.-X.L. analyzed the data. Z.W., M.D., C.Z., and Y.-X.L. contributed reagents/materials/analysis tools. Z.W., Y.-X.L., X.D., and W.J.J.S. contributed to the discussion and article. Z.W. and Y.-X.L. wrote the article.

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