Cloning and expression analysis of PtHDT903, a HD2-type histone deacetylase gene in Populus trichocarpa

Botong Tong*, Dean Xia*, Shibo Lv and Xujun Ma
State Key Laboratory of Tree Genetics and Breeding, School of Forestry, Northeast Forestry University, Harbin, PR China

ABSTRACT
Histone deacetylases (HDACs) catalyse the deacetylation of core histones and non-histone proteins. Histone deacetylases work in concert with histone acetyltransferases to modify the structure and function of chromatin, and regulate gene transcription. Histone deacetylases are critical enzymes involved in the regulation of multiple cellular processes, such as plant growth and development, stress responses and gene silencing. Plant HDACs are a supergene family and can be divided into three families, namely RPD3/HDA1, HD2 and SIR2. HD2 specifically occur in plants, not in fungi and animals. In this study, an HD2-type HDAC gene, PtHDT903, was cloned from Populus trichocarpa. Its amino acid sequence, subcellular localization and expression patterns under abiotic stresses were analysed. The results showed that PtHDT903 encodes a hydrophilic and acidic protein consisting of 305 amino acids. The predicted PtHDT903 protein has the conserved domains which found in the other HD2-type HDACs. The PtHDT903 protein was localized in the nucleus. The expression of the PtHDT903 gene was down-regulated by salt stress, whereas it was up-regulated by cold. The results provide valuable information for the further functional study of PtHDT903.

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Introduction
Multiple mechanisms regulate gene expression. One of them is epigenetic regulation, which is fast, reversible and inheritable [1]. The epigenetic regulation mechanism includes DNA methylation, histone modifications, small and non-coding RNAs, and chromatin architecture. Among the histone modifications, histone acetylation has been studied much earlier and in greater detail [2]. Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyse histone acetylation, which is associated with expanded chromatin structure. HDACs catalyse the removal of acetyl groups from chromatin, leading to contact chromatin structure, and thus are considered to be transcription repressors. Both HATs and HDACs are important for the regulation of gene expression. Recently, HDACs attract more and more attention.

HDACs are a supergene family. Based on sequence homology to yeast HDACs, plant HDACs can be classified into three families, namely reduced potassium dependency 3/histone deacetylase 1 (RPD3/HDA1), histone deacetylase 2 (HD2) and silent information regulator 2 (SIR2). RPD3/HDA1 includes a large number of members and the family proteins are Zn\(^{2+}\)-dependent [3, 4]. HD2 are plant specific and the first HD2 protein was identified in maize embryos [5–10]. SIR2 catalyse \(\text{NAD}^{+}\)-dependent deacetylation from histones and non-histone proteins [11, 12]. The studies in herbaceous plants, including maize, Arabidopsis and rice, show that HDACs play an important role in development, stress responses and gene silencing [13–33]. The aberrant expression of HD2 family genes alters the leaf morphology, flowering and seed development [16, 19, 34–37]. The over-expression of \(\text{AtHD2C}\) in \(\text{Arabidopsis}\) can reduce the transpiration and increase the tolerance of transgenic plants to salt and drought [25]. In rice, the over-expression of \(\text{HDT701}\) can enhance the salt and osmotic stress tolerance during the seedling stage [38]. To date, however, the expression and function of HD2 in cold stress is less known.

In plants, the studies on HD2 are just at the beginning. The expression and function of HD2 family genes in woody plants are still not known. In this study, a
gene encoding an HD2-type HDAC, PtHDT903, was cloned from Populus trichocarpa. The amino acid sequence, subcellular localization and expression of PtHDT903 under various types of stress were investigated in detail.

**Materials and methods**

**Plant growth and stress treatment**
We used 4-week-old seedlings of poplar (P. trichocarpa) grown at 25 °C under the 16-h light/8-h dark conditions. The stress treatments were salt and cold. For salt treatment, we supplemented the seedlings with 200 mmol/L NaCl in vermiculite for 0, 12 and 48 h. For cold treatment, we incubated the seedlings at 4 °C for 0, 1 and 3 days. After cold treatment for 3 days, we allowed the seedlings to recover for 2 days at 25 °C. After salt and cold treatments, we collected the leaves, stems and roots of the seedlings for RNA isolation and gene expression analyses. The presented data are mean values with standard deviation from three independent treatment experiments with nine plants per treatment.

**Cloning and sequence analysis of PtHDT903**
Based on the nucleic acid sequence of PtHDT903 in the Chromdb database (http://www.chromdb.org/) and the National Center for Biotechnology Information (NCBI) database, the Open Reading Frame (ORF) of the HDT903 gene in P. trichocarpa was cloned by reverse transcription-polymerase chain reaction (RT-PCR). The cDNAs were synthesized with PrimeScript RT reagent Kit (TaKaRa), using the total RNAs from young seedlings. The specific primers were designed and the primer sequences were as follows: 5′-ATGGAGTTCTGGGGTGTTGA-3′ and 5′-CTATGCAGCACTGTGCTTAG-3′. The molecular weight (MW) and the theoretical isoelectric point (pI) of the PtHDT903 protein were determined using ExPASy (Expert Protein Analysis System) ProtParam software (http://web.expasy.org/protparam/). The PtHDT903 homologous proteins were obtained by blasting NCBI. The homologous proteins include P. euphratica PeHDT1 (XP 011018878), Herrania umbratica HuHDT1 (XP 021289379), Sesamum indicum SiHDT1 (XP 011101488), Jatropha curcas JcHDT1 (XP 012076192), Manihot esculenta MeHDT1 (XP 021610077), Arachis ipaensis AiHDT1 (XP 016208108) and Cajanus cajan CcHDT1 (XP 020211823). The amino acid sequences of PtHDT903 and its homologous proteins were aligned using ClustalX 1.83 [39] and refined with Genedoc [40]. The amino acid sequence identity among the eight HD2 was analysed using DNAAM 7.0. The neighbour-joining phylogenetic tree was constructed using the MEGA 5 program [41] with a bootstrap analysis of 1000 replicates. The conserved domain of PtHDT903 was determined using the SMART program (http://smart.embl-heidelberg.de/) and by searches in the Conserved Domain Database (CDD, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

**Subcellular localization analysis**
The ORF fragment of PtHDT903 was fused to the 5′ end of the green fluorescent protein (GFP) reporter gene in the vector pCAMBIA1302. The recombinant plasmid containing the fusion gene (HDA903-GFP) and the vector (GFP) were respectively transformed into the epidermal cells of onion by particle bombardment using PDS-1000/He (BIO-RAD). After incubation in 1/2 Murashige and Skoog (MS) medium at 28 °C for 24 h, GFP fluorescence was observed under a confocal laser-scanning microscope (LSM700; Carl Zeiss).

**Gene expression analysis**
The total RNAs from leaves, stems and roots of P. trichocarpa seedlings were prepared using Trizol reagent (Invitrogen) and cDNA were obtained using PrimeScript RT reagent Kit (TaKaRa). The real-time PCR was set up using SYBR Premix Ex Taq II Kit (TaKaRa) in a volume of 20 μL. The reaction procedure was 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. The primers for PtHDT903 were: 5′-CAAAAGTTGCTGTTCTGGGTGTGGA-3′ and 5′-GTACGAGTGTTCTGGGTGTGGA-3′.

Figure 1. Phylogenetic analysis of HD2-type histone deacetylases. Note: The phylogenetic tree is based on amino acid sequences of PtHDT903 and its homologous proteins. The tree was drawn to scale (scale bar, 0.05 amino acid substitutions per site). The eight HD2 proteins are Populus trichocarpa PHDT903, Populus euphratica PeHDT1, Herrania umbratica HuHDT1, Sesamum indicum SiHDT1, Jatropha curcas JcHDT1, Manihot esculenta MeHDT1, Arachis ipaensis AiHDT1, and Cajanus cajan CcHDT1.
CAACCCGAAT-3' and 5'-TGGTTGCTTTGCTCCTCCTT-3'.

The 18S rRNA (18S) was used as the internal control. For organ-specific expression analysis, the expression level of PtHD9T03 gene was calculated by the 2^{ΔΔCt} method [42] and presented as 10^{-6}/2^{ΔΔCt}. The expression level of PtHD9T03 under salt and cold stresses was determined using 2^{ΔΔCt} calculations. The transcript level of PtHD9T03 without cold and salt treatments was indicated as 1. The transcript levels (n-fold) of PtHD9T03 under cold and salt stresses were relative to that without any treatments. The data were statistically analysed using one-way analysis of variance (ANOVA) and Tukey test. The significance level was set to p < 0.05.

Figure 2. Amino acid sequence alignment of HD2-type histone deacetylases. Note: Amino acid sequences of PtHD9T03 and its homologous proteins were aligned. Letters indicate: (a) invariable pentapeptide motif (MEFWG); (b) critical amino acid residues for histone deacetylase catalytic activity; (c) nuclear localization signal (KKAK); (d) conserved C2H2-type zinc finger domain. The extended acidic domain is underlined.
Results and discussion

Cloning, sequence and organ specific expression analysis of PtHDT903

In our study, we cloned an HD2-type HDAC gene PtHDT903 from *P. trichocarpa* (GenBank accession No. EF145859). The ORF of the cloned PtHDT903 is 918 bp in length, encoding a hydrophilic protein consisting of 305 amino acid residues. Its molecular mass is 33.35 kDa, and its isoelectric point is 4.61.

PtHDT903 has the closest relationship in evolution with PtHdT1 from *P. euphratica* (Figure 1) and their amino acid sequence identity was 97.8%. The amino acid sequences of PtHDT903 and the other seven HD2 proteins were highly conserved (Figure 2). In the N-terminal region of the predicted PtHDT903 protein, there is an invariable pentapeptide motif (MEFWG) and two conserved amino acid residues, a histidine at position 25 and an aspartate at position 70, which are postulated to be critical for HDAC catalytic activity [43]. The central part of PtHDT903, as in the other HD2 proteins [6, 22, 35, 44], is an extended acidic region enriched in Asp and Glu residues (position 162 to 208). The acidic region might be involved in nucleolar localization or association with basic tails of histones [45]. The C-terminal region of PtHDT903 contains a nuclear localization signal (KKAK) and a C2H2-type zinc finger domain. The single zinc finger might be involved in protein–protein interactions [46]. In addition to the C2H2 zinc finger (position 279 to 302), the predicted PtHDT903 also has two Low Complexity domains, from amino acids 159 to 177 and from 185 to 206 (Figure 3(A)).

The PtHDT903 gene could be expressed in all the organs examined, but at different levels. Its expression levels in leaves and stems were high, while its expression in roots was relatively low (Figure 3(B)).

Subcellular localization of PtHDT903

To know the subcellular localization of the PtHDT903 protein, the HDT903-GFP infusion gene was transformed
into the epidermal cells of onion. In the onion cells transformed with GFP alone, the green fluorescence was distributed throughout the cells (Figure 4(A–C)), while in the cells transformed with HDA903-GFP, the fluorescent signals were only detected in the nucleus (Figure 4(D–F)). The data showed that PtHDT903 is localized in the nucleus. In our experiment, the GFP fluorescence could be detected in some cells. The lack of fluorescence in the surrounding cells might be due to the low transformation efficiency of onion cells.

**Expression patterns of PtHDT903 under abiotic stresses**

We analysed the expression patterns of the PtHDT903 gene under salt and cold stresses. The results showed that the expression of PtHDT903 was regulated by both salt and cold treatment, but the expression patterns were different under both stresses. Salt stress treatment with 200 mmol/L NaCl for 48 h resulted in down-regulation of the PtHDT903 expression in leaves and stems, but not in roots (Figure 5). After such a treatment, the expression levels of PtHDT903 in the leaves and stems were 84.7 and 78.7% down-regulated, respectively. This result was consistent with the findings in rice and Arabidopsis. In rice, the expression level of OsHDT701 steadily decreased after 300 mmol/L NaCl treatment for 1 and 3 h [38]. In Arabidopsis, the expression levels of the four genes in the HD2 family, HD2A, HD2B, HD2C and HD2D, were significantly down-regulated after 250 mmol/L NaCl treatment for 6 h [47].

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**Figure 5.** Expression patterns of PtHDT903 under salt (A–C) and cold (D–F) stress in leaves (A and D), stems (B and E) and roots (C and F). Note: Real-time PCR analysis. Data represent mean values with standard deviations (±SD) of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001, significant difference between the treated and control samples.
In plants, the expression of HD2 family genes under cold stresses is not well understood and few data on their expression are available. The data available in rice show that the expression of the OsHDT701 gene was not altered after cold (4°C) treatment for 1 day [21]. In our study, the expression of PtHDT903 was not altered after cold (4°C) treatment for 1 day, but was significantly induced after long-term cold treatment (3 days). After cold treatment for 3 days, the PtHDT903 expression in the leaves, stems and roots was 2.2-, 1.4- and 1.8-fold up-regulated, respectively. After recovery for 2 days following cold treatment for 3 days, the expression of PtHDT903 in the leaves, stems and roots was almost the same as that before cold treatment. Taken together, the expression of PtHDT903 in poplar seedlings was repressed by salt, but was induced by cold. The different responses of PtHDT903 to salt and cold stimuli suggest that the up-stream regulators or down-stream target genes of PtHDT903 might be different under salt and cold stress conditions.

Conclusions

In our work, an HD2-type HDAC gene PtHDT903 was cloned from P. trichocarpa. The PtHDT903 protein was predicted to contain three conserved domains. The PtHDT903 protein is exclusively localized in the nucleus. The expression of PtHDT903 gene is responsive to abiotic stresses. Its expression was repressed by salt, but was induced by cold. Our findings provide valuable information for further functional study of PtHDT903, which might be potentially applied for the cultivation of stress-tolerant poplar varieties.

Disclosure statement

No potential conflict of interest was reported by the authors.

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