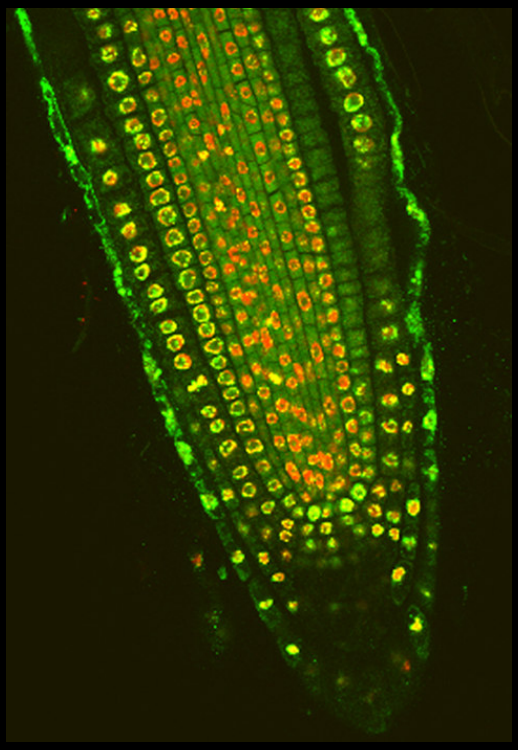
Chromatin Organisation during Arabidopsis root development



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Chromatin Organisation during Arabidopsis root development

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OUTLINE

The different cell types of a multicellular organism express different sets of genes. This is one of the oldest statements of developmental genetics. However, how different patterns of gene expression are established in genetically identical cells and maintained during subsequent cell division is still an active topic of research. Especially studies on the involvement of epigenetic modifications in development are a rapidly expending area.

The eukaryotic nucleus stores a great amount of information in an extremely compact way. This high level of compaction of the chromatin raises challenges for processes such as transcription. To access the chromatin, 'chromatin modifiers' are essential to 'open' or 'close' the chromatin and in this way control gene expression and establish epigenetic marks that can be inherited.

Chromatin modifiers can modify the DNA itself like DNA methyltransferases, which methylate cytosine; but also the histones can be modified by for example histone acetyltransferases or histones methyltransferases. Further, the nucleosomes can be repositioned by for example ATP-dependent chromatin remodelling enzymes, loosing up' or 'tightening up' the chromatin structure.

In chapter 1, I will introduce the organisation of the chromatin in relation to development of a multicellular organism. An overview will be provided on what is known about cytosine methylation; about Heterochromatin Protein 1, one of the first chromatin modifier studied in animals and its homolog LHP1 in plants; and about plant histone lysine 9 methyltransferases, which interact with HP1 in animals.

We used as model system the root of Arabidopsis thaliana. Arabidopsis has a small genome size of 150 Mbp, and a simple organisation of euchromatin and heterochromatin. The root of Arabidopsis has a simple organisation; furthermore this thin organ (150 μm in thickness) allows microscopic studies on nuclei in an intact organ.

In Chapter 2, we first studied whether the structure of the chromatin changes during differentiation of root cells. Level of DNA methylation was used as a

characteristic epigenetic mark of the heterochromatin and this was studied and quantified in a developing root. We showed that the Quiescent Center cells and surrounding stem cells have the highest level of methylation. Further, the level of DNA methylation decreases in the division zone of the root and increases again when cells differentiate.

As a second criterion to study changes of chromatin during development, Like Heterochromatin Protein 1 was chosen and analyzed in chapter 3. Its homolog in animals, HP1 was first identified as a heterochromatic protein. However, several isoforms have now been identified which localizes in euchromatin as well as heterochromatin or exclusively in the euchromatin. We showed, like others, that LHP1 is localized in the euchromatin where it forms numerous foci in the differentiation zone of the root and hardly any in the division zone. We demonstrated that LHP1 is highly mobile but still bound to chromatin in both cell types and that it colocalizes with H3K9m3 and H3K27m3, euchromatin marks in Arabidopsis.

To investigate further this difference of subnuclear patterning of LHP1 during developmental stages, we tried to identify which histone methyltransferase would provide the binding site for the LHP1 foci in the nucleus. HP1 has been shown to bind H3K9m3 and to interact with the lysine 9 histone H3 trimethyltransferase SU(VAR)3-9. In chapter 4, we screened the SUVH proteins available which are homologs of SU(VAR)3-9 in plants. However a SUVH protein able to directly interact with LHP1 could not be identified. We showed that SUVH3, SUVH7 and SUVH9 are localized in the euchromatin and part of dynamics complexes. They have tissue specific expression and SUVH3 and SUVH9 forms foci in the nucleus depending on the developmental stage of the cell.

Finally in chapter 5, I use the data described in this thesis as well as studies of others to discuss the role of certain aspects of the chromatin organisation in plants development.

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Maëlle

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General Introduction

Chapter 1: General Introduction

Chromatin organisation

Proper development of a multicellular organism depends on the establishment and maintenance of differential gene expression in cells that are genetically identical. Epigenetic modifications such as DNA methylation or histone tail modifications create a 'second code' for spatial and temporal differential expression of genes (Jenuwein et al. 2001), which is superimposed on the "primary code" based on DNA sequences and transcriptional regulators. This information is stored in the eukaryotic nucleus where a relatively great amount of DNA-protein complexes, the so called chromatin, is packed in a very compact way. The basic organisation of chromatin is formed by nucleosomes that are positioned every 200 bp on the DNA strands. About 150 bp DNA is wrapped around a histone octamer forming the nucleosomes and these are linked together by histone H1. This 'beads-on-a-string' organisation forms the 10 nm strand. Further interactions between nucleosomes and other nuclear proteins create higher-order chromatin structures such as the 30 nm chromatin fibre that occur in interphase chromatin (Hayes et al. 2001; Hsieh et al. 2005).

DNA staining of interphase nuclei distinguishes two classes of chromatin, brightly stained regions called heterochromatin and weakly stained regions called euchromatin. Euchromatin is gene-rich and decondensed during interphase, whereas heterochromatin is rich in repetitive sequences, low in gene density, and remains mostly condensed throughout the cell cycle (Hsieh et al. 2005).

The high level of compaction of chromatin raises challenges for processes such as DNA replication, transcription, recombination and repair which involve direct binding of protein factors to their target DNA sequences. To access the chromatin, 'chromatin remodelling' is necessary and can be achieved in different ways. For example, positioning of nucleosomes on DNA can be disrupted and reconfigured to 'open' the chromatin by ATP-dependent remodelling complexes. These include proteins like DDM1 (DECREASED DNA METHYLATION 1), a plant ATPase/helicase Swi2/Snf2 with several animal homologs (Brzeski et al. 2003). Composition of the

nucleosomes themselves can be modified by replacing major histones with variants (Ahmad et al. 2002; McKittrick et al. 2004; Hsieh et al. 2005). For example the histone variant H3.3 is a mark of active euchromatin. H3.3 variants are enriched in regions with high transcription activity and replacement of H3 by H3.3 provides a dynamic mechanism for rapid activation of chromatin (Henikoff et al. 2004; McKittrick et al. 2004). Post-translational modifications of histone proteins such as acetylation or methylation of histone tails residues can generate localized distinct chromosomal domains by recruiting diverse chromatin-binding protein complexes such as Polycomb proteins (Cao et al. 2004) or Heterochromatin Protein 1 (Bannister et al. 2001; Fischle et al. 2005). Finally, methylation of cytosine by DNA methyltransferases can provide a stable and heritable epigenetic mark and modulate chromatin structure by recruiting chromatin complexes that bind to methylated DNA (Hsieh et al. 2005). There is an interplay between these mechanisms and in concert they determine a specific chromatin state (Johnson et al. 2002; Hsieh et al. 2005).

In this thesis, we focused on the role of Heterochromatin Protein 1 and DNA methylation in nuclear organisation and plant development. Therefore these 2 topics will be first introduced in more details. This will be followed by an introduction of Arabidopsis (roots) as a model system to study chromatin organisation during plant development.

DNA methylation

DNA methylation is one of the most abundant epigenetic modifications in higher plants and animals (Finnegan et al. 2000; Bird 2002). It is considered to be involved in defending the genome against transposable elements and retroviruses, to control genomic imprinting and to regulate gene expression (Berger et al. 2003; Hsieh et al. 2005). In general, DNA methylation is prominently present in heterochromatin and transposons. Methylation can occur on cytosine or adenine (Ratel et al. 2006), however, we will focus on cytosine methylation, since this is the most abundant DNA methylation in plants (Finnegan et al. 1998).

Cytosine methylation (5mC) in a symmetrical CG context is an evolutionarily conserved DNA modification that is found in vertebrates, plants and some fungi. In

addition, plants have significant levels of cytosine methylation also in non-CG sequences, which include symmetrical CNG and asymmetrical CNN sequences (Finnegan et al. 2000; Bird 2002). In total, 5-25 % of all cytosines is methylated in plants (Rangwala et al. 2004).

Patterns of methylation are established by *de novo* methylation and maintained after replication by maintenance methyltransferases which copy the methylation marks onto the daughter DNA strand. Arabidopsis possesses 4 classes of DNA methyltransferases. MET1 (METHYLTRANSFERASE 1), a DNA maintenance methyltransferase, is the homolog of the animal DNMT1 (DNA METHYLTRANSFERASE 1) and maintains DNA methylation in a symmetrical CG context. It might also contribute to *de novo* CG methylation (Aufsatz et al. 2004). Null mutations in the mouse DNA methyltransferase *DNMT1* result in embryonic lethality. In Arabidopsis, *met1* mutants show pleiotropic effects including floral homeotic changes that lead to partial or complete sterility, delayed flowering time, alteration of leaf shape and reduced size (Finnegan et al. 1996; Ronemus et al. 1996; Richards 1997; Jeddeloh et al. 1999).

CMT3 (CHROMOMETHYLASE 3) is the major enzyme for CNG methylation and other non-CG methylation and it is involved in *de novo* and maintenance methylation. It combines a chromodomain motif with motifs characteristic of cytosine methyltransferases (Bartee et al. 2001; Lindroth et al. 2001). *Cmt3* mutants do not result in obvious phenotypic abnormalities, although some genes are selectively silenced. Two Arabidopsis DOMAINS REARRANGED METHYLTRANSFERASES, DRM1 and DRM2, have been identified as *de novo* methyltransferases mainly in a non-CG context**.** Like *cmt3* mutants, *drm* mutants do not display an obvious phenotype. However, a triple mutant *drm1drm2cmt3* does show pleiotropic effects. Therefore it has been proposed that DRM and CMT3 are partially functionally redundant and act to control non-CG methylation (Cao et al. 2002). Finally, Arabidopsis possesses a DNMT2 (DNA METHYLTRANSFERASE 2) gene, which is a putative methyltransferase that is a homolog of DNMT2, an animal DNA methyltransferase with unknown function (Fransz et al. 2006).

DNA methylation patterns are the result of *de novo* methylation, demethylation and maintenance of existing methylation. Demethylation can be passive or active.

Passive demethylation occurs automatically during replication if the newly synthesised DNA is not methylated. Active demethylation is performed by DNA glycosylases. In Arabidopsis only two DNA glycosylases are known. The DNA glycosylase DEMETER regulates early seed development through activation of the maternal copy of the imprinted MEDEA, a Polycomb gene (Choi et al. 2002) and FWA, a transcription factor. DEMETER relieves DNA methylation specifically of the maternal alleles of those two genes. (Kinoshita et al. 2004). The second known protein involved in demethylation of DNA is ROS1 (REPRESSOR OF SILENCING 1). It is involved in preventing transcriptional gene silencing by demethylation of promoter sequences of target genes (Agius et al. 2006).

The role of DNA methylation during plant development has not been studied in great detail, but there are a few cases that indicate its importance. Active demethylation was reported to occur in *Silene latifolia* during the transition from a dry quiescent seed in which the DNA of embryo's is hypermethylated, to a germinating seed. Demethylation of the central zone of the shoot meristem during the formation of the floral meristem was also observed *in Silene latifolia* (Zluvova et al. 2001). Cold temperatures during vernalization of Arabidopsis were shown to induce a decrease in DNA methylation, perhaps by uncoupling replication and maintenance methylation (Burn et al. 1993; Finnegan et al. 1998).

In addition to methyltransferases and demethylases, several other genes play an important role in DNA methylation. An example is chromatin remodelling gene DDM1. 70% reduction of DNA methylation was observed in the *ddm1-1* mutant (Vongs et al. 1993). DDM1 is an ATPase/helicase SWI2/SN2-like protein, it binds to the nucleosomes and repositions them to modify the chromatin (Brzeski et al. 2003). DDM1 was shown to be required for DNA methylation and its maintenance (Soppe et al. 2002).

DNA methylation and histone modifications interact to define a specific chromatin state. For example, DDM1 is not only required for DNA methylation, but also for subsequent methylation of histone H3 lysine 9 and for deacetylation of histone H4 lysine 16 (Gendrel et al. 2002; Johnson et al. 2002; Soppe et al. 2002). HDA6, a histone deacetyltransferase, was also shown to be involved in maintaining CpG methylation at specific loci (Aufsatz et al. 2002). Furthermore,

SUVH4/KRYPTONITE, an histone H3 lysine 9 dimethyltransferase provides one of the histone marks necessary to recruit the CMT3 DNA methyltransferase (Jackson et al. 2002; Jackson et al. 2004; Lindroth et al. 2004).

LHP1

Posttranslational modifications of histone proteins provide the binding marks of chromatin remodelling proteins, for example the chromodomain proteins. The chromodomain proteins include Polycomb group and Heterochromatin Protein 1 (HP1) proteins. They are thought to form multimeric complexes and to either 'open' or 'close' the chromatin. In this thesis I have especially focused on the Arabidopsis homolog of HP1.

HP1 was discovered in Drosophila (James et al. 1986), but is conserved in many organisms (Singh et al. 1991). HP1 proteins possess three distinct domains: an amino-terminal chromodomain (CD) a more flexible intervening region (the hinge region) and a specific carboxyl-terminal chromoshadow domain (CSD). The CD was shown in several systems (fission yeast (Nakayama et al. 2000), Drosophila (Bannister et al. 2001; Jacobs et al. 2002), mammals (Aagaard et al. 1999; Lachner et al. 2001) to bind to methylated histone 3 Lysine 9 (H3K9) and with the highest affinity for trimethylated H3K9 (Fischle et al. 2005).The hinge region is involved in binding of RNA, DNA and chromatin, and the CSD in protein-protein interaction. Since its discovery, several homologues of HP1 have been identified, from fission yeast (Swi6) to human, showing that HP1 is a highly conserved protein. Several isoforms were discovered as well, each with its own subnuclear location: in heterochromatin and/or in euchromatin like HP1γ (Vakoc et al. 2005). Currently HP1 is thought to serve as a bridging protein, connecting histones and non-histone chromosomal proteins (Li et al. 2002). In animals and yeast, HP1 was shown to be involved in chromatin structural organisation, maintenance of heterochromatin and gene regulation (Hiragami et al. 2005; Hediger et al. 2006) and to be a highly dynamic protein (Cheutin et al. 2003; Festenstein et al. 2003; Schmiedeberg et al. 2004; Zemach et al. 2006).

In Arabidopsis, a unique homologue of HP1 was discovered named Like Heterochromatin Protein 1 (LHP1). LHP1 contains a CD, a hinge region and a

CSD (Gaudin et al. 2001). LHP1 was shown to be located in the euchromatin and to be present in many foci (Gaudin et al. 2001; Kotake et al. 2003; Takada et al. 2003; Nakahigashi et al. 2005). In nuclei of root cells of Arabidopsis, LHP1 was described to have a diffuse pattern in dividing meristematic cells and a speckled-like pattern (foci) in differentiated cells (Gaudin et al. 2001).
Microarray analysis on *lhp1* knockout mutants reveals that LHP1 silences genes within euchromatin, but not in heterochromatin (Nakahigashi et al. 2005). In Arabidopsis, LHP1 was shown to regulate two genes involved in flowering time: Flowering Locus T (FT) and Flowering Locus C (FLC). FLC is a repressor of flowering in the vernalization pathway and negatively regulates FT, which is a floral pathway integrator. Further, LHP1 was shown to be required for epigenetic maintenance of vernalization-induced repression of FLC (Mylne et al. 2006).

SUVH and histone modifications

In Drosophila, SU(VAR)3-9 (SUPPRESSOR OF POSITION EFFECT VARIEGATION), an H3K9 trimethyltransferase, is known to provide the binding site for the chromodomain of HP1 (Lachner et al. 2001), but also was shown *in vitro* (yeast two-hybrid) to interact with HP1 through its CSD (Aagaard et al. 1999). In fission yeast, CLR4 (CRYPTIC LOCI REGULATOR) is the unique homolog of SU(VAR)3-9 and LHP1 was shown to complement the *swi6* mutant of the HP1 yeast homolog (Kotake et al. 2003) suggesting that LHP1 can bind (tri)methylated H3K9 in yeast.
Methylation of lysine residues in histones is more complex than for example lysine acetylation, as a lysine can be subjected to mono-, di or tri-methylation. Histone lysine methylation has been shown to be catalyzed almost exclusively by conserved SET domain proteins originally identified in Drosophila as Supressor of variegation [Su(var)3-9] (Tschiersch et al. 1994), Enhancer of Zeste [E(z)] (Jones et al. 1993) and Trithorax (Mozer et al. 1989). The majority of histone methyltransferases has a SET domain, but also two flanking cystein-rich regions, the so-called pre-SET and post-SET domains. The model enzyme for trimethylation of H3K9 is SU(VAR)3-9. SU(VAR)3-9 has well conserved homologs in yeast, Drosophila, human and plants. CLR4 is the unique yeast homolog; SUV39H1 and SUV39H2 are the two human homologs and they were

shown to be trimethyltransferases. In contrast, the Arabidopsis genome contains 10 *SU(VAR)3-9* homologues (*SUVH*) and five *SU(VAR)3-9* related (*SUVR*) and their function / activity remains to be established. Multiple SUVH homologues have also been identified in tobacco, rice and maize (Baumbusch et al. 2001; Shen 2001; Springer et al. 2003). In addition to the SET, pre-SET and post-SET domains, plant SUVH proteins contain a SRA domain (SET and RING finger associated) (Baumbusch et al. 2001) which could play a role in targeting SUVH proteins to specific chromatin sub-domains (Citterio et al. 2004; Yu et al. 2004).

Phylogenetic analysis of the SUV39H family proteins based on the sequence alignment of the SET domain, showed that AtSUVH SET domains are more closely related to DmSU(VAR)3-9, HsSUV39H (1 and 2), and SpCLR4 SET domains (figure 1) (Baumbusch et al. 2001). However, AtSUVR SET domains are most similar with the one of HsG9a (figure 1) (Baumbusch et al. 2001), which is also a histone H3K9 trimethyltransferase but which was shown not to be able to recruit HP1 (Stewart et al. 2005). Therefore we decided to concentrate our study on the AtSUVH proteins, which seems more likely to interact with LHP1. This phylogenetic analysis results in a classification in four distinct subgroups of the 10 SUVH Arabidopsis proteins: SUVH1 subgroup (AtSUVH1,-3,-7,-8 and -10), SUVH2 subgroup (AtSUVH2 and AtSUVH9), SUVH4 subgroup (AtSUVH4), and SUVH5 subgroup (AtSUVH5 and AtSUVH6) (figure. 1) (Baumbusch et al. 2001; Naumann et al. 2005). SUVH1 and SUVH2 proteins were shown to be located in the heterochromatin (Naumann et al. 2005; Fischer et al. 2006). Loss of SUVH1 shows a weak reduction of heterochromatic H3K9m2. *suvh2* null mutants have a significant reduction of most heterochromatic marks: DNA methylation, H3K9m, H3K9m2, H3K27m, H3K27m2 and more specifically H4K20m. However, *suvh2* null plants do not exhibit phenotypic defects. Over-expression of SUVH2 enhances gene silencing and ectopic heterochromatinization. Furthermore, SUVH2 over-expressing plants display significant growth reduction and curled cotyledons. SUVH4 is located as well in the heterochromatin (Naumann et al. 2005; Fischer et al. 2006). SUVH4 and SUVH6 were shown to catalyze the formation of H3K9m and H3K9m2 (Jackson et al. 2004). Loss of function mutant of *SUVH4* causes a strong reduction of H3K9m2 in pericentromeric heterochromatin, but less than in

suvh2 mutants (Jackson et al. 2004; Naumann et al. 2005). Loss of function mutants of *SUVH5* or *SUVH6* results in a minor reduction of H3K9 methylation. SUVH5 was shown *in vitro* to be a H3K9 mono- or dimethyltransferase (Ebbs et al. 2005; Ebbs et al. 2006). SUVH3 was shown to be localised in sub-nuclear foci in tobacco BY2 cells and to remain associated with condensed chromosomes throughout mitosis. In Arabidopsis, SUVH3 is broadly expressed during plant development with the highest levels found in proliferating cells (Casas-Mollano et al. 2006).

Arabidopsis nuclei

Arabidopsis is an ideal plant to study the relation between chromatin organisation in interphase nuclei and development. It has a relatively small genome of ~ 150 Mbp (Zhang et al. 2006) with 5 chromosomes and a simple heterochromatin organisation. The heterochromatin forms so-called chromocenters in interphase nuclei. These chromocenters contain the centromeres (major tandem repeats like 180 bp repeat) and their flanking pericentromeric regions which are rich in various transposon-like Athila retroelements. (Maluszynska et al. 1991; Heslop-Harrison et al. 1999; Fransz et al. 2002). Chromosome 2 and 4 contain also heterochromatic Nucleolar Organizer Regions (NORs) which occur in chromocenters as well and chromosome 4 has a heterochromatic knob (Fransz et al. 1998). So in Arabidopsis interphase nuclei maximally 12 chromocenters can be visualised. However, in most interphase nuclei fewer chromocenters are visible due to their tendency to cluster (Fransz et al. 2002). Recent genome-wide mapping of DNA methylation in Arabidopsis showed that about 19% of the genome is methylated including the centromeres, the pericentromeres, NORs and heterochromatin knob, but also ~ 40% of the expressed genes is methylated (Zhang et al. 2006).

Arabidopsis root

The root of Arabidopsis has a simple radial organisation. From outside to inside, concentric layers of epidermis, cortex and endodermis encircle the stele that contains the vascular system. New cells are added to the files of cells that form the different root tissues in the zone of mitotic activity, the meristem. Meristematic

cells divide in a stereotype manner facilitating studies on chromatin structure throughout development. In roots, stem cells (pluripotent cells), cells necessary to organize those stem cells (quiescent centre), dividing cells and differentiated cells are present. Quiescent Centre (QC), stem cells and dividing cells form the meristem at the apex of the root. At the end of the division zone cells start to elongate and differentiate, this is the so-called elongation zone. The cells that have reached their full size and are differentiated form the differentiation zone (figure 2) (Dolan et al. 1993; van den Berg et al. 1998). The Arabidopsis root allows us to study tissue specific as well as developmentally regulated modifications of the chromatin, also because the Arabidopsis root is enough thin (~150 μm) to be analysed under the microscope without sectioning, offering us a picture of the entire organ.

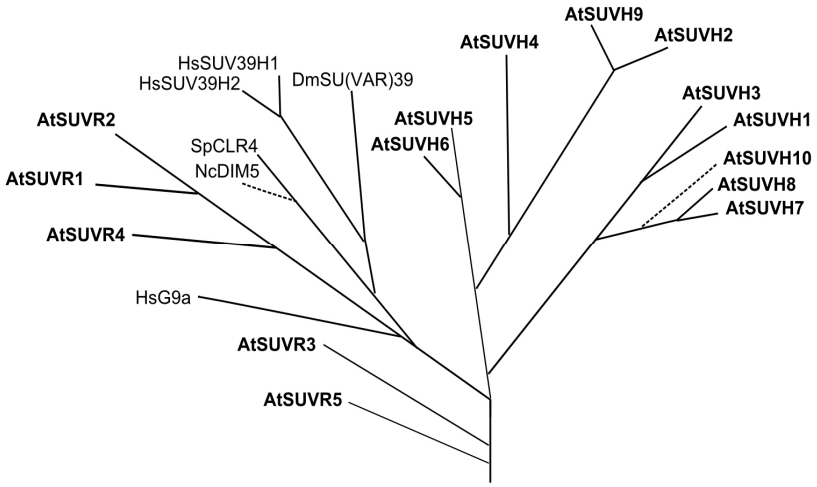

Figure 1: Phylogenetic tree of the proteins of the SUV39H family adapted from Baumbusch et al (Baumbusch et al. 2001). Protein sequences are from *Arabidopsis thaliana* (At), *Neurospora crassa* (Nc), *Drosophila melanogaster* (Dm), *Saccharomyces pombe* (Sp), and *Homo sapiens* (Hs).

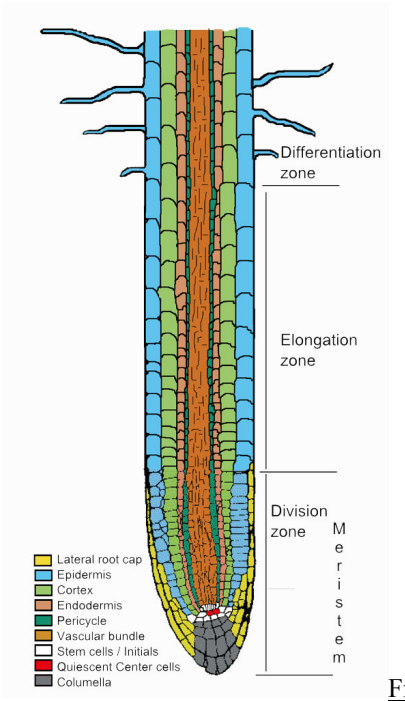

Figure 2: Arabidopsis root organisation

References

Aagaard, L., G. Laible, et al. (1999). "Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31." Embo J 18(7): 1923-38.

Agius, F., A. Kapoor, et al. (2006). "Role of the Arabidopsis DNA glycosylase/lyase ROS1 in active DNA demethylation." Proc Natl Acad Sci U S A 103(31): 11796-801.

Ahmad, K. and S. Henikoff (2002). "The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly." Mol Cell 9(6): 1191-200.

Aufsatz, W., M. F. Mette, et al. (2002). "HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA." Embo J 21(24): 6832-41.

Aufsatz, W., M. F. Mette, et al. (2004). "The role of MET1 in RNA-directed de novo and maintenance methylation of CG dinucleotides." Plant Mol Biol 54(6): 793-804.

Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature 410(6824): 120-4.

Bartee, L., F. Malagnac, et al. (2001). "Arabidopsis cmt3 chromomethylase mutations block non-CG methylation and silencing of an endogenous gene." Genes Dev 15(14): 1753-8.

Baumbusch, L. O., T. Thorstensen, et al. (2001). "The Arabidopsis thaliana genome contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes." Nucleic Acids Res 29(21): 4319-33.

Berger, F. and V. Gaudin (2003). "Chromatin dynamics and Arabidopsis development." Chromosome Res 11(3): 277-304.

Bird, A. (2002). "DNA methylation patterns and epigenetic memory." Genes Dev 16(1): 6-21.

Brzeski, J. and A. Jerzmanowski (2003). "Deficient in DNA methylation 1 (DDM1) defines a novel family of chromatin-remodeling factors." J Biol Chem 278(2): 823-8.

Burn, J. E., D. J. Bagnall, et al. (1993). "DNA methylation, vernalization, and the initiation of flowering." Proc Natl Acad Sci U S A 90(1): 287-91.

Cao, R. and Y. Zhang (2004). "The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3." Curr Opin Genet Dev 14(2): 155-64.

Cao, X. and S. E. Jacobsen (2002). "Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes." Proc Natl Acad Sci U S A 99 Suppl 4: 16491-8.

Casas-Mollano, J. A., N. T. Lao, et al. (2006). "Intron-regulated expression of SUVH3, an Arabidopsis Su(var)3-9 homologue." J Exp Bot 57(12): 3301-11.

Cheutin, T., A. J. McNairn, et al. (2003). "Maintenance of stable heterochromatin domains by dynamic HP1 binding." Science 299(5607): 721-5.

Choi, Y., M. Gehring, et al. (2002). "DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in arabidopsis." Cell 110(1): 33-42.

Citterio, E., R. Papait, et al. (2004). "Np95 is a histone-binding protein endowed with ubiquitin ligase activity." Mol Cell Biol 24(6): 2526-35.

Dolan, L., K. Janmaat, et al. (1993). "Cellular organisation of the Arabidopsis thaliana root." Development 119(1): 71-84.

Ebbs, M. L., L. Bartee, et al. (2005). "H3 lysine 9 methylation is maintained on a transcribed inverted repeat by combined action of SUVH6 and SUVH4 methyltransferases." Mol Cell Biol 25(23): 10507-15.

Ebbs, M. L. and J. Bender (2006). "Locus-specific control of DNA methylation by the Arabidopsis SUVH5 histone methyltransferase." Plant Cell 18(5): 1166-76.

Festenstein, R., S. N. Pagakis, et al. (2003). "Modulation of heterochromatin protein 1 dynamics in primary Mammalian cells." Science 299(5607): 719-21.

Finnegan, E. J., W. J. Peacock, et al. (1996). "Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development." Proc Natl Acad Sci U S A 93(16): 8449-54.

Finnegan, E. J., R. K. Genger, et al. (1998). "DNA Methylation in Plants." Annu Rev Plant Physiol Plant Mol Biol 49: 223-247.

Finnegan, E. J. and K. A. Kovac (2000). "Plant DNA methyltransferases." Plant Mol Biol 43(2-3): 189-201.

Finnegan, E. J., W. J. Peacock, et al. (2000). "DNA methylation, a key regulator of plant development and other processes." Curr Opin Genet Dev 10(2): 217-23.

Fischer, A., I. Hofmann, et al. (2006). "Heterochromatin proteins and the control of heterochromatic gene silencing in Arabidopsis." J Plant Physiol 163(3): 358-68.

Fischle, W., B. S. Tseng, et al. (2005). "Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation." Nature 438(7071): 1116-22.

Fransz, P., S. Armstrong, et al. (1998). "Cytogenetics for the model system Arabidopsis thaliana." Plant J 13(6): 867-76.

Fransz, P., J. H. De Jong, et al. (2002). "Interphase chromosomes in Arabidopsis are organized as well defined chromocenters from which euchromatin loops emanate." Proc Natl Acad Sci U S A 99(22): 14584-9.

Fransz, P., R. ten Hoopen, et al. (2006). "Composition and formation of heterochromatin in Arabidopsis thaliana." Chromosome Res 14(1): 71-82.

Gaudin, V., M. Libault, et al. (2001). "Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in Arabidopsis." Development 128(23): 4847-58.

Gendrel, A. V., Z. Lippman, et al. (2002). "Dependence of heterochromatic histone H3 methylation patterns on the Arabidopsis gene DDM1." Science 297(5588): 1871-3.

Hayes, J. J. and J. C. Hansen (2001). "Nucleosomes and the chromatin fiber." Curr Opin Genet Dev 11(2): 124-9.

Hediger, F. and S. M. Gasser (2006). "Heterochromatin protein 1: don't judge the book by its cover!" Curr Opin Genet Dev 16(2): 143-50.

Henikoff, S., T. Furuyama, et al. (2004). "Histone variants, nucleosome assembly and epigenetic inheritance." Trends Genet 20(7): 320-6.

Heslop-Harrison, J. S., M. Murata, et al. (1999). "Polymorphisms and genomic organization of repetitive DNA from centromeric regions of Arabidopsis chromosomes." Plant Cell 11(1): 31-42.

Hiragami, K. and R. Festenstein (2005). "Heterochromatin protein 1: a pervasive controlling influence." Cell Mol Life Sci 62(23): 2711-26.

Hsieh, T. F. and R. L. Fischer (2005). "Biology of chromatin dynamics." Annu Rev Plant Biol 56: 327-51.

Jackson, J. P., A. M. Lindroth, et al. (2002). "Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase." Nature 416(6880): 556-60.

Jackson, J. P., L. Johnson, et al. (2004). "Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in Arabidopsis thaliana." Chromosoma 112(6): 308-15.

Jacobs, S. A. and S. Khorasanizadeh (2002). "Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail." Science 295(5562): 2080-3.

James, T. C. and S. C. Elgin (1986). "Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene." Mol Cell Biol 6(11): 3862-72.

Jeddeloh, J. A., T. L. Stokes, et al. (1999). "Maintenance of genomic methylation requires a SWI2/SNF2-like protein." Nat Genet 22(1): 94-7.

Jenuwein, T. and C. D. Allis (2001). "Translating the histone code." Science 293(5532): 1074-80.

Johnson, L., X. Cao, et al. (2002). "Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation." Curr Biol 12(16): 1360-7.

Jones, R. S. and W. M. Gelbart (1993). "The Drosophila Polycomb-group gene Enhancer of zeste contains a region with sequence similarity to trithorax." Mol Cell Biol 13(10): 6357-66.

Kinoshita, T., A. Miura, et al. (2004). "One-way control of FWA imprinting in Arabidopsis endosperm by DNA methylation." Science 303(5657): 521-3.

Kotake, T., S. Takada, et al. (2003). "Arabidopsis TERMINAL FLOWER 2 gene encodes a heterochromatin protein 1 homolog and represses both FLOWERING LOCUS T to regulate flowering time and several floral homeotic genes." Plant Cell Physiol 44(6): 555-64.

Lachner, M., D. O'Carroll, et al. (2001). "Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins." Nature 410(6824): 116-20.

Li, Y., D. A. Kirschmann, et al. (2002). "Does heterochromatin protein 1 always follow code?" Proc Natl Acad Sci U S A 99 Suppl 4: 16462-9.

Lindroth, A. M., X. Cao, et al. (2001). "Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation." Science 292(5524): 2077-80.

Lindroth, A. M., D. Shultis, et al. (2004). "Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3." Embo J 23(21): 4286-96.

Maluszynska, J. and J. S. Heslop-Harrison (1991). "Localization of tandemly repeated DNA sequences in Arabidopsis thaliana." The Plant Journal 1(2): 159-166.

McKittrick, E., P. R. Gafken, et al. (2004). "Histone H3.3 is enriched in covalent modifications associated with active chromatin." Proc Natl Acad Sci U S A 101(6): 1525-30.

Mozer, B. A. and I. B. Dawid (1989). "Cloning and molecular characterization of the trithorax locus of Drosophila melanogaster." Proc Natl Acad Sci U S A 86(10): 3738-42.

Mylne, J. S., L. Barrett, et al. (2006). "LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of FLC." Proc Natl Acad Sci U S A 103(13): 5012-7.

Nakahigashi, K., Z. Jasencakova, et al. (2005). "The Arabidopsis heterochromatin protein1 homolog (TERMINAL FLOWER2) silences genes within the euchromatic region but not genes positioned in heterochromatin." Plant Cell Physiol 46(11): 1747-56.

Nakayama, J., A. J. Klar, et al. (2000). "A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis." Cell 101(3): 307-17.

Naumann, K., A. Fischer, et al. (2005). "Pivotal role of AtSUVH2 in heterochromatic histone methylation and gene silencing in Arabidopsis." Embo J 24(7): 1418-29.

Rangwala, S. H. and E. J. Richards (2004). "The value-added genome: building and maintaining genomic cytosine methylation landscapes." Curr Opin Genet Dev 14(6): 686-91.

Ratel, D., J. L. Ravanat, et al. (2006). "N6-methyladenine: the other methylated base of DNA." Bioessays 28(3): 309-15.

Richards, E. J. (1997). "DNA methylation and plant development." Trends Genet 13(8): 319-23.

Ronemus, M. J., M. Galbiati, et al. (1996). "Demethylation-induced developmental pleiotropy in Arabidopsis." Science 273(5275): 654-7.

Schmiedeberg, L., K. Weisshart, et al. (2004). "High- and low-mobility populations of HP1 in heterochromatin of mammalian cells." Mol Biol Cell 15(6): 2819-33.

Shen, W. H. (2001). "NtSET1, a member of a newly identified subgroup of plant SET-domain-containing proteins, is chromatin-associated and its ectopic overexpression inhibits tobacco plant growth." Plant J 28(4): 371-83.

Singh, P. B., J. R. Miller, et al. (1991). "A sequence motif found in a Drosophila heterochromatin protein is conserved in animals and plants." Nucleic Acids Res 19(4): 789-94.

Soppe, W. J., Z. Jasencakova, et al. (2002). "DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in Arabidopsis." Embo J 21(23): 6549-59.

Springer, N. M., C. A. Napoli, et al. (2003). "Comparative analysis of SET domain proteins in maize and Arabidopsis reveals multiple duplications preceding the divergence of monocots and dicots." Plant Physiol 132(2): 907-25.

Stewart, M. D., J. Li, et al. (2005). "Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment." Mol Cell Biol 25(7): 2525-38.

Takada, S. and K. Goto (2003). "Terminal flower2, an Arabidopsis homolog of heterochromatin protein1, counteracts the activation of flowering locus T by constans in the vascular tissues of leaves to regulate flowering time." Plant Cell 15(12): 2856-65.

Tschiersch, B., A. Hofmann, et al. (1994). "The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes." Embo J 13(16): 3822-31.

Vakoc, C. R., S. A. Mandat, et al. (2005). "Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin." Mol Cell 19(3): 381-91.

van den Berg, C., P. Weisbeek, et al. (1998). "Cell fate and cell differentiation status in the Arabidopsis root." Planta 205(4): 483-91.

Vongs, A., T. Kakutani, et al. (1993). "Arabidopsis thaliana DNA methylation mutants." Science 260(5116): 1926-8.

Yu, Y., A. Dong, et al. (2004). "Molecular characterization of the tobacco SET domain protein NtSET1 unravels its role in histone methylation, chromatin binding, and segregation." Plant J 40(5): 699-711.

Zemach, A., Y. Li, et al. (2006). "Different domains control the localization and mobility of LIKE HETEROCHROMATIN PROTEIN1 in Arabidopsis nuclei." Plant Cell 18(1): 133-45.

Zhang, X., J. Yazaki, et al. (2006). "Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis." Cell 126(6): 1189-201.

Zluvova, J., B. Janousek, et al. (2001). "Immunohistochemical study of DNA methylation dynamics during plant development." J Exp Bot 52(365): 2265-73.

CHAPTER 2

DNA methylation in developing Arabidopsis roots

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Introduction

DNA methylation is a conserved epigenetic silencing mechanism and is considered to be involved in for example defending the genome against transposable elements and retroviruses as well as controlling genomic imprinting and regulation of gene expression (Berger et al. 2003; Hsieh et al. 2005). DNA methylation was also shown to be critical for proper development of mammals as well as in plants (Finnegan et al. 1996; Lei et al. 1996; Jaenisch 1997; Finnegan et al. 1998).

In mammals, the global DNA methylation levels change markedly during development. For example in mouse, nuclei of blastula cells have a very low level of DNA methylation. This is the result of an active demethylation induced after fertilization of the egg cell (Jaenisch 1997). Also in mammalian embryonic stem cells the low DNA methylation level is maintained (Lei et al. 1996). However, when cells differentiate, DNA becomes methylated (Lei et al. 1996; Jaenisch 1997). This methylation is apparently essential, since for example a null mutation in the mouse DNA methyltransferase DNMT1 (DNA METHYLTRANSFERASE 1) results in embryonic lethality (Lei et al. 1996).

In plants, DNA methylation has extensively been studied in leaf cells (Finnegan et al. 1998; Soppe et al. 2002), but the relation between global DNA methylation and stem cell fate or differentiation has hardly been studied. Here we describe this relation for the different root cell types of Arabidopsis.

We focused on the root of Arabidopsis, because it is a rather thin organ in which all nuclei can be studied by confocal microscopy in an intact organ. In the apical meristem a stem cell niche contains the quiescent centre (QC), that consists of 4 cells, and is surrounded by the stem cells for the different tissues (Dolan et al. 1993). The different root tissues are from the outside to the inside: epidermis, cortex, endodermis, pericycle and vascular bundle. At the apex of the root, the columella and lateral root cap are located (figure 1.c). The QC cells do not (or very

rarely) divide and they function as an organiser of the stem cell niche as they are essential to maintain the stem cell fate in these cells (van den Berg et al. 1995). The stem cell niche together with the adjacent zone of dividing cells form the meristem at the apex of the root (figure 1.c). When the meristematic cells stop dividing, they start to elongate (elongation zone) and subsequently obtain their fully differentiated fate (differentiated zone, figure 1.c) (Dolan et al. 1993). Root tissues are composed of cell files in which cells are in subsequent stages of development and this facilitates analysis on DNA methylation levels during the development from stem cell to fully differentiated cell.

DNA methylation in Arabidopsis nuclei is most prominent in the heterochromatin (Fransz et al. 2002; Probst et al. 2003). Heterochromatin is restricted to the centromeric and pericentromeric regions and the nuclear organisers (NORs). Therefore, upon staining of the DNA with DAPI or PI, the heterochromatic regions are visible as (maximally) 12 more intensely stained structures that are named chromocentres (Fransz et al. 2002). However, in most interphase nuclei fewer chromocentres are visible due to their tendency to cluster (Fransz et al. 2002).

Also in plants, DNA methylation has been shown to be important for proper development. Mutations in for example Arabidopsis DNA methyltransferase MET1 (METHYLTRANSFERASE 1) that lead to a markedly reduced DNA methylation level cause; smaller plant size, altered leaf size and shape, decreased fertility, reduced apical dominance and altered flowering time (Finnegan et al. 1996). Several studies indicate that also during plant development, the overall DNA methylation levels markedly vary. For example, active demethylation of DNA occurs in embryo's of *Silene latifolia* when seeds start to germinate (Zluvova et al. 2001). Further, DNA demethylation of the highly methylated central zone of the shoot meristem *Silene latifolia* occurs when the transition from vegetative to floral meristem is initiated (Zluvova et al. 2001). The importance of DNA methylation in development is underlined by the observation that during vernalization of wild type Arabidopsis, a decrease in DNA methylation is induced, perhaps due to reduced maintenance methylation during replication (Burn et al. 1993; Finnegan et al. 1998). The above described studies indicate that development

is correlated with changes in DNA methylation. However, a good description of such changes during all steps of a differentiation process is lacking.
In most eukaryotes various enzymes are involved in DNA methylation. In general these enzymes add a methyl group at position 5 of the nucleotide cytosine although in a few cases also adenine can be methylated (Ratel et al. 2006). Cytosine methylation (5mC) in a symmetrical CG context is an evolutionarily conserved DNA modification that is found in vertebrates, plants and some fungi. In addition, plants have significant level of cytosine methylation also at non-CG sequences, which include symmetrical CNG and asymmetrical CNN sequences (Finnegan et al. 2000; Bird 2002).
Methyltransferase activities can be aimed at maintenance methylation or are methylating *de novo* synthesized DNA.
Arabidopsis has several DNA methyltransferases. DRM1 and DRM2 (DOMAINS REARRANGED METHYLASES) are especially involved in *de novo* methylation and are orthologs of the mammalian *de novo* methyltransferase DNMT3 (DNA METHLYTRANSFERASE 3) (Choi et al. 2002). Surprisingly knockout (double) mutants of DRM1 or DRM2 do have a wild type phenotype. MET1 and CMT3 (CHROMOMETHYLASE 3) are especially involved in maintenance methylation although MET1 and CMT3 also have *de novo* methylation activity (Aufsatz et al. 2004; Schob et al. 2006). MET1 especially methylates CG sequences, which are the most abundant methylated sequences in Arabidopsis; it is an ortholog of the mouse DNA methyltransferase DNMT1. Loss of function mutations cause pleiotropic effects (see above) (Takeda et al. 2006). CMT3 is involved in CNG methylation and loss of function mutants have a wild type phenotype (Bartee et al. 2001; Lindroth et al. 2001; Cao et al. 2002).

In this chapter we studied the changes in overall DNA methylation level during the development of the different root tissues as well as in the stem cell niche. We show that during differentiation of root cells the overall DNA methylation level increases. Surprisingly, QC and stem cells have the highest level of DNA methylation. By using a set of mutants in which DNA methylation is affected, first

insight is obtained in the enzymes that are involved in establishing the hyper DNA methylation level in the stem cell niche.

Results

Levels of cytosine methylation during development of root tissues

The global level of cytosine methylation during development of Arabidopsis root cell types was studied by whole mount immunocytology. Roots of 5 day old Arabidopsis seedlings were incubated with a 5-methylcytidine (5mC) monoclonal antibody and DNA was stained with propidium iodide (PI). In figure 1.a the meristematic region of a root is shown. The red signal represents the DNA stained by PI and the green signal the methylated DNA which appears yellow on the merged picture. The antibody is specific for 5mC as in mutants with a markedly reduced 5mC level, the signal is also reduced (see for example *ddm1*, figure 4.d). The DNA methylation level in nuclei varies in different cell types, but always the highest DNA methylation level does occur in the heterochromatic chromocentres of a nucleus, shown as bright yellow spots in the merged images (figure 1.a, c-f). The euchromatin is also labelled by the 5mC antibody albeit at a markedly lower level than in the heterochromatic chromocenters and the euchromatic signal is rather equally distributed.

Overall DNA methylation in nuclei markedly varies during development. Quiescent Centre (QCs) nuclei and nuclei of stem cells have the highest level of cytosine methylation. Figure 1.f shows the region of the stem cell niche and nuclei of QC cells (◗) and those of cortex/endodermis stem cells (*) have a similar high amount of DNA methylation. Otherwise, nuclei of cells in the division zone, (figure 1.f) especially those of the vascular tissue, have the lowest DNA methylation level. Cytosine methylation increases again in the elongation zone (figure 1.e) and stays high in the differentiation zone (figure 1.d).

The daughter cell of a columella stem cell stops dividing and rapidly obtains a fully differentiated state. This is in contrast to the formation of other root cell types as those daughter cells in these cases remain mitotically active. The nuclei of columella stem cells as well as the nuclei of all columella cells have a rather high DNA methylation level. So in contrast with the other root tissues a zone with a

reduced DNA methylation level is not present in this tissue. This is consistent with the absence of a division zone and the rapid differentiation process.

The difference in the level of cytosine methylation per nucleus might be caused by an increased methylation of the genome, but could also be due to differences in total DNA content, for example by endopolyploidy. Therefore the DNA content of nuclei was quantified by the averaging 3D method developed by Willemse et al. (Willemse et al. 2007). The columella cells are known to have 2C nuclei and are therefore used as a reference (Sugimoto-Shirasu et al. 2005). QC cells and initials (cortex/endodermis, epidermis/lateral root cap and columella initials) were shown to have a DNA content of 2C, whereas the initials of the vascular tissue were shown to have a DNA content of 4C. Most initials have a 2C (4C for vascular tissue initials) content reflecting that they are in G1. Only about 2% of the initials have a DNA content that is more than 2C, but never over 4C, indicating that this small subset is in S or G2 phase. This is consistent with the fact that initials have a lower frequency of division than other meristematic cells (Fiorani et al. 2006). The nuclei of ground tissue cells (cortex and endodermis) in the division zone have a DNA content that varies between 2C to 4C, whereas the vascular bundle nuclei in this zone have even a 4C to 8C content. Since vascular initials are endotetraploid these cells are mitotically active. A more detailed description of DNA quantification of nuclei in the root meristem is described in Willemse et al. (Willemse et al. 2007).

Since QC nuclei have a 2C content, the higher level of DNA methylation in the QC nuclei reflects that the genome in those cells is indeed more methylated than that of any other cell in the root. Furthermore, the vascular cells with the highest DNA content have the lowest methylation level and so the difference between QC and those cells is even higher than is indicated by figure 1.

Apparently the level of methylation is lower in cells that are mitotically active and increases when cells differentiate. To compare DNA replication activity and DNA methylation level, roots were incubated with 5'-Bromo-2'-deoxy-uridine (BrdU). BrdU incorporated in genomic DNA was subsequently visualized by whole mount immunocytology and appears as green signal in the nucleus (figure 1.b). As shown in figure 1.b, QCs cells, the surrounding stem cells, columella cells, lateral root cap

cells and cells in the elongation zone hardly incorporated any BrdU, whereas cells from the division zone have a high level of BrdU. The patchy pattern in this zone indicates that not all cells have gone through a complete cell cycle in the root meristem after the addition of BrdU. Comparing the DNA methylation level and the BrdU incorporation, we can conclude that the division zone has a high DNA replication activity, whereas its DNA methylation status is the lowest. This suggests that DNA methylation is not fully maintained in mitotically active cells of the meristem and that DNA methylation is re-established when cells switch from division to elongation.

This experiment also shows that cells of the division zone, including those of the vascular bundle are very well accessible for antibodies and so the observed low level of DNA methylation cannot be due to poor accessibility of these cells.

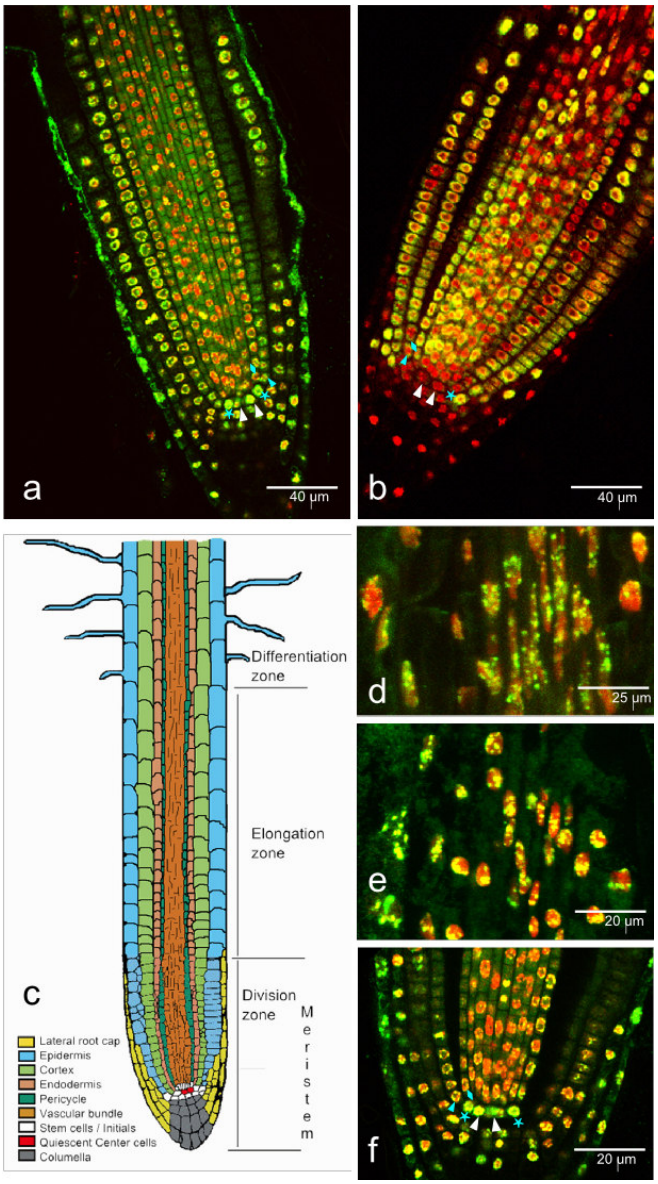

Figure 1: a,d,e,f. Whole mount immunodetection of methylated DNA in Arabidopsis seedlings. a. Root tip. d. youngest part of differentiation zone. e. Elongation zone. f. Root meristem. c. Schematic representation of an Arabidopsis root. b. Whole mount immunodetection of Bromouridine in Arabidopsis roots. White arrows represent QC cells; blue stars cortex/endodermis initials; blue arrows cortex daughters; blue rhombus endodermis daughter cells.

Transition from stem cell to daughter cell

As shown in figure 1.a, the DNA methylation level in nuclei of cells of the division zone is markedly lower than in the corresponding stem cells. To obtain clues whether this reduced methylation is caused by an active (demethylation by DNA demethylases) or passive demethylation (reduced maintenance methylation during replication), the methylation level of initials and their daughter cells is semi-quantified.

Initials of cortex/endodermis, cortex first daughter cells and endodermis first daughter cells were analyzed. Cortex/endodermis stem cells divide first anticlinally to generate a stem cell and a basal daughter cell. The basal daughter cell then undergoes a periclinal division to form the first cells of the cortex and endodermis lineages (Dolan et al. 1993). So, at least two divisions have occurred when endodermis and cortex daughter cells are formed from a cortex/endodermis initial. In case maintenance methylation would not occur during replication these daughter cells should have at least a 4 times lower level of DNA methylation than the cortex/endodermis initial.

Z-stacks of the region of the root tip including QC cells, the initials and some endodermis daughter cells were made by confocal microscopy and images were analyzed by ImageJ (v.1.37, National Institutes of Health, USA. http://rsb.info.nih.gov/ij) and the plugin Color Histogram. This plugin gives the mean and standard deviation for each channel of a selected area.

The overall level of DNA methylation of cortex/endodermis initials and their daughter cells was determined. Both the DNA methylation signal and PI signal were quantified for a square covering the entire nucleus. The DNA methylation signal was divided by the DNA intensity value (PI signal). Cortex/endodermis initials have a DNA methylation level of 0.8 (arbitrary units) that is about 2 times higher than that of their endodermis daughter cell with DNA methylation level of 0.4 and about 2,5 times higher than their cortex daughter cell with a DNA methylation level of 0.3. Since the DNA methylation level is only 2 times reduced DNA methylation has partially been maintained during replication. Therefore it seems unlikely that active demethylation plays a role in the decrease of DNA methylation level.

The present comparison has only been made for 4 initials - daughter cells pairs but in all cases the daughter cells had a 2- 2.5 times lower DNA methylation level than the initial, however this should be further confirmed in a more extended study.

Both euchromatin and heterochromatin have an increased methylation level in QC nuclei

To semi-quantify the DNA methylation level of heterochromatin and euchromatin in nuclei of QC cells, they were compared with columella cells as both are non dividing and have a similar DNA content (Willemse et al. 2007). To do this, z-stacks of confocal images were analysed with the plugin Color Histogram and squares of 10 x 10 pixels were selected for heterochromatic and euchromatic areas as well as background areas and DNA intensity as well methylation level were quantified. In both cell types, heterochromatin was shown to be 1.4±0.2 and 1.5±0.1 times more intense in PI signal than euchromatin. Ttest analysis showed that the difference between euchromatin and heterochromatin is significant with a probability of 0.0001.

The heterochromatin in nuclei of QC and columella cells has a DNA methylation level of 1.2±0.2 and 0.6±0.1 respectively; and the euchromatin 0.4±0.04 for QC nuclei and 0.2±0.04 for columella nuclei. So In both cell types, the heterochromatin has a 3 fold higher methylation level than the euchromatin. Since this relative difference is similar, it is probable that the increased higher methylation of QC nuclei is due to a global effect and is not specific for example to the heterochromatin. The increased methylation level in both euchromatin and heterochromatin of QC nuclei in comparison to columella nuclei was analysed by the Ttest and shown to have a probability in both cases much lower than 0.05, demonstrating that these differences are significant.

At first glance, the areas that are hypermethylated in QC nuclei seem larger than the chromocenters, which seems not to be the case in columella nuclei (figure 2. a-c, e-g). This could be due to methylated DNA around the heterochromatic chromocentres. Alternatively, the higher level of DNA methylation of the chromocentre might lead to overexposure which artificially suggests that the hypermethylated region is larger. To investigate this more accurately, the images of

the region surrounding the QC cells were analyzed by another plugin of ImageJ: the Color Profiler. This plugin gives the profile of each channel along a line. 4 lines were analysed along the diagonals of, and a vertical and horizontal line through the centre of the 10 x 10 pixels squares.

A total of 45 chromocentres from 8 QC cells and 22 chromocentres from 4 columella cells were analysed. 71% and 68% of the chromocentres of the nuclei of QC and columella cells, respectively, have identical profiles for the PI signal and the DNA methylation (figure 2. d, h). Whereas in about 30% of the chromocentres of both QC cells and columella cells the region of hyper DNA methylation is slightly larger than the chromocentre. Therefore the region of DNA methylation in both cell types coincides in majority of the cases with the chromocentres and in this respect there is no significant difference between QC nuclei and columella nuclei.

Hypermethylation of QC nuclei does not lead to a higher relative heterochromatin fraction

To determine whether hypermethylation of DNA in QC nuclei correlates with an increased amount of heterochromatin, we quantified the relative heterochromatin fraction (RHF) in QC and columella cells. Z-stacks were made of the root tip of PI stained Arabidopsis seedlings. The middle slide of each chromocenter as well as the middle slide of each nucleus was identified. The area of all chromocenters of a nucleus as well as of the nucleus (middle slide) was measured by using the rectangular selection tool of ImageJ (v.1.37, National Institutes of Health, USA. http://rsb.info.nih.gov/ij). The percentage of heterochromatin was calculated by dividing the sum of heterochromatin areas by the area of the nucleus.

QC cells have an RHF of 14.5±1.9% (4 nuclei) and columella cells of 17.8±1.6% (4 nuclei). This indicates that both cell types contain the same amount of heterochromatin and the higher level of cytosine methylation therefore does not cause higher heterochromatin content in QC nuclei.

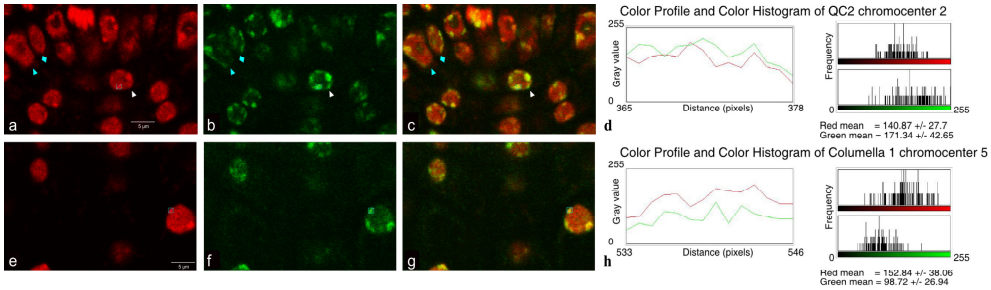

Figure 2: Whole mount immunodetection of methylated DNA on Arabidopsis seedlings. a-c. QC cells, surrounding initials and first daughter cells. White arrows indicate QC cells; blue arrows cortex daughters; blue rhombus endodermis daughter cells. d. Color profile and histogram of a chromocentre (n°2) of a QC nucleus, pink line and blue square on pictures a-c, e-g. Columella cells. h. Color profile and histogram of a chromocentre (n°5) of a columella cell, pink line and blue square on pictures e-g. a+e. Propidium Iodide signal, DNA staining. b+f. Methylated DNA signal. c-g. Merged pictures.

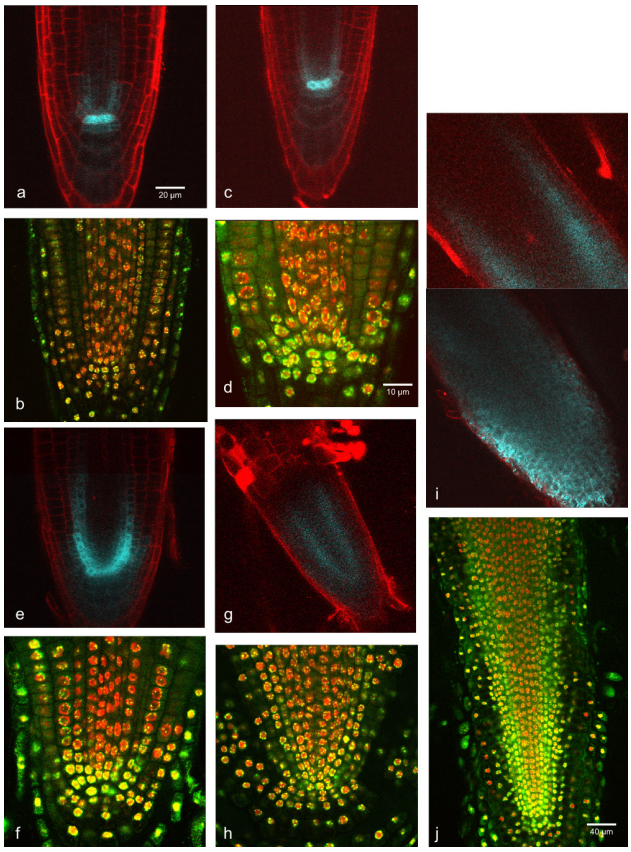

Figure 3: QC25::CFP expression (a+c+e+g+i+j) and whole mount immunodetection of methylated DNA (b+d+f+h+j) in Arabidopsis roots after induction of QC cell fate in endodermal cells by exogenous application of 1 µM 2,4 D auxin. a+b. Untreated seedlings. c+d. seedlings after 1 day of treatment. e+f. seedlings after 3 days of treatment. g+h. seedlings after 5 days of treatment. i+j. seedlings after 12 days of treatment.

QC cell fate correlates with a high level of cytosine methylation

The hypermethylation in QC nuclei might be due to a lack of cell division by which 5mC has accumulated during the lifespan of the plant. However, the high level of cytosine methylation might also be part of the QC cell identity. Therefore we induced a QC fate in the endodermal cells by the procedure developed by Sabatini et al (1999).

In roots the maximal auxin level occurs in the region around the QC. However, in roots treated with the auxin polar transport inhibitor 2,3,5-triiodobenzoic acid (TIBA), there is a shift of the auxin maximum which extends as a cup-shaped domain. This induces a QC cell fate in the former endodermal cells (Sabatini et al. 1999). The former cortical cells adjacent to the new QC cells become columella initials and the former epidermal cells obtain a columella fate. This reprogramming can also be induced by treatment with the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D), which can not be transported through the root (Sabatini et al. 1999).

2,4-D (or TIBA) was applied to 2 day old seedlings. Two markers were used to visualise the reprogramming in the primary root tip: activity of the QC25 promoter *(QC25::CFP* expression), that is specifically expressed in QC cells and starch accumulation, which is a marker of differentiated columella cells (Sabatini et al. 1999). The seedlings were analysed 1, 3, 5 and 12 days after treatment. Half of the seedlings were used to visualise the cell fate markers and the other half was fixed for whole mount immunodetection of methylated DNA (as both methods are not compatible).

The reprogramming (based on *QC25* expression) started after one day of treatment (figure 3.c) and was clearly visible after 3 days of treatment with TIBA or 2,4D. *QC25::CFP* expression was visible in the ex-cortex/endodermis initials and in some ex-endodermis cells (figure 3.e). Further starch started to accumulate in the ex-epidermal cells (data not shown). This reprogramming progressed through time and at 12 days a high amount of *QC25::CFP* expression is detected in most ex-endodermis cells of the root meristem (figure 3.i)

Immunodetection of methylated DNA from 1 to 12 days after the treatment (figure 3.b,d,f,h,j), showed that the highest amount of methylation is in the former

endodermis cells that obtained a QC identity. The former cortical cells, now columella cells, have a DNA methylation level similar to the one observed for columella initials, under normal conditions, and that is quite high as well and also showing starch accumulation (data not shown). The increase of cells expressing QC marker and the increase of cells expressing high level of DNA methylation were shown to coincide in time and to be both located in ex-endodermal cells. Therefore a high level of cytosine methylation appears to be an integral part of QC identity.

DRM1/DRM2 and HDA1 are involved in hypermethylation level of QC nuclei

Since the endodermis cells become hypermethylated when they obtain a QC fate it seems probable that *de novo* methylation plays an important role in establishing this high 5mC level. Therefore we tested whether in a *drm1drm2* double mutants hypermethylation can be induced in the endodermis by auxin/TIBA treatment. 12 days after exogenous auxin application, the *drm1drm2* double mutant showed starch accumulation in the former epidermal cells (data not shown) which suggests that a QC cell fate is induced in the former endodermal cells. However, these cells did not have a marked increased DNA methylation level (figure 4.f) suggesting that DRM1 and DRM2 are essential for *de novo* methylation in the endodermal cells that obtain a QC cell fate. Since these former endodermal cells appear to function as QC, hypermethylation in QC nuclei is most likely not essential to perform this function.

Next it was observed that in *drm1drm2* double mutant, the DNA methylation in the root is markedly reduced, especially in the QC and initials that do not appear as the highest DNA methylated nuclei anymore (figure 4.b). Root morphology and patterning as well as starch accumulation under normal growth conditions appear to be similar to that of wild-type plants suggesting that the QC is still functional in *drm1drm2* despite the reduced DNA methylation level.

Whole mount immunodetection of DNA methylation and starch staining was also performed on mutants of the maintenance DNA methyltransferases MET1 and CMT3. All loss of function mutants of these genes that we tested (see Material &

Methods) had a wild-type like hyper methylation level in their QC (data not shown). A QC identity could be induced in the endodermal cells and in these cells also a hyper DNA methylation level was induced (data not shown). We therefore conclude that the maintenance DNA methyltransferases are not essential to induce the increased DNA methylation level in QC nuclei.

Since it is known that histone deacetylases can facilitate DNA methylation (Earley et al. 2006), we started to analyse several histone deacetylase mutants. In one case, namely *hda1 (histone deacetylase 1)*, we observed a marked affect on the DNA methylation level of QC nuclei. Like in *drm1drm2* double mutant, we noticed a lower DNA methylation level more specifically for QC and initial cell nuclei (figure 4.c). In a similar way as *drm1drm2* double mutants, we tested if hypermethylation could be induced in the endodermal cells by auxin/TIBA treatment. The loss-of- function mutants show a reduced level of DNA methylation of QC and initials after 5 day treatment (figure 4.g). However after 12 days of exogenous auxin application, in contrast to the *drm1drm2* double mutant *hda1* mutants showed an intermediate level of DNA methylation in newly formed QC cells (figure 4.h).

This suggests that deacetylation of histones by HDA1 is important for DNA methylation of QC nuclei.

DDM1 (DECREASE IN DNA METHYLATION 1), is an ATPase/helicase Swi2/Snf2 involved in maintenance of DNA methylation (Jeddeloh et al. 1999) and loss of DDM1 function is known to cause a 70% reduction of genomic cytosine methylation (Vongs et al. 1993). Contrarily to *drm1drm2* or *hda1* which have a rather specific reduction in DNA methylation level in the stem cell niche, we observed in *ddm1* mutants an overall reduction of DNA methylation. However, the cells with the highest DNA methylation remain the QC and the initials (figure 4.d).

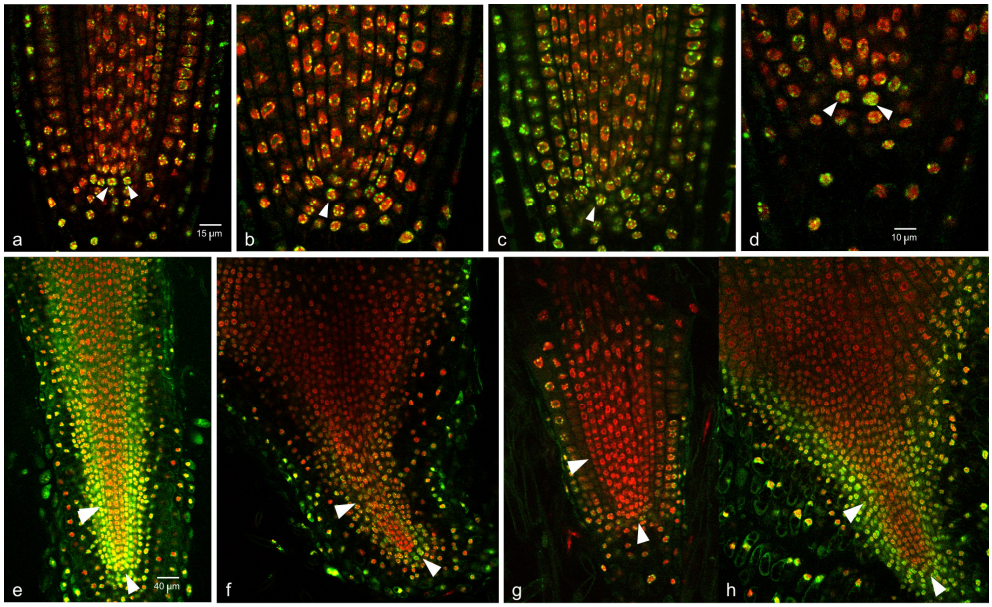

Figure 4: Whole mount immunodetection of methylated DNA on Arabidopsis mutants. a. Root meristem of Columbia wild-type 14 dpg seedling. b. Root meristem of *drm1drm2* mutant 14 dpg seedling. c. Root meristem of *hda1* mutant 7 dpg seedling. d. Root meristem of *ddm1-1* mutant 4 dpg seedling. e. Root tip of 14 dpg Columbia seedling after 12 days of treatment by 1 µM 2,4 D auxin. f. Root tip of 14 dpg *drm1drm2* seedling after 12 days of treatment by 1 µM 2,4 D auxin. g. Root tip of *hda1* mutant after 5 days of treatment by 1 µM 2,4 D auxin. h. Root tip of 14 dpg *hda1* mutant seedling after 12 days of treatment by 1 µM 2,4 D auxin. White arrows represent QC cells.

Discussion

Here we describe that during differentiation of root cell types the overall DNA methylation levels increases when the cells switch from the meristematic phase to the elongation phase. In addition, the nuclei of cells of the stem cell niche are hyper methylated, in particular those of the QC cells and the cortex/endodermis initials. Apparently, DNA hypermethylation level correlates with QC identity but it is not required for the organizer function of the QC. The *de novo* methyltransferases DRM1 and DRM2 as well as the histone deacetylase HDA1 are involved in DNA hypermethylation of QC cells and initials.

Differentiated cells appear to be highly methylated whereas dividing cells are much less methylated in the Arabidopsis root and this is consistent with a few other studies. For example Finnegan et al. reported that Arabidopsis young seedlings have a lower overall DNA methylation level when compared with mature leaves (Finnegan et al. 1998).

The high methylation level of root stem cells is counterintuitive as these cells are pluripotent. In contrast, nuclei of mammalian blastulas and embryonic stem cell are hypomethylated and these become more methylated as the cells differentiate (Lei et al. 1996; Jaenisch 1997). Therefore with respect to their low overall methylation level, blastocysts, resemble more the Arabidopsis root cells of the division zone than the root initials. It would be interesting to see whether nuclei of mammalian stem cells in adult organisms, for example those of skin and intestine, would be hyper or hypomethylated.

The hypermethylated level of QC cells may reflect the very specialised function of those cells. The QC appears to be necessary to maintain the identity of the surrounding stem cells, most likely by short distance signalling (van den Berg et al. 1995; van den Berg et al. 1997). To fulfil this role, QC cells might need the activity of fewer genes, and the higher methylation level (in the euchromatin) might contribute to gene silencing. However, transcriptome analysis of the Arabidopsis QC cells did not reveal that markedly fewer genes are expressed in these cells in comparison to other cell types (Nawy et al. 2005). However, it should also be noticed that the marker used to isolate QC protoplasts, AGL42, was not shown to

be enriched in the data set (Nawy et al. 2005). Therefore these studies may not have been accurate enough to reveal such a reduced number of expressed genes.
Overall DNA methylation levels have not yet been studied in the Arabidopsis shoot apical meristem of Arabidopsis. However, the central zone in the shoot apical meristem of *Silene* is highly methylated (Zluvova et al. 2001). The central zone of the shoot apical meristem has an organizing function like the root QC cells and so the hypermethylation of nuclei seems a more general property of within cells that function as an organizer within a stem cell niche.
DRM1/DRM2 and HDA1 were shown to be involved in the establishment of DNA hypermethylation in QC nuclei. *De novo* methylation by DRM methyltransferases might occur during early embryogenesis. Since DRM methyltransferases have been shown to be involved in non-CG methylation it is possible that QC nuclei have an exceptionally high level of methylation level of non-CG sequences.
In general when DNA is methylated, histones are hypoacetylated and when demethylated, histones are acetylated. Deacetylation of histones by HDA1 might therefore facilitate methylation of DNA The involvement of a histone deacetylase in DNA methylation suggests that methylation of histones could actually preceed DNA methylation. A link between histone methylation and DNA methylation in Arabidopsis nuclei has been previously suggested (Soppe et al. 2002).
We also showed that hypermethylation of DNA is part of the QC identity while it is not required for the acquisition of QC fate in root endodermal. This induced increase of methylation depends on the *de novo* DNA methyltransferases DRM1/DRM2. Also the nuclei of the QC cells of the DRM double mutant are not hypermethylated. This suggests that the QC pattern of hypermethylation is established by *de novo* methylation when the QC is formed in the embryo.
The root meristem of the *drm1drm2* has a similar organisation when compared to that of wild type. The QC of *drm1drm2* functions normally as an organizer of the stem cell niche. This implies that the hypermethylation status is not essential for this function. If the hypothesis that hypermethylation is used to reduce gene expression is valid, it would imply that increased expression of genes for which a low expression level is sufficient does not disturb QC functioning.

To better understand the function of hypermethylation in the stem cell niche it will be important to determine which sequences are methylated. DNA methylation mapping was performed on the genome of Arabidopsis (Zhang et al. 2006). Applying this technique on isolated QC protoplasts obtained my methods as developed by Birnbaum et al. (2003) and Naway et al. (2005), it would be possible to obtain this information and this could improve our understanding of QC cell functioning.

Material & Methods

Plant material and growth conditions

The Arabidopsis Columbia ecotype was used as wild-type. ddm1-1 mutant was kindly provided by Dr T.Kakutani; met1-3, cmt3-7, hda1 mutants in WS were kindly provided by Dr. Paul Fransz; drm1drm2, demeter, ros1 and hda1 in Columbia were obtained from the NASC stock center.

Plants were grown vertically for 4-5 days post-germination except otherwise stated on 0.8% agar plates containing 2.2g Murashige and Skoog 10 salts with vitamins (Duchefa) plus 1 % sucrose at pH 5.8 in LD (16h light/ 8h dark) conditions at 23-24 °C.

Whole mount immudetection of methylated DNA

Protocole was adapted from whole mount immunofluorescence in situ of interphase nuclei of Arabidpsis from Bauwens et al (Bauwens, 1994). 4-5 dpg old seedling were fixed in a glass vial in 1% formaldehyde and 10% DMSO in fixation buffer (1.1 x PBS; 0.067 M EGTA at pH 7.5 adjusted with NaOH) for 1h with 20 min vacuum at room temperature. They were washed 2 x 10 min with anhydrous MetOH followed by 4 x 10 min anhydrous EtOH. The last ethanol wash was discarded and the sample was covered with with fresh anhydrous EtOH. The sample was left at –20°C for no longer than 1–4 days. The EtOH was removed and

the seedlings were rinsed 2 times with anhydrous EtOH. The sample was incubated for 30-40 min in a solution of anhydrous EtOH and xylene (1:1) at room temperature. Then it was rinsed 2 times with anhydrous EtOH followed by 2 times with anhydrous MetOH. MetOH was replaced by a 1:1 mixture of methanol and PBT (PBS with 0.1% (v/v) Tween ® 20) containing 1% (v/v) formaldehyde and the sample was incubated for 5 min. It was postfixed a second time for 25 min with PBT containing 1% formaldehyde. The fixative was removed and the sample rinsed 5 times with PBT. At the last step the sample was transfered to a microcentrifuge tube with PBT. PBT was removed and the sample was incubated with 40 µg/ml proteinase K in PBT for 5 min at 37°C. The seedlings were postfixed with PBT containing 1 % formaldehyde for 25 min at room temperature. Then rinsed 5 times with PBT and washed 10 min with a 1:1 mixture of PBT and (50% formamide in 2xSSC). The sample was rinsed 2 times with 50% formamide in 2xSSC. And the DNA of the seedlings was denatured in 500 µl of 50% formamide in 2xSSC in boiling water for 7 min. The sample was placed immediately on ice for 5 min and washed in ice-cold PBT 3 times 5 min each. Seedlings were rinsed with 1% BSA in PBS and incubated in 1% BSA in PBS for 30 min at 37°C. The sample was incubated with mouse monoclonal 5-methylcytidine antibody (1:300) (Eurogentec) in the same buffer overnight at 16°C in the cold room. The following day, seedlings were washed 4 times 15 min in PBT followed by 4 washes of 15 min with 1% BSA in. The sample was incubated with anti-mouse –IgG-FITC (1:50 or 1:25) (Jackson Immunoresearch laboratories) overnight at 16°C in the cold room. The next day, sample was washed 4 times 15 min in PBT. It was then incubated with 0.5 - 1 µg/ml Propidium Iodide in PBS for 30 min at room temperature. Seedlings were mounted on slides in Citifluor. A space between slide and cover slip was created by using strips of tape to keep seedlings intact. Seedlings were observed by confocal microscopy.

Whole mount immudetection of 5-Bromo-2'-deoxy-uridine

Plants were grown vertically on normal plates till 3 dpg and then transfer for 5 to 24h on 1 mM BrdU labeling agent (Roche) agar plates (0.8% Daishin agar, 2.2g Murashige and Skoog 10 salts with vitamins (Duchefa) plus 1 % sucrose at pH 5.8) in LD (16h light/ 8h dark) conditions at 23-24 °C. Whole mount immunodetection of Arabidopsis seedlings was performed as described for detection of methylated DNA. BrdU was detected with a mouse monoclonal anti-BrdU primary antibody

(Roche) followed by detection the next day with an anti-mouse-Ig-FITC secondary antibody (Roche)

Induction of QC cell fate in endodermal cells

Plants were grown first vertically on basic agar plate and transfer on the 2 dpg to plates containing 30 µM 2,3,5-triiodobenzoic acid (TIBA) or 1 µM 2,4-dichlorophenoxy acetic acid (2,4 D). Whole mount immunodetection of methylated DNA was performed as described. QC25-CFP expression was observed by confocal microscopy. Starch staining was performed by incubating for few minutes the seedlings in Lugol solution (Merck) and they were then mounted on slides in choral hydrate solution (8 g chloral hydrate, 1 ml glycerol and 2 ml water) for clearing. Seedlings were observed on Nikon DIC Normaski microscope with a 10x or 20x objective.

Confocal Imaging

All confocal images were acquired on a Zeiss 510 inverted microscope using a 40x/1.3 oil immersion objective. Image resolution was always higher than the theoretical limit for light microscopy to insure no data was missed.

Quantification analysis

Quantification analysis was performed using ImageJ (v.1.37, Rasband, W.S, National Institutes of Health, USA. http://rsb.info.nih.gov/ij., 1997-2006) with two specific plugins: Color Profiler and Color Histogram. (Dimiter Prodanov (D.Prodanov@lumc.nl).Department of Neurosurgery. Leiden University Medical Center. The Netherlands). The Color profiler plugin gives the profile of each channel for a selected area in our case the profile was performed along a line of 1 pixel width. The Color Histogram plugin gives the mean and standard deviation for a selected area. In our case the area is a 10 x 10 pixels square or the all nucleus. Background for the 10x10 pixels area was taken in the cytoplasm of the cell; and for the all nucleus a similar square was taken outside the cell. The area of the nucleus was calculated with the Zeiss LSM software.

Ttest on means to test if the differences observed are significant and Fischer test on standard deviations to know if the Ttest should be done for 2 samples with an equal or unequal variance were calculated in Microsoft Excel.

References

Aufsatz, W., M. F. Mette, et al. (2004). "The role of MET1 in RNA-directed de novo and maintenance methylation of CG dinucleotides." Plant Mol Biol 54(6): 793-804.

Bartee, L., F. Malagnac, et al. (2001). "Arabidopsis cmt3 chromomethylase mutations block non-CG methylation and silencing of an endogenous gene." Genes Dev 15(14): 1753-8.

Berger, F. and V. Gaudin (2003). "Chromatin dynamics and Arabidopsis development." Chromosome Res 11(3): 277-304.

Bird, A. (2002). "DNA methylation patterns and epigenetic memory." Genes Dev 16(1): 6-21.

Birnbaum, K., D. E. Shasha, et al. (2003). "A gene expression map of the Arabidopsis root." Science 302(5652): 1956-60.

Burn, J. E., D. J. Bagnall, et al. (1993). "DNA methylation, vernalization, and the initiation of flowering." Proc Natl Acad Sci U S A 90(1): 287-91.

Cao, X. and S. E. Jacobsen (2002). "Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes." Proc Natl Acad Sci U S A 99 Suppl 4: 16491-8.

Choi, Y., M. Gehring, et al. (2002). "DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in arabidopsis." Cell 110(1): 33-42.

Dolan, L., K. Janmaat, et al. (1993). "Cellular organisation of the Arabidopsis thaliana root." Development 119(1): 71-84.

Earley, K., R. J. Lawrence, et al. (2006). "Erasure of histone acetylation by Arabidopsis HDA6 mediates large-scale gene silencing in nucleolar dominance." Genes Dev 20(10): 1283-93.

Finnegan, E. J., W. J. Peacock, et al. (1996). "Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development." Proc Natl Acad Sci U S A 93(16): 8449-54.

Finnegan, E. J., R. K. Genger, et al. (1998). "DNA Methylation in Plants." Annu Rev Plant Physiol Plant Mol Biol 49: 223-247.

Finnegan, E. J. and K. A. Kovac (2000). "Plant DNA methyltransferases." Plant Mol Biol 43(2-3): 189-201.

Fiorani, F. and G. T. Beemster (2006). "Quantitative analyses of cell division in plants." Plant Mol Biol 60(6): 963-79.

Fransz, P., J. H. De Jong, et al. (2002). "Interphase chromosomes in Arabidopsis are organized as well defined chromocenters from which euchromatin loops emanate." Proc Natl Acad Sci U S A 99(22): 14584-9.

Hsieh, T. F. and R. L. Fischer (2005). "Biology of chromatin dynamics." Annu Rev Plant Biol 56: 327-51.

Jaenisch, R. (1997). "DNA methylation and imprinting: why bother?" Trends Genet 13(8): 323-9.

Jeddeloh, J. A., T. L. Stokes, et al. (1999). "Maintenance of genomic methylation requires a SWI2/SNF2-like protein." Nat Genet 22(1): 94-7.

Lei, H., S. P. Oh, et al. (1996). "De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells." Development 122(10): 3195-205.

Lindroth, A. M., X. Cao, et al. (2001). "Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation." Science 292(5524): 2077-80.

Nawy, T., J. Y. Lee, et al. (2005). "Transcriptional profile of the Arabidopsis root quiescent center." Plant Cell 17(7): 1908-25.

Probst, A. V., P. F. Fransz, et al. (2003). "Two means of transcriptional reactivation within heterochromatin." Plant J 33(4): 743-9.

Ratel, D., J. L. Ravanat, et al. (2006). "N6-methyladenine: the other methylated base of DNA." Bioessays 28(3): 309-15.

Sabatini, S., D. Beis, et al. (1999). "An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root." Cell 99(5): 463-72.

Schob, H. and U. Grossniklaus (2006). "The first high-resolution DNA "methylome"." Cell 126(6): 1025-8.

Soppe, W. J. J., Z. Jasencakova, et al. (2002). "DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*
" The EMBO Journal 21(23): 6549-6559.

Sugimoto-Shirasu, K., G. R. Roberts, et al. (2005). "RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth." Proc Natl Acad Sci U S A 102(51): 18736-41.

Takeda, S. and J. Paszkowski (2006). "DNA methylation and epigenetic inheritance during plant gametogenesis." Chromosoma 115(1): 27-35.

van den Berg, C., V. Willemsen, et al. (1995). "Cell fate in the Arabidopsis root meristem determined by directional signalling." Nature 378(6552): 62-5.

van den Berg, C., V. Willemsen, et al. (1997). "Short-range control of cell differentiation in the Arabidopsis root meristem." Nature 390(6657): 287-9.

Vongs, A., T. Kakutani, et al. (1993). "Arabidopsis thaliana DNA methylation mutants." Science 260(5116): 1926-8.

Willemse, J., H. D. Jong, et al. (2007). Chapter 2: Quantification of nuclear DNA in whole mount arabidopsis roots Molecular Biology, Wageningen University. PhD: 117.

Zhang, X., J. Yazaki, et al. (2006). "Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis." Cell 126(6): 1189-201.

Zluvova, J., B. Janousek, et al. (2001). "Immunohistochemical study of DNA methylation dynamics during plant development." J Exp Bot 52(365): 2265-73.

CHAPTER 3

LHP1 forms euchromatin complexes at trimethylated lysines of histone H3

Chapter 3: LHP1 forms euchromatin complexes at trimethylated lysines of histone H3

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Introduction

Structural and functional changes in the organisation and dynamics of the chromatin state are keys to control genome function and are performed by chromatin regulators like chromodomain proteins. The chromodomain proteins are non histone chromosomal proteins and include Polycomb group and HP1 proteins.
Heterochromatin Protein 1, in *Drosophila melanogaste*r, was discovered as a protein associated with heterochromatin (James and Elgin 1986). Since its discovery, several homologues of HP1 have been identified, from fission yeast (Swi6) to human, showing that HP1 is a highly conserved protein, and several isoforms were discovered as well, each with its own subnuclear location: in heterochromatin and/or in euchromatin. HP1 proteins possess three distinct domains: an amino-terminal chromodomain (CD) a more flexible intervening region (the hinge region) and a specific carboxyl-terminal chromoshadow domain (CSD). The CD was shown in several systems (fission yeast (Nakayama, Klar et al. 2000), *Drosophila* (Bannister, Zegerman et al. 2001; Jacobs and Khorasanizadeh 2002), mammals (Aagaard, Laible et al. 1999; Rea, Eisenhaber et al. 2000)) to bind to methylated histone 3 Lysine 9 (H3K9) and with a highest affinity for trimethylated H3K9 (Fischle, Wang et al. 2003). The hinge region would be involved in binding of RNA, DNA and chromatin, and the CSD in protein-protein interaction. Currently HP1 is thought to serve as a bridging protein, connecting histones and non histone chromosomal proteins (Li, Kirschmann et al. 2002). In animals and yeast, HP1 was shown to be involved in chromatin structural organisation, maintenance of heterochromatin and gene regulation (Hiragami and Festenstein 2005; Hediger and Gasser 2006).

In Arabidopsis *thaliana*, a unique homologue of HP1 was discovered named Like Heterochromatin Protein 1(Gaudin, Libault et al. 2001). Like HP1, LHP1 contains a CD, a hinge region and a CSD (Gaudin, Libault et al. 2001).

LHP1 was shown to be located in the euchromatin and to be present in many foci (Kotake, Takada et al. 2003; Libault, Tessadori et al. 2005; Nakahigashi, Jasencakova et al. 2005). However, whether these foci represent functional chromatin- complexes is unclear as these foci could also be artificial aggregates of transgenic LHP1-GFP proteins (Waldo, Standish et al. 1999) or interchromatin nuclear bodies like nucleoli, or Cajal bodies (Shaw and Brown 2004).

In case LHP1 is part of a chromatin complex it most likely interacts with a specific histone modification as has been described for animal/yeast. Therefore we tested whether LHP1 is in close vicinity of histone/DNA. In fission yeast, LHP1 was shown to complement the swi6- mutant of the HP1 yeast homolog (Kotake, Takada et al. 2003) suggesting that LHP1 can bind (tri)methylated H3K9 in yeast.HP1.

In roots of Arabidopsis, LHP1 was described to have a diffuse pattern in dividing meristematic cells and a speckled-like pattern (foci) in differentiated cells. These foci are located in the euchromatic area of the Arabidopsis nuclei (Libault, Tessadori et al. 2005) Arabidopsis interphase nuclei have a simple organisation with only 10 to 12 heterochromatic chromocenters and so the LHP1 foci can easily be distinguished from heterochromatic chromocenters (see figure 2). The region with differentiated cells can be easily identified in roots and all nuclei within a whole mount preparation can be analyzed by CLSM. For these reasons we used the differentiated zone of Arabidopsis' roots to study whether LHP1 foci represent chromatin complexes.

We showed that like HP1 in animals, LHP1 binds to chromatin and that its chromodomain is involved in this binding through its interaction with H3K9m3. LHP1 partially colocalizes with H3K9m3 as observed in animals as well as H3K27m3. Furthermore LHP1 seems to form chromatin complexes with similar dynamics than its animal counterpart. It is a highly dynamic protein with a slightly slower mobility in the intrafoci region compared to the interfoci region. Our study points to a similar role for LHP1 as animal euchromatic HP1 proteins, possibly in gene regulation (Hiragami and Festenstein 2005; Hediger and Gasser 2006).

Results

Subnuclear localization of LHP1

Several studies have shown that in Arabidopsis nuclei LHP1 is localized in euchromatic area and is present in many foci (Kotake, Takada et al. 2003; Libault, Tessadori et al. 2005; Nakahigashi, Jasencakova et al. 2005). Whether these foci represent functional chromatin complexes, or for example interchromatin nuclear bodies (Shaw and Brown 2004) or even artefacts is unclear. To address this question, LHP1-GFP fusion constructs driven by its own promoter (pLHP1::LHP1-GFP) or by the ubiquitously expressed 35S promoter (35S::LHP1-GFP), respectively, were introduced into *lhp1* mutants. These are Arabidopsis *lhp1* knockout mutants, which show pleiotropic phenotypes such as early flowering and reduced growth (Kotake, Takada et al. 2003). In case of pLHP1::LHP1-GFP, stable transformants were obtained and these have a restored wild-type phenotype, showing that the fusion protein is biologically active. Despite numerous attempts, no transformants expressing 35S::LHP1-GFP was obtained. Therefore to obtain 35S::LHP1-GFP expressing plant material, we used the *Agrobacterium rhizogenes* hairy root transformation system on wild-type (accession Columbia) roots. *A. rhizogenes* can generate many transformed roots on one seedling within 8 to 10 days, making this transformation system a fast method to generate genetically transformed roots (Limpens, Ramos et al. 2004).

The fluorescence intensity of LHP1-GFP in plants expressing pLHP1::LHP1-GFP confirmed that LHP1 is higher expressed in the root meristem than in the differentiated zone of the root (Kotake, Takada et al. 2003). Furthermore, two different subnuclear localization patterns of LHP1-GFP were observed depending on the differentiation state of the cell. In the root meristem, LHP1 shows a diffuse distribution throughout the nucleus with sometimes 1 or 2 foci with a diameter of about 0.4 μm and is excluded from the nucleolus (figure 1.a). In the differentiated zone of the root, LHP1 formed numerous foci (~ 0.4 μm) and was also present in a diffuse manner in the interfoci region albeit at a lower level (figure 1.b). It was absent from the nucleolus.

In 35S::LHP1-GFP roots, LHP1-GFP was present in all nuclei of the root (including meristem) forming numerous foci of about 0.4 µm and show as well a diffuse distribution in the interfoci region (figure 1.c+d) (Kotake, Takada et al. 2003).So ectopic and higher expression with the 35S promoter causes an increase in foci formation.

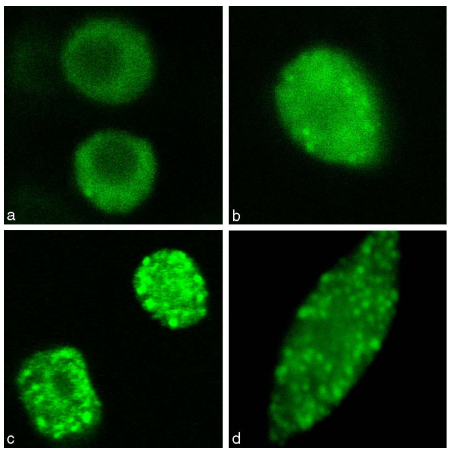

Figure 1: Subnuclear Localisation LHP1-GFP in Arabidopsis roots. a+b. Roots expressing pLHP1::LHP1-GFP. c+d. Roots expressing 35S::LHP1-GFP. a+c. Meristematic nuclei. b+d. Differentiated nuclei.

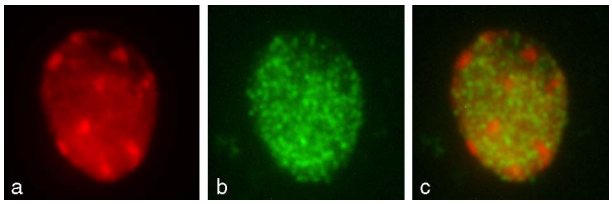

Figure 2: Immunodetection of LHP1-GFP in interphase nucleus of Arabidopsis plants expressing plhp1::LHP1-eGFP. a. Propidium Iodide staining of the DNA. b. Immunodetection of LHP1-GFP with GFP antibody. c. Merged picture of a and b.

To investigate if LHP1 was associated with heterochromatin, LHP1-GFP was detected with rabbit anti-GFP polyclonal antibody (Molecular Probes) and the DNA was stained with Propidium Iodide in roots expressing pLHP1::LHP1-GFP. In figure 2, the merged picture (2.c) clearly shows that LHP1-GFP (green signal) is excluded from the chromocenters (bright red spots) and localizes nearly

exclusively in the euchromatic regions (figure 2) as observed by Libault and Nakahigashi (Libault, Tessadori et al. 2005; Nakahigashi, Jasencakova et al. 2005). This is the case in meristematic as well as differentiated cells. Similar results were obtained for 35S::LHP1-GFP expressing plants.

LHP1 is present in chromatin complexes

In case the LHP1 foci represent chromatin complexes LHP1 molecules will be in close vicinity to DNA, especially when they interact with a specific histone modification. In contrast, when they are present in interchromatin nuclear bodies or are artefacts, LHP1 proteins will not be in such close vicinity to DNA.

FRET (Förster Resonance Energy Transfer) microscopy is a sensitive method to test whether molecules are in close vicinity. FRET is a non-radiative, dipole-dipole coupling process, whereby energy from an excited donor fluorophore is transferred to an acceptor fluorophore (Förster, 1948). FRET causes a decrease of fluorescence intensity as well as the fluorescence lifetime of the donor. Therefore both can be used to quantify the efficiency of FRET. FRET is highly dependent on the distance between donor and acceptor and in general it is only detectable when this distance is < 10 nm. FRET has been successfully used to study interactions of chromatin proteins and DNA. For example, HP1α and HP1β were shown by FRET to be in close vicinity to DNA in Hela cells (Cremazy, Manders et al. 2005).

To determine whether FRET occurs between LHP1-GFP and DNA in Arabidopsis nuclei we made use of the method developed by Cremazy *et al.* (Cremazy, Manders et al. 2005), they showed that DNA can efficiently be stained with Sytox orange and this fluorescent dye can be used as acceptor fluorophore in FRET experiments when GFP is used as the donor fluorophore.

As described above, LHP1 is not present at an equal concentration throughout the nucleus. Therefore we used Fluorescence Lifetime IMaging (FLIM) to quantify FRET efficiency as fluorescence lifetime of the fluorophore is independent of its concentration, whereas fluorescence intensity is not. When FRET occurs, the fluorescence lifetime of the donor decreases, because energy transfer to the acceptor provides an additional decay pathway for the donor. The fluorescence lifetime was measured by using two-photon excitation Time Correlated Single

Photon Counting (TCSPC) instrumentation to obtain a detailed FLIM image with voxel specific lifetime values.

The staining of DNA with Sytox orange requires that cells are first fixed. However, due to this fixation procedure, the fluorescence intensity of GFP is reduced. Therefore, Arabidopsis roots transformed with 35S::LHP1-GFP were studied as they have a higher expression level and numerous foci containing LHP1-GFP are formed. 35S::GFP expressing plants were used as a control to determine the fluorescence lifetime of GFP in the absence of FRET.

The fluorescence lifetime of GFP and LHP1-GFP, respectively, were measured in root nuclei in the absence of Sytox Orange to test whether LHP1 affects the fluorescence lifetime of GFP. Further since DNA can only be stained by Sytox orange in fixed cells, we tested as well whether the fixation procedure (see materials and methods) affected the fluorescence lifetime of GFP.

The fluorescence life time of each voxel is quantified and the average fluorescence lifetime is similar in all cases. In fixed roots, the average fluorescence lifetime of LHP1-GFP and GFP are 2.20 ± 0.06 and 2.32 ± 0.05 nanoseconds (ns) (voxels of 10 nuclei) respectively, time, which is similar to values reported for GFP fusion proteins in Hela Cells (Cremazy, Manders et al. 2005).

Lifetime values are represented by pseudocolors for each voxel of the FLIM images. Lifetime values of GFP in the absence of FRET values are pseudocolored in red; shorter lifetime values are pseudocolored in blue indicating FRET (figure 3).

As you can see in figure 3, fluorescence lifetimes of GFP as well as 35S::LHP1-GFP are equal throughout the nucleus and indicated in red (figure 3.a-f).

When DNA of 35S::GFP expressing plants was stained with Sytox Orange, the measured lifetime of GFP was 2.32 ± 0.05 ns (figure 3.g-i). So the lifetime of GFP is not affected by the Sytox dye implying that FRET does not take place between DNA and a freely mobile GFP. Similar data were obtained with GFP transfected in Hela cells (Cremazy, Manders et al. 2005).

When DNA of 35S::LHP1-GFP roots were stained with Sytox-orange, the average lifetime of LHP1-GFP is 1.85 ± 0.17 ns and the FLIM image showed uniform shorter lifetime values over the nucleus indicated by the blue-green colour (figure

3.j-l). The distribution histograms of fluorescence lifetimes of 35S::LHP1-GFP with or without Sytox show both one uniformly distributed population of the fluorescence lifetimes (figure 3.c+l) This marked reduction of the lifetime of the donor shows that FRET occurs between DNA and LHP1-GFP.

The lifetime values of LHP1-GFP are similar in foci and interfoci region (figure 3.j-k). Therefore within foci as well as in interfoci region, LHP1 is in close vicinity to the DNA and seems to be part of chromatin. The reduction in lifetime values of 16% obtained for LHP1 in our system is in the same range than the 20% reduction observed in Hela cells for HP1α-GFP and HP1β-GFP (Cremazy, Manders et al. 2005).

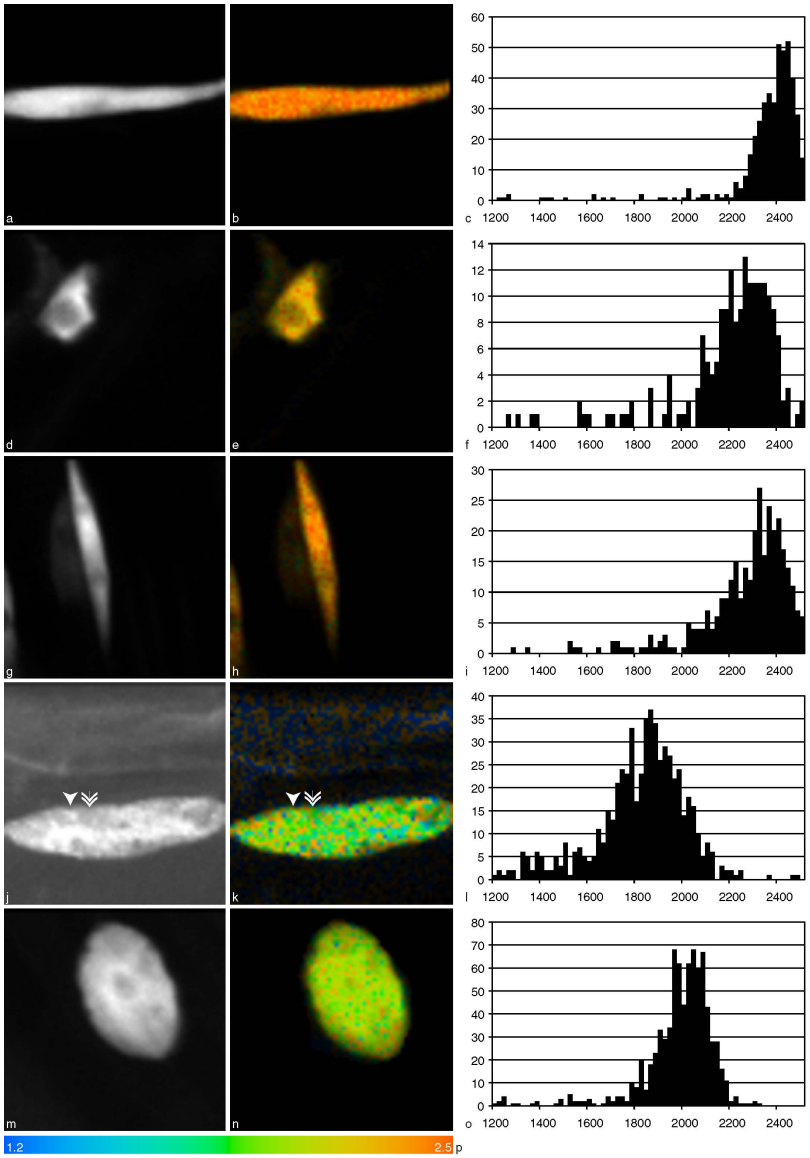

Figure 3: FRET-FLIM study on Arabidopsis differentiated root nuclei. a,d,g,j+m. Fluorescence intensity pictures. b,e,h,k+n. FLIM pictures. p. scale of fluorescence lifetimes of the FLIM pictures. c,f,i,l+o. Distribution Histogram of Fluorescence Lifetimes. a-c. 35S::LHP1-GFP without Sytox. d-f. 35S::GFP without Sytox. g-i. 35S::GFP with Sytox. j-l. 35S::LHP1-GFP with Sytox, ▸ arrow represent foci, » arrow interfoci region. m-o. 35S::LHP1ΔCD-GFP with Sytox

The chromodomain of LHP1 is necessary for foci formation

The FRET-FLIM studies strongly suggest that LHP1 is present in chromatin complexes. This could mean that LHP1 interacts with a specific histone modification. The sequence of the CD of LHP1 is homologous to the one of HP1/SWI6 and LHP1 was shown to complement a swi6- mutant in fission yeast (Kotake, Takada et al. 2003) suggesting that LHP1 can bind trimethylated H3K9 in yeast (Shilatifard 2006). If this is also the case in Arabidopsis a deletion of the CD might result in a free mobile protein. To test whether the CD was essential for the association with DNA a mutant LHP1 protein was constructed, lacking the CD. This construct was introduced by *A. rhizogenes* transformation in wild-type Arabidopsis roots. In none of the cells of these transgenic roots, foci were present in the nuclei and instead LHP1ΔCD-GFP is present in a diffuse manner throughout the nucleus (see figure 3.m). By staining these roots with Sytox orange and FLIM analysis it was tested whether LHP1ΔCD is no longer closely associated with DNA. Surprisingly, the average fluorescence lifetime of this LHP1 CD-GFP was 1.89 ± 0.21 ns showing that LHP1 lacking the CD is still in close vicinity to DNA (figure 3.m-o). Collectively these data show that the CD is essential for the formation of the chromatin complexes that are visible as foci and probably during the formation of these foci the CD of LHP1 interacts with a histone modification. The close association of LHP1and DNA in the interfoci region does not require the CD and so the association with DNA in these regions must depend on another domain (e.g. the hinge region) and is less likely to depend on a specific histone modification.

LHP1 colocalizes with H3K9m3 and H3K27m3

LHP1 CD could bind to H3K9m3 as shown for HP1/SWI6 in other systems. However, recently Turck et al showed by ChIP-chip experiments that LHP1 target genes are enriched in H3K27m3 (Turck, Roudier et al. 2007) (submitted). Therefore we tested whether LHP1 foci colocalizes with H3K9m3 or H3K27m3. In roots of Arabidopsis plants expressing pLHP1::LHP1-GFP, LHP1-GFP was detected with a rabbit anti-GFP polyclonal antibody (Molecular Probes) H3K9m3 with a mouse anti-H3K9m3 monoclonal antibody (Abcam) and H3K27m3 with a

mouse anti-H3K27m3 monoclonal antibody (Abcam). Therefore colocalisation of LHP1 with these histone modifications was studied in separate experiments.

Root nuclei from the differentiated zone were imaged with a confocal laser scanning microscope.

LHP1-GFP (green signal, figure 4.a+d), H3K9m3 (red signal, figure 4.b) and H3K27m3 (red signal, figure 4.e) are all present in multiple foci located in the euchromatin (figure 4).

LHP1 and H3K9m3 appear to overlap as well as LHP1 and H3K27m3 as shown by the yellow signal in the merged pictures. However, not all LHP1 colocalizes with H3K9m3 and vice versa as shown by the regions indicated by arrows on figure 4.c and the same is true for LHP1 and H3K27m3 (figure 4.f).

To estimate the degree of colocalisation of LHP1 and with H3K9m3 and H3K27m3, statistical analysis was performed using ImageJ (v.1.37, National Institutes of Health, USA. http://rsb.info.nih.gov/ij).

The Pearson's correlation coefficient, R, was calculated. The Pearson's correlation coefficient shows how well 2 signals relate by a linear equation. R ranges from -1 to 1 in which 1 reflects a perfect positive linear correlation, whereas -1 shows a perfect mutual exclusion. Furthermore, to eliminate the possibility that the observed colocalisation was due to chance only or to a too low resolution of the microscope, we compared the Pearson correlation coefficient with the one generated by a randomly generated picture (the green or red signal is scrambled whereas the other signal is kept intact (see material & methods). Root nuclei from the differentiated zone were imaged with a confocal laser scanning microscope and the middle section of a Z-stack of a nucleus was analyzed.

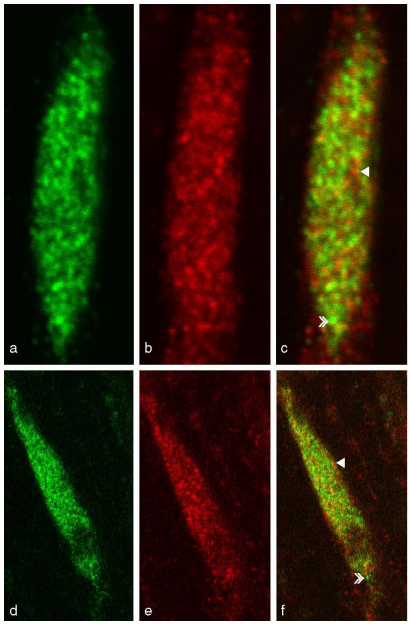

Figure 4: Whole mount immunodetection of LHP1-GFP and H3 marks on interphase root nuclei of Arabidopsis. a-c. Immunodetection of LHP1-GFP (a), H3K9m3 (b) and merged picture (c). ▸ region with H3K9m3 only, » region with LHP1-GFP only. d-f. Immunodetection of LHP1-GFP (d), H3K27m3 (e) and merged picture (f). ▸ region with H3K27m3 only, » region with LHP1-GFP only.

The average Pearson's correlation coefficient for LHP1 with H3K9m3 and H3K27m3 is 0.720 (19 nuclei) and 0.686 (39 nuclei), respectively, which corresponds to a marked degree of correlation between LHP1 and both H3 modifications (The R values for nuclei in which one of the 2 signals is scrambled are about 0.17 and 0.0). The R values are lower than 1 because not all LHP1 foci (but at least more than 50% do) colocalize with one of these H3 marks and further a perfect linearity of the LHP1 and H3K9m3/ H3K27m3 signal is not expected as the ratio of LHP1 molecules and H3 marks is not known to be constant on all their DNA targets.

LHP1 is present in dynamic complexes

To test whether the interaction of LHP1 and DNA/histone is dynamic as in animals, Fluorescence Recovery After Photobleaching experiments were

performed. FRAP makes use of the photobleaching properties of the excitation laser to selectively destroy the fluorescence of GFP in a region within the cell, after which the fluorescence intensity of the bleached region is monitored. The speed of fluorescence recovery after photobleaching provides insight in the dynamics of the molecule and the ultimate percentage of recovery shows which fraction of the molecules is dynamic.

FRAP has been used to study the dynamics of LHP1 in foci as well as in the interfoci regions in transgenic Arabidopsis roots expressing 35S::LHP1-GFP. As reference for a freely mobile protein we used transgenic plants expressing 35S::GFP. All experiments used a bleach region of 1 μm^2. As a control, the half time of recovery for free GFP was measured in 10 nuclei and is about 0.02 s.

The mobility of LHP1 was measured in 200 nuclei. In foci the half time of recovery is about 1 s whereas in the interfoci regions this is about 0.6 s (table 1). The difference between these mobilities is significant (T Student test $p = 0.0005$) underlining that the chromatin interaction of LHP1 in foci and interfoci regions is different.

35S::LHP1-eGFP (200 nuclei)				35S::eGFP (10 nuclei)	
Interfoci		Foci			
$t_{1/2}$ ($s/\mu m^2$)	Mobile fraction (%)	$t_{1/2}$ ($s/\mu m^2$)	Mobile fraction (%)	$t_{1/2}$ ($s/\mu m^2$)	Mobile fraction (%)
0.66 ± 0.44	62 ± 20	0.96 ± 0.37	71 ± 13	0.018 ± 0.003	79 ± 5

Table 1: FRAP analysis of LHP1-GFP in Arabidopsis roots. Half time recovery $t_{½}$ and mobile fraction with their standard deviation for differentiated nuclei in interfoci regions and in foci.

So the mobility of LHP1 is 30-50 times slower than that of a free mobile (GFP) protein. This confirms the FRET-FLIM experiments that showed that LHP1 is not a freely mobile protein in the regions between foci as well as in foci. In contrast, Histone 2B has a half time of recovery of ~80 $s/\mu m^2$ (Willemse, Wellink et al.

2007) showing that LHP1 complexes are markedly more dynamic than histones in nucleosomes.
Values observed for the mobility of LHP1 are in the same range as those of HP1 in mammalian cells, which are about 1 s in the euchromatin for the isoform HP1γ (80% mobile) (Schmiedeberg, Weisshart et al. 2004).

Discussion

Here we showed that LHP1 foci that are located in euchromatic area of interface nuclei are highly dynamic chromatin complexes in which relatively high levels of H3K9m3 and/or H3K27m3 occur. These LHP1 foci most likely represent chromatin complexes controlling the expression of genes.
The conclusion that LHP1 foci represent chromatin complexes is supported by the FRET-FLIM studies demonstrating that LHP1 is in close vicinity to DNA as well as the colocalisation of LHP1 with the histone modification H3K9m3 and/or H3K27m3.
The partial colocalisation of LHP1 and H3K27m3 is well in line with the recently published ChIP-chip studies of Turck et al. Since H3K9m3 as well as H3K27m3 were visualised with a mouse monoclonal antibody our studies could not reveal whether both epigenetic modifications are present in foci or whether they occur in different subsets of foci.
The chromodomain is essential for foci formation as was previously shown in Arabidopsis protoplasts (Libault, Tessadori et al. 2005). However, since major chromatin reorganisation is induced by protoplast formation (F. Tessadori, Paul Fransz submitted) this had to be confirmed in plants. It seems probable that the CD of LHP1 recognises H3K9m3 and/or H3K27m3. Turck et al indeed demonstrated that LHP1 binds H3K27m3 in vitro as well as H3K9m3. Whether LHP1 efficiently binds H3K9m3 in vivo, remains to be demonstrated. However, since LHP1 can complement a yeast swi6 mutant it is probable it will (Kotake, Takada et al. 2003).
LHP1 was shown to affect the expression of genes situated in the euchromatin but not in the heterochromatin (Nakahigashi, Jasencakova et al. 2005). Furthermore ChIP-chip experiment shows as well that LHP1 interacts with chromatin and that its chromodomain is involved in this binding. ChIP experiments performed by

Turck demonstrated that LHP1 was not found in heterochromatic sequences. Therefore it seems probable that the LHP1 foci represent chromatin complexes where target genes are regulated

The euchromatic localization of LHP1 is in agreement with most other studies done in Arabidopsis (Libault, Tessadori et al. 2005; Nakahigashi, Jasencakova et al. 2005) except one study involving Arabidopsis protoplasts where LHP1 was shown to be located in the heterochromatin (Zemach, Li et al. 2006). The latter might be due to the major global chromatin reorganisation that occurs in Arabidopsis protoplasts (Tessadori 2007 submitted).

We showed that the CD is essential for foci formation but not for the close association with DNA in the regions in between foci. The foci could be the sites where genes are regulated, whereas in the interfoci region LHP1 could be scanning the DNA searching for its target genes.

The localisation of LHP1 resembles that of HP1γ in mammals or HP1c in Drosophila that can be located in euchromatic areas and these isoforms of HP1 have been demonstrated to be involved in gene regulation (Ogawa, Ishiguro et al. 2002) (Piacentini, Fanti et al. 2003).

Our FRAP studies showed that LHP1 forms similar dynamic complexes as the animal isoform HP1γ (80% mobile) (Schmiedeberg, Weisshart et al. 2004) and this supports the conclusion that LHP1 and the "euchromatic" HP1 isoforms could fulfil a similar function in gene regulation in chromatin complexes.

Materials and methods

Plant material and growth conditions

tfl2-1 and tfl2-3, lhp1 knockout mutants in Columbia background were kindly provided by T. Kotake. The Arabidopsis Columbia ecotype was used as wild-type.

For all experiments, plants were grown vertically for 4-5 days post-germination on 0.8% agar plates containing 2.2g Murashige and Skoog 10 salts with vitamins

(Duchefa) plus 1 % sucrose at pH 5.8 in LD (16h light/ 8h dark) conditions at 23-24 °C.

Construction of LHP1 fusion genes

35S::LHP1-GFP and pLHP1::LHP1-GFP:

LHP1 cDNA was amplified with cLHP1-SalI-F (5'GTCGACCAGGAAA TGAAAGGGGCAAGTGG3') and cLHP1-XbaI-R (5'TCTAGATAAGGCGTTCG ATTGTAC3') on cDNA of Columbia and introduced into pGEMT (Promega). EGFP from pEGFP-C1 (Clontech) was digested by NheI and Sac I and cloned into the XbaI and SacI sites of pGEMT-cLHP1. EGFP was in this way cloned after the C terminal part of LHP1 creating a linker of 8 amino acids between the two proteins. After digestion of the pGEMT-cLHP1-eGFP with SalI and SacI, the cLHP1-eGFP fragment was introduced into a modified pBINPLUS binary vector (van Engelen, Molthoff et al. 1995) containing two times the constitutive 35S CaMV promoter and the NOS terminator creating pBIN35SLG plasmid.

LHP1 promoter was amplified with pLHP1-ClaI-2F (5'ATCGATATGGGTGCA GCATGG3') and pLHP1-SalI-R (5'CTGGTCGACAGTATTCGAGCCTCC3') on the Col-0 genomic P1 clone MIVA3 (81701 bp, accession number AB006706) giving a fragment of 2435 bp corresponding to the 11772-14230 MIVA3 region. After digestion with ClaI and SalI, the promoter was introduced into ClaI and SalI sites of pBIN35SLG, removing in this way the 35S CaMV promoter and creating the pBINlLG plasmid.

pBIN35SLG and pBINlLG were introduced into *Agrobacterium rhizogenus* (strain msu440) for hairy root transformation. For stable transformation pLHP1::cLHP1-eGFP was introduced into a pFluar 101 vector (Stuitje, Verbree et al. 2003) with a modified MCS called pFluar101(+2) using ClaI and PacI sites creating pFlulLG which was then introduced into *Agrobacterium tumefaciens* (strain C58).

$LHP1_{\Delta CD}$-GFP

The fragment cLHP1-eGFP was introduced into pBSK (Stratagene) by digestion with XbaI and SalI. The CD deletion was constructed with the aid of the PCR based Quicksite's mutagenesis kit hereby creating a HindIII site at the end of the CD. The CD was deleted in pBSK by a HindIII digestion of a natural occurring site at positions 304-309 in combination with the newly created HindIII site.

The primers used to create this mutation were 5'GCCTTTGAGGGAAGTTTG AAGC**TT**GGAAAGCCTGGTAGGAAACGG 3' and 5'CCGTTTCCTACCAGGCTTT CC**AA**GCT TCAAACTTCCCTCAAAGGC3', the bold letters indicating the mutation sites.

The LHP1$_{\Delta CD}$-GFP fragment was introduced into a pFluar101(+2) vector containing a 35S promoter using AgeI and SalI digestion sites in pBSK as well as pFluar. The resulting vector 35S::LHP1$_{\Delta CD}$ -GFP was introduced into *Agrobacterium rhizogenus* (strain msu440) for hairy root transformation

Hairy root transformation

4-5 dpg old Arabidopsis seedlings (accession Columbia) were transformed as described (Limpens, Ramos et al. 2004) using Färhaeus and Emergence medium instead of ½ MS.

Agrobacterium-mediated vacuum transformation

4-6 weeks old plants of lhp1 mutants (tfl2-1 and tfl2-3 in Columbia background, Kotake) were transformed as described (Bechtold, Ellis et al. 1993). The aerial part of the plants was dipped into a solution of *Agrobacterium* tumefaciens (strain C58) carrying the appropriate construct in infiltration medium (Murashige and Skoog + vitamins 2.3 g/l, sucrose (5%) 50 g/l, MES 0.5 g/l pH = 5.8 with KOH, autoclave and add 200 µl/l Silvet L77) under vacuum for 5-10 min

Localization

All confocal images were acquired on a Zeiss 510 inverted microscope using a 40x/1.3 oil immersion objective. Image resolution was always higher than the theoretical limit for light microscopy to insure no data was missed.

FRAP

All FRAP studies were performed with similar settings as described for the imaging. The ROI was kept at approximately 1 µm^2 allowing direct comparison between half-times of recovery. Recovery of fluorescence intensity was monitored in such manner that at least 10% of the obtained images were obtained before the half time of recovery.

FRET-FLIM

Two-photon microscopy was performed on a Biorad 1600 using a 60x/1.2 water immersion objective. Fixation procedures and imaging settings were identical to Cremazy et al. Two-photon excitation was used instead of single photon excitation

(870 nm). FLIM images were obtained using a 75 MHz modulated two photon laser after which Time Correlated Single Photon Counting (TCSPC) was used to determine the fluorescence lifetime.

Immunolocation

4-5 dpg old seedling roots were immunolabeled as described (Talbert, Masuelli et al. 2002; Jasencakova, Soppe et al. 2003). Roots were fixed in 4% paraformaldehyde in PBS pH 7.3 0.2% Triton for 1h with 20 min vacuum at room temperature. They were washed 2x 10 min with 1x PBS and transfer to small baskets with filters. They were digested for 40 min at 37°C with a mixture of 2.5% pectinase from *Aspergillus niger* (Sigma) and 2.5% cellulase Onozuka RS (Yakult Honsha Co., Tokyo, Japan) dissolved in PBS. Roots were washed in PBS and squashed onto slides. Slides were immersed in liquid nitrogen, the cover slips were removed, and roots were postfixed in 4% paraformaldehyde in PBS for 20 min at room temperature. After washing with 3x 5 min PBS, slides were incubated in a moist chamber at room temperature with blocking solution (3% BSA, 10% sheep serum) for 1h at 37°C. After cover slips were removed, slides were incubated with rabbit anti-GFP polyclonal antibody (1:200, A11122 Molecular Probes) in labelling solution (1% BSA, 10% sheep serum, 0.1 % Tween 20) for detection of LHP1-GFP overnight at 4°C. Cover slips were removed, and the slides were washed twice with PBS. The antibody was detected by applying Alexa 488–conjugated goat anti-rabbit secondary antibody (A11070, Molecular Probes) diluted 1:200 in labelling solution and incubated for 1-2 h, followed by two washes in PBS. The slides were stained and mounted with 2 µg/ml Propidium Iodide in Vectashield (Vector Laboratories, Burlingame, CA).

Whole mount coimmunolocalization

Immunolabeling procedure was performed as described (Friml, Benkova et al. 2003). 4 dpg old Arabidopsis seedlings were fixed in 4% paraformaldehyde in MTSB for 1h00 at + 4°C instead of room temperature. From the driselase treatment, seedlings were kept in small baskets with filters to avoid loosing the root tips during the different washing steps. They were incubated overnight at room temperature in a wet chamber with two primary antibodies: a rabbit anti-GFP polyclonal antibody (1:200, A11122 Molecular Probes) for detection of LHP1-GFP and a mouse anti-H3K9m3 monoclonal antibody (1:50, 6001 Abcam) or a mouse anti-H3K27m3 monoclonal antibody (1:50, 6002 Abcam). The seedlings after

washing were incubated with two secondary antibodies a Alexa 488 conjugated goat anti-rabbit antibody (1:200, A11070 Molecular Probes) and a Cy3 conjugated donkey anti-mouse antibody (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at room temperature. Finally the seedlings were mounted on microscopic slides in Citifluor, an antifading mounting medium.

Colocalisation analysis

Statistical analysis was performed using ImageJ (v.1.37, Rasband, W.S, National Institutes of Health, USA. http://rsb.info.nih.gov/ij, 1997-2006) with two specific plugins: Manders' coefficients and the Colocalisation test (Tony Collins, Wayne Rasband, http://www.uhnresearch.ca/facilities/wcif/imagej/).

Both plugins calculate the Pearson's correlation coefficient, R, one of the standard measures in pattern recognition. The Pearson's correlation coefficient is valid only if a linear relationship exists between the red and green signals. To check this, the Mander's coefficients plugin generate a Red-Green scatter plot, if the points scatter in a more or less linear direction then the relationship can be considered linear. Pearson's correlation coefficient is independent from the image background and the intensities of the signals. The coefficient ranges from -1 to 1. A value of 1 shows that a linear equation describes the relationship perfectly and positively, with all the data points lying on the same line and with G increasing with R. A value of -1 shows also a linear relationship between G and R but G increases as R decreases. A value of 0 shows that there is no linear relationship between G and R so no colocalisation. Furthermore, to eliminate the possibility that the observed colocalisation was due to chance only or to a too low resolution of the microscope we compared, thanks to the Colocalisation test plugin, the Pearson correlation coefficient r_p (=R) with the one generated by a randomly generated picture (the green or red signal is scrambled by randomly rearranging blocks of size equal to the point spread function of the microscope, the other signal is kept intact).

$$rp = \frac{\sum((Ri - Ravg)(gi - Gavg))}{\sqrt{\sum(Ri - Ravg)^2 \sum(Gi - Gavg)^2}}$$

Ri, Gi = intensity values of pixel I; Ravg and Gavg = average intensity R or G

References

Aagaard, L., G. Laible, et al. (1999). "Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31." Embo J 18(7): 1923-38.

Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature 410(6824): 120-4.

Bechtold, N., J. Ellis, et al. (1993). " In planta Agrobacterium-mediated gene transfer by infiltration of adult Arabidopsis thaliana plants." C. R. Acad. Sci. Paris, Life Sciences 316: 1194-1199.

Cremazy, F. G., E. M. Manders, et al. (2005). "Imaging in situ protein-DNA interactions in the cell nucleus using FRET-FLIM." Exp Cell Res 309(2): 390-6.

Fischle, W., Y. Wang, et al. (2003). "Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains." Genes Dev 17(15): 1870-81.

Friml, J., E. Benkova, et al. (2003). "Automated whole mount localisation techniques for plant seedlings." Plant J 34(1): 115-24.

Gaudin, V., M. Libault, et al. (2001). "Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in Arabidopsis." Development 128(23): 4847-58.

Hediger, F. and S. M. Gasser (2006). "Heterochromatin protein 1: don't judge the book by its cover!" Curr Opin Genet Dev 16(2): 143-50.

Hiragami, K. and R. Festenstein (2005). "Heterochromatin protein 1: a pervasive controlling influence." Cell Mol Life Sci 62(23): 2711-26.

Jacobs, S. A. and S. Khorasanizadeh (2002). "Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail." Science 295(5562): 2080-3.

James, T. C. and S. C. Elgin (1986). "Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene." Mol Cell Biol 6(11): 3862-72.

Jasencakova, Z., W. J. Soppe, et al. (2003). "Histone modifications in Arabidopsis-high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin." Plant J 33(3): 471-80.

Kotake, T., S. Takada, et al. (2003). "Arabidopsis TERMINAL FLOWER 2 gene encodes a heterochromatin protein 1 homolog and represses both FLOWERING LOCUS T to regulate flowering time and several floral homeotic genes." Plant Cell Physiol 44(6): 555-64.

Li, Y., D. A. Kirschmann, et al. (2002). "Does heterochromatin protein 1 always follow code?" Proc Natl Acad Sci U S A 99 Suppl 4: 16462-9.

Libault, M., F. Tessadori, et al. (2005). "The Arabidopsis LHP1 protein is a component of euchromatin." Planta 222(5): 910-25.

Limpens, E., J. Ramos, et al. (2004). "RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula." J Exp Bot 55(399): 983-92.

Nakahigashi, K., Z. Jasencakova, et al. (2005). "The Arabidopsis heterochromatin protein1 homolog (TERMINAL FLOWER2) silences genes within the euchromatic region but not genes positioned in heterochromatin." Plant Cell Physiol 46(11): 1747-56.

Nakayama, J., A. J. Klar, et al. (2000). "A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis." Cell 101(3): 307-17.

Ogawa, H., K. Ishiguro, et al. (2002). "A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells." Science 296(5570): 1132-6.

Piacentini, L., L. Fanti, et al. (2003). "Heterochromatin protein 1 (HP1) is associated with induced gene expression in Drosophila euchromatin." J Cell Biol 161(4): 707-14.

Rea, S., F. Eisenhaber, et al. (2000). "Regulation of chromatin structure by site-specific histone H3 methyltransferases." Nature 406(6796): 593-9.

Schmiedeberg, L., K. Weisshart, et al. (2004). "High- and low-mobility populations of HP1 in heterochromatin of mammalian cells." Mol Biol Cell 15(6): 2819-33.

Shaw, P. J. and J. W. Brown (2004). "Plant nuclear bodies." Curr Opin Plant Biol 7(6): 614-20.

Shilatifard, A. (2006). "Chromatin Modifications by Methylation and Ubiquitination: Implications in the Regulation of Gene Expression." Annu Rev Biochem.

Stuitje, A., E. Verbree, et al. (2003). "Seed-expressed fluorescent proteins as versatile tools for easy (co)transformation and high-throughput functional genomics in *Arabidopsis*." Plant Biotechnology Journal 1: 301-309.

Talbert, P. B., R. Masuelli, et al. (2002). "Centromeric localization and adaptive evolution of an Arabidopsis histone H3 variant." Plant Cell 14(5): 1053-66.

Tessadori, F. (2007 submitted). Chapter 2: Large-scale chromatin decondensation and recondensation during nuclear reprogramming in Arabidopsis protoplasts. Nuclear Organization, Swammerdam Institute for Life Sciences, University of Amsterdam: 33-69.

Turck, F., F. Roudier, et al. (2007). "Arabidopsis TFL2/LHP1 is a Polycomb Group Protein that associates with a large set of genes encoding developmental regulators." (submitted).

van Engelen, F. A., J. W. Molthoff, et al. (1995). "pBINPLUS: an improved plant transformation vector based on pBIN19." Transgenic Res 4(4): 288-90.

Waldo, G. S., B. M. Standish, et al. (1999). "Rapid protein-folding assay using green fluorescent protein." Nat Biotechnol 17(7): 691-5.

Willemse, J., J. Wellink, et al. (2007). Chapter 3: Histone 2B exchange in Arabidopsis. Molecular Biology, Wageningen University: 45-60.

Zemach, A., Y. Li, et al. (2006). "Different domains control the localization and mobility of LIKE HETEROCHROMATIN PROTEIN1 in Arabidopsis nuclei." Plant Cell 18(1): 133-45.

CHAPTER 4

Identification of Arabidopsis SUVH with trimethyltransferase activity

Chapter 4: Identification of Arabidopsis SUVH with trimethyltransferase activity

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Introduction

Heterochromatin protein 1 (HP1) is a well conserved chromatin protein that has been identified in yeast, animals and plants (Singh et al. 1991; Lorentz et al. 1994; Horsley et al. 1996; Aagaard et al. 1999; Gaudin et al. 2001). HP1 proteins possess three distinct domains; an amino-terminal chromodomain (CD), a more flexible intervening region (hinge region) and a specific carboxyl-terminal chromoshadow domain (CSD). The CD was shown in several systems (fission yeast (Nakayama et al. 2000), Drosophila (Bannister et al. 2001; Jacobs et al. 2002), mammals (Aagaard et al. 1999; Rea et al. 2000)) to bind to histone H3 with a methylated lysine 9 (H3K9m) and with the highest affinity for trimethylated H3K9 (H3K9m3) (Fischle et al. 2005). The hinge region is involved in binding of RNA, DNA and chromatin, and the CSD acts as a homodimerization interface and also binds several chromatin proteins (Maison et al. 2004). Currently, HP1 is thought to serve as a bridging protein, connecting histones and non-histone chromatin proteins (Ogawa et al. 2002). In animals and yeast, HP1 preferentially localizes at, and is involved in maintenance of constitutive heterochromatin (Maison et al. 2004). However, there are examples of a role for HP1 in the regulation of euchromatic genes in Drosophila and mammals (Nielsen et al. 2001; Hiragami et al. 2005; Liu et al. 2005; Hediger et al. 2006).

Arabidopsis has only one homolog of HP1 called LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) or TERMINAL FLOWER 2 (Gaudin et al. 2001; Kotake et al. 2003). LHP1 mutants show pleiotropic phenotypes; terminal flowers, as well as a small plant size, curled leaves and low fertility (Larsson et al. 1998; Kotake et al. 2003). LHP1 localizes in euchromatin regions where it forms foci and it is excluded from the heterochromatic chromocentres. (Libault et al. 2005;

Nakahigashi et al. 2005; Zemach et al. 2006; Lorvellec et al. Chapter 3). It was shown that LHP1 acts as a repressor of euchromatic genes related to plant development, i.e. flowering, floral organ identity, meiosis and seed maturation and does not affect expression of genes positioned in heterochromatin (Kotake et al. 2003; Chanvivattana et al. 2004; Nakahigashi et al. 2005; Mylne et al. 2006). LHP1 was shown to co-localize partially with H3K9m3 (Lorvellec et al., Chapter 3). However, the enzyme responsible for this histone modification is unknown.

Histone lysine methylation has been shown to be catalyzed almost exclusively by a family of proteins containing a conserved SET domain (Lachner et al. 2003; Marmorstein 2003; Bottomley 2004). This domain was first identified in Drosophila in three different proteins: **S**upressor of variegation [Su(var)3-9] (Tschiersch et al. 1994), **E**nhancer of Zeste [E(z)] (Jones et al. 1993) and **T**rithorax (Stassen et al. 1995). Drosophila SU(VAR)3-9 as well as its homolog in mammals (SUVH39H) and yeast (CRL4) methylate specifically H3K9, creating a binding site for the CD of HP1 (Lachner et al. 2003). Drosophila HP1 was shown to interact *in vitro* (yeast two hybrid) through its CSD with SU(VAR)3-9 (Schotta et al, 2002). Targeting HP1 to chromatin in mammalian nuclei required H3K9 methylation, but also a direct protein-protein interaction between SUVH39H1 and HP1 (Schotta et al. 2002).

The yeast CLR4 (CRYPTIC LOCI REGULATOR 4), Drosophila SU(VAR)3-9 and mammalian SUVH39H1 and SUVH39H2 are primarily involved in H3K9 methylation at constitutive heterochromatin (Lachner et al. 2003). These proteins contain a unique catalytic domain consisting of a SET domain flanked by two cystein-rich domains named pre-SET and post-SET, respectively. In addition to the catalytic domain, these proteins contain a chromodomain at their N-terminus (Jones et al. 1993; Tschiersch et al. 1994).

Yeast, Drosophila and mammals have 1-2 copies of SU(VAR)-like genes. In contrast, plants contain a much higher number of genes homologous to SU(VAR)3-9. For example, the Arabidopsis genome contains 10 of such genes. However, none of these proteins contains a chromodomain. Instead the Arabidopsis SUVH (SU(VAR)3-9 HOMOLOGS) proteins contain a SRA domain (**S**ET and **R**ING finger **a**ssociated, also called YDG domain) on their N-terminal part (Baumbusch et al.

2001; Zhao et al. 2004) that could play a role in targeting these proteins to specific chromatin sub-domains (Citterio et al. 2004; Yu et al. 2004). The questions we addressed in this study are: which methyltransferase is responsible for trimethylation of H3K9 and does it interact with the CSD domain of LHP1? To identify a H3K9 trimethyltransferase we first analysed the Arabidopsis SUVH family.

The criteria to identify an AtSUVH with trimethyltransferase activity we used are: 1) location in euchromatin; 2) possible miss-localization of LHP1 in SUVH mutants; 3) *in vitro* interaction of SUVH and LHP1. Using these criteria, we first used published data to select the best SUVH candidates.

SUVH1 and SUVH2 proteins are specifically located in the heterochromatic chromocentres (Naumann et al. 2005; Fischer et al. 2006) and are therefore not included in our study. SUVH4 is located as well in the heterochromatin (Fischer et al. 2006) and SUVH4, SUVH5 and SUVH6 were shown to catalyze mono/di-methylation of H3K9 (Jackson et al. 2004; Ebbs et al. 2005). Therefore also these AtSUVH genes are not included in our analysis.

SUVH8 and SUVH10 mRNA have not been detected in any tissue (http://www.chromdB.org). Further, SUVH10 has an internal deletion that removes a part of the SET domain and so in case it would be expressed the protein is most likely not functional (Baumbusch et al., 2001). So, by deduction we concluded that SUVH3, SUVH7 and SUVH9 are the best candidates and focused our studies on these genes.

Results

SUVH3, 7 and 9 are located within euchromatin

To determine the subcellular location of SUVH3, 7 and 9 we transformed Arabidopsis with constructs containing the 35S promoter driving the expression of an N-terminal fusion of GFP and one of these SUVH proteins (see Material and Methods). We studied the location of the fusion proteins in roots as the intact organ can be studied by CLSM. The location was studied in dividing meristematic cells where *LHP1* is expressed at the highest level. This was also done in differentiated cells where LHP1 forms foci in the euchromatic area (Chapter 3). For SUVH3 and

SUVH7 we obtained several transgenic lines. Two transgenic lines (3-1 and 3-2) carrying *35S::GFP-SUVH3* were analysed. GFP-SUVH3 was shown to be located exclusively in nuclei and was excluded from the nucleolus (figure 1). Furthermore, to better visualise the location of GFP-SUVH3 in relation to heterochromatin, DNA was stained with propidium iodide (PI) and GFP-SUVH3 was visualised by immunodetection with a polyclonal antibody against GFP. This showed that GFP-SUVH3 is also excluded from the heterochromatic chromocentres (figure 1.c). Furthermore, the subnuclear pattern depends on the developmental stage. Within the euchromatin of nuclei in the meristem, GFP-SUVH3 forms 10-20 discrete foci with a diameter of about 0.2 μm, whereas in the differentiation zone 1-2 foci of about 0.4 μm per nucleus are present (figure 1.a-b).

Two stable transgenic lines of *35S::GFP-SUVH7* (7-1 and 7-2) were used to determine the GFP-SUVH7 subnuclear location. Confocal microscopy showed that GFP-SUVH7 is only located in nuclei (figure 1.d-f). Immunodetection of GFP-SUVH7 and staining of DNA by PI showed that, in both meristematic and differentiated cells, SUVH7 is localised in the euchromatin in a uniform manner (Figure 1.f) and it is excluded from heterochromatic chromocentres. In some nuclei GFP-SUVH7 had accumulated in the centre of the nucleolus (Figure 1.d-e).

Initially, we could not obtain stable transgenic lines for *35::GFP-SUVH9*. Therefore, we made transgenic roots by *Agrobacterium rhizogenes* hairy root transformation (see Material and Methods). In transgenic roots we detected GFP fluorescence in nuclei, but also in the cytoplasm (figure 1.g). This was the case in all 15 roots analysed. In contrast we never observed a cytoplasmic location of GFP, in hairy roots expressing *35S::GFP-SUVH3* or *35S::GFP-SUVH7*. Recently, a stable line of *35S::GFP-SUVH9* (9-1) was obtained. GFP-SUVH9 had a similar location in both cytoplasm and nuclei in roots of 9-1.

The subnuclear localisation of GFP-SUVH9 in nuclei of meristematic and differentiated regions differed. In nuclei of root meristematic cells, GFP-SUVH9 forms 2-5 speckles per nucleus with a diameter of about 0.4 μm (figure 1.g) and is present in a diffuse manner in the rest of the nucleus. In the differentiated cells, SUVH9 is exclusively located in nuclei where it displays only a diffuse pattern (figure 1.h). Mostly also some empty areas are visible which might be the

chromocentres. The localization of GFP-SUVH9 in hairy roots and roots of 9-1 are similar.

According to their subnuclear localization, SUVH3, SUVH7 and SUVH9 are good candidates to interact with LHP1 as they are all located in the euchromatin.

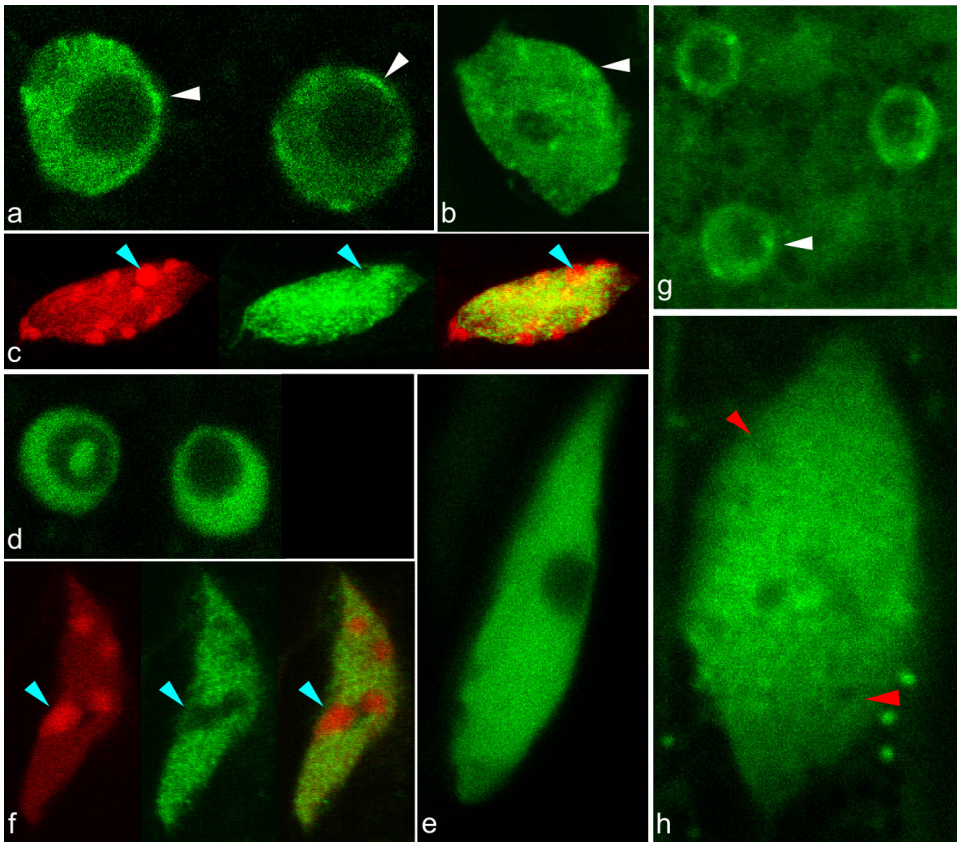

Figure 1: GFP-SUVH fluorescence in nuclei of living roots (a-b, d-e, g-h) and whole mount immunodetection of GFP-SUVH (c, f) in Arabidopsis root interphase nuclei. a-c. GFP-SUVH3 line 3-1. a. meristematic root nuclei. b. differentiation zone nucleus. c. propidium iodide staining PI (red), GFP signal (green) and merged pictures of a differentiation zone nucleus. d-f. GFP-SUVH7 line 7-1. d. meristematic root nuclei. e. differentiation zone nucleus. f. PI signal (red), GFP signal (green) and merged pictures of a differentiation zone nucleus. g-h. GFP-SUVH9 in hairy root. g. meristematic root nuclei. h. differentiation zone nucleus. White arrows indicate speckles, blue arrows chromocentres. Nucleolus is the big 'empty' area visible in the nucleus.

SUVH3 and SUVH7 are present in euchromatin complexes

To test whether SUVH3 and SUVH7 are associated with euchromatin or are freely mobile within the nucleus, Fluorescence Recovery After Photobleaching (FRAP) experiments were performed. Since the stable transgenic line 9-1 has only recently been obtained SUVH9, mobility was not yet studied. FRAP makes use of the photobleaching properties of the excitation laser to selectively destroy the fluorescence of GFP in a region within the nucleus. Subsequently, after which the recovery of fluorescence intensity of the bleached region is monitored. The speed of fluorescence recovery after photobleaching provides insight in the dynamics of the molecule and the ultimate percentage of recovery shows which fraction of the molecules is dynamic.

FRAP has been used to study the dynamics of SUVH3 and SUVH7 in transgenic Arabidopsis roots expressing *35S::GFP-SUVH3* or *35S::GFP-SUVH7*, respectively. As reference for a freely mobile protein we used transgenic plants expressing 35S::GFP. The used bleached region is 1 μm^2 in all experiments. The half time of recovery for free GFP was measured in 10 nuclei and is about 0.02 s (table 1).

The majority of SUVH7-GFP is immobile within the period measured. Only 10 % of the protein is dynamic and has a half time of recovery of 0.2 s, which is only 10 times slower than free GFP. This suggests that most GFP-SUVH7 is present in rather stable complexes, whereas a small fraction is highly dynamic. GFP-SUVH3 has a half time of recovery of 0.52 s, about 25 times slower than free GFP and the percentage of recovery is similar to that of GFP (~80%). Therefore, SUVH3 is most likely part of dynamic (euchromatin) complexes.

Sample (number measurements)	Half time recovery $t_{1/2}$ /μm^2 in s	Immobile fraction (%)
35S::GFP (10)	0.02 ± 0.03	21 ± 5
35S::GFP-SUVH4 heterochromatin (14)	5.84 ± 6.03	39 ± 25
35S::GFP-SUVH4 euchromatin (50)	0.58 ± 0.18	60 ± 11
35S::GFP-SUVH3 (17)	0.52 ± 0.24	21 ± 9
35S::GFP-SUVH7 (26)	0.20 ± 0.06	91 ± 4

Table 1: FRAP analysis of GFP-SUVH proteins in Arabidopsis roots. Half time recovery and immobile fraction with their standard deviation for each protein is measured in nuclei of differentiated nuclei.

To compare the dynamics of euchromatic SUVH proteins with that of heterochromatic SUVH proteins the mobility of SUVH4/KRYPTONITE, a H3K9 dimethyltransferase was determined. Hairy roots expressing *35S::GFP-SUVH4* were analysed. GFP-SUVH4 is located in heterochromatin as well as in euchromatin in Arabidopsis root nuclei expressing *35S::GFP-SUVH4* (data not shown). This localization is similar to that described by Fischer et al. (2006). FRAP studies showed that the mobilities of SUVH3 and euchromatic SUVH4 are in the same range whereas heterochromatic SUVH4 is about 10 times slower than euchromatic SUVH4/SUVH3 (table 1).

If LHP1 is in the same complex as a SUVH protein, they should have a similar mobility. LHP1 has a half time of 0.66 s outside its foci and 0.96 s within foci (Chapter 3), so SUVH3 has a mobility that is similar to that of LHP1.

The expression pattern of SUVH3 and SUVH9 overlap with that of LHP1

If one of the SUVH proteins and LHP1 are involved in the same process, their expression pattern should at least partly overlap. LHP1 is expressed in the root meristem, but is also expressed in the differentiation zone albeit at a lower level (Kotake et al. 2003). *LHP1* is also highly expressed in lateral root primordia (data not shown). To test whether the regions of expression of *SUVH* genes (partially) overlap with that of LHP1 we determined the pattern of expression of putative SUVH promoters.

We used the 5'- upstream region of the 3 *SUVH* genes, respectively, to drive the expression of the reporter gene *GFP-GUS*. We selected as promoter the sequence upstream of the start codon up to the 3'-end of the genes adjacent to *SUVH3* and *SUVH9*, respectively. For *SUVH7* a 2kb region upstream of its start codon was used. These promoter sequences were fused to *GFP-GUS*. The 3 resulting transgenes were introduced in hairy roots and the activity of the promoters was studied by locating GUS activity.

p*SUVH3*::*GFP-GUS* was expressed at the highest level in the root meristem and lateral root primordia (figure 2.a). p*SUVH7::GFP-GUS* is not active in the root meristem, but is expressed in the vascular tissue (figure 2.b-d). Its expression in the vascular tissue starts at the beginning of the differentiation zone and the region of expression extends to the hypocotyl. Furthermore, at the site of a lateral root primordia p*SUVH7::GFP-GUS* expression is detectable in the pericycle (figure 2.c).

p*SUVH9::GFP-GUS* is active at the highest level in the root meristem and it shows a patchy pattern (figure 2.e). This suggests that SUVH9 is only expressed during certain stages of the cell cycle. p*SUVH9::GFP-GUS* expression also occurred in the differentiated zone in the pericycle (figure 2.h) and it is also expressed at the base of lateral roots (figure 2.f-g).

So, the *SUVH3* as well as *SUVH9* promoters are active in the root meristem and there also the *LHP1* gene is active at the highest level. While we have not investigated possible colocalization of the corresponding proteins, at least the LHP1 and SUVH3/SUVH9 genes are expressed in the same cell types so the possibility exists that the proteins indeed interact in vivo.

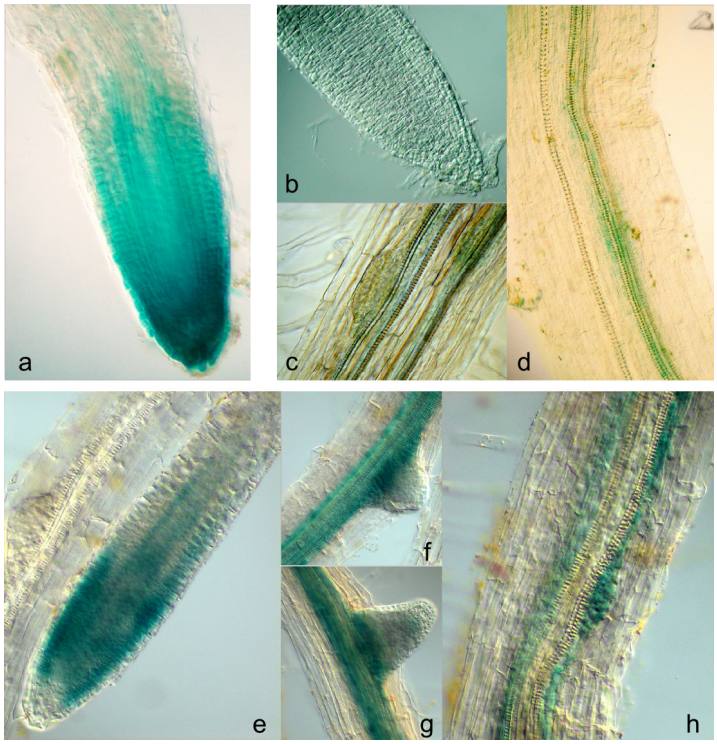

Figure 2: GUS activity in hairy roots carrying a p*SUVH::GFP-GUS* construct. a. p*SUVH3* is active in the root meristem of Arabidopsis. b-d. p*SUVH7* is active in the vascular tissue (d) and pericycle (c) of the root but not in the root meristem (b). e-h. p*SUVH9* is active in the root meristem (e), pericycle (h) and at the base of lateral roots (f, g)

Subnuclear localisation of LHP1 in *suvh* mutant backgrounds

To determine whether mutations within *SUVH3*, *-7* or *-9* proteins affect the subnuclear localisation of LHP1, we introduced by hairy root transformation a *35S::LHP1-GFP* construct into *suvh* mutants as well as in wild-type Arabidopsis. Arabidopsis T-DNA insertion mutants were available for *SUVH7* and *SUVH9*. However, no *SUVH3* T-DNA insertion mutant was available at the time we did these experiments (see Material & Methods).

In wild type hairy roots LHP1-GFP was present in all nuclei of the root (including meristem) forming numerous foci of about 0.4 µm and shows as well a diffuse distribution in the interfoci region (Kotake et al. 2003; Lorvellec et al. Chapter 3). A similar subnuclear distribution does occur in *suvh7* and *suvh9* mutants (figure 3).

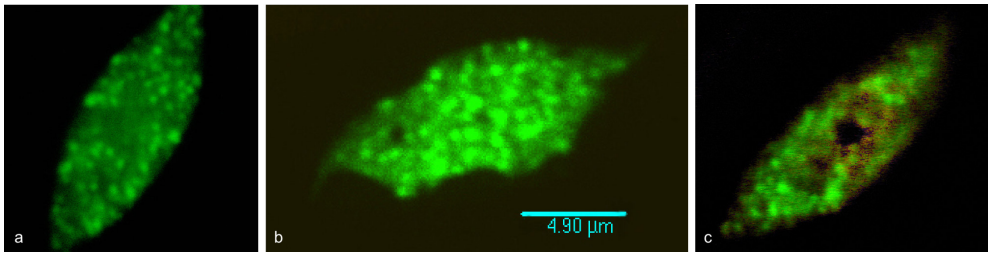

Figure 3: *35S::LHP1-GFP* expression in nuclei of differentiated cells of Arabidopsis root. a. wild-type b. *suvh7* mutant c. *suvh9* mutant

Arabidopsis *lhp1* knockout mutants display a pleiotropic phenotype such as early flowering, reduced growth, curled leaves and sterility (Kotake et al. 2003). The *suvh* mutants do not show any disturbances in morphology in comparison to Columbia wild type (data not shown). We also checked the nuclear organization of these mutants by staining seedlings with PI and did not observe any obvious difference in the distribution of heterochromatin in comparison with that of wild type (data not shown).

Overall H3K9m3 levels are not affected in *suvh9*

Western blot analysis was performed to determine the amount of H3K9m3 in *suvh9*. We studied only the *suvh9* mutant as we do not have a *suvh3* mutant. Further, *suvh7* was not analysed as it is not expressed in the root meristem. Nuclei were isolated from Arabidopsis leaves of wild type and *suvh9*. The antibody against histone H3 was used as control to visualize the total amount of histone H3 in both samples. As shown, the overall amount of H3K9m3 was not reduced for the *suvh9* mutant (figure 4).

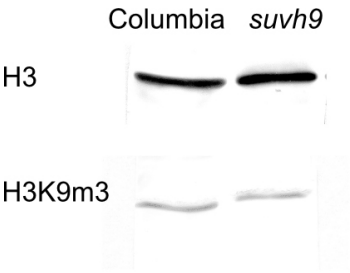

Figure 4: Western blot analysis of protein extracts from leaf nuclei of wild type Arabidopsis and *suvh9*. Top panel shows total histone H3 protein and bottom panel H3K9m3 protein.

LHP1 does not interact *in vitro* with SUVH proteins

Mammalian and yeast HP1 have been shown to interact (*in vitro*) with Suvar(3-9) homologues (e.g. Schotta et al. 2002).To test whether LHP1 can bind directly to SUVH3, SUVH7 or SUVH9 the yeast two hybrid (Y2H) system was used. For these studies we used the full-length LHP1 protein fused to the GAL4 DNA-binding domain (DB) and the different SUVH encoding parts fused to the GAL4 transcriptional activation domain (AD). If LHP1 and a SUVH protein would physically interact when co-expressed in yeast, the DB-LHP1:AD-SUVH interaction would reconstitute a functional transcription factor. This transcription factor would then activate chromosomally-integrated reporter genes *HIS3*, *ADE2* and *lacZ* driven by a promoter containing DB binding sites.

As positive control LHP1 protein was also fused to the AD domain and as expected when the DB-LHP1 protein and the AD-LHP1 protein were expressed together in yeast, the reporter genes were activated confirming that LHP1 can form dimers (Gaudin et al. 2001).

As negative control, SUVH2, SUVH3 and SUVH7, respectively, were fused to the AD domain. Y2H studies showed that they did not interact with LHP1. However, also none of the reporter genes were activated when DB-LHP1 fusion protein was co-expressed with AD-SUVH3/7/9 showing that there is no direct interaction between LHP1 and SUVH3/SUVH7/SUVH9.

Discussion

Here we showed that SUVH3, SUVH7 and SUVH9 are located in the euchromatin of Arabidopsis interphase nuclei. There they most likely are part of chromatin complexes. In our search for a histone methyl transferase that could trimethylate H3K9 and could target LHP1 to such an epigenetic mark in the chromatin, we did not identify a SUVH protein with such properties.

SUVH3 and SUVH9 GFP fusion proteins can form foci depending on the developmental stage of the cell. To a certain extend this resembles the subnuclear localization of LHP1, but coexpression of tagged proteins to demonstrate that LHP1 and SUVH foci (in part) co-localize remains to be done. In case of SUVH3 the foci seem to be highly dynamic chromatin complexes and FRAP experiments

show that that LHP1 as well SUVH3 are only 25-50 times less mobile than free GFP. Whether SUVH9 is also part of complexes that are highly dynamic remains to be demonstrated with the recently obtained stable transformed line.

Unfortunately, the *suvh* mutants that we had available have wild type phenotype. Therefore we could not test whether the fusion constructs are biologically active. Also it can not be excluded that overexpression due to the 35S promoter might have influenced the subcellular localization. For example it is possible that the occasionally observed accumulation of GFP-SUVH7 in nucleoli is caused by overexpression.

SUVH9 occurred in nuclei, but also in the cytoplasm of cells in the meristematic zone. In contrast, in the differentiation zone GFP-SUVH9 was exclusively located in nuclei. Further, GFP fusions of SUVH3 and SUVH7 never accumulated in the cytoplasm. Therefore it is possible that endogenous SUVH9 indeed in part is located in the cytoplasm. However, it can not be excluded that overexpression of this gene causes this cytoplasmic location, especially since the expression level in the differentiated cells is markedly lower than in the meristematic region and there GFP-SUVH9 exclusively is located in nuclei.

Structural analyses of a set of SET proteins have pointed out several amino acid residues that comprise the substrate (lysine) binding site and some of these could play a role in determining the number methyl groups that can be added (Zhang et al. 2003; Zhao et al. 2004; Collins et al. 2005). Mutational analysis of SET7/9, a mammalian H3K4 mono-methyltransferase and DIM-5, a H3K9 tri-methyltransferase of *Neurospora* showed that the difference in methylation multiplicity can be caused by a single amino acid. The mono-methyltransferase carries a tyrosine, whereas the tri-methyltransferase carries a phenylalanine at position 4 of the SET motif IV which is part of the catalytic site. Tyrosine residues reduce the size of the catalytic site of the enzyme allowing only one methyl group to be added to the target lysine, other residues like phenylalanine create more space permitting more methyl groups to be added (Xiao et al. 2003; Zhang et al. 2003; Collins et al. 2005). The alignment of the SET domains of the 10 SUVH proteins revealed that AtSUVH2 and AtSUVH9 have a leucine residue at position 4 of the

SET motif IV, whereas all other AtSUVH proteins have a tyrosine residue (figure 5).

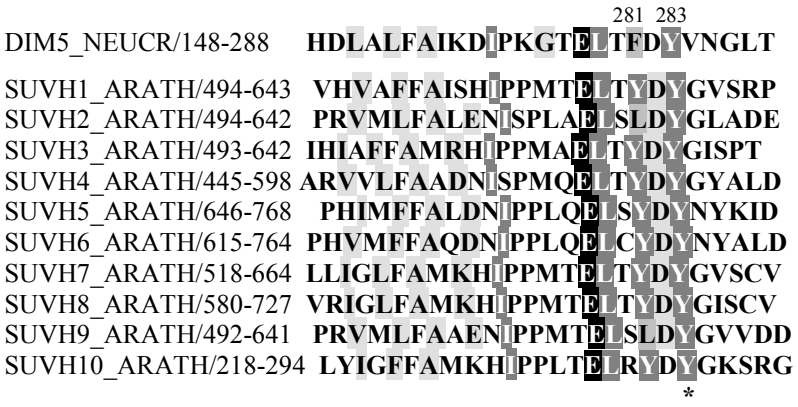

Figure 5: Multiple alignment of SET domains of SUVH1-10 proteins of Arabidopsis and DIM5 of Neurospora crassa adapted from Baumbusch et al. The asterisk * points to the important position 4 of the SET motif IV (Baumbusch et al. 2001)

This suggests that, if the latter have histone methyltransferase activity, they are mono or di-methyltransferase like SET7/9, whereas AtSUVH2 and AtSUVH9 could be tri-methyltransferases like DIM-5. This contrasts the conclusion of Ebbs et al. who stated that none of the SUVH proteins have a SET domain characteristic of a tri-methyltransferase (Ebbs et al. 2005). Indeed the Arabidopsis AtSUVH4, AtSUVH5 and AtSUVH6 proteins were shown to be H3K9 mono- or di-methyltransferases and unable to tri-methylate H3K9 (Lindroth et al. 2001; Ebbs et al. 2005). However, when the specific tyrosine was mutated into phenylalanine in AtSUVH4 and AtSUVH5, both proteins became tri-methyltransferases (Ebbs et al. 2005). Previous studies have shown that AtSUVH2 is probably a mono- or di-methyltransferase (Naumann et al. 2005). Therefore AtSUVH9 is the only putative histone tri-methyltransferase. Western blot analysis performed on leaf nuclei do not show that it indeed has histone tri-methyltransferase activity. However, it remains to be tested whether H3K9m3 levels are affected in root tips.

Since the SUVH9 expression pattern in part seems to overlap with that of LHP1, it is the best candidate among the SUVH proteins to create an epigenetic mark recognised by LHP1.

Studies on the animal H3K9 trimethyltransferases SUV39H1 and G9a showed that their enzymatic activity is not sufficient to target HP1 to chromatin (Stewart et al. 2005). In addition to enzymatic activity, also a binding of the SUV39H1 methyltransferase to HP1 turned out to be essential for this targeting (Stewart et al. 2005). Therefore we tested by yeast two-hybrid whether SUVH9 and LHP1 can interact. This turned out not to be the case and therefore this does not support that SUVH9 directly targets LHP1 to the chromatin. Further a *suvh9* loss of function mutant has a wild type phenotype in contrast to an *lhp1* mutant and the sub-nuclear location of LHP1in foci is not affected in a *suvh9* mutant background. Taken together it seems unlikely that SUVH9 is responsible for an epigenetic mark that targets LHP1 to chromatin complexes.
The trimethyltransferase responsible for trimethylating H3K9 could also be one of the 5 SUVR genes as none of them have a tyrosine at the critical position in their SET domain (Baumbusch et al. 2001). Further studies, on for example the SUVR family, are necessary to elucidate which trimethyltransferase might creates this putative binding site for LHP1.

Material & methods

Plant material growth conditions and homozygous line selection

Arabidopsis accession Columbia was used as wildtype.
Plants were grown vertically for 4-5 days post-germination except otherwise stated on 0.8% agar plates containing 2.2g Murashige and Skoog 10 salts with vitamins (Duchefa) plus 1 % sucrose at pH 5.8 in 16h light/ 8h dark at 23-24 °C.
For *suvh3* mutant (sdg19; At1g73100) only one line was available from the SALK (Alonso et al., 2003) and GABI-KAT collections (Rosso et al., 2003), where T-DNA is inserted in the 5'-UTR of the gene. By RT-PCR we determined that this T-DNA insertion does not affect the expression of the gene. So, we ordered an RNAi

line available at NASC (N24000). This mutant was produced using Agrobacterium-mediated transformation using a binary vector containing a fragment of the target gene in an inverted repeat orientation for RNAi silencing. The transgene is a single-copy insertion event, as determined by DNA gel blot analysis (data are posted at ChromDB: http://www.chromdb.org/). RT-PCR analysis also showed that the expression of SUVH3 gene was not affected. So no *suvh3* mutant was available for our analysis.

As *suvh7* mutant, we selected the GABI-KAT 037C06 line (NASC N403486), which has a T-DNA insertion ~400 bp after the ATG. Homozygous lines were selected according to their resistance to sulfadiazine. 18 lines were plated on medium containing 75 mg sulfadiazine (4-amino-N-[2-pyrimidinyl]benzene-sulfonamide-Na (Sigma S-6387)) per 10 ml. All resistant lines were checked by PCR with primers 5'- cgagaagcgtccgataatactaat-3' and 5'-tgaagccccactaagcacar-3' designed to amplify DNA sequence flanking T-DNA insertion, it was confirmed that they were homozygous lines.

For *suvh9* mutant (At4g13460) we selected a T-DNA line from the SALK collection (SALK_048033). This line was already genotyped in the B. Scheres' lab (Utrecht University, Netherlands) and we received homozygous seeds from them. T-DNA is inserted at ~280 bp from ATG (ORF is 1954 bp).

RT-PCR performed on suvh7 and suvh9 mutants confirmed that they are knock-out mutants.

Hairy root transformation

4-5 day old Arabidopsis seedlings were transformed as described (Limpens et al. 2004) using Färhaeus and Emergence medium instead of ½ MS.

Alignment of SUVH SET domain

Alignment was performed on the website http://www.expasy.ch. Of the ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) proteomics server of the Swiss Institute of Bioinformatics (SIB).

The Prosite database was used (Hulo N., Bairoch A., Bulliard V., Cerutti L., De Castro E.,Langendijk-Genevaux P.S., Pagni M., Sigrist C.J.A. *The PROSITE database.*Nucleic Acids Res. 34:D227-D230 (2006)) to search all
SET domains (PS50280).

Alignment of Swiss-Prot, true positive hit in clustal format was retrieved only for the SUVH proteins.

Cloning of the SUVH genes

SUVH3, *SUVH7* and *SUVH9* genes do not contain introns and we use genomic DNA as a template to amplify these genes. Primers used for amplification of *SUVH3* gene (2010 bp) are forward: 5'caccATGCAAGGAGTTCCTGGATT and reverse: 5'-TCATCCGAATGAACCACGACAT-3'; for *SUVH7* gene (2082 bp) Suvh7-F (5'CACCATGGATAAGTCTATTCCA3') and Suvh7-R (5'TTAGGTAAAAGAGCCACGACA3'); for SUVH9 91953 bp) F-5'caccatgggttcttctcaca 3' and R 5'-TTAATTACAAATGGCAAGCTTGG-3'. Amplified PCR fragment were purified from gel and introduced into a pENTR D-TOPO vector (Invitrogen). By recombination genes were introduced in frame with GFP in the pK7WGF2 vector (Karimi et al. 2002) creating a GFP-SUVH fusion proteins driven by the *35S CaMV* promoter.

Cloning of the SUVH promoters

To determine the expression pattern of the SUVH promoters we selected sequences the 3'-end of the adjacent gene up to the start codon for SUVH3 and SUVH9. For SUVH7 a 2kb region upstream the start codon was selected. Primers that were used are: SUVH3 promoter, 5'cacctgatatccttttaagacaaatt3' and 5'tgctaaactcctgtcaaag3'; SUVH7 promoter: 5'caccgttagaaatttaaggtagtta3' and 5'tggaaaaaaaatcctatcatt3'; SUVH9 promoter: 5'caccatcttctattcagtttgtact3' and 5'tcgcttctcgttgcaaaaa3', PCR products were amplified, purified from gel, cloned in pENTR D-TOPO vector (Invitrogen) and by recombination, promoter sequences were introduced into pKGWFS7.

Whole mount immunodetection of GFP-SUVH proteins

4-5 day old seedling roots were immunolabeled as described (Talbert et al. 2002). Roots were fixed in 4% paraformaldehyde in PBS pH 7.3 0.2% Triton for 1h with 20 min vacuum at room temperature. They were washed 2x 10 min with 1x PBS and transfered to small baskets with filters. They were digested for 40 min at 37°C with a mixture of 2.5% pectinase from *Aspergillus niger* (Sigma) and 2.5% cellulase Onozuka RS (Yakult Honsha Co.,Tokyo, Japan) dissolved in PBS. Roots were washed in PBS and squashed between slides. Slides were immersed in liquid nitrogen, the cover slips were removed, and roots were post-fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. After washing with 3x 5 min PBS, slides were incubated in a moist chamber at room temperature with blocking solution (3% BSA, 10% sheep serum) for 1h at 37°C. After cover slips

were removed, slides were incubated with rabbit anti-GFP polyclonal antibody (1:200, A11122 Molecular Probes) in labeling solution (1% BSA, 10% sheep serum, 0.1 % Tween 20 overnight at 4°C) for detection of LHP1-GFP. Cover slips were removed, and the slides were washed twice with PBS. The antibody was detected by applying Alexa 488–conjugated goat anti-rabbit secondary antibody (A11070, Molecular Probes) diluted 1:200 in labeling solution and incubated for 1-2 h, followed by two washes in PBS. The slides were stained and mounted with 2 µg/mL Propidium Iodide in Vectashield (Vector Laboratories, Burlingame, CA).

Whole mount Propidium Iodide staining

4-5 day old seedlings were incubated for 1-2h in Carnoy's solution (¼ Acetic acid + ¾ EtOH). The solution was then 5 times changed every 20 min. Samples were stored at -20°C.

Plant material was washed 3 x 10 min with water and then 2 times with PBS + 1% Triton X-100. Sample was incubated 1-2h with 2 µg/ml of Propidium Iodide in PBS + 1% Triton X-100, washed few times during 1 h with PBS + 1% Triton X-100. Seedlings were mounted on slides in citifluor for microscopic analysis.

Histochemical β-glucoronidase (GUS) staining

Transformed hairy roots were selected 4 weeks after transformation. Plantlets were washed 2 x 15 minutes with 0.1 M NaH_2PO_4 - Na_2HPO_4 pH 7.4. They were incubated 30 minutes under vacuum in the GUS reaction buffer (97.5 ml of (0.1 M NaH_2PO_4 - Na_2HPO_4 + 3% sucrose) + 1 ml of 0.5 mM EDTA + 0.5 ml of 0.1 M Kferrocyanide + 0.5 ml of 0.1 M Kferricyanide + 50 mg X-gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucororonic acid) in 500 µl DMFO (N,N dimethylformamide)) at room temperature. Incubation was then continued for 2 hours or longer till appearance of a visible blue colour at 37^0C. Reaction was stopped by washing 3 x 15 minutes with MQ water. To conserve the sample for several days, successive washes with EtOH can be performed: 20 % for 20 mn, 50% for 20 mn, 70% for 20 mn. Samples were stored at 4 ^{0}C till observation under Nikon DIC Normaski microscope.

Starch staining

Starch staining was performed by incubating seedlings for a few minutes in Lugol solution (Merck) and they were then mounted on slides in choral hydrate solution (8 g chloral hydrate, 1 ml glycerol and 3 ml water) for clearing. Seedlings were observed on Nikon DIC Normaski microscope with a 10x or 20x objective.

Confocal Laser Scanning Microscopy

All confocal images were acquired on a Zeiss 510 inverted microscope using a 40x/1.3 oil immersion objective. Image resolution was always higher than the theoretical limit for light microscopy to insure no data was missed.

FRAP

All FRAP studies were performed with similar settings as described for the imaging. The Region Of Interest was kept at approximately 1 μm^2 allowing direct comparison between half-times of recovery. Recovery of fluorescence intensity was monitored in such manner that at least 10% of the obtained images were obtained before the half time of recovery.

Western Analysis

Nuclei were isolated from wt Arabidopsis acc. Columbia or *suvh9* leaves as described (Zhong, X-B et al, 1996. Plant Molecular Biology reporter 14, 232-242). Proteins were separated on SDS-15% polyacrylamide gels. Gels were transferred to nitrocellulose membranes. Membranes were used for immunodetection with α-histone H3 rabbit polyclonal (1:1000, Abcam) and α-H3K9m3 rabbit polyclonal IgG (1:500, Upstate). Detection of primary antibodies was performed α-rabbit IgG (Fc) Alkaline Phosphatase conjugate (Promega).

Interaction of LHP1 with a SUVH protein with the Yeast two hybrid system

The full-length coding regions of LHP1 (1338 bp), SUVH2 (1956 bp), SUVH3 (2010 bp), SUVH7 (2082 bp), SUVH8 (2268 bp) and SUVH9 (1953 bp) were cloned into the pENTR D-TOPO and by recombination into the pDEST^{TM}22 vector (carrying the TRP1 gene; Invitrogen) containing the GAL4 activation domain (AD). LHP1 was also cloned into the pDEST^{TM}32 vector (carrying the LEU2 gene; Invitrogen) containing the GAL4 DNA-binding domain (DB). The transcription factors AGL74 + AGL103 and AGL24 + AGL8, fused with the BD and AD, respectively, were used for positive control of strong interaction and weak interaction…. Interaction of the encoded fusion proteins was investigated by co-transforming appropriate plasmids into the yeast reporter strain PJ69-4A (MATa trp1-90 leu2-3,112 ura3-52 his3-200 gal4D gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (James et al., 1996). Transformed yeast cells were plated onto Synthetic Complete medium (6.7 g/l yeast nitrogenbase without amino acids +

2% glucose + amino acids mix + 20g/l agar, pH 5.9), lacking leucine and tryptophan and grown at 28°C for 2-4 days to select for the presence of both plasmids. Colonies were then transferred to select for interactions to medium lacking leucine, tryptophan and adenine or lacking leucine, tryptophan and histidine with different concentration of 3-Amino-1,2,4-Triazole (0, 10, 15, 30 and 60 mM) to enhance the likelihood of detecting weak interaction. Yeast colonies grown in SC lacking leucine and tryptophane were replicated on a nitrocellulose membrane and placed on YPAD medium (10g/l bacto-yeast extract + 20g/l bacto-peptone + 2% glucose + 100 mg/l adenine sulphate, pH 6) to perform the β-galactosidase assay. After 2 days of growth at 30°C, the membranes were lifted and immerse in liquid nitrogen for 20-30 s and placed on Whatman filter papers soaked in ~ 8 ml X-gal solution :10 mg X-gal in 100 µl DMF + 60 µl β-mercaptoethanol +10 ml Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0). They were incubated at 37°C and monitored for appearance of blue colour for 24h.

References

Aagaard, L., G. Laible, et al. (1999). "Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31." Embo J 18(7): 1923-38.

Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature 410(6824): 120-4.

Baumbusch, L. O., T. Thorstensen, et al. (2001). "The Arabidopsis thaliana genome contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes." Nucleic Acids Res 29(21): 4319-33.

Bottomley, M. J. (2004). "Structures of protein domains that create or recognize histone modifications." EMBO Rep 5(5): 464-9.

Chanvivattana, Y., A. Bishopp, et al. (2004). "Interaction of Polycomb-group proteins controlling flowering in Arabidopsis." Development 131(21): 5263-76.

Citterio, E., R. Papait, et al. (2004). "Np95 is a histone-binding protein endowed with ubiquitin ligase activity." Mol Cell Biol 24(6): 2526-35.

Collins, R. E., M. Tachibana, et al. (2005). "In vitro and in vivo analyses of a Phe/Tyr switch controlling product specificity of histone lysine methyltransferases." J Biol Chem 280(7): 5563-70.

Ebbs, M. L., L. Bartee, et al. (2005). "H3 lysine 9 methylation is maintained on a transcribed inverted repeat by combined action of SUVH6 and SUVH4 methyltransferases." Mol Cell Biol 25(23): 10507-15.

Fischer, A., I. Hofmann, et al. (2006). "Heterochromatin proteins and the control of heterochromatic gene silencing in Arabidopsis." J Plant Physiol 163(3): 358-68.

Fischle, W., B. S. Tseng, et al. (2005). "Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation." Nature 438(7071): 1116-22.

Gaudin, V., M. Libault, et al. (2001). "Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in Arabidopsis." Development 128(23): 4847-58.

Hediger, F. and S. M. Gasser (2006). "Heterochromatin protein 1: don't judge the book by its cover!" Curr Opin Genet Dev 16(2): 143-50.

Hiragami, K. and R. Festenstein (2005). "Heterochromatin protein 1: a pervasive controlling influence." Cell Mol Life Sci 62(23): 2711-26.

Horsley, D., A. Hutchings, et al. (1996). "M32, a murine homologue of Drosophila heterochromatin protein 1 (HP1), localises to euchromatin within interphase nuclei and is largely excluded from constitutive heterochromatin." Cytogenet Cell Genet 73(4): 308-11.

Jackson, J. P., L. Johnson, et al. (2004). "Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in Arabidopsis thaliana." Chromosoma 112(6): 308-15.

Jacobs, S. A. and S. Khorasanizadeh (2002). "Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail." Science 295(5562): 2080-3.

Jones, R. S. and W. M. Gelbart (1993). "The Drosophila Polycomb-group gene Enhancer of zeste contains a region with sequence similarity to trithorax." Mol Cell Biol 13(10): 6357-66.

Karimi, M., D. Inze, et al. (2002). "GATEWAY vectors for Agrobacterium-mediated plant transformation." Trends Plant Sci 7(5): 193-5.

Kotake, T., S. Takada, et al. (2003). "Arabidopsis TERMINAL FLOWER 2 gene encodes a heterochromatin protein 1 homolog and represses both FLOWERING LOCUS T to regulate flowering time and several floral homeotic genes." Plant Cell Physiol 44(6): 555-64.

Lachner, M., R. J. O'Sullivan, et al. (2003). "An epigenetic road map for histone lysine methylation." J Cell Sci 116(Pt 11): 2117-24.

Larsson, A. S., K. Landberg, et al. (1998). "The TERMINAL FLOWER2 (TFL2) gene controls the reproductive transition and meristem identity in Arabidopsis thaliana." Genetics 149(2): 597-605.

Libault, M., F. Tessadori, et al. (2005). "The Arabidopsis LHP1 protein is a component of euchromatin." Planta 222(5): 910-25.

Limpens, E., J. Ramos, et al. (2004). "RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula." J Exp Bot 55(399): 983-92.

Lindroth, A. M., X. Cao, et al. (2001). "Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation." Science 292(5524): 2077-80.

Liu, L. P., J. Q. Ni, et al. (2005). "Sex-specific role of Drosophila melanogaster HP1 in regulating chromatin structure and gene transcription." Nat Genet 37(12): 1361-6.

Lorentz, A., K. Ostermann, et al. (1994). "Switching gene swi6, involved in repression of silent mating-type loci in fission yeast, encodes a homologue

of chromatin-associated proteins from Drosophila and mammals." Gene 143(1): 139-43.

Lorvellec, M., J. Willemse, et al. (Chapter 3). LHP1 forms euchromatin complexes at trimethylated lysines of histone H3. Molecular Biology. Plant Sciences, Wageningen University.

Maison, C. and G. Almouzni (2004). "HP1 and the dynamics of heterochromatin maintenance." Nat Rev Mol Cell Biol 5(4): 296-304.

Marmorstein, R. (2003). "Structure of SET domain proteins: a new twist on histone methylation." Trends Biochem Sci 28(2): 59-62.

Mylne, J. S., L. Barrett, et al. (2006). "LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of FLC." Proc Natl Acad Sci U S A 103(13): 5012-7.

Nakahigashi, K., Z. Jasencakova, et al. (2005). "The Arabidopsis heterochromatin protein1 homolog (TERMINAL FLOWER2) silences genes within the euchromatic region but not genes positioned in heterochromatin." Plant Cell Physiol 46(11): 1747-56.

Nakayama, J., A. J. Klar, et al. (2000). "A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis." Cell 101(3): 307-17.

Naumann, K., A. Fischer, et al. (2005). "Pivotal role of AtSUVH2 in heterochromatic histone methylation and gene silencing in Arabidopsis." Embo J 24(7): 1418-29.

Nielsen, A. L., M. Oulad-Abdelghani, et al. (2001). "Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins." Mol Cell 7(4): 729-39.

Ogawa, H., K. Ishiguro, et al. (2002). "A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells." Science 296(5570): 1132-6.

Rea, S., F. Eisenhaber, et al. (2000). "Regulation of chromatin structure by site-specific histone H3 methyltransferases." Nature 406(6796): 593-9.

Schotta, G., A. Ebert, et al. (2002). "Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing." Embo J 21(5): 1121-31.

Singh, P. B., J. R. Miller, et al. (1991). "A sequence motif found in a Drosophila heterochromatin protein is conserved in animals and plants." Nucleic Acids Res 19(4): 789-94.

Stassen, M. J., D. Bailey, et al. (1995). "The Drosophila trithorax proteins contain a novel variant of the nuclear receptor type DNA binding domain and an ancient conserved motif found in other chromosomal proteins." Mech Dev 52(2-3): 209-23.

Stewart, M. D., J. Li, et al. (2005). "Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment." Mol Cell Biol 25(7): 2525-38.

Talbert, P. B., R. Masuelli, et al. (2002). "Centromeric localization and adaptive evolution of an Arabidopsis histone H3 variant." Plant Cell 14(5): 1053-66.

Tschiersch, B., A. Hofmann, et al. (1994). "The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes." Embo J 13(16): 3822-31.

Xiao, B., C. Jing, et al. (2003). "Structure and catalytic mechanism of the human histone methyltransferase SET7/9." Nature 421(6923): 652-6.

Yu, Y., A. Dong, et al. (2004). "Molecular characterization of the tobacco SET domain protein NtSET1 unravels its role in histone methylation, chromatin binding, and segregation." Plant J 40(5): 699-711.

Zemach, A., Y. Li, et al. (2006). "Different domains control the localization and mobility of LIKE HETEROCHROMATIN PROTEIN1 in Arabidopsis nuclei." Plant Cell 18(1): 133-45.

Zhang, X., Z. Yang, et al. (2003). "Structural basis for the product specificity of histone lysine methyltransferases." Mol Cell 12(1): 177-85.

Zhao, Z. and W.-H. Shen (2004). "Plants Contain a High Number of Proteins Showing Sequence Similarity to the Animal SUV39H Family of Histone Methyltransferases
" Ann.N.Y.Acad.Sci. 1030: 661-669.

CHAPTER 5

General discussion

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It is increasingly clear that chromatin is not just a device for packing DNA within the nucleus, but also a dynamic material that changes that responds to developmental and environmental cues to determine the correct spatial and temporal expression of genes. During the entire life cycle of a plant, a wide variety of developmental decisions are carried out that require regulation at the chromatin level for example in meristems, during the transition from the vegetative to reproductive phase or when seeds start to germinate. The involvement of chromatin remodelling genes in such processes is for example illustrated by the fact that, components of the chromatin assembly factor 1 complex (CAF-1), FASCIATA1 and FASCIATA2 restrict WUSCHEL and SCARECROW activity, essential for shoot and root meristem maintenance (Kaya et al. 2001; Byrne et al. 2003; Ono et al. 2006). CAF-1 is a histone chaperon complex involved in chromatin reconstitution after DNA replication.
In the further paragraphs we will review how properties of heterochromatin and euchromatin change during plant development.

Relative Heterochromatin level

Heterochromatin appears as dense regions in interphase nuclei. However, heterochromatin amounts are not forever fixed, as the amount of heterochromatin varies through development. Tessadori et al, reported an increase by 27% in heterochromatin during mesophyll cell differentiation (Tessadori et al. 2004). Furthermore, a reduction in size of chromocentres was observed in Arabidopsis leave nuclei during the transition to the reproductive phase before bolting and heterochromatin levels recovered after bolting. At the stage when the relative heterochromatin levels were reduced the pericentric repeats and 5S rDNA are no longer present within the chromocentres but 45S rDNA and the 180 bp centromeric repeat do (Tessadori 2006).

DNA methylation

5-methylcytosine is in general a mark for transcriptional repression, conserved from mammals to plants and predominantly localizes at chromocentres in

Arabidopsis. Studies with *ddm1* (decreased in dna methylation) and *met1* (methyltransferase 1) mutants revealed that the level of DNA methylation and the organization of the heterochromatic chromocentres are strongly correlated. When the level of DNA methylation decreases, the relative level of heterochromatin decreases as well in most nuclei (Fransz et al. 2003). In our studies, we show that level of DNA methylation in chromocentres varies among cell types and through development. Heterochromatin is ~2 times more methylated in QC and cortex/endodermis nuclei than in differentiated columella cells. However, in this case the relative heterochromatin level is not affected (Chapter 2).
In animals, the level of DNA methylation also varies through development. During mammalian embryogenesis, after formation of the zygote, both maternal and paternal chromosomes undergo progressive demethylation by a passive mechanism. This erases, by the blastocyst stage, most of the methylation marks inherited from the gametes. However, methylation marks on imprinted genes remain (Li 2002). Upon differentiation DNA methylation increases. During the differentiation of cultured embryonic stem (ES) cell , ES cells nuclei also acquire a higher DNA methylation level (Meshorer 2007). So plants and mammals both obtain a higher DNA methylation level during differentiation.

DNA methylation is highest in heterochromatic regions of the genome. However, it is not restricted to heterochromatin as it is also occurs in euchromatic area. In our immunocytology studies, we showed that the euchromatin in Arabidopsis Quiescent Centre (QC) cells and columella cells is methylated and in QC euchromatin, this methylation is about twice as high than in columella cells. This shows that the overall methylation level of euchromatin can vary markedly between Arabidopsis cell types.
Zhang et al, showed recently that about 19% of the Arabidopsis genome is methylated and DNA methylation is also found in euchromatin. About 5% of the expressed genes were methylated within their promoter regions and 33% within their transcribed regions (body-methylated). Surprisingly, the expression level of body-methylated genes was higher than of unmethylated genes, whereas promoter-

methylated genes were lower expressed and showed a high tissue specificity (Zhang et al. 2006).
In conclusion, DNA methylation is not always a mark of gene silencing and its level is not fixed even in the heterochromatin..

Histone modifications and histone variants

Histone N-terminal tails can be acetylated, methylated, phosphorylated, ubiquitinated, glycosylated, ADP-ribosylated, carbonylated and sumoylated on different amino acids like arginine, lysine or serine. This shows that numerous variations are possible and so the 'histone' code can be very complex. However, our knowledge on to what extend this complexity is exploited during development is still scanty but some recent studies indicate that the epigenetic code participates into regulation of genes expression during development.
For example in mouse, H3K9m3, H3K27m and H4K20m3 are marks of constitutive heterochromatin, H3K9m2, H3K27m3 and H4K20m mark the facultative heterochromatin, and H3K9m, H3K27m2 and H4K20m2 mark the euchromatin. In Arabidopsis, chromocentres are enriched in H3K9m, H3K9m2, H3K27m, H3K27m2 and H4K20m. Euchromatin is marked by H3K9m3, H3K27m3, H4K20m2 and H4K20m3 (Ebert et al. 2006; Fuchs et al. 2006). This distribution pattern however differs even among plant species (Fuchs et al. 2006).
Histone acetylation and phosphorylation is also cell-cycle-dependent. In plants, H3S10ph and H3S28ph are restricted to pericentromeric regions during mitosis and meiosis II, whereas during the first meiotic division both residues are phosphorylated on the entire chromosome. H3 phosphorylation at both serine is thought to be involved in cohesion of sister chromatids during metaphase I and of sister pericentromeres during mitosis and metaphase II (Fuchs et al. 2006).
Another level of complexity in chromatin regulation is brought by the possibility to replace the major histones by histone variants. In mammals CenH3, a histone variant localised in the centromere is required for accurate chromosome segregation. Several histone H1 variants are involved in repression of transcription during differentiation (Kamakaka et al. 2005). In plants an homolog of CenH3 exists, HTR12 is localised in the centromeres in mitotic and meiotic cells and

revealed tissue and stage specific differences in centromere morphology (Talbert et al. 2002). In contrast to the genetic code, the epigenetic code is not universal to all organisms and was revealed to be highly complex and to evolve during development. A careful analysis of this code is necessary to start to comprehend how genes are regulated through development.

Chromatin complexes

A combination of DNA methylation and posttranslational modifications of histones define specific chromatin states and recruit different chromatin remodellers like Polycomb Group proteins (PcG), Heterochromatin Protein 1 proteins or Histone modifying enzymes. These proteins form large complexes that most likely play an important role in regulation of gene expression (Jones et al. 2000; McBryant et al. 2006).

Polycomb group (PcG)proteins are highly conserved chromatin proteins and were discovered in *Drosophila* (Lewis 1978). They are known to create the memory for maintaining Hox gene expression patterns during development (Bantignies et al. 2006). A much more dynamic role for the PcGb proteins has emerged in recent years as integrators and/or modulators of cell cycle checkpoints in dividing cells. For example, Cyclin A is a direct target of PcG proteins (Bantignies et al. 2006; Martinez et al. 2006). PcG proteins form multimeric complexes in the euchromatin, visible as foci (Furuyama et al. 2003). Polycomb Repressive Complex 1 (PRC1) and PRC2 are the best studied in Drosophila and bind to H3K27m3 (Bantignies et al. 2006).

Several PcG have been identified in plants. The MEA/FIE/FIS/MSI1 complex regulates the transition from gametophytic to sporophytic phase by preventing initiation of the endosperm development program in the central cell of the embryo sac (Hsieh et al. 2005). PHERES1 has been reported to be a direct target of MEA in flowers and of CURLY LEAF/SWINGER, two other PcG proteins, in leaves (Steimer et al. 2004; Makarevich et al. 2006). So different PcG complexes regulate common target genes during different developmental stages

Heterochromatin Protein 1 was discovered in Drosophila (James et al. 1986). Since its discovery, several homologues of HP1 have been identified, from fission yeast

(Swi6) to human to plants, showing that HP1 is a highly conserved protein. Further several isoforms were discovered as well, each with its own subnuclear location: in heterochromatin and/or in euchromatin like HP1γ (Vakoc et al. 2005).. Currently HP1 is thought to serve as a bridging protein, connecting histones and non histone chromosomal proteins (Li et al. 2002). HP1 was shown in several systems (fission yeast (Nakayama et al. 2000), Drosophila (Bannister et al. 2001; Jacobs et al. 2002), mammals (Aagaard et al. 1999; Rea et al. 2000) to bind to methylated histone 3 Lysine 9 (H3K9) and with a highest affinity for trimethylated H3K9 (Fischle et al. 2003). In chapter 3 we showed that this histone modification in part coincides with the speckles formed by LHP1, the Arabidopsis HP1 homologue.

In animals and yeast, HP1 was shown to be involved in chromatin structural organization, maintenance of heterochromatin and gene regulation (Hiragami et al. 2005; Hediger et al. 2006) and to be a highly dynamic protein (Cheutin et al. 2003; Festenstein et al. 2003; Schmiedeberg et al. 2004; Zemach et al. 2006).

Arabidopsis LHP1 is located in the euchromatin and forms many foci. In roots of Arabidopsis, LHP1 was described to have a diffuse pattern in dividing meristematic cells and a speckled-like pattern (foci) in differentiated cells (Chapter 3) (Gaudin et al. 2001; Kotake et al. 2003; Takada et al. 2003; Nakahigashi et al. 2005). Furthermore, we showed in this thesis by FRET/FLIM that LHP1 within the foci as well as win the regions between foci is in very close vicinity to DNA. Therefore we postulate that also in plants the HP1 homologue forms chromatin complexes. FRAP studies further showed that these complexes are highly dynamic (Chapter 3).

Microarray analyses on *lhp1* knockout mutants revealed that LHP1 silences genes within euchromatin, but not in heterochromatin(Nakahigashi et al. 2005). In Arabidopsis, LHP1 was shown to regulate two genes involved in flowering time: Flowering Locus T (FT) and Flowering Locus C (FLC). FLC is a repressor of flowering in the vernalization pathway and negatively regulates FT, which is a floral pathway integrator. Furthermore, LHP1 was shown to be required for epigenetic maintenance of vernalization-induced repression of FLC (Mylne et al. 2006). Although it is clear that LHP1 is involved in regulation of gene expression it remains to be demonstrated that the foci formed by LHP1 are involved in this

regulation. These foci are rather large and could contain tens of genes. Since LHP1 associated genes are not clustered (Turck et al. 2007), it would imply that LHP1 regulated genes would cluster in foci by forming numerous loops. In addition, LHP1 regulation seems to depend of the developmental stage of the cells as hardly any foci are formed in the meristematic cells. Histone methyltransferases form also chromatin complexes. Drosophila SU(VAR)3-9 as well as its homolog in mammals (SUVH39H1) methylates specifically residue lysine 9 of histone H3 (H3K9) creating a specific binding site for the chromodomain of HP1. This results in chromatin condensation and gene silencing (Lachner et al. 2003). This mechanism is conserved in the fission yeast *S. pombe* in which the SU(VAR)3-9 homolog CLR4 and the HP1 homolog SWI6 play orthologous functions. In Drosophila, HP1 was shown *in vitro* (yeast two hybrid) to interact through its CSD with SU(VAR)3-9 (Schotta et al. 2002). Targeting HP1 to chromatin required not only K9 methylation but also a direct protein-protein interaction between SUVH39H1 and HP1.

In chapter 4, we investigated whether a histone methyltransferase from the Arabidopsis SUVH proteins, homologs of SU(VAR)3-9, could provide for a H3K9 modification for LHP1 binding. However our studies do not provide support for the idea that any of the SUVH proteins could fulfil such function. We did show that . SUVH3, SUVH7 and SUVH9 are located in the euchromatin and SUVH3 and SUVH9 forms speckles which are highly dynamic suggesting they are part of chromatin complexes involved in gene regulation during development (Chapter 4).

So numerous foci are present within the euchromatin of Arabidopsis interphase nuclei, These foci most likely represent chromatin complexes involved in regulation of gene expression. Although the distribution of heterochromatin and euchromatin in an Arabidopsis interphase nucleus is rather simple, the occurrence of numerous euchromatic foci that are putatively involved in regulation of gene expression shows that especially the chromatin organisation in euchromatin is rather complex. This is probably required to respond to developmental and environmental clues in a proper and robust manner

References

Aagaard, L., G. Laible, et al. (1999). "Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31." Embo J 18(7): 1923-38.

Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature 410(6824): 120-4.

Bantignies, F. and G. Cavalli (2006). "Cellular memory and dynamic regulation of polycomb group proteins." Curr Opin Cell Biol 18(3): 275-83.

Byrne, M. E., C. A. Kidner, et al. (2003). "Plant stem cells: divergent pathways and common themes in shoots and roots." Curr Opin Genet Dev 13(5): 551-7.

Cheutin, T., A. J. McNairn, et al. (2003). "Maintenance of stable heterochromatin domains by dynamic HP1 binding." Science 299(5607): 721-5.

Ebert, A., S. Lein, et al. (2006). "Histone modification and the control of heterochromatic gene silencing in Drosophila." Chromosome Res 14(4): 377-92.

Festenstein, R., S. N. Pagakis, et al. (2003). "Modulation of heterochromatin protein 1 dynamics in primary Mammalian cells." Science 299(5607): 719-21.

Fischle, W., Y. Wang, et al. (2003). "Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains." Genes Dev 17(15): 1870-81.

Fransz, P., W. Soppe, et al. (2003). "Heterochromatin in interphase nuclei of Arabidopsis thaliana." Chromosome Res 11(3): 227-40.

Fuchs, J., D. Demidov, et al. (2006). "Chromosomal histone modification patterns--from conservation to diversity." Trends Plant Sci 11(4): 199-208.

Furuyama, T., F. Tie, et al. (2003). "Polycomb group proteins ESC and E(Z) are present in multiple distinct complexes that undergo dynamic changes during development." Genesis 35(2): 114-24.

Gaudin, V., M. Libault, et al. (2001). "Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in Arabidopsis." Development 128(23): 4847-58.

Hediger, F. and S. M. Gasser (2006). "Heterochromatin protein 1: don't judge the book by its cover!" Curr Opin Genet Dev 16(2): 143-50.

Hiragami, K. and R. Festenstein (2005). "Heterochromatin protein 1: a pervasive controlling influence." Cell Mol Life Sci 62(23): 2711-26.

Hsieh, T. F. and R. L. Fischer (2005). "Biology of chromatin dynamics." Annu Rev Plant Biol 56: 327-51.

Jacobs, S. A. and S. Khorasanizadeh (2002). "Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail." Science 295(5562): 2080-3.

James, T. C. and S. C. Elgin (1986). "Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene." Mol Cell Biol 6(11): 3862-72.

Jones, D. O., I. G. Cowell, et al. (2000). "Mammalian chromodomain proteins: their role in genome organisation and expression." Bioessays 22(2): 124-37.

Kamakaka, R. T. and S. Biggins (2005). "Histone variants: deviants?" Genes Dev 19(3): 295-310.

Kaya, H., K. I. Shibahara, et al. (2001). "FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems." Cell 104(1): 131-42.

Kotake, T., S. Takada, et al. (2003). "Arabidopsis TERMINAL FLOWER 2 gene encodes a heterochromatin protein 1 homolog and represses both FLOWERING LOCUS T to regulate flowering time and several floral homeotic genes." Plant Cell Physiol 44(6): 555-64.

Lachner, M., R. J. O'Sullivan, et al. (2003). "An epigenetic road map for histone lysine methylation." J Cell Sci 116(Pt 11): 2117-24.

Lewis, E. B. (1978). "A gene complex controlling segmentation in Drosophila." Nature 276(5688): 565-70.

Li, E. (2002). "Chromatin modification and epigenetic reprogramming in mammalian development." Nat Rev Genet 3(9): 662-73.

Li, Y., D. A. Kirschmann, et al. (2002). "Does heterochromatin protein 1 always follow code?" Proc Natl Acad Sci U S A 99 Suppl 4: 16462-9.

Makarevich, G., O. Leroy, et al. (2006). "Different Polycomb group complexes regulate common target genes in Arabidopsis." EMBO Rep 7(9): 947-52.

Martinez, A. M. and G. Cavalli (2006). "The role of polycomb group proteins in cell cycle regulation during development." Cell Cycle 5(11): 1189-97.

McBryant, S. J., V. H. Adams, et al. (2006). "Chromatin architectural proteins." Chromosome Res 14(1): 39-51.

Meshorer, E. (2007). "Chromatin in embryonic stem cell neuronal differentiation." Histol Histopathol 22(3): 311-9.

Mylne, J. S., L. Barrett, et al. (2006). "LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of FLC." Proc Natl Acad Sci U S A 103(13): 5012-7.

Nakahigashi, K., Z. Jasencakova, et al. (2005). "The Arabidopsis heterochromatin protein1 homolog (TERMINAL FLOWER2) silences genes within the euchromatic region but not genes positioned in heterochromatin." Plant Cell Physiol 46(11): 1747-56.

Nakayama, J., A. J. Klar, et al. (2000). "A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis." Cell 101(3): 307-17.

Ono, T., H. Kaya, et al. (2006). "Chromatin assembly factor 1 ensures the stable maintenance of silent chromatin states in Arabidopsis." Genes Cells 11(2): 153-62.

Rea, S., F. Eisenhaber, et al. (2000). "Regulation of chromatin structure by site-specific histone H3 methyltransferases." Nature 406(6796): 593-9.

Schmiedeberg, L., K. Weisshart, et al. (2004). "High- and low-mobility populations of HP1 in heterochromatin of mammalian cells." Mol Biol Cell 15(6): 2819-33.

Schotta, G., A. Ebert, et al. (2002). "Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing." Embo J 21(5): 1121-31.

Steimer, A., H. Schob, et al. (2004). "Epigenetic control of plant development: new layers of complexity." Curr Opin Plant Biol 7(1): 11-9.

Takada, S. and K. Goto (2003). "Terminal flower2, an Arabidopsis homolog of heterochromatin protein1, counteracts the activation of flowering locus T by constans in the vascular tissues of leaves to regulate flowering time." Plant Cell 15(12): 2856-65.

Talbert, P. B., R. Masuelli, et al. (2002). "Centromeric localization and adaptive evolution of an Arabidopsis histone H3 variant." Plant Cell 14(5): 1053-66.

Tessadori, F., R. van Driel, et al. (2004). "Cytogenetics as a tool to study gene regulation." Trends Plant Sci 9(3): 147-53.

Tessadori, F. (2006). Chapter 3: The FLoral Transition in Arabidopsis is accompanied by chromatin decondensation in repeat-rich and gene-rich domains. Biochemistry, Swammerdam Institute for Life Sciences. University of Amsterdam. PhD: 175.

Turck, F., F. Roudier, et al. (2007). "Arabidopsis TFL2/LHP1 is a Polycomb Group Protein that associates with a large set of genes encoding developmental regulators." (submitted).

Vakoc, C. R., S. A. Mandat, et al. (2005). "Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin." Mol Cell 19(3): 381-91.

Zemach, A., Y. Li, et al. (2006). "Different domains control the localization and mobility of LIKE HETEROCHROMATIN PROTEIN1 in Arabidopsis nuclei." Plant Cell 18(1): 133-45.

Zhang, X., J. Yazaki, et al. (2006). "Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis." Cell 126(6): 1189-201.

SUMMARY

The genetic information is stored in a highly compact manner in every nucleus. About 150 bp of DNA is packed around a histone octamer constituting a nucleosome. Nucleosomes are linked together by histone H1 and further compaction of this "beads on a string" form higher-order chromatin structures. DNA staining reveals two cytologically different chromatin states: weakly stained euchromatin and brightly stained heterochromatin. Euchromatin is gene-rich and decondensed during interphase, whereas heterochromatin is rich in repetitive sequences, low in gene density, and remains mostly condensed throughout the cell cycle. Euchromatin and heterochromatin differ also by their epigenetic modifications. Epigenetic modifications of chromatin are for example methylated cytosine and acetylation or methylation of histones tails. Acetylation of histones is in general a mark of euchromatin, whereas DNA methylation and histone methylation are marks of heterochromatin. To access the chromatin to perform processes such as DNA replication or to modify the expression of a gene, chromatin remodelling is necessary and performed by chromatin modifiers such as Heterochromatin Protein 1.

In this thesis, we studied how chromatin is organised through development of the root of Arabidopsis. This model plant has a simple organized root meristem. Further the distribution of eu- and heterochromatin in interphase nuclei is rather simple. This allows us to follow the chromatin organisation of a cell through development from stem cell into a fully differentiated cell.

DNA methylation is one of the most abundant epigenetic modifications and varies through development. It is involved in the defence of the genome against transposable elements and retroviruses, in the control of genomic imprinting and in the regulation of gene expression.

In Arabidopsis, we showed that Quiescent Center (QC) cells and stem cells are highly methylated contrarily to stem cells in animals. When cells divide their DNA methylation level decreases to increase again when cells differentiate. DRM1 and DRM2, *de novo* DNA methyltransferases, and HDA1, a histone deactyltransferase,

appear to be involved in establishing the hypermethylated DNA state in nuclei of QC and stem cells.

Heterochromatin Protein 1 in animals is a chromatin modifier first discovered as a protein involved in heterochromatin formation. Nowadays it is thought to be a bridging protein, connecting histones through its chromodomain and non-histone chromosomal proteins through its chromoshadow domain. The homologue of Heterochromatin Protein 1 in Arabidopsis is Like Heterochromatin Protein 1 (LHP1). LHP1 was shown to be located in the euchromatic part of interphase nuclei like the animal isoform HP1γ and to form foci in differentiated cells. We showed that these foci are most likely chromatin complexes bound to the DNA and that LHP1 binds probably trimethylated lysine 9 and/or trimethylated lysine 27 of histone H3.

HP1 in animal was shown to bind to trimethylated lysine 9 of histone H3 (H3K9m3) and to interact with the H3K9 trimethyltransferase SU(VAR)3-9. In Arabidopsis, we tried to identify among the family of SU(VAR)3-9 homologues, the SUVH proteins, which is responsible for trimethylating H3K9 and might interact with LHP1. We showed that SUVH3, SUVH7 and SUVH9 are tissue specifically expressed and their encoded proteins are located in the euchromatic regions where they most likely form chromatin complexes. SUVH3 and SUVH9 form foci depending on the developmental stage of the cell. SUVH9 might be a candidate for trimethylating histone H3 lysine 9 however neither SUVH3,-7 or -9 are likely to interact with LHP1.

NEDERLANDSE SAMENVATTING

In eukaryote cellen is het genomisch DNA opgeslagen in de kern. Het is daar geassocieerd met eiwitten en dit zogenaamde chromatine is compact opgevouwen. Het gehele genoom is opgevouwen rond octameren van histonen, de nucleosomen. Op iedere 200bp komt een nucleosoom vorm en deze chromatine organisatie wordt "beads on a string" genoemd. Door verdere vouwing wordt een hogere orde chromatine organisatie bewerkstelligd. Cytologische kleuring van DNA laat zien dat het chromatine ruw weg verdeeld kan worden in licht gekleurd euchromatine en meer intens gekleurd heterochromatine. Euchromatine is rijk aan genen en is gedurende de interface relatief weinig gecondenseerd. Het heterochromatine is daarentegen rijk aan repetitieve DNA sequenties, bevat weinig gene en blijft in het algemeen sterker gecondenseerd gedurende de gehele cel cyclus. Euchromatine en heterochromatine verschillen ook sterk in hun epigenetische modificaties. Dit zijn b.v. cytosine methyleing en acetylering en methylering van aminozuren in histon staarten die uit de nucleosomen steken. Acetylering van histonen is in het algemeen een kenmerk van euchromatine, terwijl methylering van DNA en histonen meer voorkomt in het heterochromatine.

Voor processen als DNA replicatie en transcriptie van genen kan het essentieelzijn dat de chromatine organisatie veranderd wordt. Eiwitten die dit kunnen doen worden "chromatin remodellers" genoemd. Heterochromatin Protein 1 (HP1) is een sterk geconserveerde "chromatin remodeller" en de *Arabidopsis thaliana* homoloog is bestudeerd in dit proefschrift.

In dit proefschrift staan mijn studies beschreven waarin ik de chromatine organisatie in een zich ontwikkelende wortel van de model plant Arabidopsis heb bestudeerd. Dit orgaan inclusief zijn meristeem heeft een simpele organisatie. Verder zijn Arabidopsis kernen gekenmerkt door een simpele distributie van euchromatine en heterochromatine. Dit maakte het mogelijk de chromatine organisatie van een cel type gedurende zijn ontwikkeling te bestuderen.

DNA methylering is een van de meest voorkomende epigenetische modificatie en het niveau van DNA methylering kan sterk variëren gedurende ontwikkeling. Het is betrokken bij b.v. ; de bescherming van het genoom tegen teveel "transposable element" activiteit, het aanbrengen van een moleculair geheugen in genen (ïmprinting) betreffende hun expressie status aan te brengen.
We hebben laten zien dat het DNA van "Quiescent Center" (QC) cellen het hoogst is. Dit is opvallend omdat dierlijke stam cellen eerder gekarakteriseerd zijn door een zeer laag DNA methylerings niveau. In de delende cellen van het meristeem die uit de stam cellen zijn ontstaan is het globale DNA methylerings niveau wel laag en dit neemt weer toe wanneer de cellen differentiëren. De DNA methyltransferases DRM1 en DRM2 alsmede de histon deacetyltransferase lijken een belangrijke rol te spelen bij het bewerkstelligen van het hoge DNA methylerings niveau in de QC en stam cellen.
HP1 is voor het eerst gevonden in Drosophila. In gist Drosophila en andere dierlijke systemen is HP1 vaak betrokken bij heterochromatine vorming. In het algemeen wordt verondersteld dat HP1 een brug kan vormen tussen histonen met een specifieke epigenetische modificatie en andere chromatine eiwitten. Het eiwit van Arabidopsis dat zeer sterk lijkt opHP1 is genaamd Like HP1 (LHP1). LHP1 is gelokaliseerd in euchromatine regio's, zoals dat ook het geval is voor een enkele speciale isoform van HP1in dieren. In gedifferentieerde cellen van Arabidopis worden eer relatief groot aantal foci gevormd. We hebben laten zien dat deze foci chromatine complexen zijn en dat de lysines op positie 9 en 27 van Histon 3 herkent als daar 3 methyl groepen aan vast zijn gezet.

In dierlijke systemen het is aangetoond dat HP1 lysine 9 met 3 methylgroepen van histon 3 herkent. Echter binding aan deze epigenetische modificatie gebeurt alleen als HP1ook kan binden aan het enzym dat deze epigenetische modificatie aanbrengt. In Drosophila heet dit enzym SU(VAR)3-9.
Arabidopsis heeft een familie van 10 *SUVH* genen die koderen voor eiwitten die lijken op dit Drosophila enzym. We hebben bestudeerd of een van deze SUVH eiwitten de betreffende enzymatische activiteit heeft en of dit proteine aan LHP1 kan binden. SUVH3, SUVH7 als ook SUV9 zijn in het euchromatine gelokaliseerd,

zoals dat ook het geval is voor LHP1. SUVH9 is mogelijk een trimethyl transferase dat lysine 9 modificeerd. Echter *in vitro* binding studies lieten zien dat het niet bindt aan LHP1. Dus SUVH9 brengt mogelijk wel de juiste histon modificatie aan, maar het lijkt onwaarschijnlijk dat LHP1 gedirigeerd wordt naar deze modificatie.

RESUME

L'information génétique est emmagasinée de manière très compacte dans chaque nucleus. Environ 150 bp d'ADN est enroulé autours d'un octamer d'histones formant un nucléosome. Les nucléosomes sont attachés entre eux par l'histone H1, cette conformation 'beads on a string' (collier de perle) est elle-même compressée formant des structures chromatiques de compaction supérieure. Au niveau cytologique, on distingue deux types de chromatine : l'euchromatin faiblement colorée par des colorants spécifiques de l'ADN est riche en gènes et décondense pendant l'interphase du cycle cellulaire, tandis que l'hétérochromatine est riche en séquences répétitives, pauvre en gènes et reste condensée pendant tout le cycle cellulaire. L'euchromatine et l'hétérochromatine se différencie aussi par leurs modifications épigénétiques. Les modifications épigénétiques de la chromatine sont par exemple, méthylation de la cytosine de l'ADN ou méthylation des extrémités N-terminale des histones. L'acétylation des histones est en général une marque de l'euchromatine, tandis que la méthylation de l'ADN ou des extrémités des histones sont des marques de l'hétérochromatine. Pour accéder à la chromatine pour effectuer des procédés comme la réplication de l'ADN ou pour modifier l'expression d'un gène, un remodelage de la chromatine est nécessaire et réalisé par des protéines régulatrices de la chromatine comme Hétérochromatine Protéine 1 (HP1).

Dans cette thèse, nous avons étudié comment la chromatine est organisée pendant le développement de la racine d'*Arabidopsis*. Cette plante modèle a une organisation relativement simple de son méristème racinaire. De plus, la distribution de l'euchromatine et de l'hétérochromatine dans un nucléus en interphase est plutôt simple. Ce qui nous permet de suivre l'organisation de la chromatine d'une cellule tout au long de son développement de cellule souche à cellule différenciée.

La méthylation de l'ADN est l'une des modifications épigénétiques la plus abondante et varie pendant le développent d'un organisme. Elle est impliquée dans

la défense du génome contre les transposons et les rétrovirus ; dans le contrôle de l'empreinte génomique et dans la régulation de l'expression des gènes.

Chez *Arabidopsis*, nous avons observés que le Centre Quiescent et les cellules souches ont un niveau de méthylation de l'ADN élevé contrairement aux cellules souches animales. Quand les cellules sont dans la zone de division du méristème racinaire, leur niveau de méthylation de l'ADN diminue pour augmenter à nouveau quand elles atteignent la zone de différentiation. DRM1 et DRM2, des *de novo* methyltransferases de l'ADN, et HDA1, une histone déacetyltransferase semblent prendre part à l'établissement du niveau élevé de méthylation de l'ADN des nuclei du Centre Quiescent et des cellules souches du méristème racinaire.

HP1 chez les animaux est une protéine régulatrice de la chromatine découverte tout d'abord comme impliquée dans la formation de l'hétérochromatine. De nos jours, HP1 est vue comme une protéine faisant le lien entre les histones grâce à son 'chromo domain' et les protéines non histosomales grâce à son 'chromoshadow domain'. L'homologue de HP1 chez Arabidopsis est 'Like Heterochromatin Protein 1' (LHP1). LHP1 a été démontré d'être localisée dans l'euchromatine du nucléus en interphase comme l'isoform animale HP1γ et de former des foci nucléaires dans les cellules différenciées. Nous avons démontré que ces foci sont très probablement des complexes chromatiques attachés à l'ADN et que LHP1 est fixée à la lysine 9 triméthylée ou la lysine 27 triméthylée de l'extrémité N-terminale de l'histone H3.

Chez les animaux, il a été établi que HP1 se fixe à la lysine 9 triméthylée de l'histone H3 et interagit avec l'histone trimethyltransferase SU(VAR)3-9. Chez Arabidopsis, nous avons essayé d'identifier parmi la famille des homologues de SU(VAR)3-9, les SUVH protéines, celle qui est responsable pour triméthyler lysine 9 d'histone H3 et pourrait interagir avec LHP1. Nous avons établi que les gènes SUVH3, SUVH7 et SUVH9 sont exprimés dans des tissus spécifiques et que leurs protéines sont localisées dans l'euchromatine où probablement elles forment des complexes chromatiques. SUVH3 et SUVH9 forment des foci selon le stade de développement de la cellule. SUVH9 protéine pourrait être une candidate pour triméthyler lysine 9 d'histone H3, cependant ni SUVH3, SUVH7 ou SUVH9 n'interagissent avec LHP1.

CURRICULUM VITAE

Maëlle Audrey Lorvellec was born in Versailles (78) in France in 1977. She obtained her Baccalauréat Scientifique with high honours in 1995 at the Lycée Rotrou of Dreux (28). She then pursued her studies at the Lycée Hoche of Versailles (78) and passed the entrance exam of the Institut Supérieur d'Agriculture de Beauvais (ISAB) (60) in 1996…. she competed her 5th year of ISAB at Wageningen University in the Netherlands in 2000 thanks to a programme exchange. In July 2001, she started her master thesis in the department of Molecular Biology of Wageningen University (WUR) under the supervision of Dr. Tom Ruttink, Dr. Henk Franssen and Prof. Dr. A.H.J. Bisseling.

In her master thesis, she studied the Influence of Enod40 on levels and patterns of phytohormones in Arabidopsis. In 2002, she obtained her Master in Biotechnology with distinction from WUR and her diploma of 'Ingénieur en Agriculture' from ISAB. In April 2002, she began her PhD in the Chromatin group of the Molecular Biology department of WUR on the functional analysis of *Arabidopsi*s chromatin remodelling genes throughout root development. Here are presented the results of this study.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Maelle A. Lorvellec**
Date: **16 April 2007**
Group: **Molecular Biology, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Heterochromatin remodelling throughout Arabidopsis root tip development- LHP1 in Arabidopsis' root	Nov 11, 2002
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Chromatin dynamics in Arabidopsis root development (submitted to Current Opinion in Plant Biology)	2007
► **MSc courses**	
Molecular development. MOB-31303	2003
► **Laboratory use of isotopes**	
Working safely with radioactive materials and sources. PRI and Molecular Biology, WUR	2001
Safe handling of Radio-active Materials and Sources 5B. Larenstein	Jun 16, 2005
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
PhD Student day 2003, Utrecht University	Mar 27, 2003
PhD Student day 2004, Vrije Universiteit Amsterdam	Jun 3, 2004
► **EPS theme symposia**	
Theme symposium 'Genome Plasticity', Wageningen University	Dec 20, 2002
Theme symposium 'Developmental Biology of Plants', Leiden University	Feb 2, 2002
Theme symposium 'Genome Plasticity', Radboud University, Nijmegen	Dec 10, 2003
Theme symposium 'Developmental Biology of Plants',	Feb 6, 2003

	Leiden University	
	Theme symposium 'Genome Plasticity', Wageningen University	Dec 9, 2004
	Theme symposium 'Genome Plasticity', Wageningen University	Dec 9, 2005
►	**NWO Lunteren days and other National Platforms**	
	ALW meeting Lunteren 2002, Plant Sciences	Apr 15-16, 2002
	ALW meeting Lunteren 2003, Plant Sciences	Apr 7-8, 2003
	ALW meeting Lunteren 2004, Plant Sciences	Apr 5-6, 2004
	ALW meeting Lunteren 2004, Nucleic Acids	Dec 6-7, 2004
	ALW meeting Lunteren 2005, Plant Sciences	Apr 4-5, 2005
	ALW meeting Lunteren 2005, Nucleic Acids	Nov 28-29, 2005
	ALW meeting Lunteren 2006, Plant Sciences	Apr 3-4, 2006
►	**Seminars (series), workshops and symposia**	
	Flying Seminars (11-10-02, 8-11-02, 22-10-02, 11-03-03, 23-09-03, 26-09-05, 24-10-05, 23-10-06)	2002-2005
	NVBMB Spring Symposium - Utrecht	Apr 18, 2002
	Frontiers in Plant Science (27-02-03, 06-03-03, 22-09-05, 13-10-05)	2003-2005
	Symposium on Systems Biology, in Honour of Prof.dr.Pierre de Wit	Nov 4, 2004
	1st Dutch Chromatin Meeting 2004 - Amsterdam	Apr 23, 2004
	Special lecture Science editor Dr.Pam Hines	Oct 5, 2004
	SILS seminar Prof.Dr.Gunter Reuter- Amsterdam	Oct 7, 2004
	2nd Dutch Chromatin Meeting 2005 - Wageningen	Jan 21, 2005
	3rd Dutch Chromatin Meeting 2005 - Kerkrade	Dec 7-8, 2005
►	**Seminar plus**	
►	**International symposia and congresses**	
	ICC XV. The 15th International Chromosome Conference - Brunel University, London, UK.	Sept 5-10, 2004
	Epigenetics and the Dynamic Genome Conference - Babraham Institute, Cambridge, UK	Jun 30-Jul 2, 2005
►	**Presentations**	
	Theme symposium IV, 2003, Nijmegen (oral)	Dec 10, 2003
	ALW meeting Lunteren 2005, Plant Sciences (oral)	Apr 4-5, 2005
	Epigenetics and the Dynamic Genome Conference - Babraham Institute, Cambridge, UK (poster)	Jun 30-Jul 2, 2005
	3rd Dutch Chromatin Meeting 2005 - Kerkrade (oral)	Dec 8, 2005
►	**IAB interview**	Jun 2004

► **Excursions**	
Subtotal Scientific Exposure	*15.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Embo Course 'Advanced Molecular and Immuno Cytogenetics on Chromosomes and Nuclei in Plants'	Oct 13-20, 2003
FEBS Advanced Workshop- Nuclear Architecture:"Chromatin Structure & Gene Control Plant vs.Animal vs.Yeast'	Nov 14-17, 2003
► **Journal club**	
Literature Discussions, Once a Week	2002-2006
► **Individual research training**	
Subtotal In-Depth Studies	*5.4 credits**

4) Personal development	*date*
► **Skill training courses**	
Scientific Publishing: An Introductory Workshop for PhD students and Young Authors	Oct 12, 2004
Teaching and Supervising Thesis Students. OWU	Oct 26-27, 2004
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Member PhD Council	2003
Subtotal Personal Development	*1.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31,8**

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 credits

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

PUBLICATION LIST

T. Ruttink; **M. Lorvellec**, T. Bisseling, H. Franssen. '*Arabidopsis thaliana*: useful for functional analysis of ENOD40?' Molecular plant-microbe interactions: new bridges between past and future, @Proceedings of the 11th international congress on molecular plant-microbe interactions, St. Petersburg, Russia, July 18-26, 2003 / Tikhonovich, I., Lugtenberg, B., Provorov, N., - St. Paul, Minnesota, USA: Internat. Society for Molecular Plant-Microbe Interactions, 2004 (Biology of plant-microbe interactions 4) - ISBN 0965462536 - p. 332 - 337.

To be submitted or in preparation

M. Lorvellec, J. Willemse, O. Kulikova, J. Verver & T. Bisseling. 'DNA methylation in Arabidopsis root through development'

M. Lorvellec, J. Willemse, O. Kulikova, H. de Jong & T. Bisseling**.**
'LHP1 forms euchromatin complexes at trimethylated lysines of histone H3'

O. Kulikova, **M. Lorvellec**, T. Bisseling. 'Characterization of the SUVH proteins localised in the euchromatin of *Arabidopsis thaliana*.'

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