The Distinct Roles of Class I and II RPD3-Like Histone Deacetylases in Salinity Stress Response[1][OPEN]

Minoru Ueda,[a,b] Akihiro Matsui,[a] Maho Tanaka,[a] Tomoe Nakamura,[a,c] Takahiro Abe,[a,d] Kaori Sako,[a] Taku Sasaki,[a,b] Jong-Myong Kim,[a] Akihiro Ito,[e] Norikazu Nishino,[e] Hiroaki Shimada,[c] Minoru Yoshida,[e] and Motoaki Seki[a,b,d,2]

[a]Plant Genomic Network Research Team, RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa 230-0045, Japan
[b]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan
[c]Department of Biological Science and Technology, Tokyo University of Science, Katsushika-ku, Tokyo 125-8585, Japan
[d]Kihara Institute for Biological Research, Yokohama City University, Yokohama, Kanagawa 244-0813, Japan
[e]Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, Wako, Saitama 351-0198, Japan

ORCID IDs: 0000-0002-7750-6056 (M.U.); 0000-0002-3979-3947 (A.M.); 0000-0001-8288-0467 (M.S.).

Histone acetylation is an essential process in the epigenetic regulation of diverse biological processes, including environmental stress responses in plants. Previously, our research group identified a histone deacetylase (HDAC) inhibitor (HDI) that confers salt tolerance in Arabidopsis (*Arabidopsis thaliana*). In this study, we demonstrate that class I HDAC (HDA19) and class II HDACs (HDA5/14/15/18) control responses to salt stress through different pathways. The screening of 12 different selective HDIs indicated that seven newly reported HDIs enhance salt tolerance. Genetic analysis, based on a pharmacological study, identified which HDACs function in salinity stress tolerance. In the wild-type Columbia-0 background, *hda19* plants exhibit tolerance to high-salinity stress, while *hda5/14/15/18* plants exhibit hypersensitivity to salt stress. Transcriptome analysis revealed that the effect of *HDA19* deficiency on the response to salinity stress is distinct from that of *HDA5/14/15/18* deficiencies. In *hda19* plants, the expression levels of stress tolerance-related genes, late embryogenesis abundant proteins that prevent protein aggregation and positive regulators such as ABI5 and NAC019 in abscisic acid signaling, were induced strongly relative to the wild type. Neither of these elements was up-regulated in the *hda5/14/15/18* plants. The mutagenesis of HDA19 by genome editing in the *hda5/14/15/18* plants enhanced salt tolerance, suggesting that suppression of HDA19 masks the phenotype caused by the suppression of class II HDACs in the salinity stress response. Collectively, our results demonstrate that HDIs that inhibit class I HDACs allow the rescue of plants from salinity stress regardless of their selectivity, and they provide insight into the hierarchal regulation of environmental stress responses through HDAC isoforms.

Histones are DNA-packaging proteins that provide stability to the genome by preventing physical genotoxicity (e.g. DNA breaks; Luger et al., 1997; Downs et al., 2007). They also have been considered to originally function as regulators of mRNA expression before the divergence of the Archaea and Eukarya (Ammar et al., 2012). A variety of chemical modifications (acetylation, methylation, phosphorylation, etc.) to the N tails of histones are one of the properties that enable the regulation of mRNA expression, which is generally conserved in eukaryotes (Jenuwein and Allis, 2001; Kouzarides, 2007). Chromatin possesses a diverse array of chemical moieties that allows it to contain and transmit information that is independent of the genetic code (i.e. epigenetic) and regulate gene expression levels. Epigenetic regulation is considered to be profoundly associated with plant development and adaptation to the environment. A complete understanding of the coordinated regulation of gene expression by histone modifications, however, is still lacking in plants. The role of epigenetic regulation in the abiotic stress response has gradually been elucidated, starting with McClintock (1984), who first recognized the relationship between epigenetics and stress (Kim et al., 2015; Provart et al., 2016; Asensi-Fabado et al., 2017).

[1] This work was supported by grants to M.U. and M.S. from RIKEN and by grants to M.S. from the Core Research for Evolutional Science and Technology, Japan Science and Technology Agency (grant no. JPMJCR13B4) and the Ministry of Education, Culture, Sports, Science, and Technology of Japan (KAKENHI on Innovative Areas; grant no. 16H01476).

[2] Address correspondence to motoaki.seki@riken.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Motoaki Seki (motoaki.seki@riken.jp).

M.S. and M.Y. conceived the original screening and research plans; M.U. and M.S. supervised the experiments; M.U., A.M., and M.T. performed most of the experiments; T.A., T.N., K.S., T.S., J.-M.K., A.I., N.N., and H.S. provided technical assistance to M.S. and M.Y.; M.U. and A.M. designed the experiments and analyzed the data; M.U. conceived the project and wrote the article with contributions of all the authors; M.S. and M.Y. supervised and complemented the writing.

[OPEN] Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.17.01332

Among histone modifications, the mode of action of histone acetylation is relatively well understood. The positively charged Lys residues within the N tails of histones are often targets for histone acetylation. These positively charged amino acid residues can bind to the negatively charged region of the nucleosome (proteins and phosphate groups of DNAs), thus affecting chromatin structure. The acetylation of histones, however, neutralizes the positive charges on Lys residues and reduces the binding to nucleosomes, resulting in a relaxed chromatin structure. The open chromatin facilitates the recruitment of transcriptional factors to DNA and enhances transcription (Shahbazian and Grunstein, 2007). Acetylation levels are balanced by histone acetyltransferases and histone deacetylases (HDACs). These enzymes have the ability to write or erase an acetylation mark, respectively. The suppression of HDAC activity results in hyperacetylation, which, in turn, generally leads to transcriptional activation. HDACs are categorized into zinc-dependent and NAD [NAD(+)] types based on their catalytic domains. The Reduced Potassium Deficiency3 (RPD3)-like family and Silent Information Regulator2 (SIR2)-like (sirtuin) family are zinc dependent and NAD (+) dependent, respectively. The RPD3-like family is divided into three classes (I, II, and IV), based on their homology to yeast HDACs (Bolden et al., 2006; Seto and Yoshida, 2014; Verdin and Ott, 2015). Plants also have evolved a plant-specific HDAC (HD-tuin) family (Brosch et al., 1996; Lusser et al., 1997; Hollender and Liu, 2008). Members of the HD-tuin family are considered to be zinc-dependent HDACs (Lee and Cho, 2016). The Arabidopsis (*Arabidopsis thaliana*) genome encodes 18 genes representing three HDAC families (12 RPD3-like family proteins, two sirtuin family proteins, and four HD-tuin family proteins; Hollender and Liu, 2008).

The existence of multiple HDAC gene families implies that a functional diversification of these genes has occurred. Previous studies revealed that HDAC genes exhibit diversified responses when plants are exposed to abiotic stresses and that their independent functions play a pivotal role in plant responses to various environmental stresses. (Chinnusamy et al., 2008; Ma et al., 2013; Kim et al., 2015; Asensi-Fabado et al., 2017). The involvement of HDACs in response to salinity stress also has been documented. For example, HDA9 and HD2D negatively regulate the salt response (Han et al., 2016; Zheng et al., 2016), whereas HDA6 and HD2C positively regulate it (Chen et al., 2010; Chen and Wu, 2010; Luo et al., 2012). In the case of HDA19, previous studies reported controversial data demonstrating that an *hda19* knockout mutant in the Wassilewskija (Ws) background shows sensitivity to salt stress, whereas an *hda19* knockdown mutant in the Columbia-0 (Col-0) background exhibits the opposite phenotype (Chen and Wu, 2010; Mehdi et al., 2016). Taking these previous results into consideration, HDACs appear to activate positive and negative responses in salinity stress, with some discrepancies.

HDAC activity can be suppressed in not only genetic but also pharmacological manners. Many different HDAC inhibitors (HDIs) are currently available, and a variety of different inhibitory effects on each of the classes of HDACs within the RPD3-like family have been demonstrated based on their selectivity to mammalian HDAC proteins. Differences in their selectivity are believed to alter the activity of HDACs targeted specifically by the HDIs (Bolden et al., 2006; Seto and Yoshida, 2014). Although the inhibition of HDACs may lead to an increased sensitivity to salinity stress (as shown in HDA6), Ky-2 and SAHA treatments enhance salinity stress tolerance in Arabidopsis (Sako et al., 2016) and cassava (*Manihot esculenta*; Patanun et al., 2017), respectively. These observations strongly support the potential for using HDIs to improve salt tolerance. In accordance with what occurs commonly with the use of pharmacological compounds, HDIs occasionally have off-target effects. For example, Tubacin, a human HDAC6-selective inhibitor, impairs the enzymatic activity of Ser palmitoyltransferase (Siow and Wattenberg, 2014). Therefore, it is still unclear whether HDAC inhibition is the cause of increased salt tolerance or which HDAC is responsible for inducing salinity tolerance when an HDI treatment is applied.

In this study, 12 different selective HDIs were tested to determine whether their application allows Arabidopsis plants to tolerate salinity stress. The screening revealed that inhibition of the class I HDACs appears to be essential for inducing salinity stress tolerance. A genetic analysis further indicated that dysfunction of HDA19, a class I HDAC, is responsible for conferring the increase in salinity tolerance, whereas dysfunction of four class II HDACs does not. Transcriptome analysis revealed the antagonistic response to a high level of salinity stress between *hda19* and the quadruple *hda5/14/15/18* (*quad*) mutant. Furthermore, we generated the quintuple mutant for class I (*HDA19*) and class II (*HDA5/14/15/18*) HDACs by a gene-driven method using clustered regularly interspaced short palindromic repeats (CRISPR)/associated 9 endonuclease (Cas9). Consistent with a pharmacological study, the *hda5/14/15/18/19* (*quint*) mutant, which is partially analogous to the broad pharmacological inhibition of different class HDACs by nonselective HDIs, shows tolerance to salinity stress. The phenotype of *quint* indicates a hierarchal regulation of HDACs in Arabidopsis: specifically, class I HDAC inhibition hides the sensitivity induced by the inhibition of class II HDACs. In this study, we provide a strategy for chemical screening and a design for discovering plant-specific HDIs to increase environmental stress tolerance. We also discuss the potential role of HDI-like compounds in nature and the functional diversification of HDACs to the salinity stress response.

RESULTS

Selective HDIs Confer Tolerance to High-Salinity Stress in Arabidopsis

Twelve HDI compounds were screened in liquid culture in order to determine which HDI enhances tolerance to high-salinity stress (Fig. 1A). The first

screening was conducted using three dilution series according to the half-maximal inhibitory concentration value (IC_{50}) of each HDI (Supplemental Table S1). This enabled the optimization of any HDI that exhibited evidence of increasing salt stress tolerance. The results of this initial screening revealed that seven HDIs (FK228, JNJ-26481585, LBH-589, MC1293, MS-275, sodium butyrate [NaBT], and trichostatin A [TSA]) clearly increased salinity stress tolerance in Arabidopsis (Fig. 1B).

Global acetylation levels in histone H3 (K9/14Ac) were evaluated by immunoblotting in order to confirm to what extent each of the HDIs increasing salinity tolerance affected histone acetylation status. The accumulation of acetylated histones H3 in total protein extracts was detected in all of the plants treated with HDIs, and the extent to which each HDI increased the acetylation level varied. HDIs having relatively lower IC_{50} values, such as FK228, JNJ-26481585, LBH589, and TSA, were applied to plants at a 5 μM concentration. HDIs with higher IC_{50} values were applied at 100 μM (MC1293 and MS275) or 1 mM (NaBT). FK228, LBH589, NaBT, and TSA HDIs exhibited significant induction of histone acetylation. JNJ-26481585 induced some level of hyperacetylation (Fig. 2). Treatment with MS-275 and MC1293 HDIs induced no significant alteration in the levels of histone H3 acetylation at 16 h after treatment (Fig. 2). However, their treatment induced significant acetylation of histone H3 at different incubation periods (MC1293, 3 h [$P = 0.02$, Student's t test], and MS-275, 24 h [$P = 0.03$, Student's t test]; Supplemental Fig. S1), suggesting that they function as HDIs in plants, although their maximum inhibitory effect on HDACs seems to be exerted at a shorter or longer time than 16 h of incubation. The results from immunoblot analyses suggested that the HDIs that increase salt tolerance also are capable of inducing histone hyperacetylation in Arabidopsis.

A Class I HDAC, *HDA19*, Plays an Important Role in Increasing Salinity Tolerance

HDAC proteins in plants are classified into three types, RPD3-like, sirtuin, and HD-tuins. The RPD3-like HDACs are divided further into three subclasses (Hollender and Liu, 2008). The HDIs used in the previously described screening have the ability to inhibit RPD3-like HDAC enzyme activity in a class-selective or nonselective manner. The seven HDIs that were effective in increasing salt stress tolerance fall into three types based on their selectivity to mammalian HDACs. FK228 and MS-275 are class I-selective HDIs, JNJ-26481585 and NaBT are class I- and II-selective HDIs, and LBH-589 and TSA are nonselective HDIs (Bolden et al., 2006). Based on their selectivity, there was a tendency for class I-selective HDIs to increase salinity tolerance and for class II-selective HDIs to have no effect on salinity tolerance. These observations imply that the inhibition of class I HDACs is imperative in order for Arabidopsis to increase salinity tolerance. Arabidopsis plants, however, might acquire salinity tolerance in an HDAC-independent manner, because chemicals occasionally have off-target effects. In order to exclude the possibility that an HDAC-independent pathway was responsible for enhancing salinity tolerance and to confirm that the increased salinity tolerance resulting from the application of these HDIs is due to the dysfunction of HDAC activity, we investigated whether an *hdac* mutant exhibited tolerance to salinity stress by using transfer DNA (T-DNA) insertion mutants and genome-edited plants by the CRISPR/Cas9 method (Supplemental Fig. S2).

The results indicated that *hda19-3* plants were less sensitive to high-salinity conditions and exhibited a 79.4% survival ratio under the salt stress conditions used in our experiments (Fig. 3A). An attempt at complementation failed because the mutant exhibited an extremely low transformation efficiency. As an alternative, we generated another allele (*hda19-5*) using the CRISPR/Cas9 gene-driven method in order to examine the functional role of *HDA19* deficiency for the salt-tolerant phenotype (Jinek et al., 2012; Fauser et al., 2014; Schiml et al., 2014). A thymine was inserted 79 nucleotides downstream from the HDA19 translational initiation codon, resulting in the generation of a nonsense mutation at nucleotide position 211 (Supplemental Fig. S2). Similar to the *hda19-3* T-DNA insertion mutant, the results indicated that the *hda19-5* mutant exhibited a similar level of salinity tolerance (77.8%; Fig. 3A). *HDA19* transcripts were reduced significantly in both mutants, and those in the *hda19-3* and *hda19-5* mutants were reduced 0.02% and 8.6%, respectively, in relative comparison with the transcript levels of Col-0 plants (Fig. 3B). These data indicate that two lines of plants with independent mutations in HDA19, resulting in two recessive alleles, were both insensitive to salinity stress. This implies that HDA19 plays a pivotal role in tolerance to salinity stress.

Class II HDACs Play an Antagonistic Role in Salinity Stress Tolerance

Plants treated with the class II-selective HDIs (Tubastatin A, MC1568, TMP195, and TMP269; Fig. 1A) did not exhibit any increase in salinity tolerance (Fig. 1B). However, it is possible that these compounds are not bioavailable in planta (e.g. impermeability, instability, etc.) or that they have no effect on plant HDACs because they have a contrasting protein conformation compared with their animal homologs. Therefore, the salt stress response of the *quad* mutant for class II HDAC genes was analyzed in order to determine whether the suppression of class II HDAC activity had any effect on salinity tolerance. Hollender and Liu (2008) categorized HDA5, HDA15, and HDA18 as class II HDACs. Tran et al. (2012) reported that HDA14 has the ability to deacetylate α-tubulin in vitro. Human HDAC6, a class II HDAC, also has been demonstrated to participate in α-tubulin deacetylation (Hubbert et al., 2002). Therefore, α-tubulin deacetylation mediated by

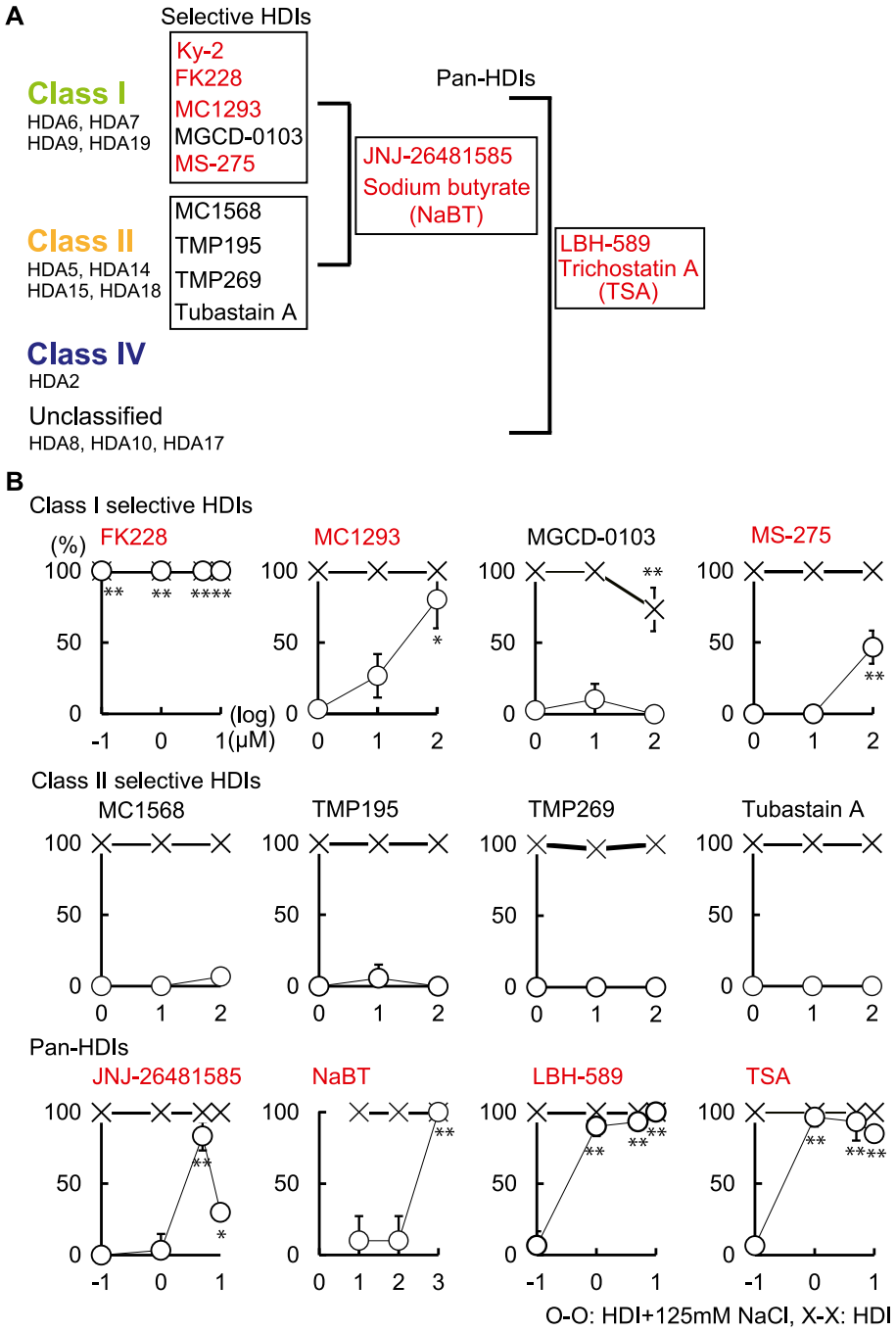

Figure 1. Pharmacological study using HDIs to increase salinity stress tolerance. A, Summary of HDI selectivity based on human HDACs as reported by Bolden et al. (2006) and Lobera et al. (2013). HDIs conferring salinity tolerance are shown in red letters. Ky-2 has been reported previously to confer salinity tolerance in Arabidopsis (Sako et al., 2016). Classification of Arabidopsis RPD3-like HDACs is based on Hollender and Liu (2008) and Tran et al. (2012). B, Increased tolerance to salinity stress by HDI treatments. The survival rate (%) of different HDI-treated plants was evaluated 5 d after treatment with NaCl (means ± SD; $n = 3$, where each biological replicate was a collection of 10 plants). Asterisks indicate significantly different means (*, $P < 0.05$ and **, $P < 0.01$) as determined with Student's t test. Lines with crosses and circles designate the survival rates of HDI-treated plants under normal and salt stress growth conditions, respectively. Statistical significance in survival rates under normal and salt stress conditions is shown above and below the lines, respectively.

class II HDACs is likely to be conserved in eukaryotes. The LEGGY motif, which is broadly conserved in class IIb HDACs in eukaryotes (Tran et al., 2012), also is found in HDA5, HDA14, HDA15, and HDA18 (Arabidopsis Genome Initiative, 2000). Considering these reports, HDA5, HDA14, HDA15, and HDA18 proteins were defined as class II HDACs in this study.

In order to prepare multiple knockout lines of class II HDACs, *hda18-3* was generated by the CRISPR/Cas9 gene-driven method in the *hda5/14/15* background because HDA5 and HDA18 are tandemly located on the same chromosome (Arabidopsis Genome Initiative, 2000). An adenosine was inserted 228 nucleotides downstream from the HDA18 translational initiation codon, which resulted in the generation of a nonsense mutation in the proximity of the A insertion (Supplemental Fig. S2). *HDA18* transcripts were decreased significantly in the *hda18-3* allele. In the *quad* mutant, HDA18 transcripts were at 17.8% of the transcript level of Col-0 plants (Fig. 3C). Consistent with the previous experiment using class II-selective HDIs, the *quad* mutant (which exhibited decreased class II HDAC

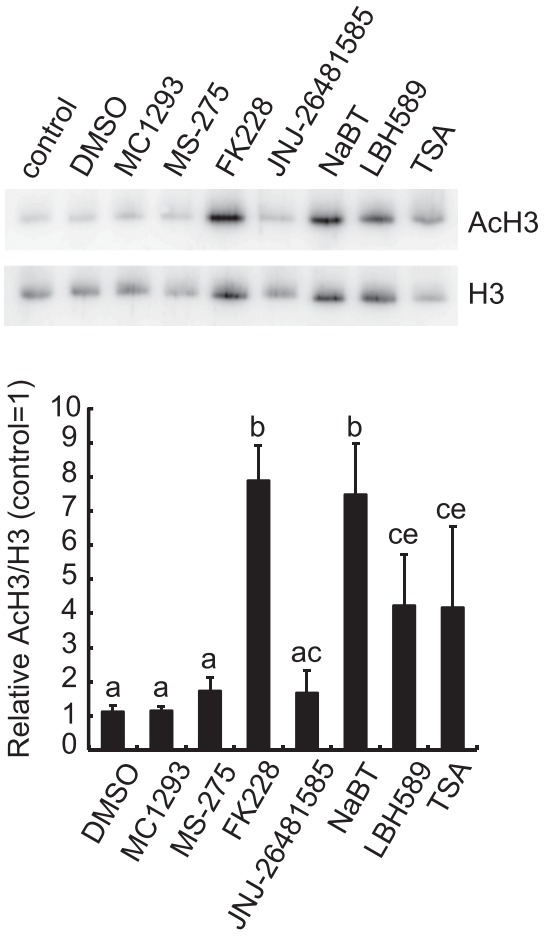

Figure 2. Impact of HDIs on H3 histone acetylation level. Total protein extracts were obtained from samples collected at 16 h after HDI treatment (5 μM FK228, 5 μM JNJ-26481585, 5 μM LBH-589, 100 μM MC1293, 100 μM MS-275, 1 mM NaBT, and 5 μM TSA). HDIs were applied to 4-d-old seedlings after germination. Multiple comparisons of acetylation levels in Col-0 plants treated with each HDI were performed with one-way ANOVA. $P < 0.05$ was considered significant. Means with the same letter are not significantly different from each other.

activity) did not enhance tolerance to salt stress, whereas the *hda19* mutants did. Unlike the *hda19* plants, the *quad* mutant appeared to become more sensitive to salt stress. The *quad* mutant showed a lower survival rate than wild-type (Col-0) plants at each time point (2 and 3 d after the addition of 100 mM NaCl; Fig. 3D). These data suggest that deficiency in class II HDAC activity enhanced the sensitivity to salinity stress.

Collectively, these data indicate that the inhibition of HDA19 and class II HDACs has an antagonistic response in plants exposed to salinity stress.

Transcriptome Analysis of Salt-Responsive Genes in the *hda19* and Class II *hda5/14/15/18* Mutants

In relative comparison with wild-type plants, a 1.6-fold increase in the level of acetylation of histone H3 was detected in *hda19-3* ($n = 4$; Student's t test, $P = 0.02$) and no significant difference in acetylation was observed in the *quad* mutant ($n = 4$; Student's t test, $P > 0.1$; Fig. 3E). Collectively, these data suggest that deficiencies of HDA19 and HDA5/14/15/18 may impact gene expression in different manners. Therefore, the mRNA profile of genes altered by their deficiency was evaluated using a genome-wide microarray in order to reveal how HDAC deficiencies alter the response to salinity stress.

In concert with our immunoblot analysis, which detected a hyperacetylation of histone H3 at the whole-genome level in *hda19-3* plants, a microarray analysis also revealed that the number of up-regulated genes in the *hda19-3* plants was larger than that in the *quad* plants, 824 versus 115 up-regulated genes ($\log_2$ ratio > 0.5, false discovery rate [FDR] < 0.05), respectively (Gene Expression Omnibus [GEO] identifier GSE90562). Nearly completely different sets of genes were up-regulated in the *hda19-3* and *quad* plants, with only 23 and 14 up-regulated genes being common to both types of mutants under normal and salt stress growth conditions, respectively (Fig. 4, B and C; Supplemental Tables S2 and S3). These results suggest that HDA19 and class II HDACs partly overlap in their function of regulating the status of histone and/or nonhistone acetylation. Regardless of this possibility, transcriptome analysis indicated a clear functional divergence between HDA19 and class II HDACs in their regulation of gene expression.

Whole-genome transcriptome analysis under the growth conditions used in this study identified 2,485 up-regulated genes when plants were subjected to salinity stress (Fig. 4). Inhibition or absence of either HDA19 or class II HDAC expression enhanced mRNA levels of 39 or 26 salt-responsive genes, respectively (Fig. 4C; Supplemental Tables S4 and S5). An examination of the up-regulated genes in the two types of mutants revealed that each one is likely to have an opposite response to salt stress. In *hda19-3* plants, ABI5, a bZIP transcriptional factor, was up-regulated under both nonstress and salinity stress conditions (Fig. 5; Supplemental Table S6). *ANAC019* expression, a NAC transcriptional factor, also was induced in response to salinity stress (Fig. 5; Supplemental Table S6). These two transcription factors are considered to be positive regulators in the abscisic acid signaling pathway (Finkelstein, 2013; Podzimska-Sroka et al., 2015). The activation of these transcriptional factors may lead to an up-regulation in the expression of genes encoding proteins or enzymes that allow plants to ameliorate or prevent injury resulting from salinity and other environmental stresses. For example, HDA19 deficiency enhanced the mRNA expression of late embryogenesis abundant (LEA) proteins, which function in preventing protein aggregation (Goyal et al., 2005), and also increased the expression of a rate-limiting enzyme, Δ1-pyrroline-5-carboxylate synthase1 (P5CS1), involved in the synthesis of Pro, an osmoprotectant that accumulates in plants when they are exposed to various

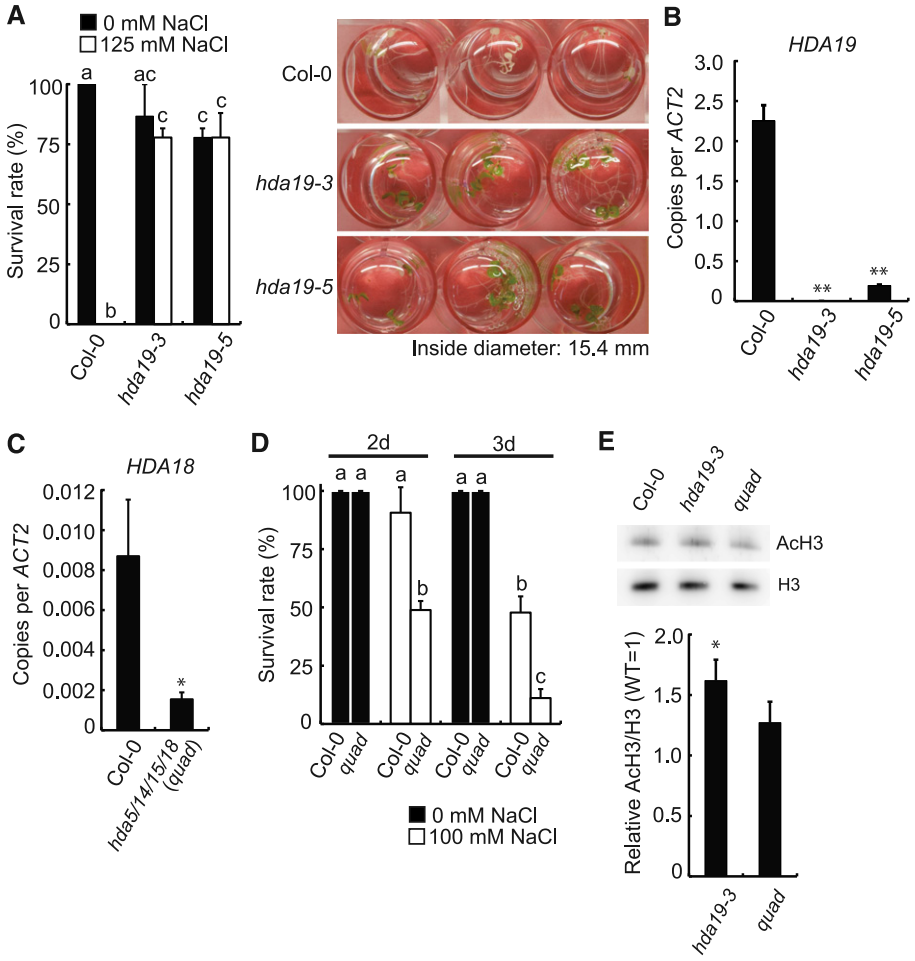

Figure 3. Distinct roles of HDA19 and class II HDACs in the salinity stress response. A, HDA19 deficiency enhances tolerance to salinity stress in Col-0. Images show one replication of each line under salinity stress conditions (125 mM NaCl). B, *HDA19* transcript levels in the *hda19* mutants. The abundance of mRNAs was measured in the *hda19* mutants using reverse transcription-quantitative PCR (RT-qPCR). C, *HDA18* transcript levels in the *quad* mutant. The abundance of mRNAs from the *hda18-3* allele was measured in the *quad* mutant using RT-qPCR. D, HDA5/14/15/18 deficiencies (*quad*) enhance the sensitivity to salinity stress. E, Differences in histone H3 acetylation levels in the *hda19-3* and *quad* mutants. Acetylation levels in *hda19-3* were affected significantly ($n = 4$; Student's t test, $P < 0.05$). For information of the *hda19-3*, *hda19-5*, and *quad* mutants, see Supplemental Figure S2. Survival rates were determined in groups of 15 plants subjected to salinity stress (125 mM NaCl in A and 100 mM NaCl in D; means ± SD; $n = 3$). Multiple comparisons of survival rate were performed with one-way ANOVA. $P < 0.05$ was considered significant. Means with the same letter are not significantly different from each other in A and D. RT-qPCR data in B and C are representative of three independent experiments. *ACTIN2* (AT3G18780) was used as a reference gene. *P* values were calculated using Student's t test (*, $P < 0.05$ and **, $P < 0.01$) in B, C, and E.

environmental stresses (Verbruggen and Hermans, 2008). Ectopic expression of these two genes in various plants increases their tolerance to abiotic stresses, including salinity stress (Verbruggen and Hermans, 2008, and refs. therein; Candat et al., 2014). Among the 51 *LEA* genes encoded in the Arabidopsis genome (Hundertmark and Hincha, 2008), the mRNA expression levels of four of them (AT5G44310, AT2G23110, *LEA4_2* [AT2G35300], and AT1G72100; Supplemental Table S6) and *P5CS1* increased in response to salinity stress relative to the levels in salinity-stressed wild-type plants. The transcript abundance of *LEA4_2* and *P5CS1* was confirmed by RT-qPCR (Fig. 5; Supplemental Table S6). Among the 51 *LEA* genes, only embryonic cell protein63 (AT2G36640) was significantly down-regulated in the *hda19-3* plants (Supplemental Table S7).

ANAC016, a NAC transcriptional factor that is positioned in a different phylogenetic clade from ANAC019 (Takasaki et al., 2015), was strongly up-regulated in the *quad* mutant (Fig. 5; Supplemental Tables S5 and S6). In comparison with wild-type plants, transgenic Arabidopsis plants overexpressing *NAC016* (*ANAC016-OX*) rapidly turn white when subjected to either salt or oxidative stress (Kim et al., 2013). Furthermore, an analysis of drought stress sensitivity in *ANAC016-OX* and *ANAC019-OX* transgenic plants revealed opposite phenotypes, where

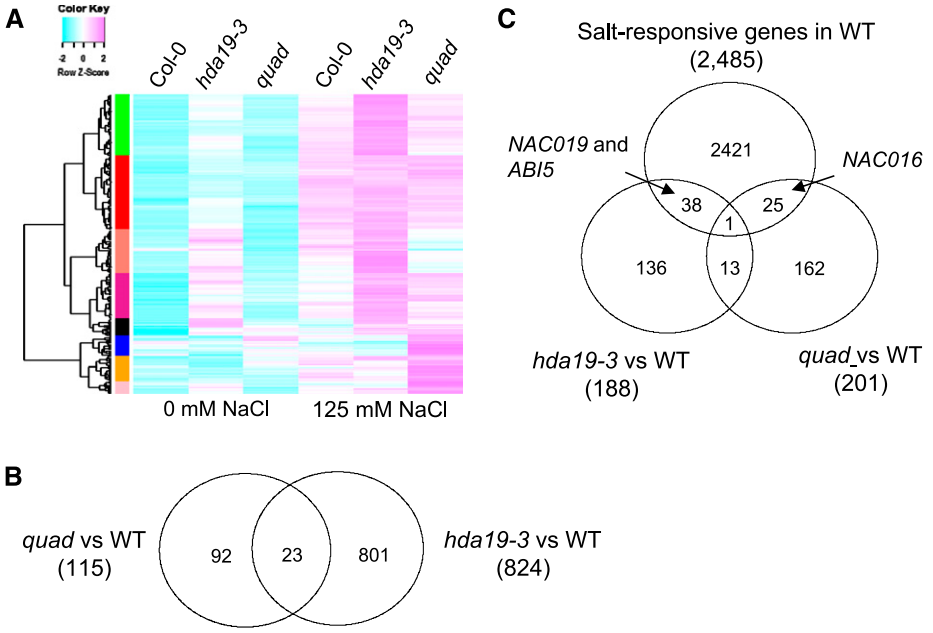

Figure 4. Microarray analysis of genome-wide transcription. A, Hierarchical cluster analysis of 2,485 salt-responsive genes in wild-type (Col-0), *hda19-3*, and *quad* seedlings. Only salt-inducible genes were analyzed in wild-type Col-0 plants, because HDAC disruption causes hyperacetylation and acetylation levels are positively correlated with mRNA expression levels in general (Shahbazian and Grunstein, 2007). The genome-wide mRNA profiles determined by microarray analysis were obtained from 5-d-old plants treated with or without 125 mM NaCl for 2 h. Transcript data were generated from three biological replicates. The heat map represents the Z-score, with bars showing values from −2 to 2. Red represents up-regulated genes, while blue represents down-regulated genes. Genes with a significant change in expression were selected using the following criteria: an expression $\log_2$ ratio greater than 0.5 and a controlled *P* value (FDR; Benjamini and Hochberg, 1995) from Student's *t* test analysis less than 0.05. B, Venn diagram of up-regulated genes in *hda19-3* and *quad* seedlings under nonstressed conditions. C, Venn diagram of up-regulated genes in *hda19-3* and *quad* seedlings subjected to a salinity (125 mM NaCl) stress. WT, Wild type.

ANAC016-OX plants were drought sensitive and *ANAC019-OX* plants were drought tolerant (Tran et al., 2004; Sakuraba et al., 2015). In contrast to the microarray results obtained using the *hda19-3* plants, no significant induction of *LEA* gene expression or *P5CS1* was observed in *quad* plants, relative to wild-type plants, in response to salinity stress (Fig. 5; Supplemental Tables S5–S7). These results indicate that the pattern of mRNA expression found in *quad* reflects its sensitivity to abiotic stress.

Most of the HDIs were basically developed against human HDACs for the sake of cancer therapy (Bolden et al., 2006). In some cases, HDIs have been developed against plant HDACs, and one example is MC1568, which has class II HDAC selectivity against maize (*Zea mays*) HDACs (Mai et al., 2003, 2005). Consistent with the genetic analysis, the mRNA expression of *NAC016* was pharmacologically induced to some extent by MC1568 treatment as in the *quad* mutant (Supplemental Fig. S3).

Collectively, our data indicate that salinity tolerance-related and salt sensitivity-related genes are up-regulated in *hda19* and *quad* plants, respectively. In short, HDA19 and class II HDACs regulate plant responses to salinity stress in opposite and antagonistic manners.

Genetic Manipulation to Mimic the Effect of Nonselective HDI Treatment by Generation of the *hda5/14/15/18/19* Mutant

The interpretation of transcriptome analyses suggests that the reason why any class II HDAC selective inhibitors do not enhance salinity stress tolerance is that class II HDACs positively activate the salinity stress response. In addition, our pharmacological study also implies the possibility that the inhibition of class I HDAC masks the sensitivity to salinity stress that is induced by that of class II HDACs. In order to functionally assess this possibility, the *quint* mutant was generated. Mutagenesis of HDA19 in *quad* resulted in the generation of the *hda19-6* allele by inserting an adenosine 79 nucleotides downstream from the HDA19 translational initiation codon instead of a thymine (which is observed in *hda19-5*). Both single-nucleotide insertions introduced by genome editing resulted in a nonsense mutation 211 nucleotides from the translational initiation codon (Supplemental Fig. S2). Similar to the *hda19* plants, the *quint* plants exhibited a comparable level of salinity tolerance (91.1%; Fig. 6). To further support our hypothesis, the *quad* plants were treated with MC1293 and MS-275, class I-selective HDIs.

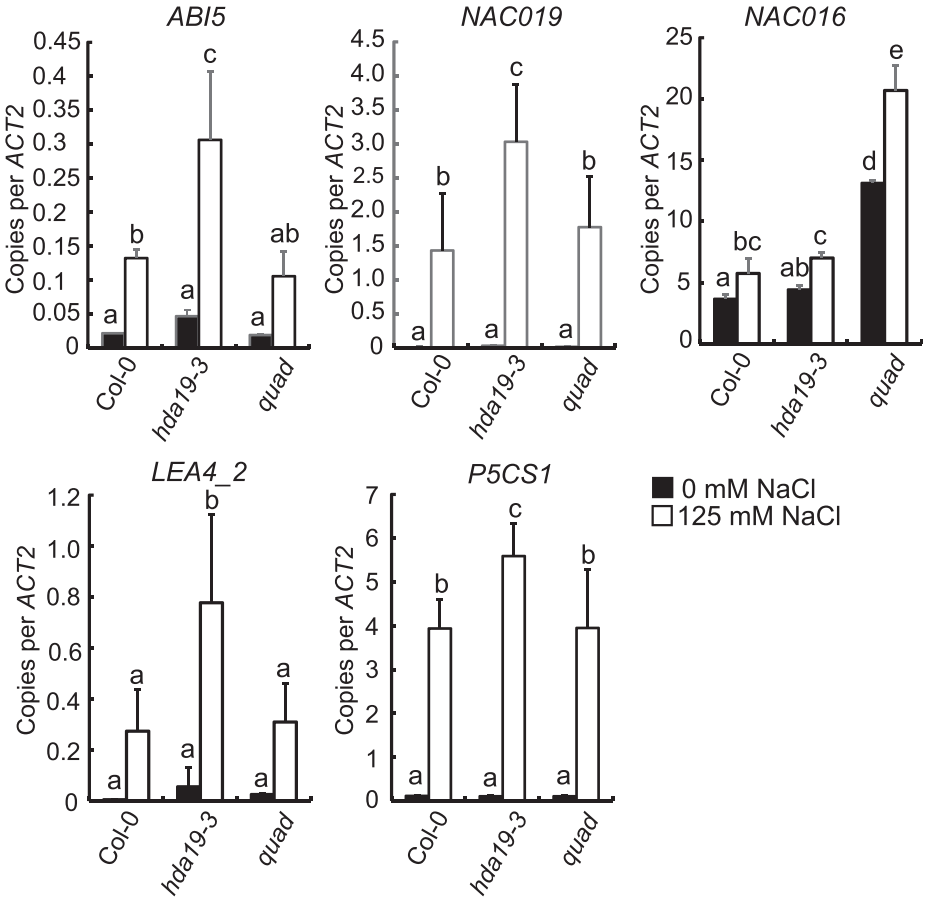

Figure 5. RT-qPCR analysis of *ABI5*, *NAC016*, *NAC019*, *LEA4_2*, and *P5CS1* gene expression. Tissue samples of 5-d-old seedlings of wild-type (Col-0), *hda19-3*, and *quad* plants growing under the salinity (125 mM NaCl) stress condition or the nonstressed condition for 2 h were collected and analyzed by RT-qPCR. Multiple comparisons of RT-qPCR scores were performed with one-way ANOVA. $P < 0.05$ was considered significant. Means with the same letter are not significantly different from each other. *ACTIN2* (AT3G18780) was used as a reference gene. Three independent biological replicates of each line were analyzed for each condition.

As a result, treatment with these HDIs enhanced tolerance to salinity stress in them (Supplemental Fig. S4).

These data suggest that the class II HDAC-dependent pathway was controlled by class I HDAC (HDA19) inhibition and that the hierarchal regulation enables the enhancement of salinity stress tolerance in Arabidopsis via the non-class-selective inhibition of HDAC proteins.

Background-Dependent Phenotype of HDA19 Deficiency in the Salinity Stress Response between Arabidopsis Accessions

In contrast to this study, an opposite role of HDA19 in the salinity stress response was observed in the salt-sensitive *athd1* mutant (Chen and Wu, 2010), which was isolated from the Ws background (Tian et al., 2003). In addition, *hda19-2* also was identified from a T-DNA pool that was produced in the Ws background (Long et al., 2006). To reveal the effect of an HDA19 defect on the salinity stress response in *athd1* and *hda19-2* under our experimental conditions, we functionally characterized their survival rates under salinity stress conditions. Consistent with the previous report, *athd1* showed sensitivity to salinity stress, although wild-type plants in the Ws background showed a higher tolerance to salinity stress (125 mM NaCl) than Col-0. The *athd1* and Ws wild-type plants exhibited 60% and 93.3% survival ratios, respectively (Fig. 7A). In the *hda19-2* mutant, *HDA19* transcripts were decreased significantly (11.7%) relative to the wild type, and these plants showed a similar survival rate as compared with wild-type plants (90%; Fig. 7, A and B). In contrast to *hda19-3*, the up-regulation of *ABI5* was never detected in *athd1* and *hda19-2* (Fig. 7C; Supplemental Table S6), suggesting that *ABI5* expression is not dominantly regulated by HDA19 in young Ws seedlings under salinity stress conditions. Collectively, these data suggest that HDA19 deficiency does not enhance tolerance to salinity stress in the Ws accession and that the role of HDA19 in the salinity stress response has been diversified at least between the Col-0 and Ws accessions. With regard to the noncorrelation between the mRNA levels of HDA19 and the phenotype in the *athd1* and *hda19-2* mutants (Fig. 7), transgene activation via a promoter encoded in the T-DNA for the expression of a selectable marker might occur in *athd1* (see "Discussion").

MS-275, the class I-selective HDI, rescued *athd1* from salinity stress (Supplemental Fig. S5), suggesting that the inhibition of single or multiple class I HDACs, except for HDA19, contributes to increased tolerance to salinity stress in the Ws accession.

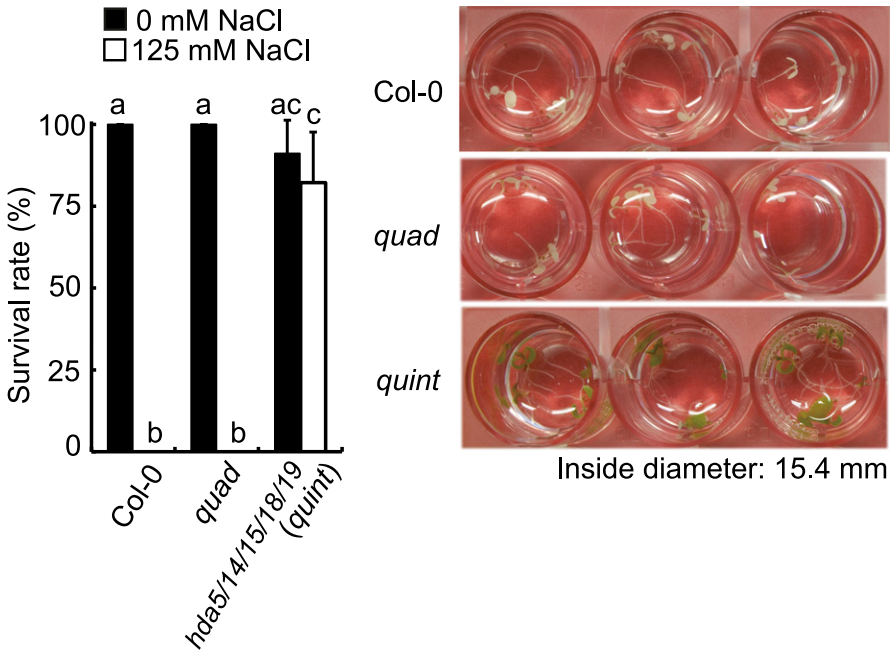

Figure 6. Increased tolerance of *quint* plants to salinity stress conditions. The survival rate (%) of each plant was evaluated 5 d after treatment with 125 mM NaCl or without NaCl (means ± SD; $n = 3$, where each biological replicate was a collection of 15 plants). Multiple comparisons of survival rate were performed with one-way ANOVA. $P < 0.05$ was considered significant. Means with the same letter are not significantly different from each other. Images show the results of one replication of each plant under salinity stress conditions.

DISCUSSION

This study demonstrates that class I HDAC (HDA19) is a possible target enzyme to enhance salinity stress tolerance mediated through the chemical inhibition of HDAC enzymatic activity. In addition, the class-selective inhibition is not necessary for increasing salinity stress tolerance, at least between class I and II HDACs. This evidence provides useful information on chemical screening or design for a plant-specific HDI to enable a practical use for chemical breeding approaches.

Among the four salt tolerance-enhancing HDIs (FK228, JNJ-26481585, LBH589, and TSA) with lower IC_{50}, the optimum concentration of JNJ-26481585 to increase survival rate under high salinity stress was limited (Fig. 1B). JNJ-26481585 did not induce a significant hyperacetylation (Fig. 2). These data suggest that JNJ-26481585 is a relatively unstable and low-persistence compound, relative to the other HDIs that were tested. HDIs capable of inhibiting class I HDAC activity, such as MGCD-0103, had a relatively high IC_{50}, indicating that higher concentrations may be required to inhibit HDAC activity (Davie, 2003; Fournel et al., 2008; Huber et al., 2011). As the hyperacetylation of histone H3 in plants treated with MC1293 and MS-275 HDIs was detected at shorter or longer times than 16 h of incubation (Fig. 2; Supplemental Fig. S1), the maximum inhibitory effect of MGCD-0103 on HDACs might be exerted at much shorter or longer times than its 16 h of incubation. Therefore, although MGCD-0103 did not confer salinity tolerance in our study, MGCD-0103 may have the ability to enhance salinity stress tolerance under different experimental conditions (concentration, incubation time, etc.) and/or growth stages than were used in our study. Of course, it is plausible that the ineffectiveness of MGCD-0103 might account for the different levels of bioavailability and variations in the capacity of the compounds to permeate membranes.

Pharmacological inhibition of HDAC for an extended period of time may be difficult to achieve, as in the case of JNJ-26481585. However, this reversible artificial manipulation in histone acetylation level through the use of chemical compounds that act as HDIs is an effective way to enhance salinity stress tolerance. The *hda19-3* knockout mutants exhibit a sterile phenotype and, thus, rarely produce seeds (Hollender and Liu, 2008). Additionally, the ectopic expression of stress-responsive genes, such as *ABI5* and *P5CS*, which was observed in *hda19-3* plants, often induces growth inhibition (Himmelbach et al., 2003) and toxic levels of Pro accumulation (Verbruggen and Hermans, 2008). HDACs are involved not only in the stress response but also in development (Hollender and Liu, 2008). There is a possibility that multiple and constitutive deficiencies of HDACs may cause severe growth inhibition. In order to avoid or limit these undesired phenotypes and still enhance salinity tolerance, the use of HDI treatment just when plants are subjected to salinity stress would be more appropriate than the constitutive inhibition generated by genetic manipulation, such as in the *hda19* mutants.

TSA was identified originally as a compound produced by *Streptomyces hygroscopicus* with antifungal activity (Tsuji et al., 1976; Yoshida et al., 1990), but it also exhibits HDI activity. Other compounds with HDI activity also have been shown to exist widely in nature. Previous studies have reported the defense-related properties of compounds with HDI activity, which inhibits the growth of competing organisms. Venturelli et al. (2015) demonstrated that plants release aminophenoxazinone compounds, such as 2-amino-3*H*-phenoxazin-3-one

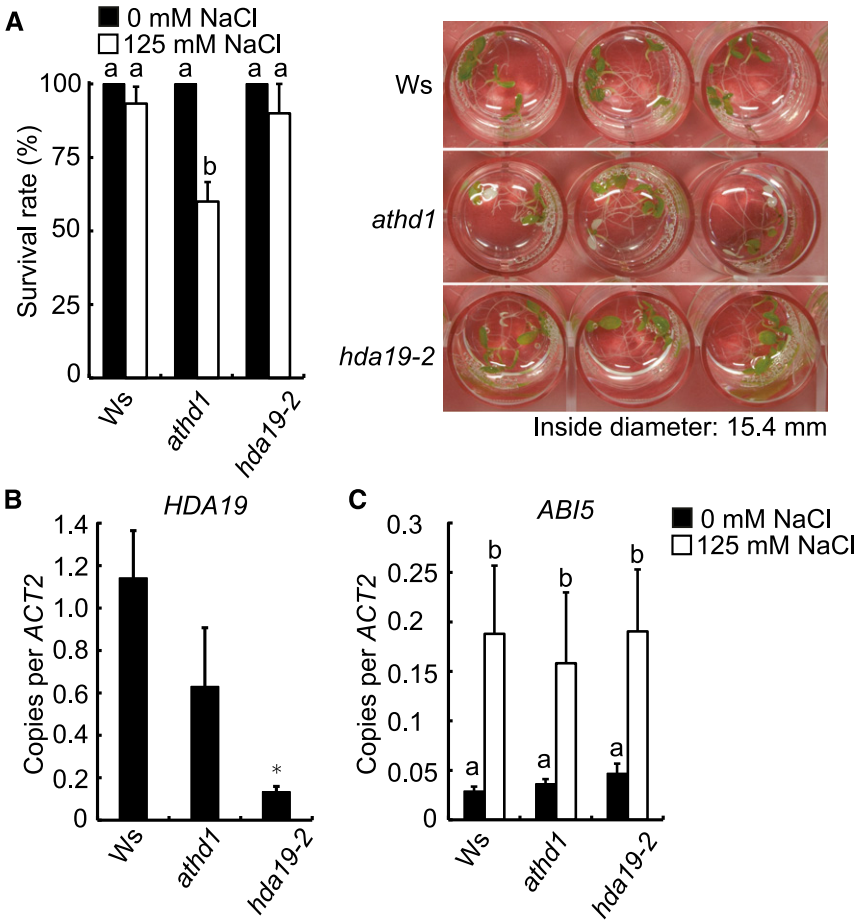

Figure 7. Different responses of *athd1* and *hda19-2* mutants in the Ws background to salinity stress from that of Col-0 *hda19* mutants. A, The survival rate (%) of each plant was evaluated 5 d after treatment with NaCl (means ± SD; $n = 3$, where each biological replicate was a collection of 15 plants). Multiple comparisons of survival rate were performed with one-way ANOVA. $P < 0.05$ was considered significant. B, *HDA19* transcript levels in the *athd1* and *hda19-2* mutants (Ws background). The abundance of mRNAs was measured in the *hda19* mutants using RT-qPCR. The asterisk indicates a significantly different mean ($P < 0.05$) as determined by Student's *t* test. C, RT-qPCR analysis of *ABI5* mRNA expression. Multiple comparisons of *ABI5* expression in different conditions were performed with one-way ANOVA. $P < 0.05$ was considered significant. Means with the same letter are not significantly different from each other in A and C. *ACTIN2* (AT3G18780) was used as a reference gene in B and C.

and 2-amino-7-methoxy-3*H*-phenoxazin-3-one, that have broad HDAC inhibition activity and function as growth inhibitors against competitors. A biological interaction between maize and *Cochliobolus carbonum* via HC toxin, which is a host-selective HDI, also has been reported (Brosch et al., 1995). In both cases, the recipients of the HDI-like compounds appear to be at a disadvantage. This study, however, provides another viewpoint of the involvement of HDIs in biological interactions: namely, that HDAC inhibition in recipient organisms, either directly from donor organisms or by the application of HDI compounds, can be beneficial and help the recipient organisms adapt to and survive adverse environmental conditions, such as salinity stress. Currently, however, there is no evidence demonstrating that recipient organisms use naturally produced HDIs from donor organisms to enhance stress tolerance. This and previous studies (Sako et al., 2016; Zheng et al., 2016) raise the possibility of the existence of HDI-mediated, symbiotic relationships that enhance abiotic stress tolerance. Further studies are necessary to elucidate the role of naturally produced metabolites with HDI properties in natural habitats.

Our data suggest that class I HDAC inhibition contributes to enhanced tolerance to salinity stress. However, it is possible that each HDAC may control the response to salt stress in a different manner among class I HDACs. *hda9* and *hda19* knockdown lines lead to the derepression of salt stress-responsive genes (Mehdi et al., 2016; Zheng et al., 2016). Considering the previous results from the functional analysis of *HDA9* and the *hda19* mutants in this study, both of which belong to class I HDACs, HDA9 and HDA19 negatively regulate salt stress-responsive genes. In contrast to these two class I HDACs, HDA6 appears to positively regulate the salt response (Chen et al., 2010; Chen and Wu, 2010), although HDA6 often acts redundantly with HDA19 (Kim et al., 2012) and HDA9 also interacts with both of them (Zheng et al., 2016). Previous biochemical studies revealed that HDA19/AtHD1, HDA5, HDA6, and HDA15 possess HDAC enzyme activity and that their activities are inhibited by TSA in RPD3-like HDACs (Earley et al., 2006; Fong et al., 2006; Liu et al., 2013; Luo et al., 2015). Therefore, a phenotype in salt stress response conferred by HDA6 inhibition might be hidden by the inhibition of HDA19, as in the case of class II HDACs under treatment with HDIs or in the *quint* mutant.

athd1 is characterized as a null allele of *HDA19* at the protein level (Tian et al., 2003). On the other hand, HDA19 protein accumulation was not observed in *hda19-2*, although both the *athd1* and *hda19-2* mutants showed

similar phenotypes of root formation in the shoot pole during Arabidopsis embryogenesis (Long et al., 2006). This raises the possibility that HDA19 might be weakly expressed at the protein level in *hda19-2*, because a T-DNA is inserted upstream from the translational initiation codon of HDA19 in *hda19-2* (Long et al., 2006). Therefore, the *athd1* and *hda19-2* mutants might show different sensitivities to salinity stress (Fig. 7). Regarding some residual expression in *athd1*, a promoter encoded in the T-DNA for the expression of a selectable marker might accidentally work in transgene activation (Yoo et al., 2005), resulting in noncorrelation between the mRNA levels of HDA19 and the phenotype in the *athd1* and *hda19-2* mutants. Although the sensitivity induced by HDA19 deficiency to salinity stress might be debatable in the Ws background, it appears that the role of HDA19 in response to salinity stress is functionally diversified among Arabidopsis ecotypes (Fig. 7). It is possible that different functional interactions might occur between class I HDACs in the Ws background. Further functional analyses are warranted and necessary in order to clearly elucidate the discrepancies between accessions.

Multiple inhibition of class I HDAC activity (at least HDA9 and HDA19) by HDIs may occur in HDI-treated plants, although the inhibition of HDA19 alone nearly explains the observed salinity stress tolerance as a result of the HDI treatment. The analysis of Ky-2 HDI revealed that *SOS1* gene activation contributes to an increase in salinity tolerance (Sako et al., 2016). In this study, the genome-wide transcriptomic analysis of *hda19* plants did not detect a significant induction of *SOS1* (data not shown). The multiple inhibition of the activity of HDACs could explain why discrepancies occurred between the *hda19* mutants and HDI-treated plants.

In this study, we consider the possibility that the increased expression levels of stress tolerance-related genes, such as *LEA* (Candat et al., 2014), *ABI5* (Skubacz et al., 2016), and *P5CS1* (Verbruggen and Hermans, 2008), might explain why the *hda19* plants showed tolerance to salinity stress. There is another possibility, that the acetylation of nonhistone proteins may control the response to salinity stress in the *hda19* mutants, to explain the effect of HDAC inhibition on increasing salinity stress tolerance. HDA6, a class I HDAC, regulates the level of nonhistone acetylation in the GSK3-like kinase BR-INSENSITIVE2, which is a key negative regulator in the BR signaling pathway, resulting in the control of phytohormone balance (Hao et al., 2016). Some HDACs, such as HDA14 and SRT2, are actually targeted to organelles (Alinsug et al., 2012; König et al., 2014), suggesting that HDACs might be involved in the acetylation of nonhistone protein and regulate enzymatic activity or protein stability like HDA6. Furthermore, HDA19 forms a complex with Histone Deacetylase Complex1 and MSl1, and they control HDA19 activity in stress responses (Perrella et al., 2013; Mehdi et al., 2016; Asensi-Fabado et al., 2017). It is also reported that the BES1/TPL/HDA19 repressor complex mediates the inhibitory action of brassinosteroids on abscisic acid responses (Ryu et al., 2014). Considering the above, the identification of a target with which HDA19 interacts directly or indirectly, including histones and nonhistone proteins, is needed in order to reveal how the suppression of HDA19 activity contributes to enhancing salinity tolerance.

In the case of *quad*, the significant acetylation could not be detected by immunoblotting (Fig. 3E; $P = 0.29$), although previous studies reported that at least HDA5 and HDA15 are involved in the acetylation of histone H3 (Luo et al., 2015; Gu et al., 2017). Our transcriptome analysis revealed that 824 genes are up-regulated in *hda19-3*. In *quad*, only 115 genes were up-regulated (Fig. 4B), which suggests that HDA5/14/15/18 deficiencies might have less impact on the levels of histone acetylation than HDA19 deficiency. High-resolution analyses such as chromatin immunoprecipitation sequencing and/or tissue- and stage-specific analyses may be necessary to detect the targets where each class II HDAC regulates predominantly. Alternatively, the absence of significant induction in *quad* might indicate either that the substrate of HDA5/14/15/18 is not histone proteins or is histones whose acetylation residues were not tested. Further analysis also is needed to reveal how class II HDACs regulate sensitivity to salinity stress.

In conclusion, this study clearly demonstrated genetically, and by chemical HDI experiments, that inhibition of HDACs confers increased salinity tolerance in Arabidopsis plants. A genome-wide transcriptomic analysis revealed the antagonistic regulation of HDACs in response to salinity stress, and the class II HDAC-dependent pathway was controlled by another HDAC, implying that there is a recessive epistasis of class II HDACs to class I HDACs in conferring salt stress tolerance by HDIs. The epistasis probably allows nonselective HDIs to confer salinity stress tolerance. This information will be useful for the identification of new compounds that can be applied to plants to increase salinity tolerance.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*; Col-0 and Ws ecotypes), *hda5*; FLAG_351H04 (Ws), *hda14*; SALK_097005 (Col-0), *hda15*; SALK_004027 (Col-0; Xu et al., 2005), *hda18-3* (a genome-edited allele in *quad*), *athd1* (Ws; Tian et al., 2003), *hda19-2* (Ws; Long et al., 2006), *hda19-3*; SALK_139445 (Col-0; Kim et al., 2008), *hda19-5* (a genome-edited allele in Col-0); and *hda19-6* (a genome-edited allele in *quint*) were used in the course of this study (Supplemental Fig. S2). The T-DNA insertional mutants were obtained from the Arabidopsis Biological Resource Center (Samson et al., 2002; Alonso et al., 2003). The *quad* mutant was generated to prepare a genetically homozygous line (*hda5* is the Ws background) as follows. The *hda5* plant was backcrossed to wild-type plants (Col-0) twice, *hda14*, and *hda15*, successively. Homozygous *hda5/14/15* mutants segregated from heterozygous *hda5/14/15* mutants were subjected to CRISPR/Cas9-mediated mutagenesis. After the mutagenesis, the plant was backcrossed to wild-type plants (Col-0), and a homozygous *hda5/14/15/18* was obtained from a self-fertilized population. The crossing also was performed to eliminate the constitutive expression of the CRISPR/Cas9 protein and eliminate any alternative targets being affected by the CRISPR/Cas9 protein. The sequences of the primers used to genotype the mutants are listed in Supplemental Table S8. After surface sterilization with sodium hypochlorite, followed by two rinses with distilled water, seeds were floated on 1 mL of liquid medium (one-half-strength

Murashige and Skoog medium with 0.5% MES and 0.1% agar, pH 5.7) at 4°C for 48 h on 24-well tissue culture plates (TPP). After germinating, the plants on the 24-well tissue culture plates were placed in a growth chamber at 22°C with a long-day photoperiod (16-h/8-h light/dark cycle) at 50 to 100 μE m^{-2} s^{-1}.

Generation of Mutant Arabidopsis Lines Using the CRISPR/Cas9 System

The *hda18-3*, *hda19-5*, and *hda19-6* alleles were generated by genome editing as described in previous studies (Fauser et al., 2014; Schiml et al., 2014). To express a single guide RNA (sgRNA) and CRISPR/Cas9 protein, pZH_OsU3-gYSA_FFCas9 and pUC_AtU6oligo vectors were used for targeted mutagenesis in HDA18 and HDA19. The sgRNA information used to target HDA18 (P15/P16) and HDA19 (P17/P18) is presented in Supplemental Table S8. The *hda19-5* and *hda19-6* alleles were generated from the same sgRNA. The sgRNAs used for mutagenesis in the generation of them were designed using the CRISPR-P program (Lei et al., 2014).

Evaluation of Salinity Tolerance after Application of HDIs in the *hdac* Mutants

The list of HDIs used in the experiments includes FK228 (Narita et al., 2009), MGCD-0103 (Mocetinostat; ChemieTek; CT-MGCD), Tubastatin A (Selleck Chemicals; S8049), LBH-589 (Panobinostat; Selleck Chemicals; S1030), TMP195 (Axon Medchem; Axon 2180), TMP269 (Cellagen Technology; C8626-2s), NaBT (Wako Pure Chemical Industries; 193-01522), MC1293 (Enzo Life Sciences; ALX-270-344-M005), MC1568 (AdooQ BioScience; A10560-10), MS-275 (AdooQ BioScience; A10611-50), JNJ-26481585 (AdooQ BioScience; A10492-10), and Trichostatin A (Sigma-Aldrich; T1952). The inhibitor treatments were applied to 4-d-old plants (counted after seeds had germinated) in liquid culture. At 16 h after the inhibitor had been applied and absorbed, 125 mM NaCl was added to the growth medium, and the percentage survival was determined 5 d later (three biological replicates consisted of 15 plants; means ± SD). In the evaluation of salinity tolerance in the *hda19-3*, *hda19-5*, and *quad* mutants, all conditions and sample sizes were the same, with the exception that 5-d-old plants (counted after germination) were used instead of 4-d-old plants. Significant differences between the survival values of the experimental plants, relative to untreated wild-type plants, were determined using Student's *t* test ($P \leq 0.05$).

Microarray Analysis

Total RNAs were extracted from 5-d-old plants using the RNeasy Plant Mini Kit (Qiagen). RNAs isolated at 2 h after treatment with or without 125 mM NaCl served as controls. All RNAs were further purified by incubation with RNase-free DNase I (Qiagen) according to the manufacturer's instructions. RNAs were reverse transcribed into cDNAs using 400 ng of total RNA. cDNA was labeled with a single color (Cy3) using the Quick Amp labeling kit (Agilent Technologies) and hybridized to an Arabidopsis custom microarray (Nguyen et al., 2015; GEO array platform GPL19830; Agilent Technologies). Arrays were scanned with a microarray scanner (G2505B; Agilent Technologies). The resulting microarray data were deposited in and are available on the GEO Web site. The R program version 3.2.3 was used for the analysis of the microarray data. The fluorescence intensities of the microarray probes were normalized by quantile normalization using the limma package (Smyth, 2004). Genes with a significant change in expression were selected using the following criteria: an expression $\log_2$ ratio greater than 0.5 and a controlled *P* value (FDR; Benjamini and Hochberg, 1995) from Student's *t* test analysis less than 0.05. For construction of the heat map, a Z-score was computed for each of the selected genes using gplots. Pairwise distances between all expression data were calculated using the euclidean method, and hierarchical clustering on this distance matrix was constructed using the ward method. The information from the microarray data are available on the GEO Web site (GEO identifier GSE90562).

RT-qPCR Analysis

First-strand cDNA was synthesized from 500 ng of total RNA with random primers. ReverTra Ace (Toyobo) was used for the reverse transcription reaction according to the manufacturer's instructions. Transcript levels were assayed using Fast SYBR Green Master Mix (Applied Biosystems) and the StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocols. Gene-specific primers were designed using the PrimerQuest tool (http://sg.idtdna.com/primerquest/Home/Index). Melting-curve analysis was conducted to validate the specificity of the PCR amplification. At least three biological replicates were used in each RT-qPCR assay. *ACTIN2* was used as a reference gene to normalize data. The RT-qPCR scores and relevant primers are listed in Supplemental Tables S6 and S9, respectively. Changes in gene expression were statistically analyzed with Student's *t* test or one-way ANOVA from data obtained from more than three biological repeats.

Immunoblotting

Total protein from 10 4-d-old plants treated with each of the HDIs was solubilized in 100 μL of 2× Laemmli buffer under reducing conditions and heated at 95°C for 3 min. Proteins were separated by SDS-PAGE on 12.5% or 15% bis-Tris gels (Nacalai Tesque) and subsequently transferred to an Immobilon-P polyvinylidene difluoride membrane (Merck Millipore). Loading protein volume in each lane was confirmed by histone H3 levels. The membranes were blocked for 1 h with 5% skim milk, followed by overnight incubation with a primary antibody at 4°C, and final incubation for 1 h with anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences). Primary antibody dilutions were as follows: acetylated histone H3, 1:2,000 (Merck Millipore; 06-599); acetylated histone H4, 1:4,000 (Merck Millipore; 06-866); H3, 1:5,000 (Abcam; 1791); and H4, 1:3,000 (Abcam; 10158). Immunoreacted proteins were detected by Chemi-Lumi One Super (Nacalai Tesque) and image analysis on a LAS4010 (GE Healthcare Bio-Sciences) scanner. Labeling intensities on the images were quantified using ImageQuant TL software (GE Healthcare Bio-Sciences). The histone acetylation level was normalized to correspond to the level of the histone variant. The results were generated from three technical repeats for statistical analysis by one-way ANOVA or Student's *t* test.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *HDA5*, AT5G61060; *HDA14*, AT4G33470; *HDA15*, AT3G18520; *HDA18*, AT5G61070; *HDA19*, AT4G38130; *ACTIN2*, AT3G18780; *UBC21*, AT5G25760; *ABI5*, AT2G36270; *NAC016*, AT1G34180; *NAC019*, AT1G52890; *LEA4_2*, AT2G35300; and *P5CS1*, AT2G39800.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Impact of MC1293 and MS-275 treatment on H3 histone acetylation levels at different time points.

Supplemental Figure S2. Construction of mutants used to identify the distinct functions of class I and class II HDACs in plants subjected to salinity stress.

Supplemental Figure S3. RT-qPCR analysis of *NAC016* gene expression in MC1568-treated plants.

Supplemental Figure S4. Increased tolerance of *quad* plants to salinity stress by MC1293 and MS-275 treatment.

Supplemental Figure S5. Increased tolerance of *athd1* plants to salinity stress by MS-275 treatment.

Supplemental Table S1. Characteristic information of HDIs applied in this study.

Supplemental Table S2. List of genes up-regulated in both *hda19-3* and *quad* under normal growth conditions.

Supplemental Table S3. List of genes up-regulated in both *hda19-3* and *quad* under salinity stress conditions.

Supplemental Table S4. List of genes up-regulated in *hda19-3* under salinity stress conditions.

Supplemental Table S5. List of genes up-regulated in *quad* under salinity stress conditions.

Supplemental Table S6. RT-qPCR scores.

Supplemental Table S7. mRNA expression profiles of 51 *LEA* genes as determined by microarray analysis.

Supplemental Table S8. Primers used for genotyping and the generation of various constructs.

Supplemental Table S9. Primer sequences used in RT-qPCR analyses.

ACKNOWLEDGMENTS

We thank Dr. F. Fauser, Dr. S. Schiml, and Prof. H. Puchta at the Karlsruhe Institute of Technology and Dr. M. Endo and Dr. S. Toki at the National Institute of Agrobiological Sciences for providing the pZH_OsU3gYSA_FFCas9 and pUC_AtU6 oligonucleotide vectors for the CRISPR/Cas9 endonuclease constructs that were used in this study. We also thank Prof. T. Katoh at Tohoku Pharmaceutical University, Prof. Z.J. Chen at the University of Texas, and Prof. L.A. Long at the University of California, Los Angeles, for providing FK228, *athd1*, and *hda19-2*. We also thank C. Torii (Plant Genomic Network Research Team) and S. Maeda (Chemical Genomics Research Group) for technical support.

Received September 15, 2017; accepted October 6, 2017; published October 10, 2017.

LITERATURE CITED

Alinsug MV, Chen FF, Luo M, Tai R, Jiang L, Wu K (2012) Subcellular localization of class II HDAs in *Arabidopsis thaliana*: nucleocytoplasmic shuttling of HDA15 is driven by light. PLoS ONE **7:** e30846

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

Ammar R, Torti D, Tsui K, Gebbia M, Durbic T, Bader GD, Giaever G, Nislow C (2012) Chromatin is an ancient innovation conserved between Archaea and Eukarya. eLife **1:** e00078

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796–815

Asensi-Fabado MA, Amtmann A, Perrella G (2017) Plant responses to abiotic stress: the chromatin context of transcriptional regulation. Biochim Biophys Acta **1860:** 106–122

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B **57:** 289–300

Bolden JE, Peart MJ, Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov **5:** 769–784

Brosch G, Lusser A, Goralik-Schramel M, Loidl P (1996) Purification and characterization of a high molecular weight histone deacetylase complex (HD2) of maize embryos. Biochemistry **35:** 15907–15914

Brosch G, Ransom R, Lechner T, Walton JD, Loidl P (1995) Inhibition of maize histone deacetylases by HC toxin, the host-selective toxin of *Cochliobolus carbonum*. Plant Cell **7:** 1941–1950

Candat A, Paszkiewicz G, Neveu M, Gautier R, Logan DC, Avelange-Macherel MH, Macherel D (2014) The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in *Arabidopsis* offers tailored protection against abiotic stress. Plant Cell **26:** 3148–3166

Chen LT, Luo M, Wang YY, Wu K (2010) Involvement of *Arabidopsis* histone deacetylase HDA6 in ABA and salt stress response. J Exp Bot **61:** 3345–3353

Chen LT, Wu K (2010) Role of histone deacetylases HDA6 and HDA19 in ABA and abiotic stress response. Plant Signal Behav **5:** 1318–1320

Chinnusamy V, Gong Z, Zhu JK (2008) Abscisic acid-mediated epigenetic processes in plant development and stress responses. J Integr Plant Biol **50:** 1187–1195

Davie JR (2003) Inhibition of histone deacetylase activity by butyrate. J Nutr (Suppl) **133:** 2485S–2493S

Downs JA, Nussenzweig MC, Nussenzweig A (2007) Chromatin dynamics and the preservation of genetic information. Nature **447:** 951–958

Earley K, Lawrence RJ, Pontes O, Reuther R, Enciso AJ, Silva M, Neves N, Gross M, Viegas W, Pikaard CS (2006) Erasure of histone acetylation by *Arabidopsis HDA6* mediates large-scale gene silencing in nucleolar dominance. Genes Dev **20:** 1283–1293

Fauser F, Schiml S, Puchta H (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. Plant J **79:** 348–359

Finkelstein R (2013) Abscisic acid synthesis and response. The Arabidopsis Book **11:** e0166, doi/10.1199/tab.0166

Fong PM, Tian L, Chen ZJ (2006) *Arabidopsis thaliana* histone deacetylase 1 (AtHD1) is localized in euchromatic regions and demonstrates histone deacetylase activity *in vitro*. Cell Res **16:** 479–488

Fournel M, Bonfils C, Hou Y, Yan PT, Trachy-Bourget MC, Kalita A, Liu J, Lu AH, Zhou NZ, Robert MF, et al (2008) MGCD0103, a novel isotype-selective histone deacetylase inhibitor, has broad spectrum antitumor activity *in vitro* and *in vivo*. Mol Cancer Ther **7:** 759–768

Goyal K, Walton LJ, Tunnacliffe A (2005) LEA proteins prevent protein aggregation due to water stress. Biochem J **388:** 151–157

Gu D, Chen CY, Zhao M, Zhao L, Duan X, Duan J, Wu K, Liu X (2017) Identification of HDA15-PIF1 as a key repression module directing the transcriptional network of seed germination in the dark. Nucleic Acids Res **45:** 7137–7150

Han Z, Yu H, Zhao Z, Hunter D, Luo X, Duan J, Tian L (2016) *AtHD2D* gene plays a role in plant growth, development, and response to abiotic stresses in *Arabidopsis thaliana*. Front Plant Sci **7:** 310

Hao Y, Wang H, Qiao S, Leng L, Wang X (2016) Histone deacetylase HDA6 enhances brassinosteroid signaling by inhibiting the BIN2 kinase. Proc Natl Acad Sci USA **113:** 10418–10423

Himmelbach A, Yang Y, Grill E (2003) Relay and control of abscisic acid signaling. Curr Opin Plant Biol **6:** 470–479

Hollender C, Liu Z (2008) Histone deacetylase genes in *Arabidopsis* development. J Integr Plant Biol **50:** 875–885

Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF, Yao TP (2002) HDAC6 is a microtubule-associated deacetylase. Nature **417:** 455–458

Huber K, Doyon G, Plaks J, Fyne E, Mellors JW, Sluis-Cremer N (2011) Inhibitors of histone deacetylases: correlation between isoform specificity and reactivation of HIV type 1 (HIV-1) from latently infected cells. J Biol Chem **286:** 22211–22218

Hundertmark M, Hincha DK (2008) LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. BMC Genomics **9:** 118

Jenuwein T, Allis CD (2001) Translating the histone code. Science **293:** 1074–1080

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science **337:** 816–821

Kim JM, Sasaki T, Ueda M, Sako K, Seki M (2015) Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. Front Plant Sci **6:** 114

Kim JM, To TK, Seki M (2012) An epigenetic integrator: new insights into genome regulation, environmental stress responses and developmental controls by histone deacetylase 6. Plant Cell Physiol **53:** 794–800

Kim KC, Lai Z, Fan B, Chen Z (2008) *Arabidopsis* WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell **20:** 2357–2371

Kim YS, Sakuraba Y, Han SH, Yoo SC, Paek NC (2013) Mutation of the Arabidopsis NAC016 transcription factor delays leaf senescence. Plant Cell Physiol **54:** 1660–1672

König AC, Hartl M, Pham PA, Laxa M, Boersema PJ, Orwat A, Kalitventseva I, Plöchinger M, Braun HP, Leister D, et al (2014) The Arabidopsis class II sirtuin is a lysine deacetylase and interacts with mitochondrial energy metabolism. Plant Physiol **164:** 1401–1414

Kouzarides T (2007) Chromatin modifications and their function. Cell **128:** 693–705

Lee WK, Cho MH (2016) Telomere-binding protein regulates the chromosome ends through the interaction with histone deacetylases in *Arabidopsis thaliana*. Nucleic Acids Res **44:** 4610–4624

Lei Y, Lu L, Liu HY, Li S, Xing F, Chen LL (2014) CRISPR-P: a web tool for synthetic single-guide RNA design of CRISPR-system in plants. Mol Plant **7:** 1494–1496

Liu X, Chen CY, Wang KC, Luo M, Tai R, Yuan L, Zhao M, Yang S, Tian G, Cui Y, et al (2013) PHYTOCHROME INTERACTING FACTOR3 associates with the histone deacetylase HDA15 in repression of chlorophyll biosynthesis and photosynthesis in etiolated *Arabidopsis* seedlings. Plant Cell **25:** 1258–1273

Lobera M, Madauss KP, Pohlhaus DT, Wright QG, Trocha M, Schmidt DR, Baloglu E, Trump RP, Head MS, Hofmann GA, et al (2013)

Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group. Nat Chem Biol **9:** 319–325

Long JA, Ohno C, Smith ZR, Meyerowitz EM (2006) TOPLESS regulates apical embryonic fate in *Arabidopsis*. Science **312:** 1520–1523

Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389:** 251–260

Luo M, Tai R, Yu CW, Yang S, Chen CY, Lin WD, Schmidt W, Wu K (2015) Regulation of flowering time by the histone deacetylase HDA5 in Arabidopsis. Plant J **82:** 925–936

Luo M, Wang YY, Liu X, Yang S, Lu Q, Cui Y, Wu K (2012) HD2C interacts with HDA6 and is involved in ABA and salt stress response in *Arabidopsis*. J Exp Bot **63:** 3297–3306

Lusser A, Brosch G, Loidl A, Haas H, Loidl P (1997) Identification of maize histone deacetylase HD2 as an acidic nucleolar phosphoprotein. Science **277:** 88–91

Ma X, Lv S, Zhang C, Yang C (2013) Histone deacetylases and their functions in plants. Plant Cell Rep **32:** 465–478

Mai A, Massa S, Pezzi R, Rotili D, Loidl P, Brosch G (2003) Discovery of (aryloxopropenyl)pyrrolyl hydroxyamides as selective inhibitors of class IIa histone deacetylase homologue HD1-A. J Med Chem **46:** 4826–4829

Mai A, Massa S, Pezzi R, Simeoni S, Rotili D, Nebbioso A, Scognamiglio A, Altucci L, Loidl P, Brosch G (2005) Class II (IIa)-selective histone deacetylase inhibitors. 1. Synthesis and biological evaluation of novel (aryloxopropenyl)pyrrolyl hydroxyamides. J Med Chem **48:** 3344–3353

McClintock B (1984) The significance of responses of the genome to challenge. Science **226:** 792–801

Mehdi S, Derkacheva M, Ramström M, Kralemann L, Bergquist J, Hennig L (2016) The WD40 domain protein MSI1 functions in a histone deacetylase complex to fine-tune abscisic acid signaling. Plant Cell **28:** 42–54

Narita K, Kikuchi T, Watanabe K, Takizawa T, Oguchi T, Kudo K, Matsuhara K, Abe H, Yamori T, Yoshida M, et al (2009) Total synthesis of the bicyclic depsipeptide HDAC inhibitors spiruchostatins A and B, 5′-epi-spiruchostatin B, FK228 (FR901228) and preliminary evaluation of their biological activity. Chemistry **15:** 11174–11186

Nguyen AH, Matsui A, Tanaka M, Mizunashi K, Nakaminami K, Hayashi M, Iida K, Toyoda T, Nguyen DV, Seki M (2015) Loss of Arabidopsis 5′-3′ exoribonuclease AtXRN4 function enhances heat stress tolerance of plants subjected to severe heat stress. Plant Cell Physiol **56:** 1762–1772

Patanun O, Ueda M, Itouga M, Kato Y, Utsumi Y, Matsui A, Tanaka M, Utsumi C, Sakakibara H, Yoshida M, et al (2017) The histone deacetylase inhibitor suberoylanilide hydroxamic acid alleviates salinity stress in cassava. Front Plant Sci **7:** 2039

Perrella G, Lopez-Vernaza MA, Carr C, Sani E, Gosselé V, Verduyn C, Kellermeier F, Hannah MA, Amtmann A (2013) Histone deacetylase complex1 expression level titrates plant growth and abscisic acid sensitivity in *Arabidopsis*. Plant Cell **25:** 3491–3505

Podzimska-Sroka D, O'Shea C, Gregersen PL, Skriver K (2015) NAC transcription factors in senescence: from molecular structure to function in crops. Plants (Basel) **4:** 412–448

Provart NJ, Alonso J, Assmann SM, Bergmann D, Brady SM, Brkljacic J, Browse J, Chapple C, Colot V, Cutler S, et al (2016) 50 years of Arabidopsis research: highlights and future directions. New Phytol **209:** 921–944

Ryu H, Cho H, Bae W, Hwang I (2014) Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of *ABI3*. Nat Commun **5:** 4138

Sako K, Kim JM, Matsui A, Nakamura K, Tanaka M, Kobayashi M, Saito K, Nishino N, Kusano M, Taji T, et al (2016) Ky-2, a histone deacetylase inhibitor, enhances high-salinity stress tolerance in *Arabidopsis thaliana*. Plant Cell Physiol **57:** 776–783

Sakuraba Y, Kim YS, Han SH, Lee BD, Paek NC (2015) The Arabidopsis transcription factor NAC016 promotes drought stress responses by repressing *AREB1* transcription through a trifurcate feed-forward regulatory loop involving NAP. Plant Cell **27:** 1771–1787

Samson F, Brunaud V, Balzergue S, Dubreucq B, Lepiniec L, Pelletier G, Caboche M, Lecharny A (2002) FLAGdb/FST: a database of mapped flanking insertion sites (FSTs) of *Arabidopsis thaliana* T-DNA transformants. Nucleic Acids Res **30:** 94–97

Schiml S, Fauser F, Puchta H (2014) The CRISPR/Cas system can be used as nuclease for *in planta* gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. Plant J **80:** 1139–1150

Seto E, Yoshida M (2014) Erasers of histone acetylation: the histone deacetylase enzymes. Cold Spring Harb Perspect Biol **6:** a018713

Shahbazian MD, Grunstein M (2007) Functions of site-specific histone acetylation and deacetylation. Annu Rev Biochem **76:** 75–100

Siow D, Wattenberg B (2014) The histone deacetylase-6 inhibitor tubacin directly inhibits *de novo* sphingolipid biosynthesis as an off-target effect. Biochem Biophys Res Commun **449:** 268–271

Skubacz A, Daszkowska-Golec A, Szarejko I (2016) The role and regulation of ABI5 (ABA-Insensitive 5) in plant development, abiotic stress responses and phytohormone crosstalk. Front Plant Sci **7:** 1884

Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol **3:** Article3

Takasaki H, Maruyama K, Takahashi F, Fujita M, Yoshida T, Nakashima K, Myouga F, Toyooka K, Yamaguchi-Shinozaki K, Shinozaki K (2015) SNAC-As, stress-responsive NAC transcription factors, mediate ABA-inducible leaf senescence. Plant J **84:** 1114–1123

Tian L, Wang J, Fong MP, Chen M, Cao H, Gelvin SB, Chen ZJ (2003) Genetic control of developmental changes induced by disruption of Arabidopsis histone deacetylase 1 (*AtHD1*) expression. Genetics **165:** 399–409

Tran HT, Nimick M, Uhrig RG, Templeton G, Morrice N, Gourlay R, DeLong A, Moorhead GB (2012) *Arabidopsis thaliana* histone deacetylase 14 (HDA14) is an α-tubulin deacetylase that associates with PP2A and enriches in the microtubule fraction with the putative histone acetyltransferase ELP3. Plant J **71:** 263–272

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the *early responsive to dehydration stress 1* promoter. Plant Cell **16:** 2481–2498

Tsuji N, Kobayashi M, Nagashima K, Wakisaka Y, Koizumi K (1976) A new antifungal antibiotic, trichostatin. J Antibiot (Tokyo) **29:** 1–6

Venturelli S, Belz RG, Kämper A, Berger A, von Horn K, Wegner A, Böcker A, Zabulon G, Langenecker T, Kohlbacher O, et al (2015) Plants release precursors of histone deacetylase inhibitors to suppress growth of competitors. Plant Cell **27:** 3175–3189

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids **35:** 753–759

Verdin E, Ott M (2015) 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. Nat Rev Mol Cell Biol **16:** 258–264

Xu CR, Liu C, Wang YL, Li LC, Chen WQ, Xu ZH, Bai SN (2005) Histone acetylation affects expression of cellular patterning genes in the *Arabidopsis* root epidermis. Proc Natl Acad Sci USA **102:** 14469–14474

Yoo SY, Bomblies K, Yoo SK, Yang JW, Choi MS, Lee JS, Weigel D, Ahn JH (2005) The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. Planta **221:** 523–530

Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J Biol Chem **265:** 17174–17179

Zheng Y, Ding Y, Sun X, Xie S, Wang D, Liu X, Su L, Wei W, Pan L, Zhou DX (2016) Histone deacetylase HDA9 negatively regulates salt and drought stress responsiveness in Arabidopsis. J Exp Bot **67:** 1703–1713