Histone Deacetylase Complex1 Expression Level Titrates Plant Growth and Abscisic Acid Sensitivity in Arabidopsis

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Histone deacetylation regulates gene expression during plant stress responses and is therefore an interesting target for epigenetic manipulation of stress sensitivity in plants. Unfortunately, overexpression of the core enzymes (histone deacetylases [HDACs]) has either been ineffective or has caused pleiotropic morphological abnormalities. In yeast and mammals, HDACs operate within multiprotein complexes. Searching for putative components of plant HDAC complexes, we identified a gene with partial homology to a functionally uncharacterized member of the yeast complex, which we called Histone Deacetylase Complex1 (HDC1). HDC1 is encoded by a single-copy gene in the genomes of model plants and crops and therefore presents an attractive target for biotechnology. Here, we present a functional characterization of HDC1 in Arabidopsis thaliana. We show that HDC1 is a ubiquitously expressed nuclear protein that interacts with at least two deacetylases (HDA6 and HDA19), promotes histone deacetylation, and attenuates derepression of genes under water stress. The fast-growing HDC1-overexpressing plants outperformed wild-type plants not only on well-watered soil but also when water supply was reduced. Our findings identify HDC1 as a rate-limiting component of the histone deacetylation machinery and as an attractive tool for increasing germination rate and biomass production of plants.

INTRODUCTION

Biochemical modifications of DNA and histones regulate gene expression in eukaryotes at a level that is superimposed onto regulation of promoter activity through transcription factors (Berger, 2007). Modifications of specific histone tail residues, alone and in combination, constitute a histone code that is recognized by the transcriptional machinery. Within this context, deacetylation of Lys residues in histones 3 and 4, catalyzed by histone deacetylases (HDACs), establishes a repressive mark (Kouzarides, 2007; Roudier et al., 2009). In plants, histone deacetylation supports fundamental life functions, including maintenance of genome stability (Probst et al., 2004; To et al., 2011; Liu et al., 2012), determination of cell-type specificity (Xu et al., 2005; Hollender and Liu, 2008), and transition between developmental stages (Tanaka et al., 2008; Yu et al., 2011). Gene repression through histone deacetylation is also an important part of the hormonal signaling pathways that orchestrate plant responses to biotic or abiotic stress factors in the environment (Zhou et al., 2005; Chen et al., 2010; Chen and Wu, 2010). The Class1-REDUCED POTASSIUM DEPENDENCY3 (Rpd3)-type HISTONE DEACETYLASE6 (HDA6; often acting redundantly with another enzyme of the same family, HDA19) has emerged as a major player in many of these functions (Kim et al., 2012). For example, HDA6 silences transposons and repetitive elements in a process that is at least partially coupled to DNA methylation (Probst et al., 2004; To et al., 2011; Liu et al., 2012). HDA6 and HDA19 redundantly silence embryonic genes after germination, and in mature plants, HDA6 induces flowering by repression of the flowering inhibitor FLOWERING LOCUS C (FLC; Tanaka et al., 2008; Yu et al., 2011). Furthermore, HDA6 and HDA19 are required for jasmonate/ethylene-mediated defense responses to pathogens (Zhou et al., 2005; Zhu et al., 2011) and for abscisic acid (ABA)-mediated responses to drought or salt (Chen et al., 2010; Chen and Wu, 2010).

Current mechanistic models of how histone deacetylation modulates gene transcription are primarily based on work performed in yeast and mammalian cells showing that de novo nucleosome assembly, chromatin compaction, and recruitment of transcriptional repressors all depend on the acetylation status of histone proteins, which affects the electrostatic histone–DNA interaction and higher-order folding of the chromatin (Kurdistan and Grunstein, 2003; Shahbazian and Grunstein, 2007). Histone deacetylation can occur globally, affecting contiguous regions of the euchromatin, or locally, after recruitment of HDACs by site-specific DNA binding proteins. To enable these processes, HDACs interact with other proteins that establish a structural link between the core deacetylation enzymes, the histones, and the DNA. Several such multiprotein complexes have been biochemically purified in yeast and mammalian cells (Carrozza et al., 2005a, 2005b; Roguev and Krogan, 2007; Yang and Seto, 2008; Chen et al., 2012).
Each of them comprises a distinct set of proteins. For example, so-called Sin3 complexes contain a core deacetylase (Rpd3 and HDAC1/2), a corepressor protein (SWI-INDEPENDENT3 [SIN3]), SIN3-associated proteins, histone binding proteins, and DNA binding proteins. Plant HDAC complexes have not yet been biochemically purified, but several genes with homology to the above-listed HDAC-interacting proteins have been functionally characterized in Arabidopsis thaliana, including SIN3 (Song et al., 2005) and SAP18 (Song and Galbraith, 2006), as well as histone binding proteins MULTICOPY SUPPRESSOR OF IRA1 4/5 (Gu et al., 2011) and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES15 (Zhu et al., 2008). Protein interaction assays and mutant analysis firmly placed these proteins into HDA6/19-regulated pathways, thus providing strong evidence that plant HDACs, as their counterparts in other eukaryotes, operate within multiprotein complexes.

HDACs provide interesting targets for epigenetically engineering stress responses in plants, bypassing the requirement to manipulate many individual components of a complex signaling network. However, the fact that they operate within multiprotein complexes represents a problem for achieving quantitative effects. Indeed, no phenotypes have been reported for overexpression of HDA6. Furthermore, a high degree of redundancy can be expected because HDACs and interacting proteins are encoded by large gene families, suggesting that different complexes assemble depending on tissue type, developmental stage, and environmental condition.

With the aim to identify potential candidate genes for epigenetic manipulation of stress sensitivity in plants, we performed a comprehensive search for plant homologs of confirmed components of yeast and mammalian histone deacetylation complexes. We identified one gene that occurred as a single-copy gene in all sequenced plant genomes, which we called Histone Deacetylation Complex1 (HDC1). HDC1 has partial homology to Regulator of transcription3 (Rxt3), a 34-kD protein of unknown function that coelutes with the large Rpd3 complex in yeast (Carrozza et al., 2005b). However, the function of HDC1 cannot be inferred without further analysis. The functions of Rxt3-type and HDC1-type genes have not been clarified, and neither of them contains any known functional motifs. Furthermore, the plant genes are considerably longer than the ancestral Rxt3 genes and could have acquired new functions.

Here, we present a functional characterization of HDC1 in Arabidopsis. We show that HDC1 interacts with HDACs, promotes histone deacetylation, and regulates known downstream processes, such as ABA sensitivity and flowering. Furthermore, we found that HDC1 overexpression increases plant biomass production. Our results identify HDC1 as a nonredundant, ubiquitous, rate-limiting component of the histone deacetylation
machinery, which offers opportunities for improving plant performance with limited water supply.

RESULTS

**HDC1 Is a Nonredundant, Ubiquitous, Nuclear Protein**

HDC1 (At5g08450) is a single-copy gene in *Arabidopsis*. Predicted splice variants only differ in the upstream untranslated region. Unique HDC1 homologs are also present in all other plant species for which genome information is currently available, including important crops, such as maize (*Zea mays*) and rice (*Oryza sativa*; Figure 1A). The ~900-amino-acid-long sequence of the predicted plant HDC1 proteins contains an ~300-amino-acid-long sequence in the C-terminal half that is highly similar to Rxt3 proteins, which are ubiquitously present in fungi, algae, and protozoa but remain functionally uncharacterized (Figure 1B). Particularly high sequence similarity occurs in a Protein domain family signature (PF08642) labeled as "histone deacetylation Rxt3" (box in Figure 1B). The term derives from biochemical evidence that yeast Rxt3 coelutes with the large Rpd3 complex (Garrozza et al., 2005b), but the region has no homology to catalytic domains of HDACs. Based on sequence similarity, no obvious function can be assigned to this or any other part of the HDC1 sequence. The more variable extended N-terminal part of HDC1 has no counterpart in non-plant genomes. Sequence extension from Rxt3 to HDC1 occurred between algae and land plants, with mosses showing intermediate length (see sequence alignment in Supplemental Data Set 1 online).

The notion of a conserved nonredundant function of HDC1 is supported by ubiquitous expression within the plant. Histological analysis of stable *Arabidopsis* lines expressing β-glucuronidase (GUS) under the control of the HDC1 promoter revealed HDC1 promoter activity in all vegetative tissues, including seed, root, cotyledon, rosette leaf, and flower bud (Figures 2A to 2E). However, GUS was not detected inside anthers and stigmas (Figures 2F), indicating that HDC1 is silenced during reproduction. This is in accordance with a general resetting of chromatin status during reproduction (Paszkowski and Grossniklaus, 2011).

Visualization of a green fluorescent protein (GFP)–HDC1 fusion protein in transiently expressing tobacco (*Nicotiana tabacum*) plants and in stable transgenic *Arabidopsis* plants showed exclusive presence of HDC1 in the nucleus (Figures 2G and 2H) but not in the nucleolus (Figure 2I).

**HDC1 Physically Interacts with HDA6 and HDA19 and Promotes Histone Deacetylation**

To investigate whether HDC1 is a member of HDAC protein complexes in plants, we tested colocalization and direct interaction of HDC1 with known HDACs of *Arabidopsis*. Coexpression of full-length GFP-HDC1 with red fluorescent protein (RFP)–HDA6 or RFP-HDA19 in epidermal tobacco cells indicated tight colocalization of HDC1 with HDA6 and HDA19 in different locations within the nucleus (see Supplemental Figure 1 online). Direct interaction was investigated by bimolecular fluorescence complementation (BiFC). To avoid misinterpretation of background fluorescence, we used a new ratiometric BiFC assay (Grefen and Blatt, 2012) in which N- and C-terminal halves of yellow fluorescent protein (YFP), fused to HDC1 and HDA6/19, respectively, and a full-length RFP are expressed from a single vector (Figure 3A). In RFP-producing cells, a strong YFP signal was recorded for HDA6 and for HDA19, indicating successful BiFC and, hence, interaction of HDC1 with both HDACs. BiFC was also successful when HDA19 was coexpressed with SIN3 previously shown to interact with HDA19 in yeast two-hybrid assays (Song et al., 2005). By contrast, no YFP signal was recorded for HDC1 and SIN3, indicating that HDC1 does not interact with all HDAC complex proteins. Normalization of the obtained YFP signal to the RFP signal from the same cell (Figure 3B) provided statistically significant, quantitative evidence for a strong and specific interaction of HDC1 with the two deacetylases in the heterologous system (Figure 3C).

In vitro pull-down experiments using glutathione S-transferase (GST)– and His-tagged recombinant proteins further confirmed...
the ability of HDC1 to physically interact with HDA6 and HDA19 (Figure 4A). Using GST-HDA6 or GST-HDA19 as bait, HDC1 was pulled down in nuclei-enriched protein samples obtained from leaves of mature Arabidopsis plants (Figure 4B). A single band for HDC1 was detected in these assays, indicating that additional modified or truncated forms of HDC1 in the in vitro system (triple band in Figure 4A) were not produced in planta. HDC1 was not recovered in pull-down assays with GST alone. No HDC1 was detected when the same assays were performed with protein extract from a T-DNA insertion knockout line, hdc1-1 (for mutant description, see below).

To test whether HDC1 had an influence on histone deacetylation activity in the plant, we probed leaf protein extracts from wild-type and mutant lines with a commercial antibody that recognizes acetylated Lys residues 9 and 14 in histone 3 (anti-H3K9K14ac), a predominant target of HDA6 (To et al., 2011). As shown in Figure 4C, hdc1-1 knockout plants produced a significantly higher H3K9K14ac:H3 signal ratio than wild-type plants, indicating higher levels of the acetylated form of H3 over the deacetylated form. HDC1 transcript levels in the other T-DNA insertion lines were similar to those in the wild type or even higher (see Supplemental Figures 3A and 3B online). Some partial mRNA but no HDC1 protein (full-length or partial) was detected in hdc1-1 plants (see Supplemental Figure 2C online). HDC1c complementation lines were obtained by expressing genomic HDC1 under its own promoter (646-bp upstream sequence) in hdc1-1 background. We also produced stable homozygous HDC1-overexpressing lines in Col-0 background using either 35S or Ubiquitin-10 promoter (HDC1-OX1 and HDC1-OX2, respectively). Both lines produced ~30-fold higher HDC1 mRNA levels than the Col-0 wild type (see Supplemental Figure 2D online).

HDC1 Determines the Set Point of ABA Sensitivity during Germination

It was previously reported that hda6 and hda19 mutant lines are hypersensitive to ABA during germination (Chen et al., 2010; Chen and Wu, 2010). Germinating seeds arrest growth and development if they encounter low water potentials in the environment (Finkelstein et al., 2008). The postimbibition response is mediated by ABA and can be mimicked by external application of
ABA. Gibberellin (GA) antagonizes ABA in this response and, hence, seedling growth arrest also occurs if the GA biosynthesis inhibitor paclobutrazol (PAC) is applied (Daszkowska-Golec, 2011). To test a function of HDC1 in this process, seeds of Arabidopsis wild-type, hdc1-1, and HDC1-OX lines were imbibed to break dormancy and subsequently plated out on agar plates containing different concentrations of sodium chloride (NaCl), mannitol, ABA, or PAC. A cumulative germination rate (encompassing all postimbibition stages of seedling development) was scored as the number of seedlings that had developed cotyledons after 6 d. In control conditions, all lines germinated similarly well (close to 100%), and germinated seedlings were similar in size and shape (Figure 5; see Supplemental Figure 4A online). All lines showed a decrease in germination rates with increasing concentrations of NaCl, mannitol, ABA, or PAC; however, compared with the wild type, hdc1-1 was significantly more sensitive, whereas the OX lines were significantly less sensitive to the treatments. Hyposensitivity was observed in both OX lines, independent of promoter or insertion site. Homozygous lines derived from Salk150126C and SAIL1263E05 displayed similar or slightly decreased ABA sensitivity during germination in accordance with a moderate increase of HDC1 mRNA in these lines (see Supplemental Figure 3C online). We conclude that the expression level of HDC1 quantitatively determines the set point of ABA sensitivity in germinating seeds.

The fact that HDC1 overexpression had a desensitizing effect on ABA-dependent germination was interesting because HDA6 overexpression had not been proven to produce physiological phenotypes. We therefore assessed ABA sensitivity in seedlings of an HDA6-overexpressing line previously generated for biochemical studies (Gu et al., 2011). 35S:HDA6 seedlings showed similar ABA sensitivity as wild-type plants, and they were considerably more sensitive to ABA than HDC1-OX seedlings despite a similar increase in transcript level (see Supplemental Figures 5A and 5B online).

To test whether histone deacetylation was required for ABA dependence of seed germination and for the effect of HDC1 on

Figure 4. HDC1 Interacts with HDACs in Planta and Facilitates H3K9/14 Deacetylation.

(A) Anti-His protein gel blots of recombinant HDC1-His after in vitro pulldown with recombinant GST-HDA6 (second lane) and GST-HDA19 (third lane). The first lane contains a positive control (recombinant HDC1-His), and the last lane contains a negative control (pull down with GST alone).

(B) Anti-HDC1 protein gel blots of native HDC1 after pulldown from nuclei-enriched protein samples of wild-type (WT, left) or HDC1 knockout plants (hdc1-1, right) with recombinant GST-HDA6 (second lanes) or GST-HDA19 (third lanes). HDC1 is recognized in the untreated protein samples from the wild type (input) and in wild-type samples after pulldown with GST-HDA6/19 but not with GST alone. HDC1 is not found in protein samples (input or pulldowns) from knockout plants. The bottom panel shows the membrane reprobed with anti-GST, confirming presence of the bait.

(C) Protein gel blot with anti-H3K9K14ac shows increased amounts of acetylated H3K9K14 in protein extract from hdc1-1 plants compared with the wild type (left blot). After complementation of hdc1-1 with HDC1 (HDC1c), H3K9K14ac is reverted to the wild-type level (right blot). Total H3 (loading control) was detected with anti(α)-H3. H3K9K14Ac/H3 signal ratios in wild-type, hdc1-1, and HDC1c lines were determined after quantification of bands with Image J. Bars are means ± se from at least three protein gel blots. Asterisk indicates significant (P < 0.05) difference to the wild type and HDC1c.

Figure 5. HDC1 Desensitizes Seedlings to Salt, Mannitol, ABA, and PAC.

Germination rates of Arabidopsis wild-type (black), hdc1-1 knockout (white), and HDC1-overexpressing (OX) lines (gray) on agar containing different concentrations of salt (NaCl; [A]), mannitol (B), ABA (C), or GA biosynthesis inhibitor PAC (D). Germination rates in percentages reflect the number of seedlings that had developed cotyledons on day 6 after sowing, normalized to the total number of seeds sown. Bars are means ± se of at least three plates containing 50 seeds each. Asterisks indicate significant differences (P < 0.05) to the wild type. A photo of the seedlings is shown in Supplemental Figure 4 online.
this process, we subjected germinating seeds to the HDAC inhibitor trichostatin A (TSA). Unlike higher TSA concentrations tested before (Tanaka et al., 2008), the low micromolar concentrations of TSA applied in our experiments had no effect on seed germination in the absence of ABA (Figure 6). Nevertheless, TSA increased the ABA sensitivity of wild-type plants in a dose-dependent manner, with 0.3 µM producing a significant effect at 0.2 µM ABA and 3 µM TSA producing a significant effect at 0.4 µM ABA. Furthermore, addition of TSA increased ABA sensitivity of the HDC1-overexpressing lines. Thus, ABA sensitivity of germinating seeds and desensitization of seedlings toward ABA by HDC1 overexpression depend on the catalytic activity of HDACs.

HDC1 Does Not Affect Vegetative Development but Is Required for Flowering

Several developmental phenotypes have been reported for HDAC mutants. For example, hda6 hda19 double mutants display embryonic structures on mature leaves and do not repress embryonic-specific transcription factors, such as LEAFY COTYLEDON1 (LEC1), FUSCA3 (FUS3), and ABA INSENSITIVE3 (ABI3) after germination (Tanaka et al., 2008). By contrast, leaves of hdc1-1 plants were normal, and LEC1 and FUS3 were effectively repressed already 2 d after germination (DAG; see Supplemental Figure 4B online). ABI3 transcript was still present at 2 DAG, with hdc1-1 plants expressing higher levels and HDC1-OX plants expressing lower levels than wild-type plants, but was reduced to very low levels in all lines by 6 DAG. We conclude that in control conditions, HDC1 is not required for successful progression of seedlings into the vegetative growth phase.

During vegetative growth, leaf development was normal in hdc1-1 and HDC1-OX plants. New leaves appeared at a similar rate in all lines (Figure 7A). When grown in long-day conditions, wild-type and HDC1-OX plants started to bolt within 4 weeks, whereas hdc1-1 plants continued to produce rosette leaves and flowered—2 weeks later (Figure 7B) at considerably higher rosette leaf number (Figure 7C). The flowering phenotype was reflected in a high transcript level of the flowering inhibitor FLC in hdc1-1 knockout plants on day 28 compared with low levels in the wild-type and HDC1-OX plants (Figure 7D). It can be concluded that HDC1 does not affect vegetative development but is required for the transition to the reproductive stage.

HDC1 Promotes Plant Growth

Despite normal vegetative development, HDC1 mutant lines showed a clear growth phenotype (Figure 8). Differences in root length and leaf size started to appear within 2 weeks after germination (see Supplemental Figure 6 online) and led to significant differences of shoot and root weights in older plants, particularly when the vegetative growth phase was extended by applying short-day conditions (Figure 8). With a similar number of leaves,
4-week-old HDC1-OX plants had produced 20% more and hdc1-1 plants had produced 10% less fresh weight than wild-type plants, and the differences increased to 50% (more or less weight) after 5 weeks (Figure 8A). All lines had a similar relative water content of 92% ± 1%; hence, differences in fresh weight were primarily caused by differences in dry matter. Both HDC1-overexpressing lines showed enhanced growth, with OX2 (Ubi10) being consistently slightly bigger than OX1 (35S) plants. A positive correlation between HDC1 expression level and growth was further confirmed in hdc1-1:HDC1 complementation lines. Plant sizes and weights reflected the HDC1 protein levels in the lines (Figure 8B).

Arabidopsis HDAC mutants have not been shown to have obvious growth phenotypes. We therefore reassessed growth of hda6 knockdown (axe1-5) plants in our growth conditions. Indeed, axe1-5 plants produced less fresh and dry weight than the corresponding wild-type plants (Col-0 DR5) despite slightly higher leaf number (see Supplemental Figure 7 online). By contrast, HDA6-overexpressing plants had similar weights as wild-type plants (see Supplemental Figure 5C online) and therefore did not phenocopy HDC1-overexpressing lines.

HDC1 Alters Transcript Levels and Acetylation Status of Salt Stress–Regulated Genes

To examine a function of HDC1 in transcriptional regulation, we treated 4-week-old hydroponically grown wild-type and mutant plants with 150 mM NaCl for 24 h and determined transcript levels of several known salt stress–responsive genes, including ABA biosynthesis genes ABA DEFICIENT1 (ABA1) and ABA DEFICIENT3 (ABA3), transcription factors RESISTANT TO DESSICATION29A/B (RD29A/B), dehydrin RESPONSIVE TO ABA18, and ABI FIVE BINDING PROTEIN3 (AFP3) (Yamaguchi-Shinozaki and Shinozaki, 2006). We found that after the salt treatment, transcript levels showed a consistent profile across the lines with higher levels in hdc1-1 and/or lower levels in HDC1-OX plants than in wild-type plants (Figure 9). In control conditions, transcript levels of the genes were similarly low in all lines apart from ABA1 transcript, which was increased in hdc1-1. Analysis of shoot ABA levels confirmed that ABA biosynthesis was efficiently induced by salt in all lines, but attained levels were slightly higher/lower in hdc1-1/OX lines (see Supplemental Figure 8 online). ABA receptor PYR1-LIKE4 (PYL4; Lackman et al., 2011) and drought-repressed protein protease-like DROUGHT-REPRESSSED4 (DR4)
are examples of genes that are downregulated by osmotic stress (Kilian et al., 2007). We found that both genes were efficiently repressed by salt stress in all lines, but in control conditions, hdc1-1 and HDC-OX plants produced higher and lower transcript levels than the wild type, respectively.

To assess whether and which of the observed transcriptional changes were a consequence of altered histone acetylation status, we performed anti-H3K9ac/H4ac chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR) on regions encompassing the start codons of the above genes. For ABA1, RD29B, PYL4, and DR4, we recovered less ChIP-DNA from HDC1-OX plants and more from ChIP-DNA hdc1-1 plants than from wild-type plants (Figure 10). By contrast, no change was found for ABA3 (see Supplemental Figure 9 online), suggesting that the transcriptional changes in this gene are the result of positive feedback regulation through ABA (Barrero et al., 2006). The acetylation status of other genes remains to be tested. The results obtained here identify ABA1, RD29B, PYL4, and DR4 as potentially direct targets of HDC1-facilitated histone deacetylation, and they provide a mechanistic explanation for the altered transcriptional responses of these genes in the mutants.

The Growth-Enhancing Effect of HDC1 Overexpression Is Maintained under Moderate Drought and Salt Stress

The combination of enhanced growth with lower expression of stress-inducible genes in HDC1-OX lines raised our curiosity about the net outcome of these potentially counterproductive features on plant performance under water or salt stress. We therefore subjected HDC1 mutant lines and wild-type plants to a controlled water-limiting regime in short-day conditions that started on day 14 and imposed a continuous relative soil water content of 50% of the control condition for the remainder of the experiment (Figure 11A). Differences in growth between the lines were apparent in larger (HDC1-OX) and smaller (hdc1-1) rosettes of younger plants, recorded on days 14 and 28. In older plants, rosette diameters differed less due to maximal extension of the outer leaves, but significant differences of total shoot fresh and dry weights were found when the plants were harvested on day 40 (before flowering). In well-watered conditions, shoot fresh weights were 20% higher in HDC1-OX plants and 40% lower in hdc1-1 plants than in wild-type plants. Limited water supply slowed the growth of all lines (by 30% on day 28 and 80% on day 40; see insets with absolute wild-type data in Figure 11A), yet HDC1-OX plants still produced significantly higher (20%) biomass than wild-type plants, and hdc1-1 knockout plants were still significantly smaller than wild-type plants (although the difference in fresh weight had narrowed to 10%; Figure 11A).

In a second experiment, hydroponically grown plants were subjected for 6 d to salt stress (Figure 11B). Addition of 80 mM NaCl is a physiologically relevant sublethal salt stress dose for moderately salt-tolerant plants such as Arabidopsis (Wang et al., 2006) and did not cause severe chlorosis or desiccation over the course of this experiment (see photos in Figure 11B). However, the treatment reduced shoot water content (from 92% ± 1% to

![Figure 9. HDC1 Knockout/Overexpression Deregulates Salt-Responsive Genes.](image-url)

Transcript levels of salt-responsive genes in the wild type (WT; black), hdc1-1 knockout (KO; white), and HDC1 overexpressing line (OX; gray). Plants were grown for 4 weeks in short-day conditions and subjected (+) or not (−) to 150 mM NaCl for 24 h in hydroponics. mRNA was pooled from three independently treated plant batches of five plants each. Each replicate treatment resulted in a significant increase of ABA (see Supplemental Figure 8 online). Transcript levels were normalized to those of tubulin 9 (TUB9). Bars are means of four technical qPCR replicates ± se. Asterisks indicate significant differences to the wild type (P < 0.05). RAB18, RESPONSIVE TO ABA18.
Here, we presented the functional characterization of a plant gene with an important role in fundamental life processes, including germination, vegetative growth, and flowering. The identification of HDC1 was based on its partial homology with the yeast gene Rxt3. Present in fungi, protozoa, and plants, but not in animals and humans, Rxt3/HDC1 homologs are potentially interesting targets for drug and crop development, but their role in non-plant species has remained obscure. In yeast, Rxt3 coelutes with other proteins of the large Rpd3 histone deacetylation complex, but the protein is neither required for deacetylation of known Rpd3 targets nor for in vitro reassembly of a minimal functional Rpd3 complex (Carrozza et al., 2005a; Chen et al., 2012). The protein sequence of Rxt3 does not contain any regions with known function in enzymatic activity or histone/DNA binding properties. Nevertheless, the Rxt3 part and its core signature are well conserved in the plant proteins (Figure 1B). Clearly, a detailed structure-function analysis of this class of proteins is long overdue. The ABA-hypersensitive germination of Arabidopsis hdc1-1 mutants provides a quick functional assay for such studies.

HDC1-like proteins in land plants are considerably larger than the ancestral Rxt3 proteins (Figure 1B), suggesting additional functions or regulatory sites. Publicly available microarray data indicates that HDC1 transcript is unchanged across a large number of environmental conditions, including salt, drought, and pathogen infection (eFP browser in The Arabidopsis Information Resource; Winter et al., 2007). Whether the HDC1 protein is subject to posttranslational regulation and under which conditions should now be explored. The fact that sequence extension from Rxt3 to HDC1 occurred between algae and land plants is interesting as it coincides with the need of plants to adapt to water stress on land and with the promotion of ABA from growth regulator to plant hormone (Takezawa et al., 2011). It is tempting to speculate that the development of HDC1 as an ABA-stat made an important contribution to this evolutionary progress.

HDC1 Is a Titratable Component of Histone Deacetylation Complexes

hdc1-1 knockout phenocopied knockout/knockdown of HDACs HDA6 or HDA19 with respect to ABA-sensitive germination and flowering (Figures 5 and 7). The fact that hdc1 knockout inhibited both of these HDAC-mediated processes (Song et al., 2005; Yu et al., 2011) although they involve different repressors (ETHYLENE RESPONSE FACTOR7 and FLOWERING LOCUS D, respectively) and different transcriptional targets (ABA-inducible genes and FLC, respectively) positions HDC1 action upstream of the respective signaling pathways. A third phenotype consisting in reduced vegetative growth was also found to be shared between hdc1-1 and hda6 (axe1-1-5) mutants (Figure 8; see Supplemental Figure 7 online), thus adding further evidence for a closely related function. However, hdc1 knockout did not reproduce aberrant developmental phenotypes observed in hda6 hda19 double mutants (Tian and Chen, 2001; Tanaka et al., 2008) suggesting that basal activity of at least one of the two HDACs is maintained in the absence of HDC1.

We further showed that HDC1 physically interacts with HDA6 and HDA19 and is required for deacetylation of Lys 9 and 14 in histone 3 (H3K9K14) (Figures 3, 4, and 10). Based on its general occurrence as a single-copy gene and its ubiquitous expression within the plant, HDC1 is likely to function as a universal scaffolding protein that enhances the apparent HDAC activity by stabilizing the interaction of the enzymes with the substrate or with other regulatory proteins (Figure 12). In yeast and animals, interaction of HDACs with downstream repressors is achieved through corepressor proteins, such as SIN3, and in Arabidopsis,

**DISCUSSION**

**Plant HDC1-Type Proteins Have Extended from Smaller Rxt3 Proteins in Fungi and Algae**

Here, we presented the functional characterization of a plant gene with an important role in fundamental life processes, including germination, vegetative growth, and flowering. The identification of HDC1 was based on its partial homology with the yeast gene Rxt3. Present in fungi, protozoa, and plants, but not in animals and humans, Rxt3/HDC1 homologs are potentially interesting targets for drug and crop development, but their role in non-plant species has remained obscure. In yeast, Rxt3 coelutes with other proteins of the large Rpd3 histone deacetylation complex, but the protein is neither required for deacetylation of known Rpd3 targets nor for in vitro reassembly of a minimal functional Rpd3 complex (Carrozza et al., 2005a; Chen et al., 2012). The protein sequence of Rxt3 does not contain any regions with known function in enzymatic activity or histone/DNA binding properties. Nevertheless, the Rxt3 part and its core signature are well conserved in the plant proteins (Figure 1B). Clearly, a detailed structure-function analysis of this class of proteins is long overdue. The ABA-hypersensitive germination of Arabidopsis hdc1-1 mutants provides a quick functional assay for such studies.

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**HDC1 Is a Titratable Component of Histone Deacetylation Complexes**

hdc1-1 knockout phenocopied knockout/knockdown of HDACs HDA6 or HDA19 with respect to ABA-sensitive germination and flowering (Figures 5 and 7). The fact that hdc1 knockout inhibited both of these HDAC-mediated processes (Song et al., 2005; Yu et al., 2011) although they involve different repressors (ETHYLENE RESPONSE FACTOR7 and FLOWERING LOCUS D, respectively) and different transcriptional targets (ABA-inducible genes and FLC, respectively) positions HDC1 action upstream of the respective signaling pathways. A third phenotype consisting in reduced vegetative growth was also found to be shared between hdc1-1 and hda6 (axe1-1-5) mutants (Figure 8; see Supplemental Figure 7 online), thus adding further evidence for a closely related function. However, hdc1 knockout did not reproduce aberrant developmental phenotypes observed in hda6 hda19 double mutants (Tian and Chen, 2001; Tanaka et al., 2008) suggesting that basal activity of at least one of the two HDACs is maintained in the absence of HDC1.

We further showed that HDC1 physically interacts with HDA6 and HDA19 and is required for deacetylation of Lys 9 and 14 in histone 3 (H3K9K14) (Figures 3, 4, and 10). Based on its general occurrence as a single-copy gene and its ubiquitous expression within the plant, HDC1 is likely to function as a universal scaffolding protein that enhances the apparent HDAC activity by stabilizing the interaction of the enzymes with the substrate or with other regulatory proteins (Figure 12). In yeast and animals, interaction of HDACs with downstream repressors is achieved through corepressor proteins, such as SIN3, and in Arabidopsis,
one of six SIN3-like proteins in Arabidopsis has been shown to interact with both HDA19 and ERF7 (Song et al., 2005). Our quantitative BiFC assay showed SIN3 does not directly interact with HDC1, suggesting that HDC1 operates in histone binding rather than DNA binding. In yeast, Rxt3 and the histone binding protein Pho23 form a submodule that is linked to the core complex via another functionally uncharacterized protein, Rxt2 (Carrozza et al., 2005b). Both Pho23 and Rxt2, as well as other putative members of the complex, have multiple homologs in Arabidopsis. A systematic dissection of HDAC multiprotein complexes in plants is now needed to explore how HDC1 affects their composition and stability in vivo.

Overexpression of HDC1 produced significant phenotypes both at the physiological level (Figures 5 and 8) and at the molecular level (Figures 9 and 10), which were opposite to those of hdc1-1 mutants and hence directly linked to HDC1. To date, no phenotypes have been reported for overexpression of HDA6, and our own analysis of 35S:HDA6 lines did not reveal any phenotypic differences to the wild type (see Supplemental Figure 5 online).

Overexpression of an HDA19 homolog in rice, on the other hand, increased growth but also produced a range of developmental abnormalities that did not occur in HDC1-overexpressing plants. Thus, indirect manipulation of HDAC activity via titration of HDC1 expression levels provides a means to effectively regulate plant growth and stress sensitivity without developmental side effects (see also below).

From a mechanistic point of view, opposite effects of knockout and overexpression of HDC1 indicate that the amount of HDC1 protein is a limiting factor in the histone deacetylation machinery that determines the rate and sensitivity of downstream processes (Figure 12). Viability and normal development of hdc1 mutants also suggests that the role of HDC1 is in fine-tuning rather than essential maintenance.

HDC1 Titrates Transcript Levels of Stress-Responsive Genes

In accordance with a repressive function of histone deacetylation, we found that transcript levels of several known
HC1 lowers the amount of stimulus required for derepression of a gene upon stress, thereby reducing its stress sensitivity (see Supplemental Figure 10A online). Absence of HC1 knockout/overexpression in both well-watered and water-limited conditions should be addressed in the future by monitoring biomass, relative water content, and poststress recovery in hdc1 and HC1-OX plants over a wide range of increasing stress severity.

In conclusion, the discovery of HC1 adds a quantitative element to the histone deacetylation machinery in plants. Several physiological outputs of histone deacetylation that are highly relevant for plant biotechnology can be titrated through HC1 expression, without producing any of the morphological abnormalities observed for manipulation of HDACs in Arabidopsis, rice, and maize (Tian and Chen, 2001; Jang et al., 2003; Zhou et al., 2005; Rossi et al., 2007; Tanaka et al., 2008). Thus, HC1 opens avenues to exploit epigenetic regulation for crop improvement without risking unwanted side effects caused by interference with fundamental roles of chromatin modifications in tissue differentiation. The growth-enhancing effect of HC1 overexpression in both well-watered and water-limited conditions is particularly promising in the context of sustainable, water-efficient agriculture.
METHODS

Plant Materials

All transgenic lines for HDC1 were generated in our laboratory in Arabidopsis thaliana Col-0 background. The stable homozygous knockout line hdc1-1 was obtained from progeny of GABI-Kat 054G03. Stable, homozygous complementation lines were identified from the progeny of hdc1-1 plants transformed with genomic HDC1, including the native promoter (see cloning procedures). Stable, homozygous HDC1-overexpressing lines were generated from the progeny of wild-type Col-0 plants transformed with HDC1 under the control of 35S or Ubiquitin-10 promoters (see cloning procedures). Seeds for 35S:HDA6 (Gu et al., 2011) and ax1-5 (Probst et al., 2004) were kindly provided by Yuehui He and Ortrun Mittlesten Scheid, respectively.

Growth Conditions and Treatments

All experiments were performed in controlled growth rooms at a temperature of 20 to 22°C and a light intensity of 120 to 150 µmol PAR. Plants were grown either in long days (16-h light) or in short days (10-h light) as indicated in text and figure legends. Seeds of Arabidopsis wild-type and transgenic lines were sterilized, stratified, and germinated on soil or on agar plates. Agar plates contained half-strength Murashige and Skoog media with 1% Suc and 0.8% agar at pH 5.7. For germination assays, media were supplemented with NaCl, ABA (Sigma-Aldrich), PAC (Fluka), or TSA (Sigma-Aldrich) at the concentrations given in the figures. Germination rate was scored on day 6 after sowing by counting seedlings that had developed green cotyledons. Experiments with adult plants were performed on soil or in hydroponic culture. For the latter, seeds were germinated on agar plates and 2- to 3-week-old seedlings were transferred to perforated lids of black 0.7-liter plastic containers. The growth medium consisted of a minimal sufficient nutrient medium (Kellermeier et al., 2013). For salt treatment, NaCl powder was stirred directly into the growth container to obtain the desired concentration (as stated in the figure). Control media were stirred without adding NaCl. For controlled drought experiments, plants were grown on soil in pots according to a randomized design. Using previously reported methodology (Granier et al., 2006; Skirycz et al., 2011), controlled watering was used to impose moderate water stress. After 14 d of plant growth in well-watered soil, watering was reduced so that the relative soil water content of the stressed plants was maintained at 50% of the normal watering regime. Control plants were watered normally.

Sequence Alignment

BLASTp searches were performed with the predicted amino acid sequence of HDC1 against the database of nonredundant protein sequences at the National Center for Biotechnology Information using default parameters (matrix BLOSUM62, existence 11, and extension 1). Representative sequences from different kingdoms (listed in Supplemental Data Set 1 online) were subsequently aligned using the COBALT tool (Papadopoulos and Agarwala, 2007) with default parameters (gap penalties (−11/−1 and end-gap penalties −5/−1 for opening/extension). The extracted cluster dendrogram (COBALT tree) reflects the overall similarity between the aligned sequences (note that this is not a phylogenetic tree). An additional sequence alignment was performed with the full-length sequence of the yeast Rxt3 protein and the corresponding parts in the Arabidopsis and Brachypodium distachyon HDC1 proteins.

Cloning Procedures

Entry clones with full-length HDC1, HDA6, HDA19, and SIN3 with or without stop codon were generated by PCR amplification using primers that contained attB1and attB2 sites or attB3 and attB4 as 5’ modifications. Gel-purified PCR products were introduced into pDONR207/221 (Life Technologies) using BP-clonase II according to the manufacturer’s instructions and transferred to destination vectors by recombination using LR-clonase II (Life Technologies). The reaction product was used to transform Top10 bacterial cells. Antibiotic marker-resistant colonies were isolated and verified by restriction digest analysis and sequencing. The following plasmids were generated and used in this study: 35S:HDA6/HDA19-RFP in pB7RWG2, HDC1 (646 bp upstream) promoter in pMDC163, HDC1 genomic DNA (including 646-bp upstream sequence) in pMDC123, 2X35S:HDC1 in pMDC32 (Curtis and Grossniklaus, 2003), Ub10:HDC1 in pUB-Dest, 35S:GFP-HDC1 in pU7GW2 (Karim et al., 2002), Ub10:GFP-HDC1 pUBN-GFPDest (Grefen et al., 2010), 35S:MYFP-HDC1/cYFP-HDA6/HDA19/SIN3 in pBIFCt-2in1-NN, and 35S:MYFP-SIN3/cYFP-HDA19 in pBIFCt-2in1-NN (Grefen and Blatt, 2012).

Antibodies

HDC1 antibody was raised in rabbit (Agrisera), using a synthetic peptide matching amino acids 341 to 356 in the HDC1 sequence, and affinity purified. An extra Cys was added to the N terminus to improve binding capacity. H3K9/K14Ac and H3 antibodies were purchased from Diagenode (pAb-005-044) and Abcam (ab1791). His-tag antibody was obtained from New England Biolabs (No. 2366).

Plant Transformation

Plasmids were inserted by heat shock into Agrobacterium tumefaciens strain GV3101 pMP90 (Koncz and Schell, 1986). Agrobacterium-mediated transformation of Arabidopsis was performed by the floral dip method (Clough and Bent, 1998). Homozygous T2 progenies were used for germination tests. Agrobacterium-mediated transient transformation of Nicotiana tabacum and Nicotiana benthamiana was achieved by leaf infiltration (Geelen et al., 2002). For ratiometric BiFC assays and colocalization studies, each construct was coexpressed with p19 protein of tomato bushy stunt virus, encoding for a suppressor of gene silencing (Voinnet et al., 2003).

PCR

Total genomic DNA was extracted according to Edwards et al. (1991). All PCR reactions were performed with 0.4 units of Taq polymerase (Promega). Total RNA was extracted using hot phenol. cDNA was obtained with the iScript cDNA synthesis kit (BioRad). For quantitative RT-PCR, primer sequences are given in Supplemental Table 1 online. Reactions were performed in four technical replicates on three biological replicates.

ChiP

Chromatin extraction and ChiP were performed using published protocols (Gendrel et al., 2002; Saleh et al., 2008; Sani et al., 2013). Tissue samples were incubated in 1% (w/v) formaldehyde for 15 min under vacuum. To stop cross-linking, 125 mM Gly was added, and tissues were rinsed, blotted dry, and frozen. Chromatin extracts were incubated with antibody against H3K9/K14Ac (Diagenode pAb-005-044) following the manufacturer’s instructions. Immunoprecipitated chromatin-DNA (IP-DNA) and input chromatin-DNA were reverse cross-linked, and residual protein was removed by proteinase K treatment. DNA was extracted with phenol and chloroform and ethanol precipitated. DNA was resuspended and purified by MiniElute Reaction Cleanup kit (Qiagen). Before proceeding to ChiP-qPCR, DNA samples were amplified using GenomePlex Complete Whole
Genome Amplification (WGA2; Sigma-Aldrich) following the manufacturer’s protocol. As a quality control for successful ChiP, existence or absence of sequences previously found to be associated (positive control) or not (negative control) with H3K9K14Ac (Zhou et al., 2010; To et al., 2011) in the ChiP samples was confirmed by PCR. Supplemental Table 1 online lists primer pairs and positions of the amplified regions for Actin2 (positive control), AT1G37110 (negative control), and the tested genes.

Protein Extraction and Protein Gel Blotting
Nuclei-enriched protein extracts were prepared according to a published protocol (Gendrel et al., 2002). The chromatin was extracted twice with 0.4 M H2SO4 and proteins precipitated with 20% trichloroacetic acid. All buffers were supplemented with 100 mM PMSF and proteinase inhibitors (Complete Mini, Roche). Samples were boiled and loaded onto SDS-PAGE gels.

Production of Recombinant Tagged Protein and GST Pull-Down Assays
GST- or His-tagged proteins were expressed in Escherichia coli BL21 cells. Following induction with 1 mM isopropyl β-D-1-thiogalactopyranoside, cells were harvested and sonicated in lysis buffer. The soluble HDC1-His, GST-HDA6, and GST-HDA19 proteins were affinity-purified using the nickel-nitritotriacetic acid (Sigma-Aldrich) and Glutathione-Sepharose resin (GE Healthcare) according to the manufacturer’s instructions. For pull-down assays, GST-tagged proteins were bound to Glutathione-Sepharose resin and applied to a microcolumn. Recombinant HDC1-His or nuclei-enriched plant lysates (Gendrel et al., 2002) were combined with 1X protein inhibitor (Complete Mini, 11836153001; Roche) in Tris(hydroxymethyl) aminomethane-NaCl buffer. Samples were incubated overnight on ice. After several washes, pulled-down protein was eluted in 1X Laemmli buffer.

GUS Assay
Plant tissues from independent primary transformants expressing GUS under control of the HDC1 promoter were infiltrated in a solution containing 2 mM 5-Bromo-4-chloro-1H-indol-3-yl-β-D-glucopyranosiduronic acid. The samples were incubated overnight at 37°C, followed by ethanol washes to remove excess dye. Photos were taken on a stereomicroscope (MBA 166305; Wild Heerbruggi).

Confocal Microscopy
Fluorescence in tobacco epidermal cells was assessed 2 d after infiltration using a CLSM-510-META-UV confocal microscope (Zeiss). For single protein localization, GFP fluorescence was excited at 488 nm with light from an argon laser and collected after passage through an NFT545 dicroic mirror with a 505-nm long-pass filter. For colocalization experiments, GFP fluorescence was collected with a 505- to 530-nm band-pass filter. RFP fluorescence was excited at 543 nm with light from a helium neon laser and was collected after passage through an NFT545 dicroic mirror and a 560- to 615-nm band-pass filter. YFP fluorescence was excited at 514 nm with light from an argon laser and was collected using lambda mode between 520 and 550 nm. Colocalization plane and line scans were evaluated using Zeiss LSM 510 AIM software (v3.2).

Determination of ABA Content
ABA in methanol extracts from dried leaf sample was quantified by liquid chromatography–mass spectrometry (Page et al., 2012) at the University of Exeter Mass Spectrometry Facility (Exeter, UK) using 1200 series HPLC (Agilent Technologies; 3.5 μm, 2.1 × 150-mm Eclipse Plus C18 column) and a 6410B enhanced sensitivity triple quadruple mass spectrometer (Agilent Technologies). [2H6]( + ) -cis.trans-ABA (Chemil) was included as a standard.

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL libraries and in The Arabidopsis Information Resource under the following accession numbers: ABA1, AT5G67030; ABA3, AT1G16540; ABI3, AT3G24680; AFP3, AT3G29575; DR4, AT1G73330; FLC, AT5G10140; FUS3, AT3G26790; HDC1, AT5G08450; HDA6, AT5G63110; HDA19, AT4G38150; LEC1, AT1G21970; PYL4, AT2G38310; RESPONSIVE TO ABA18, AT5G366400; RESPONSIVE TO DESSICATION29, AT1G16540; RD29B, AT5G52300; and SIN3 (also called SIN3-LIKE3), AT1G24190.

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

Supplemental Figure 1. Colocalization of HDC1 with HDA6 and HDA19 within Nuclei of Transiently Expressing Tobacco Epidermis Cells.

Supplemental Figure 2. Confirmation of hdc1-1 Knockout and HDC1-Overexpressing Lines.

Supplemental Figure 3. Salk150126 and Sail1263E05 Are Not hdc1 Knockouts.

Supplemental Figure 4. Without ABA, HDC1 Does Not Alter Germination or Progression of Seedlings to Vegetative Stage.

Supplemental Figure 5. HDA6 Overexpression Does Not Affect Germination or Growth.

Supplemental Figure 6. Root and Leaf Growth Phenotypes in Young Plants.

Supplemental Figure 7. HDA6 Knockdown Affects Plant Growth without Delaying Leaf Development.

Supplemental Figure 8. HDC1 Has a Small Effect on ABA Content after Salt Treatment.

Supplemental Figure 9. HDC1 Alters H3K9K14 Acetylation Levels in ABA1 but Not ABA3.

Supplemental Figure 10. Scenarios for the Effects of HDC1 on ABA-Dependent Gene Expression.

Supplemental Figure 11. Effects of HDC1 Expression on Stress Sensitivity during Vegetative Growth.

Supplemental Table 1. Primers for Genotyping gDNA.

Supplemental Data Set 1. Alignment of HDC1 and Rxt3 Sequences from Different Organisms.

ACKNOWLEDGMENTS
We thank Amparo Ruiz-Prado and Naomi Donald (University of Glasgow) for horticultural assistance. We thank Yuehui He (National University of Singapore), Ortrun Mittelenstedi Scheid (Gregor Mendel Institute Vienna), and Christopher Greifen (University of Glasgow) for supplying seeds and vectors. We thank Hannah Florance and Nick Smirnoff from the Exeter Mass Spectrometry Facility for measuring ABA levels. We thank all members of the Glasgow laboratory for constructive comments on the article. The project was funded by the Leverhulme Trust and by Bayer CropScience.
AUTHOR CONTRIBUTIONS
G.P., M.A.L.-V., M.A.H., and A.A. designed the research. G.P., M.A.L.-V., C.C., E.S., V.G., C.V., and F.K. performed the experiments and analyzed the data. A.A. wrote the article.

Received June 15, 2013; revised August 23, 2013; accepted August 28, 2013; published September 20, 2013.

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