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Characterization and expression analysis of histone deacetylases family RPD3/HDA1 in *Populus trichocarpa*

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Abstract

Histone deacetylases (HDACs) catalyze histone deacetylation and play an important role in suppression of gene transcription in multiple cellular processes. HDACs are widely distributed in eukaryotes, however, detailed characterization of HDACs in woody plants is not available. In this study, the sequences of reduced potassium dependency 3/ histone deacetylase 1 (RPD3/HDA1) family proteins in black cottonwood (*Populus trichocarpa* Torr. & A. Gray) were characterized and their expression patterns in response to cold and salt stresses were determined. The RPD3/HDA1 proteins had conserved HDAC domains and can be divided into three classes based on sequence similarity and phylogenetic analysis. The transcripts of the *HDAC* genes were detected in different amounts in leaves, stems, and roots. The expressions of *HDAC* genes *HDA902*, *HDA903*, *HDA904*, *HDA909*, and *HDA912* were up-regulated in a cold stress. Interestingly, in a salt stress, most of the *HDAC* genes were down-regulated. These results indicate that the poplar *HDAC* genes were regulated by the cold and salt stresses, and the members of the RPD3/HDA1 family play a role in stress responses.

Additional key words: black cottonwood, cold, gene expression, salinity.

Introduction

Histone acetylation and deacetylation are major post-translational modifications of core histones. They are dynamic and reversible processes. Histone acetylation and deacetylation are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Histone acetyltransferases add acetyl groups to lysines, whereas HDACs counteract the effect of HATs and remove acetyls from histones. Hypoacetylation mediated by HDACs is associated with the condensed structure of chromatin and is generally considered to be associated with repression/silencing of genes (Hollender and Liu 2008). Based on the sequence homology to yeast HDACs, plant HDACs are classified into three major groups, namely reduced potassium dependency 3 / histone deacetylase 1 (RPD3/HDA1), histone deacetylase 2 (HD2), and silent information regulator 2 (SIR2) (www.chromdb.org; Pandey *et al*. 2002). The RPD3/HDA1 is a large family, and members in the family are related to yeast RPD3 and HDA-1. The RPD3/HDA1-type histone deacetylases require a zinc ion for catalytic activity and their enzyme activities can be inhibited by an HDAC specific inhibitor trichostatin A or sodium butyrate (Hollender and Liu 2008).

The RPD3/HDA1-type histone deacetylases are important enzymes to regulate plant growth, development, and stress responses. Aberrant expression of *HDAC* genes result in various morphological and developmental abnormalities such as slow growth, late flowering, and impaired root development (Xu *et al.* 2005, Rossi *et al.* 2007, Hu *et al.* 2009, Ma *et al*. 2013). The RPD3/HDA1-type histone deacetylases are also involved in stress responses. The *Arabidopsis* AtHDA6 and AtHDA19 are involved in abscisic acid (ABA) response and salt stress tolerance. An *AtHDA6* mutant, *axe1-5*, and

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Abbreviations: 18S - 18S rRNA; ABA - abscisic acid; HAT - histone acetyltransferase; HDA1 - histone deacetylase 1; HDAC - histone deacetylase; MEGA - molecular evolutionary genetic analysis; NCBI - National Center for Biotechnology Information; ORF - open reading frame; RPD3 - reduced potassium dependency 3; PCR - polymerase chain reaction.

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AtHDA6 RNA-interfering plants are hypersensitive to ABA and salt stress (Chen *et al*. 2010). An *Arabidopsis AtHDA19* T-DNA insertion mutant, *AtHDA19-1*, is also hypersensitive to ABA and salt stress (Chen and Wu 2010). Histone deacetylation has recently been shown to play an essential role in plant acclimation/tolerance to cold stress. In *Arabidopsis*, highly expressed osmotic stress responsive (HOS15) is a component of the protein complex which can catalyze histone deacetylation. *Arabidopsis hos15* mutants are hypersensitive to freezing temperature but not to salt, ABA, or oxidative stresses (Zhu *et al*. 2008). A recent work of To *et al*. (2011) provides a direct evidence that HDAC is involved in cold stress responses. In the study, the expression of the *Arabidopsis AtHDA6* gene is induced by a cold stress (2 °C), and *AtHDA6* mutation renders the transgenic plants a high sensitivity to a freezing temperature (-18 °C) after cold acclimation at 2 °C (To *et al*. 2011). These findings show that the RPD3/HDA1-type histone deacetylases play an important regulatory role in salt and cold stress responses.

In the past decade, several HDACs in *Arabidopsis* and some crops have been deeply studied. However, little is known about HDACs in woody plants. Woody plants are different from herbaceous plants in height, structure, life cycle, and multiple life processes. Their growth, development, and responses to ambient stimuli might be different as well. Thus, it is important to understand the function of HDACs in woody plants. In this study, we comprehensively characterized the sequences of cottonwood RPD3/HDA1-type HDACs and examined their expression profiles in response to cold and salt stresses.

Materials and methods

Nucleotide sequences and amino acid sequences of 11 HDACs in the cottonwood RPD3/HDA1 family were obtained from the plant *ChromDB* database (http://www.chromdb.org) and from the search in the National Center for Biotechnology Information (*NCBI*). Using these sequences, chromosomal localization and sequence identity were analyzed by informatics tools. The prediction of chromosomal localization was performed at the *Phytozome* website (http://www.phytozome.net). To determine the sequence identity among HDACs, full-length protein sequences of the 11 HDACs were aligned and compared using the *DNAMAN 6* software (*Lynnon Biosoft*, San Ramon, CA, USA).

For phylogenetic analysis, the protein sequences of HDACs from seven different plant species (*Populus trichocarpa*, *Prunus persica*, *Malus* × *domestica*, *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, and *Physcomitrella patens*) were retrieved from the plant *ChromDB* database. The amino acid sequences of 71 HDACs from these species in the *FASTA* format were aligned with *Clustal W* (Thompson *et al*. 1994), and then a neighbor-joining phylogenetic tree was constructed using the *MEGA 5* program (Tamura *et al*. 2011) with a bootstrap analysis of 1 000 replicates.

Recognizable conserved domains of cottonwood HDACs in the RPD3/HDA1 family were identified with *Pfam* (http://pfam.sanger.ac.uk) and also verified by the conserved domain database *CDD* (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), *InterPro* (http://www.ebi.ac.uk/interpro), and *UniProtKB/TrEMBL* (http://www.uniprot.org/uniprot/). The amino acid sequences of the HDAC domains were aligned using the *ClustalX 1.83* software (Thompson *et al*. 1997) and refined with *Genedoc* (Nicholas and Nicholas 1997).

Four-week-old seedlings of *Populus trichocarpa* Torr. & A. Gray were used for salt and cold treatments. For salt treatment, the seedlings were grown at a temperature of 25 °C, a 16-h photoperiod, an irradiance of 100 μmol m^{-2} s^{-1}, and a relative humidity of 75 % in *Vermiculite* supplemented with 200 mM NaCl for 12 and 48 h. For cold treatment, the seedlings were transferred to a 4 °C incubator and grown under the 16-h photoperiod for 1 and 3 d. For cold de-acclimation test, the seedlings were subsequently grown at 25 °C for another 2 d following the 3-d cold treatment. After the salt and cold treatments, the treated seedlings were blotted dry on tissue paper. Then, leaves, stems, and roots were immediately frozen in liquid nitrogen and stored at -80 °C before RNA isolation and gene expression analyses.

The total RNA from leaves, stems, and roots was extracted using a *Trizol* reagent (*Invitrogen*, Carlsbad, USA). The prepared RNA was treated with DNase I and then used for synthesis of cDNA. For cDNA synthesis, the total RNA was reverse transcribed with random primers using a *PrimeScript* reverse transcription reagent kit (*TaKaRa*, Dalian China) according to the manufacturer's instructions. The diluted cDNA obtained from reverse transcription was used as template for real-time PCR analysis.

The real-time PCR was set up using a *SYBR Premix Ex Taq II* kit (*TaKaRa*) in a volume of 0.02 cm^3. The reactions were performed in triplicate for each run and three biological replicates were included. The conditions for all the PCR reactions were as follows: 95 °C for 5 min, followed by 44 amplification cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Specific primers used for the 11 *HDAC* genes are listed in Table 1 Suppl. Values "Ct" obtained for all the genes were normalized to that of an internal control 18S rRNA (18S). For spatial expression analysis, the expression level of each HDAC gene was relative to 18S, calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and presented as $10^6 \times 2^{-\Delta Ct}$. For expression analysis of HDACs in response to the salt and cold stresses, transcript amount was determined using $2^{-\Delta\Delta Ct}$ calculations. The transcription of each gene in plants

without the cold and salt treatments was indicated as 1. Statistical significances ($\alpha = 0.05$) of differences between the treated and control plants were determined by one-way *ANOVA* and Tukey's test. In order to test the specificity of primers for *HDA902*, *HDA903*, *HDA908*, and *HDA909* genes which share a high sequence identity, the real-time PCR products were sequenced in *Shenzhen Huada Gene Sci-Tech Company* (Shenzhen, China).

Results

Nucleic acid sequences of 11 cottonwood RPD3/HDA1-family genes and the corresponding amino acid sequences were analyzed using bioinformatics tools. Table 1 shows ORF length, encoded protein size, genomic length, number of introns, and chromosomal localizations of the *HDACs*. The *HDAC* genes were distributed in 7 chromosomes and encoded proteins ranged from 292 to 646 amino acids. However, *HDA912* was not yet assigned to a particular chromosome. In order to know homology among the cottonwood RPD3/HDA1 proteins, the whole protein sequences of these HDACs were aligned using the *DNAMAN 6* software, and Table 2 shows their sequence identity. The HDA901 and HDA912 were 55.7 % identical at the amino acid level. The HDA902, HDA903, HDA904,

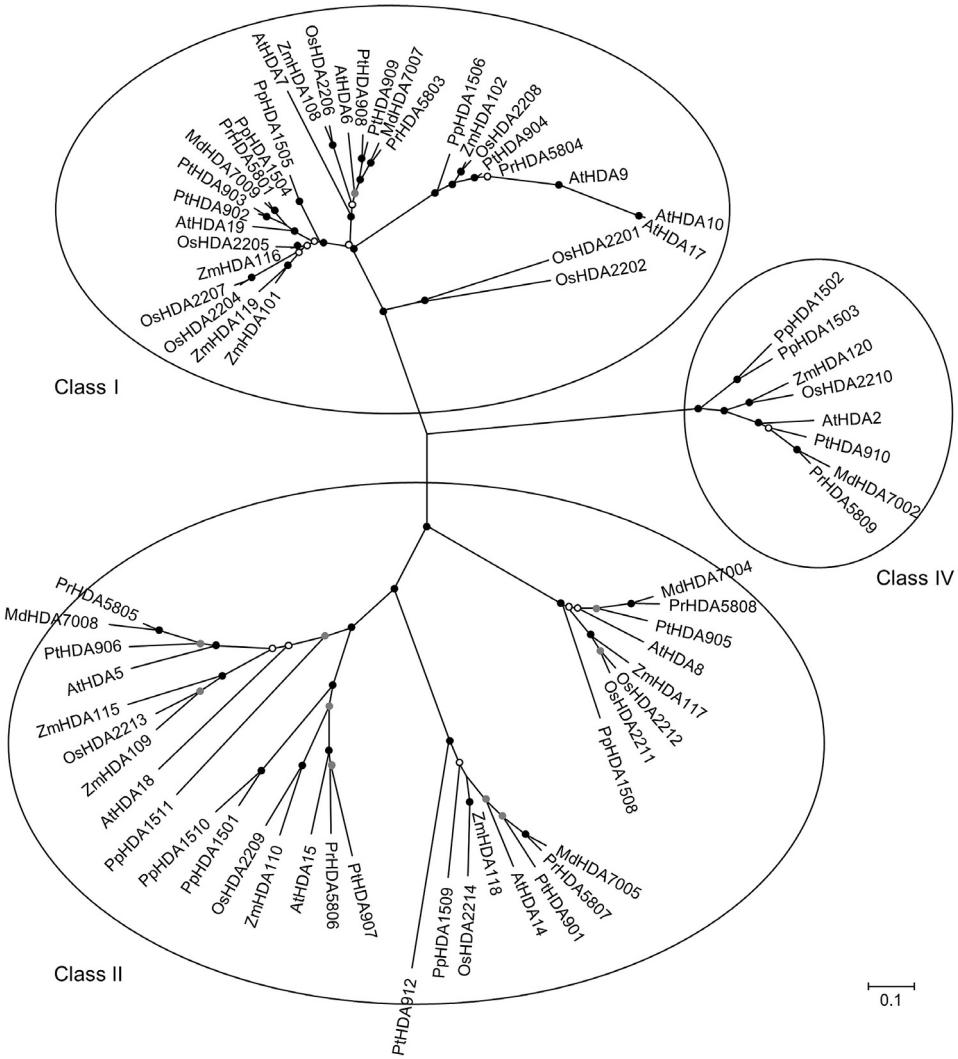

Fig. 1. Phylogenetic analysis of reduced potassium dependency 3 / histone deacetylase 1 family proteins. An unrooted neighbor-joining phylogenetic tree of 71 histone deacetylase sequences from 7 different plant species was constructed using the *MEGA5* program with a bootstrap analysis of 1 000 replicates (*scale bar* - 0.1 amino acid substitutions per site; bootstrap replicas: > 95 % - excellent support (*filled circles*); > 80 % - good support (*gray circles*); > 50 % - majority support (*empty circles*). Abbreviations for species are as follows: *Arabidopsis thaliana* (At), *Populus trichocarpa* (Pt), *Prunus persica* (Pr), *Malus × domestica* (Md), *Oryza sativa* (Os), *Zea mays* (Zm), and *Physcomitrella patens* (Pp).

Table 1. A list of *histone deacetylase* genes in *Populus trichocarpa* (ND - not detectable, NA - not available).

Gene name	ORF length [bp]	Number of amino acids	Genomic length [bp]	Chromosome	No. of introns	NCBI acc. No.
HDA901	1245	414	6993	5	8	XM_002306999
HDA902	1500	499	3110	9	6	XM_002313528
HDA903	1506	501	3822	4	6	XM_006384822
HDA904	1293	430	4523	1	13	XM_002300518
HDA905	1140	379	5536	9	2	XM_002313443
HDA906	1941	646	6475	4	13	XM_006384112
HDA907	1779	592	11303	12	16	XM_006376691
HDA908	1404	467	4265	15	5	XM_002322156
HDA909	1323	440	4899	12	4	XM_002318625
HDA910	1044	347	8114	6	12	XM_006381960
HDA912	879	292	4670	ND	8	NA

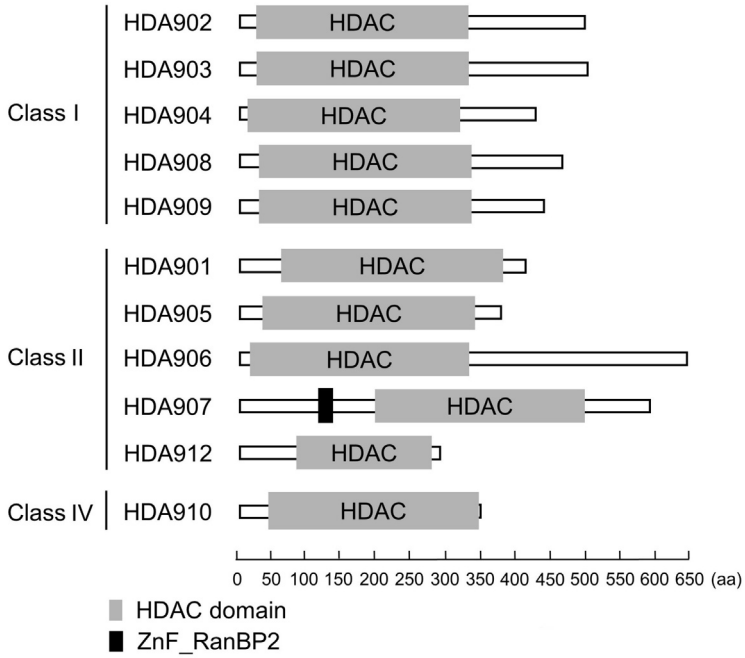

Fig. 2. Conserved domains of reduced potassium dependency 3 / histone deacetylase 1 (RPD3/HDA1) types of histone deacetylases (HDACs) in *Populus trichocarpa*. Recognizable conserved domains of 11 RPD3/HDA1 types of HDACs in *Populus trichocarpa* were identified. The size and locations of the HDAC domains are shown using the same shape. The RPD3/HDA1 proteins were divided into three classes.

HDA908, and HDA909 belonged to one group sharing over a 55 % amino acid identity with each other. Of the five HDACs, HDA902 had a very high degree of identity with HDA903 (94.8 %), and HDA908 was closely related to HDA909 with a sequence identity of 89.3 %. The high amino acid identity suggests the possibility of similar functions between HDA902 and HDA903, and between HDA908 and HDA909.

To examine evolutionary relationships among plant HDACs, protein sequences of 71 HDACs from 7 different species were obtained from the *ChromDB* database and a phylogenetic tree was constructed (Fig. 1). Based on sequence homology to HDACs in yeast and *Arabidopsis* (Alinsug *et al*. 2009), the cottonwood RPD3/HDA1 type of histone deacetylases were grouped into three clusters, namely Class I, Class II, and Class IV. The HDA902, HDA903, HDA904, HDA908, and HDA909 belonged to Class I representing RPD3-like HDACs. The HDA901, HDA905, HDA906, HDA907, and HDA912 belonged to Class II as HDA1-like HDACs, whereas HDA910 belonged to Class IV. Inspection of the phylogenetic tree shows that dicot HDACs from *Arabidopsis*, cottonwood, peach, and apple were grouped into one branch and monocot HDACs from rice and maize were clustered into another branch. The HDACs from peach and apple had a close phylogenetic relationship with the homologues in *Populus trichocarpa* indicating that HDACs in woody plants are closely related in evolution.

Recognizable conserved domains of the 11 cottonwood RPD3/HDA1 proteins were identified using different databases such as *Pfam*, *CCD*, *InterPro*, and *UniProtKB/TrEMBL*. Each of the 11 HDACs had a

conserved HDAC domain (Fig. 2). The HDAC domains generally ranged from 296 to 318 amino acids, whereas the HDAC domain of HDA912 was short containing only 194 amino acids. The HDAC domains of HDA902, HDA903, HDA904, HDA906, HDA908, and HDA909 were located at the N-terminus of the proteins, the HDAC domains of HDA910 and HDA912 were located close to the C-terminus, whereas the HDAC domains of HDA901, HDA905, and HDA907 were located in the middle regions of the sequences. In addition to an HDAC domain, a zinc-finger domain at amino acids 116 - 135 was identified in the HDA907 protein by the *InterPro* program. The alignment analysis of the HDAC domain sequences shows that the central regions of the HDAC domains were more conserved (Fig. 1 Suppl.) and contained several conserved residues assumed to be essential for histone deacetylase activity (Hassig *et al*. 1998).

Table 2. Sequence identity for reduced potassium dependency 3 / histone deacetylase 1 proteins in *Populus trichocarpa*.

	HAD901	HDA902	HDA903	HDA904	HDA905	HDA906	HDA907	HDA908	HDA909	HDA910	HDA912
HDA901	-	21.7	21.4	21.3	24.0	26.2	22.5	22.3	21.4	18.1	55.7
HDA902		-	94.8	56.8	23.3	17.7	19.0	65.0	65.8	22.9	16.4
HDA903			-	56.8	23.3	18.0	19.0	64.6	65.8	22.6	16.1
HDA904				-	25.5	19.4	18.7	57.1	56.4	23.5	17.4
HDA905					-	27.2	25.7	21.8	21.8	17.5	20.1
HDA906						-	31.9	17.8	18.9	19.7	19.1
HDA907							-	17.4	17.9	15.7	20.3
HDA908								-	89.3	21.4	17.5
HDA909									-	21.1	17.5
HDA910										-	14.4
HDA912											-

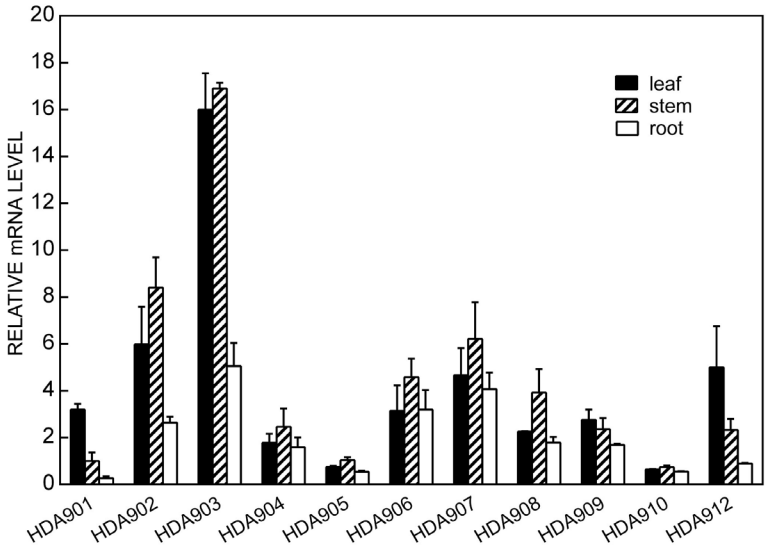

Fig. 3. The expressions of *histone deacetylase* (*HDAC*) genes in different organs. The 18S rRNA (18S) was used as internal control. The transcriptions of all the *HDAC* genes were relative to 18S and presented as $10^6 \times 2^{-\Delta Ct}$. Means ± SDs were calculated from data obtained from three biological replicates.

The spatial expression of the *HDAC* genes in the RPD3/HDA1 family was analyzed by real-time quantitative PCR (Fig. 3). Due to a high sequence identity between HDA902 and HDA903 and between HDA908 and HDA909, the real-time PCR products for the four genes were sequenced and the data show that the primers for these genes were very specific. The mRNAs of the 11 *HDAC* genes were detected at different amounts in leaves, stems, and roots. Of the genes examined, *HDA903* was most highly expressed in the organs examined, whereas the transcriptions of *HDA905* and *HDA910* were much lower in comparison with the other members in the RPD3/HDA1 family.

In order to know the response of the HDAC genes to the cold stress, the transcriptional profiles of the 11 *HDAC* genes were examined by real-time PCR (Fig 4). Under the cold stress, most of the *HDAC* genes were only slightly changed in the first 24 h but strongly up-regulated after 3 d; after recovery at 25 ° C for 2 d, the expressions of these genes almost returned back to the levels without the cold treatment (0 d). Of the 11 genes, *HDA903*, *HDA904*, and *HDA909* in leaves (Fig. 4*A*), *HDA902*, *HDA903*, *HDA904*,

HDA909, and *HDA912* in stems (Fig. 4*B*), and *HDA902*, *HDA903*, *HDA904*, *HDA909*, and *HDA912* in roots (Fig. 4*C*) were significantly up-regulated (over 1.9-fold) after the cold treatment for 3 d.

To examine response of the *HDAC* genes to the salt stress, the cottonwood seedlings were treated with 200 mM NaCl for different periods (0, 12, and 48 h), and then expression patterns of the *HDAC* genes were determined by real-time PCR (Fig. 5). Most of the *HDAC* genes started to be down-regulated after the NaCl treatment for 12 h and reached the lowest level 48 h after the salt treatment. A few genes including *HDA901*, *HDA90*8, *HDA909*, and *HDA912* were up-regulated after the short-term salt treatment (12 h) in some organs. The

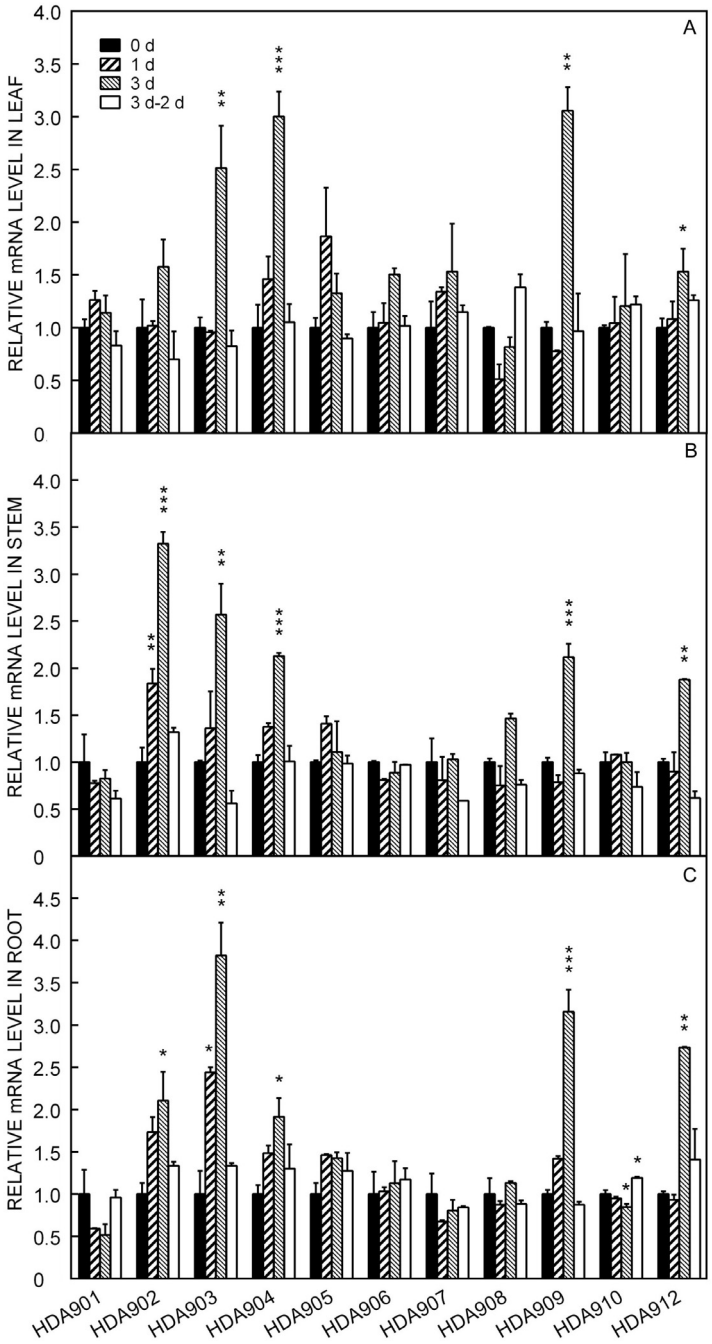

Fig. 4. The transcriptional profiles of histone deacetylase genes in response to cold stress (4 °C) for 0, 1, and 3 d and for 3 d + 2 d of recovery. Levels of mRNA in leaves (*A*), stems (*B*), and roots (*C*) were evaluated by real-time PCR. The 18S rRNA was used as internal control. The transcription of samples without the cold treatment (0 d) were indicated as 1. Means ± SDs were calculated from data pooled from three biological replicates. *, **, and *** indicate significant difference between the treated and control plants at $P \leq 0.05$, 0.01, and 0.001, respectively.

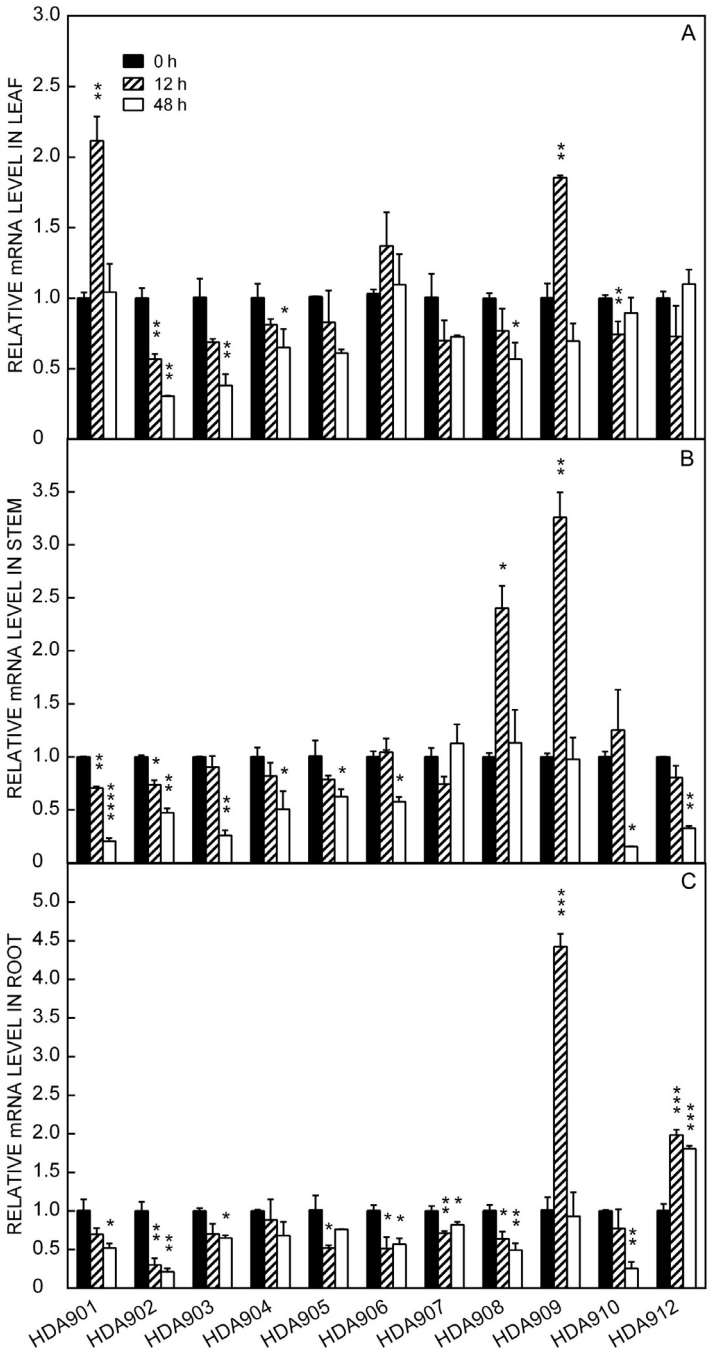

Fig. 5. The transcriptional profiles of histone deacetylase genes in leaves (*A*), stems (*B*), and roots (*C*) in response to salt stress for 0, 12, and 48 h evaluated by real-time PCR. The 18S rRNA was used as internal control. The transcription of each gene in samples without the salt treatment (0 h) was indicated as 1. Means ± SDs for three biological replicates. *, **, ***, and **** indicate significant differences between the treated and control plants at $P \leq 0.05$, 0.01, 0.001, and 0.0001, respectively.

expressions of the *HDA902* and *HDA903* genes in leaves (Fig. 5*A*), *HDA901*, *HDA902*, *HDA903*, *HDA904*, *HDA910*, and *HDA912* in stems (Fig.5*B*), and *HDA902*, *HDA908,* and *HDA910* in roots (Fig. 5*C*) were significantly down-regulated (over 2-fold) 48 h after the salt treatment. It is interesting to note that *HDA909*, unlike the other members of the family, was induced after 12 h of the salt treatment in all the organs examined suggesting that *HDA909* might have unique functions in the early response to the salt stress.

Discussion

Histone deacetylases play a key role in plant growth, development, and stress responses (Ma *et al*. 2013). However, to date, only HDACs in herbaceous plants, such as *Arabidopsis*, rice, and maize, have been well characterized. Histone deacetylases in woody plants remain to be investigated. In our experiments, the sequences and gene expressions of *Populus trichocarpa* HDACs in the RPD3/HDA1 family were characterized in detail. Sequence analysis shows that two pairs of genes sharing a high sequence identity were identified: *HDA902* and *HDA903* were 94.8 % identical in amino acid sequence and *HDA908* shared a high sequence identity with *HDA909* (89.3 % identity). It would be very interesting to know the reason why cottonwood has these pairs of homologous genes. These paired genes might be duplicated and modified in evolution and obtained novel functions or, due to their importance, have redundant functions. According to the expression patterns in response to the cold and salt stresses, we speculate that *HDA902* and *HDA903* might have overlapped functions, whereas *HDA908* and *HDA909* perhaps function differently in stress responses. The future functional study of these genes might better answer the question.

Hu *et al.* (2009) reported that expression of rice *HDACs* are less affected by cold compared with drought and salt stresses. On the other hand, To *et al*. (2011) reported that *Arabidopsis AtHDA6* and *AtHDA19* are up-regulated by cold. In cottonwood, *HDA908* and *HDA909* are homologues of *Arabidopsis AtHDA6*, whereas *HDA902* and *HDA903* are homologues of *Arabidopsis AtHDA19* based on sequence similarity and phylogenetic analysis (Ma *et al*. 2013). In our experiment, the expressions of the *HDA902*, *HDA903*, and *HDA909* genes were highly induced after the long-term cold treatment (3 d), which is consistent with the finding in *Arabidopsis* (To *et al*. 2011). Our data indicate that homologous *HDAC* genes from herbaceous and woody plants have similar expression patterns in response to cold stress.

In our study, the transcription of most of the *HDAC* genes was up-regulated under the cold treatment, whereas most of the genes were down-regulated under the salt treatment. It is very interesting that the responses of *HDACs* to the cold and the salt were different. *HDACs* are generally associated with depression/silencing of genes. Thus, we speculate that under cold stress, a set of cold-responsive genes might be repressed due to the up-regulation of *HDAC* genes, whereas under salt stress many genes might be induced as a result of the *HDAC* repression. The expression patterns of *HDAC* genes in response to cold and salt stresses provide useful information for further functional study of the genes.

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