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HD2C interacts with HDA6 and is involved in ABA and salt stress response in *Arabidopsis*

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Abstract

HD2 proteins are plant specific histone deacetylases. Four HD2 proteins, HD2A, HD2B, HD2C, and HD2D, have been identified in *Arabidopsis*. It was found that the expression of *HD2A*, *HD2B*, *HD2C*, and *HD2D* was repressed by ABA and NaCl. To investigate the function of HD2 proteins further, two *HD2C* T-DNA insertion lines of *Arabidopsis*, *hd2c-1* and *hd2c-3* were identified. Compared with wild-type plants, *hd2c-1* and *hd2c-3* plants displayed increased sensitivity to ABA and NaCl during germination and decreased tolerance to salt stress. These observations support a role of HD2C in the ABA and salt-stress response in *Arabidopsis*. Moreover, it was demonstrated that HD2C interacted physically with a RPD3-type histone deacetylase, HDA6, and bound to histone H3. The expression of ABA-responsive genes, *ABI1* and *ABI2*, was increased in *hda6*, *hd2c*, and *hda6/hd2c-1* double mutant plants, which was associated with increased histone H3K9K14 acetylation and decreased histone H3K9 dimethylation. Taken together, our results suggested that HD2C functionally associates with HDA6 and regulates gene expression through histone modifications.

Key words: ABA, *Arabidopsis*, HD2C, HDA6, histone deacetylases.

Introduction

In eukaryotes, genomic DNA is tightly compacted into a complex structure known as chromatin. To facilitate cellular activities, the accessibility of chromatin is dynamically regulated during growth and development (Berger, 2002). Two types of chromatin modification that correlate with either positive or negative transcriptional states are DNA methylation and histone post-translational modifications. The amino-terminal tails of core histones protruding from the nucleosomes interact with DNA and thereby facilitate the chromatin assembly via post-translational modifications including acetylation, phosphorylation, methylation, ubiquitination, and sumoylation. Histone acetylation and deacetylation are catalysed by histone acetyltransferases and histone deacetylases (HDAs), respectively. The acetylation of conserved lysine residues neutralizes the positive charge of the histone tails and decreases their affinity for negatively charged DNA; thereby promoting the accessibility of chromatin to transcriptional regulators. Conversely, removing the acetyl group by histone deacetylation can result in a reduction of gene expression. The level of histone acetylation can, therefore, be used as a marker to understand the activity of target genes (Earley *et al.*, 2006).

Four types of HDAs have been identified in various species of plants (Lusser *et al.*, 2001; Pandey *et al.*, 2002; Alinsug *et al.*, 2009). Three of them are homologous to yeast RPD3 (reduced potassium dependency protein 3), HDA1 (histone deacetylase 1 protein), and SIR2 (silent information regulator protein 2) proteins. The fourth type of HDAs, the HD2-like proteins, appears to be plant-specific HDAs (Lusser *et al.*, 1997). HD2-type histone deacetylase was first

discovered in maize as an acidic nucleolar phosphoprotein in a high molecular weight complex (Brosch *et al.*, 1996; Lusser *et al.*, 1997). It was found that the maize HD2 accepted all core histones as substrate *in vitro*, and it was highly sensitive to deacetylase inhibitors, Trichostain and cyclic tetrapeptides. Four HD2 proteins, HD2A, HD2B, HD2C, and HD2D, have been identified in *Arabidopsis* (Wu *et al.*, 2000; Dangl *et al.*, 2001; Zhou *et al.*, 2004). HD2A, HD2B, and HD2C can mediate repression of a reporter gene (Wu *et al.*, 2003). Furthermore, HD2 proteins may be involved in fertilization (Lagace *et al.*, 2003), seed development (Wu *et al.*, 2000; Zhou *et al.*, 2004), ABA response (Sridha and Wu, 2006), and leaf development (Ueno *et al.*, 2007). However, the molecular mechanism of how HD2 proteins are involved in these processes is not clear. HD2 proteins do not show sequence homologies with other known HDA proteins, but they share some sequence similarities with nuclear FK506-binding protein family (FKBP) members (Aravind, 1998). The findings that some FKBPs are required for the reduction of gene expression and can interact with RPD3-type HDAs raise the possibility that HD2 proteins may also interact with other known HDAs (Yang *et al.*, 2001; Kuzuhara and Horikoshi, 2004; Earley *et al.*, 2006).

Recent studies indicated the involvement of histone acetylation and deacetylation in the plant abiotic stress response. Both tobacco and *Arabidopsis* cells show a nucleosomal response to high salinity and cold stress, manifested by transient up-regulation of H3 phosphoacetylation and histone H4 acetylation (Sokol *et al.*, 2007). Furthermore, it was found that histone acetylation on the histone H3 was altered with gene activation on the coding regions of drought stress-responsive genes under drought stress conditions (Kim *et al.*, 2008). Both abscisic acid (ABA) and salt stress can induce histone H3K9K14 acetylation and H3K4 trimethylation but can decrease the H3K9 dimethylation of some ABA and abiotic stress-responsive genes, suggesting that functionally related gene groups are regulated co-ordinately through histone modifications in response to abiotic stress in plant cells (Chen *et al.*, 2010).

In this study, the function of a HD2-type HDA, HD2C, was investigated in ABA- and abiotic-stress responses as well as its interaction with a RPD3-type HDA, HDA6. Bimolecular fluorescence complementation (BiFC), *in vitro* pull-down assays, and co-immunoprecipitation (Co-IP) indicated that HD2C interacts physically with HDA6. Moreover, HD2C can bind to histone H3 and affect the levels of histone H3K9K14 acetylation, H3K4 trimethylation, and H3K9 dimethylation. In addition, the *HD2C* T-DNA insertion plants, *hd2c-1* and *hd2c-3*, are more sensitive to ABA and NaCl. The expression and histone modification of ABA-related genes, *ABI1*, *ABI2*, and *AtERF4*, were affected in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. Our studies provided evidence indicating that HD2C is involved in the ABA and salt-stress response by interacting with HDA6 and modulating stress-responsive genes.

Materials and methods

Plant materials

Plants were germinated and grown at 23 °C under a long day condition (16/8 h light/dark cycle). The T-DNA insertion mutants, *hd2c-1* (HD2CT99, Salk_129799.19.60N) and *hd2c-3* (HD2CT84, Salk_039784.52.90x) were obtained from the *Arabidopsis* Resource Centre (http://www.arabidopsis.org/). An *hda6* mutant line, *axe1-5*, is a splice site mutant that has a base change at an intron splice site resulting in two HDA6 transcripts with altered lengths (Murfett *et al.*, 2001; Yu *et al.*, 2011).

Measurement of germination rates

Seeds were treated with 50% bleach solution for 15 min and then rinsed three times with distilled water. The surface-sterilized seeds were incubated at 4 °C for 3 d to synchronize germination and then planted in Petri dishes containing different growth media. The media contained half-strength Murashige and Skoog salts, 1% sucrose, and 0.8% agar, and were supplemented with or without ABA and NaCl. All plates were transferred to a growth chamber and incubated at 23 °C under long-day conditions. For germination-rate tests, seeds were germinated on media with or without ABA (0.5 μM, 1 μM, and 2 μM) or NaCl (100 mM, 125 mM, and 150 mM) in a growth chamber under long-day conditions, and seed germination rates were analysed after 2 d.

For the per cent survival of seedlings under high salinity treatment, 5-d-old seedlings growing in Perti dishes were transferred to a medium containing 150 mM NaCl, and the per cent survival of seedlings was measured after 5 d.

Semi-quantitative RT-PCR analysis

To isolate total RNA, 0.1–0.2 g of *Arabidopsis thaliana* leaves were ground with liquid nitrogen in a mortar and pestle and mixed with 1 ml TRI$_{ZOL}$ Reagent (Invitrogen, Carlsbad, CA, USA) to isolate total RNA. One microgram of total RNA was used for the first-strand cDNA synthesis after incubation at 65 °C for 10 min. cDNA was synthesized in a volume of 20 μl that contained the MoMLV reverse transcriptase buffer (Promega, Madison, Wisconsin, USA), 10 mM dithiothreitol, 1.5 μM poly(dT) primer, 0.5 mM dNTPs, and 2 U of MoMLV reverse transcriptase at 37 °C for 1 h. All PCR reactions were performed with 0.5 U of *Taq* polymerase, the buffer provided by the supplier, 0.2 mM dNTPs, and a pair of primers (0.1 μM each) in a final volume of 20 μl. The thermocycling conditions were 94 °C for 4 min, followed by 22–35 cycles of 94 °C for 30 s, 50–65 °C for 1 min, and 72 °C for 1 min, with a final polymerization step at 72 °C for 7 min.

Quantitative real-time PCR (qPCR)

cDNAs (diluted ×100) obtained from RT-PCR were used as a template to run real-time PCR. The following components were added to a reaction tube: 9 μl of iQ^{TM} SYBR Green Supermix solution (Bio-Rad; Catalogue no. 170-8882), 1 μl of 5 μM specific primers, and 8 μl of the diluted template. *UBIQUITIN* was used as an internal control in real-time quantitative RT-PCR. The thermocycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s, with a melting curve detected at 95 °C for 1 min, 55 °C for 1 min, and the denature time detected from 55 °C to 95 °C. The gene-specific primer pairs are listed in Supplementary Table S1 at *JXB* online. Each experiment was repeated with three biological and three technical replicates.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed as described by (Gendrel *et al.* (2005). The chromatin extract was prepared from 18-d-old leaves. Antibodies specific for histone

H3K9K14Ac and H3K9Me2 (Millipore) were used in this study. The primers used for real-time PCR analysis in ChIP assays are listed in Supplementary Table S2 at *JXB* online. Each of the immunoprecipitations was replicated three times, and each sample was quantified at least in triplicate.

Bimolecular fluorescence complementation assay

To generate the construct for BiFC assay, full-length coding sequences of *HD2A*, *HD2B*, *HD2C*, *HD2D*, and *HDA6* were PCR-amplified. The PCR products were subcloned into the pENTR/SD/D-TOPO or pCR8/GW/TOPO vector, and then recombined into the pEarleyGate201-YN and pEarleyGate202-YC vectors (Lu *et al.*, 2010). The resulting constructs were used for transient assays by PEG transfection of *Arabidopsis* protoplasts (Yoo *et al.*, 2007). Transfected cells were imaged using an Olympus BX51 fluorescence microscope, or a Leica SP5 confocal microscope.

Protein expression and purification

The full-length HDA6, HD2C, and AtFKBP53 cDNA were subcloned into pET25b+ and pGEX-4T-3 expression vectors to generate HDA6-His, GST-HD2C, and GST-AtFKBP53 fusion protein constructs and expressed in *E. coli* strain BL21 (DE3). The His- and GST-fusion proteins expressed in bacteria were induced by 0.1 mM isopropylthio-β-galactoside at 20 °C for 18 h. For protein extraction, cells were collected by centrifugation and then sonicated in a lysis buffer (50 mM phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM β-mercaptoethanol, 0.1% Triton X-100 and 10 mM imidazole for the His-fusion protein, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.3 for the GST-fusion protein). The HDA6-His, GST-HD2C, and GST-AtFKBP53 recombinant fusion proteins were purified by Ni-NTA resin and GST Bind Resin, respectively.

In vitro pull-down assay

The pull-down assay was performed as previously described by Yang *et al.* (2008). GST-HD2C and HDA6-His fusion proteins or HeLa Core Histones (Active Motif) were incubated in a binding buffer (50 mM TRIS-Cl, pH 7.5, 100 mM NaCl, 0.25% Triton X-100, and 35 mM β-mercaptoethanol) for 2 h at 4 °C, then the binding reaction was performed by mixing 30 μl of GST binding resin for an additional 2 h. After extensive washing (at least six times), the pulled-down proteins were eluted by boiling , separated by 10% SDS-PAGE, and detected by Western blotting using an anti-His or an anti-H3 antibody.

Co-immunoprecipitation assay

The co-immunoprecipitation assay was carried out using the tobacco transient expression system as described previously by Yu *et al.* (2011). Tobacco leaves were infiltrated with *Agrobacterium tumefaciens* carring 35S-HDA6-Myc and 35S:HD2C-HA, and the leaf extracts were used to perform co-immunoprecipitation. The co-immunoprecipitated proteins were analysed by Western blotting using anti-HA and anti-Myc antibodies (Sigma).

Determination of histone H3 modifications

Total histone proteins were prepared according to the method of Lu *et al.* (2011). Proteins were separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Antibodies specific for histone H3K9K14Ac, H3K4Me3, and H3K9Me2 (Millipore), were used as a primary antibody. Signals were detected by using the Millipore Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore).

Results

Expression of HD2A, HD2B, HD2C, and HD2D was repressed by ABA and NaCl

The mRNA accumulation patterns of *HD2A*, *HD2B*, *HD2C*, and *HD2D* in *Arabidopsis* plants treated with ABA and NaCl were examined by quantitative PCR. Under 100 μM ABA treatment, the expression of *HD2A*, *HD2B*, *HD2C*, and *HD2D* was reduced after 6 h (Fig. 1A). For salt treatment, plants were watered with 250 mM NaCl. As shown in Fig. 1B, the expression of *HD2A*, *HD2B*, *HD2C*, and *HD2D* was repressed when treated with NaCl at 6 h.

HD2C T-DNA insertion mutants were hypersensitive to ABA and NaCl

Two *Arabidopsis lines*, hd2c-1 (Salk_129799.19.60N) and hd2c-3 (Salk_039784.52.90), that contain a T-DNA insertion in the 5th intron and 7th exon of *HD2C*, respectively (Fig. 2A) were identified. It was not possible to obtain the knock-out mutants for other *HD2* genes, so this study was therefore focused on *HD2C*. The T-DNA insertions were confirmed by PCR screening and sequencing. As shown in Fig. 2B, homozygous hd2c-1 and hd2c-3 T-DNA insertion lines were identified using LBa1/HD2C pr4 and HD2C-RT1/
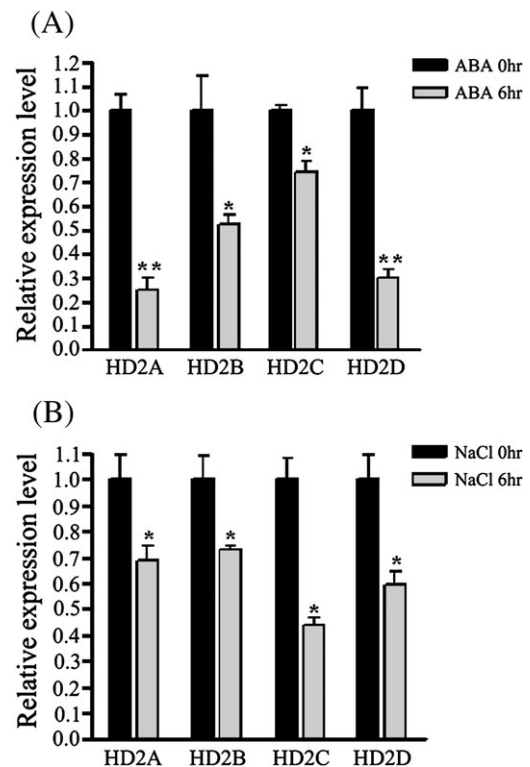
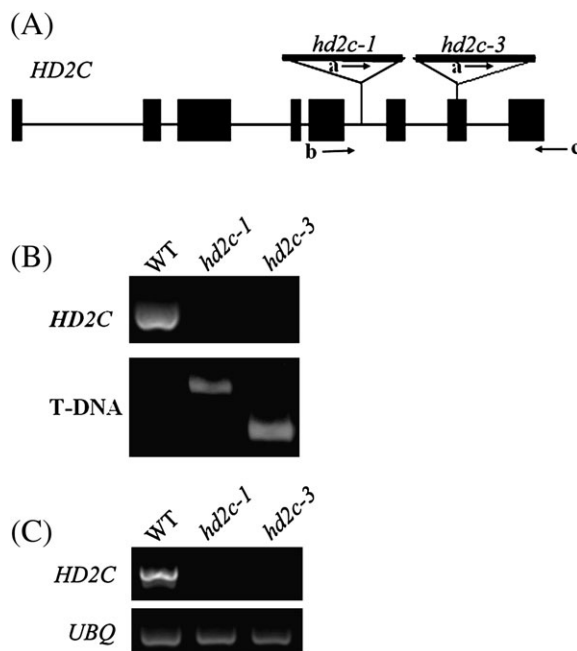**Fig. 1.** Expression of *HD2A*, *HD2B*, *HD2C*, and *HD2D* in response to ABA and NaCl. 2-week-old plants were treated with 100 μM of ABA (A) or 250 mM NaCl (B). Relative mRNA levels were determined by quantitative RT-PCR analysis. Two biological replicates were carried out and gave similar results. Asterisks mark values that are significantly different from the wild type (*t* test, *P <0.05, **P <0.01).

HD2C pr4 primer pairs. Moreover, our sequencing analysis showed that the sites of T-DNA insertion are at 1253 bp and 1732 bp in the 5th intron and 7th exon of *HD2C* in *hd2c-1 and hd2c-3* T-DNA mutants, respectively. In addition, RT-PCR analysis indicated that the *HD2C* transcript was absent in *hd2c-1* and *hd2c-3* homozygous plants (Fig. 2C).

To analyse the involvement of *HD2C* in the ABA and abiotic stress response, *hd2c-1* and *hd2c-3* seeds were tested for their response to ABA and NaCl during seed germination. The germination rates were measured after 2 d planting on media containing different concentrations of ABA or NaCl. Without ABA and NaCl treatment, no difference was found in the germination rates among the wild-type and mutant lines. Compared with the wild type, *hd2c-1* and *hd2c-3* mutants displayed decreased germination rates under ABA and salt treatment conditions (Fig 3). At 2 μM ABA, the germination rate of the wild type was 48%, whereas those of *hd2c-1* and *hd2c-3* were 25% and 21%, respectively (Fig. 3A). At 150 mM NaCl, the germination rate of the wild type was 52%, whereas those of *hd2c-1* and *hd2c-3* were 25%, respectively (Fig. 3B).

The response of *HD2C* T-DNA insertion plants to high salinity stress was also investigated. 5-d-old *Arabidopsis* seedlings were transferred to a medium containing 150 mM NaCl for 2 d. The leaf survival rates were measured as the percentages of green leaves left after treatment. As shown in Fig. 4, *hd2c-1* and *hd2c-3* plants were more sensitive to salt stress and displayed a lower survival rate. The survival rate of wild-type plants was 67%, whereas the survival rates of both *hd2c-1* and *hd2c-3* plants were 51% only (Fig- 4B).

HD2C interacted with HDA6 in vitro *and* in vivo

HD2 proteins share some sequence similarities with nuclear FK506-binding protein family members that interact with RPD3-type histone deacetylases, HDAC1 and HDAC2, in human (Aravind, 1998; Yang *et al.*, 2001). To determine whether HD2C could interact with HDA6 in *Arabidopsis*, BiFC was used to determine their *in vivo* protein interactions. HD2C was fused to the N-terminal 174 amino acid portion of YFP in the pEarleyGate201 vector (pEarleyGate201-YN) (Lu *et al.*, 2010), whereas HDA6 was fused to the C-terminal 66 amino acid portion of YFP in the pEarleyGate202 vector (pEarleyGate202-YC). The corresponding constructs were co-delivered into protoplasts of *Arabidopsis*, and fluorescence was observed using a confocal microscope. As shown in Fig. 5A, HD2C interacted with HDA6 in nucleoli, which was in accordance with the localization of HD2C and HDA6 (Pontes *et al.*, 2007; Earley *et al.*, 2010). Similar results were also obtained when the HD2C was fused to YC and HDA6 was fused to YN (Fig. 5A). As the negative controls, no YFP signals were detected when HD2C fused with YN/YC or HDA6 fused
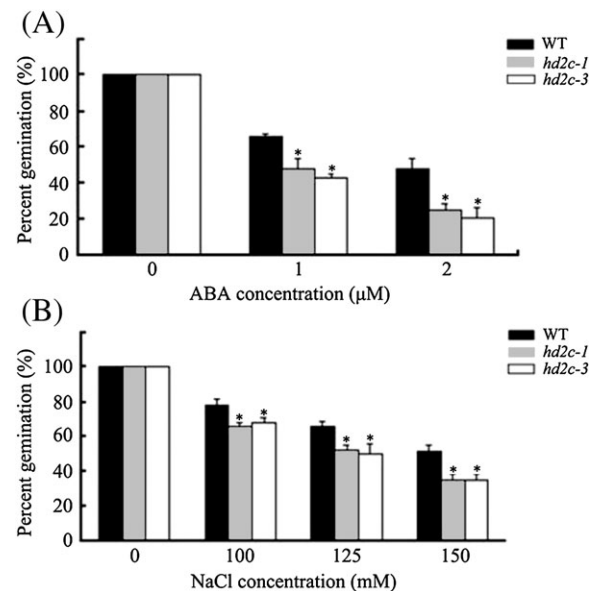

Fig. 2. Identification of T-DNA insertion mutants of *HD2C*. (A) Schematic structure of *HD2C* and T-DNA insertion sites of *hd2c-1* and *hd2c-3*. The relative locations of PCR primers were indicated by the arrows. (a) LBa1 (5′-GTTCACGTAGTGGGCCATCG-3′), (b) HD2C-RT1 (5′-TGACGCTGACGGTAGTGAAG-3′), and (c) HD2C pr4 (5′-AATTAGATCTGCACTGTGTTTGGCCTTTG-3′). (B) The *HD2C* wild-type allele was detected in the wild type using HD2C-RT1 and HD2C pr4 primers, whereas T-DNA alleles were detected in *hd2c-1* and *hd2c-3* using LBa1 and HD2C pr4 primers. (C) RT-PCR analysis indicated that the *HD2C* transcript was detected in wild-type plants, but absent in *hd2c-1* and *hd2c-3*.

Fig. 3. Seed germination rates of *hd2c-1* and *hd2c-3* plants treated with ABA and NaCl. (A) Germination rates of 2-d-old wild-type (WT), *hd2c-1*, and *hd2c-3* seedlings treated with ABA. (B) Germination rates of 2-d-old wild-type (WT), *hd2c-1*, and *hd2c-3* seedlings treated with NaCl. Three biological replicas were performed with three technical replicates for each treatment (*n* ≥100), Asterisks mark values that are significantly different from the wild type (*t* test, *P <0.05, **P <0.01).
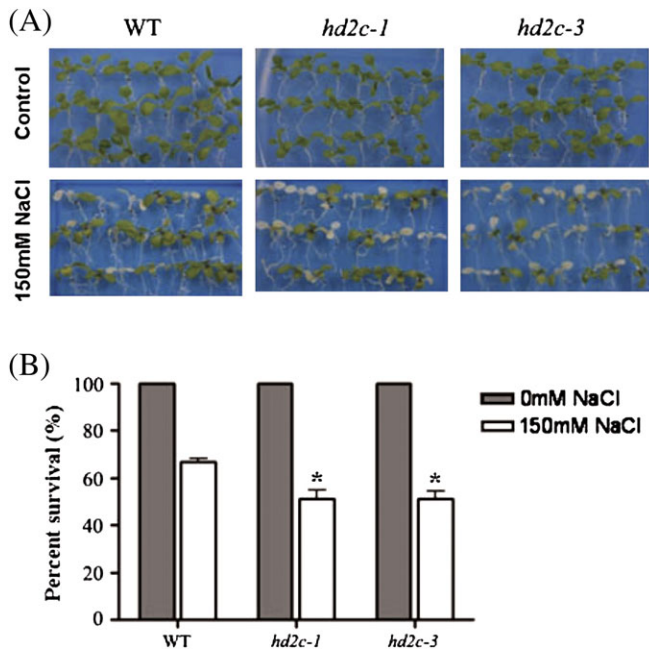
Fig. 4. Phenotype comparison of *hd2c-1* and *hd2c-3* plants in response to salt stress. (A) 5-d-old seedlings of the wild type (WT), *hd2c-1*, and *hd2c-3* were transferred to a medium containing 150 mM NaCl for 5 d. (B) 5-d-old seedlings were transferred to a medium containing 150 mM NaCl, and the percentage survival of seedlings was measured after 5 d. Three biological replicas were performed with three technical replicates for each treatment ($n \geqslant 100$). Asterisks mark values that are significantly different from the wild type (*t* test, *$P <$0.05).

with YN/YC was co-transfected with empty vectors (YN and YC) (see Supplementary Fig. S1 at *JXB* online).

An *in vitro* GST pull-down assay was also performed to examine the interaction between HD2C and HDA6. Purified His-HDA6 recombinant protein was incubated with GST-HD2C protein. As shown in Fig. 5B, HDA6-His was pulled down by GST-HD2C, indicating that HD2C can directly associate with HDA6. Moreover, the interaction between HD2C and HDA6 was further confirmed by the co-immunoprecipitation (co-IP) assay. As shown in Fig. 5C, the HD2C-HA was co-immunoprecipitated by HDA6-Myc. Taken together, these results indicate that HD2C can physically interact with HDA6.

hda6/hd2c-1 double mutants displayed hypersensitivity to ABA and NaCl

The *hda6* mutant *axe1-5* is a *HDA6* splicing mutant which carries a point mutation in the *HDA6* splicing site (Murfett *et al.*, 2001; Wu *et al.*, 2008; Yu *et al.*, 2011). To study the genetic interaction between *HDA6* and *HD2C*, *hda6* and *hd2c-1* were crossed to generate the *hda6/hd2c-1* double mutant. The seed germination rates of *hda6*, *hd2c-1*, and *hda6/hd2c-1* in response to ABA and NaCl were compared. Without ABA and NaCl treatment, no difference was found in the germination rates among the wild-type and mutant lines. As shown in Fig. 6, *hda6*, *hd2c-1*, and *hda6/hd2c-1*

double mutants displayed lower germination rates compared with the wild type when treated with ABA and NaCl.

Expression of ABI1, ABI2, and AtERF4 was increased in hda6, hd2c-1, and hda6/hd2c-1 double mutant plants

The expression of the ABA-responsive genes, *ABI1* and *ABI2*, in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants was analysed further. *ABI1* and *ABI2* encode serine/threonine phosphatease 2C (PP2C) that negatively regulate the ABA response (Merlot *et al.*, 2001). As shown in Fig. 7, the expression of *ABI1* and *ABI2* were higher in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants compared with the wild type. Furthermore, it was found that the AP2-domain transcriptional repressor *AtERF4* was also up-regulated in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. In addition, two transcriptional activators *MYB2* and *MYC2* were down-regulated in *hd2c* mutant plants (see Supplementary Fig. S2 at *JXB* online). Since HDAs are usually associated with the repression of gene expression, the down-regulation of *MYB2* and *MYC2* in the *hd2c* mutants suggested that HD2C may affect the expression of *MYB2* and *MYC2* indirectly.

Histone H3K9K14Ac and H3K9Me2 levels of ABI1, ABI2, and AtERF4 were changed in hda6, hd2c-1, and hda6/hd2c-1 double mutant plants

To determine whether the higher expression of *ABI1*, *ABI2*, and *AtERF4* in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants was related to histone acetylation and methylation in the chromatin, a chromatin immunoprecipitation (ChIP) assay was used to analyse the gene activation mark histone H3K9K14Ac and the gene repression mark H3K9Me2 of the up-regulated genes, *ABI1*, *ABI2*, and *AtERF4*. The levels of histone H3K9K14Ac in the promoter regions of *ABI1* and *ABI2* in *hda6*, *hd2c-1*, and hda6/*hd2c-1* double mutant plants were higher than those in the wild-type plants (Fig. 8A; Supplementary Fig. S3 at *JXB* online). By contrast, no significant changes were found in the levels of histone H3K9K14Ac at *AtERF4*. These data indicate that HD2C and HDA6 may regulate *ABI1* and *ABI2* expression by histone acetylation. In addition, our ChIP data also showed that the levels of H3K9Me2 were decreased in the promoter and exon regions of *ABI1*, *ABI2*, and *AtERF4* in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants (Fig. 8B). These data revealed that the higher expression of *ABI1*, *ABI2*, and *AtERF4* was associated with increased H3K9K14Ac and/or decreased H3K9Me2.

HD2C physically associated with histone H3 and affected histone modifications

To investigate whether HD2C can interact with histone H3, an *in vitro* pull-down assay was performed using purified GST-HD2C protein with a HeLa core histone protein. AtFKBP53, a FK506-binding protein that was previously shown to physically interact with histone H3 (Li and Luan, 2010), was taken as a positive control. The result showed

(A)

(B)

(C)
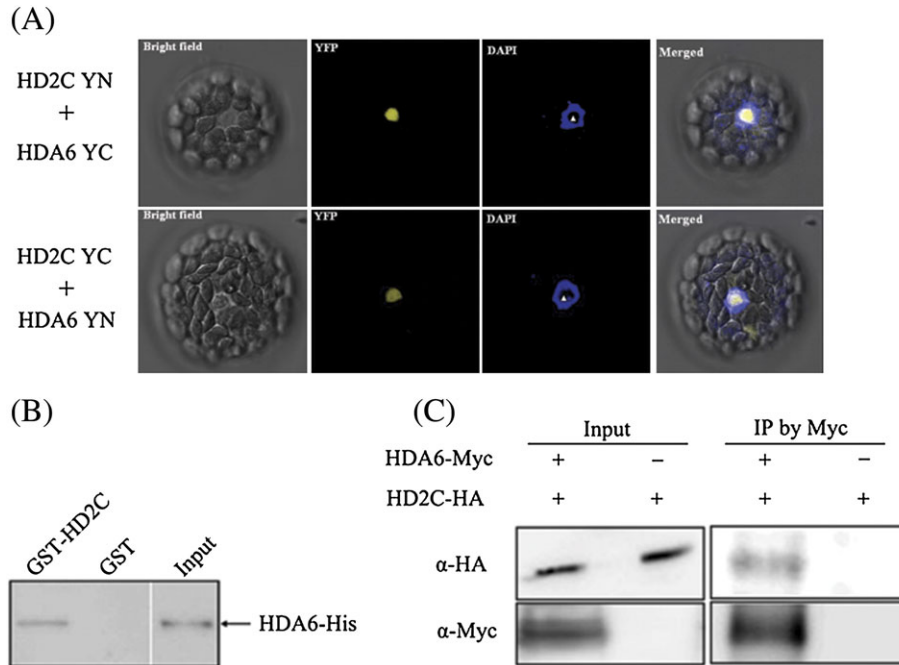
Fig. 5. HD2C interacted with HDA6. (A) BiFC assays. HD2C and HDA6 fused with N-terminal (YN) or C-terminal (YC) of YFP were co-transfected into protoplasts, and visualized using confocal microscope. The nucleolus is indicated by white triangle. (B) *In vitro* pull-down assays. Purified His-HDA6 recombinant protein was incubated with GST or GST-HD2C protein. After extensive washing, the pulled-down proteins were eluted and the retention of HDA6-His proteins by GST-HD2C was detected by Western blotting using an anti-His antibody. (C) Co-immunoprecipitation analysis of HD2C interaction with HDA6 in *N. benthamiana* transient expression system. *Agrobacterium* cultures carrying 35S-HDA6-Myc and 35S:HD2C-HA were co-infiltrated into tobacco leaves. Crude extracts (input) were immunopreciped (IP) with an anti-Myc antibody and analysed by Western blotting.

(A)

(B)
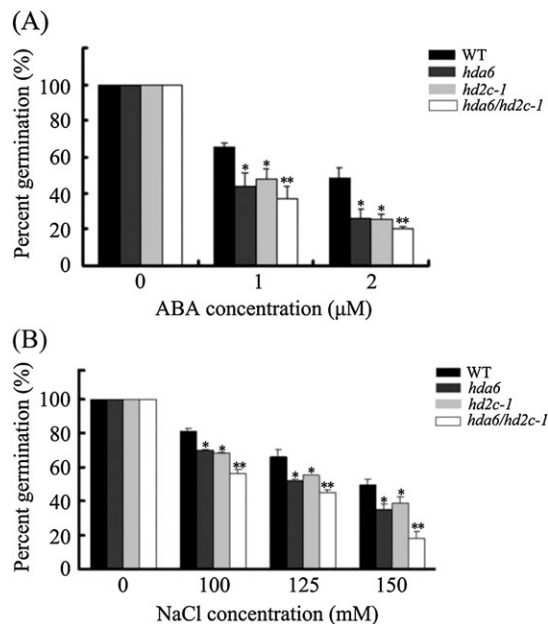
Fig. 6. Seed germination rates of *hda6*, *hd2c-1*, and *hda6/hd2c-1* plants treated with ABA and NaCl. (A) Germination rates of 2-d-old wild-type (WT), *hda6*, *hd2c-1*, and *hda6/hd2c-1* seedlings treated with ABA. (B) Germination rates of 2-d-old wild-type (WT), *hda6*, *hd2c-1*, and *hda6/hd2c-1* seedlings treated with NaCl. Asterisks mark values that are significantly different from the wild type (*t* test, *P <0.05, **P <0.01). Three biological replicas were performed with three technical replicates for each treatment ($n \geqslant 100$).

that histone H3 can interact with AtHD2C as well as AtFKBP53, but not GST (Fig. 9A).

To determine whether HD2C can affect histone H3 modifications globally, the levels of histone H3K9K14Ac, H3K4Me3, and H3K9Me2 were analysed in *hd2c-1* as well as in *hda6* and *had6/hdc2c-1* plants. Western blot analysis was performed using Anti-acetyl-Histone H3K9K14, Anti-trimethyl-Histone H3K4, and Anti-dimethyl-Histone H3K9 as the primary antibody. As shown in Fig. 9B, the levels of acetylated histone H3K9K14Ac and H3K4Me2 were increased in *hd2c-1*, *hda6*, and *had6/hdc2c-1* compared with the wild type (Col-0). By contrast, the level of H3K9Me2 was decreased in *hd2c-1*, *hda6*, and *had6/hdc2c-1* mutant plants.

Discussion

Histone acetylation is involved in plant abiotic stress responses

The involvement of RPD3-type HDAs, HDA6 and HDA19, in ABA and abiotic stress has been reported (Chen *et al.*, 2010; Chen and Wu, 2010). It was found that *hda6* mutant and HDA6 RNA-interference (HDA6-RNAi) plants displayed a phenotype that is more sensitive to ABA and salt stress. Compared with wild-type plants, the expression of ABA and abiotic stress-responsive genes was decreased in *hda6* and HDA6-RNAi plants (Chen *et al.*,
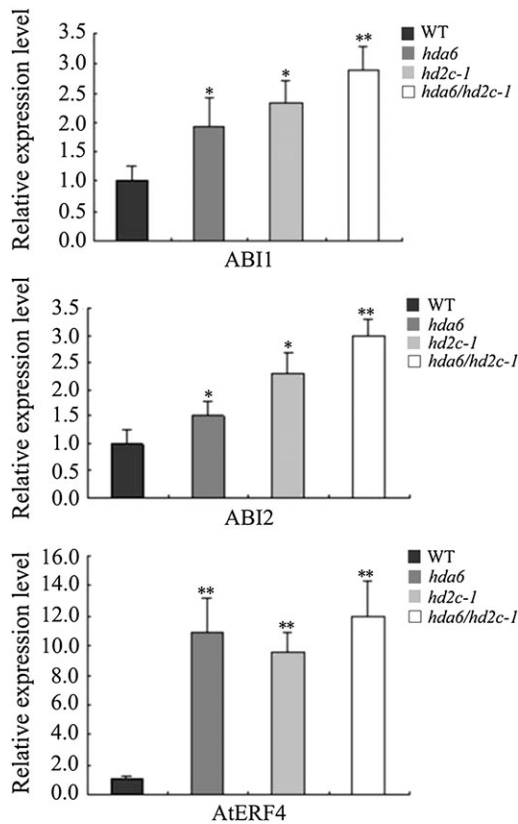
Fig. 7. Expression of *ABI1*, *ABI2*, and *AtERF4* in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. Total RNA was isolated from leaf tissues and the expression of *ABI1*, *ABI2*, and *AtERF4* was determined by real-time RT-PCR. Asterisks mark values that are significantly different from the wild type (*t* test, **P <0.01, *P<0.05). The experiment was repeated three times with similar results.

2010). Similarly, the *Arabidopsis* HDA19 T-DNA insertion mutant, *hda19-1*, was hypersensitive to ABA and salt stress, and the expression of ABA-responsive genes was decreased in *hda19-1* plants (Chen and Wu, 2010). *Arabidopsis* HDA19 may act in a protein complex of AtERF7, a transcription repressor (Song *et al.*, 2005; Yang *et al.*, 2005), to regulate the abiotic stress response genes. Furthermore, AtERF7 interacts with the *Arabidopsis* homologue of a human global corepressor of transcription, AtSin3, which, in turn, may interact with HDA19. It was proposed that AtERF7, AtSin3, and HDA19 can form a transcriptional repressor complex to regulate ABA and drought response in *Arabidopsis* (Song *et al.*, 2005).

In this study, it was found that *hd2c-1* and *hd2c-3* plants displayed increased sensitivity to ABA and NaCl during germination, and decreased tolerance to salt stress during seedling growth. In addition, over-expression of *HD2C* conferred an ABA-insensitive phenotype and enhanced tolerance to salt and drought stresses in transgenic *Arabidopsis* plants (Sridha and Wu, 2006). Furthermore, a rice SIR2-related HDA, *OsSRT1*, could enhance tolerance to oxidative stress when over-expressed in transgenic rice plants (Huang *et al.*, 2007). Taken together, the data

described above demonstrate that histone acetylation and deacetylation regulate the plant responses to abiotic stresses, and HDAs could be essential players in such regulation. The observations that both *hda6* and *hd2c-1* mutants displayed hypersensitivity to ABA and salt suggested that both *HD2C* and *HDA6* are involved in the ABA and salt response of *Arabidopsis*. Therefore, HD2C may function with an RPD3-type HDA such as HDA6 to regulate gene expression in the ABA and abiotic stress-response pathways.

HD2C regulates the expression of abiotic stress-responsive genes

The *HD2C* T-DNA knock-out lines, *hd2c-1* and *hd2c-3*, were hypersensitive to ABA and NaCl. *hd2c-1* and *hd2c-3* plants showed lower germination and survival rates under NaCl treatment. Similarly, Colville *et al.* (2011) also reported that a *hd2c* mutant was hypersensitive to ABA and NaCl in seed germination. The expression of ABA-responsive genes, *ABI1* and *ABI2*, which were found to be protein phosphatases (Merlot *et al.*, 2001) that negatively regulate the ABA response (Sheen, 1998), were increased in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. A previous study reported that the transcription factor *AtERF4* is a negative regulator involved in the ABA response (Yang *et al.*, 2005). The expression of *AtERF4* was up-regulated in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. Taken together, our results suggest that HD2C might regulate the expression of abiotic stress-related genes to affect plant stress tolerance. In this study, it was found that the expression of *HD2A*, *HD2B*, *HD2C*, and *HD2D* can be repressed by ABA and NaCl. Similarly, ABA also represses the expression of HD2-type HDAs, HDT701 and HDT702, in rice (Fu *et al.*, 2007). More recently, Colville *et al.*, (2011) reported that *hd2a* and *hd2c* mutants responded differently to ABA and NaCl. It remains to be determined whether HD2 proteins function differently in the abiotic stress response.

The levels of the gene activation mark histone H3K9K14Ac were increased in the promoter regions of *ABI1* and *ABI2* in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. In addition, the levels of the gene repression mark H3K9Me2 of *ABI1*, *ABI2*, and *AtERF4* were decreased in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. These results revealed that the high expression of *ABI1*, *ABI2*, and *AtERF4* in the mutants is associated with the increased levels of H3K9K14Ac and/or decreased levels of H3K9Me2. More recently, genome-wide analyses were performed in *Arabidopsis*, rice, and maize by ChIP-chip or ChIPseq (Lauria and Rossi, 2011). These studies showed that histone H3K9Ac is invariably correlated with transcriptional activation and biased towards the 5′-end of genes, whereas the gene repression mark H3K9me2 was found in repressed genes, spanning both the promoter and the gene body. Similarly, it was found that H3K9K14Ac increased in the promoter regions of *ABI1* and *ABI2*, but H3K9Me2 decreased in the promoter and exon regions of
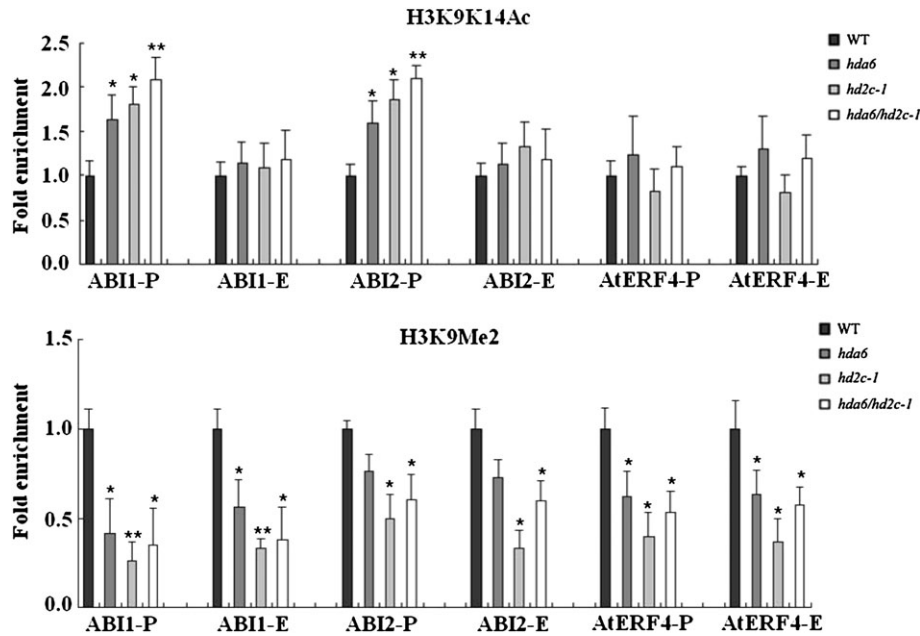
Fig. 8. Histone acetylation and methylation of genes up-regulated in *hda6*, *hd2c-1*, and *hda6/hd2c-1* double mutant plants. Relative levels of H3K9K14Ac (A) and H3K9Me2 (B) in *ABI1*, *ABI2*, and *AtERF4* promoter and first exon regions were determined. The amounts of DNA after ChIP were quantified and normalized to an internal control *ACTIN2* for H3K9K14Ac or *Ta3* for H3K9Me2. Error bars represent standard errors. Asterisks mark values that are significantly different from the wild type (*t* test, **P <0.01, *P<0.05). The experiment was repeated three times with similar results.

ABI1, *ABI2*, and *AtERF4* in the *hda6* and *hd2c* mutants. These data suggested that HD2C and HDA6 may regulate *ABI1*, *ABI2*, and *AtERF4* expression through histone modifications.

HD2C affects histone acetylation by interacting with HDA6

HD2-type histone deacetylases were identified as a plant-specific HDACs (Lusser *et al.*, 1997), and sequence analysis revealed that HD2 proteins share some sequence similarities with FKBP family pepridylprolyl *cis-trans* isomerases (PPIase) and a trypanosomal RNA-binding protein (Aravind, 1998). It has been shown that human FKBP25 shows 17% identity in protein sequence to the maize histone deacetylase HD2, with the most striking homology (22% identity) in residues 15–200 of human FKBP25 and residues 56–283 of maize HD2. FKBP25 physically associates with the histone deacetylases HDAC1 and HDAC2 and with HDA-binding transcriptional regulator YY1 in human cell (Yang *et al.*, 2001). Similarly, it was found that HD2C can interact with HDA6 in *Arabidopsis*, suggesting that HD2 proteins, like FKBP25 in human cells, might form a complex with RPD3-type HDAs.

Increased histone H3 acetylation was found in *hd2c-1* and *hda6* plants, suggesting that HD2C may affect the level of histone H3 acetylation by interacting with HDA6. In addition, increased H3K4Me3 and decreased H3K9Me2 were also observed in *hd2c-1* and *hda6* plants. Modulation of gene expression through cross-talk between histone
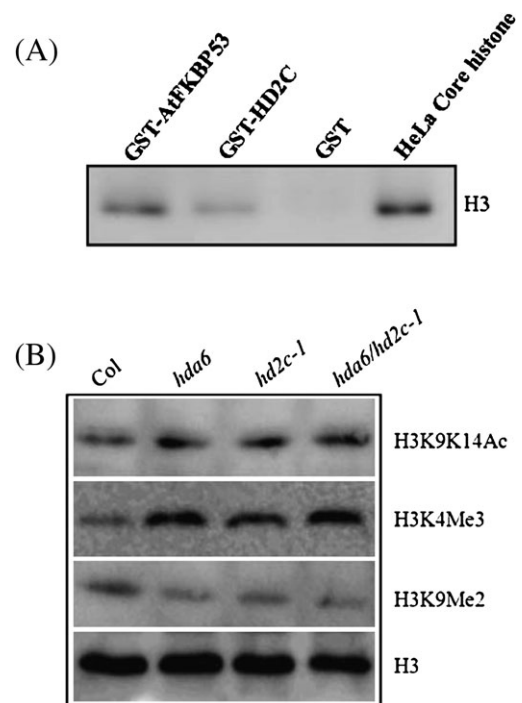
Fig. 9. Levels of histone H3K9K14Ac, H3K4Me3, and H3K9Me2 in *hda6*, *hd2c-1*, and *hda6/hd2c-1* plants. (A) The H3 was pulled down by GST-HD2C and GST-FKBP53 and analysed by Western blotting using anti-H3 antibody. (B) The levels of histone H3K9K14Ac, H3K4Me3, and H3K9Me2 in Col wild-type, *hda6*, *hd2c-1*, and *hda6/hd2c-1* plants were determined by Western blot analysis. The levels of H3 were shown as a loading control.

acetylation and methylation has been reported previously (Lee *et al.*, 2006; Chen *et al*, 2010). Our recent study indicates that HDA6 can interact with histone demethylase FLD and regulate both histone acetylation and methylation (Yu *et al.*, 2011). The observations that HD2A/HDT1 and HDA6 knock-down plants displayed a similar phenotype with respect to rRNA gene derepression, promoter cytosine methylation, histone modifications, and NOR condensation also support the functional association of HD2 proteins with RPD3-type HDAs (Lawrence *et al.*, 2004; Earley *et al.*, 2006). Our study indicated that HD2C physically associated with histone H3 and affected the levels of histone H3 acetylation and methylation. These results suggest that HD2 proteins may be a part of the chromatin remodelling complexes, including HDAs and other histone modification proteins. Our double mutant analysis indicated that the *hda6*/*hd2c* double mutant had enhanced phenotypes compared with the *hda6* and *hd2c* single mutants in *ABI1* and *ABI2* expression, histone acetylation, and germination, suggesting a synergistic effect. These results indicated that HDA6 and HD2C proteins could indeed cooperate in these responses (Capaldi *et al.*, 2008). Further research is required to determine the molecular mechanisms of HD2C and HDA6 interaction involved in the regulation of ABA-responsive genes and abiotic stress response.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Negative controls in BiFC assays.

Supplementary Fig. S2. Expression of *MYB2 and MYC2* determined by real-time RT-PCR.

Supplementary Fig. S3. Two additional independent analyses of histone acetylation and methylation of *ABI1* and *ABI2* in *hda6* plants.

Supplementary Table S1. Primers used for qRT-PCR analysis.

Supplementary Table S2. Primers used for Chip assay.

Acknowledgements

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