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Caractérisations moléculaires et fonctionnelles des gènes codant des protéines de la famille de 'Nucleosome Assembly Protein 1' (NAP1) chez les plantes

Soutenue le 17 Novembre 2006 devant le jury composé de:

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ZHU Yan

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TABLE OF CONTENTS

I Abbreviations

AP	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CTAB	cetyl trimethyl ammonium bromide
Da	Dalton
DAPI	4',6-Diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EB	ethidium bromide
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol tetra acetic acid
GFP	green fluorescent protein
GST	Glutathion-S-Transferase
hr	hour
IPTG	isopropyl-beta-D-thiogalactopyranoside
min	minute
MS	Murashige and Skoog
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenyl methyl sulphonyl fluoride
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulphate
sec	second
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBS	Tris buffered saline
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UV	ultra violet
YFP	yellow fluorescent protein

II INTRODUCTION

II-1 Chromatin

In eukaryotic cells, chromatin comprises huge DNA information ranging from 10 million to 100 billion bp. This “database” is confined in a “library” of nucleus with only a few micrometers in diameter (Richmond, 2006). The marvelous engineering of hierarchical chromatin folding is hence critical for its condensation and package to fit into this small volume. Chromatin folding is participated by mammoth amounts of basic protein histones and other functional nuclear proteins, mainly through largely unknown and complex mechanisms of protein-protein and protein-DNA interactions (Luger and Hansen, 2005). All the cellular processes targeting DNA as substrates, such as replication, recombination, repair and transcription, should overcome the structural barrier of chromatin structure. It becomes clear that any mechanism with the potential ability to alter the levels of chromatin compaction could inherently regulate DNA accessibility (Luger, 2006).

II-1-1 Heterochromatin and euchromatin

By using DNA coloration, chromatin in interphase can be visualized at microscopic level into two relatively distinct forms, heterochromatin and euchromatin (Fransz *et al.*, 2003). Heterochromatin is initially referred as those chromatin regions that remain densely stained and highly condensed throughout the cell cycle. With the increasing knowledge on chromatin, heterochromatin is assigned with several additional features, such as inactive in transcription and late in replication (Hennig, 1999). On the contrary, euchromatin is loosely packed during interphase and contains genes actively transcribed.

Heterochromatin can be either constitutive or facultative (Brown, 2002). Constitutive heterochromatin is highly condensed and consists of high amount of repeated DNA sequences, such as those present in the centromeric, pericentromeric and subtelomeric regions, whose decondensation only occurs during DNA replication in late S-phase. Facultative heterochromatin is not a permanent feature but is seen in some cells at certain time, and

contains loci activated in special stage and repressed in others (Brown, 2002). In recent years, heterochromatin studies have led to a dramatic advance in understanding epigenetic control of gene activity (Lund *et al.*, 2004; Bernstein and Allis, 2005). Nevertheless, the precise molecular interactions and structural changes at chromatin higher-order folding remain barely known (Grigoryev *et al.*, 2006).

II-1-2 Chromatin folding

The fundamental unit of chromatin is the nucleosome, which introduces supercoil responsible for the first level of DNA packaging and results in condensation for over 5 times in length. With the help of linker histones occupying the positions between nucleosomes, nucleosomes in the structure of “beads-on-a-string” are further compacted into fiber structure with 30 nm of diameter, in a way currently undetermined (Luger and Harsen, 2005). This level of compaction reflects the major euchromatic structure containing loci actively transcribed, and it is considered that most nuclear machineries targeting DNA carry on their functions efficiently on this level of “workshops” (Hayes and Harsen, 2001). Those domains embracing inert loci or intergenic repeat sequences are further condensed into higher hierarchical structures with the participation of other nuclear proteins, forming heterochromatin (Woodcock, 2006) (**see Figure II-1**).

II-2 Nucleosome

II-2-1 Nucleosome core

Nucleosome core is composed of histone octamer wrapped by approximate 146 bp of DNA in about 1.75 turns. Between nucleosome cores are found the linker histones (named as H1/H5) and linker DNA that is about 160 bp (yeast) to more than 200 bp (higher organisms) in length (Hayes and Harsen, 2001; Luger, 2006).

Histone octamer consists of two molecules of each of core histones H2A, H2B, H3 and H4. As the most slowly evolved proteins, histones present similar structures containing 2 distinct domains: the histone fold domain formed by three α-helices connected by two loops, and the

Hierarchical DNA compaction in nucleus

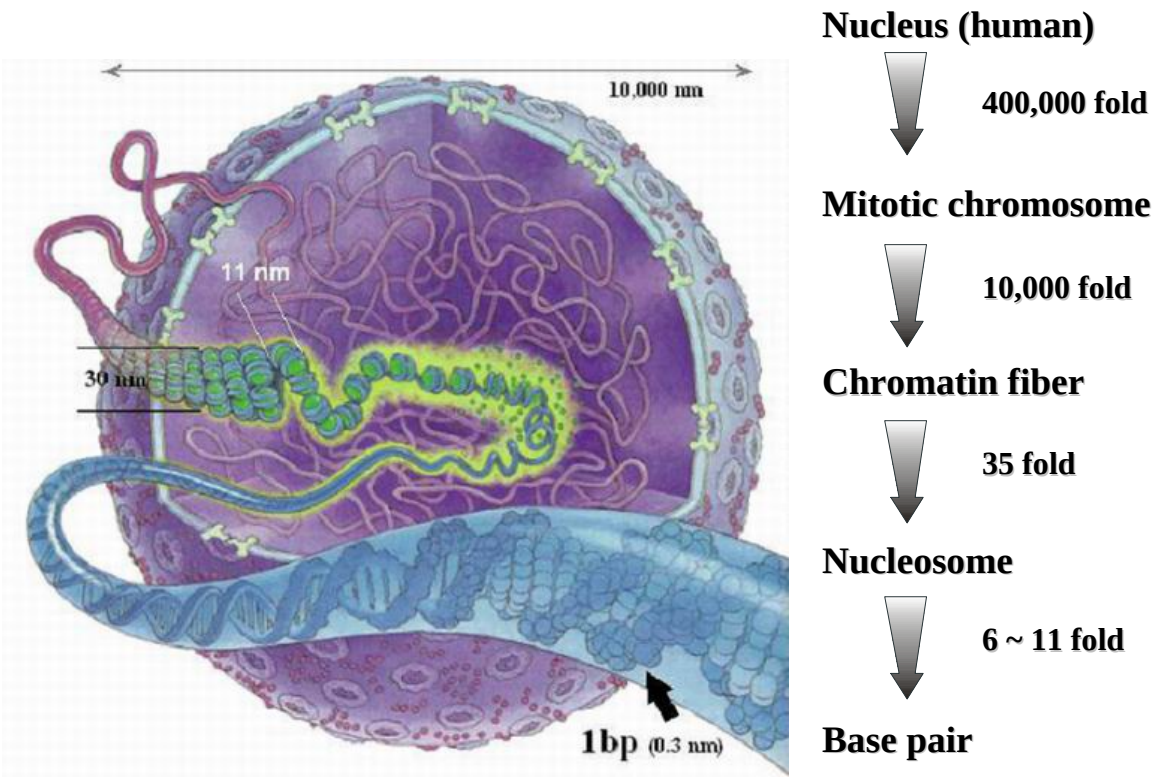

Figure II-1 DNA compaction in nucleus

Each level of DNA strucuture and compaction are shown, and size of corresponding organization are illustrated.

N-terminal tail domain consisting of 15-30 basic amino acids and protruding out of nucleosome surface (Khorasanizadeh, 2004). Biochemical studies have shown that in solutions of moderate salt and in the absence of DNA, the H3-H4 complex forms a tetramer and H2A-H2B complex forms a dimer in the “handshake” arrangement. These components then associate together to form the histone octamer in the presence of DNA or in buffered solutions containing more than 1M NaCl. H3 has a unique role within the nucleosome, as there is a 2-fold symmetry in the nucleosome organized directly along the dimer interface of the two H3 histones. Apart from heterodimerization with H4, H3 also forms direct contacts with histone H2A whereby the H2A-H2B dimers position themselves (Luger, 2006) (**see Figure II-2**).

More than 120 direct atomic interactions between histones and DNA backbone, and nearly equal number of water-mediated interactions are distributed at the 14 super-helix locations at the interface of these sub-complexes, while multiple electrostatic effect, hydrophobic effect and hydrogen bonds are required for nucleosome formation (Luger, 2003; Kamakaka and Biggins, 2005). Although nucleosome is the basic and repeating unit of chromatin and the sequence of DNA wrapped around histone octamer greatly varies, no obvious change in overall nucleosome structure depending on DNA sequence has been found in the crystal studies in the past decades (Luger, 2006). Currently it is considered that solvent water contributes greatly to the structural similarity by adapting histone surfaces to conformational variations in DNA (Davey *et al*., 2002). Nevertheless, as the basic components of nucleosome, histones have critical contribution in nucleosome structure. It is certain that modification on histone residues or alteration in primary sequence would affect nucleosome dynamics possibly via causing subtle structural variation or recruiting different chromatin-modifying factors, and hence influence the DNA accessibility and thereafter higher architecture.

II-2-2 Nucleosome surface

Histone N-terminal tails that protrude from nucleosome core contain the main sites for several covalent post-translational modifications, such as methylation on lysines and arginines, acetylation on lysines, phosphorylation on serines and threonines (Peterson and Laniel, 2004).

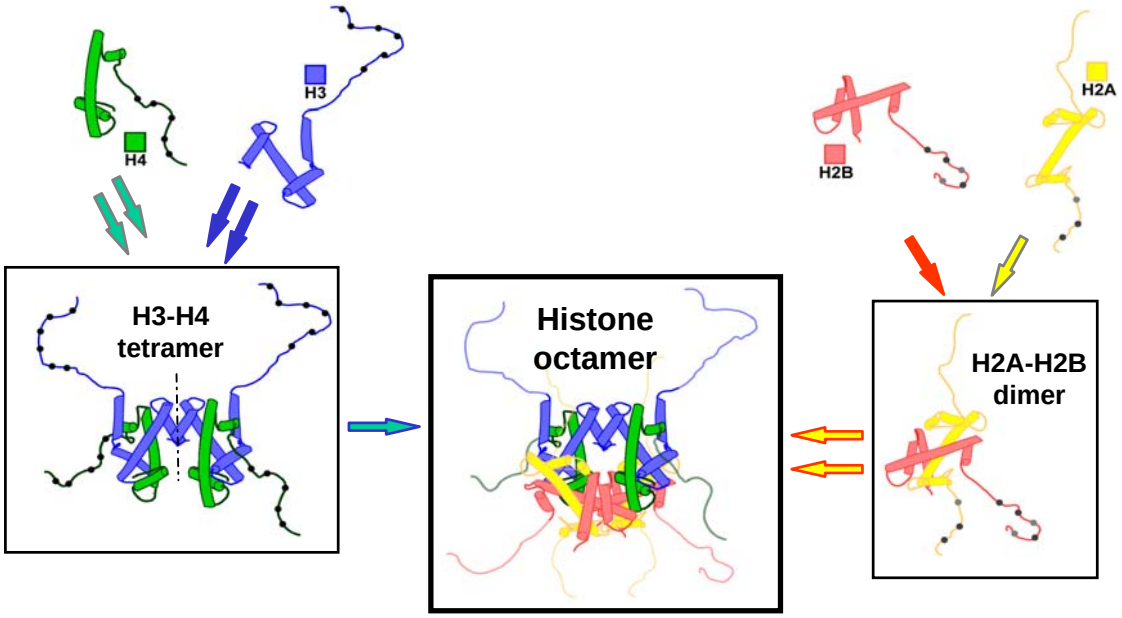

Figure II-2 Histone octamer assembly

Histone H3 and H4 form tetramer, while H2A and H2B form dimer. The 2-fold symmetry organized directly along the dimer interface of the two H3 histones is marked in dashed. Via interaction between H2A and H3, two dimers of H2A-H2B position themselves and form histone octamer.

These modifications are considered to be involved in the regulation of chromatin dynamics, in the ways of controlling the folding of the nucleosomal array into higher order structures, and mediating signaling for cellular processes. It is widely accepted that these covalent modifications function as “a histone code” in two levels: as a short-term signal to activate or repress specific gene(s) in response to cellular signaling, and as a stable marking system during cellular differentiation to inheritably determine specific chromatin states in an epigenetic manner (Loyola and Almouzni, 2004).

II-2-3 Variant nucleosomes

Besides canonical histones, most eukaryotes have evolved various histone variants that are non-allelic with the major ones. In general, canonical histone genes have multiple copies highly similar in sequence, primary expression during S-phase in cell cycle, and the products are incorporated in the chromatin throughout the genome. In contrast, histone variants usually present as single-copy genes that escape the strict restriction in S-phase and can be expressed during the whole cell cycle (Kamakaka and Biggins, 2005). After transferred into nucleus, histone variants are destined to being incorporated in specific functional domains in chromatin to carry out their corresponding cellular functions. In some cells, histone variants can especially replace the canonical histone positions and promote nucleosomal and further chromatic structure changes resulting in differentiated cellular functions. Variants of histones H2A and H3 have been known for decades, and recently they have emerged to the forefront of chromatin research because they are intimately involved in the specification of alternative chromatin states (Henikoff *et al.*, 2004).

II-3 Nucleosome assembly and histone chaperones

The whole chromatin is replicated during S-phase in cell cycle. Pre-existed nucleosomes are randomly transferred to both daughter strands after the passing of replication fork machinery, while the other half of the nucleosome complement is made from newly synthesized and nuclear-imported histones in a reaction known as *de novo* nucleosome assembly (Verreault, 2000).

Histones are highly positively charged due to basic residues rich in composition. While directly interacting with naked DNA in physiologic conditions, histones would cause precipitation due to opposing charges rather than functional interaction. Various histone chaperones are evolutionally introduced to prevent the electrostatic dysfunction. These chaperones, mainly as negatively charged molecules, interact with histones possibly via masking basic charges and promote the intrinsic character of histones for their deposition onto DNA and nucleosome assembly. Importantly, they are not the components consisted in the final assembly products (Polo and Almouzni, 2006). To date, increasing number of histone chaperones are successively isolated and identified (**see Table II-1**).

II-3-1 Nucleosome assembly

In S-phase, the bulk of canonical histones are imported into nucleus and deposited onto nascent DNA for nucleosome assembly tightly coupled with DNA replication, which is termed as replication-coupled (RC) pathway. Besides RC, canonical histones can also be incorporated during processes independent of replication, such as transcription and DNA repair, which is correspondingly termed as replication-independent (RI) pathway (Verreault, 2000). Histone variants are generally incorporated into nucleosomes mainly in RI pathway because of its constitutive expression pattern and specific cellular functions.

No matter in pathway of RC or RI, as well as by use of the canonical or variant, histones are assembled into mature and functional nucleosomes in a stepwise course, as presented in current models. First, $(H3\text{-}H4)_2$ tetramer is deposited onto naked DNA to form sub-nucleosomal particles via histone chaperone with preference to H3-H4, due to its higher affinity for DNA as compared to histone chaperone. Second, two dimers of H2A-H2B are deposited onto tetramer via histone chaperone with preference to H2A-H2B, due to their higher affinity for sub-nucleosomal particles as compared to either histone chaperone or DNA. However, this nascent state of nucleosome is immature and irregularly positioned along the template, and needs chromatin remodeling to form regular nucleosome array and functional regions, which is finally processed in the presence of ACF (ATP-utilizing chromatin

Table II-1, Histone Chaperones

Histone Chaperone	**Human**	**Mouse**	***Xenopus***	***Drosophila***	**Yeast**	***Arabidopsis***	**Related function**
CAF-1	p150	p150	p150	p150	Cac1	FAS1	chromatin assembly coupled to DNA synthesis, stable maintenance of silent chromatin states, DNA repair
	p60	p60	p60	p105	Cac2	FAS2	
	RbAp48	RbAp48	RbAp48	NURF-55	Cac3	MSI1	
Asf1	ASF1a	ASF1a	---	ASF-1	ASF-1	ASF1a	putative histone transfer, synergy with CAF-1 activities
	ASF1b	ASF1b				ASF1b	
HIRA	HIRA	HIRA	HIRA	HIRA	Hir1	HIRA	chromatin assembly independent of DNA replication
					Hir2		
N1/N2	---	---	N1/N2	---	---	---	storage of H3/H4 pools in *Xenopus* oocyte to be used for chromatin assembly
NAP-1	NAP-1	NAP-1	NAP-1	NAP-1	NAP-1	---	deposition of H2A/H2B, exchange of H2A/H2B variant
SET	TAF1α	TAF1α	TAF1α	SET	---	---	deposition of H2A/H2B, PP2A inhibitor, INHAT
	TAF1β	TAF1β	TAF1β				
Nucleoplasmin	---	---	Nucleoplasmin	Nucleoplasmin	---	---	storage of H2A/H2B pools in *Xenopus* oocyte to be used for chromain assembly, sperm chromatin decondensation

remodeling factors) (Nakagawa *et al.*, 2001; Tyler, 2002) (**see Figure II-3 and following Introduction**).

II-3-2 H3-H4 histone chaperones

There are many currently identified chaperones preferentially binding H3 and H4, such as CAF-1 (Chromatin Assembly Factor-1), RCAF (Replication-Coupling Assembly Factor), HIRA (Histone Regulation A) and N1/N2. CAF-1 is the best-documented chaperone well studied in divergent species, and is considered to be involved in the RC chromatin assembly, DNA repair and heterochromatin silencing; while RCAF is an intermediate and co-operator with CAF-1 participating in the replication. HIRA was found in the nucleosome assembly complex containing H3 variant H3.3, and responsible for its deposition and the corresponding RI pathway. N1/N2 role is speculated as species-specific histone storage, for its peculiar abundance in *Xenopus* oocyte.

II-3-2-1 CAF-1 (Chromatin Assembly Factor-1)

CAF-1 was initially identified by biochemical fractionation of extracts derived from human HeLa cells as an activity that allowed chromatin assembly coupled to DNA replication in the SV40 viral system (Smith and Stillman, 1989). With H3 and specifically acetylated isoforms of H4, i.e. acetylated lysine 5 and 12 in histone tail, CAF-1 forms CAC (Chromatin Assembly Complex), which was isolated from nuclei of human cells and could assemble nucleosomes in a RC manner (Verreault *et al.*, 1996). CAF-1 is evolutionarily conserved, and in most species, CAF-1 consists of three components: the large, mid and small subunits. In humans, the subunits correspond to p150, p60 and p48, in budding yeast to Cac1, Cac2 and Cac3, and in *Arabidopsis* to FAS1, FAS2 and MSI1 (Ridgway and Almouzni, 2000).

p150, the largest subunit of CAF-1, interacts directly with PCNA (Proliferating Cell Nuclear Antigen), an accessory factor in DNA polymerase complex, which elucidates the molecular mechanism whereby CAF-1 is coupled to replication fork, and provides the first molecular link between nucleosome assembly and DNA replication (Shibahara and Stillman, 1999).

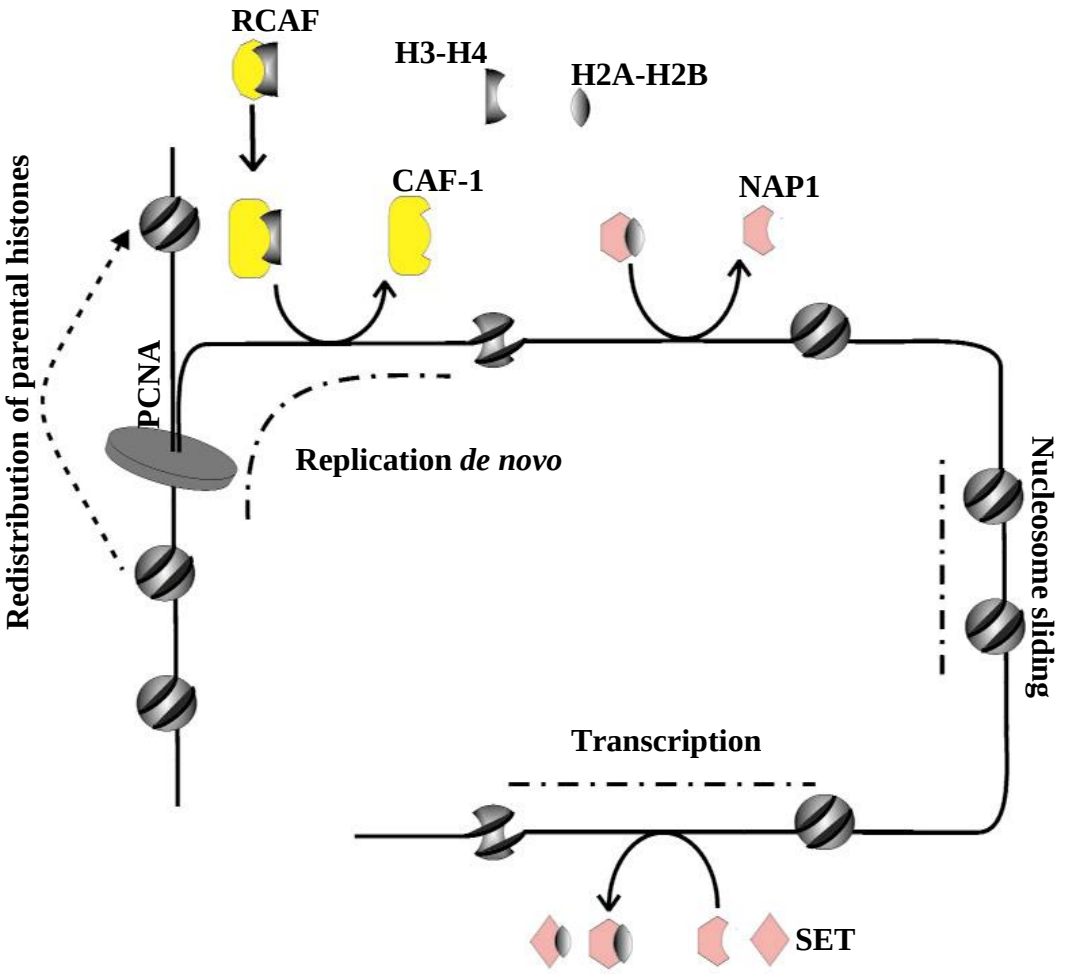

Figure II-3 Current model of participation of histone chaperone in nucleosome assembly

Parental nucleosomes are redistributed into daughter strands at random. For *de novo* assembly, $(H3\text{-}H4)_2$ tetramer is firstly deposited onto naked DNA facilitated by histone chaperones with preference to H3-H4; next, two dimers of H2A-H2B are deposited onto tetramer facilitated by histone chaperone with preference to H2A-H2B; finally, nascent nucleosomes are slided by ATP-utilizing chromatin remodeling complex for regular nucleosome array. Transcription activation needs the initial removal of H2A-H2B dimers for consequential activities.

Deletion of mammalian CAF-1 causes S-phase arrest and cell death, indicating the vital importance of CAF-1 replication-dependent assembly activity (Hoek and Stillman, 2003; Ye *et al.*, 2003; Nabatiyan and Krude, 2004). In contrast, loss of CAF-1 is not lethal in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*, implying uncertain pathways bypass CAF-1 function in plant and yeast cells to survive (Schönrock *et al.*, 2006).

Loss of CAF-1 is not lethal in yeast, but results in transcriptional release of some silenced genes at telomeres and increases the UV radiation sensitivity, suggested CAF-1 is required for the stable inheritance of transcriptionally repressed chromatin and responses to DNA damage (Monson *et al.*, 1997; Enomoto and Berman, 1998; Game and Kaufman, 1999). Functional genomic analysis of *fas1* and *fas2* mutants showed that CAF-1 was needed for the complete compaction of heterochromatin in *Arabidopsis* (Schönrock *et al.*, 2006), and loss of CAF-1 function could cause the release of certain repressive transcription such as *TSI*, as well as the increased sensitivity to genotoxic stress (Takeda *et al.*, 2004). Furthermore, phenotype analysis of *fas* mutants shows that CAF-1 maintains the cellular and functional organization of both shoot apical meristem (SAM) and root apical meristem (RAM). The mutants are defective in maintenance of the expression states of *WUSCHEL (WUS)* in SAM and *SCARECROW (SCR)* in RAM, suggesting that CAF-1 plays a critical role in the organization of SAM and RAM during post-embryonic development by facilitating stable maintenance of gene expression states (Kaya *et al.*, 2001).

II-3-2-2 ASF1 (Anti-Silencing Function 1)

The analysis of factors that are required in addition to CAF-1 for DNA replication-coupled chromatin assembly led to the identification of RCAF (Replication-Coupling Assembly Factor) complex from crude *Drosophila* embryo extract (Tyler *et al.*, 1999). *Drosophila* RCAF complex comprises histone H3 and H4 and the homologue of yeast ASF1 (Anti-Silencing Function 1), which was previously identified as a late G_1 phase-specific gene that derepresses transcriptional silencing when over-expressed (Le *et al.*, 1997). The specific acetylation patterns on H3 and H4 in RCAF complex are identical to those of newly synthesized histones to be assembled onto replicating DNA.

The eukaryotic ASF1 proteins are highly conserved through evolution in structure and function (Tamburini *et al.*, 2005). Yeast and *Drosophila* encode one ASF1 protein, while human genome contains two Asf1 homologues, Asf1a and Asf1b. In contrast to CAF-1 complex, RCAF/ASF1 is unable on its own to promote RC nucleosome deposition (Loyola and Almouzni, 2004). It functions synergistically with CAF-1 complex during *in vitro* chromatin assembly coupled to DNA replication and repair (Tyler *et al.*, 2001). Although 2 Asf1 homologues, Asf1a and Asf1b, are present in the *Arabidopsis* genome, no functional studies have been reported yet.

Currently, RCAF/ASF1 is proposed to transfer nascent histones to CAF-1, the specialized histone deposition factor complex. Thus it would be placed in a perfect position to monitor the fluctuation in the histone pools in a cycling somatic cell, and represent the prototype of a histone donor chaperone that could effectively ensure a constant supply of histones at sites of nucleosome assembly (Loyola and Almouzni, 2004).

II-3-2-3 HIRA (Histone Regulation A)

HIR/HIRA (Histone Regulation) genes were initially identified in yeast as negative regulators of histone gene expression (Sherwood and Osley, 1991). They represent a conserved family of proteins found in different species including yeast, *Drosophila*, *Xenopus*, mouse and human (Loyola and Almouzni, 2004). *Saccharomyces cerevisiae* contains two homologues, *HIR1* and *HIR2*; while in vertebrates there is only one, HIRA. Its ectopic over-expression leads to a transcriptional down-regulation of histone genes (Nelson *et al.*, 2002). HIRA was found in complex with histone variant H3.3 to facilitate chromatin assembly independent of DNA synthesis, distinct from the RC histone chaperone CAF-1 (Ray-Gallet *et al.*, 2002; Tagami *et al.*, 2004). The knockout of HIRA in *Arabidopsis* is embryo lethal. However, a decrease in the level of HIRA resulted in dramatic modifications in leaves in transgenic plant (Phelps-Durr *et al.*, 2005)

II-3-3 H2A-H2B histone chaperones

In the current knowledge, the main histone chaperones of H2A-H2B are nucleoplasmin and NAP1 (Nucleosome Assembly Protein 1). Nucleoplasmin is the typical *Xenopus* chaperone involved in histone storage in cooperation with N1/N2, and is not conserved in yeast and plants. NAP1 is considered as a highly conserved histone chaperone from yeast to human with preference to H2A-H2B dimer to facilitate the chromatin assembly and play roles in chromatin remodeling during cell proliferation and gene regulation.

II-3-3-1 NAP1 (Nucleosome Assembly Protein 1) family

NAP1 was first identified as a cellular factor that facilitates the nucleosome assembly *in vitro* by introducing supercoils into relaxed circular DNA in the presence of purified core histones (Ishimi *et al.*, 1983). Only one NAP1 gene is present in yeast, however, higher eukaryotes, from *Drosophila* to human, have evolved NAP1 families comprising multiple members.

Based on the result from yeast NAP1 protein crystal structure recently determined (Park and Luger, 2006a), while its N- and C-terminal regions are largely disordered, the central region is considered as stable structural entity, which was previously proved sufficient for histone binding and nucleosome assembly. It can be divided into two domains: domain I consists of one long α-helix flanked by two shorter α-helices, responding for protein dimerization. Domain II is a four-stranded anti-parallel β-sheet that is protected by α-helices at its underside, responding for protein-protein interaction (Park and Luger, 2006a).

According to the protein structure, another protein SET (SE Translocation) can also be classified into NAP1 family since it shares the central region, with difference in N- and C-terminus (Park and Luger, 2006b) (**see Figure II-4**). SET protein is not found in yeast, but conserved in multicellular eukaryotes. It was initially identified as the product of a gene that was fused to the *can* gene as a result of a translocation in a patient with acute undifferentiated leukemia (von Lindern *et al.*, 1992). In addition, it was independently characterized as a cellular factor required for the replication of purified adenovirus core particles *in vitro*,

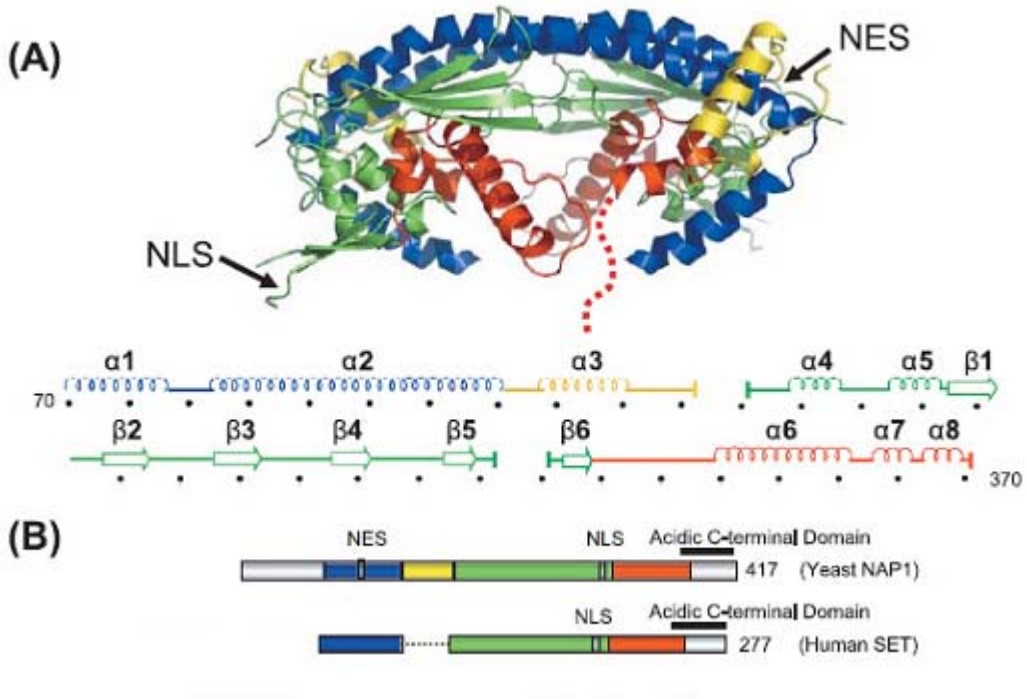

Figure II-4 Structural organization of NAP1 family (Park and Luger, 2006b)

(A) Overall structure of yeast NAP1. Subdomains are represented in different colors. Secondary structure assignments and amino acid numbers (in intervals of 10) are indicated below. The unstructured acidic C-terminal domain that is not a part of current model is indicated with a red dotted line. (B) Alignment of NAP family member, yeast NAP1 and human SET are shown. The position and length of the acidic C-terminal domain are indicated.

whereby it acquired the nomenclature of TAF1 (Template Activating Factor 1) (Matsumoto *et al.*, 1993).

II-3-3-2 Histone-binding activities of NAP1 family

The ability to bind histones is the prerequisite property for the histone chaperones to carry on their function. *Drosophila* NAP1 protein was found associated with histone H2A and H2B in a crude whole-embryo extract by co-immunoprecipitation assay (Ito *et al.*, 1996). Furthermore, density gradient analysis showed that, although capable of binding all four histones *in vitro*, NAP1 exhibited a greater affinity for histone H2A-H2B, however in the presence of H3-H4, H2A-H2B were transferred from NAP1 to the sub-nucleosomal particles (Nakagawa *et al.*, 2001). Similar results were obtained for mouse and human proteins, showing that those NAP1 homologues are conserved in this activity (Ishimi *et al.*, 1987). Nevertheless, yeast NAP1 preferentially binds the H3-H4 tetramer *in vitro*, which is mediated by the N-terminal histone tails (McBryant *et al.*, 2003); in addition, it is also capable of binding linker histone H1 (Kepert *et al.*, 2005). Human SET interacts with core histones with a preference for histone H3-H4, which is also considered via the interaction with N-terminus of H3 (Schneider *et al.*, 2004). The significance of these findings for the *in vivo* function of NAP1 family remains to be determined.

II-3-3-3 Subcellular localization of NAP1 family

As a histone chaperone, NAP1 family members should not only interact with free histones in the cytoplasm, but also participate in the assembly of nucleosomes in the nucleus. Studies of NAP1 localization in *Drosophila* embryo revealed that NAP1 is present in the nucleus during S phase but predominantly in the cytoplasm during G_2 phase (Ito *et al.*, 1996). Similar result was found for hNAP1L4 (human NAP1-like 4) (Rodriguez *et al.*, 2000).

Import of nascent histones into nucleus is mediated by importin Kap (Karyopherin) proteins. The N-terminal tails of H2A and H2B serve as NLS (Nuclear Localization Signal) recognized by certain Kap proteins in yeast, while NAP1 plays a role to bridge the Kap-histone

interaction to increase specificity of histone cargo (Mosammaparast *et al.*, 2002). Intriguingly, yeast NAP1 was immuno-stained predominantly in the cytoplasm. Deletion analysis has uncovered that its N-terminal leucine-rich NES (Nuclear Export Signal) was functional to export the protein out of nucleus via exportin Crm1p, resulting in its nucleocytoplasmic shuttling, which is important for its function during mitotic progression (Mosammaparast *et al.*, 2002; Miyaji-Yamaguchi *et al.*, 2003). Mammalian SET was predominantly located in the nucleus (Nagata *et al.*, 1998).

II-3-3-4 Post-translational modification of NAP1 family

Post-translational modifications on protein could bring influence over its function and/or cellular localization. The prominent modification on NAP1 group members is phosphorylation (Adachi *et al.*, 1994; Kellogg *et al.*, 1995a; Li *et al.*, 1999; Rodriguez *et al.*, 2000). *Xenopus* NAP1 could be specifically phosphorylated by Cyclin B/$p34^{cdc2}$ *in vitro*, but not by Cyclin A/ $p34^{cdc2}$, suggesting its potential modulation by specific cyclin during cell cycle for further cellular functions (Kellogg *et al.*, 1995a). It was found that *Drosophila* NAP1, human NAP1 and hNAP1L4 could be phosphorylated by CK2 (Casein Kinase 2) *in vitro* and *in vivo*, a ubiquitous second message-independent serine/threonine-specific kinase. Furthermore, the subcellular localization of hNAP1L4 is associated with its phosphorylation state: it is phosphorylated and localized in the cytoplasm at the G_0/G_1 boundary; while at G_1/S boundary it is dephosphorylated and transported into nucleus to carry out its histone chaperone function (Rodriguez *et al.*, 2000). Although human SET was phosphorylated *in vivo*, the kinase and its influence on SET activity still remain obscure (Adachi *et al.*, 1994).

Poly-glutamylation is an unusual modification by adding variable number of glutamyl units on protein side chains, which is previously only detected on tubulin. Interestingly, various animal NAP1 proteins, from *Drosophila* to human, were found poly-glutamylated *in vivo*, however, by distinct pathway and for potentially distinct functions (Regnard *et al.*, 2000). The discovery of poly-glutamylation on NAP1, which was previously considered as tubulin-specific, implies their potential cytological relevance.

II-3-3-5 Chaperone activity of NAP1 family

NAP1 family members alone could deposit core histones onto DNA and assembly nucleosome *in vitro* (Kawase *et al.*, 1996; Gamble *et al.*, 2005). However, except yeast NAP1, most members could reconstitute nucleosomes along DNA template only with random distribution and failed to result in periodic arrays of nucleosomes resembling those functional and native chromatin *in vivo* (Tyler, 2002). Although yeast NAP1 could accomplish regularly spaced nucleosome assembly, the nucleosomal array presented relatively short spacing interval distinct from the normal ones observed *in vivo* and unlikely rendered functional structure and dynamics. These results suggested that, as histone chaperone, NAP1 family members along could not remodel the nascent nucleosomes into active and functional arrays, which should be cooperated with ACF (Tyler, 2002).

One important characteristic of NAP1 family is that they can bind basic protein other than histones. During spermatogenesis in *Xenopus*, histones, especially H2A-H2B, are largely replaced with various SP (Sperm-specific basic Protein), which is tightly related to the highly condensation in sperm chromatin structure. Incubation of NAP1 family member can result the interaction of chaperone with all kinds of SP proteins and their release from the highly packaged structure, and hence the final decondensation of sperm chromatin (Matsumoto *et al.*, 1999).

The adenovirus genome complexed with viral core proteins is the template for transcription of early genes and the first round of replication in adenovirus-infected human cells. NAP1 family members can interact with adenovirus core protein and be involved in remodeling of the adenovirus chromatin-like structure for replication and transcription (Kawase *et al.*, 1996).

II-3-3-6 Transcriptional regulation by NAP1 family

During transcription, chromatin regions have to adopt a more open conformation, and transcription factor and nucleosomal histones compete for the regulatory region on the template. Removal of one H2A-H2B dimer from the histone octamer seems to be essential for

the transcriptional initiation and elongation (Belotserkovskaya *et al.*, 2003). Yeast NAP1 could reversibly remove and replace histone H2A-H2B dimer or variant dimer from assembled nucleosomes, resulting in active histone exchange (Park *et al.*, 2005). Similar property was also found in human NAP1 and SET (Okuwaki *et al.*, 2005).

It was proved that NAP1 family members interact with p300/CBP (Corticosteroid-Binding Protein) *in vivo* and *in vitro*, one transcriptional co-activator with intrinsic histone acetyltransferases activity (Shikama *et al.*, 2000; Asahara *et al.*, 2002; Karetsou *et al.*, 2005). They form multi-component ternary complex with histones and regulate gene expression, likely via modifying acetylation on histones followed by transferring H2A-H2B dimers from nucleosomes to NAP1 family members (Ito *et al.*, 2000).

Besides the common regulation, other examples of respective involvement of NAP1 in promoter-specific transcription regulation have been published. In a yeast two-hybrid screen, human NAP1 was shown to form a complex with the transcriptional activator E2 (Rehtanz *et al.*, 2004). A complex of human NAP1, E2 and p300 was shown to activate transcription *in vitro*. Over-expression of *Xenopus* NAP1 affects the expression of several potential target genes in a tissue-specific manner (Steer *et al.*, 2003). Human SET plays as a cofactor along with other transcription factors to specifically regulate P450c17 transcription (Compagnone *et al.*, 2000). It also regulates KA11 gene-promoter-specific transcription by binding adaptor protein Fe65 (Telese *et al.*, 2005).

II-3-3-7 *In vivo* function of NAP1 family

NAP1-regulated gene transcription in chromatin context is exemplified by the genome-wide expression analysis of *nap1* in *Saccharomyces cerevisiae*. In Δ*nap1* strain about 10% of all yeast open reading frames change the expression level more than 2-fold compared with wild-type strain in micro-array analysis. These genes are generally clustered, suggesting the specific manner of NAP1 regulation (Ohkuni *et al.*, 2003).

Knockout targeting of *NAP1* gene in *Drosophila* resulted in embryonic lethal or poorly viable

adult escapers (Lankenau *et al.*, 2003). mNAP1L2 (mouse NAP1-like 2) is an X-chromosome linked gene specifically expressed in neurons. Inactivated mNAP1L2 by gene targeting would lead to embryonic lethality from mid-gestation onwards, suggesting it represents a class of tissue-specific factors to regulate neuronal cell proliferation (Rogner *et al.*, 2000).

Presenilin plays critical roles in the genesis of Alzheimer's disease and in LIN-12/Notch signaling during development. While in screen for genes that influence presenilin level or activity in *Caenorhabditis elegans*, it was found that substitution mutant of *spr-2* (suppressor of presenilin-2) suppresses the egg-laying defective phenotype caused by a null allele of the *sel-12* presenilin gene. SPR-2 was identified as the homologue of SET (Wen *et al.*, 2000).

II-4 Histone chaperones beyond nucleosome assembly

II-4-1 Small subunit of CAF-1

As the small subunit of CAF-1, p48/Cac3/MSI1 is a conserved protein from yeast to human, and has also been found associated with chromatin regulatory complexes. HAT-B (B-type histone acetyltransferases), which acetylate K5 and K12 of newly synthesized histone H4, are heterodimeric complexes composed of a catalytic subunit Hat1p and an accessory protein p48 that facilitates contact between the complex and histones (Parthun *et al.*, 1996). In addition, p48 protein binds to RPD3 histone deacetylases and is part of several RPD3-containing complexes (Vermaak *et al.*, 1999).

p48/Cac3/MSI1 also functions together with Enhancer of Zeste [E(Z)]-type Polycomb group histone methyltransferases in mammals, insects and plants. Polycomb group proteins are required for the maintenance of transcriptional repression of genes for developmental regulators, which often are Homeobox transcription factors in animals and MADS box transcription factors in plants (Ringrose and Paro, 2004). p48/Cac3/MSI1 and RPD3 histone deacetylases interact with Retinoblastoma tumor suppressor protein pRB in mammals and plants, and mediate the transcriptional repression of pRB target genes (Nicolas *et al.*, 2001; Kennedy *et al.*, 2001). Taken together, p48/Cac3/MSI1 activities, which are beyond

nucleosome assembly, are of central importance for cell proliferation and differentiation.

II-4-2 Interaction of NAP1 family with B-type cyclin

In addition to the histone chaperone activity, NAP1 was also found involved in the mitotic event via interaction with B-type cyclins (Kellogg *et al.*, 1995a). Yeast NAP1 was found to play an important role in budding yeast cyclin Clb2 function in suppressing polar bud growth (Kellogg and Murray, 1995b). Yeast cells lacking *NAP1* showed in clumps of connected cells with elongated shapes, and the deleting *nap1* in *Clb2*-dependent cells had a much more dramatic effect that formed large clumps of interconnected cells that had an unusual elongated morphology and temperature sensitive for growth (Kellogg *et al.*, 1995a). This abnormal phenotype is greatly related to the microtubule dynamics that is regulated by B-type cyclins during mitosis, and NAP1 may play a role in controlling microtubule stability (Kellogg and Murray, 1995b).

Although no SET exists in yeast, *Xenopus* SET can interact specially with cyclin B, similar with NAP1 (Kellogg *et al.*, 1995a), suggesting that SET may also be involved in the regulation pathway of cyclin B on cell cycle. Cyclins perform function via interacting with distinct CDK (cyclin-dependent kinase) at appropriate period, and modulating its substrate specificity to activate corresponding pathways. CDK activity can be regulated by CKI (CDK Inhibitor) that associates with cyclin-CDK complexes, such as $p21^{Cip1}$. SET along, or in cooperation with $p21^{Cip1}$, can inhibit cyclin B-CDK1 activity and regulate the transition of G_2/M in cell cycle (Canela *et al.*, 2003). Furthermore, SET can interact with GAPDH (glyceraldehyde 3-phosphate dehydrogenase), which can reverse the SET inhibition on cyclin-CDK1 activity in a dose-dependent manner (Carujo *et al.*, 2006). Finally, SET can reverse the inhibition of cyclin E-CDK2 induced by $p21^{Cip1}$, and hence regulate the transition of G_1/S in cell cycle (Estanyol *et al.*, 1999).

II-4-3 PP2A inhibitory activity of SET

A truncated form of SET was first isolated as a potent heat-stable protein inhibitor of PP2A

(Protein Phosphatase 2A) (Li *et al.*, 1995). PP2A is a major serine/threonine phosphatase that regulates diverse cellular processes. Physiological targets of this phosphatase include cell-surface receptors and ion channels, protein kinases involved in mitogenic signaling and cell cycle, key regulatory enzymes and proteins involved in metabolism, and numerous transcription factors (Al-Murrani *et al.*, 1999). Subsequent study indicated that SET could potently and specifically inhibit PP2A activity at nanomolar concentrations similar with the truncated form (Li *et al.*, 1996). This inhibition is likely conserved since studies in human malaria parasite, *Plasmodium falciparum*, showed that SET can also inhibit its parasitic PP2A activity potently (Dobson *et al.*, 2003).

c-Jun is one proto-oncogene, as a major component of AP-1 (Activator Protein-1) transcription factor complex. PP2A can markedly down-regulate the concentration and DNA binding of c-Jun and subsequent AP-1 transcriptional activity, while SET can reverse this repression via its inhibition on PP2A. Further experiments showed that the effect of SET and PP2A might be mediated, in part, by changing the phosphorylation of c-Jun at Ser^{63} (Al-Murrani *et al.*, 1999). SET can also modulate the PP2A effect on the balance of cytochrome P450c17 metabolism between cortisol synthesis and sex steroids production (Pandey *et al.*, 2003).

Transcriptional activation of the heat shock genes during the heat shock response in *Drosophila* has been intimately linked to phosphorylation of histone H3 at serine 10, whereas repression of non-heat-shock genes correlates with dephosphorylation of histone H3, which is accomplished by PP2A. Mutants in PP2A display reduced genome-wide H3 dephosphorylation, and sites of H3 phosphorylation that do not contain heat shock genes remain transcriptionally active during heat shock in PP2A mutants. Interestingly, SET is found recruited to the heat shock gene loci following heat shock, and potentially prevent the PP2A repression and promote the activation. Aberrant SET activity results in misregulation of the catalytic activity of PP2A, producing abnormal patterns of gene expression (Nowak *et al.*, 2003). Human leukemic HRX fusion proteins derived from a chromosomal translocation in acute leukemia formed a heterocomplex with SET and PP2A. HRX fusion proteins might

function in conjunction with SET and PP2A to deregulate cell proliferation and cell cycle controls, resulting in leukemia (Adler *et al.*, 1997).

II-4-4 INHAT activity of SET

SET is found as a subunit of human cellular complex INHAT (Inhibitor of Acetyltransferases) that potently inhibits the histone acetyltransferase activity of p300/CBP and PCAF (Seo *et al.*, 2001). However, SET alone, also contain the INHAT activity attributed by its C-terminal acidic tail, which is essential for histone binding. It was proposed that SET inhibits histone acetylation by masking histone tails from the histone acetyltransferases such as p300. In addition, SET was shown to associate with histone deacetylases *in vitro* and *in vivo*, suggesting that SET could integrate chromatin hypo-acetylation and transcriptional repression (Kutney *et al.*, 2004).

Histone hypo-acetylation and DNA hyper-methylation are hallmarks of gene silencing. SET, as an inhibitor of histone acetyltransferase, can inhibit active demethylation of DNA resulting in gene silencing, integrating DNA methylation and transcriptional silencing (Cervoni *et al.*, 2002).

SET INHAT activity is not limited in histones but also directly on transcription factor. The Sp/KLF (for Sp1- and Krüppel-like factor) family of zinc finger transcription factors is important in development, differentiation, and oncogenic processes. SET can interact with the DNA-binding domain (DBD) of transcription factors Sp1 and KLF5, and mask them from acetylation by co-activator/acetylase p300 and hence from activation. It is a new pathway for regulation of DNA-binding transcription factor on DBD through interaction and coupled acetylation by two opposing regulatory factors of a coactivator/acetylase and a negative cofactor harboring activity to inhibit acetylation (Miyamoto *et al.*, 2003; Suzuki *et al.*, 2003). This notion is further supported by the interaction of SET with estrogen receptor α-associated proteins via DBD, and the inhibition of acetylation and hence transactivation (Loven *et al.*, 2003).

II-5 Objectives of thesis

As histone chaperones, NAP1 family members contain histone-binding and chromatin assembly and remodeling activities, as well as functions in many other biologic processes. Although much progress has been achieved in molecular and biochemical characterization of NAP1 family member in yeast and animal, intriguingly, no study had been published in the field of plant, except one report on the isolation of soybean cDNA encoding a NAP1 homologue (Yoon et al., 1995). My thesis work focused on molecular and functional characterization of plant NAP1 homologues. We first want to know the conservation of NAP1, their histone binding activity and their expression during the cell cycle to insight into H2A/H2B chaperones in plants. This work uncovers a complex pattern of intracellular localization of different plant NAP1 proteins, which intrigued us to investigate nuclear transport of these proteins and their binding proteins. Finally, by reverse genetics in the model plant Arabidopsis, we investigated function of NAP1 genes in plant growth and development. Together my thesis work provides novel insights into the dynamic regulation of chromatin structure for plant development.

III RESULTS

III-1 Molecular characterization of rice and tobacco NAP1 proteins

My thesis starts from the molecular characterization of plant NAP1 proteins. The monocotyledon plant rice and the dicotyledon plant tobacco were initially chosen to characterize. My contributions to the work included cDNA isolation, gene expression analysis, plasmid construction, recombinant protein production, protein phosphorylation *in vitro* and microscopy observation. The corresponding results were published in Article 1 and Article 2.

(Article 1-*Planta*)

(Article 2-*Plant Physiology*)

Interacting Proteins and Differences in Nuclear Transport Reveal Specific Functions for the NAP1 Family Proteins in Plants

Aiwu Dong, Ziqiang Liu**, Yan Zhu,** Fang Yu, Ziyu Li, Kaiming Cao, et Wen-Hui Shen

Plant Physiology, 2005, Vol. 138, Pages 1446–1456

Pages 1446 à 1456 :

III-2 Molecular and functional characterization of *Arabidopsis* NAP1-related proteins (NRPs)

Due to the poor genetic approaches in rice and tobacco, for further analysis of biological roles of NAP1, I chose to work on the model plant *Arabidopsis thaliana*, which contains 4 genes encoding NAP1 and 2 genes encoding NAP1-related proteins (*NRP1* and *NRP2*).

One or several T-DNA mutant lines corresponding to each gene were searched from the *Arabidopsis* Stock Centers and identified. Single mutant with loss-of-function of any of these genes did not show any phenotype under our *in vitro* culture and greenhouse growth conditions, then I crossed single mutants to generate double mutants. The *NRP* double mutant clearly showed short-root phenotype. My work was subsequently focused on the characterization of this mutant. The results obtained were described in Article 3 and Article 4.

(Article 3-*Plant Cell*)

(Article 4, submitted)

Addenda

Chromatin remodeling in *Arabidopsis* root growth

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KEY WORDS : *Arabidopsis thaliana*, histone chaperone, chromatin, transcription, root growth

Addendum to : ***Arabidopsis NRP1 and NRP2 encode histone chaperones and are required for maintaining post-embryonic root growth***

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ABSTRACT

The basic structural unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around the histone octamer constituted by two molecules each of histones H2A, H2B, H3 and H4. Nucleosome assembly/disassembly/reassembly processes occur primarily during DNA replication and also during transcription, DNA repair and recombination. Several chromatin-remodeling factors had been previously shown to have pleiotropic roles in different processes of plant growth and development. We have recently demonstrated that the *Arabidopsis NRP1* and *NRP2* genes encode H2A/H2B chaperones and are required for the maintenance of post-embryonic root growth. The *nrp1-1nrp2-1* double mutant plants specifically showed a short-root phenotype in normal growth conditions. They were also hypersensitive to DNA damage and showed release of transcriptional gene silencing. We propose that NRP1 and NRP2 act as histone H2A/H2B chaperones in nucleosome assembly, playing critical roles for a correct genome transcription in the maintenance of root growth.

In plants as in animals, development is regulated by differential gene expression whereby cells acquire specific fates (1). Plant development takes place essentially postembryonically. During embryogenesis, only the basic body plan is established, with small groups of cells called apical meristems at both ends of the body axis. During postembryonic development, the shoot apical meristem (SAM) at the apical end and the root apical meristem (RAM) at the basal end continuously provide cells to maintain stem cells as well as to initiate organogenesis which leads to the development of the aerial parts and the subterranean root system of the plant, respectively. Chromatin structure is fundamental to transcription. Similar chromatin gene mutations that cause embryonic lethality in animals frequently result in plants that are detectably modified but viable, making it relatively easy to study the effects of these genes in development in plants. Chromatin-remodeling factors have been shown to play crucial roles in divers processes of plant development, including leaf patterning, shoot organogenesis, flowering time control, gametogenesis and seed reproduction (2-5).

By reverse genetic analysis, we demonstrated that the *nrp1-1nrp2-1* mutant containing loss-of-function of both *NRP1* and *NRP2* genes has a short-root phenotype. NRP1 and NRP2 proteins show highest homology with the animal SET/TAF-I/I_2^{PP2A} proteins, which are involved in nucleosome assembly by chaperoning histones H2A and H2B (6). Consistently, NRP1 and NRP2 were found to bind histones, preferentially H2A and H2B than H3. They are localized primarily in the nucleus and they bind chromatin in *planta*. Additional supports of NRP1 and NRP2 in chromatin remodeling were the observations that the *nrp1-1nrp2-1* mutant plants exhibit perturbed genome transcription, release of heterochromatic gene silencing and hypersensitive response to DNA damage.

In spite of the fundamental role of NRP1 and NRP2 in chromatin remodeling, the phenotype of the *nrp1-1nrp2-1* mutant is remarkably root specific, the embryos and the aerial organs (leaves, rosettes, inflorescences, flowers and fruits) developed normally in the mutant plants. Arrest of cell cycle progression at G2/M and disordered cellular organization were observed in the mutant roots. In this addendum, we show that the mutant root segments, when cultured *in vitro*, had similar capacity in callus formation than the wild-type root segments (Figure 1A), and that roots regenerated *in vitro* from hypocotyls were also arrested in elongation in the mutant but not in the wild-type (Figure 1B). These new data further strengthen the root specificity of requirement of *NRP1* and *NRP2* in the maintenance of cell proliferation. The specific short-root phenotype of the *nrp1-1nrp2-1* mutant is in sharp contrast to the pleiotropic phenotypes of the *fas1* and *fas2* mutants, which include stem fasciation, abnormal phyllotaxy, modified leaf shape, reduced growth and size of all organs (7, 8). *FAS1* and *FAS2* encode subunits of the CAF1 complex, which chaperones histones H3 and H4 in nucleosome assembly. It is possible that H2A/H2B and H3/H4 contribute to different levels of nucleosome assembly/disassembly and more importantly additional chaperones are likely involved in chaperoning histones in nucleosome assembly in *Arabidopsis*.

Several ethylene-responsive genes encoding transcription factors were upregulated in the *nrp1-1nrp2-1* mutant seedlings. However, the mutant seedlings showed normal ethylene triple response (data not shown) and the treatment by the ethylene biosynthesis inhibitor AVG (L-a-(2-amino-ethoxyvinyl)-glycine) could not sufficiently rescue the short-root phenotype (Figure 1C). These latter observations indicate that the modifications of ethylene-responsive genes are not enough to explain the *nrp1-1nrp2-1* mutant phenotype. Among the other differentially expressed

genes found in the *nrp1-1nrp2-1* mutant seedlings, *GLABRA2* (*GL2*) was downregulated whereas *PLETHORA2* (*PLT2*) was upregulated. *GL2* encodes a homeodomain transcription factor and represses root hair formation (9), its downregulation correlated with the high proliferation of root hairs in the *nrp1-1nrp2-1* mutant. *PLT2* encodes an AP2-type transcription factor and plays crucial roles in stem cell specification and maintenance in the RAM (10), its perturbed expression could have significantly contributed to the *nrp1-1nrp2-1* mutant root phenotype. NRP1 and NRP2 proteins were found to bind chromatin at *GL2* and *PLT2*, thus might regulate directly expression of these genes. Chromatin organization at *GL2* was also affected in *fas2* mutant roots (11). In addition to dynamics of nucleosome assembly/disassembly, histone acetylation/deacetylation and ATP-dependent chromatin remodeling also play important roles in root growth and development (12, 13). Future experiments will further explore epigenetic regulation in root growth and development, particularly in response to intrinsic and environmental factors.

NRP1 and *NRP2* are expressed not only in roots but also in leaves, stems and flowers. In addition, NRP1 and NRP2 can form homomeric and heteromeric protein complexes, and *NRP1* and *NRP2* show functional redundancy in root growth. Nonetheless, molecular phenotype differed between the *nrp1-1* and *nrp2-1* single mutants: a significantly higher number of genes was detected by transcriptome analysis to be differentially expressed in the *nrp2-1* than in the *nrp1-1* mutant seedlings. Further work is required to understand both redundant and unique molecular functions of NRP1 and NRP2. The close homologues of NRP1 and NRP2 are NAP1-group proteins, which include four members (AtNAP1;1 to AtNAP1;4) in *Arabidopsis*. Different members of the NAP1-group proteins in rice and tobacco have distinct subcellular localizations and show different affinity to different types of

histones (14, 15). Genetic and molecular characterization of this group of genes in *Arabidopsis* will help to understand their roles in epigenetic inheritence, particularly interesting in comparison with *NRP1* and *NRP2* in plant growth and development.

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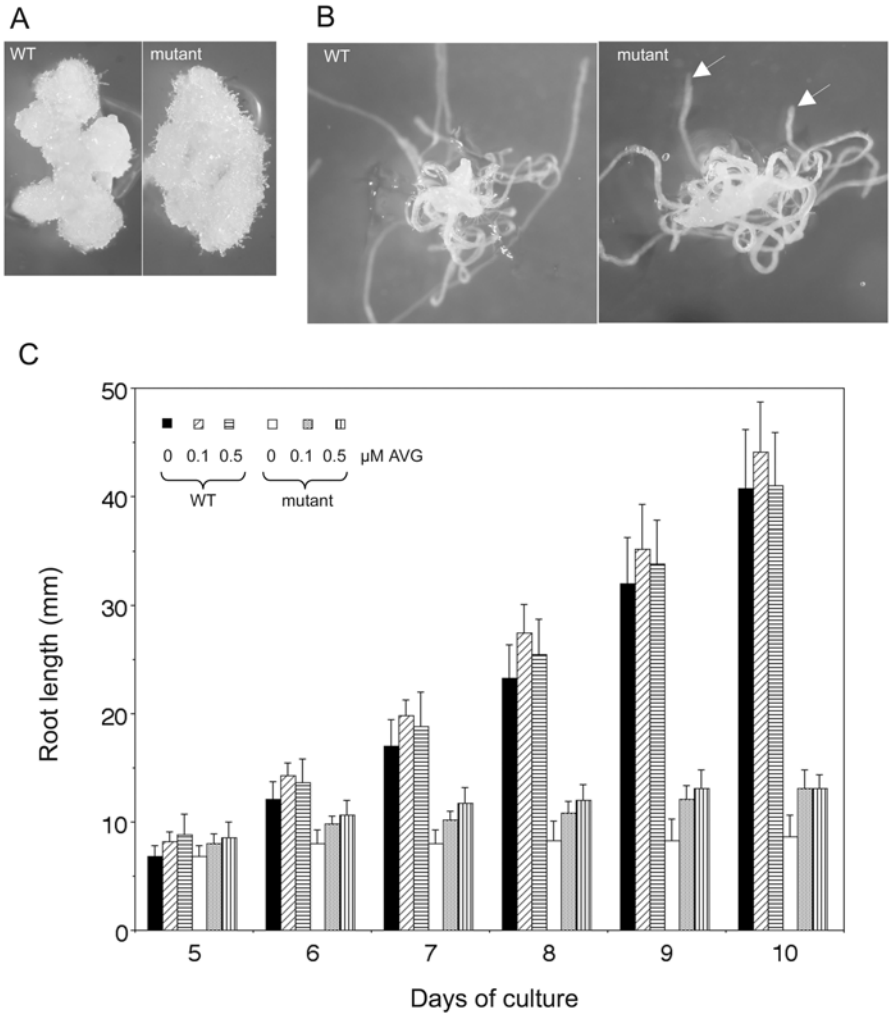


Figure 1. Comparison of callus and root growth between the wild-type (WT) and the *nrp1-1nrp2-1* mutant. (A) Representatives of callus regeneration and growth from root segments cultured in the presence of 2.3 µM 2,4-D, 11.4 µM IAA and 3.2 µM BAP. (B) Representatives of root regeneration and growth from hypocotyls cultured in the presence of 5.7 µM IAA and 1 µM IBA. Arrows indicate roots arrested in growth. Photographs were made 2 weeks after culture. (C) Root elongation of plants grown at different concentrations of the ethylene biosynthesis inhibitor AVG. The mean value from 20 plants is shown. Vertical bars represent standard deviations.

IV GENERAL DISCUSSION AND PERSPECTIVES

IV-1 Histone-binding and self-association of NAP1 family proteins in plant

Our pull-down assays showed that NRP proteins, as well as tobacco NAP1 proteins, can bind histone H2A and H2B. Furthermore, this interaction seems preferential, since NRP proteins interact barely with H3. *In vitro* binding assay showed that, despite of distinct relative binding strengths, plant NAP1 proteins bind core histones as well as the linker histone H1. Since animal NAP1 can modulate the binding of H1 to chromatin, it will be interesting to test the role of plant NAP1 family in the construction and remodeling of chromatin mediated by H1 in the cellular processes and plant growth. Taken together, the histone-binding property of plant NAP1 family members we analyzed support their role as histone chaperones.

We also showed that NRP1 and NRP2 could form homo- and heteromeric protein complexes, as well as tobacco NAP1 proteins. Consistently, previous work showed that human SET proteins could form homo- and heterodimer (Matsumoto *et al.*, 1999; Miyaji-Yamaguchi *et al.*, 1999), while yeast and *Drosophila* NAP1 proteins could form polymeric complexes (McBryant and Peersen, 2004; Ito *et al.*, 1996). It is considered that the long α-helix in N-terminal NAP domain is responsible for forming protein dimer (Park and Luger, 2006a), and interestingly, this long α-helix is well conserved in plant NAP1 group. It was considered that animal NAP1 proteins exist as dimer to participate in the H2A-H2B nuclear import and transcription regulation (Tóth *et al.*, 2005); in addition, dimerization of human SET protein is critical for its chromatin remodeling activity (Miyaji-Yamaguchi *et al.*, 1999). It is likely that NAP1 family members in plant also carry on their function in the form of dimer.

IV-2 Subcellular localization of plant NAP1 family

By using GFP-tagged fusion protein, the subcellular localization analysis shows that NRP1 and NRP2 proteins are mainly localized in the nucleus, like the animal homologue SET. This is quite consistent with their function of histone chaperone in chromatin remodeling and transcription regulation.

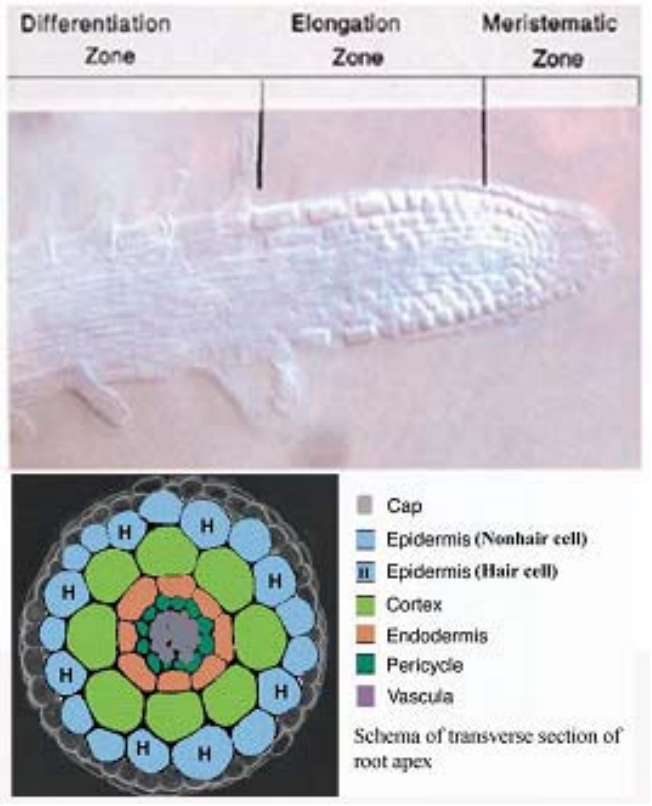
Except OsNAP1;3, all the plant NAP1 proteins we analyzed contain a conserved and typical NLS (KKKPKK), in addition, there is a second NLS (KPKKK) in C-terminal tail of OsNAP1;1. The common NLS (KKKPKK) does not seem functional since it could not import its proteins into the nucleus. The nuclear localization of OsNAP1;1 depends on the second NLS, which is supported by substitution mutant, while the mechanism of nuclear-import of OsNAP1;3 remains unclear yet.

We showed that OsNAP1;1 and NtNAP1;1 could shuttle between the cytoplasm and the nucleus. Approaches of mutagenesis and use of inhibitor proved that the nuclear-export of the two proteins is mediated by NES (Nuclear Export Signal), and the result indirectly proved that NtNAP1;1 can also be imported into nucleus, however, this nuclear localization seems transient and quickly reversed by exportins. Surprisingly, the other nucleocytoplasmic protein OsNAP1;3 is not affected by NES mutagenesis and inhibitor treatment. There are two explanations for the distinct subcellular localization of NAP1 proteins. One is that all the NAP1 proteins can be imported into nucleus but are exported by distinct mechanisms. OsNAP1;1 and NtNAP1;1 are via their NES recognized by exportins while others are via unknown and independent pathways, however with inefficiency in OsNAP1;3. The other explanation is that besides OsNAP1;1, only NtNAP1;1 and OsNAP1;3 can be imported into the nucleus to some extent, while the mechanisms of their nuclear exporting are still distinct. Further study on differential NAP1-binding proteins may help to explain it. After all, the divergent compartmentation of NAP1 proteins suggests that, as a whole family, the activities of NAP1 proteins are beyond nucleosome assembly.

Our result is the first systematic research on plant NAP1 family, and for the first time presents the variation in subcellular compartmentation of its members in plant, helps to guide the future research to discover the functional regulation of individual plant NAP1 family member.

IV-3 Chromatin remodeling in root development

Arabidopsis roots are tip-growing structures that grow in length through the action of tip meristems. Behind the root cap, the root tip is organized along its length into successive



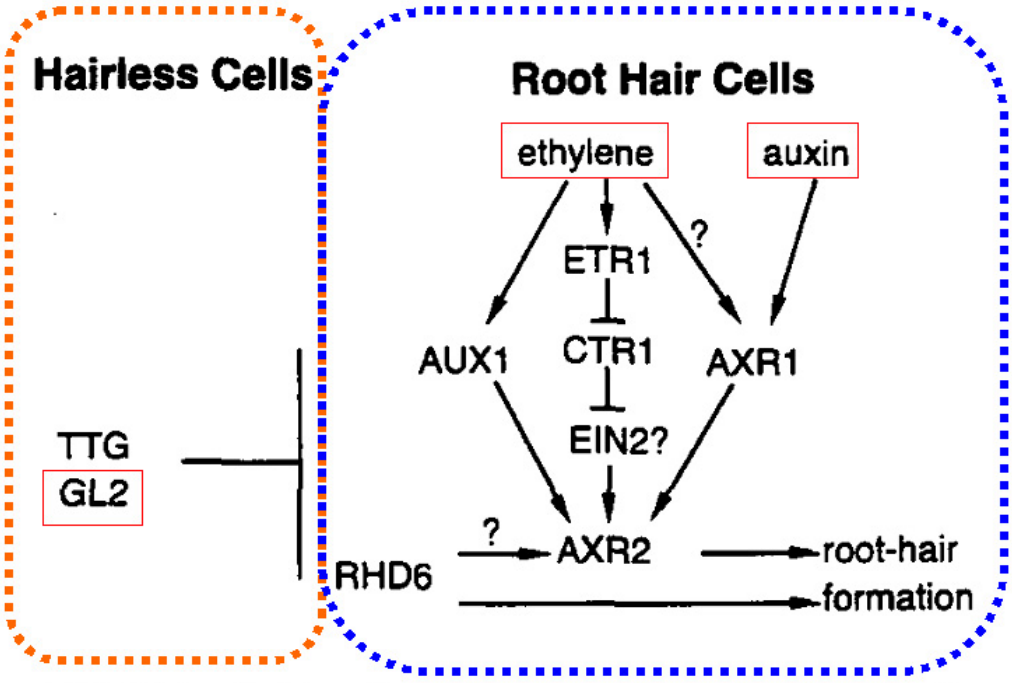
meristematic zone, elongation zone and differentiation zone (Scheres *et al.*, 2002). Furthermore, transverse sections showed that the radial organization of the mature tissues in wild-type roots consists of the epidermis, cortex, endodermis and pericycle, which surround the vascular cylinder.

The elongation zone in the *nrp1-1nrp2-1* double mutant is greatly reduced compared to that of wild-type roots. Transverse sections revealed that cell division in mutant roots was abnormal and cell organization in each layer was irregular, which were much more pronounced in sections of the differentiation zone. We introduced cell division marker *CYCB1:GUS* into double mutant by cross to analyze the role of *NRP1* and *NRP2* in cell cycle progression in proximal meristem cells. It was found that higher number of GUS positive cells were observed in the mutant root tips at later growth stages (from 8 DAG) compared to the wild-type root tips, suggesting that G_2/M arrest occurred in the mutant.

Since human SET protein can interact with Cyclin B and inhibit Cyclin B-CDK1 activity and regulate the transition of G_2/M in cell cycle (Canela *et al.*, 2003), we initially want to verify the conservation of this pathway in plant. In our pull-down assays, tobacco cyclin Nicta;CYCB1;1 binds the NAP1 proteins, but fails to bind NRP1 or NRP2. In addition, expression pattern analysis shows that, like Cyclin B, NRP are ubiquitously expressed in all the tissues tested. The model that direct interaction of NRP proteins with Cyclin B exerts the subsequent inhibitory effect on its activity seems unlikely to explain the current short-root phenotype. The occurrence of G_2/M arrest in the mutant should be contributed by other mechanisms.

Ectopic long hairs are found irregularly positioned in the mutant root, in parallel, ectopic expression of *GL2 (GLABRA 2)* gene is also found in the mutant by RT-PCR. *GL2* is a homeobox gene involved in development of root epidermis in *Arabidopsis* (Masucci *et al.*, 1996a). Wild-type epidermis is composed of two cell types, root-hair cells and hairless cells, which are located at distinct positions within the root, implying that positional cues control cell-type differentiation. *GL2* gene is preferentially expressed in the differentiating hairless cells of the wild-type, and appears to act in a cell-position-dependent manner to suppress hair formation in differentiating hairless cells (Masucci *et al.*, 1996a). The phenotype of the double mutant is quite consistent with the down-regulation of *GL2* gene in our findings

It is considered that hormones ethylene and auxin act downstream of *GL2* to promote root hair outgrowth during epidermis development in the *Arabidopsis* root (Masucci and Schiefelbein, 1996b). Furthermore, ethylene and auxin are known to regulate root hair elongation (Pitts *et al.*, 1998), as well as root elongation (Swarup *et al.*, 2002; Alonso *et al.*, 2003), while the molecular mechanisms by which ethylene and auxin interact to regulate these



A Model for the Genetic and Hormonal Control of Root Epidermis Development (Masucci and Schiefelbein, 1996b)

The genes proposed to act in differentiating hairless epidermal cells are shown at left, whereas the genes and hormones proposed to act in differentiating root hair epidermal cells are shown at right. Arrows indicate positive action; blunted lines indicate negative regulation. Question marks indicate an uncertain step in the pathway.

processes, agonistically in some cases and antagonistically in others, remain largely unknown (Stepanova *et al.*, 2005). Interestingly, 3 ethylene-responsive proteins are up-regulated to more than 2-fold in the double mutant in our microarray. This finding promotes us to investigate the involvement of ethylene and auxin in the NRP-dependent root growth.

We have tried several chemicals related to ethylene and auxin in the rescue assays, however, neither the ethylene biosynthesis inhibitor AVG, nor the synthetic auxins or auxin transport inhibitors, can rescue the short-root phenotype of *nrp1-1nrp2-1* double mutant. Furthermore, the mutant shows similar triple response with the wild-type, such as apical hook maintenance in the dark growing environment, and opened hook and shortened hypocotyl in the presence of auxin transport inhibitor NPA (Achard *et al.*, 2003). Taken together, these results show that simple regulation of ethylene and auxin concentration or compartmentation cannot rescue the mutant phenotype caused by the loss-of-function of NRPs. There are two possibilities: NRPs likely accomplish their function in the pathways independent of ethylene or auxin signaling, or, NRPs function in the downstream and loss-of-function of NRPs constitutively activate or repress some consequent pathways resulting in the phenotype.

IV-4 *In vitro* and *in vivo* chromatin-based functions

Our results established the interaction of plant NRP proteins with chromatin. YFP:NRP fusion protein can interact with chromatin region corresponding to the loci whose expression are regulated in the double mutant, such as *GL2*; however, exception is also detected at some genes whose expression are unchanged. At the pericentromeric heterochromatin, YFP:NRP can bind *TSI* but to a less extent *Ta3* and the centromeric 180 bp-repeats. Taken together, NRP protein interacts with chromatin with no uniform distribution over the genome.

Study of animal SET showed that it was a multifunctional protein. It alone, or forming complex INHAT with other subunits, could mask histone tail from acetylation by histone acetyltransferase p300/CBP and PCAF, and hence repress *in vitro* transcription mediated by the participation of co-activators with histone acetyltransferase activity (Seo *et al.*, 2001). During the heat shock response in *Drosophila*, repression of non-heat-shock genes correlates

with PP2A activities by dephosphorylation of histone H3 Ser10, while SET is found recruited to the heat shock gene loci and potentially prevents the PP2A repression and promotes the activation (Nowak *et al.*, 2003). SET is required for transcription *in vitro* of chromatin templates as histone chaperone (Gamble *et al.*, 2005), and is also found associated with various transcription factors and chromatin remodeling factors to influence their activities in distinct mechanisms (Al-Murrani *et al.*, 1999; Pandey *et al.*, 2003; Loven *et al.*, 2003; Miyamto *et al.*, 2003; Suzuki *et al.*, 2003; Kutney *et al.*, 2004). In summary, the chromatin-association state of SET is quite divergent depending on its histone-binding activities and/or mediation via other factors, and its function in gene expression depends on its dynamic state and divergent associated partners. It may help to explain our failure to set up one concise platform to link the NRP binding and NRP-regulated genes.

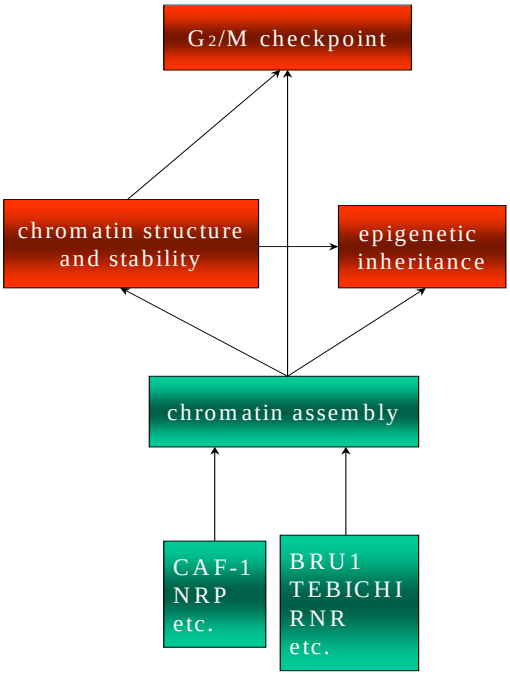
In *nrp1-1nrp2-1* double mutant, 102 genes were found differentially expressed, some of which are good candidates responsible for the phenotype. However, this number is lower than those reported for *fas* mutants, 483 genes in *fas1* and 204 genes in *fas2* (Schönrock *et al.*, 2006). In addition, the specific short-root phenotype of *nrp1-1nrp2-1* double mutant is distinct from the phenotype of *fas1* and *fas2* which is pleiotropic in the whole plant including fasciation. In addition, the identified genes barely overlap in these two categories of mutants, suggesting that they likely act independently, and NRP1 and NRP2 function in maintaining post-embryonic growth in a more specific manner.

Perturbed gene expression in double mutant spreads into the heterochromatic region, such as *TSI*, in the presence of genotoxic stress. This release was previously found in mutants defective in factors participated in DNA/chromatin replication and assembly, such as *FAS1*, *FAS2*, *TSK/MGO3/BRU1* and *RNR*, which encode the larger two subunits of CAF-1, one novel factor involved in histone chaperoning together with CAF-1, and ribonucleotide reductase respectively (Takeda *et al.*, 2004; Wang and Liu, 2006), implying NRP proteins are involved in the maintenance of heterochromatin silencing in epigenetic inheritance. Previous research has suggested that stochastic release of TGS in *tsk/mgo3/bru1* and mutants of *fas* and *rnr* caused meristem fasciation resulting from the ectopic expression of *WUSCHEL (WUS)*, a

key meristem regulator (Mayer *et al.*, 1998; Kaya *et al.*, 2001; Takeda *et al.*, 2004; Wang and Liu, 2006). It is also likely that ectopic release of some critical transcription factor(s) in double mutant would be collectively responsible for the short-root phenotype.

It is considered that chromatin assembly/disassembly is essential for DNA damage response and epigenetic inheritance in DNA repair. *tsk/mgo3/bru1*, and mutants of *rnr* and *fas*, all show higher sensitivity to DNA damage, which is also found in mutant defective in *TEBICHI* (*TEB*) that encodes protein with helicase and DNA polymerase domains (Inagaki *et al.*, 2006). Our result shows that n5n6 double mutant is more sensitive to genotoxic stress such as bleomycin and UV treatment, indicating that the mutant genome is also more sensitive to DNA damage. In view of the common release of TGS and genotoxic sensitivity, it is reasonable to postulate that affected chromatin assembly would influence not only the epigenetic inheritance, but also the maintenance of chromatin architecture and global stability.

The results of *CYCB1:GUS* accumulation in the above-mentioned mutants furthermore give support to the critical role of chromatin assembly. Mutant *tsk/mgo3/bru1*, *tebichi* and *fas*, as well as n5n6, all show the accumulation of GUF-positive cells, indicating the arrest of G_2/M in these mutants. G_2/M checkpoint ensures correct and complete DNA synthesis and chromatin duplication before the entry into mitosis and cytokinesis, and B-type cyclins are involved in this checkpoint activation (Stark and Taylor, 2006). The accumulation of *CYCB1:GUS* and activation of G_2/M checkpoint, together with the unstable chromatin structure to genotoxic stress, and incorrect epigenetic inheritance, could be considered as an interplaying consequences, resulting from the inhibition of chromatin duplication caused by affected chromatin assembly. This potential interplay is illustrated in the following model. As histone chaperones, NRP and CAF-1 cooperate with other factors, such as BRI1, TEBICHI and RNR, to guarantee the correct chromatin assembly and further influence chromatin structure, as well as epigenetic inheritance and cell cycle checkpoint.



IV-5 Perspectives

Despite that our work provides novel insight into dynamic regulation of chromatin structure for plant development, to fully understand H2A-H2B histone chaperone, we still lack their biochemical and functional characterizations. Obviously, those direct proofs of their activities in nucleosome assembly and involvement in transcription regulation would greatly help us to better understand the plant H2A-H2B histone chaperones function and their effect in chromatin dynamics during plant growth and development.

Our initial approach of reverse genetics in studying NAP1 function in *Arabidopsis* showed that single mutant of NAP1 family member didn't show any phenotype. It will be interesting for the further analysis of double, triple and quadruple mutants of this gene group to clarify their biological functions. In addition, as H2A-H2B histone chaperone, the phenotype of *nrp1-1nrp2-1* double mutant is much less severe that those of any of H3-H4 histone chaperones: the loss-of-function of *HIRA* or *ASF1* in *Arabidopsis* results in lethality, and mutation in *CAF-1* function shows pleiotropic effect in the shoot and root. Considering the high similarity of protein sequence of plant NAP1 family, and the redundant function of NAP1 and SET in animal, it will be interesting to test the overlap and redundancy of function

of plant NAP1 family in plant growth and development.

Animal SET has many unique activities beyond histone chaperone, which include INHAT, PP2A inhibition. It was reported that ectopic histone acetylation can affect the expression of root patterning genes *GL2* and other genes involved in epidermal cell differentiation in *Arabidopsis* (Xu *et al.*, 2005). Ectopic PP2A activity can cause pleiotropic effects including inhibition of root growth (Garbers *et al.*, 1996). Whether these activities, as well as the potential ones unknown but unique to plants, contribute to the function of either NRP protein in a similar or preferential manner, needs further investigation.

V MATERIALS AND METHODS

V-1 Materials

V-1-1 Plant materials

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

Tobacco BY2 cell line shows high synchronization ability and growth rate, by 80-100 fold once a week. High cell cycle synchrony can be easily obtained after aphidicolin treatment. In addition, it is easy for transformation and stable transgenic maintenance, making it powerful in exploring molecular and cellular biology of plant cells.

b) *Arabidopsis thaliana* Columbia (Col-0)

As the model organism of flowering plants, *Arabidopsis thaliana* has many advantages: small genome, rapid life cycle and prolific seed production, as well as efficient transformation approaches and amounts of available mutant lines, which facilitate studies of cellular and molecular biology in plant. Distinct *Arabidopsis* ecotypes show different organ shapes and growth characteristics due to different genetic background. Columbia (Col), with sequenced genome in *Arabidopsis* Genome Initiative (AGI), is one most popular ecotype, and Col-0, one of Col accessions, is used here as background line.

V-1-2 Bacteria strains

V-1-2-1 *Escherichia Coli*

a) Top10

Strain Top10 is ideal for high-efficiency cloning and plasmid propagation. In addition to support of blue/white screening, mutations *rec*A1 and *end*A1 in Top10 increase insert stability and improve plasmid DNA quality in preparation.

b) DH5α

Strain DH5α is *rec*A⁻ *end*A⁻ mutant like Top10 and gives high transformation efficiency and plasmid yield. It can also be used as host for protein expression of pGEX series of vectors such as pGEX-4T-1.

c) BL21 (DE3)

Strain BL21 (DE3) allows highly efficient protein expression under control of T7 promoter. It is used as host for protein expression of pET system such as pET14b.

V-1-2-2 *Agrobacterium tumefaciens*

a) GV3101

C58, Ti plasmid cured, resistant to rifampicin, in use for mediating *Arabidopsis* transformation.

b) LBA4404

pAL4404 plasmid, resistant to rifampicin, in use for mediating tobacco BY2 cells transformation or others.

V-1-3 Cloning and expression vectors

a) pGEM®-T and pGEM®-T Easy Vector (Promega)

Both vectors are prepared for PCR product cloning by use of 3' terminal thymidine. They contain multiple restriction sites within α-peptide coding region of *β*-galactosidase, allowing color screening on indicator plates for insertional inactivation. Ampicillin resistance gene is for selection.

b) pEYFP-pEYFP

pEYFP-pEYFP contains two *EYFP* cDNAs in tandem under promoter *lac*. Three multiple cloning sites are located between these cDNAs and promoter, allowing selective construction of EYFP-fusion protein. Ampicillin resistance gene is for selection.

c) pET-14b (Novagen)

pET system is powerful for cloning and expression of recombinant proteins. pET-14b is one vector that allows N-terminal fusion to cleavable His-tag sequence for rapid affinity purification. Ampicillin resistance gene is for its selection. After cloning, plasmids are transformed into expression hosts such as DE3 containing a chromosomal copy of T7 RNA polymerase gene under *lacUV5* control, and induced by IPTG for expression.

d) pGEX-4T-1 (Amersham Biosciences)

pGEX-4T-1 vector is for high-level expression of GST-fusion protein under *tac* promoter inducible by IPTG. One recognition site for thrombin is introduced for GST-tag cleavage. Ampicillin resistance gene is for selection.

e) pER8

pER8 is one binary expression vector developed for plant transformation, which adopts the XVE, LexA-Vp16-ER system strictly induced by estrogen receptor-based chemical. Spectinomycin and hygromycin resistance genes are for selection in agrobacteria and plants, respectively.

V-1-4 Antibiotics

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

V-1-5 Chemicals

Name	Full Name	Company	M.W.	Function
1-NAA	naphthalene-1-acetic acid	SERVA	186.2	Synthetic auxin, auxin carrier-independent, diffused into nucleus
1-NOA	1-naphthoxyacetic acid	ALDRICH	202.2	Auxin influx inhibitor
2,4-D	2,4-dichlorophenoxyacidic acid	SERVA	221.0	Synthetic auxin, auxin carrier-mediated, blocked by 1-NOA
6-BAP	6-benzylaminopurine	SERVA	225.3	Cytokinin growth regulator
AVG	(S)-trans-2-Amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride	SIGMA	196.6	Ethylene synthesis inhibitor
β-Estradiol	β-Estradiol	ICN Biomedicals, Inc.	272.4	Transgenic plant gene induction
Cantharidin	Cantharidin	SIGMA	196.2	Inhibitor of PP2A
IAA	Indole-3-acetic acid	SERVA	175.2	Cytokinin growth regulator
IBA	Indole-3-butyric acid	SERVA	203.2	Auxin growth regulator
NPA	N-1-naphthylphthalamic acid	DUCHEFA	291.3	Auxin efflux inhibitor

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)

PCR reaction comprises following components: 1x amplification buffer, 1.5-3 mM $MgCl_2$, 200 μM dNTPs Mix, 0.4 μM forward primer, 0.4 μM reverse primer, 1-5 unit(s) thermostable DNA polymerase and suitable template DNA. Reaction volume is usually adjusted up to 20-50 μl. After first denaturation treatment, PCR reaction enters repeating cycles including denaturation (94°C, 30 sec), annealing (30 sec) and elongation (72°C). Optimal elongation time and annealing temperature varies in distinct reaction conditions. Normally, every 1 kb more of PCR product needs about 1 min more of elongation, and PCR products are purified by the kit “Nucleospin Extract II” (Macherey-Nagel).

V-2-1-1-2 Semi-quantitative PCR

Same PCR reactions are established in several tubes. During PCR program, at the end of desired cycle, pause the PCR machine and pick out one tube quickly, then continue the program to the next step.

V-2-1-1-3 Restriction enzyme digestion

Digestion reaction comprises approximately 200 ng of DNA with 1-2 unit(s) of restriction enzyme(s) in optimal reaction buffer at suggested temperature. Generally, 1 hr is sufficient for most digestion. Released DNA fragment is purified by the kit “Nucleospin Extract II” (Macherey-Nagel).

V-2-1-1-4 Gel electrophoresis

DNA samples are resolved in agarose gel prepared in TAE or TBE buffer. The higher

concentration of agarose gel resolves the smaller fragment of DNA sample. With the staining of ethidium bromide, agarose gel can be read under UV light.

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

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

V-2-1-1-5 Ligation

DNA ligation comprises approximate 60 ng of insertion DNA and 20 ng of linear vector DNA in 1 x ligation buffer with 1 unit of T4 DNA ligase (Fermentas). Usually overnight at 16°C results in maximal ligation efficiency. The ligation product can be transformed into competent *E. coli* host by heat shock or electroporation. Before electroporation, ligation product needs to be desalted usually via ethanol precipitation.

V-2-1-1-6 Competent cell preparation and transformation

V-2-1-1-6-1 Heat shock using calcium chloride

A single bacterial colony is inoculated in 3 ml of LB and incubated by vigorous agitation at 37°C overnight. 0.5 ml of overnight culture is inoculated and propagated in 50 ml of fresh LB in a 250-ml flask at 37°C for about 2 hr until OD_{600} reaches 0.2-0.3. The culture is fully chilled on ice and centrifuged at 3,000 rpm for 10 min at 4°C. The pellets are gently resuspended in 10 ml of ice-cold fresh 0.1 M $CaCl_2$ solution, and kept on ice for 30 min. After another centrifugation at 3,000 rpm for 10 min at 4°C, the pellets are resuspended gently in 2 ml of ice-cold 0.1 M $CaCl_2$ solution. The $CaCl_2$-treated cells can be used directly for transformation or dispensed into aliquots and frozen at -70°C.

Mix each 200 μl of competent cells with plasmid DNA or ligation product in sterile tubes by swirling gently and store on ice for 30 min. Transfer the tubes in a preheated 42°C water bath and keep for exactly 90 sec. Do not shake the tubes. Rapidly transfer the tubes to an ice bath and allow cells to chill for 1-2 min. Add 800 μl of LB medium to each tube and incubate cultures for 1 hr at 37°C to allow the bacteria to recover and express antibiotic resistance

marker encoded by plasmid. Transfer the appropriate volume of transformed competent cells onto agar LB medium containing the appropriate antibiotic. Invert the plates and incubate at 37°C. Transformed colonies should appear in 12-16 hr.

V-2-1-1-6-2 Electroporation

A single bacterial colony is inoculated in 3 ml of LB. After overnight agitation at 37°C, 3 ml of culture is diluted 100 times with fresh LB and incubated by agitation at 37°C until OD_{600} reaches 0.5. The culture is chilled on ice for 10 min and centrifuged at 5,000 rpm for 10 min at 4°C. The cells are washed twice by ice-cold pure H_2O, and once by ice-cold sterile 10% glycerol. Finally cells are resuspended in 1 ml of ice-cold 10% glycerol. The electrocompetent cells can be used immediately. For storage, 40 µl aliquots of cell suspension are dispensed in each tubes, frozen quickly in liquid nitrogen and stored in a -70°C freezer.

40 µl of electrocompetent cells mixed with plasmid DNA or purified ligation products are pipetted into pre-cooled electroporation cuvettes. 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 added with 1 ml of LB medium and removed from electroporation cuvette, followed by incubation for 1 hr at 37°C to allow bacteria to recover. 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

Inoculate a single bacterial colony in 3 ml of LB containing appropriate antibiotic and incubate by vigorous shaking at 37°C overnight. Harvest bacteria by centrifugation at maximum speed for 30 sec and resuspend in ice-cold 100 µl of alkaline lysis solution I by vigorous vortexing. Add 200 µl of freshly prepared alkaline lysis solution II and mix by inverting tube 6-8 times and keep on ice for 3-5 min. Do not vortex. Add 150 µl of ice-cold solution III, invert tube 6-8 times and keep on ice for another 3-5 min. Centrifuge bacterial

lysate at maximum speed for 5 min and transfer the supernatant to a fresh tube. Add an equal volume of phenol:chloroform (1:1 v/v), mix organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 min. Transfer the aqueous upper layer to a fresh tube and precipitate by adding 2 volumes of 100% ethanol. DNA pellet is visible after centrifugation at maximum speed for 5 min. Wash DNA pellet with 70% ethanol. After air-dry, DNA pellet is finally dissolved in 50 µl of distilled H_2O containing 10 µg/ml DNase-free RNase A (Fermentas).

Alkaline Lysis Solution I: 50 mM Glucose; 10 mM EDTA; 25 mM Tris-Cl (pH 8.0)
Alkaline Lysis Solution II: 0.2 M NaOH; 1% (w/v) SDS.
Alkaline Lysis Solution III: 3 M potassium acetate; 11.5% acetic acid.

Alternatively, plasmid DNA can be prepared via the kit “NucleoSpin Plasmid QuickPure” (Macherey-Nagel).

V-2-1-2 Genomic DNA isolation

V-2-1-2-1 CTAB method

Grind plant tissue in liquid nitrogen to fine powder. Homogenize about 200 mg of fine powder in 600 µl of pre-heated 2x CTAB Buffer and incubate in 65°C for 15 min. Add equal volume of chloroform/isoamyl alcohol (24:1, v/v) to form an emulsion by thorough vortex. After centrifugation at 11,000 g for 1 min, transfer supernatant to a new tube, and add 1/5 volume of 5% CTAB Solution and mix well. Perform another chloroform/isoamyl alcohol extraction. Mix final supernatant gently with equal volume of CTAB Precipitation Buffer and keep for 15 min. After centrifugation at 11,000 g for 1 min, re-hydrate DNA pellet in high-salt TE Buffer. Perform another precipitation by adding 2 volumes of 100% ethanol and keep at -20°C for more than 30 min. Centrifuge DNA pellet at 11,000 g for 15 min and wash with 80% ethanol. Finally the pellet is briefly dried and dissolved in 50 µl of 0.1x TE containing 100 µg/ml DNase-free RNase A (Fermentas). The quantity and quality of genomic DNA are analyzed by gel electrophoresis in 0.3% agarose.

2x CTAB Buffer: 2% CTAB; 100 mM Tris-Cl (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-Cl (pH 8.0); 10 mM EDTA.

High-Salt TE Buffer: 10 mM Tris-Cl (pH 8.0); 1 mM EDTA; 1 M NaCl.

0.1x TE: 1 mM Tris-Cl (pH 8.0); 0.1 mM EDTA.

V-2-1-2-2. Urea method (for large scale of T-DNA insertion mutants screening)

Grind one rosette leaf or one inflorescence in a tube with 600 µl of Urea Lysis Buffer, and extract once by equal volume of phenol:chloroform (1:1, v/v) by vigorous vortex. After centrifugation at 12,000 rpm for 7 min, transfer supernatant to a new tube and mix with 1/10 volume of 3 M sodium acetate and 1 volume of isopropyl alcohol, and keep at -20°C for 20 min. Centrifuge DNA pellet at 12,000 rpm for 5 min and wash by 70% ethanol. After air-dry, re-hydrate pellet with 50 µl of distilled H_2O.

Urea Lysis Buffer: 7.5 M Urea; 0.2 M Tris-Cl (pH 8.0); 50 mM EDTA; 2% Sodium Lauroyl Sarcosine (SLS)

V-2-1-3 RNA isolation and analysis

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

Grind plant tissue in liquid nitrogen to fine powder. Homogenize 100 mg of fine powder in 1 ml of Trizol Reagent (Invitrogen) by vigorous vortex and keep at room temperature for 5 min. Add 200 µl of chloroform and keep at room temperature for 2-3 min. After centrifugation at 12,000 g for 15 min at 4°C, transfer supernatant to a new tube and add 500 µl of isopropyl alcohol for precipitation at room temperature for 10 min. After centrifugation at 12,000 g for 10 min at 4°C, RNA pellet is visible and washed with 1 ml of 75% ethanol by centrifugation at no more than 7,500 g for 5 min at 4°C. Finally, air-dry and dissolve RNA pellet in 20 µl RNase-free H_2O; 55°C water bath for 5 min helps the RNA dissolution. 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 RT-PCR

To avoid DNA interference, Reaction Mixture I contains 5 μg of RNA, 5 μl of 5x Buffer and 1 μl of RQ1 DNase (Promega), with H_2O up to 18.75 μl. After incubation at 37°C for 10 min, DNase activity is inactivated by incubation at 56°C for 10 min. Add Reaction Mixture II then: 2.5 μl of 0.1 M DTT, 2.5 μl of 10 mM dNTPs, 1 μl of 0.5 μg/μl Oligo poly-dT and 0.25 μl of 40 U/μl RNase Inhibitor (Promega). After incubation at 42°C for 2 min, add 1 μl of Improm-II Reverse Transcriptase (Promega) into the mixture. Keep reaction of reverse transcription at 42°C for 60 min and then at 70°C for 10 min to inactivate RTase activity. The cDNA synthesized in the reaction can be used as template in following PCR.

V-2-1-4 Recombinant protein expression, purification and detection

V-2-1-4-1 Protein expression

Transform constructed expression plasmids into appropriate host cells. Propagate fresh transformants in fresh liquid LB medium in large flask to provide aeration. After culture reaches OD_{600} 0.4~0.6, add IPTG to a final concentration of 0.4-1 mM and keep induction at 28°C for 2 hr more. Harvest cells for immediate purification or frozen at -70°C. Expression of recombinant protein is detected by SDS-PAGE.

V-2-1-4-2 Protein purification

V-2-1-4-2-1 His-tag fusion proteins

Collect bacteria (about 1000 OD_{600}) by centrifugation at 3,000 rpm for 10 min at 4°C and then resuspend in 20 ml of ice-old Balance Buffer plus 0.5 mM EDTA, 100 μg/ml lysozyme and protease inhibitor cocktail (Roche), and lyse by French Press. After centrifugation at 20,000 g for 30 min at 4°C, load supernatant onto Ni^{2+}-Sepharose Fast Flow column (Amersham Biosciences), which has been previously coupled with 0.1 M $NiSO_4$ and pre-equilibrated with Balance Buffer. Wash column extensively with Balance Buffer and Wash Buffer. The retained fusion protein is eluted from the column with Elution Buffer.

Balance Buffer: 20 mM Tris–Cl (pH 8.0); 0.5 M NaCl; 0.2% Tween 20; 5% glycerol; 1 mM DTT; 1mM PMSF

Wash Buffer: His-tag lysis buffer plus 60 mM imidazole

Elution Buffer: 20 mM Tris-Cl (pH 8.0); 0.15 M NaCl; 0.3 M imidazole; 1 mM DTT; 1 mM PMSF; 10% glycerol.

V-2-1-4-2-2 GST fusion proteins

Harvest cells and resuspend in 20 ml of ice-cold Lysis Buffer plus 100 μg/ml lysozyme and protease inhibitor cocktail (Roche), and lyse by French Press. After centrifugation at 20,000 g for 20 min at 4°C, load supernatant onto a glutathione-Sepharose-4B column (Amersham Biosciences), which has been pre-equilibrated with Lysis Buffer. After extensive wash by Lysis Buffer without glycerol, the retained GST-fusion protein is eluted from the column with Elution Buffer.

PBS: 140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; pH 7.3

Lysis buffer: 1 x PBS; 0.1% Triton X-100; 1 mM EDTA; 1 mM DTT; 1 mM PMSF; 10% Glycerol

Elution Buffer: 50 mM Tris-Cl; 10 mM glutathione; pH 7.5

V-2-1-4-2-3 SDS-PAGE

Using “Mini-protein II” system (Bio-Rad), SDS-PAGE is performed to separate proteins mainly based on molecular weight. The protein samples are treated in 1x SDS loading buffer at 95°C for 10 min. After preparation of resolving gel and stacking gel, load protein samples onto the gel and run in 1x SDS electrophoresis buffer at 100 V to separate samples. To visualize proteins, the gel is stained with coomassie blue staining buffer and destained with destaining buffer.

Resolving Gel: 10-15% Acrylamide/Bis-acrylamide (29:1); 375 mM Tris-Cl (pH8.8); 0.1% SDS; 0.1% AP; 0.4 μl/ml TEMED

Stacking Gel: 5% Acrylamide/Bis-acrylamide (29:1); 125 mM Tris-Cl (pH6.8); 0.1% SDS; 0.1% AP; 1 µl/ml TEMED

1x SDS Loading Buffer: 50 mM Tris-Cl (pH6.8); 100 mM DTT; 2% SDS; 0.1% Bromophenol blue; 10% glycerol

1x SDS Electrophoresis Buffer: 25 mM Tris; 250 mM Glycine; 0.1% SDS

Coomassie blue staining buffer: 0.1% Commassie Blue R-250, 40% ethanol, 2% Acetic acid

Destaining Buffer: 7% ethanol, 7% acetic acid

V-2-1-4-2-4 Western blot

After SDS-PAGE, the gel is equilibrated in Transfer Buffer for at least 10 min before transfer. 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 transfer proteins from gel to PVDF membrane at 270 mA for 2 hr at 4°C.

Wash membrane in TTBS for 5 min, block in milk-TTBS for 1 hr, then incubate in primary antibody diluted in milk-TTBS at 4°C overnight. After washing 3 times with milk-TTBS, incubate membrane in secondary antibody diluted in milk-TTBS at room temperature for 1 hr, then wash membrane once with milk-TTBS, 3 times with TTBS and once with TBS. Finally detect signal on membrane 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.

Milk-TTBS: TTBS buffer containing 5% non-fat milk powder.

V-2-2 Tobacco BY2 (TBY2) cells culture and transformation

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 Thiamine; 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

Inoculate a single colony of *Agrobacterium tumefaciens* LBA4404 in 2 ml of YEB in presence of antibiotics and incubate at 28˚C for 24-48 hr. Then the bacteria are activated by inoculating 50 µl of culture into 2 ml of fresh of YEB with the same antibiotics and incubated at 28˚C overnight. Harvest bacteria cells by centrifugation at 3,000 rpm for 10 min, and wash once with 10 mM $MgSO_4$. Finally resuspend bacteria pellet in 2 ml of 10 mM $MgSO_4$. Acetosyringone is added to bacteria suspension to a final concentration of 200 μM and kept at least 2 hr before use.

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

V-2-3 *Arabidopsis thaliana* culture, callus regeneration and transformation

V-2-3-1 *Arabidopsis* culture

Treat *Arabidopsis* seeds with 70% ethanol containing 0.2% Tween-20 by rotating on a wheel for no more than 10 min. After that, wash seeds with 96% ethanol twice and dry up completely. Then spread seeds carefully on the plate containing *Arabidopsis* medium. Wrap plates with breathing tape and keep in cold room for a couple of days before transferring to growth room.

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

V-2-3-2 Explant and callus regeneration

After seedlings are grown until the first 3 true leaves appear, cut segments from cotyledon, leaves(1^{st}–3^{rd}), hypocotyls and root, and further cut by knives for more wound areas. Sterilely transfer these segments to *Arabidopsis* Medium containing corresponding phytohormones to induce distinct regeneration. For root induction, 1 mg/L IAA and 0.2 mg/L IBA are included; for shoot induction, 0.54 μM 1-NAA and 3.2 μM BAP are included; and for callus regeneration, 2.3 μM 2,4-D, 11.4 μM IAA and 3.2 μM BAP are included. After transfer, these segments are maintained in growth room for 2 weeks more.

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

Inoculate a single colony of transformed *Agrobacterium tumefaciens* in 3 ml YEB in presence of antibiotics, and incubate at 28˚C overnight. This 3 ml of overnight culture is then diluted in 300 ml YEB with the same antibiotics and incubated at 28˚C for another 16-24 hr until the OD_{600} reaches about 1.2-1.6. Harvest bacteria cells by centrifugation at 5,000 rpm for 10 min, and wash once with 100 ml of 10 mM $MgSO_4$ and finally resuspended in 10 ml of 10 mM $MgSO_4$. Acetosyringone is added to bacteria suspension to a final concentration of 200 μM and treated at least 20 min before flower dip.

Dilute 10 ml of bacteria suspension into 200~250 ml with Infiltration Medium. Following dipping flowers in Infiltration Medium for 1-2 min, put the plant immediately in small “growth chamber”, which helps to keep a high humidity. After 24 hr, open the door of the “growth chamber” 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 Pull down assay

Stably transformed and estradiol-inducible tobacco BY2 cells or *Arabidopsis* expressing GFP

or 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 chromatin proteins. The whole cell lysate is centrifuged at 20,000 g for 30 min at 4°C. Collect supernatant and mix with Glutathion-sepharose beads pre-fixed with GST or GST fusion proteins. After rotating for 2 hr at 4°C on a wheel, the beads are washed for 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 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 *In vitro* Casein kinase 2α (CK2α) kinase assay

V-2-5-1 Kinase and substrate

CK2α cDNA is obtained by RT-PCR from *Oryza sativa*, and cloned into pGEX-4T-1 by *Bam*H1 and *Xho*I, resulting as pGEX-4T-1-*Os*CK2α. After freshly transformed into DH5α host cell, recombinant proteins are expressed and purified as methods mentioned above.
Coding regions of rice and tobacco NAP1 cDNAs are cloned into pET-14b by using *Nde*I and *Bam*HI restriction sites. After freshly transformed into BL21 (DE3), recombinant proteins are expressed and purified as methods mentioned above.

V-2-5-2 Kinase assay

Incubate 2 μg of recombinant NAP1 protein with 2 ng of purified recombinant GST-OsCK2α in 20 μl of Kinase Buffer in presence of 5 μCi ^{32}P-γ-ATP at 30°C for 20 min. Casein (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France) is used as a control substrate. Reaction products are separated on 12% to 15% SDS-PAGE gels. Proteins on the gel are visualized by Coomassie Brilliant Blue R250 staining, while radioactive products by

auto-radiography.

Kinase Buffer: 50 mM Tris-Cl (pH 7.5); 10 mM $MgCl_2$; 0.1 M NaCl; 1 mM DTT

V-2-6 Chromatin immunoprecipitation (CHIP)

20-day-old *Arabidopsis* seedlings are used for CHIP analysis. Dip seedlings in Fix Buffer in vacuum for 10 min for crosslink, and then add glycine to a final concentration of 0.125 M and for additional 5 min in vacuum. 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, centrifuge the whole lysate at 3,500 rpm at 4°C for 10 min. Resuspend the pellet in 1 ml Lysis Buffer. DNA is sheared by sonication to approximately 500~1000 bp fragments. After centrifugation at maximum speed for 10 min at 4°C, pre-clear the supernatant by adding 40 μl of Protein A beads (Amersham Biosciences) and rotate at 4°C for 1 hr. After centrifugation at 500 g for 2 min at 4°C, mix the supernatant with appropriate antibody at 1:100 dilution, followed by incubation at 4°C overnight. Collect the immune complexes by adding 40 μl of Protein A beads and rotate at 4°C for 1 hr. Wash Protein A beads 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-mercaptoethanol.

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-mercaptoethanol.

Elution buffer: 1% SDS; 0.1 M $NaHCO_3$.

V-2-7 Comet assay

Harvest plant material and briefly rinse in liquid MS medium, carefully dry with a paper towel and then immediately use for comet assay or freeze in liquid nitrogen and store at –80°C. Microscopic slides were pre-coated with a layer of 1% normal melting point agarose dissolved in water and thoroughly dried (such as air-dried overnight).

Slice plant material within 300-400 μl of PBS containing 50 mM EDTA on ice with a fresh razor blade. Filter suspension with 100 μm nylon mesh to remove visible pellet. Mix 40 μl of filtered suspension of nuclei with same volume of liquid 1% low melting point agarose at 42°C and quickly drop separately on 2 pre-coated slides, then cover with a 22 mm*22 mm cover-glass and keep on ice.

After 30 min, take off the cover-glass, then subject the nuclei to lysis in High Salt Buffer for 20 min at room temperature. Equilibrate the slides with ice-cold TBE buffer for 5 min for 3 times, then electrophorese at room temperature in TBE at 17 mA for 10 min.

To clear gels of starch grains present in the nuclear suspension, keep the slides for 10 min in 1% Triton X-100 prior to dehydration for 2*5 min in 70 and 96% ethanol and air-drying(such as air-dried overnight). These slides could be kept for a long time at room temperature. Dry agarose gels are stained with 20 µl of fresh EtBr solution, covered with a cover-glass and used for evaluation. The comets were viewed with a Nikon E800 epifluorescence microscope and analyzed with the CometScore. (http://autocomet.com)

Each experimental point is represented by the mean value(standard deviation =S.D.) of 400 data from 4 comet gels, based on the median values of the percentage of DNA tail of 100 individual comets per gel.

High Salt: 2.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 100 mM EDTA

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