Functional Analyses of Plant-specific Histone Deacetylases

**Their Role in Root Development, Stress
Responses and Symbiotic Interactions**

Huchen Li

PROPOSITIONS

Plant-specific histone deacetylases regulate the switch from cell division to expansion during Arabidopsis root development.

(this thesis)

Environmental stimuli determine the subcellular localization of plant-specific histone deacetylases.

(this thesis)

The conclusion that hypomethylation of the *Karma* locus underlies the mantled phenotype in oil palm fruit requires genetic evidence.

(Ong-Abdullah et al., 2015. Nature 525, 533-537)

Science should not be driven only by our needs of today.

The increase of communication options has led to reduced connections.

The time of pursuing a PhD career abroad compromises that of maintaining domestic partnership.

Propositions belonging to the thesis, entitled

“Functional Analyses of Plant-specific Histone Deacetylases: Their Role in Root Development, Stress Responses and Symbiotic Interactions”

Huchen Li

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Functional Analyses of Plant-specific Histone Deacetylases

Their Role in Root Development, Stress
Responses and Symbiotic Interactions

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Functional Analyses of Plant-specific Histone Deacetylases

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Huchen Li

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OUTLINE

Plants being sessile organisms experience a constantly changing environment. They have to adapt their growth and development in order to cope with multiple environmental stimuli. Some stresses, either biotic such as pathogenic bacteria and fungi, or abiotic for instance salinity or nutrient deprivation, in general result in transient reduction of growth, so plants can retain energy to acquire resistance. Roots of some land plants could also develop beneficial interactions with soil microorganisms like arbuscular mycorrhiza or rhizobia. During these interactions, new structures can be formed on plant roots. These are arbuscule containing root cortical cells in the case of symbiotic interaction with arbuscular mycorrhiza and root nodules in the case of the legume rhizobium symbiosis.

The ability of plants to cope with (a)biotic environmental stresses relies on flexible mechanisms for reprogramming gene expression. Transcriptional factors (TFs) and chromatin remodelling factors play important roles in reprogramming genes expression in response to environmental stimuli, as well as during developmental transitions. The role of TFs in this reprogramming is intensively studied. However the knowledge on chromatin remodelling factors is elusive. The objective of this thesis is to investigate the role of plant-specific histone deacetylases (HDTs) in adaptation of plant development to biotic and abiotic factors.

In **Chapter 1** an overview of chromatin modifications mediated plant response to environmental factors, as well as regulated cell fate transitions are presented. *Arabidopsis thaliana* (Arabidopsis) roots and *Medicago truncatula* (Medicago) root nodules are model systems to study cell fate transitions, therefore the current knowledge concerning chromatin remodelling factors controlled cell fate switches during these two organs development is introduced.

In **Chapter 2** we studied how in Arabidopsis roots the switch from cell division to expansion is controlled by chromatin modification. We characterize two paralogs encoding plant-specific histone deacetylases, AtHDT1 and AtHDT2, involved in the regulation of this switch. The regulation framework of AtHDT1/2 is investigated by transcriptome analyses and ChIP. The results show that AtHDT1/2 repress *C₁₉-gibberellin 2-oxidase 2 (AtGA2ox2)* expression and in this way regulate the switch from cell division to expansion. Most likely AtHDT1/2 are involved in fine tuning gibberellin level in root tips.

In **Chapter 3** we explored whether the AtHDT1/2 play a role in halotropism, that is a response of plant roots to avoid a saline environment. We show that *AtHDT1/2* knock-down mutants display a more severe halotropic response. Interestingly, during halotropism AtHDT1/2 levels are reduced, whereas expression of one of their targets, *AtGA2ox2*, is induced at the high salt side. Constitutive expression of *AtHDT2*, as well as exogenous application of GA reduces halotropic response. We discuss that an asymmetric distribution of AtHDT1/2 that is formed during halotropism most likely decreases the GA concentration at the high salt side and through this way is involved in halotropism.

AtHDT1/2 proteins are nuclei localized but more abundant in nucleolus than in nucleoplasm. In spite of this they regulate the transcription of a plethora of genes in nucleoplasm. In **Chapter 4** we studied a mechanism that regulates the shuttle of HDT proteins between nucleolus and nucleoplasm under biotic stress. Previously Heribert Hirt's laboratory (Institute of Plant Sciences, Evry, France) discovered that mitogen-activated protein kinases (MPKs) can phosphorylate many proteins including AtHDT2. We collaborated with this group to study whether the phosphorylation status of AtHDT2 determines its sub-nuclear localization. We show that AtHDT2 directly interacts with AtMPK3 and is phosphorylated by it. Upon flagellin22 treatment that activates MPK signalling pathway, translocation of AtHDT2 from nucleolus to nucleoplasm occurs, leading to transcriptional reprogramming of defense genes. This provides a protein synthesis independent mechanism by which AtHDT2 becomes available for gene regulation upon biotic stress.

During symbiotic interactions, Medicago can form nitrogen fixing root nodules with rhizobia and develop cortical cells with arbuscules. In **Chapter 5** and **Chapter 6**, we explored the function of HDTs in controlling these two symbiotic interactions.

In **Chapter 5**, we studied the role of all 3 Medicago *HDT* genes (*MtHDTs*) in nodule development. Functional analyses of the *MtHDT* genes using a RNA interference approach show that they are functionally redundant in regulating nodule development. Knock-down of *MtHDTs* blocks the development of nodule primordia. The number of formed nodules is very low and in these nodules meristem is mal-functional and rhizobial infection is hampered. Transcriptome analyses show that MtHDTs are involved in controlling the expression of *3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (MtHMGR)* genes. This

includes as well *MtHMGR1* whose encoded protein is known to interact with MtDMI2, a receptor like kinase which is a component of the common signalling pathway for nodule symbiosis. It has been shown that MtHMGR1 is required to initiate Ca^{2+} spiking and symbiotic gene expression in *Medicago* roots in response to rhizobia. These data suggest that MtHDTs are involved in regulating *MtHMGR1* expression to control nodule development.

The role of MtHDTs during arbuscules development was studied in **Chapter 6**. We show that in mycorrhiza colonized root segments, *MtHDT2* is expressed whereas *MtHDT1* and *MtHDT3* are not. *MtHDT2* is especially activated in arbuscule containing cells and in these cells MtHDT2 displays variable sub-nuclear localization patterns. Knock-down of MtHDT2 significantly reduces arbuscule formation in mycorrhized root segments, and possibly affects AM symbiosis maintenance.

In **Chapter 7**, I discussed the data obtained in this thesis, integrated them with published data and outlined perspectives for future research.

CHAPTER 1

General Introduction

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Unlike animals, plants are sessile organisms that cannot escape from their environment. Therefore they evolved sophisticated mechanisms to adapt their growth and development in response to environmental cues, including (a) biotic stresses (Boyer, 1982; Grativol et al., 2012). These stresses reprogram gene expression in specific tissues or cells, along with alterations in enzymatic activity, metabolism and cell cycle progression (Cutler et al., 2010). Transcription factors (TFs) and chromatin remodelling factors contribute to these alterations by regulating expression of genes. Over recent years the stress-responsive TF networks have been intensively studied (Jisha et al., 2015; Hu et al., 2016; Song et al., 2016; Zhu, 2016). In most stress responses, the transcriptional reprogramming is also accompanied by changes in chromatin structure (Mirouze and Paszkowski, 2011; Luo et al., 2012a; Asensi-Fabado et al., 2017; Jiang and Berger, 2017). Here I describe mechanisms that contribute to the regulation of gene expression by altering chromatin structure and discuss how these mechanisms are involved in stress responses, developmental transitions and symbiotic interactions.

Chromatin and Transcription

Eukaryotic genomic DNA is packed together with histone proteins in chromatin. The fundamental repeating unit of chromatin is the nucleosome, which is constituted of 147 base pairs of DNA wrapped around an octamer of histones (Figure 1A). A standard histone octamer consists of two pairs of each core histone proteins H2A, H2B, H3 and H4. These core particles are connected by 20 base pairs of linker DNA. One additional H1 protein is associated with linker DNA (Venkatesh and Workman, 2015). A long chain of nucleosomes constitutes chromatin and its additional level of condensation determines whether a gene is accessible for TFs and the basal transcriptional machinery (Ernst and Kellis, 2012; Liu et al., 2015). Chromatin functions as an extra strata of gene regulation above DNA sequences recognized by TFs and in some cases modifications of chromatin structure can be inherited to the next generations. Therefore their modifications have been named as epigenetic mechanisms (Cosgrove and Wolberger, 2005).

The formation of a specific chromatin structure can be mediated by four type of modifications: (1) DNA methylation that occurs on cytosine (C) in CG, CHG and CHH (H represents A, C, T) and in general leads to gene repression. It is catalyzed by DNA methyltransferases and demethylases (Teixeira and Colot,

Figure 1. Structure, Assembly and Modifications of the Nucleosome Unit.

(A) Two histones H3-H4 heterodimers form a tetramer and two H2A-H2B dimers associate with H4 to form the protein scaffold, which is wrapped by 147 base pairs of nucleotides, forming a nucleosome unit.

(B) Amino acid residues in N-terminal tails of H2A, H2B, H3 and H4, representing potential post-translational modification (PTM) sites. N, amino terminus; C, carboxyl terminus; K, lysine; S, serine; E, glutamic acid; A, acetylation; M, methylation and P, phosphorylation. Modified from (Marks et al., 2001; Venkatesh and Workman, 2015).

2009; Matzke and Mosher, 2014; Bewick et al., 2017; Bewick and Schmitz, 2017); (2) Repositioning (loop, twist or slide) of nucleosomes along the DNA which is controlled by ATP-dependent chromatin remodelling factors (Saha et al., 2006b, a; Henikoff, 2008; Clapier et al., 2017); (3) Histone variants, that exchange with regular histones and result in an active or silent chromatin (Kamakaka and Biggins, 2005; Stroud et al., 2012; Yelagandula et al., 2014;

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1 Zhang et al., 2017) and (4) Post-translational modifications (PTMs) of residues in the N-terminal histone tails, such as acetylation, methylation, phosphorylation and others (Figure 1B) (Strahl and Allis, 2000; Kouzarides, 2007; Xiao et al., 2016). Acetylation of the lysine residues at the N-terminus of histone proteins is one of the major post-translation modification of proteins controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs add the acetyl group to histones and in general this causes an “open” chromatin preferentially associated with active genes. HDACs remove acetyl group from histones and are responsible for a “closed” chromatin configuration and inactivation of genes (Marks et al., 2001; Shahbazian and Grunstein, 2007). Histone methyltransferases catalyze the transfer of 1 to 3 methyl groups to the H3 or H4 histones. Their activities are counteracted by the histone demethylases (Liu et al., 2010; Du et al., 2015). Depending on position of the methyl groups, methylated histones can either contribute to activation (such as H3K4me2/3) or repression (such as H3K27me2/3 and H4K20me3) of genes (Sanchez and Gutierrez, 2009; Le Masson et al., 2012; Gan et al., 2015). Phosphorylation of histone H3 serine 10/28 leads to chromosome condensation and repression of gene expressions (Ozawa, 2008; Sawicka and Seiser, 2012). These post-translational modifications of histones constitute the so-called ‘histone code’ (Jenuwein and Allis, 2001). Together with the other three types of modifications, they affect chromatin status and determine the expression level of genes.

Environmental Stresses and Chromatin Structure

Upon perceiving environmental cues, some plant cells change their transcriptional program. Alterations in chromatin structure are a prerequisite for this, or a consequence of this transcriptional reprogramming.

DNA Methylation Affects Stress Responses

A number of studies have shown that different environmental stresses alter the methylation status of DNA. This depends on the type of stresses as well as plant species involved (Table 1) (Peng and Zhang, 2009). For example, cold stress induces the expression of a cold-responsive gene *ZmMI1* in maize, and this induction correlates with a hypomethylation of the gene (Steward et al., 2002). In tobacco leaves cold stress also causes demethylation in the coding sequence of a glycerophosphodiesterase-like gene (*NtGDPL*). The induced *NtGDPL* demethylation correlates with its higher expression level, this also

Table 1. Studies that have analyzed the function of chromatin remodelling factors in response to stresses. The stresses applied, the specific chromatin modification monitored, the species and genotype and the regulated genes are indicated.

Stress	Chromatin modification type	Species, genotype	Genes regulated	Reference
Cold	DNA methylation	Maize	<i>ZmMI1</i>	(Steward et al., 2002)
Cold, salt, aluminium and oxidative	DNA methylation	Tabacco	<i>NtGDPL</i>	(Choi and Sano, 2007)
Drought	DNA methylation	Pea		(Labra et al., 2002)
Salt	DNA methylation	Arabidopsis, <i>met1</i>	<i>HKT1</i>	(Baek et al., 2011)
Virus infection	DNA methylation	Tabacco		(Kovalchuk et al., 2003)
Avirulent bacteria	DNA methylation	Arabidopsis, <i>drm1/drm2/drm3</i>		(Zhang, 2012)
<i>Fusarium oxysporum</i>	DNA methylation	Arabidopsis, <i>ros1/dml2/dml3</i>		(Le et al., 2014)
SA	nucleosomal reposition	Arabidopsis	<i>PR1</i>	(Duque, 2016)
Drought	nucleosomal reposition	Arabidopsis, <i>brm</i>	<i>ABI5</i>	(Han et al., 2012)
Heat	nucleosomal reposition	Arabidopsis, <i>brm</i>		(Brzezinka et al., 2016)
JA, Ethylene, <i>Pseudomonas syringae</i>	nucleosomal reposition	Arabidopsis, <i>syd</i>		(Walley et al., 2008)
Osmotic stress	nucleosomal reposition	Arabidopsis, <i>pkl</i>		(Perruc et al., 2007; Han and Wagner, 2014)
Drought, salt and heat	nucleosomal reposition	Arabidopsis, <i>MINU1^{OE}</i> , <i>CHR12^{OE}</i> , <i>CHR23^{OE}</i>		(Mlynarova et al., 2007; Leeggangers et al., 2015)
Drought, ABA	histone variant	Arabidopsis, <i>His1-3</i>	<i>RAB18</i>	(Ascenzi and Gantt, 1997, 1999)

(continued on next page)

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Table 1 (continued)

Stress	Chromatin modification type	Species, Genotype	Genes regulated	Reference
Dehydration, low light intensity	histone variant	Arabidopsis, <i>h1.3</i>		(Rutowicz et al., 2015)
Heat, <i>Pseudomonas syringae</i>	histone variant	Arabidopsis, <i>hta9/11</i>	<i>HSP70</i>	(March-Diaz et al., 2008; Kumar and Wigge, 2010)
Salt, drought	histone acetylation	Arabidopsis, <i>hda9</i>		(Zheng et al., 2016)
Cold	histone acetylation	Maize	<i>ZmDREB1</i> , <i>ZmCOR413</i>	(Hu et al., 2011)
Cold	histone acetylation	Rice	<i>OsDREB1</i>	(Roy et al., 2014)
Drought	histone acetylation	Rice		(Fang et al., 2014)
Wounding, <i>Alternaria brassicicola</i>	histone acetylation	Arabidopsis, <i>HDA19^{OE}</i> , <i>HDA19-RNAi</i>		(Zhou et al., 2005)
<i>Magnaporthe oryzae</i> , <i>Xanthomonas oryzae pv oryzae</i>	histone acetylation	Rice, <i>OsHDT701^{OE}</i> , <i>OsHDT701-RNAi</i>		(Ding et al., 2012a)
Submergence	histone acetylation/methylation	Rice	<i>OsADH1</i> , <i>OsPDC1</i>	(Tsuji et al., 2006)
Salt, heat, cold and <i>Pseudomonas syringae</i>	histone acetylation/methylation	Arabidopsis, <i>hac1-1</i>	<i>WRKY53</i> , <i>FRK1</i> , <i>NHL10</i>	(Singh et al., 2014)
Drought	histone acetylation/methylation	Arabidopsis	<i>RD29A/B</i> , <i>RD20</i> , <i>RAP2.4</i>	(Kim et al., 2008; Kim et al., 2012)
Salt	histone methylation	Arabidopsis, <i>JMJ15</i>	<i>RD29A/B</i> , <i>COR15A</i> , <i>P5CS1/2</i>	(Shen et al., 2014)
<i>Alternaria brassicicola</i> , <i>Botrytis cinerea</i>	histone methylation	Arabidopsis, <i>sdg8-1</i> , <i>sdg25-2</i> , <i>sdg8/25</i>	<i>LAZ5</i> , <i>CCR2</i> , <i>CER3</i>	(Berr et al., 2010; Palma et al., 2010; Lee et al., 2016)
Cold	histone methylation	Arabidopsis	<i>FLC</i>	(Shindo et al., 2006)
Dehydration	histone methylation	Arabidopsis	<i>RD29A/B</i>	(Ding et al., 2012b)

occurs upon salt, aluminium and oxidative stresses (Choi and Sano, 2007). While under drought stress or after pathogenic infection, root tips of the pea or tobacco show a hypermethylation DNA status (Labra et al., 2002; Kovalchuk et al., 2003).

The involvement of DNA methylation in response to environmental cues is underlined by mutations in genes that encode DNA methyltransferases (*MET1*, *CMT3*, *DRM1* and *DRM2*) or demethylases (*ROS1*, *DME*, *DML2* and *DML3*) as the mutants display altered responses to stresses (Rout and Das, 2013; Sanchez et al., 2016). An Arabidopsis *met1* mutant is hypersensitive to salt stress due to the hypomethylation of *HKT1* which encodes a sodium transporter (Baek et al., 2011). *drm1/drm2/cmt3* mutants are more resistant to bacterial pathogens, and a hypomethylation of pathogen-responsive genes may be responsible for this (Zhang, 2012). In contrast, *ros1/dml2/dml3* mutants show increased susceptibility to the fungal pathogen *Fusarium oxysporum* (Le et al., 2014).

Repositioning of Nucleosomes During Stress Responses

Unlike DNA methylation that mediates gene repression at the DNA level, repositioning of nucleosomes alters their density. In general, higher expression levels correlate with lower nucleosome occupancy (Clark and Wolffe, 1991; Kwon et al., 2009; Han et al., 2015). Changes in nucleosome density occurs when plants encounter stress. For example, salicylic acid (SA) reduces nucleosome occupancy of *PR1*, a gene that is highly induced upon SA treatment or pathogenic attack (Duque, 2016). Under drought stress, *ABI5*, an ABA response regulator, is de-repressed by a reduced nucleosome occupancy (Han et al., 2012). This is most likely mediated by BRM, a chromatin remodelling ATPase which is part of SWI/SNF complexes. BRM is also involved in heat stress by interacting with FGT1, this protein is known to be associated with the promoter regions of actively expressed genes in a heat-dependent fashion (Brzezinka et al., 2016). Other known chromatin remodelling ATPase members, such as SYD, MINU1, PKL and CHR12/23 that affect nucleosome reposition also play a role in stress responses (Mlynarova et al., 2007; Perruc et al., 2007; Walley et al., 2008; Leeggangers et al., 2015). For example, *syd* mutants are hypersensitive to pathogenic fungus (Walley et al., 2008) and *pk1* mutants display a hypersensitive germination response to ABA (Perruc et al., 2007; Han and Wagner, 2014). Overexpression of *MINU1* causes growth arrest under drought, salt and heat

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stresses. While overexpression of *CHR12/23* results in a pronounced reduction in germination under salt and heat stresses (Leeggangers et al., 2015), (Table 1).

Histone Variants and Stress Responses

Each histone type has a number of variants with some differences in amino acid sequence and structure. Histone variants differ in their affinity for DNA and for histone binding proteins. They can substitute their canonical counterpart and in this way change properties of nucleosomes (Talbert and Henikoff, 2014). It has been shown that genes encoding diverse histone variants are induced under various environmental stresses. For instance, in *Arabidopsis*, Histone H1.3 is induced by ABA and drought stress and it replaces H1 in nucleosomes of *RAB18* and this facilitates the activation of *RAB18* expression (Ascenzi and Gantt, 1997, 1999). This H1.3 variant is also required for plant adaptive responses to water deficiency combined with low light intensity (Rutowicz et al., 2015). Another example is the transcriptional activation of temperature sensitive genes in *Arabidopsis*. Upon heat stress Histone H2A.Z becomes part of nucleosomes in the vicinity of transcriptional start sites of these genes (Kumar and Wigge, 2010; Jarillo and Pineiro, 2015). This enrichment of H2A.Z is correlated with lower transcriptional activity of these genes (Coleman-Derr and Zilberman, 2012). In line with this, loss-of-function mutations in *H2A.Z* result in a constitutive expression of *SAR* which enhances resistance to pathogenic bacteria (March-Diaz et al., 2008), (Table 1).

Histone Post-translational Modification During Stress Responses

While histone variants have one or a few amino acid differences compared with their conventional counterparts, histone post-translational modifications only result in a reversible covalent modification at certain amino acids. Histone post-translational modifications (PTMs) are extremely important in the controlling of condensation degree of chromatin. Histone PTMs such as acetylation and methylation, are the most commonly used chromatin remodelling strategy by plants during (a)biotic stress (Grativol et al., 2012; Asensi-Fabado et al., 2017).

Upon salt and drought stress, a number of *Arabidopsis* genes involved in response to water deprivation are induced rapidly, possibly due to an increased H3K9 acetylation level at their promoters (Zheng et al., 2016). In maize, cold stress induces the expression of HDACs and this leads to a global deacetylation

of H3 and H4 within 24h. Repression of HDACs expression under cold stress, inhibits the expression of *DREB1* and *COR413*, two cold-responsive genes (Hu et al., 2011). The elevated acetylation level of H3K9 and H3K14 at the rice *DREB1* promoter occurs within 4h upon cold stress (Roy et al., 2014). Four rice HATs are significantly induced within 33 hours by drought stress and their overexpression increases the acetylation level on H3K9, H3K18, H3K27 and H4K5 (Fang et al., 2014). The alteration in acetylation level of histones occurs during biotic stress as well (Gomez-Diaz et al., 2012). Expression of *HDAC19* is induced during the interaction between Arabidopsis and pathogenic bacteria, and its expression is accompanied with changes in expression of jasmonic acid (JA) and ethylene-regulated genes (Zhou et al., 2005). A plant-specific HDAC, HDT701, regulates plant innate immunity after fungal pathogen infection in rice. Silencing of *HDT701* causes an increased H4 acetylation level and elevates transcription of defense-related genes (Ding et al., 2012a), (Table 1).

Besides acetylation, methylation of histones is also involved in regulating gene expression in response to various stresses. In Arabidopsis, salt stress alters genome-wide the H3K27me3 landscape within 24h (Sani et al., 2013). It also alters the H3K4me2/3 levels at salt-responsive gene loci, such as *RD29A/B*, *COR15A* and *P5CS1/2* which are preferentially modified by JMJ15, a histone H3K4 demethylase. The importance of JMJ15 is illustrated by its overexpression as this enhances salt tolerance (Shen et al., 2014). The histone methyltransferases SDG8/25 play a role in plant defense against fungal pathogens by modifying H3K4me2/3, H3K36me2/3 levels of a subset of genes which are involved in JA and ethylene signalling pathways in Arabidopsis. They are also required to trigger plant defense to pathogenic bacteria in tomato (Berr et al., 2010; Palma et al., 2010; Lee et al., 2016). Interestingly, plants could “remember” the stress by retaining H3K4/27me3 levels at certain genes (Kwon et al., 2009; Ding et al., 2012b; Espinas et al., 2016). One well known example is vernalization in which case increased H3K27me3 levels in the promoter of *FLC*, a negative regulator of flowering, correlates with exposure time to cold and results in gene repression (Shindo et al., 2006). A similar “memory” is also observed after dehydration stress. The H3K4me3 level at *RD29B* and *RAB18* is increased in response to dehydration stress, and this increase is retained during recovery (Ding et al., 2012b). This memory of stress by histone methylation enables plants to fortify their defense responses rapidly when the same stress re-occurs.

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Several studies indicate that (de)acetylation and (de)methylation of histones can occur simultaneously and they together determine gene expression upon stresses. In rice submergence-inducible *ADH1* and *PDC1* genes are activated in a biphasic manner. The first induction occurs after 2h of submergence and is associated with a change from a H3K4me2 state to K3K4me3 of these genes. The second step occurs after 12h and an increased H3ac level of these genes might be responsible for this (Tsuji et al., 2006). Repetitive mild abiotic stress such as salt, heat and cold, as well as pathogen attack increase H3K4me2/3 and H3K9ac levels at pattern-triggered immunity genes, like *WRKY53/FRK1/NHL10* and promote their transcription (Singh et al., 2014). In addition, a simultaneously induced enrichment of H3K4me3 and H3K9ac at drought-inducible genes, such as *RD29A/B*, *RD20* and *RAP2.4* is also observed under drought stress and they together promote the expression of these genes (Kim et al., 2008; Kim et al., 2012).

Enzymes that are involved in histone PTMs operate within multi-protein complexes of which at least one of the subunits has a DNA binding domain. A number of studies identified components of such complexes. In Arabidopsis, a plant-specific HDAC, HDT3, recruits a RPD3-type HDAC, HDA6, and they together regulate ABA responses (Sridha and Wu, 2006; Luo et al., 2012b). Silencing of *HDT3/HDA6* increases H3K9ac as well as H3K14ac levels and decreases H3K9me2 levels which is associated with the induction of *ABI1/2*, two ABA-responsive genes. HDA6/19 interacts with HDC1 which belongs to HDAC complex 1. This promotes histone deacetylation and attenuates depression of genes under drought stress (Perrella et al., 2013). HDA19 has also been shown to interact with MSI1 which belongs to a family of histone binding WD40-repeat proteins. The HDA19-MSI1 complex maintains low level of H3K9ac at ABA receptor genes and fine-tunes ABA signalling (Mehdi et al., 2016). While ADA2b, interacts with a HAT GCN5 that is required to maintain H3/4ac level at *RD29B* and its mutation causes a reduced salt sensitivity (Kaldis et al., 2011).

Developmental Transitions and Chromatin Modifications

Chromatin structure changes not only in response to environmental stimuli, but also during developmental transitions (Kouzarides, 2007; Boycheva et al., 2014; Cao et al., 2015; Perino and Veenstra, 2016; Wang et al., 2016). When a transition occurs, cells alter their fate and gain a new identity that is accomplished by transcriptional reprogramming which can involve chromatin

modifications (Costa and Shaw, 2006; Kornet and Scheres, 2008; She et al., 2013; Moris et al., 2016). Chromatin modification mechanisms that regulate cell fate switches are well studied during the development of Arabidopsis roots.

Arabidopsis roots consist of three distinct zones, root meristem (RM), the elongation zone and differentiated zone (Verbelen et al., 2006), (Figure 2A). Cell fate switches occur not only at the transition from the RM to elongation zone, and from the elongation to differentiated zone, but also within the RM.

The RM is maintained by the quiescent center (QC) and its surrounding stem cells [Figure 2B, (van den Berg et al., 1995; Scheres, 2007)]. The stem cells divide to renew themselves and provide daughter cells which continue to divide for a while before differentiating into the various tissues that form the root. This switch from stem cell identity to differentiation is suppressed by two independent pathways controlled by transcription factors; the SHORT-ROOT(SHR)/SCARECROW (SCR) pathway and the PLETHORA (PLT) pathway (van den Berg et al., 1997; Sabatini et al., 2003; Aida et al., 2004; Cruz-Ramirez et al., 2013). It is also suppressed by chromatin remodelling factors, such as the histone acetyltransferase GCN5 and the chromatin remodelling ATPase BRM. Both GCN5 and BRM are required for proper regulation of *PLT* gene expression (Kornet and Scheres, 2009; Yang et al., 2015). Knock-out of BRM and GCN5 results in differentiation of QC and stem cells .

Some chromatin remodelling factors are required for suppression of the switch to differentiation of specific stem cells. For example, a layer of columella stem cells is located below the QC (Figure 2B). Upon division one of the daughter cells will differentiate into a columella cell (Dolan et al., 1993). This switch is suppressed by the histone deacetylase HDA19 as in the knock-down *hda19* mutant columella stem cells are differentiated (Pi et al., 2015). The chromatin remodelling factor DECREASED DNA METHYLATION 1 (DDM1) might also play a role at this switch, as DDM1 is absent in the columella, this results in the most hyper-methylated genome in columella cells, compared with other cell types of the RM (Kawakatsu et al., 2016).

Transit amplifying cells divide a few times before they switch to expansion in the elongation zone (Scheres et al., 1994; Kang et al., 2003; Ishida et al., 2010), (Figure 2A). Although chromatin remodelling factors associated with the switch from cell division to expansion are unknown, transit amplifying cells are

Figure 2. Longitudinal Schematic Views of the Arabidopsis Root and Medicago Nodule Primordium.

(A) Arabidopsis root consists of the meristem zone, elongation zone and differentiated zone. Boundaries between three zones are indicated by arrowheads. The dividing cells are in the meristem zone, expanding cells are in the elongation zone. Cells in the differentiated zone are visualized by emergence of root hairs in epidermis.

(continued on next page)

characterized by weaker and more dynamic histone-DNA interactions than cells from elongation zone. This is at least in part determined by histone acetylation levels (Rosa et al., 2014).

The expanding cells form the elongation zone and reach their final size at the beginning of the differentiated zone (Figure 2A). There in specific epidermal cells root hairs emerged. A histone deacetylase HDA18 is shown to affect the differentiation of hair and non-hair epidermal cells. Both knock-out and overexpression of *HDA18* result in epidermal cells with root hairs at the non-hair position (Xu et al., 2005; Liu et al., 2013).

Legume Root Nodule Development and Chromatin Modifications

Legumes can interact with rhizobium bacteria that results in the formation of a new root organ, the nodule. Nodule formation starts with perception of Nod factors, which are secreted by rhizobia. This induces transcriptional reprogramming in different root cell types and changes their cell fates. In the model legume *Medicago* the first cell fate switch occurs in pericycle, cortical and endodermal cells. These cells are mitotically activated and form a nodule primordium [Figure 2C, (Timmers et al., 1999; Xiao et al., 2014)]. The inner cortical cell layers (C4 and C5) form about 8 cell layers of the central tissue. Infection threads are initiated in root hairs and they grow towards the nodule primordia and enter the 8 layers of the future central tissue and there rhizobia are released into these cells. The middle cortical cell layer (C3) is also mitotically activated and from this the

(continued)

(B) Magnification of boxed area in **(A)** and marked in colour, indicating the organization of quiescent center and its surrounding initial cells that will differentiate to different cell types.

(C) A stage V nodule primordium (provided by T. Xiao). The Pericycle/Endodermis derived cells contribute to uninfected cell layers at the basal of the nodule. The inner cortex (C4 and C5) form around 8 layers of infected cells at the central tissue and the middle cortex (C3) gives rise to the future nodule meristem. The outer cortex (C1 and C2) and Ep have limited role in nodule ontology. The arrow indicates the infection thread. Pe, pericycle; En, endodermis; C1-5, cortical cell layers and Ep, epidermis.

CHAPTER 1

nodule meristem is formed. The nodule meristem continuously adds cells to the central tissue and the peripheral tissues and this is the second cell fate switch, as the new added cells stop dividing and start to express early nodulation genes (Lohar et al., 2006). These new added cells form the infection zone where cells are infected by rhizobia and undergo endoreduplication (Vinardell et al., 2003).

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Recently, a DNA demethylase gene *MtDME* has been shown to be critical for the induction of endoreduplication of infected cells as well as differentiation of rhizobia (Satge et al., 2016; Montiel et al., 2017). This is the first chromatin remodelling factor that is shown to play a role during nodule development.

Concluding Remarks

Plants must coordinate their growth and development when exposed to stresses. It is obvious that a crosstalk between developmental pathways and stress signalling pathways exist. Chromatin modifications most likely function as a part of this crosstalk, as chromatin structure changes accompany developmental transitions, and chromatin structure is continuously modulated in response to environmental stimuli. However, their role in connecting environmental cues and developmental transitions is still elusive. This is possibly due to a few reasons: 1) Functional redundancy of chromatin remodelling factors. For example, 18 HDACs have been identified in Arabidopsis and they can be divided into three subfamilies: RPD3/HDA (12 members), HDT (4 members) and SIR2 (2 members) (Pandey et al., 2002; Hollender and Liu, 2008; Alinsug et al., 2009). Their catalytic substrates are histones and one HDAC might easily take over the function of the other; 2) Unknown spatial expression patterns of chromatin remodelling factors under control conditions and whether their spatial expression patterns will be affected by environmental stimuli. When these are known we could investigate the phenotype of specific cells/tissues/organs where genes are expressed and exclude secondary effects of others; 3) Severe/lethal phenotype of knockout mutants. Although single gene knockout mutants are available for most factors, they do not display any phenotype, and stacked combination could induce a severe/detrimental effect on plants and have not often been studied (Huang et al., 2007).

In our studies, we first focus on the role of HDTs in regulating Arabidopsis root development under control condition (**Chapter 2**). *HDTs* encode plant-specific HDAC with no sequence homology to the *RPD3/HDA* and *SIR2* subfamilies

(Lusser et al., 1997). This phylogenetic divergence makes probable that there is no functional redundancy with other type of HDAC. 4 *HDT* genes present in Arabidopsis genome, therefore it is possible to determine their functional redundancy. We study their expression patterns under control (**Chapter 2**) and abiotic stress (**Chapter 3**) and biotic stress (**Chapter 4**) conditions. We then perform *RNA* interference to knockdown *HDT*(s) expression specifically to study their role in root growth and development under different conditions. The response of a *HDT* mutant under biotic stress is studied in (**Chapter 4**). Medicago has 3 *HDT* genes and the role of HDTs in regulating nodule (**Chapter 5**) and arbuscular mycorrhiza (**Chapter 6**) formation is explored.

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CHAPTER 2

Plant-specific Histone Deacetylases HDT1/2 Regulate *GIBBERELLIN 2-OXIDASE 2* Expression to Control Arabidopsis Root Meristem Cell Number

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ABSTRACT

2 Root growth is modulated by environmental factors and depends on cell production in the root meristem (RM). New cells in the meristem are generated by stem cells and transit-amplifying cells, which together determine RM cell number. Transcription factors and chromatin-remodelling factors have been implicated in regulating the switch from stem cells to transit-amplifying cells. Here we show that two *Arabidopsis thaliana* paralogs encoding plant-specific histone deacetylases, HDT1 and HDT2, regulate a second switch from transit-amplifying cells to expanding cells. Knockdown of *HDT1/2* (*hdt1,2i*) results in an earlier switch and causes a reduced RM cell number. Our data show that HDT1/2 negatively regulate the acetylation level of the *C₁₉-GIBBERELLIN 2-OXIDASE 2* (*GA2ox2*) locus and repress the expression of *GA2ox2* in the RM and elongation zone. Overexpression of *GA2ox2* in the RM phenocopies the *hdt1,2i* phenotype. Conversely, knockout of *GA2ox2* partially rescues the root growth defect of *hdt1,2i*. These results suggest that by repressing the expression of *GA2ox2*, HDT1/2 likely fine-tune gibberellin metabolism and they are crucial for regulating the switch from cell division to expansion to determine RM cell number. We propose that HDT1/2 function as part of a mechanism that modulates root growth in response to environmental factors.

INTRODUCTION

Root architecture and growth is regulated through the activity of root meristems. The root growth rate correlates with the cell number in the root meristem (RM), as this determines the number of cells that can differentiate in a given time. The RM includes the stem cell niche (SCN), which is composed of the quiescent center (QC) and its surrounding stem cells (van den Berg et al., 1995; Scheres, 2007). The daughter cells of the stem cells divide a few times before they switch to expansion. These dividing cells are the transit-amplifying cells and together with the SCN they form the RM, whereas the expanding cells form the elongation zone (Moubayidin et al., 2009; Perilli et al., 2010). The RM cell number is determined by the activity of stem cells, as well as by the number of times transit-amplifying cells divide before switching to expansion.

The genes and mechanisms involved in regulating the stem cell activity or the switch from cell division to expansion are becoming clear (Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004; Dello Ioio et al., 2007; Galinha et al., 2007; Dello Ioio et al., 2008; Bennett and Scheres, 2010; Moubayidin et al., 2010). In *Arabidopsis thaliana* roots, the stem cell activity depends on two independent pathways controlled by transcription factors; the SHORT-ROOT (SHR)/SCARECROW (SCR) pathway and the PLETHORA (PLT) pathway (Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004; Galinha et al., 2007). The expression domains of *SHR* and *SCR* overlap with the distal *PLT* transcript accumulation area to position the SCN (Aida et al., 2004). Loss-of-function mutations in *SHR*, *SCR* or *PLT1/PLT2*, cause a loss of stem cell maintenance and this results in a reduced RM cell number (Helariutta et al., 2000; Sabatini et al., 2003; Aida et al., 2004; Galinha et al., 2007). The switch from cell division to expansion is controlled by transcription factors *ARR12*, *ARR1* and *SHY2* (Dello Ioio et al., 2007; Bennett and Scheres, 2010; Moubayidin et al., 2010). *ARR1* and *ARR12* activate the expression of *SHY2*, a transcriptional repressor that triggers the switch from cell division to expansion (Dello Ioio et al., 2008). Gain-of-function mutations in *ARR1* or *SHY2* cause a reduced RM cell number due to an earlier switch to expansion (Dello Ioio et al., 2008; Moubayidin et al., 2010).

Beside transcription factors, chromatin-remodelling factors also contribute to transcriptional reprogramming by creating an active or silent chromatin configuration (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Kouzarides, 2007; Shahbazian and Grunstein, 2007). Some of them are essential for maintaining

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stem cell activity in Arabidopsis. For example, the chromatin-remodelling ATPase BRAHMA is indispensable to maintain the SCN by controlling *PIN* gene expression (Yang et al., 2015). GCN5, a histone acetyltransferase, positively regulates PLT-mediated SCN maintenance (Kornet and Scheres, 2009). In loss-of-function mutants of *brm* or *gcn5*, RM cell number is reduced due to gradual loss of stem cells.

2 Recently, it has been demonstrated that the switch from cell division to expansion in Arabidopsis roots is accompanied by changes in level of histone acetylation (Rosa et al., 2014). Histone acetylation level is regulated by histone acetyltransferases and histone deacetylases (Shahbazian and Grunstein, 2007). In the region where the switch from cell division to expansion is initiated, among the highest expressed histone acetylation genes in Arabidopsis roots are the four plant-specific histone deacetylase genes, named *HDT1–HDT4* [Supplemental Dataset 1, (Birnbaum et al., 2003)]. This suggests that these HDTs may play a role in controlling the switch from cell division to expansion. However, the function of HDTs in root development has not been investigated. It has been shown that HDTs are involved in responses to biotic and abiotic stress (Sridha and Wu, 2006; Bourque et al., 2011; Ding et al., 2012; Luo et al., 2012) and they can repress the expression of defence-related genes by altering their chromatin acetylation status (Bourque et al., 2011; Ding et al., 2012). We studied whether HDTs regulate the switch from cell division to expansion, and in this way contribute to the mechanisms controlling RM cell number and subsequently root growth.

Here we investigated the RM phenotype of *hdt* mutants. We show that two members of the HDT family, HDT1/2, determine the RM cell number by affecting the switch from cell division to expansion. Down-regulation of their expression (*hdt1,2i*) reduces RM cell number and results in a markedly changed transcriptome. Genetic analyses indicate that the strongly increased expression of *C₁₉-GIBBERELLIN 2-OXIDASE 2 (GA2ox2)* in *hdt1,2i* is a cause of this reduced RM cell number. HDT1/2 negatively regulate the level of histone H3 acetylation of *GA2ox2* and possibly in this way repress the transcription of this gene. These data indicate that HDT1/2 repression of *GA2ox2* expression contributes to regulation of the switch from cell division to expansion in Arabidopsis roots.

RESULTS

HDT1 and HDT2 Control Root Growth

To test whether HDTs control Arabidopsis root growth, we analyzed T-DNA insertion mutants of all four *HDT* genes [designated *hdt1*, 2, 3-1, 3-2 (Luo et al., 2012) and 4, respectively; Supplemental Figure 1]. Reverse transcription PCR (RT-PCR) analyses showed that *hdt1*, 3-1 and 4 were null mutants, as transcript of the mutated genes was not detectable (Figure 1A). *hdt2*, however, was a knockdown mutant that retained 20% of transcript (Figure 1A and 1B). *hdt1*, *hdt3-1*, *hdt3-2* and *hdt4* displayed no root phenotype. However, *hdt2* showed a 12% reduced root length, compared to WT (Figure 1C, Supplemental Figure 2), at 7 days after germination (DAG).

To determine if the other three HDTs contribute to root growth in the *hdt2* mutant background, we crossed *hdt2* with *hdt1*, 3-1 and 4 to generate double mutants. *hdt2 hdt3-1* and *hdt2 hdt4* homozygous plants were morphologically indistinguishable from *hdt2* (Supplemental Figure 2), suggesting that neither HDT3 nor HDT4 contributes to root growth. In contrast, selfing of *HDT1/hdt1 HDT2/hdt2* did not result in any homozygous *hdt1 hdt2* double mutants among more than 200 daughter plants tested. This indicates that loss of function of both *HDT1* and *HDT2* is lethal, by which the role of HDT1 in root growth could not be studied. In order to study this role, we first determined the *HDT1/2* expression patterns in roots, by creating *HDT1/2* promoter/GUS fusions (*pHDT1:GUS* and *pHDT2:GUS*) as well as translational GFP fusions (*pHDT1:HDT1-GFP* and *pHDT2:HDT2-GFP*). The latter two constructs were functional, as they complemented the *hdt1 hdt2* lethal phenotype and fully restored root growth in the corresponding background (Supplemental Figure 2). This indicates that both promoter regions are sufficient to drive genes expression at the right place. Transgenic *pHDT1:GUS* and *pHDT2:GUS* plants showed that both promoters were especially active in the RM. They displayed patchy expression patterns, suggesting a cell cycle phase dependent expression (Figure 1D and 1E). Their high activity in the RM is consistent with transcriptome data (Birnbaum et al., 2003) and supports the idea that both *HDT* genes are involved in controlling RM activity. To study this, we made use of the root-specific *ROOT CLAVATA HOMOLOGUE 1 (RCH1)* promoter to knockdown both *HDT1/2*. The *RCH1* promoter is specifically active in the RM and it is first activated during the torpedo stage of embryo development (Casamitjana-Martinez et al., 2003). So

Figure 1. Silencing of *HDT1* and *HDT2* Leads to Reduced Root Growth.

(A) Expression of *HDT* genes in WT and *hdt* mutants. RT-PCR analysis was performed with cDNA prepared from seedlings at 6 DAG. The *TUBULIN* gene was used as a loading control.

(B) Reverse transcription quantitative PCR (RT-qPCR) analyses of *HDT1* and *HDT2* expression in WT, *hdt1*, *hdt2*, *hdt1,2i-1* and *hdt1,2i-8* root tips at 6 DAG. All panels show mean $\pm$ SEM values determined from three independent experiments.

(C) Primary root length of WT, *hdt1*, *hdt2*, *hdt1,2i-1* and *hdt1,2i-8* seedlings at 7 DAG. Data shown are average $\pm$ SD (n>20). Asterisks indicate significant differences compared with the WT (* p <0.05, *** p <0.001; Student's *t* test).

(D) and **(E)** Expression patterns of *pHDT1:GUS* (**D**, n=22) and *pHDT2:GUS* (**E**, n=25) in root tips of seedlings at 6 DAG. Arrows indicate the SCN and arrowheads indicate the boundary between the RM and elongation zone. Bars=50μm.

if HDT1/2 are essential for developmental processes preceding this embryonic stage, these will not be affected. Five independent lines (designated *hdt1,2i-1*, 4, 6, 8 and 9) were generated. The levels of *HDT1/2* transcript, in these lines, were between 15% and 40% of that of the WT (Figure 1B, Supplemental Figure 3). In comparison with *hdt2*, all *hdt1,2i* lines showed more severely reduced root growth (Figure 1C, Supplemental Figure 2), indicating that both HDT1 and HDT2 control root growth. The level of root length reduction in these *hdt1,2i* lines correlates with decreasing levels of *HDT1/2* transcript (Figure 1B and 1C, Supplemental Figure 2, Supplemental Figure 3), indicating that HDT1/2 positively regulate root growth.

Reduced Root Growth of *hdt1,2i* Involves a Lower RM Cell Number

To investigate how HDT1/2 affect root growth, the kinematic growth of roots was analyzed in WT and two *hdt1,2i* lines, *hdt1,2i-1* and *hdt1,2i-8*, which had the strongest reduction of *HDT1/2* mRNA levels. Root growth rate from 6 to 7 DAG was determined (see Methods). In comparison with the WT, *hdt1,2i-1* or *hdt1,2i-8* roots grew 56% and 60% slower, respectively (Table 1, Supplemental Table 1). Root growth rate is determined by number of cells that are added from the RM to elongation zone within a defined time period and to what extent these cells subsequently expand (De Veylder et al., 2001). Therefore the reduced root growth rate of *hdt1,2i* could be due to fewer cells being added to the elongation zone, or reduced expansion of newly added cells. The length of cortical cells

Table 1. Kinematic Analyses of Root Growth in WT and *hdt1,2i-1* Seedlings.

Parameter	WT	<i>hdt1,2i-1</i>	Difference (%)	<i>p</i> value
Root growth rate ($\mu\text{m/h}$, <i>M</i>)	320.6 $\pm$ 35.0	141.8 $\pm$ 24.7	-56	<0.001
Fully expanded cell length (μm , <i>N</i>)	191.0 $\pm$ 9.2	195.2 $\pm$ 9.0	-	NS
Cell production per day (<i>X</i>)	40.3 $\pm$ 4.3	17.4 $\pm$ 2.9	-57	<0.001
Cell number in the RM (<i>Y</i>)	38.0 $\pm$ 3.9	17.3 $\pm$ 1.7	-54	0.001
Cell cycle duration (h, <i>Z</i>)	22.8 $\pm$ 2.4	24.4 $\pm$ 4.7	7	0.107

The analyses were performed based on WT and *hdt1,2i-1* root cortical cell file. Data shown are average $\pm$ SD. *p* values were determined by Student's *t* test (*n*>20). NS, no statistical significance. $X=24*M/N$, $Z=24*Y/X$.

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in the differentiated zone of both *hdt1,2i-1* and *hdt1,2i-8* roots was measured and shown to be the same as in WT. However, the number of cells added to the elongation zone was reduced in *hdt1,2i-1* and *hdt1,2i-8* (Table 1, Supplemental Table 1). This reduced cell number could be caused either by the presence of fewer cells in *hdt1,2i* RM, or by a prolonged cell cycle duration. We inferred cell cycle duration from kinematic growth analyses and this showed that it was only 7% or 6% longer in *hdt1,2i-1* and *hdt1,2i-8* compared to WT. However, RM cell number was reduced by 54% in *hdt1,2i-1* and by 59% in *hdt1,2i-8*. This reduction equals the difference in root growth rate (Table 1, Supplemental Table 1).

The reduced cell number in RM can be caused either by the loss of the SCN or by a reduced number of divisions of the transit-amplifying cells. To distinguish between these two possibilities, we first investigated whether the SCN was lost in *hdt1,2i*. The columella stem cells in *hdt1,2i-1* and *hdt1,2i-8* were present; these are the cells adjacent to the QC and not containing starch granules, which occur in differentiated columella cells (Figure 2A, 2B, Supplemental Figure 4A). Also, *SCR* which is required for SCN maintenance (Sabatini et al., 2003), had a WT-like expression pattern in the QC and endodermis of *hdt1,2i-1* roots (Figure 2D and 2E). QC divisions were more frequently observed in *hdt1,2i-8*, but not in *hdt1,2i-1* (Supplemental Figure 4A, 4I). However, expression of *WOX5*, another QC marker (Sarkar et al., 2007), was maintained in *hdt1,2i-8*, like in WT and *hdt1,2i-1* (Supplemental Figure 4D-4F). This suggests that QC cells are maintained in *hdt1,2i-8*. In addition, the RMs of *hdt1,2i-1* and *hdt1,2i-8* were still present at 21 DAG (Supplemental Figure 4J-4L). Together these data indicate that the reduced cell number in *hdt1,2i* RM is not caused by loss of the SCN.

RM cell number increases after germination, and it reaches a maximum when the number of cells added by cell division is equal to the number of cells that switch to expansion (Dello Ioio et al., 2008). By monitoring at what time the maximum RM cell number is reached, we determined when cell division and cell expansion have reached a balance. The maximum RM cell number was established at 4 DAG in *hdt1,2i-1*, whereas this was first reached at 6 DAG in WT (Figure 2F). Similar results were also obtained for the *hdt1,2i-8* line (Supplemental Figure 4C). This demonstrates that HDT1/2 are involved in regulating the switch from cell division to expansion. The reduced RM cell number indicates that an earlier switch occurs after fewer divisions of the transit-amplifying cells in *hdt1,2i*.

Figure 2. Reduced Cell Number in *hdt1,2i-1* and *GA2ox2^{OE1}* RM Is Caused by an Earlier Switch from Cell Division to Expansion.

(A) to (C) mPS-PI-stained root tips of the WT (A), *hdt1,2i-1* (B) and *GA2ox2^{OE1}* (C) seedlings at 6 DAG. Arrows indicate columella stem cells which did not display any starch granule.

(D) and (E) Expression pattern of the *pSCR:SCR-GFP* in the WT (D) and *hdt1,2i-1* (E) root tips at 7 DAG (n=10; representative images are shown). Identical confocal microscope settings were used in (D) and (E).

(F) Cell number of the RM in WT, *ga2ox2*, *hdt1,2i-1*, *ga2ox2hdt1,2i-1* and *GA2ox2^{OE1}* seedlings measured daily after germination during 8 days. Data shown are average $\pm$ SD (n>20).

Bars=50 μ m in (A) to (E).

Possible Targets of HDT1/2 Revealed By Transcriptome Analyses

2 In the RM and elongation zone, HDT1/2 were located in both nucleolus and nucleoplasm (Supplemental Figure 5B and 5C), where they could alter the expression level of genes by changing chromatin acetylation levels (Wu et al., 2003; Zhou et al., 2004; Ding et al., 2012). We performed Illumina RNA sequencing to determine the transcriptome differences between WT and *hdt1,2i-1* to obtain insight in how RM cell number might be reduced in *hdt1,2i-1*. HDT1/2 proteins accumulated highest in the meristem, markedly lower in the elongation zone and were hardly detectable in the differentiated zone (Figure 3A, Supplemental Figure 5A). We compared transcriptomes from the WT and *hdt1,2i-1* in these three zones (See Methods). This resulted in 90, 114 and 42 differentially expressed genes (DEGs) in the meristem, elongation and differentiated zones, respectively (Figure 3B, Supplemental Dataset 2).

In the meristem zone 75 genes were up-regulated in *hdt1,2i-1*, representing about 83% of DEGs in this zone. This is consistent with a function of HDTs in repressing gene expression (Wu et al., 2003; Zhou et al., 2004). In the elongation and differentiated zones, the number of up-regulated genes was 63 and 25, representing about 55% and 60% of DEGs, respectively (Figure 3C). 24 DEGs were shared between the meristem and elongation zones, 5 DEGs were shared between the elongation and differentiated zones, and only 1 DEG was shared between the meristem and differentiated zones. In total, 217 DEGs were identified (Figure 3B, Supplemental Dataset 2).

Gene Ontology (GO) enrichment analyses for the 217 DEGs showed that the up-regulated genes in *hdt1,2i-1* were enriched in genes involved in “response to chemical/stimulus”. The down-regulated genes were enriched in genes involved in regulating root development (Supplemental Figure 6). As HDT1/2 most likely repress the expression of genes (Wu et al., 2003; Zhou et al., 2004; Ding et al., 2012), we expected that the earlier switch to expansion of transit-amplifying cells in *hdt1,2i-1* was at least in part caused by genes with increased expression. The most up-regulated gene from the *hdt1,2i-1* meristem zone encodes a MATE protein that belongs to a proton-dependent efflux transporter family, which contains at least 54 members in Arabidopsis (Eckardt, 2001). However, the expression level of several paralogs was very high in WT and was not changed in *hdt1,2i-1* (Supplemental Dataset 2). Assuming functional redundancy of these genes, the up-regulation of one *MATE* gene probably did not have a

Figure 3. HDT1/2 Orchestrate Transcriptional Reprogramming.

(A) Expression pattern of *pHDT2:HDT2-GFP* that complements *hdt1hdt2* lethal phenotype in root of 7 DAG seedling. Bar=100μm.

(B) DEGs overlapped between the meristem zone (M), elongation zone (E) and differentiated zone (D) of WT and *hdt1,2i-1* roots.

(C) The number of up-regulated and down-regulated DEGs in M, E and D of *hdt1,2i-1* roots, compared to the WT.

major effect on root growth. *GA2ox2* was the second most up-regulated gene (Supplemental Dataset 2). This gene, the highest expressed member from its family in root tips, was a good candidate to contribute to the reduced RM cell number in *hdt1,2i-1*. *GA2ox2* encodes an oxidase that inactivates bioactive C₁₉ gibberellins via 2-oxidation. This is a major gibberellin (GA) inactivating pathway in Arabidopsis, and GA delays the switch from cell division to expansion in

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Arabidopsis roots (Rieu et al., 2008; Ubeda-Tomas et al., 2008; Ubeda-Tomas et al., 2009; Moubayidin et al., 2010).

***hdt1,2i-1* Root Tips Have a Gibberellin Deficient Phenotype**

To determine whether GA level is indeed reduced in *hdt1,2i-1* root tips we made use of a DELLA reporter construct (*pRGA:GFP-RGA*). GA destabilizes DELLA proteins such as RGA (REPRESSOR OF *ga1-3*) (Peng et al., 1997; Silverstone et al., 2001). *hdt1,2i-1* plants were crossed with *pRGA:GFP-RGA* to generate homozygous plants. RGA protein level was quantified by measuring GFP intensity in WT (n=11) and *hdt1,2i-1* (n=12) roots. In each root, GFP intensity in nuclei of five cortical cells, at the transition from division to elongation, were measured and the average values were determined. This showed that in *hdt1,2i-1*, RGA protein level was around 50% increased (Figure 4A-4C), indicating that *hdt1,2i-1* root tips have a reduced GA level. Consistent with this, PIN1 and PIN2 levels which are positively regulated by GA (Willige et al., 2011), were considerably reduced in *hdt1,2i-1*, compared to WT (Supplemental Figure 7A-7D).

To investigate whether the reduced GA level in *hdt1,2i-1* root tips is caused by the up-regulation of *GA2ox2*, WT and *hdt1,2i-1* seedlings were treated with exogenous GA_4 or GA_3 . GA_4 is a substrate of *GA2ox2* whereas GA_3 is not (Thomas et al., 1999; Hedden and Phillips, 2000; Yamauchi et al., 2007). RM cell number in both WT and *hdt1,2i-1* was increased significantly after GA_3 (1 and 10 μ M) application (Figure 4D). A similar increase in RM cell number was also observed in WT after GA_4 (1 and 10 μ M) application in WT. However, in *hdt1,2i-1* RM cell number was not affected by applying 1 μ M GA_4 and it was only increased slightly by application of 10 μ M GA_4 . This suggests that the up-regulated *GA2ox2* in *hdt1,2i-1* root tips rapidly degrades exogenously applied GA_4 but not GA_3 .

In line with the reduced GA level in *hdt1,2i-1* root tips, the exogenously applied paclobutrazol (PAC, 0.1 and 1 μ M), a GA biosynthesis inhibitor (Wang et al., 1986), significantly reduced RM cell number in WT. Whereas RM cell number in *hdt1,2i-1* was not affected (Figure 4E). This suggests that the GA level in *hdt1,2i-1* is so low that PAC has no additional inhibitory effect on RM cell number. Collectively, these data indicate that the up-regulation of *GA2ox2* reduces GA level in *hdt1,2i-1* root tips and this contributes to the decreased RM cell number.

Figure 4. The *hdt1,2i-1* Root Tip Has Gibberellin Deficient Phenotype.

(A) and (B) Expression of *pRGA:GFP-RGA* in WT (A) and *hdt1,2i-1* (B) root tips at 6 DAG. Arrowheads indicate the boundary between the RM and elongation zone. Closeup of boxed areas show 5 cortical cells that were used for quantification in (C). Identical confocal microscope settings were used to image *pRGA:GFP-RGA* proteins in (A) and (B), representative images are shown. Bars=50μm.

(C) Quantification of fluorescence intensity of *pRGA:GFP-RGA* in WT (n=11) and *hdt1,2i-1* (n=12). Arbitrary unit was used for GFP intensity. Root cells used for quantification are indicated in (A) and (B). Data shown are mean ± SEM. Asterisk indicates significant difference between WT and *hdt1,2i-1* (***p*<0.01, Student's *t* test).

(D) and (E) Cell number in WT and *hdt1,2i-1* RM after treatment with 1μM and 10μM GA₄/GA₃ (D) or with 0.1μM and 1μM PAC (E) at 6 DAG. Data shown are average ± SD (n>40). Asterisks indicate significant differences compared with the mock [(-); (***p*<0.01, ****p*<0.001; Student's *t* test)].

HDT2 Regulates the Expression of GA2ox2 Directly

To examine whether there is a correlation between the expression levels of *HDT1/2* and *GA2ox2*, we compared the *GA2ox2* mRNA level in root tips of several *hdt* mutants/lines. This included the *hdt1* and *hdt2* mutants, the *hdt1 hdt2* double mutant complemented by *pHDT2:HDT2-GFP*, *hdt1,2i-1* and *hdt1,2i-8*. The *hdt1* as well as the complemented line had a similar *GA2ox2* mRNA level as WT, whereas in *hdt2* and two *hdt1,2i* lines the expression level of *GA2ox2* was increased (Figure 5A). Lower level of *HDT2* mRNA (Figure 1B) correlates with higher expression level of *GA2ox2*. This inverse correlation suggests that the expression of *GA2ox2* is negatively regulated by HDT2.

HDTs can mediate transcriptional repression by deacetylating genes (Ding et al., 2012). To investigate whether HDT2 binds to the *GA2ox2* locus, we performed ChIP-quantitative PCR (ChIP-qPCR) on WT and *pHDT2:HDT2-GFP* complemented seedlings using an anti-GFP antibody. Five different DNA fragments were amplified spanning a region from 850 bp upstream to 618 bp downstream of the *GA2ox2* start codon. These included three fragments from the promoter region and a part of the first and the second exon, respectively (Figure 5B). The *MATE* gene (*AT2G04050*) was used as positive control. It is also up-regulated in *hdt1,2i-1* (Supplemental Dataset 2) and HDT2 binds to its first exon (David Latrasse, personal communication). A randomly selected Arabidopsis intergenic region of chromosome 5 was used as negative control. A marked enrichment of HDT2 on the positive control, as well as on the first exon of *GA2ox2* was found in *pHDT2:HDT2-GFP* complemented seedlings, compared to WT seedlings. In addition, a statistically significant enrichment was also found for one promoter fragment (TATA box) and the second exon of *GA2ox2*. No enrichment was observed for the negative control and the other two fragments of *GA2ox2* (Figure 5C). This indicates that HDT2 binds to *GA2ox2*.

Subsequently we determined whether knockdown of *HDT1/2* affects the acetylation level of the HDT2 bound *GA2ox2* regions. Since histone H3ac generally marks actively transcribed chromatin (Kouzarides, 2007), we determined H3ac levels of *GA2ox2* in *hdt1,2i*. We carried out ChIP-qPCR on *hdt1,2i-1* and WT roots (see Methods) using an anti-H3ac antibody. An increase in H3ac level was found for the positive control in *hdt1,2i-1* roots. Further, at the HDT2 binding sites, a 2.6-fold increase was found for the first exon of *GA2ox2* and a statistically significant increase in H3ac level was also found for the TATA

Figure 5. HDT2 Directly Regulates the Expression of *GA2ox2*.

(A) RT-qPCR analyses of *GA2ox2* expression in root tips of the WT, *hdt1*, *hdt2*, *pHDT2:HDT2-GFP* complemented *hdt1hdt2* mutant, *hdt1,2i-1* and *hdt1,2i-8* lines at 6 DAG.

(B) Schematic representation of the *GA2ox2* genomic region. Black boxes represent exons and grey box indicates TATA box. The bent arrow indicates the transcription start site. Bars below with numbers indicate regions used in ChIP-qPCR experiments. Bar=100bp.

(C) and **(D)** ChIP-qPCR analyses of *pHDT2:HDT2-GFP* complemented *hdt1hdt2* and WT seedlings using anti-GFP antibody **(C)**, and *hdt1,2i-1* and WT roots using anti-H3ac antibody **(D)**. PC or NC represents positive or negative control.

Data shown in **(A)**, **(C)** and **(D)** are mean $\pm$ SEM values determined from three independent experiments. Asterisks in **(C)** and **(D)** indicate significant differences compared with the WT (** $p < 0.01$, Student's *t* test).

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box region (1.9-fold) and the second exon (1.9-fold) (Figure 5D). Furthermore, there was no increase in H3ac level at the negative control locus and the HDT2 non-binding sites in *hdt1,2i-1*. This shows that knockdown of *HDT1/2* results in an increased H3ac level at regions to which HDT2 binds. Taken together, these results indicate that HDT1/2, repress the expression of *GA2ox2* likely by deacetylating histone H3 at specific regions within the *GA2ox2* locus.

***GA2ox2* Is a Determinant in HDT1/2 Regulated Switch from Cell Division to Expansion**

2 To further study whether the reduced root growth of *hdt1,2i* plants is caused by the up-regulation of *GA2ox2*, we generated four *GA2ox2* overexpression lines driven by the root-specific *RCH1* promoter (*GA2ox2^{OE1-OE4}*) (Casamitjana-Martinez et al., 2003). In these lines, the level of *GA2ox2* transcripts was 30 to 100 times higher than that of the WT (Figure 6A). All four *GA2ox2^{OE}* lines formed shorter roots than the WT. The level of reduction was correlated with the level of up-regulation of *GA2ox2* (Figure 6B). To test whether overexpression of *GA2ox2* causes the short root phenotype in a similar way as *hdt1,2i*, the SCN and the length of fully differentiated cells were analyzed, the RM cell number was determined daily from 1 to 8 DAG in *GA2ox2^{OE1}* and *GA2ox2^{OE2}*. In both lines, the SCN was shown not to be disturbed at 6 DAG and expression of *WOX5* was WT-like. (Figure 2A and 2C; Supplemental Figure 4B, 4D and 4G-4I). This was also supported by the maintenance of the RMs till 14 DAG (Supplemental Figure 4J, 4M and 4N). The length of fully differentiated cortical cells in both *GA2ox2^{OE1}* and *GA2ox2^{OE2}* roots was measured and shown to be identical to WT (Figure 6C). The maximum RM cell number was established at 5 DAG in both *GA2ox2^{OE1}* and *GA2ox2^{OE2}* (Figure 2F, Supplemental Figure 4C), whereas in WT this was first reached at 6 DAG. RM cell number of *GA2ox2^{OE1}* and *GA2ox2^{OE2}* was reduced significantly at 6 DAG, compared to that of WT (Figure 7A, 7C and 2F, Supplemental Figure 4C). These results suggest that the switch from cell division to expansion occurs earlier in *GA2ox2^{OE}* lines, like in *hdt1,2i* lines. The similarity of the *GA2ox2^{OE}* RM to that of *hdt1,2i* further supports that lower GA levels in *hdt1,2i-1* root tips contribute to RM size regulation.

We next determined whether knockout of *GA2ox2* (*ga2ox2*, Supplemental Figure 1) could rescue the RM defect in *hdt1,2i-1*. *ga2ox2* plants were crossed with *hdt1,2i-1* plants to generate *ga2ox2 hdt1,2i-1* homozygous plants. At 6

Figure 6. GA2ox2 Controls Root Growth.

(A) RT-qPCR analyses of GA2ox2 expression in root tips of the WT and four independent GA2ox2 overexpression lines at 6 DAG. All panels show mean $\pm$ SEM values determined from three independent experiments.

(B) Primary root length of the four GA2ox2 overexpression lines and WT at 7 DAG. Data shown are average $\pm$ SD ($n > 20$). Asterisks indicate significant differences between mutants and WT (* $p < 0.05$, *** $p < 0.001$; Student's t test).

(C) The length of fully differentiated cortical cells in WT, GA2ox2^{OE1} and GA2ox2^{OE2} at 7 DAG. Data shown are average $\pm$ SD ($n > 20$) with no significant difference between the WT and mutants (Student's t test).

Figure 7. *GA2ox2* Is a Gene Repressed by HDT1/2 to Determine RM Cell Number.

(A) to (E) Root tip phenotypes of the WT (A), *hdt1,2i-1* (B), *GA2ox2^{OE1}* (C), *ga2ox2* (D) and *ga2ox2/hdt1,2i-1* (E) at 6 DAG. Arrowheads indicate the boundary between the RM and elongation zone. Bars=50µm.

DAG, *ga2ox2* plants had a similar RM cell number as WT (Figure 7A, 7D and 2F). In *ga2ox2 hdt1,2i-1* plants the averaged RM cell number was 24.0. This is a substantially increased cell number in comparison to 16.6 in *hdt1,2i-1* (Figure 7B, 7E and 2F). The maximum cell number was established at 5 DAG in *ga2ox2 hdt1,2i-1*, compared to 4 DAG in *hdt1,2i-1* plants (Figure 2F). Therefore, knockout of *GA2ox2* rescues (in part) the RM defect of *hdt1,2i-1*. These findings suggest that HDT1/2 repress *GA2ox2* expression to fine tune gibberellin homeostasis in order to determine RM cell number.

GA2ox2* Expression Is Increased in All Transit-amplifying Cells in *hdt1,2i-1

In roots of *hdt1,2i-1* plants the switch from cell division to expansion occurs earlier than in WT, as caused by the up-regulation of *GA2ox2*. Therefore we

Figure 8. Enhanced Expression of *GA2ox2* in *hdt1,2i-1* Root Tip.

(A) and (B) Expression pattern of *pGA2ox2:GUS* in root tips of the WT (A) and *hdt1,2i-1* (B) seedlings at 6 DAG (n=15 for each; representative images are shown). Arrowheads indicate the boundary between the RM and elongation zone. Bars=50µm.

determined in which cells *GA2ox2* expression was increased. We created a *GA2ox2* promoter/GUS reporter line (*pGA2ox2:GUS*) and crossed it with *hdt1,2i-1* to generate homozygous *pGA2ox2:GUS hdt1,2i-1* plants. In WT roots, a low level of expression of *pGA2ox2:GUS* occurred in vascular tissue and pericycle at the transition from the RM to elongation zone, but it was not detected in endodermis, cortex and epidermis (Figure 8A). This expression pattern is consistent with the gene expression map of the Arabidopsis roots (Birnbaum et al., 2003). In *hdt1,2i-1* GUS activity was markedly increased in both RM and the distal part of elongation zone, and it was not restricted to vascular tissue and pericycle. Instead, we observed high GUS activity in the RM within all cell files (Figure 8B). The level of GUS activity gradually decreased from the distal to the proximal elongation zone where it became undetectable. This is consistent with the expression level of *GA2ox2* in these zones in *hdt1,2i-1* (Supplemental Dataset 2). These data indicate that HDT1/2 determine *GA2ox2* expression level in the RM and elongation zone.

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DISCUSSION

In this study, we demonstrate that HDT1/2 influence the switch from cell division to expansion in the root tip by repressing *GA2ox2* expression. Knockdown of *HDT1/2* causes an earlier switch to cell expansion and results in a reduced RM cell number. The up-regulation of *GA2ox2* is a contributing factor to this phenotype. We show that HDT2 binds to several regions of *GA2ox2*, and at these binding sites the acetylation level is negatively regulated. Up-regulation of *GA2ox2* in *hdt1,2i* most likely causes a reduced GA level that results in an earlier switch from cell division to expansion of transit-amplifying cells.

2 *pHDT1:GUS* and *pHDT2:GUS* exhibit a patchy accumulation pattern suggesting cell cycle-dependent expression. However, HDT1/2-GFP fusion proteins are present at an equal level in transit-amplifying cells. It seems unlikely that they have a function in a specific stage of the cell cycle. This is consistent with the observation that cell cycle duration is not affected in *hdt1,2i-1*. We expect that HDT proteins are especially made when the chromatin is duplicated, similar to histone transcription and translation. The equal levels of HDTs might be caused by the relatively stable nature of these proteins. This is supported by the occurrence of HDT1/2-GFP in expanding cells whereas GUS is not detectable. An alternative explanation is that HDT1/2 are transported to adjacent cells. This could explain why low levels of HDT1/2-GFP are detected in the SCN, whereas GUS is below the detection level ($n > 20$).

We show that *HDT1* and *HDT2* determine the RM cell number. Notably, treatment with trichostatin A (TSA), an inhibitor of certain zinc binding motif containing histone deacetylases (Finnin et al., 1999), increases the RM cell number (Rosa et al., 2014). This seems in disagreement with our finding that decreased HDT activity leads to a reduced cell number. However, although HDTs contain a zinc binding domain it has not been demonstrated that their activity is targeted by TSA. Furthermore, other studies show that application of TSA inhibits root growth and triggers QC divisions (Nguyen et al., 2013; Pi et al., 2015). To determine whether TSA could target HDT1/2 we compared the effect of TSA on QC cell division in WT and *hdt1,2i-1*. This showed that a TSA treatment and *HDT1/2* knockdown increase the frequency of QC divisions in a similar way (Supplemental Figure 4I), indicating that TSA-mediated QC divisions do not involve HDT1/2 but involve other targets.

Overexpression of *GA2ox2* causes a similar RM phenotype as *HDT1/2* knockdown (Figure 2F, 7B, 7C). Therefore, in *hdt1,2i* the up-regulation of *GA2ox2*, (in part) causes the decreased RM cell number. Up-regulation of *GA2ox2* in *hdt1,2i* root tips (Figure 8A, 8B) causes most likely a rapid degradation of GA. In agreement with this, the *hdt1,2i-1* RM cell number is partially restored by GA_3 , and hardly affected by GA_4 (Figure 4D). In addition, PAC which decreases RM cell number in WT that can be restored by GA_3 (Ubeda-Tomas et al., 2009), has no additional effect on *hdt1,2i-1* RM cell number (Figure 4E). This suggests that GA level in *hdt1,2i-1* is very low. The most direct way to show that GA level is reduced in *hdt1,2i-1* would be by GA quantification, which is technically difficult. However, the increased accumulation of RGA, as well as the decreased PIN1/2 abundance in *hdt1,2i-1* root tips (Figure 4A-4C, Supplemental Figure 7) are consistent with a reduced GA level. Similar phenotypes are also observed in other GA-deficient mutants or PAC-treated seedlings (Ubeda-Tomas et al., 2009; Moubayidin et al., 2010). A transit-amplifying cell exits the mitosis as it enters into the elongation zone (Blilou et al., 2002; Vanstraelen et al., 2009). GA is required to promote mitotic activity (Peng et al., 1997; Silverstone et al., 2001; Achard et al., 2009; Ubeda-Tomas et al., 2009). In line with this, GA biosynthesis mutants *ga1* and *ga3ox1 ga3ox2*, as well as a non-GA-degradable DELLA mutant have a reduced RM cell number due to an earlier exit from the mitotic cell cycle (Ubeda-Tomas et al., 2009; Moubayidin et al., 2010). We postulate that in *hdt1,2i* the up-regulation of *GA2ox2*, causes most likely a reduced GA level that results in an earlier switch from division to expansion and in this manner (in part) decreases RM cell number.

Previously it has been shown that *GA2ox6* transcript level in Arabidopsis roots is higher than that of *GA2ox2* (Dugardeyn et al., 2008). However, *GA2ox6* is only expressed in the differentiated zone (Supplemental Dataset 2). In WT both genes are hardly expressed in root tips, whereas in *hdt1,2i-1*, *GA2ox2* is remarkably up-regulated in RM and its expression starts to decrease in the distal elongation zone and becomes undetectable in the proximal elongation zone and differentiated zone (Figure 8B, Supplemental Dataset 2). This suggests that GA levels will only be disturbed in RM and the distal elongation zone. GA controls the size of fully differentiated root cells (Band et al., 2012; Shani et al., 2013). This is in agreement with the observation that the size of fully differentiated root cells is not affected (Table 1, Supplemental Table 1).

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SHY2 is an AUX/IAA-type transcriptional repressor that triggers the switch from cell division to expansion (Dello loio et al., 2008; Moubayidin et al., 2010). *SHY2* expression is induced in this transition zone by *ARR1* and *ARR12*, two cytokinin-responsive transcription factors, which are expressed in the transition zone as well. GA represses the expression of *ARR1* and in this manner delays the accumulation of *SHY2* (Mason et al., 2004; Mason et al., 2005; Dello loio et al., 2008). However, we did not observe any effect on *ARR1/SHY2* transcript levels in *hdt1,2i-1* RM and elongation zone. Possibly the reduced GA level in *hdt1,2i-1* transit-amplifying cells causes *ARR1/SHY2* transcript to accumulate so the proteins will reach the threshold level required to induce the switch from cell division to expansion earlier than in WT. This most likely does not affect their transcript levels in total root extracts (Dello loio et al., 2007; Dello loio et al., 2008), as the size of the region where they are expressed is most likely not affected.

The fact that *hdt1,2i* lines have a stronger phenotype than the *hdt2* mutant points to an additive effect of *HDT1* and *HDT2* in controlling root growth (Figure 1B, 1C, Supplemental Figure 2). They both presumably repress *GA2ox2* expression, as in *hdt1,2i-1/8* lines this gene is induced much higher than in *hdt2* (Figure 5A), whereas *HDT2* mRNA levels in these mutants are very similar (Figure 1B). This could mean that *HDT1* and *HDT2* repress the expression of same genes, but together have to be present at a sufficient level. This is supported by the observation that the *hdt1* mutant has no root phenotype, but has an increased *HDT2* expression level (Figure 1B, 1C).

GA2ox2 is only one gene out of 217 DEGs in *hdt1,2i-1*. However, knockout of *GA2ox2* rescues *hdt1,2i-1* root growth significantly and *GA2ox2* overexpression phenocopies *hdt1,2i-1* (Figure 2F, 7B, 7E). This suggests that *GA2ox2* up-regulation is a cause of the short-root phenotype in *hdt1,2i*. *HDT2* binds to 2 exons of *GA2ox2*, as well as to the TATA box (Figure 5B and 5C). The identified *HDT2* binding sites from *GA2ox2* are also potential *HDT1* binding sites, as *HDT1* and *HDT2* are most likely functionally redundant. Binding to the TATA box appears to be important as *pGA2ox2:GUS* is markedly enhanced in *hdt1,2i-1* background. This reporter construct does contain the TATA box but not the 2 exons (see Methods). The TATA box is considered to be the binding site of basal transcriptional machinery (Patikoglou et al., 1999; Bernard et al., 2010; Liu et al., 2015a). Knockdown of *HDT1/2* results in increased histone acetylation

levels of the *GA2ox2* locus. We postulate that this increases the accessibility of the *GA2ox2* promoter to basal transcription factors.

Remarkably, the DEGs in *hdt1,2i-1* are enriched in genes involved in response to various stimuli and root development (Supplemental Figure 6). This suggests that HDT1/2 play a role in adaptation of root development to environmental stimuli. It has been shown that mild abiotic stress conditions in general lead to transiently reduced growth that is reversible. The hormone abscisic acid (ABA) plays a key role in regulating stress responses and exogenously applied ABA and salt reduce RM cell number in *Arabidopsis* seedlings (Yang et al., 2014; Liu et al., 2015b). Expression of the four *HDTs* is repressed by ABA and salt (Luo et al., 2012). We postulate that the *HDTs* may be part of a mechanism that modulates root growth upon response to abiotic stress. Repression of *HDT1/2* results in retarded root growth by reducing the number of transit-amplifying cells. However, the SCN is well maintained (Figure 2A, 2B, Supplemental Figure 4A) and thus the root can re-establish growth upon release of the stress.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* accession Columbia-0 was used as WT. The T-DNA insertion lines used are the following: GK355_H03 for *HDT1* (*At3g44750*), SAIL_1247_A02 for *HDT2* (*At5g22650*), SAIL_240_C08 and SALK_129799 (Luo et al., 2012) for *HDT3* (*At5g03740*), GK_279_D04 for *HDT4* (*At2g27840*) and SALK_051749 for *GA2ox2* (*At1g30040*), (Alonso et al., 2003; Rieu et al., 2008). All seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The T-DNA insertions were verified by genotyping with primers listed in Supplemental Table 2.1. The *Arabidopsis* GFP reporter lines used are *pSCR:SCR-GFP* (Helariutta et al., 2000), *pRGA:GFP-RGA* (Silverstone et al., 2001), *pPIN1:PIN1-GFP* (Benkova et al., 2003) and *pPIN2:PIN2-GFP* (Blilou et al., 2005).

Plants were grown vertically on ½ Murashige and Skoog medium including vitamins (Duchefa) and 1% sucrose under long-day conditions (16h light/8h dark) at 22 °C. For the GA and PAC treatment, 2-days-old seedlings were transferred to the same medium containing GA₃, GA₄ or PAC (Duchefa).

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Root Growth Phenotype Analyses

Root length of *Arabidopsis* plants was measured at 7 DAG. The cell number in root meristem was determined by counting cortical cells from the cortical-endodermal initial cell to the first expanding cortical cell daily from 1 to 8 DAG. For the kinematic growth analyses, root tips of ~ 30 seedlings were marked at 6 and then at 7 DAG, and the length increase within this 24-hour interval was determined by measuring the distance between these two marks. The size of fully differentiated cells was determined by measuring the length of cortical cells in the differentiated zone. All measurements were done by using Image Lab 2.0 Software (Bio-Rad, USA).

Histology and Microscopy

Arabidopsis roots were mounted on slides in chloral hydrate solution and analyzed under Axio Imager A1 microscope (Zeiss) with Nomarski optics. β -glucuronidase (GUS) activity was visualized after incubation of transgenic plants for 1 hour at 37°C in 0.1 M NaH_2PO_4 - Na_2HPO_4 (pH 7) buffer including 3% sucrose, 0.05 mM EDTA, 0.5 mg/ml X-gluc, 2.5 mM potassium ferrocyanide and potassium ferricyanide (Jefferson et al., 1987). All confocal images were acquired using Leica SP2 confocal laser scanning microscope (Leica, Germany). Fresh transgenic roots were mounted on slides with 10 μM propidium iodide for cell wall staining. GFP was detected with an excitation wavelength of 488 nm and propidium iodide was detected with an excitation wavelength of 543 nm. GFP intensity was measured by using Image Lab 2.0 Software (Bio-Rad, USA). To visualize starch in columella cells, mPS-PI staining was performed according to (Truernit et al., 2008).

Constructs and Plant Transformation

To generate *pHDT1:HDT1-GFP* and *pHDT2:HDT2-GFP* constructs the genomic sequences of *HDT1* and *HDT2* including their putative promoters (1.5 kb for *HDT1* and 0.5 kb for *HDT2*) were PCR amplified from genomic DNA using Phusion High-Fidelity DNA Polymerase (FINNZYMES), and directionally cloned into pENTR-D-TOPO (Invitrogen). Subsequently, these pENTR-D-TOPO constructs, the pENTR 4-1 vector (Invitrogen) and the pENTR 2-3 vector (Invitrogen) containing a GFP open reading frame and a CaMV 35S terminator were recombined into the binary destination pBnRGW vector by Multisite Gateway reaction. The pBnRGW vector is a modified vector based on PKGW

(Karimi et al., 2002) including the *pNAP:RFP* expression cassette from pFluar101 (Stuitje et al., 2003) for easy selection of red fluorescent transgenic seeds. For *pHDT1:GUS*, *pHDT2:GUS* and *pGA2ox2:GUS* constructs, the promoters (1.8 kb for *GA2ox2*) were cloned into the pENTR-D-TOPO vector. The pENTR-D-TOPO constructs, the pENTR4-1 vector (Invitrogen) and the pENTR 2-3 vector containing a GUS were recombined into the binary destination pBnRGW vector by Multisite Gateway reaction. The primers used for making these constructs are listed in Supplemental Table 2.2.

To build the *pRCH1:HDT1HDT2 RNAi* construct (*hdt1,2i*), the CaMV 35S promoter in pK7GWIWG2 vector (Limpens et al., 2005) was replaced with *RCH1* promoter [pK7GWIWG2(II)]. The *RCH1* promoter was first amplified on genomic DNA and cloned into pENTR-D-TOPO. Then it was cut out from the vector by HindIII (partial digestion) and Xba I and recombined with two fragments of the pK7GWIWG2(II) vector in a three-point ligation. The two fragments of pK7GWIWG2(II) vector were obtained by digestion either with HindII and Nco I, or with Spe I (compatible with Xba I) and HindIII. The whole *RNAi* cassette including the *RCH1* promoter was cut out from the vector using Apa I and HindIII and ligated into the pBnRGW binary vector digested with the same enzymes and in such way creating pBnRRGWIWG vector. The *RNAi* target sequences of *HDT1* and *HDT2* coding sequences (0.6 kb for each) were combined in one amplicon using a two-step PCR, following the procedure described in (Franssen et al., 2015). In brief, the first step PCRs introduced short overlaps (15 bp) in PCR fragments of *HDT1* and *HDT2* coding sequences with *HDT1nai-F* and *HDT1nai-R* primers, or with *HDT2nai-F* and *HDT2nai-R* primers. These two fragments were used as templates in the second step PCR with *HDT1nai-F* and *HDT2nai-R* primers. The final PCR fragment was introduced into pENTR-D-TOPO vector and recombined in inverse-repeat orientation into the pBnRRGWIWG binary vector by a LR Gateway reaction (Invitrogen). The primers used for *RNAi* are presented in Supplemental Table 2.3.

To construct *pRCH1:GA2ox2 (GA2ox2^{OE})*, the *RCH1* promoter was cut out of the pENTR-D-TOPO vector using the Not I and Asc I restriction sites, and then ligated with a Bsa I -digested pENTR4-1 vector. *GA2ox2* cDNA was cloned into pENTR-D-TOPO vector. These two vectors and the CaMV 35S terminator containing pENTR 2-3 vector were recombined in a Multisite Gateway reaction

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into the binary destination pBnRGW vector. The primers used are shown in Supplemental Table 2.4.

All constructed binary vectors were introduced into *Arabidopsis* through *Agrobacterium tumefaciens* (strain C58)-mediated transformation by floral dipping method (Clough and Bent, 1998). The *Arabidopsis* (Columbia-0) plants were transformed with *pHDT1:GUS*, *pHDT2:GUS*, *pGA2ox2:GUS*, *pRCH1:HDT1HDT2 RNAi* and *pRCH1:GA2ox2* constructs. To test whether *pHDT1:HDT1-GFP* and *pHDT2:HDT2-GFP* could complement the *hdt1 hdt2* mutant, homozygous line for *hdt1* and heterozygous for *HDT2* plants were transformed with *pHDT1:HDT1-GFP* and *pHDT2:HDT2-GFP*. The primers used for confirming the complementation are listed in Supplemental Table 2.5.

Gene Expression Analyses

For RT-PCR, ~10 WT and *hdt* seedlings were collected at 6 DAG. For study of gene expression by RT-qPCR, ~100 root tips (including meristem and elongation zone) were collected at 6 DAG and this was done in three independent experiments (three biological replicates). The collected plant material was used for total RNA extraction using the E.Z.N.A. Plant RNA Kit (Omega, USA). cDNA was synthesized from 1 µg of RNA by reverse transcription with random hexamer primers using the iScript Select cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. RT-qPCR was performed in a 10-µl reaction with MyiQ SYBR Green Super-mix (Bio-Rad, USA). Each sample was quantified in triplicates using CFX3.0 software (Bio-Rad, USA) and normalized using *TUBULIN* as a reference. The normalization using *ACTIN* as a reference shows similar results. The primers used for RT-PCR or RT-qPCR are listed in Supplemental Table 2.6 or S2.7.

RNA-Seq

Root tips of ~1000 WT and ~2000 *hdt1,2i-1* seedlings at 6 DAG were cut and collected in three parts: the meristem zone (M, from columella till the disappeared lateral root cap, approximate 500 or 300µm in the WT or *hdt1,2i-1*), elongation zone (E, till the appeared root hairs, approximate 900 or 800µm in the WT or *hdt1,2i-1*) and differentiated zone (D, approximate 1 mm for both, to avoid lateral root primordia). The experiment was performed three times (three biological replicates). Total RNA from each zone was extracted using RNeasy Micro Kit (QIAGEN, Germany). RNA was sequenced at BGI Tech Solutions (Hong Kong)

using low-input Hiseq2000 instrument. This generated approximately 12 million 50 bp clean single-end reads for each sample. Sequencing data were analyzed by mapping to the *Arabidopsis thaliana* genome (TAIR10) using CLC Genomics Workbench (Denmark). Gene expression levels were determined by calculating the RPKM [(Reads Per Kilobase per Million mapped reads), (Consortium et al., 2006)]. Differentially expressed genes (DEGs) are defined based on relatively stringent statistics and filtering (fold change>2, FDR P value<0.05) within the CLC. GO enrichment analyses was performed using agriGO v2.0 (Tian et al., 2017).

Chromatin Immunoprecipitation (ChIP) Assay

Approximately 1 gram of WT and *pHDT2:HDT2-GFP* (in *hdt1hdt2* background) complemented seedlings at 6 DAG were used for each anti-GFP ChIP assay. Approximately 0.5 gram of WT and *hdt1,2i-1* roots (approximately 5mm from the tip) at 6 DAG were used for each anti-H3ac ChIP assay. For both assays 3 independent ChIP experiments were performed as described in (Kaufmann et al., 2010), with some modifications. For anti-GFP assays, sonicated chromatin was incubated with GFP-Trap agarose beads (Chromotek) for 90 min at 4°C. For anti-H3ac assays, sonicated chromatin was immunoprecipitated with an anti-H3ac antibody (06-599, Millipore) for 1 h at 4°C and then incubated with Protein A/G PLUS-Agarose beads (SantaCruz) for 50 min at 4°C. Immunoprecipitated DNA was recovered using the IPure kit v2 (Diagenode) and quantified using Qubit dsDNA HS Assay Kit (Life Technologies). An aliquot of non-treated sonicated chromatin was processed in parallel and used as total input DNA control. ChIP-qPCR data analyses was performed according to (Frank et al., 2001). All ChIP enrichments were calculated as % DNA immunoprecipitated at the locus of interest relative to the corresponding input samples, and normalized to the % DNA immunoprecipitated at that locus in WT plants. The primers used for ChIP-qPCR are listed in Supplemental Table 2.8.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *HDT1* (At3g44750); *HDT2* (At5g22650); *HDT3* (At5g03740); *HDT4* (At2g27840); *MATE* (At2g04050) and *GA2ox2* (At1g30040).

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Supplemental Data (available online: <http://www.plantcell.org/content/early/2017/08/30/tpc.17.00366>).

Supplemental Figure 1. Genome Structure of *HDT1-4* and *GA2ox2* Genes.

Supplemental Figure 2. HDT1/2 Control Root Growth.

Supplemental Figure 3. Expression of *HDT1* and *HDT2* Genes in WT and *hdt1,2i* Lines.

Supplemental Figure 4. Reduced Cell Number in *hdt1,2i* and *GA2ox2^{OE}* RM Is Caused by an Earlier Switch from Cell division to Expansion.

Supplemental Figure 5. Expression Pattern of *pHDT1:HDT1-GFP* and *pHDT2:HDT2-GFP* in Roots.

Supplemental Figure 6. Gene Ontology (GO) Enrichment Analyses of DEGs in *hdt1,2i-1* Roots.

Supplemental Figure 7. PIN1 and PIN2 Proteins Are Reduced in *hdt1,2i-1*.

Supplemental Table 1. Kinematic Analyses of Root Growth in WT and *hdt1,2i-8* Seedlings.

Supplemental Table 2. Primers Used in This Study.

Supplemental Dataset 1. Expression of Histone Acetylation Genes from 8 Subzones of the Root.

Supplemental Dataset 2. Gene Expression Map in the WT and *hdt1,2i-1* Roots.

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AUTHOR CONTRIBUTIONS

H.L. conceived this project, designed the research, analyzed the data and wrote the article. H.L., J.T., S.S and W.Z. conducted the experiments. D.L., M.B. and H.H contributed to ChIP studies. O.K. and T.B. modified the article.

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CHAPTER 3

Plant-specific Histone Deacetylases Are Involved in Halotropism in Arabidopsis

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ABSTRACT

Plants evolve sophisticated mechanisms to coordinate their growth and development with salt stress. Halotropism is a response of plant root to avoid a saline environment. However, the mechanism of halotropism is still elusive. Here we show that silencing of *Arabidopsis thaliana* *AtHDT1* and *AtHDT2*, encoding plant-specific histone deacetylases, causes a more severe response of the roots in halotropism. By contrast, constitutively activated *AtHDT2* expression reduces halotropic response. We further show that in halotropism *AtHDT1/2* are reduced at the high salt side of root but their target *C₁₉-GIBBERELLIN 2-OXIDASE 2* (*AtGA2ox2*) is induced there, in comparison to the side opposites to high salt. These asymmetric patterns most likely cause a lower GA level at the high salt side. Exogenously applied GA inhibits halotropic response. Collectively, our data strongly suggest that the asymmetric *AtHDT1/2* pattern is required for root halotropic response.

INTRODUCTION

Plants growing in nature encounter various environmental cues. To cope with these cues, they have a flexible morphology and growth potential. For example, upon salt stress, which is one of the major abiotic factors hindering root growth (Zhu, 2002; Liu et al., 2015), roots can acclimate by using halotropism to bend away from a salty environment (Galvan-Ampudia et al., 2013).

The establishment of halotropism depends on a signal transduction pathway which is activated after the perception of salt. It has been shown that in halotropism a re-distribution of auxin results in increased auxin levels at the side of the root opposite to high salt while higher auxin inhibits cell expansion (Rayle et al., 1970). The re-distribution of auxin is dependent of PIN-mediated auxin transport, like occurs in gravitropism and phototropism (Tanimoto, 2005; Petrasek et al., 2006; Wisniewska et al., 2006; Liscum et al., 2014). In gravitropism also an asymmetric distribution of gibberellin (GA) occurs in *Arabidopsis*, Rice, Maize and Barley (Rood et al., 1987; Cui et al., 2005; Wolbang et al., 2007; Lofke et al., 2013). Probably this contributes to halotropism as well.

Previously, we showed that in *Arabidopsis* root tips two plant-specific histone deacetylases, AtHDT1 and AtHDT2 repress the transcription of *C₁₉-Gibberellin 2-OXIDASE 2 (AtGA2ox2)* to fine-tune GA homeostasis (Chapter 2). Therefore we ask the question whether AtHDT1/2 play a role in halotropism.

Here, we show that the *AtHDT1/2 RNAi* knockdown mutant, *hdt1,2i-8*, has a more severe halotropic response than WT. During this response the level of AtHDT1/2 protein is reduced, and by contrast expression of *AtGA2ox2* is induced at the high salt side. Disrupting the asymmetric accumulation of AtHDT2 by constitutive expression of *AtHDT2* or by applying exogenous GA reduce the halotropic response. Our preliminary data indicate that the asymmetric distribution of AtHDT1/2 in a root contributes to halotropism most likely by decreasing the GA concentration at the high salt side.

RESULTS

AtHDT1/2 Have a Function in Halotropism

To study whether AtHDT1/2 are involved in halotropism, we performed salt gradient assay with 200mM NaCl as a highest concentration according to [(Galvan-Ampudia et al., 2013), see Methods] on WT and *hdt1,2i-8* seedlings. In this assay we could study halotropic response by measuring root bending angle. In both cases roots changed their growth direction to circumvent high salt and later on gravitropism was re-established (Figure 1A, 1B). Quantification of the root bending angle showed that this was significantly increased in *hdt1,2i-8*, compared to that in the WT (Figure 1C, 1D). This shows that knockdown of *AtHDT1/2* leads to a more severe response to high salt.

Halotropism Induces an Asymmetric AtHDT1/2 Distribution in Root Tips

To test whether the halotropic response is due to a regulation of *AtHDT1/2* expression by salt, we transferred 6-days-old WT seedlings from a plate without NaCl to a plate containing 100mM NaCl and examined expression of *AtHDT1/2* in root tips by qRT-PCR. The control seedlings were transferred to plates with 0 mM NaCl. This showed that at 3 hours(h) after transfer (Figure 2A), expression of *AtHDT1* and *AtHDT2* were 50% and 30% reduced, respectively. Their expression levels were not changed at 6h after salt treatment, compared to 3h. This indicates that salt stress affects the expression of *AtHDT1/2* rapidly and the major difference occurs as an early response to salt.

As *AtHDT1/2* expression responds to salt, we ask the question whether AtHDT1/2 obtains an asymmetric distribution during halotropism. To examine this, Arabidopsis seedlings expressing *pHDT1:HDT1-GFP* or *pHDT2:HDT2-GFP* were exposed to the salt gradient. AtHDT1/2 levels in root tips at the high salt side was compared with that of the side opposites to high salt. AtHDT1/2 levels were studied by confocal microscopy. Both proteins are nuclear localized (Chapter 2). To ensure the whole nucleus is included we made z-stacks (~8μm) of the epidermal cells. The moment when the formation of salt gradient was started was defined as 0h. This showed that at 6h after exposure to the salt gradient, both AtHDT1 and AtHDT2 were lower at the high salt side (Figure 2B, 2C), likely caused by salt induced repression of *AtHDT1/2* expression. Both proteins were mainly localized in nucleoli, like occurred under normal

Figure 1. AtHDT1/2 Inhibit Halotropic Response Likely by Disruption of the Establishment of Asymmetric Gibberellin Distribution.

(A), (B), (E) and (F) Halotropic response of seedlings after exposing to salt gradients for 48h. 6-days-old WT (A), *hdt1,2i-8* (B), or *HDT2^{C1}* (E) seedlings were transferred to mock. 2-days-old WT seedlings were pre-treated by 1mM GA₃ for 4 days and then transferred to the medium containing 1mM GA₃ (F). Then salt gradients were generated by 200mM NaCl according to (Galvan-Ampudia et al., 2013). Arrowheads indicate the position of root tip at the start point of exposing to salt gradient.

(C) and (D) Quantification of WT (C) and *hdt1,2i-8* (D) root angles in salt gradient after 24h. Bars represent percentage of root bending angle with 10 degrees range. The number indicates how many roots were measured. Different letters indicate a significant difference is present between the two groups ($p < 0.001$, Student's *t* test).

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growth conditions (Chapter 2). These data indicate that during the halotropic response, salt does not affect AtHDT1/2 sub-nuclear localization, but reduces their expression levels at the high salt side.

Constitutive Activation of *AtHDT2* Reduces Halotropism

Our data indicate that the reduction of *AtHDT1/2* expression at the high salt side of root tips is part of the mechanism of halotropism. This would mean that a constitutive expression of *AtHDT1/2* will decrease the halotropic response. To test this, we generated *35S:AtHDT2* lines that constitutively express *AtHDT2*, by fusing *AtHDT2* coding sequence with the *CaMV 35S* promoter (Odell et al., 1985). This promoter does not respond to salt, therefore exposure to a salt gradient will not reduce *AtHDT2* expression (Hou et al., 2016). Four lines (*HDT2^{C1-4}*) were generated and they did not show a root phenotype, compared to the WT under control condition (data not shown). We exposed *HDT2^{C1}* seedlings to a salt gradient and monitored their root growth directions. This line was randomly selected out of four lines, and the halotropic response of this line was markedly reduced, compared to the WT (Figure 1A, 1E). This indicates that the asymmetric distribution of *AtHDT2* contributes to the halotropic response.

Halotropism Induces an Asymmetric *AtGA2ox2* Expression in Root Tips

Previous studies showed that AtHDT1/2 repress the transcription of *AtGA2ox2* in root tips. To test whether the role of AtHDT1/2 in halotropism involves *AtGA2ox2* repression, we determined expression level of *AtGA2ox2* in root tips by qRT-PCR after transferring 6-days-old seedlings to medium containing 100 mM NaCl. The control seedlings were transferred to plates with 0mM NaCl. This showed that at 3h after transfer, a 10 times up-regulation of *AtGA2ox2* was observed (Figure 2A). Its expression level was also not changed at 6h after salt treatment, compared to 3h. This indicates that *AtGA2ox2* also responds to salt stress early.

To study whether *AtGA2ox2* obtains an asymmetric distribution during halotropism, we first generated a *pAtGA2ox2:3YFP_{nls}* reporter line by fusing the *AtGA2ox2* promoter to three consecutive copies of Yellow Fluorescent Protein containing a nuclear localization signal. This protein has a higher fluorescence intensity than regular YFP and also remains in the cell where it is produced due to its high molecular weight (Mathieu et al., 2007). Under control condition, a low level of expression of *pAtGA2ox2:3YFP_{nls}* occurred in vascular tissue and

Figure 2. Salt Stress Reduces *AtHDT1/2* Expression and Accumulation, But Induces *AtGA2ox2* Expression.

(A) Quantitative real time PCR (qRT-PCR) analyses of *AtHDT1/2* and *AtGA2ox2* expression in root tips of 6-days-old WT seedlings after 100mM NaCl treatment for 0 hour (h, Mock), 3h and 6h. All data show mean $\pm$ SE values determined from three independent experiments (each experiment was estimated as the average of three technical replicates).

(B) to (D) Asymmetric patterns of *AtHDT1* **(B)**, *AtHDT2* **(C)** and *AtGA2ox2* **(D)** in root tips during halotropic response. Arabidopsis seedlings expressing *pHDT1:HDT1-GFP* **(B)**, *pHDT2:HDT2-GFP* **(C)** or *pAtGA2ox2:3YFPnls* **(D)** were grown in salt gradients for 6h **(B and C)** or 24h **(D)**. White arrows indicate the high salt side. For each reporter ~10 seedlings were imaged and representative data were shown. Bars=50 μ m.

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pericycle at the transition from root meristem to elongation zone (data not shown). This expression pattern is consistent with that of *pAtGA2ox2:GUS* (Chapter 2). We next exposed these plants to a salt gradient and compared *AtGA2ox2* expression levels at the high salt side with that at the side opposite to high salt. This showed that at 6h, no difference in *AtGA2ox2* expression was observed between the two sides. However, after 24h at the high salt side, expression of this gene was markedly increased, compared to the opposite side of root (Figure 2D). This indicates that high salt induces *AtGA2ox2* expression. The induction of *AtGA2ox2* was later than the reduction of *AtHDT1/2* which supports the conclusion that high salt reduces the *AtHDT1/2* level and this subsequently leads to an increased expression of *AtGA2ox2*.

Symmetric GA Distribution Reduces Halotropism

3 Both *AtHDT1/2* and *AtGA2ox2* obtain asymmetric patterns during halotropism. This most likely results in a low GA level at the high salt side (Chapter 2). To investigate whether this contributes to the halotropic response we exogenously applied 1 μ M GA₃ to WT seedlings to monitor their root growth direction. GA₃ is not a substrate of *AtGA2ox2* and has previously been shown to rescue root phenotype in GA-deficient *ga1-3* mutants (Thomas et al., 1999; Hedden and Phillips, 2000; Yamauchi et al., 2007; Ubeda-Tomas et al., 2009). Therefore we hypothesize that its application avoids the establishment of asymmetric GA distribution when roots are exposed to a salt gradient. This showed that application of GA₃ caused a markedly reduced halotropic response (Figure 1A, 1F), indicating that an asymmetric distribution of GA in root tips contributes to halotropism.

DISCUSSION

We show that during the halotropic response *AtHDT1/2* become asymmetrically distributed with the lower level at the high salt side. The mechanism by which the *AtHDT1/2* control halotropism most likely involves regulation of asymmetric GA accumulation. The involvement of *AtHDT1/2* in controlling halotropism seems to be in disagreement with the observation that in the *AtHDT1/2 RNAi* line the halotropic response is more severe than in WT. This implies that with asymmetric GA distribution in the meristem the additional mechanisms can contribute to halotropism. For example, study of PIN1 and PIN2 accumulation

in the *AtHDT1/2 RNAi* line exposed to salt gradient might shed light on this.

The involvement of *AtHDT1/2* in halotropism is consistent with the fact that an exposure to (homogenous) salt represses *AtHDT1/2* expression in root tips (Figure 2A), as well as in seedlings (Luo et al., 2012). In line with this, during halotropic response, at 6h after exposure to the salt gradient *AtHDT1/2* proteins are lower in root tips at the side of high salt (Figure 2B, 2C). Bending is initiated after ~9h (Christa Testerink, personal communication), which means that the decrease of *AtHDT1/2* levels most likely precedes the start of bending. Whether the increase of *AtGA2ox2* expression precedes bending cannot yet be concluded as we only measured its expression level at 6 and 24h after exposure to the salt gradient.

The importance of the asymmetric accumulation of *AtHDT1/2* and subsequently GA in halotropism is underlined by the reduced halotropism when *AtHDT2* is constitutively expressed (Figure 1C). It is also supported by the observation that exogenously application of GA also reduces the halotropic response (Figure 1D). The involvement of asymmetric accumulation of GA during halotropism is shared with that in gravitropism (Lofke et al., 2013). This indicates that the mechanisms controlling bending in both tropic responses share common aspects. A more degradation of PIN2 at the upper than the lower side of root plays an important role in gravitropism (Abas et al., 2006; Kleine-Vehn et al., 2008). *AtHDT1/2* appear to play a role in stabilizing PIN2 under control condition (Chapter 2). We hypothesize that in halotropism the reduction of *AtHDT1/2* is linked to observed endocytosis of PIN2 at high salt side (Galvan-Ampudia et al., 2013).

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis* accession Columbia-0 was used as WT. The *Arabidopsis pHDT1:HDT1-GFP* and *pHDT2:HDT2-GFP* reporter lines used were described as before (Chapter 2).

Plants were grown vertically on ½ Murashige and Skoog medium (pH 5.8) including vitamins (Duchefa), 0.5% sucrose and 1% Daishin agar (Duchefa) under long-day conditions (16h light/8h dark) at 22 °C. At 6 days after

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germination, seedlings were transferred to the medium containing homogenous 100mM NaCl for a corresponding period, or to plates in which the formation of the salt gradient was started when root tips were above the diagonal border below which 200mM salt was applied according to (Galvan-Ampudia et al., 2013). For the GA₃ treatment, 2-days-old seedlings were pre-treated with GA₃ for 4 days before transferring them to the in the salt gradient with mock side containing GA₃.

Root Growth Phenotype Analyses and Microscopy

After transferring, only those seedlings with a 5-10 mm distance from root tips to media interphase at the moment of generating in gel salt gradient were studied. Root growth direction was monitored on vertical plates by marking the root tip at 0h and 24h. 0h was defined as the moment when the salt gradient medium was set. Root bending degree was defined by the angle between vertical line and the straight line between the two marks. The measurement was done by using Image Lab 2.0 Software (Bio-Rad, USA).

Roots imaged during halotropism were cut out together with the gel to keep orientation and mounted on cover slides with or without 10 μ M propidium iodide for cell wall staining. All confocal images were acquired using Leica SP8 confocal laser scanning microscope (Leica, Germany). GFP, YFP and propidium iodide were detected with an excitation wavelength of 488 nm, 514nm and 543 nm, respectively.

Constructs and Plant Transformation

To construct *pAtGA2ox2:3YFPnls*, *AtGA2ox2* promoter was cut out of the pENTR-D-TOPO vector (Chapter 2) using the Not I and Asc I restriction sites, and then ligated with a Bsa I digested pENTR4-1 vector (Invitrogen). This vector, the pGEM-T easy 221 entry vector containing 3 consecutive copies of YFP with nucleus localization signal (Ben scheres), and the CaMV 35S terminator containing pENTR 2-3 vector (Invitrogen) were recombined in a Multisite Gateway reaction into the binary destination pBnRGW vector (Karimi et al., 2002; Stuitje et al., 2003).

To generate *35S:AtHDT2* (*HDT2^C*) construct, the coding sequence of AtHDT2 was first amplified by primers, Forward: CACCATAAAATAGCCCCAAACCCACTGCC and Reverse: AGCTCTACCCTTTCCCTTGC, and then directionally cloned into

pENTR-D-TOPO vector (Invitrogen). Subsequently, this vector, the pENTR 4-1 vector containing CaMV 35S promoter (Invitrogen) and the pENTR 2-3 vector containing a CaMV 35S terminator (Invitrogen) were recombined into the binary destination pBnRGW vector by Multisite Gateway reaction (Karimi et al., 2002; Stuitje et al., 2003).

The two constructed binary vectors were introduced into *Arabidopsis* (Columbia-0) through *Agrobacterium tumefaciens* (strain C58)-mediated transformation by floral dipping method (Clough and Bent, 1998).

Gene Expression Analyses

Total RNA from root tips (including meristem and elongation zone) was extracted using the plant RNA easy kit (Omega, USA). cDNA was synthesized from 1 µg of RNA by reverse transcription with random hexamer primers using the iScript Select cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. qRT-PCR was performed in a 10 µl reaction system with MyiQ SYBR Green Super-mix (Bio-Rad, USA). Each sample was quantified in triplicates using CFX3.0 software (Bio-Rad, USA) and normalized using *TUBULIN* as a reference. The primers used for qRT-PCR are listed in Chapter 2.

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CHAPTER 4

MAPK-triggered chromatin reprogramming by histone deacetylase in plant innate immunity

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ABSTRACT

Microbial-associated molecular patterns (MAMPs) activate several MAP Kinases (MAPKs), which are major regulators of the innate immune response in Arabidopsis that induce large-scale changes in gene expression. Here, we determined whether MAMP-triggered gene expression involves modifications at the chromatin level. Our results show that histone acetylation and deacetylation are major regulators of MAMP-triggered gene expression and implicate the histone deacetylase HD2B (named as HDT2 in Chapter 2 and 3) in the reprogramming of defense gene expression and innate immunity. The MAPK MPK3 directly interacts with and phosphorylates HD2B, thereby regulating the intra-nuclear compartmentalization and function of the histone deacetylase. By studying a number of gene loci that undergo MAMP-dependent activation or repression, our data reveal a mechanistic model for how protein kinase signaling directly impacts chromatin reprogramming in plant defense.

INTRODUCTION

Due to their sessile nature, plants have developed sophisticated ways to respond and adapt to a variety of external stress factors that would otherwise compromise proper development, reproductive success and ultimately survival. Numerous cellular proteins interact and communicate in response to extracellular stimuli and, through multiple signaling networks, transmit signals to the nucleus for reprogramming chromosomal gene expression. These dynamic regulatory mechanisms contribute to the capacity of plants to adapt to the onslaught of both biotic and abiotic challenges. Indeed, the success of photosynthetic eukaryotes is influenced by the adaptive dynamics of chromatin regulatory mechanisms, like histone modifications, which are rapid and reversible.

The choice of gene expression ultimately determines the fate of cells, forming the basis of biological diversity. The regulation of gene expression is closely coupled to chromatin structure and its modifications, which determine the accessibility of many regulatory proteins and non-coding RNAs to the DNA, adding a further layer of complexity to the genetic information encoded by the DNA sequence (Kouzarides, 2007; Saze et al., 2012; Mercer and Mattick, 2013). Chromatin is a tightly contained higher order structure that compacts genomic DNA to fit within the nucleus. The fundamental unit of chromatin is the nucleosome, which is composed of DNA that is wrapped around an octamer of histone proteins. Chromatin structure is modulated by a variety of mechanisms including DNA methylation catalyzed by DNA cytosine methyltransferases, histone post-translational modifications, such as acetylation and methylation, catalyzed by a wide range of enzymes specific for each modification, alterations in histone-DNA interactions that facilitate nucleosome sliding and are catalyzed by chromatin remodeling complexes, histone variants, and long and small noncoding RNAs (ncRNAs) that can act directly on chromatin and induce RNA-dependent DNA methylation (RdDM) (Bannister and Kouzarides, 2011; Bender, 2012; Keller et al., 2013).

Many developmental and environmental cues induce changes in chromatin structure. Plants sense pathogens through the perception of MAMPs, which induce signaling cascades to activate transcription factors and invoke chromatin regulatory mechanisms to reorganize the chromatin structure and, ultimately, provoke the changes in gene expression necessary for plant defense. Thus, in response to different stimuli, a single eukaryotic genome (DNA sequence) can

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give rise to distinct epigenomes.

Here, we examine the role of chromatin remodeling in *Arabidopsis thaliana* upon challenge with a synthetically produced 22 amino-acid long flagellin peptide (flg22) that mimics the response to bacterial pathogens. Flg22 is recognized in *Arabidopsis* by the plasma membrane Leucine-Rich Repeat-Receptor Kinase (LRR-RK) FLS2 and activates two MAPK signaling pathways that initiate an array of defense responses including the production of several hormones, reactive oxygen species and the induction of a large set of defense genes, processes generally referred to as MAMP-triggered immunity (MTI). These two main cascade branches involve the successive recruitment of MAP kinase kinase kinases (MAP3Ks), which phosphorylate and activate MAP kinase kinases (MAP2Ks) that phosphorylate and activate the MAP kinases MPK3, 4, 6 and 11 (Asai et al., 2002; Gao et al., 2008; Bethke et al., 2012). Ultimately, these MAPKs phosphorylate and thereby regulate protein factors responsible for the increased or decreased expression of specific gene sets with the goal to counteract the pathogen assault. Although specific transcription factors have been identified as targets for MPK3 (Djamei et al., 2007), MPK4 (Andreasson et al., 2005) and MPK6 (Bethke et al., 2009), much of the protein machinery orchestrating gene regulation in response to flagellin has not been identified.

Here, we report a role for the MAP kinase MPK3 in chromatin modulation and dissect the defense mechanism during the response to flagellin. We show that MPK3 interacts directly with and phosphorylates the histone deacetylase HD2B, which has been shown to deacetylate the lysine 9 residue of Histone 3 (H3K9), a modification generally linked to the compaction of chromatin. We also show that, upon flagellin perception, HD2B is re-localized from the nucleolus to the nuclear compartment, leading to global genome-wide shifts in the H3K9 acetylation landscape. We identified flg22-regulated defense genes targeted by the MPK3-HD2B regulatory module and show that HD2B is directly implicated in bacterial defense in plants. Our results mechanistically define how a MAMP-activated MAP kinase regulates global changes in the chromatin landscape.

RESULTS

MPK3 Interacts with and Phosphorylates HD2B *in vivo*

Our previous phosphoproteomic approach aimed at identifying MAPK substrates revealed 303 *in vivo* phosphorylation sites in proteins isolated from Arabidopsis root cells (de la Fuente van Bentem et al., 2008). Among these sites, 91 matched the proline-directed motifs pS/pT-P that commonly serve as phosphorylation sites for MAPKs. Because we were interested to identify global regulators of gene expression that play a role in pathogen defense, we selected the histone deacetylase (HDAC) HD2B, which was phosphorylated at the amino acid positions T249 and S266, matching the S/T-P consensus motif for MAPK substrates (Supplemental Figure S1-S5).

We first assayed whether HD2B was a substrate of the canonical MAPK pathways activated by pathogen recognition. To this end, we immuno-purified endogenous flg22-activated MPK3, MPK4 and MPK6 from a root cell culture extract and tested whether the MAPKs phosphorylated HD2B *in vitro*. Using MBP and GST as positive and negative controls, respectively, MPK3 showed the highest phosphorylation activity toward GST-tagged HD2B among the 3 tested MPKs (Figure 1A), whereas MPK4 and MPK6 showed a weaker substrate preference to HD2B when compared with the artificial substrate MBP (Figure 1A). To control our MAPK antibody specificity, we performed kinase assays with protein extracts from *mpk3*, *mpk4*, *mpk6* and *mpk7* mutants and their respective wild type lines after immunoprecipitation with the corresponding antibodies (Supplemental Figure S6). MPK7 antibody was used as a negative control as this kinase is not activated upon flg22 treatment. The results indicated that the antibodies specifically immunoprecipitated the respective kinases (Supplemental Figure S6). To confirm the substrate specificity of the phosphorylation of HD2B by MPK3, we also tested several additional substrates that were identified in the phosphoproteome screen. As shown in Supplemental Figure S7, PI-4K β 1 (At5g64070) was specifically phosphorylated by MPK3 and MPK6, SCF (At5g13300) was not phosphorylated by any of the three immune MAPKs, MSL9 (At5g19520) and GOS12 (At2g45200) were preferentially phosphorylated by MPK6. To determine whether MPK3 phosphorylated the previously identified HD2B *in vivo* sites T249 and S266 (de la Fuente van Bentem et al., 2008), the T249 amino acid residue was mutated individually to glutamate or in combination with S266 to aspartate residues (HD2B-T249E

Figure 1. MPK3 Phosphorylates HD2B *in vitro* and Interacts with HD2B.

(A) *In vitro* phosphorylation assays of MBP (left), GST (middle) and HD2B-GST (right) by MPK3 (3), MPK4 (4) and MPK6 (6). MPK3, 4 and 6 were immunoprecipitated from flagellin-treated root cell suspensions. For each assay, Coomassie staining of the gel (CBB) is shown on the left as a protein loading control and the autoradiography (Autorad) is shown on the right. MPK3, 4 and 6 were able to phosphorylate MBP, but not GST. HD2B phosphorylation was observed with all three MPKs, but MPK3 showed the highest activity. Arrows

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and HD2B-T249E/S266D, respectively; denominated further as HD2B-ED), and these mutant HD2B proteins together with WT HD2B were tested for their ability to serve as MPK3 substrates (Figure 1B). In comparison to WT HD2B, HD2B-T249E showed reduced and HD2B-T249E/S266D complete lack of phosphorylation by MPK3, indicating that the *in vivo* phosphorylation sites T249 and S266 can be targeted by MPK3 *in vitro* (Figure 1B).

To better understand where the MPK3 and HD2B interaction takes place, we investigated the subcellular localization of the HD2B and MPK3 proteins. A GFP-HD2B fusion protein was localized in the nucleolus (Zhou et al., 2004; Pendle et al., 2005), whereas MPK3 was observed in both cytoplasmic and nuclear cell compartments (Figure 1C and Supplemental Figure S8). Using indirect immunofluorescence staining, we found that MPK3 and HD2B were co-localized in the nucleoli of the same plant tissue (Figure 1D). To determine if MPK3 and HD2B can interact *in vivo*, we performed a bimolecular fluorescence

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indicate full-length forms of all substrate proteins. IgG indicates immunoglobulin G used for immunoprecipitation of the MAPKs. Asterisks indicate an unknown phosphoprotein in the MPK4 lanes, or MPK6 autophosphorylation in the MPK6 lanes.

(B) *In vitro* phosphorylation assays of wild type HD2B (WT) and the mutant HD2B protein forms HD2B-T249E and HD2B-T249E/S266D. Coomassie staining of the gel (CBB) is shown as a protein loading control.

(C) Cell fractionation assay of MPK3 localization. Proteins from total, cytoplasmic and nuclear cell extracts were subjected to SDS-PAGE and analyzed by immunoblotting with anti-MPK3 antibody. Anti-H3 and anti-PEPC antibodies were used as controls for nuclear and cytoplasmic proteins, respectively.

(D) Immuno-staining of MPK3 and HD2B performed on plants expressing a GFP-HD2B fusion. Antibodies used to reveal the presence of the proteins are noted above the panels. DAPI staining was used to label the nuclei. MPK3 could be detected both in the cytoplasm and in the nucleus while GFP-HD2B was only observed in the nucleus.

(E) BiFC analysis of HD2B interaction with MPK3 in epidermal cells of *Agrobacterium*-infiltrated *Nicotiana benthamiana*. Empty vectors were used as controls. Fluorescence indicates that YFPc-HD2B interacts with YFPn-MPK3. YFPc-HD2B does not interact with empty YFPn vector and YFPn-MPK3 does not interact with empty YFPc vector. Overlay indicates merging the YFP and light transmission images.

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complementation assay (BiFC). To this end, the MPK3 and HD2B cDNAs were inserted into binary vectors, containing the split YFP N-terminal fragment (YFPn) and the C-terminal fragment (YFPc), respectively. When YFPc-HD2B and YFPn-HD2B were expressed together in transiently transformed *Nicotiana benthamiana* leaves, YFP fluorescence was observed inside nuclei revealing an *in planta* interaction between the two proteins (Figure 1E, upper panels). No fluorescence was observed when each tagged protein was expressed separately with the corresponding empty vector (Figure 1E, middle and bottom panels). Because both MPK4 and MPK6 showed a capacity to phosphorylate HD2B, but with a weaker preference than MPK3, we tested their interaction with HD2B by BiFC. As expected we found that as MPK4 and MPK6 can also interact with HD2B but with a lower efficiency (supplemental Figure S9). Due to the fact that MPK3 showed the highest phosphorylation activity we decided to focus our study on this specific MAPK. To confirm the MPK3/HD2B interaction *in vivo* we performed co-immunoprecipitation experiments. First, protein extracts obtained from protoplasts transiently expressing *HD2B-c-Myc* and *MPK3-HA* tagged proteins were immunoprecipitated with an anti-c-Myc antibody and analyzed by immunoblotting with anti-c-Myc or anti-HA antibodies. We observed that HD2B interacts with MPK3 (Supplemental Figure S10A) consistent with our *in vitro* kinase assays (Figure 1A). Second, co-IP assays performed with transgenic plants expressing both *GFP-HD2B* and *MPK3-c-Myc* tagged proteins (Supplemental Figure S10B) confirmed our BiFC assays (Figure 1 E) that HD2B interacts with MPK3 *in vivo*. Taken together, our results showed that MPK3 interacts with and phosphorylates HD2B.

The MPK3-HD2B Module Controls the Transcription of Biotic Stress Response Genes by Modulating H3K9ac Levels

To gain insight into the role of HD2B and its interaction with MPK3 in gene regulation and plant defense, we examined the gene expression consequences of HD2B and MPK3 loss-of-function mutants in resting conditions and in flagellin-challenged plants. To this end, we performed RNA-seq analyses on *hd2b* and *mpk3* mutants and wild type (WT) plants (Supplemental Table I and II) either mock-treated for 30 minutes or treated for the same period of time with 1 μ M of flg22. First, when compared to WT, we observed that 1714 genes were deregulated in *hd2b* with a fold change >2 and p-value <0.05. From these 1714 genes 74% were up-regulated and 26% down-regulated (Figure 2A). This

pattern is consistent with the role of HD2B as a repressor. A functional annotation of *hd2b* up-regulated genes under control conditions showed a clear enrichment in genes involved in transcription (Figure 2A and Supplemental Figure S11). By contrast, an annotation of down-regulated genes showed enrichment in genes involved in transport activity (Supplemental Figure S12A and S12B). Because HD2B is a histone deacetylase and as such a putative repressor of gene expression, we focused our subsequent analyses on the set of genes that was up-regulated in the *hd2b* mutant. Comparative analysis between the *hd2b* and *mpk3* transcriptomes revealed an overlap among the identity of the deregulated genes in the two mutants. Indeed, a total of 414 genes were commonly up-regulated in the two mutants (Figure 2B) representing 32% of the *mpk3* up-regulated genes. This result was not surprising because MPK3 likely has many substrates in addition to HD2B that contribute to the gene expression modifications observed in the transcriptome of our mutants. Reciprocally, HD2B likely is involved in diverse cellular processes that include, but are not limited to, pathogen defense. Gene ontology analysis of this class of genes revealed a significant enrichment in RNA biosynthetic processes and transcription (Figure 2C).

To put these responses in the context of defense responses, the transcriptome results of untreated *hd2b* and *mpk3* mutants were compared to data obtained on WT plants after flagellin treatment, which allowed us to distinguish 150 genes that were up-regulated in the *mpk3* and *hd2b* mutants and upon flagellin treatment (Figure 2D), suggesting that the HD2B-MPK3 module normally represses the expression of these genes but that the inhibitory effect is alleviated during pathogen defense.

To further dissect the mechanisms by which MPK3 and HD2B regulate these genes, and based on the changes in their expression levels from the RNA-seq data, we selected four genes, *AT2G31290*, *AT5G03350*, *AT3G11380* and *AT2G28180*, for further analysis. Quantitative RT-PCR confirmed the RNA-seq results indicating an over-expression of *AT2G31290*, *AT5G03350*, *AT3G11380* and *AT2G28180* in *mpk3* and *hd2b* mutants (Figure 2E).

Because HD2B is a histone deacetylase, we hypothesized that HD2B could be directly interacting with the gene loci to repress their transcription. To test if HD2B directly binds the four regulated genes, we performed chromatin immunoprecipitation (ChIP)-qPCR on seedlings expressing a GFP-HD2B fusion

Figure 2. HD2B and MPK3 Regulate the Basal Expression of a Subset of Defense Genes.

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protein under the control of the endogenous HD2B promoter (*pHD2B::GFP-HD2B*). As a negative control, we included the *AT1G07690* gene that is up-regulated in *hd2b* mutants (Supplemental figure S13A) but was not identified as a HD2B target by ChIP-seq. A marked enrichment of HD2B binding on each of the four candidate genes but not on *AT1G07690* was found in the absence of flagellin treatment (Figure 2F and Supplemental Figure S13B). Therefore, the HD2B protein is associated with chromatin and binds to these four genes, suggesting that HD2B directly regulates their transcription.

Acetylation and deacetylation are dynamic processes that can be rapidly reprogrammed, depending on the signals received by the cell. To determine

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(A) Annotation of *hd2b* deregulated genes. 26% of *hd2b* deregulated genes are downregulated (green, 452 genes) whereas 74% are up-regulated (red, 1263 genes). Generation of a hierarchical tree graph with the Agrigo GO Analysis Toolkit shows that up to 63% of the up-regulated genes code for proteins with significant enrichment in “transcription” and “RNA biosynthetic process”.

(B) Comparisons among the transcriptomes of *hd2b* and *mpk3* mutants. 32% of up-regulated (414) and 29 % of down-regulated (126) genes in *mpk3* are in common with *hd2b* mutants.

(C) Gene Ontology (GO) analysis of commonly up-regulated genes in *hd2b* and *mpk3* mutants with the Agrigo GO Analysis Toolkit. Histograms of the values highlight the enrichment of genes involving RNA biosynthesis and transcription. P-values for each enriched class are indicated (*p*-v).

(D) Comparisons of the 414 commonly up-regulated genes in *hd2b* and *mpk3* mutants with the genes up- or down-regulated in WT seedlings by flg22 treatment for 30 minutes.

(E) Validation of transcriptomic data by RT-qPCR. The up-regulation of 4 genes (of the 150 commonly up-regulated genes in *hd2b* and *mpk3* and flg22-treated WT in Figure 2D) was confirmed by RT-qPCR.

(F) HD2B protein binding to flg22-inducible genes in mock conditions. ChIP-qPCR assays with anti-GFP antibodies were performed on *pHD2B::GFP-HD2B* seedlings (Figure 2E). An IgG antibody was used as a negative control.

(G) MPK3 and HD2B promote H3K9 deacetylation of flg22-inducible genes in mock conditions. Using an anti-H3K9ac antibody, ChIP-qPCR assays were performed in *hd2b*, *mpk3* and WT seedlings. Compared to WT, the 4 loci (Figure 3E and 3F) were hyperacetylated in *hd2b* and *mpk3* mutants.

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the level of H3K9 acetylation, we performed ChIP-qPCR assays on these representative genes. Interestingly, we found hyper-acetylation on these four genes in both *mpk3* and *hd2b* mutants when compared to WT (Figure 2G). Altogether, our results indicate that the HD2B-MPK3 module is required to regulate the basal expression of a subset of genes. In *hd2b* and *mpk3* mutants, the absence of deacetylation results in the constitutive induction of these genes (Figure 2E). Thus, in addition to responding to pathogen stress, MPK3 activity appears to be required under normal conditions to promote HD2B-directed histone deacetylation to repress unwanted expression of these genes (Figure 2E, 2F and 2G).

HD2B is Involved in Pathogen Defense

Because the MPK3-HD2B module regulates pathogen response genes, we investigated the direct relevance of HD2B during biotic stress. In Arabidopsis, MPK3 has been shown to negatively regulate the basal activity of defense genes in the absence of MAMPs, but to be required for full expression of defense-related genes upon pathogen challenge (Frei dit Frey et al., 2014). Because MPK3 interacts and phosphorylates HD2B, we hypothesized that HD2B might also be implicated in defense against pathogens. To test this hypothesis, *hd2b* mutants were challenged with the non-pathogenic bacterial strain *Pseudomonas syringae* pv. *tomato* *hrcC*- (*Pst hrcC*-) that carries a mutation in the type 3 secretion apparatus and is hence deficient in effector deployment. Mutants showed increased sensitivity compared to wild-type plants (Figure 3A). As a control, we also tested the *hd2b* mutant complemented by expression of *HD2B* under its own promoter (*pHD2B::GFP-HD2B*). *hd2b* complemented with *pHD2B::GFP-HD2B* showed infection levels of *Pst hrcC*- that were similar to wild type col-0 plants. We also compared the resistance of the *hd2b* mutant and HD2B over-expressing lines to wild type Col-0 plants upon infection to virulent *Pseudomonas syringae* pv. *tomato* (*PstDC3000*). *hd2b* mutants were also more susceptible than wild type, whereas plants over-expressing *HD2B* under the constitutive 35S cauliflower mosaic virus promoter (*35S::HD2B*) were more resistant to *Pst DC3000* (Supplemental Figure S14). These results show that HD2B is implicated in the defense against pathogens probably by regulating defense genes. We hypothesized that HD2B is not involved in MAMP signaling upstream of the MAP Kinase pathway. To confirm this, we first analyzed the MPKs activation after flg22 treatment in both WT and *hd2b* mutant and no

Figure 3: HD2B Plays a Role in Plant Immunity and Shuttles from the Nucleolus to the Nucleoplasm.

(A) Susceptibility of *hd2b* mutant and independent *hd2b* transgenic lines expressing an unphosphorylatable version of HD2B-AA (*hd2b pHD2B::GFP-HD2B-AA* #1 and #2) to *Pseudomonas syringae* pv. *tomato hrcC*- was compared to that of wild type Col-0 and to a complemented mutant line (*hd2b pHD2B::GFP-HD2B*) as a control. 14-day-old in vitro seedlings were incubated with a suspension of *Pst hrcC*-, and bacteria were quantified 2 hrs (day 0) and two days (day 2) after inoculation. *hd2b* mutant plants and GFP-HD2B-AA lines showed enhanced susceptibility compared to col-0 and to the nonmutated GFP-HD2B complemented line. Average values and standard deviations were calculated from three independent experiments. For each condition (day 0, day 2), a one way ANOVA followed by an all pairwise multiple comparison procedure was performed (Holm-Sidak method, $23 > n > 8$, $p < 0.05$).

(B) Immunofluorescence staining was performed on *pHD2B::GFP-HD2B* rosette leaves either mock-treated or elicited with flg22 for 30 minutes. In mock-treated tissues, GFP-HD2B was localized to the nucleolus while GFP-HD2B was observed in the whole nucleus after flg22 treatment. Scale bars, 5μm.

(C) GFP-HD2B-ED that mimics a constitutively phosphorylated version of HD2B was stably expressed in Arabidopsis plants and accumulated in the nucleoplasm while GFP-HD2B-AA was localized in the nucleolus. Scale bars, 5μm.

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changes were observed (Supplemental Figure S15). This result suggested that the signaling cascade is not affected in the *hd2b* mutant. We next asked if flagellin sensitivity is affected in this mutant. We did not observe any difference in growth inhibition (Supplemental Figure S16), suggesting that HD2B is not involved in MAMP-induced growth inhibition which is MAPK-independent.

The HD2B Phosphorylation Status Determines Its Sub-nuclear Localization and Choice of Target Genes.

Having established that HD2B contributes to pathogen defense and that it can be phosphorylated by MPK3, we investigated in more detail the consequences of MPK3-dependent HD2B phosphorylation in response to flagellin. MPK3-mediated phosphorylation could affect HD2B activity in several ways, such as modulating its catalytic activity, its stability or cellular localization. To discern if MPK3-mediated phosphorylation affects any of these parameters, we first investigated the subcellular localization of HD2B after flagellin-induced MPK3 activation. In *pHD2B::GFP-HD2B* leaves, GFP-HD2B was observed to be relocated from the nucleolus to the nucleoplasm (diffuse and speckle pattern) uniquely after flg22 recognition but not after mock treatment (Figure 3B). This relocalization was confirmed also to occur in protoplasts (Supplemental Figure S17), indicating that HD2B moves from the nucleolus to other sites in the nuclear compartment in response to flg22 treatment. To examine the role of HD2B phosphorylation by MPK3 on the sub-nuclear localization of HD2B, we analyzed the localization of WT and mutated HD2B. Consistent with phosphorylation playing a role in HD2B localization, YFP-HD2B-ED (HD2B-T249E/S266D), a mutated form of HD2B that mimics a constitutively phosphorylated version of the HD2B protein, accumulated in the whole nucleus of Arabidopsis protoplasts even in the absence of flagellin treatment (Supplemental Figure S18). We confirmed this nuclear re-localization in stably transformed Arabidopsis lines expressing *pHD2B::GFP-HD2B-ED* (Figure 3C and Supplemental figure S19) suggesting that flg22-triggered MPK3 phosphorylation of HD2B induces the nuclear re-localization of HD2B. To test the role of HD2B phosphorylation in vivo, we introduced a non-phosphorylatable HD2B version HD2B-AA (HD2B-T249A/S266A) in the *hd2b* mutant. This mutated version of the protein accumulated only in the nucleolus (Figure 3C). We next tested the sensitivity of HD2B-AA complemented *hd2b* lines to *Pst hrcC*-. These plants displayed enhanced sensitivity to the pathogen when compared to plants expressing

wild type *HD2B* (Figure 3A), further confirming that phosphorylation of HD2B is required for proper activation of defense mechanisms. Consistently, the HD2B-AA protein fails to restore a normal expression level to HD2B target genes that are constitutively activated in the *hd2b* mutant (Supplemental Figure S20).

To determine if the change in HD2B localization has an impact on the genes that this HDAC targets, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) in *pHD2B::GFP-HD2B* seedlings after a 30 min flagellin or mock treatment. First we controlled the HD2B enrichment after ChIP-seq (Figure 4A) and then performed a peak detection using MACS2. In control mock conditions, HD2B bound 5460 genomic loci, whereas it bound 8149 loci in flg22-treated plants (Supplemental Table IV), indicating that flg22 treatment induced a recruitment of HD2B on chromatin. These differences could be attributed to a relocalization of the protein rather than to modifications of its accumulation since the expression of *HD2B* remains constant upon flagellin treatment (Supplemental Figure S21). 4431 HD2B target sites overlapped between mock- and flg22-treated plants, revealing a redistribution of HD2B on chromatin.

In mock- and flg22-treated conditions, most HD2B peaks were found approximately 300 base pairs (bp) downstream of the transcription start site (TSS) (Figure 4C and 4D), and most target genes (84% in mock- and 78% in flg22-treated plants) were protein-coding genes (Figure 4B). Eight of these ChIP-seq identified targets were confirmed by ChIP-qPCR in this study (Figure 2F and 6C). A Gene Ontology (GO) analysis of the protein-coding HD2B-targeted genes revealed a significant enrichment for genes involved in defense response under mock conditions, whereas HD2B targeted genes after flg22 treatment were mainly involved in plastid organization and interestingly these genes are down-regulated after a pathogen attack (Figure 4E and supplemental figure S22).

To correlate HD2B binding to changes in gene expression, we compared the ChIP-seq results obtained for HD2B binding sites with the transcriptome of the *hd2b* mutant. 277 of HD2B direct targets were up-regulated in the *hd2b* mutant, whereas only 79 were down-regulated (Supplemental Figure S23). This low level of correlation could be explained by the redundancy in the HD2 protein family. Together, these results indicate that after pathogen recognition, MPK3 modulates HD2B localization and activity to promote its dynamic redistribution on a new set of target genes.

Figure 4. Flagellin Recognition Leads to HD2B Redistribution to a New Set of Genes.

(A) Comparison of HD2B tag density in the regions of ± 1 kb around the HD2B-occupied loci upon flg22 treatment. HD2B binding was determined by ChIP-seq experiments with an anti-GFP antibody in *pHD2B::GFP-HD2B* seedlings upon either mock or flg22 treatment. A comparison with the Input tag density confirmed HD2B enrichment after ChIP-seq in mock and flg22 conditions.

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Modulation of H3K9ac Levels Is a Hallmark of the Flagellin Response

Because HD2B targets are a highly varied set of genes before and after flg22 treatment, it is possible to assume that changes in histone acetylation are an important component of the cellular response to pathogens. To analyse the contribution of histone acetylation to the flg22-induced immune response, we analyzed the H3K9ac landscape before and after 30 min of flg22 treatment using a ChIP-seq approach. We identified about 15000 peaks both in control and flg22-treated plants (Supplemental Table III). A subset of these ChIP-seq results was validated by ChIP-qPCR (Figure 6C). Most of these peaks were observed at core promoters or protein-coding genes where they predominantly localized to the first nucleosome after the TSS (Supplemental Figure S24A-E). According to the agriGO toolkit, genes harbouring the H3K9ac mark were associated with developmental processes, responses to abiotic and biotic stimulus, signalling

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(B) Pie chart representation of the classification of HD2B target genes upon mock or flg22 treatment. Genomic annotation of the detected HD2B peaks was performed using the Genomic Position Annotation Tool (GPAT).

(C) HD2B global profiles. The majority of HD2B peaks were located at approximately 300 bp downstream of the transcription start site. HD2B peaks in both mock- and flg22-treated plants are represented to compare their relative position to the TSS.

(D) Genome Browser view of ChIP-seq data across a region of chromosome 3 targeted by HD2B.

(E) Flagellin treatment induces HD2B targeting to a new set of genes involved in plastid organization which is repressed by biotic stresses. The Venn diagram represents the overlap between HD2B targets in mock and flg22 conditions (*left*). The hierarchical tree graph shows a significant enrichment of the specific flg22-HD2B-targets in “plastid organization” with Agrigo GO Analysis Toolkit (*middle*). The heatmap generated with publically available microarrays data and the Genevestigator software 56 indicates that specific flg22-HD2B target genes are repressed after biotic or elicitor stresses. Each column represents a gene from the list, and each line represents a particular microarray experiment. Red, green and black colors indicate up- or down-regulation or no change of gene expression, respectively (*right*).

Figure 5. Flagellin Recognition Modulates the Genome-wide H3K9 Acetylation Landscape.

(A) Flagellin triggers hyper- and hypo-acetylation of two different peak clusters. H3K9ac ChIP-seq data from mock- and flg22-treated plants were compared using seqMINER. From these analyses 3 classes of peaks emerged: Class I and II contain hyper- and hypo- acetylated peaks, respectively, after flg22 treatment and the third class contains peaks that do not change.

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cascades and responses to hormone stimuli, both in control conditions and after flg22 treatment (Supplemental Figure S24F).

We next compared the two ChIP-seq data sets using seqMINER and diffReps, which allows qualitative and quantitative comparisons to be made between a reference set of genomic positions and multiple ChIP-seq datasets (Ye et al., 2011). Using this approach, we observed 3 classes of genes. The first class, consisting of 731 genes (Supplemental Table III), was hyper-acetylated after flg22 treatment (Figure 5A and 5B). Gene ontology analysis of this class revealed

continued

(B) Tag read densities of class I H3K9ac peaks are stronger upon flg22 treatment. The graph represents the average of tag reads relative to the H3K9 peak summit positions for all the peaks from cluster I.

(C) GO analysis of H3K9 flg22 hyper-acetylated genes. Genomic annotation of the H3K9 hyper-acetylated peaks was performed using the Genomic Position Annotation Tool (GPAT). GO data were extracted with the Agrigo GO Analysis Toolkit. A histogram of the values highlights the enrichment of the GO classes. P-values for each enriched class are indicated (p -v).

(D) H3K9 flg22 hyper-acetylated genes mainly are induced by biotic or elicitor stresses. The gene expression of flg22-induced H3K9 hyper-acetylated genes was analyzed using publically available microarrays data and the Genevestigator software 56. Each column represents a gene from the list, and each line represents a particular microarray experiment. Red, green and black colors indicate an up- or down-regulation or no change of gene expression, respectively.

(E) Tag read densities of class II peaks are stronger in mock conditions. The graphic represents the average of tag reads relative to the H3K9 peak summit positions for all the peaks from cluster II.

(F) GO analysis of H3K9 flg22 hypo-acetylated genes. Genomic annotation of the H3K9 hypo-acetylated peaks was performed as in Figure 5C. A histogram highlights the enrichment of the GO classes. P-values for each enriched class are indicated (p -v).

(G) H3K9 flg22 hypo-acetylated genes are repressed mainly by biotic or elicitor stresses. The gene expression of flg22-induced H3K9 hypo-acetylated genes was analyzed and displayed as in Figure 5D.

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a significant enrichment in genes involved in defense and immune responses and phosphorylation (class I, Figure 5C and Supplemental Figure S26A). The second class of 13159 genes did not display any obvious changes in H3K9ac marks (class II, Figure 5A). The third class totaling 787 genes was hypo-acetylated after flg22 treatment (class III, Figure 5A and 5E and Supplemental Table III) and mainly consisted of genes involved in chloroplast and plastid organization and in metabolic processes (Figure 5F and Supplemental Figure S26B). Gene expression analysis revealed that most hyper-acetylated genes were induced (Figure 5D) and most hypo-acetylated genes were repressed by diverse biotic stresses (Figure 5G), providing strong evidence for a positive correlation between the acetylation and the expression level of biotic stress responsive genes. Consistently, comparing these results with the transcriptome of WT plants treated with flg22 revealed that about 98% of hyper-acetylated genes were induced by flg22 treatment whereas only 2% were repressed, and about 64% of hypo-acetylated genes were repressed by flg22 treatment, whereas only 36% were induced (Supplemental Figure S27), clearly showing that H3K9ac is associated with gene activation. Our results indicated that flg22-induced genes were regulated mainly by *de novo* acetylation, whereas flg22-repressed genes were regulated by dynamic deacetylation, highlighting the role of HDAC proteins in the control of flg22-regulated genes.

HD2B Is a Major Contributor of Flg22-induced Changes in Histone Acetylation

Because histone acetylation appears to be a major factor controlling the transcriptional reprogramming induced by pathogen recognition, we investigated whether HD2B could be a central contributor to this process. Among the 787 genes hypo-acetylated after flg22 treatment, 60% were HD2B targets, indicating that HD2B plays an important role in the transcriptional reprogramming induced by pathogens (Figure 6A). As expected among the 731 hyper-acetylated genes only 14% were HD2B targets (Figure 6A).

To clarify the mechanism by which the MPK3-HD2B module acts on genes after flg22 treatment, we selected four HD2B target genes that were hypo-acetylated after flg22 treatment. We first confirmed their acetylation status (Figure 6B), and then verified that they were bound by HD2B after flg22 treatment (Figure 6C). Secondly, we analyzed their expression and acetylation levels before and after flg22 treatment in WT plants and compared them to the levels in *mpk3* and *hd2b*

Figure 6. The MPK3-HD2B Module Contributes to the Regulation of H3K9 Acetylation Dynamics in Response to Flagellin.

continued on next page

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mutants (Figure 6D). As expected, in WT control plants, hypo-acetylated genes were repressed and were further hypo-acetylated after flg22 treatment, but neither gene repression nor a change in acetylation occurred in the *mpk3* and *hd2b* mutants, confirming the role of the MPK3-HD2B module in the repression and acetylation dynamics of these genes (Figure 6B, 6C and 6D). Furthermore, to functionally prove that HD2B phosphorylation plays a major role in this process we analyzed the expression levels of these specific four genes by qRT-PCR in our HD2B-AA line. We observed that this repression is not restored in the HD2B-AA line, providing further evidence for the functional relevance of HD2B phosphorylation (Supplemental Figure S28).

continued

(A) 60 % of H3K9 flg22-induced hypo-acetylated genes are HD2B targets (*left*) compared to only 14% of H3K9 flg22-induced hyper-acetylated (*right*).

(B) MPK3 and HD2B promote H3K9 deacetylation of hypo-acetylated genes after flg22 treatment. ChIP-qPCR assays on 4 selected loci using an anti-H3K9ac antibody were performed in WT and *hd2b* and *mpk3* mutant seedlings either mock- or flg22-treated for 30 minutes. The 4 genes were hypo-acetylated after flagellin treatment in WT plants, and this deacetylation failed to occur in the *hd2b* or *mpk3* mutants. Asterisks indicate significantly different values (ANOVA test, $P < 0.05$).

(C) HD2B is bound to hypo-acetylated genes after flg22 treatment. ChIP-qPCR assays with anti-GFP antibodies were performed on *pHD2B::GFP-HD2B* seedlings using oligonucleotides in the proximal promoter region of the 4 selected loci. IgG antibody was used as a negative control. Asterisks indicate significantly different values (Student's t test, $P < 0.05$).

(D) MPK3 and HD2B are required for gene repression of hypo-acetylated genes after flg22 treatment. Expression of the 4 selected genes was analyzed by qRT-PCR in WT, *hd2b* and *mpk3* seedlings mock or flg22 treated for 30 minutes. Genes were repressed in the wild-type but not in *hd2b* and *mpk3* mutants. Asterisks indicate significantly different values (ANOVA test, $P < 0.05$).

DISCUSSION

Plants are sessile organisms that constantly have to respond to changes in environmental conditions such as biotic or abiotic stress. In the case of biotic stresses, plants have adapted the capacity to recognize pathogens through MAMPs via specific receptors. The multi-faceted responses of plants to pathogens have been examined extensively, and these studies have revealed that receptor-mediated pathogen recognition triggers MAP kinase signaling cascades, which, through the activation and repression of large gene sets, ultimately result in the establishment of MAMP-triggered immunity.

Previous studies have identified several transcription factors targeted by MAP kinases. For example, the bZIP (basic leucine zipper) transcription factor VIP1 (VirE1-INTERACTING PROTEIN 1) is specifically phosphorylated by MPK3 upon flg22 treatment and activates a number of defense-related genes such as *PR1* (*PATHOGENESIS-RELATED 1*) (Djamei et al., 2007; Pitzschke et al., 2009). However, extensive changes in the transcriptional program of cells likely rely not only on the activation of specific transcription factors but also on chromatin modifications that can dynamically and reversibly regulate gene expression. Consistently, we show here that flagellin-activated MAP kinases interact with and phosphorylate the histone deacetylase HD2B to control the expression level of biotic stress-regulated genes through modulation of the H3K9ac histone mark.

To date, a handful of histone modifiers have been implicated in plant innate immunity. For example, salicylic acid (SA) signaling plays an essential role in plant pathogen resistance and is controlled partially by the HDAC SIRTUIN2 (SRT2), which represses the expression of several SA biosynthetic genes such as *PAD4* and *SID2* (Wang et al., 2010). Consistently, *srt2* mutant Arabidopsis plants were reported to be more resistant to pathogen infection than WT control plants, whereas an SRT2 over-expressing line was more susceptible. In addition, it was reported in Arabidopsis that mutations in the HDAC *HDA19* result in enhanced basal expression of several biotic responsive genes (Tian et al., 2005) and improve tolerance to *P. syringae* (Choi et al., 2012), although contradictory results have been described previously (Kim et al., 2008). Moreover, the rice HDAC HDT701 negatively regulates innate immunity by directly binding and modulating the histone H4 acetylation levels of PRR and defense-related genes (Ding et al., 2012).

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Here, we measured the resistance of *hd2b* mutants to the both virulent and non-pathogenic *Pseudomonas syringae* strains and concluded that *hd2b* mutants were more susceptible than wild type control plants, whereas *HD2B* over-expressing plants were more resistant. Hence, different HDACs can lead to similar or contrasting outcomes for plant immunity likely depending on the genes they target, and our results and those reported by others clearly highlight the crucial contribution of chromatin regulation to pathogen defense.

Although several studies provide evidence for the involvement of histone modifiers in pathogen response, the way in which their activity is connected to biotic stress signaling cascades has not been unraveled. In this work, we connected MAPK-induced signal transduction to HDAC-dependent histone modifications. Indeed, our results show that *H2DB* is a direct target of MAPKs and that *HD2B* regulates a large number of genes involved in pathogen defense. Although our study is the first to describe a general histone modifying protein as a direct target of a MAPK in plants, such mechanisms seem to be highly conserved in eukaryotes. Indeed, in animals, MAPKs drive histone modifications and direct chromatin remodeling. For example, phosphorylation of the histone acetyl-transferase p300 by the MAP kinase ERK2 promotes its localized histone binding (Chen et al., 2007). Likewise, ERK1 and ERK2 modulate the assembly of chromatin remodeling complexes and, thereby, control the expression of vitamin D-responsive genes (Oya et al., 2009).

The Arabidopsis genome codes for 18 HDACs, which fall into four groups (Dangl et al., 2001; Wu et al., 2003): Class I and II HDACs correspond to yeast RPD3 and HDA1, respectively, class III enzymes are NAD-dependent HDACs related to yeast SIR2 and class IV HDACs are plant-specific and consist of four members called HD2A-D (Dangl et al., 2001). The four class IV HDACs all contain a conserved N-terminal catalytic and a central acidic domain and a divergent C-terminal region that suggest specific functioning of the different proteins (Dangl et al., 2001). In contrast to the other three Arabidopsis HD2s, *HD2B* does not contain a Zn-finger motif in its C-terminus. However, with the exception of Arabidopsis *HD2A*, the two MAPK-targeted C-terminally located *HD2B* *in vivo* phosphorylation sites are highly conserved in other species, including the equivalent rice and maize HD2s (Dangl et al., 2001). Our data indicate that phosphorylation of these sites is important for the molecular function of *HD2B* and that the phosphorylation status of the two residues

determines the intra-nuclear localization of the HD2B. Possibly, a nucleolar protein could anchor unphosphorylated HD2B to this sub-nuclear compartment, and MAPK-directed phosphorylation of HD2B could release HD2B to enter the remaining nuclear compartment and associate with novel chromosomal loci. In this way, the nucleolus could serve as a stocking center to prevent HD2B from potentially associating with certain labile nuclear sites and could provide a protein synthesis-independent mechanism to make HD2B rapidly available for gene regulation upon signaling.

Our results show that MPK3 likely modulates HD2B activity by altering its nuclear distribution. Indeed, flagellin treatment induces the relocalization of HD2B in an MPK3-dependent manner, and this effect can be obtained by expressing a phosphomimetic version of HD2B. Consistently, control of the intracellular location has been described in yeast and mammals as an important regulatory mechanism of HDAC activity (Bjerling et al., 2002; Segre and Chiocca, 2011). As expected from their ability to deacetylate histones, HDACs are found generally in the nucleus of most organisms. Nevertheless, in mammals, the Class IIa histone deacetylases (HDACs) were found both in the cytoplasm and in the nucleus. Indeed, specific phosphorylation of HDAC7 induces its nuclear exclusion, rendering it unable to impact transcription (Dequiedt et al., 2006). In the case of HD2B, phosphorylation leads to a sub-nuclear relocalization from the nucleolus to the nucleoplasm in specific chromatin regions. In agreement with the flg22-induced relocalization of HD2B, our ChIP-seq analyses revealed a drastic shift in the HD2B chromatin targeted sites upon flagellin treatment. In mammals, it has been reported that the phosphorylation of HDAC2 leads to its redistribution on chromatin and increased recruitment to promoters (Sun et al., 2002; Sun et al., 2007), suggesting that HDAC phosphorylation could be a mechanism conserved from mammals to plants to drive their redistribution in response to a stimulus.

HD2B is a HDAC and is predicted to function as a repressor of gene expression. As expected, a proportion of HD2B target genes were constitutively up-regulated in *hd2b* mutants. Furthermore, 64% of the genes that are hypo-acetylated after flagellin treatment are direct HD2B targets, indicating that HD2B contributes substantially to MAMP-triggered transcriptional reprogramming. The study of pathogen-induced histone modifications is a fledgling field of research. In mammals, although four bacteria have been reported to modulate histone

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acetylation levels upon infection, the underlying mechanisms are not known (Wang et al., 2005; Basu et al., 2007; Garcia-Garcia et al., 2009; Eskandarian et al., 2013). In plants, modulation of histone acetylation levels in response to biotic stress has also been described, but again the underlying mechanisms remained unclear (Kim et al., 2010; Wang et al., 2010; Bourque et al., 2011; Choi et al., 2012; Ding et al., 2012). Our data show that genome-wide modulation of H3K9ac levels is an early chromatin response to a biotic stress. Histone acetylation is a labile chromatin mark (Waterborg, 2001; Scott, 2012), and, as such, is a fast and reversible post-translational process that can allow plants to rapidly modulate gene expression. Thus, this mark is ideally suited for biological programs that require an immediate response to fluctuating environmental conditions. In line with this characteristic, we observed a strong correlation between up-regulation of gene expression and hyper-acetylation, whereas down-regulation correlated with hypo-acetylation. Altogether, our data suggest that modulation of the genome-wide H3K9ac landscape is a hallmark of the flg22 response, and that HD2B is a key player in this process. However, our studies do not exclude the involvement of additional HDACs in this process.

4 Finally, our detailed analysis of the expression levels, HD2B binding and histone acetylation of HD2B target genes under control and flg22 treatment conditions revealed that HD2B has several roles. We have shown that HD2B regulates the basal expression level of a subset of genes in the absence of pathogen challenge, probably by acting as a counter-balance to the activity of HATs. Indeed, expression of these HD2B-targeted genes in unchallenged plants is constitutively high in *hd2b* mutants. These genes can be distributed into two classes after flagellin treatment: Class I genes are repressed by flagellin due to increased HD2B recruitment on these sites, whereas Class II genes are induced by flagellin due to eviction of HD2B from these loci.

In summary, our work defines the first example of a MAP kinase-regulated chromatin mechanism and details how MAMP-triggered MAP kinase signaling regulates global changes in the chromatin landscape. As such, it sets the stage for other large-scale studies examining the contribution of protein kinase-mediated chromatin regulation in plants and mammals.

METHODS

Plant Material, Growth Conditions and Treatments

T-DNA insertion lines *hd2b* (At5g22650) from SAIL collection (Sessions et al., 2002) *Sail_1247_A02* (Supplemental Figure S29) and *mpk3* SALK_151594 (Wang et al., 2007) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). To produce transgenic plants expressing a 35S::GFP:MPK3 construct, the coding region of MPK3 (At3g45640) was amplified from cDNA of Col-0 by PCR using the following primers: forward, 5'gc gga tcc atg aac acc ggc ggt ggc3', reverse 5'gc act agt cta acc gta tgt tgg ctt gag3'. The restriction enzyme sites, set in bold, that had been added to the primers were utilized to ligate the open reading frame to BamHI/SpeI sites of pCAT-GFP, resulting in *p35S::GFP-MPK3*. For the generation of stably transformed *A. thaliana* plants, the expression cassette was excised by Sse8387I and cloned into the PstI site of the binary vector pCB302 (Xiang et al., 1999). This vector was transformed to *A. tumefaciens* strain GV3101 by electroporation. *A. thaliana* Col-0 plants were transformed by floral dip method according to Clough and Bent (Clough and Bent, 1998). Transformed plants were selected on BASTA.

Both *pHD2B::GFP-HD2B* and *35S::GFP-HD2B* constructs were made by using Gateway technology (Invitrogen). The CDS of HD2B was PCR amplified from cDNA with forward primer 5'-caccATGGAGTTCTGGGGAGTTGC-3' and reverse primer 5'-AGCTCTACCCTTTCCCTTGC-3' using Phusion High Fidelity DNA polymerase (New England BioLabs). The putative promoter (1.055 kb upstream of the start codon) was PCR amplified from genomic DNA with forward primer 5'-caccGTTTTGGATCTGCAGACAAGG-3' and reverse primer 5'-TGTTGTTGAACGAGGAAGAGAG-3'. Both PCR fragments were cloned into pENTR/D-TOPO (Invitrogen). The promoter was then re-cloned from the pENTR/D-TOPO into a pENTR4-1 vector (Invitrogen) in front of a GFP using the NotI and Ascl restriction sites. The pENTR4-1 containing the HD2B promoter and GFP, the pENTR-D-TOPO-AtHD2B vector and a pENTR2-3 vector containing a CaMV 35S terminator were recombined by a multisite gateway reaction (Invitrogen) into the binary destination pBnRGW vector. This is a modified vector based on pKGW (Karimi et al., 2002) in which the kanamycin resistance was replaced with basta resistance and the NAP::DsRed expression cassette from pFluar 101 (Stuitje et al., 2003) was introduced for easy selection of red fluorescent transformed seeds. To create *35S::GFP-HD2B* construct

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the same strategy was used but pENTR4-1 contained CaMV35S promoter. *A. tumefaciens* strain C58 mediated transformation for both constructs was performed as described by Bechtold and Pelletier (Bechtold and Pelletier, 1998). Seed-specific expression of red fluorescent protein DsRed permitted the identification of mature transformed seeds by fluorescence stereo microscopy (Leica).

For flagellin experiments, seeds were surface-sterilized by treatment with bayrochlore and then soiled in sterile half-strength MS liquid medium, placed for 2-4 days at 4°C to obtain homogeneous germination, and plants were grown in chambers at 20°C in long-days (16h of light) conditions. After 14 days, flg22 peptide was added in the medium to a final concentration of 1µm when necessary.

Infection assays in Arabidopsis rosette leaves with *Pseudomonas syringae* pv. *tomato* PstDC3000 were performed as described previously (Kemmerling et al., 2007) on leaves of 4-5 week old plants grown on soil in environmental chambers at 22°C under short-day conditions (8h of light).

For *Pseudomonas* pathogen assays in Arabidopsis seedlings, ½ MS plates containing 14-day old Arabidopsis plantlets were flooded with a bacterial suspension of the *hrcC*- mutant (defective in type III secretion system) of *Pseudomonas syringae* pv. *tomato* DC3000 during 3 minutes. The bacteria were grown ON at 30°C in LB broth+Rif, and re-suspended in 10 mM MgCl₂ after several washings for the removal of the media and the antibiotic. The final concentration of the suspension was adjusted to an OD₆₀₀=0.1 and supplemented with 0.025% Silwet L-77. After discarding the bacterial suspension, the plates were sealed with micropore and put back in the incubation room. Day 0 samples were taken 2 hours post-inoculation and day 2 samples after 48 hours. For the sampling process the rosettes of 4 plantlets were cut and weighed together, registering each time the fresh weight in mg. Afterwards, the rosette surface was sterilized by a 5-seconds wash in 70% ethanol, followed by 2 washes in sterile water. The samples were ground in order to release the bacteria, and serial dilutions performed. 10 µl of each dilution was plated on plates with LB+Rif and incubated during 2 days at 30°C. The growth of the bacterial population inside the plantlets was determined by calculating the total amount of bacterial CFU per mg of fresh weight. Each experiment was performed in quintuplicate, analyzing 8 samples per accession on each experiment.

Kinase Assays

Full-length *HD2B* (At5g22650) was amplified and cloned into pGEX4T-1. For mutagenesis of *HD2B*, PCR was performed on plasmids with Pfu Ultra. PCR mixtures were digested for 2 hours with *DpnI* and transformed into *Escherichia coli*. Clones were sequenced and each mutation was transformed into *E. coli* BL21. GST protein expression was induced for 4 hours at 37°C with 1 mM IPTG, and purified according to the manufacturer's protocol. For producing *HD2B* T249E and S266D, the following primers were used: GGAGGACACACCGCC**GAAC**-CACACCCAGCT, AGCTGGGTGTGGT**TC**GGCGGTGTGTCCTCC, GTGAAT-GCTAACCAG**GA**CCCCAAGTCTGGA and TCCAGACTTGGGG**TC**CTGGT-TAGCATTCAC, respectively.

Radioactive kinase assays were performed with MPK3, -4 and -6 immunoprecipitated from extracts of *A. thaliana* cell cultures that were treated for 10 minutes with 1 μ M flg22. Non-radioactive kinase assays for phosphosite mapping were performed the same for 45 minutes at 30°C.

Protein Localisation in Protoplasts

Full-length *HD2B* and *HD2B-DD* mutated form were cloned in a pGreen plasmid behind a yellow fluorescent protein (YFP) gene driven by a 35S promoter. Arabidopsis protoplasts were prepared from a suspension culture as described (Cardinale et al., 2000). One day after transformation, flg22 peptide was added in the medium to a final concentration of 2 μ M for 15 minutes when necessary and localization was checked by fluorescence microscopy.

Immunofluorescence Labelling

Leaves of plants stably transformed with *p35S::GFP-HD2B* construct were treated during 30 min with or without 1 μ M flg22 and fixed in PFA 4% in PHEM (PIPES 60mM ; HEPES 25mM ; EGTA 10mM ; MgCl₂ 2mM pH 6.9) during 1 hour at room temperature (apply vacuum 20 min to facilitate uptake of the fixation solution). Seedlings were washed 5 min in PHEM and 5 min in PBS pH 6.9 and chopped on a Petri dish in PBS supplemented with 0.1% Triton X-100 (w/v). The mixture was filtered (50 μ m) and centrifuged 10 min at 2,000 g. The supernatant was carefully removed and the pellet washed once with PBS, gently resuspended in 20 μ L PBS and a drop was placed on a poly-lysine slide and air dried. Slides were rehydrated with PBS and permeabilized 2 times by 10

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min incubation in PBST (PBS, 0.1% Tween-20 v/v). Slides were placed in a moist chamber and incubated overnight at 4°C with primary antibody anti-GFP (Clontech, ref. 632592) in PBST supplemented with BSA (3% w/v). Slides were washed 5 x 10 min in PBST (at RT) and incubated 1 h at RT in the dark with the secondary antibody (A11037 Invitrogen, Alexa Fluor 594 goat anti-rabbit) diluted (1/400 v/v) in PBST, 3% BSA. Slides were washed 5x 10 min in PBST and then mounted with a drop of Vectashield with DAPI and observed as described for pollen mitosis analysis with the suitable cube fluorescence filters (BP340-380, DS 400, BP 450-490 for DAPI) (BP570-590, DS595, BP 605-655 for A594).

For whole mount immunofluorescence labeling, six-day-old *pHD2B::GFP-HD2B* seedlings were labeled according to the method of Sauer et al. (Sauer et al.) with small modifications: seedlings were fixed in 1.5% paraformaldehyde and 0.5% glutaraldehyde in ½ MTSB buffer (50 mM K-PIPES, 5 mM MgSO₄·7H₂O, 5 mM EGTA) at pH 6.8; cell-wall digestion enzyme mixture contained 1% meicelase, 1% cellulase and 1% macerozyme in PBS; samples were incubated with rabbit polyclonal anti-AtMPK3 (SIGMA-ALDRICH) primary antibody diluted 1:350 in PBS supplemented with 2% BSA at 4°C overnight, and subsequently with secondary antibody Alexa-Fluor 546 goat anti-rabbit IgGs (H+L) (Invitrogen) diluted 1:500 in PBS containing 2% BSA for 3 h (1.5 h at 37°C and 1.5 h at room temperature). Finally, samples were counterstained with DAPI and mounted in one drop of 0,1% [w/v] para-phenylenediamine prepared in 90% [v/v] glycerol in PBS. Microscopic analysis of immunolabeled samples was performed using a Zeiss LSM710 (Carl Zeiss Jena, Germany). DAPI was excited at 405 nm and emission was detected between 410 and 476 nm. GFP was excited at 488 nm and emission was detected between 500 and 535 nm. Alexa 546-conjugated antibody was excited at 561 nm, and fluorescence was detected between 566 and 591 nm. The post-processing of images was done using ZEN 2010 software, Photoshop 6.0/CS, and Microsoft PowerPoint.

BiFC Experiment

LR recombinations of *HD2B* and *MPK3* coding sequences in pENTR3C were done with split-YFP destination vectors pBiFC3 and pBiFC2 that allow N-terminal fusion with the C- and N-terminal YFP moieties, respectively (Azimzadeh et al., 2008). Recombined vectors were transformed into the *Agrobacterium* C58C1 strain. *Nicotiana benthamiana* leaves were agro-infiltrated as previously described (Colcombet et al., 2013). After 3 days, the YFP fluorescence was

visualized using a confocal laser scanning microscope (Leica, Germany).

Live-Cell Imaging

For microscopy, 6-days-old plants stably transformed with *p35S::GFP-MPK3* construct were transferred to microchambers mounted between microscopic slides and coverslips with one Parafilm layer as a spacer. The chambers were filled with liquid half-strength MS medium. Microscopic analysis was performed using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss Jena, Germany). All images were acquired using a 40X objective lens (NA 1.42). GFP was excited at 488 nm and detected between 500 and 535 nm. Post-processing of images was done with the aid of Zeiss ZEN software (Ver.2010b) and Microsoft PowerPoint applications.

Cell Fractionation

Two grams of 16-day-old Col-0 seedlings were ground in a mortar with liquid nitrogen and were resuspended in buffer A containing 2.5% Ficoll type 400 (F-4375, Sigma-Aldrich), 5% Dextran (D1662, Sigma-Aldrich), 0.4 M sucrose, 25 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT (D0632, Sigma-Aldrich), protease inhibitors (Complete cocktail, Roche) and phosphatase inhibitors (1 mM NaF, 0.5 mM Na₃VO₄, 15 mM B-glycerophosphate, 15 mM 4-nitrophenyl phosphate, Sigma-Aldrich chemicals). After a few minutes incubation on ice, the samples were filtrated through one layer 62 µm nylon mesh by centrifugating for 3 min at 212 g at 4°C. Triton X-100 was then added to a final concentration of 0.5% and the samples were gently mixed and incubated on ice for 15 min. An aliquot was kept at this step, hereafter referred to as Input. The samples were then centrifuged for 5 min at 1,500 g at 4°C and an aliquot of the supernatants was kept at this step, hereafter referred to as Cytoplasmic fraction. The pellets were gently resuspended in buffer B (buffer A and 0.1% Triton X-100) and centrifuged for 5 min at 1,500 g at 4°C. The pellets were then resuspended in buffer A and centrifuged for 5 min at 2,000 g at 4°C. The pellets were finally resuspended in SDS-sample buffer; they are hereafter referred to as nuclear fractions. SDS-sample buffer was also added to the Input and Cytoplasmic fractions and all three kinds of aliquots were heated at 95°C for 10 min for complete denaturation. Protein samples were resolved by SDS-PAGE at a constant amperage of 15 mA per gel and transferred onto methanol-activated PVDF membranes (GE Healthcare) for 1 h at a constant voltage of 100 V. Blots were blocked with 5%

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non-fat dry milk in 1x TBST for 1 h and probed with primary antibodies overnight at 4°C: anti-H3 (Abcam ref. Ab1791, diluted 1:10,000), anti-PEPC (Tebu-bio ref. 100-4163, diluted 1:15,000) and anti-MPK3 (described in Nakagami et al., 2006, diluted 1:4,000). The membranes were washed four times with 1x TBST. Goat anti-rabbit antibodies, at a dilution of 1:20,000 in 5% non-fat dry milk in 1x TBST, conjugated to horseradish peroxidase (A6154, Sigma-Aldrich) were used as secondary antibodies for 1 h at RT. The membranes were washed again four times with 1x TBST and the antigen-antibody interaction was detected with enhanced chemiluminescence reagent (ECL Prime, GE Healthcare) using a GeneGnome imaging system (Syngene).

Co-immunoprecipitation

For co-immunoprecipitation assays in protoplasts, protein extracts were prepared from protoplasts as described (Cardinale et al., 2002). For co-immunoprecipitation, samples were extracted in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40 (NP-40), and proteinase inhibitor mix (Complete EDTA free, Roche). Three independent protoplast transformation 100 µL of each were pulled and used for protein extraction. Protein extracts were precleared with 15 mL of protein A–Sepharose beads for 2 h at room temperature, then immunoprecipitated overnight in the presence of antibodies with 25 µL of beads. Samples were washed three times with wash buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, and 0.1% Tween 20) and subjected to immunoblotting.

For co-immunoprecipitation experiments *in planta*, transgenic lines expressing *p35S::GFP-HD2B* or *p35S::MPK3-c-Myc* constructs were crossed together to obtain plants expressing both constructs. Plant leaves were ground to a fine powder in liquid nitrogen. Cells were homogenized and lysed in Chris buffer containing 50 mM Tris-HCl pH 8.0, 0.5% NP-40, 200 mM NaCl, 0.1 mM EDTA, 10% glycerol, 10 mM NEM and protease inhibitor mix (Complete EDTA-free, Roche). GFP-HD2B and MPK3-c-Myc were then immunoprecipitated using respectively a polyclonal anti-GFP antibody (Clontech) and a polyclonal anti-c-Myc antibody (Sigma). Immune complexes were collected by incubation for 2 h at 4°C with Protein A/G Ultralink Resin (Thermo Scientific) and washed six times in lysis buffer. Immunoprecipitated proteins were detected by immuno-blot using a polyclonal anti-c-Myc antibody (Sigma).

Immunoblotting

About 200 mg of 14-day-old seedlings were ground with liquid nitrogen and resuspended in 500 μ L of a buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP40, 5 mM EGTA, 0.1 mM DTT (Sigma-Aldrich chemicals), protease inhibitors (Complete cocktail, Roche), and phosphatase inhibitors (1 mM NaF, 0.5 mM Na_3VO_4 , 15 mM β -glycerophosphate, 15 mM 4-nitrophenyl phosphate, Sigma-Aldrich chemicals). The suspension was centrifuged at 20,000 g for 15 min at 4°C, and the supernatant was collected. Protein quantification was carried out by Bradford method (Protein Assay Kit, Thermo Fisher Scientific), and the normalized protein amounts of all samples were denatured with SDS-sample buffer by boiling them at 95°C for 10 min. Protein samples were resolved by SDS-PAGE and transferred onto PVDF membranes (Bio-Rad). Blots were blocked with 5% BSA in 1x TBST and then probed with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP rabbit monoclonal antibody (#4370, Cell Signaling), hereafter referred to as anti-pTpY antibody. Goat anti-rabbit antibodies conjugated to horseradish peroxidase were used as secondary antibodies (A6154, Sigma-Aldrich). The antigen-antibody interaction was detected with chemiluminescent reagents (Clarity ECL substrate, Bio-Rad) using an imaging system (ChemiDoc MP System, Bio-Rad). Coomassie blue staining of blots was then carried out for protein visualization.

Gene Expression Analysis

Total RNA were extracted from seedlings with the RNeasy MiniPrep kit (Qiagen), according to the manufacturer's instructions. First strand cDNA was synthesized from 2 μ g of total RNA using Improm-II reverse transcriptase (A3802, Promega) according to the manufacturer's instructions. 1/25th of the synthesized cDNA was mixed with 100nM of each primer and LightCycler® 480 Sybr Green I master mix (Roche Applied Science) for quantitative PCR analysis. Products were amplified and fluorescent signals acquired with a LightCycler® 480 detection system. The specificity of amplification products was determined by melting curves. UBQ10 was used as internal control for signals normalization. Exor4 relative quantification software (Roche Applied Science) automatically calculated relative expression level of the selected genes with algorithms based on $\Delta\Delta\text{Ct}$ method. Data were from duplicates of at least two biological replicates. The sequences of primers can be found in Supplemental Table V.

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Transcriptomic analyses were performed RNA-seq at the URGV platform (Evry, France). Three biological replicates were run for each condition.

For Gene Ontology analyses, the GO Analysis Toolkit and Database for Agriculture Community (Du et al., 2010) was used.

Chromatin Immunoprecipitation Experiments

ChIP assays were performed on 14-day-old *in vitro* seedlings using anti-GFP (Santa Cruz), IgG control (Millipore), or anti-H3K9ac (Millipore) antibodies, using a procedure adapted from Gendrel et al. (Gendrel et al., 2005). Briefly, after plant material fixation in 1% (v/v) formaldehyde, tissues were homogenized, nuclei isolated and lysed. Cross-linked chromatin was sonicated using a water bath Bioruptor UCD-200 (Diagenode, Liège, Belgium) (30 s on/30 s off pulses, at high intensity for 60 min). Protein/DNA complexes were immunoprecipitated with antibodies, overnight at 4°C with gentle shaking, and incubated for 1h at 4°C with 50 µL of Dynabeads Protein A (Invitrogen, Ref. 100-02D). Immunoprecipitated DNA was then recovered using the IPure kit (Diagenode, Liège, Belgium) and analyzed by quantitative real-time PCR. An aliquot of untreated sonicated chromatin was processed in parallel and used as the total input DNA control.

For ChIP-qPCR experiments, fold enrichment of targets in ChIPed DNA relative to input was calculated from an average of three replicate qPCR reactions. The sequences of primers can be found in Supplemental Table V. Positions of the amplified regions on the different loci are indicated in supplemental figure S30.

Chromatin Immunoprecipitation-sequencing Analysis

After immunoprecipitation of the chromatin, ChIP-Seq libraries were generated and sequenced. Alignment was performed using Bowtie (Langmead et al., 2009) v0.12.7 on *Arabidopsis thaliana* genome TAIR10. Default Bowtie parameters were used except for : -best -strata (used to get the best mapping position with the minimum of mismatches). Peak calling was performed with MACS (<http://liulab.dfci.harvard.edu/MACS/>) (Zhang et al., 2008). Gene annotation and peak distribution relative to annotated Arabidopsis transcription start site was performed with GPAT (http://bips.u-strasbg.fr/GPAT/Gpat_home.html).

Global clustering of the H3K9ac ChIP-seq data and quantitative comparisons were performed using the seqMINER program (<http://bips.u-strasbg.fr/seqminer/>) (Ye et al., 2011). As reference coordinates, we used the MACS

determined peaks for H3K9ac. Tag densities from each ChIP-seq data set were collected in a window of 1 kb around the reference peak. The collected values were subjected to k-means clustering coupled to linear-based normalization. The normalization procedure reduces bias in the clustering due to inherent differences between ChIP-seq experiments.

Supplemental Data (available online: <https://genomebiology.biomedcentral.com/articles/10.1186/s13059-017-1261-8>).

Supplemental Figure 1-5. HD2B Phosphorylation Sites.

Supplemental Figure 6. MPK3, MPK4, MPK6 and MPK7 Antibody Specificity.

Supplemental Figure 7. MPK3, MPK4 and MPK6 Substrate Specificity.

Supplemental Figure 8. Subcellular Localization of MPK3.

Supplemental Figure 9. BiFC Analysis of HD2B Interaction with MPK3, 4 and 6 in Epidermal Cells of *Agrobacterium*-infiltrated *Nicotiana benthamiana*.

Supplemental Figure 10. Co-immunoprecipitation of HD2B with MPK3.

Supplemental Figure 11. GO Analysis of Transcription Factors Up-regulated in *hd2b* Mutant.

Supplemental Figure 12. GO Analysis of Down-regulated Genes in *hd2b* Mutant.

Supplemental Figure 13. Validation of Transcriptomic Data by RT-qPCR.

Supplemental Figure 14. Susceptibility of *hd2b* Mutant and *HD2B* Overexpressing lines.

Supplemental Figure 15. Flg22-induced Activation of MPK3, MPK4 and MPK6 Is Similar in Col-0 and *hd2b* Mutant.

Supplemental Figure 16. Flg22 Growth Inhibition Assay of *hd2b* Mutant.

Supplemental Figure 17. Flagellin-induced Relocalization of HD2B.

Supplemental Figure 18. Nucleolar HD2B and Nuclear HD2B-ED Localization.

Supplemental Figure 19. Nuclear HD2B Localization.

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Supplemental Figure 20. Gene Expression Analyses of *hd2b* and *mpk3* Over-expressed Genes in the HD2B-AA line.

Supplemental Figure 21. Flagellin-independent Expression of *HD2B*.

Supplemental Figure 22. Specific HD2B Targets in Mock Conditions Are Involved in Defense Response.

Supplemental Figure 23. Correlation Between HD2B Binding and Gene Expression in *hd2b* Mutants in Mock and flg22 Conditions.

Supplemental Figure 24. Characterization of H3K9 Acetylated Regions in Mock- and flg22-treated Seedlings.

Supplemental Figure 25. Comparison Between HD2B and H3K9ac Peak Positions Relative to TSS.

Supplemental Figure 26. GO Analysis of H3K9-hyper and H3K9-hypo acetylated Genes After flg22 Treatment.

Supplemental Figure 27. H3K9 Acetylation Levels Are Directly Correlated with Expression Levels After flg22 Treatment.

Supplemental Figure 28. Gene Expression Analyses of *hd2b* and *mpk3* flg22 De-regulated Genes in the HD2B-AA Line.

Supplemental Figure 29. Characterization of *hd2b* Mutant Line.

Supplemental Figure 30. Positions of the Regions Analyzed by ChIP-qPCR Experiments on the Different Loci.

Additional Files:

List of HD2B Target Genes in Mock and flg22 Conditions. (XLSX 167 kb)

List of H3K9 Hyper- and Hypo-acetylated Genes After flg22 Treatment. (XLSX 60 kb)

List of Deregulated Genes in *hd2b* and *mpk3* Mutants. (XLSX 51 kb)

List of Deregulated Genes After flg22 Treatment in Wt. (XLSX 52 kb)

List of Primers Used in this Study. (XLSX 13 kb)

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CHAPTER 5

The Function of Medicago Plant-specific Histone Deacetylases During Nodule Development

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ABSTRACT

Legumes and rhizobia interact to develop nitrogen-fixing root nodules. The nodule formation is accomplished through transcriptional reprogramming of root cells. In this study, we show that *Medicago truncatula* (Medicago) plant-specific histone deacetylases (*MtHDTs*) are required for this. *MtHDTs* are induced in early stages of nodule primordia and stay active during the following nodule development stages. Knock-down of their expression by using *RNAi* strategy blocks nodule primordia development. A few nodules still can be formed but their nodule meristems are mal-functional and rhizobial release into the meristem derived cells is markedly reduced. Transcriptome analyses indicate that a decreased expression of 3-hydroxy-3-methylglutaryl coenzyme a reductase 1 (*MtHMGR1*) and its paralogs is possibly responsible for this phenotype.

INTRODUCTION

Legumes, of which *Medicago truncatula* (Medicago) is a model, can establish an endosymbiosis with nitrogen-fixing bacteria, known as rhizobia. This symbiosis culminates in the formation of root nodules where rhizobia differentiate into bacteroids which are able to convert atmospheric nitrogen into ammonia, that is used by the plant and in return get carbohydrates (Geurts and Bisseling, 2002).

The legume-rhizobia symbiosis requires a molecular dialogue between plants and bacteria. The process is initiated when plant roots secrete flavonoids which can be recognized by rhizobia. The bacteria respond with the production and secretion of lipochitooligosaccharide molecules, called Nod factors. The recognition of Nod factors by the plant activates both, infection process in epidermis, where bacteria enter the root hair through an infection thread, and mitotic division of pericycle, endodermis and cortical cells that contributes to the formation of the nodule primordia (Denarie et al., 1996; Timmers et al., 1999; Oldroyd and Downie, 2008; Xiao et al., 2014). The infection threads grow into primordium cells where release of rhizobia from infection threads is initiated. *Medicago* forms indeterminate nodules with a persistent meristem at its apex (Libbenga et al., 1973). This meristem is derived from the middle cortical cell layer and continuously adds cells to the infection zone where cells enter endoreduplication process (Vinardell et al., 2003; Mergaert et al., 2006). The infected cells in the fixation zone are specialised to accommodate thousands of fully differentiated bacteroids (Perret et al., 2000; Mergaert et al., 2006; Gavrin et al., 2014).

Transcriptional reprogramming occurs during consecutive stages of nodule development. Transcription factors contribute to this reprogramming and their roles during *Medicago* nodule development are well documented. Some of them are involved in Nod factor signalling pathway. For example, in epidermis Nod factors perception activates Cyclops/IPD3, a transcriptional activator that induces the expression of *NIN* (Yano et al., 2008; Singh et al., 2014). *NIN* in its turn induces expression of *NF-YA1* that encodes the CCAAT box-binding transcription factor (Soyano et al., 2013). *NIN* and *NF-YA1* together with transcription regulators NSP1 and NSP2 control infection thread formation (Schauser et al., 1999; Kalo et al., 2005; Smit et al., 2005; Laporte et al., 2014). In addition, they play a pivotal role in initiating pericycle, endodermis and cortical cell divisions as ectopic expression of *NIN* or *NF-YA1* is sufficient to stimulate

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cortical cell division. Besides, the AP2 family transcription factors, PLT1-4 control nodule meristem formation and maintenance (Franssen et al., 2015).

Transcriptional reprogramming relies on chromatin remodelling factors. A recent study indicates that Medicago demethylase gene (*MtDME*) that modifies DNA methylation is critical for endoreduplication of nodule infected cells and differentiation of rhizobia (Satge et al., 2016). So far the roles of other chromatin remodelling factors in nodule development, especially at early stages has not been studied. There are strong evidences that nodules are evolutionary related to lateral roots (Hirsch and LaRue, 1997; Gualtieri and Bisseling, 2000). During Arabidopsis lateral root primordium initiation, before the first cell division takes place 4 members of plant-specific histone deacetylases (*AtHDTs*) are markedly up-regulated in founder cells, and they stay active in dividing cells (De Smet et al., 2008). Medicago contains 3 *HDT* members (*MtHDT1/2/3*) (Grandperret et al., 2014), and we hypothesize that they might be induced in cells of young nodule primordia. In addition, laser capture microdissection RNA sequencing (LCM-RNA-seq) analyses indicate that all 3 *MtHDTs* are expressed in mature nodules (Roux et al., 2014).

Here we investigated the function of *MtHDTs* in nodule development. The study on the temporal and spatial expression patterns of the 3 *MtHDTs* during nodule development shows that they are induced in young nodule primordia and stay active till the nodule meristem becomes functional. In mature nodules *MtHDTs* are expressed in nodule meristem and infection zone. Knock-down of *MtHDTs* expression blocks nodule primordia development. In the few formed mature nodules, meristem functioning and rhizobial infection are affected. Transcriptome analyses indicates that *MtHDTs* are required for *MtHMGRs* expression and in this way they might affect Nod factor signalling.

RESULTS

***MtHDTs* Are Expressed During Nodule Development**

3 *HDT* genes are identified in Medicago [*Medtr4g055440*, *Medtr2g084815* and *Medtr8g069135*, which are designated as *MtHDT1*, *MtHDT2* and *MtHDT3*, respectively, (Li et al., 2012; Grandperret et al., 2014)]. Sequence homology analyses by BLAST showed that the *MtHDT2* coding sequence was 60.2% and

55.1% identical to that of *MtHDT1* and *MtHDT3*, respectively (Pearson et al., 1997). All 3 MtHDT proteins contain the conserved N-terminal EFWG motif and a central acidic domain. Both MtHDT2 and MtHDT3 also have a C-terminal putative zinc finger domain (Supplemental Figure 1).

To determine the spatial expression pattern of the 3 *MtHDTs*, we performed RNA *in situ* hybridization on longitudinal sections of the nodules using probes specific for each *MtHDTs*. This showed that all 3 *MtHDTs* were mainly expressed in the nodule meristem and infection zone (Figure 1A-1C). *MtHDT2* was the highest, while *MtHDT3* was the lowest expressed gene. The expression of *MtHDT2* is at an equal level in the meristem and infection zone. In the latter it is expressed in both infected and uninfected cells. The transition of infection to fixation zone is, among others, characterized by the sudden accumulation of starch in the infected cells (Gavrin et al., 2014). At this transition the expression of *MtHDT2* dropped dramatically, which underlines that this is a major developmental switch in nodules and expression of *MtHDTs* is tightly regulated. Promoter-GUS (*pMtHDTs:GUS*) studies showed a similar expression pattern as the *in situ* experiments (Figure 1D, Supplemental Figure 3A-3C). In addition, it also showed that all 3 *MtHDTs* were expressed in the nodule vascular meristem, and *MtHDT1* and *MtHDT2* were also expressed in nodule vasculature.

Nodule primordia development is divided into six stages (Xiao et al., 2014). To determine expression patterns of the 3 *MtHDTs* during nodule primordium formation, we made use of the promoter-GUS constructs. At stage I all 3 *MtHDTs* were expressed in dividing pericycle and cortical cells and slightly activated in cells that have not yet divided but most likely have entered the cell cycle (Figure 1E, Supplemental Figure 3D-3F). At primordia stage V all 3 *MtHDTs* were also expressed in dividing cells. In the infected cells *MtHDT2* and *MtHDT1* were expressed, but *MtHDT3* expression was not detectable (Figure 1F, Supplemental Figure 3G, 3H). During primordium development, the expression level of *MtHDT2* was highest and *MtHDT3* lowest which is similar to their expression levels in nodules. The high DNA sequence homology and similar expression patterns of *MtHDTs* suggest that they are functionally redundant in controlling nodule development.

Subcellular Localization of MtHDTs in Nodule Cells

To study the subcellular localization of MtHDTs in nodule cells, we created

Figure 1. Expression of *MtHDTs* During Nodule Development.

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N-terminal GFP translational fusions with their coding sequences and these fusion constructs are driven by their own promoters (*pMtHDTs:GFP-HDTs*). To test whether the *pMtHDT2:GFP-HDT2* fusion is biologically functional, we introduced it into the Arabidopsis *HDT1hdt1HDT2hdt2* mutants, as Medicago *hdt* mutants are not available. The Arabidopsis *hdt1hdt2* mutant is lethal (Chapter 2). This mutant was rescued by introducing *pMtHDT2:GFP-HDT2* (Supplemental Figure 2), confirming that this fusion is functional. In Medicago root cells, MtHDT2 protein was detected in nuclei and especially in the nucleoli (Figure 2A). In cells of the nodule meristem and infection zone, MtHDT2 was also present in nucleoli, but in comparison to root cells, the level in the nucleoplasm was markedly increased (Figure 2B). In nuclei of lateral root primordia, MtHDT2 was also primarily located in nucleoli, whereas in nodule primordia an increased level of MtHDT2 was observed in nucleoplasm (Supplemental 5A, 5B). These data indicate that MtHDT2 displays slightly different sub-nuclear localization patterns during nodule and root development.

The expression level of *MtHDT1:GFP-HDT1* was rather low in root nodules and therefore we performed immuno-cytology using anti-GFP antibodies. This showed that MtHDT1 was located in the meristem and the infection zone (Supplemental Figure 5C, 5D). The *MtHDT3:GFP-HDT3* fusion was not detectable in the nodule even by using immuno-cytology with anti-GFP antibodies.

continued

(A) to (C) *In situ* localization of *MtHDT1* **(A)**, *MtHDT2* **(B)** and *MtHDT3* **(C)** transcripts in WT *M. truncatula* nodules. Nodules were harvested at 21dpi.

(D) to (F) Expression pattern of *MtHDT2* in the nodule and nodule primordia. **(D)** A mature nodule harvested at 21dpi. **(E)** A stage I nodule primordium. **(F)** A stage V nodule primordium. GUS activity was visualized after incubation with GUS buffer for 6 hours.

Longitudinal nodules/nodule primordia sections were shown. Bars=100µm.

Figure 2. The Sub-nuclear Localization of MtHDT2 in Root and Nodule Cells.

(A) Localization pattern of *pMtHDT2:GFP-HDT2* in root cells. Epidermal cells at the transition from root meristem to elongation zone were shown. GFP signal was mainly detected in nucleoli.

(B) Localization pattern of *pMtHDT2:GFP-HDT2* in nodule cells. Arrowhead or arrow indicate cells from nodule meristem or infection zone with considerable GFP signal in nucleoplasm. M, nodule meristem; I, infection zone and F, fixation zone.

Longitudinal root/nodule sections were shown. Bars=25μm.

Knock-down of *MtHDTs* Affects Nodule Formation

To test whether *MtHDTs* control nodule formation, we made conditional RNA interference (*RNAi*) constructs specific for individual genes, or different combinations of the two or all 3 *MtHDT* genes simultaneously. The constructs are driven by *ENOD12* promoter which is activated in all nodule primordia cells and remains active in nodule meristem and infection zone of mature nodules. This pattern covers the expression domains of the *MtHDTs* (Pichon et al., 1992; Limpens et al., 2009; Limpens et al., 2013; Franssen et al., 2015). Due to the high homology of *MtHDTs*, we first tested the specificity of the *RNAi* constructs that aimed to reduce the expression of the corresponding *MtHDT* gene(s). The knock-down level of *MtHDT(s)* mRNA in nodules was determined by qRT-PCR. This showed that in the transgenic nodules interfered *MtHDT(s)* was specifically knocked-down with a 3 to 8-fold reduction (Figure 3A), while the expression of non-interfered *MtHDT(s)* was not affected.

Control roots at 21 days post inoculation (dpi), formed about 6 nodules per root (Figure 3B). Knock-down of any individual *MtHDT* gene or simultaneously knock-down of *MtHDT1/3*, *MtHDT2/3* did not reduce the nodule number significantly. In contrast, nodule number on transgenic *MtHDT1/2 RNAi* roots was reduced significantly. A slightly higher reduction was observed on *MtHDT1,2,3 RNAi* (*MtHDTs RNAi*) roots. These data indicate that *MtHDTs* and especially *MtHDT1* and *MtHDT2* are required for nodule formation. It also suggests that *MtHDTs* have functional redundancy in controlling nodule formation.

Expression of *MtHDTs* in nodule primordia suggests that the reduced nodule formation on *MtHDTs RNAi* roots could be caused either by the reduced initiation or by a block of nodule primordia development at an early stage. To examine this, we transformed Medicago *ENOD11:GUS* plants (Journet et al., 2001) with *ENOD12-EV* (Empty Vector, see Methods) as a control or *MtHDTs RNAi* constructs. The *ENOD11* promoter is active in dividing cells of nodule primordia, but it is only slightly expressed in pericycle derived dividing cells of lateral root primordia (Supplemental Figure 4). This allows us to distinguish between nodule primordia and lateral root primordia. At 6 dpi, 37 and 29 nodule primordia were identified in 5 comparable transgenic control and *MtHDTs RNAi* roots, respectively (Figure 3C). This shows that knock-down of *MtHDTs* does not reduce nodule primordia initiation. On the transgenic control roots more than 80% (30 out of 37) of nodule primordia passed the stage II and a relatively

Figure 3. Silencing *MtHDTs* Reduces Nodule Formation.

(A) Quantitative real time PCR (qRT-PCR) analyses of *MtHDT1*, *MtHDT2* and *MtHDT3* expression in control (*ENOD12-EV*) and *MtHDT(s) RNAi* nodules. Nodules were harvested at 21dpi. All panels show mean $\pm$ SE values determined from three biological replicates (each replicate was estimated as the average of three technical replicates).

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high number of them (35%, 13 out of 37) developed into or passed stage V. In contrast, on *MtHDTs RNAi* transgenic roots, the majority of nodule primordia (62%, 18 out of 29) were in stage I or stage II, and only a few nodule primordia had developed into or passed stage V (7%, 2 out of 29). The latter is similar to the number of nodules that are formed. These data indicate that knock-down of *MtHDTs* blocks nodule primordia development and in this way affects nodule formation.

Meristem Functioning and Rhizobial Release Require *MtHDTs*

Although *MtHDTs RNAi* markedly reduces nodule number, some nodules can be formed due to the *MtHDTs* expression level probably above the threshold level required for the early stages of nodule development. This allows to study the role of *MtHDTs* in mature *Medicago* nodules. *MtHDTs RNAi* nodules were in general smaller than control nodules. To determine the cause of this size reduction, we analyzed longitudinal sections of these nodules. At 21 dpi, both the control (n=24) and *MtHDTs RNAi* (n=30) nodules had meristems (Figure 4A, 4B). Meristems of control nodules consisted of about 6 cell layers, however the number of cell layers in *MtHDTs RNAi* meristems was reduced to about 3. In agreement with this, expression of *MtPLT3* and *MtPLT4*, two genes that are expressed throughout the nodule meristem (Franssen et al., 2015), was markedly reduced in *MtHDTs RNAi* nodules (Figure 4C). In mature *Medicago* nodules about 8 proximal cell layers of infected cells are directly derived from the primordium cells and not from the nodule meristem (Xiao et al., 2014), whereas the other infected cells at the distal part of the nodule are derived from the nodule meristem. In *MtHDTs RNAi* nodules the proximal 8 layers of cells were

continued

(B) Number of nodules formed on control (*ENOD12-EV*) roots and *MtHDTs RNAi* roots at 21dpi. Only those roots with a length between 8-10cm were considered for analysis. More than 20 roots were analyzed for each line.

(C) Number of different stages of nodule primordia formed on control (*ENOD12-EV*) and *MtHDTs RNAi* roots in *ENOD11:GUS* background at 6dpi. 5 comparable roots from each line were collected and sectioned.

Figure 4. Knock-down of *MtHDTs* Affects Nodule Meristem Activity and Rhizobial Infection.

(A) and **(B)** Longitudinal sections of 3-weeks old control (*ENOD12-EV*, **A**) and *MtHDTs RNAi* (**B**) nodules. Representative figures were shown. Both control and *MtHDTs RNAi* nodules have ~8 central tissue layers (below the red line) infected by rhizobia. *MtHDTs RNAi* nodule has a relatively small nodule meristem, and in the infection zone cells are less infected. M, nodule meristem; I, infection zone and F, fixation zone. Bars=100 μ m.

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control-like, whereas the number of cell layers derived from the nodule meristem was markedly reduced (Figure 4D). In addition, in cells derived from the nodule meristem rhizobial release was reduced. These data indicate that knock-down of *MtHDTs* reduces rhizobial release in cells derived from the meristem but not from primordia. Most likely release in the former cells requires a higher threshold levels of *MtHDTs* than in the latter. These data suggest that meristem functioning and rhizobial release are affected in the *MtHDT RNAi* nodules.

Knock-down of *MtHDTs* Results in Transcriptome Changes

HDT proteins are known to regulate gene transcription (Wu et al., 2003; Ding et al., 2012). To investigate which genes might be regulated by *MtHDTs* during nodule development, we performed illumina RNA sequencing to compare transcriptome differences in the nodule meristem and infection zone, as *MtHDTs* are preferentially expressed there. To dissect the nodule meristem and infection zone from the rest, transgenic control and *MtHDTs RNAi* roots were inoculated with rhizobia expressing *nifH::GFP*. The *nifH* promoter is activated in bacteria that fix atmospheric nitrogen (Gavrin et al., 2014). This gene is switched on at the transition from infection to fixation zone where *MtHDT2* is switched off. So, the nodule meristem and infection zone can be distinguished and dissected from the fixation zone under fluorescent microscope.

We detected the expression of ~20,000 genes in control and *MtHDTs RNAi* nodules (Supplemental Data Set 1, see Methods). The differences in expression level of *MtHDT1/2* and *MtPLT3/4* between control and *MtHDTs RNAi*

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continued

(C) Quantitative real time PCR (qRT-PCR) analyses of *MtPLT3* and *MtPLT4* expression in control (*ENOD12-EV*) and *MtHDTs RNAi* nodules. Nodules were harvested at 21dpi. All panels show mean $\pm$ SE values determined from three biological replicates (each replicate was estimated as the average of three technical replicates).

(D) Number of cell layers added by nodule meristem in 3-weeks old control (*ENOD12-EV*) and *MtHDTs RNAi* nodules. Data shown are average $\pm$ SD ($n > 20$). Asterisks indicate significant differences (***) $p < 0.001$; Student's *t* test).

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transcriptomes are consistent with qRT-PCR data. This indicates that RNA-seq data are most likely reliable. To identify differential expressed genes (DEGs), we performed relatively stringent statistics and filtering (fold change>4 and FDR p-value<0.05). In total 49 DEGs were identified between control and *MtHDTs RNAi* (Supplemental Data Set 1).

To obtain insight in the biological functions of these 49 DEGs, we performed Gene Ontology (GO) analyses. This showed that some of these genes display oxidoreductase activity (Supplemental Figure 6) and two of them encode 3-hydroxy-3-methylglutaryl-coenzyme A reductases (*MtHMGR1* and *MtHMGR4*). *MtHMGR1* has been shown to interact with SymRK (*MtDMI2*) which is an important component of the Nod factor signalling cascade (Kevei et al., 2007; Venkateshwaran et al., 2015). Noteworthy its expression is down-regulated in *MtHDTs RNAi* nodules. As Nod factor signalling is important for bacterial release (Moling et al., 2014), it suggests that the decreased expression of *MtHMGR1* might explain the reduced bacterial release in *MtHDTs RNAi* nodule cells.

It has previously been shown that knock-down of *MtHMGR1* blocks nodule primordia development (Kevei et al., 2007), this is similar to our *MtHDTs RNAi* studies (Figure 3C). We ask the question whether *MtHDTs RNAi* affects *MtHMGR1* expression in nodule primordia. The expression of *MtHMGR1* was determined in *MtHDTs RNAi* late stages of nodule primordia by qRT-PCR and it showed that its was also reduced (Figure 5). This suggests that the reduced expression of *MtHMGR1* is sufficient to explain the phenotype of *MtHDTs RNAi* nodule primordia.

DISCUSSION

In this study we show temporal and spatial expression patterns of *MtHDTs* during nodule formation. Conditional knock-down of *MtHDTs* blocks nodule primordia development and in mature nodules meristem functioning and rhizobial release are affected. Transcriptome data suggest that *MtHDTs* control Nod factor signalling pathway by regulating expression of *MtHMGR1* and its paralogs and in this way control nodule development.

Figure 5. Expression of *MtHMGR1* and Its Paralogs in Nodule Primordia

Quantitative real time PCR (qRT-PCR) analyses of *MtHMGRs* in control (*ENOD12-EV*) and *MtHDTs* RNAi nodule primordia. Nodule primordia were harvested at 6dpi. All panels show mean $\pm$ SE values determined from three biological replicates (each replicate was estimated as the average of three technical replicates).

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Knock-down of *MtHDTs* reduces nodule meristem activity (Figure 4B-4D). This is similar to the function of Arabidopsis HDT1/2 in maintaining root meristem size by repressing *AtGA2ox2* (Chapter 2). However, transcriptome studies indicate that expression of *MtGA2oxs* is not affected in *MtHDTs* RNAi nodules (Supplemental Data Set 1). This suggests that HDTs regulate meristem activity in these two organs, root and nodule, but most likely in a different manner. This is to some extent in line with the different sub-nuclear localization patterns of *MtHDT2* in the meristematic cells of nodule and root (Figure 2). Noteworthy, a cell cycle gene *cyclinB1* expression (Gutierrez, 2009) is not affected by knocking-down of *MtHDTs*, pointing to the existence of a potential post-transcriptional regulation in *MtHDTs* RNAi nodule.

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Rhizobial release from infection threads into the nodule cells requires Nod factor receptors NFP and LYK3, as well as a plasma membrane located LRR-type receptor kinase MtDMI2. In *MtNFP RNAi* and *MtDMI2 RNAi* nodules rhizobial release is markedly reduced (Limpens et al., 2005; Moling et al., 2014), similar to that in *MtHDTs RNAi* nodules (Figure 4B), suggesting that MtHDTs might affect Nod factor signalling. We show that MtHDTs regulate the expression of *MtHMGR1*, encoding an interactor of MtDMI2 (Kevei et al., 2007), and likely in this way affects rhizobial release in nodules. *MtHMGR1* is also down-regulated in *MtHDTs RNAi* nodule primordia (Figure 5). It has been shown that knock-down of *MtHMGR1* expression blocks nodule primordia development (Kevei et al., 2007), which is consistent with the main phenotype of *MtHDTs RNAi* (Figure 3B). In both cases rhizobial release is blocked and nodule meristems are not formed, indicating that MtHDTs and MtHMGR1 are required for both, rhizobial release and nodule organogenesis.

Besides *MtHMGR1*, all other members of the *MtHMGR* family were down-regulated in *MtHDTs RNAi* nodule primordia and nodules (Figure 5, Supplemental Data Set 1), suggesting that knock-down of *MtHDTs* decreases mevalonate biosynthesis. This might also contribute to the reduction of nodule formation, as pharmacological inhibition of HMGR enzymatic activity by applying lovastatin reduces nodule formation as well (Alberts et al., 1980; Kevei et al., 2007). Intriguingly, five down-regulated *MtHMGR* paralogs are localized in tandem on chromosome 5 (Supplemental Figure 7). Similar to this, five *(3S)-linalool/(E)-nerolidol/(E, isoflavone-7-O-methyltransferase* genes and five *cytochrome P450 family 71* genes grouped by Gene Ontology analyses are also tandem and all down-regulated in *MtHDTs RNAi* nodules (Supplemental Data Set 1). The clustering could facilitate regulation by histone modification genes, such as *MtHDTs*. We conclude that MtHDTs control nodule formation, in part by regulating the transcription of *MtHMGR1* and its paralogs.

METHODS

Plant Growth, Transformation and Inoculation

Medicago ecotype Jemalong A17 was used as wild-type in all experiments. *ENOD11:GUS* stable line was described in (Journet et al., 2001). For transformation, *Agrobacterium rhizogenes* MSU440 mediated hairy root

transformation was used according to (Limpens et al., 2004). The plants with transgenic roots were grown at 21°C in a 16 h : 8 h, light : dark regime in perlite at low nitrate conditions in the presence of *Sinorhizobium meliloti* 2011 or the rhizobial strain expressing *nifH::GFP* to form nodules as described by (Limpens et al., 2004b). Nodule primordia were harvested at 6 days after inoculation (dpi). Mature nodules were harvested at 21 dpi.

Constructs

To make *pMtHDTs::GUS* constructs, DNA fragments of putative promoter regions of *MtHDTs* genes were generated by PCR using *Medicago* genomic DNA as a template and Phusion high-fidelity DNA polymerase (Finnzymes) and specific primers indicated in Supplemental Table 1.1. Fragments were cloned into pENTR-D-TOPO (Invitrogen), verified by nucleotide sequence analysis, and recombined into the modified Gateway vector pKGWFS7 containing the *UBQ10::DsRED* selection marker and GUS reporter gene (Karimi et al., 2002).

N-terminal fusions of *MtHDTs* with GFP under the control of their own promoter were constructed using Multisite Gateway (Invitrogen). The coding sequence (CDS) of each *MtHDT* was PCR amplified and introduced into a pENTR-D-TOPO vector (Invitrogen). The primers used were indicated in Supplemental Table 1.2. The *MtHDT* promoters were re-cloned from the pENTR-D-TOPO vectors into a pENTR4-1 vector (Invitrogen) in front of a GFP open reading frame. The pENTR4-1 vector with the *MtHDT* promoter and GFP, the corresponding pENTR-TOPO *MtHDT* CDS vector and a pENTR2-3 vector containing a CaMV35S terminator were recombined into the binary destination vector pKGW-RR-MGW, thereby creating *pMtHDT::GFP-MtHDT*.

To create *MtHDT(s) RNAi* constructs, the PCR fragments of about 400-500bp of cDNA for each *MtHDTs* were amplified and then combined by subsequential PCR steps using primers with a complementary 15 bp overhang to generate one amplicon of either *MtHDT1-MtHDT3* or the two, or all three *MtHDTs*. The following primers were indicated in Supplemental Table 1.3. These fragments were introduced into a pENTR-TOPO vector (Invitrogen) and recombined in inverted repeat orientation into the Gateway compatible binary vector pK7GWIWG2(II) driven by nodule specific *MtENOD12* promoter (Limpens et al., 2005b). The control vector [*ENOD12::Empty Vector (EV)*] contained no DNA coding sequence.

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Gene Expression and RNA-Seq

Total RNA from transgenic nodules or nodule primordia was isolated using the plant RNA Easy Kit (Qiagen). cDNA was synthesized with 1 µg of isolated total RNA by reverse transcription with random hexamer primers using the iScript Select cDNA synthesis kit (BioRad) according to the manufacturer's instructions. Quantitative PCR was performed on a MyiQ (Biorad) using the Quantitative PCR kit for SYBR green (Eurogentec). *UBIQUITIN* and *ACTIN* were used as reference genes and showed similar results. Primers used for real-time PCR are listed in Supplemental Table 1.4.

For RNA-Seq analyses, nodule meristem and infection zone of *ENOD12-EV* and *MtHDTs RNAi* was cut and collected. Total RNA was extracted as described above. RNA was sequenced at BGI Tech Solutions (Hong Kong) using Hiseq2000 instrument. Sequencing data were analyzed by mapping to the Medicago genome using CLC Genomics Workbench (Denmark). Gene expression levels were determined by calculating the RPKM [(Reads Per Kilobase per Million mapped reads), (Consortium et al., 2006)]. Differentially expressed genes (DEGs) are defined based on relatively stringent statistics and filtering (fold change > 4, FDR P value < 0.05) within the CLC. GO enrichment analyses was performed using agriGO (Du et al., 2010).

RNA *in situ* Hybridization

The nodules were fixed with 4% paraformaldehyde mixed with 5% glutaraldehyde in 50 mM phosphate buffer (pH 7.4) and embedded in paraffin (Paraplast X-tra, McCormick Scientific). Nodule sections of 7 µm were prepared by RJ2035 microtome. RNA ISH was conducted according to the Affymetrix user manual for ViewRNA ISH Tissue 2-plex Assay (<http://www.panomics.com/UserDocs>). RNA ISH probe sets were designed and produced by Affymetrix. Each set contains 20 oligonucleotide probes, each consisting of a target-specific region and a unique sequence upon which signal amplification is built. Probe sets for *MtHDT1* covered the region 2-799 nt (804 nt), for *MtHDT2* the region 93-972 nt (1171 nt), for *MtHDT3* the region 411-968 nt (984 nt) the full-length mRNAs. Slides were analyzed with an AU5500B microscope equipped with a DFC425c camera (Leica).

Histological Analysis and Microscopy

For GUS staining, plant tissues containing promoter-GUS fusions were incubated at 37°C in 0.1 M NaH₂PO₄-Na₂HPO₄ (pH 7) buffer including 3% sucrose, 0.05 mM EDTA, 0.5 mg/ml X-gluc, 2.5 mM potassium ferrocyanide and potassium ferricyanide. Incubation time varied depending on tissues and different promoter-GUS fusions. For plastic sections, root material and nodules were fixed in mixture of 4% paraformaldehyde and 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight. After that, they were washed with 0.1 M phosphate buffer four times for 15 min each, once with water for 15 min, and dehydrated for 10 min in 10%, 30%, 50%, 70%, 90% and 100% ethanol, and sequentially embedded in plastic Technovit 7100 (Heraeus Kulzer). Sections were made of 5-10 µm using a microtome (RJ2035, Leica), stained either with 0.05% Toluidine Blue (Sigma) or 0.1% Ruthenium Red (Sigma), mounted in Euparal (Carl Roth), and analyzed with a Leica AU5500B microscope equipped with a DFC425c camera (Leica).

Immuno-detection and Confocal Microscopy

Transgenic *pMtHDT1:GFP-HDT1* nodules were fixed in 1% paraformaldehyde in PBS on ice for 1h. They were hand-sectioned using a double-sided razor blade and washed 3 times 0.5h each with PBS + 0.3% Triton X100. Then nodules were incubated in blocking solution (3% BSA in PBS) for 1h. Primary anti-GFP antibody (Thermo Fisher Scientific) were added to blocking solution (1: 200) and incubation was done during night in a cold room. Next day nodules were washed and incubated with secondary goat anti-rabbit Alexa~488 antibodies (1: 200, Thermo Fisher Scientific) for 3h. After washing nodules were counterstained with 0.5 µg/ml Propidium Iodide in PBS and imaged with a Leica TCS SP8 HyD 8 confocal microscope.

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Supplemental Figure 1. Domain Organization of Medicago HDTs.

The red box represents the conserved EWFG motif, the blue box the central acidic region and the yellow box the zinc finger domain.

Supplemental Figure 2. *MtHDT2-GFP* Fusion Complements Arabidopsis *hdt1hdt2* Phenotype.

Phenotype of the wild-type (WT), *AtHDT1* loss-of-function (*hdt1*), *AtHDT2-GFP* complemented (*AtHDT2-GFP/hdt1hdt2*) and *MtHDT2-GFP* complemented (*pMtHDT2:GFP-HDT2*) seedlings at 6 days after germination.

Supplemental Figure 3. Expression of *MtHDTs* During Nodule Formation.

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Supplemental Figure 4. Expression Pattern of *ENOD11* in Lateral Root Primordium (left) and Nodule Primordium (right).

Data shown was obtained in *ENOD11:GUS* stable line after rhizobium inoculation for 6 days. GUS activity was visualized after incubation with GUS buffer for 2 hours. Bar=100μm.

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continued

(A) to (H) Expression patterns of *MtHDT1* (A, D and G), *MtHDT2* (B and E) and *MtHDT3* (C, F and H) in mature nodules (A to C), stage I/II nodule primordia (D to F) and stage V nodule primordia (G and H). (E) is a zoom out image of Figure 1E.

Nodules or nodule primordia were harvested at 21dpi or 6dpi, respectively. Arrowheads in (A to C) or arrows in (A) and (B) indicate nodule vascular meristems or vasculature. GUS activity was visualized after incubation with GUS buffer for 6 hours (A, B, D, E and G) or for 24 hours (C, F and H). Longitudinal nodules/nodule primordia sections were shown. Bars=100μm.

Supplemental Figure 5. The Sub-nuclear Localization Pattern of MtHDT2 and MtHDT1 Proteins.

(A) and **(B)** Localization pattern of *pMtHDT2::GFP-HDT2* in later root primordium **(A)** and nodule primordium **(B)**. The boxed areas were magnified, showing a considerable GFP signal in nucleoplasm was observed in cells of nodule primordium, but not in cells of lateral root primordium.

(C) and **(D)** Localization pattern of *pMtHDT1::GFP-HDT1* in mature nodules. GFP signal was detected using α -GFP immunocytology in nodule meristem and young infection zone **(C)**. A weak nucleoplasm signal was detected **(D)**. M, nodule meristem; I, infection zone and F, fixation zone.

Longitudinal sections were shown. Bars=100 μ m.

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Supplemental Table 1. Primers Used in This Study.

	Primer name	Sequence
1.1	pHDT1-F	CACCTCTTTTTGAATTAGTTTCTTATTTTGG
	pHDT1-R	TTGAAAGGGAGAGCGAAACG
	pHDT2-F	CACCGTTTTTACTTCCTTCTGCCAAAC
	pHDT2-R	TGGAAACAGTGAGACAGAGAGAG
	pHDT3-F	CACCGGTTTGGGTGATTTTGGATTG
	pHDT3-R	GACTGCAGAGAGAGAGCAAGC
1.2	HDT1-F	CACCATGGATCGACCAATGGAGTT
	HDT1-R	ACGGCGATCTTGCTTAGACT
	HDT2-F	CACCATGGAGTTCTGGGGTGCTG
	HDT2-R	CTTAGCACCATGCTTGGCCT
	HDT3-F	CACCATGGAGTTTGGGGTGTTGA
	HDT3-R	GGCATCTTCAGTCTTAAAGGC
1.3	HDT12 RNAi F	CCACCCTATGAAGAAATCCCATTGATTACC
	HDT12 RNAi R	GGTAATCAATGGGATTCTTCATAGGGTGG
	HDT13 RNAi F	CCACCCTATGAAGAAATGGGAGCGTCTTCT
	HDT13 RNAi R	AGAAGACGCTCCCATTCTTCATAGGGTGG
	HDT23 RNAi F	AAAACCTCCAAGTCTATGGGAGCGTCTTCT
	HDT23 RNAi R	AGAAGACGCTCCCATAGACTTGGGAGTTTT
1.4	HDT1qPCR-F	GGATGAGGGCAAGAACAAAA
	HDT1qPCR-R	TTGGTGGTAGCAGGCTTCTT
	HDT2qPCR-F	TCTGGGGTGCTGAGGTTAAG
	HDT2qPCR-R	TTTGTCTTGGTCAGGGTTC
	HDT3qPCR-F	CTGAAGGGAGCAACAAGAGG
	HDT3qPCR-R	TGGCTGCTTGTTATTTGCAG
	PLT3qPCR-F	TGACGTGGAAGCGATAATGA
	PLT3qPCR-R	GTTGCTGCTGCTGCTGTTAAG
	PLT4qPCR-F	TCACGAGGTGCATCCATTTACCGA
	PLT4qPCR-R	ACATCATATGCCTCTGCTGCCTCT
	HMGR1qPCR-F	CAGCAGGATTCACAGTCACAAC
	HMGR1qPCR-R	GGATCTTTTCACGCCACCTG
	HMGR2qPCR-F	CTCGCTCTTGTTCCCTCATC
	HMGR2qPCR-R	GGTTTTTGCAGCAATGTCTTC
	HMGR3qPCR-F	CCGGAGTCGCACAGAAGC
	HMGR3qPCR-R	CGGACAACCTCGTCATCTTCC
	HMGR4qPCR-F	CGAAAACCACCAAAGTCATGC
	HMGR4qPCR-R	GAATTGATCCAGACACCACTGC
	HMGR5qPCR-F	TGCACCCGTGAATCTCACAC
	HMGR5qPCR-R	TTATCGTTTGCACCGCTGAAC
	HMGR6qPCR-F	ATCGTTTGCTACACGCGCTTC
	HMGR6qPCR-R	AGTTTGCGGAGAGGCTGATTG
	UBQqPCR-F	CCCTTCATCTTGCTTCGTCG
	UBQqPCR-R	CACCTCCAATGTAATGGTCTTTCC
	ACTINqPCR-F	ACGAGCGTTTCAGATG
	ACTINqPCR-R	ACCTCCGATCCAGACA

Supplemental Data Set 1. Gene Expression Map of the *ENOD12-EV* and *MtHDTs RNAi* Nodule Meristem and Infection Zone. (XLSX 18.6 Mb; available upon request).

CHAPTER 6

MtHDT2 Regulates Arbuscule Formation in the *Medicago truncatula* (Medicago) – Arbuscular Mycorrhizal (AM) Symbiosis

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ABSTRACT

Arbuscular mycorrhizal (AM) symbiosis enables plants to acquire phosphate that is usually limited in soil. Establishment of this symbiosis starts from the colonization of roots by fungal hyphae, followed by their entry into the root and eventually highly branched fungal structures called arbuscules are formed inside inner cortical cells. During this symbiotic interaction, transcriptional reprogramming occurs. Chromatin remodelling factors contribute to the regulation of gene expression and therefore most likely are involved in the establishment of AM symbiosis. Here we show that in *Medicago* a plant-specific histone deacetylase *MtHDT2* is induced in mycorrhized roots, especially in arbuscule containing cells. In these cells MtHDT2 protein displays variable sub-nuclear localization patterns. Knock-down of MtHDT2 significantly reduces arbuscule formation in mycorrhized root segments, and possibly affects AM symbiosis maintenance.

INTRODUCTION

More than 80% of the land plants, including most crops, are able to form arbuscular mycorrhizal (AM) symbiosis with fungi that belong to the *phylum Glomeromycota* (Schussler et al., 2011; Berruti et al., 2016). This mutualistic interaction enables plants access to phosphorus and other nutrients, and in return the fungus obtains carbohydrates and lipids from the host (Hodge et al., 2001; Harrison et al., 2002; Helber et al., 2011; Jiang et al., 2017; Luginbuehl et al., 2017). Given the limitation of phosphorus in natural soils, the AM symbiosis plays an important role in plant growth and therefore contributes to sustainable agriculture (Fester and Sawers, 2011).

AM symbiosis is initiated at the root epidermis through a signalling pathway that is triggered by Myc factor that are secreted by the fungus (Levy et al., 2004; Imaizumi-Anraku et al., 2005; Yano et al., 2008; Ried et al., 2014). This signalling ultimately activates transcription factors, such as CYCLOPS (IPD3), NSP2 and RAM1 (Yano et al., 2008; Maillet et al., 2011; Gobbato et al., 2012; Lauressergues et al., 2012) that induce transcriptional reprogramming. This allows the fungus to colonize host roots and triggers cellular rearrangement of host cells for hyphae infection through the epidermis and for the formation of arbuscles in cortical cells (Harrison, 2012). In loss-of-function *cyclops/ipd3*, *nsp2* and *ram1* mutants, mycorrhizal colonization is significantly reduced (Yano et al., 2008; Maillet et al., 2011; Gobbato et al., 2012; Lauressergues et al., 2012; Pimprikar et al., 2016).

In addition to transcription factors chromatin organization plays a major role in regulation of gene expression. Chromatin remodelling factors modify chromatin organization (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Kouzarides, 2007; Shahbazian and Grunstein, 2007). However, their function in controlling AM symbiosis has not been studied so far. Previously, we showed that *Medicago truncatula* plant-specific histone deacetylases (MthDTs) are required for the development of the nodule symbiosis. They might also control AM symbiosis since there is a close evolutionary relationship between the two symbionts (Manchanda and Garg, 2007; Oldroyd, 2013; Zipfel and Oldroyd, 2017). Here we demonstrate that *MthDT2* is expressed during AM symbiosis formation. Its transcript and protein are abundant in arbuscule-containing cells. In these cells the MthDT2 protein has variable sub-nuclear localization patterns that probably are associated with arbuscular developmental stages.

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Knock-down of *MtHDT2* does not affect the colonization of roots by hyphae, but significantly reduces arbuscule formation in colonized root segments and possibly affects arbuscule maintenance.

RESULTS

***MtHDT2* Expression Is Induced During Arbuscule Formation**

The Medicago *MtHDT* family contains 3 members (Grandperret et al., 2014). To test whether they are induced during arbuscule formation, we inoculated transgenic roots expressing *pMtHDT1:GUS*, *pMtHDT2:GUS* and *pMtHDT3:GUS* with *Rhizophagus irregularis*. In non-inoculated transgenic roots *MtHDT1* and *MtHDT2* were highly expressed in root meristems and their expression level was markedly decreased in the root elongation zone (Supplemental Figure 1A, 1C). *MtHDT3* showed the same expression pattern whereas this pattern could only be detected after a prolonged incubation time in GUS buffer (Supplemental Figure 1B, 6h for *MtHDT1* and *MtHDT2*, 24h for *MtHDT3*). In the differentiated zone, the expression of all 3 genes was below the detection level. At 4 weeks post inoculation (wpi) expression patterns of *MtHDT1* and *MtHDT3* were similar to those of non-inoculated transgenic roots (Figure 1A, 1C). Arbuscules were formed on *pMtHDT1:GUS* and *pMtHDT3:GUS* transgenic roots (Supplemental Figure 1D, 1E), pointing that these 2 genes are not induced during AM symbiosis. In the inoculated *pMtHDT2:GUS* transgenic roots, the expression pattern of *MtHDT2* in root meristem and elongation zone was the same as in the non-inoculated roots, however, in some regions of the differentiated zone a marked increase of *MtHDT2* expression was observed (Figure 1B). This indicates that root colonization by AM fungi induces *MtHDT2* expression. To better visualize the spatial expression pattern of *MtHDT2* in mycorrhized roots, we embedded and sectioned these roots and this showed that *MtHDT2* was highly expressed in arbuscule containing cells. It was also expressed in outer cortex and endodermis albeit at a markedly lower level (Supplemental Figure 1F). RNA *in situ* hybridization, by using a *MtHDT2* specific probe, confirmed the expression of this gene in arbuscule containing cells. In addition, it also showed that *MtHDT2* had variable expression levels in these cells within a single root segment (Figure 1D). This was consistent with *pMtHDT2:GUS* data after a short incubation time (2h) in GUS buffer when the intensity of blue colour is still

Figure 1. Expression of *MtHDTs* in Roots During Arbuscule Formation.

(A) to (C) Expression patterns of *pMtHDT1:GUS* (A), *pMtHDT2:GUS* (B) and *pMtHDT3:GUS* (C) in mycorrhized roots.

(D) RNA *in situ* hybridization of *MtHDT2* in arbuscule containing cells. Experiments were done as described in Chapter 5.

(E) Longitudinal section of mycorrhized *pMtHDT2:GUS* transgenic roots.

Arrow or arrowhead in (D) and (E) indicates arbuscule containing cells with strong or weak expression of *MtHDT2*, respectively. All transgenic roots were inoculated with *R. irregularis* for 4 weeks. β -glucuronidase (GUS) activity was visualized after incubation with GUS buffer for 6 hours (A and B) or 24 hours (C), or 2 hours (E). Bars=500 μ m in (A) to (C), or 50 μ m in (D) and (E).

Figure 2. The Sub-nuclear Localization Patterns of MtHDT2 Protein.

(A) Localization pattern of *pMtHDT2::GFP-HDT2* in control roots.

(B) to (E) Localization patterns of *pMtHDT2::GFP-HDT2* in mycorrhizal roots. In arbuscule containing cells, strong GFP signal was observed either in nucleoplasm (B and C), or in nucleolus (D). Weak nucleolar GFP signal was observed in the cell containing collapsing arbuscule (E).

Black arrows or arrowheads indicate nuclei from cortex or endodermis/pericycle, respectively. White arrow in (D) indicates the nucleus of an outer cortical cell that adjacent to the hyphae which is indicated by a white arrowhead. Identical confocal microscope settings were used to image GFP signal in (A), (D) and (E). Bars=10 μ m in (A) to (E).

not saturated (Figure 1E). This shows that the level of *MtHDT2* in arbuscule containing cells varies and probably depends on the age of arbuscules. So *MtHDT2* expression is induced during arbuscule formation, whereas expression of the other 2 *MtHDT* genes are not.

MtHDT2 Protein Displays Variable Sub-nuclear Localization Patterns in Arbuscule Containing Cells

During the interaction of plants with pathogens or rhizobia HDT proteins display a sub-nuclear localization pattern different from non-infected plants (Chapter 4, 5). To study whether the sub-nuclear localization of MtHDT2 protein is changed during arbuscule formation, we inoculated transgenic *pMtHDT2:GFP-HDT2* roots with *R. irregularis* and analyzed MtHDT2 localization pattern in the differentiated zone where arbuscules had been formed. In the control roots, MtHDT2 was present at a low level in nucleoli (and not in nucleoplasm) of vascular and endodermal cells and was hardly detectable in cortical cells of the differentiated zone (Figure 2A). However, in inoculated roots MtHDT2 accumulated at a relatively high level in the inner cortical cells containing arbuscules (Figure 2B-2D). Further, in some of these cells a markedly increased MtHDT2 level was observed in nucleoplasm (Figure 2B, 2C). In cells containing collapsing arbuscules, the level of MtHDT2 protein was dramatically reduced (Figure 2E). These data suggest that the sub-nuclear localization patterns of MtHDT2 are associated with the arbuscule developmental stages. MtHDT2 is also detectable in outer cortical cells that are adjacent to hyphae, albeit at a low level (Figure 2D). Like in control roots MtHDT2 was detected in nucleoli of vascular and endodermal cells (Figure 2B, 2D and 2E). This shows that mycorrhizal infection results in an accumulation of MtHDT2 in cortical cells, as well as a relatively high level of the protein in the nucleoplasm in cells containing arbuscules.

Knock-down of *MtHDT2* Reduces AM Formation in Mycorrhized Root Segments

To examine whether MtHDT2 plays a role during AM symbiosis, we used RNA interference to knock-down *MtHDT2* expression. We expressed the RNAi construct (*MtHDT2i*) driven by the Arabidopsis *EF1 α* promoter which is highly expressed in Medicago roots (Axelos et al., 1989; Auriac and Timmers, 2007). The knock-down level of *MtHDT2* mRNA in root tips was determined by

Figure 3. Silencing of *MthD22* Reduces AM Symbiosis Formation in Infected

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qRT-PCR, this showed a ~5-fold reduction of its expression level in *MtHDT2i*, compared with that in control [(*EF-EV*, see Methods, (Figure 3A)].

MtHDT2i roots did not display an altered phenotype in comparison to control roots (data not shown). At 4wpi with *R. irregularis*, we compared mycorrhizal root colonization of control and *MtHDT2i* roots according to (Trouvelot A, 1986). This showed that the frequency of root fragments that are mycorrhized (F), as well as the intensity of infection in all root fragments (M) and the intensity of infection in mycorrhized root parts (m) were all slightly higher in *MtHDT2i* roots (Figure 3B). However, in *MtHDT2i* the abundance of arbuscules in mycorrhized root parts (a) was significantly reduced (75% in control, compared with 40% in *MtHDT2i*, Figure 3B-3D). While the abundance of arbuscules in all root fragments (A) was

continued

Root Segments.

(A) Quantitative real-time PCR (qRT-PCR) measurement of *MtHDT2* expression in tips of transgenic roots expressing *EF-EV* or *MtHDT2i* constructs. Both panels show mean $\pm$ SE values determined from three biological replicates (each replicate was estimated as the average of three technical replicates). *MtUBIQUITIN* or *MtACTIN* was used as the references and showed similar results.

(B) Quantification of mycorrhizal events in *EF-EV* and *MtHDT2i* transgenic roots. Parameters in percentages (%) used are F, the frequency of analyzed root fragments that are mycorrhized; M, the intensity of infection in all root fragments; m, the intensity of infection in mycorrhized root fragments; a, arbuscule abundance in mycorrhized root parts; A, arbuscule abundance in the total root system as described in (Trouvelot et al., 1986). Data shown were mean $\pm$ SE values determined from three biological replicates (each replicate contained at least 4 transgenic roots for each construct). Asterisk indicates significant differences (* $p < 0.05$, Student's *t* test).

(C) and **(D)** Light microscopy images of *EF-EV* **(C)** and *MtHDT2i* **(D)** transgenic roots colonized by *R. irregularis*. Root segments shown were with the first observed arbuscule close to the root tips. Black arrows indicate arbuscules.

(E) and **(F)** Sections of *EF-EV* **(E)** and *MtHDT2i* **(F)** transgenic roots colonized by *R. irregularis* at 4wpi. Arrows or arrowheads in **(F)** indicate mature or collapsed arbuscules, respectively.

Bars=50 μ m in **(C)** to **(F)**.

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equal to the control roots (Figure 3B). Sections of mycorrhized roots showed that *MtHDT2i* roots contained more collapsed arbuscules (collapsed : mature= 220 : 174, Figure 3F) than the control (collapsed : mature= 53 : 352, Figure 3E). Together, these data indicate that knock-down of *MtHDT2* does not affect hyphal infection in the whole roots, but significantly reduces arbuscule formation and/or maintenance.

DISCUSSION

In this study aiming to determine the role of MtHDTs in Medicago – AM symbiosis we have shown that MtHDT2 expression is induced during arbuscule formation, whereas *MtHDT1* and *MtHDT3* are not. In addition, MtHDT2 protein sub-nuclear localization in arbuscule containing cells is markedly different from non-infected cells. An increased MtHDT2 level is observed in nucleoplasm and this might be correlated with arbuscule age. Knock-down of *MtHDT2* reduces arbuscule formation in mycorrhized root segments and likely shortens arbuscule lifespan.

In contrast to our observation that *MtHDT2* is expressed during AM symbiosis, a laser capture microdissection (LCM) based transcriptome comparison between arbuscule containing cells and cortical cells of non-mycorrhized root (Gaude et al., 2012) does not indicate a significant increase in *MtHDT2* expression. Therefore we speculate that *MtHDT2* is only transiently expressed in arbuscule containing cells. This might occur during a specific stage of arbuscule development. In *Arabidopsis* roots the *HDT* genes appear to have a cell cycle stage related expression pattern. Therefore we hypothesize that *MtHDT2* expression coincides with endoreduplication which occurs during AM symbiosis (Berta et al., 2000). The transient expression is supported by RNA *in situ* hybridization data and the *pMtHDT2:GUS* study that both show that *MtHDT2* displays very variable expression levels in arbuscule containing cells of the same root segment (Figure 1D, 1E).

The occurrence of arbuscules in the infected *MtHDT2i* root segments is significantly reduced. This is either caused by a shorter lifespan of arbuscules which is supported by an increased number of collapsed arbuscules (Figure 3E, 3F), or by a reduced intracellular infection events of mycorrhizal hyphae, or combination of both. The reduced intracellular infections is in analogy with the reduced release of rhizobia in *MtHDTs RNAi* nodules (Chapter 5). During

root infection by AM fungi, hyphae cross the outer cortex intercellularly, while in the inner cortex the hyphae grow intracellularly into cells and form highly branched arbuscules (Genre et al., 2008; Parniske, 2008). *MtHDT2* has a higher expression level in these arbuscule containing cells, compared to the outer cortical cells that are adjacent to mycorrhizal hyphae (Figure 2D, Supplemental Figure 1F). This suggests that the intracellular infection of the hyphae requires more *MtHDT2* proteins than the intercellular hyphal growth.

MtHDT2 displays different sub-nuclear localization patterns in different cell types. In root cells *MtHDT2* is primarily present in nucleoli whereas in arbuscule containing cells, *MtHDT2* protein can occur both in nucleoli and in nucleoplasm. The ratio between the two is variable. Possibly these different sub-nuclear localization patterns are associated with certain stages of arbuscule development. In line with this, a considerable amount of *MtHDT2* protein could also be observed in nucleoplasm of nodule cells. Further, a shift in sub-nuclear localization pattern of *AtHDT2* was induced in leaf cells upon exposure to the immune response inducing elicitor flg22. *MtHDT2* could complement *AtHDT2* in controlling Arabidopsis plants development (Chapter 5), suggesting *MtHDT2* is an orthologous of *AtHDT2* and their encoded proteins might have the same response to biotic factor. Upon flg22 perception, *AtMPK3* phosphorylates *AtHDT2*, resulting in a translocation of *AtHDT2* from nucleolus to nucleoplasm. There it contributes to the suppression of flg22-regulated defense genes (Chapter 4). As *MPK3* is also activated by fungal elicitor Pep13 (Cardinale et al., 2002; Wang et al., 2007; Taj et al., 2010), it is interesting to determine whether the nucleoplasm location of *MtHDT2* in arbuscule containing cells depends on *MtMPK3* phosphorylation.

The abundance of arbuscules in *MtHDT2i* mycorrhized roots is reduced significantly, whereas it is not affected in the total root segments which might be caused by a compensated intercellular mycorrhization of *MtHDT2i* roots (Figure 3B).

It has been shown that in nodule symbiosis *MtHDTs* positively regulate the expression of *MtHMGR1*. This gene encodes an interactor of *MtDMI2* that is a receptor-like kinase required for establishment of root endosymbiosis with rhizobia as well as arbuscular mycorrhiza. In loss-of-function *dmi2* mutant, infection of mycorrhiza and rhizobia are blocked (Limpens et al., 2005; Maillet et

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al., 2011). In *MtHDT(s)* knock-down mutants, infection by mycorrhiza and rhizobia are both reduced (Figure 3B, Chapter 5, Figure 4B), suggesting the mycorrhizal phenotype in *MtHDT2i* roots is caused by a reduced expression of *MtHMGR1* as well. Noteworthy, *MtHDT1* and *MtHDT2* have functional redundancy in controlling nodule development, whereas only *MtHDT2* is expressed during arbuscular formation. This suggests that the common signalling cascade shared by both symbionts is instructive to *MtHDT2* expression, while a rhizobia-specific branch is initiating also *MtHDT1* expression.

METHODS

Plant Growth Conditions

Medicago ecotype Jemalong A17 was used in all experiments. For mycorrhizal inoculation, plants were placed into pots containing a 1:1 (v/v) ratio of clay and sand evenly mixed with or without ~ 5,00 *Rhizophagus irregularis* spores. Each pot contained 6 plants and for mycorrhizal root colonization comparison, 3 control plants and 3 plants with *RNAi* transgenic roots were placed into one pot. All plants were irrigated with modified Hoagland medium containing 20μM potassium phosphate (Huisman et al., 2016), and were grown at 21°C in a 16 h : 8 h, light : dark regime and harvested at 4 weeks.

Constructs and Plant Transformation

The *pMtHDT1/2/3:GUS* and *pMtHDT2:GFP-MtHDT2* constructs were generated as described in Chapter 5. To knock-down *MtHDT2* expression, *RNAi* target sequence of *MtHDT2* coding sequence was amplified and introduced into a pENTR-D-TOPO vector (Invitrogen) and subsequently recombined in pK7GWIGG2(II) (Limpens et al., 2005) driven by the Arabidopsis *EF1α* promoter (*MtHDT2i*). The same vector but without *MtHDT2* coding sequence (Empty Vector) was used as the control (*EF-EV*). *Agrobacterium rhizogenes* MSU440 mediated hairy root transformation was used to generate transgenic roots, according to (Limpens et al., 2004). Only one transgenic root was kept for each plant before placing into the pot.

RNA Isolation and qRT-PCR

Total RNA was extracted from transgenic root tips using the plant RNA easy kit

(Omega, USA). cDNA was synthesized from 0.5–1 µg of total RNA by reverse transcription with random hexamer primers using the iScript Select cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad, USA). qRT-PCR was performed in a 10 µl reaction system with MyiQ SYBR Green Super-mix (Bio-Rad, USA). Expression of *MtHDT2* (Forward primer: TCTGGGGTGCTGAGGTTAAG; Reverse primer: TTTGTCCTTGGTCAGGGTTC) was quantified in triplicates using CFX3.0 software (Bio-Rad, USA) and normalized using *ACTIN* (Forward primer: ACGAGCGTTTCAGATG; Reverse primer: ACCTCCGATCCAGACA) or *UBIQUITIN* (Forward primer: CCCTTCATCTTGTCTTCGTCTG; Reverse primer: CACCTCCAATGTAATGGTCTTTCC) as a reference.

Staining of Mycorrhizal Roots and Quantification of Mycorrhizal Events

The mycorrhizal roots were first incubated in 10% (w/v) potassium hydroxide for 20 minutes at 90°C and then rinsed with deionized water for three times. After that, the roots were stained in Trypan Blue solution [25ml 2% (w/v) Trypan Blue, 300ml Lactic acid, 300ml Glycerol and 400ml MQ water] for 5 min at 90°C. At the last, decant the Trypan Blue solution and the roots were submerged in glycerol. For quantification, roots were mounted in glycerol on slides labelled with 1cm scale. The mycorrhizal root colonization with three biological replicates were scored and calculated as described by (Trouvelot et al., 1986). The significance of differences was analyzed using Student's *t*-test and determined by SPSS v.22 (IBM, Armonk, NY, USA).

Tissue Embedding and Sectioning

The root segments were fixed in 4% (v/v) paraformaldehyde, 3% (v/v) glutaraldehyde and 3% (w/v) sucrose dissolved in phosphate buffer (7.0) for 3 h at room temperature. The roots were then dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus-Kulzer, Hanau, Germany) for polymerization according to the manufacturer's protocol. 5 µm longitudinal sections were cut on a microtome (Leica Microsystems 2035) and stained with 0.05% Toluidine Blue (Sigma). For *GUS* stained roots 10 µm longitudinal sections were made and stained with 0.1% Ruthenium Red (Sigma).

Histology and Microscopy

β-glucuronidase (*GUS*) activity was visualized after incubation of transgenic roots at 37°C in *GUS* buffer [0.1 M NaH₂PO₄-Na₂HPO₄ (pH 7) buffer including 3%

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sucrose, 0.05mM EDTA, 0.5 mg/ml X-gluc, 2.5 mM potassium ferrocyanide and potassium ferricyanide] (Jefferson et al., 1987). The root sections were analyzed using a Leica DM5500 light microscope equipped with DFC425C camera (Leica Microsystems, Wetzlar, Germany). For GFP visualisation confocal microscopy (Leica SP8) was used. Fresh roots were manually and longitudinally sectioned and GFP was detected with an excitation wavelength of 488 nm.

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Supplemental Figure 1. Expression of *Medicago truncatula* HDTs in Root Tips and Mycorrhizal Roots.

(A) to (C) Expression patterns of *MtHDT1* (A), *MtHDT3* (B) and *MtHDT2* (C) in tips of non-inoculated transgenic roots.

(D) and (E) Light microscopy images of *pMtHDT1:GUS* (D) and *pMtHDT3:GUS* (E) transgenic roots colonized by *R. irregularis* at 4wpi.

(F) Longitudinal section of mycorrhizal *pMtHDT2:GUS* transgenic roots.

GUS activity was visualized after incubation with GUS buffer for 6 hours (A, C and F) or for 24 hours (B). Arrowheads in (D) to (F) indicate arbuscules. Bars=50μm in (A) to (F).

CHAPTER 7

General Discussion

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CHAPTER 7

Plants have to adapt their growth and development to cope with environmental cues. These include abiotic and biotic stresses among them symbiotic interactions. Abiotic and biotic stresses can result in a transient reduction of plants growth which enables plants to retain energy in order to acquire resistance. The role of the chromatin modifiers of the Arabidopsis *HDT* gene family, encoding plant-specific histone deacetylases, in response to abiotic stress has been studied (Sridha and Wu, 2006; Luo et al., 2012). However, the mechanisms involved in coping with abiotic stress to adapt plant development are still far from understood. In **Chapter 2** and **Chapter 3** of this thesis I try to bridge this gap by studying the role of Arabidopsis (*Arabidopsis thaliana*) AtHDT1/2 in regulating cell fate switches during root development under control and salt stress conditions. The role of HDTs in response to biotic stress has been studied in rice and tobacco (Bourque et al., 2011; Ding et al., 2012), but never in Arabidopsis. In **Chapter 4** we show that AtHDT2 plays a role in the reprogramming of defence gene expressions in response to flagellin22 in Arabidopsis leaf cells. The role of HDTs in establishing legume-rhizobium and plant-arbuscular mycorrhiza symbiosis are studied in Medicago (*Medicago truncatula*) (**Chapter 5** and **Chapter 6**). In this chapter, I will discuss these results in a broader perspective.

Regulation of *HDT* Expression Varies Between (A) Biotic Stresses

Abiotic factors in general reduce expression of *HDTs* (Stockinger et al., 2001; Sridha and Wu, 2006; Luo et al., 2012). For example, ABA treatment, salt stress and dehydration stress reduce expression of *AtHDTs* in Arabidopsis seedlings (Sridha and Wu, 2006; Luo et al., 2012). In line with this, the expression of *AtHDT1/2* in Arabidopsis root tips is down-regulated under salt stress (Chapter 3). Biotic factors can either induce or reduce expression of *HDTs* (Demetriou et al., 2009; Bourque et al., 2011; Ding et al., 2012). For example, Medicago *MtHDT(s)* are up-regulated during symbiotic interactions with rhizobia as well as arbuscular mycorrhiza (Chapter 5, 6). In rice leaves, transcription of *OsHDT701* is increased during a compatible interaction, but decreased in an incompatible interaction, with fungal pathogens (Ding et al., 2012). It has been shown that in tobacco leaves *NtHDT1/2* transcript levels are rapidly reduced upon treatment with cryptogin, a fungal elicitor inducing a hypersensitive response (Bourque et al., 2011). By contrast, both salicylic acid and jasmonic acid increase *HvHDT1/2* expression in barley (Demetriou et al., 2009). These observations demonstrate

that expression of *HDTs* is tightly regulated when plants are exposed to stresses. This points to their important role in the process of plant adaptation to stresses.

The Sub-cellular Localization of HDTs Depends on Environmental Cues

HDT proteins display different sub-nuclear localization depending on the stress or type of biological interaction. Under optimal growth conditions, AtHDT1/2 are mainly localized in nucleoli of both root and leaf cells. This is consistent with the fact that they all contain a nucleolar localization domain (Koonin, 1998; Grandperret et al., 2014). Salt stress does not affect their localization in root cells. However, in leaf cells a translocation of AtHDT2 from nucleolus to nucleoplasm occurs upon flagellin 22 treatment (Chapter 2, 3, 4). A similar translocation can also occur during symbiotic interactions, as a considerable amount of MtHDT2 is observed in nucleoplasm of arbuscule containing cells, but not in non-infected root cells (Chapter 6).

The translocation of HDTs from nucleolus to nucleoplasm indicates that a mechanism exists that controls their sub-nuclear localization. Part of this mechanism is identified in Arabidopsis leaf cells after flagellin treatment (Chapter 4). AtHDT2 interacts with MAP kinase AtMPK3 and two amino acids, T249 and S266, are phosphorylated by AtMPK3 *in vivo*, upon exposure to flagellin22. This phosphorylation results in the translocation of AtHDT2 from nucleolus to nucleoplasm. AtHDT1 is a substrate of AtMPK6 and might be translocated like AtHDT2 (de la Fuente van Bentem et al., 2008). It would be interesting to determine whether the increased nucleoplasm localization of MtHDT2 in arbuscule containing cells also depends on MtMPK3 phosphorylation. In addition, salt stress also induces *AtMPK3* and *AtMPK6* expression in Arabidopsis seedlings, but no translocation is observed for AtHDT1/2 (Ichimura et al., 2000; Droillard et al., 2002). However, under salt stress the HDT genes are repressed and this seems most relevant for the adaptive response. Further, whether these *MPK* genes are induced in cells where HDT expression is maintained in the halotropic response is not known.

Similar to AtHDT1/2 and MtHDT2, AtHDT3 and tobacco NtHDT are also mainly localized in nucleoli of protoplasts or leaf cells under control conditions (Sridha and Wu, 2006; Bourque et al., 2011; Luo et al., 2012). HDTs located in nucleoli could function either in repressing ribosomal genes, or as a storage to be used for a fast response to stimuli. The former, at least for AtHDT1/2 and

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MtHDT2 in meristematic cells, is less likely, as cell proliferation depends on a continuous supply of ribosomes (Hannan and Rothblum, 1995; Medina et al., 2000). The latter is supported by a rapid response to flagellin 22 which causes AtHDT2 translocation to nucleoplasm to regulate expression of defense genes (Chapter 4). During nodule formation a relatively high level of MtHDT2 occurs in nucleoplasm before rhizobia infect cells. These are the nodule meristem cells as well as nodule primordium cells. The latter might be caused by a signal generated during the symbiotic interaction. The translocation of HDTs can also occur from nucleus to cytosol. It has been shown that NtHDTs are translocated from nucleus to cytosol upon treatment of tobacco leaves with cryptogein, an elicitor of cell death (Bourque et al., 2011; Grandperret et al., 2014). Similarly, HDA15, a RPD3 class histone deacetylase, is also translocated from nucleus to cytosol for degradation in response to light (Alinsug et al., 2012). Whether the AtHDTs and MtHDTs are targeted to the cytoplasm for degradation is not yet known.

HDTs, like other chromatin remodelling factors, have to recruit transcription factors to target specific DNA regions (Kuang et al., 2012; Asensi-Fabado et al., 2017). It has been shown that in the longan HDT interacts with ERF1, an ethylene-responsive factor that controls fruit senescence (Kuang et al., 2012). Whether HDTs interact with transcriptional factors in root, nodule and arbuscule containing cells, and how these interactions are triggered by environmental cues is still unknown.

Specificity and Functional Redundancy of HDTs

Several studies demonstrate that certain HDTs can have functional redundancy in one process but have specificity in others. It has been shown that AtHDT1 and AtHDT2 have functional redundancy in the establishment of Arabidopsis leaf polarity (Ueno et al., 2007). In agreement with this, I show that they both regulate root growth. Knock-down studies in Medicago nodules showed that silencing of two or more *HDT* members is essential to obtain a phenotype. This functional redundancy might be due to the fact that *AtHDTs* and *MtHDTs* are the result of recent gene duplication. In some monocots this duplication has not occurred (Pandey et al., 2002; Grandperret et al., 2014) and the knock-out of a single *HDT701* gene in rice results in increased pathogen resistance (Ding et al., 2012). Despite this functional redundancy in certain processes the same *HDT* genes can also have specialised functions. For example, salt stress

reduces seeds germination rate in loss-of-function *Arabidopsis hdt3* single mutant (Luo et al., 2012). We show that *Arabidopsis hdt2* single mutant can display a phenotype in response to pathogenic infection in leaves. In addition, arbuscular mycorrhizal infection only induces *MtHDT2* expression in roots.

It has been shown that *Arabidopsis* AtHDT1-3 repress transcription of genes (Wu et al., 2003). This raises the question whether the repression ability of HDTs depends on deacetylation. AtHDT1 is required for H3K9 deacetylation (Lusser et al., 1997; Lawrence et al., 2004). Similar to this, in rice HDT701 is required for H4 deacetylation (Ding et al., 2012). This is in agreement with our observations that AtHDT1/2 deacetylate the *AtGA2ox2* gene to repress its expression (Chapter 2) and AtHDT2-MPK3 module promotes deacetylation of some genes under normal conditions (Chapter 4).

Gibberellin Degradation by C₁₉-GA 2-oxidases Is Part of Adaptation Mechanisms in Response to Abiotic Stress

Because plants must coordinate their growth and development to adapt to stress, it is not surprising that a crosstalk exists between stress and hormonal signalling pathways (Zhu, 2016). For example, salt, dehydration and osmotic stress result in accumulation of abscisic acid (ABA) which binds to receptor PYLs, and this regulates inhibition of growth (Boudsocq et al., 2004; Ma et al., 2009; Park et al., 2009). Pathogen infection results in jasmonic acid (JA) and salicylic acid (SA) accumulation and this inhibits plant growth as well (Vicente and Plasencia, 2011; Yang et al., 2012).

In this paragraph, I will focus on gibberellin (GA) as it appears a major target in HDT mediated responses. Recently, gibberellin (GA) inactivation is shown to play a role in cold and salt stress induced responses resulting in reduced plant growth (Skirycz et al., 2011; Claeys et al., 2012). In *Arabidopsis* the most prominent mechanism for GA inactivation involves up-regulation of GA2-oxidases (Sponsel and Macmillan, 1978; Thomas et al., 1999; Rieu et al., 2008). Seven *AtGA2oxidase* genes (*AtGA2ox1-4*, 6-8; *AtGA2ox5* is a pseudogene) have been identified (Yamaguchi, 2006; Hedden and Sponsel, 2015). Cold stress leads to an up-regulation of *AtGA2ox3* and *AtGA2ox6* in *Arabidopsis* seedlings within 4h (Achard et al., 2008). Similarly, high salinity induces the transcription of six *AtGA2oxidase* genes (1,2,4,6-8) with *AtGA2ox7* being the most-upregulated in seedlings. However, salt induced *AtGA2ox7* transcripts

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do not accumulate in roots (Magome et al., 2008). This suggests that *AtGA2-oxidases* have their effect on GA levels in a tissue/organ specific manner.

However, mechanisms that induce *AtGA2oxidases* expression upon abiotic stresses are not well understood. *AtGA2ox2* is a direct target of *AtHDT2* and its expression is repressed by *AtHDT1/2*. Repression of *AtHDT1/2* occurs during salt stress, possibly by ABA, as salt stress induces ABA accumulation and ABA is known to reduce *AtHDT1/2* expression (Jia et al., 2002; Zhu, 2002; Luo et al., 2012). Their reduction likely induces *AtGA2ox2* in root meristem (Chapter 2, 3). In addition, *AtGA2ox8* is supposed to be regulated by this mechanism as well, as it is also up-regulated in *hdt1,2i* roots and upon salt stress [(Chapter 2, Supplemental Dataset 2) (Magome et al., 2008)]. Another mechanism comes from the study showed that *AtERF6*, an ethylene-response factor, can induce *AtGA2ox6* in growing leaves upon mild osmotic stress (Dubois et al., 2013). Noteworthy, knock-down of *AtHDT1/2* also induces *AtERF6* in root tips (Chapter 2, Supplemental Dataset 2). Therefore it will be interesting to test whether *AtHDT1/2* functions upstream of *AtERF6*.

Drought stress results in an up-regulation of *GmGA2ox4* and *OsGA2ox6* and causes a dwarf phenotype in soybean and rice, respectively (Suo et al., 2012; Lo et al., 2017). Whether this involves a repression of HDTs is not known.

As a negative correlation of HDTs and *GA2oxs* levels occurs under different abiotic stresses and in different plant species, it is probable that HDTs mediated repression of *GA2oxs* expression is a general mechanism in response to abiotic stresses.

***AtHDT1/2-GA2ox2* module plays a role in halotropism**

Natural environments are not homogenous and plants can acclimate to any change to a single environmental factor by using tropisms, such as gravitropism, halotropism, hydrotropism and phototropism, to link the direction of growth to environmental cues (Jaffe et al., 1985; Galvan-Ampudia et al., 2013; Goyal et al., 2013; Daniela Dietrich, 2017). Halotropism is a response of roots to avoid a saline environment (Galvan-Ampudia et al., 2013). Mechanisms of halotropism are not completely understood, however, mechanisms used to create asymmetric responses in roots during for example gravitropism might be operational when roots are exposed to non-homogenous salt stress.

During gravitropism, auxin accumulates more at the lower side of the root. This asymmetric distribution is consolidated by an increased gibberellin accumulation along the lower side which stabilizes PIN2 (Rahman et al., 2010; Lofke et al., 2013). This results in a higher auxin concentration that locally blocks cell elongation. As this occurs in an asymmetric manner the root will bend. An asymmetric distribution of auxin occurs also during halotropism (Galvan-Ampudia et al., 2013). This correlates with an endocytosis of PIN2 at the high salt side of root. This indicates that the mechanisms that control the two tropisms share common aspects. My data suggests that gibberellin also plays a role in halotropism. When GA asymmetric distribution is eliminated the halotropic response is alleviated.

The asymmetric GA distribution in halotropism is likely caused by asymmetric induction of *AtGA2ox2* which leads to a reduced GA at the high salt side. This is likely results from a reduction of *AtHDT1/2* (Chapter 2, 3). In agreement with this, a homogenous salt stress reduces expression of *AtHDT1/2*, but induces expression of *AtGA2ox2* (Magome et al., 2008; Luo et al., 2012).

It is known that high auxin level in root inhibits cell elongation (Rayle et al., 1970). Therefore during halotropism at the side opposite to high salt an increased auxin concentration likely inhibits cell elongation. This contributes to root bending away from high salt. Besides this, an earlier switch from cell division to elongation at the high salt side, which is in line with *AtHDT1/2* down-regulation and *AtGA2ox2* up-regulation, will increase root length rapidly and might also contribute to root bending.

Concluding Remarks

Plant development results from specific patterns of gene expression that are tightly regulated in spatio-temporal manner. Chromatin remodelling factors play an important role in the establishment of these patterns and also provide regulatory flexibility for gene expression in response to environmental signals. In this thesis I show that *AtHDT1/2*-*GA2ox2* module is operational in control of root growth and halotropism. How the *AtHDT1/2*-*AtGA2ox2* module is regulated is still unknown. This can be the subject for future studies.

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SUMMARY

Plants have a sessile lifestyle. To ensure survival, they develop a potential to respond to environmental cues to set up an adaptive growth and development. This adaptation involves transcriptional reprogramming of the genome through chromatin-based mechanisms relying on the dynamic interplay of transcription factors (TFs), post-translational modification of histones, the deposition of histone variants, DNA methylation, and nucleosome remodeling. This thesis is focused on a role of one group of histone post-translational modifiers, plant-specific histone deacetylases (HDTs), in plant development under control condition and variable stresses/symbiotic interactions.

It is well known that HDTs are involved in plant responses to environmental stresses. However, whether they play a role in regulating plant growth and development is elusive. In this thesis it is shown that *Arabidopsis thaliana* AtHDT1/2 regulate the cell fate switch from division to expansion in the *Arabidopsis* root. Knock-down of *AtHDT1/2* (*hdt1,2i*) causes that this switch occurs earlier and results in less cells in the root meristem. This process slows down root growth. One target of AtHDT1/2, *AtGA2ox2*, is identified here. Its overexpression displays the same root phenotype as *hdt1,2i*, and its knock-out partially rescues *hdt1,2i* root meristem phenotype. *AtGA2ox2* inactivates gibberellin (GA₄) whose application increases root meristem cell number in WT, but not in *hdt1,2i*. Based on these data, we conclude that AtHDT1/2 repress the transcription of *AtGA2ox2*, and likely fine-tunes GA homeostasis to regulate the switch from cell division to expansion in root tips.

HDTs respond to salt stress in *Arabidopsis* seedlings. Halotropism is a novel reported tropism allowing roots to avoid a saline environment. Whether the AtHDT1/2-*AtGA2ox2* module is operational in halotropism is studied here. We show that *hdt1,2i* mutants respond more severe in halotropism. AtHDT1/2, as well as *AtGA2ox2* display asymmetric localization patterns in halotropism with AtHDT1/2 reduced and *AtGA2ox2* induced at high salt side of root tips. Our data indicate that their asymmetric patterns likely results in less GA at high salt side of root tips and this is required for halotropism establishment. In line with this, both constitutive expression of *AtHDT2* and exogenous GA application reduce halotropic response. A reduction of GA in root tips causes an earlier switch from cell division to expansion. We discuss that this earlier switch enables roots rapidly to bend away from saline environment.

It has been shown that HDTs play a role under biotic stress in rice and tobacco leaves. We demonstrate that they are also involved in response to biotic stress in *Arabidopsis* leaves. *Arabidopsis hdt2* mutants are more susceptible to virulent *Pseudomonas syringae* pv. *tomato PstDC3000*, whereas mutants with *AtHDT2* constitutive expression are more resistant. In addition, we detected a translocation of AtHDT2 from nucleolus to nucleoplasm after the perception of flagellin22 in *Arabidopsis* leaf cells. This translocation is not observed under abiotic stress. A mechanism controlling this translocation is identified. AtMPK3 is activated under biotic stress, it interacts with and phosphorylates AtHDT2. This leads to the accumulation of AtHDT2 in nucleoplasm where it contributes to the repression of defense genes.

During the interaction with symbiotic microorganisms, plants could develop a symbiotic organ/structure. For example, legumes of which *Medicago truncatula* is a model, can form root nodules or arbuscules by interacting with rhizobia or arbuscular mycorrhiza.

We show that nodule-specific knock-down of *MtHDT1/2/3* (*MtHDTs RNAi*) blocks nodule primordia development and affects the function of nodule meristem. This is consistent with their roles in controlling cell division during root development and suggests that the function of nodule and root meristems is closely related. However, MtHDT2 gains a new sub-nuclear localization pattern in nodule meristem by using a not yet known mechanism, different from that in root meristem. This suggests that these two meristems have different transcriptional landscapes. In the nodule infection zone *MtHDTs* are also expressed and in *MtHDTs RNAi* the intracellular release of rhizobia is markedly reduced. Expression of *MtHMGR1* and its paralogs, encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductases are down-regulated in *MtHDTs RNAi*. It has been shown MtHMGR1 interacts with MtDMI2, a component of Nod factor signalling pathway, to control rhizobial infection. Knock-down of *MtHMGR1/MtDMI2*, as well as inhibiting MtHMGRs enzymatic activity blocks nodule primordia development and rhizobial infection in nodule primordia/mature nodules. This phenotype partially resembles *MtHDTs RNAi* phenotype. We discuss that MtHDTs regulate expression of *MtHMGRs* and in this way affect Nod factor signalling and control nodule development.

Similar to nodule symbiosis, during arbuscular mycorrhizal symbiosis cells in the cortex are also intracellularly infected. We show that *MtHDT2* is also induced in these arbuscule containing cells. Knock-down of *MtHDT2* (*MtHDT2i*)

significantly reduces the intracellular infection of the hyphae on the mycorrhized root segments, indicating that MtHDT2 control mycorrhizal intracellular infection. We discuss whether MtHDTs can regulate mycorrhizal/rhizobial infection in a similar way.

The data obtained in this thesis and the published information related to these subjects are discussed at the end. HDTs are key players in plant responses to environmental cues, whereas they respond to abiotic factors and biotic factors differently. They are also key regulators of plant growth and development that is clearly demonstrated in this thesis on examples of root and nodule development. I also propose a role of AtHDT1/2 in response to salt signal to fine-tune the switch from cell division to expansion in root tips during halotropism.

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我的家人!

CURRICULUM VITAE

Huchen Li was born on October 21, 1986 in Liaocheng, China. He finished his high school education in 2004, and in the same year he started his four years education in College of Biological Science, Shandong Normal University. There he obtained his bachelor degree in science in 2008 and then worked as a biology teacher at a middle school for half year. In 2009 he moved to Beijing and started his Msc study in Beijing Normal University. During his three years study on Chinese traditional medicine, including theoretical and practical courses, like plant tissue culture, microbial fermentation and animal pharmacology, he started to show his interests toward plant science. In 2012 he obtained his master degree and got the scholarship from China Scholarship Council for supporting his PhD study in the Netherlands. There, from 2012 till 2016, he worked in the Laboratory of Molecular Biology of Wageningen University, under the supervision of Prof. dr Ton Bisseling and Dr. Olga Kulikova, focusing on the research topic "Functional analyses of plant-specific histone deacetylases". From September, 2016, he started as a post-doc in the same group and extended his research topic into "Environmental stresses regulated plant growth and development".

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Huchen Li
 Date: 16 October 2017
 Group: Laboratory of Molecular Biology
 University: Wageningen University & Research

1) Start-up phase	<u>date</u>
▶ First presentation of your project	
Title: Plant-specific Histone Deacetylases in Lateral Root and Nodule Development	28 Jun 2013
▶ Writing or rewriting a project proposal	
Title: Plant-specific Histone Deacetylases in Lateral Root and Nodule Development	Sep-Nov 2012
▶ MSc courses	
▶ Laboratory use of isotopes	
Subtotal Start-up Phase	
	4.5 credits*
2) Scientific Exposure	<u>date</u>
▶ EPS PhD student days	
EPS PhD student day, Amsterdam, NL	30 Nov 2012
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student day 'Get2Gether', Soest, NL	29-30 Jan 2015
▶ EPS theme symposia	
EPS Theme 4 Symposium "Genome Biology", Nijmegen, NL	07 Dec 2012
EPS Theme 1 Symposium "Developmental Biology of Plants", Wageningen, NL	17 Jan 2013
EPS Theme 1 Symposium "Plant Development Biology", Wageningen, NL	24 Jan 2014
EPS Theme 4 Symposium "Genome Biology", Wageningen, NL	03 Dec 2014
EPS Theme 1 Symposium "Developmental Biology of Plants" Leiden, NL	08 Jan 2015
EPS Theme 2 Symposium "Interactions between Plant and Biotic Agents", Utrecht, NL	20 Feb 2015
EPS Theme 4 Symposium "Genome Biology", Amsterdam, NL	15 Dec 2015
EPS Theme 1 Symposium "Developmental Biology of Plants", Wageningen, NL	21 Jan 2016
EPS Theme 3 Symposium "Metabolism and Adaption", Wageningen, NL	14 Mar 2017
▶ National meetings (e.g. Lunten days) and other national platforms	
Annual meeting 'Experimental Plant Sciences', Lunten, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunten, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunten, NL	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunten, NL	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunten, NL	10-11 Apr 2017
Joint Dutch Chromatin Meeting and NVBMB Fall Meeting, Rotterdam, NL	29 Oct 2013
The 13th Dutch Chromatin Meeting, Nijmegen, NL	22 Oct 2015
▶ Seminars (series), workshops and symposia	
Start symposium Plant Development Biology	14 Oct 2013
Symposium: Chinese association of life science in the Netherlands annual meeting	09 Nov 2013
Mini-symposium - Plant pathology	24 Nov 2014
EPS Symposium "Omics Advances for Academic and Industry - Towards True Molecular Plant Breeding"	11 Dec 2014
Symposium "The underground Labyrinth: Roots, Friends and Foes"	08 Feb 2017
Celebrative Symposium - Gene regulation and epigenetics	14 Jun 2017
Workshop - ExPectationS (EPS career day), Creativity and inspiration in science	01 Feb 2013
Seminar: Chromatin dynamics and cell fate in plants: From Genetics to Epigenetics	16 Jan 2013
Seminar: Writing for high impact journals	08 Feb 2013
Seminar: Plant stem cell system	15 Apr 2013
Seminar: Auxin and plasmodesmata; measuring fluxes in the root	02 Jul 2013
Seminar: Advanced Microfluidics for the Control of Living Cells	07 Jan 2014
Seminar: Self-organising tissues: root regeneration as a case study	10 Mar 2014
Seminar: Plant Sciences Seminar 11 March: Mutualism in Action	11 Mar 2014
Seminar: The Dynamics of Root Stem Cell Regulation: Old Players with New Function?	11 Mar 2014
Seminar: Genome and effector evolution in the Irish potato famine pathogen lineage	28 May 2014
Seminar: Arbuscular mycorrhiza, and their yin-yang position in the plant-soil microbiome	04 Jun 2014
Seminar: Dissecting the interaction between Phytophthora sojae and soybean: making sense out of signalling and effectors	16 Jul 2014
Seminar: Spindle mechanics and chromosome segregation	04 Sep 2014
Seminar: Peptide signaling in lateral root and root nodule development	15 Sep 2014
Seminar: PIN-ning down the function of D6 PROTEIN KINASES in auxin transport	21 Oct 2014
Seminar: Seminar Series Plant Sciences - Plant Bioinformatics	04 Nov 2014
Seminar: EPS Flying Seminar Ortrun Mittelsten Scheid-Genetics and epigenetics: a complex relationship	19 Nov 2014
Seminar: Flying seminar. Seasonal flowering in annual and perennial plants	19 Jan 2015

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Seminar: 'The evolutionary significance of gene and genome duplications'	03 Feb 2015
Seminar: WEES seminar: Mycoheterotrophy and mixotrophy : plants eating mycorrhizal fungi	26 Nov 2015
Seminar: Genomics-enabled natural products discovery	31 Mar 2016
Seminar: Auxin Signaling: Inputs and Outputs'	07 Apr 2016
Seminar: Evolution of symbiotic gene networks in land plants	08 Apr 2016
Seminar: The evolution of branching mechanisms'	12 May 2016
Seminar: Perianth evolution in Ranunculaceae: are petals ancestral in the family?	20 May 2016
Seminar: Genetic basis of anatomical variety in plant ground tissue. A lesson from Cardamine hirsuta	29 Aug 2016
Seminar: Clocks across taxa: Conserved cellular timekeeping mechanisms in plants, algae and other eukaryotes	29 May 2017
Seminar: The immune receptor Rx1 remodels chromatin and chromatin interactors in immunity	11 Jul 2017
► Seminar plus	
► International symposia and congresses	
6th European Plant Science Retreat 2014, Amsterdam	01-04 Jul 2014
Conference on Epigenetic & Chromatin Regulation of Plant Traits, Strasbourg, French	14-15 Jan 2016
5th European workshop on plant chromatin, Vienna, Austria	01-02 Jun 2017
► Presentations	
Poster: Joint Dutch Chromatin Meeting and NVBMB Fall Meeting, Rotterdam, NL	29 Oct 2013
Poster: 6th European Plant Science Retreat 2014, Amsterdam, NL	01-04 Jul 2014
Talk: EPS Theme 1 Symposium "Plant Development Biology" Leiden, NL	08 Jan 2015
Talk: EPS Theme 4 Symposium "Genome Biology", Amsterdam, NL	15 Dec 2015
Flash talk: Conference on Epigenetic & Chromatin Regulation of Plant Traits, Strasbourg, France	14-15 Jan 2016
Talk: NWO Plant Sciences meeting, Lunteren, NL	11-12 Apr 2016
► IAB interview	
► Excursions	
Scientific Excursion to Rijk Zwaan	27 Sep 2013
<i>Subtotal Scientific Exposure</i>	
<i>19.1 credits*</i>	
3) In-Depth Studies	
► EPS courses or other PhD courses	<u>date</u>
Bioinformatics - A user's Approach, Wageningen, NL	25-29 Aug 2014
Introduction to R for statistical analysis, Wageningen, NL	18-19 May 2015
Phylogenetics: principles and methods, Wageningen, NL	17-19 May 2016
► Journal club	
participation in a literature discussion group at Mol. Biology	2012-2016
► Individual research training	
<i>Subtotal In-Depth Studies</i>	
<i>6.0 credits*</i>	
4) Personal development	
► Skill training courses	<u>date</u>
PhD Competence Assessment, Wageningen, NL	23 Oct 2012
Course: High-Impact Writing Course, Wageningen, NL	18-21 Nov 2013
Information literacy PhD including Endnote Introduction, Wageningen, NL	28-29 Oct 2014
Course: Techniques for writing and presenting a scientific paper, Wageningen, NL	01-04 Sep 2015
► Organisation of PhD students day, course or conference	
► Membership of Board, Committee or PhD council	
<i>Subtotal Personal Development</i>	
<i>3.1 credits*</i>	
TOTAL NUMBER OF CREDIT POINTS*	
32.7	

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* A credit represents a normative study load of 28 hours of study.

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