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HDACs MADS-domain protein interaction: a case study of HDA15 and XAL1 in *Arabidopsis thaliana*

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ABSTRACT

Cellular behavior, cell differentiation and ontogenetic development in eukaryotes result from complex interactions between epigenetic and classic molecular genetic mechanisms, with many of these interactions still to be elucidated. Histone deacetylase enzymes (HDACs) promote the interaction of histones with DNA by compacting the nucleosome, thus causing transcriptional repression. MADS-domain transcription factors are highly conserved in eukaryotes and participate in controlling diverse developmental processes in animals and plants, as well as regulating stress responses in plants. In this work, we focused on finding out putative interactions of *Arabidopsis thaliana* HDACs and MADS-domain proteins using an evolutionary perspective combined with bioinformatics analyses and testing the more promising predicted interactions through classic molecular biology tools. Through bioinformatic analyses, we found similarities between HDACs proteins from different organisms, which allowed us to predict a putative protein-protein interaction between the *Arabidopsis thaliana* deacetylase HDA15 and the MADS-domain protein XAANTAL1 (XAL1). The results of two-hybrid and Bimolecular Fluorescence Complementation analysis demonstrated *in vitro* and *in vivo* HDA15-XAL1 interaction in the nucleus. Likely, this interaction might regulate developmental processes in plants as is the case for this type of interaction in animals.

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Introduction

Acetylation and deacetylation are important post-translational modifications of histones that are critical to gene expression regulation. While acetylation is associated with gene activation, deacetylation promotes the interaction of histones with DNA; causing nucleosome compaction, thus causing gene transcription to be repressed. The enzymes that participate in these processes are known as histone acetylases (HATs) and histone deacetylases (HDACs), respectively. Contrary to the HAT enzymes, which catalyze the transfer of an acetyl group from acetyl-CoA to an ε-amino group of histone lysine residues, HDACs remove an acetyl group. The deacetylation carried out by HDACs contributes to various eukaryote development processes such as embryogenesis, cell differentiation, apoptosis, and others.[1–3]

In humans, HDACs can be classified into two families. The first one comprises proteins related to Reduced Potassium Dependency 3 (RPD3) proteins that are grouped into four classes (I, IIa, IIb, and IV). The second family is the Silent Information Regulator 2 (SIR2) family which also consists of four classes (I-IV).[4] In plants, there are three subfamilies of HDACs: 1) RPD3/histone deacetylase 1 (HDA1), includes proteins with a zinc finger motif in addition to the conserved histone deacetylase domain; 2) SIR2, is a NAD^+-dependent histone deacetylase with two distinct domains that bind NAD, and 3) Histone deacetylase 2 (HD2) which is specific of plants and include proteins with the deacetylase domain in its N-terminus. Additionally, the subfamily RPD3/HDA1 has three classes: class I (HDA6, HDA7, HDA9, HDA10, and HDA19) involved in germination, flowering, signaling, and stress tolerance; class II (HDA5, HDA8, HDA14, HDA15, HDA18) implicated in germination, ABA signaling, autophagy, heat tolerance, sperm cell formation, and photosynthesis; and class III sirtuin-like HDACs (SRT1, SRT2) involved in ethylene signaling, energy metabolism, cell division cycle and cellular response to DNA damage.[1,5]

The role of HDACs in transcriptional repression is conserved among plants and animals. Furthermore, phylogenetic analysis of HDACs revealed a conserved deacetylation domain and several other similarities among their orthologs.[3,6] HDACs can work as part of co-repressor protein complexes or in

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association with chromatin remodeling proteins as modulators of accessibility to DNA.[7] However, due to the large family number, it is primordial to unravel how their function is regulated to achieve specificity between the different HDACs. One possibility is that they associate with transcription factors that determine the specificity binding to specific DNA sites. For instance, it has been reported that HDACs can form complexes with MADS-domain proteins,[8–10] which function as transcriptional regulators in a broad spectrum of developmental stages and processes in animals, plants, and fungi.[8,11–13]

Two lineages of MADS-box genes have been described in eukaryotes: type I, or serum response factor (SRF)-like members, which play an essential role during early development in animals and in plant reproduction; and type II myocyte enhancer factor 2 (MEF2)-like, whose members are involved in muscle development and, in plants, participate in different developmental processes along the life cycle.[14,15] Plant type II members or MIKC have been well described as modular proteins with several functional domains, including the highly conserved MADS-domain implicated in DNA binding (M), the intervening domain (I), the keratin-like (K) domain that participates in dimer formation and the variable C-terminal domain (C).[16]

In animals, HDAC4 and HDAC5, belonging to class IIa, interact with the MADS-domain transcription factor, MEF2A, inhibiting its transcriptional activity *in vivo* and *in vitro* during muscle cell differentiation. HDAC4 binds and represses MEF2A, regulating the specification of cardiomyoblasts[17] whereas the N-terminal in HDAC5 is sufficient to repress MEF2A.[10] Additionally, HDAC9 acts as a negative regulator of muscle differentiation and cardiac tissue development, at least in the latest, by binding to MEF2 and suppressing fetal cardiac gene expression. All these data suggests that HDACs class II from animals have a crucial role in different cell proliferation/differentiation processes.

Growing evidence indicates that HDACs play a key regulatory role in plant development and the plant's responses to abiotic and biotic stresses.[5] However, few reports have been devoted to uncovering HDAC-MADS-domain protein interactions and their role in developmental regulation. The *Arabidopsis thaliana* (from now on Arabidopsis) MADS-domain protein AGL15 is known to interact with members of the Swi-independent 3/histone deacetylase (SIN3/HDAC) complex; this interaction is required to regulate AGL15 target genes.[18] Moreover, during the floral transition, SAP30 FUNCTION-RELATED 1 (AFR1) and SAP30 FUNCTION-RELATED 2 (AFR2) form a complex with AGL18 to be recruited to a HDAC complex and inhibit the *FLOWERING LOCUS T* (*FT*) expression via deacetylation. FT is an important component of the photoperiodic regulation of flowering.[19] In tomato, three MADS-domain proteins expressed during flowering and fruit formation *Lycopersicon esculentum* MADS1, 5, and 6 (LeMADS1, LeMADS5, and LeMADS6) possess a histone deacetylase binding domain which is needed to interact with mammalian HDA5.[8] In addition, *Solanum lycopersicum* HDA1, 3, and 4 (SlHDA1, SlHDA3 and SlHDA4; members of the RPD3/HDA1 family) interacted with TOMATO AGAMOUS1 (TAG1), and TOMATO MADS BOX29 (TM29).[20]

To have a global knowledge of the HDAC-MADS protein interactions in Arabidopsis, we analyzed the sequences of HDACs and MADS-domain proteins and compared their putative interaction domains. Sequence analyses and hydrophobic cluster analyses (HCA) methods delineated a similarity between the MADS-domain binding motifs of human HDACs and those of Arabidopsis. Here, we demonstrate the interaction of XAANTAL 1 (XAL1)/AGL12, a key regulator of root cell proliferation and flowering transition,[21–23] with HDA15 *in vitro* and *in vivo*. BiFC analysis revealed a primarily nuclear location of this protein-protein interaction, specifically in the nucleolus. We suggest that the interaction between XAL1 and HDA15 could be involved in regulating cell proliferation/differentiation similar to what is reported in animals for homologs of XAL1 and HDA15.

Materials and methods

Plant material and growth conditions

All experiments used wild type *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). Sterilized and scarified (3 days incubation at 4°C in darkness) seeds were grown on vertical plates with medium containing 0.2X MS (Murashige & Skoog) salt, 1% (w/v) sucrose, and 1% (w/v) agar in a controlled environment chamber in a 16 h light/8 h dark cycle with 22–24°C temperature, 45% relative humidity and illumination of 120 mmol m^2s^{-1}.

Gene expression analysis

Arabidopsis Col-0 roots on different days after sowing (3, 5, 8, 10) were collected and frozen immediately with liquid nitrogen to isolate RNA. Total RNA was extracted from 100 mg of roots by homogenizing the tissue in liquid nitrogen and using TRIzol reagent (Invitrogen™) according to the manufacturer's instructions.

From 1–2 micrograms of RNA, the first-strand cDNA was synthesized using the SuperScript™II kit (Invitrogen™) according to the manufacturer's specifications. Reversed transcription products were then used as the template for PCR reactions. The endpoint PCR analysis was performed using a thermocycler (2720 Thermal Cycler, Applied Biosystems). Reactions contained a master mix in a final volume of 20 μL with appropriated primers for *HDA5*, *HDA15*, *HDA18* and *XAL1* (Table S1). PCR conditions were 94°C for 3 min, followed by 24 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Each experiment included three independent biological replicates. Samples were normalized using the *At-TUB* expression.

Sequences sampling and amino acid sequences alignment

Amino acid sequences were retrieved from Genbank to represent a diversity of Class I and Class II *HDAC* genes in plants and animals, a total of 150 sequences (Table S2) were selected. Sequences were aligned in MAFFT 7[24] using the -auto option; the resulting alignment was visually inspected and corrected in Mesquite 3.6.[25]

Phylogenetic analysis

Support values were estimated in a maximum likelihood (ML) frame with a non-parametric bootstrap[26] in IQ-TREE.[27] For ML analyses, amino acids model selection was conducted with the ModelFinder strategy implemented in IQ-TREE[28]; once the model was selected, an ultra-fast bootstrap strategy with 1000 replicates was conducted.[29] A Bayesian inference tree search was conducted in MrBayes 3.2.6,[30] the amino acid substitutions model was selected with a reversible jump MCMC method,[31] rates were set to equal, MCMCMC chain ran 15,000,000 generations, sampling trees and parameters every 1,000 generations. The analysis was conducted in CIPRES online portal.[32]

Bioinformatics analysis: hydrophobic cluster analysis

Hydrophobic Cluster Analysis (HCA) analyzes patterns in protein primary sequence to find motifs based on the conservation of amino acid hydrophobic/hydrophilic properties rather than sequence conservation *per se*.[33] HCA has proven useful in the detection of distant homologs of domains with uncommon properties without prior information. To perform an HCA, we built a set of putative HDACs. The Human HDAC4 protein sequence[9] was used as a query in BLASTp against the NCBI nr protein database (all non-redundant GenBank CDS translations-PDB-Swiss Prot-PIR-PRF excluding environmental samples from WGS projects). Human HDAC4 was chosen because it presents a motif for interaction with MEF2. Subsequently, the sequences recovered during the BLASTp were used as a query to search all Arabidopsis HDACs using PSI-BLAST. Convergence was attained in the 50th PSI-BLAST round. The next step was to analyze the HCA with the HCA-Analyze program[34] to compare hydrophobic patterns and choose the proteins with a similar pattern to the one in human HDAC4. Additionally, 56 Arabidopsis MADS-domain protein sequences were searched and aligned using BLASTp, with a custom substitution matrix, as described by Silva,[34] in order to identify hydrophobic patterns similar to the one found in the HDAC4 interaction domain of MEF2B (Fig. S1). Finally, to gain further insights into the conservation patterns of hydrophobic clusters among selected HDACs and MADS-domain proteins, hydrophobic clusters of the following proteins were drawn and visually compared using the program HCADraw[35] (available on https://bioserv.impmc.jussieu.fr/hca-form.html at the time of analysis): Human HDACs, HDAC4 (UniProt entry P56524); Arabidopsis HDACs, HDA5 (UniProt entry Q8RX28), HDA15 (Q8GXJ1), HDA18 (Q8LRK8); Human MADS-domain proteins, MEF2B (UniProt entry Q02080); Arabidopsis MADS-domain proteins, XAANTAL1 (XAL1, UniProt entry Q38841), PISTILLATA (PI, UniProt entry P48007), AGL15 (UniProt entry Q38847), AGL19 (UniProt entry O82743), and AGL64 (UniProt entry Q7XJK9).

Protein-protein docking simulations

To gain further insight into the possible interactions between XAL1 and HDA15, docking simulations were performed. First, we obtained a predicted 3D structure for the XAL1 monomer from the AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/); specifically, we used AlphaFold's prediction for Uniprot protein Q38841 (prediction retrievable at https://alphafold.ebi.ac.uk/entry/Q38841). Then, we constructed a homo-oligomer for XAL1 using the GalaxyHomomer service from GALAXYweb (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=HOMOMER) without specifying the expected oligomeric state. All the five best *ab initio* oligomerization models were 2-mers, with oligomer docking scores ranging from 1563.96 to 1394.14; all further work was pursued with the single best scoring model. Docking simulations between XAL1 and HDA15 were done on the HDOCK server (http://hdock.phys.hust.edu.cn/,[36]) which carries out a hybrid algorithm of template-based and *ab initio* free docking. We used as input the predicted XAL1 dimer from the previous steps and PDB crystal structure 6J6T, which contains the histone deacetylase domain of HDA15, as a proxy for HDA15 (https://www.rcsb.org/structure/6J6T).[37] The XAL1 homodimer was designated as the receptor molecule, and the HDA15 homotetramer was the ligand molecule. The docking run was allowed to perform both free *ab initio* and template-guided dockings. As a positive control for the ability of HDOCK to detect *bona fide* interactions, we ran a docking simulation in which we tried to reproduce the union between the human MADS-domain transcription factor MEF2b and the MEF2-binding domain of murine histone deacetylase HDAC9, as reported in PDB file 1TQE. Namely, we used chains E and F from 1TQE (corresponding to a MEF2b dimer) as a receptor and chain G (corresponding to the MEF2-binding domain of HDAC9) as a ligand on an HDOCK docking simulation to try to obtain the same interaction surfaces as reported in 1TQE.

Y2H assays

For two-hybrid assays, the bait and prey clones used were cloned into the Gateway version of pGBKT7-GW (bait) and pGADT7-GW (prey) vectors using the recombination-based Gateway cloning system (Invitrogen™). Sets of constructs were co-transformed into Y2H Gold yeast strain (Clontech). The transformed yeasts were selected in a minimal synthetic medium without tryptophan and leucine. Protein interactions were evaluated in a triple dropout medium without histidine, tryptophan, and leucine or in a quadruple dropout medium without histidine, tryptophan, leucine, and adenine with X-α-Gal. At least three clones with three repetitions were analyzed, giving similar results.

Bimolecular fluorescence complementation assay

The *XAL1* and *HDA15* cDNA were cloned in pDONR201 (Invitrogen, now Life Technologies). All clones were recombined with pYFN43[38] to generate N-terminal fusions with the N-terminal part of YFP. XAL1 (pENTRY/D) was also recombined with pYFC43[38] to generate an N-terminal fusion with the C-terminal part of the YFP protein. The constructs were individually introduced in *Agrobacterium tumefaciens* GV2260 and cultured on Luria Bertani (LB) with 100-µg/ml kanamycin and 25µg/ml rifampicin. Overnight cultures of *Agrobacterium* (OD 1.2–1.6) were collected and re-suspended in a similar volume of infiltration medium [10 mM $MgCl_2$, 10 mM 2-(N-morpholine)-ethane sulfonic acid (MES), pH 5.6, 200 mM acetosyringone], the OD was adjusted to 1.0 and incubated at 25°C for three h with weak shaking. Before the co-infiltration, *Agrobacterium*

containing pYFC43-XAL1 was mixed with a similar volume of *Agrobacterium* with pYFN43-HDA15. This mixture was introduced in the abaxial air space of young leaves of *Nicotiana benthamiana* using a needleless syringe. YFP fluorescence was assayed 2–3 days after infiltration using an inverted LSM 510 META confocal laser-scanning microscope (Zeiss). YFP was excited using a 488-nm line of an argon laser, and emission was filtered using a BP 500–550 nm filter.

Results

Phylogenetic analysis of histone deacetylases class I and II

A broad phylogenetic analysis of representative HDAC sequences was performed using sequences of homolog proteins from bacteria, animals, and plants (Fig. S2). A Markov chain Monte Carlo search of the phylogenetic tree with the highest posterior probability was performed with the MrBayes program. After 15,000,000 generations the chains converged (the average standard deviation between chains was 0.01), and all parameters had an adequate sample size of 6,150 or above. To have a clear perspective of the topology and relationships between sequences, the maximum clade credibility tree was selected from the sampled trees using TreeAnnotator 2.6.[39] Class I and Class II HDAC sequences were recovered in reciprocally monophyletic groups (Fig. S2). Sequences from Arabidopsis HDA5 and HDA18 were recovered as a monophyletic group, followed by other plant HDA sequences, and as a sister group with a monophyletic group of animal HDAC sequences (Figure 1). The Arabidopsis HDA15 sequence was recovered in another clade, with a very similar inner topology. While all the plant sequences were recovered in a monophyletic group, animal sequences were in a paraphyletic topology (Figure 1). Although plant HDACs do not belong to the same clade as animal HDACs, they belong to a sister clade, suggesting functional conservation. Our data were consistent with those previously reported by Gregoretti et al.[6] where the catalytic domain of histone deacetylases was shown to be conserved between plants and animals.

Arabidopsis HDAC and MADS-domain proteins conserve a human interaction domain

To study the interactions of HDACs and MADS-domain proteins in Arabidopsis, bioinformatic tools were used to detect putative members of the animal class II HDACs. The class II (HDAC 5, 15, and 18 in Arabidopsis) was selected due to its high conservation and the interaction between HDACs class II and MEF2 in humans, as reported by Han et al.[9]; in fact, plant HDACs are similar to human class II HDACs in our phylogeny (Figure 1). Since the MEF2-binding domain is conserved in the animal class II HDAC (HDACs 4, 5, 7 and 9)[9], multiple alignments between animals and Arabidopsis class II HDACs were performed. However, the conservation of this domain was not evident at the primary sequence level (Fig. S3). Therefore, a Hydrophobic Cluster Analysis (HCA) was carried out. This methodology can detect domains with unusual characteristics following patterns of amino acids with similar biochemical characteristics in secondary structures of proteins[34]. Our results showed that Arabidopsis HDA5 and HDA18 conserve a similar hydrophobic pattern of the MEF2-binding domain compared to the human HDAC4 (yellow shaded area in Fig. S4a, c), while HDA15, does not conserve the hydrophobic pattern of MEF2-binding domain. The hydrophobic pattern between HDA5, HDA18 and HDAC4 is highly conserved, even though HDAC4 is much more divergent at the primary sequence level

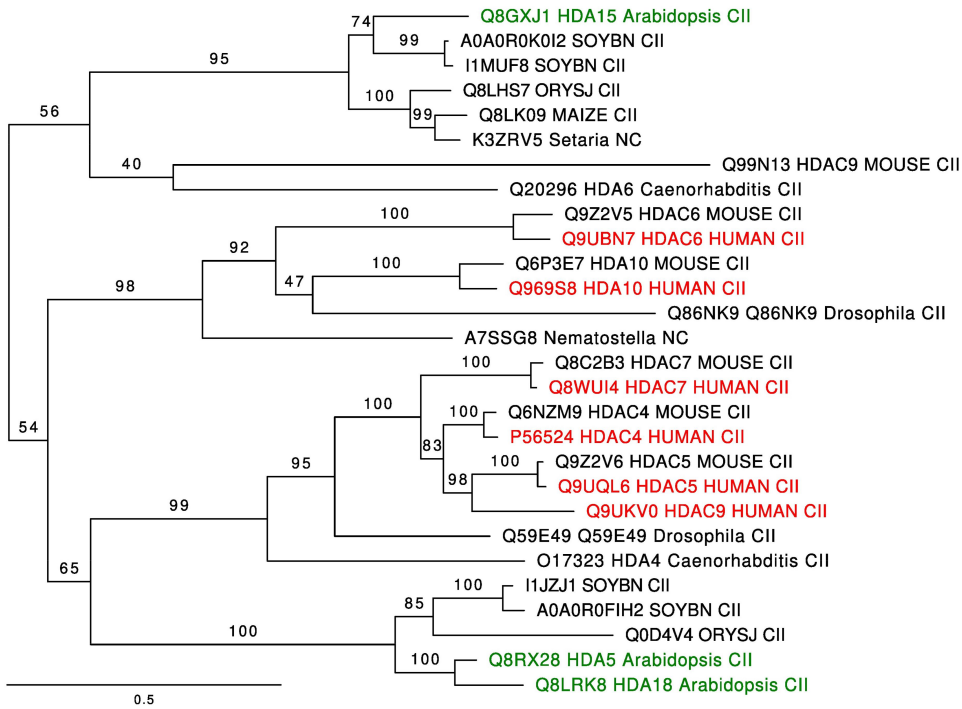


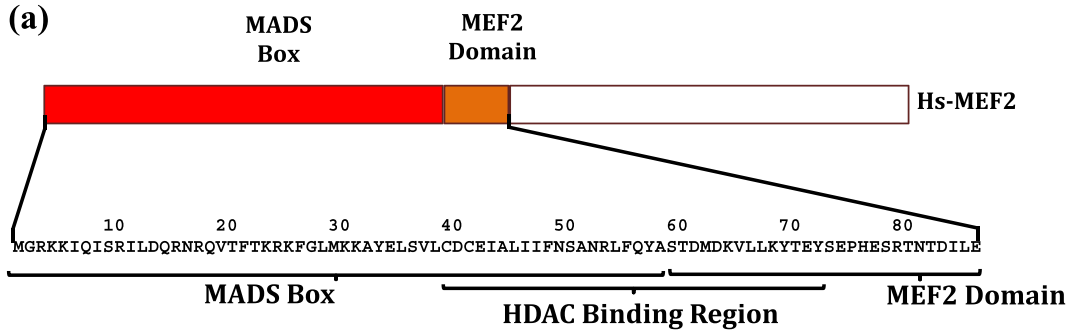
Figure 1. Phylogeny of class II histone deacetylase. HDA5 and HDA18 were recovered as a monophyletic group, and HDA15 in the sister clade. The sequences of animals were in a paraphyletic topology, but HDA15 of Arabidopsis and HDAC of humans belong to a sister clade suggesting functional conservation; in green HDAC class II of Arabidopsis and red HDAC class II of *Homo sapiens*. Soybean (SOYBN), *Oriza sativa japonica* (ORYSJ).

(Fig. S4b). Although HDA15 does not retain a similar hydrophobic pattern, its primary sequence shows some conserved amino acids with HDA5 and 18 (Fig. S4c), suggesting a possible conserved interaction between class II HDACs with MADS-domain proteins in Arabidopsis. To pursue this possibility, we explored whether the hydrophobicity pattern of the MEF2 domain is conserved in some Arabidopsis MADS-domain proteins. To this end, multiple alignments were first performed with MEF2-domain from MEF2B and MADS-domain of 40 Arabidopsis MADS-domain proteins (Figure 2a and Fig. S5). We selected some of them with a score > 30 to analyze the hydrophobicity pattern; however, neither AGL15, which contains the major similarity score (42.7), nor the other ones, including the MADS-box with a lower score (AGL64), showed a hydrophobic pattern similar to the one in the MEF2 domain (Fig. S5). Since Gaffe et al.[8] reported the binding site of the tomato MADS-domain proteins (LeMADS1, LeMADS5 and LeMADS6) involved in the interaction with HDAC5 in tomato; this domain was also analyzed in the Arabidopsis MADS-domain dataset, showing that XAL1 the MADS-domain protein with higher similarity (Figure 2b). Additionally, as XAL1 regulates root development by controlling cell proliferation of the root meristem,[23] could interact with HDACs in Arabidopsis.

Han et al.[9] reported an analysis that revealed the interaction between murine HDAC9 and human MEF2B. To further establishes a possible interaction between HDACs and XAL1 in Arabidopsis, we selected HDA15, which has a primary sequence homologous to HDA5, to make a docking simulation between a XAL1 homodimer and an HDA15 homotetramer.[41] The simulation yielded the ten most probable interaction modes, presenting confidence scores varying between 0.8612 and 0.8827, as well as docking energy score between −250.93 and −241.25, respectively (more negative values represent a more stable predicted interaction between proteins). In accordance with the suggestions from Yan et al. (2020)[36], we are including the ten best models produced by HDOCK docking energy score, as well as the best predicted model for the MEF2B/HDAC9 interface (Table 1). The structure of the first two models with confidence of 08827 and 0.8799 as well as its comparation with the docking analysis (0.8387) reported by Han et al.[9] can be seen in Table 1. These results showed that an interaction between HDA15 and XAL1 is possible.

The HDACs 15 and XAL1 genes are expressed in Arabidopsis roots

Considering the hypothesis of an interaction between three members of class II HDAC and XAL1, we investigated whether *HDA5*, *15* and *18* are expressed in the same tissues where *XAL1* is expressed. An analysis of microarrays data from the Genevestigator platform,[42] showed that *HDA15* is expressed in hypocotyl, root, cotyledon, and seed tissues, while *HDA18* is expressed in the same tissues but at lower levels in comparison with *HDA5* and *HDA15*. The high levels of *XAL1* transcript levels found in root and hypocotyl (Fig. S6), opens a possible interaction between *HDACs* and *XAL1* in hypocotyl and root tissues. In fact, previous reports indicated that XAL1 has an



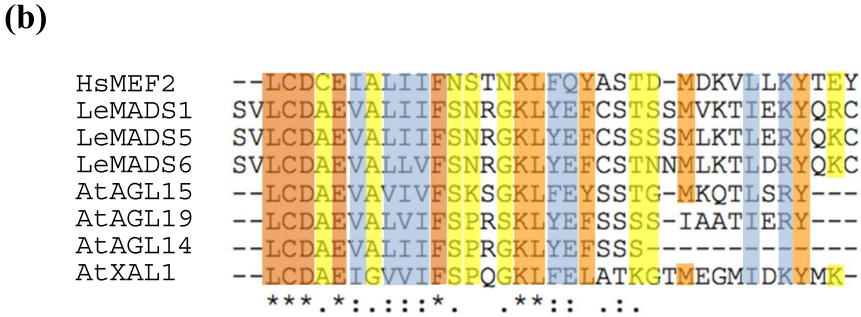
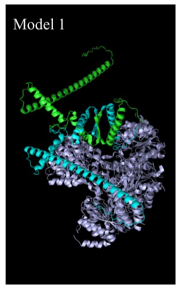
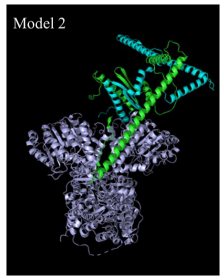
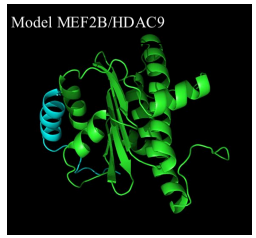
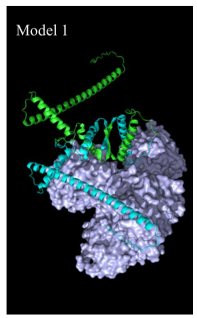
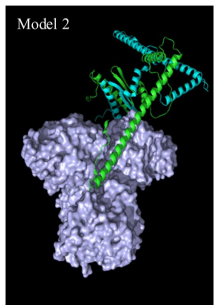
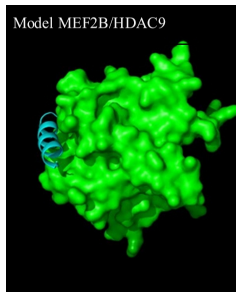


Figure 2. MADS-domain/MEF2 motif is conserved in some MADS-domain proteins of Arabidopsis. (a) Graphic representation of MEF2 protein, in red the MADS-domain and in orange the MEF2-domain. Below the model representation, there is the linear amino acid sequence indicating the MADS-domain, HDAC binding region and MEF2 domain (Figure was modified from Lu et al. 2000[40]). (b) Multiple alignments of the MEF2C motif sequence, tomato MADS-domain proteins, and some Arabidopsis MADS-domain proteins. In orange are identical amino acids, in yellow less conserved substitutions, and in blue conservative substitutions. *Homo sapiens* (Hs), *Lycopersicon esculentum* (Le), *Arabidopsis thaliana* (At).

Table 1. Interaction of XAL1 with HDA15 in Arabidopsis as predicted by HDOCK docking simulations. The table shows the top ten models that docking analysis generated and the reported interaction for humans of MEF2B with HDAC9. The two last rows show the images of the structure of the interactions. HDA15 is in light lilac, XAL1 is in helices green and aquamarine, and the last column is HDAC9 and MEF2B in aquamarine and green, respectively.

Organism	*Arabidopsis thaliana*										*Homo sapiens/Mus musculus*
Model rank	1	2	3	4	5	6	7	8	9	10	Model 1 from MEF2B/HDAC9
HDOCK docking energy score	−250.93	−249.58	−248.81	−248.53	−245.33	−245.20	−244.55	−242.01	−241.76	−241.25	−232.43
Confidence score	0.8827	0.8799	0.8783	0.8777	0.8706	0.8703	0.8689	0.8630	0.8624	0.8612	0.8387
Ligand RMSD (Å)	106.61	107.81	74.14	78.76	73.30	111.37	109.35	65.89	85.03	113.06	0.79
Struture (helices)											
Struture (helices-surface)											

essential function in root development,[21,23] therefore we compared the expression kinetics of *HDA5, 15* and *18* and *XAL1* during root development. *HDA5* is highly expressed from 5 to 10 days after sowing (DAS), in contrast to *HDA18*, which is only expressed at 10 DAS but at a lower level, whereas *HDA15* is expressed at 3, 5, and 10 DAS. *XAL1* has an increased expression from 5–10 DAS (Figure 3). Therefore, *HDA5* is expressed similarly to *XAL1* during root development. In contrast, *HDA15* and *HDA18* are expressed preferentially at 10 DAS. These results indicate that all three *HDACs* are co-expressed with *XAL1* at a certain root development stage.

HDA15 interacts with XAL1

Given the prediction of interaction between HDA15 and XAL1 and the co-expression at some development stage, we performed the yeast two-hybrid assay to evaluate this interaction. Our results showed that HDA15 can bind to XAL1 (Figure 4a) since the yeast strain carrying the BD-HDA15/AD-XAL1 plasmid grew in a restrictive medium (SD-Leucine-Tryptophan-Histidine and SD-Leucine-Tryptophan-Histidine with 3-AT). The interaction was not observed when XAL1 was cloned in the BD and HDA15 in the AD plasmid (Figure 4b), indicating that although XAL1 is a transcriptional factor, it has no transactivation activity in this system. Besides, when different concentrations of 3AT were used (0.25–1.5 mM), it was observed an inhibition of colony growth, in a concentration dependent manner. When dilutions were made, growth reduced significantly, as expected (Figure 4c). These results were confirmed by Lac-Z activity (Figure 4d). In addition, the negative interactions found between HDA15 and other MADS-box genes (BD-HDA15/AD-AGL11 and BD-HDA15/AD-AGL77) (Fig. S7) confirmed that the interaction of HAD15 with XAL1 is not due to an artifact of HDA15 self-activation. Together these data showed an interaction between HDA15 and XAL1 in this heterologous system.

Nucleolar localization of the HAD15-XAL1 interaction

The biomolecular fluorescence complementation (BiFC) approach was used to verify the HAD15-XAL1 interaction *in planta*. HDA15 and XAL1 were fused to either the N-terminal or C-terminal portion YFP in the pYFN43 and pYFC43 vector which were introduced to *Agrobacterium tumefaciens* to perform transient expression in *Nicotiana benthamiana* leaves. The interaction of HDA15 with XAL1 was observed in the nucleus, confirming the interaction *in planta* (Figure 5a). Interestingly, the observed fluorescence intensity was strongly confined in an area within the nucleus, possibly the nucleolus (Figure 5c). For the BiFC assay, the interaction between PISTLLATA (PI) and APETALA (AP3) was used as a positive control, which co-localize, giving a fluorescent signal observed with confocal microscopy (Figure 5b,d). Otherwise, β subunit of sucrose non-fermenting-1-related protein kinase 1 (AKINβ), a subunit of the SnRK1 kinase, with XAL1 and YFN (empty vector) with XAL1 did not show fluorescence signal. As a negative control we used the interaction between the SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) with AGAMOUS (AG), as these two proteins do not interact[38,43,44] (Fig. S8). All these results confirm an interaction of HDA15 with XAL1, however future work will be necessary to determine the function of this specific interaction during the plant development.

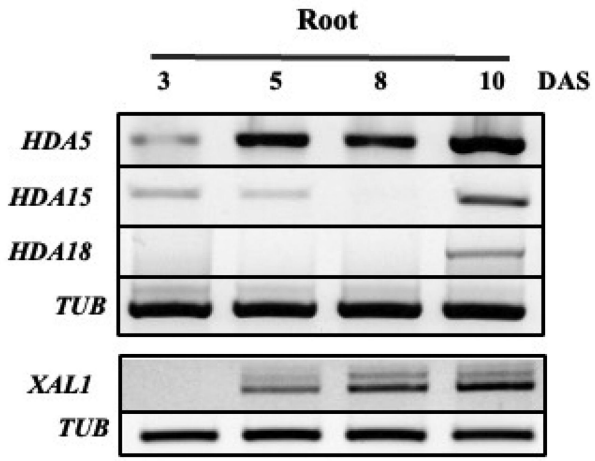


Figure 3. Expression of MADS and HDACs in root of *Arabidopsis thaliana*. *XAL1* expression HDACs and different times in the development of the root from Arabidopsis (PCR endpoint); days after sowing (DAS), tubulins (*TUB*).

Discussion

HDACs are crucial players in all aspects of plant development, including embryogenesis, abaxial/adaxial polarity determination, flowering, senescence, responses to day length, and environmental stresses.[5] However, most studies have been performed in Arabidopsis, and relatively few HDACs have been characterized in other plant species.[1,45] Still, there is not enough information about the functional redundancy of HDACs. However, it has been shown that HDA19 and HDA6 participate in germination, embryonic development, and salt resistance with a partially redundant function.[1] Considering that the specificity in regulating other genes may be related to the interaction of HDACs with transcription factors, it is essential to know what these interactions are, the cell types where they occur, and the processes in which they participate. In this work, a bioinformatic and experimental analysis was carried out to identify the interaction between HDA15 and XAL1.

The tools used here to analyze the protein structure similarities in a native condition allowed us to detect conserved domains not found by conventional primary sequence conservation analysis. The hydrophobic cluster analysis graphs allowed us to identify a common pattern between Arabidopsis class II HDAC protein sequences since the primary sequence of the HDAs did not present an apparent conserved domain. Although this methodology does not perfectly predict an exact structure of the protein-protein interaction, it allowed us to find structures with characteristics similar to those of human HDAC and MADS-domain proteins, namely MEF2B and class II HDACs[9]. Thus, we identified a putative binding site within the MADS-domain with the consensus sequence, which may participate in the interaction with class II HDAC proteins. This putative MADS-box/MEF2S interaction site was studied by comparative analysis with a domain reported in the MADS-domain protein from

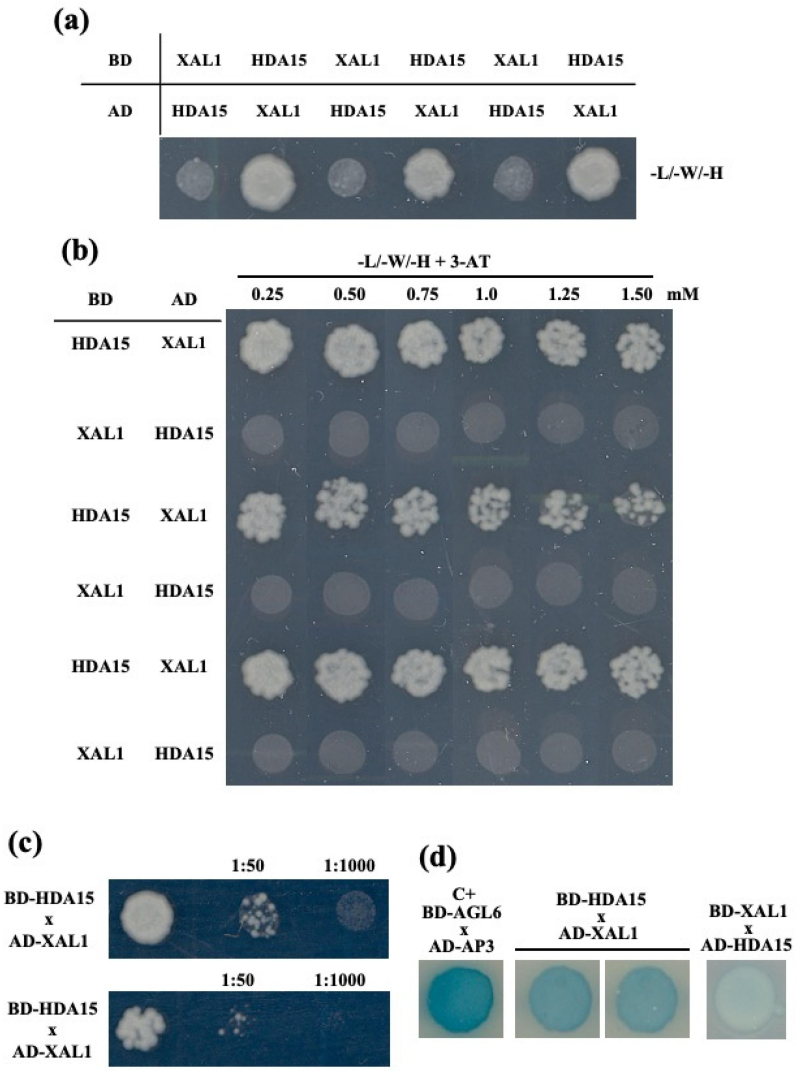


Figure 4. Protein-protein interaction between HDA15 and XAL1. A two-hybrid assay indicates an interaction between HDAC and XAL1 in Arabidopsis. (a) SD/-L/-W/-H correspond to dropout medium lacking L, W, H and positive interactions, BD HDA15 and AD XAL1, resulting in yeast growth on the SD/-L/-W/-H plate. (b) HDA15 and XAL1 interaction with different concentrations of 3-AT (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mM). (c) HDA15 interaction with XAL1 at different dilutions (1:50 and 1: 1000). (d) Lac-Z expression in interaction HDA15 and XAL1. AGAMOUS LIKE 6 (AGL6) and APETALA 3 (AP3) was used as a positive control.

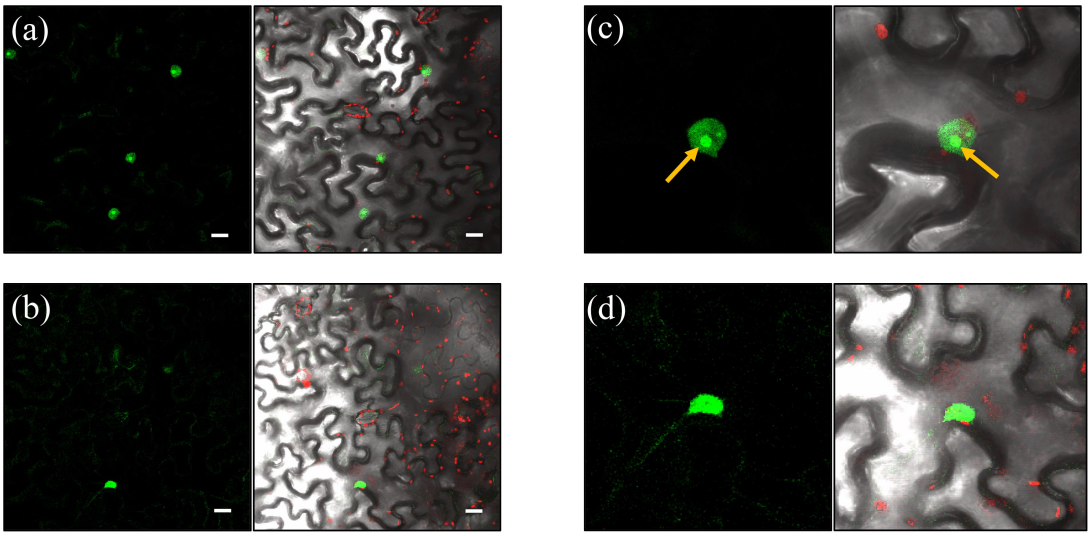


Figure 5. Subcellular localization of the interaction of HDA15 and XAL1. Bimolecular fluorescence complementation assay in tobacco leaf cell nuclei between transiently expressed HDA15 and XAL1. (a) HDA15-XAL1 interaction in the nucleus (YFC-XAL1 + YFN-HDA15). (b) Positive control, PISTILLATA interaction with APETALA3 (YFC-PI + YFN-AP3). In c and d is a zoom of a and b, respectively. In c, the yellow arrow points to the possible nucleolus. The GFP spectrum is shown in the left column panels. Merged visible and fluorescent signals are shown in the right column panel. Scale bar: 20 μm.

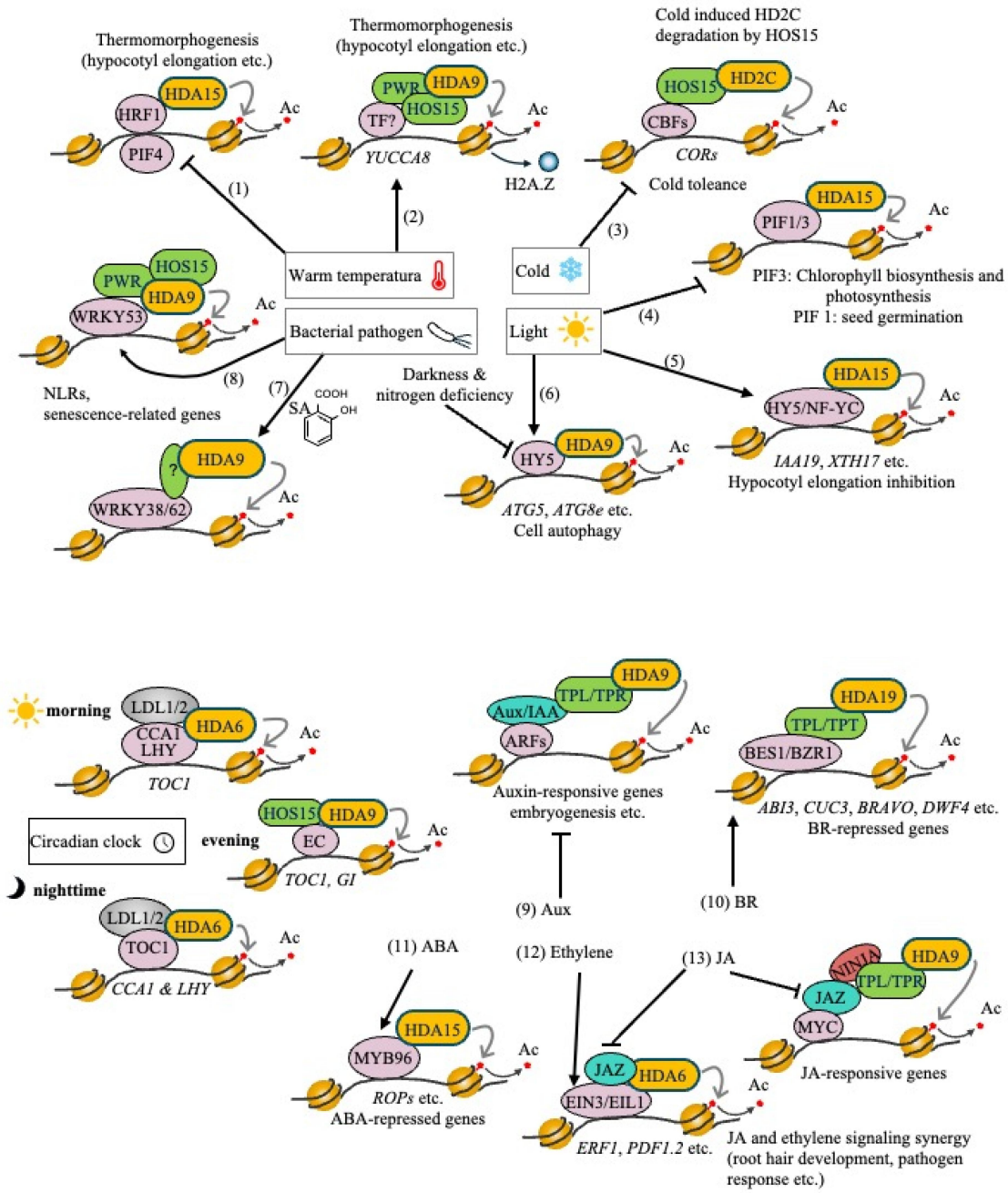


Figure 6. Summary of principal function of histone deacetylation in plants. 1, repression thermal-responsive genes and warm temperatures induce PIF4 inhibiting HDA15/HFR1 interaction. 2, hypoacetylation at the *YUCCA8* by HDA9/PWR/HOS15 complex in warm temperatures. 3, HOS15/CBFs/HD2C complex repress COR genes at normal temperature; the cold induces HD2C degradation. 4, HDA15, PIF1, and PIF3 are associated in dark conditions, deacetylating histones (3 and 4) to repress chlorophyll biosynthetic/photosynthetic and seed germination genes. In light, HDA15 is released to degradation of PIF1 and PIF3. 5, in light, the HDA15-NFYCs-HY5 complex inhibits the expression of *IAA19* and *XTH17* genes, which are related to auxin biosynthetic, signaling, and cell wall organization. 6, in light, the HY5/HDA9 interaction inhibits *ATG5* and *ATG8e* (cell autophagy genes). In Darkness and nitrogen deficiency, these genes are released for the degradation of HY5-inducing autophagy. In bacterial infection: 7, SA induction occurs, *WRKY38/62* is expressed, and WRKY38/62 recruits HDA19 to elicit a basal defense response.; 8, the expression of WRKY53 is induced, recruiting the HDA9/PWR/HOS15 complex. This complex suppresses NLR genes and leaf senescence genes. Hormone signaling: 9, HDA19 and Aux/IAA – TPL/TPR induced deacetylation, and auxin-responsive genes were repressed without Aux. In the presence of Aux, the Aux/IAA protein is degraded, and ARFs are released; 10, BR stimulates BES1 and BZR1 activity to recruit TPL/TPR/HDA19 complex and repress BR-repressed genes; 11, High concentrations of ABA stimulate the MYB96 - HDA15 interaction, regulating ABA-repressed genes for hyperacetylation of histones (3 and 4); 12, JAZ and HDA6 repress transcription factors EIN3/EIL1, and ethylene stabilizes EIN3/EIL1. JAZ is degraded for JA to express ethylene-responsive genes; 13, the interaction of JAZ and NINJA with TPL/TPR recruits HDA19 and HDA6 for transcriptional repression in the absence of JA. Circadian clock: 14 morning, HDA6/LDL1–2/CCA1/LHY complex repress *TOC1*; 14 evening, Evening Complex (EC), HDA9 and HOS15 repress *TOC1* and *GI* expression; 14 nighttime, HDA6, LDL1/2, TOC1 repress the expression of *CCA1/LHY*. *ABA INSENSITIVE3* (*ABI3*). AUTOPHAGY-RELATED GENES (*ATG5/8*). *BRASSINOSTEROIDS at VASCULAR and ORGANIZING CENTER* (*BRAVO*). *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*). *COLD-RESPONSIVE* (*COR*). *CUP SHAPED COTYLEDON3* (*CUC3*). *DWARF4* (*DWF4*). *ETHYLENE RESPONSE FACTOR1* (*ERF1*). INDOLE-3-ACETIC ACID INDUCIBLE 19 (*IAA19*). *LATE ELONGATED HYPOCOTYL* (LHY). *PLANT DEFENSIN 1.2* (*PDF1.2*). *RHO of PLANTS* (*ROP*). *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 17* (*XTH17*). *TIMING of CAB EXPRESSION 1* (*TOC1*). Auxin (Aux). Abscisic Acid (ABA). Salicylic Acid (SA). AUXIN RESPONSE FACTOR (ARFs). Brassinosteroid (BR). BRI1-EMS-SUPPRESSOR1 (BES1). BRASSINAZOLE-RESISTANT1 (BZR1). C-REPEAT BINDING FACTORSs (CBFs). *CIRCADIAN CLOCK-ASSOCIATED1* (CCA1). ELONGATED HYPOCOTYL5 (HY5). ETHYLENE INSENSITIVE 3 (EIN3). ETHYLENE INSENSITIVE 3-LIKE 1 (EIL1). HIGH EXPRESSION of OSMOTICALLY RESPONSIVE GENES15 (HOS15). HD2-type HDACs (HD2C). VARIANT HISTONE 2A (H2A.Z). INDOLE-3-ACETIC ACID (IAA). JASMONATE ZIM DOMAIN (JAZ). Jasmonic Acid (JA). LONG HYPOCOTYL in FAR-RED1 (HRF1). LSD1-LIKE1/2 (LDL1/2). LYSINE-SPECIFIC DEMETHYLASE1 (LSD). *LATE ELONGATED HYPOCOTYL* (LHY). TRANSCRIPTION FACTOR MYB 96 (MYB96). TRANSCRIPTION FACTOR MYC (MYC). NOVEL INTERACTOR of JAZ (NINJA). NUCLEAR FACTOR-YC HOMOLOGS (NF-YCs). PHYTOCROME-INTERACTING FACTOR (PIF). POWERDRESS (PWR). TRANSCRIPTION FACTOR (TF). TOPLESS (TPL). TOPLESS- RELATED (TPR). *TIMING of CAB EXPRESSION* (TOC). TRANSCRIPTION FACTOR WRKY (WRKY). Figure was modified [53, 54].

tomato.[8] From those analyses, we found that XAL1 contains this conserved domain and is able to interact with HDA15. Although we do not know if this domain is responsible for the interaction with HDA15, this interaction may be relevant to the function of XAL1, regulating cell proliferation in the root meristem.[23] Future studies generating double mutant of *xal1-hda15* could elucidate the possible role of the interaction between XAL1 and HDA15.

Protein-protein docking is a bioinformatics tool that can be useful in exploring interactions that are difficult to determine experimentally.[46] Many works have used docking simulation to model interactions, mainly between proteins and drugs or small molecules. This is the case of HDAC1 and HDAC2, interacting with flavones that regulate the activity of HDACs.[47] Similarly, docking has been used as a research tool to explore protein-protein interactions with significant results[48] and a steady ongoing improvement of methods and results.[49] In this study we performed a protein-protein docking analysis, and we presented ten models with different conformations in the interaction. The HDOCK predictor provides models of the possible interactions between two proteins by adjusting the structure and testing shape complementarity and bonding energy between the amino acids that could participate in the interface between the two proteins (Tables S3 and S4); this adjustment causes proteins in each model to have different conformations and occasionally to make it appear that the predicted interactions are very different from model to model. Also, these differences arise because the analysis predicts many conformations if no restrictions are given. The ten models with confidence and docking energy scores similar to MEF2B/HDAC9 indicate that HDA15 and XAL1 could interact. Moreover, we found that there is substantial overlap in the interface regions among the different models (Tables S3 and S4). Thus, the results from docking simulations are consistent with the rest of the bioinformatics approaches that indicated a putative interaction between XAL1 and HDA15 and congruent with both molecular assays we performed.

Perspectives

The HDA15-XAL1 interaction was demonstrated by yeast two hybrid and by BiFC assays, indicating that this interaction occurs *in planta*. Moreover, HDA15 is localized in the nucleus,[50] and we found that HDA15-XAL1 interaction is in the nucleus too, suggesting that HDA15-XAL1 may have a role in deacetylating histone or non-histone proteins. Future work could be carried out to elucidate the function of this interaction during plant development. In animals, it has been found that MEF2 genes participate in the balance between proliferation and differentiation in *in vitro* cell cultures. While in plants, MADS-box genes have been attributed to different functions, some of them related to the determination of cell type or identity of organs, and others in diverse developmental processes from embryogenesis to floral development[51] and they have been implicated in the networks that underlie the balance between cellular proliferation and differentiation.[21,52] Our data revealed that *HDA15* and *XAL1* are expressed during root development, supporting a possible interaction between HDA15-XAL1. *XAL1* is involved in the regulation of several cell-cycle genes such as *CYCLIN D3;1* (*CYCD3;1*), *CYCLIN A2;3* (*CYCA2;3*), *CYCLIN B1;1* (*CYCB1;1*), *CYCLIN-DEPENDENT KINASE* (*CDKB1;1*) and *CHROMATIN LICENSING AND DNA REPLICATION FACTOR 1* (*CDT1a*),[21] therefore it will be important to study if HDA15 contributes to *XAL1* function in cell-cycle gene regulation. Besides, HDACs are also involved in several biological processes in plants (Figure 6) and can coincide spatially with MADS. Likewise, it has been reported that HDACs, specifically class II, interact with MEF2 in animals. Assuming all the contexts of HDAC15 and XAL1, we could explore the function of the HDA15 with XAL1 interaction in other biological processes of Arabidopsis.

In docking, it is advantageous to use or predict hot spots (small sets of residues that contribute significantly to protein-protein interaction formation), as mentioned by Tsuchiya et al.,[55] to increase the accuracy and reliability of docking predictions. In the future, we will analyze interaction hot spots to provide more grounding to the potential interactions between HDA15 and XAL1.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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