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Evolutionary diversification of type-2 HDAC structure, function and regulation in *Nicotiana tabacum*

Valérie Nicolas-Francès[a,1], Vincent Grandperret[a,1], Benjamin Liegard[a], Sylvain Jeandroz[b], Damien Vasselon[a], Sébastien Aimé[c], Agnès Klinguer[c], Olivier Lamotte[d], Emilie Julio[e], François Dorlhac de Borne[e], David Wendehenne[a], Stéphane Bourque[a,*]

[a] Université de Bourgogne-Franche Comté, UMR 1347 Agroécologie, BP 86510, F-21000, Dijon, France
[b] Université de Bourgogne-Franche Comté, UMR 1347 Agroécologie, AgroSup Dijon, BP 86510, F-21000, Dijon, France
[c] INRA, UMR 1347 Agroécologie, BP 86510, F-21000, Dijon, France
[d] ERL CNRS 6300, UMR 1347 Agroécologie, BP 86510, F-21000, Dijon, France
[e] Institut du Tabac, Domaine de la Tour, LBCM, 24100, Bergerac, France

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ABSTRACT

Type-2 HDACs (HD2s) are plant-specific histone deacetylases that play diverse roles during development and in responses to biotic and abiotic stresses. In this study we characterized the six tobacco genes encoding HD2s that mainly differ by the presence or the absence of a typical zinc finger in their C-terminal part. Of particular interest, these *HD2* genes exhibit a highly conserved intron/exon structure. We then further investigated the phylogenetic relationships among the *HD2* gene family, and proposed a model of the genetic events that led to the organization of the *HD2* family in Solanaceae. Absolute quantification of *HD2* mRNAs in *N. tabacum* and in its precursors, *N. tomentosiformis* and *N. sylvestris*, did not reveal any pseudogenization of any of the *HD2* genes, but rather specific regulation of *HD2* expression in these three species. Functional complementation approaches in *Arabidopsis thaliana* demonstrated that the four zinc finger-containing HD2 proteins exhibit the same biological function in response to salt stress, whereas the two HD2 proteins without zinc finger have different biological function.

1. Introduction

In eukaryotes, genomic DNA is packaged into chromatin. Gene transcription regulation depends on DNA sequences and also on epigenetic events such as DNA methylation and chromatin condensation. Changes in chromatin structure between the two relaxed and condensed states are tightly associated with transcriptional activity. Condensed chromatin prevents transcription, whereas relaxed chromatin allows DNA to interact with RNA polymerases and transcription factors. In plants, responses to environmental stresses imply the chromatin opening at the target gene loci and in turn the transcription of the corresponding genes [1–5]. Histone acetylation/deacetylation is one of the mechanisms involved in chromatin remodeling and changes in transcriptional program. The level of nucleosomal histone acetylation depends on the antagonistic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Both classes of enzymes were initially termed that way because they were first thought to act only on histones. Since then, increasing evidence have shown that they can also act on non-histone proteins; it was therefore Yang et al., proposed to rename these proteins acetyltransferases and protein deacetylases, respectively [6]. Among living organisms, only two conserved HAT families, GCN5 and MYST (Lee and Workman; Shen et al.), and two HDAC families, RPD3/HDA1 and SIR2 (Grandperret et al.), are commonly found. A third family, named type-2 HDAC (HD2), contains plant-specific HDACs first identified in maize [6–8]. Four HD2 proteins, HD2A (also known as HDT1), HD2B (HDT2), HD2C (HDT3), and HD2D

Abbreviations: bp, base pair; Ca, *Capsicum annum*; Gr1&2, group 1 & 2; HDAC, histone deacetylase; HD2, type-2 HDAC; Ns, *Nicotiana sylvestris*; Nt, *Nicotiana tabacum*; Ntom, *Nicotiana tomentosiformis*; nt, nucleotide; SGN, sol genomics network; Sl, *Solanum lycopersicum*; Sm, *Solanun melongena*; St, *Solanum tuberosum*; ZnF, zinc finger

* Corresponding author.
E-mail addresses: valerie.frances@u-bourgogne.fr (V. Nicolas-Francès), vincent.grandperret@ifpen.fr (V. Grandperret), benjamin.liegard@inra.fr (B. Liegard), sylvain.jeandroz@inra.fr (S. Jeandroz), damien.vasselon@inra.fr (D. Vasselon), sebastien.aime@inra.fr (S. Aimé), agnes.klinguer@inra.fr (A. Klinguer), olivier.lamotte@inra.fr (O. Lamotte), Emilie.JULIO@fr.imptob.com (E. Julio), Francois.DORLHAC@fr.imptob.com (F.D. de Borne), david.wendehenne@inra.fr (D. Wendehenne), stephane.bourque@inra.fr (S. Bourque).
[1] Co-first author.

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(HDT4) have been found in Arabidopsis. In addition to the model plant Arabidopsis, HD2 proteins have also been experimentally described in other species such as barley [9], rice [10], tomato [11], longan fruit [12], and tobacco [13]. Recently, we performed an extensive screening of HD2 based on the transcriptomic data from more than 1300 green plants generated by the 1000 plants consortium (www.onekp.com) and showed that these proteins appeared early in green plant evolution [14]. Large protein sequence alignments (over 1200 sequences) led to propose a general model for HD2 organization. HD2s are built on the same model: a well conserved MEFWG N-terminal pentapeptide, a 100-amino-acid-long catalytic domain, an acidic central domain that contains putative serine/threonine residues specifically targeted by casein kinase 2α and mitogen-activated protein kinases, and a monopartite nuclear localization signal. Over 97% of these HD2 sequences contained in the C-terminal region a TFIIA-type zinc finger (ZnF) and were defined as Group 1 (Gr1). HD2 sequences without the C-terminal ZnF were defined as Group 2 (Gr2). Gr2 HD2s were found only in angiosperms and result from several independent deletion of ZnF from Gr1 HD2s. So far, there has been no experimental analysis investigating the putative different functional activities of Gr1 and Gr2 HD2s.

This HD2 family is really intriguing. All HD2s analyzed so far share more than 70% sequence identity, but no similarity with members of the other families, RPD3/HDA1 and SIR2 [15]. The specific role of HD2s is still unclear as plants also possess and express the other two families shared with other eukaryotes. A reasonable hypothesis could be that HD2s deacetylate plant-specific substrates; however, such targets have not been identified yet. Another possibility is that HD2s might control specific plant physiological processes. In that way HD2s are involved in leaf [16] and fruit development [12], in the control of seed dormancy and germination [5,17–19], and in plant defence responses [20].

Chromatin-bound HD2-containing complexes were first purified from germinating maize embryos [6,21]. Once highly purified, these complexes displayed *in vitro* deacetylase activity against all four acetylated core histones. Indirect evidence of HD2 in vivo deacetylase activity was provided by [22], who showed that overexpression of the rice HD2 HDT701 decreased histone H4 acetylation, and by [13], who reported that the silencing of NtHD2a and NtHD2b, two *Nicotiana tabacum* HD2 isoforms, caused the accumulation of hyperacetylated nuclear proteins. In this latter case, silencing of both NtHD2s strongly enhanced the hypersensitive response induced by cryptogein, a proteinaceous elicitor produced by the oomycete *Phytophthora cryptogea*.

Free access to *N. tabacum* genomic and transcriptomic data offered an opportunity to improve our knowledge of the HD2 family within the genus *Nicotiana,* both at the genome and protein levels [23]. Based on data obtained from the *N. tabacum* progenitors (*N. sylvestris* and *N. tomentosiformis)* and from other Solanaceaes, we propose an updated model of HD2 evolution in the Solanaceae family. This study is strengthened by a functional analysis investigating the involvement of HD2s in salt stress tolerance and pointing out the putative role of HD2 ZnF in their function.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana T-DNA insertion mutants, *hd2c-1* (HD2CT99 – Salk_129799.19.60N) and Sail_240_C08 and their corresponding wild types Col-0 and Col-3, respectively, were provided by the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/). Arabidopsis plants were grown in a phytotron with 10 h light/14 h dark photoperiod at 20 °C/18 °C. *N. tabacum*, *N. sylvestris* and *N. tomentosiformis* plants were grown in a greenhouse with a 12 h light/12 h dark photoperiod at 23 °C. The organ-specific series of samples (leaf, stem, root, and flower) were collected from flowering plants. Seeds were collected later.

2.2. Germination rate measurements

A. thaliana seeds were sowed on Petri dishes containing Hoagland growth medium with 1% agar and with or without 150 mM NaCl. Seeds were incubated for 2 days in the dark at 4 °C to synchronize germination, and then for 4 days in a growth chamber under a long day photoperiod (16 h light/8 h dark) at 23 °C.

2.3. Arabidopsis thaliana transformation

pB2GW7 vectors containing HD2-coding sequences were used to transform competent *Agrobacterium tumefaciens* strain GV3101 (pMp90), using the quick protocol published online by DNA Cloning Service (http://www.dna-cloning.com/), except that bacteria were frozen in liquid nitrogen instead of at −80 °C. Transformed bacteria were selected on Luberia Broth agar plates containing gentamicin (25 μg mL^{-1}), rifampicin (50 μg mL^{-1}), and spectinomycin (100 μg mL^{-1}). Plants were transformed using the floral dipping method [24]. Transgenic plants were selected by spraying phosphinothricin (50 μg mL^{-1}). To select homozygous plants for transgene integration, T2 seeds were sowed on Petri dishes containing Hoagland growth medium with 1% agar. After one week incubation, the plantlets were sprayed with phosphinothricin (50 μg mL^{-1}). Batches of seeds with 100% resistance to phosphinothricin provided for homozygous plants. The transgene expression was verified at the RNA level by semi-quantitative RT-PCR with GoTaq® (Promega) using total RNAs extracted from leaves and oligonucleotides specific of each coding sequence (see below for RNAs extraction and cDNA synthesis). Wild type NtHD2a and mutant HD2a^{C270AC273A} proteins were detected in the leaves by western blot with a homemade antibody as described previously [13].

2.4. RNA extraction, cDNA synthesis and q-PCR

Plant organs were ground in liquid nitrogen, and total RNAs were purified using SV Total RNAs Isolation System (Promega) according to the manufacturer's protocol. cDNAs were synthesized from 500 ng of total RNAs using the DyNAmo cDNA Synthesis Kit (Thermo Fisher). Real-Time PCR was performed with GoTaq qPCR Master Mix using the ViiA 7 Real-Time PCR System (Applied Biosystems). Two technical replicates were performed on three independent biological samples. The *N. tabacum L25* ribosomal protein gene (accession number L18908) was used as an internal control. Relative expression levels were calculated using the linear regression of efficiency method [25]. Normalized *HD2* expression was calculated using the geometric mean of *L25* expression for each tissue type. Using the standard curves described below, the results were expressed as numbers of *HD2* mRNA copies *per* ng of total RNAs. Primers used in this study are presented in Table S1.

2.5. Standard curves

For each pair of primers specific to each cDNA, amplicons were cloned into pCR4-TOPO vectors using a TOPO TA cloning kit (Invitrogen). Vectors containing the amplicons were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher), and diluted to a final concentration of 2×10^8 copies μL^{-1}. Ten-fold serial dilutions were performed down to 20 copies μL^{-1}. Five μL of each dilution were used in qPCR reactions. Standard curves were thus performed for each primer pair to correlate amplicon copy numbers to Ct values.

2.6. Plasmid constructs

Coding sequences of *NtHD2s* and *AtHD2c* were amplified from cDNAs obtained from tobacco and Arabidopsis leaves, respectively (see RNA extraction and cDNA synthesis), using Phusion High-Fidelity DNA Polymerase (Thermo Scientific), and cloned into either pENTR/D-TOPO or pDONR221 vectors (Invitrogen). NtHD2 and AtHD2c coding