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Sugar signaling is involved in histone deacetylase-mediated repression of embryonic characteristics after germination

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Abstract Sugars are important regulators of germination and seedling development, and sugar signaling is thought to be involved in the repression of embryonic characteristics after germination. In contrast, chromatin remodeling induced by histone modification plays an important role in the control of plant development. We found that *HDA6* and *HDA19* redundantly repress embryo-specific gene expression during germination in *Arabidopsis*. We analyzed the effects of sucrose-containing or sucrose-free medium on post-germination growth arrest in an *HDA6/19* double repression line (*HDA6*/*19*:RNAi). Although *HDA6*/*19*:RNAi seeds showed post-germination growth arrest when grown in sucrose-containing medium, sucrose-free medium relieved growth arrest, indicating the suppression of embryo-specific genes. Thus, sugar signaling may prevent the HDAC-mediated repression of embryonic characteristics after germination. Based on expression analyses of *VSP2* in *HDA6*/*19:*RNAi seedlings, the repression of *HDA6* and *HDA19* may not affect sugar signaling. During germination, HDA6 and HDA19 repress embryonic properties *via* regulation of embryo-specific transcription factors. Sugar signaling may also contribute to this repression of embryonic properties.

Key words: Embryonic property, germination, HADC, post-germination growth arrest, sucrose.

Plant cells undergo drastic changes during seed germination. Before germination, plant cells are dormant and possess some seed reserve substances. After germination, the cells use the seed reserve substances to initiate growth and greening. During the transition from germination to post-germination growth, embryonic properties are lost as an autotrophic nutrition system is established. In multicellular organisms, the defining characteristics of each organ and each developmental stage are determined by the expression of a specific gene cluster. During germination in higher plants, the gene cluster responsible for embryonic properties is suppressed and the cells lose embryonic characters, resulting in a phase transition from embryogenesis to vegetative growth.

Chromatin remodeling induced by histone modification plays an important role in several biological phenomena in eukaryotes, and genetic studies in model plants have demonstrated the importance of chromatin remodeling factors in the control of plant development (Köhler and Grossniklaus 2002; Wanger 2003). Histonedeacetylase (HDAC) catalyzes histonedeacetylation and is involved in various phenomena associated with transcriptional repression. Several studies have examined the putative functions of HDACs in plant development (Chen and Tian 2007). In *Arabidopsis*, the embryo-specific transcription factors LEAFY COTYLEDON 1 (LEC1), FUSCA3 (FUS3), and ABSCISIC ACID INSENSITIVE 3 (ABI3) play key roles in embryogenesis (To et al. 2006; Suzuki et al. 2007). Although the repression of embryogenesis-related genes during germination is thought to occur, the role of HDAC in this process has not been elucidated. In a previous study, we revealed that the treatment of *Arabidopsis* seeds with an HDAC inhibitor resulted in growth arrest and increased transcription of *LEC1*, *FUS3*, and *ABI3* during germination with 90 mM sucrose (Tanaka et al. 2008). Among HDAC mutants, an *HDA6* repression line showed growth arrest and the expression of embryo-specific transcription factors after germination in the presence of low concentrations of HDAC inhibitor. In addition, an *HDA6*/*HDA19* double repression line (*HDA6*/*19*:RNAi) displayed post-germination growth arrest even in the absence of an HDAC inhibitor with 90 mM sucrose (Tanaka et al. 2008).

HDAC-deficient seedlings that undergo growth arrest after germination show strong similarities to seedlings that undergo growth arrest as a result of treatment with high concentrations of sugar (Price et al. 2003).

Abbreviations: ABI3, ABSCISIC ACID INSENSITIVE 3; FUS3, FUSCA3; HDAC, histonedeacetylase; LEC1, LEAFY COTYLEDON 1; PHE1, PHRES1; VSP2, VEGETATIVE STORAGE PROTEIN 2; Ws, ecotype Wassilewskija (wild type).
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Furthermore, the disruption of a gene involved in sugar signaling induces post-germination growth arrest in synchrony with the ectopic expression of *LEC1*, *FUS3*, and other embryogenesis-related genes; these phenotypes are dependent upon the presence of sugars (Tsukagoshi et al. 2007). Based on these results, sugar signaling is thought to be involved in the post-germination growth arrest observed in an *HDA6*/*19*:RNAi line. In this paper, we examined the contribution of sugar signaling to the repression of embryogenic properties during germination.

Materials and methods

Plant materials

The *Arabidopsis thaliana* ecotype Wassilewskija (Ws; the wild type), *HDA6*/*19*:RNAi-1 (CS24039/CS30925), and *HDA6*/*19*:RNAi-1*lec1-1*were examined (Tanaka et al. 2008). For germination, seeds were surface-sterilized in a sodium hypochlorite solution (with an available chlorite concentration of 1%) for 5 min and then rinsed five times in sterile distilled water. The surface-sterilized seeds were incubated at 4°C for 4 days, sown in plastic Petri dishes (9 cm diameter) containing 20 ml of phytohormone-free Gamborg's B5 solid medium (0.8% agar, w/v), and incubated under a 16-h : 8-h light : dark cycle at 25°C. To analyze the effects of sucrose on post-germination growth, surface-sterilized Ws and *HDA6*/*19*:RNAi seeds were sown in B5 solid medium in the presence or absence of 90 mM sucrose.

RT-PCR expression analysis

Total RNA was isolated using an RNAqueous Kit (Ambion, Austin, TX, USA). cDNAs were synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Each gene was amplified under the following conditions: 94°C for 10 min, followed by 25–40 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min. The forward and reverse primers were as follows: *LEC1*, 5′-AGACGGCAGAGAAACAATGG-3′ and 5′-ATTCATCTT-GACCCGACGAC-3′; *VSP2*, 5′-ACGCAAAATATGGATACG-GA-3′ and 5′-AAGGTACGTAGTAGAGTGGA-3′; *UBQ10*, 5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′ and 5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′.

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed using a LightCycler (Roche Applied Science, Indianapolis, IN, USA) and the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Applied Science). *PHE1* transcripts were amplified using the primers 5′-CGCAT-GTGCGGTCATCC-3′ and 5′-TCCAACACCGAAAACTCC-AT-3′. The amount of cDNA produced was calculated using LightCycler 3.1 (Roche Applied Science). *PHE1* expression was normalized against that of *UBQ10*, which was amplified using the primers 5′-GTACTTTGGCGGATTACAACA TC-3′ and 5′-GAATACCTC CTT GTCCTGGAT CT-3′.

Staining with fat red 7B

Seven-day-old seedlings from the Ws and *HDA6*/*19*:RNAi lines were germinated in medium with or without 90 mM sucrose, and then stained with a 0.1% (w/v) solution of fat red 7B (Sigma) as described by Brundrett et al. (1991). Briefly, a 0.1% solution of fat red 7B was prepared by dissolving the dye in polyethylene glycol (average molecular mass=400 D, Sigma) for 1 h at 90°C. An equal volume of 90% (v/v) glycerol was added. Before staining, chlorophyll was removed from seedlings by incubating overnight in 70% (v/v) ethanol. Seedlings were stained for 1 h at room temperature, rinsed several times in water, and then observed under a Leica DMR microscope using bright-field Nomarski optics.

Generation of PHE1::GUS transgenic plants

The generation of a *PHE1::GUS* transgenic line was performed as described by Köhler et al. (2003). The *PHE1* promoter, contained within a 2961-bp sequence of the 5′ flanking region, was amplified by PCR using the primers 5′-CACCGAATTCG-ACTTTAAAATAGTAGAAAAGCTTG-3′ and 5′-AATTCCA-TGGATCTCTTATCTTTTTCTTTTGTG-3′. The amplified PCR product was cloned into pENTR-D-TOPO (Invitrogen). This construct was subcloned into the binary vector pKGWFS7 (Karimiet al. 2002), positioning the *PHE1* promoter upstream of the β-glucuronidase gene, and then introduced into *Agrobacterium tumefaciens* strain GV2260. Wild-type Ws plants were transformed *via* the floral dip method. *PHE1::GUS* plants were crossed with *HDA6*/*19*:RNAi plants to introduce the *PHE1::GUS* transgene.

Staining for GUS activity

Seven-day-old seedlings and siliques that had been longitudinally sliced were fixed for 1 h at −20°C in 90% acetone and then washed three times in 50 mM phosphate buffer (pH 7.0). The specimens were incubated at 37°C in reaction buffer (0.19 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 10 mM EDTA, 0.1% Triton X-100, 0.1 mM KFeCN, 50 mM phosphate buffer, pH 7.2) for 24 h. Whole seeds were observed after clearing in Hoyer's medium (Grossniklaus et al. 1998) using a Leica DMR microscope under bright-field Nomarski optics. Seedlings were observed without clearing in Hoyer's medium.

Results

Effects of sugar on post-germination growth arrest in an HDA6/19 double repression line

Wild-type and *HDA6*/*19*:RNAi-1 seeds were sown in solid medium in the presence or absence of 90 mM sucrose. In wild-type seeds, no difference was observed in the rate of germination or post-germination growth in response to sucrose. In contrast, the rate of post-germination growth in *HDA6*/*19*:RNAi seeds more than doubled when they were sown in sucrose-free medium (Figures 1 and 2). Because growth arrest is coupled with the continued expression of embryo-specific genes and some embryonic properties (Tanaka et al. 2008), *LEC1* expression was analyzed in wild-type and *HDA6*/*19*:RNAi seeds germinated in medium with or without sucrose. *LEC1* expression was not observed in *HDA6*/*19*:RNAi seedlings when they were germinated in

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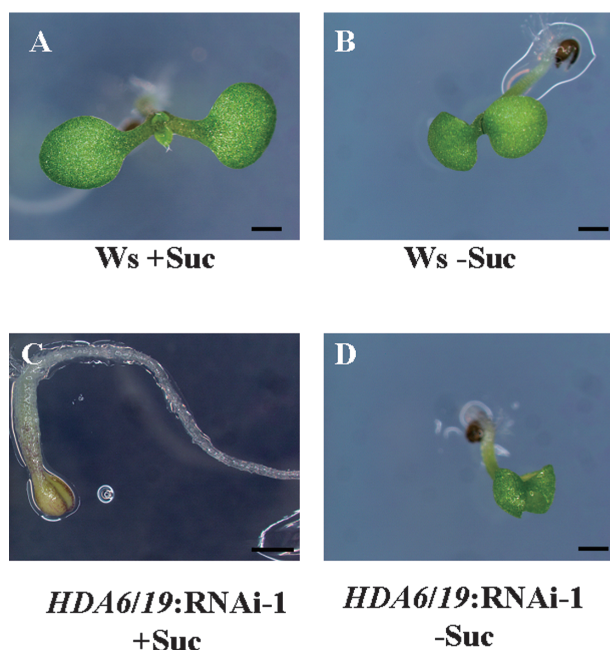

Figure 1. Effect of 90 mM sucrose on post-germination growth in wild-type (ecotype Wassilewskija, Ws) and *HDA6/19* double repression (*HDA6/19*:RNAi) seedlings. (A) Ws seedlings sown in sucrose-containing medium. (B) Ws seedlings sown in sucrose-free medium. (C) *HDA6/19*:RNAi-1 seedlings sown in sucrose-containing medium. (D) *HDA6/19*:RNAi-1 seedlings sown in sucrose-free medium. Photographs were taken 7 days after sowing. Bar=1 mm.

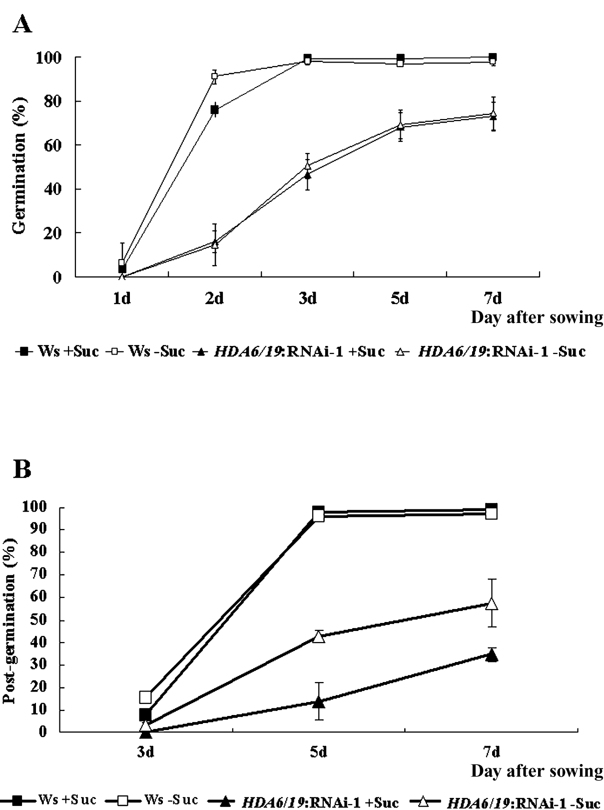

Figure 2. Effect of 90 mM sucrose on germination rate (A) and post-germination growth (B) in wild-type (ecotype Wassilewskija, Ws) and *HDA6*/19 double repression (*HDA6/19*:RNAi) seeds. Closed squares, Ws seeds sown in sucrose-containing medium; open squares, Ws seeds sown in sucrose-free medium; closed triangles, *HDA6/19*:RNAi-1 seeds sown in sucrose-containing medium; open triangles, *HDA6/19*:RNAi-1 seeds sown in sucrose-free medium. The values represent the mean value±standard deviation from three experiments.

sucrose-free medium (Figure 3). Furthermore, we used fat red 7B to confirm that these germinated seedlings had lost their embryonic properties. Because fat red 7B interacts with neutral lipid stores (Brundrett et al. 1991), positive staining indicates the presence of large amounts of triacylglycerol, an embryonic characteristic (Henderson et al. 2004). Fat red 7B staining was not observed in wild-type seedlings germinated in medium with or without sucrose (Figure 4). Dense staining was observed in the closed cotyledon and the hypocotyl of *HDA6/19*:RNAi seeds germinated in sucrose-containing medium, but not in the growing root (Figure 4). In contrast, staining intensity was much weaker in *HDA6/19*:RNAi seedlings germinated in sucrose-free medium.

To examine whether the repression of *HDA6* and *HDA19* was involved in sucrose signaling, we examined the expression of *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), a sugar-inducible gene (Dejardin et al. 1999). *VSP2* expression did not differ between wild-type and *HDA6/19*:RNAi seeds germinated in medium with or without 90 mM sucrose (Figure 3).

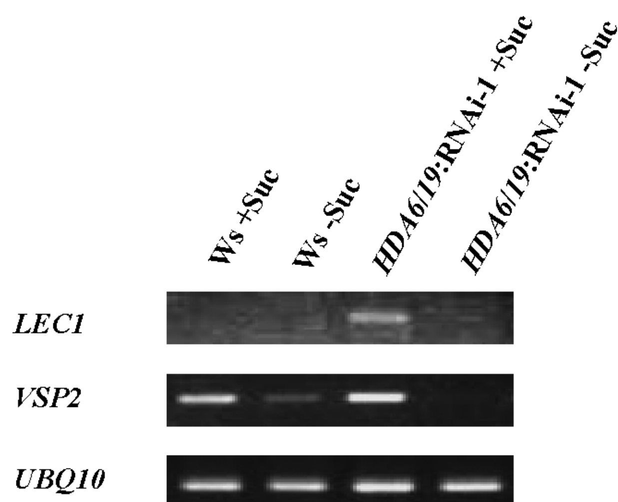

Figure 3. Effect of 90 mM sucrose on *LEC1* and *VSP2* expression in wild-type (ecotype Wassilewskija, Ws) and *HDA6/19* double repression (*HDA6/19*:RNAi, CS24039/CS30925) seeds germinated in sucrose-containing (+Suc) or sucrose-free (−Suc) medium. *UBQ10* was used as an internal standard.

Contribution of HDA6 and HDA19 to the regulation of PHE1 expression during embryogenesis

We also analyzed the expression of another embryo-specific gene, *PHRES1* (*PHE1*), which encodes a

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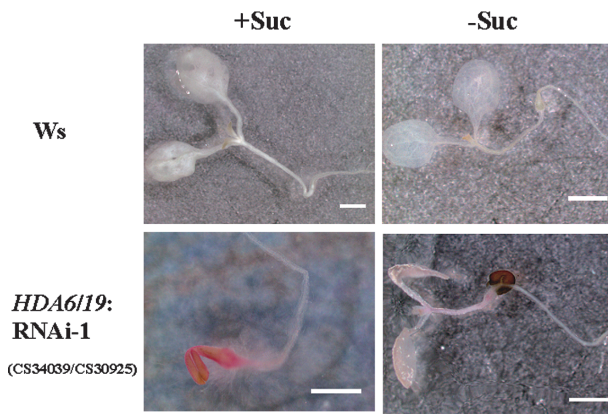

Figure 4. Fat red 7B staining in wild-type (ecotype Wassilewskija, Ws) and *HDA6/19* double repression (*HDA6/19*:RNAi, CS24039/CS30925) seeds germinated in sucrose-containing (+Suc) or sucrose-free (−Suc) medium 7 days after sowing. Bar=1 mm.

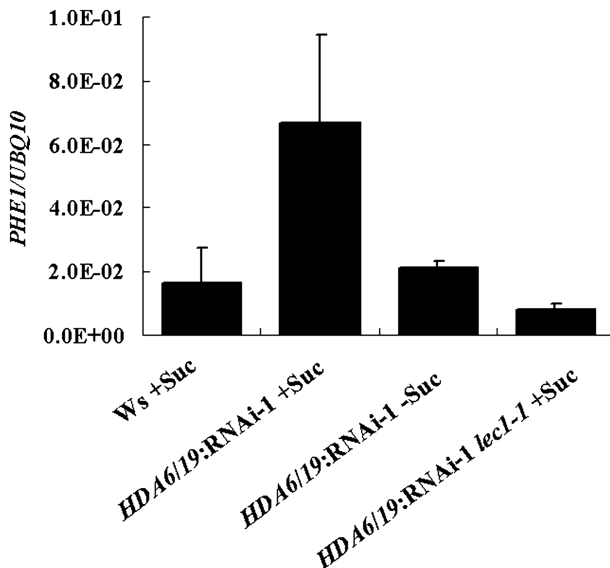

Figure 5. Effect of 90 mM sucrose on *PHE1* expression in germinated seeds. *PHE1*expression was analyzed 7 days after sowing via quantitative RT-PCR. The vertical lines represent *PHE1* expression normalized against *UBQ10* expression. The values represent the mean ±standard deviation from three experiments. Ws, wild-type (ecotype Wassilewskija) seeds; *HDA6/19*:RNAi-1, *HDA6*/19 double repression line; *HDA6/19*:RNAi-1 *lec1-1*, *HDA6/HDA19* double-repression line with an additional *lec1* mutation; +Suc, seeds germinated in sucrose-containing medium; −Suc, seeds germinated in sucrose-free medium.

MADS-box transcription factor. The ectopic expression of *PHE1* and *LEC1* occurs in *pkl* mutants, which retain embryonic characteristics in the root even after germination (Li et al. 2005). *PHE1* expression was examined via quantitative RT-PCR in wild-type and *HDA6/19*:RNAi seeds sown in medium with or without 90 mM sucrose (Figure 5). In wild-type plants, *PHE1* expression was not observed in seedlings germinated in either medium. In contrast, high levels of *PHE1* expression were observed in *HDA6/19*:RNAi seeds germinated in sucrose-containing medium; however,

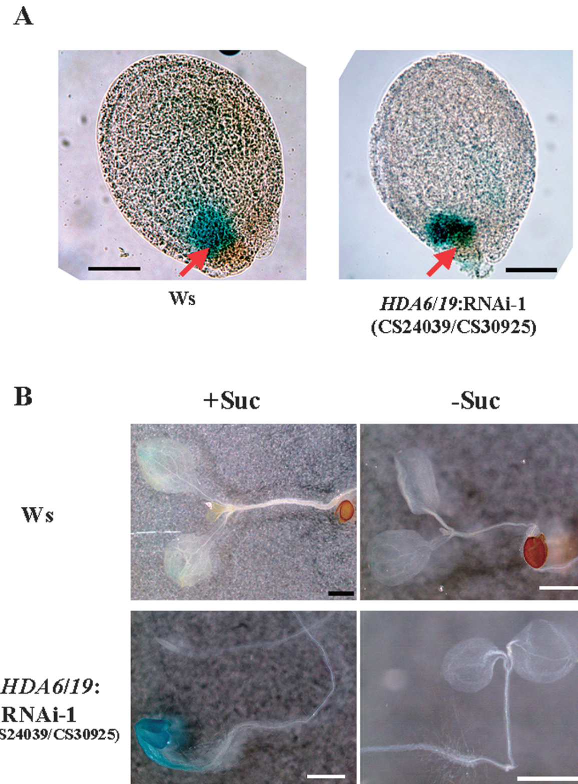

Figure 6. Localization of *PHE1* expression in seeds and germinated seedlings. (A) GUS staining in wild-type (ecotype Wassilewskija, Ws) and *HDA6/19*:RNAi-1 (CS24039/CS30925) seeds transformed with the *PHE::GUS* transgene. Arrows indicate the chalazal endosperm. Bar=0.1 mm. (B) GUS staining in Ws and *HDA6/19*:RNAi-1 seedlings germinated in sucrose-containing (+Suc) or sucrose-free (−Suc) medium. Ws and *HDA6/19*:RNAi-1 seeds were transformed with the *PHE::GUS* transgene and sown in B5 solid medium with or without sucrose. GUS staining was performed 7 days after sowing. Bar=1 mm.

similar to *LEC1* expression, *PHE1* expression was reduced in *HDA6/19*:RNAi seedlings germinated in sucrose-free medium. To examine the association between the ectopic expression of *PHE1* and *LEC1* in *HDA6/19*:RNAi seedlings, *PHE1* expression was analyzed in *HDA6/19*:RNA*i-1lec1-1* seedlings germinated in sucrose-containing medium. As a result, the introgression of a *lec1* mutation abolished the previously observed increase in *PHE1* expression in *HDA6/19*:RNAi seedlings.

The specific expression of *PHE1* in gametophytes and developing embryos is regulated by MEDEA (MEA) via histone methylation, and the ectopic expression of this factor has been observed in the endosperm of *mea* mutants (Köhler et al. 2003; Makarevich et al. 2006). To determine whether HDA6 and HDA19 contribute to the regulation of *PHE1* expression during embryogenesis, the promoter sequence of the *PHE1* gene was ligated upstream of the β-glucuronidase reporter gene to create a *PHE1::GUS* construct. This construct was then introduced into wild-type and *HDA6/19*:RNAi plants. *PHE1* is specifically expressed in gametophytes and developing embryos (Köhler et al. 2003). In wild-type

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plants, GUS staining was observed in developing seeds in the chalazal endosperm (Figure 6A), which is consistent with previous reports (Köhler et al. 2003, 2005). Furthermore, *HDA6*/*19*:RNAi seeds showed a similar staining pattern to that of wild-type seeds. After germination, however, GUS staining was not observed in wild-type seedlings germinated in sucrose-containing or sucrose-free medium (Figure 6B). In the case of *HDA6*/*19*:RNAi seeds, GUS staining was observed in growth-arrested seeds that had been germinated in sucrose-containing medium, but not in seedlings germinated in sucrose-free medium (Figure 6B). Similar to fat red 7B staining, GUS staining was particularly intense in the closed cotyledon and the hypocotyl, but not in the growing root (Figure 6B).

Discussion

The overexpression of *LEC1* in seedlings causes growth arrest after germination (Lotan et al. 1998). Furthermore, ectopic *LEC1* expression in seedlings alters hypocotyl structure and induces the ectopic accumulation of starch and lipids, which is further enhanced in the presence of exogenous auxin and sugars, but not in the presence of GA_3 or ABA (Casson and Lindsey 2006). Sugars are important regulators of germination and seedling development (Gibson et al. 2005). In addition, sugar signaling may be involved in the repression of embryonic properties after germination (Tsukagoshi et al. 2007). Based on these previous studies, sugar signaling is thought to be involved in the expression of embryo-specific transcription factors and the observed post-germination growth arrest in the double repression *HDA6*/*19*:RNAi line. In the present study, the presence of 90 mM sucrose did not affect germination (radicle emergence) in *HDA6*/*19*:RNAi seeds, but did have a negative effect on cotyledon expansion and greening. These arrested seeds were partially rescued when they were germinated in sucrose-free medium (Figures 1 and 2). Because the presence or absence of sugar did not affect the expression profile of the sugar-inducible gene VSP2 in *HDA6*/*19*:RNAi seeds (Figure 3), the repression of *HDA6* and *HDA19* may not affect sugar signaling.

LEC1 expression in *HDA6*/*19*:RNAi seeds was also dependent upon the presence of sucrose during germination (Figure 3). Fat red 7B staining, which indicates the presence of large amounts of triacylglycerol and the maintenance of embryonic properties (Henderson et al. 2004), was observed in the closed cotyledon and hypocotyl of *HDA6*/*19*:RNAi seeds germinated in sucrose-containing medium. However, the staining intensity was much weaker when the seeds were germinated in sucrose-free medium (Figure 4). These results suggest that both the expression of *LEC1* and the maintenance of embryonic properties during post-germination growth are dependent upon the presence of sugar during germination. This notion is supported by the observed increase in *PHE1* expression in *HDA6*/*19*:RNAi seedlings germinated in sucrose-containing medium (Figures 5 and 6). Growth-arrested *HDA6*/*19*:RNAi seeds germinated in sucrose-containing medium showed high *PHE1::GUS* transgene expression; however, GUS staining decreased when seeds were germinated in sucrose-free medium (Figure 6B). In *HDA6*/*19*:RNAi seeds germinated in sucrose-containing medium, *PHE1::GUS* expression and fat red 7B staining were observed in the closed cotyledon and the hypocotyl, but not in the growing root (Figures 4 and 6B); growth arrest was observed in the cotyledon, but not in the root (Figure 1C). These results are consistent with a previous report (Figure 7D and Supplemental Figure 4D in Tanaka et al. 2008). Taken together, these results suggest that post-germination growth is associated with the ectopic expression of embryonic properties.

PHE1 encodes a MADS-box transcription factor that shows transient expression during early embryogenesis (Köhler et al. 2003). Embryo-specific expression of *PHE1* is directly regulated by MEA, a member of the polycomb group proteins, which mediates histone methylation (Köhler et al. 2003; Makarevich et al. 2006). Because histone methylation is occasionally coupled with histonedeacetylation (Peterson and Laniel 2004), and because *PHE1* is ectopically expressed in the endosperm and induces seed abortion in *mea* mutants (Köhler et al. 2003), the misregulation of *PHE1* may also occur in the endosperm of *HDA6*/*19*:RNAi seeds. However, we did not observe the expression of the *PHE1::GUS* transgene in the endosperm of *HDA6*/*19*:RNAi seeds (Figure 6A). In addition, *PHE1* expression in germinated *HDA6*/*19*:RNAi seedlings was three times higher than that in wild-type seedlings germinated in sucrose-containing medium; however, *PHE1* expression disappeared when *HDA6*/*19*:RNAi seeds were sown in sucrose-free medium (Figure 5).

Previous studies showed that the ectopic expression of *PHE1*and *LEC1* occurred in *pkl* mutants that retained embryonic characteristics in the root even after germination (Li et al. 2005). In the present study, the introgression of a *lec1* mutation abolished the previously observed increase in *PHE1* expression in *HDA6*/*19*:RNAi seeds germinated in sucrose-containing medium. These results exclude the possibility of direct HDA6- or HDA19-mediated *PHE1* inhibition via histonedeacetylation at the *PHE1* locus during embryogenesis. It is more likely that this sucrose-dependent *PHE1* expression is triggered by the expression of embryo-specific transcriptional factors. In conclusion, our results indicate that HDA6 and HDA19 repress embryonic properties via the regulation of embryo-specific transcription factors. Sugar signaling

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may also contribute to this repression of embryonic properties.

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