Epigenetic control of root and nodule development

The role of plant-specific histone deacetylases and LHP1 in root cell reprogramming

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CHAPTER 1

General Introduction

Plant organogenesis

Plants are sessile organisms and therefore have to have the ability to adjust their development to be able to grow in and to adapt to a constantly changing environment. For example, a root system is formed to supply plants with anchorage and for the uptake of nutrients and water. The root architecture varies greatly depending not only on the plant species but also on the availability of nutrients and water, and other environmental factors like temperature, wind and light (López-Bucio et al., 2003; Malamy, 2005). The ability of plants to adapt to the environment depends on their post-embryonic development. Thus in plants, new organs can develop during their entire lifespan, unlike in animals where organs are formed predominantly during embryo development. As a result plant architecture is variable.

The formation of new organs in plants requires reprogramming of differentiated cells into pluripotent cells that will form a primordium, which develops further into an organ.

Root meristems and stem cells

Plants grow predominantly through apical and lateral meristems of the root and shoot. The root apical meristem (RAM) will give rise to the subterranean root system and the shoot apical meristem (SAM) will give rise to aerial tissues (Weigel and Jürgens, 2002). In these highly organized meristems, plants maintain a small population of undifferentiated cells, which are called stem cells. The stem cells can both renew themselves and give rise to specific daughter cells to build the different plant tissues and organs. The stem cells are present in a specific stem cell niche in which they are regulated by extrinsic signals, similar to what has been described for animal stem cells (Scheres, 2007). In plants new meristems can also be established post embryonically, during the formation of for example lateral roots or other lateral root organs like nodules, and these processes are the focus of the research described in this thesis.

In general roots of different plant species have a similar anatomy. The central vascular bundle is surrounded by files of cells of 4 different cell types, from inside to outside the pericycle, endodermis, cortex and epidermis (figure 1) (Dolan et al., 1993). Different plant species can have a varying number of files of certain cell types. Young Arabidopsis roots, for example, have only one file of each cell type, whereas *Medicago truncatula* roots have up to 5 cortical cell files (Péret et al., 2009a; Timmers et al., 1999). Each cell file originates from a limited number of stem cells in the root meristem through a specific

number of periclinal and anticlinal divisions. In this meristem the stem cells are present in a highly controlled microenvironment called the stem cell niche and are maintained by a small number of mitotically inactive central cells called the Quiescent Center (QC). In Arabidopsis, the QC consists of about 4 cells and is surrounded by a single layer of stem cells, the initials, which give rise to specific daughter cells that can differentiate into the various root cell types (van den Berg et al., 1997). The stem cell daughter cells divide several times and eventually elongate and differentiate to contribute to the growing root.

After germination and initial growth, the main root starts to branch to form an elaborate root system. The formation of these lateral roots with their own apical meristem is a clear example of post embryogenic organ formation.

Lateral root formation

Through the formation of lateral roots a more elaborate root system is generated. Characteristic for dicots is a tap root system, which consists of one primary root on which shortly after generation lateral roots emerge (Lloret and Casero, 2002). In monocots, the primary root only plays a role shortly after germination, and post-embryonic shoot-borne roots will generate the fibrous root system of the adult plant (Feldman, 1994; Hochholdinger et al., 2004).

Lateral root formation is best studied in Arabidopsis. As in most other plants, the Arabidopsis lateral root originates from the pericycle cell layer. However, in maize and rice lateral roots originate from both pericycle and endodermis cell layers (Fahn, 1990). In Arabidopsis lateral roots develop from pericycle cells at the xylem poles in roots. These cells turn into pluripotent cells and will give rise to a complete lateral root with its own new meristem (Dolan et al., 1993; Malamy and Benfey, 1997). The two adjacent pericycle cells that will form the lateral root are called the lateral root founder cells (figure 1A). Both cells first undergo an asymmetric anticlinal division, resulting in two smaller cells, flanked by two larger cells (figure 1B). In total three pericycle cell files are dividing but only the middle cell file will contribute significantly to the lateral root primordium (Kurup et al., 2005). Next, several rounds of anticlinal divisions take place of the two smaller inner cells, resulting in up to 10 small cells. These cells divide periclinaly to form two parallel cell layers, an outer and inner layer. Following several rounds of periclinal and anticlinal divisions, a lateral root primordium is formed (Dolan et al., 1993; Malamy and Benfey, 1997).

The phytohormone auxin is a key regulator of plant growth and development with functions in for example embryogenesis, root formation and

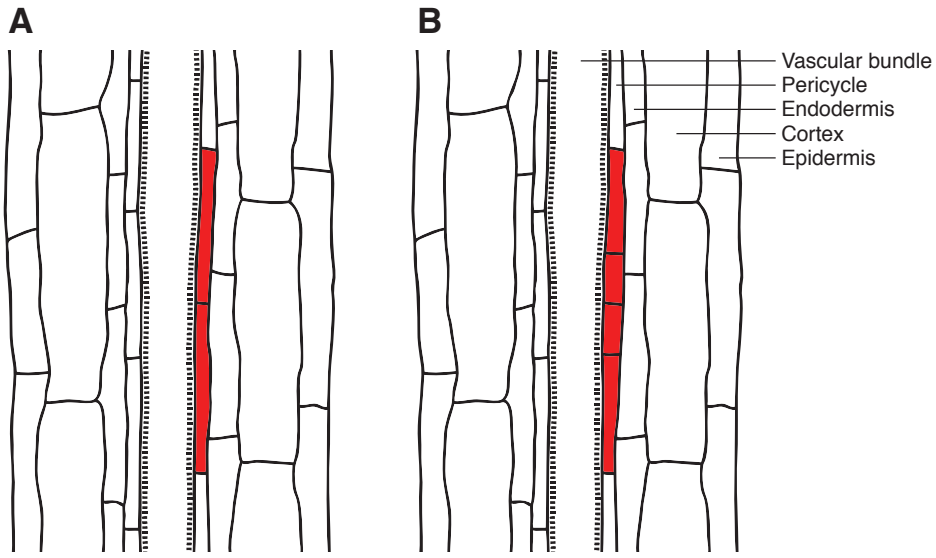

Figure 1: Schematic longitudinal section of an Arabidopsis root during lateral root initiation. Neighboring pericycle lateral root founder cells before the first division (A) and after the first asymmetric division (B) are marked in red. The different tissues that make up a root are indicated in (B).

apical dominance (Vanneste and Friml, 2009). Auxin also plays an important role in lateral root initiation. Several mutants affected in lateral root formation have been characterized and in a number of cases the mutated gene has been found to have a role in auxin transport, biosynthesis or perception (De Smet et al., 2006; Péret et al., 2009b). Furthermore, external application of auxin can induce lateral root formation, whereas treatment with the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) inhibits the formation of lateral roots (Laskowski et al., 1995; Casimiro et al., 2001). Auxin is also thought to be involved in priming of pericycle cells in the basal meristem. There, oscillating auxin levels specify and prime pairs of pericycle cells resulting in a regular spacing of lateral roots (De Smet et al., 2007). Not only auxin priming can position lateral root formation also mechanical forces through for example root bending can induce lateral roots (Laskowski et al., 2008). Lateral root spacing therefore is influenced by both endogenous signals and environmental stimuli.

Legume symbiosis and root nodule formation

Another example of postembryonic organ development is the formation of root nodules. Plants are in need of nitrogen for their growth and development. Although the air consists of more than 80 percent of nitrogen, only micro-

organisms are able to make use of this nitrogen source. Some of these micro-organism can do this in an intracellular symbiosis with plants. In specific organs called root nodules, atmospheric nitrogen is fixed by the bacteria. The most efficient examples are legume plants that can form a symbiosis with diverse nitrogen fixing bacteria collectively called rhizobia. One of the model legumes is Medicago that is used in the experiments described in this thesis.

During the initiation of this symbiosis in Medicago, rhizobium bacteria secrete specific lipo-chitooligosaccharides named Nod factors (Geurts and Bisseling, 2002). These are perceived by the plant in a specific region of the root called the susceptible zone. The bacteria induce root hair curling and enter the plant through tube like structures made by the host, called infection threads (Brewin, 2004; Gage, 2004). Simultaneously, the reprogramming of root cortical cells is triggered and mitotic activation of these cells leads to the formation of a nodule primordium (Timmers et al., 1999). Nodules, like lateral roots, mainly are initiated opposite of protoxylem poles in roots. When growing infection threads reach cells at the base of the primordium the bacteria are released in an endocytosis like process (Ivanov et al., 2010). After release the primordium differentiates into a new organ; the root nodule. The bacteria are surrounded by a membrane from the host and differentiate into their nitrogen fixing symbiotic form. These membrane compartments harboring rhizobium are called symbiosomes (Ivanov et al., 2010). The central tissue of the nodule is composed of cells packed with symbiosomes interspersed with non-infected cells. Medicago forms indeterminate nodules with a meristem at the apex. Through division, the meristem maintains itself and adds cells to the different tissues of the nodule. Subsequently new cells are penetrated by infection threads and after infection with rhizobia symbiosomes are formed. This results in a gradient of developmental stages in nodules, with the youngest cells close to the meristem and the oldest in the basal part of the nodule.

Chromatin remodeling during organogenesis

To be able to develop a new organ like lateral roots or nodules, some cells in the plant need to change their fate and enter a different developmental program. Cell fate depends on the establishment and maintenance of specific transcriptional programs, and this is mainly regulated by transcription factors in interaction with chromatin in the nucleus of the cell.

The basic unit of chromatin is the nucleosome. Nucleosomes are composed of two copies of each of the histone proteins, H2A, H2B, H3 and H4, which are assembled into an octamer that has about 150 base

pairs of DNA wrapped around it (Luger et al., 1997). Histones can carry a variety of posttranslational modifications, which influence chromatin structure and compaction of the nucleosomes. The chromatin environment is highly dynamic and is tightly regulated by a multitude of protein complexes. The three-dimensional architecture of chromatin determines accessibility of the DNA for transcription factors and the transcription machinery.

Historically two types of chromatin are distinguished cytologically; heterochromatin and euchromatin. Heterochromatin was originally defined as dark staining regions of the genome whereas euchromatin is weakly stained (Heitz, 1928). Heterochromatic regions are in general rich in repetitive sequences and low in gene density and are often associated with telomeres and pericentric regions of chromosomes (Hsieh and Fischer, 2005). In contrast, euchromatin is found in gene rich regions, which are readily transcribed. Heterochromatin can further be subdivided into constitutive heterochromatin, which refers to condensed and permanently inactive chromosomal regions and facultative heterochromatin, which exists in different locations of euchromatic regions and is tissue or cell type specific (Hsieh and Fischer, 2005). Facultative heterochromatin can loose its condensed structure and become transcriptionally active and vice versa, and histone modifying enzymes, chromatin binding proteins, histone chaperones and ATP-dependent chromatin-remodellers have an important role in this process (He and Amasino, 2005).

Histone modifications can occur through incorporation of specific histone variants in the nucleosomes or through post-translational modification (PTM) of the histones themselves. The majority of these PTMs occur on the N-terminal histone tails and include methylation, acetylation, phosphorylation, sumoylation and ubiquitination (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Because of the large number of different possibilities to modify the chromatin, combinations of these PTMs were thought to form a specific code that regulates chromatin organization and in this way transcription and other nuclear processes. The PTMs are added or removed by histone modifying enzymes and these enzymes either have a broad specificity and add or remove modifications at several different histone amino acid residues or only modify one specific histone amino acid residue. The effect of the PTMs on chromatin can be direct as for example acetylation of lysines and arginines in the histone tails will neutralize the positive charge of these residues, thereby directly decreasing the interaction between nucleosomes and the DNA. The PTMs can also create new binding sites for chromatin binding proteins such as Heterochromatin Protein 1 (HP1), which contains a chromo domain that is able to recognize methylated lysine residues and causes compaction of chromatin.

DNA can also be methylated and between 5% and 40% of the cytosines in an organism can be modified into 5-methylcytosine by DNA-methyltransferases. In plants and mammals heterochromatic DNA is hypermethylated. Three classes of cytosine modifications can be distinguished, CpG, CpNpG, and asymmetric Cytosine methylation respectively (Cao and Jacobsen, 2002; Vanyushin, 2005). All these modifications of the chromatin that are inheritable and do not involve changes in the DNA sequence itself are called epigenetic modifications. Chromatin modifiers like histone modifying enzymes, histone chaperones and ATP-dependent chromatin-remodellers, play an essential role in establishing these epigenetic modifications and controlling gene expression.

Recently, epigenetic genome-wide mapping of frequently occurring histone modications and DNA methylation have revealed that in Arabidopsis 4 main chromatin states are found; representing active genes, repressed genes, silent repeat elements and intergenic regions (Roudier et al., 2011), similar to Drosophila where only 5 subtypes were described (Filion et al., 2010). Epigenetic regulation of chromatin states strongly regulate the transcription program and therefore cell fate choices. An example is the transition from vegetative to reproductive growth, which requires large changes of gene expression to induce the formation of flowers. Several chromatin remodeling mutants, with for example mutations in genes coding for histone methyltransferases or subunits of the polycomb complex, show early or late flowering phenotypes indicating that chromatin modifiers have an important role in this reprogramming (He and Amasino, 2005; Reyes, 2006).

Histone (de)acetylation

One of the best understood and studied epigenetic modification is histone acetylation. Acetylation of histones is usually associated with active genes (Hebbes et al., 1988; Grunstein, 1997). Histon acetylation is reversible and is maintained by two groups of antagonistic enzymes; histone acetyl transferases (HATs) and histone deacetylases (HDACs).

Plant HDACs can be grouped into three different classes, RDP3/HDA1, SIR2 and HDT (Pandey et al., 2002). The last group, HDT, is plant specific and has no homology to HDACs in animals or fungi (Lusser et al., 1997). HDAC function in plants has been best studied in Arabidopsis. Arabidopsis contains 18 histone deacetylases. Twelve belong to the RPD3/HAD-like family, two to the SIR2 family and four are plant-specific HDTs.

Plant HDACs play important roles in plant development. For example,

inhibition of histone deacetylases by treatment with trichostatin A (TSA) in Arabidopsis, affects root hair patterning and results in changes in expression of cellular patterning genes (Xu et al., 2005). Another example is the inactivation of the RPD3-like AtHDA19 (HD1) by using antisense *AtHDA19* transgenic plants that was shown to be associated with pleiotropic effects on plant gene regulation and development (Tian and Chen, 2001; Tian, 2004). Furthermore, AtHDA19 interacts with the histon acetyl transferases GCN5 and TAF1 to regulate histone acetylation required for light-responsive gene expression (Benhamed et al., 2006). The acetyl transferase GCN5, an antagonist of histone deacetylases, is essential for maintenance of the stem cell niche in the root meristem (Kornet and Scheres, 2009)

The plant specific HDTs AtHDT1, AtHDT2 and AtHDT3 are involved in reproductive development. Knockdown of AtHDT1 expression for example, resulted in seed abortion and over-expression of AtHDT1 introduced pleiotropic developmental abnormalities (Wu et al., 2000; Zhou et al., 2004). AtHDT1 and AtHDT2 both are required for the establishment of leaf polarity (Ueno et al., 2007). Two Tobacco HDTs play a role in cell fate determination as negative regulators of programmed cell death (Bourque et al., 2011; Dahan et al., 2011). These examples indicate a strong association between chromatin modifiers like histone deacetylases and determination of cell fates in developmental programs.

LHP1

One of the most studied histone binding proteins is Heterochromatin protein 1 (HP1). HP1 was first characterized in Drosophila as a protein associated with heterochromatin (James and Elgin, 1986; James et al., 1989). Since then, multiple HP1 isoforms have been found in drosophila, with each having a specific localization in hetero or euchromatic regions.

All HP1 proteins consist of a Chromodomain (CD) and Chromo-shadowdomain (CSD) separated by a hinge region. The CD of drosophila and mammalian HP1 have been shown to bind to trimethylated histone H3 lysine9 (H3K9me3) (Bannister et al., 2001; Lachner et al., 2001; Jacobs and Khorasanizadeh, 2002; Fischle et al., 2003). The hinge is a more variable domain, both in sequence as in length, which is used for DNA binding (Sugimoto et al., 1996; Meehan et al., 2003). The most C-terminal domain is the CSD, this domain is used for dimerization as well as binding to other proteins (Aasland and Stewart, 1995; Le Douarin et al., 1996). Unlike metazoans and yeast which contain multiple isoforms of HP1, plants contain in general

only a single HP1 (like) gene. The first characterized plant HP1 homolog in Arabidopsis is called Like Heterochromatin Protein 1 (LHP1). Unlike its name, it localizes predominantly to euchromatin in characteristic speckles (Gaudin et al., 2001; Kotake et al., 2003; Takada and Goto, 2003; Libault et al., 2005; Nakahigashi et al., 2005). ChIP-chip and DamID-chip studies showed that LHP1 is associated with histone H3 lysine27 trimethylation (H3K27me3). Therefore it has been postulated that LHP1 might be functionally similar to Polycomb (Pc) which is a part of Polycomb Repressive Complex-1 (PRC1) and recognizes H3K27me3, a silencing epigenetic mark deposited by the PRC2 complex (Turck et al., 2007; Zhang et al., 2007; Hennig and Derkacheva, 2009). Polycomb Repressive Complexes (PRC) modulate the epigenetic status of key cell fate and developmental regulators in eukaryotes (Schwartz and Pirrotta, 2007). Two recent findings support a role of LHP1 in PRC1. Firstly, two proteins, AtRING1a and AtRING1b, have been identified as homologs of the animal PRC1 core component RING1 (Xu and Shen, 2008). AtRING1a binds to itself and to AtRING1b, to CURLY LEAF (CLF) and to LHP1. Secondly, it has been shown that a LHP1 mutant with a defective chromodomain has strongly reduced binding to H3K27met3 in vivo, and phenotype of this mutant is very similar to an *lhp1* null allele, indicating that chromodomain-mediated binding of LHP1 to H3K27Me3 is essential for LHP1 function (Exner et al., 2009).

In Arabidopsis LHP1 plays a role in several developmental processes. The *lhp1* mutant, also known as terminal flower 2 (*tfl2*) has a pleiotropic phenotype. *Lhp1/tfl2* plants are affected in overall plant architecture, leaf morphology, flower determinacy and flowering time (Larsson et al., 1998; Gaudin et al., 2001; Kotake et al., 2003; Takada and Goto, 2003). LHP1 is required for transcriptional repression of several floral homeotic genes and genes regulating flowering time, like for example epigenetic silencing of the flowering repressor Flowering Locus C (Kotake et al., 2003; Mylne et al., 2006; Sung et al., 2006).

Thesis outline

Although histone modifying and chromatin binding proteins are widely studied in plants, until now a role in cell fate changes has not been established. In this thesis we focused on reprogramming events in lateral root organ formation, for which large changes in gene expression are needed and most likely chromatin modifiers are involved. In chapter 2 we showed that in Arabidopsis all 4 plant specific histone deacetylases, AtHDT1, AtHDT2, AtHDT3 and AtHDT4, are involved in the reprogramming of pericycle cells during early steps of the

formation of lateral roots. Furthermore, ATHDT1 and AtHDT2 are necessary for maintaining the root stem cell niche and meristem. In chapter 3 and 4 we characterized the plant specific histone deacetylases MtHDT1, MtHDT2 and MtHDT3 in Medicago. We showed that these histone deacetylases are essential for the formation of root nodules in Medicago. These MtHDTs are involved in early steps of reprogramming of the cortical cells in nodule initiation as well as in later steps of nodule development. To study this, a fate map of nodule development was created. Cells originating from the newly formed nodule meristem are disturbed in differentiation. In chapter 5 we characterized the LHP1 homolog in Medicago. MtLHP1 forms chromatin complexes that regulate gene expression in nodule development and it might be involved in proper differentiation of cells originating from the nodule meristem as well. Thus, in this thesis we established a role for chromatin modifiers in cell fate changes.

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CHAPTER 2

Arabidopsis plant-specific histone deacetylases and (lateral) root development

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Introduction

Organogenesis in plants, unlike in animals, can occur during its entire lifespan. New organs originate post-embryonically from, for example, the shoot meristem or by reprogramming of differentiated somatic cells. An example of the latter is the initiation of lateral root formation in the pericycle of the differentiated zone of the root. During the initiation of lateral root formation, these pericycle cells are “reprogrammed” and start to divide to form a primordium from which all root tissues can develop. Also in animals somatic cells can be programmed. A classical example is the regeneration of limbs in amphibian. Although regeneration does not occur in mammals, somatic cells of mammals can be reprogrammed into stem cells by ectopic expression of only 4 specific transcription factors (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Studies on the underlying mechanism have revealed that reprogramming of somatic mammalian cells involves chromatin remodeling (Li, 2002). However, whether this is the case in plants is not known. Therefore we studied whether chromatin modifiers play a role in the switch from pericycle to lateral root primordium fate.

In Arabidopsis, lateral root primordia are formed from two adjacent pericycle cells, the so-called lateral root founder cells. These founder cells are activated by auxin and undergo several rounds of division to form a lateral root primordium (De Smet et al., 2007). These primordia eventually develop into lateral roots with an apical meristem (Malamy and Benfey, 1997). During lateral root primordium formation large changes in gene expression occur (Vanneste et al., 2005; De Smet et al., 2008). De Smet *et al.,* 2008 identified 1920 Arabidopsis genes with differential expression during this process. They made use of fluorescently labeled pericycle cells, using an enhancer trap line, and growth conditions by which lateral root formation was induced synchronously (Himanen et al., 2002). In this system lateral root formation was first inhibited by germinating seeds on plates containing the auxin transport inhibitor 1-N- naphthylphthalamic acid (NPA). After 72h, the seedlings were transferred to plates containing synthetic auxin analog 1-naphthaleneacetic acid (NAA), which induced massive and synchronized cell division in the pericycle after about six hours.

We hypothesize that the large change in gene expression very early after lateral root initiation requires chromatin remodeling. Therefore, we searched their database for genes encoding chromatin modifiers that showed strong up-regulation during the initiation of lateral roots. This showed that all four members of the plant specific histone deacetylase family (HDT) of Arabidopsis are markedly upregulated in pericycle cells already at two hours

after application of auxin, so preceding the first cell divisions.

Histone deacetylases are together with histone acetyl transferases key regulators of the chromatin state and can reversibly remove or add acetyl groups from histones, respectively. The addition of acetyl groups to histone tails is thought to facilitate transcription by opening up the chromatin. Removal of an acetyl group results, in general, in a more compact chromatin and therefore repression of transcription (Berger, 2002; Narlikar et al., 2002). Arabidopsis has 18 histone deacetylases, which belong to three gene families. Twelve of them belong to the conserved RDP3/HDA1-like family, found in all eukaryotes. Two histone deacetylases belong to the Silent Information Regulator 2 (SIR2) family. All four histone deacetylases which are strongly upregulated during lateral root initiation belong to the HDT family, which is found only in plants and was first identified in maize in 1997 (Lusser et al., 1997). These Arabidopsis AtHDTs, named AtHDT1 – AtHDT4, have no homologues in animals and fungi. They contain a conserved N-terminal EWGF motif and a conserved histidine at position 25, both are important for repressor activity (Zhou et al., 2004). Phylogenetic analysis of HDTs showed that a gene duplication occurred early in diversification of dicots resulting in a split in HDT3 and HDT1, 2 and 4. Furthermore, AtHDT1 and AtHDT2 are the result of a recent gene duplication (Pandey et al., 2002; Chapter 3, this thesis). AtHDT1, AtHDT2 and AtHDT3 were shown to repress transcription when targeted to a reporter gene (Wu et al., 2000; Wu et al., 2003)

HDTs are involved in several developmental programs and responses to (a)biotic factors. AtHDT1, AtHDT2 and AtHDT3 have been studied in most detail and showed to be involved in reproductive development (AtHDT1-3), establishment of leaf polarity (AtHDT1-2), nucleolar dominance (AtHDT1) and ABA and abiotic stress responses (AtHDT3) (Wu et al., 2000; Zhou et al., 2004; Sridha and Wu, 2006; Ueno et al., 2007). HDTs of tobacco play a role in pathogen response as negative regulators of elicitor-induced cell death (Bourque et al., 2011).

Here we characterized the role of the Arabidopsis HDTs in lateral root initiation and in root development. We showed that all four AtHDTs are induced in the lateral root founder cells before they divide. However, none of the (double) *hdt* mutants that we tested was blocked in lateral root formation. In contrast, AtHDT1 and AtHDT2 are essential for maintenance of the root stem cell niche.

Results

AtHDT expression during lateral root initiation

It has been suggested that pericycle founder cells become primed in the meristem and first at a later stage of development form a lateral root (Beeckman et al., 2001). The induction of pericycle cells by exogenously applied auxin is therefore rather artificial. Therefore we determined whether the *AtHDTs* are expressed during normal lateral root initiation. Furthermore, it is studied whether they are first expressed shortly before the founder cells divide or whether they are already induced in cells when pericycle cells become primed in the meristem. To determine when and where these *AtHDTs* are induced, we generated transgenic plants that express an N-terminal fusion of GFP with one of the AtHDTs. These constructs were driven by the corresponding *AtHDT* promoter. We analyzed 10 day old seedlings for expression of GFP-AtHDT during lateral root initiation. We first identified young root primordia. The cells of these primordia do express these *AtHDT* genes (figure 1D,E GFP-AtHDT2, data not shown). Subsequently, we followed the pericyle cell layer towards the root tip, where younger lateral root primordia and activated founder cells are present. The GFP-AtHDTs are expressed in these primordia as well as in founder cells that have divided once. Slightly closer to the root tip often two adjacent pericycle cells express GFP-AtHDT, whereas the expression in surrounding pericycle cells is below detection level. This expression pattern was observed for all 4 AtHDTs and in figure 1 the results are shown for GFP-AtHDT1 and GFP-AtHDT2 (figure 1A,B respectively). Note that the distance between the nuclei in divided founder cells is markedly smaller than in the non-divided pericycle cells due to cell division in absence of cell elongation (figure 1C, arrows). The position of the 2 adjacent pericycle cells in which GFP-AtHDT is expressed as well as the fact that 1 or 0 of these pairs occur in a root support the conclusion that most likely they are activated founder cells. Thus the *AtHDT* genes are first expressed shortly before division of the founder cells and expression is not already induced in the meristem during priming.

Expression of all 4 *AtHDTs* was maintained in the tip (meristem) of fully emerged lateral roots, as shown for GFP-AtHDT2 (figure 1F). All 4 AtHDTs locate predominantly in the nucleolus as has been previously described for AtHDT1, 2 and 3 (Zhou et al., 2004).

Are HDTs required for (lateral) root development?

The highly regulated induction of expression of all four *AtHDTs* during lateral root formation indicates an important role in this process. To test this, T-DNA

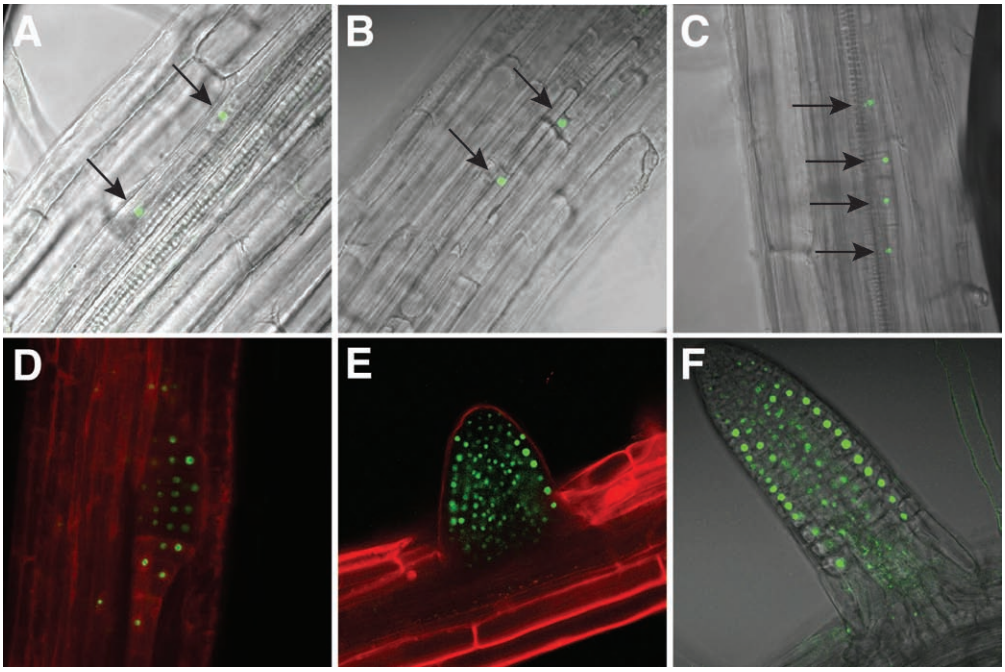

Figure 1: Expression of GFP-AtHDTs in lateral root formation. GFP-AtHDT1 (A) and GFP-AtHDT2 (B) fusions are expressed in two lateral root founder cells in the pericycle prior to division (nuclei indicated with arrows). Expression remains in these pericycle cells after the first division (C, GFP-AtHDT2, arrows) and after several rounds of division forming the lateral root primordium (D and E, GFP-AtHDT2) and emerging lateral root (F, GFP-AtHDT2). Confocal microscopy images of GFP after Propidium Iodide (PI) staining (D,E) or bright field microscopy (A,B,C,F).

insertion mutants for all four *AtHDT* genes were analyzed. RT-PCR and qPCR analysis showed that all *hdt1, 3* and *4* mutants were null mutants as the transcripts of the mutated gene are not detectable. However, *hdt2*, where the T-DNA is located in the second intron of the gene, has an expression level of about 10% of the wild type (data not shown).

We studied whether lateral root formation was affected in these *hdt* mutants. The number of (emerged) lateral roots per centimeter in *hdt1-4* and wild type Columbia plants at 14 days after germination were determined. A slight but significant reduction in lateral root number was found only for *hdt1* (figure 2). Interestingly, during the studies on lateral root formation we noticed that *hdt2* seedlings have slightly shorter roots. Therefore we measured the root length of *hdts* and wt Columbia seedlings. Indeed, a significant reduction of about 20% in average root length was observed between *hdt2* and Columbia at 14 days after germination (figure 3). Similar results were obtained in 2 other

experiments. Thus, some slight effect on lateral root formation and root growth were observed in the hdt1 and hdt2 mutant, respectively. As the HDTs are highly homologous (especially AtHDT1 and 2) and have similar expression patterns, it is probable that they are functionally redundant. To test this double mutants were made.

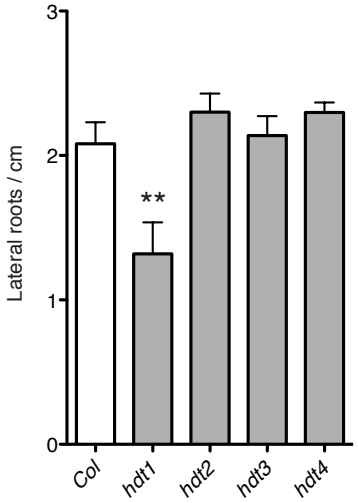

Figure 2: Lateral root formation in hdt mutants at 14 DAG. Only hdt1 showed a slight but significant reduction in lateral root number per centimeter compared to wild type Columbia plants. Error bars represent standard error. (n=22, 16, 23, 25 and 24 respectively) (** p≤0.01)

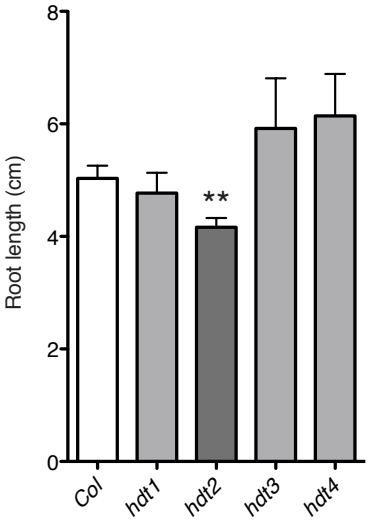

Figure 3: Average root length of hdt mutants compared to Columbia wild type at 14 DAG. hdt2 showed a significant reduction in root length compared to wild type Columbia (Col). Error bars represent standard error. (n=22, 16, 23, 25 and 24 respectively) (** p≤0.01)

Redundancy of HDTs?

To determine whether AtHDT1, 3 and 4 contribute to root growth we crossed the *hdt2* mutant with the other *hdt* mutants to obtain double mutants. We successfully obtained homozygous double mutants of *hdt2-hdt3* and *hdt2-*

hdt4. However, selfing of plants homozygous for *hdt1* and heterozygous for *hdt2* did not result in any homozygous *hdt1hdt2* double mutants among more than 50 plants tested. This suggests that the double mutant is lethal.

Analysis of the average root length in the double mutants (*hdt2hdt3* and *hdt2hdt4)* did result in a similar decrease in root length as in the *hdt2* mutant (data not shown). This indicates that either only AtHDT2 is involved root growth or that the AtHDT3 and AtHDT4 genes are functionally redundant and triple or quadruple HDT mutants might be needed.

The double mutant *hdt1hdt2* appeared to be lethal and therefore it could not be studied whether AtHDT1 and AtHDT2 are functionally redundant in root growth. To test this an RNAi construct to knockdown AtHDT1 as well as AtHDT2 was generated. This construct was driven by the root specific *RCH1* promoter, which is active in the root meristem but not in the embryo upto the torpedo stage (Casamitjana-Martínez et al., 2003). Columbia wild type plants were transformed and transgenic seeds (T0), were selected through the expression of the red fluorescent selection marker. These seeds were germinated and at 7 days after germination (DAG) roots of the vast majority of the RCH1::RNAi-HDT1HDT2 transgenic seedlings were much smaller than the control for which we used non-transgenic seeds (figure 4A). Average root length of these transgenic seedlings at 7 DAG was 6.6 ± 2.8 mm (n=21), whereas roots from non-transgenic seedlings were on average 16.5 ± 2.2 mm (n=25).

Root growth is depending on proliferation rate of the root meristem and elongation rate of cells leaving the root meristem. The stem cell niche in the root meristem consist of 4 organizing cells called the Quiescent Center (QC) surrounded by a single layer of stem cells, the initials. These stem cells give rise to specific daughter cells that can differentiate into the various root cell types (van den Berg et al., 1997). The stem cell daughter cells divide several times. These transiently amplifying cells eventually elongate and differentiate.

The meristem in RCH1::RNAi-HDT1HDT2 seedlings (7 DAG) is very short and cell elongation starts close to the QC and surrounding stem cells (figure 4C and D) as compared to wild type seedlings (figure 4B, note that the scale of B is smaller than in C). In wild type roots (7 DAG) the cortex cell file of the meristem consists of about 30-35 cells counted from the QC up to the transition zone where elongation starts (Dello Ioio et al., 2007). In RCH1::RNAi-HDT1HDT2 seedlings only about 10 cortical cells are present (figure 4C and D). The low number of cells in the meristem indicates that, like in other mutants with reduced root growth, the stem cell niche is not maintained. The presence of starch granules in columella cells can be used as a marker for differentiation. In wild type roots a single layer of columella stem cells is present between the

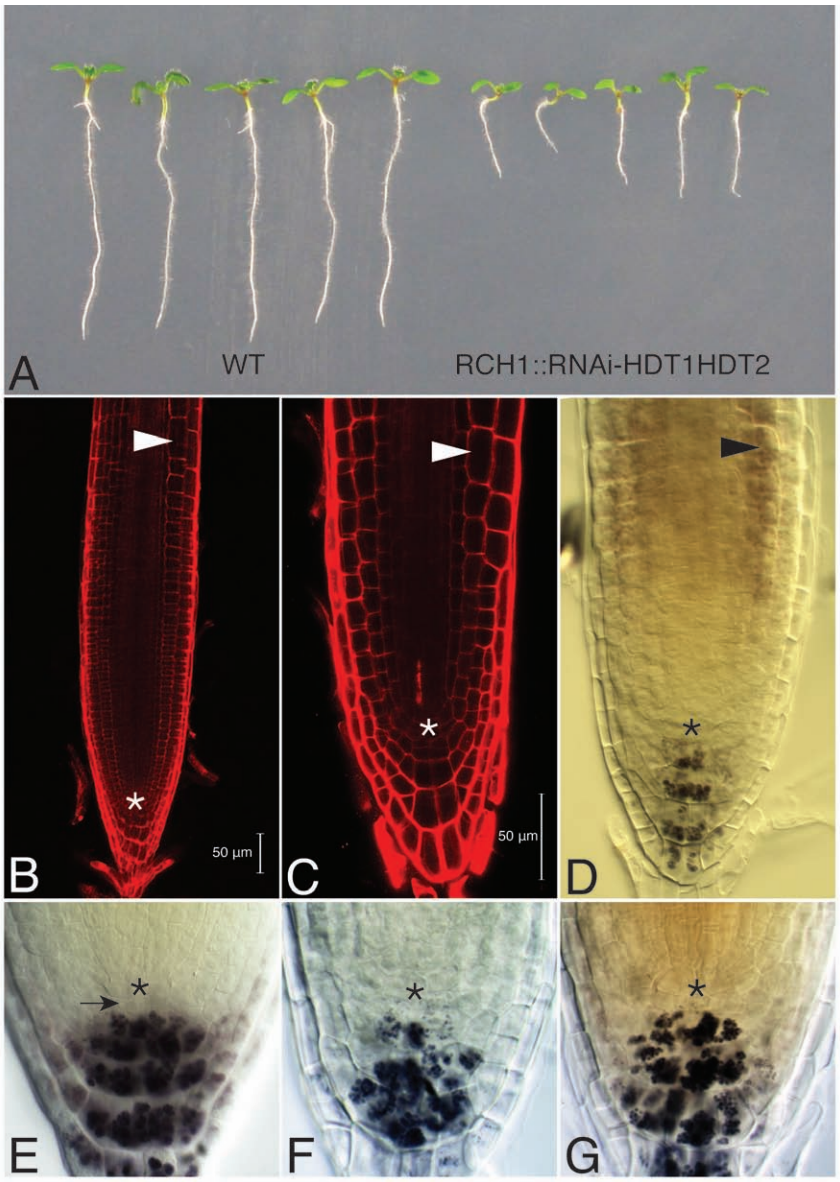

Figure 4. RNAi knock-down of AtHDT1 and AtHDT2. Reduced root length of RCH1::RNAi-HDT1HDT2 compared to wild type (WT) seedlings at 7 DAG (A). The meristem in RCH1::RNAi-HDT1HDT2 (C and D) at 7 DAG is much smaller than WT (B). The QC is marked with an asterisk. Arrowheads indicate first elongating cells in the root tip. Lugol staining of starch granules indicates diffenentiated columnella cells. In RCH1::RNAi-HDT1HDT2 seedlings (F and G) starch granules appear in the columella layer next to the QC (asterisk), whereas in WT (E) a layer of columella stem cells (marked with an arrow) is present next to the QC (asterisk). Confocal image with PI staining (B and C), Nomarski image with lugol starch staining in columella cells (D-G)

QC and differentiated columella cells containing starch granules (figure 5E). In the majority of the RCH1::RNAi-HDT1HDT2 transgenic seedlings at 7 DAG starch granules were present in the cells adjacent to the QC, as shown in figure 5F and G. This indicates that these cells are differentiated and have lost stem cell identity. Thus, AtHDT1 and 2 are essential for stem cell maintenance in the root meristem and these highly homologous HDTs are functionally redundant.

At 14 DAG the main root has stopped to grow and several lateral roots have emerged, indicating that lateral root initiation is not inhibited (data not shown). However, the *RCH1* promoter is first active when lateral roots emerge and not during earlier stage root primordia (Scheres and Heidstra, 2004)

HDT expession in the root apical meristem

To study wether *HDTs* could be involved in stem cell niche maintenance in a cell autonomous manner we characterized the expression of *AtHDT1* and *AtHDT2* in the root tip. Surprisingly, in plants expressing GFP-AtHDT1, GFP fluorescence in the main root meristem was close to the detection limit, whereas during lateral root formation it was clearly detectable. GFP-AtHDT2 expression in the main root locates predominantly to the meristem and elongation zone (figure 5A). In the differentiated zone expression is rapidly reduced. GFP-AtHDT2 expression is very low in the QC and surrounding stem cells and in their first daughter cells (figure 5B). Furthermore, expression is very low in all cells of the columella. However, published root expression data indicate that both *AtHDT1* and *AtHDT2* are expressed in the root meristem and QC (Birnbaum et al., 2003; Nawy et al., 2005). This suggests that the protein is less stable in the stem cell niche or that the genomic region that we cloned as putative promoter does not contain all information for proper expression. Therefore we tested whether the GFP-AtHDT2-GFP construct could rescue the mutant phenotype.

The lethality of the *hdt1hdt2* double mutant was used to study the biological activity of the GFP fusion protein. We transformed *hdt1hdt1-hdt2HDT2* plants with the *AtHDT2::GFP-HDT2* construct and among the T1 progeny we found plants that express *AtHDT2::GFP-HDT2* and are homozygous for both *hdt1* and *hdt2.* In addition, root length was as in wt showing that the construct is biologically active (data not shown). Therefore, at least AtHDT2 does not appear to control stem cell niche maintenance in a cell autonomous manner.

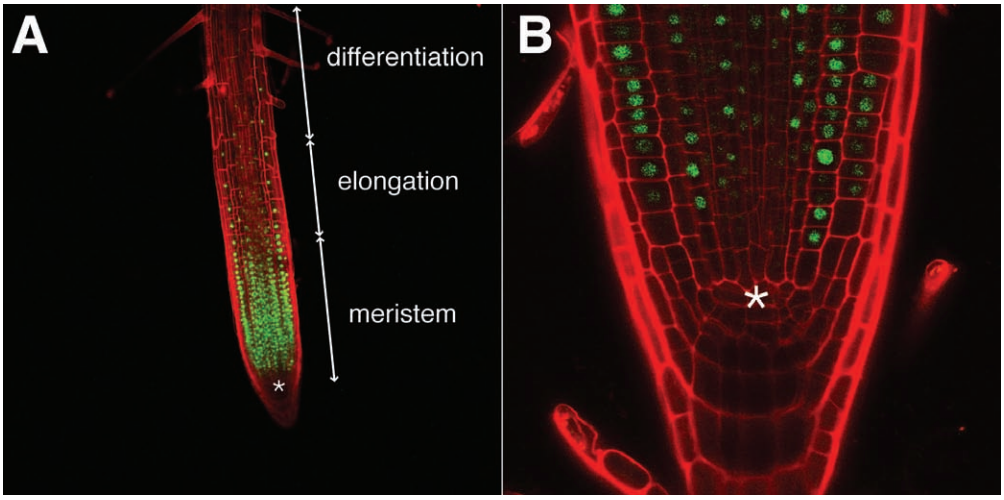

Figure 5: GFP-AtHDT2 localization in roots. Strong expression of GFP-AtHDT2 in the meristem and elongation zone (A). Magnification of the stem cell organizing region (B) shows that GFP-AtHDT2 is lower expressed in QC (marked by asterisk), surrounding single layer of stem cells and in columella cells. Confocal image of GFP with PI counter staining.

Discussion

During lateral root formation the expression of all four Arabidopsis HDTs is induced in the lateral root founder cells shortly before they start dividing. The expression is maintained in the lateral root primordium and root meristem. The highly homologous AtHDT1 and AtHDT2 are essential for the maintenance of the stem cell niche of the root. Whether the AtHDTs are essential for lateral root formation remains unclear.

Although the induction of expression of the four AtHDTs precedes the first division of these lateral root founder cells, only in the *hdt1* mutant we found a small but significant reduction in lateral root number. We showed that in other processes AtHDT1 and AtHDT2 are functionally redundant. This is the case in maintenance of the root stem cell niche and the process that is blocked in the *hdt1hdt2* double mutant that causes lethality. Therefore it seems very likely that AtHDT1 and AtHDT2 are also functionally redundant in lateral root initiation. However, we were unable to test this because on one hand the double *AtHDT* mutant is lethal. In addition, the root specific promoter *RCL1* that we used for the RNAi silencing of both genes is most likely first active when the lateral root meristem is formed (Heidstra, personal communication) and so too late to block lateral root initiation. Moreover, the block of growth of the main root by RNAi of *AtHDT1,2* will even stimulate lateral root formation.

We were unable to obtain a *hdt1hdt2* double mutant which is most

likely due to embryo lethality or an effect on the formation of gametes. Therefore an RNAi approach was applied using a root specific promoter that is first induced when the embryo has reached the torpedo stage. The loss of stem cell maintenance was observed in more than 15 primary transformants. When lines will be obtained from these transformants, the effect on the growth retardation will be analyzed in more detail. Our studies on primary transformants show that the stem cell niche is disappearing 7 DAG. However the effect on for example cell division rate and cell size remains to be studied.

The *AtHDTs* are switched on at the onset of lateral root formation. Developmental programs require a specific set of genes that are activated or repressed. Histone deacetylases are known for their role in regulation of gene expression, by deacetylation of histones gene expression is repressed. An example is the repression of embryonic traits during germination by AtHDA19, a histone deacetylase of the RFD3/HDAC family (Tanaka et al., 2008).

All four GPF-AtHDTs locate to the nucleolus as has been previously been reported for AtHDT1, AtHDT2 and AtHDT3 (Zhou et al., 2004). Based on this nucleolar location it has been proposed that HDTs are involved in the repression of rRNA expression. However, it seems highly unlikely that repression of rRNA genes does occur in the metabolically very active meristematic cells nor seems it probable that in this way stem cell maintenance can be regulated. In addition to the nucleolar location, AtHDTs also occur in the nucleoplasm albeit at a much lower level. We expect that there they contribute to a gene expression pattern that is essential for stem cell maintenance.

Recently a histone acetyl transferase, an antagonist of histone deacetylases, has been shown to be involved in regulation of root growth. The histone acetyl transferase GCN5 is as AtHDT1 and AtHDT2 essential for root stem cell niche maintenance (Kornet and Scheres, 2009). Roots of the *gcn5* mutant were short and the meristem is not maintained. The root stem cell niche specification is dependant on two independent pathways. The SHORT-ROOT/SCARECROW/RETINABLASTOMA RELATED pathway and the PLETHORA (PLT) pathway, where auxin responsive *PLT* expression regulates stem cell maintenance (Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003; Aida et al., 2004; Blilou et al., 2005; Wildwater et al., 2005). GCN5 acts on the PLT pathway by disturbing the PLT gradient. In which pathway AtHDT1 and AtHDT2 act needs to be elucidated. To test this, combinations of RNAi knockdown of *AtHDT1,2* expression with *shr* or *plt* mutants need to be made. However, it is unlikely that GCN5, AtHDT1 and AtHDT2 regulate maintenance of the stem cell niche in a similar manner. GCN5 is expressed in the stem cell niche whereas the expression of AtHDT2 is very low in the stem cell niche and markedly higher in the transiently amplifying cells of the meristem. Therefore

it is probable that AtHDT2 regulates the maintenance of the stem cell niche in a non-cell autonomous manner. The expression level of GFP-AtHDT1 appears to be very low therefore it does not provide a clue whether this is also the case for AtHDT1. However, since AtHDT1 and AtHDT2 are functionally redundant we expect that this is the case. The maintenance of the stem cell niche depends on a high level of auxin in the stem cell niche. This high level of auxin is formed by polar transport of auxin towards the tip. Therefore we hypothesis that knock down of AtHDT affects somehow the mechanisms by which a high concentration of auxin is created in the stem cell niche.

Methods

Plant material and growth conditions.

The *Arabidopsis thaliana* Columbia ecotype was used as wild type. For the HDT mutants T-DNA insertion lines were used from the SALK (Alonso et al., 2003), Sail (Sessions et al., 2002) and GABI-KAT (Rosso et al., 2003) collections and obtained from the Nottingham Arabidopsis Stock Centre (NASC). For *hdt1* (At3g44750) GK355_H03; *hdt2* (At5g22650) Sail_1247A02; *hdt3* (At5g03740) SALK_129799 and *hdt4* (At2g27840) GK279_D04. Double mutants were obtained by crossing of these mutants. AtHDT2 and AtHDT3 are located on the same chromosome arm. Therefore a crossover event was needed to obtain the double mutant *hdt2hdt3* after crossing. For this cross we used the at that time available *hdt3* mutant Sail_240_C08.

Null mutants were verified using RT-PCR. *AtHDT2* expression was quantified using quantitative-PCR (qPCR) in *hdt2* and wt seedlings. For this, total RNA was isolated using the plant RNA easy kit (Qiagen) and cDNA was synthesized of 1ug 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. qPCR was performed on a MyiQ (Biorad) using the Quantitative PCR kit for SYBR green (Eurogentec). Ubiquitin was used as a reference. qHDT2 and qUBQ primer sequences used for qPCR are listed in table 1.

Plants were grown vertically on 0.8% agar plates containing 2.2g Murashige and Skog 10 salts with vitamins (Duchefa) with 1% sucrose at pH 5.8 under LD 16h light / 8h dark conditions at 23-24 ^{o}C. For the lateral root number and root length essays plates without sucrose were used.

Plant transformations

Agrobacterium tumefaciens strain AGL0 (GFP fusion constructs) and strain C58 (RNAi construct and complementation) mediated transformation was performed as described by (Bechtold et al., 1993).

Root analysis

Roots of RCH1::RNAi-HDT1HDT2 and Columbia 7 day old seedlings were stained for a few minutes in Lugol solution (Merck) and mounted on slides in chloral hydrate-water-glycerol (8:2:1) for clearing and subsequently analyzed with a Nikon DIC Nomarski microscope.

Cell wall staining was performed by mounting roots 7 DAG on slides in a 10 mg/ml propidium iodide (PI) solution in water and analyzed using a Zeiss 510 confocal scanning laser

microscope.

Constructs

The CDS of each AtHDT was PCR amplified from cDNA and the AtHDT putative promoter sequences (1kb upstream of the start codon) were PCR amplified from genomic DNA using Phusion High Fidelity Taq polymerase (New England Biolabs) with HDT and pHDT primers listed in table 1 and directionally cloned into pENTR-D-TOPO (Invitrogen). The promoters were then cloned from the pENTR-D-TOPO vector into a pENTR4-1 vector (Invitrogen) in front of a GFP open reading frame using the NotI and AscI restriction sites. The pENTR4-1 containing the AtHDT1 promoter and GFP, the pENTR-D-TOPO-AtHDT1 vector and a pENTR2-3 vector containing a CaMV 35S terminator were recombined in 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::RFP expression cassette from pFluar 101 (Stuitje et al., 2003) was introduced for easy selection of red fluorescent transformed seeds. The same procedure was used to obtain AtHDT2::GFP-AtHDT2, AtHDT3::GFP-AtHDT3 and AtHDT4::GFP-AtHDT4.

The RCH1 promoter was PCR amplified from Arabidopsis genomic DNA using primers RCH1-HindIII-F and RCH1-XbaI-R (table 1) and directionally cloned into the pENTR-D-TOPO vector creating pENTR-RCH1. The RCH1 promoter was cut out of the pENTR-RCH1 vector using the HindIII (partial digestion) and XbaI restriction sites and combined with two fragments of the pK7GWIWG2(II) vector (Limpens et al. 2005) in a three-point ligation, thereby replacing the CaMV 35S promoter. The two fragments of the pK7GWIWG2(II) vector were obtained by digestion with either HindII and NcoI or with SpeI (compatible with XbaI) and HindIII. The whole RNAi cassette including the RCH1 promoter was cut out using ApaI and HindIII restriction sites and ligated into the pBnRGW binary vector, modified to contain a red fluorescent seed selection marker (NAP::RFP), creating pBnRRGWIWG.

The RNAi target sequences of 600bp of AtHDT1 and AtHDT2 were combined using a two-step PCR process. First, fragments of HDT1 and HDT2 were amplified from the pENTR-D-TOPO~HDT vectors in a single PCR reaction with primers HDT1rnai-F, HDT2rnai-R, HDT1rnai-R and HDT2rnai-F, of which the latter two contain a 15pb complementary overhang. Second, the PCR product was diluted (1:500) and in a second PCR step using only HDT1rnai-F and HDT2rnai-R primers, the fragments were combined and subsequently introduced by directional cloning into the pENTR-D-TOPO vector. The combined fragment of HDT1HDT2 was recombined in inverse-repeat orientation into the pBnRRGWIWG binary vector in a LR Gateway reaction (Invitrogen). Primer sequences are listed in table 1.

Table 1: Primers used.

qHDT2-F	GCCATTCAACTCAGGCAAAC
qHDT2-R	CGAAAACCCAAAACCTCTCC
qUBQ-F	TTAGAGATGCAGGCATCAAGAGCGC
qUBQ-R	CATATTTCTCCTGTCTTGAAATGAA
HDT1-F	CACCATGGAGTTCTGGGGAATTGAAG
HDT1-R	CTTGGCAGCAGCGTGCTT
pHDT1-F	CACCGAAGCCCTTGACGATGAGAT
pHDT1-R	GGCTAAGAATCAAGGTTGAGAAA
HDT2-F	CACCATGGAGTTCTGGGGAGTTGC
HDT2-R	AGCTCTACCCTTTCCCTTGC
pHDT2-F	CACCGTTTTGGATCTGCAGACAAGG
pHDT2-R	TGTTGTTGAACGAGGAAGAGAG
HDT3-F	CACCATGGAGTTCTGGGGTGTTGA
HDT3-R	AGCAGCTGCACTGTGTTTG
pHDT3-F	CACCGCACTCAACGCATTTTTGTC
pHDT3-R	TGTTGTGCGAGGTAGTGTGA
HDT4-F	CACCATGGAGTTTTGGGGTATCGA
HDT4-R	CTTTTTGCAAGAGGGACCAC
pHDT4-F	CACCGGCCATTTTTACCGGAATCT
pHDT4-R	AGCTAGTGAAAGAGGAAGATGTG
RCH1-HindIII-F	CACCAAGCTTCCATCAGTTGCAATGTACAG
RCH1-XbaI-R	TCTAGAGGATCCAGAGTTTTTTTCTTTG
HDT1rnai-F	CACCATGGAGTTCTGGGGAATTGAAGTTAAATCA
HDT1rnai-R	CGCAACTCCCCCAGAACTCATCTGTTTTCTG
HDT2rnai-F	CAGAAAACAGATGAGTTCTGGGGAGTTGCG
HDT2rnai-R	CAGAAAACAGATGAGTTCTGGGGAGTTGCG

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CHAPTER 3

The role of plant-specific histone deacetylases in reprogramming of root cortical cells in the Rhizobium-legume symbiosis

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Introduction

During plant development, in contrast to animals, most organs are formed post-embryonically originating from clusters of undifferentiated dividing cells that form so-called meristems. In some cases differentiated cells can be activated to enter the cell cycle and to ultimately give rise to new meristems. These differentiated cells reprogram to become pluripotent cells that are able to give rise to all cell types of the new organ. A typical example is lateral root formation from pericycle cells. These are mitotically activated, and in Arabidopsis from 2 (pericycle) founder cells a primordium is formed (Dubrovsky et al., 2001). Ultimately at the apex of such primordium a new root meristem is formed. While lateral root formation is common in the plant kingdom, legumes have the ability to form another, unique lateral root organ, the nodule.

Legumes can establish a symbiosis with bacteria that collectively are named rhizobium. This symbiosis culminates in the formation of the root nodules. There, bacteria are able to reduce atmospheric nitrogen in ammonia (Geurts and Bisseling, 2002). During the initiation of root nodule formation, rhizobium bacteria secrete specific lipo-chitooligosaccharides named Nod factors. These trigger the reprogramming of root cortical cells and this leads to the formation of a nodule primordium from which, after a meristem is established, a nodule is formed. These meristem cells will give rise eventually to all different tissue types of the nodule and so, they are pluripotent stem cells (Timmers et al., 1999). This reprogramming of root cortical cells is unique to legumes, as cortical cells in other plants are not able to change their cell fate (Geurts and Bisseling, 2002).

Plant specific histone deacetylases (HDTs) play an important role in lateral root initiation in Arabidopsis (Chapter 2, this thesis). During the activation of the pericycle cells, large changes of gene expression occur (Vanneste et al., 2005; De Smet et al., 2008). The four Arabidopsis *HDT* genes are among the up-regulated genes. Their expression is activated in root pericycle cells within 6 hrs after auxin addition that triggers lateral root initiation. This is even before the first pericycle cell divisions take place (Vanneste et al., 2005; De Smet et al., 2008 ; Chapter 2, this thesis).

In general, acetylation of histone tails by a histone acetyl transferase is associated with active chromatin, open for transcription. Removal of acetyl groups of histone tails by histone deacetylases results in a more closed conformation of the chromatin and therefore gene silencing (Hebbes et al., 1988; Grunstein, 1997). Therefore it is plausible that chromatin reorganization plays a role in the activation of pericycle cells during the initiation of lateral root formation.

Plants contain in general three types of histone deacetylases. The yeast homologous of the RPD3/HDA1 type, the Sir2 type and a third type of histone deacetylases called the plant specific histone deacetylases (HDT-type) (Pandey et al., 2002). These HDTs were first described in maize and have no structural homology to histone deacetylases found in other organisms (Lusser et al., 1997). All HDTs contain a conserved N-terminal EFWG motif and a central acidic domain. Most HDTs also have a C - terminal zinc finger motif (Dangl et al., 2001; Hollender, 2008). Four different homologs have been identified in Arabidopsis (Wu et al., 2000; Dangl et al., 2001). They were found to be most highly expressed in meristems and reproductive tissues and to be located predominantly in the nucleolus (Chapter 2, this thesis) (Wu et al., 2000; Dangl et al., 2001; Pandey et al., 2002; Zhou et al., 2004; Schmid et al., 2005).

To determine whether this histone deacetylase family also plays a role in the reprogramming of legume root cortical cells during the formation of nodules, we characterized the HDT family of Medicago. Medicago is one of the legume model systems for which the whole genome sequence is available (Cook, 1999; Oldroyd and Geurts, 2001; Young et al., 2011). In this chapter we identified and characterized the Medicago HDT family and show that these HDTs are essential for the reprogramming of root cortical cells and nodule primordium formation.

Results

Medicago HDT histon deacetylase family

We identified three Medicago HDT-like histone deacetylases, using the Medicago genomic DNA and EST sequences. All three MtHDTs contain the conserved N-terminal EFWG motif and, a central acidic domain (figure 1). MtHDT2 and MtHDT3 have both a C-terminal putative single zinc finger, like found in AtHDT1 and AtHDT3 and in most other HDTs. Phylogenetic analysis, using the Arabidopsis and poplar *HDT* sequences as well as the *HDTs* of two other legumes, soybean and Lotus, indicated the existence of two distinct *HDT* subgroups (figure 2). In the subgroup containing *AtHDT3* all analyzed species, with the exception of soybean have only one member. The Medicago gene in this subgroup is named *MtHDT3* and is most likely orthologous to *AtHDT3*. The second subgroup has two Medicago *HDT* genes, *MtHDT1* and *MtHDT2*, and the orthologs in Soybean and Lotus underwent additional duplications that are genus specific. The *HDT* duplication that is shared by the three legumes is most likely part of the whole genome duplication (marked by a dot) that occurred shortly after the rise of legumes, as this event is neither

seen in poplar or in Arabidopsis (Young et al., 2011). Three Arabidopsis genes are present in this subgroup and they are the result of two branch specific duplications resulting in *AtHDT1*, *AtHDT2* and *AtHDT4*.

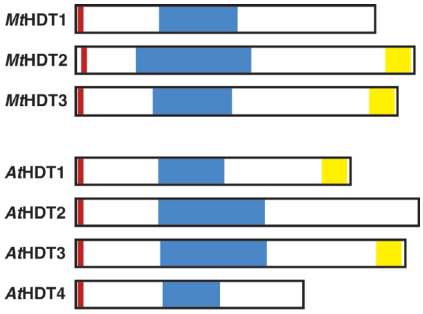

Figure 1: Domain organization of Medicago and Arabidopsis plant-specific HDTs. The red box represents the conserved EWFG motif, the blue box the central acidic region and the yellow box the zinc finger domain.

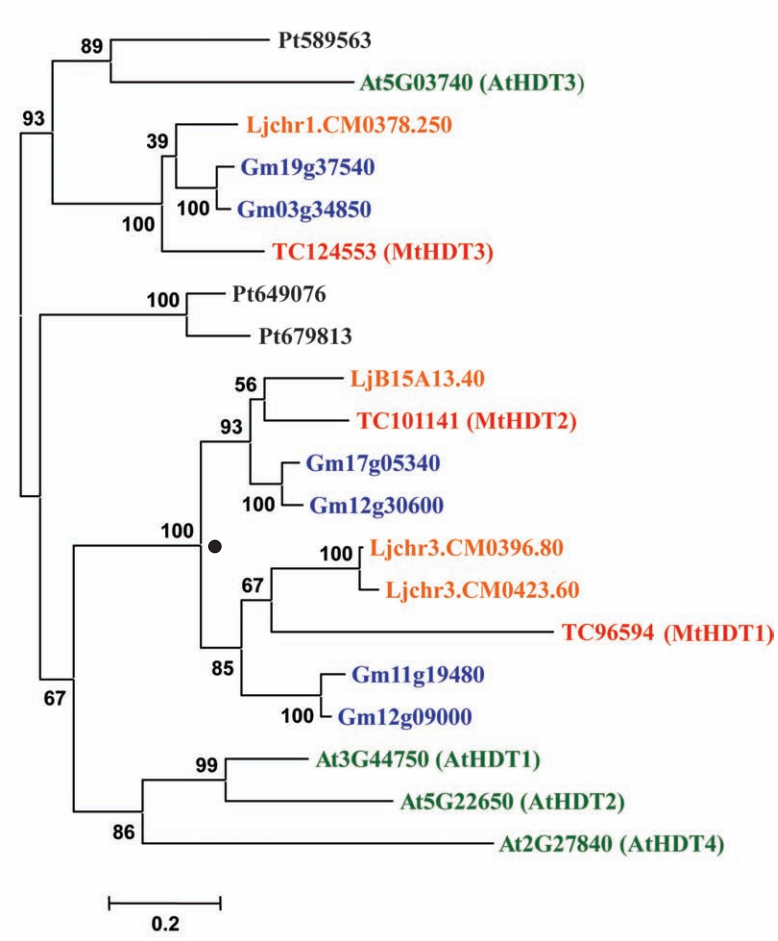

Figure 2: Phylogenetic tree of plant specific *HDT* sequences in Medicago (TC/Mt), Arabidopsis (At), Soybean (Gm), Lotus (Lj) and Poplar (Pt) based on Maximum-Likelyhood, bootstrap value of 100 replicates, ClustalW alignment of CDS sequences. Legume *HDTs* form two distinct groups, one group with *MtHDT3* and a second group with both *MtHDT2* and *MtHDT3*. The black dot marks the whole genome duplication shortly after the rise of legumes.

HDT expression in roots

The four Arabidopsis *AtHDT* genes are all expressed in the root meristem and their expression is markedly reduced when cells start to elongate. Furthermore, their expression is induced very early during the formation of lateral root primordia in the pericycle founder cells (chapter 2, this thesis). Before studying the role of the MtHDTs during nodule initiation, we first investigated whether the Medicago genes have a similar root expression pattern as Arabidopsis *HDTs*. We isolated RNA from 2 parts of 4-day old Medicago roots; the root apex (1 cm long segments), including the root meristem, elongation zone and from the rest of the root consisting of only differentiated cells. Total RNA was extracted, cDNA was synthesized and the amount of the *MtHDT* mRNAs was determined with qPCR. Relative expression levels were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). All three *MtHDTs* genes, but especially *HDT1* and *HDT2*, were expressed at higher levels in the apex of the root containing the meristem and the elongation zone than in the differentiated part, similar to Arabidopsis (figure 3).

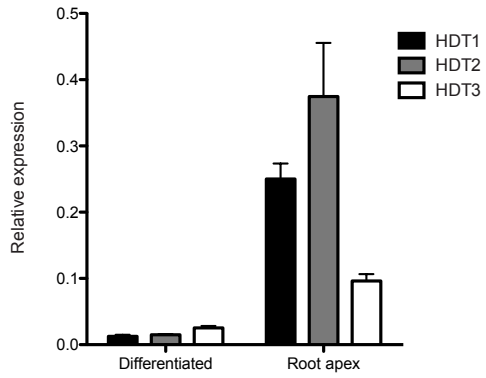

Figure 3: Relative expression of MtHDTs in roots. Expression of HDT1 and HDT2 genes is much higher in root apex compared to the differentiated part of the root. For HDT3 the difference in expression level is less. Expression was normalized to ubiquitin.

Expression of MtHDTs during nodule initiation

Medicago nodule formation starts with a few rounds of cell division in the pericycle followed by mitotic activation of groups of inner cortical cells. The latter do form the nodule primordium from which the nodule develops. The division of cortical cells is preceded by the migration of the nucleus from the

periphery of the cell to the center, by which these cells can easily be recognized before initiation of cell division (Timmers et al., 1999).

To further analyze the expression of *HDTs* during nodule initiation, we cloned the promoters of *MtHDT1, MtHDT2* and *MtHDT3* in front of the beta-glucuronidase (GUS) reporter gene. Only *MtHDT1* has been annnotated in the sequenced genome of Medicago (Young et al., 2011). Therefore, we screened the Mth-2 BAC library for *MtHDT2* and *MtHDT3* gene-containing BAC clones and identified their promoter regions by sequencing. To express these constructs in roots, we made use of the *Agrobacterium rhizogenes* hairy root transformation system that results in composite plants with transgenic roots (Limpens et al., 2004). Subsequently, transgenic roots expressing one of the *MtHDT::GUS* genes were spot inoculated with rhizobium bacteria to induce nodule primordium formation. Roots were harvested 25-30 hrs after inoculation, embedded and sections were screened for GUS reporter gene expression. All three *MtHDT:GUS* are expressed early during the early stages of nodule primordum formation (shown for *MtHDt2::GUS* in figure 4). Expression was detected in the dividing pericyle cell layer and in activated and dividing inner cortical cells. These cells give rise to the nodule primordium and for all three HDTs expression remained in older nodule primordia (data not shown).

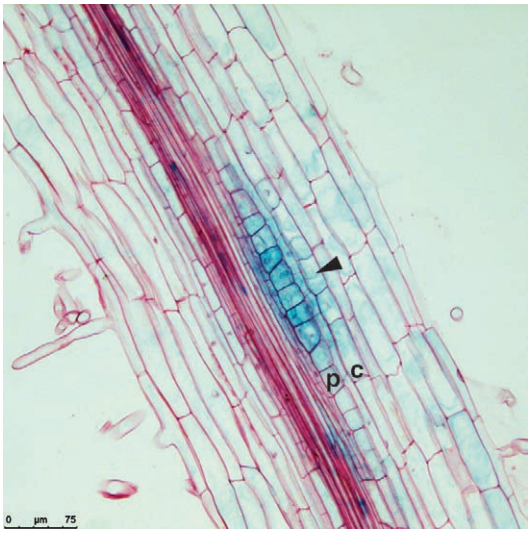

Figure 4: Root section with MtHDT2::GUS expression during nodule initiation observed at 25-30 hours after spot inoculation with rhizobium. GUS blue staining can be seen in the divided pericycle cell file (p) and in activated and dividing cortical cells (c, arrowhead). Longitudinal section stained with ruthenium red.

MtHDTs are essential for nodule primordium formation

To test whether these MtHDTs are essential for nodule initiation we used an RNAi approach. We created RNAi constructs of which the expression was controlled by the symbiotic *ENOD12* promoter (E12). The constructs contained

specific inverted-repeat sequences targeted to each HDT to knockdown their expression. The *ENOD12* promoter is active in early stages of nodule primordium initiation and in the apex of mature nodules (Pichon et al., 1992). By using this symbiotic specific promoter silencing of *HDTs* should only occur during nodule formation and not in the root meristem or during lateral root formation. Transgenic roots harboring the RNAi construct were obtained using the hairy root transformation system and these roots were inoculated with rhizobium bacteria to induce nodule formation. The number of nodules per root was counted 21 days after inoculation. No reduction in nodule numbers was detected in transgenic roots with knockdown of any single HDTs (data not shown). To exclude functional redundancy we also generated double and triple RNAi constructs to knockdown the two closely related HDT1 and HDT2 or all three HDTs simultaneously. The number of nodules on transgenic roots with the double HDT1/HDT2 and triple HDT1/HDT2/HDT3 RNAi constructs was significantly reduced (figure 5A).

We tested expression levels of each *MtHDT* in transgenic nodules with triple HDT1/HDT2/HDT3 RNAi constructs by qPCR and found a significant reduction (3 to 4 fold) of expression of all these *HDTs* compared to wild type nodules (figure 5B).

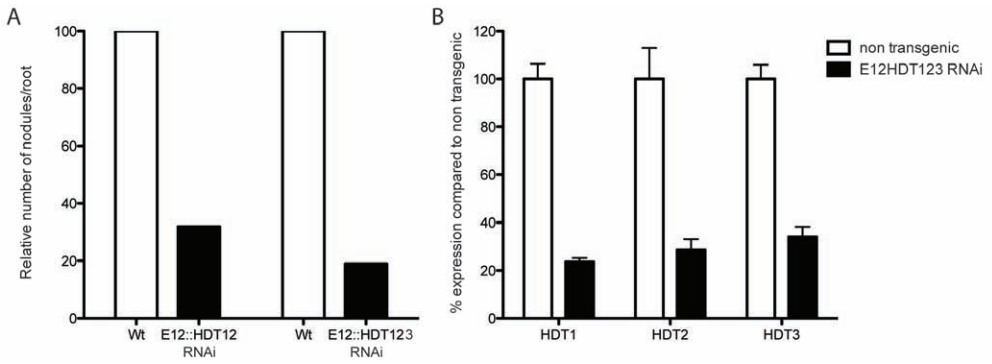

Figure 5. Reduction in nodule number and *HDT* gene expression in HDT RNAi knockdown nodules. Nodule number was strongly reduced on transgenic roots with double knockdown of HDT1 and HDT2 (n=30 roots) or triple knockdown of HDT1, HDT2 and HDT3 (n= 25 roots) in comparison with nodule number on wild type roots (n=38, n=19 roots respectively) (A). The qPCR analysis detected strong reduction of expression for each MtHDT in the triple RNAi knockdown (B).

Discussion

Our study showed that the Medicago MtHDTs play an important role in reprogramming of differentiated root cortical cells which marks the start of root nodule formation in legumes.

Phylogenic analysis indicated 2 distinct groups of HDTs in legumes as is the case in Arabidopsis. One group with *MtHDT1* and *MtHDT2,* which are closest related to *AtHDT1*, *AtHDT2* and *AtHDT4* and a second group with *MtHDT3* that is the orthologue of *AtHDT3*. *MtHDT1* and *MtHDT2* are the result of a duplication that seems legume-specific, and genes that result from this duplication are also maintained in Soybean and Lotus. A whole genome duplication has occurred shortly after the legumes arose and this whole genome duplication has probably contributed to the evolution of nodulation (Young et al., 2011). In Medicago about 900 gene pairs that originate from this whole genome duplication are maintained. Both *MtHDT1* and *MtHDT2* are expressed during nodule formation as well as in non- symbiotic organs such as the root. This suggests that they both are important for nodule formation as well as have maintained their ancestral function in for example root formation.

Reduction of the expression level of either *MtHDT1* or *MtHDT2* by RNAi during the initiation of nodule development had no effect on nodule formation. In contrast, when a RNAi construct was used that resulted in knock down of both or all three *HDTs* simultaneously, a 70 to 80% reduction of nodule number was observed. Furthermore knockdown of all three HDTs resulted in only a slightly stronger reduction in nodule number compared to the double knockdown. These data suggest that *MtHDT1* and *MtHDT2* are functionally redundant and essential for nodule formation. Although *MtHDT3* is also expressed in nodule primordia and could have a function different from *MtHDT1* and *MtHDT2* the RNAi data indicate that *MtHDT3* might be of less importance in nodule initiation.

For the RNAi experiments, we used the *MtENOD12* promoter that is nodule specific and is induced when cortical cells are reactivated (Pichon et al., 1992). In this way we avoided that knockdown of *MtHDTs* would affect root development, which indirectly might influence the ability of root cortical cells to dedifferentiate. *MtENOD12* is induced in dividing root cortical cells and so it is probable that the RNAi construct will be expressed in these cells and thus influence nodule initiation. To determine the effect of the RNAi construct on *MtHDT* expression, *MtHDT* mRNA levels should ideally be determined in such dividing cortical cells but this is technically not possible. As the *MtENOD12* promoter is also active in the apex of Medicago nodules we determined the knock-down levels in nodules that developed on the transgenic roots. In these

nodules the *MtHDT* mRNA levels are 3-4 fold reduced. However, the number of nodules that is formed is highly reduced, which indicates that MtHDT silencing probably also has an effect on the root cortical cell divisions. It is therefore quite reasonable to assume that in places where the cortical cell divisions are arrested, the *MtHDT* mRNA levels are more severely reduced.

Nodules originate from cortical cells that dedifferentiate and re-enter the cell cycle to form a nodule primordium. This reprogramming of fully differentiated cortical cells is unique for legumes, as cortical cells from other plants do not have this ability to form new secondary root organs. The nodule primordium cells can differentiate in all nodule cell types, which includes infected and uninfected cells of the central tissue, the peripheral nodule cortex and parenchyma that are separated by an endodermis and vascular bundles surrounded by a pericycle and endodermis. Therefore, the nodule primordium cells are pluripotent. *MtHDT* genes are activated in the cortical cells before cytokinesis. This is similar to Arabidopsis root pericycle cells, where the *AtHDT* genes are activated prior to division (Vanneste et al., 2005; De Smet et al., 2008 ; Chapter 2, this thesis). In these cells the AtHDTs are upregulated 2 hrs after auxin application and this coincides with the start of S-phase. This could mean that the HDTs are important for mitotic activity. However, the dividing pericycle cells form a primordium from which all root cell types are formed. Thus, like nodule primordium cells, also lateral root primordium cells have a pluripotent nature. Therefore it is also possible that HDTs play a role in initiating or maintaining the pluripotent nature of the primordium cells.

The chromatin state of pluripotent stem cells in mammals differs significantly from that of differentiated cells. These stem cells have for example a much more homogenous chromatin structure (Meshorer and Misteli, 2006; Efroni et al., 2008). Furthermore, in induced pluripotent stem (iPS) cells, which are somatic cells that are reprogrammed to a pluripotent state through over-expression of a defined set of transcription factors, chromatin modifiers play a key function in establishing and maintaining pluripotency (Gaspar-Maia et al., 2011). Also, maintenance of Arabidopsis root stem cells involves a specific histone acetyl transferase, which is an antagonist of histone deacetylases (Kornet and Scheres, 2009). Therefore, in Medicago histone deacetylases could play an important role in the reprogramming and induction of pluripotency of differentiated cortical cells during the initiation of nodules.

Methods

Gene identification and characterization

We identified the *MtHDT1* (TC96594), *MtHDT2* (TC101141) and *MtHDT3* (TC124553) sequences using homology search with Arabidopsis and other available plant HDT sequences. The genomic sequence, including promoter, for MtHDT1 was present in available DNA sequences of the Medicago genome. The genomic sequences of the MtHDT2 and MtHDT3 were obtained by sequencing of PCR fragments amplified using cDNA primers and genomic DNA as template. Putative promoter sequences of MtHDT2 and MtHDT3 were identified by screening the Medicago Mth2 BAC library using of 500-600bp DNA fragments of the genomic sequence as a probe. MtHDT2 was detected on BAC 35O10 and MtHDT3 on BAC 86M06 and BAC 83O10. Local sequencing of these BACs resulted in the genomic DNA sequences upstream of the start codon.

Phylogenetic analysis

Multiple alignments of HDT sequences were performed using Molecular Evolutionary Genetics Analysis 4 (MEGA 4) software (Tamura et al., 2007). Phylogenetic analysis was performed using the full coding sequences (CDS) of each HDT. The tree was drawn based on maximum likelihood with a bootstrap value of 1000 replicates.

Quantitative expression analysis

Medicago truncatula, Jemalong A17 accession, plants were grown vertically for 4 days on Fåhraeus plates (Fåhraeus, 1957). Root tips (5mm) and remaining roots were collected. Total RNA was isolated using the plant RNA easy kit (Qiagen). cDNA was synthesized from 1ug 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 was used as a reference. Primers used for real-time PCR were: HDT1-F GGATGAGGGCAAGAACAAAA; HDT1-R TTGGTGGTAGCAGGCTTCTT; HDT2-F TCTGGGGTGCTGAGGTTAAG; HDT2-R TTTGTCCTTGGTCAGGGTTC; HDT3-F CTGAAGGGAGCAACAAGAGG; HDT3-R TGGCTGCTTGTTATTTGCAG; UBQ-F GGTTGATTGCTCTTCTCTCCCC and UBQ-R AAGTGATTGCTCGTCCAACCC.

Histochemical analyses

About 1kb (2kb for MTHDT1) upstream region of the ATG start codon containing the putative promoter of the gene was amplified using primers; pHDT1-F CACCTCTTTTTGAATTAGTTTCTTATTTTGG; pHDT1-R TTGAAAGGGAGAGCGAAACG; pHDT2-F CACCGTTTTTACTTCCTTCTGCCAAAC; pHDT2-R TGGAAACAGTGAGACAGAGAGAG; pHDT3-F CACCGGTTTGGGTGATTTTGGATTG; pHDT3-R GACTGCAGAGAGAGAGCAAGC.

The PCR fragments were introduced into a pENTR-D-TOPO vector (Invitrogen) and subsequently recombined into the pKGWFS7-RR destination vector containing a GUS-GFP reporter and a dsRED1 expression cassette for easy selection of transgenic roots (Karimi et al., 2002). The destination vector was introduced into Medicago A17 roots through *Agrobacterium rhizogenes*, strain msu440 (Sonti et al., 1995), mediated transformation as described by (Limpens et al., 2004). Plants containing transgenic roots expressing the HDTpromoter-GUS were selected based on dsRED1 expression and inoculated with *Sinorhizobium meliloti* strain

Sm2011 to induce nodulation according to (Limpens et al., 2004). Transgenic roots were harvested after 25-30h post inoculation and GUS stained in 0.1 phosphate buffer (pH 7.0) containing 1mM x-gluc (DMSO), 3% sucrose, 0.5mM EDTA 0.1M K ferricyanide and 0.1M K ferrocyanide. Stained roots were cleared in 70% ethanol and subsequently embedded in Technovit 7100 (Heraeus Kulzer). Seven µm thin longitudinal sections were cut using a microtome, stained with ruthedium red and analyzed with a Leica DM5500B microscope.

Knockdown of HDT expression

RNAi target sequences of about 400-500bp of each MtHDTs were amplified from the pENTR-D-TOPO vectors containing the mtHDT CDS (described in chapter 4) using primer combinations HDT13-F+HDT12-R, HDT12-F+HDT23-R and HDT23-F+HDT12R. These fragments were combined in a subsequent PCR step using a mixture of the three PCR products as template and making use of the complementary 15bp overhang of the primers used in the first step. The MtHDT1 and MtHDT2 fragments were combined in this second PCR step using primers HDT13-F and HDT23-R and the MtHDT1, MtHDT2 and MtHDT3 fragments were combined using primers HDT13F and HDT13R. The following primers were used: HDT13-F CCACCCTATGAAGAAATGGGAGCGTCTTCT; HDT13-R AGAAGACGCTCCCATTTCTTCATAGGGTGG; HDT12-F CCACCCTATGAAGAA-ATCCCATTGATTACC; HDT12-R GGTAATCAATGGGAT-TTCTTCATAGGGTGG; HDT23-F AAAACTCCCAAGTCT-ATGGGAGCGTCTTCT; HDT23-R AGAAGACGCTCCCAT-AGACTTGGGAGTTTT.

The single or combined MtHDT fragments were introduced into a pENTR-D-TOPO vector (Invitrogen) and subsequently recombined in inverted repeat orientation in to the gateway vector pK7GWIWG2(II) driven by the nodule specific MtEnod12 promoter and containing the dsRED1 selection marker (Limpens et al., 2005). The constructs were transformed into plants using *Agrobacterium* mediated transformation as described above. After transformation the plants were grown on perlite in low nitrate conditions and inoculated with Sm2011 as described by (Limpens et al., 2004). 21 days after inoculation nodule numbers on transgenic, recognizable by dsRED1 expression, and non-transgenic roots were counted. Reduced expression of *MtHDTs* was verified by qPCR on cDNA from E12::HDT123RNAi transgenic nodules and compared to non-transgenic nodules of these plants using primers and method as described above.

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CHAPTER 4

The function of Medicago plant-specific histone deacetylases during nodule development

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Introduction

The Rhizobium-legume symbiosis involves the formation of a new organ, the root nodule. In specialized nodule cells rhizobium bacteria are hosted intracellular and there they have a niche that allows them to reduce atmospheric nitrogen into ammonia. Root nodules are formed by the dedifferentiation of root cortical cells. These dividing cortical cells form a nodule primordium from which, upon infection by Rhizobium, the nodule is formed (Geurts and Bisseling, 2002). The dedifferentiation of root cortical cells and the formation of a primordium, in *Medicago truncatula* (Medicago), are accompanied by the expression of three genes encoding histone deacetylases (chapter 3, this thesis), which belong to a plant specific histone deacetylase family (MtHDTs). By an RNAi approach it was shown that these MtHDTs are important for nodule formation as nodule number is reduced to about 20% when all three genes are silenced (chapter 3, this thesis).

Medicago forms nodules together with *Sinorhizobium meliloti.* The formation of these new symbiotic root organs starts with the formation of a primordium. Cells in the root dedifferentiate and re-enter the cell cycle. Eventually at the distal part of this primordium the nodule meristem is formed and the proximal part of the primordium will differentiate into all other initial tissues of the nodule. How this occurs will be addressed in this chapter. During further growth of the nodule, the meristem at the apex continuously adds cells to the different nodule tissues resulting in an indeterminate growth. For this reason these tissues are of graded age, with the youngest cells adjacent to the meristem and the oldest cells near the root attachment point (Geurts and Bisseling, 2002).

Nodules can be divided in zones that reflect the subsequent steps of development. This is especially clear in the central tissue, the tissue that contains so-called infected cells that are filled with nitrogen fixing rhizobia. A second cell type in this tissue is the uninfected cell that has a special function in N assimilation. The most distal zone of the nodule is the persistent meristem. This meristem divides by which it maintains itself and adds cells to, among others, the central tissue. These cells can be penetrated by an infection thread. Bacteria are released from these infection threads, during this process they become surrounded by a host membrane and the organelle-like symbiosomes are formed. Subsequently, symbiosomes divide and start to differentiate (Ivanov et al., 2010). In Medicago this differentiation of the symbiosomes is accompanied with a marked increase of their volume. Infection, division and differentiation of symbiosomes occurs in a zone of about 15 cell layers and it is named Infection zone. When symbiosomes are fully differentiated they have

become N fixing “organelles”. The switch from Infection zone into Fixation zone is marked by the induction of Nif genes that encode the subunits of the N fixing enzyme nitrogenase (Limpens et al., 2005). A more convenient marker for the start of the Fixation zone is the accumulation of starch granules in the host cells (Geurts and Bisseling, 2002).

The switch from meristem to Infection zone is marked by the block of cell division. However, DNA replication continuous and by this endoreduplication polyploid cells (up to 64n) are formed. This endoreduplication is essential for normal nodule development. When it is blocked, for example by a mutation in CCS52a, nodule development becomes arrested at a stage when symbiosomes are hardly developed (Vinardell et al., 2003).

Histone deacetylases are, together with histone acetyl transferases, key regulators of the chromatin state and can reversibly remove or add acetyl groups on histones, respectively (Shahbazian and Grunstein, 2007). The histone deacetylase genes of Medicago belong to 3 families of which the *HDT* family is plant specific. Medicago has three *MtHDT* genes and these are all upregulated during nodule primordium formation (chapter 3, this thesis). Knock-down of *MtHDTs* results in a markedly reduced nodule number and most of these nodules remain small. Here, we studied in more detail the function of the Medicago *MtHDT* genes during nodule development.

Results

Fate map of root (cortical) cell layers during nodule formation

Our aim is to determine whether *MtHDT* genes are essential for the fate shift of cells of the root nodule primordium or nodule meristem, when they differentiate in specialized nodule cells. The most proximal nodule primordium cells will directly differentiate into nodule cells, whereas distal primordium cells will form a meristem that subsequently, by division, maintains itself and adds cells to the nodule tissues (Timmers et al., 1999). To determine which primordium cells contribute to the formation of the meristem and which directly differentiate into specialized nodule cells we made a fate map. This concerns the fate of the root cell layers that contribute to the formation of the nodule primordium as well as the fate of the different parts of the primordium.

Medicago roots have from outside inwards the following cell layers; epidermis, 5 layers of cortical cells, endodermis and the pericycle that surrounds the vascular bundle. The outermost cortical layer is named C1 and the innermost C5. We spot-inoculated Medicago roots with *Sinorhizobium meliloti*. At different time points (24-72hrs) post inoculation (hpi) roots were fixed, embedded and longitudinal or transverse sections were made. At all

time points at least 5 spot inoculated roots were analyzed. Here the general picture that emerged from these analyses is presented.

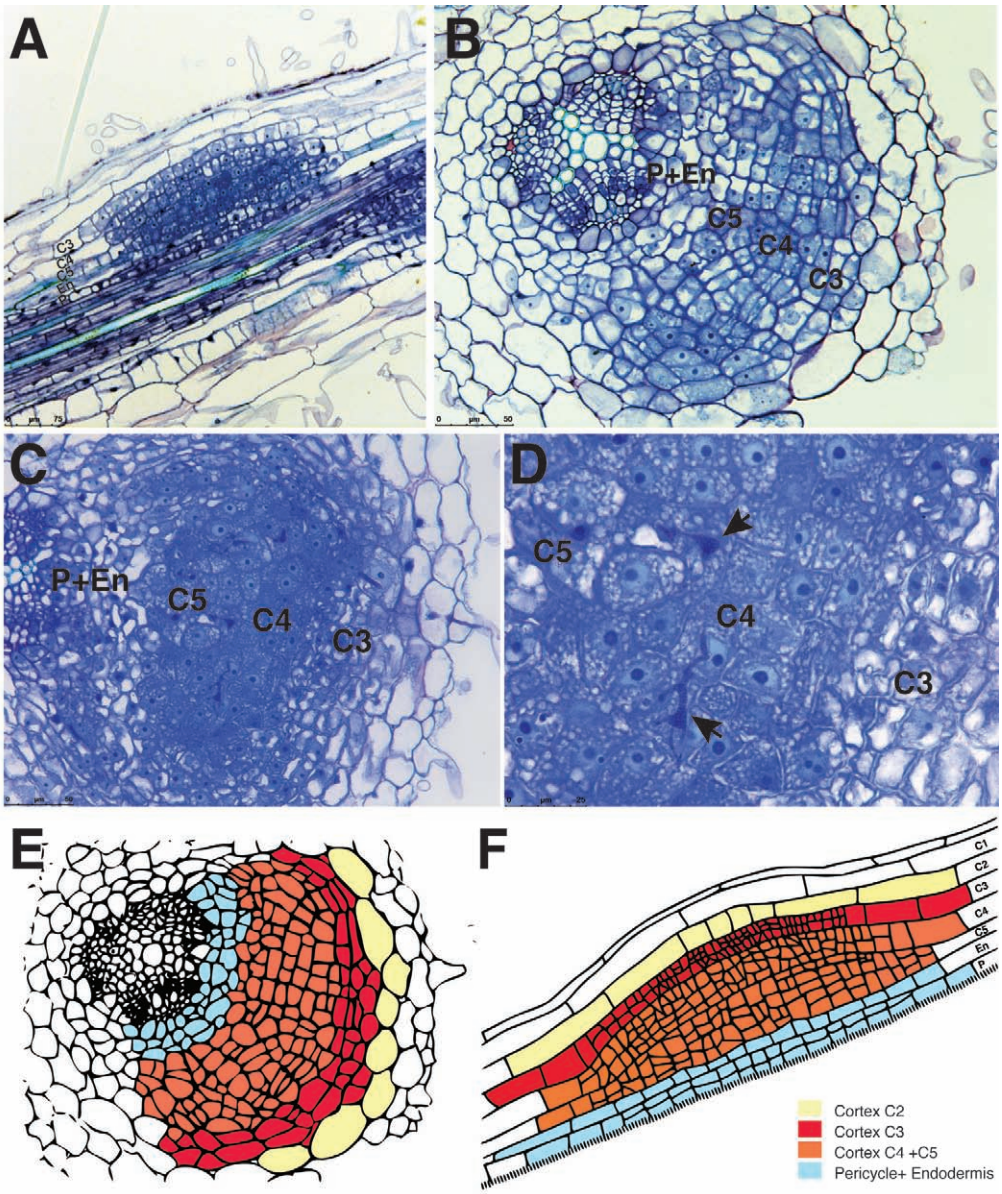

Figure 1: Longitudinal (A,F) and transverse (B,C,D,E) sections of nodule primordia.
A: 32 hpi. Divisions start in the pericycle cell layer (P), followed by divisions in C5, C4 and first anticlinal divisions in the C3 cortical layer. Proximal primordial cells are differentiated and increase in size. At the distal side, cells continue to divide and form the nodule meristem.
B: 42 hpi. 6-8 cell layers originate from the C5 and C4 layers and have stopped dividing.
C: 67 hpi. The C3 layer continued to divide to form the nodule meristem.

D: Magnification of C. Cells from C4 an C5 have many small vacuoles and are surrounded by rhizobia infection threads (arrows) and morphologically different from cells of C3 which will form the meristem.
E: Transverse schematic view of the nodule primordium of B at 42 hpi. The future nodule meristem originates from cells from the C3 layer (red). C4 and C5 cells enlarge after several round of division and can be infected and will eventually differentiate into the central tissue of the nodule (orange).
F: Longitudinal schematic view of a 72 hpi nodule primordium. The nodule meristem is formed from C3 (red). C4 and C5 will form the central tissue of the nodule (orange).
Sections were stained with toluidine blue

Rhizobia first trigger cell divisions in the pericycle within 24 hpi. This is the inner most layer that contributes to the formation of the nodule primordium. It starts with anticlinal division (data not shown), which are followed by periclinal divisions. At 32 hpi 2 cell layers are present that originated from the pericycle and furthermore anticlinal divisions have also occurred in the endodermis (figure 1A). This is best seen in the cross section 42 hpi (figure 1B, E). At this time point divisions have ceased in these layers, as there are still 3-4 cell layers that originated from the pericycle and endodermis (indicated in blue in figure 1E).

At 24 hpi cells of the cortical layer C5 are activated as their nuclei have migrated to the center of the cells (data not shown). Subsequently, anticlinal divisions are induced and cells of C4 are activated and anticlinal divisions are induced as well. This is followed by a few rounds of division that include both periclinal and anticlinal divisions. These are divisions by which the cells become smaller and growth hardly occurs. Therefore this is similar to the cleavage divisions that occur during amphibian embryogenesis (Gilbert, 2000). At 32 hpi about 8 nodule primordium cell layers have been formed from C5 and C4 and a similar number is present at 72 hpi (figure 1C). Also in the cross section (42 hpi), shown in fig 1B, it can be seen that C5 and C4 have resulted in about 8 nodule primordium cell layers (indicated in orange in schematic section in figure 1E and 1F). At 32 hpi the first divisions occur in C3. These are anticlinal divisions that can be well seen in the longitudinal section (figure 1A), but as the plane of division runs in parallel with the plane of sectioning, are not well visible in the cross section. In the about 8 cell layers that are already formed from C5 and C4 cell division ceases as the number cells has hardly increased at 72 hpi. Most likely the C4/5 derived cells enter endo-reduplication as their nuclei enlarge.

Cell division in C3 start with anticlinal divisions at about 32 hpi and “cleavage” division continue in all directions. Eventually around 72 hpi a meristem, composed of very small cells, is formed from C3 (figure 1C and 1F).

At this stage some anticlinal divisions have been induced in C2 and this will from the nodule cortex that protects the nodule meristem. Further, an infection thread, which was initiated in the epidermis, has already reached the cells that originated from C4 and C5 and in several cells bacteria released from the infection thread can be seen (figure 1D). Subsequently, the cells of C4 and C5 enlarge and form the about 8 most proximal layers of the central tissue and contain infected and uninfected cells. In figure 1D a clear distinction can be seen between cells originating from C4 and C5, which are larger in size and contain more dense cytoplasm with many small vacuoles compared to cells from C3 with larger vacuoles and which are still dividing and will form the nodule meristem. This nodule meristem continues to divide by which it adds cells to the different nodule tissues, especially the central tissue, and maintains itself. So in a mature nodule the about 8 proximal layers of the central tissue have differentiated directly from the nodule primordium, whereas all other layers of the central tissue originate from the nodule meristem.

Knock-down of MtHDTs results in disturbed differentiation from the nodule meristem

Knock-down of both *MtHDT1* and *MtHDT2* or of all three *MtHDTs* simultaneously using an RNAi construct under the control of the *ENOD12* nodule symbiosis specific promoter resulted in a up to 80% reduction in nodule numbers on transgenic roots (chapter 3, this thesis). In addition, the nodules that were formed remained rather small. The *ENOD12* promoter that is used in this study is active in the meristem as well as in the Infection zone of the Medicago nodule and therefore is suited to study the effect of *MtHDT* knock-down on nodule differentiation (Pichon et al., 1992). Fourteen dpi nodules that were formed on triple RNAi knock-down transgenic roots were embedded and longitudinal sections were made and analyzed by light microscopy. We performed this experiment twice and in both cases a very low number of nodules were still formed of which the majority showed an aberrant appearance. The proximal cell layers of the Fixation zone are similar to those in wt nodules (figure 2A-D). The infected cells are large and are fully packed with elongated rhizobia. This “wt-like” zone of about 8 cell layers is the region that presumably differentiates directly from the nodule primordium (figure 2A). In wt nodules a meristem is present at the apex and this adds cells to the Infection zone. This zone is composed of cells that gradually increase in size and have reached their maximum size at the transition to the Fixation zone (figure 2C and D). In the knock-down nodules a meristem is present at the apex and an Infection zone is formed. In this Infection zone infection threads are present. However, release of rhizobia is absent or markedly reduced and the cells of the Infection zone

remain rather small, which creates a clear discontinuity between the proximal region of the central tissue with large infected cells and this Infection zone with small cells (figure 2B). In other words, the cells of the meristem form an Infection zone but cells of this zone do not become part of the Fixation zone as growth of the nodule becomes arrested when they are rather small. Also the differentiation of the meristem in the distal direction, forming the nodule cortex, is disturbed as more cortical cell layers can be present (~6 instead of ~3) (figure 2A and C).

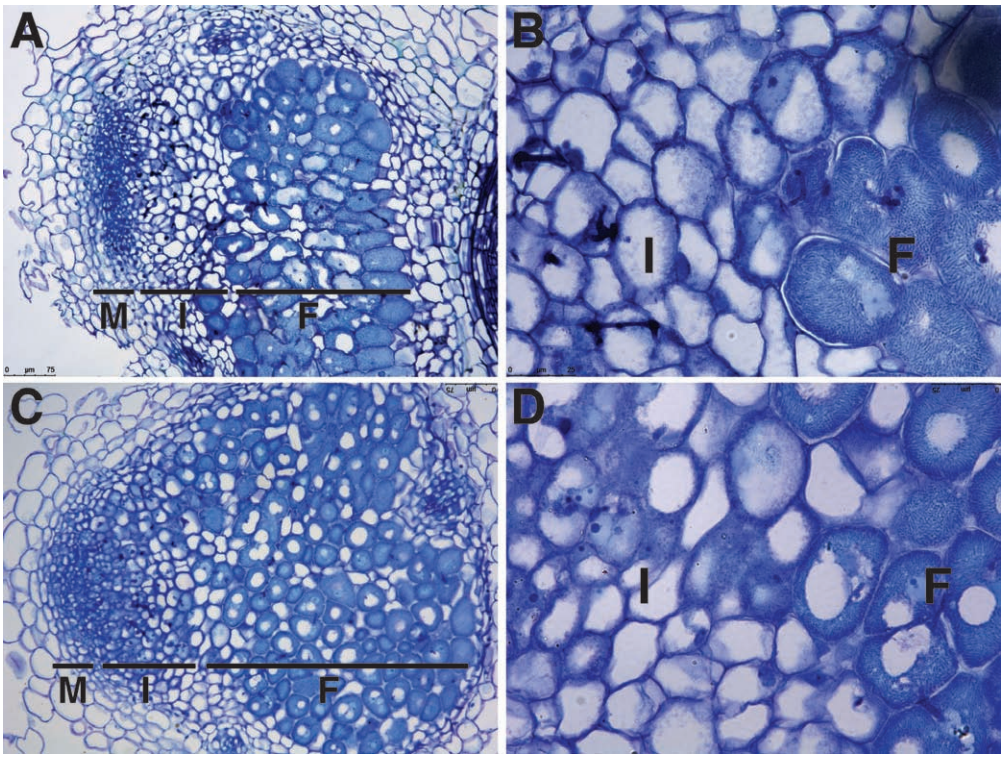

Figure 2: Sections of transgenic and wt nodules 14 dpi. Nodules with knock-down expression of MtHDT1, MtHDT2 and MtHDT3 (A and higher magnification in B) have a reduced number of layers (~8) with fully developed infected cell compared to wt nodules (C and higher magnification in D). Meristem (M), Infection zone (I) and Fixation zone (F) are marked. Sections were stained with toluidine blue.

The reduced *MtHDT* expression after RNAi knock-down results in disturbed differentiation and infection with rhizobia of those cells added from the meristem to the central tissue, whereas this is not the case for cells coming directly from the primordium. To get more insight in the process in which these *MtHDTs* are involved, we looked in more detail at their expression in nodules.

Expression of the MtHDT-like genes in nodules

To analyze the expression patterns of the 3 *MtHDTs* in nodules, we first determined the overall expression levels of these genes in nodules and compared it to expression levels in roots by qPCR using gene specific primers. RNA was isolated from nodules 14 dpi and from the remaining roots and relative expression was calculated using the deltadeltaCt method (Livak and Schmittgen, 2001). Expression level of each *MtHDT* is similar in roots and nodules, with strongest expression of *MtHDT2* in both roots and nodules (figure 3).

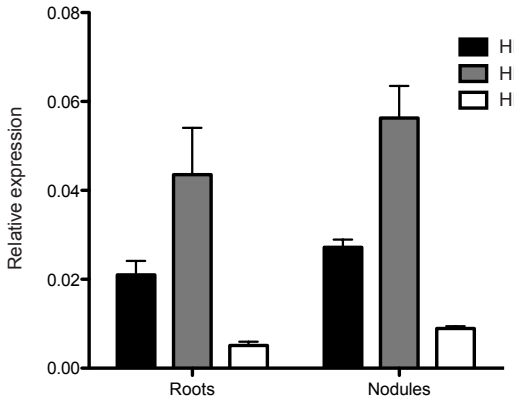

Figure 3: Relative expression of MtHDTs in roots and nodules. Relative expression, compared to ubiquitin, of each MtHDT is similar in roots and nodules. MtHDT2 is most abundantly expressed in roots and nodules.

Next, to find out where in root nodules the *MtHDT* genes are expressed we made use of beta-glucuronidase (GUS) reporter constructs driven by an *MtHDT* promoter. The putative promoter of each *MtHDT* gene was cloned in front of the *GUS* reporter gene and Medicago roots were transformed with these constructs using *Agrobacterium rhizogenes* by which composite plants with transgenic roots were obtained. Transgenic roots expressing one of the 3 *MtHDT::GUS* constructs were inoculated with *S. meliloti*. Expression of the *GUS* reporter gene was analyzed in nodules 14 dpi. This showed that all 3 genes are predominantly expressed at the apex of the nodule (figure 4 A-F).

To better analyze the expression of these *MtHDTs,* nodules were embedded and sections were made. This showed that their expression patterns are clearly different. *MtHDT1::GUS* expression is limited to the meristem and 2-3 cell layers of the Infection zone (figure 4A). The expression level in the few infection zone cell layers is equal to or even higher than in the meristem. *MtHDT2::GUS* is expressed in the meristem, Infection zone and nodule cortex (figure 4C). The expression level in the meristem and nodule cortex is equal and in the Infection zone it is slightly higher. In contrast to *MtHDT1*, *MtHDT2* is expressed in the entire Infection zone. Expression of

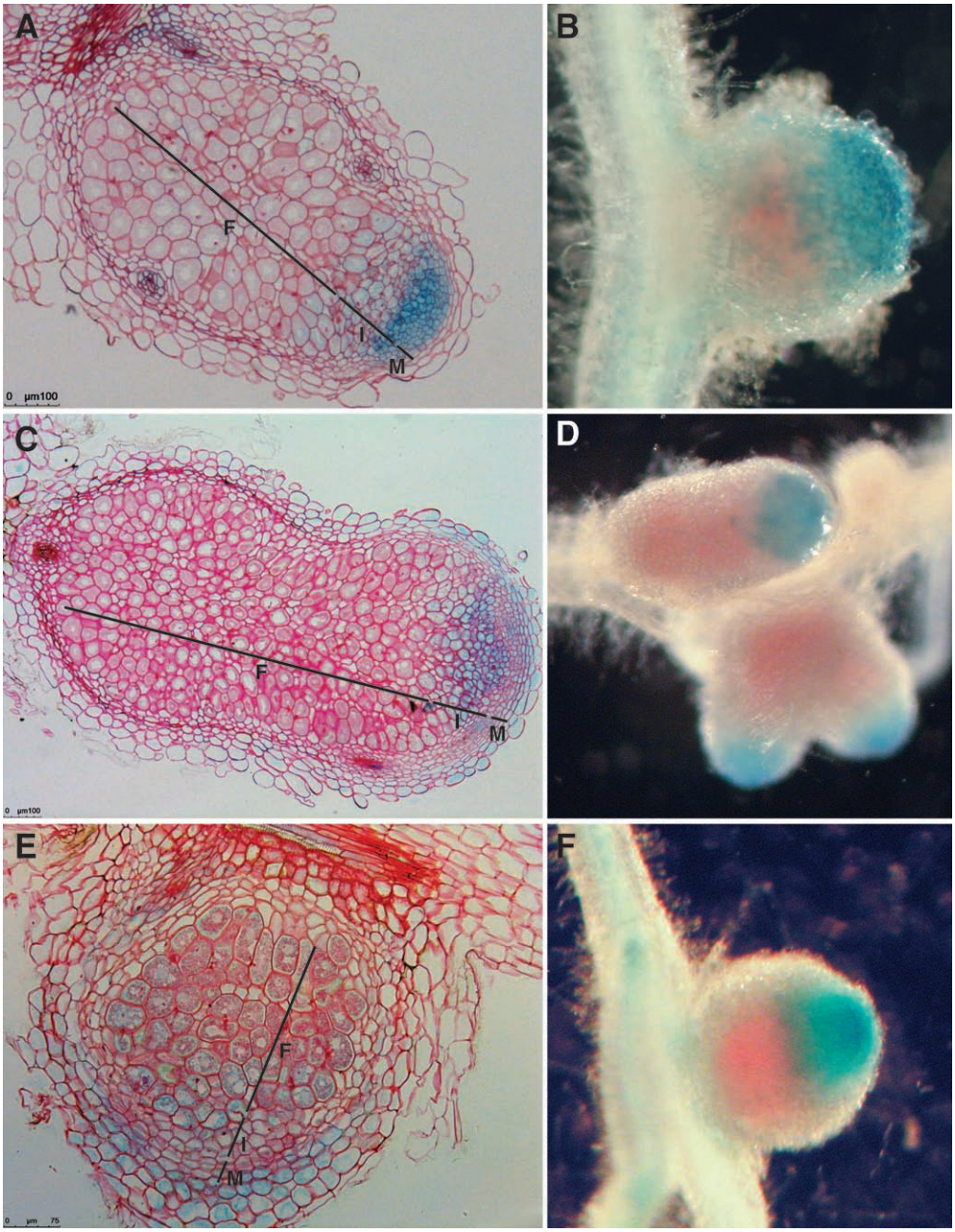

Figure 4: MtHDT expression in nodules. Longitudinal sections and whole transgenic nodules with GUS reporter gene expression of MtHDT1::GUS (A,B), MtHDT2::GUS (C,D), and MtHDT3::GUS (E,F). Meristem (M), Infection zone (I) and Fixation zone (F) are marked.

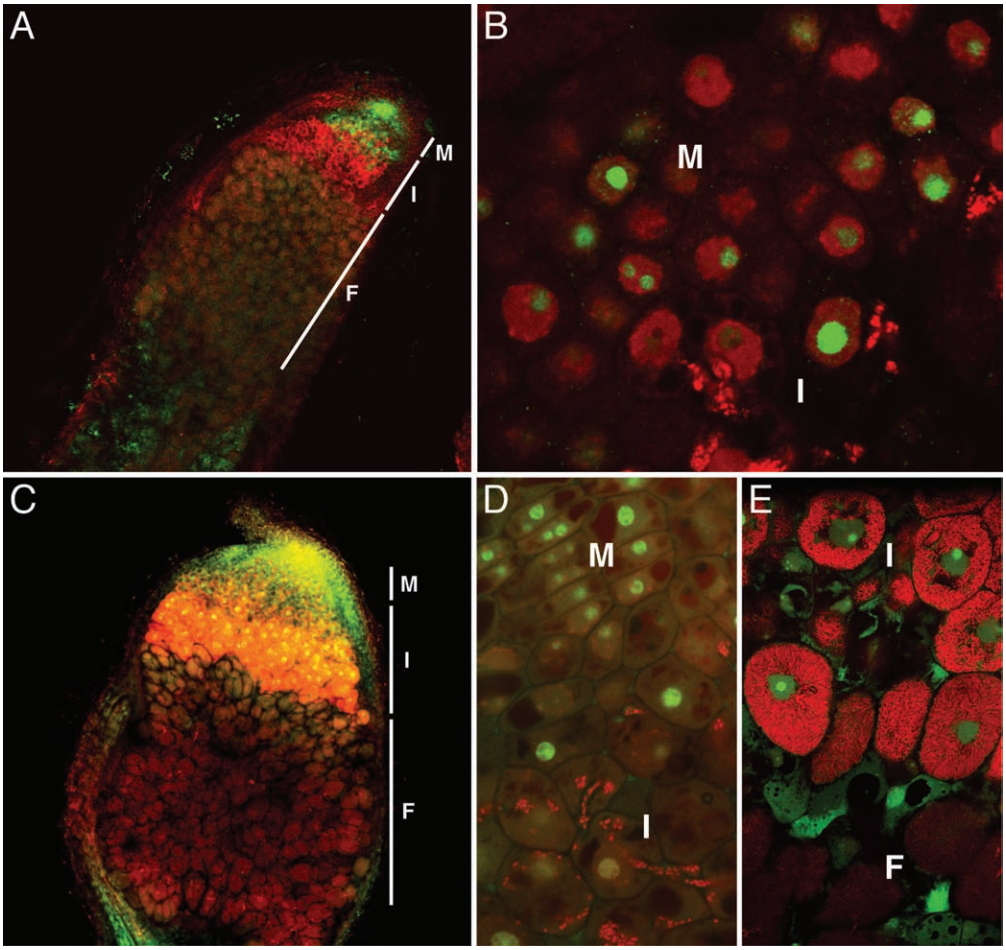

Figure 5: Confocal images of HDT-GFP localization in longitudinal sections of whole nodules. HDT1-GFP was detected using α-GFP immunocytology in the meristem and early Infection zone (A,B). HDT1-GFP locates to the nucleolus with weak nucleoplasm localization. HDT2-GFP was detected in the meristem and in the complete Infection zone (C-E). In the meristem and early Infection zone HDT2-GFP locates to the nucleolus (D), in the older Infection zone it localizes in both nucleoplasm and nucleolus (E). Auto fluorescence causes the green signal in between cells in E. Nodules were fixed, cut in half and stained with propidium iodide (PI) to visualize rhizobia and nuclei. At the transition between Infection zone and Fixation zone the intensity of the PI fluorescence of the differentiated rhizobia markedly drops within one cell layer (A,C). This rapid change in PI staining of the intracellular rhizobia is well visible in the magnification showing infected cells from Infection and Fixation zone (E). Note that during this "molecular switch" the levels of MtHDT2 in the nucleoplasm is relatively high. Meristem (M), Infection zone (I) and Fixation zone (F) are marked.

MtHDT3::GUS was detected globally at the distal half of the nodule, including the meristem, Infection zone, nodule cortex and a part of the Fixation zone, but overall expression was much lower compared to *MtHDT1* and *MtHDT2* (figure 4E).

Furthermore, the subcellular location was studied using translational fusion constructs of each *MtHDT* with GFP. N-terminal fusions of cDNA of each *MtHDT* with GFP under the control of the *MtHDT* promoter were constructed and composite plants with transgenic roots were created. The fluorescence intensity of the GFP-MtHDT1 was rather low and therefore GFP was detected by immunocytology using confocal microscopy (figure 5A and B). This showed that GFP-MtHDT1 is located in the nucleolus, in the meristem as well as in the youngest cell layers of the Infection zone. Further, it also occurs in the nucleoplasm, albeit at a markedly lower level (figure 5B). Expression of *MtHDT2::GFP-MtHDT2* could be seen by confocal microscopy and GFP-MtHDT2 was found in the meristem, in the complete Infection zone and in the nodule cortex at the distal part of the nodule (figure 5C-E). Like MtHDT1 it is located in the nucleolus, but also in the nucleoplasm. This is especially the case in the oldest cells of the Infection zone (figure 5E). GFP-MtHDT2 is present in a much larger region of the nodule, consistent with the *MtHDT2::GUS* expression pattern (figure 4C and D). We were not able to detect *GFP-MtHDT3* expression by neither confocal microscopy nor by immunocytology using GFP antibodies in Medicago roots or nodules. This might suggest that MtHDT3 protein levels like MtHDT1 are negatively regulated in nodules. This conclusion is supported by the fact that the expression of this GFP-MtHDT3 construct in tobacco leaves leads to clearly detectable GFP fluorescence in nucleoli (data not shown).

Discussion

In this study we showed that the MtHDTs are essential for proper nodule development. Knock-down of MtHDTs resulted in small nodules in which the proximal cell layers, with infected cells, developed as in wt, whereas the formation of the distal part is disturbed. This distal part encompasses the Infection zone. Release of bacteria as well as cell size increasement are disturbed. At first glance it is hard to understand this “hybrid” phenotype. However, by studying the fate map of the root cortical cells that form the nodule primordium we obtained insight in how such hybrid nodules could be formed.

We confirmed that the pericycle is the first cell layer that divides, despite that it is the inner most layer that responds to rhizobial signaling (Timmers et al., 1999). This is followed by mitotic reactivation of cortical cell layer 5 (C5)

and subsequently C4. The infection thread that is initiated in curled root hairs has reached the C5 and C4 derived cells before divisions are induced in C3. The C3 derived cells give rise to the meristem.

In nodules with a persistent apical meristem, infection threads penetrate cells that are released from the meristem, but never genuine meristematic cells. In this line it seems essential that the infection thread has passed C3 before divisions occur in this layer. This is consistent with previous studies that showed that infection threads get arrested in outer cortical cell layers when a meristem has formed before they have reached the C5 and C4 derived cells (Timmers et al., 1999). At the time that cell divisions are induced in C3 the infection threads enter C4/5 derived cells that than will stop dividing. This is consistent with the fact that also cells that leave the root nodule meristem and become infected will stop dividing and start endoreduplication. We assume that endoreduplication is also initiated in the C4/5 derived cells as their nuclei enlarge. The time between mitotic activation of C4 and C3, respectively, is only a couple of hours. This implies that induction of cell division and infection thread growth have to be strictly coordinated.

When C3 is mitotically activated C4/5 have already formed about 8 cell layers and this number of layers is similar at the time a meristem is formed (~65 h). So in mature nodules, about 8 cell layers of the central tissue developed directly from the primordium, whereas the additional cell layers are formed from the apical meristem. As nodules are relatively small organs a substantial part of the central tissue of the nodule developed directly from the primordium.

Also during the formation of other organs that grow via apical meristems, the meristem will only add cells to the organ when organ tissues with the right patterning have been formed in parallel with the meristem. For example, a rootlet is formed during embryogenesis and the root tissues are established in parallel with the apical meristem (Mansfield and Briarty, 1991; Scheres et al., 1994; Laux, 2004). Only when the seed germinates the meristem starts to add cells to the root tissues. The same holds for the formation of a lateral root. In the lateral root primordium a meristem and the root tissues are formed in parallel (Malamy and Benfey, 1997).

Knock-down of *MtHDTs* also results in a marked reduction in nodule number. This suggests that MtHDTs are important for early steps of nodule primordia formation. The few primordia that escape from MtHDT knock-down can form wt like central tissue cells, but the meristem that is formed adds cells that do not develop properly to the central tissue. We have not studied at which stage nodule primordium formation is arrested, but it might well be that nodule primordium formation requires a certain threshold level of MtHDTs. Each transgenic root is the result of an independent transformation

event. Therefore, each root has a different RNAi induced reduction in *MtHDT* expression. Above the "primordium" MtHDT threshold level a normal primordium is formed. However, when nodule meristem formation or functioning requires a higher MtHDT threshold level a hybrid nodule is formed with a wt like proximal part and a disturbed distal part. This explanation would imply that certain RNAi roots have wt number nodules, whereas others have none. This is not the case as RNAi roots make only few nodules (in general only 0-2 per root). A more likely explanation therefore is that MtHDTs are essential for several different processes and the processes they control during primordium formation are different from those involved in establishing nodule meristem formation or the differentiation from meristematic cells. In case MtHDTs would control the strictly controlled timing of infection thread growth and division in C3/4/5, it would reduce the number of successful infections but those that are successful would develop normal, up to the process that is blocked in nodule functioning.

The 3 *MtHDTs* are expressed at the apex of the nodule. Nevertheless they have clearly distinct expression patterns. All 3 *MtHDTs* are expressed in the meristem and a few cell layers of the adjacent Infection zone. The latter are the only cell layers in which rhizobia are released from infection threads. *MtHDT1* expression is restricted to this small zone, whereas *MtHDT2* is further expressed in the complete Infection zone as well as in the nodule cortex at the apex of the nodule. *MtHDT3* is expressed in the same regions as *MtHDT2* and further in the young part of the Fixation zone.

Despite their different expression pattern, MtHDT1 and MtHDT2 are probably in part functionally redundant as RNAi of both genes causes a nodule phenotype, whereas knock-down of one of these genes does not lead to a clear phenotype. Both are expressed in the meristem and the adjacent cells where rhizobia are released from the infection thread. Therefore we hypothesize that these MtHDTs have somehow a function in the release of rhizobia from the infection thread. This might be indirect, for example by controlling the proper differentiation from meristem cells into nodule cells that can be infected.

The mechanisms by which HDTs control development remains a mystery. Their nucleolar location has led to the hypothesis that they are involved in controlling rRNA biogenesis. However, the expression of MtHDT2/3 in fully differentiated nodule cortical cells and of MtHDT3 in young fully differentiated infected cells is not consistent with a simple function in rRNA biosynthesis.

Our studies show that the MtHDTs also occur in the nucleoplasm, and that the amount of HDT protein present in the nucleoplasm appears to be stage dependent. For example the level of nucleoplasmic MtHDT2 is highest in the oldest infected cells of the Infection zone. Therefore we hypothesize that

the HDTs can be stored in the nucleolus and their target genes are located in the nucleoplasm. In conclusion MtHDTs are essential for nodule development. The proper development of nodules relies at multiple stages on the correct expression of these HDTs, as reduced expression results in disturbed nodule formation or inhibits the formation of nodules completely.

Methods

Spot inoculation

Medicago truncatula, accession Jemalong A17, germinated seedlings were grow vertically on top of filter a paper placed on buffered Nod medium (BNM) with 1.2% agar and 0.1 µM AVG (Ehrhardt et al., 1992) under low nitrate conditions for 5-7 days. Roots were then inoculated with a small droplet of *Sinorhizobium meliloti* (Sm2011) bacterial suspension in water (OD_{600} = 0.1) at the susceptible zone of the root. At time points 24-72h after spot inoculation, root parts were harvested and fixed and embedded as described below.

Tissue embedding and sectioning

Roots parts and nodules were fixed for at least 1h under vacuum in 4% paraformaldehyde (v/v), 3% glutaraldehyde (v/v) and 3% sucrose (w/v) dissolved in phosphate buffer (pH7.0). The fixed material was dehydrated in an ethanol series and subsequently embedded in Technovit 7100 (Heraeus Kulzer) according to the manufacturer's protocol. Five µm thin longitudinal or transverse sections were cut using a microtome, stained with toluidine blue and analyzed on a Leica DM5500B microscope. For GUS stained nodules 7 µm longitudinal sections were cut and stained with ruthedium red.

RNAi construct

RNAi target sequences of about 400-500bp of each *MtHDTs* were amplified from the pENTR-D-TOPO vectors containing the mtHDT CDS (described below) using primer combinations HDT13-F+HDT12-R, HDT12-F+HDT23-R and HDT23-F+HDT12R. These fragments were combined in a subsequent PCR step using a mixture of the three PCR products as template and making use of the complementary 15bp overhang of the primers used in the first step. The MtHDT1 and MtHDT2 fragments were combined in this second PCR step using primers HDT13-F and HDT23-R and the MtHDT1, MtHDT2 and MtHDT3 fragments were combined using primers HDT13F and HDT13R. The following primers were used: HDT13-F CCACCCTATGAAGAAATGGGAGCGTCTTCT; HDT13-R AGAAGACGCTCCCATTTCTTCATAGGGTGG; HDT12-F CCACCCTATGAAGAA-ATCCCATTGATTACC; HDT12-R GGTAATCAATGGGAT-TTCTTCATAGGGTGG; HDT23-F AAAACTCCCAAGTCT-ATGGGAGCGTCTTCT; HDT23-R AGAAGACGCTCCCAT-AGACTTGGGAGTTTT.

The combined MtHDT fragments were introduced into a pENTR-D-TOPO vector (Invitrogen) and subsequently recombined in inverted repeat orientation in to the Gateway vector pK7GWIWG2(II) driven by the nodule specific MtEnod12 promoter and containing the Q10::dsRED1 selection marker (Limpens et al., 2005). These constructs were transformed into plants using *Agrobacterium rhizogenes*, strain msu440 (Sonti et al., 1995), mediated transformation as described by (Limpens et al., 2004). After transformation the plants, were grown on perlite in low nitrate conditions and inoculated with *Sinorhizobium meliloti* strain Sm2011 to induce nodulation according to (Limpens et al., 2004). At 14 days after inoculation

nodules from transgenic roots, recognizable by dsRED1 expression, were harvested, fixed and after embedding sections were cut as described above.

Quantitative expression analyses

Medicago A17, germinated seedlings were grown in perlite under low nitrate conditions as described in Limpens et al., 2004. After 7 days the plants were inoculated with 2ml of *Rhizobium Sm2011* bacterial suspension in water (OD600=0.1). Fourteen days after inoculation roots and nodules were collected. Total RNA was isolated using the plant RNA easy kit (Qiagen). cDNA was synthesized of 1ug 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 was used as a reference. Primers used for real-time PCR were: HDT1-F GGATGAGGGCAAGAACAAAA, HDT1-R TTGGTGGTAGCAGGCTTCTT, HDT2-F TCTGGGGTGCTGAGGTTAAG, HDT2-R TTTGTCCTTGGTCAGGGTTC, HDT3-F CTGAAGGGAGCAACAAGAGG, HDT3-R TGGCTGCTTGTTATTTGCAG, UBQ-F GGTTGATTGCTCTTCTCTCCCC and UBQ-R AAGTGATTGCTCGTCCAACCC.

Histochemical expression analysis

About a 1kb (2kb for MTHDT1) upstream region of the ATG start codon containing the putative promoter of the gene was amplified using primers; pHDT1-F CACCTCTTTTTGAATTAGTTTCTTATTTTGG; pHDT1-R TTGAAAGGGAGAGCGAAACG; pHDT2-F CACCGTTTTTACTTCCTTCTGCCAAAC; pHDT2-R TGGAAACAGTGAGACAGAGAGAG; pHDT3-F CACCGGTTTGGGTGATTTTGGATTG; pHDT3-R GACTGCAGAGAGAGAGCAAGC.

The PCR fragments were introduced into a pENTR-D-TOPO vector (Invitrogen) and subsequently recombined into the pKGWFS7-RR destination vector (Karimi et al., 2002), containing a GUS-GFP reporter and modified by introducing a Q10::dsRED1 expression cassette for easy selection of transgenic roots. The destination vector was introduced into Medicago A17 roots through *A. rhizogenes* mediated transformation and inoculated with *S. meliloti* Sm2011 as described above. Nodules on transgenic roots, selected based on dsRED1 expression, were harvested at 14 days post inoculation and GUS stained in 0.1 phosphate buffer (pH 7.0) containing 1mM X-Gluc (DMSO), 3% sucrose, 0.5mM EDTA, 0.1M K ferricyanide and 0.1M K ferrocyanide. Stained nodules were fixed and subsequently embedded as described above.

Localization studies

N-terminal fusions of HDTs 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), creating pENTR1-2~MtHDT1, 2 and 3. The following primers were used: MtHDT1-F CACCATGGATCGACCAATGGAGTT; MtHDT1-R ACGGCGATCTTGCTTAGACT; MtHDT2-F CACCATGGAGTTCTGGGGTGCTG; MtHDT2-R CTTAGCACCATGCTTGGCCT; MtHDT3-F CACCATGGAGTTTTGGGGTGTTGA; MtHDT3-F GGCATCTTCAGTCTTAAAAGGC.

The MtHDT promoters were re-cloned from the pENTR-D-TOPO vectors generated for the histochemical studies (described above) into a pENTR1-4 vector (Invitrogen) in front of a GFP open reading frame. The pENTR4-1 vector with the HDT promoter and GFP, the corresponding

pENTR1-2~MtHDT vector and a pENTR2-3 vector containing a CaMV35S terminator were recombined into the binary destination vector pKGW-RR-MGW in a Multisite Gateway reaction (Invitrogen), thereby creating pKGW-RR~MtHDT::GFP-MtHDT. pKGW-RR-MGW contains a dsRED1 marker for easy selection of transgenic roots (Limpens et al., 2004). These constructs were introduced in Medicago roots through *A. rhizogenes* mediated root transformation and inoculated with *S. meliloti* Sm2011 as described above. At 14 days after inoculation transgenic nodules were hand-sectioned using a double-sided razorblade and GFP immuno-detection was performed as described by (Limpens et al., 2009). Nodule sections were counterstained with propidium iodide.

The of MtHDT3::GFP-MtHDT3 construct was tested by injecting *Nicotiana tabacum* leaves with a suspension (OD_{600} 0.1) of Agrobacterium, containing the pKGW-RR_ MtHDT3::GFP-MtHDT3 vector, in MMAi medium (20g sucrose, 5g MS basal salts, 2g MES, 1ml 200mM acetosyringone in a total volume 1L MQ (pH5.6)). After two days GFP fluorescence was analyzed using a Zeiss 510 confocal laser scanning microscope.

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CHAPTER 5

LHP1 speckles represent (repressive) chromatin regions and these appear important for proper nodule meristem functioning in Medicago root nodules

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Introduction

Legume plants, like *Medicago truncatula* (Medicago), are able to form new organs, so-called root nodules, in which symbiotic rhizobium bacteria can fix atmospheric nitrogen. These nodules are formed by reprogramming of fully differentiated inner cortical cells, by which a nodule primordium is formed. The mitotic activation of these root cells is induced by specific lipo-chitooligosaccharides secreted by Rhizobium (Geurts and Bisseling, 2002). Simultaneously, rhizobia enter the root by tube-like structures made by the host, which are named infection threads. When the infection threads have reached the primordia, rhizobium bacteria are released into the plant cells that then stop dividing. Subsequently, cells of the middle cortical layer (there are 5 cortical layers in Medicago roots) divide and from these a non-infected meristem is formed. The meristem keeps on dividing during the life span of the nodule. By division it adds cells to the different nodule tissues and maintains itself. In this way a pool of stem cells is formed that controls nodule growth (Chapter 4, this thesis, Timmers et al., 1999). So, the Medicago nodulation process includes two interesting reprogramming events: First, inner root cortical cells that start to divide and form a root nodule primordium. When rhizobium bacteria are released in these primordium cells, they stop dividing and differentiate. Second, a nodule meristem is formed by subsequent division of cells in the middle of the cortex. These meristem cells differ from primordium cells as they; 1 can not be infected, 2 remain mitotically active by which the meristem is maintained and daughter cells are produced that differentiate in nodule tissue cells and of which a part can become infected. (Hirsch, 1992; Timmers et al., 1999; Complainville et al., 2003 , Chapter 4, this thesis).

To be able to develop an organ like lateral roots or nodules, cells in the plant need to change their fate and enter a different developmental program in which specific gene expression patterns are required. This occurs for example during reprogramming of pericycle cells when lateral roots are initiated (Chapter 2, this thesis; De Smet et al., 2008). Further, we showed that histone deacetylases are involved in cell fate switches during both initiation of nodule formation as well as the proper functioning of the Medicago nodule meristem (Chapter 3 and 4, this thesis).

LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) is another chromatin modifier, that is involved in several developmental processes. As chromatin remodeling often plays a role in reprogramming during developmental processes, we studied whether LHP1 plays a role in nodulation. LHP1 is the plant homologue of heterochromatin protein 1 (HP1). HP1 was first discovered in Drosophila as a component of constitutive heterochromatin and involved

in position effect variegation (James and Elgin, 1986). Ever since, several homologues have been identified in mammals, fission yeast and fruit flies and in all these species it is associated with heterochromatin. In metazoans and fission yeast multiple isoforms of HP1 are found, with each a specific function and distinct localization pattern. Many plants species contain only a single gene coding for LHP1 (Guan et al., 2011). The function of LHP1 is best studied in Arabidopsis.

Arabidopsis *lhp1* mutants, also known as *tfl2* (*terminal flower 2*), show pleiotropic effects, altered flowering time, abnormal leaf morphology and the overall plant architecture is affected (Larsson et al., 1998; Gaudin et al., 2001; Kotake et al., 2003; Takada and Goto, 2003). It controls for example the switch from vegetative growth to flowering by suppressing the expression of genes that control this fate switch (Kotake et al., 2003; Libault et al., 2005; Nakahigashi et al., 2005).

All (L)HP1 proteins contain two conserved structural domains: a chromo domain (CD) and a chromo-shadow domain (CSD) separated by a more variable “hinge” region. The CD can recognize and bind specific methylated lysine residues on histone tails. The CD of HP1 of fission yeast and metazoans specifically binds methylated H3K9 (Bannister et al., 2001; Lachner et al., 2001; Fischle et al., 2003). The CSD domain is thought to be involved in protein–protein interaction (Aasland and Stewart, 1995; Gaudin et al., 2001).

AtLHP1 was found to be associated *in vivo* with regions in the Arabidopsis genome containing H3K27Me3 modifications (Turck et al., 2007; Zhang et al., 2007; Exner et al., 2009). Therefore it has been postulated that LHP1 is functionally similar to Polycomb, which is a subunit of Polycomb Repressive Complex-1 (PRC1) (Sung et al., 2006). Furthermore, LHP1 binds to homologues of the animal PRC1 subunit RING1 (Chen et al., 2010).

Animal PRC1 proteins accumulate in characteristic nuclear foci, called Polycomb bodies. These are proposed to be concentrated areas of transcriptional repression, possibly containing multiple Polycomb group (PcG) complexes (Grimaud et al., 2006). Cytological studies have shown that LHP1 locate mainly in a diffuse manner in nuclei of the Arabidopsis root meristem. In differentiated cells it is present at a lower level, but there it forms speckles (nuclear foci) (Kotake et al., 2003; Libault et al., 2005; Nakahigashi et al., 2005). These speckles are exclusively formed in the euchromatin and LHP1 does not occur in the heterochromatic chromocenters. Whether these speckles represent repressive chromatin complexes or are artificial aggregates is unclear. In contrast, expression of Drosophila HP1 in Arabidopsis locates to heterochromatic chromocenters (Naumann et al., 2005).

In this study we identified and characterized the Medicago LHP1 (MtLHP1) homologue. We showed that in Arabidopsis as well as Medicago LHP1 forms speckles that co-localize with H3K27Me3. Further, by studying FRET between GFP tagged LHP1 and fluorescently labeled DNA it is shown that in speckles LHP1 complexes are bound to DNA (Lorvellec, 2007). Deletion of the CD domain, that is essential for binding to H3K27me3, blocks speckle formation. Collectively, these data suggest that speckles represent repressive chromatin complexes.

The MtLHP1 RNAi silencing studies indicate that MtLHP1 has no function in nodule primordium formation as nodule number is not reduced. However, the meristem that is formed in such transgenic nodules appears to be severely hampered in its function. Therefore we hypothesize that MtLHP1 is required for proper initiation and functioning of the nodule meristem.

Results and discussion

Identification and sequence analysis of Medicago LHP1

We identified one LHP1 homolog (MtLHP1) in Medicago, based on the Expressed Sequence Tag (EST) library (The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/)) search and screening of the Medicago genome BAC library (Mth-2). The recently published Medicago genome sequence confirmed that Medicago has a single LHP1 gene (Young et al., 2011).

Sequence comparison with LHP1 of other plants showed that the identified MtLHP1 protein has a similar domain organization as all other LHP1s (Guan et al., 2011). The MtLHP1 protein is 275 amino acids (AAs) long and consists of the typical N-terminal Chromo Domain (CD) and a C-terminal Chromo Shadow Domain (CSD) separated by a hinge region, as found in all other (L)HP1 proteins. MtLHP1 is shorter than other LHP1 proteins and this is due to a smaller hinge region and the lack of a pronounced stretch of acidic amino acid residues at the N- terminal end of the protein. This stretch of acidic glutamic (E) or aspartic acid (D) residues at the N-terminal end is called the acidic domain or E/D domain. In general this stretch is more pronounced in plants compared to HP1 from metazoans and fungi. This acidic domain is also found in LHP1s of close relatives of Medicago, like soybean and Lotus, but surprisingly in MtLHP1 it is absent. We confirmed the transcription initiation site of MtLHP1 by 5'RACE to rule out an incomplete mRNA sequence (data not shown).

Subnuclear localization of Medicago and Arabidopsis LHP1

In most organisms HP1 is associated with heterochromatin, although some

HP1 isoforms, like for example HP1γ in mammals or HP1c in Drosophila, are also present in euchromatin (Smothers and Henikoff, 2001; Dialynas et al., 2007). In Arabidopsis, however, AtLHP1 locates only in euchromatin and there it can be present in many speckles (Kotake et al., 2003; Libault et al., 2005; Nakahigashi et al., 2005).

We tested whether MtLHP1 also locates to such characteristic speckles by constructing a MtLHP1-GFP translational fusion driven by the CaMV 35S promoter (35S::MtLHP1-GFP). We used an *Agrobacterium rhizogenes* mediated root transformation method (Limpens et al., 2004) to obtain transgenic roots of Medicago expressing the MtLHP1-GFP fusion construct. As a control, we made a similar Arabidopsis AtLHP1-GFP translational fusion under the control of its own promoter (AtLHP1::AtLHP1-GFP) and introduced it by stable transformation into the *lhp1*(*tfl2)* Arabidopsis mutant background. This construct rescued the mutant phenotype indicating that it is biologically functional. The complemented mutant was used for further studies.

Transgenic roots from Arabidopsis and Medicago were fixed and stained with propidium iodide (PI). AtLHP1-GFP fluorescence in the differentiated part of the root was rather low. Therefore we used anti-GFP antibodies to detect AtLHP1-GFP. The euchromatic localization in a speckled pattern of AtLHP1 and its absence at the heterochromatic chromocenters (figure 1A-C) is in agreement with other studies in Arabidopsis (Libault et al., 2005; Nakahigashi et al., 2005). The chromatin organization in Medicago nuclei is similar to that in Arabidopsis, with heterochromatin consisting of (peri)centromeric sequences that in interphase nuclei are predominantly present in chromocenters (Kulikova et al., 2004). MtLHP1-GFP localizes in a similar speckled pattern in nuclei of differentiated root cells (figure 1D-F) and is absent from the intensely propidium iodine (PI) stained chromocenters (figure 1A-F, marked by arrows). To determine whether these Arabidopsis and Medicago LHP1 speckles represent chromatin complexes we first determined whether they co-localize with a specific histone modification.

Speckles are chromatin complexes

Chromatin immuno-precipitation-chip and DamID-chip studies of AtLHP1 showed that almost all LHP1 target genes co-localize with the histone mark H3K27Me3 (Turck et al., 2007; Zhang et al., 2007). In case the LHP1 speckles in Arabidopsis and Medicago represent chromatin complexes, we expect that they will co-localize with H3K27Me3. AtLHP1~GFP fluorescence in the *AtLHP::LHP1-GFP* expressing plants was very low. Therefore we made a new fusion construct by replacing its own promoter by the strong 35S promoter. We used the *A. rhizogenes* root transformation system to obtain Arabidopsis

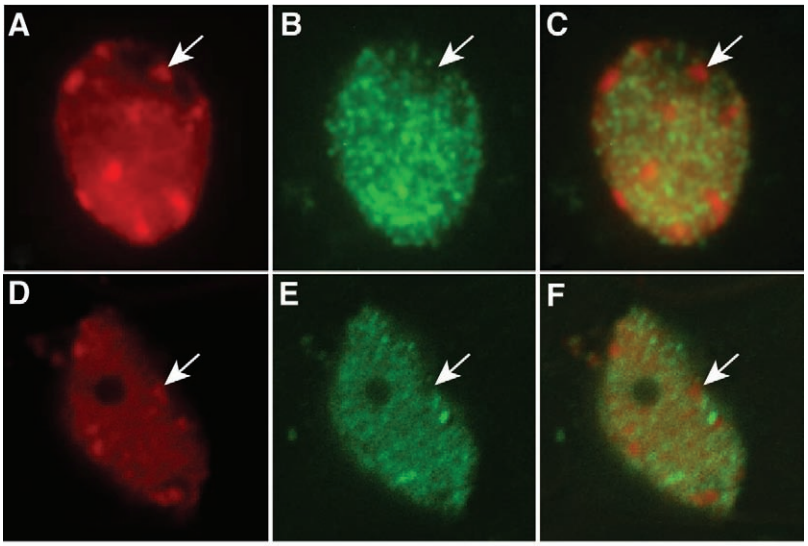

Figure 1: AtLHP1-GFP (A-C) and MtLHP1-GFP (D-F) localization in roots. Interphase nuclei stained with Propidium Iodide (PI) (A and D), detection of LHP1-GFP with anti-GFP antibodies (B and E) and the merged picture (C and F). Chromocenters are marked by arrows. In B and E the chromocenters are visible as dark holes and in the merged image the chromocenters have the red PI fluorescence showing that they do not co-localize with LHP1-GFP.

transgenic roots expressing 35S::AtLHP1-GFP (Limpens et al., 2004). Transgenic roots were fixed, thereby preserving GFP fluorescence, and the histone mark was detected using H3K27Me3 antibodies. In Arabidopsis (figure 2A-C) H3K27Me3 is present in a speckled pattern (red) and they co-localize very well with the LHP1 foci (green) as can be seen in the merged picture, where the combined signal appears in yellow. A similar experiment was done to test whether also the Medicago MtLHP1 speckles co-localize with H3K27Me3. In figure 2D-F it is shown that they also co-localize.

LHP1 and Polycomb proteins are both involved in epigenetic silencing and share the conserved chromodomain (CD) that can bind to histone modifications. *In vitro* experiments showed that the CD of AtLHP1 can bind H3K27Me3 with high affinity (Zhang et al., 2007). As speckles co-localize with H3K27Me3 it seems probable that the CD is essential for speckle formation. We made a construct in which we deleted the CD from AtLHP1 (35S::AtLHP1ΔCD-GFP) and transformed wild type Arabidopsis plants, using *A. rhizogenes* as described above, to generate transgenic roots. AtLHP1ΔCD-GFP did no longer form speckles, but has a diffuse distribution in the nucleus (figure 2G). This shows that the CD is essential for speckle formation and confirms the observations of others that AtLHP1 with a deleted or mutated

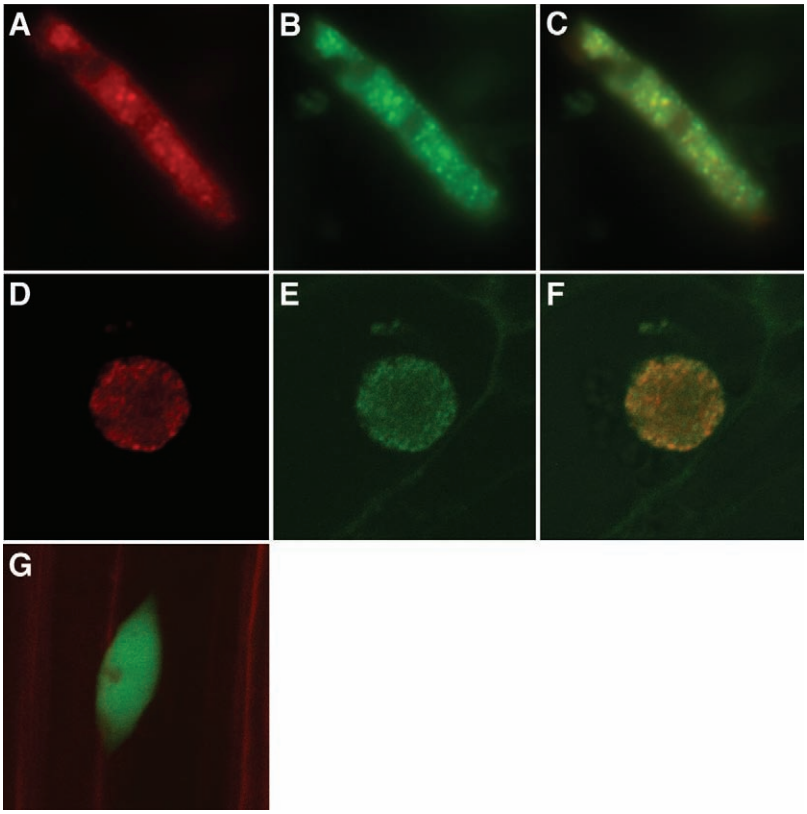

Figure 2: AtLHP1-GFP (A-C) and MtLHP1-GFP (D-F) co-localization with H3K37me3 and AtLHP1ΔCD-GFP localization in interphase nuclei in roots. H3K27Me3 immuno-detection (A and D), AtLHP1-GFP (B) and MtLHP1-GFP (E) immunodetection and the merged picture (C and F). Interphase nucleus with diffuse AtLHP1ΔCD-GFP localization (G).

CD no longer forms speckles (Libault et al., 2005; Exner et al., 2009). The co-localization of LHP1 with H3K27Me3 in speckles, together with the CD being essential in the formation of these speckles gives a strong indication that these foci are indeed chromatin complexes.

An alternative approach to show that the LHP1 speckles are chromatin complexes is to determine the distance between LHP1 and DNA. When LHP1 binds to H3K27Me3 it has to be very close to the DNA that is wrapped around the nucleosomes. We tested if this is indeed the case for LHP1. For this we made use of Foerster Resonance Energy Transfer (FRET), a method to determine whether two molecules are in close physical proximity (Foerster, 1948; Gadella et al., 1999). FRET occurs when energy of the exited donor fluorophore is transferred to an acceptor fluorophore, resulting in a decrease of fluorescence intensity and fluorescence lifetime of the donor. This only

occurs if the distance between donor and receptor is small enough (<10nm) and the reduction in lifetime can then be measured using for example Fluorescence Lifetime IMaging (FLIM) microscopy (Lakowicz et al 1992). To test whether LHP1 is closely associated with DNA it requires that DNA is stained with a fluorescent dye that can serve as acceptor for LHP1-GFP. In a study on DNA binding of HP1 variants in Hela cells it is shown that sytox orange is a good fluorophore to stain DNA (Cremazy et al., 2005). In our study we used LHP1-GFP as donor and Sytox Orange stained DNA as acceptor fluorophore. We could study in Arabidopsis whether FRET occurs between these fluorophores, whereas in Medicago such studies are difficult due to rather high autofluorescence.

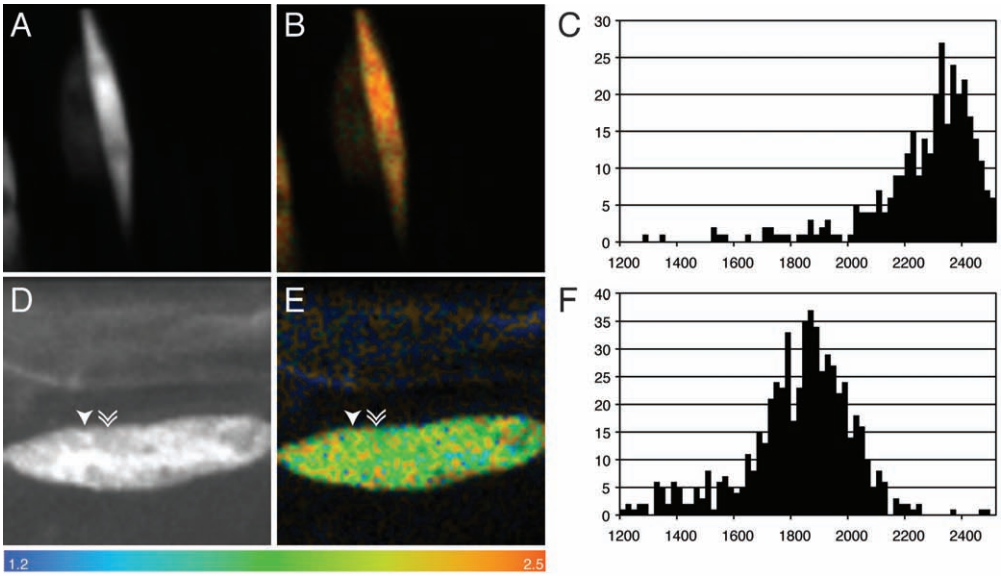

Figure 3: FRET-FLIM in differentiated root nuclei of 35S::GFP (A-C) and 35S::LHP1-GFP (D-F) in the presence of Sytox Orange. Fluorescence intensity (A and D) and fluorescence lifetime FLIM pictures (B and E). Colors represent the fluorescence lifetime of each voxel in ns. Distribution histogram (C and F) of fluorescence lifetimes of voxels of 1 nucleus. Arrow and double arrow indicate speckle and inter-speckle regions, respectively.

Arabidopsis roots were transformed with 35S::LHP1-GFP or 35S::GFP, which is used as a control to determine the fluorescence lifetime in the absence of FRET. Transgenic roots were fixed and stained with Sytox Orange. The fluorescence lifetime of GFP and LHP1-GFP was measured in root nuclei using two-photon excitation Time Correlated Single Photon Counting (TCSPC) instrumentation to obtain a detailed FLIM image with voxel specific lifetime values. This is done in the presence or absence of Sytox Orange

staining. We determined the fluorescence lifetime as it is independent of the local concentration of the fluorophore and so it is not hampered by intensity differences due to the presence of speckles.

The measured average lifetime of free GFP in the absence and presence of Sytox Orange were 2.32 ± 0.05ns and 2.32 ± 0,05ns, respectively (voxels of 10 nuclei). This shows that Sytox Orange DNA staining does not affect the lifetime of free GFP. The average lifetime of LHP1-GFP in the absence of Sytox Orange was 2.20 ± 0.06ns (voxels of 10 nuclei), so very similar to that of free GFP. When the DNA is stained with Sytox Orange the lifetime of LHP1-GFP is markedly reduced to 1.85 ± 0.17ns. The reduction in lifetime was in the same range as described for HP1-DNA FRET studies in Hela cells (Cremazy et al., 2005). Fluorescence lifetime FLIM images and distribution histograms of these lifetimes measured in voxels are shown in figure 3. The data are shown for 35S::GFP and 35S::LHP1-AtLHP1 in the presence of Sytox Orange. The histograms show a clear overall reduction of the life this is also seen in the pseudocolored nucei (figure 3D and E). These FLIM images also show that lifetime values were similar in speckles and the region in between speckles.

LHP1 in speckles is in close vicinity to DNA and this supports the conclusion that LHP1 speckles represent chromatin complexes. Since the life time of LHP1-GFP in between the speckles is reduced in a similar manner, LHP1 is also closely associated with DNA in this part of the euchromatin. To test whether this binding is independent of H3K27Me3 recognition we determined the lifetime of LHP1-GFP (when DNA is stained with Sytox) with a deleted CD. This lifetime is 1.89 ± 0.21ns (voxels of 10 nuclei). So while it is unable to recognize and bind the H3K27Me3 histone mark, it remains in close association with DNA. Thus, LHP1 is in close vicinity to DNA and in the speckles it co-localizes with the H3K27Me3 histone mark. The CD domain of LHP1 might be essential for recruiting LHP1 to this histone mark. It has previously been shown that LHP1 suppresses flowering time genes in an epigenetic manner (Sung et al., 2006). Therefore it seems probable that the LHP1 speckles represent chromatin clusters in which gene expression is repressed epigenetically.

Expression of MtLHP1 in root nodules.

Our major aim was to determine whether MtLHP1 plays a role during nodule development. Therefore we first determined the expression pattern of *MtLHP1* in Medicago root nodules. Available expression data from the Medicago Gene Expression Atlas indicated that *MTLHP1* is strongly expressed in young developing nodules as wells as in mature nodules (Benedito et al., 2008; He et al., 2009). To determine where in nodules *MtLHP1* is expressed we made

a β-glucuronidase (GUS) reporter construct under the control of the *MtLHP1* putative promoter consisting of the 1.3kb upstream region of the *MtLHP1* start codon (MtLHP1::GUS). We used the *A. rhizogenes* root transformation method to obtain transgenic roots of Medicago expressing MtLHP1::GUS and transformed roots were inoculated with Rhizobium to induce nodule formation. Twenty one day old nodules were harvested, stained for GUS activity and after embedding sections were made. GUS is expressed especially in the nodule apex (figure 4A). The expression region includes the nodule meristem (M) and the infection zone (I). In this last zone rhizobium bacteria are released from infection threads, they subsequently divide and differentiate into enlarged bacteroids. At the transition of infection zone into the fixation zone they fill the infected cells almost completely and start to fix nitrogen. In the first cell layers of the fixation zone the expression level of GUS rapidly decreases.

To determine the subcellular location of MtLHP1, we made a GFP translational fusion driven by the *MtLHP1* promoter. Nodules on *MtLHP1::MtLHP1-GFP* expressing roots were obtained as described above. The MtLHP1-GFP fluorescence was below detection; therefore it was detected by GFP antibodies. Like in roots, MtLHP1 localizes primarily in euchromatic speckles (figure 4B-D). Interestingly, LHP1 is present in pronounced speckles in the nodule meristem (figure 4C) as well as in the cells of the infection and fixation zone (figure 4D). This localization of LHP1 in speckles in the nodule meristem indicates that numerous regions with a repressing chromatin state are already present in the meristem. In Arabidopsis roots LHP1 is expressed at the highest level in the meristem, but there very few speckles are detected. These speckles are much more pronounced and increase in number in differentiated cells where the LHP1 concentration is markedly lower (Libault et al., 2005). The formation of these repressing chromatin complexes in the nodule meristem might be essential for proper gene regulation and nodule development.

Knock-down of MtLHP1 expression results in disturbed nodule formation

To study the role of MtLHP1 in nodulation, some preliminary RNAi studies were performed. To knock-down MtLHP1 expression we generated transgenic roots expressing an MtLHP1-RNAi construct driven by the 35S promoter. Only few transgenic roots were obtained and these were inoculated with Rhizobium and nodules were analyzed 21 days after inoculation. Reduced expression levels of MtLHP1 in transgenic roots did not inhibit nodule formation, but resulted in abnormal nodule development. Nodules from transgenic roots were fixed and after embedding longitudinal sections were made. 9 transgenic nodules were sectioned, 6 of these were much smaller than nodules on control roots (figure

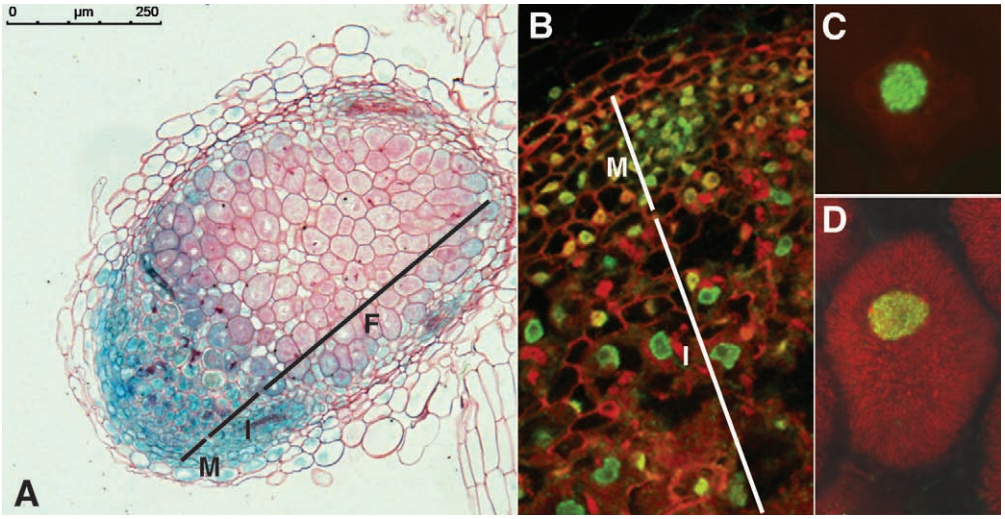

Figure 4: Expression and localization of MtLHP1 in nodules. Expression of MtLHP1::GUS in mature Medicago nodules (A). Immunodetection of MtLHP1-GFP (green) and PI (red) staining after hand sectioning of the nodule apex (B). Magnification of a nodule meristem cell (C) and an infected cell (D) of the fixation zone. PI counterstaining of cell walls (B and C) and rhizobium bacteria (D). Meristem (M), infection zone (I) and fixation zone (F) of nodules are marked.

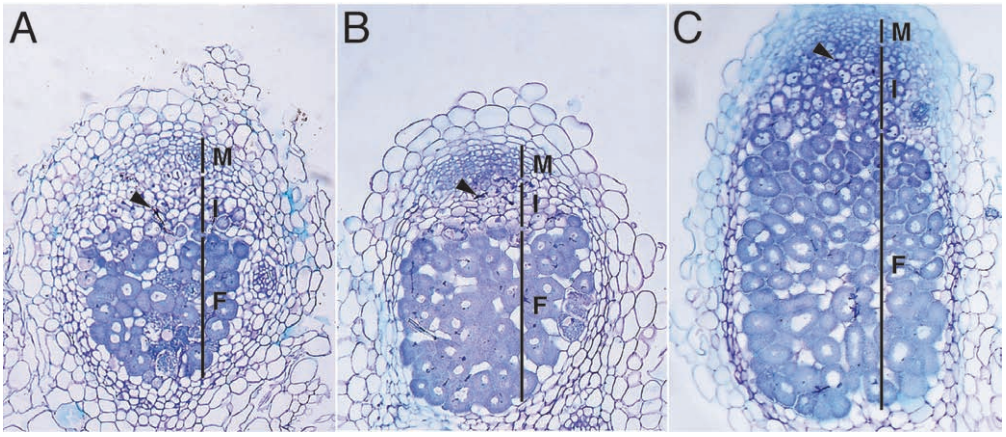

Figure 5: MtLHP1-RNAi knock-down. Longitudinal sections stained with toluidine blue of LHP1-RNAi knock-down (A and B) and non-transgenic (C) nodules, 21days after inoculation. The meristem (M), infection (I) and fixation zone (F) are indicated. Infection threads are marked with arrowheads.

5). The smaller knock-down nodules have about 8 layers of fully infected cells at the proximal part of the nodule. These cells originate from the primordium and are not derived from the nodule meristem. So, infection of nodule primordium cells is not affected. A relatively large meristem is present at the

apex of these RNAi nodules, but the cells derived from this meristem remain small and release of Rhizobium from infection threads appears to be blocked. It appears that MtLHP1 is involved in the initiation of the nodule meristem and proper differentiation and infection of cells originating from this meristem. The disturbed phenotype of the knock-down nodules is similar as MtHDTs RNAi nodules described in Chapter 4.

The pronounced speckles formed by MtLHP1 in the nodule meristem could indicate that in the nodule meristem specific transcriptional programs need to be repressed to allow the cells to change their fate. LHP1, possibly as a plant analog of PRC1, is known to be involved in several other developmental programs where it responsible for maintaining epigenetic repression at specific loci encoding floral homeotic genes and genes involved in regulation of flowering time (Kotake et al., 2003; Germann et al., 2006; Mylne et al., 2006; Sung et al., 2006). However, which genes are targets of LHP1 in the nodule developmental process remains to be elucidated.

Concluding remarks

We characterized the LHP1 homolog of Medicago and we found that MtLHP1, like AtLHP1, locates in a speckled pattern in euchromatin. These speckles represent, most likely, as part of the plant analog of PRC1, chromatin repressing complexes that regulate gene expression. Knock-down of MtLHP1 expression results in disturbed nodule development, Especially meristem formation is disturbed and cells derived from the nodule meristem appear not to enter the correct developmental program. However, further experiments with for example RNAi knock-down of MtLHP1 using a nodule specific promoter are needed to confirm our preliminary findings.

Methods

Gene identification

We identified the MtLHP1 cDNA sequence (TC178027) in the EST library (http://compbio.dfci.harvard.edu/tgi/) using homology search with AtLHP1 and other available lhp1 sequences. Primers designed for cloning of the CDS of MtLHP1 were used to amplify its genomic DNA sequence, which has then been sequenced. A fragment of the LHP1 genomic DNA of about 400bp was used as a probe for screening the Mth2 BAC library. Local sequencing of the identified positive BAC clones (31N4, 37K22, 42O23, 42O24, 78H8, 79E16 and 95J14) resulted in the corresponding putative promoter DNA sequence upstream of the start codon. The 5' end of the LHP1 mRNA was verified using the 5' RACE kit (Roche) using the nested primers GAGCATTAGTAGTTTGTGGTGGAAGG, CCTCCTGCTGCTGATGTTG and GCATGAATGAGATCGGGAACAGAC. MtLHP1 has been annotated in the recently published Medicago genome as Medtr3g163840 (Young et al., 2011).

Plant material

The *Medicago truncatula* Jemalong A17 accession and the *Arabidopsis thaliana* Columbia ecotype were used in this study. The *tfl2-1* (*lhp1*) knockout mutant in Columbia background was kindly provided by T. Kotake.

Constructs

The AtLHP1 CDS was amplified from cDNA using primers AtLHP1-SAL1-F (GTCGACCAGGAAATGAAAGGGGCAAGTGG) and AtLHP1-Xba1-R (TCTAGATAAGGCGTTCGATTGTAC) and this fragment was introduced into pGEM-T (Progema). EGFP from pEGFP-C1 (Clontech) was digested by Nhe1 ands Sac1 and cloned into the Xba1 and Sac1 sites of pGEM-T-AtLHP1 to obtain a C-terminal fusion of AtLHP1 with GFP. AtLHP1-GFP was introduced by digestion with Sal1 and Sac1 into a modified pBINPLUS binary vector (van Engelen et al., 1995) containing the CaMV 35S promoter and the NOS terminator creating pBIN35SLG. The AtLHP1 promoter was PCR amplified on genomic DNA with primers pAtLHP1-ClaI-F (ATCGATATGGGTGCAGCATGG) and pAtLHP1-SalI-R (CTGGTCGACAGTATTCGAGCCTCC). This promoter was introduced into pBIN35SLG by digestion with ClaI and SalI replacing the 35S promoter and creating pBINlLG. For stable transformation AtLHP1::AtLHP1-GFP was introduced into a MCS (Multi Cloning Site) of the modified pFlur101 vector (Stuitje et al., 2003) using ClaI and PacI sites creating pFlulLG.

AtLHP1ΔCD-GFP was constructed by introducing the AtLHP-GFP fragment into a pBSK (Stratagene) using XbaI and SalI sites. The CD deletion was made by the PCR based Quicksite mutagenisis kit (Stratagene) by creating a HindIII site at the end of the CD, using primers GCCTTTGAGGGAAGTTTGAAGC**TT**GGAAAGCCTGGTAGGAAACGG and CCGTTTCCTACCAGGCTTTCC**AA**GCTTCAAACTTCCCTCAAAGGC. Digestion with HindIII, a restriction site which also naturally occurres in pBSK, resulted in deletion of the CD. The AtLHP1ΔCD-GFP fragment was introduced into a MCS modified pFluar101 vector containing a 35S promoter using Age1 and Sal1 digestion sites.

The MtLHP1 CDS was PCR amplified from cDNA using primers (CACCATGAGAAAGACGAAGAAGAGCAGC and AGTAGGATTGTACCGGAGATGC) and introduced into the pENTR-D-TOPO vector (Invitrogen) creating pENTR-MtLHP1. MtLHP1 was recombined using LR clonase (Invitrogen) into the Gateway compatible binary vector 35S-pK7FWG2-R (Smit et al., 2005), containing the CaMV 35S promoter, GFP and a Q10::dsRED1 selection marker, to obtain the 35S::MtLHP1-GFP construct.

The 1.3kb region upstream of the start codon of MtHP1 (containing the putative promoter) was PCR amplified from one of the positive BAC clones using primers pMtLHP1-NotI-F CACGCGGCCGCTTAAATTTTATTTACCAAAAGCTTCG and pMtLHP1-AscI-R CTCGGCGCGCCTTCTTGCTTCCTCCCAACAC. The PCR fragment was cloned into a pENTR1-2 vector (Invitrogen) using NotI and AscI restriction sites, creating pENTR-pMtLHP1, and subsequently recombined using LR clonase (Invitrogen) into the pKGWFS7-RR destination vector, which contains a GUS-GFP reporter and a Q10::dsRED1 expression cassette for easy selection of transgenic roots (Karimi et al., 2002).

The C-terminal fusion of MtLHP1 with GFP under the control of its own promoter (MtLHP1::MtLHP1-GFP) was constructed using Multisite Gateway (Invitrogen). The MtLHP1 promoter from the pENTR-pMtLHP1 vector (described above) was cloned into a pENTR4-1 vector (invitrogen) using NotI and AscI restriction sites. This pENTR4-1 vector with the MtLHP1 promoter, the pENTR-MtLHP1 vector and a pENTR2-3 vector containing a GFP open reading

frame and a CaMV35S terminator were recombined into the binary destination vector pKGW-RR-MGW (Invitrogen). The pKGW-RR-MGW also contains a Q10::dsRED1 marker (Limpens et al., 2004).

For the RNAi knockdown of MtLHP1 expression a fragment of about 400bp of MtLHP1 was PCR amplified from cDNA using primers MtLHP1-RNAi-F CACCGCAGCTTGATGATGGTTTCTTTGA and MtLHP1-RNAi-F GATTTTCTGCGGTTGGTTCCTCT. This PCR fragment was introduced into a pENTR-D-TOPO vector (Invitrogen) and subsequently recombined in inverted repeat orientation into the Gateway vector pK7GWIWG2(II) driven by the CaMV 35S promoter and containing the Q10::dsRED1 selection marker (Limpens et al., 2005).

Plant transformation and nodulation

Arabidopsis transgenic roots expressing 35S::AtLHP1-GFP (pBIN35SLG) or AtLHP1ΔCD-GFP and Medicago transgenic roots expressing 35S::MtLHP1-GFP, MtLHP1::GUS-GFP, MtLHP1::MtLHP1-GFP or 35S::MtLHP1-RNAi were generated using *Agrobacterium rhizogenes* strain MSU440 (Sonti et al., 1995) mediated root transformation as described by (Limpens et al., 2004). Nodulation was done using 1 ml *Sinorhizobium meliloti* strain Sm2011 suspension (OD_{600} = 0.1) per plant, as described by (Limpens et al., 2004). Stable transformation of the Arabidopsis *tfl2-1* mutant with AtLHP1::AtLHP1-GFP (pFlulLG) was performed using *Agrobacterium tumefaciens* strain C58 as described by (Bechtold et al., 1993).

Immuno-localization of H3K27met3 on interface nuclei of Medicago

Nuclei from the 35S::MtLHP1~GFP transgenic Medicago roots were isolated and immuno-labeled as described (Talbert et al., 2002; Jasencakova et al., 2003). Roots were fixed in 4% paraformaldehyde in PBS with 0.2% Triton for 1h at room temperature, then washed 2x 10 min with PBS and digested for 40 min at 37°C with a mixture of 2.5% pectinase (Sigma) and 2.5% cellulase Onozuka RS (Yakult Honsha Co., Tokyo, Japan) dissolved in PBS. Roots were washed in PBS, placed on slide in a drop of PBS and squashed, then slides were immersed in liquid nitrogen and the cover slips were removed. Slides were first incubated in a moist chamber at room temperature with blocking solution (3% BSA) for 1h and then with rabbit anti-H3K27met3 polyclonal antibody (1:200, Invitrogen) overnight at 4°C. The antibody was detected by applying Cy3–conjugated goat anti-rabbit secondary antibody (1:200, Invitrogen) and incubated for 1-2 h at root temperature, followed by two washes in PBS. The slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and analyzed with a Zeiss LSM 510 confocal laser scanning microscope.

Whole mount immuno-localization

Immuno-labeling procedure was performed as described by (Friml et al., 2003) with some modifications. To co-localize LHP1~GFP and the histone mark H3K27me3 the 4 days old 35S::LHP1~GFP transgenic Arabidopsis roots were fixed in 4% paraformaldehyde in PBS for 1h00 at + 4°C thereby preserving GFP fluorescence. After the driselase treatment, roots were kept in small baskets with filters. They were incubated overnight at room temperature in a moist chamber with primary anti-H3K27met3 antibodies: After washing roots were incubated with secondary antibodies, a Cy3 conjugated goat anti-rabbit antibody (1:200, Invitrogen) overnight at room temperature. Finally the seedlings were mounted on microscopic slides in Citifluor, and analyzed with a Zeiss LSM 510 confocal laser scanning microscope. .

To study the nuclear localization pattern of MtLHP1, transgenic roots of Medicago expressing 35S::MtLHP1-GFP were fixed in 1% paraformaldehyde for 1h, then washed in PBS and stained with propidium iodide (PI). In roots from the complemented *lhp* mutant expressing the pFlulLG vector, AtLHP1-GFP was detected using immunodetection with rabbit anti-GFP polyclonal antibodies (1:200, Invitrogen) and visualized by using the secondary goat anti-rabbit~Alexa488 antibodies. Roots were stained with propidium iodide (PI 1 mg/ml). GFP and PI fluorescence were analyzed with a Zeiss LSM 510 confocal laser scanning microscope.

FRET-FLIM

Two-photon microscopy was performed on a Biorad 1600 using 60x/1.2 water immersion objective. Fixation procedures and imaging settings were identical to Cremazy et al 2005. Two-photon excitation was used instead of single photon excitation (870nm). FLIM images were obtained using a 75Mhz modulated two-photon laser after which Time Correlated Single Photon Counting (TCSPC) was used to determine the fluorescence lifetime.

Histochemical analyses and nodule sectioning

Nodules on transgenic roots expressing MtLHP1::GUS were generated as described above. 21days after inoculation, nodules were harvested and GUS stained up to 4h in 0.1 phosphate buffer (pH 7.0) containing 1mM x-gluc (DMSO), 3% sucrose, 0.5mM EDTA 0.1M K ferricyanide and 0.1M K ferrocyanide. Stained nodules were fixed in 4% paraformaldehyde, 3% glutaraldehyde, and 3% sucrose in phosphate buffer (pH7.0) by vacuum infiltration for at least 1h and subsequently dehydrated using an ethanol series. Dehydrated nodules were embedded in Technovit 7100 (Heraeus Kulzer) according to the manufacturer's protocol. 7 µm sections were cut using a microtome, stained with ruthedium red and analyzed with a Leica DM5500B microscope.

The MtLHP1 RNAi construct was transformed into Medicago roots and inoculated as described above. 21 days after inoculation nodules on transgenic roots and non-transgenic roots were fixed and embedded in technovit 7100 (Heraeus Kulzer) as described above. 5 µm longitudinal sections were cut, stained with toluidine blue and analyzed with a Leica DM5500B microscope.

MtLHP1-GFP localization in nodules

Nodules on transgenic roots, expressing MtLHP1::MtLHP1-GFP, were generated as described above. 21 days after inoculation, nodules on transgenic roots were hand-sectioned using a double sided razorblade and GFP immunodetection was performed as described by (Limpens et al., 2009). Nodule sections were counterstained using propidium iodide and analyzed with a Zeiss LSM 510 confocal laser scanning microscope.

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CHAPTER 6

General Discussion

The role of chromatin remodeling in plant development has mainly been studied in Arabidopsis. The knowledge generated by these studies can now be translated to other systems in which other biological questions can be addressed. In this thesis I especially have studied Rhizobium induced root nodule organogenesis in Medicago. The formation of this organ from fully differentiated root cortical cells involves two cell fate shifts and the role of some chromatin modifiers during these shifts was studied. Two well-studied chromatin modifiers in Arabidopsis are the HDT gene family, which encodes plant specific histon deacetylases and LHP1, the plant homolog of Heterochromatin protein 1 (HP1) (Gaudin et al., 2001; Pandey et al., 2002). I first analyzed the role of the Arabidopsis HDT family in root development. The expression of all 4 *AtHDT* genes was shown to be induced in the lateral root founder cells shortly before they divide. Furthermore, the highly homologous AtHDT1 and 2 are essential for maintenance of the root stem cell niche and meristem. The Medicago HDT homologs were identified and it is shown that during the fate shifts that occur during Medicago root nodule organogenesis the MtHDTs play a crucial role. Moreover, it is probable that Medicago LHP1 plays a similar role.

Legume nodule primordium formation and Nod factor signaling

Legume root nodule organogenesis starts with the mitotic activation of pericycle cells as well as root cortical cells. In this way fully differentiated cells are reprogrammed and a nodule primordium is formed (Geurts and Bisseling, 2002). Concomitantly with the formation of a primordium, rhizobia invade the root via cell wall bound infection structures, called infection threads (Brewin, 2004; Gage, 2004; Jones et al., 2007). In general, such infection threads start in curls that are formed by root hairs and subsequently they grow towards nodule primordia. The cortical layers that have to be traversed before reaching the primordium, have entered the cell cycle, but are arrested in G2. In this way cytoplasmic structures, named pre-infection threads, are formed that form the tracks by which the infection threads can reach the primordia (Yang et al., 1994). There, rhizobia are released by an endocytosis-like process (Ivanov et al., 2010). Infection and nodule primordium formation are both induced by specific lipochito-oligosaccharides made by rhizobia and are called Nod factors (Geurts and Bisseling, 2002). Depending on the rhizobial species, specific decorations can be present and the activation of root cortical cells as well as the infection process have tight demands towards Nod factor structure (Geurts and Bisseling, 2002).

Nod factors are perceived by LysM-domain containing receptor

kinases such as NFP and LYK3 in Medicago (Limpens et al., 2003; Arrighi et al., 2006). These receptors trigger ion fluxes leading to a depolarization of the plasma membrane, calcium influx at the plasma membrane and Ca^{2+} oscillations (Ca^{2+} spiking) in and around the nucleus (Oldroyd and Downie, 2006; Capoen et al., 2009). The Ca^{2+} spiking response also depends on a plasma membrane LRR receptor kinase MtDMI2 (SymRK) (Ané et al., 2002; Endre et al., 2002; Stracke et al., 2002) and a putative cation channel MtDMI1 located at the nuclear envelope (Ané et al., 2002; Charpentier et al., 2008). The resulting Ca^{2+} spiking pattern is recognized by a nuclear localized Ca^{2+}/calmodulin-dependent kinase MtDMI3/CCaMK (Lévy et al., 2004; Mitra et al., 2004). CCaMK activation is essential for root hair curling and infection thread formation as well as cortical cell divisions. The induction of these processes involves the induction of expression of specific genes and this requires among others the transcription factors NSP1, NSP2, and NIN (Schauser et al., 1999; Kaló et al., 2005; Smit et al., 2005). The induction of cortical cell division further requires the activation of a cytokinin-receptor, called CRE1 in Medicago (Gonzalez-Rizzo et al., 2006) and LHK1 in Lotus (Tirichine et al., 2007). Further, cytokinin is generated in the root upon Nod factor perception (op den Camp, 2012) and thus it might be a mobile signal that induces the formation of nodule primordia.

Infection of nodule cells by Rhizobium

Upon release of rhizobia in nodule primordium cells, the primordium develops in a nitrogen-fixing root nodule. In Medicago indeterminate nodules are formed with a meristem at their apex. Nodules are composed of several tissues of which the central tissue contains the cells that are infected by rhizobia. The meristem adds cells to, among others, the central tissue and these cells become penetrated by an infection thread from which rhizobia are released. During this process the bacteria become surrounded by a membrane of the host and this membrane compartment containing a Rhizobium is called symbiosome (Ivanov et al., 2010).

Several studies indicate that the Nod factor signaling pathway is also active in the nodule and is essential for symbiosome formation. All Nod factor signaling genes are expressed in the region of the central tissue that is adjacent to the meristem. This region is called infection zone and here symbiosome formation and division occurs (Bersoult et al., 2005; Capoen et al., 2005; Limpens et al., 2005; Mbengue et al., 2010). SymRK/DMI2, CCaMK/DMI3 and a protein (IPD3) interacting with DMI3 are essential for release of rhizobia from infection threads. When the expression of these genes is affected

(specifically) in nodules, nodules are still formed and these contain numerous infection threads. However, the bacteria are not released from these cell wall bound infection threads, pointing to an important role for Nod factor signaling in symbiosome formation.

Fate map of root nodules

The nodule phenotype that we obtained with the RNAi experiments of the Medicago *HDT* genes prompted us to study the fate map of nodules in more detail. Mature Medicago nodules have an apical meristem that remains mitotically active. In this way it maintains itself and adds new cells to the central tissue. This contains the infected cells that become fully packed with symbiosomes. Thus a gradient of developmental stages is present in a nodule with the youngest cells adjacent to the meristem (distal) and the oldest in the proximal part of the nodule. In general it is assumed that all infected cells in a nodule originate from the apical meristem. An example is the RNAi study on the nodule specific transcription factor HAP2 (Combier et al., 2006). Ten days after inoculation, HAP2 RNAi nodules lack an apical meristem and the nodule endodermis fully surrounds the nodule even at the apex. However, several layers of fully infected cells are present at the proximal part of such nodules. Therefore it is concluded that the nodule meristem was formed, but has rapidly disappeared during development.

MtHDT RNAi nodules also have a proximal part with wild type like infected cells, whereas the apical part is markedly disturbed (chapter 4). To determine whether these proximal cell layers originate from the apical meristem, we studied the fate of nodule primordium cells (chapter 4). About one day after inoculation the first divisions are induced in the pericycle and this is followed by divisions in the root cortex. This is consistent with previous studies of Timmers et al. (Timmers et al., 1999). In the root cortex the divisions start in the most inner layer (#5) and this is rapidly followed by divisions in cortical layer 4. Simultaneously, infection threads are formed by which the rhizobia reach the dividing cells of cortical layers 4 and 5 and they are released form these infection threads. At about the same time mitotic activity of the cells derived from the 4th and 5th cortical cell layer stops and anticlinal divisions are now induced in layer 3. This layer keeps on dividing, does not become infected and forms the apical nodule meristem. Thus the meristem is derived from a specific root cortical cell layer and is formed at a later stage of development. Therefore I propose to name the group of cells derived from the 3rd cortical cell layer nodule meristem, from the moment division starts in this cortical cell layer. Neither the cells from the 4th and 5th cortical cell layers, nor pericycle

and endodermis cells contribute to the nodule meristem. Therefore I propose to name the cells derived from these cell layers nodule primordium up to the moment the meristem starts to add cells to the nodule tissue.

The cells derived from the 4th and 5th cortical layer form about 8 cell layers before they stop dividing and several of these cells develop into fully infected cells. Therefore in a mature nodule about 8 cell layers of the central tissue are not derived from the meristem, but originate from the primordium. For the published HAP2 RNAi nodule phenotype it implies that the wt-like infected cells at the proximal part of the nodule are most likely derived from the primordium cells and a nodule meristem has most probably not even been formed. This results in a small nodule that stops growing due to the absence of a meristem. This phenotype strongly suggests that mechanisms controlling the formation of primordium and meristem, respectively, are different.

Cell fate switches during root nodule formation

During nodule formation 2 processes involve a cell fate switch. This is on one hand the formation of a nodule primordium by mitotic activation of root cortical cells and the second process is the formation of a meristem.

The primordium is formed from fully differentiated cells that most likely lose their cortex identity when cell division is induced. They probably remain in an undifferentiated state up to the moment they become penetrated by an infection thread. When rhizobia are released from these infection threads the primordium cells differentiate into infected cells that are fully packed with rhizobia. This differentiation process is accompanied by endoreduplication (and block of mitosis) and the induction of several genes. Nod factor signaling is essential for the release of rhizobia from infection threads as for example is shown by knock-down studies of SymRK in Sesbania as well as in Medicago (Capoen et al., 2005; Limpens et al., 2005). In Medicago MtSymRK RNAi nodules, infected cells are completely absent, although many cells of the central tissue contain an infection thread. This implies that cells of the primordium become penetrated by an infection thread, but release of rhizobia does not occur.

The nodule meristem is formed from the 3rd cortical cell layer by the mitotic activation of these cells. These cells differ from the primordium cells as they remain mitotically active and cannot be infected by Rhizobium. By division they on one hand maintain a pool of these meristematic cells and on the other hand add cells to the different nodule tissues. In this way the nodule meristem cells have stem cell properties. The majority of cells added to the central tissue become penetrated by an infection thread, symbiosomes

are released and these cells differentiate into infected cells. Also here upon penetration of the infection thread and release of rhizobia, division is stopped and cells start endoreduplication. Like in primordium cells, formation of symbiosomes in meristem-derived cells also involves Nod factor signaling. The demand for Nod factor signaling in this process seems more stringent than during release in primordium cells. For example, in pea mutant *sym41* the majority of the SymRK pre-mRNA is spliced in the wrong manner, which results in only about 10% properly spliced *SymRK* mRNA. This 10% level of wt SymRK mRNA is sufficient to induce efficient release of symbiosomes in the nodule primordium, but leads to a block of symbiosome formation in cells that are added by the meristem to the central tissue (Ovchinnikova, 2012).

Genes involved in nodulation have been identified by forward genetic screens and by selecting genes that are markedly up-regulated during nodule formation. These approaches have not identified chromatin modifiers as key regulators of steps in this process. This is probably due to the absence of nodule specific variants and the involvement of chromatin modifiers in various processes. I selected 2 different chromatin modifiers that were known to be expressed in root nodules (Benedito et al., 2008; He et al., 2009) and by a reverse genetics approach I studied their function in nodulation.

Nodule formation by itself is an ideal system to study reprogramming. Unlike (lateral) roots and most other plant organs, root nodules are non-essential organs and are only formed in nitrogen limiting growth conditions. Furthermore, several genes involved in nodulation have nodule specific expression patterns. Since they are expressed at specific stages of development, their promoters provide a tool to study, by reverse genetics, chromatin modifiers in specific tissues and developmental stages of the nodule. Another advantage of nodule formation is that it requires signaling by Rhizobium or exogenously applied Nod factor. This makes it possible to induce reprogramming of cells in a very controlled manner. In contrast, lateral roots develop from so-called lateral root founder cells in the pericycle cell layer and these cells are thought to be already primed in the meristem (Malamy and Benfey, 1997; De Smet et al., 2007). Therefore it is less clear when pericycle cells are reprogrammed. Also the main function of the pericycle is the formation of lateral roots. Therefore, in comparison to the cortical cells it is less clear whether they are even reprogrammed.

Thus, the availability of methods to induce reprogramming of cortical cells in a very controlled manner, the possibility to affect gene expression specifically during nodule development and nodulation being not essential for plants, makes nodulation an ideal system to study reprogramming of cells.

Plant specific histone deacetylases

The induction of expression of *AtHDTs* at the start of lateral root formation in Arabidopsis stimulated us to study whether these histone deacetylases could play a role in reprogramming events during nodule formation in Medicago. In this thesis I identified 3 plant specific histone deacetylases (HDTs) in Medicago (chapter 3). RNAi knock-down of the expression of all three *MtHDTs* resulted in a strong reduction (80%) of nodule formation. However, at which early stage nodule formation is precisely blocked remains to be studied.

Knock-down of *HDT* expression in Medicago did not only affect the initiation of nodule formation, but nodule development itself as well. Due to our plant transformation system, each transgenic root is the result of an independent transformation event and RNAi knock-down levels vary as such. This can explain why nodules are still formed on some transgenic roots where knock-down levels were insufficient to block nodule formation at an early stage. Most of these nodules that were still formed were small. The proximal part of these nodules developed normally. These cells originate from the nodule primordium and consist of about 8 cell layers filled with symbiosomes. However, at the nodule apex, cells originating from the meristem do not differentiate and are not infected by rhizobia. This indicates that although expression levels are sufficient to start nodule initiation, local expression during the formation of the meristem or during differentiation of meristem-derived cells is not. What functions these HDTs could have in reprogramming events will be discussed below.

HDT redundancy

Phylogenetic analysis showed that MtHDT1 and 2 as well as AtHDT1 and 2 are very homologous and are the result of an independent duplication (chapter 3, Pandey et al., 2002). In both species HDT1 and 2 are at least in part functionally redundant. MtHDT1 and 2 for example, proved to be most important for nodule initiation and in part functionally redundant because RNAi induced reduction of expression of both *MtHDT1* and *MtHDT2* gives a marked reduction of nodule number, whereas knockdown of only MtHDT1 or 2 has no effect. In Arabidopsis we were unable to generate a double *hdt1hdt2* mutant, as it appeared to be lethal. RNAi knock-down of *AtHDT1* together with *AtHDT2* in Arabidopsis roots resulted in a severe disturbance of root growth and the root meristem is not maintained, whereas in the *hdt2* single mutant only a small reduction in root length was observed. It was not well possible to determine whether lateral root formation depends on AtHDT1 and 2 in these knock-down plants, because these plants completely lose the root apical

meristem after two weeks and loss of the root apical meristem stimulates the formation of lateral and adventitious roots. Further the promoter that is used to express the RNAi construct is first active when the lateral roots emerge and not in the lateral root founder cells (Heidstra pers comm.)

HDT function

All plant specific histone deacetylases characterized so far localize predominantly to the nucleolus (Lusser et al., 1997; Zhou et al., 2004; chapter 2). In the nucleolus primarily transcription and processing of ribosomal RNAs and assembly of ribosome subunits takes place, but in recent years more and more additional functions have been postulated like for example cell cycle progression, mitotic regulation, and stress responses (Pendle et al., 2005; Boisvert et al., 2007). Can the HDT mutant phenotypes be explained if their only function would be the regulation of rDNA genes? For AtHDT1 it is known that it is involved in regulation of rRNA expression in nucleolar dominance that occurs in genetic hybrids. There, rRNA genes inherited from one parent are transcribed, but the other parental rRNA genes are silenced (Lawrence et al., 2004). In this process AtHDT1 is required for deacetylation of histone 3 lysine 9 and subsequently methylation of histone 3 lysine 9 occurs, thereby inducing transcriptional silencing. For AtHDT1, AtHDT2 and AtHDT3 it has been shown that they can induce transcriptional silencing when targeted to a promoter of a reporter gene (Wu et al., 2000; Wu et al., 2003).

Besides the plant specific histone deacetylases, the RDP3/HDA1 type AtHDA6 also locates to the nucleolus (Earley et al., 2006; Wu et al., 2008). AtHDA6 is required for rRNA silencing and mutation of AtHDA6 results in decondensation of chromatin containing rDNA repeats (Probst et al., 2004). AtHDA6 is involved in removing of histone acetylation in the process of nucleolar dominance too (Earley et al., 2006). Thus, there are indications that histone deacetylation play a role in regulation of transcription of rDNA genes. It is known that ribososome production is strongly increased in proliferating and growing cells (Grummt, 2003). Histone deacetylation is associated with gene silencing. Therefore it is not likely that the HDTs expressed for example in root and nodule meristems silence rRNA genes.

Furthermore, the nucleolar localization does not rule out a function in nucleoplasmic gene regulation. Other nucleolar proteins like for example *Nucleostemin-like 1*, a GTP binding protein involved in flower development, locates to the nucleolus, but can shuttle to and is functional in the nucleoplasm where it regulates the expression of the floral homeotic gene *AGAMOUS* (Wang et al., 2012).

HDTs have been shown to be involved in several developmental processes. For example AtHDT1 plays a role in reproductive development (Wu et al., 2000) and AtHDT1 and AtHDT2 are both involved in the establishment of leaf polarity (Ueno et al., 2007). Further, AtHDT3 is involved in abscisic acid controlled abiotic stress responses (Sridha and Wu, 2006). It seems unlikely that HDTs can fulfill these functions only by repression of rRNA transcription during these (developmental) processes. Therefore HDTs might be involved in regulation of transcription of genes in the nucleoplasm.

Reprogramming of cells and the start of a specific developmental programs need the activation of a specific set of genes. Like for example the infection of meristem-derived cells in nodule development that relies on the expression of specific Nod factor signaling genes (see above). However, simultaneously other developmental programs need to be repressed and it might be that HDTs repress genes involved in these other programs. In a similar way, as for example the RDP3/HDA1 type histone deacetylases AtHDA19 and AtHDA6, which are involved in repression of embryonic properties after germination through the repression of embryo-specific transcription factors (Tanaka et al., 2008). Where AtHDA6 locates predominantly to the nucleolus, AtHDA19 locates to the nucleoplasm (Zhou et al., 2005). However, both are involved in repression of transcription factors in the nucleoplasm, underlining that histone deacetylases with a nucleolar localization can be important for the repression of genes located in the nucleoplasm.

LHP1

In this thesis we also investigated the role of MtLHP1 in nodule development. Arabidopsis *lhp1/tfl2* mutants have severe developmental defects, like altered flowering time and the plant architecture is severely affected (Gaudin et al., 2001). LHP1 epigenetically represses the transcription of several floral homeotic genes and genes involved in determination of flowering time (Kotake et al., 2003; Germann et al., 2006; Mylne et al., 2006; Sung et al., 2006). The MtLHP1 RNAi silencing studies indicate that MtLHP1 has no function in nodule primordium formation, as nodule number is not reduced (chapter 5). However, the majority of the nodules showed a phenotype similar to MtHDT1, 2 and 3 RNAi knock-down nodules (chapter 4). The proximal part of these nodules appear normal, with about 8 layers of fully infected cells, but at the nodule apex the cells originating from the meristem do not differentiate correctly. Therefore we hypothesize that MtLHP1 is required for proper initiation and functioning of the nodule meristem.

LHP1 as part of the polycomb complex

AtLHP1 was found to associate *in vivo* with regions in the genome containing H3K27m3 modifications (Turck et al., 2007; Zhang et al., 2007). These studies have proposed that the CD of LHP1 could be responsible for the recognition of H3K27Me3. Therefore it has been postulated that LHP1 is part of the Polycomb repressive complex 1 (PRC1) in plants, unlike its HP1 homolog in animals and yeast where it functions in heterochromatin formation (Hall et al., 2002; Cheutin et al., 2003). Polycomb group (PcG) complexes maintain epigenetic repression of genes and control gene expression in development (Kohler and Villar, 2008; Hennig and Derkacheva, 2009). Hundreds of potential targets for PcG have been identified in recent years. In mammals and Drosophila, gene silencing by Polycomb group proteins occurs through the trimethylation of a lysine of H3 (H3K27Me3) by a methyltransferase present in the Polycomb repressing complex 2 (PRC2). This chromatin mark is then recognized by PRC1, which maintains the transcriptional silenced state. The chromo domain (CD) of the Polycomb protein of PRC1 is thought to be involved in recognition of the H3K27Me3 modification. In plants no homologues of Polycomb have been indentified, however LHP1 might have a similar function.

In this thesis we showed that LHP1 of Medicago as well as of Arabidopsis locates to euchromatic speckles in the nucleus that strongly co-localize with H3K27me3 and which most likely represent chromatin complexes. This is similar to PRC1 in animals that locates in characteristic nuclear foci, called PcG bodies (Zhang et al., 2004; Hernández-Muñoz et al., 2005).

One of the few interacting partners of LHP1 identified so far is the transcription factor *SCARECROW* (SCR) (Cui and Benfey, 2009). SCR is required for stem cell maintenance and cellular patterning of the cortex and endodermis cell layers in the root meristem (Sabatini et al., 2003; Heidstra et al., 2004). It is required for the first asymmetric division of the stem cell daughter cell that generate the cortex and endodermis cell layer, but represses further longitudinal asymmetric divisions thereby maintaining a single cortical layer. In *lhp1* mutants this longitudinal division, creating two cortical layers, occurs much earlier than in wild type plants (Cui and Benfey, 2009). In wild type plants this usually happens about 2 weeks after germination (Baum et al., 2002; Paquette and Benfey, 2005). Interestingly, treatment of roots with Trichostatin A (TSA), an inhibitor of RPD3/HDA1 and HDT histone deacetylases, results in a second cortical layer as well, indicating a similar function of LHP1, SCR and histone deacetylases in suppression of longitudinal divisions in the cortex (Cui and Benfey, 2009).

Another process in which both LHP1 and histone deacetylases are

involved in is the repression of *FLOWERING LOCUS C* (FLC). This gene is involved in vernalization, a process that promotes flowering after prolonged exposure to cold. The RPD3/HDA1 type histone deacetylase HDA6 is involved in silencing of FLC and *LHP1* is necessary for maintaining the epigenetically repressed state after exposure to cold (Sung et al., 2006)(Wu et al., 2008).

This might indicate an interplay between LHP1 and histone deacetylases in repression of gene transcription.

Concluding remarks

In this thesis we characterized two chromatin modifiers; the HDT gene family, which encodes plant specific histone deacetylases and LHP1 in especially Rhizobium induced root nodule organogenesis in Medicago. I showed that during the fate shifts that occur in Medicago root nodule organogenesis the HDTs play an important role. Further, it is probable that Medicago LHP1 does as well. In Arabidopsis HDTs are important in maintenance of the stem cell niche and meristem of roots.

The HDTs are plant specific histone deacetylases. Our finding that these HDTs are involved in meristem maintenance and a cell fate switch at the start of the post embryonic formation of organs, like nodules, shows that they are involved in processes that are plant specific.

A typical characteristic of multicellular organisms is that they are composed of different cell types present in a specific pattern Since multicellularity evolved independently in plants and animals also the mechanism controlling for example patterning and post–embryonic development are independently derived (Meyerowitz, 2002). This is clearly reflected in the master regulators that control patterning. In animals this are HOX homeobox transcription factors whereas in plants the master regulators controlling patterning belong to the MADS box transcription family. Thus in the plant and animal lineage master regulatory processes controlling spatial pattern formation led to different but logically similar mechanism and gene families present in their common ancestor were recruited.

A major difference between (multicellular) plant and animal development is the prominent role in plants of post-embryonic development driven by meristems. We showed that some HDTs have a role in meritem maintenance. Interestingly HDTs have not been detected in the genome of unicellular algae and even not in Volvox. However, Physcomitrella does have HDT genes. Therefore I hypothesize that this novel gene family of histone deacetylases evolved to support plant specific processes like meristem maintenance. In contrast to master regulators of patterning this gene family was not present

in common ancestor of plants and animals and even appears to be absent in unicellular plants.

Future studies will have to elaborate on the mechanisms by which the HDTs and LHP1 control for example properties of meristems. The New Biology approaches will give major novel possibilities to do this. It is now within reach to determine the transcriptome of specific cells and also the dynamics of this can be studied. It will for example be possible to isolate and analyze nodule meristem cells from the moment the third cortical cell layer in Medicago starts to divide up to the time point that a meristem adds cells to the cells. For these studies good Medicago mutant will be very important, but fortunately a good transposon tagged Medicago collection has been developed. Similarly, the dynamics of genome wide chromatin modifications should be studied.

Such New Biology approaches will be of pivotal importance to gain more insight into the molecular mechanisms of the epigenetic control of root and nodule development.

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Summary

In plants, unlike in animals, most organs develop post embryonically. These organs originate from clusters of undifferentiated dividing cells that form so-called meristems. Differentiated cells can be re-activated to enter the cell cycle and to ultimately give rise to new meristems. These differentiated cells reprogram to become pluripotent cells that are able to give rise to all cell types of the new organ. Examples are the formation of lateral roots from pericycle cells in Arabidopsis and the formation of root nodules from cortical cells in Medicago. In the latter organ rhizobia bacteria in symbiosis with Medicago fix atmospheric nitrogen. The reprogramming of differentiated cells is associated with large changes in gene expression. In this thesis the role of two chromatin modifiers in these reprogramming events is studied; the plant specific histone deacetylases (HDTs) and LIKE HETEROCHROMATIN PROTEIN I (LHP1).

In Chapter 2 we show that in Arabidopsis during lateral root formation all four members of the AtHDT family are strongly induced in lateral root founder cells already before the first divisions occur. Furthermore, AtHDT1 and AtHDT2 were shown to be necessary for maintenance of the root stem cell niche and meristem.

Nodules are formed from fully differentiated root cortical cells. The reprogramming of cortical cells during the formation of nodules is unique to legumes, as cortical cells in other plants are not able to change their cell fate. In Medicago we characterized 3 HDTs and showed that these MtHDTs are involved in cell fate changes and are essential for the formation of nodules (Chapter 3). These MtHDTs are involved in early steps of reprogramming of the cortical cells in nodule initiation, since RNAi knock-down expression of all 3 *MtHDTs* simultaneously, strongly reduced nodule formation. Microscopic analysis of the small nodules that were still formed revealed these had a normal wt-like proximal part and a disturbed distal part.

To explain this hybrid phenotype we created a fate map of nodule development by analyzing early stages of nodule formation (Chapter 4). This analysis revealed that the proximal part of nodules contain about 8 layers of fully differentiated cells that directly originate from the nodule primordium, which is derived from the two innermost cortical layers. The nodule meristem originates from the middle cortical layer and further growth of the nodule depends of differentiation and infection of cells originating from this meristem. At the disturbed distal part of the RNAi hybrid nodules, meristem-derived cells do not properly differentiate and are not infected with rhizobia indicating a disturbed meristem function.

The second chromatin modifier we studied is LHP1, which is known to

be involved in several developmental processes in plants, possibly as part of the plant analog of Polycomb Repressive Complex 1 (PRC1). We characterized the Medicago LHP1 homolog (Chapter 5) and showed that LHP1 forms euchromatic complexes that most likely regulate gene expression. MtLHP1 might be involved in proper differentiation of meristem-derived cells in the nodule since knock-down of MtLHP1 resulted in a similar phenotype as seen in MtHDT knock-down nodules.

Our findings provide new insights on the role of the chromatin modifiers HDTs and LHP1 in reprogramming events in Arabidopsis and Medicago organ development.

Samenvatting

Gedurende de ontwikkeling van (zaad)planten worden de meeste (laterale) organen postembryonaal na het kiemen van het zaad gevormd. Dit in tegenstelling tot de situatie bij dieren waar alle lichaamsdelen al in het embryo aanwezig zijn. Deze plantorganen worden gevormd uit meristemen die bestaan uit clusters van ongedifferentieerde cellen. Ook kunnen gedifferentieerde cellen opnieuw geactiveerd worden om te gaan delen om zo een nieuw meristeem te vormen. Het herprogrammeren van deze gedifferentieerde cellen leidt tot de vorming van pluripotente cellen die uiteindelijk tot elk celtype van het nieuw te vormen orgaan kunnen differentiëren.

Voorbeelden van dit proces zijn de vorming van zijwortels uit pericykelcellen in Arabidopsis en de vorming van wortelknolletjes uit corticale cellen in Medicago. In de symbiotische wortelknolletjes die zich kunnen vormen bij vlinderbloemigen, kunnen rhizobium bacteriën stikstofgas uit de lucht vastleggen. Zijwortels en wortelknolletjes ontstaan door herprogrammeren van gedifferentieerde cellen wat gepaard gaat met een grote verandering in de expressie van genen. In dit proefschrift worden twee typen chromatine modificerende eiwitten bestudeerd die betrokken zijn bij deze herprogrammerings processen; namelijk de plant specifieke histon deacetylases (HDTs) en LIKE HETEROCHROMATIN PROTEIN I (LHP1).

Zijwortels in Arabidopsis ontstaan uit twee naast elkaar gelegen pericykel cellen. In hoofdstuk 2 wordt aangetoond dat in deze twee cellen alle vier AtHDT genen sterk geactiveerd worden voordat de eerste celdelingen plaatsvinden. Ook blijken AtHDT1 en AtHDT2 noodzakelijk te zijn voor het in stand houden van het wortelmeristeem.

Wortelknolvorming in Medicago is een interessant systeem om herprogrammering van cellen tijdens de vorming van nieuwe organen te bestuderen. Tijdens de vorming van deze wortelknolletjes worden gedifferentieerde corticale cellen aangezet tot de vorming van een knol primordium. Dit herprogrammeren van corticale cellen is uniek voor vlinderbloemigen. In hoofdstuk 3 zijn drie MtHDTs gekarakteriseerd en laten we zien dat deze essentieel zijn voor de vorming van wortelknolletjes. Deze MtHDTs zijn betrokken bij een vroege stap in de ontwikkeling van knolletjes, omdat een gelijktijdige reductie in het nivo van expressie van alle 3 *MtHDTs* met behulp van RNAi resulteert in een sterke reductie in het aantal wortelknolletjes op wortels. Bovendien zijn de enkele wortelknolletjes die nog wel gevormd worden verstoord in de ontwikkeling van het distale deel, terwijl het proximale deel van deze wortelknolletjes zich normaal ontwikkeld. Om dit hybride fenotype te verklaren is allereerst een ontwikkelingskaart van wortelknollen gemaakt

door te kijken naar vroege stadia van knol ontwikkeling (hoofdstuk 4). Hieruit blijkt dat het proximale deel met volledig gedifferentieerde en met Rhizobium geïnfecteerde cellen van deze hybride wortelknolletjes direct ontstaat uit het knolprimordium, dat gevormd wordt uit de twee meest centrale corticale cellagen in de wortel. Het apicale meristeem van wortelknolletjes ontstaat uit de corticale cellaag uit het midden van de cortex. Groei van wortelknollen is afhankelijk van differentiatie en infectie van cellen afkomstig van dit meristeem. In het verstoorde distale deel van deze hybride wortelknolletjes differentiëren cellen afkomstig van het meristeem onvolledig en worden ze niet geïnfecteerd met Rhizobium. Dit duidt op een verstoorde werking van het meristeem.

LHP1 is betrokken bij verscheidene ontwikkelingsprocessen in planten, mogelijk als onderdeel van een complex analoog aan PRC1 in dieren. In hoofdstuk 5 is de LHP1 homoloog van Medicago gekarakteriseerd en wordt aangetoond dat LHP1 euchromatische complexen vormt die waarschijnlijk betrokken zijn bij regulatie van gentranscriptie. Mogelijk speelt MtLHP1 ook een rol in het ontstaan of functioneren van het meristeem van wortelknolletjes, omdat door RNAi gereduceerde expressie van MtLHP1 resulteert in kleine wortelknolletjes met een vergelijkbaar hybride fenotype als in MtHDT-RNAi wortelknolletjes.

Onze bevindingen tonen een nog niet eerder beschreven rol aan van HDTs en LHP1 in het herprogrammeren van cellen in Arabidopsis en Medicago bij het aanleggen van zijorganen in wortels.

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Stefan

Curriculum Vitae

Stefan Schilderink was born on the 11th of May 1981 in Lichtenvoorde, the Netherlands. After obtaining his VWO diploma at the Marianum College in Lichtenvoorde and Groenlo in 1999, he started his study Bioprocess Technology at Wageningen University in Wageningen where he specialized in Cellular and Molecular Biology. His first thesis was performed at the Laboratory of Virology, Wageningen University, where he worked on WSSV virus in shrimps. Afterwards he did an internship at CSIRO livestock industries in Brisbane, Australia, studying RNAi induced virus resistance in Drosophila. For his final thesis he joined the Laboratory of Molecular Biology, Wageningen University. There he worked on the role of RNAi in heterochromatin formation in Arabidopsis. In July 2005 he obtained his MSc degree and in August 2005 he started as a PhD-student at the Laboratory of Molecular Biology under the supervision of Ton Bisseling, Olga Kulikova and Joan Wellink. After his PhD he moved to Vienna, Austria, where he and his spouse Leonie Smeenk continue their scientific careers.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Stefan Schilderink
Date: 11 December 2012
Group: Molecular Biology, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Does RNA interference play a role in heterochromatin formation?	Dec 21, 2005
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1,5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS student day 2006, Wageningen University	Sep 19, 2006
EPS student day 2007, Wageningen University	Sep13, 2007
EPS PhD student retreat 2008, Wageningen University	Oct 02-03, 2008
EPS student day 2009, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS Theme 4 symposium 'Genome Plasticity', Wageningen University	Dec 09, 2005
EPS Theme 4 symposium 'Genome Plasticity', Radboud University Nijmegen	Dec 08, 2006
EPS Theme 1 symposium 'Developmental Biology of plants', Wageningen University	Oct 11, 2007
EPS Theme 4 symposium 'Genome Plasticity', Wageningen University	Dec 07, 2007
EPS Theme 4 symposium 'Genome Plasticity', Wageningen University	Dec 12, 2008
EPS Theme 4 symposium 'Genome Plasticity', Radboud University Nijmegen	Dec 11, 2009
EPS Theme 1 symposium 'Developmental Biology of plants', Wageningen University	Jan 28, 2010
► **NWO Lunteren days and other National Platforms**	
NWO, Nucleic Acid Meeting, Lunteren	Nov 28-29, 2005
Chromatin III meeting, Kerkrade	Dec 07-08, 2005
Chromatin IV meeting, Rotterdam	Sep 07, 2006
NWO, Nucleic Acid Meeting, Lunteren (one day meeting), 2006 and 2007	Dec 2006, 2007
Chromatin VI meeting, Nijmegen	Oct 16, 2008
NWO-ALW, Experimental Plant Sciences, Lunteren (two day meetings), 2006, 2007, 2008,2009 and 2010)	Apr 2006, 2007, 2008, 2009, 2010
► **Seminars (series), workshops and symposia**	
Seminar Gunter Reuter	Oct 13, 2005
Seminars (Erika Eiser, Jiri Friml, Veronica Grieneisen, Ingo Schubert)	Feb-Sep, 2006
Seminar Paulien Hogeweg	Jun 06, 2007
EPS flying seminars (Joe Ecker, Phil Benfey, Masahiro Yano, Rob Martienssen, Scott Poethig, Hiroo Fukada, Richard Fierstra, Simon Gilroy, Zhenbiao Yang)	2005-2008
Seminars (Jean-Philippe Combier, Alexey Borisov, Stephen Adams, Enrico Scarpella, Thorsten Neurnberger)	Apr 15, 2008
Pamela Hines mini-symposium, Wageningen	Nov 08, 2008
Annual Lab Seminar Day, Molecular Biology (2005, 2006, 2007 and 2008)	Dec 2005, 2006, 2007, 2008
► **Seminar plus**	
Ecker, Benfey, Martienssen, Poethig and Gilroy	2005-2008
► **International symposia and congresses**	
PGEM, Amsterdam	Sep 20-23, 2005
International Chromosome Conference, Amsterdam	Aug 25-29, 2007
MAPD, Vienna, Austria	Feb 23-26, 2010
EPIGENOME Meeting, Vienna, Austria	Mar 16-19, 2010
► **Presentations**	
EPS Theme 4 symposium 'Genome Plasticity', Radboud university Nijmegen (oral)	Dec 11, 2009
EPS PhD student retreat 2008, Wageningen University (poster)	Oct 02-03, 2008
Gregor Mendel Institute, Vienna (oral)	Apr 07, 2010
► **IAB interview**	Sep 14, 2007
► **Excursions**	
Subtotal Scientific Exposure	*21,7 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD Course Models take root: the role of mathematics in plant biology	Feb 19-24, 2006
► **Journal club**	
Literature discussions at Molecular Biology	2005-2010
► **Individual research training**	
Subtotal In-Depth Studies	*5,0 credits**

4) Personal development	date
► **Skill training courses**	
EPS Career perspective Seminars, Wageningen	Nov 04, 2008
Scientific Writing, Wageningen University	Jan 15 -Mar 05, 2009
► **Organisation of PhD students day, course or conference**	
PhD Student Day 2007 (organization)	Sep 13, 2007
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31.5**

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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