UNIVERSITE LOUIS PASTEUR – STRASBOURG 1

THESE DE DOCTORAT

Discipline: Sciences du Vivant

Spécialité: Aspects Moléculaires et Cellulaires de la Biologie

Présentée par:

Yu YU

Caractérisations fonctionnelles de la cycline, Nicta;CYCA3;2, et des protéines à domaine SET chez les plantes

Soutenue le 24 Avril 2006 devant le jury composé de:

M. Léon OTTEN	Rapporteur interne
Mme. Valérie GAUDIN	Rapporteur externe
Mme. Sylvette TOURMENTE	Rapporteur externe
M. André STEINMETZ	Examinateur
M. Wen-Hui SHEN	Directeur de thèse

Unité de Recherche:

Institut de Biologie Moléculaire des Plantes

UPR2357, CNRS

Acknowledgements

I am deeply indebted to my supervisor Dr. SHEN Wen-Hui, whose guidance, suggestions and encouragement helped me in all the time of research and writing of this thesis. His wide knowledge and logical way of thinking have been of great value for me.

I would like to express my sincere gratitude to Dr. STEINMETZ André. He helped me to get the fellowship from Luxembourg. Without his support it would be impossible to finish my thesis research. I also acknowledge him for his close reading of the draft version of the thesis and providing a lot of detailed and constructive comments for improvement.

I would also like to thank the other member of my thesis committee: Prof. OTTEN Léon, Dr. GAUDIN Valérie, and Dr. TOURMENTE Sylvette, who accepted to be the "Rapporteur" and took effort in reading and writing.

Gratitude also goes to my former advisor Dr. DONG Aiwu in Fudan University (Shanghai, China) for her various suggestions and help in operations and also for the encouragement during the thesis research. More importantly, I would not have the opportunity to work on this project without her recommendation.

Needless to say, it is difficult to survive in France for a foreigner like me who can not understand french. I am bound to thank Michel and Denise for everything they have done for me, from careful reading and correction of the french abstract of my thesis to small things in the daily life. They are always available when I need help.

All my colleagues in lab 606 gave me the feeling of being at home at work. They are: ZHAO Zhong, WANG Ning, XU Lin, ZHU Yan, RUAN Ying, LIU Ziqiang, WU Chengjun, YU Fang, and LIU Shiming. I want to thank them for all their help, support, interest and valuable hints.

Many more persons helped me in various ways during my thesis research. I wish to thank Marie claire, Yves, Esther, and Alexis in the lab 612 for their friendly help, particularly when I just came to IBMP. I have furthermore to thank Herrade for her assistance on BY2 cell culture, Jérome for confocal observation, Malek for DNA sequencing, and all the gardeners for their hard work.

Finally, I would like to give my special thanks to my husband whose constant encouragement and patient love enabled me to complete this research. I wish to share this moment of happiness with him and also with my parents.

Yu YU

March 2006

TABLE OF CONTENTS

I

ABBREVIATIONS

I Abbreviations

AP	ammonium persulfate
ATP	adenosine triphosphate
BAP	Benzylamino Purine
bp	base pair
BSA	bovine serum albumin
CTAB	cetyl trimethyl ammonium bromide
Da	Dalton
Dam	DNA adenine methyltransferase
DAPI	4',6-Diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EB	ethidium bromide
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetra acetic acid
GFP	green fluorescent protein
GST	Glutathion-S-Transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
IPTG	isopropyl-beta-D-thiogalactopyranoside
MAB	methylation activity buffer
MES	2-(N-Morpholino)ethanesulfonic Acid
min	minute
MOPS	3-(N-morpholino)propanesulphonic acid
MS	Murashige and Skoog
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pipes	piperazine-N,N'-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulphate
sec	second
SSC	sodium chloride-sodium citrate buffer
SSPE	sodium chloride-sodium phosphate-EDTA buffer
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBS	Tris buffered saline
TCA	Trichloroethanoic acid
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	(hydroxymethyl)methylamine

UV	Ultra Violet
YFP	yellow fluorescent protein

II

INTRODUCTION

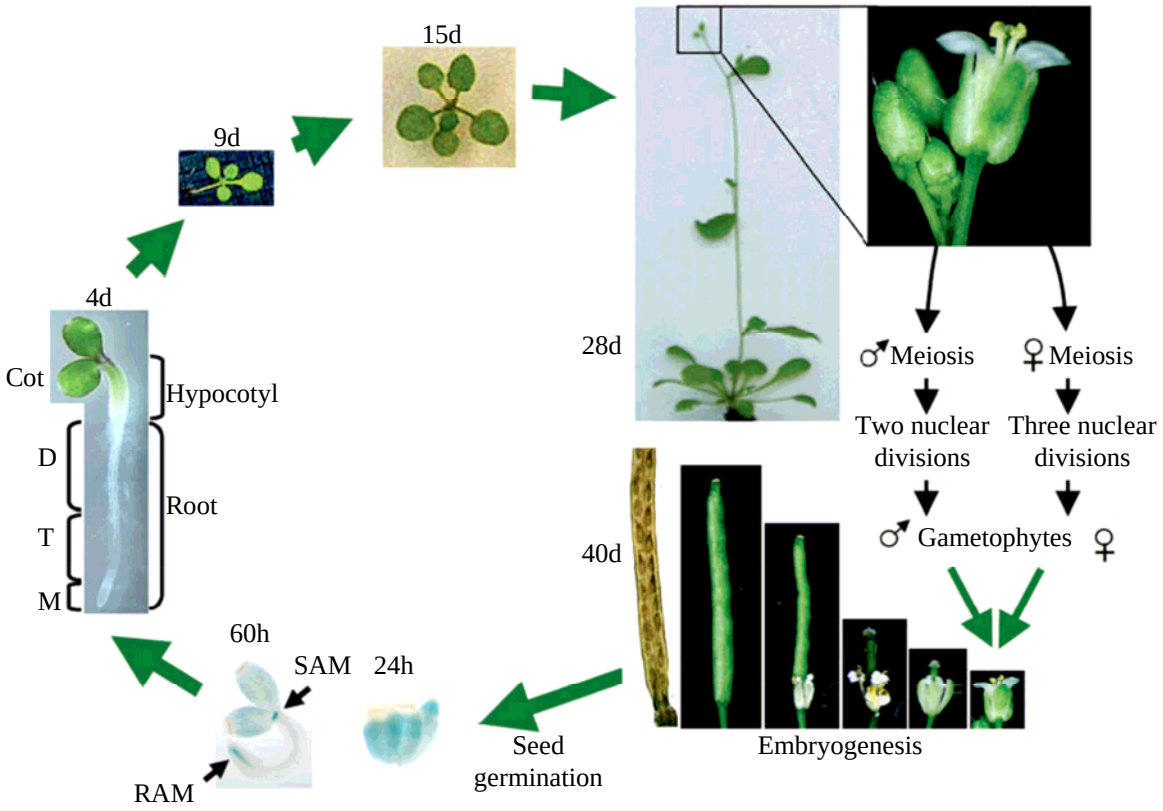


Figure II-1, The life cycle of *Arabidopsis thaliana* (Gutierrez, 2005)

Simplified scheme showing the basic stages throughout the life cycle of an *Arabidopsis thaliana* plant. After fertilization, embryogenesis occurs and seeds and fruits (siliques) develop and mature. Embryos are fully developed within the mature seeds. After germination, they rapidly grow, and SAM and RAM can be seen here by labeling with a reporter gene (60h). Four days after germination the root already contains three regions, the meristem (M), the transition zone (T) where cells start to differentiate and the mature zone (D) with fully differentiated cells. In addition, the hypocotyl and the two expanding cotyledons (cot) can be distinguished in the aerial part. Rosette leaves progressively develop in the following days and later flowers appear (28d). Inside the flowers, some cells specialize to generate the germinal cells that undergo meiosis, and the resulting haploid cells divide and give rise to both the male and female gametophytes. A new life cycle starts after fertilization.

II-1 Plant cell division and plant development

II-1-1 Plant development

Plant development is a biphasic process (reviewed in Jurgens, 2003). During embryogenesis, the development of a plant begins with division of a fertilized egg to form an embryo. The plant embryo is a relatively simple structure consisting of a shoot and a root primordium at the apical and basal end, respectively. In contrast to that of animals, plant development is largely post-embryonic and occurs over the entire lifespan of the organism. During post-embryonic development, continuous cell division of the shoot apical meristem (SAM) and root apical meristem (RAM) throughout the plant life is primarily responsible for plant growth and morphogenesis. New organs - such as roots, stems, leaves and flowers - originate from iterative cell divisions, followed by cell expansion and differentiation.

Figure II-1 illustrates a life cycle of *Arabidopsis thaliana* (Gutierrez, 2005), which serves as a primary model organism for the analysis of plant development. After fertilization, embryogenesis occurs and seeds and fruits (siliques) develop and mature. Embryos are fully developed within the mature seeds. Four days after germination the root already contains three regions: the meristem, the transition zone where cells start to differentiate and the mature zone with fully differentiated cells. In addition, the hypocotyl and the two expanding cotyledons can be distinguished in the aerial part. Rosette leaves progressively develop in the following days and later flowers appear. Inside the flowers, some cells specialize to generate the germinal cells that undergo meiosis, and the resulting haploid cells divide and give rise to both the male and female gametophytes. A new life cycle starts after fertilization.

The development of plants, compared with that of animals, is influenced much more by the environment (such as nutrients, light, temperature and humidity), which adds another layer of complexity to the plant development. It suggests that plants have evolved mechanisms that relay environmental signals to control development and growth.

Since plant development is unique compared with animals, it suggests that plants have evolved and distinct mechanisms to control cell division and ultimately plant growth.

II-1-2 Plant cell division

A typical plant cell cycle division is illustrated in **Figure II-2**, which is divided into four

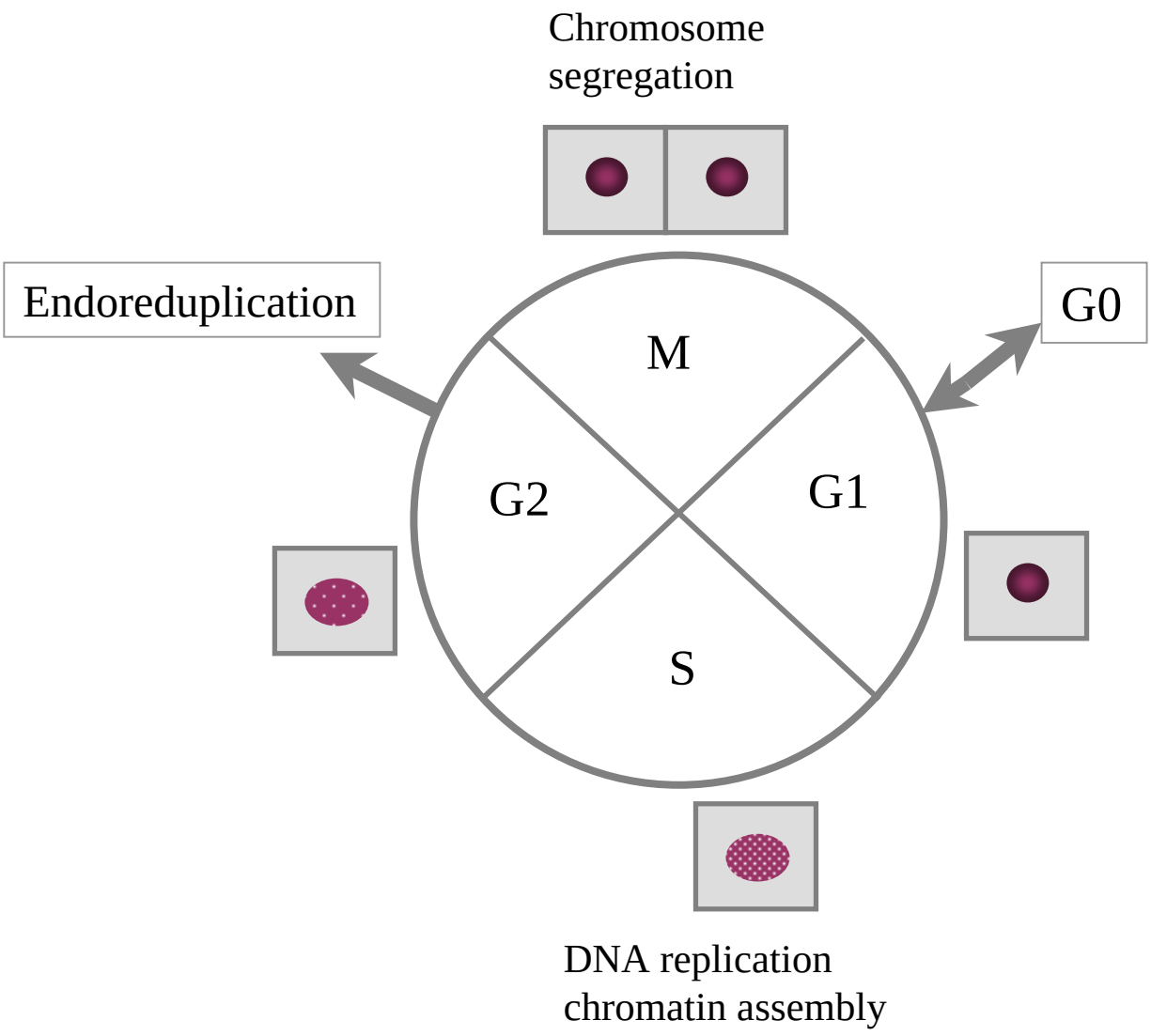


Figure II-2, The overview of the plant cell Cycle

The cell cycle is divided into four major phases: S, G2, M, and G1. Genomic DNA is duplicated and assembled into chromatin during the S phase. In the M phase, the duplicated genome segregates into two daughter cells. Plant cells can exit the mitotic cell cycle and differentiate from both G1 and G2 phases.

major phases: S, G2, M, and G1. Genomic DNA is duplicated and assembled into chromatin during the S (synthesis) phase. The completion of DNA synthesis is followed by the G2 (gap 2) phase, during which cell growth continues and new proteins are synthesized for the subsequent progression of the cell cycle. After progressing through the G2 phase, cells begin the complex process of mitosis, also called the M phase.

The M phase is further divided into four subphases: prophase, metaphase, anaphase and telophase. In early prophase, the sister chromatids become condensed and visible in the nucleus. In late prophase, the nuclear envelope breaks down and the sister chromatids interact with the microtubules that emanate from the two spindle poles. A plant specific cytoskeletal structure known as the preprophase band (PPB), which is formed in late G2 phase and defines the future division plane of the cell, disappears upon establishment of the mitotic spindle. Plants do not have centrosomes, but their spindle poles contain proteins related to those found in the centrosomal matrix of animal cells (Varmark, 2004). During metaphase, the sister chromatids become aligned at the equator of the spindle. Anaphase begins when the pairs of sister chromatids separate and move to opposite poles of the cell. The chromosomes arrive at the poles in telophase, and new nuclear membranes form around them. During cytokinesis, small vesicles from the Golgi carry polysaccharides and cell wall proteins to the phragmoplast, which is centered between the two poles at the site previously marked by the PPB, to form the plasma membrane and joins with the new cell wall. The process of mitosis results in two daughter cells, each with an identical set of chromosomes.

The M phase is followed by the G1 (gap 1) phase. A cell proceeding through G1 phase faces a key control point of commitment to cell division - either to reenter a new cell division cycle by passing through the G1/S restriction point, or to exit from the cell cycle temporarily or permanently to differentiate. Thus the G1/S transition is a crucial crossroad at the interface between cell proliferation and differentiation.

II-1-3 G1/S regulation

In all eukaryotes, regulation of cell cycle progression depends on the cyclin-dependent kinase complexes, the heterodimeric serine/threonine kinases, which consist of a catalytic CDK subunit and a regulatory cyclin subunit (**Figure II-3**). Specific CDKs and cyclins are involved in the control of plant G1/S transition; the activities of cyclin/CDK complexes can be regulated by several additional factors, including CDK phosphorylation and CDK-inhibitory proteins (**Figure II-4**). Furthermore, the E2F-Rb pathway plays an essential role in regulation

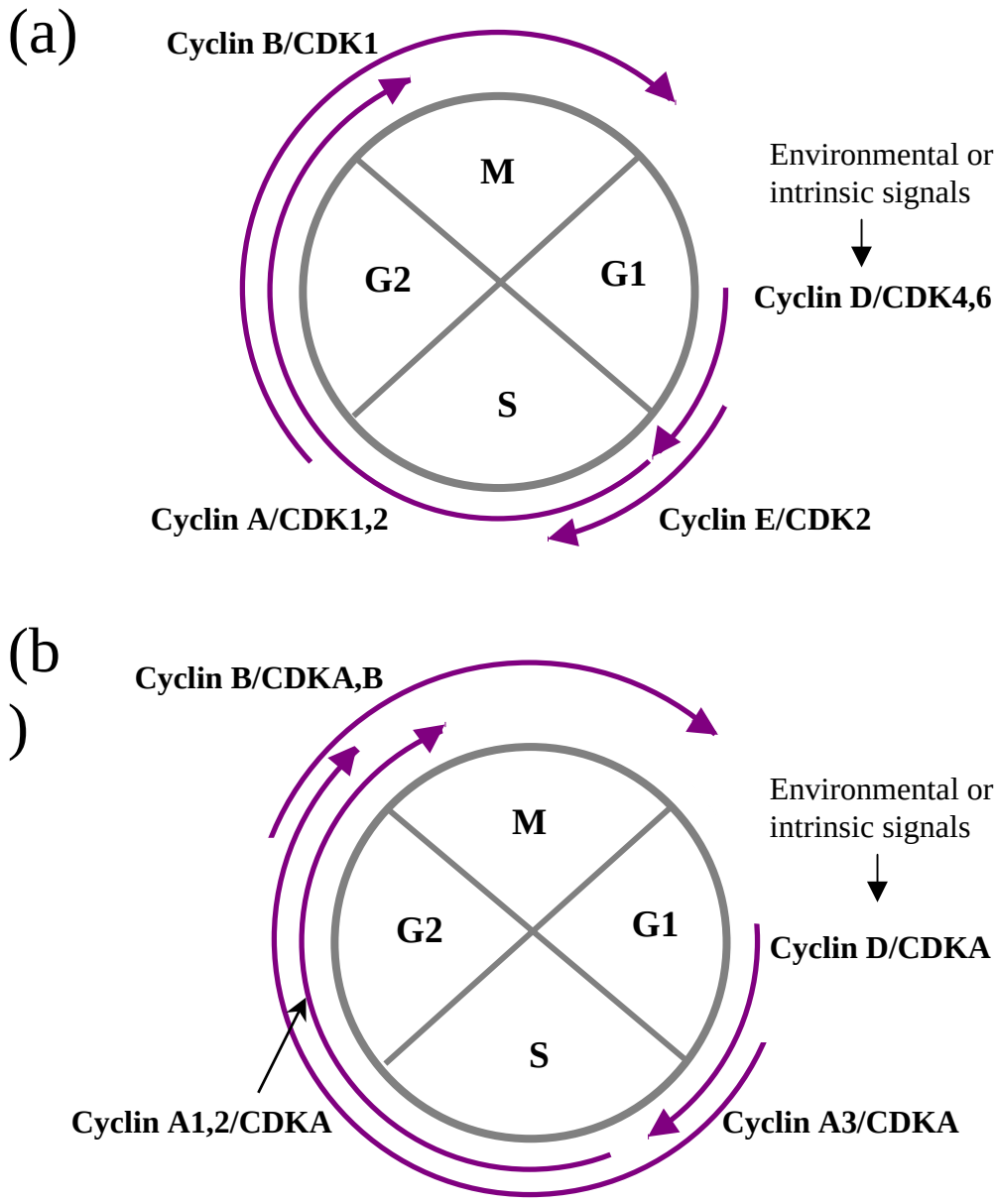


Figure II-3, A current view of the variations in cyclins/CDKs activities throughout the cell cycle

(a), A mammalian cell cycle . Cyclin D/CDK4,6 start accumulating at mid-G1, whereas cyclin E/CDK2 appears later, just prior to the G1/S transition. Progression through S and G2 phase requires cyclin A, associated with CDK2 first and then with CDK1, while cyclin B/CDK1 trigger to enter mitosis. (b), A plant cell cycle involves the sequential activity of D-, A- and B-type cyclins in combination with CDKA and CDKB. No cyclin E homologue has been identified in plants. Instead of cyclin E, Cyclin A3 subgroup genes are induced at the G1/S transition.

of G1/S transition (**Figure II-4**).

II-1-3-1 CDKs

Plants contain a diverse family of CDK subunits including CDK-A, -B, -C, and -E. During G1 phase, CDKA and CDKE are expressed (Joubès *et al*., 2000).

The CDKA, which contains the PSTAIRE-peptide motif in the cyclin-binding domain, is well studied and characterized to exhibit central functions in the plant cell cycle control. The CDKs in this family are most closely related to yeast CDC2/CDC28 and to animal CDK1 and CDK2. Plant A-type CDKs can functionally complement the temperature-sensitive mutants in the yeast *cdc2/cdc28* gene. In yeast, the CDC2/CDC28 protein is involved in G1/S and G2/M controls, suggesting a pivotal role for plant CDKA in both the G1/S and G2/M transitions. Consistently, the member of CDKA has been demonstrated to have the ability to interact with different cyclin partners (reviewed in Shen, 2001a). In agreement with a central role in the cell cycle, overexpression of a dominant-negative CDKA had been demonstrated to strongly interfere with the cell cycle, resulting in elongated cells in tobacco cells and in severe embryonic defects in the plant (Joubès *et al*., 2004; Hemerly *et al*., 2000). These effects are presumably due to a competition of the mutant proteins for the association with rate-limiting interacting proteins, such as cyclins.

Currently, little is known about the roles of CDKE during G1/S progression. *HUA ENHANCER3* (*HEN3*) gene, which encodes the only *Arabidopsis* CDKE, is required for the specification of floral organ identity and for the proper termination of stem cells in the floral meristem, suggesting a role for CDKE in cell differentiation in plants (Wang and Chen, 2004).

II-1-3-2 Cyclins

To be active, CDKs must associate with cyclins that are key regulatory subunits controlling the activity of the CDK complexes. All the cyclins share a large region of homology called the cyclin box, an about 100 amino acids central domain which is responsible for binding to the CDK (Noble *et al*., 1997). As animal cells proceed through the cycle, four major cyclins are produced sequentially (D, E, A, and B) and couple with distinct CDKs to form active cyclin/CDK complexes (**Figure II-3a**). Cyclin D starts accumulating at mid-G1, whereas cyclin E appears later, just prior to the G1/S transition. Progression through S and G2 phase

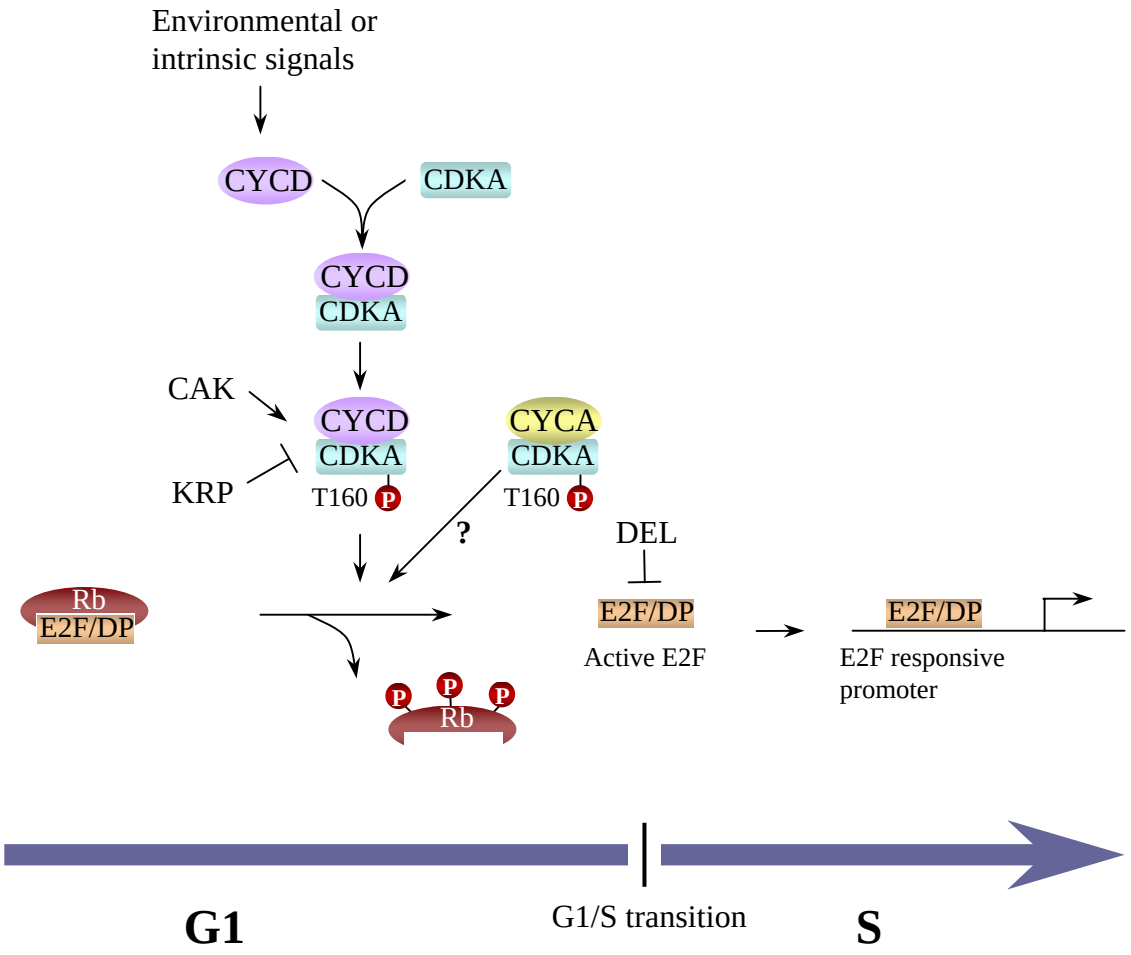


Figure II-4, Schematic overview of G1/S transition control of the plant cell cycle

Environmental and intrinsic signals result in an increase in CYCD, which then associate with CDKA. The activity of the complex is modulated by KRPs (CDK inhibitors) and CAKs (CDK activating kinases). Late in G1 phase, the increased levels of CYCD/CDKA phosphorylate (represented by P) the Rb proteins, resulting in the release of E2F/DP transcription factors. DEL acts as negative regulators of the E2F-Rb pathway by competing with E2F-DP for binding sites The activation of the E2F-responsive genes results in progression into S-phase. CYCA/CDK complexes may also involve in G1/S transition.

requires cyclin A, while cyclin B plays primary roles in mitosis. Plants contain many more cyclins than previously described in other eukaryotes (Vandepoele *et al*., 2002; Wang *et al*., 2004a). In plants, the cyclins have been classified, based on sequence similarity with the animal counterparts, into A-, B-, and D-type cyclins, whereas no cyclin E homologue has been identified in plants (Renaudin *et al*., 1996; Vandepoele *et al*., 2002, **Figure II-3b**). During G1 phase, D-type cyclins and some members of the A-type cyclins are expressed (Rossi and Varotto, 2002).

D-type cyclins have been identified in a variety of plant species; in *Arabidopsis*, ten D-type cyclins sequences have been identified, and these can be classified according to Vandepoele *et al*. (2002) into seven subgroups. Expression of several CYCDs is regulated by nutrient availability and hormones. When analyzed in synchronous cell cultures, expressions of most CYCDs increase during cell cycle re-entry, consistent with a primary role in responding to external signals (de Jager *et al*., 2005). Moreover, it has been reported that members of all three classes of D-type cyclins can bind Arath;CDKA;1 *in vitro*, and that Arath;CDKA;1 co-immunoprecipitates with CYCD2 and CYCD3 in *Arabidopsis* cell extracts (Rossi and Varotto, 2002). Therefore, it is likely that D-type cyclins may function in the G1 phase by binding to A-type CDKs in plants. In support of this, overexpression of CYCD is sufficient to drive cells into S-phase, resulting in a shortened G1-phase and dramatic modifications in plant development. In *Arabidopsis*, overexpression of *Arath;CYCD2;1* increases growth rate by shortening G1 in meristematic areas (Cockcroft *et al*., 2000), whereas that of *Arath;CYCD3;1* induces ectopic divisions, producing leaves with more but smaller cells, and results in differentiation defects in leaf tissues (Dewitte *et al*., 2003). The expression of *Antma;CYCD;1* from *Antirrhinum majus* leads to the early activation of CDKA activity and accelerates the cell cycle progression in tobacco BY2 cells (Koroleva *et al*., 2004).

Plants contain many A-type cyclins, which belong to three different subgroups (CYCA1, CYCA2, and CYCA3). Representative members of the three subgroups of plant A-type cyclins have been found to date in several species, including *Arabidopsis* and tobacco (Chaubet-Gigot, 2000). Using synchronized tobacco BY2 cells, the transcription levels of the tobacco A-type cyclins were analyzed. *CYCA1* subgroup and *CYCA2* subgroup genes are induced around mid-S phase and their transcripts declined around mid-mitosis like their animal counterparts. Interestingly, *CYCA3* subgroup genes, whose transcripts appear significantly earlier than the *CYCA1* and *CYCA2* genes, are induced at the G1/S transition and

decrease to very low levels when cells enter mitosis (Reichheld *et al*., 1996; Chaubet-Gigot, 2000). These findings indicate that CYCA3 subgroup genes may be components of the cell cycle machinery that commits a cell to enter S phase. However, little is known about the CYCAs function in plants. Medsa;CYCA2;2 is auxin-regulated and involved in meristem formation, but no effect on plant development is detected when overexpressed (Roudier *et al*., 2003). As described in the Article 1 of the RESULT section of this thesis, we showed that Nicta;CYCA3;2 plays a crucial role in cell division and differentiation (Yu *et al*., 2003). More recently, Arath;CYCA2;3 is reported to act as a key regulator of ploidy levels in *Arabidopsis* endoreplication (Imai *et al*., 2006).

II-1-3-3 CDK phosphorylation and dephosphorylation

Besides cyclin binding, the activity of CDKs is regulated by phosphorylation/dephosphorylation mechanisms.

The activating phosphorylation of the CDKs, which occurs at the conserved CDK threonine 160 (T160) in plants, is catalyzed by an enzyme called CAK (Cdk-activating kinase). In animals, CAKs are a heterotrimeric complex composed of a catalytic kinase subunit CDK7 and a regulatory subunit cyclin H. A complex of CDK7 and cyclin H is also associated with transcription factor TFIIH through a phosphorylated carboxy-terminal domain (CTD) of RNA polymerase II, suggesting that the animal CAKs may have a dual function in cell cycle regulation and basal transcription. In plants, two distinct classes of CAK have been identified: one is known as CDKD and functionally related to the animal homologue CDK7, while another one is named as CDKF and is a plant-specific CAK (reviewed in de Jager *et al*., 2005). There are three *CDKD* genes in the *Arabidopsis* genome: *Arath;CDKD;1*, *Arath;CDKD;2* and *Arath;CDKD;3* (Umeda *et al*., 2005). Analysis of their enzyme activities demonstrated that, while Arath;CDKD;2 and Arath;CDKD;3 phosphorylate both human CDK2 and *Arabidopsis* CTD when associated with *Arabidopsis* cyclin H, Arath;CDKD;1 lacks this kinase activity *in vitro* (Shimotohno *et al*., 2003). Therefore, Arath;CDKD;1 may be an inactive CAK variant, or it may have a different substrate specificity. The plant specific CAK Arath;CDKF;1 in *Arabidopsis* is shown to have unique enzymatic characteristics. Independent of cyclin H, Arath;CDKF;1 phosphorylates Arath;CDKD;2 and Arath;CDKD;3 and activates the CTD-kinase activity of Arath;CDKD;2 *in vitro* and in root protoplasts (Shimotohno *et al*., 2004).

The activity of CDKs is also negatively regulated by phosphorylation on threonine 14 (T14) and/or tyrosine 15 (Y15), which is carried out by a protein kinase called WEE1 in animals. Reversibly, dephosphorylation of T14/Y15 by CDC25 phosphatase reactivates the CDK kinase activity. Orthologues of both WEE1 and CDC25 have been identified in *Arabidopsis* (de Jager *et al*., 2005). The WEE1/CDC25-mediated phosphorylation/dephosphorylation plays an important role in triggering the G2/M transition. However, the role of CDK phosphorylation/dephosphorylation in G1/S remains currently unknown.

II-1-3-4 CDK inhibitors

In addition, the activities of cyclin/CDK complexes can also be controlled by CDK-inhibitory proteins (called CKIs for CDK inhibitors). In mammalian cells, two families of CKI (INK4 and Kip/Cip family) exist, each with its own CDK-binding specificity and protein structure.

In the *Arabidopsis* genome, there are seven genes encoding Kip/Cip-like proteins, designated KRP1 to KRP7 (Kip-Related Proteins), while no plant counterparts of the INK4 family inhibitors have been found (Vandepoele *et al*., 2002). All KRPs share a conserved region at their C terminus with the mammalian Kip/Cip proteins, which is essential for the interaction with cyclin/CDK complexes. KRPs have been shown to interact directly with both CDKA and Cyclin D and to inhibit CDK kinase activity (reviewed in Dewitte and Murray, 2003). The inhibitory activity of KRPs depends on the binding ability between KRP proteins and cyclin/CDK complexes (Nakaia *et al*., 2006). Expression patterns of KRPs vary during the cell cycle, suggesting different roles of KRPs (Ormenese *et al*., 2004). Overexpression of *KRP1* (Wang *et al*., 2000) or *KRP2* (de Veylder *et al*., 2001) negatively affects cell division, leading to changes in leaf morphology, with fewer but larger cells. These effects are partially reversed by the overexpression of cyclin D3 (Zhou *et al*., 2003), suggesting a potential role for KRPs in G1/S transition. The tobacco CKI NtKIS1a was identified and shown to inhibit *in vitro* the kinase activity of cyclin/CDK complexes and to interact with tobacco D-type cyclins and Nicta;CDKA;1 (Jasinski *et al*., 2002a). Gain of NtKIS1a function caused decreased organ size and increased cell size, and blocked endoreplication (Jasinski *et al*., 2002b). Furthermore, it restored essentially normal development in plants overexpressing *Arath;CycD3;1* (Jasinski *et al*., 2002b), providing the evidence of cyclin D-CKI co-operation within the context of a living plant.

II-1-3-5 The E2F-Rb pathway

E2F, as a cellular transcription factor, has been show to regulate a wide variety of genes, including genes involved in cell cycle regulation, DNA replication and repair, and chromatin dynamics. The tumour suppressor retinoblastoma (Rb) protein binds E2F and functions as a transcriptional repressor. The E2F-Rb pathway plays an essential role in the regulation of G1/S transition. During early G1 phase, heterodimeric E2F-DP (dimerization partner of E2F) activity is repressed by Rb. Upon growth stimulation, Rb is phosphorylated during late G1 phase by cyclin/CDK complexes and consequently loses its affinity for E2F-DP. The release of Rb triggers the activation of E2F-target genes, which irreversibly commits cells to undergo DNA replication (Harbour and Dean, 2000). In mammals, highly orchestrated Rb phosphorylation is initiated by CYCD/CDK complexes, then carried on by CYCE/CDK, and later by CYCA/CDK during S phase, resulting in the dissociation of Rb from E2F-DP during late G1 and S phase.

The mechanism that regulates the G1/S transition seems to be conserved between mammals and plants, since homologues of most of the key players of the E2F-Rb pathway have been identified in plants (reviewed in Shen, 2002).

II-1-3-5-1 Plant E2F and DP proteins

Six *E2F* genes (*a to f*) and two *DP* genes (*DPa* and *DPb*) are present in the *Arabidopsis* genome (reviewed in Shen, 2002). All of them contain a highly conserved DNA-binding domain, suggesting that plant E2F-DP factors bind the same DNA-sequence motifs as their mammalian counterparts. Indeed, plant E2Fs (a, b and c) can bind DNA sequences containing E2F-binding sites, and this binding requires the heterodimerization partner DP protein (Ramirez-Parra and Gutierrez, 2000). A genome-wide analysis in transgenic *Arabidopsis* ectopically overexpressing E2Fa and DPa led to the identification of 181 putative E2F target genes, some of which are upregulated during G1/S transition (Vandepoele *et al*., 2005). The individual *Arabidopsis* E2Fs differ in their function: two (E2Fa and E2Fb) act as activators and one (E2Fc) acts as repressor. E2Fa, in cooperation with DPa, upregulates S-phase genes and induces ectopic cell divisions in several post-proliferative organs (de Veylder *et al*., 2002). E2Fb represses cell enlargement and endoreduplication in auxin-free conditions, resulting in very small-sized cells (Magyar *et al*., 2005). In contrast, overexpression of E2Fc results in decreased expression of the S-phase genes, and it also inhibits cell division leading

to enlarged cells (Del Pozo *et al*., 2002).

In contrast, three other E2Fs (d, e and f) in *Arabidopsis*, also termed *DP-E2F-like* (*DEL*), differ from the E2Fs (a, b and c) by lacking the C-terminal transactivation domain embracing a conserved Rb-binding site. Thus these three proteins are postulated to act as negative regulators of the E2F-Rb pathway by competing with E2F-DP for binding sites (reviewed in de Veylder *et al*., 2003). A homologue of DEL proteins, designated E2F7, has also been discovered in mammals (reviewed in Bracken *et al*., 2004). Recently, E2Ff/DEL3 has been shown to play a role in repressing cell wall biosynthesis during cell elongation in differentiated cells (Ramirez-Parra *et al*., 2004) and *E2Fe*/*DEL1* appears to control the endocycle in *Arabidopsis* (Vlieghe *et al*., 2005).

II-1-3-5-2 Plant Rb protein

In animals, Rb binds E2F at the Rb-binding site within the C-terminal transactivation domain through its pocket domains and consequently inhibits the E2F transcriptional activity (Harbour and Dean, 2000). Plant Rb proteins exhibit a similar domain organization as their animal counterparts. An *Rb*-related gene (*RBR*) is first discovered in maize and later in other plant species, including *Arabidopsis* (Grafi *et al*., 1996; Vandepoele *et al*., 2002).

Increasing evidence suggests that the E2F-RBR pathway plays an important role in controlling G1 to S phase transition in plant cells. By using the yeast two hybrid system, the interaction between the plant E2F and RBR has been demonstrated (Ramirez-Parra *et al*., 1999). Besides E2F, the plant RBR protein was shown to bind plant D-type cyclins, and the CYCD/CDKA complex can phosphorylate the plant RBR proteins, which has been demonstrated in tobacco and *Arabidopsis* (Nakagami *et al*., 1999; Boniotti and Gutierrez, 2001). Moreover, phosphorylation is cell cycle dependent, peaking in G1/S transition and progressively decreasing until G2 phase (Nakagami *et al*., 2002). Thus, it seems that plant RBR proteins are the targets of the plant CYCD/CDKA complexes during G1/S transition. Additional evidence was obtained from a recent study in tobacco (Uemukai *et al*., 2005) which showed that transcriptional activation of NtE2F is repressed by co-transfection with NtRBR1, while cyclin D expression overcomes this repressor activity.

Furthermore, a loss-of-function of the *Arabidopsis* RBR gene has been shown to be gametophytically lethal (Ebel *et al*., 2004). Using an inducible system to inactivate RBR

function, AtRBR was shown to restrict cell division during early leaf development when cell proliferation predominates, while it regulates endocycle occurrence at later stages (Desvoyes *et al*., 2006). AtRBR also regulates the size of the stem cell population in *Arabidopsis* roots in a canonical E2F-Rb pathway (Wildwater *et al*., 2005). Together, these results suggest that the E2F-Rb pathway plays a crucial role in the regulation of cell proliferation, differentiation and ultimately in plant development.

II-2 Chromatin and epigenetic regulation

Once the cell is committed to pass through the G1/S restriction point, DNA synthesis is initiated until the entire genome is replicated. During DNA replication, new nucleosomes must be assembled onto the daughter DNA strands to propagate or modify chromatin configurations. Regulation of the organization of DNA and the histones into nucleosomes, as well as regulation of the higher-order condensation of chromatin, not only plays a role in transcriptional activation and repression but also is required for stable silencing, cell differentiation and development. Thus the establishment and maintenance of specific chromatin states provides an epigenetic mechanism for inheriting expression states throughout plant development.

II-2-1 The basic structure of chromatin

II-2-1-1 Heterochromatin and euchromatin

Chromatin can be divided into two distinct forms, heterochromatin and euchromatin. Heterochromatin is defined as chromosome regions that remain densely stained and highly condensed throughout the cell cycle and is generally transcriptionally inert, whereas euchromatin is loosely packed during interphase containing most of the actively expressed genes.

Heterochromatin can be either constitutive or facultative. Constitutive heterochromatin is permanently condensed and contains highly repeated DNA sequences, such as those present at centromeres as well as pericentromeric, and subtelomeric regions. Facultative heterochromatin exists in different locations within chromosomes and contains sequences that are not transcribed in the cell being examined, but are transcribed in other cell types. Thus it is developmentally regulated and spends some of its life as euchromatin. The regulation of transcription through heterochromatin-mediated repression is important in multicellular eukaryotes and is under intensive investigation.

II-2-1-1-1 Centromeres

The centromere plays a critical role in ensuring the correct distribution of duplicated chromosomes to daughter cells during mitosis. Both the DNA in the centromere regions, and

the proteins attached to it, have special features (Henikoff *et al*., 2001).

The centromere of *Saccharomyces cerevisiae* is approximately 125 base pairs consisting of three sequence elements: two short sequences of 8 and 25 base pairs separated by 78 to 86 base pairs of very AT-rich DNA. However, this short centromere sequence is not conserved in *Schizosaccharomyces pombe*, *Drosophila*, humans, nor in plants. Instead of this short centromere sequence, the centromeres consist of highly repetitive sequences. For instance, in humans the primary centromeric sequence is satellite DNA, which is a 171-base-pair sequence arranged in tandem repeats spanning up to millions of base pairs. In the plant centromeres investigated, the sizes of satellite repeat units are relatively consistent, ranging from ~150 to ~180 bp (e.g., 155 bp for rice, 156 bp for maize, and 180 bp for *Arabidopsis*). However, the sequences of satellite repeats are quite different among species (reviewed in Hall *et al*., 2004).

In addition, centromeres of humans and other mammals have been identified by the binding of special centromere-associated proteins (Warburton, 2001), including CENP-A, CENP-B, Mis12, CENP-C, CENP-H and CENP-I. These centromeric proteins have various roles and mutual interactions, and their associations with centromeric DNA create structural domains that support the different functions of the centromere. Homologues of CENP-A and CENP-C have been characterized in maize, rice and *Arabidopsis* (Talbert *et al*., 2004). In addition, a Mis12 homologue in *Arabidopsis* has been characterized and seems to be a constitutive component of the *Arabidopsis* centromere (Sato *et al*., 2005). It suggests that similar centromeric proteins (and perhaps machineries) are being used for centromere assembly in plants.

II-2-1-1-2 Telomeres

Telomeres, the sequences at the ends of eukaryotic chromosomes, fulfill unique and essential functions in genome integrity. Telomeres mark the ends of chromosomes and therefore enable the cell to distinguish a real end from an unnatural end caused by chromosome breakage - an essential requirement because the cell must repair the latter but not the former.

The telomeric DNA sequences of a variety of eukaryotes are similar, consisting of an array of short repeats of a simple-sequence DNA containing clusters of $T_{2\text{-}3}AG_3$ and varying from the repeat time of T and the size of telomere (reviewed in LeBel and Wellinger, 2005). For

example, human telomeres comprise less than 20 kb of (TTAGGG)n repeats, while mouse telomeres are exceptionally long extending to approximately 60 kb. In almost all plants, telomeres are composed of tandem repeat of the sequence TTTAGGG, but differ in length: 0.5 kb in the green alga *Chlorella vulgaris*; 3 kb in *Arabidopsis*; and reaching 150 kb in tobacco (Riha and Shippen, 2003). Telomeres have a distinctive structure, which has been reported in some species, composed of a G-rich and a C-rich strand, and the directionality of the repeats is conserved such that the 3' end of the chromosome is on the G-rich strand. Furthermore, the G-rich strand is longer than the C-rich strand leading to G-rich extensions at the very end of chromosomes that fold back to invade the double-stranded region of the telomere, creating a T-loop. T-loops are postulated to enhance telomere protection and facilitate telomeric DNA replication (reviewed in LeBel and Wellinger, 2005).

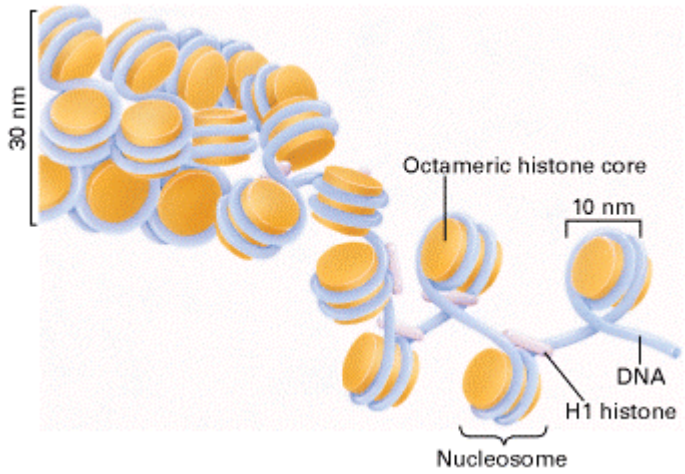
Telomere binding proteins (TBPs), which have been classified into two groups, seem to provide further protection for the chromosome ends and to control telomere length and gene silencing (reviewed in LeBel and Wellinger, 2005). The first group consists of proteins interacting with double-stranded telomeric DNA, e.g. human TRF1, TRF2 and yeast Rap1p protein. The second group binds to single-stranded G-rich tails, such as human POT1 and yeast Cdc13p. Proteins that are able to bind double-stranded telomeric DNA have been found in rice, maize, tobacco and *Arabidopsis* and shown to bind to plant telomeric DNA repeats *in vitro* (Hwang *et al*., 2005). The candidate proteins for binding single-stranded tails are AtPot1 and AtPot2 in *Arabidopsis*, which function in telomere length homeostasis and chromosome end protection (Shakirov *et al*., 2005).

II-2-1-1-3 Nucleolus Organizing Region (NOR)

The somatic cells of higher eukaryotes contain tens, hundreds or, as in plant cells, even thousands of ribosomal RNA genes. The genes are tandemly repeated consisting of a transcribed sequence and an external non-transcribed spacer, and form arrays in one or several chromosomes. In *Arabidopsis*, pericentromeric regions and nucleolus organizing regions (NORs) contain all major tandem repeats of the genome. The positions of NORs of chromosomes 2 and 4 in *Arabidopsis* are also marked as heterochromatin regions (Fransz *et al*., 1998, 2002). In interphase, nucleolar fusion takes place during which NORs from the chromosomes often participate in the formation of a given nucleolus.

At the molecular level, NOR is composed of tandemly arranged repeated units of ribosomal

(a)



(b)

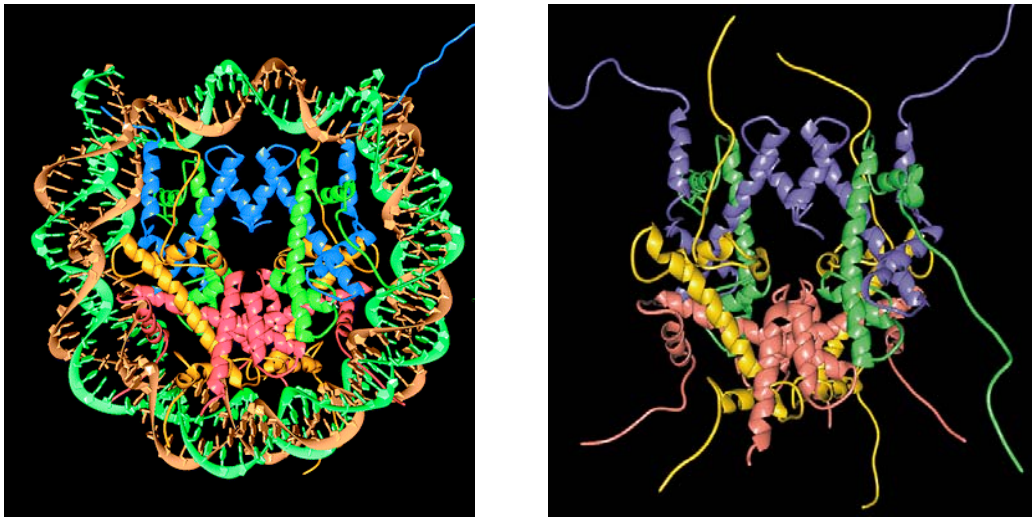


Figure II-5, Structure of the chromatin and nucleosome

(a) “Beads-on-a-string” form of chromatin. (b) Ribbon diagram of the nucleosome (left) and the histone octamer (right) (Luger *et al*., 1997). One DNA strand is shown in green and the other in brown. H2A is yellow; H2B, red; H3, blue; H4, green.

RNA genes coding for 18S, 5.8S, and 28S rRNA. The transcription of these genes is driven by the nucleolar RNA polymerase I (pol I), which synthesizes the long precursor 45S rRNA. The biogenesis of mature ribosomal RNAs is a complex process and necessitates the presence of a large variety of factors. Additionally, 5S rRNA, synthesized in the nucleoplasm, and ribosomal proteins, synthesized in the cytoplasm, are recruited into the nucleolus and become integrated with the 45S rRNA into complete ribosomal particles, which then move to the cytoplasm (reviewed in Raska *et al*., 2004).

It is generally agreed that most ribosomal RNA genes are transcriptionally inactive, such as the tandem repeats of NORs and 5S rDNA. For instance, in the *Arabidopsis* accession Columbia, 5S rDNA is located in the pericentromeric heterochromatin of chromosomes 3, 4, and 5. Both a major and some minor 5S rRNA species are expressed from chromosomes 4 and 5, whereas the genes on chromosome 3 are not transcribed (Mathieu *et al*., 2003). How can it be achieved that so many ribosomal genes are inactive? Studies have brought clues that rRNA gene silencing is based on the structural features of ribosomal chromatin and modulated by the various chromatin modifications (Raska *et al*., 2004; Mathieu *et al*., 2003).

II-2-1-2 Nucleosome assembly

The basic structural unit of chromatin is the nucleosome (**Figure II-5**), which was described by Kornberg in 1974. Each individual nucleosome core particle consists of 146 base pairs of DNA wrapped 1.65 times around an octamer of the core histones. The histone octamer is composed of a central heterotetramer of histones H3 and H4, flanked by two heterodimers of histones H2A and H2B (Luger *et al*., 1997). Thus, it is suggestive in the structure of the nucleosome that H3 and H4 must be deposited prior to the incorporation of H2A and H2B. In agreement with this, nucleosome assembly is shown to be a stepwise process (Krude and Keller, 2001): the chromatin assembly factor 1 (CAF1) binds H3 and H4, and targets these histones to the DNA replication fork via direct binding to PCNA, a DNA polymerase processivity factor that forms a protein clamp around DNA; the nucleosome assembly protein 1 (NAP1) then mediates the subsequent deposition of histone H2A-H2B.

A homologue of CAF1 has been identified in *Arabidopsis.* The importance of CAF1 in plant development is evidenced by findings that a null mutation of *CAF1* genes caused disorganization of both the root and the shoot apical meristems (Kaya *et al*., 2001). NAP1 is conserved in all eukaryotes. In addition to their role in nucleosome assembly, NAP1 proteins

were hypothesized as a core histone shuttle to deliver the histones from the cytoplasm to the nucleus. In higher plants, several NAP1 homologues have been identified in a given species including tobacco, rice, maize, and *Arabidopsis*. Tobacco and rice NAP1 family proteins can bind histones *in vitro* (Dong *et al*., 2003). It was also demonstrated that tobacco NAP1 proteins bind histone H2A and H2B and that Nicta;NAP1;1 and Orysa;NAP1;1 shuttle between the cytoplasm and the nucleus (Dong *et al*., 2005), suggesting that the function of NAP1 proteins are conserved in plants, and providing additional evidence for a possible role of plant NAP1 proteins as histone shuttles.

II-2-2 Regulation of chromatin structure

II-2-2-1 ATP-dependent remodeling complex

One of the important protein complexes involved in the regulation of chromatin structure is the SWI (SWITCH)/SNF (SUCROSE NON-FERMENTING) ATP-dependent remodeling complex.

The SWI/SNF family is subdivided into several classes based on the presence of other protein motifs in addition to the ATPase domain: SWI2/SNF2, ISWI (Imitation Switch), and CHD (Chromodomain Helicase DNA-binding protein) (reviewed in Lusser and Kadonaga, 2003). The distinct biochemical mechanism each subfamily uses and the varied substrate requirement suggest the unique biological functions in remodeling activity of each complex.

The *Arabidopsis* genome encodes more than 40 SWI/SNF-like proteins, of which only a few have been functionally characterized and shown to be essential for *Arabidopsis* development (reviewed in Hsieh and Fischer, 2005). *SYD* (*SPLAYED*), encoding a SWI2/SNF2 subfamily ATPase, was shown to regulate homeotic gene expression and floral transition, and maintain meristem during the reproductive phase (Wagner and Meyerowitz, 2002). Another SWI2/SNF2 homologue, AtBRM, is mostly expressed in meristems and proliferating tissues, and is required for normal vegetative and reproductive development (Farrona *et al*., 2004). An ISWI-type ATPase gene, *PIE1 (PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1)*, was identified to play a role in flowering time control in *Arabidopsis* (Noh and Amasino, 2003): *pie1* mutants flower earlier and PIE1 is required to upregulate the MADS box transcription factor FLC (Flowering Locus C), which represses floral transition. *CHR11* (*Chromatin-remodeling protein 11*) encodes an ISWI-type ATPase abundantly expressed

during female gametogenesis and embryogenesis in *Arabidopsis* (Huanca-Mamani *et al*., 2005). RNAi lines in which *CHR11* was specifically silenced had defective female gametophytes arrested before the completion of the mitotic haploid nuclear divisions, indicating that *CHR11* is essential for haploid nuclear proliferation during megagametogenesis (Huanca-Mamani *et al*., 2005). PKL (PICKLE), a CHD-like ATPase, was shown to repress LEC1 (LEAFY COTYLEDON1), a transcriptional regulator of embryonic identity (Ogas *et al*., 1999). Activation of *LEC1* in *pkl* mutants leads to inappropriate expression of embryo-specific genes after germination and causes expression of storage proteins and accumulation of storage lipids in the adult resulting in a "pickle root".

II-2-2-2 DNA methylation and demethylation

II-2-2-2-1 DNA methylthansferases

In most eukaryotes, particularly in plants, DNA methylation provides a heritable epigenetic mark and is involved in genomic imprinting and regulation of gene transcription. The reaction involves covalent addition of a methyl group to the 5-position carbon of cytosine by a family of enzymes called DNA methyltransferases. Cytosine methylation is mostly restricted to symmetrical CG dinucleotide context in vertebrates. By contrast, plant DNA methylation occurs at CG, CNG (where N is any nucleotide) and CHH (an asymmetric site, where H is A, C or T) sequences, each of which has different genetic requirements for the maintenance of its methylation (reviewed in Chan *et al*., 2005).

Arabidopsis contains the full members of the DNA methyltransferase types that are found in mammals, including DNMT1, DNMT2 and DNMT3 (reviewed in Chan *et al*., 2005). *Arabidopsis* MET1 (METHYLTRANSFERASE1) is the most extensively studied plant DNA methyltransferase and orthologous to the mammalian DNMT1 enzyme, both of which function in maintaining CG methylation. Null *met1* mutants display immediate effects on gene silencing owing to a loss of CG methylation in the gametophytic generation (Saze *et al*., 2003), suggesting a crucial role for CG methylation in epigenetic inheritance during the gametophytic phase of plant development. The function of second type of methyltransferase DNMT2 is highly conserved in mammals and *Arabidopsis*. Instead of DNA, a small RNA, $tRNA^{Asp}$, is the target of DNMT2 (Goll *et al*., 2006). *Arabidopsis* DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE2) is orthologous to the mammalian DNMT3 type of methyltransferase and both function to control *de novo* methylation (Cao *et al*., 2000).

The plant-specific methyltransferase CMT3 (CHROMOMETHYLASE3) controls the maintenance of non-CG methylation (Bartee *et al*., 2001; Lindroth *et al*., 2001). In contrast to *met1* mutants, mutations in either DRMs or CMT3 have no apparent phenotypes; however, the release of gene silencing at specific loci has been observed.

II-2-2-2-2 DNA glycosylases

The establishment and maintenance of DNA cytosine methylation are well studied in both mammals and plants. By contrast, the existence of a demethylation pathway has been an enduring controversy. Recent work in plants has identified a family of DNA glycosylases that can remove DNA methylation and alleviate silencing (reviewed in Chan *et al*., 2005).

Mutations in the DNA glycosylase ROS1 (*REPRESSOR OF SILENCING1*) in *Arabidopsis* cause DNA hypermethylation and transcriptional silencing of specific genes (Gong *et al*., 2002). Recombinant ROS1 protein has a DNA glycosylase activity on methylated but not unmethylated DNA substrates, inducing strand breaks in DNA containing 5-methylcytosine (Gong *et al*., 2002). A base excision repair mechanism (BER) may follow, resulting in the replacement of a 5-methylcytosine with an unmethylated cytosine (Krokan *et al*., 1997). So, ROS1 might be directly involved in DNA demethylation through a base excision repair mechanism. Recently, *Arabidopsis* DME (DEMETER), another member of the DNA glycosylase family (Choi *et al*., 2002), was shown to excise 5-methylcytosine *in vitro* and when expressed in *E. coli* (Gehring *et al*., 2006). DME is responsible for removing DNA methylation from MEA (MEDEA), which is imprinted in the endosperm, allowing the maternal MEA allele to be expressed (Gehring *et al*., 2006).

II-2-2-3 Histone variants

A fraction of histones are nonallelic variants that are distinguished by sequence differences. Some variants are simply polymorphic versions of the major histones and thought to alter the properties of nucleosomes, while others deposit to specific chromatin regions, leading to the suggestion that the histone variants have specialized functions regulating chromatin structure. In core histones, histone H2B and H4 are markedly deficient in variants (Kamakaka and Brggins, 2005). Histone H3 and H2A variants are currently the best studied.

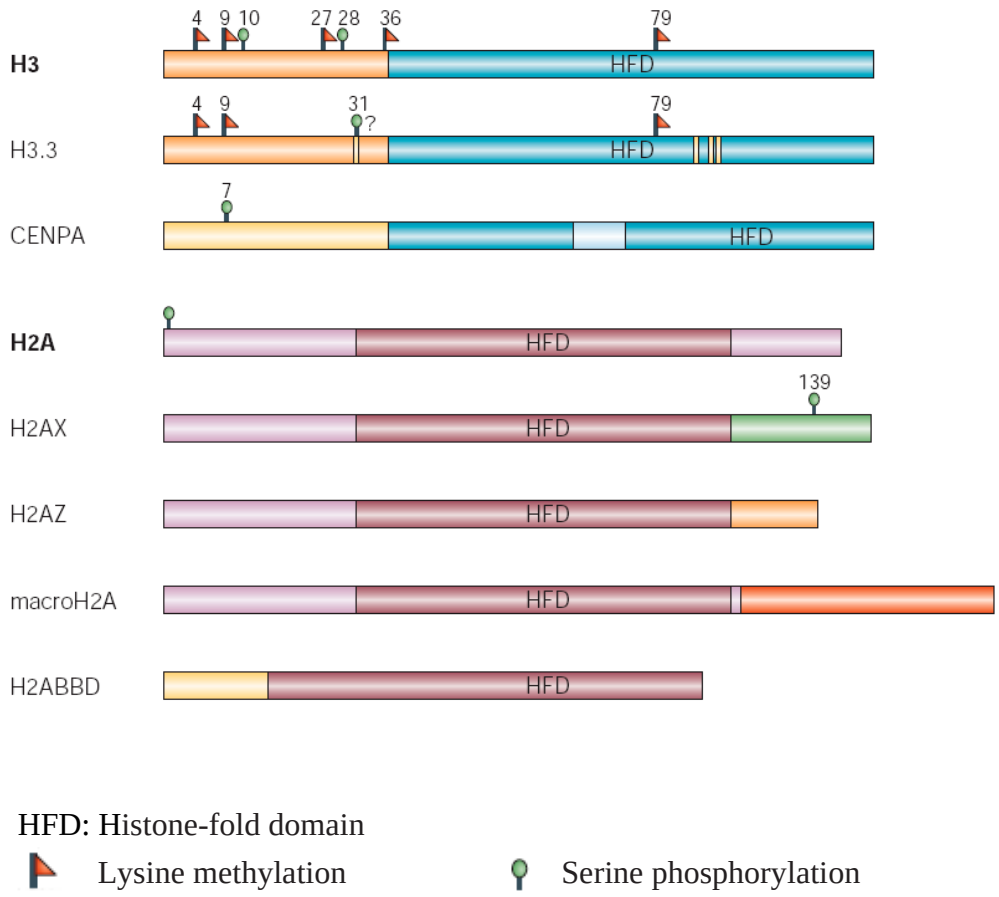


Figure II-6, Core histones H3, H2A, and their variants (Sarma and Reinberg, 2005)

In the histone H3.3 variant, the residues that differ from the major histone H3 are highlighted in yellow. The residue Ser31 has been speculated to be a potential site for phosphorylation on H3.3. The centromeric histone CENP-A has a unique N-terminus containing the residue Ser7. The region in the globular domain that is required for targeting CENPA to the centromere is highlighted in light blue. Histone H2A variants differ significantly from the major core H2A in their C terminus. The C terminus of H2AX harbours a conserved serine residue (Ser139). MacroH2A has an extended C-terminal macro domain. H2ABBD is the smallest of the H2A variants and only contains a distinct N terminus. For simplicity, only well-established sites for lysine methylation and serine phosphorylation are shown.

II-2-2-3-1 H2A variants

Four H2A variants have been reported so far in animals - H2AZ, H2AX, macroH2A and H2ABBD (H2A-bar-body-deficient) (reviewed in Sarma and Reinberg, 2005). The H2A variants are distinguished from the major H2A histones by their C-terminal tails that diverge in both length and sequence (**Figure II-6**), as well as in their genome distribution. H2AZ and H2AX are the minor H2A variants that appear constitutively expressed and localize throughout the genome. MacroH2A is a vertebrate-specific variant, which has two distinct domains - the N-terminus, which is similar to H2A, and a large C-terminus, which has no similarity to other histones. MacroH2A localizes predominantly to the inactive X (Xi) chromosome in mammalian female cells. H2ABBD is the smallest H2A variant only containing a distinct N terminus, which is excluded from the Xi chromosome in mammalian cells.

However, little is known for the plant H2A variants. A homologue of H2AZ variant has been identified in *Arabidopsis*, indicating that H2AZ variants seem to be conserved in plants. The expression of this *Arabidopsis* H2AZ is tightly correlated with cell proliferation in suspension culture cells (Callard and Mazzolini, 1997). Recently, it was demonstrated that phosphorylated H2AX in *Arabidopsis*, known as γ-H2AX, forms "foci" at DNA double-strand breaks (DSBs) induced by ionizing radiation (IR). Moreover, both *AtATM* (Ataxia Telangiectasia Mutated) and *AtATR* (Ataxia Telangiectasia mutated and Rad3-related), which are involved in DNA damage response, were shown to have complementary roles in the IR-induced γ-H2AX formation (Friesner *et al*., 2005). Additionally, a novel histone variant specifically expressed in male gametic cells within the pollen of *Lilium longiflorum* was isolated, designated as gH2A (Ueda *et al*., 2000).

II-2-2-3-2 H3 variants

Four different isoforms of histone H3 have been reported: the major histone H3.1, the variants H3.2 and H3.3, and CENP-A, the centromeric histone H3. CENP-A shows a wide variability in amino-acid composition between species and even within the same species when compared with the other H3 isoforms (reviewed in Sarma and Reinberg, 2005).

Studies in *Drosophila* showed that unlike the major H3.1, the variant histone H3.3 is deposited to active chromatin throughout the cell cycle by the replication independent

nucleosome assembly, suggesting that H3.3 displaces H3.1 during transcription (Ahmad and Henikoff, 2002). In the *Arabidopsis* genome, fifteen histone H3 genes are present, of which three are H3.3 variants (Okada *et al*., 2005). H3.3 genes exhibited an extremely high level of expression in most of the tissues examined and displayed replication-independent expression at the transcription level, suggesting that the mechanism of H3.3 displacement for gene activity might be conserved in plants.

The variant histone CENP-A shares a similar C-terminal histone-fold domain with H3, but varies extensively in its N-terminal region. Human CENP-A is localized exclusively to centromeres. Although not much is known about how CENP-A is targeted to centromeres, it is known to carry out an essential function, as knockout of this gene in mice results in lethality. Until now, homologues of CENP-A have been characterized in maize (maize CENH3), rice (rice CENH3) and *Arabidopsis* (HTR12). *Arabidopsis* HTR12 was found as a major protein that binds to centromeres and is thought to replace H3 in the centromeric nucleosomes (Talbert *et al*., 2002). Fang and Spector used transgenic *Arabidopsis* lines expressing a fusion protein of HTR12 with Venus, a brighter variant of YFP, and showed that *Arabidopsis* centromeres were observed as small, bright HTR12-Venus foci in nuclei (Fang and Spector, 2005). Thus, it is assumed that this H3 variant confers special properties on centromeric nucleosomes in plants.

Furthermore, tissue-specific histone H3 variants, which show cell-specific expression in the male gametes, have been identified in *Arabidopsis* (AtMGH3) and *Lilium longiflorum* (gH3) (Okada *et al*., 2005; Ueda *et al*., 2000). These histone H3 variants as well as other male gamete-specific histone variants are expected to be specifically synthesized in the male gametic cells and to cause chromatin condensation or remodeling of chromatin structure.

II-2-2-4 Covalent modifications of histones

The flexible tails of histones that protrude from the nucleosome core are subject to numerous posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, glycosylation, ADP ribosylation, sumoylation and biotinylation. These histone modifications represent additional epigenetic information on chromatin that alters the functional properties of the underlying genetic information, and have been proposed to form a "histone code" (Jenuwein and Allis, 2001; Turner, 2002).

II-2-2-4-1 Histone acetylation and deacetylation

Of the different types of histone modifications, acetylation is most extensively characterized. Epsilon-amino groups of lysine residues in the N-terminal tails of core histones (K9, 14, 18, 23 of H3; K5, 8, 12, 16 of H4) are the predominant sites for acetylation (Turner, 2002), and can be acetylated by histone acetyltransferases (HATs) using acetyl-CoA as a co-factor. Acetylation can be reverted by histone deacetyltransferases (HDACs). In plant, additional lysine residues in the N-terminal histone regions are subject to acetylation compared with animals and fungi. Plant H4 can be acetylated at lysine 20 (Waterborg, 1992), which is only subject to methylation in animals and fungi.

Some HATs and HDACs have preferential sites, whereas others seem to have a broader substrate range and acetylate various histone lysine residues. In addition, many non-histone regulatory proteins e.g. proto-oncogene products like c-Myc, tumor suppressors like p53 and the tumor suppressor Rb protein and tubulin are also substrates of some HATs and HDACs (reviewed in Loidl, 2004).

II-2-2-4-1-1 HATs

The various HATs are well conserved in their catalytic domains and recruit proteins with homologous functions in different species. By sequence analysis, four distinct families of HATs can be distinguished: (i) the GNAT-MYST family; (ii) the p300/CBP coactivator family; (iii) the TAFII250-related family; and (iv) the nuclear receptor coactivator family, which is present in vertebrates but not in plants or fungi (Pandey *et al*., 2002).

The *Arabidopsis* genome encodes at least 12 HATs (Pandey *et al*., 2002). The AtGCN5 (Stockinger *et al*., 2001), a GNAT-type HAT and PCAT2 (Bordoli *et al*., 2001), a p300/CBP-type HAT exhibit HAT activity *in vitro*. The AtGCN5 is associated with the transcriptional adaptor protein ADA2; mutations disrupting *ADA2* or *GCN5* have pronounced effects on growth, development and gene expression patterns (Stockinger *et al*., 2001; Vlachonasios *et al*., 2003). Additionally, by characterizing a mutation in the *AtGCN5*, Bertrand *et al*. studied the regulatory function of this gene in controlling floral meristem activity. Homeotic transformations of flower organs occur in the mutant flowers, which correlate with an expansion of the expression domains within floral meristems of the key regulatory genes *WUSCHEL* (*WUS*) and *AGAMOUS* (*AG*), suggesting that *AtGCN5* is required for regulation

of the floral meristem activity through the *WUS/AG* pathway (Bertrand *et al*., 2003).

Strong correlations have been established between transcriptional activity and the distribution patterns of histone acetylation. The activation of the pea plastocyanin gene (*PetE*) is associated, in a tissue-dependent manner, with hyperacetylation of both H3 and H4 in the enhancer/promoter region (Chua *et al*., 2001). The transcriptional enhancer of this gene activates transcription by associating with the nuclear matrix, thereby triggering the acetylation of histones on the promoter and altering chromatin structure (Chua *et al*., 2003). Recent analysis on the *phaseolin* (*phas*) promoter also showed that H3 and H4 acetylation at the target chromatin significantly increased when the *phas* promoter was actively transcribing (Ng *et al*., 2006).

II-2-2-4-1-2 HDACs

Three families of HDACs have been identified: (i) members of the Rpd3/HDA gene family; (ii) the Silent Information Regulator2 (SIR2) family; and (iii) the Heading Date2 (HD2) family (Pandey *et al*., 2002). The HD2 class, seems to be plant-specific. In general, HDACs are often correlated with transcriptional repression and gene silencing.

The *Arabidopsis* genome encodes at least 17 HDACs (Pandey *et al*., 2002), of which the Rpd3-type HDACs are well characterized. Suppression of the Rpd3-type HDAC *AtHD1* in *Arabidopsis* by anti-sense inhibition resulted in elevated levels of acetylated histones and various growth and developmental defects (Tian and Chen, 2001). Mutations in *AtHDA6*, another Rpd3-type HDAC of *Arabidopsis* caused loss of transcriptional silencing of the transgene (Murfett *et al*., 2001) and repetitive rDNA repeats (Probst *et al*., 2004). Moreover, characterization of the *rts1* (RNA-mediated transcriptional silencing) mutation reveals that *RTS1* encodes AtHDA6 and is required for RNA-directed *de novo* DNA methylation in *Arabidopsis* (Aufsatz *et al*., 2002). HDA19 (AtRPD3A), also belonging to the Rpd3-type HDACs, was shown to regulate gene expression involved in jasmonic acid and ethylene signaling of the pathogen response in *Arabidopsis* (Zhou *et al*., 2005). A plant specific HD2-type HDAC *AtHD2A* was also functionally characterized. Antisense inhibition of *AtHD2A* in *Arabidopsis* leads to aborted seed development, consistent with its expression in flowers and siliques (Wu *et al*., 2000).

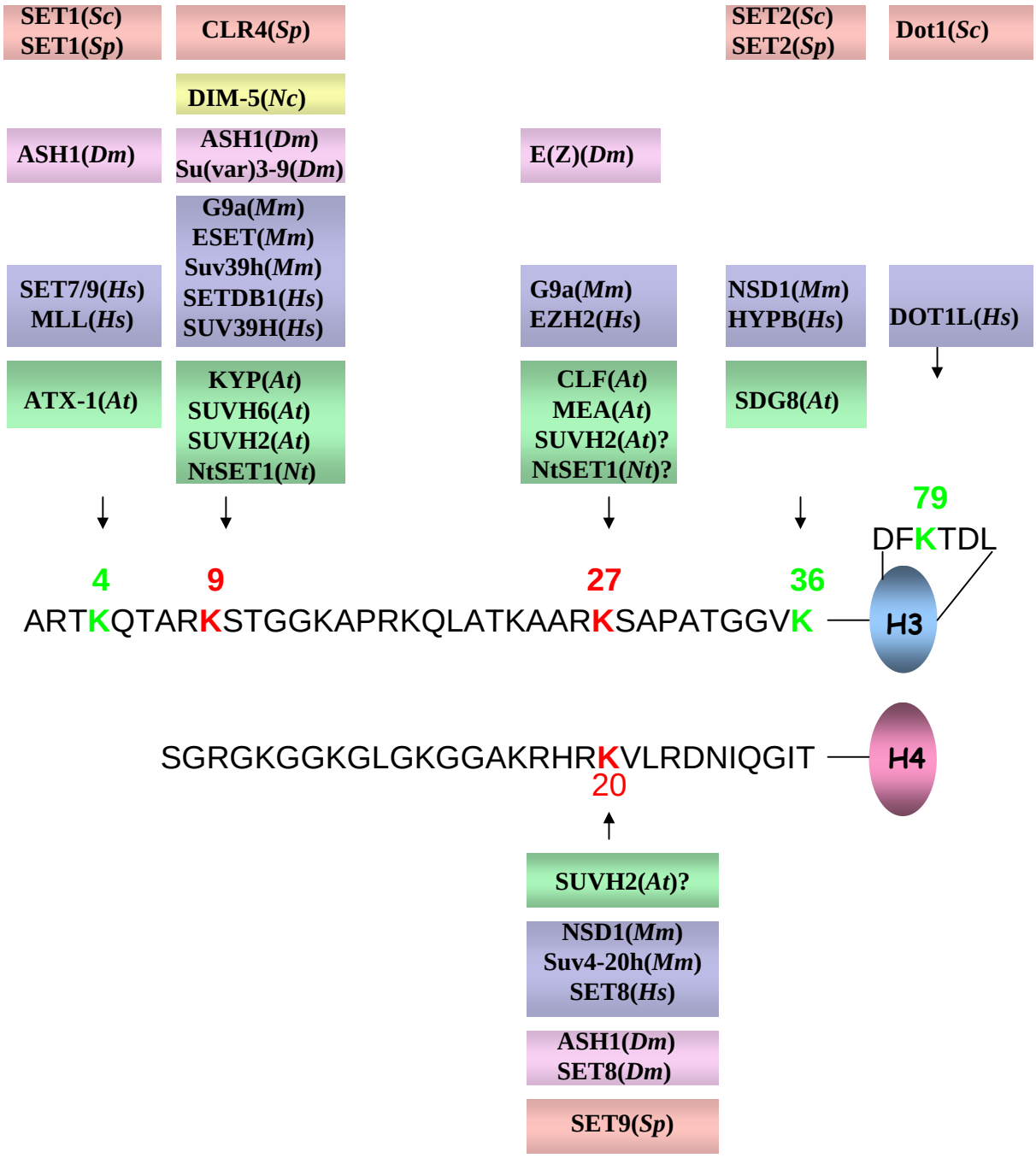


Figure II-7, Lysine residues for the methylation on the core histones, and the responsible histone lysine methyltransferases

Histone lysine methylation occurs on lysine residues 4, 9, 27, 36 and 79 in H3, and on lysine 20 in H4. The histone lysine methyltransferases are grouped according to the specific lysine residue targeted for modification and color coded according to their origin (yeast, red; *Neurospora*, yellow; fly, purple; mammalian, blue; plant, green). Species abbreviations: *Sc*, *Saccharomyces cerevisiae*; *Sp*, *Schizosaccharomyces pombe*; *Nc*, *Neurospora crassa*; *Dm*, *Drosophila melanogaster*; *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*; *At*, *Arabidopsis thaliana*; *Nt*, *Nicotiana tabacum*.

II-2-2-4-2 Histone methylation

In the N-terminal extensions of histones H3 and H4, both arginine and lysine residues are subject to various degrees of methylation.

II-2-2-4-2-1 Arginine methylation

Arginines can be either mono- or dimethylated in a symmetric (both side-chain amino groups are methylated) or asymmetric (only one side-chain amino group is dimethylated) manner by protein arginine methyltransferases (PRMTs), which transfer methyl groups from S-adenosyl methionine to the guanidino nitrogen atoms of the side chain. PRMTs are grouped into two types with the type I enzymes catalyzing the formation of N^G-monomethylarginine and asymmetric N^G, N^G-dimethylarginine residues, and the type II enzymes catalyzing the formation of N^G-monomethylarginine and symmetric N^G, N^G-dimethylarginine residues. Arginine methylation has been linked mainly with transcriptional activation (reviewed in Zhang and Reinberg, 2001). However, arginine methylations in plants remain to be uncovered.

II-2-2-4-2-2 Lysine methylation

Histone lysine methylation occurs on lysine residues 4, 9, 27 and 36 in H3, and on lysine 20 in H4 (reviewed in Loidl, 2004, **Figure II-7**). H3 can also be methylated at K79 inside the globular domain (Feng *et al*., 2002; van Leeuwen *et al*., 2002, **Figure II-7**). Generally, methylation of H3K4, K36, and K79 correlate with active transcription, whereas methylation of H3K9, K27, and H4K20 are associated with silenced chromatin. In addition, lysine residues can be mono-, di-, or trimethylated, which adds another layer of complexity to the histone code. Recent evidence suggests that there may be functional differences between these methylation states (Dutnall, 2003). For instance, in mammalian cells it was shown that trimethyl H3K9 is preferentially localized to pericentromeric heterochromatin, while mono- and dimethyl H3K9 are localized to euchromatin (Peters *et al*., 2003; Rice *et al*., 2003). However, for H3K9 methylation, plants have a distinct pattern: abundant dimethyl H3K9 but little trimethyl H3K9 is found at silent loci in *Arabidopsis*, suggesting that dimethylation of H3K9, instead of the trimethyl state, is the critical mark for gene silencing and heterochromatin (Jackson *et al*., 2004).

Table II-1, Sites and functions of histone lysine methylation

Site	HMTs	Function	References
H3			
K4	SET1(*Sc*)	Transcriptional activation	Santos-Rosa *et al*., 2002; Bernstein *et al.*, 2002
		rDNA silencing, telomeric silencing	Briggs *et al*., 2001; Bryk *et al*., 2002
	SET1(*Sp*)	Transcriptional activation	Noma and Grewal, 2002
	Ash1(*Dm*)	Trithorax activation	Beisel *et al.*, 2002
	SET7/9(*Hs*)	Transcriptional activation	Wang *et al.*, 2001a; Nishioka *et al*., 2002a
	MLL(*Hs*)	Trithorax activation	Milne *et al*., 2002; Nakamura *et al*., 2002
	ATX-1(*At*)	Trithorax activation	Alvarez-Venegas *et al*., 2003
K9	CLR4(*Sp*)	Centromeric/mating-type silencing	Bannister *et al*., 2001; Nakayama *et al*., 2001
	Su(var)3-9(*Dm*)	Pericentric heterochromatin	Schotta *et al*., 2002
	Ash1(*Dm*)	Trithorax activation	Beisel *et al*., 2002
	G9a(*Mm*)	Transcriptional repression	Tachibana *et al*., 2001; Tachibana *et al.*, 2002
	ESET(*Mm*)	Transcriptional repression	Yang *et al*., 2002
	Suv39h1(*Mm*)	Pericentric heterochromatin	Lachner *et al*., 2001; Peters *et al.*, 2001
	Suv39h2(*Mm*)	Pericentric heterochromatin	Lachner *et al*., 2001; Peters *et al.*, 2001
	SETDB1(*Hs*)	Transcriptional repression	Schultz *et al*., 2002
	SUV39H1(*Hs*)	Pericentric heterochromatin	Rea *et al*., 2000; Lachner *et al.*, 2001
	DIM-5(*Nc*)	DNA methylation	Tamaru and Selker, 2001
	KYP(*At*)	DNA methylation	Jackson *et al.*, 2002; Malagnac *et al*., 2002
	SUVH6(*At*)	DNA methylation at *PAI1-PAI4* locus	Ebbs *et al*., 2005
	SUVH2(*At*)	Pericentric heterochromatin	Naumann *et al*., 2005
	NtSET1(*Nt*)	putative role in heterochromatic formation	Yu, *et al.*, 2004
K27	E(Z)(*Dm*)	Polycomb repression	Cao *et al*., 2002
	EZH2(*Hs*)	Polycomb repression	Kuzmichev *et al.*, 2002
	G9a(*Mm*)	?	Tachibana *et al*., 2001
	MEA(*At*)	Silencing of the *MEA* paternal allele	Gehring *et al*., 2006
	CLF(*At*)	Regulation of *MEA* imprinting	Jullien *et al*., 2006
K36	SET2(*Sc*)	Transcription elongation	Schaft *et al.*, 2003; Xiao *et al.*, 2003
		Transcriptional repression	Carrozza *et al.*, 2005; Keogh *et al.*, 2005
	SET2(*Sp*)	Transcription elongation	Morris *et al.*, 2005
	NSD1(*Mm*)	Transcriptional regulation	Rayasam *et al.*, 2003
	HYPB(*Hs*)	Transcriptional regulation	Sun *et al.*, 2005
	SDG8(*At*)	Transcriptional regulation, flower time control	Zhao *et al.*, 2005
K79	Dot1(*Sc*)	Transcriptional regulation	van Leeuwen *et al.*, 2002
	DOT1L(*Hs*)	Transcriptional regulation	Feng *et al.*, 2002;
H4			
K20	SET9(*Sp*)	Recruitment of checkpoint protein Crb2 to sites of DNA damage	Sanders *et al.*, 2004
	Ash1(*Dm*)	Trithorax activation	Beisel *et al.*, 2002
	SET8(*Dm*)	cell cycle dependent transcriptional silencing	Fang *et al*., 2002; Karachentsev *et al.*, 2005
	SET8(*Hs*)	cell cycle dependent transcriptional silencing	Nishioka *et al.*, 2002b; Couture *et al.*, 2005
	NSD1(*Mm*)	Transcriptional regulation	Rayasam *et al.*, 2003
	Suv4-20h(*Mm*)	Pericentric heterochromatin	Schotta *et al.*, 2004
Species abbreviations: *Sc*, *Saccharomyces cerevisiae*; *Sp*, *Schizosaccharomyces pombe*; *Nc*, *Neurospora crassa*; *Dm*, *Drosophila melanogaster*; *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*; *At*, *Arabidopsis thaliana*; *Nt*, *Nicotiana tabacum*.			

Many proteins responsible for the histone methylation of specific lysine residues have been characterized (**Figure II-7**; **Table II-1**). Except the DOT1 and DOT1L proteins which methylate H3K79 in the globular region (Feng *et al*., 2002; van Leeuwen *et al*., 2002), all histone methyltransferases (HMTases) contain a conserved SET domain and thus make up the SET domain protein methyltransferase family.

The SET domain, which is approximately 130 amino acids in length, was originally identified as a conserved sequence in three *Drosophila* proteins: Suppressor of variegation 3-9 [Su(var)3-9], Enhancer of zest [E(z)], and the Trithorax-group protein Trithorax (Trx) (Jenuwein *et al*., 1998), and shown to be the methyltransferase catalytic core (Rea *et al*., 2000). In addition to the unique catalytic SET domain, the cysteine-rich regions flanking the SET domain are found in most SET domain proteins, which are thought to be important for the enzymatic activity of certain HMTases (Rea *et al*., 2000; Kouzarides, 2002). Interestingly, although each of these proteins contains a conserved SET domain, the regions flanking the SET domain are not strictly conserved, and are used for their classification.

The structure of the SET domain has been solved by crystal structure analysis of some SET domain proteins (**Figure II-8**), including human SET7/9 (Wilson *et al*., 2002), *Neurospora* DIM-5 (Zhang *et al*., 2002) and *Schizosaccharomyces pombe* CLR4 (Min *et al*., 2002). Crystal structures revealed that the SET domain forms a knot-like structure, which is formed by the most highly conserved residues within the SET domain. The histone substrate and the methyl-donor cofactor bind on opposite surface grooves of the SET domain, and the catalytic reaction occurs within the narrow channel that connects the grooves.

According to the sequence conservation in the SET domain, Springer *et al.* (2003) classified the plant SET domain proteins into five classes, named class I to V. The class V (SUV39 class) comprises the largest number of plant SET domain proteins (Springer *et al*., 2003; Zhao and Shen, 2004). The functionally characterized *Arabidopsis* members in class V include KRYPTONITE (KYP, also named as SUVH4), SUVH6 and SUVH2. KYP/SUVH4 protein methylates specifically H3K9 (Jackson *et al*., 2002). Genetic analyses revealed that KYP/SUVH4 is involved in transcriptional silencing of euchromatic loci, including the *SUPERMAN* floral homeotic gene and the *PAI2* and *PAI3* tryptophan biosynthetic gene (Jackson *et al*., 2002; Malagnac *et al*., 2002), as well as in H3K9 methylation within the heterochromatic chromocenters (Jasencakova *et al*., 2003). In contrast, mutations in

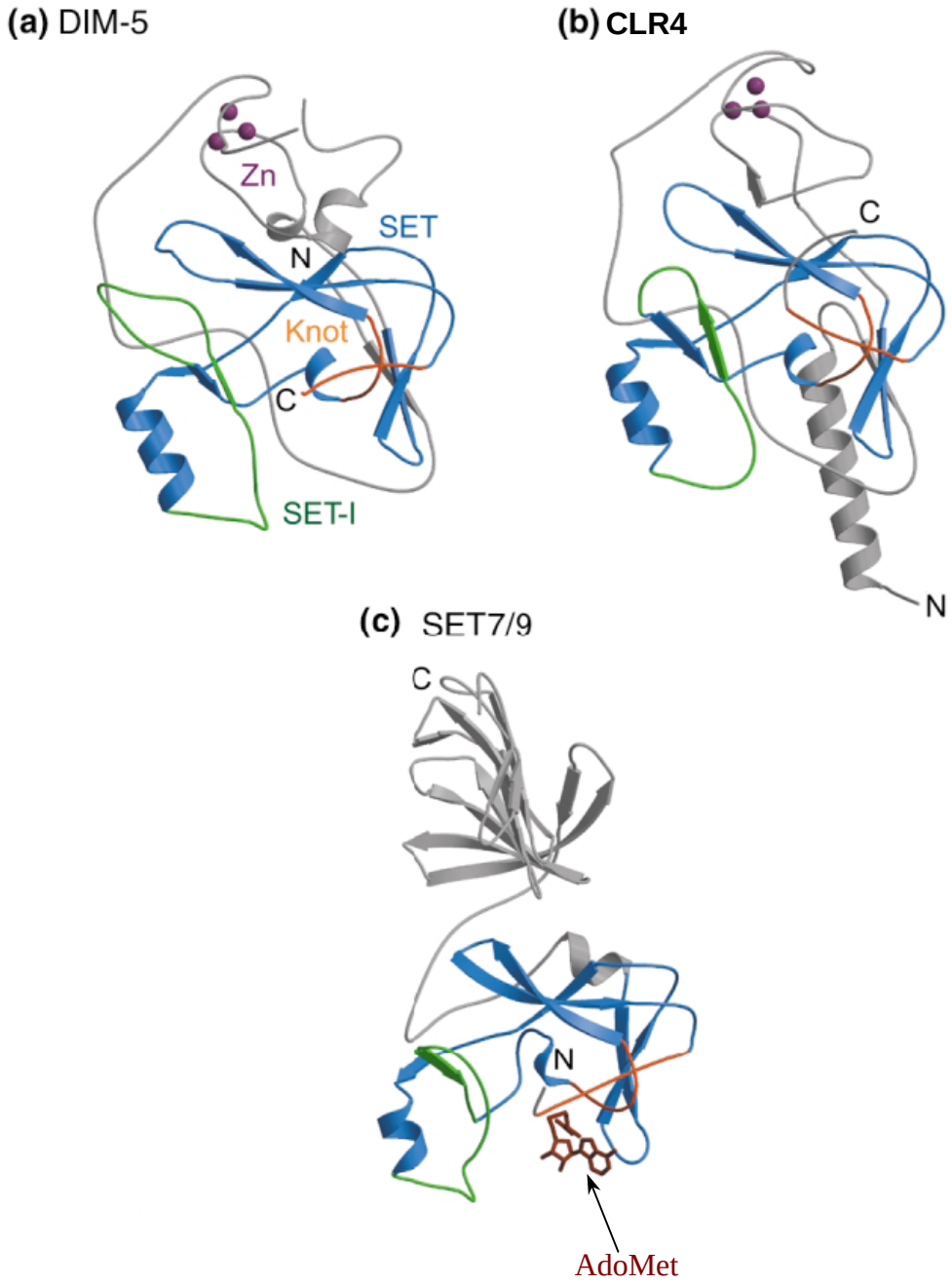


Figure II-8, Structure of SET domain proteins (Marmorstein, 2003)

Structures of (a) *Neurospora* DIM-5, (b) *Schizosaccharomyces* CLR4, and (c) human SET7/9 are shown. In each protein, the SET-N and SET-C subdomains are shown in blue, the SET-I region is shown in green, the unusual knot structure is shown in orange, bound zinc atoms are shown in purple and the N- and C-terminal regions flanking the SET domain are shown in gray.

KYP/*SUVH4* do not affect H3K9 methylation and DNA methylation at the *PAI1-PAI4* transcribed inverted repeat. However, both epigenetic modifications are reduced at this locus in a *suvh4suvh6* double mutant (Ebbs *et al*., 2005), suggesting that H3K9 methylation and DNA methylation are maintained in the transcribed inverted repeat by combined action of KYP/SUVH4 and SUVH6 HMTase. Another characterized *Arabidopsis* member of this class is SUVH2. In *SUVH2* null plants mono- and dimethyl H3K9, mono- and dimethyl H3K27, and mono-methyl H4K20, the histone methylation marks of *Arabidopsis* heterochromatin, are significantly reduced, suggesting that SUVH2 has as a central function in heterochromatic gene silencing in *Arabidopsis* (Naumann *et al*., 2005). The tobacco SET domain protein NtSET1, which was previously found to associate with chromatin (Shen, 2001b), belongs also to class V. As described in the Article 2 of the RESULT section of this thesis, we showed that NtSET1 also methylates H3K9, and that ectopic expression of NtSET1 induces chromosome-segregation defects in tobacco BY2 cells (Yu *et al*., 2004). Furthermore, NtSET1 can bind LHP1, the *Arabidopsis* homologue of animal heterochromatin protein1 (HP1), suggesting a role for NtSET1 in heterochromatic function (Yu *et al*., 2004). Genetic studies in *Arabidopsis* revealed that CLF (Goodrich *et al*., 1997) and MEA (Grossniklaus *et al*., 1998), both belonging to class I (E(Z) class), are involved in the repression of expression of the floral homeotic gene *AGAMOUS* (*AG*) and in the suppression of the parthenogenetic development of endosperm, respectively. H3K27 methylation is strongly reduced both in the *clf* mutant and *mea* mutant (Jullien *et al.*, 2006; Gehring *et al.*, 2006). The only member of class II (ASH1 class) SET domain proteins which is functionally characterized, is SDG8 in *Arabidopsis*, which is described in the Article 3 of the RESULT section of this thesis. The *sdg8* mutant showed a reduced level of dimethylated H3K36 and reduced *FLC* (*FLOWERING LOCUS C*) expression, and thus displayed early flowering phenotype (Zhao *et al*., 2005). The *Arabidopsis* ATX-1, belonging to class III (TRX class), can methylate H3K4 *in vitro*, and is required for maintaining the expression of several floral homeotic genes, including *AG* (Alvarez-Venegas *et al*., 2003).

II-2-2-4-2-3 Histone demethylation

Histone methylation had been considered as a stable modification until recently. A human arginine deiminase, PADI4/PAD4, has been shown to antagonize methylation on the arginine residues by converting arginine to citrulline (Cuthbert *et al*., 2004; Wang *et al*., 2004b). And the first experimental evidence for histone lysine demethylase is from human LSD1 (lysine-specific demethylase1), which induces amine oxidation of methylated H3K4 to generate

unmodified lysine and formaldehyde (Shi *et al*., 2004). Recently, human JHDM1 (JmjC domain-containing histone demethylase1) protein was identified to specifically demethylate H3K36 and generate formaldehyde and succinate (Tsukada *et al*., 2006). This demethylase activity of the JmjC domain-containing proteins is conserved in yeast, suggesting that the JmjC domain may be a novel demethylase signature motif and endue a protein demethylation activity (Tsukada *et al*., 2006). In *Arabidopsis*, EARLY FLOWERING6 (ELF6) and RELATIVE OF EARLY FLOWERING6 (REF6), containing jmjC domains, were identified (Noh *et al*., 2004) and proposed to have putative functions in histone demethylation (Reyes, 2005). ELF6 and REF6 were shown to play divergent roles in the regulation of *Arabidopsis* flowering (Noh *et al*., 2004).

II-2-2-4-3 Histone phosphorylation

Histone phosphorylation that involves the hydroxyl groups of serine and threonine is another chromatin mark and is mediated by specific kinases.

Histone phosphorylation has long been considered to associate with condensed chromosomes and is linked to chromosome condensation during mitosis and meiosis (Wei *et al*., 1999; Barber *et al*., 2004). However, studies in maize indicate that histone H3 Ser10 (H3S10) phosphorylation during mitosis and meiosis begins late in prophase, after chromosome condensation has been initiated, and appears to be associated with chromosome cohesion rather than condensation (Kaszas and Cande, 2000). In addition to H3S10, phosphorylations occur at H3S28 and CENH3S50 in maize. And these two phosphoserines demarcate pericentromere and centromere respectively during mitosis (Zhang *et al*., 2005).

Furthermore, evidence has accumulated indicating that histone phosphorylations have an important role in transcriptional regulation in various organisms (reviewed in Nowak and Corces, 2004). In the case of phosphorylation at serine 10, it has been shown in mouse that phosphorylation occurs on the same histone H3 tail together with acetylation and associates with the active *IE* (*immediate-early*) gene (Clayton *et al*., 2000). A correlation between histone H3 phosphorylation and the induction of the heat-shock loci in *Drosophila* has also been established (Nowak and Corces, 2000). Recent studies in mammalian cells show that H3S10 phosphorylation by the principal mitotic kinase Aurora B causes the dissociation of HP1 (heterochromatin protein1) from mitotic heterochromatin, even though the methylation levels of H3K9 remain unchanged (Fischle *et al*., 2005; Hirota *et al*., 2005). Therefore it is

proposed that histone phosphorylation controls the binding of proteins to chromatin, thereby regulating gene expression, chromatin packaging and heterochromatin formation. Aurora-like kinases have been characterized in *Arabidopsis*, being very similar in structure to those of mammals. AtAurora1 preferentially phosphorylates H3S10 *in vitro* and colocalizes with phosphorylated H3S10 during mitosis (Demidov *et al*., 2005).

In addition to chromosome condensation and transcription regulation, histone phosphorylation is also linked to other cellular processes. An H2A variant in mammalian cells, H2AX, is rapidly phosphorylated at its C-terminal tail (at Ser139) upon exposure to ionizing radiation (IR), suggesting a function in response to DNA damage (Rogakou *et al*., 1998, 1999). The mechanism seems conserved in plants, since a recent study in *Arabidopsis* demonstrated that phosphorylated H2A.X in *Arabidopsis* forms "foci" at DNA double-strand breaks (DSBs) induced by IR (Friesner *et al*., 2005). Phosphorylation on serine 1 of histone H4 (H4S1) was shown to associate with MMS- or phleomycin-induced DSBs but not with UV-induced DNA damage (Cheung *et al*., 2005).

II-2-2-5 siRNA

In plants, dsRNAs can also target their homologous sequences for gene silencing (Baulcombe, 2004). This RNA interference (RNAi) mechanism involves the generation of small interfering RNA molecules (siRNAs), ~22 nucleotides long, from longer dsRNAs by an RNase-III-like enzyme called Dicer. These siRNAs are then recognized, unwound and incorporated as single stranded RNAs into a protein complex known as RISC (RNA-induced silencing complex) containing Argonaute (AGO) family proteins, then serve to direct silencing of homologous sequences. RNA-dependent RNA polymerases (RdRPs) are also involved in amplification of the RNAi response by generating more double stranded RNA from single-stranded targets that can then enter and continue to stimulate the RNA silencing pathway.

The first indication that RNA plays a role in transcriptional repression came from the observation of RNA-mediated DNA methylation (RdDM) in tobacco, triggered by infection with viroid RNA (Wassenegger *et al*., 1994). Following this observation, it was demonstrated in *Arabidopsis* that RdDM and stable transcriptional gene silencing can be induced by dsRNAs that are degraded into siRNAs (Mette *et al*., 2000). The presence of dsRNAs homologous sequences in the promoter or the coding region of a gene results in heritable silencing of that gene (Mette *et al*., 2000; Jones *et al*., 2001), suggesting the involvement of

the RNAi mechanism in establishing silent chromatin. Recent studies in *Arabidopsis* indicate that siRNA may direct chromatin modifications, including DNA methylation and histone methylation. Vaistij *et al*. showed that virus vectors carrying parts of a GFP transgene targeted RNA silencing in *Nicotiana benthamiana* and *Arabidopsis* against the entire GFP RNA, accompanied by methylation of the corresponding GFP DNA, which was dependent on the putative RdRP, SDE1/SGS2 (Vaistij *et al*., 2002). As in the *Arabidopsis sde1*/*sgs2* mutant, the *ago1* mutant exhibited reduced DNA methylation of the transgenes (Morel *et al*., 2002). Furthermore, mutations in *Arabidopsis AGO4* also decrease DNA methylation, particularly at non-CG positions, and histone H3K9 dimethylation, causing derepression of the silent *SUPERMAN* (*SUP*) epialleles. In addition, the *ago4* mutant also eliminates cytosine methylation and histone H3K9 dimethylation in a constitutive heterochromatin locus, *AtSN1* (the *Arabidopsis thaliana* short interspersed element 1), due to failure of siRNA accumulation (Zilberman *et al*., 2003).

Recent findings suggest that RNAi-mediated silencing pathways play a role in heterochromatin assembly in fission yeast *S. pombe* (Volpe *et al*., 2002; Verdel *et al*., 2004). Central to the mechanism is a protein complex, known as RITS (RNA-induced transcriptional silencing) composed of three protein subunits (an Argonaute family protein, Ago1; a centromere-associated chromodomain protein, Chp1; and a protein named Tas3), which is associated with siRNA molecules that are homologous to repeat sequences found at sites of heterochromatin formation. RITS may attract CLR4 to methylate histone H3K9 on target DNA, and the nucleosomes bearing the H3K9 methylation lead to the recruitment of Swi6, the fission yeast homologue of mammalian HP1, resulting in stable heterochromatin formation (Verdel *et al*., 2004; Almeida and Allshire, 2005). However, the contribution of the RNAi pathway in mammalian and plant heterochromatin formation has not yet been established. In contrast to *S. pombe*, mutations in *AGO4* in *Arabidopsis* decrease DNA methylation and histone H3K9 methylation, but do not obstruct centromeric heterochromatin or centromere function (Zilberman *et al*., 2003). It suggests that the mechanism involved in heterochromatin formation in *Arabidopsis* might differ with *S. pombe*, although siRNA can direct chromatin modifications, including DNA methylation and histone methylation.

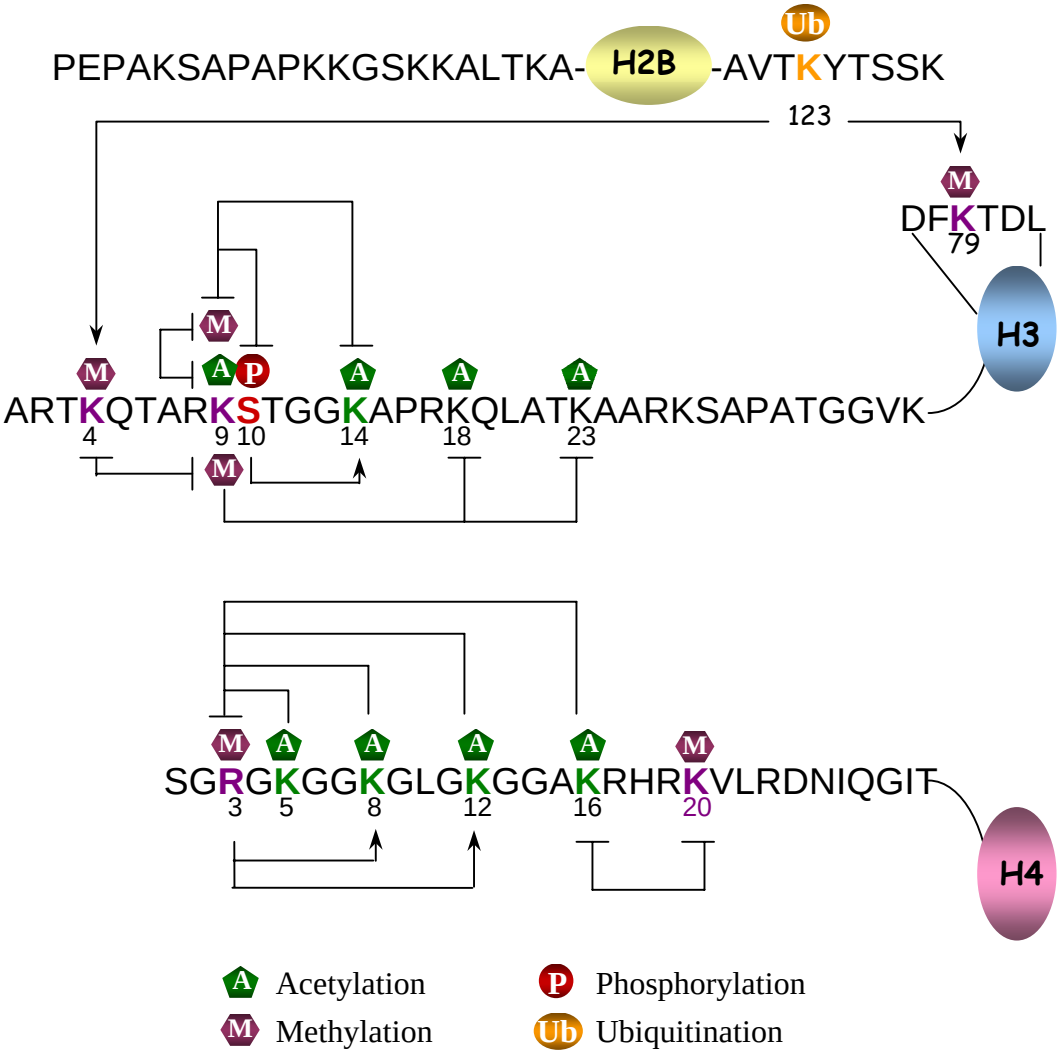


Figure II-9, Interplay between different posttranslational modifications occurring on histone H2B, H3 and H4 tails.

II-2-2-6 Cross-talk among chromatin modifiers

II-2-2-6-1 Interplay between different histone modifications

Increasing evidence indicates that the different modifications can affect each other (reviewed in Zhang and Reinberg, 2001; Fischle *et al*., 2003; Margueron *et al*., 2005, **Figure II-9**). However, while most of them are derived from the studies in yeast, *Drosophila* and animals, little is known in plants.

In yeast and mammals, phosphorylation of H3S10 could promote GCN5-mediated acetylation of H3K14 at certain promoters (Cheung *et al*., 2000; Lo *et al*., 2000). Inversely, acetylation of H3K14 had no effect on phosphorylation of H3S10 (Lo *et al*., 2000). Phosphorylation on histone H3S10 also plays an important role in regulating lysine methylation. It was reported that methylation of histone H3K9 by SUV39H1 is inhibited when an H3 tail peptide phosphorylated at S10 is used as a substrate. Similarly, methylation on H3K9 inhibits subsequent phosphorylation of H3S10 (Rea *et al*., 2000). In addition, H3K9 methylation can inhibit acetylation of the H3 tail (on K14, K18 and K23) by histone acetyltransferases (HATs) (e.g. p300), and methylation of H3 on K4 by HMTs (e.g. Set7) (Fischle *et al*., 2003; Margueron *et al*., 2005). This observation is not surprising since methylation of H3K9 correlates with gene silencing, whereas histone acetylation correlates with transcriptional activation. By contrast, H3K4 methylation inhibits H3K9 methylation by Su(var)3-9, but promotes acetylation of H3 by p300 (Fischle *et al*., 2003; Margueron *et al*., 2005).

Similar to histone H3, multiple modifications also exist on the histone H4 tail. Methylation on H4R3 by PRMT1 facilitates acetylation on histone H4K8 and H4K12 by p300, which is consistent with a role for the arginine methylation and acetylation in transcriptional control: both p300-mediated acetylation and PRMT1-mediated arginine methylation are found to stimulate transcription (Wang *et al*., 2001b). In contrast, acetylation on any of the four lysines on the histone H4 (K5, K8, K12 and K16) inhibits subsequent methylation on H4R3 by PRMT1 (Wang *et al*., 2001b). Moreover, H4K20 methylation and H4K16 acetylation are found to preclude each other (Nishioka *et al*., 2002b).

Furthermore, several groups have uncovered an interplay between ubiquitination of the H2B and methylation of H3 in yeast. Ubiquitination of histone H2B at K123 is required for efficient methylation of histone H3K4 by the SET1 and K79 by DOT1 (Dover *et al*., 2002;

Sun and Allis, 2002; Ng *et al*., 2002).

II-2-2-6-2 DNA methylation and Histone methylation

Histone methylation is a key process involved in maintaining patterns of DNA methylation. Recently, two alternative models are discussed. Either DNA methylation is triggered by histone methylation, or *vice versa* histone methylation is initiated after DNA methylation (Tariq *et al*., 2003).

In *Neurospora*, mutations in *DIM-5,* which is shown to encode a H3K9-specific methyltransferase, result in abnormal growth and complete loss of DNA methylation, suggesting that H3K9 methylation is required for DNA methylation (Tamaru and Selker, 2001; Tamaru *et al*., 2003). The same causal relationship is also reported in plants. The *Arabidopsis* KYP is specific for methylation of histone H3K9. Loss-of-function alleles of *kyp* results in reduced DNA methylation specifically at non-CG sites, as in *cmt3* mutants, suggesting that CMT3 activity occurs downstream of H3K9 methylation (Jackson *et al*., 2002). In addition, it has been shown that simultaneous methylation of H3 at K9 and K27 is recognized by CMT3, implying that methylation of H3K9 and H3K27 form a combinatorial histone mark required for non-CG methylation (Lindroth *et al*., 2004).

However, it was shown that the methylation level of H3K9 in heterochromatin is drastically reduced after removal of CG methylation in an *Arabidopsis met1* mutant lacking the DNA methyltransferase MET1 (Soppe *et al*., 2002; Tariq *et al*., 2003). These results seem to contradict previous results in *Neurospora crassa* and *Arabidopsis*, and suggest that DNA methylation at CG sites is required for the retention of H3K9 methylation at chromocenters. In contrast, H3K4 dimethylation and H3K27 trimethylation, which are both euchromatic marks in *Arabidopsis*, are enriched upon loss of CG methylation, independently of H3K9 methylation (Mathieu *et al*., 2005). However, unlike methylation of H3K9, mono- and dimethylation of H3K27, which are the heterochromatin marks in *Arabidopsis*, are not influenced by CG methylation of the DNA (Mathieu *et al*., 2005).

The discrepancies may be due to the functional differences of DNA methyltransferases involved and the distinct regulation of diverse histone modifications. CG methylation maintained by MET1 represses H3K27 trimethylation and H3K4 dimethylation, which mark active chromatin. Conversely, methylated CG stimulates H3K9 methylation, a hallmark of

silent chromatin, which in turn can induce non-CG methylation via CMT3. The induction of non-CG methylation might require the additional heterochromatin marks H3K27 mono- and dimethylation, which are not influenced by CG methylation and may be modulated by as yet unknown factors. Methylation of H3K9 and H3K27 thus form a combinatorial histone mark required for the recognition of CMT3, which are required for non-CG methylation.

II-2-2-6-3 Chromatin remodeling factors, DNA methylation and histone methylation

In a screens for *Arabidopsis* mutants impaired in DNA methylation and transgene silencing, the *ddm1* (decrease in DNA methylation 1) mutant was isolated. *ddm1* mutants show a global reduction of DNA methylation throughout cytosine residues that is independent of their sequence context, which leads to reactivation of transcriptionally silent transposable elements and silent transgenic loci (Jeddeloh *et al*., 1999; Singer *et al*., 2001). DDM1 protein is a member of the ISWI family and was shown to exhibit remodeling activity *in vitro* (Brzeski and Jerzmanowski, 2002). It has also been shown that DDM1 is required to maintain H3K9 methylation in heterochromatin (Gendrel *et al*., 2002). Therefore, a frequently postulated model assumes that DDM1 unwinds nucleosomal DNA to permit modification of DNA cytosine and histone H3K9. It also suggests that the ATP-dependent remodeling complex may participate indirectly in other chromatin remodeling pathways such as histone modifications and DNA methylation.

III

RESULTS

III-1 Functional characterization of the tobacco A-type cyclin, Nicta;CYCA3;2

The first part of my thesis focuses on functional characterization of the tobacco A-type cyclin, Nicta;CYCA3;2. This work had been started before my arrival in the laboratory. My contribution in Article 1 majorly concerns construction of the *GFP-Nicta;CYCA3;2* fusion gene, transformation of *Arabidopsis*, and molecular and cellular analysis of the transgenic *Arabidopsis* plants.

The Tobacco A-Type Cyclin, *Nicta;CYCA3;2*, at the Nexus of Cell Division and Differentiation

Yu Yu, Andre Steinmetz, Denise Meyer, Spencer Brown and Wen-Hui Shen

The Plant Cell, 2003, Vol. 15, Pages 2763–2777

Pages 2763 à 2777 :

III-2 Molecular and functional characterization of the tobacco SET domain protein NtSET1

The major part of my thesis focused on the molecular and functional characterization of the tobacco SET domain protein NtSET1. NtSET1 was previously identified and found to associate with chromatin (Shen, 2001). However, the histone methylation activity of NtSET1, as well as its specificity in chromatin binding, was not characterized. Thus my studies first focused on these two objects and the results obtained were described in Article 2.

III-3 Identification of *in vivo* DNA targets of NtSET1 and LHP1 in plants

Our previous results suggest that LHP1, the sole *Arabidopsis* homologue of animal HP1, functions analogously as HP1 and can be recruited to/by dimethylated H3K9 in heterochromatin in tobacco BY2 cells. Furthermore, the direct interaction between NtSET1 and LHP1 was demonstrated *in vitro*, suggesting a role for NtSET1 in heterochromatin function.

To gain better understanding of how NtSET1 and LHP1 function in chromatin structure and organization, I studied the targets of NtSET1 and LHP1 by chromatin immunoprecipitation (CHIP) and Dam identification (DamID) assays. The results obtained were described in the preliminary version in view of a publication.

(Manuscript in preparation)

Identification of *in vivo* DNA targets of NtSET1 and LHP1 in plants

Yu Yu, …, and Wen-Hui Shen

Abstract

Previous results suggested that LHP1, the sole *Arabidopsis* homologue of animal HP1, functions analogously as HP1 and can be recruited to/by dimethylated H3K9 in heterochromatin in tobacco BY2 cells. Furthermore, the direct interaction between NtSET1 and LHP1 was demonstrated *in vitro*, suggesting a role for NtSET1 in heterochromatin function. To gain better understanding of how NtSET1 and LHP1 function in chromatin structure and organization, we studied the direct target loci of NtSET1 and LHP1 in transgenic tobacco BY2 cells and in *Arabidopsis*. By chromatin immunoprecipitation (CHIP), we showed that both NtSET1-YFP and LHP1-YFP bind subtelomere-like sequences in tobacco, and that this association for NtSET1 depends on the C-terminal SET domain. Similarly, in *Arabidopsis* the enrichment of the 180bp centromeric repeat, the retrotransposon *Ta3* and the telomere sequences is specifically detected in the LHP1-YFP and to a lower extend in the NtSET1-YFP immunoprecipitated fractions, which was revealed by both CHIP and DamID assays, suggesting the involvement of NtSET1 and LHP1 in heterochromatin function. In addition to the heterochromatin regions, we also showed that *FT* is specifically enriched in the fraction by LHP1-YFP in *Arabidopsis*, suggesting a role for LHP1 in euchromatin gene silencing.

Introduction

In eukaryotes, there are two major types of chromatin: heterochromatin and euchromatin. The loosely packed euchromatin contains most of the actively expressed genes while the more condensed heterochromatin predominantly associates with transcriptionally inactive, gene-poor sequences. Heterochromatin can be either constitutive or facultative. The permanently condensed and largely inactive constitutive heterochromatin is concentrated at the centromere and pericentromere, and subtelomere regions. The facultative heterochromatin exists in different locations within chromosomes and could be transcriptionally silenced in one tissue type, cell lineage, or developmental stage while active in others. The regulation of

transcription through heterochromatin-mediated repression is important in multicellular eukaryotes and is under intensive investigation.

The mammalian Suv39h1 as well as its *Drosophila* homologue SU(VAR)3-9 specifically methylate the residue H3K9, creating a specific binding site for heterochromatin protein 1 (HP1) and resulting in heterochromatin formation (Lachner and Jenuwein, 2002). This mechanism is conserved in the fission yeast *S. pombe* where the CLR4 H3K9 methyltransferase and the HP1 homologue SWI6 play orthologous functions (Bannister *et al*., 2001; Nakayama *et al*., 2001). HP1 proteins are highly conserved through evolution from yeast to human, showing high sequence homology and similar domain organization, consisting of a conserved N-terminal chromo domain (CD), followed by a variable hinge region and a conserved C-terminal chromo-shadow domain (CSD). Although CD and CSD have very similar domain structures, they seem to serve distinct functions. CD is known to be the chromatin-binding domain. Lack of or mutations in this domain results in dislocalization of HP1 from heterochromatin (Cheutin *et al*., 2003).

In humans, the three members of the HP1 family (HP1α, HP1β, and HP1γ) display different patterns of subnuclear localization: HP1α and HP1β tend to be associated with condensed chromatin, whereas HP1γ is associated with euchromatic regions (Hiragami and Festenstein, 2005). *Drosophila* contains five genes encoding proteins with HP1-like domain organization. The *HP1A* gene encodes the prototype HP1 protein required for heterochromatin maintenance. The other four genes show different expression patterns. HP1B is found in both heterochromatin and euchromatin; HP1C is restricted to euchromatin; and HP1D/Rhino is localized within the heterochromatic domain in tissue culture cells, although its localization does not overlap with H3K9 methylation (Vermaak *et al*., 2005). *Arabidopsis* possesses a sole gene encoding the homologue of HP1, named LHP1, for Like-HP1 protein (Gaudin *et al*., 2001). The LHP1-GFP protein is localized in subnuclear foci and expression of the *LHP1* gene complemented yeast *swi6* mutants (Gaudin *et al*., 2001; Kotake *et al*., 2003). Also, it has been shown that LHP1 can bind *in vitro* to the methyl H3K9 (Jackson *et al*., 2002). Our previous data also showed that the LHP1-YFP fusion proteins co-localize with heterochromatic foci enriched of dimethylated H3K9 in tobacco BY2 cells, and that LHP1 binds to the H3K9 methyltransferase NtSET1 (Yu *et al*., 2004). More recently, Zemach *et al*. showed that LHP1-GFP colocalizes with the intensely DAPI-stained chromocenters in *Arabidopsis* (Zemach *et al*., 2006). Together, these data support that LHP1 plays similar

function as HP1 in animals.

However, *lhp1*/*tfl2* mutants, the mutants at the *LHP1* locus in *Arabidopsis,* show an early-flowering phenotype as well as several other pleiotropic phenotypes: a small plant size, terminal flowers, curled leaves and low fertility (Gaudin *et al*., 2001; Kotake *et al*., 2003). The early flowering phenotype of *tfl2* is caused mainly by ectopic expression of the *FLOWERING LOCUS T* (*FT*) gene, a floral pathway integrator that promotes flowering (Kotake *et al*., 2003). In addition to *FT,* the expression of some other floral homeotic genes is upregulated in *tfl2* mutants (Kotake *et al*., 2003; Nakahigash *et al*., 2005), whereas the expression of genes positioned within heterochromatin is not activated (Kotake *et al*., 2003; Nakahigash *et al*., 2005). In the *lhp1*/*tfl2* mutant, no effect could be detected on chromocenter organization, as revealed by DAPI staining and FISH with CEN180 (Libault *et al*., 2005). Thus it is supposed that LHP1 is involved in the repression of specific euchromatic genes but not heterochromatic genes in *Arabidopsis*.

To gain better understanding of how NtSET1 and LHP1 function in chromatin structure and organization, we studied the direct target loci of NtSET1 and LHP1 in transgenic tobacco and *Arabidopsis*. By chromatin immunoprecipitation (CHIP), we show that both NtSET1-YFP and LHP1-YFP bind subtelomere-like sequences in tobacco, and this association depends on the C-terminal SET domain. Similarly, in *Arabidopsis* the enrichment of the 180bp centromeric repeat, the retrotransposon *Ta3* and the telomere sequence is specifically detected in the LHP1-YFP and to a lower extend in the NtSET1-YFP immunoprecipitated fractions. We also show that FT is specifically enriched in the fraction by LHP1-YFP in *Arabidopsis*.

Results

NtSET1-YFP and LHP1-YFP bind subtelomere-like sequences in tobacco BY2 cells

Tobacco BY2 cells expressing *YFP*, *NtSET1-YFP* and *LHP1-YFP* (Yu *et al*., 2004) were used for chromatin immunoprecipitation with an anti-GFP antibody. To identify the *in vivo* DNA targets, differential hybridization was performed as shown in Figure 1. Immunoprecipitates from NtSET1-YFP and LHP1-YFP cells were amplified by linker-mediated PCR and cloned into pCR II-Topo vector respectively. The colonies from NtSET1-YFP and LHP1-YFP were transferred onto nitrocellulose membranes and screened by differential hybridization with probe pairs of YFP/NtSET1-YFP and YFP/LHP1-YFP respectively, which were also prepared

from chromatin immunoprecipitates. Specific clones which gave signals specifically in NtSET1-YFP and LHP1-YFP samples but not in the YFP control were selected and sequenced. Table 1 shows the specific target loci of NtSET1-YFP and LHP1-YFP revealed by differential hybridization. Five out of 14 clones for NtSET1-YFP and 16 out of 22 clones for LHP1-YFP showed about 90% identity with the tobacco genomic RETS (repeats containing telomeric stem) sequence, which contains tandem repeats of a 45-bp sequence found abundantly around the telomeres of tobacco chromosomes (Suzuki *et al*., 1994). To confirm the target loci revealed by differential hybridization, the enrichment of the RETS sequences was tested by PCR directly using ChIP-precipitated DNA fragments as the template. In addition, the (TTTAGGG)n telomere sequence was also analyzed since the RETS sequence was found abundantly at the ends of chromosomes. Figure 2 shows the enrichment of the telomere sequence and the RETS sequence in the NtSET1-YFP and in the LHP1-YFP immunoprecipitated fraction compared to the YFP control. It suggests that NtSET1-YFP and LHP1-YFP bind subtelomere-like sequences in tobacco BY2 cells.

Tobacco seedlings expressing *GFP*, *NtSET1-GFP* and its SET domain deletion mutant *NtSET1D1-GFP* (Shen and Meyer, 2004) were also used for chromatin immunoprecipitation with the anti-GFP antibody. Figure 3 shows the enrichment by PCR of the telomere and RETS sequences in the NtSET1-GFP-precipitated fraction. It suggests that the association of NtSET1-GFP with subtelomere-like sequences depends on the C-terminal region including the conserved SET domain, which functions to methylate histones H3K9.

NtSET1-YFP and LHP1-YFP bind centromeric, pericentromeric and telomere-like sequences in *Arabidopsis*

To identify the target loci of NtSET1 and LHP1 in *Arabidopsis*, the YFP fusion constructs pER8:NtSET1-YFP and pER8:LHP1-YFP were introduced into *Arabidopsis* plants by *Agrobacterium* through the flower-dip method. The fusion construct YFP alone was used as the control. YFP was found to localize in both the cytoplasm and the nucleus of *Arabidopsis* cells (data not shown), while NtSET1-YFP and LHP1-YFP localized exclusively in the nucleus (Figure 4a and 4b). In addition, in some cells LHP1-YFP was not uniformly distributed but showed speckles in the nucleus (Figure 4c and 4d). From more than 1000 cells examined, 7.2% of cells expressing LHP1-YFP showed nuclear speckle localization, which are detected primarily in cells within regions close to the hypocotyl-root junction, including

hypocotyl cells, the fully differentiated root cortex and hair cells.

CHIP analyses and differential hybridization were performed as previously described in tobacco and the results obtained are shown in Table 2. Ten out of 15 clones for NtSET1-YFP and 16 out of 18 clones for LHP1-YFP showed very high identity (about 95%) with an *Arabidopsis* DNA chromosome centromeric region. Sequence analysis showed that these sequences correspond to a 180 bp centromeric repeat, which is the primary centromeric sequence and found as tandem arrays spanning the core centromeres of all five chromosomes in *Arabidopsis* (Hall *et al*., 2004). The finding of a 180 bp centromeric repeat gives a hint that NtSET1 and LHP1 may associate with heterochromatin *in vivo*. Given that the constitutive heterochromatin is concentrated at pericentromere and subtelomere regions in addition to the centromeres, the sequences located at these regions may also be candidate target sites. To confirm the 180 bp centromeric repeats revealed by differential hybridization, the enrichment of this sequence was tested by PCR using the ChIP-precipitated DNA fragment as the template. In addition, several other silenced loci were selected for enrichment analysis: retrotransposon *Ta3* which is located in the pericentromeric region of chromosome 1; and the (TTTAGGG)n telomere sequence which is characteristic of telomere regions and was shown to be bound by NtSET1-YFP and LHP1-YFP in tobacco cells. PCR (Figure 5a) shows the enrichment of the 180 bp centromeric repeat, telomere sequence and *Ta3* specifically in the LHP1-YFP and to a lower amount in the NtSET1-YFP immunoprecipitated fraction compared to YFP control. The quantitative PCR (Figure 5b and 5c), showing the percentage of the selected sequences to be precipitated relative to the input, also suggested that LHP1 as well as NtSET1 bind centromeric and pericentromeric heterochromatin in *Arabidopsis*.

LHP1-YFP binds *FT* in *Arabidopsis*

Considering that LHP1/TFL2 was reported as a repressor of specific genes within euchromatin such as *FT*, the promoter region of *FT* was tested by PCR in CHIP fractions. As shown in Figure 6, the promoter region of *FT* is enriched specifically in the LHP1-YFP fraction. For comparison, no significant changes in the promoter region of *TSF* (*TWIN SISTER OF FT*) were detected after PCR amplification in LHP1-YFP precipitated fraction when compared with YFP and NtSET1-YFP. This result is expected since the transcription level of *TSF* is not affected in the *lhp1*/*tfl2* mutant, although *TSF* is the closest homolog of *FT* in *Arabidopsis* (Yamaguchi *et al*., 2005).

Dam-tagged LHP1-YFP binds both heterochromatin and euchromatin in *Arabidopsis*

An alternative approach used to identify target loci of chromatin proteins is the technique termed DamID, which had been successfully used in *Drosophila* (Greil *et al*., 2003). In brief: this technique involves *in vivo* expression of trace amounts of a chromatin protein of interest fused to *E. coli* DNA adenine methyltransferase (Dam). As a result, DNA around the target loci of the chromatin protein is preferentially methylated by the tethered Dam. Subsequently, the DNA fragments containing adenine methylation caused by Dam fusion proteins will be specifically cut out by methylation-sensitive enzyme and selectively amplified by a PCR-based method.

For this purpose, N-terminal Dam fused constructs with *GFP*, *NtSET1-GFP*, and *LHP1-YFP* were made under the control of the estradiol-inducible promoter in pER8 vector and were introduced into *Arabidopsis* plants. Figure 7a shows the RNA levels of the transgene expression of the plants used for adenine methylation analysis by RT-PCR. And the specific DNA fragments containing adenine methylation caused by Dam fusion proteins were selectively amplified (Figure 7b) by a linker-mediated PCR. Using these PCR products as the templates, a second PCR was performed. Consistent with the results in CHIP-based PCR analysis, retrotransposon *Ta3*, 180 bp *centromeric* repeat and the promoter region of *FT* were also specifically amplified in the Dam-LHP1-YFP fraction (Figure 7b), suggesting the direct binding of Dam-LHP1-YFP with these regions in *Arabidopsis*. In addition, 180 bp *centromeric* repeat was also amplified in the Dam-NtSET1-GFP fraction, though at a lower level than in Dam-LHP1-YFP fraction.

Discussion

Functions of NtSET1 and LHP1 in heterochromatin

In fission yeast and in animals, the H3K9 dimethylation by CLR4 and SUV39H1 HMTases creates a specific binding site recognized by SWI6 and HP1, playing an essential role in heterochromatin function (Bannister *et al*., 2001; Nakayama *et al*., 2001; Lachner and Jenuwein, 2002). Previous studies suggested that LHP1, the sole *Arabidopsis* homologue of animal HP1, functions analogously as HP1 and can be recruited to/by dimethylated H3K9 in heterochromatin (Jackson *et al*., 2002; Yu *et al*., 2004). Furthermore, NtSET1 binds LHP1 *in vitro*, suggesting a role for NtSET1 in heterochromatin function (Yu *et al*., 2004).

By CHIP analyses, we show that ectopically expressed NtSET1-YFP, as well as LHP1-YFP, can bind tandem repeats of telomere and RETS sequences in tobacco. We could not conclude that NtSET1 and LHP1 target to telomere as tandem arrays of telomere-similar sequence had been found, in addition to telomeric regions, in centromeric regions of the *Solanum* species (Tek and Jiang, 2004). Nevertheless, NtSET1 and LHP1 were shown to localize in heterochromatic regions, supporting a similar mechanism in tobacco as in yeast and animals in which the binding of the NtSET1 H3K9 HMTase to LHP1 is involved in heterochromatin formation.

In agreement with previous reports, the LHP1-YFP fusion protein is localized exclusively in the nucleus in *Arabidopsis* cells, and concentrated in nuclear speckles. Similar to those described in tobacco, in transgenic *Arabidopsis* ectopically expressed LHP1-YFP also binds directly to heterochromatin regions in chromatin immunoprecipitation and DamID assays. However, the role played by LHP1 in heterochromatin is not clear. In the *Arabidopsis lhp1*/*tfl2* mutant, no effect could be detected on chromocenter organization, as revealed by DAPI staining and FISH with CEN180 (Libault *et al*., 2005), and on the expression of genes within heterochromatin (Nakahigashi *et al*., 2005). Thus, it is possible that LHP1 functions in heterochromatin redundantly with other proteins and/or that LHP1 plays an as yet unknown role at these heterochromatin regions. As to NtSET1-YFP, although the fusion proteins showed uniformly distributed localization within the nucleus, and were not localized to specific regions, NtSET1-YFP can also bind to heterochromatin regions, albeit at a lower level than LHP1-YFP. It suggests that the ectopically expressed NtSET1-YFP might also be involved in heterochromatin function in *Arabidopsis*.

LHP1 acts as a repressor of *FT*

The mutants at the *LHP1* locus in *Arabidopsis* resulted in upregulated expression of the floral homeotic gene *FT*. Thus it is proposed that *Arabidopsis* LHP1 protein is a component of euchromatin repressing specific homeotic gene expression. By using chromatin immunoprecipitation and DamID, our results revealed that LHP1-YFP can bind directly the promoter region of *FT*, but not that of *TSF*, which was not upregulated in the *lhp1*/*tfl2* mutant. It suggests that LHP1 might repress specific gene expression by binding to the promoter of this gene. However, the possible mechanism for the repression remains unknown. It seems that LHP1 might be recruited to a specific site by interaction with other proteins, since

sequence-specific DNA binding of the HP1 family proteins is not known. In *Arabidopsis*, it was shown that dimethyl H3K9 is found abundant in heterochromatin, while trimethyl H3K9 is enriched in euchromatin regions (Jackson *et al*., 2004). The binding of HP1 to both di- and trimethyl H3K9 peptides (Bannister *et al*., 2001; Jacobs *et al*., 2002; Lachner *et al*., 2001) suggests that LHP1 might be recruited in euchromatin gene silencing by binding trimethyl H3K9. Recent studies show that the recruitment of HP1 to chromatin requires not only H3K9 methylation but also a direct protein-protein interaction between HP1 and HMTase such as SUV39H1 (Stewart *et al*., 2005). The tobacco H3K9 HMTase NtSET1 has been shown to bind LHP1 *in vitro* (Yu *et al*., 2004). Thus it is likely that the *Arabidopsis* NtSET1-like HMTases are the candidate partner proteins of LHP1 in *Arabidopsis*.

Dynamic function of LHP1 throughout plant development

Two main localization patterns of LHP1-YFP were observed in transgenic *Arabidopsis*: centromere-like speckle localization or uniform fluorescence distribution in nucleus, but no punctuated nucleus localization with small fluorescence foci as previously described (Libault *et al*., 2005; Zemach *et al*., 2006) was observed. This is probably because of the different expression systems used: the expression of LHP1-YFP in our experiments was under the control of an estradiol-inducible promoter, while in previous studies the expression of GFP-fused LHP1 was driven by a 35S promoter. Nevertheless, the two main localization patterns were observed in different cell types: the centromere-like speckle localization was detected primarily in the fully differentiated cells including older hypocotyl cells, the older root cells, and the older root hair cells; while the uniform distribution pattern was observed in the young cells such as the proliferating root cells in the region of the meristem, and the transition zone where cells start to differentiate, which was also described by Libault *et al*. (2005). It suggests that LHP1 may have different roles throughout plant development.

Materials and Methods

Plant Materials

Tobacco (*Nicotiana tabacum* cv SR1) and *Arabidopsis thaliana* (Columbia (Col-0) ecotype) plants were grown under 12h light/12h dark cycle at 25°C, and 16h light/8h dark cycle at 22°C respectively. Tobacco BY2 (*Nicotiana tabacum* L. cv Bright Yellow 2) cell suspension was maintained by weekly subculture as described (Nagata *et al*., 1992). Induction of

transgene expression from pER8-based vectors was performed according to Zuo *et al*. (2000), using 4 μM estradiol, while transgene expression from pTA-based vectors was induced as described previously (Shen, 2001), by the addition of dexamethasone (DEX) to a final concentration of 5 μM.

Microscopy

Cell imaging was performed using a confocal laser scanning microscope, Zeiss model LSM510 (Carl Zeiss, Jena, Germany), as previously described (Shen, 2001).

Chromatin immunoprecipitation

3-day-old TBY2 cells, 30-day-old tobacco seedlings and 20-day-old *Arabidopsis* seedlings were used for CHIP experiments as previously described (Johnson *et al*., 2002). Antibody against GFP (Molecular Probes, Catalogue no. A-11122) was used to detect GFP/YFP fusion proteins.

Differential hybridization

The chromatin-immunoprecipitated DNA fragments were blunted with T4 DNA polymerase (Fermentas), and ligated to a double stranded catch linker (top strand: 5'-GCGGTGACCCGGGAGATCTGAATTC-3', bottom strand: 5'-GAATTCAGATC -3'). The linker-ligated DNA fragments were then amplified by PCR using the oligo specific to the linker and ligated into pCR II-Topo vector (Invitrogen). The colonies were transferred to two the Protran BA 85 Nitrocellulose membranes (Schleicher & Schuell). Following lysis of the bacteria, the DNA was fixed to the membranes by UV crosslinking. The two replicated membranes were hybridized with two different ^{32}P-labeled probes (one of them serving as the control), which were prepared from the CHIP-precipitated fragments. Hybridization and probe labeling were performed as described (Shen, 2001). After hybridization and washing, membranes were autoradiographed using X-ray film. The signals from the two replicated membranes were compared, and the specific colonies that show signals with the desired probe but not with the control were selected and sequenced.

CHIP PCR

PCR was used to detect the enrichment of DNA immunoprecipitated in the ChIP experiments.

The primer pairs of *180-bp CEN* repeats and *Ta3* were as described (Johnson *et al*., 2002). Other primer pairs used were as follows: *Actin* (5'-AAGTCATAACCATCGGAGCTG-3' and 5'-ACCAGATAAGACAAGACACA C-3'); *FT1* (5'-TTCTCTATAAACTTGGCGGTAC-3' and 5'-TAACTCGGGTCGGTGAAATCA-3'); *FT2* (5'-CTACAGTTGTTAGGCTATGGTT A-3' and 5'-GCGGCCATATTATGGAAAAGTGA-3'); *TSF1* (5'-TGACGTACGCAAATAT GCAGCA-3' and 5'-CTTACCTTATGAACCCTTACTTGC-3'); *TSF2* (5'-CTGAACATAA CGTCCCACGTATG-3' and 5'-CCTAAAAGCTCAACCGTGTATTC-3'); *Telomere* repeats (5'-TTTAGGGTTTAGGGTTTAGGG-3' and 5'-TAAACCCTAAACCCTAAACCC-3'); tobacco *RETS* sequence (5'-TCAGTTTAGGGTTTAAAACC-3' and 5'-AGTCAGCATTAG GGTTTTAA-3'); tobacco *Actin* (5'-GTGCGACGTGGATATCAGAAAG-3' and 5'-CATCATATTCTGCCTTTGCAATCCAC-3'). The amount of immunoprecipitate used in each assay was determined empirically such that an equal amount of *Actin* was amplified.

Quantitative PCR was performed to determine the amounts of enrichment of DNA immunoprecipitated in the ChIP experiments using qPCR Mastermix plus for SYBR green I (Eurogentec). For each sample, two PCR were performed, and each PCR was in triplicate using fixed amounts of template DNA. PCR was carried out using the following conditions: 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles of 30 sec at 95ºC, 30 sec at 60ºC, and 30 sec at 72ºC. The threshold value was set such that the fluorescence signal was above the baseline noise but as low as possible in the exponential amplification phase. And the B/I ratios were determined by taking a fixed aliquot of the DNA extracted from the Input and Bound (immunoprecipitated) samples.

Dam fusion gene constructs and plant transformation

To construct the *Dam* fusion gene, the entire coding region of *E. coli Dam* gene was amplified by PCR from the vector pNDamMyc (van Steensel and Henikoff, 2000) using the primers 5'-cgctcgagcATGAAGAAAAATCGCGCTT-3' and 5'-cgctcgaggcCAGATCCTCTTCAGA GAT-3'. The PCR product was cloned into the pER8:GFP and pER8:NtSET1-GFP (Yu *et al*., 2004) vectors using XhoI restriction sites, resulting in the in-frame fusion of pER8:Dam-GFP and pER8:Dam-NtSET1-GFP. To obtain pER8:Dam-LHP1-YFP construct, the *Dam* gene was amplified using the primers 5'-cgctcgagcATGAAGAAAAATCGCGCTT-3' and 5'-cgctcgagCAGATCCTCTTCAGAGAT-3', and cloned into the pER8:LHP1-YFP vector (Yu *et al*., 2004). Transgenic *Arabidopsis* plants were obtained by *Agrobacterium tumefaciens*-

mediated transformation using the floral-dip method (Clough and Bent, 1998).

RT-PCR

Total RNA was prepared from *Arabidopsis* seedlings using the Trizol reagent kit (Invitrogen). RT-PCR was performed as described (Zhao *et al*., 2005), using the Improm-II Reverse Transcriptase (Promega). The primers used in RT-PCR were 5'-GCAACATACGGAAAACTTACCC-3' and 5'-CAAACTCAAGAAGGACCATG TGG-3' for *GFP* fused genes, and 5'-CGACGTAAACGGCCACAAGTTC-3' and 5'-GTTCTGCTGGTAGTGGTCGGC-3' for *YFP* fused gene. The same primer pair for *Actin* used in CHIP PCR was used as normalization.

Specific amplification of Dam methylated DNA

Genomic DNA was isolated from *Arabidopsis* seedlings according to the method described by Rogers and Bendich (1988). A PCR-based method that selectively amplifies adenine-methylated DNA fragments was performed as described (Greil *et al*., 2003). Isolated genomic DNA was digested with Dpn I first, which selectively cuts the GATC sequence where A is methylated. The exposed DNA ends were then ligated to the catch linker which was used in differential hybridization. Subsequently, DNA fragments with one or more non-methylated GATC sites were destroyed by Dpn II digestion, which only recognizes unmethylated GATC. Finally, PCR was performed to amplify the linker-ligated fragment using the primer specific to the linker plus TC at its 3' end (5'-TGACCCGGGAGATCTGAATTCTC-3'). The second PCR was performed using the primers for *Ta3*, *180-bp CEN* repeats, *FT1*, *FT2*, *TSF1*, and *TSF2*, which were previously used for CHIP PCR.

References

Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, and Kouzarides T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* 410, 120-124.

Cheutin, T., McNairn. A.J., Jenuwein, T., Gilbert, D.M., Singh, P.B., and Misteli, T. (2003) Maintenance of stable heterochromatin domains by dynamic HP1 binding. *Science* 299, 721-725.

Clough, S.J., and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735-743.

Eissenberg, J.C., and Elgin, S.C. (2000) The HP1 protein family: getting a grip on chromatin. *Curr. Opin. Genet. Dev.* 10, 204-210.

Gaudin, V., Libault, M., Pouteau, S., Juul, T., Zhao, G., Lefebvre, D., and Grandjean, O. (2001) Mutations in *LIKE HETEROCHROMATIN PROTEIN 1* affect flowering time and plant architecture in *Arabidopsis*. *Development* 128, 4847-4858.

Greil, F., van der Kraan, I., Delrow, J., Smothers, J.F., de Wit, E., Bussemaker, H.J., van Driel, R., Henikoff, S., and Van Steensel, B. (2003) Distinct HP1 and Su(var)3-9 complexes bind to sets of developmentally coexpressed genes depending on chromosomal location. *Genes Dev.* 17, 2825-2838.

Hall, A.E., Keith, K.C., Hall, S.E., Copenhaver, G.P., and Preuss, D. (2004) The rapidly evolving field of plant centromeres. *Curr. Opin. Plant Biol.* 7, 108-114.

Hiragami, K., and Festenstein, R. (2005) Heterochromatin protein 1: a pervasive controlling influence. *Cell Mol. Life Sci.* 62, 2711-2726.

Jackson, J.P., Johnson, L., Jasencakova, Z., Zhang, X., PerezBurgos, L., Singh, P.B., Cheng, X., Schubert, I., Jenuwein, T., and Jacobsen, S.E. (2004) Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. *Chromosoma* 112, 308-315.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* 416, 556-560.

Jacobs, S.A, and Khorasanizadeh, S. (2002) Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science* 295, 2080-2083.

Johnson, L.M., Cao, X., and Jacobsen, S.E. (2002) Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. *Curr. Biol.* 12, 1360-1367.

Kotake, T., Takada, S., Nakahigashi, K., Ohto, M., and Goto, K. (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, 555-564

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410, 116-120.

Libault, M., Tessadori, F., Germann, S., Snijder, B., Fransz, P., and Gaudin, V. (2005) The *Arabidopsis* LHP1 protein is a component of euchromatin. *Planta* 222, 910-925.

Nagata, T., Nemoto, Y., and Hasezawa, S. (1992) Tobacco BY-2 cell line as the "Hela" cell in the cell biology of higher plants. *Int. Rev. Cytol.* 132, 1-30.

Nakahigashi, K., Jasencakova, Z., Schubert, I., and Goto, K. (2005) The *Arabidopsis* HETEROCHROMATIN PROTEIN1 homolog (TERMINAL FLOWER 2) silences genes within euchromatic region but not genes positioned in heterochromatin. *Plant Cell Physiol.*

46, 1747-1756.

Nakayama, J.-I, Rice, J.C., Strahl, B.D., Allis, C.D., and Grewal, S.I.S. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 292, 110-113.

Nielsen, S.J., Schneider, R., Bauer, U.M., Bannister, A.J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R.E., and Kouzarides, T. (2001) Rb targets histone H3 methylation and HP1 to promoters. *Nature* 412, 561-565.

Rogers, S.O., and Bendich, A.J, (1988) Extraction of DNA from plant tissues. *Plant molecular biology manual*. A6, 1-10.

Ryan, R.F., Schultz, D.C., Ayyanathan, K., Singh, P.B., Friedman, J.R., Fredericks, W.J., and Rauscher, F.J., 3rd. (1999) KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol. Cell Biol.* 19, 4366-4378.

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, 371-383.

Stewart, M.D., Li, J., and Wong, J. (2005) Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol. Cell Biol.* 25, 2525-2538.

Suzuki, K., Yamagiwa, Y., Matsui, T., and Yoshida, K. (1994) Restriction enzyme-resistant high molecular weight telomeric DNA fragments in tobacco. *DNA Res.* 1, 129-138.

van Steensel, B., and Henikoff, S. (2000) Identification of *in vivo* DNA targets of chromatin proteins using tethered dam methyltransferase. *Nat. Biotechnol.* 18, 424-428.

Vermaak, D., Henikoff, S., and Malik, H.S. (2005) Positive selection drives the evolution of rhino, a member of the heterochromatin protein 1 family in *Drosophila*. *PLoS Genet.* 1, 96-108.

Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M., and Araki, T. (2005) TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. *Plant Cell Physiol.* 46, 1175-1189.

Yu, Y., Dong, A., and Shen, W.-H. (2004) Molecular characterization of the tobacco SET domain protein NtSET1 unravels its role in histone methylation, chromatin binding, and segregation. *Plant J.* 40, 699-711.

Zemach, A., Li, Y., Ben-Meir, H., Oliva, M., Mosquna, A., Kiss, V., Avivi, Y., Ohad, N., and Grafi, G. (2006) Different domains control the localization and mobility of LIKE HETEROCHROMATIN PROTEIN1 in *Arabidopsis* nuclei. *Plant Cell* 18, 133-145.

Zhao, Z., Yu, Y., Meyer, D., Wu, C., and Shen, W.-H. (2005) Prevention of early flowering by expression of *FLOWERING LOCUS C* requires methylation of histone H3 K36. *Nat. Cell Biol.* 7, 1156-1160.

Zuo, J., Niu, Q.W., and Chua, N.-H. (2000) Technical advance: an estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* 24, 265-273.

Table 1. Specific target loci of NtSET1-YFP and LHP1-YFP in tobacco BY2 cells revealed by CHIP and differential hybridization

Protein of interest	Target	Frequency
NtSET1-YFP	RETS (repeats containing telomeric stem)	5/14
	BamHI repeat element	1/14
	retrotransposon Tto1	1/14
	short interspersed repetitive element	1/14
	not found in the database	6/14
LHP1-YFP	RETS (repeats containing telomeric stem)	16/22
	not found in the database	6/22

Table 2. Specific target loci of NtSET1-YFP and LHP1-YFP in *Arabidopsis* revealed by CHIP and differential hybridization

Protein of interest	Target	Frequency
NtSET1-YFP	chromosome centromere region	10/15
	At3g01990 genomic sequence	1/15
	intergenic sequence between At2g39860 and At2g39870	1/15
	At3g14830 genomic sequence	1/15
	At3g51880 genomic sequence	1/15
	not found in database	1/15
LHP1-YFP	chromosome centromere region	16/18
	T-DNA flanking sequence, AJ834625.1	1/18
	At5g09890 genomic sequence	1/18

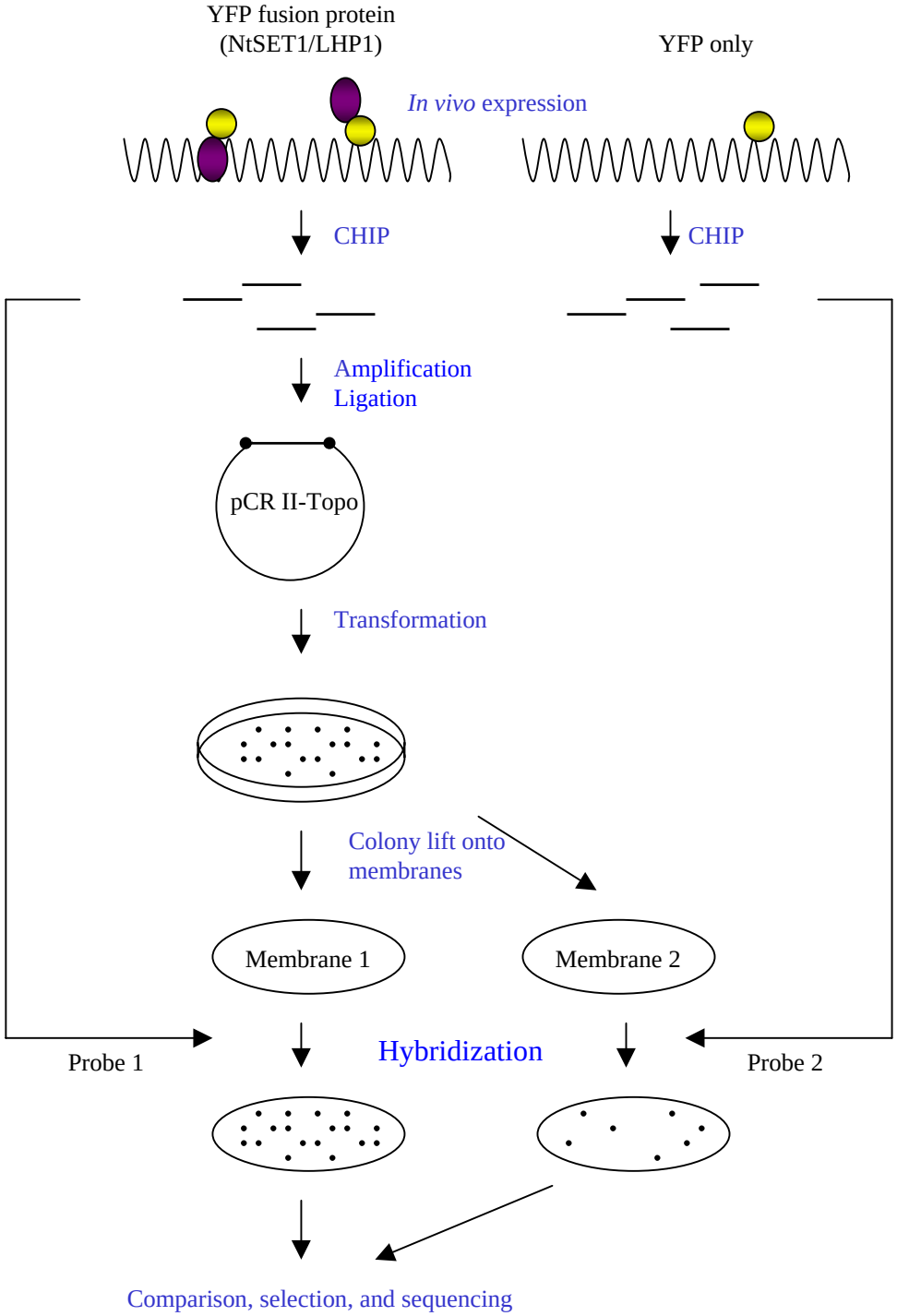


Figure 1 Experimental procedure for differential hybridization.
To identify the *in vivo* DNA targets, immunoprecipitates from cells expressing YFP fusion proteins were amplified and cloned into pCR II-Topo vector. The colonies were transferred onto nitrocellulose membranes and screened by differential hybridization with probe pairs of YFP fusion and YFP respectively, which were also prepared from chromatin immunoprecipitates. Specific clones which gave signals specifically in YFP fusion fractions but not in YFP controls were selected and sequenced.

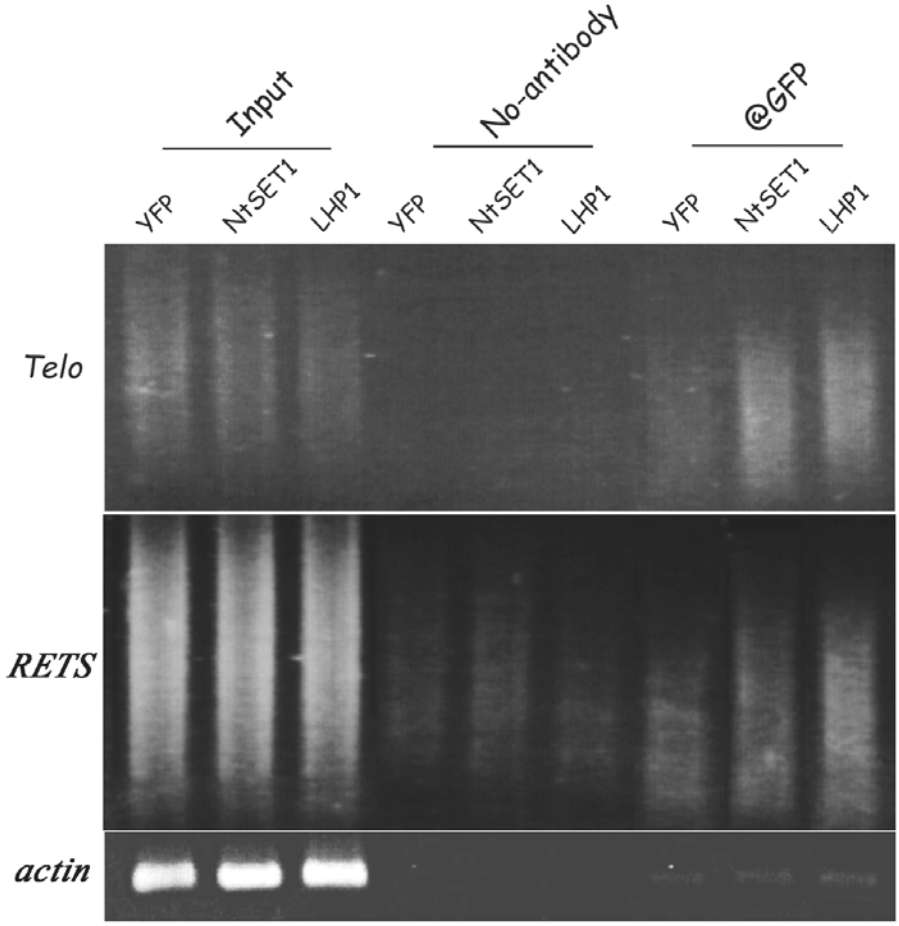


Figure 2 CHIP analysis shows the enrichment of telomere sequences and *RETS* sequences in transgenic tobacco BY2 cells expressing NtSET1-YFP and LHP-YFP. *Actin* serves as internal control.

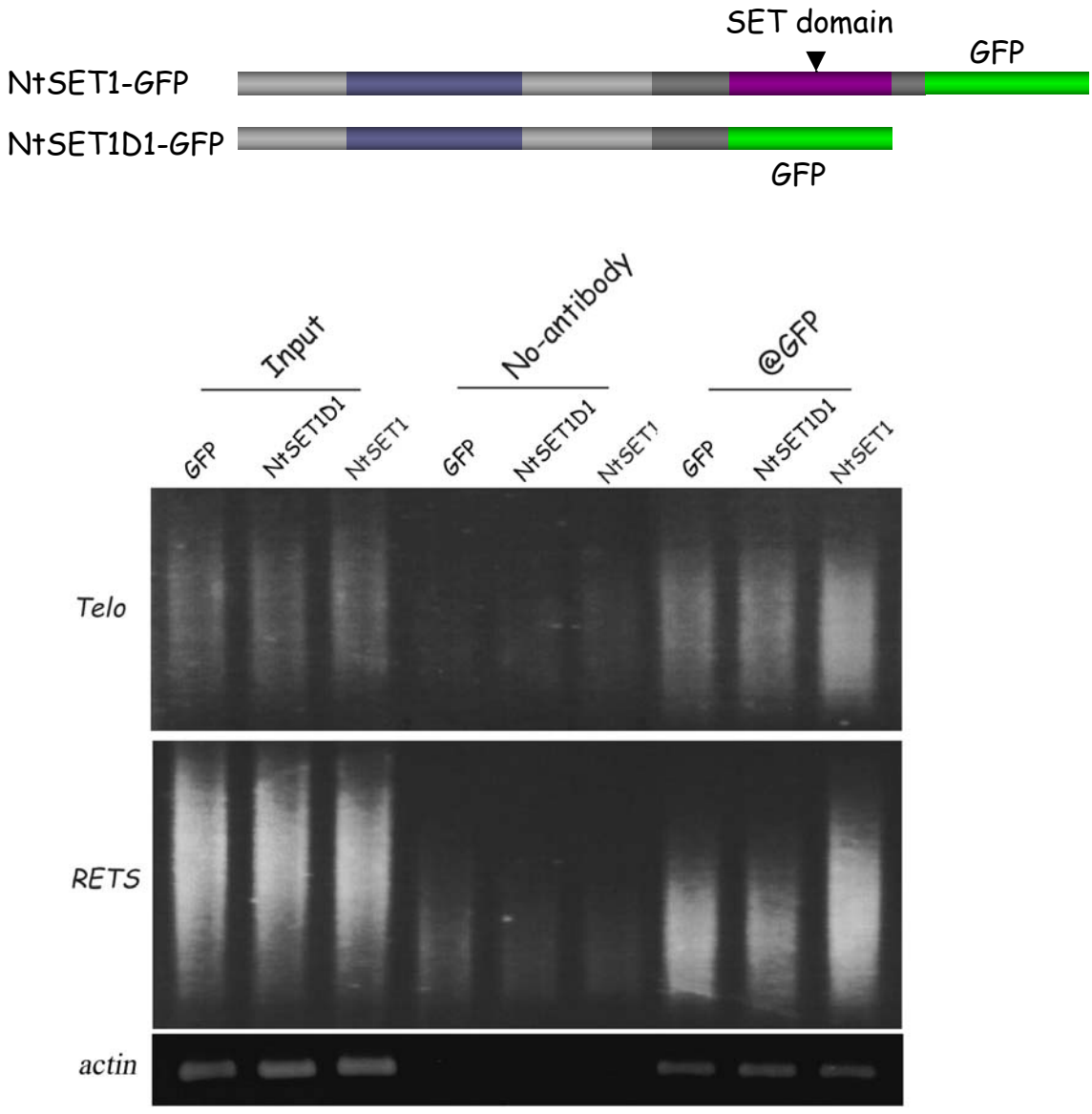


Figure 3. CHIP analysis shows the enrichment of telomere sequences and *RETS* sequences in transgenic tobacco plant expressing NtSET1-GFP. *Actin* serves as internal control.

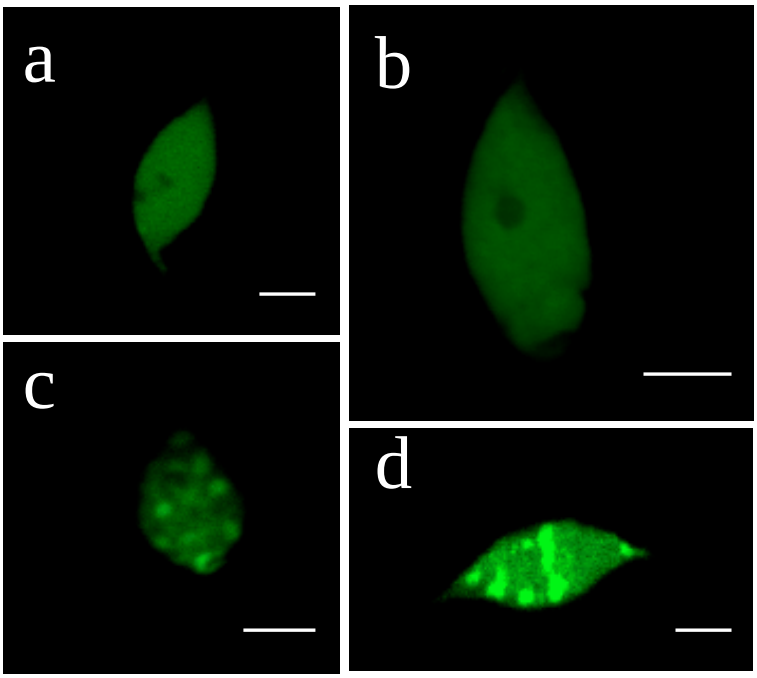


Figure 4 Subcellular localization of NtSET1-YFP and LHP1-YFP in transgenic *Arabidopsis*. a, root hair cell expressing *NtSET1-YFP*; b, root cortex cell expressing *LHP1-YFP*; c, root hair cell expressing *LHP1-YFP*; and d, root cortex cell expressing *LHP1-YFP*; Scale bar: 5 μm.

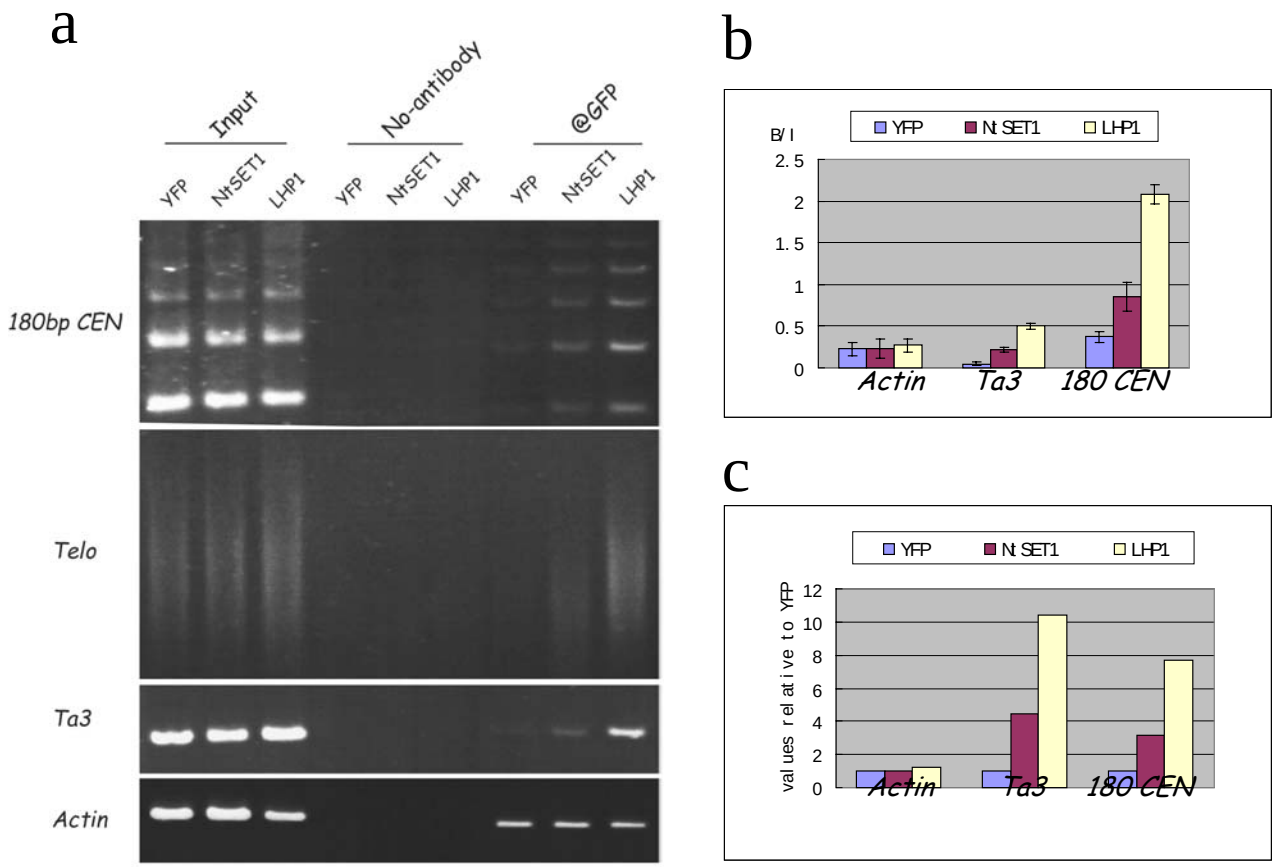


Figure 5 CHIP analysis shows the enrichment of the heterochromatic sequences in transgenic LHP1-YFP and to a lower amount in NtSET1-YFP in transgenic *Arabidopsis* by PCR (a) and quantitative PCR (b and c). The B/I values in (b) are indicated as percentage precipitation relative to the input. The values in (c) are indicated as the relative value by referencing YFP=1.

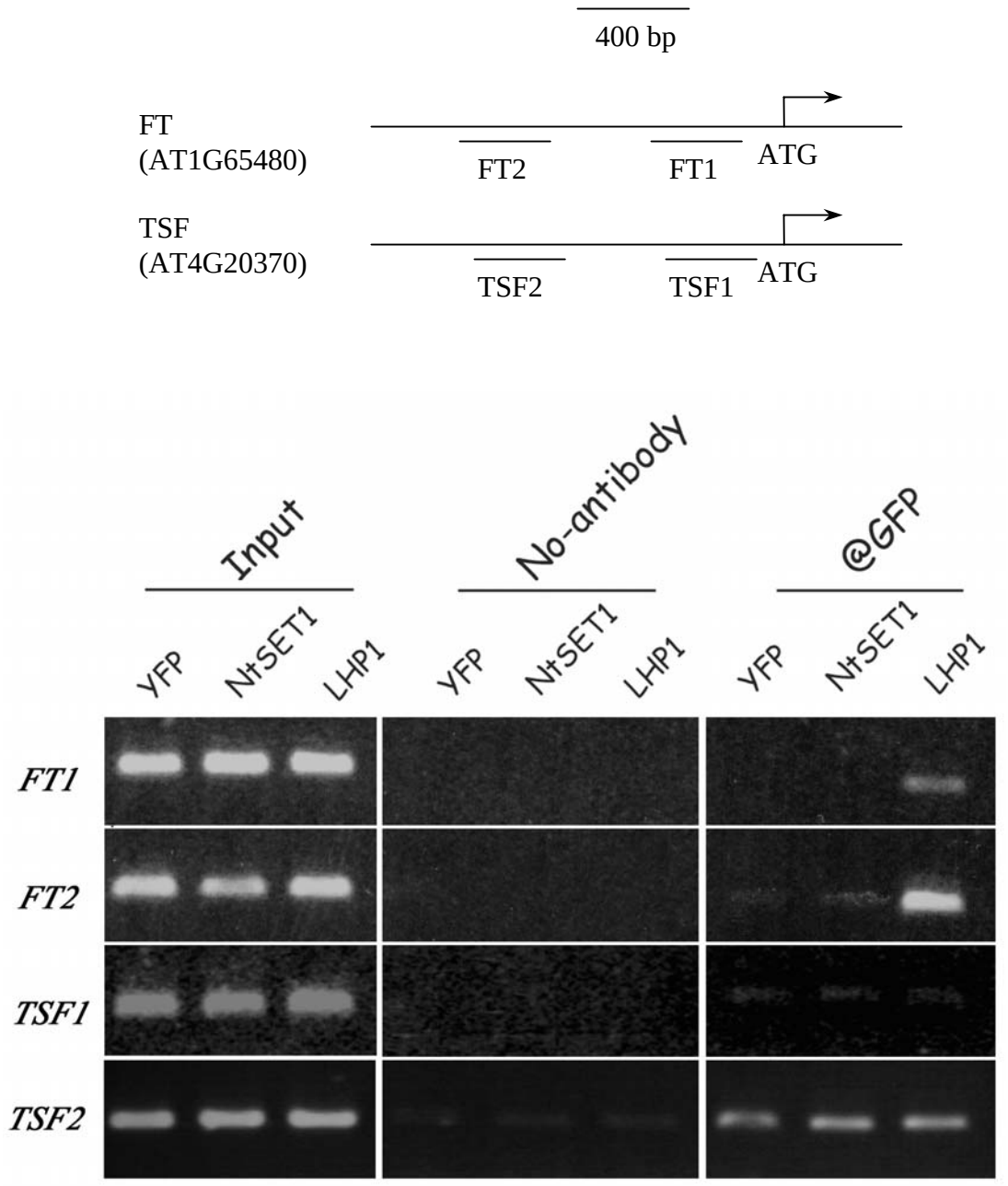


Figure 6 CHIP analysis shows the promoter region of *FT* but not that of TSF is enriched specifically in the LHP1-YFP precipitated fraction.

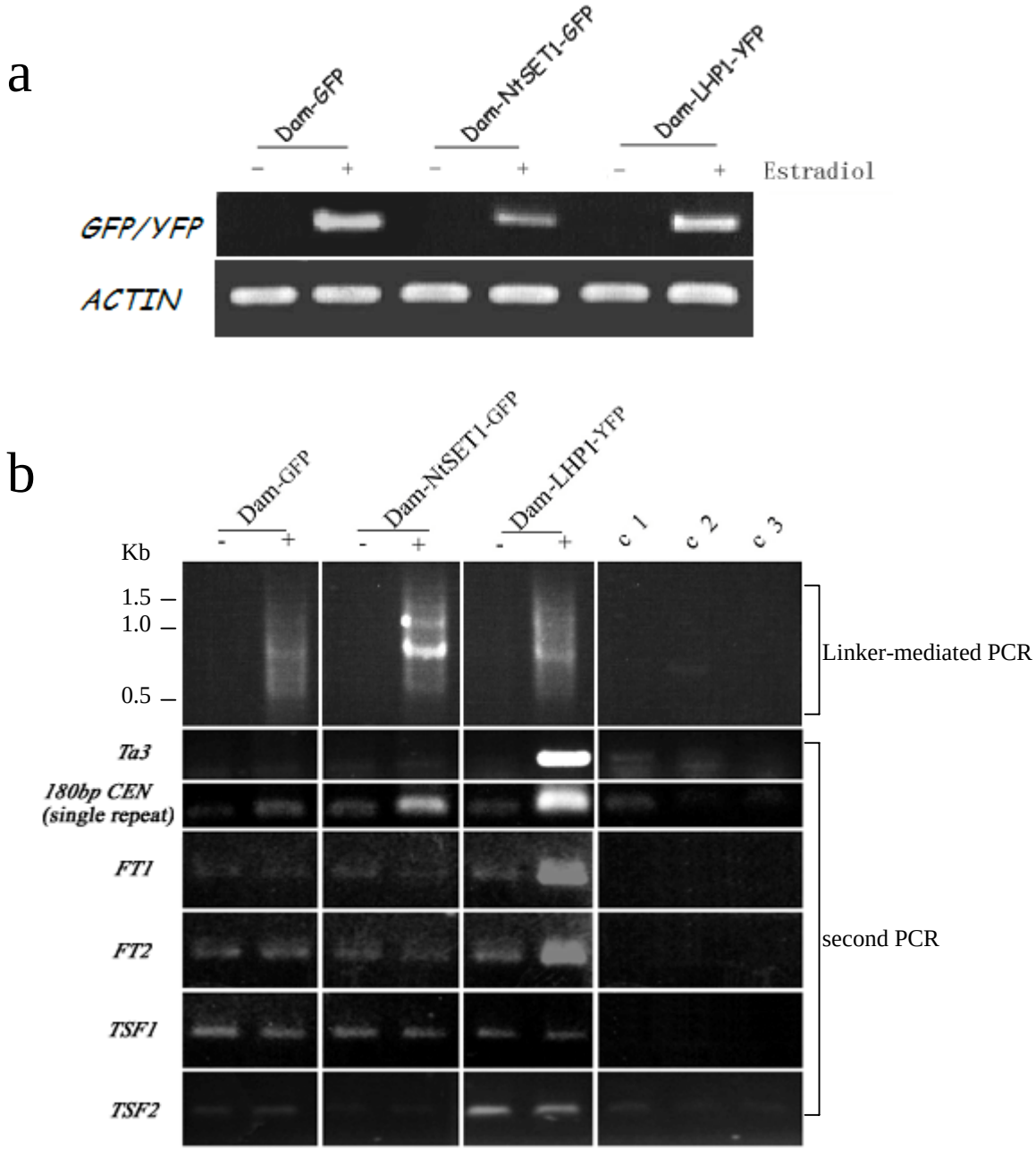


Figure 7 *In vivo* target analysis by DamID in transgenic *Arabidopsis* expressing Dam fusion proteins. (a) RT-PCR shows the RNA level of the transgene expression of the plant used for adenine methylation analysis. (b) The fragments containing adenine methylation caused by Dam fusion proteins were selectively amplified by a link-mediated PCR. Using the link-mediated PCR products as the templates, a second PCR was performed, showing *Ta3*, 180 bp *CEN* and the promoter region of *FT* were specifically amplified in Dam-LHP1-YFP(+) fraction. c1: Wt plant; c2: Dam-LHP1-YFP(+), DpnI digestion omitted; c3: Dam-LHP1-YFP(+), linker ligation omitted.

III-4 Analysis of the *Arabidopsis* mutants of *SDG8* (*SET DOMAIN GROUP8*) gene

Parallel to the studies of NtSET1, I also participated in the work on the characterization of *SET DOMAIN GROUP8* (*SDG8*) gene in *Arabidopsis*. My contribution in Article 3 majorly concerns western analysis of histone H3 methylations and CHIP analysis of histone methylations at *FLC* (*Flowering Locus C*) locus in wild-type and *sdg8* mutant plants.

IV

GENERAL DISCUSSION AND PERSPECTIVES

IV-1 Cell division, cell differentiation and plant development

IV-1-1 Role of cyclins in plant cell division and differentiation

Cell division plays a vital role in plant growth and development. Obviously, division is essential in generating the cells that constitute tissues and organs. Moreover, decisions leading to cell differentiation may be made during the cell division cycle. Cyclins, as the key regulatory proteins to regulate CDK activities, are thought to be essential for cell division, cell differentiation, and ultimately plant development.

Plants encode a complex set of cyclins, e.g. more than 40 in *Arabidopsis* (Wang *et al.*, 2004a). The expression of *Antma;CYCD;1* from *Antirrhinum majus* leads to the early activation of CDKA activity and accelerates cell cycle progression in tobacco BY2 cells (Koroleva *et al*., 2004). Overexpression of *Arabidopsis Arath;CYCD2;1* increases growth rate by shortening G1 in meristematic areas (Cockcroft *et al*., 2000). Overexpression of *Arath;CYCD3;1* induces ectopic divisions, producing leaves with more but smaller cells, and results in differentiation defects in leaf tissues (Dewitte *et al*., 2003). A distinct type of cyclin D, CYCD4;2, is also involved in the activation of cell division in *Arabidopsis* (Kono *et al*., 2006). Using both mutant and overexpression analysis, D-type cyclins were shown to activate cell division in the root meristem to promote seed germination in *Arabidopsis* (Masubelele *et al*., 2005). Although these CYCDs are all shown to activate cell division and promote cell cycle progression, the mechanisms involved seem to be distinct. For instance, coupled with ectopic divisions, overexpression of *Arath;CYCD3;1* induces hyperplasia with more but smaller cells in leaves. This effect is also observed in trichomes, which become multicellular when *CYCD3;1* is upregulated (Walker *et al.,* 2000). Distinct from *Arath;CYCD3;1*, overexpression of *GFP-Nicta;CYCA3;2* did not induce hyperplasia. Our results show that ectopic overproduction of GFP-Nicta;CYCA3;2 impairs shoot as well as root regeneration from transgenic *Arabidopsis* calli. In addition to the overexpression experiments, we found that antisense expression of *Nicta;CYCA3;2* inhibits callus formation and shoot regeneration from transgenic tobacco leaf explants (Yu *et al*., 2003). The defects in organogenesis indicated the requirement of Nicta;CYCA3;2 in cell division and differentiation. Nevertheless, antisense expression of *Medsa;CYCA2;2* did not interfere with callus and somatic embryo formation from transgenic alfalfa leaf explants, whereas subsequent shoot and root development was inhibited; when Medsa;CYCA2;2 is overexpressed no effect is detected on plant development

(Roudier *et al*., 2003). Thus, it is likely that different cyclins play distinct roles in cell division and differentiation.

To gain better understanding the regulatory mechanisms of plant cell division and differentiation, further characterizations of cyclins and other cell cycle genes are required. This will offer a unique opportunity to address the mechanisms that control these processes in the context of plants.

IV-1-2 Endoreplication and plant development

Endoreplication is a different cell cycle mode in which cells undergo iterative DNA replications without any subsequent mitosis and cytokinesis. The presence of endoreplication has been reported in many eukaryotes, and it is more frequent in plants in which endoreplication is activated in response to developmental and environmental cues that control a variety of processes, including cell differentiation, cell expansion, and metabolism. Endoreplicated cells do not reenter the mitotic cell cycle, and is thus characteristic of a terminal differentiation phase.

However, how and why cells are triggered to undergo endoreplication in plants is poorly understood. Endoreplication might require the absence of mitotic cyclin/CDK activity and the oscillation of S-phase cyclin/CDK activity, in conjunction with various factors that regulate the cyclin/CDK activity (Joubès and Chevalier, 2000; Larkins *et al*., 2001). For instance, overexpression of *E2Fa-DPa* activates the expression of S-phase genes and induces endoreplication in *Arabidopsis* and tobacco (De Veylder *et al*., 2002). *Arabidopsis* plants overexpressing *Arath;CYCD3;1*, an activator of the E2F-Rb pathway, exhibited a reduced level of endoreplication (Dewitte *et al*., 2003). Our results from transgenic *Arabidopsis* demonstrate that the ectopic overexpression of *GFP-Nicta;CYCA3;2* results in a dramatically decreased level of endoreplication in leaves, indicating an inhibition of cell differentiation (Yu *et al*., 2003). This reduces cell, organ and plant size and modifies the morphology of the plant, revealing an important function of endoreplication. Another A-type cyclin Arath;CYCA2;3 has also been reported very recently to negatively regulate endocycles and act as a key regulator of ploidy levels in *Arabidopsis* endoreplication (Imai *et al*., 2006).

However, the role of G1/S regulators in endoreplication is not completely understood. It was shown that overexpression of *E2Fa-DPa* induces endoreplication while overexpression of D-

type cyclin and A-type cyclins reduce it. Nevertheless, the effects of endoreplication, either decreasing or increasing, lead in both cases to a profound perturbation in plant development (De Veylder *et al*., 2002; Dewitte *et al*., 2003). Further analysis will be required to add our understanding of the mechanism of endoreplication in plant development.

IV-1-3 Role of chromatin modifiers in plant cell division and proliferation

In mammals, various chromatin modifiers play an important role in controlling G1-S progression by regulating the E2F-Rb pathway, including histone deacetylases (HDACs), ATP-dependent remodeling complex SWI/SNF, and the human H3K9 HMTase SUV39H1 (reviewed in Shen, 2002). The E2F-Rb complex is shown to recruit the SUV39H1 and the sequential HP1 to E2F-responsive promoters to repress transcription (Nielsen *et al*., 2001; Vandel *et al*., 2001). On the contrary, NtSET1, the H3K9 HMTase from tobacco, did not bind the Rb protein in the yeast two-hybrid assay (Yu *et al*., 2004). Furthermore, the expression of *NtSET1-GFP* does not modify the expression level of tobacco *RNR2* (*ribonucleotide reductase 2*) (Yu *et al*., 2004), which is regulated by E2F-Rb pathway and is necessary to initiate DNA replication (Chabouté *et al*., 2000). These results suggest that NtSET1 may not be directly involved in the plant E2F-Rb-mediated cell cycle gene expression.

Using the plant protoplast regeneration system, the biochemical and molecular basis of cellular dedifferentiation was studied (Williams *et al*., 2003). It was shown that the acquisition of competence for fate switch to reenter the cell cycle correlated with chromatin reorganization, including the redistribution of HP1 protein and H3 modifications. The human HP1γ fused to GFP was strictly localized in the nucleus when expressed in tobacco cells, whereas in dedifferentiated protoplasts a fraction of GFP-HP1γ was released into the cytoplasm (Williams *et al*., 2003). For the *Arabidopsis* homologue LHP1, the cell cycle-dependent distribution was also demonstrated (Libault *et al*., 2005). During prophase LHP1-GFP fluorescence foci disappeared progressively and dropped to being hardly visible on metaphase chromosomes; progressively, the fluorescent foci reappeared concomitant with the segregation of the chromosomes and their decondensation. The temporal and dynamic redistribution of LHP1 during mitosis suggests a potential role for LHP1 during plant cell division.

In dedifferentiated protoplasts, the release of GFP-HP1γ from chromatin was associated with modifications of H3K9 and K14 acetylation and H3K9 methylation (Williams *et al*., 2003),

suggesting that additional chromatin modifiers are involved in the acquisition of competence for fate switch to reentry into cell cycle. So far the role of histone methyltransferases in plant cell division is uncovered. However, although no significant effects of NtSET1-GFP expression are observed on tobacco BY2 cell cycle progression, 1–2% of cells expressing *NtSET1-GFP* exhibit chromosomal segregation defects resulting in poly-/micronuclei (Yu *et al*., 2004). Consistent with the results obtained in tobacco BY2, abnormal nuclear divisions are also observed in about 1% of cells within the tobacco leaf (Shen and Meyer, 2004), indicating that ectopic expression of *NtSET1-GFP* interferes with chromatin structure which results in chromosome segregation defects.

Our results show that the loss of function mutants in *SDG8* gene, which is allelic to *EARLY FLOWERING IN SHORT DAYS* (*EFS*), cause an early flowering phenotype. In addition, *sdg8*/*efs* mutants show a dramatically reduced plant size (Zhao *et al*., 2005; Kim *et al*., 2005) and other pleiotropic phenotypes (unpublished data in the lab). Observation of the cells of mature rosette leaves and petals showed that the cell size was the same in wild-type and *sdg8* mutant (unpublished data in the lab), indicating that the smaller plant size of the mutant is due to fewer total cells rather than a normal number of smaller cells. Therefore, it appears that SDG8 is required for maintaining the cell proliferation.

However, the relation between chromatin modifications and cell cycle division and cell proliferation, particularly in plants, is presently unclear. Further studies unraveling plant-specific aspects could help to better understand the regulation of cell expansion, division and proliferation.

IV-1-4 Role of chromatin modifiers in plant cell differentiation and development

Compared with animals, epigenetic switches controlled by chromatin modifiers occur not only during embryogenesis but also during postembryonic developmental transitions such as organogenesis and floral transition, which occur at a relatively late stage of development in plants (Guyomarc'h *et al*., 2005).

Most plant organs are formed during the post-embryonic stages from meristems. SAM and RAM are organized pools of undifferentiated cells maintained by a dynamic balance between cell division and cell differentiation. Mutations in chromatin factors have been found to lead to dysfunctions of the SAM and RAM. *Arabidopsis* mutants in *fasciata1* (*fas1*) and *fasciata2*

(*fas2*), which encode two of the three subunits of the *Arabidopsis* chromatin assembly factor 1 (CAF-1) complex, cause disorganization of both SAM and RAM and display aberrant shoot development and deficient root growth (Kaya *et al*., 2001). AtBRM, the *Arabidopsis* homologue of SWI2/SNF2 ATPase, is preferentially expressed in meristems, organ primordia and tissues with active cell division (Farrona *et al*., 2004). Underexpression of AtBRM or SYD, another *Arabidopsis* SWI2/SNF2 ATPase, leads to alterations in shoot development, including disruption of SAM maintenance, reduced size of organs and twisted leaves (Farrona *et al*., 2004; Wagner and Meyerowitz, 2002).

In addition, chromatin modifiers have been shown to participate in the regulation of the transition from the vegetative to the reproductive development (floral transition). During the last few years, an increasing number of developmental mutants with defects in floral transition have been identified in *Arabidopsis*, in which genes encoding chromatin modifiers are affected. Mutations in the SWI/SNF homologues such as *SPY* and *PIE1*, or in SET domain-encoding genes such as *CLF*, cause alterations in the flowering time. Furthermore, *lhp1*/*tfl2* mutants, the mutants in the *Arabidopsis LHP1*, show an early-flowering phenotype as well as several other pleiotropic phenotypes: a small plant size, terminal flowers, curled leaves and low fertility (Gaudin *et al*., 2001; Kotake *et al*., 2003). The early flowering phenotype of *lhp1*/*tfl2* is caused mainly by ectopic expression of the *FT* gene, a floral pathway integrator that promote flowering (Kotake *et al*., 2003). The *sdg8*/*efs* mutants also cause an early flowering phenotype due to, primarily, a reduction in the expression of *FLC* (Zhao *et al*., 2005; kim *et al*., 2005). In addition, *SDG8* was shown to be involved not only in flowering time control but also in vegetative growth and in flower differentiation.

Taken together, these genetic and molecular data suggest that the defects during *Arabidopsis* development are affected by factors that modify chromatin structure. It is therefore tempting to speculate that plants have evolved chromatin-based activation and repression mechanisms to finely control plant development. Plants will also help considerably in gaining a better understanding of the mechanism of chromatin-mediated developmental processes. Mutations in many chromatin modifiers result in lethality in animals, but are viable in plants, reflecting the advantage of using plants, particularly *Arabidopsis*, as a model system.

IV-2 H3K9 methylation and establishment of silenced chromatin

IV-2-1 Heterochromatin formation

Constitutive heterochromatin is stably inherited and thus must contain one or more epigenetic marks to direct its maintenance during cell division. The histones found in heterochromatin are characterized by specific posttranslational modifications, where one of the best-characterized modifications is methylation of H3K9. An intrinsic relationship between H3K9 methylation and heterochromatin is established by the discovery of the mammalian SET domain protein Suv39h1 (Lachner *et al*., 2001; Peters *et al*., 2001). The mammalian Suv39h1 as well as its homologue in the *Drosophila* SU(VAR)3-9 methylate specifically histone H3K9, creating a specific binding site for the chromodomain of HP1 and resulting in heterochromatin formation (Lachner and Jenuwein, 2002). This mechanism is conserved in the fission yeast *S. pombe* where CLR4 and the HP1 homologue SWI6 play orthologous functions, respectively (Bannister *et al*., 2001; Nakayama *et al*., 2001).

Unlike in animal cells where trimethyl H3K9 is preferentially localized to heterochromatin (Peters *et al*., 2003; Rice *et al*., 2003), plants seem to have a distinct distribution pattern of H3K9 methylation: abundant dimethyl H3K9 but little trimethyl H3K9 is found at silent loci in *Arabidopsis*, suggesting that dimethylation of H3K9, instead of the trimethyl state, is the critical mark for heterochromatin (Jackson *et al*., 2004). Our studies in tobacco cells suggest that LHP1, the sole *Arabidopsis* homologue of animal HP1, functions analogously as HP1 and can be recruited to/by dimethylated H3K9 in heterochromatin (Yu *et al*., 2004). Furthermore, the H3K9 HMTase NtSET1 binds LHP1 *in vitro*, suggesting a role for NtSET1 in heterochromatin function (Yu *et al*., 2004). Using chromatin immunoprecipitation it was shown that ectopically expressed NtSET1-YFP, as well as LHP1-YFP, can bind subtelomere-like heterochromatic sequences directly in tobacco BY2 cells. In *Arabidopsis*, as shown by Zemach *et al*. (2006), the LHP1-YFP fusion protein displayed centromeric localization in some cells. Similar to those described in tobacco BY2 cells, in transgenic *Arabidopsis*, ectopically expressed LHP1-YFP and NtSET1-YFP also bind directly heterochromatin regions. Together, these results in plant cells support the previous described mechanism in yeast and animals in which H3K9 methylation caused by NtSET1 may recruit LHP1 in heterochromatin formation. However, in the *Arabidopsis lhp1* mutant, no effect could be detected on chromocenter organization (Libault *et al*., 2005) and on the expression of genes

within heterochromatin (Nakahigashi *et al*., 2005). Thus, it is possible that LHP1 functions in heterochromatin redundantly with other proteins and/or that LHP1 plays an as yet unknown role at these heterochromatic sites.

Nevertheless, although NtSET1-YFP can bind heterochromatin regions in *Arabidopsis*, the ability is less efficient compared with LHP1-YFP. It suggests the presence of the *Arabidopsis* endogenous H3K9 HMTases that may function in heterochromatin formation. The *Arabidopsis* genome contains 15 genes encoding the same class (SUV39 class) SET domain proteins as NtSET1 (Zhao and Shen, 2004), where several of them have been shown to be involved in heterochromatin silencing. KYP/SUVH4, identified as a H3K9 HMTase, is required for maintaining H3K9 methylation and DNA methylation within heterochromatin regions (Jackson *et al*., 2002; Jasencakova *et al*., 2003). Another functionally characterized *Arabidopsis* member of this class is SUVH2. In *SUVH2* null plants mono- and dimethyl H3K9, the *Arabidopsis* heterochromatin marks, are significantly reduced, suggesting that SUVH2 has as a central function in heterochromatin silencing in *Arabidopsis* (Naumann *et al*., 2005).

In addition to the histone H3K9 methylation and HP1 binding, heterochromatin is characterized by another epigenetic mark, DNA cytosine methylation (Martienssen and Colot, 2001). A direct connection between histone H3K9 methylation and DNA cytosine methylation has been established in plant. Furthermore, RNAi-mediated silencing pathways were shown to play a role in heterochromatin assembly in fission yeast *S. pombe* (Volpe *et al*., 2002; Verdel *et al*., 2004). However, the contribution of the RNAi pathway in plant heterochromatin formation has not yet been established, although siRNA can direct chromatin modifications, including DNA methylation and histone methylation (Zilberman *et al*., 2003).

IV-2-2 Euchromatin gene silencing

H3K9 methylation is not restricted to constitutive heterochromatin and is also involved in transcriptional gene repression at euchromatic targets. G9a, the member of the SUV39 family HMTase in mammals, was shown to be responsible for H3K9 mono- and dimethylation, which are excluded from pericentromeric regions and to mark silent domains within euchromatin, and involved in the repression of euchromatic genes (Tachibana *et al*., 2002; Rice *et al*., 2003). In addition to G9a, evidence comes from the finding that the E2F-Rb complex can recruit Suv39h1/HP1 to the cell cycle-controlling gene *cyclin E* to repress

transcription (Neilsen *et al*., 2001). In cells lacking Rb, the *cyclin E* promoter is under-methylated at H3K9 and the association of HP1 with the *cyclin E* promoter is disrupted, showing that Rb is necessary to deliver these activities. In addition, it was also shown that Suv39h1 is necessary for repression other Rb-regulated genes (Vandel *et al*., 2001). Since the transcriptional repression effect of HP1 occurs on many euchromatic sites in *Drosophila*, HP1 is unlikely to be involved only in the transcriptional regulation at E2F/Rb-controlled genes (Hwang *et al*., 2001). More likely HP1 is involved in a number of different transcriptional regulator-controlled promoters, as suggested by the fact that HP1 is found associated with several transcriptional regulators (Jones *et al*., 2000).

Our results show that NtSET1-GFP protein is found broadly distributed in tobacco BY2 cells, covering in part both heterochromatic foci and euchromatin regions (Yu *et al*., 2004), suggesting a possible role for NtSET1 in euchromatin gene silencing. However, genes that are targeted by NtSET1 within euchromatin are not known. In *Arabidopsis*, KYP was also shown to be involved in silencing of euchromatic loci, including the *SUPERMAN* floral homeotic gene and the *PAI2* tryptophan biosynthetic gene (Jackson *et al*., 2002; Malagnac *et al*., 2002). At the *PAI1-PAI4* transcribed inverted repeat locus, both H3K9 methylation and DNA methylation are maintained by combined action of KYP/SUVH4 and SUVH6 HMTases (Ebbs *et al*., 2005).

A function of LHP1 in repression of gene expression is also evidenced. The mutants at the *LHP1* locus in *Arabidopsis* display upregulated expression of specific floral homeotic genes (Kotake *et al*., 2003; Nakahigash *et al*., 2005). Furthermore, additional genes within euchromatin are activated, including the genes related to seed maturation, meiosis, and cell cycle regulation (Nakahigash *et al*., 2005). Thus it is proposed that the *Arabidopsis* LHP1 protein is a component of euchromatin to repress gene expression. Our results revealed that LHP1-YFP can bind directly the promoter region of *FT*, but not that of *TSF*, which was not upregulated in *lhp1* mutant. This suggests that LHP1 might repress specific gene expression by binding with the promoter of target genes. However, the possible mechanism for the repression remains unknown. It seems that LHP1 might recruit to a specific site by interaction with other proteins, since sequence-specific DNA binding of the HP1 family proteins is not known. In *Arabidopsis*, abundant dimethyl H3K9 was found at heterochromatin, while trimethyl H3K9 was rich in euchromatin regions (Jackson *et al*., 2004). Since HP1 binds both di- and trimethyl H3K9 peptides (Bannister *et al*., 2001; Jacobs *et al*., 2002; Lachner *et al*.,

2001), it suggests that LHP1 might be recruited in euchromatin gene silencing by binding trimethyl H3K9. Recent studies show that the recruitment of HP1 to chromatin requires not only H3K9 methylation but also a direct protein-protein interaction between HP1 and HMTase such as SUV39H1 (Stewart *et al*., 2005). However, the candidate partner proteins of LHP1 in *Arabidopsis* have not been identified yet. The tobacco H3K9 HMTase NtSET1 has been shown to bind LHP1 *in vitro* (Yu *et al*., 2004). Thus it is likely that the *Arabidopsis* NtSET1-like HMTases are the candidate partner proteins of LHP1 in *Arabidopsis*.

Until now, the number of identified target genes directly controlled by chromatin modifies to transcriptional silencing is still small. Furthermore, reversibility of silenced chromatin state is of broad interest at present. These provide us a rich research field and a new frontier.

V

MATERIALS AND METHODS

V-1 Materials

V-1-1 Plant materials

a) *Nicotiana tabacum* L. cv. Bright Yellow 2 (BY2) cell

The tobacco BY2 cell line, often cited as the HeLa cell line of higher plant, is well characterized by Nagata *et al*. (1992). This cell line is highly homogeneous and shows an exceptionally high growth rate, multiplying 80- to 100-fold in one week. After treatment with aphidicolin, a high cell cycle synchrony can be easily obtained. In addition, BY2 cells can be easily transformed and stable transgenic calli and suspension-cultured cells are easily obtained. These features make this cell line a powerful tool for exploring the molecular and cellular biology of plant cells.

b) *Nicotiana tabacum* cv SR1

The tobacco (*Nicotiana tabacum* cv SR1) seedlings expressing *GFP*, *NtSET1-GFP* and its SET domain deletion mutant *NtSET1D1-GFP* (Shen and Meyer, 2004) are used for chromatin immunoprecipitation.

c) *Arabidopsis thaliana* Columbia (col-0)

Arabidopsis thaliana is a small flowering plant with small genomes, a rapid life cycle, and prolific seed production. Moreover efficient transformation methods and a large number of mutant lines are available. Such advantages have made *Arabidopsis* a model organism for studies of the cellular and molecular biology of flowering plants. Different *Arabidopsis* ecotypes have a different genetic background and thus show different organ shapes and growth characteristics. Columbia (Col) is one of the most popular ecotypes of *Arabidopsis thaliana*, which is being sequenced in the *Arabidopsis* Genome Initiative (AGI). One of the Columbia accessions, col-0, is used here as the background line.

V-1-2 Bacteria strains

V-1-2-1 *Escherichia coli*

a) *Escherichia coli* Top10

The Top10 strain is ideal for high-efficiency cloning and plasmid propagation. This strain is used as the host for initial cloning of target DNA and for maintaining plasmids. In addition to supporting blue/white screening, the *rec*A1 and *end*A1 mutations in Top10 increase insert stability and improve the quality of plasmid DNA prepared from mini-preps.

b) *Escherichia coli* DH5α

DH5α is a versatile strain. As Top10, DH5α is *rec*A– *end*A– and gives high transformation efficiencies and good plasmid yields. Here, DH5α is used as the host for initial cloning and also for GST and GST fused protein expression in pGEX vectors.

c) *Escherichia coli* BL21 (DE3)

The BL21 (DE3) strain allows high-efficiency protein expression under the control of a T7 promoter. This strain is lysogenic for carrying the lambda DE3 lysogen, which contains the T7 bacteriophage gene I, encoding IPTG inducible T7 RNA polymerase under the control of the *lac* UV5 promoter. BL21 (DE3) is used as the host for the recombinant protein expression in pET system.

V-1-2-2 *Agrobacterium tumefaciens*

a) *Agrobacterium tumefaciens* GV3101

This *Agrobacterium* strain, containing C58C1 plasmid, is resistant to gentamicin and rifampicin and used to mediate *Arabidopsis* transformation by flower dip method.

b) *Agrobacterium tumefaciens* LBA4404

This *Agrobacterium* strain, containing pAL4404 plasmid, is resistant to rifampicin and used to mediate transformation of tobacco BY2 cells.

V-1-3 Cloning and expression vectors

a) pCR II-Topo vector (Invitrogen)

pCR II-Topo vector with single 3'-T overhang is designed for cloning Taq-amplified PCR products faster and more successfully. The key to the pCR II-Topo vector is the enzyme DNA

topoisomerase I covalently bound to the 3' phosphate group of the 3'-T overhang, which functions to linearize the vectors for readily ligation of PCR products with compatible ends. T7 and SP6 promoters flank a multiple cloning region within the α-peptide coding region for β-galactosidase (*lacZα*). Insertional inactivation of *lacZα* allows recombinant clones to be directly identified by blue/white colony screening. Kanamycin and ampicillin resistance genes are for the choice of selection in *E. coli*.

b) pSK-GFP

This vector contains the full-length of *GFP* with stop code under the control of *lac* promoter. It was made to facilitate the creation of GFP fusion proteins, with GFP linked to the C-terminus of the fused protein. Ampicillin resistance gene is for selection in *E. coli*.

c) pEYFP-EYFP

The pEYFP-EYFP vector contains two tandem copies of *EYFP* under the control of *lac* promoter: first one without stop code and the second one with stop code. The multiple cloning sites locate at the 5' terminus of the first copy, between the two copies, and at the 3' terminus of the second copy. Ampicillin resistance gene is for selection in *E. coli*.

d) pET32a (Novagen)

In addition to the cleavable His-Tag and S-Tag sequences for purification and detection, the pET32a vector also incorporates fusion tag sequence of a 109 aa Trx-Tag thioredoxin protein specifically designed to enhance the solubility of target proteins in the *E. coli* cytoplasm. The tag sequences are driven by the T7*lac* promoter, and ampicillin resistance gene is for selection in *E. coli*.

e) pGEX4T-1 (Amersham Biosciences)

In pGEX4T-1, GST gene is driven by the IPTG inducible tac promoter for high-level expression, followed by a thrombin recognition site and multiple cloning sites. An internal *lac* I*q* gene is for use in any *E. coli* host. Ampicillin resistance gene is for selection in *E. coli*.

f) pER8 (Zuo *et al*., 2000)

pER8 is a plant expression vector using in transgenic plants by an estrogen receptor-based chemical-inducible system, termed XVE for LexA-Vp16-ER. The XVE activator is strictly regulated by estradiol in transgenic plants, with undetectable transactivating activity in absence of the inducer. Spectinomycin and hygromycin resistance genes are for the selection in bacteria and in plants respectively.

g) pNDamMyc (van Steensel and Henikoff, 2000)

This vector contains the full-length ORF of *E. coli Dam* (*DNA adenine methyltransferase*) gene, followed by a myc epitope tag, under the *Drosophila* heat-shock promoter. Here, it is used to amplify Dam and myc tag to create the Dam fusion proteins in plant expression vector, with Dam at the N-terminus.

V-1-4 Antibodies

Name	Host	Company
anti-Histone-H3 monoclonal antibody	Mouse	Upstate
anti-dimethyl-Histone-H3-(K4) antibody	Rabbit	Upstate
anti-dimethyl-Histone-H3-(K9) antibody	Rabbit	Upstate
anti-Acetyl-Histone-H3-(K9) antibody	Rabbit	Upstate
anti-dimethyl-Histone-H3-(K27) antibody	Rabbit	Upstate
anti-GFP antibody	Rabbit	Molecular Probes
anti-GFP monoclonal antibody	Mouse	Roche
anti-cdc2 (PSTAIRE) antibody	Rabbit	Santa Cruz Biotechnology

V-1-5 Antibiotics

Name	Stock Concentration	Company
Ampicillin	100 mg/l	Duchefa
Carbenicillin	500 mg/l	Duchefa
Chloramphenicol	34 mg/l	Duchefa
Gentamicin	20 mg/l	Duchefa
Hygromycin	40 mg/l	Duchefa
Kanamycin	100 mg/l	Duchefa
Rifampicin	50 mg/l	Duchefa
Spectinomycin	100 mg/l	Duchefa
Tetracycline	12 mg/l	Duchefa

V-1-6 Oligos

Name	Sequence (From 5'-3')
FT1F	TTCTCTATAAACTTGGCGGTAC
FT1R	TAACTCGGGTCGGTGAAATCA
FT2F	CTACAGTTGTTAGGCTATGGTTA
FT2R	GCGGCCATATTATGGAAAAGTGA
TSF1F	TGACGTACGCAAATATGCAGCA
TSF1R	CTTACCTTATGAACCCTTACTTGC
TSF2F	CTGAACATAACGTCCCACGTATG
TSF2R	CCTAAAAGCTCAACCGTGTATTC
Ta3MF	GATTCTTACTGTAAAGAACATGGCATTGAGAGA
Ta3MR	TCCAAATTTCCTGAGGTGCTTGTAACC
CACTA1F	CTGCTGCCAGGAGTGTAAACAG
CACTA1R	CACCAATGATATCCTCAGGACCA
180bpF	ACCATCAAAGCCTTGAGAAGCA
180bpR	CCGTATGAGTCTTTGTCTTTGTATCTTCT
TEL3G	TTTAGGGTTTAGGGTTTAGGG
TEL3C	TAAACCCTAAACCCTAAACCC
tRETS1	TCAGTTTAGGGTTTAAAACC
tRETS2	AGTCAGCATTAGGGTTTTAA

tACTf	GTGCGACGTGGATATCAGAAAG
tACTr	CATCATATTCTGCCTTTGCAATCCAC
SUVH1-1	cgctcgagcaacATGGAAAGAAATGGTGGTCAC
SUVH1-2	tgccatggcTCCAAATGAGCCACGGCAATA
SUVH1-3	TGATGCGTTAAGAAGAAGG
SUVH1-4	GAGTCATGGGTAGAGAAAGG
DAM-1	cgctcgagcATGAAGAAAAATCGCGCTT
DAM-2	cgctcgaggcCAGATCCTCTTCAGAGAT
DAM-3	cgctcgagCAGATCCTCTTCAGAGAT
Linker1	GCGGTGACCCGGGAGATCTGAATTC
Linker2	GAATTCAGATC
DamLK	TGACCCGGGAGATCTGAATTCTC

V-2 Methods

V-2-1 General techniques in molecular biology

V-2-1-1 Vector construction

V-2-1-1-1 PCR (Polymerase Chain Reaction)

In a sterile PCR tube, PCR reaction is performed by mixing the following: 10 x amplification buffer 5 µl, 50 mM $MgCl_2$ 1.5 µl, 10 mM solution of four dNTPs 1 µl, 10 µM forward primer 1 µl, 10 µM reverse primer 1 µl, thermostable DNA polymerase 1-5 units (Stratagene; BD Biosciences), and adding template DNA and distilled H_2O to total volume 50 µl. The amount of template DNA required varies according to the complexity of its sequence. PCR is performed 20-30 cycles to amplify the nucleic acids using the denaturation (30 sec at 94°C), annealing (30 sec at 55°C), and polymerization (1 min at 72°C). Times and temperatures need to be adapted to suit the particular reaction conditions. Polymerization should be carried out for 1 min for every 1000 bp of length of the target DNA. PCR products are purified by the kit "Nucleospin Extract II" (Macherey-Nagel).

V-2-1-1-2 Restriction enzyme digestion

In a 20 µl reaction mixture, approximately 200 ng of DNA is digested with 1-2 units of the relevant restriction enzyme(s) (Roche, Biolabs, Fermentas). The reaction is incubated for 1 hr at the optimum temperature for digestion. After digestion, restriction enzyme is inactivated by heating. Digested DNA is purified by the kit "Nucleospin Extract II" (Macherey-Nagel).

V-2-1-1-3 Agarose gel electrophoresis

The electrophoresis is performed on an agarose gel with appropriate concentration for separating the particular size fragments in TAE or TBE buffer. DNA fragments are then visible by UV light after staining with EB solution (30 µg/l).

1 x TAE: 40 mM Tris-acetate; 2 mM EDTA.
1 x TBE: 89 mM Tris-borate; 2 mM EDTA.

V-2-1-1-4 Ligation

The 10 μl ligation mixture, containing target DNA 60 ng, plasmid DNA 20 ng, 10 x ligation buffer 1 μl, T4 DNA ligase (Fermentas) 1 unit, is incubated overnight at 16°C. The ligation products can be used for *E. coli* transformation directly (by heat shock) or be purified first and then transformed by electroporation. To purify the ligation products, 3 M sodium acetate (pH 5.2) is added to the ligation mixture to achieve a final concentration of 0.3 M. The DNA is precipitated by adding 2 volumes of 100% ethanol, and then washed by 70% ethanol. Finally, the DNA is dissolved in 10 μl of distilled H_2O.

V-2-1-1-5 Competent cell preparation

a) Competent cell preparation for heat shock using $CaCl_2$

A single bacterial colony is inoculated in 3 ml LB and incubated by shaking at 37°C overnight. 0.5 ml overnight culture is inoculated in 50 ml LB and incubated by shaking at 37°C until OD_{600} reaches 0.3. The culture is chilled on ice for 10 min, and centrifugated at 3,000 rpm for 10 min at 4°C. The cells are resuspended in 30 ml of ice-cold 0.1 M $CaCl_2$ solution, and kept on ice for 30 min. After centrifugation at 3,000 rpm for 10 min at 4°C, the cells are resuspended gently in 2 ml of ice-cold 0.1 M $CaCl_2$. The $CaCl_2$ treated cells can be used directly for transformation or dispensed into aliquots and frozen at -70°C.

LB: 10 g/l Tryptone; 5 g/l Yeast extract; 10 g/l NaCl; pH 7.0.

b) Competent cell preparation for electroporation

A single bacterial colony is inoculated in 3 ml LB/YEB. After overnight culture by shaking, 3 ml overnight culture is diluted 100 times and incubated by shaking until OD_{600} reaches 0.5. The culture is chilled on ice for 10 min and centrifugated at 5,000 rpm for 10 min at 4°C. The cells are washed twice by ice-cold distilled H_2O, and once by ice-cold 10% glycerol. Finally the cells are resuspended in 1 ml of ice-cold 10% glycerol. The electrocompetent cells can be used immediately. For storage, 40 μl aliquots of the cell suspension are dispensed in each tubes, dropped into a bath of liquid nitrogen, and stored in a -70°C freezer.

YEB: 5 g/l Beef extract; 5 g/l Peptone; 1 g/l Yeast extract; 2 mM $MgSO_4$; 5g/l Sucrose; pH 7.2.

V-2-1-1-6 Transformation of competent cells

a) By heat shock

200 µl the $CaCl_2$-treated competent cells are mixed with plasmid DNA or ligation products in a sterile tube. After keeping on ice for 30 min, the tube is transferred into a preheated 42°C water bath and kept for exactly 90 sec, and then rapidly transferred on ice and kept for 1-2 min. 300 µl of LB medium is added to each tube and the mixture is incubated for 1 hr at 37°C to allow the bacteria to recover. The recovered culture is transferred onto agar LB medium containing appropriate antibiotics. Transformed colonies should appear in 12-16 hr.

b) By electroporation

40 µl the electrocompetent cells are mixed with plasmid DNA or purified ligation products. Then the DNA/cell mixture is pipetted into a cold electroporation cuvette. Transformation is carried by Bio-rad electroporation apparatus delivering an electrical pulse of 25 µF capacitance, 2.5 kV, and 200/400 Ω resistance (200 Ω for *E. coli*, and 400 Ω for *Agrobacterium*). The DNA/cell mixture is removed from the electroporation cuvette by adding 1 ml of LB medium, and the mixture is incubated for 1 hr at 37°C to allow the bacteria to recover. The recovered culture is then transferred onto agar LB medium containing appropriate antibiotics. Transformed colonies should appear in 12-16 hr (*E. coli*) or after 36 hr (*Agrobacterium*).

V-2-1-1-7 Mini-preparation of plasmid DNA by alkaline lysis with SDS

A single bacterial colony is inoculated in 3 ml LB and incubated by shaking at 37°C overnight. The bacteria are harvested and resuspended in ice-cold 100 µl of solution I by vigorous vortex. 200 µl of freshly prepared solution II is added, and the content is mixed by inverting the tube 6-8 times and kept on ice for 3-5 min. 150 µl of ice-cold solution III is added by inverting the tube 6-8 times, and kept on ice for another 3-5 min. The bacterial lysate is centrifuged at maximum speed for 5 min in a microfuge. After extracting the supernatant by an equal volume of phenol/chloroform (1:1), the aqueous upper layer is precipitated by adding 2 volumes of 100% ethanol and washed by 70% ethanol. Finally, the DNA pellet is dissolved in 50 µl of distilled H_2O containing 10 µg/ml DNase-free RNase A (Fermentas).

Solution I: 50 mM Glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0).
Solution II: 0.2 M NaOH, 1% SDS.

Solution III: 3 M KAc, 11.5% acetic acid.

Alternatively, the plasmid DNA is prepared using the kit "NucleoSpin Plasmid QuickPure" (Macherey-Nagel).

V-2-1-2 Genomic DNA isolation

After grinding the plant tissues in liquid nitrogen, 200 mg of the fine powder is homogenized in 600 μl 65°C pre-heated 2 x CTAB buffer and incubated in 65°C for 15 min. An equal volume of chloroform/isoamyl alcohol (24:1) is added to form an emulsion by thorough vortex. After centrifugation at 11,000 g for 1 min, 1/5 volume of the 5% CTAB solution is added to the supernatant. Another chloroform/isoamyl alcohol extraction is then performed. The supernatant is precipitated for 15 min by adding an equal volume of CTAB precipitation buffer. After centrifugation at 11,000 g for 1 min, the DNA pellet is dissolved in high-salt TE buffer. Another precipitation is performed by adding 2 volumes of 100% ethanol and centrifugation at 11,000 g for 15 min. After washing with 80% ethanol, the pellet is dried and dissolved in 50 μl 0.1 x TE containing 100 μg/ml DNase-free RNase A (Fermentas). To inactivate the DNase, the solution is incubated at 65°C for 1 hr, which also facilitates the solubilization of the genomic DNA pellet.

The concentration of the genomic DNA is measured by absorbance at 260 nm (1 unit of OD_{260} = 50 μg/ml of DNA). The quality of the DNA is analyzed by gel electrophoresis.

2 x CTAB buffer: 2% CTAB; 100 mM Tris-HCl (pH8.0); 20 mM EDTA; 1.4 M NaCl.
5% CTAB solution: 5% CTAB in 0.35 M NaCl.
CTAB precipitation buffer: 1% CTAB; 50 mM Tris-HCl (pH 8.0); 10 mM EDTA.
High-salt TE buffer: 10 mM Tris-HCl (pH 8.0); 1 mM EDTA; 1 M NaCl.
1 x TE: 10 mM Tris-HCl (pH 8.0); 1 mM EDTA.

V-2-1-3 RNA isolation and analysis

V-2-1-3-1 Total RNA extraction

After grinding the plant tissues in liquid nitrogen, 100 mg of the fine powder is homogenized in 1 ml Trizol Reagent (Invitrogen) by vortex immediately and kept on ice for 5 min. 200 μl chloroform is then added and kept on ice for another 5 min. After centrifugation at 12,000 g

for 15 min at 4°C, the supernatant is transferred to a new 1.5 ml tube and precipitated by adding 500 µl isopropanol for 10 min. Centrifugation is performed at 12,000 g for 10 min at 4°C. The RNA pellet is then washed with 1 ml 75% ethanol by centrifuging at 7,500 g for 5 min at 4°C. Finally, the RNA pellet is dissolved in 20 µl RNase-free water. The concentration of RNA is measured by absorbance at 260 nm (1 unit of OD_{260} = 40 µg/ml of RNA).

V-2-1-3-2 Reverse transcription

To avoid the DNA interference, the reaction mixture containing 5 µg RNA (12.25 µl), 5 µl 5 x RTase Buffer, and 1 µl RQ1 DNase (Promega) is incubated at 37°C for 10 min, followed by the incubation at 56°C for 10 min to inactivate DNase. The following is then added into DNase treated RNA: 2.5 µl 0.1 M DTT, 2.5 µl 10 mM dNTPs, 1 µl 0.5 µg/µl Oligo dT, and 0.25 µl 40 U/µl RNase Inhibitor (Promega). After incubation at 42°C for 2 min, 1 µl Improm-II Reverse Transcriptase (Promega) is added into the mixture. The reaction mixture is incubated at 42°C for 60 min, and then in 70°C for 10 min. The synthesized cDNA can be used as the templates in PCR.

V-2-1-3-3 Electrophoretic separation of RNAs

The 20 µl denaturation reaction is set up by mixing the following: RNA 10-30 µg, 10 x MOPS buffer 2 µl, formaldehyde 3.5 µl, formamide 10 µl. The mixture is incubated at 65°C for 15 min, and then chilled on ice. After that, 2 µl of 10 x loading buffer and 1 µl EB (200 µg/ml) is added to each sample. The RNA samples are then loaded onto the agarose gel prepared by 1 x MOPS and 0.7 M formaldehyde. Following electrophoresis running in 1 x MOPS buffer, RNAs can be visualized by UV light.

10 x MOPS buffer: 200 mM MOPS; 80 mM sodium acetate; 10 mM EDTA; pH 7.0.

V-2-1-3-4 Transfer of RNA to membrane

The gel is rinsed with RNase-free water, soaked in 0.05 N NaOH for 20 min and then in 20 x SSC for 40 min. Using 20 x SSC as the transfer buffer, RNA is transferred onto the nylon membrane Hybon-N+ (Amersham Biosciences) by capillary transfer system. After overnight transfer, the membrane is rinsed in 2 x SSC, and then fixed by UV crosslink.

20 x SSC: 3 M NaCl ; 0.3 M Tri-sodium citrate.

V-2-1-3-5 Northern hybridization

To label the probes, the kit "Prime-a-Gene" Labeling system (Promega) is used. The 50 µl reaction contains: 5 x labeling buffer 10 µl; 1.5 mM dATP 1 µl; 1.5 mM dGTP 1 µl; 1.5 mM dTTP 1 µl; denatured DNA template 25 ng; nuclease-free BSA 2 µl; [α-^{32}P]dCTP 5 µl (50 µCi); DNA polymerase I, large (klenow) fragment 5 units. After incubating at room temperature for 1 hr, the reaction is terminated by heating at 95°C for 5 min and subsequently chilling on ice. After incubating the membrane for 2 hr at 42°C in 20 ml of prehybridization buffer, the denatured radiolabeled probe is added directly to the prehybridization buffer. Continuous incubation is performed for 12-16 hr at 42°C. After hybridization, the membrane is washed at 42°C twice with 2 x SSC, 0.1% SDS, twice with 0.2 x SSC, 0.1% SDS, and autoradiographed using X-ray film.

Prehybridization buffer: 10% Dextran sulfate; 50% formamide; 5 x SSPE; 1% SDS; 5 x denhardt's solution; 100 µg/ml salmon sperm DNA.
20 x SSPE: 3.6 M NaCl; 0.2 M NaH_2PO_4; 0.2 M EDTA.
100 x denhardt's solution: 2% BSA; 2% Ficoll 400; 2% polyvinylpyrrolidone (PVP).

V-2-1-4 Differential hybridization

The colonies are transferred to two the Protran BA 85 Nitrocellulose membranes (Schleicher & Schuell). To isolate the DNA, the membranes are rinsed in denaturation buffer, neutralization buffer I, and neutralization buffer II for 5 min respectively. After washing the membranes briefly in 2 x SSC, the isolated DNA is fixed to the membranes by UV crosslink. The two replicated membranes are hybridized with two different ^{32}P-labeled probes (one of them serving as the control). Hybridization and probe labeling are performed as described previously in "V-2-1-3-5, Northern hybridization". After hybridization and washing, membranes are autoradiographed using X-ray film. The signals from the two replicated membranes are compared, and the specific colonies that show signals with the desired probe but not with the control are selected and sequenced.

Denaturation buffer: 0.5 N NaOH; 1.5 M NaCl.
Neutralization buffer I: 1 M Tris-HCl (pH 8.0).
Neutralization buffer II: 1 M Tris-HCl (pH 7.5); 2 x SSC.

V-2-1-5 Quantitative PCR analysis

The quantitative PCR reaction contains 12.5 μl 2 x reaction buffer [qPCR Mastermix plus for SYBR green I (Eurogentec)], 5 pmol forward primer and 5 pmol reverse primer. Template DNA and distilled H_2O are added to total volume 25 μl. For each sample, PCR is performed in triplicate using fixed amounts of template DNA as well as the no template control. PCR is carried out using the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. Melting curves of the PCR products are checked for the quality of PCR. The threshold (CT) value is set such that the fluorescence signal is above the baseline noise but as low as possible in the exponential amplification phase.

V-2-1-6 Protein expression, purification and detection

V-2-1-6-1 Protein expression in *E. coli*

The expression vectors containing target genes are transformed into *E. coli* BL21 (DE3) or *E. coli* DH5α cells. The next day, the bacteria on the plate are transferred to liquid LB medium. After incubating the culture to reach OD_{600} 0.4~0.6, IPTG is added to a final concentration of 0.1-0.4 mM. To induce the expression of proteins, the culture is incubated at 28°C for another 2 hr. The cells are harvested and resuspended in 1 x SDS loading buffer. Protein expression is detected by SDS-PAGE.

V-2-1-6-2 Purification of expressed proteins from *E. coli*

a) His-tag fusion proteins

A large-scale expression of his-tag fusion proteins is performed in *E. coli* BL21 (DE3). The bacteria are collected by centrifugation at 3,000 rpm for 10 min and then resuspended in 20 ml of ice-old his-tag lysis buffer plus 100 μg/ml lysozyme and protease inhibitor cocktail (Roche), and lysed by French Press. After centrifugation at 20,000 g for 20 min at 4°C, the supernatant is loaded onto a Sepharose Fast Flow column (Amersham Biosciences), which has been coupled with 0.1 M Ni_2SO_4 and pre-equilibrated with lysis buffer. The column is then washed extensively with his-tag lysis buffer and his-tag wash buffer. The his-tag fusion proteins are retained on beads and determined yield by SDS-PAGE.

His-tag lysis buffer: 20 mM Tris–HCl (pH 8.0); 0.5 M NaCl; 0.2% Tween 20; 5% glycerol.
His-tag wash buffer: His-tag lysis buffer plus 40 mM imidazole.

b) GST fusion proteins

A large-scale expression of GST fusion proteins is performed in *E. coli* DH5α. The cells are harvested and resuspended in 20 ml of ice-old GST lysis buffer plus 100 μg/ml lysozyme and protease inhibitor cocktail (Roche). After disrupting the cells by French Press, the cell lysate is centrifugated at 20,000 g for 20 min at 4°C. The supernatant is mixed with settled glutathion-Sepharose-4B beads (Amersham Biosciences), which is pre-washed 3 times with H_2O and 3 times with 1 x PBS. Following incubation of the suspension on a rotation wheel for 2 hr at 4°C, beads are spinned down, washed once in 1 x PBS plus 0.1% Triton X-100 and 3 times in 1 x PBS plus 10% glycerol. Finally, 1 x PBS plus 10% glycerol is added to make 10% slurry. Proteins fixed to the beads are determined by SDS-PAGE.

PBS: 140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO4; 1.8 mM KH_2PO4; pH 7.3.
GST lysis buffer: 1 x PBS; 0.1% Triton X-100; 1 mM DTT; 10% Glycerol.

V-2-1-6-3 Separation of proteins by SDS-PAGE

Using the "Mini-protein II" system (Bio-rad), SDS-PAGE is performed to separate the proteins mainly based on molecular weight. The protein samples are treated in 1 x SDS loading buffer at 95°C for 5 min. After preparation of the resolving gel and the stacking gel, the protein samples are loaded onto the gel and run in the 1 x SDS electrophoresis buffer at 100 V to allow the samples to separate. To visualize the proteins, the gel is stained with coomassie blue staining solution and destained with destain solution.

Resolving gel: 10-15% Acrylamide/Bis-acrylamide (29/1); 375 mM Tris-HCl (pH8.8); 0.1% SDS; 0.1% AP; 0.4 μl/ml TEMED.
Stacking gel: 5% Acrylamide/Bis-acrylamide (29/1); 125 mM Tris-HCl (pH6.8); 0.1% SDS; 0.1% AP; 1 μl/ml TEMED.
1 x SDS loading buffer: 50 mM Tris-HCl (pH6.8); 100 mM DTT; 2% SDS; 0.1% bromophenol blue; 10% glycerol.
1 x SDS electrophoresis buffer: 25 mM Tris; 250 mM glycine; 0.1% SDS.
Coomassie blue staining solution: 0.1% Commassie Blue R-250; 40% ethanol; 2% Acetic

acid.
Destain solution: 7% ethanol; 7% acetic acid.

V-2-1-6-4 Western blot

After separation of proteins by SDS-PAGE, the gel is equilibrated in transfer buffer for at least 10 min before transference. Immobilon-p PVDF transfer membrane (Millipore) is pre-wetted in 100% methanol, rinsed in water for 5 min and equilibrated in transfer buffer for at least 10 min before use. Then proteins are transferred from gel to PVDF membrane at 270 mA for 2 hr at 4°C.

The membrane is washed in TTBS for 5 min, blocked in 5% non-fat milk in TTBS for 1 hr, incubated in diluted primary antibody at 4°C overnight. After washing 3 times with the milk-TTBS, the membrane is incubated in the diluted secondary antibody for 1 hr. Then the membrane is washed once with milk-TTBS, 3 times with TTBS, and once with TBS. Finally the membrane is detected by the ECL western blot detection kit (Amersham Biosciences).

Transfer buffer: 25 mM Tris; 192 mM Glycine; 15% Methanol.
TBS buffer: 20 mM Tris-HCl (pH7.4); 150 mM NaCl.
TTBS buffer: TBS buffer plus 0.1% Triton X-100.

V-2-1-7 *In vitro* histone H1 kinase and histone methyltransferase assays

a) Histone H1 kinase assay

For histone H1 kinase activity, the reaction mixture containing 0.5 μg/μl H1, 0.5 μCi/μl ^{32}P-ATP and enzymes is incubated at room temperature for 30 min in H1 kinase activity buffer. The reaction products are separated by 12–15% SDS-PAGE. After coomassie blue staining and destaining, the gel is vacuum dried at 70°C for 2 hr and exposed by autoradiography.

H1 kinase activity buffer: 20 mM Tris-HCl (pH 7.5); 15 mM $MgCl_2$; 5 mM EGTA; 1 mM DTT.

b) Histone methyltransferase assay

30 μl reaction mixture containing substrates (histones), enzymes (HMTases), and 250 nCi S-

adenosyl-[methyl-^{14}C]-L-methionine (Amersham Biosciences) in MAB buffer is incubated at 37°C for 1 hr with shaking. The reaction products are separated by 12–15% SDS-PAGE gel electrophoresis. After coomassie blue staining and destaining, the gel is vacuum dried at 70°C for 2 hr and exposed by autoradiography.

1 x MAB buffer: 50 mM Tris-HCl (pH 8.5); 20 mM KCl; 10 mM $MgCl_2$; 250 mM Sucrose; 100 μM $ZnCl_2$; 10 mM β-mercaptoethanol.

V-2-2 Tobacco BY2 cells culture, transformation and synchronization

V-2-2-1 TBY2 cell culture

TBY2 cell suspension is maintained by weekly subculture, agitating on a rotary shaker at 130 rpm at 27°C in the dark.

TBY2 medium: 4.3 g/l MS (M 0221, Duchefa); 30 g/l Sucrose; 100 mg/l Myo-Inositol; 1 mg/l Thyamine; 0.2 mg/l 2,4-D; 200 mg/l KH_2PO_4; (9 g/l Agar); pH 5.8.

V-2-2-2 TBY2 cell transformation

A single colony of *Agrobacterium tumefaciens* LBA4404 is inoculated in 2 ml YEB in presence of antibiotics and incubated at 28˚C for 24-48 hr. Then 50 μl of the pre-culture is inoculate in 2 ml fresh YEB (supplemented with the same antibiotics) and incubated at 28˚C overnight. The bacteria cells are harvested by centrifugation at 3,000 rpm for 10 min, and washed once with 10 mM $MgSO_4$. Finally the bacteria pellet is resuspended in the same volume (2 ml) of 10 mM $MgSO_4$. Acetosyringone is added to the bacteria suspension to a final concentration of 200 μM and the suspension is treated at least 2 hr before use.

For transformation, 5 ml 3-day-old TBY2 cells are incubated with 100 μl of acetosyringone treated *Agrobacterium.* After incubation for 48 hr at 27°C in the dark, the cells are washed 4 times in 25 ml TBY2 medium. The antibiotics are added in the last wash. The cells are then plated onto solid TBY2 medium complemented with the antibiotics. Calli, which appear after 2-3 weeks, are transferred onto new plates and are cultured independently.

V-2-2-3 TBY2 cell synchronization

For synchronization of the cell cycle, 20 ml of stationary phase TBY2 cells (7-8 days old) is transferred into 80 ml of the fresh TBY2 medium and cultured at 27°C for 24 hr in the dark with 3 μg/ml aphidicolin (Sigma), an inhibitor of DNA polymerase to block the cells at early S phase. The cells are washed with 10 volumes of 3% sucrose and then resuspended in 100 ml fresh TBY2 medium. Generally, the enrichment of cells of S phase, G2 phase, M phase and G1 phase can be observed at 1 hr, 5 hr, 8-9 hr, and 12-14 hr after release from the aphidicolin treatment. Mitotic index (MI) are checked for the quality of the synchronization.

V-2-3 *Arabidopsis thaliana* culture and transformation

V-2-3-1 *Arabidopsis* culture

Arabidopsis seeds are treated with 1 ml of 70% ethanol containing 0.2% Tween-20 by rotating on a wheel for no more than 10 min. After that, the seeds are washed with 96% ethanol twice and dried up completely. Then the seeds are spread carefully on the plate containing *Arabidopsis* medium. The plates are wrapped with the breathing tape and kept in the cold room for a couple of days before transferring to the growth room.

Medium for *Arabidopsis*: 4.9 g/l MS (M 0255, Duchefa); 10 g/l Sucrose; 9 g/l Agar; pH 5.7.

V-2-3-2 *Arabidopsis* transformation by flower dip method

A single colony of *Agrobacterium tumefaciens* GV3101 is inoculated in 3 ml YEB in presence of antibiotics, and incubated at 28˚C overnight. This 3 ml overnight culture is then diluted in 300 ml YEB (supplemented with the same antibiotics) and incubated at 28˚C for another 16-24 hr. The bacteria cells are harvest by centrifugation at 5,000 rpm for 10 min, and washed once with 100 ml 10 mM $MgSO_4$. Finally the bacteria pellet is resuspended in 10 ml 10 mM $MgSO_4$. Acetosyringone is added to the bacteria suspension to a final concentration of 200 μM and the suspension is treated at least 20 min before flower dip.

10 ml bacteria suspension is diluted into 200~250 ml with infiltration medium. Following dipping the flowers in infiltration medium for 1-2 min, the plant is put immediately in the small “growth chamber”, which helps to keep a high humidity. After 24 hr, the door of the “growth chamber” is opened to allow the dipped flowers and the coming-up flowers from the same stem to set seeds. The seeds are collected after plants dry up.

Infiltration medium: 2.15 g/l MS (M 0221, Duchefa); 5 g/l Sucrose; 0.01 mg/l BAP; 0.05% Sylwett; pH 5.7.

V-2-4 GST pull down assay

Stably transformed, estradiol-inducible tobacco BY2 cells expressing GFP and GFP fusion proteins are induced by 4 μM estradiol for 24 hr and harvested. After grinding cells in liquid nitrogen, 2 g fine powder is homogenized in 10 ml GST pull down buffer plus protease inhibitor cocktail (Roche). DNase I (Roche) is added to 10 μg/ml as a final concentration to release the chromatin proteins. The whole cell lysate is centrifugated at 20,000 g for 30 min at 4°C. The supernatant is collected and mixed with Glutathion-sepharose beads fixed with GST or GST fusion proteins. After rotating for 2 hr at 4°C on a wheel, the beads are washed four times with GST pull down buffer. After washing, specifically bound proteins are eluted from the beads by GST pull down buffer containing 1 M NaCl and then precipitated by 10% TCA. The bound proteins are analyzed by SDS-PAGE and western blot using the polyclonal antibody against GFP (Molecular Probe Inc.) with 1:5000 dilution.

GST pull down buffer: 25 mM Tris-HCl (pH 7.5); 100 mM NaCl; 15 mM $MgCl_2$; 10 mM EDTA; 5 mM EGTA; 1 mM NaF; 1 mM DTT; 0.1% Tween-20; 5% glycerol.

V-2-5 Histone extraction

After grinding 0.5 g tissues in liquid nitrogen, the fine powder is homogenized in 10 ml buffer A. The resulting slurry is filtered through 100 μm nylon mesh. The filtrate is centrifuged at 3,500 rpm for 20 min. The pellet is resuspended in 2 ml of buffer B and centrifuged at 12,000 g for 10 min. Following this wash, the pellet is homogenized in 1 ml of buffer C and centrifuged at 16,000 g for 1 hr. Histones are extracted from the resulting purified chromatin with 0.4 M H_2SO_4 twice, 45 min for each time. The extracted histones in H_2SO_4 are precipitated with 5 volumes of ethanol at -20°C for 48 hr, and washed with 80% ethanol. The pellet is dried and resuspended in 50-100 μl of 0.01 N HCl overnight. All procedures are carried out at 4°C, and all solutions should be added PMSF to a final concentration of 0.1 mM as a protease inhibitor.

Buffer A: 0.4 M sucrose; 10 mM Tris-HCl (pH8.0); 10 mM $MgCl_2$; and 5 mM β-mercaptoethanol.

Buffer B: 0.25 M sucrose; 10 mM Tris-HCl (pH8.0); 10 mM $MgCl_2$; 1% Triton X-100; and 5 mM β-mercaptoethanol.
Buffer C: 1.7 M sucrose; 10 mM Tris-HCl (pH8.0); 2 mM $MgCl_2$; 0.15% Triton X-100; and 5 mM β-mercaptoethanol.

V-2-6 Immunofluorescence staining

5 ml of 3-day BY2 cells is transferred into a 15 ml Falcon tube and centrifuged at 1,000 rpm for 5 min. After removing the liquid medium, 4.5 ml of fixation buffer is added to fix the cells for 1 hr by mild mixing. The cells are washed 4 times with MSB/glycerol buffer containing 0.05% Triton X-100 (30 min for each time). The cells are deposited on a coverslip treated previously with polylysine (Sigma). Digestion buffer containing enzymes (0.02% pectolyase, 0.1% macerozyme and 0.3% caylase) is added on the cells to digest the cell wall for 10 min. The coverslip is then washed 3 times with PBS, and once with PBS plus 0.1% BSA. PBS containing 0.1% BSA and 5% Normal Goat Serum is added onto the coverslip for saturation for 20 min. The primary antibody (10 times amount of that for western blot) is added for incubation at 4°C overnight. After washing 4 times with PBS plus 0.1% BSA, the secondary antibody conjoint with specific dye is added for incubation for 1 hr. After washing 4 times with PBS, PBS containing 1 μg/ml DAPI is used to stain the nucleus for 5 min. After washing, 60 μl DABCO (100 mg/ml in Mowiol) is added onto the coverslip, which finally covers onto the microscope slide.

Fixation buffer: 3.7% paraformaldehyde (PFA); 0.25% Triton X-100 in MSB/glycerol buffer.
MSB/glycerol buffer: 50 mM pipes (pH6.9); 5 mM EGTA; 1 mM $MgCl_2$; 2% glycerol.
Digestion buffer: 25 mM MES (pH 5.5); 8 mM $CaCl_2$; 600 mM Mannitol.

V-2-7 Chromatin immunoprecipitation (CHIP)

3-day-old TBY2 cells, 30-day-old tobacco seedlings and 20-day-old *Arabidopsis* seedlings are used for CHIP analysis. After crosslink in fix buffer, glycine is added to a final concentration of 0.125 M. 2 g of plant material is ground for each immunoprecipitation and is homogenized in 10 ml lysis buffer plus 10% glycerol and protease inhibitor cocktail (Roche). After homogenization, the whole lysate is centrifugated at 3,500 rpm at 4°C for 10 min. The pellet is resuspended in 1 ml lysis buffer. DNA is sheared by sonication to approximately 500~1000 bp fragments. After centrifugation at top speed for 10 min at 4°C in a microfuge,

the supernatant is pre-cleared by adding 40 µl of Protein A beads (Amersham Biosciences) and rotating at 4°C for 1 hr. After centrifugation at 500 g for 2 min at 4°C, the supernatant is mixed with the appropriate antibody at 1:100 dilution, followed by an overnight incubation at 4°C. The immune complexes are collected by adding 40 µl of Protein A beads and rotating at 4°C for 1 hr. The Protein A beads are washed with 1 ml of each of the following wash buffers at 4°C for 5 min: 1 x lysis buffer; 1 x high salt wash buffer; 1 x LiCl wash buffer; and 2 x TE. The immune complexes are then eluted twice with 200 µl of elution buffer from the beads, by incubation at 65°C for 15 min with agitation. A total of 16 µl 5 M NaCl is then added to about 400 µl elution products, and protein-DNA crosslink is reversed by incubation at 65°C for 5~6 hr. Following phenol/chloroform extraction and ethanol precipitation, the pellet is washed with 70% ethanol and dissolved in 30 µl H_2O.

Fix buffer: 0.4M sucrose; 10 mM Tris-HCl (pH 8.0); 1 mM EDTA; 1 mM PMSF; 1.0% formaldehyde.
Lysis buffer: 50 mM HEPES (pH 7.5); 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 5 mM β-mercaptoethano.
High salt wash buffer: lysis buffer containing 500 mM NaCl.
LiCl wash buffer: 0.25 M LiCl; 0.5% NP-40; 1 mM EDTA; 10 mM Tris-HCl (pH 8.0); 5 mM β-mercaptoethano.
Elution buffer: 1% SDS; 0.1 M $NaHCO_3$.

V-2-8 Dam identification (DamID)

40 µg of the isolated genomic DNA is digested for 16 hr at 37°C with 40 units *Dpn*I (Fermentas), which selectively cuts the GATC sequence where A is methylated, in a total volume of 50 µl. After inactivation of *Dpn*I at 80°C for 20 min, 5 µg of the *Dpn*I digested genomic DNA is ligated to the double stranded catch linker (top strand: 5'-GCGGTGACCCGGGAGATCTGAATTC-3', bottom strand: 5'-GAATTCAGATC-3') at 16°C overnight with 5 units of T4 Ligase (Fermentas) in a total volume of 20 µl. To prevent amplification of DNA fragments containing unmethylated GATCs, 5 µg of the linker-ligated DNA is cut with 10 units *Dpn*II (Biolabs) for 2 hr at 37°C in a total volume of 20 µl, which only cuts the unmethylated GATC. PCR is then performed to amplify the methylated DNA fragments using 0.5 µg *Dpn*II-cut DNA, and the primer specific to the linker plus TC at its 3'

end (5'-TGACCCGGGAGATCTGAATTCTC-3'). To test the enrichment of the targets, second PCR is performed using the corresponding primers.

VI

REFERENCES

References

Ahmad, K., and Henikoff, S. (2002) The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol. Cell* 9, 1191-1200.

Almeida, R., and Allshire, R.C. (2005) RNA silencing and genome regulation. *Trends Cell Biol.* 15, 251-258.

Alvarez-Venegas, R., Pien, S., Sadder, M., Witmer, X., Grossniklaus, U., and Avramova, Z. (2003) ATX-1, an *Arabidopsis* homolog of Trithorax, activates flower homeotic genes. *Curr. Biol.* 13, 627-637.

Aufsatz, W., Mette, M.F., van der Winden, J., Matzke, M., and Matzke, A.J.M. (2002) HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. *EMBO J.* 21, 6832-6841.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* 410, 120-124.

Barber, C.M., Turner, F.B., Wang, Y., Hagstrom, K., Taverna, S.D., Mollah, S., Ueberheide, B., Meyer, B.J., Hunt, D.F., Cheung, P., and Allis, C.D. (2004) The enhancement of histone H4 and H2A serine 1 phosphorylation during mitosis and S-phase is evolutionarily conserved. *Chromosoma* 112, 360-371.

Bartee, L., Malagnac, F., and Bender, J. (2001) *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes Dev.* 15, 1753-1758.

Baulcombe, D. (2004) RNA silencing in plants. *Nature* 431, 356-363.

Baumbusch, L.O., Thorstensen, T., Krauss, V., Fischer, A., Naumann, K., Assalkhou, R., Schulz, I., Reuter, G., and Aalen, R.B. (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, 4319-4333.

Beisel, C., Imhof, A., Greene, J., Kremmer, E., and Sauer, F. (2002) Histone methylation by the *Drosophila* epigenetic transcriptional regulator ASH1. *Nature* 419, 857-862.

Bernstein, B.E., Humphrey, E.L., Erlich, R.L., Schneider, R., Bouman, P., Liu, J.S., Kouzarides, T., and Schreiber, S.L. (2002) Methylation of histone H3 Lys 4 in coding regions of active genes. *Proc. Natl. Acad. Sci. USA* 99, 8695-8700.

Bertrand, C., Bergounioux, C., Domenichini, S., Delarue, M., and Zhou, D.-X. (2003) *Arabidopsis* histone acetyltransferase AtGCN5 regulates the floral meristem activity through the *WUSCHEL/AGAMOUS* pathway. *J. Biol. Chem.* 278, 28246-28251.

Boniotti, M.B., and Gutierrez, C. (2001) A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in *Arabidopsis*, a CDKA/cyclin D complex. *Plant J.*

28, 341-350.

Bordoli, L., Netsch, M., Luthi, U., Lutz, W., and Eckner, R. (2001) Plant orthologs of p300/CBP: conservation of a core domain in metazoan p300/CBP acetyltransferase-related proteins. *Nucleic Acids Res*. 29, 589-597.

Bracken, A.P., Ciro, M., Cocito, A., and Helin, K. (2004) E2F target genes: unraveling the biology. *Trends Biochem. Sci.* 29, 409-417.

Briggs, S.D., Bryk, M., Strahl, B.D., Cheung, W.L., Davie, J.K., Dent, S.Y., Winston, F., and Allis, C.D. (2001) Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. *Genes Dev.* 15, 3286-3295.

Bryk, M., Briggs, S.D., Strahl, B.D., Curcio, M.J., Allis, C.D., and Winston, F. (2002) Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in *S. cerevisiae* by a Sir2-independent mechanism. *Curr. Biol.* 12, 165-170.

Brzeski, J., and Jerzmanowski, A. (2002) Deficient in DNA methylation 1 (DDM1) defines a novel family of chromatin remodeling factors. *J. Biol. Chem.* 278, 823-828.

Callard, D., and Mazzolini, L. (1997) Identification of proliferation-induced genes in *Arabidopsis thaliana. Plant Physiol.* 115, 1385-1395.

Cao, X., Springer, N.M., Muszynski, M.G., Phillips, R.L., Kaeppler, S., and Jacobsen, S.E. (2000) Conserved plant genes with similarity to mammalian *de novo* DNA methyltransferases. *Proc. Natl Acad. Sci. USA* 97, 4979-4984.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298, 1039-1043.

Carrozza, M.J., Li, B., Florens, L., Suganuma, T., Swanson, S.K., Lee, K.K., Shia, W.-J., Anderson, S., Yates, J., Washburn, M.P., and Workman, J.L. (2005). Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. *Cell* 123, 581-592.

Chabouté, M.E., Clement, B., Sekine, M., Philipps, G., and Chaubet-Gigot, N. (2000) Cell cycle regulation of the tobacco ribonucleotide reductase small subunit gene is mediated by E2F-like elements. *Plant Cell* 12, 1987-2000.

Chan, S.M., Henderson, I.R., and Jacobsen, S.E. (2005) Gardening the genome: DNA methylation in *Arabidopsis Thaliana*. *Nat. Rev. Genet.* 6, 351-360.

Chaubet-Gigot, N. (2000) Plant A-type cyclins. *Plant Mol. Biol.* 43, 659-675.

Cheung, P., Tanner, K.G., Cheung, W.L., Sassone-Corsi, P., Denu, J.M., and Allis, C.D. (2000) Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol. Cell* 5, 905-915.

Cheung, W.L., Turner, F.B., Krishnamoorthy, T., Wolner, B., Ahn, S.H., Foley, M., Dorsey, J.A., Peterson, C.L., Berger, S.L., and Allis, C.D. (2005) Phosphorylation of histone H4 serine 1 during DNA damage requires Casein Kinase II in *S. cerevisiae. Curr. Biol.* 15, 656-660.

Choi, Y., Gehring, M., Johnson, L., Hannon, M., Harada, J.J., Goldberg, R.B., Jacobsen, S.E., and Fischer, R.L. (2002) DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in *Arabidopsis*. *Cell* 110, 33-42.

Chua, Y.L., Brown, A.P., and Gray, J.C. (2001) Targeted histone acetylation and altered nuclease accessibility over short regions of the pea plastocyanin gene. *Plant Cell* 13, 599-612.

Chua, Y.L., Watson, L.A., and Gray, J.C. (2003) The transcription enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. *Plant Cell* 15, 1468-1479.

Clayton, A.L., Rose, S., Barratt, M.J., and Mahadevan, L.C. (2000) Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. *EMBO J.* 19, 3714-3726.

Cockcroft, C.E., den Boer, B.G., Healy, J.M., and Murray, J.A. (2000) Cyclin D control of growth rate in plants. *Nature* 405, 575-579.

Couture, J.F., Collazo, E., Brunzelle, J.S., and Trievel, R.C. (2005) Structural and functional analysis of SET8, a histone H4 Lys-20 methyltransferase. *Genes Dev.* 19, 1455-1465.

Cuthbert, G.L., Daujat, S., Snowden, A.W., Erdjument-Bromage, H., Hagiwara, T., Yamada, M., Schneider, R., Gregory, P.D., Tempst, P., Bannister, A.J., and Kouzarides, T. (2004) Histone deimination antagonizes arginine methylation. Cell *118*, 545-553.

de Jager, S.M., Maughan, S., Dewitte, W., Scofield, S., and Murray, J.A. (2005) The developmental context of cell-cycle control in plants. *Semin. Cell Dev. Biol.* 16, 385-396.

de Veylder, L., Beeckman, T., Beemster, G.T., de Almeida Engler, J., Ormenese, S., Maes, S., Naudts, M., Van Der Schueren, E., Jacqmard, A., Engler, G., and Inzé, D. (2002) Control of proliferation, endoreduplication and differentiation by the *Arabidopsis* E2Fa-DPa transcription factor. *EMBO J.* 21, 1360-1368.

de Veylder, L., Beeckman, T., Beemster, G.T., Krols, L., Terras, F., Landrieu, I., Van Der Schueren, E., Maes, S., Naudts, M., and Inzé, D. (2001) Functional analysis of cyclin-dependent kinase inhibitors of *Arabidopsis*. *Plant Cell* 13, 1653-1658.

de Veylder, L., Joubès, J., and Inzé, D. (2003). Plant cell cycle transitions. *Curr. Opin. Plant Biol.* 6, 536-543.

Del Pozo, J.C., Boniotti, M.B., and Gutierrez, C. (2002) *Arabidopsis* E2Fc functions in cell division and is degraded by the ubiquitin-SCF (AtSKP2) pathway in response to light. *Plant Cell* 14, 3057-3071.

Demidov, D., VanDamme, D., Geelen, D., Blattner, F.R., and Houben, A. (2005) Identification and dynamics of two classes of Aurora-like kinases in *Arabidopsis* and other plants. *Plant Cell* 17, 836-848.

Desvoyes, B., Ramirez-Parra, E., Xie, Q., Chua, N.-H., and Gutierrez, C. (2006) Cell type-specific role of the retinoblastoma/E2F pathway during *Arabidopsis* leaf development. *Plant Physiol.* 140, 67-80.

Dewitte, W., and Murray, J.A.H. (2003) The plant cell cycle. *Annu. Rev. Plant Biol.* 54, 235-264.

Dewitte, W., Riou-Khamlichi, C., Scofield, S., Healy, J.M., Jacqmard, A., Kilby, N.J., and Murray, J.A. (2003) Altered cell cycle distribution, hyperplasia, and inhibited differentiation in *Arabidopsis* caused by the D-type cyclin CYCD3. *Plant Cell* 15, 79-92.

Dong, A., Liu, Z., Zhu, Y., Yu, F., Li, Z., Cao, K., and Shen, W.-H. (2005) Interacting proteins and differences in nuclear transport reveal specific functions for the NAP1 family proteins in plants. *Plant Physiol.* 138, 1446-1456.

Dong, A., Zhu, Y., Yu, Y., Cao, K., Sun, C., and Shen, W.-H. (2003) Regulation of biosynthesis and intracellular localization of rice and tobacco homologues of nucleosome assembly protein 1. *Planta* 216, 561-570.

Dover, J., Schneider, J., Tawiah-Boateng, M.A., Wood, A., Dean, K., Johnston, M., and Shilatifard, A. (2002) Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. *J. Biol. Chem.* 277, 28368-28371.

Dutnall, R.N. (2003) Cracking the histone code: one, two, three methyls, you're out! *Mol. Cell* 12, 3-4.

Ebbs, M.L., Bartee, L., and Bender, J. (2005) H3 lysine 9 methylation is maintained on a transcribed inverted repeat by combined action of SUVH6 and SUVH4 methyltransferases. *Mol. Cell. Biol.* 25, 10507-10515.

Ebel, C., Mariconti, L., and Gruissem, W. (2004) Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. *Nature* 429, 776-780.

Fang, J., Feng, Q., Ketel, C.S., Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Tempst, P., Simon, J.A., and Zhang, Y. (2002) Purification and functional characterization of SET8, a nucleosomal histone H4-lysine 20-specific methyltransferase. *Curr. Biol.* 12, 1086-1099.

Fang Y., and Spector, D.L. (2005) Centromere positioning and dynamics in living *Arabidopsis* plants. *Mol. Biol. Cell* 16, 5710-5718.

Farrona, S., Hurtado, L., Bowman, J.L., and Reyes, J.C. (2004) The *Arabidopsis thaliana* SNF2 homolog AtBRM controls shoot development and flowering. *Development* 131, 4965-4975.

Feng, Q., Wang, H., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Struhl, K., and Zhang, Y. (2002). Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET

domain. *Curr. Biol.* 12, 1052-1058.

Fischle, W., Tseng, B.S., Dormann, H.L., Ueberheide, B.M., Garcia, B.A., Shabanowitz, J., Hunt, D.F., Funabiki, H., and Allis, C.D. (2005) Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature* 438, 1116-1122.

Fischle, W., Wang, Y., and Allis, C.D. (2003) Histone and chromatin cross-talk. *Curr. Opin. Cell Biol.* 15, 172-183.

Fransz, P., Armstrong, S., Alonso-Blanco, C., Fischer, T.C., Torres-Ruiz, R.A., and Jones, G. (1998) Cytogenetics for the model system *Arabidopsis thaliana*. *Plant J.* 13, 867-876.

Fransz, P., De Jong, J.H., Lysak, M., Castiglione, M.R., and Schubert, I. (2002) Interphase chromosomes in *Arabidopsis* are organized as well defined chromocenters from which euchromatin loops emanate. *Proc. Natl. Acad. Sci. USA* 99, 14584-14589.

Friesner, J.D., Liu, B., Culligan, K., and Britt, A.B. (2005) Ionizing radiation-dependent γ-H2AX focus formation requires ataxia telangiectasia mutated and ataxia telangiectasia mutated and Rad3-related. *Mol. Biol. Cell* 16, 2566-2576.

Gaudin, V., Libault, M., Pouteau, S., Juul, T., Zhao, G., Lefebvre, D., and Grandjean, O. (2001) Mutations in *LIKE HETEROCHROMATIN PROTEIN 1* affect flowering time and plant architecture in *Arabidopsis*. *Development* 128, 4847-4858.

Gehring, M., Huh, J.H., Hsieh, T.-F., Penterman, J., Choi, Y., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (2006) DEMETER DNA glycosylase establishes *MEDEA* polycomb gene self-imprinting by allele-specific demethylation. *Cell* 124, 495-506.

Gendrel, A.V., Lippman, Z., Yordan, C., Colot, V., and Martienssen, R.A. (2002) Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. *Science* 297, 1871-1873.

Goll, M.G., Kirpekar, F., Maggert, K.A., Yoder, J.A., Hsieh, C.-L., Zhang, X., Golic, K.G., Jacobsen, S.E., and Bestor, T.H. (2006) Methylation of $tRNA^{Asp}$ by the DNA Methyltransferase Homolog Dnmt2. *Science* 311, 395-398.

Gong, Z., Morales-Ruiz, T., Ariza, R.R., Roldan-Arjona, T., David, L., and Zhu, J.K. (2002) ROS1, a repressor of transcriptional gene silencing in *Arabidopsis*, encodes a DNA glycosylase/lyase. *Cell* 111, 803-814.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997) A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis. Nature* 386, 44-51.

Grafi, G., Burnett, R.J., Helentjaris, T., Larkins, B.A., DeCaprio, J.A., Sellers, W.R., and Kaelin, W.G.Jr (1996) A maize cDNA encoding a member of the retinoblastoma protein family: involvement in endoreduplication. *Proc. Natl. Acad. Sci. USA* 93, 8962-8967.

Grossniklaus, U., Vielle-Calzada, J.P., Hoeppner, M.A., and Gagliano, W.B. (1998) Maternal control of embryogenesis by *MEDEA*, a Polycomb group gene in *Arabidopsis. Science* 280, 446-450.

Gutierrez, C. (2005) Coupling cell proliferation and development in plants. *Nature Cell Biol.* 7, 535-541.

Guyomarc'h, S., Bertrand, C., Delarue, M., and Zhou, D.-X. (2005) Regulation of meristem activity by chromatin remodelling. *Trends Plant Sci.* 10, 332-338.

Hall, A.E., Keith, K.C., Hall, S.E., Copenhaver, G.P., and Preuss, D. (2004) The rapidly evolving field of plant centromeres. *Curr. Opin. Plant Biol.* 7, 108-114.

Harbour, J.W., and Dean, D.C. (2000) The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev.* 14, 2393-2409.

Hemerly, A.S., Ferreira, P.C.G., van Montagu, M., Engler, G., and Inzé, D. (2000) Cell division events are essential for embryo patterning and morphogenesis: studies on dominant negative *cdc2aAt* mutants of *Arabidopsis. Plant J.* 23, 123-130.

Henikoff, S., Ahmad, K., and Malik, H.S. (2001) The centromere paradox: stable inheritance with rapidly evolving DNA. *Science* 293, 1098-1102.

Hirota, T., Lipp, J.J., Toh, B.-H., and Peters, J.-M. (2005) Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature* 438, 1176-1180.

Hsieh, T.F., and Fischer, R.L. (2005) Biology of chromatin dynamics. *Annu. Rev. Plant Biol.* 56, 327-351.

Huanca-Mamani, W., Garcia-Aguilar, M., Leon-Martinez, G., Grossniklaus, U., and Vielle-Calzada, J.-P. (2005) CHR11, a chromatin-remodeling factor essential for nuclear proliferation during female gametogenesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 102, 17231-17236.

Hwang, K.-K., Eissenberg, J.C., and Worman, H.J. (2001) Transcriptional repression of eurchromatic genes by *Drosophila* heterchromatin protein 1 and histone modifiers. *Proc. Natl. Acad. Sci. USA* 98, 11423-11427.

Hwang, M.G., Kim, K., Lee, W.K., and Cho, M.H. (2005) AtTBP2 and AtTRP2 in *Arabidopsis* encode proteins that bind plant telomeric DNA and induce DNA bending *in vitro*. *Mol. Genet. Genomics* 273, 66-75.

Imai, K.K., Ohashi, Y., Tsuge, T., Yoshizumi, T., Matsui, M., Oka, A., and Aoyama, T. (2006) The A-type cyclin CYCA2;3 is a key regulator of ploidy levels in *Arabidopsis* endoreduplication. *Plant Cell* 18, 382-396.

Jackson, J.P., Johnson, L., Jasencakova, Z., Zhang, X., PerezBurgos, L., Singh, P.B., Cheng, X., Schubert, I., Jenuwein, T., and Jacobsen, S.E. (2004) Dimethylation of histone H3 lysine 9 is a

critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. *Chromosoma* 112, 308-315.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002) Control of CpNpG DNA methylation by the KRYPONITE histone H3 methyltransferase. *Nature* 416, 556-560.

Jacobs, S.A, and Khorasanizadeh, S. (2002) Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science* 295, 2080-2083.

Jasencakova, Z., Soppe, W.J., Meister, A., Gernand, D., Turner, B.M., and Schubert, I. (2003) Histone modifications in *Arabidopsis* - high methylation of H3 lysine 9 is dispensable for constitutive heterochromatin. *Plant J.* 33, 471-480.

Jasinski, S., Perennes, C., Bergounioux, C., and Glab, N. (2002a) Comparative molecular and functional analyses of the tobacco cyclin-dependent kinase inhibitor NtKIS1a and its spliced variant NtKIS1b. *Plant Physiol.* 130, 1871-1882.

Jasinski, S., Riou-Khamlichi, C., Roche, O., Perennes, C., Bergounioux, C., and Glab, N. (2002b) The CDK inhibitor NtKIS1a is involved in plant development, endoreduplication and restores normal development of cyclin D3; 1-overexpressing plants. *J. Cell Sci.* 115, 973-982.

Jeddeloh, J., Stokes, T., and Richards, E. (1999) Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nat. Genet*. 22, 94-97.

Jenuwein, T., and Allis, C.D. (2001) Translating the histone code. *Science* 293, 1074-1080.

Jenuwein, T., Laible, G., Dorn, R., and Reuter, G. (1998) SET domain proteins modulate chromatin domains in eu- and heterochromatin. *Cell Mol. Life Sci.* 54, 80-93.

Jones, D.O., Cowell, I.G., and Singh, P.B. (2000) Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays* 22, 124-137.

Jones, L., Ratcliff, F., and Baulcombe, D.C. (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr. Biol.* 11, 747-757.

Joubès, J., De Schutter, K., Verkest, A., Inzé, D., and De Veylder, L. (2004) Conditional, recombinase-mediated, expression of genes in plant cell cultures. *Plant J.* 37, 889-896.

Joubès, J., and Chevalier, C. (2000) Endoreduplication in higher plants. *Plant Mol. Biol.* 43, 735-745.

Joubès, J., Chevalier, C., Dudits, D., Heberle-Bors, E., Inzé, D., Umeda, M., and Renaudin, J.P. (2000) CDK-related protein kinases in plants. *Plant Mol. Biol.* 43, 607-620.

Jullien, P.E., Katz, A., Oliva, M., Ohad, N., and Berger, F. (2006) Polycomb group complexes self-regulate imprinting of the Polycomb group gene *MEDEA* in *Arabidopsis*. *Curr. Biol.* 16, 486-492.

Jurgens, G. (2003) Growing up green: cellular basis of plant development. *Mech. Dev.* 120, 1395-1406.

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

Karachentsev, D., Sarma, K., Reinberg, D., and Steward, R. (2005) PR-Set7-dependent methylation of histone H4 Lys 20 functions in repression of gene expression and is essential for mitosis. *Genes Dev.* 19, 431-435.

Kaszas, E., and Cande, W.Z. (2000) Phosphorylation of histone H3 is correlated with changes in the maintenance of sister chromatid cohesion during meiosis in maize, rather than the condensation of the chromatin. *J. Cell Sci.* 113, 3217-322.

Kaya, H., Shibahara, K., Taoka, K., Iwabuchi, M., Stillman, B., and Araki, T. (2001) FASCIATA genes for chromatin assembly factor-1 in *Arabidopsis* maintain the cellular organization of apical meristems. *Cell* 104, 131-142.

Keogh, M.-C., Kurdistani, S.K., Morris, S.A., Ahn, S. H., Podolny, V., Collins, S.R., Schuldiner, M., Chin, K., Punna, T., Thompson, N.J., Boone, C., Emili, A., Weissman, J.S., Hughes, T.R., Strahl, B.D., Grunstein, M., Greenblatt, J.F., Buratowski, S., and Krogan, N.J. (2005) Cotranscriptional Set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. *Cell* 123, 593-605.

Kim, S.Y., He, Y., Jacob, Y., Noh, Y.-S., Michaels, S., and Amasino, R. (2005) Establishment of the vernalization-responsive, winter-annual habit in *Arabidopsis* requires a putative histone H3 methyl transferase. *Plant cell* 17, 3301-3310.

Kono, A., Ohno, R., Umeda-Hara, C., Uchimiya, H., and Umeda, M. (2006) A distinct type of cyclin D, CYCD4;2, involved in the activation of cell division in *Arabidopsis*. *Plant Cell Rep.* (Published online: 12 January 2006, DOI: 10.1007/s00299-005-0075-4)

Kornberg, R.D. (1974) Chromatin structure: A repeating unit of histones and DNA. *Science* 184, 868-871.

Koroleva, O.A., Tomlinson, M., Parinyapong, P., Sakvarelidze, L., Leader, D., Shaw, P., and Doonan, J.H. (2004) CycD1, a putative G1 cyclin from *Antirrhinum majus*, accelerates the cell cycle in cultured tobacco BY-2 cells by enhancing both G1/S entry and progression through S and G2 phases. *Plant Cell* 16, 2364-2379.

Kotake, T., Takada, S., Nakahigashi, K., Ohto, M., and Goto, K. (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, 555-564.

Kouzarides, T. (2002) Histone methylation in transcriptional control. *Curr. Opin. Genet. Dev.* 12, 198-209.

Krokan, H.E., Standal, R., and Slupphaug, G. (1997) DNA glycosylases in the base excision

repair of DNA. *Biochem. J.* 325, 1-16.

Krude, T., and Keller, C. (2001) Chromatin assembly during S phase: contributions from histone deposition, DNA replication and the cell division cycle. *Cell Mol. Life Sci.* 58, 665-672.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev.* 16, 2893-2905.

Lachner, M., and Jenuwein, T. (2002) The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.* 14, 286-298.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410, 116-120.

Larkins, B.A., Dilkes, B.P., Dante, R.A., Coelho, C.M., Woo, Y., and Liu, Y. (2001) Investigating the hows and whys of DNA endoreduplication. *J. Exp. Biol.* 52, 183-192.

LeBel, C., and Wellinger, R.J. (2005) Telomeres: what's new at your end? *J. Cell Sci.* 118, 2785-2788.

Libault, M., Tessadori, F., Germann, S., Snijder, B., Fransz, P., and Gaudin, V. (2005) The *Arabidopsis* LHP1 protein is a component of euchromatin. *Planta* 222, 910-925.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. (2001) Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science* 292, 2077-2080.

Lindroth, A.M, Shultis, D., Jasencakova, Z., Fuchs, J., Johnson, L., Schubert, D., Patnaik, D., Pradhan, S., Goodrich, J., Schubert, I., Jenuwein, T., Khorasanizadeh, S., and Jacobsen, S.E. (2004) Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3. *EMBO J.* 23, 4286-4296.

Lo, W.S., Trievel, R.C., Rojas, J.R., Duggan, L., Hsu, J.Y., Allis, C.D, Marmorstein, R., and Berger, S.L. (2000) Phosphorylation of serine 10 in histone H3 is functionally linked *in vitro* and *in vivo* to Gcn5-mediated acetylation at lysine 14. *Mol. Cell* 5, 917-926.

Loidl, P. (2004) A plant dialect of the histone language. *Trends Plant Sci.* 9, 84-90.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 389, 251-260.

Lusser, A., and Kadonaga, J.T. (2003) Chromatin remodeling by ATP-dependent molecular machines. *Bioessays* 25, 1192-1200.

Magyar, Z., De Veylder, L., Atanassova, A., Bako, L., Inze, D., and Bogre, L. (2005) The role of the *Arabidopsis* E2FB transcription factor in regulating auxin-dependent cell division. *Plant Cell* 17, 2527-2541.

Malagnac, F., Bartee, L., and Bender, J. (2002) An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation. *EMBO J.* 21, 6842-6852.

Margueron, R., Trojer, P., and Reinberg, D. (2005) The key to development: interpreting the histone code? *Curr. Opin. Genet. Dev*. 15, 163-176.

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

Martienssen, R.A., and Colot, V. (2001) DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science* 293, 1070-1074.

Masubelele, N.H., Dewitte, W., Menges, M., Maughan, S., Collins, C., Huntley, R., Nieuwland, J., Scofield, S., and Murray, J.A. (2005) D-type cyclins activate division in the root apex to promote seed germination in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 102, 15694-15699.

Mathieu, O., Jasencakova, Z., Vaillant, I., Gendrel, A.V., Colot, V., Schubert, I., and Tourmente, S. (2003) Changes in 5S rDNA chromatin organization and transcription during heterochromatin establishment in *Arabidopsis*. *Plant Cell* 15, 2929-2939.

Mathieu, O., Probst, A.V., and Paszkowski, J. (2005) Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG meth ylation in *Arabidopsis*. *EMBO J.* 24, 2783-2791.

Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A., and Matzke, A.J. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J.* 19, 5194-5201.

Milne, T.A., Briggs, S.D., Brock, H.W., Martin, M.E., Gibbs, D., Allis, C.D., and Hess, J.L. (2002) MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol. Cell* 10, 1107-1117.

Min, J., Zhang, X., Cheng, X., Grewal, S.I., and Xu, R.M. (2002) Structure of the SET domain histone lysine methyltransferase Clr4. *Nat. Struct. Biol.* 9, 828-832.

Morel, J.B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F., and Vaucheret, H. (2002) Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* 14, 629-639.

Morris, S.A., Shibata, Y., Noma, K., Tsukamoto, Y., Warren, E., Temple, B., Grewal, S.I.S., and Strahl, B.D. (2005) Histone H3 K36 methylation is associated with transcription elongation in *Schizosaccharomyces pombe*. *Eukaryot. Cell* 4, 1446-1454.

Murfett, J., Wang, X.J., Hagen, G., and Guilfoyle, T.J. (2001) Identification of *Arabidopsis* histone deacetylase *HDA6* mutants that affect transgene expression. *Plant Cell* 13, 1047-1061.

Nagata, T., Nemoto, Y., and Hasezawa, S. (1992) Tobacco BY-2 cell line as the "Hela" cell in the cell biology of higher plants. *Int. Rev. Cytol.* 132, 1-30.

Nakagami, H., Kawamura, K., Sugisaka, K., Sekine, M., and Shinmyo, A. (2002) Phosphorylation of retinoblastoma-related protein by the cyclin D/cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. *Plant Cell* 14, 1847-1857.

Nakagami, H., Sekine, M., Murakami, H., and Shinmyo, A. (1999) Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D *in vitro*. *Plant J.* 18, 243-252.

Nakahigashi, K., Jasencakova, Z., Schubert, I., and Goto, K. (2005) The *Arabidopsis* HETEROCHROMATIN PROTEIN 1 homolog (TERMINAL FLOWER2) silences genes within euchromatic region but not genes positioned in heterochromatin. *Plant Cell Physiol.* 46, 1747-1756.

Nakaia, T., Katoa, K., Shinmyoa, A., and Sekine, M. (2006) *Arabidopsis* KRPs have distinct inhibitory activity toward cyclin D2-associated kinases, including plant-specific B-type cyclin-dependent kinase. *FEBS Letters* 580, 336-340.

Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C.M., and Canaani, E. (2002) ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol. Cell* 10, 1119-1128.

Nakayama, J.-I, Rice, J.C., Strahl, B.D., Allis, C.D., and Grewal, S.I.S. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 292, 110-113.

Naumann, K., Fischer, A., Hofmann, I., Krauss, V., Phalke, S., Irmler, K., Hause, G., Aurich, A.C., Dorn, R., Jenuwein, T., and Reuter, G. (2005) Pivotal role of AtSUVH2 in heterochromatic histone methylation and gene silencing in *Arabidopsis*. *EMBO J*. 24, 1418-1429.

Ng, D.W., Chandrasekharan, M.B., and Hall, T.C. (2006) Ordered histone modifications are associated with transcriptional poising and activation of the *phaseolin* promoter. *Plant Cell* 18, 119-132.

Ng, H.H., Xu, R.M., Zhang, Y., and Struhl, K. (2002) Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. *J. Biol. Chem.* 277, 34655-34657.

Nielsen, S.J., Schneider, R., Bauer, U.M., Bannister, A.J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R.E., and Kouzarides, T. (2001) Rb targets histone H3 methylation and HP1 to promoters. *Nature* 412, 561-565.

Nishioka, K., Chuikov, S., Sarma, K., Erdjument-Bromage, H., Allis, C. D., Tempst, P., and Reinberg, D. (2002a) Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. *Genes Dev.* 16, 479-489.

Nishioka, K., Rice, J.C., Sarma, K., Erdjument-Bromage, H., Werner, J., Wang, Y., Chuikov, S., Valenzuela, P., Tempst, P., Steward, R., Lis, J.T., Allis, C.D., and Reinberg, D. (2002b) PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is

associated with silent chromatin. *Mol. Cell* 9, 1201-1213.

Noble, M.M., Endicott, J.A., Brown, N.R., and Johnson, L.N. (1997) The cyclin box fold: protein recognition in cell cycle and transcription control. *Trends Biol. Sci*. 22, 482-487.

Noh, B., Lee, S.H., Kim, H.J., Yi, G., Shin, E.A., Lee, M., Jung, K.J., Doyle, M.R., Amasino, R.M., and Noh, Y.S. (2004) Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of *Arabidopsis* flowering time. *Plant Cell* 16, 2601-2613.

Noh, Y.S., and Amasino, R.M. (2003) PIE1, an ISWI family gene, is required for *FLC* activation and floral repression in *Arabidopsis*. *Plant Cell* 15, 1671-1682.

Noma, K.I., and Grewal, S.I.S. (2002) Histone H3 lysine 4 methylation is mediated by Set1 and promotes maintenance of active chromatin states in fission yeast. *Proc. Natl. Acad. Sci. USA* 99, 16438-16445.

Nowak, S.J., and Corces, V.G. (2004) Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet.* 20, 214-220.

Ogas, J., Kaufmann, S., Henderson, J., and Somerville, C. (1999) PICKLE is a CHD3 chromatin-remodeling factor that regulates the transcription from embryonic to vegetative development in *Arabidopsis. Proc. Natl. Acad. Sci. USA* 96, 13839-13844.

Okada, T., Endo, M., Singh, M.B., and Bhalla, P.L. (2005) Analysis of the histone H3 gene family in *Arabidopsis* and identification of the male-gamete-specific variant AtMGH3. *Plant J*. 44, 557-568.

Ormenese, S., de Almeida Engler, J., De Groodt, R., De Veylder, L., Inze, D., and Jacqmard, A. (2004) Analysis of the spatial expression pattern of seven Kip related proteins (KRPs) in the shoot apex of *Arabidopsis thaliana*. *Ann. Bot.* 93, 575-580.

Pandey, R., Muller, A., Napoli, C.A., Selinger, D.A., Pikaard, C.S., Richards, E.J., Bender, J., Mount, D.W., and Jorgensen, R.A. (2002) Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res.* 30, 5036-5055.

Peters, A.H., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J.H., and Jenuwein, T. (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol. Cell* 12, 1577-1589.

Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schöfer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M., and Jenuwein, T. (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 107, 323-337.

Probst, A.V., Fagard, M., Proux, F., Mourrain, P., Boutet, S., Earley, K., Lawrence, R.J., Pikaard,

C.S., Murfett, J., Furner, I., Vaucheret, H., and Scheid, O.M. (2004) *Arabidopsis* histone deacetylase HDA6 is required for maintenance of transcriptional gene silencing and determines nuclear organization of rDNA repeats. *Plant Cell* 16, 1021-1034.

Ramirez-Parra, E., and Gutierrez, C. (2000) Characterizationof wheat DP, a heterodimerization partner of the plant E2F transcription factor which stimulates E2F-DNA binding. *FEBS Lett.* 486, 73-78.

Ramirez-Parra, E., Lopez-Matas, M.A., Frundt, C., and Gutierrez, C. (2004) Role of an atypical E2F transcription factor in the control of *Arabidopsis* cell growth and differentiation. *Plant Cell* 16, 2350-2363.

Ramirez-Parra, E., Xie, Q., Boniotti, M.B., and Gutierrez, C. (1999) The cloning of plant E2F, a retinoblastoma-binding protein, reveals unique and conserved features with animal G1/S regulators. *Nucleic Acids Res*. 27, 3527-3533.

Raska, I., Koberna, K., Malinsky, J., Fidlerova, H., and Masata, M. (2004) The nucleolus and transcription of ribosomal genes. *Biol. Cell* 96, 579-594.

Rayasam, G.V., Wendling, O., Angrand, P.O., Mark, M., Niederreither, K., Song, L., Lerouge, T., Hager, G.L., Chambon, P., and Losson, R. (2003) NSD1 is essential for early post-implantation development and has a catalytically active SET domain. *EMBO J.* 16, 3153-3163.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000) Regulation of chromatin structure by sitespecific histone H3 methyltransferases. *Nature* 40, 593-599.

Reichheld, J.P., Chaubet, N., Shen, W.-H., Renaudin, J.P., and Gigot, C. (1996) Multiple A-type cyclins express sequentially during the cell cycle in *Nicotiana tabacum* BY2 cells. *Proc. Natl. Acad. Sci. USA* 93, 13819-13824.

Renaudin, J.P., Doonan, J.H., Freeman, D., Hashimoto, J., Hirt, H., Inze, D., Jacobs, T., Kouchi, H., Rouze, P., Sauter, M., Savoure, A., Sorrell, D.A., Sundaresan, V., and Murray, J.A. (1996) Plant cyclins: a unified nomenclature for plant A-, B- and D-type cyclins based on sequence organization. *Plant Mol. Biol.* 32, 1003-1018.

Reyes, J.C. (2005) Chromatin modifiers that control plant development. *Curr. Opin. Plant Biol.* 8, 1-7.

Rice, J.C., Briggs, S.D., Ueberheide, B., Barber, C.M., Shabanowitz, J., Hunt, D.F., Shinkai, Y., and Allis, C.D. (2003) Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol. Cell* 12, 1591-1598.

Riha, K., and Shippen, D.E. (2003) Telomere structure, function and maintenance in *Arabidopsis*. *Chromosome Res.* 11, 263-275.

Rogakou, E.P., Boon, C., Redon, C., and Bonner, W.M. (1999) Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. *J. Cell Biol.* 146, 905-915.

Rogakou, E.P., Pich, D., Orr, A., Ivanova, V., and Bonner, W.M. (1998) DNA double stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* 273, 5858-5868.

Rossi, V., and Varotto, S. (2002) Insights into the G1/S transition in plants. *Planta* 215, 345-356.

Roudier, F., Fedorova, E., Lebris, M., Lecomte, P., Gyorgyey, J., Vaubert, D., Horvath, G., Abad, P., Kondorosi, A., and Kondorosi, E. (2003) The *Medicago* species A2-type cyclin is auxin regulated and involved in meristem formation but dispensable for endoreduplication-associated developmental programs. *Plant Physiol.* 131, 1091-1103.

Sanders, S.L., Portoso, M., Mata, J., Bahler, J., Allshire, R.C., and Kouzarides, T. (2004) Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. *Cell* 119, 603-614.

Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature* 419, 407-411.

Sarma, K., and Reinberg, D. (2005) Histone variants meet their match. *Mol. Cell Biol.* 6, 139-149.

Sato, H., Shibata, F., and Murata, M. (2005) Characterization of a Mis12 homologue in *Arabidopsis thaliana*. *Chromosome Res.* 13, 827-834.

Saze, H., Mittelsten-Scheid, O., and Paszkowski, J. (2003) Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nat. Genet.* 34, 65-69.

Schaft, D., Roguev, A., Kotovic, K.M., Shevchenko, A., Sarov, M., Shevchenko, A., Neugebauer, K.M., and Stewart, A.F. (2003) The histone 3 lysine 36 methyltransferase, SET2, is involved in transcriptional elongation. *Nucleic Acids Res.* 31, 2475-2482.

Schotta, G., Ebert, A., Krauss, V., Fischer, A., Hoffmann, J., Rea, S., Jenuwein, T., Dorn, R., and Reuter, G. (2002) Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.* 21, 1121-1131.

Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D., and Jenuwein, T. (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev*. 18, 1251-1262.

Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., and Rauscher, F.J., 3rd (2002) SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev.* 16, 919-932.

Shakirov, E.V., Surovtseva, Y.V., Osbun, N., and Shippen, D.E. (2005) The *Arabidopsis* Pot1 and Pot2 proteins function in telomere length homeostasis and chromosome end protection. *Mol. Cell. Biol.* 25, 7725-7733.

Shen, W.-H. (2001a) The plant cell cycle: G1/S regulation. *Euphytica.* 118, 223-232.

Shen, W.-H. (2001b) 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, 371-383.

Shen, W.-H. (2002). The plant E2F-Rb pathway and epigenetic control. *Trends Plant Sci.* 7, 505-511.

Shen, W.-H., and Meyer, D. (2004) Ectopic expression of the NtSET1 histone-methyltransferase inhibits cell expansion, and affects cell division and differentiation in tobacco plants. *Plant Cell Physiol.* 45, 1715-1719.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., and Shi, Y. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941-953.

Shimotohno, A., Matsubayashi, S., Yamaguchi, M., Uchimiya, H., and Umeda, M. (2003) Differential phosphorylation activities of CDK-activating kinases in *Arabidopsis thaliana*. *FEBS Lett.* 534, 69-74.

Shimotohno, A., Umeda-Hara, C., Bisova, K., Uchimiya, H., and Umeda, M. (2004) The plant-specific kinase CDKF;1 is involved in activating phosphorylation of cyclin-dependent kinase-activating kinases in *Arabidopsis*. *Plant Cell* 16, 2954-2966.

Singer, T., Yordan, C., and Martienssen, R.A. (2001) Robertson's mutator transposons in *A. thaliana* are regulated by the chromatin-remodeling gene decrease in DNA methylation (DDM1). *Genes Dev.* 15, 591-602.

Soppe, W.J., Jasencakova, Z., Houben, A., Kakutani, T., Meister, A., Huang, M.S., Jacobsen, S.E., Schubert, I., and Fransz, P. (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. *EMBO J*. 21, 6549-6559.

Springer, N.M., Napoli, C.A., Selinger, D.A., Pandey, R., Cone, K.C., Chandler, V.L., Kaeppler, H.F., and Kaeppler, S.M. (2003) Comparative analysis of SET domain proteins in maize and *Arabidopsis* reveals multiple duplications preceding the divergence of monocots and dicots. *Plant Physiol.* 132, 907-925.

Stewart, M.D., Li, J., and Wong, J. (2005) Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol. Cell Biol.* 25, 2525-2538.

Stockinger, E.J., Mao, Y., Regier, M.K., Triezenberg, S.J., and Thomashow, M.F. (2001) Transcriptional adaptor and histone acetyltransferase proteins in *Arabidopsis* and their interactions with CBF1, a transcriptional activator involved in cold-regulated gene expression. *Nucleic Acids Res.* 29, 1524-1533.

Strahl, B.D., Grant, P.A., Briggs, S.D., Sun, Z.W., Bone, J.R., Caldwell, J.A., Mollah, S., Cook,

R.G., Shabanowitz, J., Hunt, D.F., and Allis, C.D. (2002) Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. *Mol. Cell Biol.* 22, 1298-1306.

Sun, X., Wei, J., Wu, X., Hu, M., Wang, L., Wang, H., Zhang, Q., Chen, S., Huang, Q., and Chen, Z. (2005) Identification and characterization of a novel human histone H3 lysine 36-specific methyltransferase. *J. Biol. Chem.* 280, 35261-35271.

Sun, Z.W., and Allis, C.D. (2002) Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* 418, 104-108.

Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. (2001) Set domain containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. *J. Biol. Chem.* 276, 25309-25317.

Tachibana, M., Sugimoto, K., Nozaki, M., Ueda, J., Ohta, T., Ohki, M., Fukuda, M., Takeda, N., Niida, H., Kato, H., and Shinkai, Y. (2002) G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev*. 16, 1779-1791.

Talbert, P.B., Bryson, T.D., and Henikoff, S. (2004) Adaptive evolution of centromere proteins in plants and animals. *J. Biol.* 3, 18.

Talbert, P.B., Masuelli, R., Tyagi, A.P., Comai, L., and Henikoff, S. (2002) Centromeric localization and adaptive evolution of an *Arabidopsis* histone H3 variant. *Plant Cell* 14, 1053-1066.

Tamaru, H., and Selker, E.U. (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* 414, 277-283.

Tamaru, H., Zhang, X., McMillen, D., Singh, P.B., Nakayama, J., Grewal, S.I., Allis, C.D., Cheng, X., and Selker, E.U. (2003) Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*. *Nat. Genet.* 34, 75-79.

Tariq, M., Saze, H., Probst, A.V., Lichota, J., Habu, Y., and Paszkowski, J. (2003) Erasure of CpG methylation in *Arabidopsis* alters patterns of histone H3 methylation in heterochromatin. *Proc. Natl. Acad. Sci. USA* 100, 8823-8827.

Tian, L., and Chen, J.Z. (2001) Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. *Proc. Natl. Acad. Sci. USA* 98, 200-205.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P., and Zhang, Y. (2006) Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 439, 811-816.

Turner, B.M. (2002) Cellular memory and the histone code. *Cell* 111, 285-291.

Ueda, K., Kinoshita, Y., Xu, Z.J., Ide, N., Ono, M., Akahori, Y., Tanaka, I., and Inoue, M. (2000) Unusual core histones specifically expressed in male gametic cells of *Lilium longiflorum*. *Chromosoma* 108, 491-500.

Uemukai, K., Iwakawa, H., Kosugi, S., de Uemukai, S., Kato, K., Kondorosi, E., Murray, J.A., Ito, M., Shinmyo, A., and Sekine, M. (2005) Transcriptional activation of tobacco E2F is repressed by co-transfection with the retinoblastoma-related protein: cyclin D expression overcomes this repressor activity. *Plant Mol. Biol*. 57, 83-100.

Umeda, M., Shimotohno, A., and Yamaguchi, M. (2005) Control of cell division and transcription by cyclin-dependent kinase-activating kinases in plants. *Plant Cell Physiol*. 46, 1437-1442.

Vaistij, F.E., Jones, L., and Baulcombe, D.C. (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* 14, 857-867.

van Leeuwen, F., Gafken, P.R., and Gottschling, D.E. (2002) Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* 109, 745-756.

van Steensel, B., and Henikoff, S. (2000) Identification of *in vivo* DNA targets of chromatin proteins using tethered dam methyltransferase. *Nature Biotechnol.* 18, 424-428.

Vandel, L., Nicolas, E., Vaute, O., Ferreira, R., Ait-Si-Ali, S., and Trouche, D. (2001) Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyltransferase. *Mol. Cell Biol.* 21, 6484-6494.

Vandepoele, K., Raes, J., De Veylder, L., Rouze, P., Rombauts, S., and Inzé, D. (2002) Genome-wide analysis of core cell cycle genes in *Arabidopsis*. *Plant Cell* 14, 903–916.

Vandepoele, K., Vlieghe, K., Florquin, K., Hennig, L., Beemster, G.T., Gruissem, W., Van de Peer, Y., Inzé, D., and De Veylder, L. (2005) Genome-wide identification of potential plant E2F target genes. *Plant Physiol*. 139, 316-328.

Varmark, H. (2004) Functional role of centrosomes in spindle assembly and organization. *J. Cell. Biochem.* 91, 904-914.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* 303, 672-676.

Vlachonasios, K.E., Thomashow, M.F., and Triezenberg, S.J. (2003) Disruption mutations of *ADA2b* and *GCN5* transcriptional adaptor genes dramatically affect *Arabidopsis* growth, development, and gene expression. *Plant Cell* 15, 626-638.

Vlieghe, K., Boudolf, V., Beemster, G.T.S., Maes, S., Magyar, Z., Atanassova, A., de Almeida Engler, J., De Groodt, R., Inzé, D., and De Veylder, L. (2005) The DP-E2F-like gene *DEL1* controls the endocycle in *Arabidopsis thaliana*. *Curr. Biol.* 15, 59-63.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* 297, 1833-1837.

Wagner, D., and Meyerowitz, E.M. (2002) SPLAYED, a novel SWI/SNF ATPase homolog, controls reproductive development in *Arabidopsis*. *Curr. Biol*. 12, 85-94.

Walker, J.D., Oppenheimer, D.G., Concienne, J., and Larkin, J.C. (2000) *SIAMESE*, a gene controlling the endoreduplication cell cycle in *Arabidopsis thaliana* trichomes. *Development* 127, 3931-3940.

Wang, G., Kong, H., Sun, Y., Zhang, X., Zhang, W., Altman, N., dePamphilis, C.W., and Ma, H. (2004a) Genome-wide analysis of the cyclin family in *Arabidopsis* and comparative phylogenetic analysis of plant cyclin-like proteins. *Plant Physiol.* 135, 1084-1099.

Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Borchers, C., Tempst, P., and Zhang, Y. (2001a) Purification and functional characterization of a histone H3 - lysine 4 - specific methyltransferase. *Mol. Cell* 8, 1207-1217.

Wang, H., Huang, Z.Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B.D., Briggs, S.D., Allis, C.D., Wong, J., Tempst, P., and Zhang, Y. (2001b). Methylation of histone H4 at arginine 3 facilitates transcriptional activation by nuclear hormone receptor. *Science* 293, 853-857.

Wang, H., Zhou, Y., Gilmer, S., Whitwill, S., and Fowke, L.C. (2000) Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. *Plant J.* 24, 613-623.

Wang, W., and Chen, X. (2004) HUA ENHANCER3 reveals a role for a cyclin-dependent protein kinase in the specification of floral organ identity in *Arabidopsis*. *Dev.* 131, 3147-3156.

Wang, Y., Wysocka, J., Sayegh, J., Lee, Y.H., Perlin, J.R., Leonelli, L., Sonbuchner, L.S., McDonald, C.H., Cook, R.G., Dou, Y., Roeder, R.G., Clarke, S., Stallcup, M.R., Allis, C.D., and Coonrod, S.A. (2004b) Human PAD4 regulates histone arginine methylation levels via demethylimination. *Science* 306, 279-283.

Warburton, P.E. (2001) Epigenetic analysis of kinetochore assembly on variant human centromeres. *Trends Genet.* 17, 243-247.

Wassenegger, M., Heimes, S., Riedel, L., and Sanger, H.L. (1994) RNA-directed *de novo* methylation of genomic sequences in plants. *Cell* 76, 567-576.

Waterborg, J.H. (1992) Identification of five sites of acetylation in alfalfa histone H4. *Biochem.* 31, 6211-6219.

Wei, Y., Yu, L., Bowen, J., Gorovsky, M.A., and Allis, C.D. (1999) Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* 97, 99-109.

Wildwater, M., Campilho, A., Perez-Perez, J.M., Heidstra, R., Blilou, I., Korthout, H., Chatterjee,

J., Mariconti, L., Gruissem, W., and Scheres, B. (2005) The *RETINOBLASTOMA-RELATED* gene regulates stem cell maintenance in *Arabidopsis* roots. *Cell* 123, 1337-1349.

Williams, L., Zhao, J., Morozova, N., Li, Y., Avivi, Y., and Grafi, G. (2003) Chromatin reorganization accompanying cellular dedifferentiation is asssociated with modifications of histione H3, redistribution of HP1, and activation of E2F-target genes. *Dev. Dyn.* 228, 113-120.

Wilson, J.R., Jing, C., Walker, P.A., Martin, S.R., Howell, S.A., Blackburn, G.M., Gamblin, S.J., and Xiao, B. (2002) Crystal structure and functional analysis of the histone metyltransferase SET7/9. *Cell* 111, 105-115.

Wu, K., Tian, L., Malik, K., Brown, D., and Miki, B. (2000) Functional analysis of HD2 histone deacetylase homologues in *Arabidopsis thaliana*. *Plant J.* 22, 19-27.

Xiao, T., Hall, H., Kizer, K.O., Shibata, Y., Hall, M.C., Borchers, C.H., and Strahl, B.D. (2003) Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. *Genes Dev.* 17, 654-663.

Yang, L., Xia, L., Wu, D.Y., Wang, H., Chansky, H.A., Schubach, W.H., Hickstein, D.D., and Zhang, Y. (2002) Molecular cloning of ESET, a novel histone H3-specific methyltransferase that interacts with ERG transcription factor. *Oncogene* 21, 148-152.

Yu, Y., Dong, A., and Shen, W.-H. (2004) Molecular characterization of the tobacco SET domain protein NtSET1 unravels its role in histone methylation, chromatin binding, and segregation. *Plant J.* 40, 699-711.

Yu, Y., Steinmetz, A., Meyer, D., Brown, S., and Shen, W.-H. (2003) The tobacco A-type cyclin, *Nicta;CYCA3;2,* at the nexus of cell division and differentiation. *Plant Cell* 15, 2763-2777.

Zemach, A., Li, Y., Ben-Meir, H., Oliva, M., Mosquna, A., Kiss, V., Avivi, Y., Ohad, N., and Grafi, G. (2006) Different domains control the localization and mobility of LIKE HETEROCHROMATIN PROTEIN 1 in *Arabidopsis* Nuclei. *Plant Cell* 18, 133-145.

Zhang, X., Li, X., Marshall, J.B., Zhong, C.X., and Dawe, R.K. (2005) Phosphoserines on maize CENTROMERIC HISTONE H3 and histone H3 demarcate the centromere and pericentromere during chromosome segregation. *Plant Cell* 17, 572-583.

Zhang, X., Tamaru, H., Khan, S.I., Horton, J.R., Keefe, L.J., Selker, E.U., and Cheng, X. (2002) Structure of the *Neurospora* SET domain protein DIM-5, a histone H3 lysine methyltransferase. *Cell* 111, 117-127.

Zhang, Y., and Reinberg, D. (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev.* 15, 2343-2360.

Zhao, Z., and Shen, W.-H. (2004) Plants contain a high number of proteins showing sequence similarity to the animal SUV39H family histone-methyltransferases. *Ann. N.Y. Acad. Sci.* 1030, 661-669.

Zhao, Z., Yu, Y., Meyer, D., Wu, C., and Shen, W.-H. (2005) Prevention of early flowering by expression of *FLOWERING LOCUS C* requires methylation of histone H3 K36. *Nat. Cell Biol.* 7, 1156-1160.

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wua, K. (2005) HISTONE DEACETYLASE 19 is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. *Plant Cell* 17, 1196-1204.

Zhou, Y., Wang, H., Gilmer, S., Whitwill, S., and Fowke, L.C. (2003) Effects of co-expressing the plant CDK inhibitor ICK1 and D-type cyclin genes on plant growth, cell size and ploidy in *Arabidopsis thaliana*. *Planta* 216, 604-613.

Zilberman, D., Cao, X., and Jacobsen, S.E. (2003) ARGONAUTE4 control of locus specific siRNA accumulation and DNA and histone methylation. *Science* 299, 716-719.

Zuo, J., Niu, Q.W., and Chua, N.-H. (2000) Technical advance: an estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* 24, 265-273.

Résumé

Contrairement à celui des animaux, le développement des plantes est pour une grande part post-embryonnaire et beaucoup plus influencé par l'environnement. Ceci suggère que les plantes ont évolué en élaborant différents mécanismes pour contrôler leur développement et leur croissance.
La division cellulaire joue un rôle essentiel dans le développement et la croissance de la plante. Durant cette division, la transition G1-S est une étape décisive vers la prolifération cellulaire ou la différenciation. Par des approches génétiques, physiologiques et moléculaires, nous avons montré que Nicta;CYCA3;2 a des fonctions importantes, d'une maniére analogue à la cycline E (un régulateur essentiel à la transition G1/S) chez les animaux, dans le contrôle de la division et différentiation cellulaire.
Une fois la phase S initiée, l'ADN soit répliqué et la chromatine soit assemblé. La mise en place et la conservation d'états spécifiques de la chromatine fait appel à un mécanisme épigénétique pour assurer la transmission aux cellules filles d'une identité similaire de la cellule mère. Nos travaux sur NtSET1, une protéine à domaine SET chez le tabac, ont montré que cette protéine est capable de méthyler H3K9, qui marque majoritairement l'hétérochromatine. L'expression ectopique de *NtSET1* augmente la diméthylation de H3K9 et induit des défauts de ségrégation chromatinienne dans les cellules du tabac BY2. En plus, j'ai mis en évidence une interaction protéique entre NtSET1 et la protéine LHP1, l'homologue unique d'*Arabidopsis* de "heterochromatin protein 1" chez les animaux. Par l'immunolocalisation et l'identification des cibles ADN, j'ai montré que NtSET1-YFP et LHP1-YFP lient des régions hétérochromatiniennes, suggérant une implication dans la formation d'hétérochromatine. L'étude du gène *SET DOMAIN GROUP 8* (*SDG8*) chez l'*Arabidopsis* a montré que *SDG8* code une enzyme importante dans le contrôle de la méthylation en H3K36 chez l'*Arabidopsis*, qui régule positivement la transcription de *FLC* pour empêcher une floraison précoce.
En conclusion, l'ensemble de mes travaux de thèse m'avaient permis d'acquérir une bonne connaissance en développement de plantes. Les connaissances et techniques acquises en biologie moléculaire et cellulaire me permettraient non seulement d'envisager une poursuite dans la recherche végétale mais également de s'adapter avec facilité à une recherche sur un autre organisme.

Summary

The development of plants, compared with that of animals, is largely post-embryonic and influenced much more by the environment, suggesting that the plants have evolved and distinct mechanisms to control plant development and growth.
Cell division plays a vital role in plant development and growth. During the cell division, the G1-S transition is a crucial crossroad at the interface between cell proliferation and differentiation. By genetic, physiological, and molecular approaches, we demonstrate that Nicta;CYCA3;2 has important functions, analogous to those of cyclin E (an important regulator involved in G1-S transition) in animals, in the control of plant cell division and differentiation.
Once S phase is initiated, DNA is replicated and chromatin is assembled. The establishment and maintenance of specific chromatin states provides an epigenetic mechanism for inheriting expression states throughout plant development. Our studies show that the tobacco SET domain protein NtSET1 methylates H3K9, which marks primarily heterochromatin. Ectopic expression of *NtSET1* increases the amount of H3K9 dimethylation and induces chromosome segregation defects in tobacco BY2 cells. Furthermore, NtSET1 is shown to bind LHP1, the only *Arabidopsis* homologue of animal heterochromatin protein 1. By immunolocalization and *in vivo* target analysis, it shows that both NtSET1-YFP and LHP1-YFP bind heterochromatic regions, suggesting a mechanism of function in heterochromatin formation. Study of *Arabidopsis* SET domain group gene *SDG8* reveals that *SDG8* encodes a major enzyme controlling methylation of H3K36 in *Arabidopsis*, which positively regulates *FLC* transcription to prevent early flowering.
In conclusion, my thesis work allows me to acquire a better understanding of plant development. The knowledge and techniques acquired in molecular and cellular biology will not only enable me to continue the research in plants but also adapt with the research on other organisms.

Key words:

Nicotiana tabacum, *Arabidopsis thaliana*, cell cycle, cyclin, epigenetics, chromatin, histone methylation, SET domain