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Chromatin Structure and Transcriptional Regulation in *Arabidopsis*

（シロイヌナズナにおけるクロマチン構造と転写制御）

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INTRODUCTION

In the eukaryotic nucleus, DNA is wrapped around histone octamers to form nucleosomes. Nucleosome arrays are further organized into higher-order chromatin structures to a variable level of condensation. During M phase of cell cycle, the chromatin is organized into cytologically highly-condensed structure called mitotic chromosome (Fig. 1). This chromatin structure is fundamental for transcriptional regulation of eukaryotic genes in following two aspects. First, dynamic range of transcriptional regulation is about 10-fold above basal level with naked DNA and transcription regulators, however, chromatin structure can expand this range to approximately 25,000-fold by reducing the basal transcription level by inhibiting the accessibility of these regulators to the DNA (Bird and Wolffe 1999). This expansion of the dynamic range is essential to precisely and dynamically control gene transcription in eukaryotic cells. Second, heritable DNA sequence is virtually unchanged during cell cycle, in different tissues or developmental stages. Precise transcription from specific genes can be achieved by changing chromatin structure of the virtually unchanged DNA. The importance of these two aspects of chromatin in transcriptional regulation mainly comes from research in yeast, insects and animals. Because almost all genes are transcribed on chromatin template *in vivo*, to fully understand the transcription, it is

essential to analyze the influence of chromatin structure on transcription. In plants, importance of chromatin structure in transcriptional regulation starts to be shown mainly by genetic analysis. In this thesis, to understand the relationship between chromatin structure and transcription in plants, I directly analyzed chromatin condensation states (Chapter I), locally nucleosome-free regions and histone modifications of various types of genes (Chapter II), and examined relationship between presence of these nucleosome-free regions and transcriptional competency of genes (Chapter III) in *Arabidopsis*. Based on my results and other reports, I would like to overview the roles of chromatin structure in transcriptional regulation of genes in *Arabidopsis*.

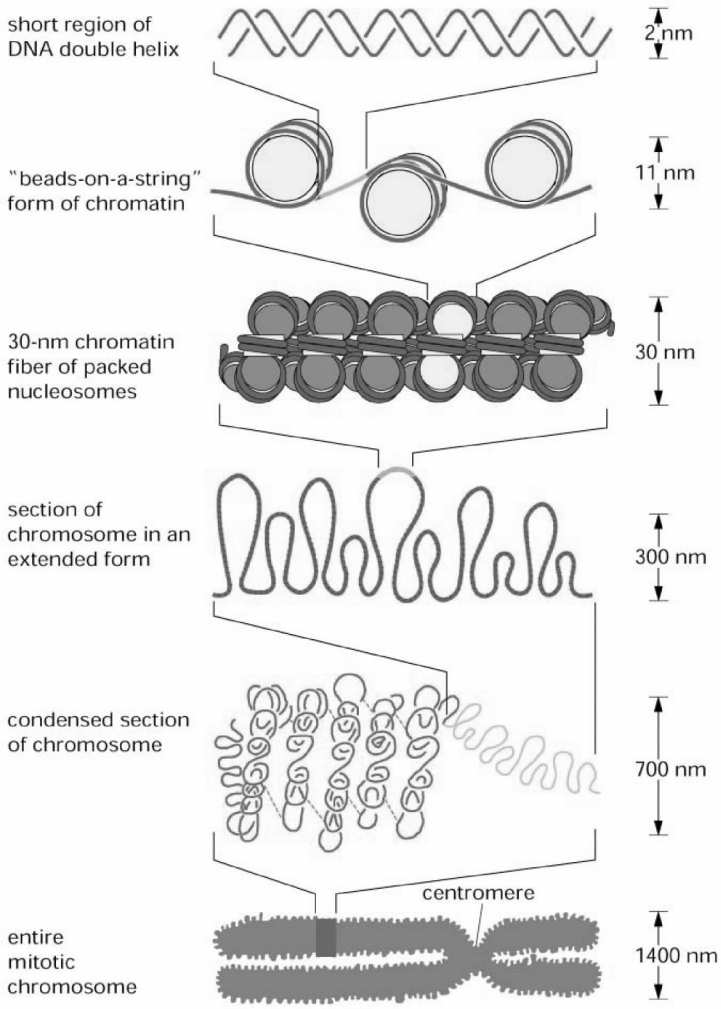


Fig. 1 Packaging of DNA into nucleosome, chromatin and chromosome in eukaryotic nucleus. Figure is adopted from Molecular Biology of the Cell, 4th edition, Garland publishing, p 230.

CHAPTER I: Chromatin Condensation and Gene Expression State in *Arabidopsis*

1.1 Introduction

In the eukaryotic nucleus, DNA is wrapped around histone octamers to form nucleosomes. Nucleosome arrays are further organized into higher-order chromatin structures to a variable level of condensation (Horn and Peterson, 2002). The higher-order chromatin can be roughly divided into two major types by their cytological appearance: heterochromatin and euchromatin. In general, heterochromatin consists of repetitive elements such as centromeres and telomeres, is inactive, and is constitutively highly condensed. On the other hand, euchromatin where most genes reside, is relatively decondensed. In addition to these general differences, euchromatic regions exhibit regionally varying degrees of condensation, as measured by their nuclease sensitivity. Inactive genes are embedded in condensed, inaccessible chromatin, whereas active genes are found in more open, accessible chromatin. For example, in chicken erythrocytes, the active *β-globin* locus resides in a 30 kb, DNase I-sensitive, open chromatin domain, whereas the inactive *ovalbumin* gene is contained in DNase I-resistant, closed chromatin (Caplan et al. 1987, Fisher and Felsenfeld 1986, Kimura et al. 1983). These chromatin condensation states are believed to regulate gene

expression by controlling the accessibility of transcription regulators to the underlying DNA.

The chromatin condensation states of the plant genome are cytologically different as in other eukaryotes, typical heterochromatin regions such as centromere, nucleolus organizing regions and telomere are highly condensed. The regions outside of these typical heterochromatin are relatively decondensed in *Arabidopsis* (Fransz et al. 2002, 2006, Houben et al. 2003). However, these low-resolution microscopic analyses cannot assess the chromatin condensation states of inactive and active genes within euchromatin or of genes within heterochromatin region. Therefore, relationship between chromatin condensation and gene expression levels is largely unknown in plants. In this study, to understand this relationship in plants, I evaluated chromatin condensation states by measuring DNase I sensitivity at 500 bp resolution, at 30 variably expressed genes in an 80 kb genomic region, a gene thought to be repressed by polycomb group and heterochromatin protein 1-like genes, two genes located near the centromere and telomere, and a retrotransposon within genetically-defined centromere in *Arabidopsis thaliana*.

1.2 Materials and methods

1.2.1 Plant materials and growth conditions

A. thaliana ecotype Columbia Col-0 was grown in potting soil at 22°C under a 16-h-light/8-h-dark cycle.

1.2.2 Standard molecular biological techniques

Standard molecular biological techniques were performed essentially as described (Sambrook et al. 2001) unless otherwise stated. Restriction enzymes, other modifying enzymes and DNA polymerases were purchased from TOYOBO (Osaka, Japan), Takara (Kyoto, Japan), or New England Biolabs (NEB, County Road, MA), and used according to the manufacturer's recommendations unless otherwise stated.

1.2.3 Digestion with nucleases and DNA purification

Digestion with DNase I was performed using a modification of a previously described method (Vega-Palas and Ferl 1995). Leaves of six-week-old plants were collected and ground in a cold mortar with ice-cold nuclei isolation buffer (NIB; 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.3 M sucrose, 1 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulphonyl fluoride). The resulting slurry was homogenized

with a motor-driven homogenizer, and the homogenate was filtered through one layer of a 105-micron nylon mesh filter. The permeabilized cells were pelleted by centrifugation at 2000 g at 4°C for 10 min, washed twice with NIB and resuspended in an appropriate volume of NIB. The resulting suspensions were pre-incubated at 37°C for 2 min and digested with DNase I (Sigma, St. Louis, MO) at 0, 0.025, 0.25, 0.5, 1, and 2 U ml^{-1} at 30°C for 10 min. The reaction was stopped by adding EDTA to 16 mM. The samples were pelleted by brief centrifugation at 4500 g at 4°C and resuspended in nuclei extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 500 mM NaCl, 1% SDS and 10 mM β-mercaptoethanol) containing 0.35 mg ml^{-1} Proteinase K (Wako pure chemical, Osaka, Japan), the preparations were incubated at 65°C for 1 h. After the addition of 1/5 volume of 5 M potassium acetate, pH 5.5, suspensions were set on ice for 30 min. The debris were removed by centrifugation at 18000 g at 4°C for 30 min, the supernatants were extracted with chloroform and the DNA was then precipitated by the addition of 0.7 volume of isopropanol. The DNA was pelleted by centrifugation at 2000 g at 25°C for 30 min, washed with 70% ethanol and dissolved in appropriate volume of TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA) containing 100 μg ml^{-1} RNase (Roche, Indianapolis, IN). After incubation at 37°C for 1 h, the samples were extracted with phenol/chloroform, and the DNA was precipitated by adding 1/10 volume

of 3 M sodium acetate and 2.5 volume of ethanol, washed with 70% ethanol, and finally dissolved in 0.1 x TE buffer (1 mM Tris, pH 8.0 and 0.1 mM EDTA) and used as *in vivo* digested chromatin DNA. The purified intact genomic DNA was also digested *in vitro* with DNase I at 0.01 and 0.025 U ml^{-1} at 30°C for 10 min in NIB and used as *in vitro* digested naked DNA.

1.2.4 Preparation of DNA probes

All DNA probes used for nuclease sensitivity assays were 500 bp long and were prepared as below. Bacterial artificial clones (BACs) containing target genes were divided into continuous, non-overlapping 500 bp segments from the first nucleotide base (designated coordinate number 1) of each clone. Each 500 bp segment was numbered consecutively from number 1, preceded by two letters designating the clone from which it originated (for example, probe XK101 corresponds to bases 50001 to 50500 in the MXK3 clone). The BAC clones used were as follows: MXK3, F13C5 (*AGAMOUS*), F19I11 (ribosomal protein S1), and T25K16 (NAC1) (underlined letters are those used to name the corresponding probe series). The ALA region was amplified from the BAC clone T8H11 by PCR with the primers

5’-GGTTACATGTTATTTCAAGAGATCATAGAC-3’ and

5’-GGATGAGTAAGAGGTTGTTGATGAAGAGGA-3’ (Kumekawa et al. 2000). Other 500 bp DNA fragments were amplified from the *Arabidopsis* genome by PCR using 30 mer primer pairs at 500 bp intervals. The amplified 500 bp fragments were inserted into the *Hin*cII site of pUC19, and the identity of the inserted DNA was verified by sequencing. The resulting plasmid was used as a template for PCR amplification with M13 forward and reverse primers, and amplified DNA fragments were used as a DNA probe. All information about gene position, gene annotation and expressed sequence tag (EST) is based on the Munich Information Center for Protein Sequences (MIPS) *A. thaliana* database (MAtDB; http://mips.gsf.de/proj/thal/db).

1.2.5 Total DNA isolation

Genomic DNA was isolated from *Arabidopsis* leaves or suspension cells according to the CTAB (cetyltrimethylammonium bromide) method (Rogers and Bendich 1994). Leaves or suspension cells were ground into a fine powder in the presence of liquid nitrogen with a mortar and pestle. The resulting powder was suspended in 2 x CTAB buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 2% CTAB, 1% polyvinylpyrrolidone, 20 mM EDTA) pre-warmed to 65°C. The suspension was incubated at 65°C for 1 h and extracted twice with chloroform. The aqueous phase was mixed with 0.1 volume of

10% CTAB solution (10% CTAB, 0.7 M NaCl) and further extracted with chloroform. To precipitate the total DNA, same volume of CTAB precipitation buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% CTAB) was added to the aqueous phase, and the solution was centrifuged at 2,000 g at R.T. for 30 min. The pelleted DNA was dissolved in high-salt TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1 M NaCl) and the DNA was precipitated with same volume of iso-propanol. After centrifugation at 2,000 g for 30 min, the DNA pellet was dissolved in TE buffer and treated with RNase (Roche) at 37°C. After the RNase treatment, the DNA was ethanol precipitated, washed with 70% ethanol, and finally dissolved in 0.1 x TE buffer for subsequent use.

1.2.6 Southern blot analysis for DNase I sensitivity and DNA methylation assays

The DNase I sensitivity of the specific genomic DNA regions was evaluated by Southern blot using the corresponding DNA probes. DNase I-treated DNA fragments were separated on 1% agarose gels and transferred to nylon membranes (Hybond-N+, Amersham Biosciences, Piscataway, NJ). The membrane was pre-hybridized in Denhart's hybridization buffer (0.375 M NaCl, 25 mM NaH_2PO_4, 40 mM EDTA, 0.25% SDS, 0.05% (w/v) Ficoll, 0.05% (w/v) polyvinylpyrrolidone, 0.05% (w/v) BSA) containing 100 μg/ml denatured salmon sperm DNA for 1 h at 60°C. DNA probe was

random-labeled with [α-^{32}P]dCTP using a *Bca*BEST Labeling Kit (Takara), added to the hybridization buffer, and further hybridized overnight at 60°C. The blot was washed once with 2 x SSC (0.3 M NaCl, 30 mM sodium acetate, pH 7.0) and 0.1% SDS for 20 min at 60°C, followed by a final wash with 1 x SSC and 0.1% SDS for 20 min at 60°C. The blot was visualized by exposure to X-ray film (New RX, Fuji Photo Film, Tokyo, Japan).

For the DNA methylation analysis, genomic DNA was digested with the methylation-insensitive enzyme or its isoschizomer; *Msp*I or *Hpa*II, *Mbo*I or *Sau*3AI, respectively. DNA methylation was assessed by comparing *Msp*I and *Hpa*II, *Mbo*I and *Sau*3AI digests of genomic DNA by Southern hybridization with DNA probes used in the DNase I sensitivity assay.

1.2.7 RT-PCR analysis

RNA was extracted from leaves and flowers using RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For RT-PCR analysis, total RNA was treated with DNase I. One μg of the DNA-free RNA was reverse transcribed using an oligo dT primer and MMLV reverse transcriptase (Applied Biosystems, Foster City, CA). The generated cDNA (equivalent to 25 ng of total RNA)

was used as a template for PCR amplification with gene-specific primers (primer sets are listed in Table 1-1). The PCR was performed for 24-26 cycles at 95°C for 1 min, 60°C for 30 sec, and 72°C for 1 min. The cycles of the PCR reaction were confirmed to occur within a linear amplification range.

1.3 Results

1.3.1 DNase I sensitivity is similar irrespective of gene expression level in the 80 kb MXK3 region

To systematically investigate chromatin condensation states at various genomic regions in the *Arabidopsis*, I used the DNase I sensitivity assay. This is a reliable method to assess chromatin condensation in a particular region in terms of overall accessibility to the 31 kDa protein DNase I (DNase I sensitivity) for many eukaryotes (Krebs and Peterson 2000, Yaniv and Cereghini 1986). I evaluated DNase I sensitivity as the extent of DNA digestion visualized by Southern blotting using 500 bp DNA probes to allow the measurement of chromatin condensation at higher resolution in the *Arabidopsis* genome. I analyzed an euchromatic 81,494 bp genomic region on chromosome V covered by BAC clone MXK3 and an *Athila* retrotransposon within the centromeric heterochromatin of chromosome V (ALA region) (Fig. 1-1A). The MXK3

region lies about 1 Mb from the end of the long arm of chromosome V (Fig. 1-1A), and contains 30 protein-coding genes with expression levels in leaves varying over 500-fold as measured previously (Hanano et al. 2002), two tRNA genes and one pseudogene (Fig. 1-1B). For direct comparison of the DNase I sensitivity of different genomic segments, hybridization was performed using a set of membranes prepared with genomic DNA that had been subjected to the same DNase I digestion series using the *Arabidopsis* leaf. To confirm specific hybridization of a probe to its target genomic region, *Eco*RI-, *Eco*RV- or *Hin*dIII-digested genomic DNA was also included in the blot (Fig. 1-2A, lanes E, V and H, respectively). DNase I sensitivity was analyzed using continuous and non-overlapping 500 bp DNA probes. A total of 163 probes covering the entire 81,494 bp MXK3 region were used.

The DNase I digestion profiles of bulk chromatin, as well as of the *Unknown* (At5g64890, XK66 probe), *β-1,3-glucanase* (At5g64790, XK3 probe), *cdc2-like protein kinase* (At5g64960, XK107 probe) and *ABC transporter protein 1-like* (At5g64840, XK30 probe) coding regions in leaves are shown in Fig. 1-2A, as representatives of the entire set of 163 autoradiographs (probe positions are indicated in Fig. 1-1B). The *Unknown* gene gave a very weak expression signal (Hanano et al. 2002), and only one EST has been identified according to the MAtDB database, suggesting that this gene is

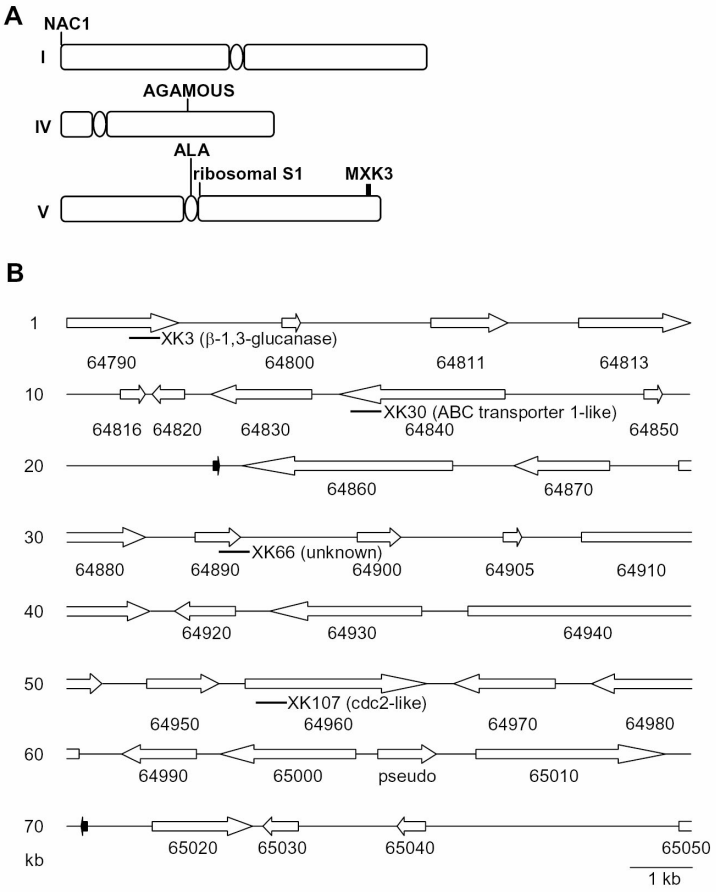


Fig. 1-1 Map of the target regions and genes on chromosomes and in MXK3 BAC clone. (A) Chromosomal positions of the MXK3 clone, ALA region, *AGAMOUS*, *ribosomal protein S1*, and *NAC1* genes. Roman numerals indicate chromosome identities and ovals represent core centromere regions. (B) Gene organization in the MXK3 region. The entire MXK3 region is represented by eight horizontal lines (each line corresponds to a 10 kb segment). The 30 protein-coding genes are represented by open arrows, with a five-digit number below each arrow indicating their AGI (Arabidopsis Genome Initiative) locus codes (At5gxxxxx). One pseudogene (open arrow, pseudo) and two tRNA-coding genes (closed arrows) are also shown. The positions of the XK3, 30, 66 and 107 segments are indicated by thick horizontal bars with the corresponding gene names. Bar indicates 1 kb. The map is drawn to scale.

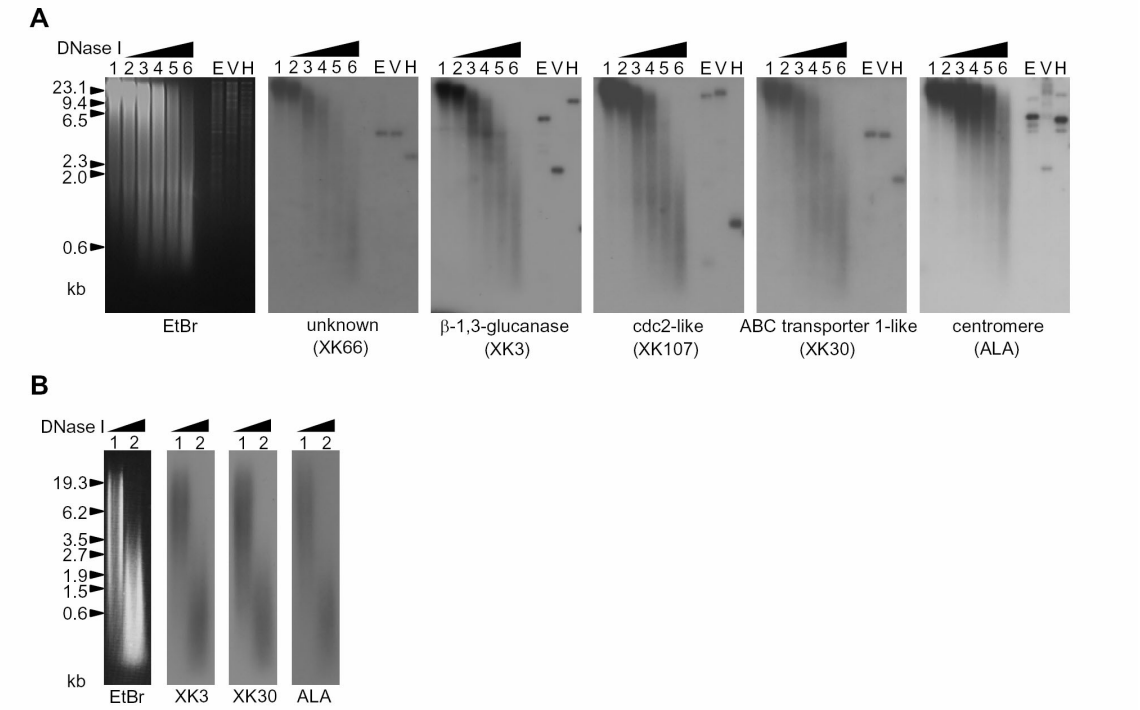


Fig. 1-2 DNase I sensitivity of the *Unknown*, *β-1,3-glucanase*, *cdc2-like protein kinase* and *ABC transporter protein 1-like* genes, and the core centromere region of chromosome V in leaves. (A) Chromatin of leaves was digested with DNase I at 0, 0.025, 0.25, 0.5, 1, and 2 U ml^{-1} (lanes 1-6, respectively), and DNA was isolated and 20 μg was fractionated on an agarose gel. Intact genomic DNA was digested with *Eco*RI, *Eco*RV, or *Hin*dIII (lanes E, V and H, respectively). An ethidium bromide-stained gel is shown at the left with molecular weight markers on the left side. The general DNase I sensitivity of the *Unknown*, *β-1,3-glucanase*, *cdc2-like protein kinase*, *ABC transporter protein 1-like* genes, and of the centromeric ALA region of chromosome V, is shown with the corresponding probe names below. (B) Naked genomic DNA was digested with DNase I at 0.01 and 0.025 U ml^{-1} (lanes 1 and 2), and DNA was isolated; 12 μg was fractionated, transferred to membranes and hybridized. An ethidium bromide-stained gel is shown at the left with molecular weight markers on the left side. The *in vitro* DNase I digestion profiles of the XK3, X30 and ALA probes are shown, with the corresponding probe names below.

hardly expressed in leaves. The *β-1,3-glucanase* gene gave a weak expression signal, whereas the *cdc2-like protein kinase*, *ABC transporter protein 1-like* genes are expressed at about 30- and 220-fold higher levels than the *β-1,3-glucanase* gene, respectively (Hanano et al. 2002). In spite of the differences in expression levels, these four segments showed similar DNase I sensitivity, the majority of the DNA being digested to fragments smaller than 2 kb as seen in lane 6 of each membrane (Fig. 1-2A). In the other regions, all 500 bp MXK3 segments containing the coding and regulatory regions showed similar DNase I sensitivity to those of the XK3, 30, 66 and 107 segments (data not shown).

The DNase I sensitivity of the typical heterochromatin region was also analyzed. To avoid intense cross-hybridization to repetitive elements, one of the double-copy *Athila* retrotransposons (named ALA, Fig. 1-1A) within the genetically defined core centromere region of chromosome V was used as a probe (Copenhaver et al. 1999, Kumekawa et al. 2000). As expected, the centromeric ALA segment showed significantly decreased DNase I sensitivity compared to those of the XK3, 30, 66 and 107 segments (Fig. 1-2A, compare lanes 5 and 6).

Because DNase I has a little sequence specificity, I assessed the contribution of sequence-specific cleavage by DNase I to *in vivo* DNase I sensitivity assays for the XK3,

XK30 and ALA segments by Southern hybridization with DNase I-digested naked DNA. The XK3, XK30 and ALA probes gave almost identical digestion profiles upon Southern hybridization with DNase I-digested naked DNA (Fig. 1-2B), confirming that the signals observed in the DNase I sensitivity assays reflect the *in vivo* chromatin structure of the DNA.

1.3.2 DNase I sensitivity of *AGAMOUS* gene and of genes near heterochromatin

In addition to the genes in the MXK3 and the ALA region, I also analyzed the floral homeotic gene *AGAMOUS* (*AG*; At4g18960; Fig. 1-1A) on chromosome IV. In *Arabidopsis* leaf, the *AG* gene is repressed by polycomb-group (PcG) genes, *Curly leaf*, *Embryonic flower2, Fertilization independent endosperm*, and heterochromatin protein 1 (HP1)-like gene *Terminal flower 2* (Goodrich et al. 1997, Katz et al. 2004, Kinoshita et al. 2001, Kotake et al. 2003, Moon et al. 2003, Nakahigashi et al. 2005). PcG proteins are known to repress homeotic genes by reducing accessibility of their chromatin in *Drosophila* (Fitzgerald and Bender 2001, Zink and Paro 1995), and HP1 is a critical component of heterochromatin that causes chromatin condensation in animals (Maison and Almouzni 2004, Verschure et al. 2005). To directly investigate the condensation state of the *AG* gene, I examined the DNase I sensitivity of the repressed *AG* gene in

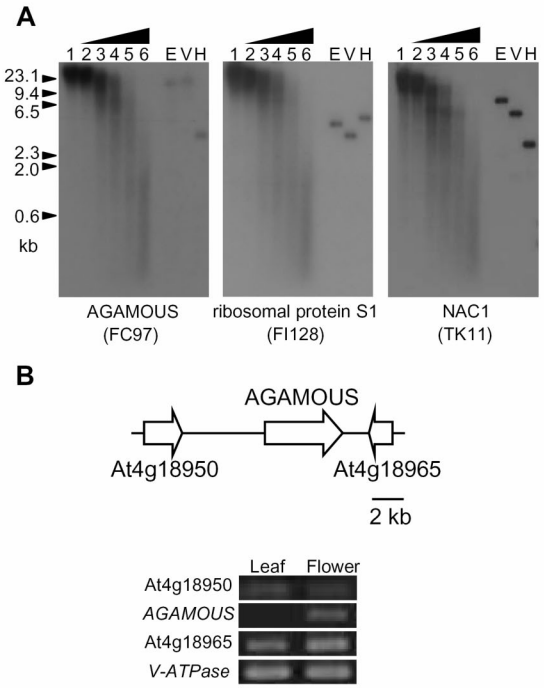


Fig. 1-3 DNase I sensitivity of the *AGAMOUS* gene and genes near heterochromatin in leaves. (A) DNase I sensitivity of the *AGAMOUS* gene and genes near heterochromatin in leaves. The general DNase I sensitivity of the *AGAMOUS*, *ribosomal protein S1* and *NAC1* genes in leaves is shown, with the corresponding probe names below. Symbols are as in Fig. 1-2. (B) Expression levels of the two neighboring genes At4g18950 and At4g18965, *AGAMOUS* and *V-ATPase* genes in leaves and flowers. Positions of the *AGAMOUS* and two neighboring genes are shown. Symbols are as in Fig. 1-1. Bar indicates 2 kb. RT-PCR was performed using cDNAs prepared from leaves or flowers as templates with gene-specific primer sets. The resulting PCR products were separated on agarose gels and stained with ethidium bromide.

leaves. Sensitivity of the *AG* gene was comparable to that in the MXK3 region and was significantly higher than that in the ALA region (Figs. 1-2A, 1-3A). Because the chromatin structure condensed by the PcG or HP1 proteins has been shown to spread into nearby regions and silence neighboring genes in *Drosophila* (Maison and Almouzni 2004, Zink and Paro 1995), expression levels of the two genes At4g18950 (*protein kinase like*, located at 5.1 – 7.6 kb upstream from the 5' end of the *AG* gene) and At4g18965 (*unknown*, located at 1.1 – 2.6 kb downstream from the 3' end of the *AG*

gene) surrounding the *AG* gene were examined in leaves and flowers (Fig. 1-3B). The *AG* gene was specifically expressed in flowers and unexpressed in leaves (Fig. 1-3B) as reported (Yanofsky et al. 1990). In contrast, the two neighboring genes were expressed both in leaves and flowers (Fig. 1-3B), suggesting that the expression of two genes is not affected by the PcG- and/or HP1-mediated repression at the *AG* locus.

In the *Arabidopsis* genome, a number of functional genes have been found in typical heterochromatin regions (Copenhaver et al. 1999, CSHL/WUGSC/PEB *Arabidopsis* Sequencing Consortium 2000, Kumekawa et al. 2000, Haupt et al. 2001). To gain insight into the chromatin structure of genes in heterochromatin regions, the *ribosomal protein S1* gene (*S1*; At5g30510) in the pericentromeric region of chromosome V (Kumekawa et al. 2000), and the *NAC1* gene (At1g01010) within the cytologically-condensed subtelomeric region of chromosome I (Avivi et al. 2004) (Fig. 1-1A), were analyzed in leaves. Both the *S1* and *NAC1* genes are surrounded by dispersed repetitive elements, the *S1* gene is located 0.3 kb apart from clusters of retrotransposons, and the *NAC1* gene is located 4 kb apart from the telomeric repeats. The *S1* gene is expressed in leaves (data not shown), and the *NAC1* gene is not expressed in leaves but is activated by protoplasting leaf cells (Avivi et al. 2004). As a result, sensitivity of the *S1* and *NAC1* genes was comparable to that in the MXK3 region

and was significantly higher than that in the ALA region (Figs. 1-2A, 1-3A).

1.3.3 DNA methylation is not correlated with gene expression levels

In addition to the chromatin condensation, to investigate the relationship between DNA methylation and gene expression levels, DNA methylation states were also analyzed. In general, DNA methylation is known to be linked to gene inactivation and DNA is heavily methylated in highly-condensed heterochromatin (Bird and Wolffe 1999). To this end, total genomic DNA from leaves was digested with the methylation-insensitive enzyme or its isoschizomer; *Msp*I or *Hpa*II, *Mbo*I or *Sau*3AI, respectively. DNA methylation was assessed by comparing *Msp*I and *Hpa*II, *Mbo*I and *Sau*3AI digests of genomic DNA by Southern hybridization with DNA probes used in the DNase I sensitivity assay. In the centromeric ALA region, DNA was heavily methylated as detected by the upward shift of the *Hpa*II- and *Sau*3AI-digested bands compared with those of their methylation-insensitive isoschizomers (Fig. 1-4D). However, the DNA methylation was not detected in the highly-expressed *ABC transporter 1-like* gene as well as unexpressed *β-1,3-glucanase* gene in the MXK3 (Figs. 1-4A, B). In addition, the DNA methylation was detected as the upward shift of the *Hpa*II-digested bands in the mediumly-expressed *cdc-2 like* gene (Fig. 1-4C). In the

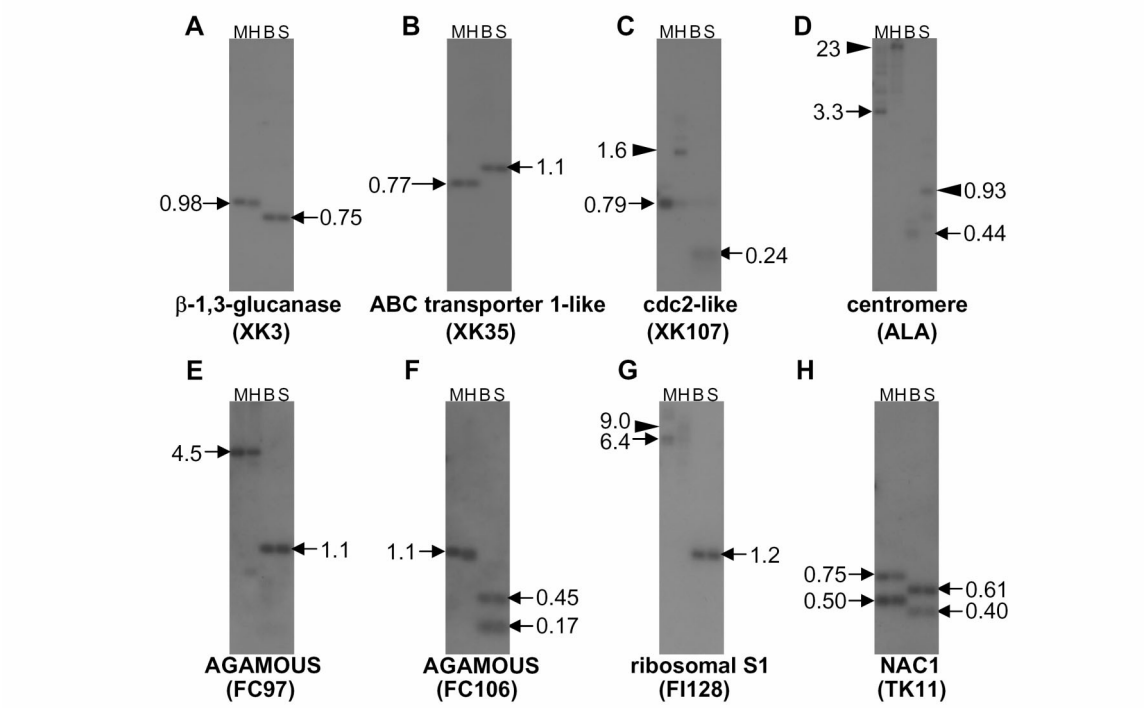


Fig. 1-4 DNA methylation states of the *β-1,3-glucanase*, *ABC transporter 1-like*, *cdc2-like*, *AGAMOUS*, *ribosomal S1*, *NAC1* genes and centromere region (ALA). The total DNA from leaves was digested completely with *Msp*I (lane M), *Hpa*II (H), *Mbo*I (B) or *Sau*3AI (S), and resolved on an agarose gel. The fractionated DNA was transferred to nylon membranes and used for subsequent Southern hybridization. The DNA methylation states of the *β-1,3-glucanase* (A), *ABC transporter 1-like* (B), *cdc2-like* (C), centromeric region (D), *AGAMOUS* (E and F), *ribosomal S1* (G), and *NAC1* (H) genes is shown with the corresponding probe names below. The *Msp*I-digested (left) and *Mbo*I-digested (right) bands are indicated by arrows, and upward shifted *Hpa*II-digested (left) and *Sau*3AI-digested (right) bands are indicated by arrowheads, with band sizes in kb. The other minor bands not indicated by arrows or arrowheads are derived from weak cross-hybridization of the probes.

other MXK3 regions, the DNA was basically unmethylated and in the methylated regions clear correlation with gene expression levels were not observed (data not shown). Similarly, in the regions other than the MXK3, the DNA was unmethylated in the *AG* gene and in the *NAC1* gene located near telomere (Figs. 1-4E, F, H). In the *ribosomal S1* gene located within the pericentromeric region, its DNA was methylated (Fig. 1-4G), however, this gene is expressed in leaves (data not shown).

1.4 Discussion

In this Chapter I, by analyzing DNase I sensitivity at 500 bp resolution over an 80 kb genomic region, I have shown that chromatin condensation state was not correlated with gene expression level (Figs. 1-1, 1-2). In the *Arabidopsis* genome, most genes are expressed to varying extents in multiple tissues and under various environmental conditions (Yamada et al. 2003). No large domains of hundreds of kilobases devoid of gene activity appear to exist aside from the regions of constitutive heterochromatin. At the chromosomal level, strong dimethylation of lysine 9 of histone H3 (H3K9me2, an epigenetic marker of heterochromatin) is restricted to constitutive heterochromatin, and is not detected in euchromatin (Houben et al. 2003). These observations, together with the present results, strongly suggest that the condensation states of higher-order chromatin are uniform and have little influence on gene expression within *Arabidopsis* euchromatin. Consistent with this notion, large number of *Arabidopsis* transgenic lines harboring same single-copy transgenes integrated at different positions of the genome, show uniform expression levels (De Buck et al. 2004, Nagaya et al. 2005, Schubert et al. 2004).

In leaf, the *AG* gene is thought to be repressed by heterochromatin formation analogous to *Drosophila* and mammalian cells (Hsieh et al. 2003), however, its chromatin

showed similar sensitivity to that of the MXK3 (Fig. 1-3A). It is possible that the *AG* gene is repressed by the PcG and HP1-like proteins through slightly-condensed heterochromatic structure which cannot be detected in my sensitivity assay. However, in the *Arabidopsis* leaf, the upstream At4g18950 and downstream At4g18965 genes were expressed (Fig. 1-3B). In the mutant lacking the HP1-like gene, the *AG* gene is up-regulated but expression of these two genes next to the *AG* is not affected (Nakahigashi et al. 2005). Recently, it was reported that the *AG* gene was repressed through H3K27me3 by the PcG proteins, however, this repressive epigenetic mark was not detected at these neighboring genes (Schubert et al. 2006). Collectively, these data suggest that the PcG and HP1-mediated heterochromatic structure of the *AG* gene is neither condensed nor propagated into nearby regions as seen in *Drosophila* and mammalian cells. The *S1* and *NAC1* genes are located near heterochromatin, however, their chromatin showed similar sensitivity to that in the MXK3 in leaves (Fig. 1-3A). In addition, the *S1* gene is expressed (data not shown) in leaves and the expression of the *NAC1* gene can be induced by protoplasting leaf cells (Avivi et al. 2004). These results suggest that genes can adopt decondensed chromatin structure even located within or near the constitutive heterochromatin.

In the analyzed regions, clear correlation between gene expression levels and DNA

methylation states were not found in the relatively rough DNA methylation assay using restriction enzymes (Fig. 1-4). This result is in contrast with the roles of the DNA methylation which is generally believed to function in gene inactivation (Bird and Wolffe 1999). Recently, genome-wide study of the DNA methylation at base pair resolution in *Arabidopsis* genome revealed that constitutive heterochromatin and transposons are heavily methylated as expected, however, over one third of expressed genes contain methylation within transcribed regions, while only ~5% of genes show methylation within promoter regions (Zhang et al. 2006). Interestingly, genes methylated in transcribed regions are highly expressed and constitutively active, whereas, promoter-methylated genes show a greater degree of tissue-specific expression (Zhang et al. 2006). The cytosine methylation which could not be detected in my assay using restriction enzymes, possibly show these reported patterns.

In this Chapter I, I have shown that the condensation states of higher-order chromatin are relatively uniform within *Arabidopsis* euchromatin. This finding suggests that transcription is not regulated by global changes in accessibility of higher order chromatin within *Arabidopsis* euchromatin. Local nucleosome positioning, histone modifications or DNA methylation not influencing the condensation states of higher-order chromatin may play a principal role in transcriptional regulation of the

Arabidopsis genes.

I summarized these results of the Chapter I into a conceptual model shown in Fig. 1-5. Previously, inactive gene is generally believed to be repressed by condensed higher-order chromatin, repressive histone modifications including hypoacetylation, H3K9me2 of histones and/or DNA methylation extending over the entire locus and neighboring genes (Fig. 1-5A). To activate this repressed gene, global changes in higher-order chromatin structure including decondensation of chromatin, acetylation of histones, removal of repressive histone modifications and/or DNA methylation are thought to be required over the entire locus. These global changes in chromatin structure may influence the expression of neighboring genes. Contrary to this, in my model based on the results of the Chapter I and other studies mentioned above, genes are not repressed by condensation of higher-order chromatin rather by local repressive histone modifications and/or DNA methylation mainly at promoter and coding regions of genes in *Arabidopsis*. To activate this repressed gene, local changes in chromatin structure such as changes in histone modifications and/or DNA methylation not affecting the higher-order chromatin structure are only necessary in *Arabidopsis*. These local changes in chromatin structure do not influence the expression of neighboring genes.

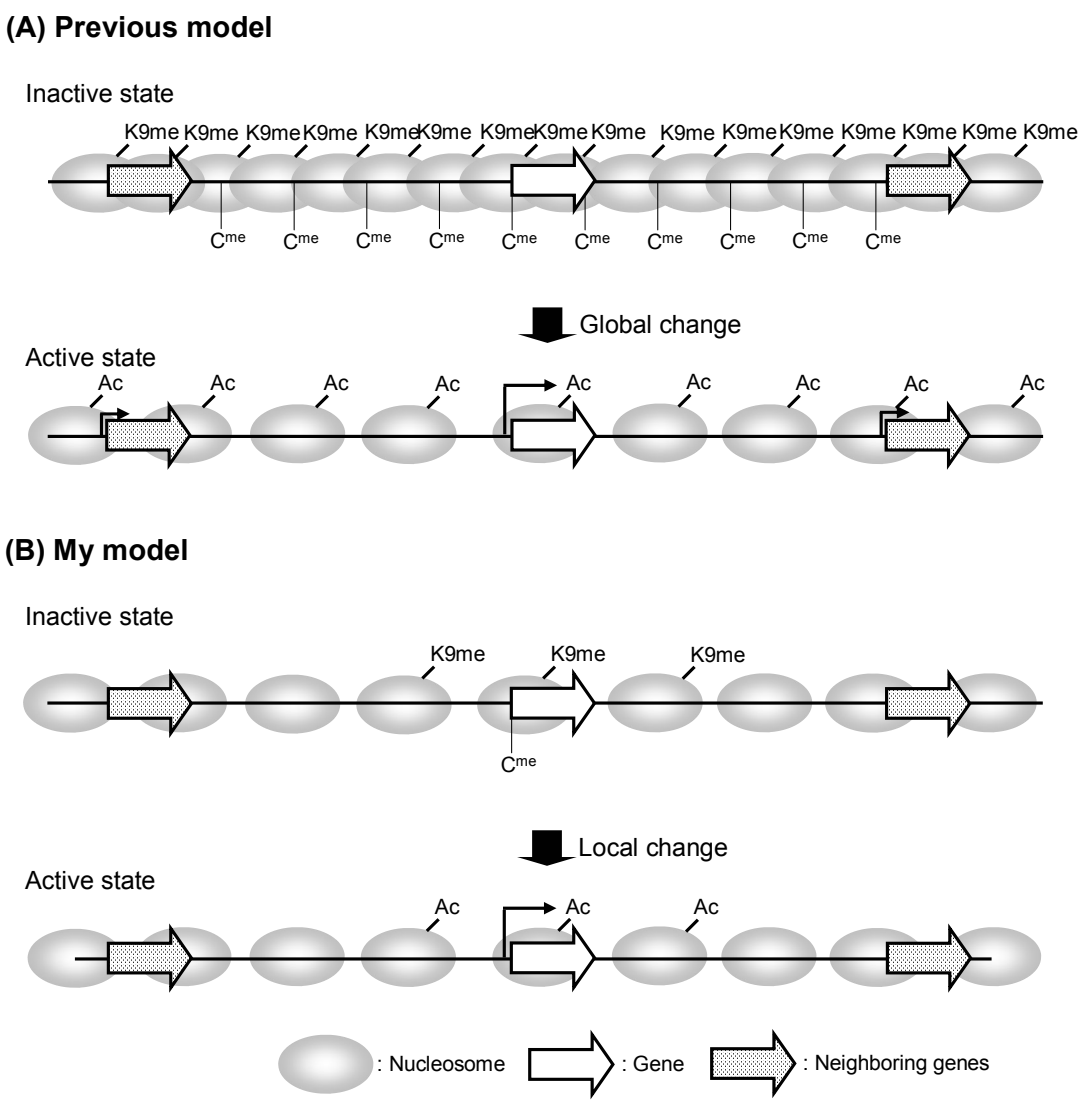


Fig. 1-5 Previous and my conceptual models of chromatin structure of inactive and active genes in *Arabidopsis*. (A) Previous model. At inactive state, gene (open arrows) is repressed by highly-condensed chromatin (depicted by dense nucleosome array) and repressive epigenetic marks H3K9me2 (nucleosomes with K9me) and DNA methylation (C^{me}). This repressive chromatin structure is extended over the entire locus and neighboring genes (dotted arrows). Global changes in chromatin structure (changes extend over entire locus), decondensation of higher-order chromatin, acetylation of histones (nucleosomes with Ac), removal of H3K9me2 or DNA methylation are needed to activate this repressed gene. This higher-order chromatin structure influences expression of neighboring genes. (B) My model based on results in the Chapter I and other studies. At inactive state, repressed gene is not highly-condensed and repressive epigenetic marks, H3K9me2 and DNA methylation are present mainly at promoter and coding regions of the gene in *Arabidopsis*. To activate this inactive gene, only local changes in chromatin structure (not affecting higher-order chromatin structure), acetylation of histones, removal of H3K9me2 or DNA methylation around promoter region are necessary. These changes in local chromatin structure do not influence expression of neighboring genes. Nucleosomes are indicated by gray ovals.

1.5 Tables

Table 1-1

Gene specific primer sets used in the RT-PCR assay.

Gene	Name	Sequence (5' to 3')
At4g18950	F	CTCATTTGCATTGATCGTTCAGG
	R	CAAATGTGAACCGGATGATGAG
AGAMOUS	F	CAACAGCAATCACGGCGTACC
	R	TCTCTAATCTGCCTTCCAAG
At4g18965	F	CAAAACAGCAGAGACGGGGTG
	R	CACTCTGTGTTTGCTTGGAGG
V-ATPase	F	CAAGCATCGCTTAGGAGATCTTTTCTACCG
	R	GCTCACAGAGCTAAAACTCAATCGATCGAC

CHAPTER II: DNase I Hypersensitive Site and Transcriptional Activation

2.1 Introduction

In the Chapter I, condensation states of the higher-order chromatin structure are relatively uniform irrespective of gene expression levels within *Arabidopsis* euchromatin. This result suggests that most genes are not regulated by changes in condensation state of higher-order chromatin in *Arabidopsis*. This finding also suggests that *Arabidopsis* gene transcription is primarily regulated by changes in local nucleosome structure and histone modifications.

The fundamental unit of chromatin is the nucleosome, which consists of 147 bp of DNA wrapped 1.7 times around a core histone octamer in a left-handed superhelix (Luger et al. 1997). The packaging of DNA into nucleosomes affects sequence accessibility to many DNA binding proteins, and the positioning of nucleosomes along the genome has been implicated in the regulation of gene expression (Anderson and Widom 2000, Venter et al. 1994, Wallrath et al. 1994). In the eukaryotic genome, nucleosome-free regions with enhanced chromatin accessibility are detected as DNase I hypersensitive sites (DNase I HSs), and these have been found predominantly in gene regulatory regions including promoters, enhancers and locus control regions (Gross and

Garrard 1988). DNase I HSs are thought to participate in transcriptional regulation by enhancing the access of transcription factors to resident *cis*-binding elements (Gross and Garrard 1988). For example, in the stress-inducible yeast *PHO5* and *HSP82* gene promoters, the presence of DNase I HSs at *cis*-elements at their inactive states allows their rapid transcriptional activation under stress conditions by facilitating the access of these *cis*-elements to transcription factors (Gross et al. 1993, Lu et al. 1993, Svaren et al. 1994, Venter et al. 1994). Recently, the genome-wide mapping of DNase I HSs in yeast and human was undertaken, and the prevalence of DNase I HSs was much higher than previously thought (Bernstein et al. 2004, Crawford et al. 2004, 2006a, b, Dorschner et al. 2004, Ercan and Simpson 2004, Sabo et al. 2004, 2006, Yuan et al. 2005). In yeast chromatin, DNase I HSs are found at *cis*-regulatory elements in the promoters of most genes, but they are not found in the coding regions (Bernstein et al. 2004, Ercan and Simpson 2004, Yuan et al. 2005). In human chromatin, DNase I HSs are also enriched in gene regulatory regions such as promoters, CpG islands, transcription termination sites, however, in contrast with yeast, half of the HSs are found in introns (Crawford et al. 2004, 2006a, b, Dorschner et al. 2004, Sabo et al. 2004, 2006).

In plants, DNase I HSs have been described in only a few genes including *Arabidopsis Adh* (Vega-Palas and Ferl 1995), tomato *Proteinase inhibitor I* (Conconi

and Ryan 1993), *Chlamydomonas HSP70A* and *RBCS2* genes (Lodha and Schroda 2005). These DNase I HSs were also found near *cis*-regulatory elements in the promoters of these genes (Lodha and Schroda 2005, Vega-Palas and Ferl 1995). To further understand and characterize the role of DNase I HSs in regulating plant gene expression, it is necessary to analyze the presence of HSs in numerous genes. In this study, I analyzed the chromatin structure of an 80 kb genomic region containing variably-expressed 30 genes by DNase I sensitivity assay at 500 bp resolution in *Arabidopsis*. Furthermore, I analyzed the position of DNase I HSs in the promoters of several well-characterized genes, and I further characterized changes in HS during the transcriptional activation of a heat-inducible gene in terms of chromatin accessibility, histone acetylation and transcription activator binding.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

Arabidopsis suspension cell strain T87 (Axelos et al. 1992) were grown in modified LS medium (Nagata 1992) with constant agitation at 84 rpm and 22°C under a 16-h-light/8-h-dark photoperiod. Six ml of suspension culture was transferred into 95 ml of fresh medium every week.

2.2.2 Preparation of DNA probes

All DNA probes used for nuclease sensitivity assays were 500 bp long and prepared as described in the Chapter I. For indirect-end labeling analysis (Wu 1980), probe H (51443-51942 on MTH12 clone, abutting the *Ase*I site), probe R (6832-7331 on K24M7, abutting the *Eco*RV site), probe P (78642-79141 on F22G5, abutting the *Xho*I site) and prove V (15668-16167 on F9K20, abutting the *Ase*I site) were used for the *HSP18.2*, *RD29b*, *PCNA1* and *V-ATPase* genes, respectively.

2.2.3 Nuclease sensitivity assays

Nuclease sensitivity assays were performed as described in the Chapter I. For DNase I digestion of suspension cells, 6-day-old suspension cells were collected and ground into a fine powder in the presence of liquid nitrogen with a mortar and pestle. The resulting powder was suspended in NIB and treated as described for leaves in the Chapter I. DNase I digestion was performed at 0, 5, 10, 20, 30, and 50 U ml^{-1}. For Micrococcal nuclease (MNase) digestion of suspension cells, the permeabilized cells were suspended in MNase buffer (50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 1.5 mM NaCl, 1 mM $CaCl_2$, 0.3 M sucrose, and 0.005 mM β-mercaptoethanol), pre-incubated at 37°C for

2 min, and digested with MNase (USB, Cleveland, OH) at 0, 10, 20, 50, 100, and 200 U ml^{-1} at 37°C for 10 min.

2.2.4 ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed using modifications of previously described methods (Gendrel et al. 2002). To crosslink suspension cells, formaldehyde was directly added to culture medium to a final concentration of 1% and shaked at 22°C for 20 min. Crosslinking was stopped by adding glycine to a final concentration of 0.1 M and incubating at 22°C for 5 min. Crosslinked cells were collected by vacuum infiltration and ground into a fine powder in the presence of liquid nitrogen with a mortar and pestle. The resulting powder was suspended in cold NIB and filtered through one layer of a 20-micron nylon mesh filter. The disrupted cells were pelleted by centrifugation at 2,000 g at 4°C for 10 min, washed once with NIB and suspended in a small volume of NIB. The suspension (equivalent to 24 mg of wet weight) was transferred to a 2 ml microcentrifuge tube and pelleted by centrifugation at 2,000 g at 4°C for 2 min and the supernatants were removed. The pelleted cells were lysed in 1.4 ml of Nuclei Lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS) containing proteinase inhibitor cocktail (Complete EDTA-free, Roche), and the

chromatin was sheared to an average fragment size of approximately 0.4 kb by 8 times of sonication (Astrason, MISONIX at level 4, Farmingdale, NY) for 10 sec pulse at 1 min intervals in ice. Any remaining cellular debris were removed by centrifugation. And the 0.5 ml of the chromatin solution was recovered for an input sample. The remaining chromatin solution was then diluted 10-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.0, 167 mM NaCl, 1 x proteinase inhibitor cocktail) and pre-cleared by incubating 40 μl of Protein-A sepharose (50% slurry in ChIP dilution buffer containing 100 μg ml^{-1} salmon sperm DNA; Protein-A sepharose 4 fast flow, Amersham Biosciences) for 1 h at 4°C. The pre-cleared chromatin solution was immunoprecipitated with histone H3 (#ab1791, Abcam, Cambridge, UK), H3-acetyl-K9, 14 (#06-599, Upstate), histone H4 (#ab10158, Abcam), H4-acetyl-K5, 8, 12, 16 (#06-866, Upstate), or Heat shock factor 1 (HSF1; rabbit polyclonal antibody raised against the unique C-terminus peptides, CNDFPTENYMDTEK and CRHMDKLIEELGL) antibodies overnight at 4°C. The immune complexes were collected by centrifugation at 2,000 g at 4°C for 2 min, washed with each of the following buffer, Low salt wash buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA), High salt wash buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA), LiCl wash buffer

(10 mM Tris, pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and TE buffer (twice). After final wash, to elute the immune complexes, 0.4 ml of elution buffer (1% SDS, 100 mM $NaHCO_3$) was added, mixed and incubated at 65°C for 15 min with agitation. The sepharose beads were pelleted and the supernatant was recovered. To reverse crosslinking, the eluate and input sample were incubated overnight at 65°C with 100 μg ml^{-1} proteinase K. The eluate and input sample were extracted with phenol/chloroform and the DNA was precipitated with ethanol in the presence of the precipitation carrier (Dr. GenTLE, Takara). The DNA pellet was washed with 70% ethanol and dissolved in an appropriate volume of the TE buffer.

For *Bcl*I digestion of crosslinked chromatin, disrupted cells (equivalent to 24 mg of wet weight) were suspended in 200 μl of NIB containing 60 mM NaCl, and incubated with 1,500 U ml^{-1} of *Bcl*I (NEB) at 40°C for overnight. The digested cells were lysed by adding 1.3 ml of NLS and treated as described above.

2.2.5 Quantitative measurement of immunoprecipitated DNA by realtime PCR

Immunoprecipitated DNA was quantitatively measured in triplicates by the ABI PRISM 7700 sequence detection system (Applied Biosystems) according to the manufacturer's recommendations. The realtime PCR amplification was performed

using a 5 μl aliquot of immunoprecipitated DNA as a template with 7.5 pmoles primers and 6.25 pmoles Taqman probe (primers and Taqman probes are listed in Table 2-1) in 25 μl reaction mix using Taqman Universal PCR Master Mix kit (Applied Biosystems). The standard curve was generated using 10, 1, 0.1 ng of the input DNA which had been measured using PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR).

2.3 Results

2.3.1 DNase I HSs are present at the 5' and/or 3' ends of most genes irrespective of their expression levels

I analyzed the DNase I sensitivity of an 81,494 bp-long region of the *Arabidopsis* genome covered by BAC clone MXK3 residing about 1 Mb from the end of the long arm of chromosome V at 500 bp resolution. This region contains 30 protein-coding genes with expression levels in leaves varying over 500-fold (Hanano et al. 2002), two tRNA genes and one pseudogene (for gene organization, see Fig. 2-1B). DNase I sensitivity was analyzed by Southern blotting with 500 bp DNA probes using membranes which were prepared with leaf genomic DNA that had been subjected to the same DNase I digestion series. To confirm the specific hybridization of a probe to its target genomic region, *Eco*RI-, *Eco*RV- or *Hin*dIII-digested genomes were also included

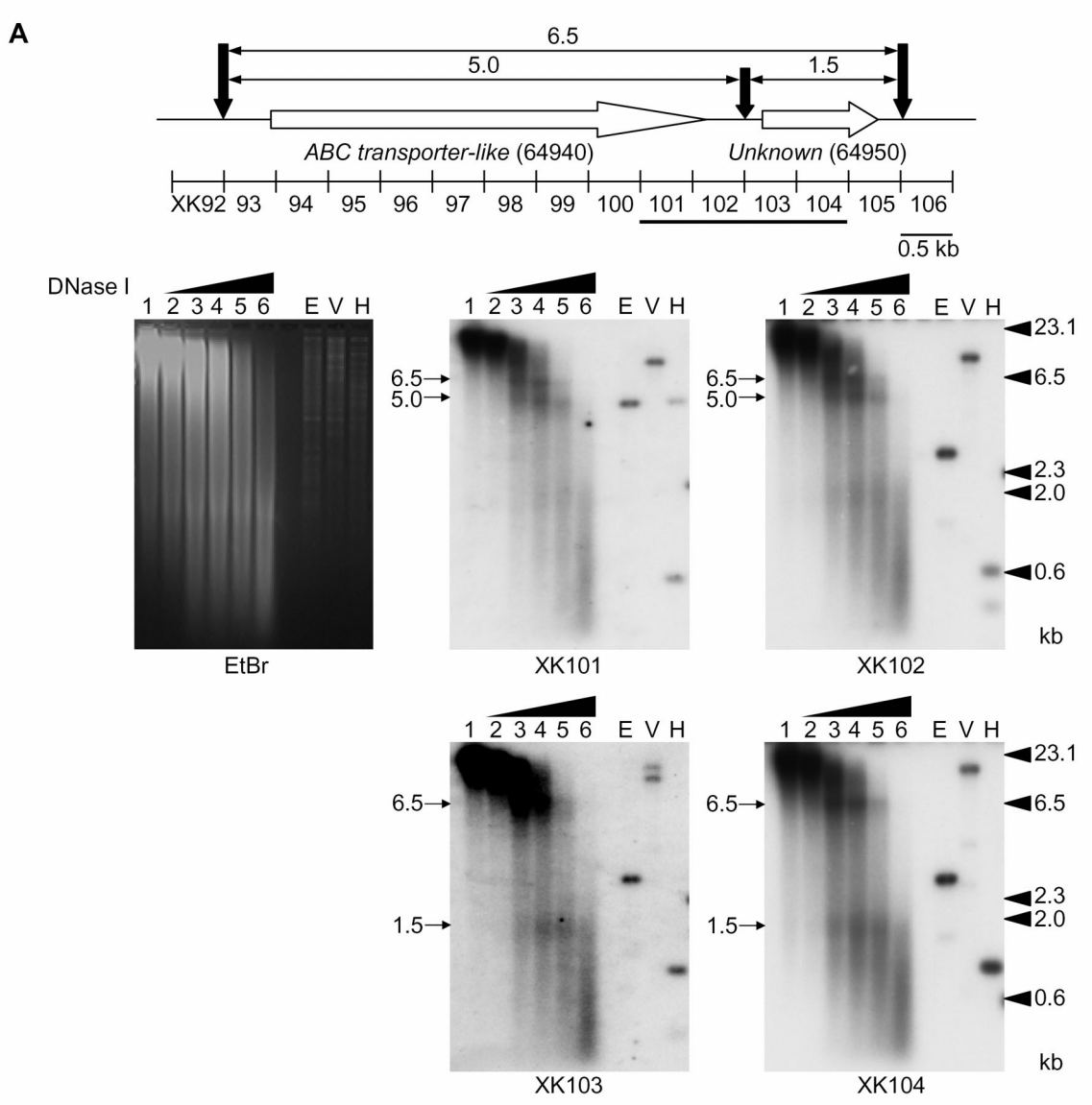


Fig. 2-1 (continue to next page)

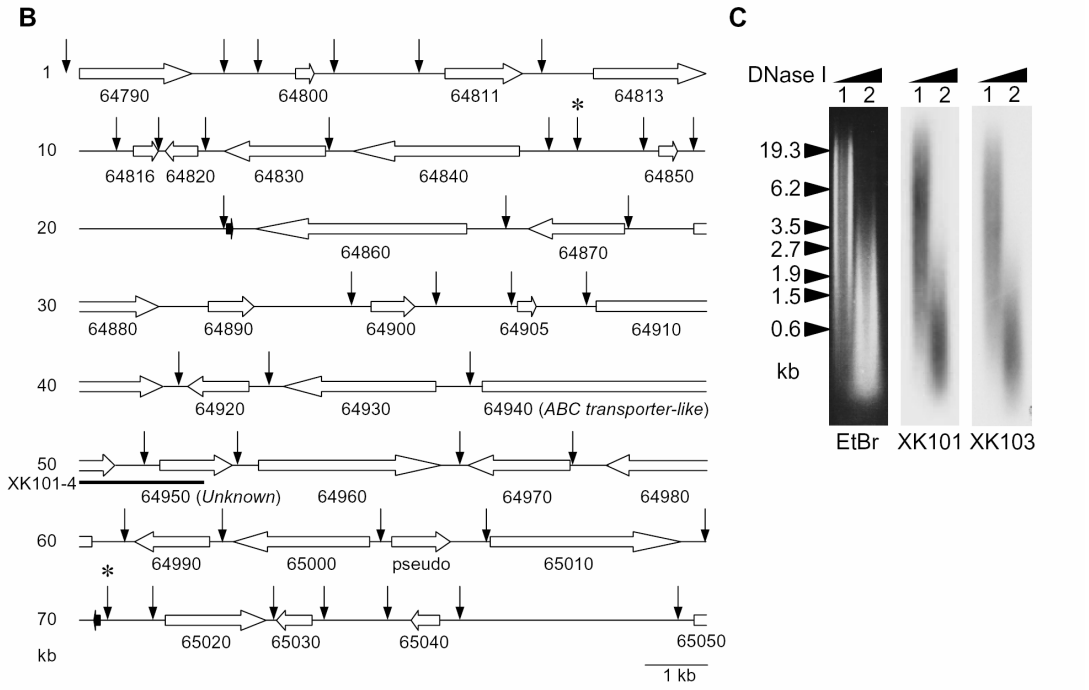


Fig. 2-1 (continued from previous page) Mapping of the DNase I HSs in the 80 kb MXK3 region. (A) A map of the DNase I HSs around the *ABC transporter-like* (At5g64940) and the *Unknown* (At5g64950) genes and the DNase I sensitivity of the XK101-104 region. Chromatin of mature leaves was digested with increasing amounts of DNase I (lanes 1-6). The intact genome was digested completely with *Eco*RI, *Eco*RV, or *Hin*dIII (lanes E, V and H, respectively). The isolated DNA (20 μg) was fractionated, transferred and hybridized. An ethidium bromide-stained gel is shown at the left. Molecular weight markers are indicated by closed arrowheads on the right sides. The arrows on the left sides of the autoradiographs indicate the positions of the bands. The genes are indicated by open arrows with their names and their five-digit AGI locus codes (At5gxxxxx) below. The positions of the probes (analyzed segments are underlined) are indicated by horizontal bars under the genes. The DNase I HSs are indicated by vertical arrows, and the distance between each site is indicated in kb. The Bar indicates 0.5 kb. (B) A map of the DNase I HSs over the MXK3 region. The entire MXK3 region is represented by the eight horizontal lines (each line corresponds to a 10 kb segment). The 30 protein-coding genes are represented by open arrows with their five-digit number AGI locus codes (At5gxxxxx). The *ABC transporter-like* (At5g64940) and the *Unknown* (At5g64950) genes are indicated with their names. The XK101-4 region is indicated by horizontal thick bar. One pseudogene (open arrow, pseudo) and two tRNA genes (closed arrows) are also shown. The positions of the DNase I HSs are marked by vertical arrows. Two DNase I HSs not associated with protein-coding genes are marked with asterisks. Bar indicates 1 kb. The map is drawn to scale. (C) Naked genomic DNA (6.5 μg) was digested with DNase I at 0.01 and 0.025 U ml^{-1} (lanes 1 and 2). The *in vitro* DNase I digestion profiles of the XK101 and XK103 regions, and an ethidium bromide-stained gel are shown with size markers at left sides.

in the membranes (Fig. 2-1A, lanes E, V and H, respectively). The DNase I sensitivity was analyzed using a total of 163 continuous and non-overlapping 500 bp DNA probes covering the entire 81,494 bp MXK3 region.

The DNase I digestion profiles of bulk chromatin and the XK101-104 region corresponding to a portion of the coding and intergenic regions of the *ABC transporter-like* (At5g64940) and *Unknown* (At5g64950) genes are shown in Fig. 2-1A as representatives of the entire set of 163 autoradiographs. In the XK101-104 region, bands derived from DNase I HSs were detected at 6.5 and 5.0 kb in the XK101-102 segment, and at 6.5 and 1.5 kb in the XK103-104 segment (Fig. 2-1A). This pattern indicates that a DNase I HS is located near the border of the XK102 and XK103 segments, and that two more sites exist 5.0 kb 5' of the *ABC transporter-like* gene and 1.5 kb 3' to this site (Fig. 2-1A). As predicted, different band patterns were observed in the XK92 and XK106 segments (data not shown). These three DNase I HSs were mapped to the 5' and 3' ends of the *ABC transporter-like* and *Unknown* genes (Fig. 2-1A). In total, I found 40 DNase I HSs and mapped them to the entire MXK3 region at 500 bp resolution (Fig. 2-1B). All DNase I HSs were located at the 5' and/or 3' ends of genes, with 28 of 30 genes possessing such sites, and sites were never found within the coding regions of genes. Single DNase I HS located between neighboring genes

may be associated with one of the adjacent genes, with both genes, or an additional site may exist. Given the high gene-density of the *Arabidopsis* genome and the limited resolution of our mapping method, I cannot distinguish between these possibilities for most of the genes contained in the region examined. The presence of 5' located DNase I HSs did not correlate with the expression level of the associated gene. For example, DNase I HSs were present at the 5' ends of the highly-expressed At5g64840 (*ABC transporter protein 1-like*) gene as well as the unexpressed At5g64811 (*WRKY transcription factor*) and At5g64900 (unknown) genes (Fig. 2-1B; gene expression levels are based on Hanano et al. 2002). In contrast to the DNase I HSs located at the 5' or 3' ends of genes, two sites were not associated with protein-coding genes (marked with asterisks in Fig. 2-1B). The only genes to lack DNase I HS were the At5g64880 and At5g64890 genes, which are both of unknown function (Fig. 2-1B). The bands observed in the XK101 and XK103 segments were not observed in DNase I-digested naked DNA (Fig. 2-1C), confirming that these bands are dependent upon the *in vivo* protein-bound structure of the DNA.

2.3.2 DNase I HSs are present near *cis*-regulatory elements in the promoters of well-characterized genes

In yeast chromatin, DNase I HSs are formed at *cis*-regulatory elements in gene promoters (Ercan and Simpson 2004, Yuan et al. 2005). To examine the position of the DNase I HSs in *Arabidopsis* gene promoters, I analyzed the well-characterized *HSP18.2* (*heat shock protein 18.2*; At5g59720) (Takahashi and Komeda 1989, Zhang et al. 2001), *RD29b* (*responsive to desiccation 29b*; At5g52300) (Uno et al. 2000), *PCNA1* (one of two copies of the *proliferating cell nuclear antigen* gene; At1g07370) (Kosugi and Ohashi 2002, Tremousaygue et al. 1999, 2003, Uemukai et al. 2005) and *V-ATPase* (*vacuolar-type proton ATPase subunit A*; At1g78900) (Magnotta and Gogarten 2002) genes in differentiated mature leaves and actively dividing suspension cells. The heat-inducible *HSP18.2* promoter has six heat shock factor (HSF) binding sites known as heat shock elements (HSEs), three HSEs (HSE1) distal and three HSEs (HSE2) proximal to the transcription initiation site (Takahashi and Komeda 1989, Zhang et al. 2001). The *RD29b* promoter has two abscisic acid (ABA)-responsive elements (ABREs) required for its transcriptional activation by ABA (Uno et al. 2000). The *PCNA1* gene has one E2F binding site and one AtPurα binding site known as telo-box, which are both involved in *PCNA* gene activation during the G1/S phase of the cell cycle

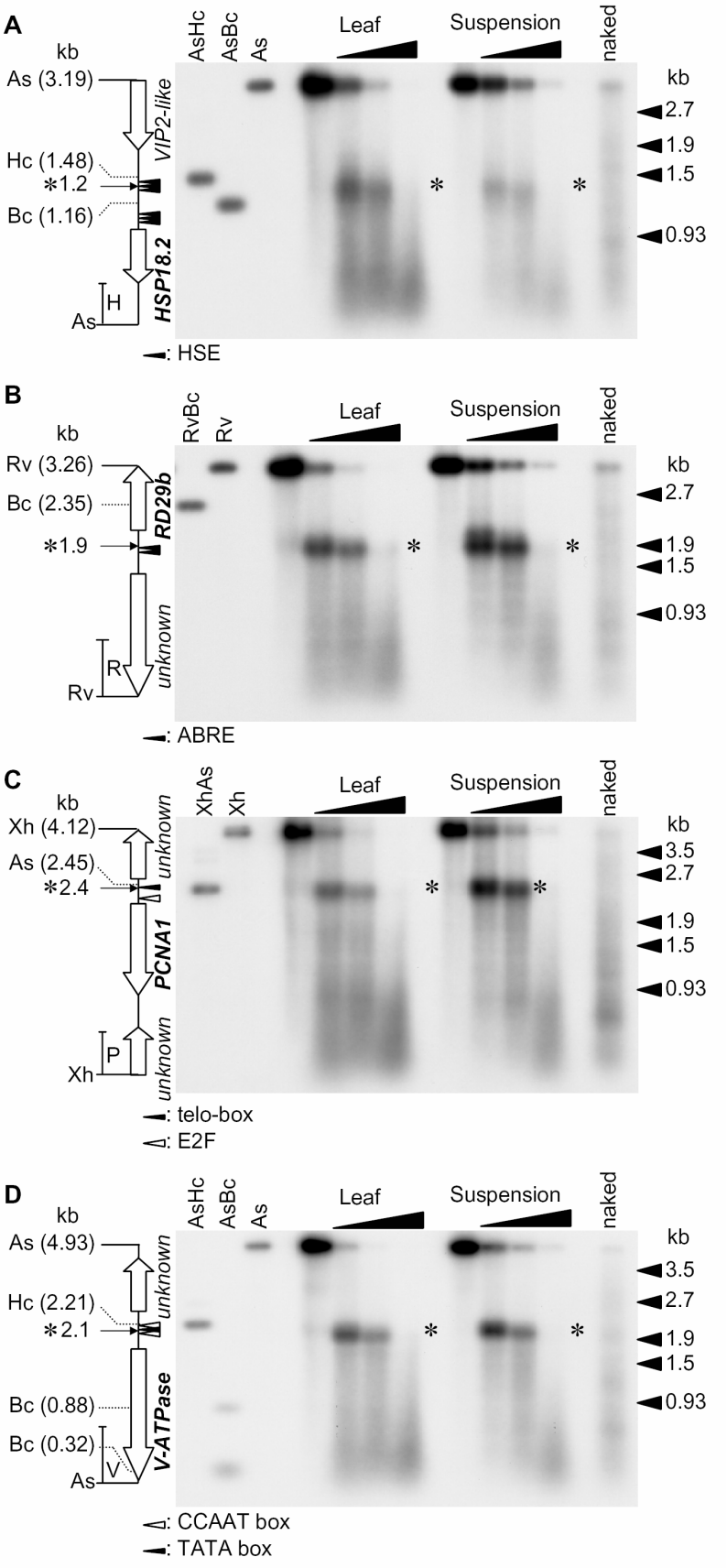


Fig. 2-2 (legend of this figure is on next page)

Fig. 2-2 (continued from previous page) Indirect-end labeling analysis of the *HSP18.2*, *RD29b*, *PCNA1* and *V-ATPase* gene promoters in leaves and suspension cells. (A-D) Chromatin of leaves or suspension cells were digested with DNase I at 0, 0.25, 0.5 and 2 U ml^{-1} (leaves) or at 0, 10, 20 and 50 U ml^{-1} (suspension cells). Naked genomic DNA (6.5 μg) was digested with DNase I at 0.01 U ml^{-1} (lanes naked). The isolated DNA (6.5 μg) was further digested with *Ase*I, *Eco*RV or *Xho*I. The blot containing *Ase*I, *Eco*RV or *Xho*I-treated DNA was hybridized with probe H, R, P or V to detect the 5'-region of *HSP18.2* (A), *RD29b* (B), *PCNA1* (C) and *V-ATPase* (D) genes. The positions of the genes (open arrows), DNase I HSs (horizontal arrows with sizes in kb and asterisks at right), *Ase*I, *Bcl*I, *Eco*RV, *Hin*cII and *Xho*I restriction sites (As, Bc, Rv, Hc and Xh with band sizes in kb, respectively), and probes H, R, P and V (vertical bars with H, R, P and V, respectively) are shown at the left sides. The positions of the six HSEs (A), two ABREs (B), one telo-box (C) and one TATA box (D) are indicated by horizontal closed arrowheads, one E2F-binding site (C) and two CCAAT boxes (D) are indicated by horizontal open arrowheads at left. The lanes As, AsBc, AsHc (A and D), Rv, RvBc (B), Xh and XhAs (C) indicate *Ase*I, *Ase*I-*Bcl*I, *Ase*I-*Hin*cII (A and D), *Eco*RV, *Eco*RV-*Bcl*I (B), *Xho*I and *Xho*I-*Ase*I (C) digests of intact genome included as size standards.

(Kosugi and Ohashi 2002, Tremousaygue et al. 1999, 2003, Uemukai et al. 2005). The *V-ATPase* promoter has two CCAAT boxes surrounding one TATA box (Higo et al. 1999), and it is constitutively expressed in various tissues reflecting its housekeeping function (Magnotta and Gogarten 2002).

To analyze the position of DNase I HSs at higher resolution, the HSs in the promoters of these four genes were mapped by the indirect-end labeling method. Using this procedure, I detected DNase I HSs as distinct bands near the promoter distal HSE1 in *HSP18.2*, near the two ABREs in *RD29b*, and near the telo-box in the *PCNA1*, and near the two CCAAT boxes and TATA box in the *V-ATPase* gene promoters in both leaves and suspension cells (Figs. 2-2A-D). The bands were detected at same position in these promoters using DNA probes abutting the other restriction enzyme sites (data not shown). Thus, DNase I HSs are located near transcription factor binding motifs in

these promoters. The DNase I digestion profiles of naked DNA gave smears or faint bands (Figs. 2-2A-D, lanes naked), confirming that bands observed *in vivo* are dependent on chromatin structure.

2.3.3 DNase I HSs are extended upon transcriptional activation of the *HSP18.2* gene

In yeast, DNase I HSs are reported to show structural alteration upon transcriptional activation of stress-inducible gene promoters (Venter et al. 1994). To characterize the roles of DNase I HSs in the transcriptional activation of an *Arabidopsis* gene, I analyzed the chromatin structure of the heat-inducible *HSP18.2* gene as a model. The *HSP18.2* gene was not expressed under normal conditions, but its transcription was rapidly induced by heating suspension cells to 30°C (Fig. 2-3A). I selected 0.5 h of heating at 30°C for further study because the mRNA amount was in steady increase at this time point. The DNase I hypersensitivity and nucleosome structure of the *HSP18.2* promoter were analyzed in both inactive (- Heat) and active states (+ Heat) by indirect-end labeling using the downstream probe abutting the *Ase*I site (probe H in Fig. 2-3B). In its inactive state, a DNase I HS was detected near the distal HSE1 (upstream of the *Bcl*I site in Fig. 2-3B, left); this site was also hypersensitive to micrococcal

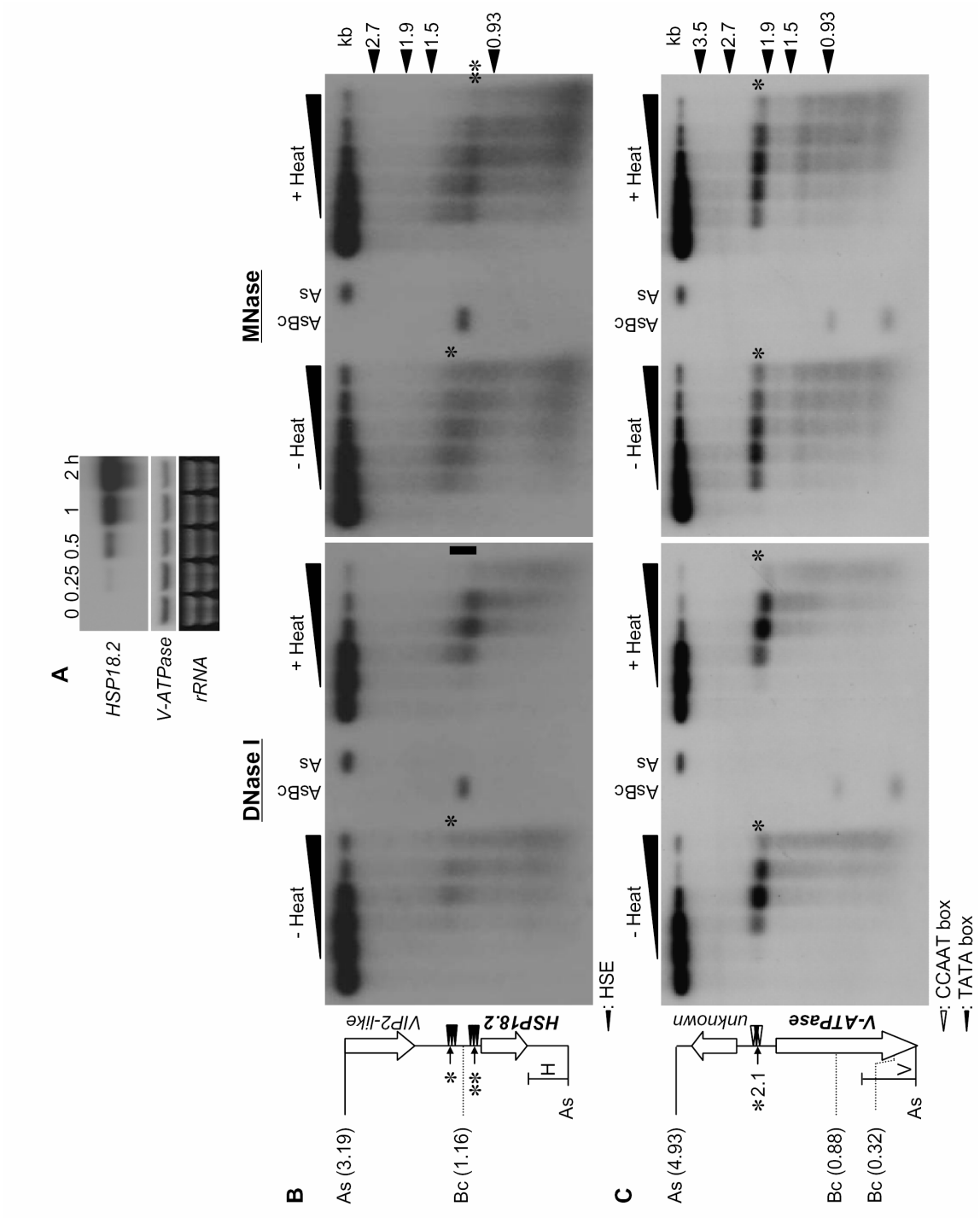


Fig. 2-3 (legend of this figure is on next page)

Fig. 2-3 (continued from previous page) Chromatin structure of the *HSP18.2* promoter in its inactive and active states. (A) Expression of the *HSP18.2* and *V-ATPase* genes following heat treatment of six-day-old suspension cells. Northern blot analysis was performed using total RNA prepared from suspension cells heat-treated at 30°C for the indicated times. An ethidium bromide-stained agarose gel of rRNA is shown as a loading control. (B and C) Chromatin from untreated (-Heat) and heat-treated (+Heat) cells was digested with increasing concentrations of DNase I (left) or MNase (right). DNA (15 μg) was isolated and further digested with *Ase*I. The blot was hybridized with probe H to visualize the 5'-region of the *HSP18.2* gene and reprobed with probe V to detect the 5'-region of the *V-ATPase* gene. The corresponding positions of the nuclease-hypersensitive sites are indicated by the asterisks in the autoradiograph. At the active *HSP18.2* promoter, the extended DNase I hypersensitive region is marked by vertical bars, and the shifted MNase HS is marked with double asterisks in the autoradiograph. Other symbols are as in Fig. 2-2.

nuclease (MNase) digestion (Fig. 2-3B, right). Upon heat activation, the DNase I HS extended downstream beyond the *Bcl*I site located between the HSE1 and HSE2, and the MNase HS also shifted downstream (Fig. 2-3B). An identical pattern of nuclease sensitivity was observed when using the upstream probe abutting another *Ase*I site (data not shown). These changes were not due to differences in sample preparations, because the nuclease digestion profiles of the same samples were similar at the constantly-expressed *V-ATPase* gene promoter before and after heat treatment (Figs. 2-3A, C). The MNase digestion profiles of naked DNA gave smears overlapped by a few faint bands (data not shown), confirming that bands observed *in vivo* are dependent on chromatin structure. Except for the MNase HSs, bands derived from well-positioned nucleosomes were detected in the *V-ATPase* gene (bands detected below 1.5 kb in Fig. 2-3C, right) but not in the *HSP18.2* gene (Fig. 2-3B, right) in the MNase sensitivity assay. This may be due to the less firmly positioned nucleosomes in the

HSP18.2 coding region.

2.3.4 Histones are removed and/or acetylated upon transcriptional activation of the *HSP18.2* gene

In addition to nuclease sensitivity, I analyzed the histone occupancy and acetylation state at the *HSP18.2* promoter in both its inactive and active state by chromatin immunoprecipitation (ChIP) assay. In the yeast *PHO5* promoter, histone acetylation plays an essential role in transcriptional activation by enhancing the removal of nucleosomes adjacent to DNase I HS (Reinke and Horz 2003).

I first measured the histone occupancy at the *HSP18.2* promoter (primer and Taqman probe set 2F, 2R and 2T in Fig. 2-4A) and constitutively-expressed *V-ATPase* coding region (primer and Taqman probe set VF, VR and VT in Fig. 2-4A) as a control by ChIP assay using antibodies against unmodified histone H3 and H4 in heat-untreated (Heat -) and treated (Heat +) cells. The absolute histone H3 occupancy at the *HSP18.2* promoter was decreased upon activation (Fig. 2-4B, left). To compare the signals from heat-untreated and treated cells, the absolute histone H3 occupancy at the *HSP18.2* promoter was normalized to the histone occupancy at the constantly-expressed *V-ATPase* coding region (Fig. 2-4B, right). The normalized H3 occupancy at the

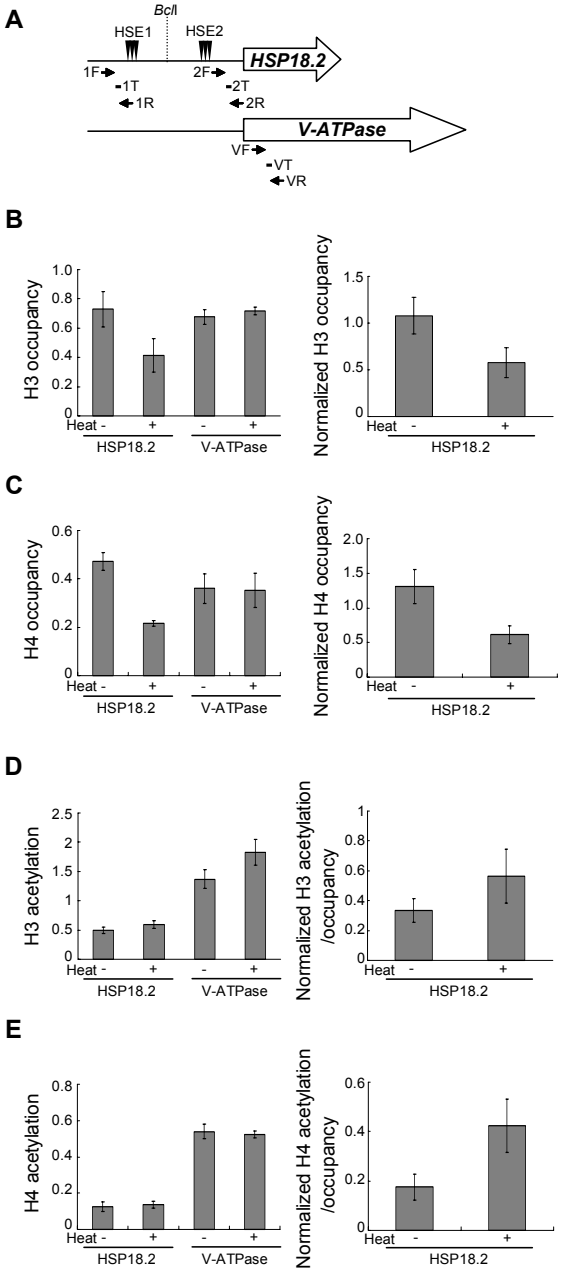


Fig. 2-4 ChIP analysis of histone occupancy and histone acetylation at the *HSP18.2* promoter at its inactive and active states. (A) Schematic diagram showing the primer positions at the *HSP18.2* promoter and *V-ATPase* coding region used in the ChIP assays. The promoter (horizontal thin lines) and coding regions (horizontal open arrows) of the *HSP18.2* and *V-ATPase* genes are shown with the positions of primer (horizontal closed arrows) and Taqman probe (horizontal closed bars) sets 1F, 1R and 1T (-304 to -221 relative to the *HSP18.2* ATG), 2F, 2R and 2T (-86 to -3 relative to the *HSP18.2* ATG), and VF, VR and VT (+2 to +70 relative to the *V-ATPase* ATG) below. The positions of the HSE1, HSE2 (vertical closed arrowheads), and *Bcl*I site (vertical dashed line) are also indicated. (B-E) Suspension cells before (Heat -) and after the heat treatment (Heat +) at 30°C for 0.5 h were fixed with formaldehyde. Chromatin fragments were precipitated with antibodies specific for unmodified histone H3, H4 or acetylated H3 or H4. The amounts of immunoprecipitated DNA were determined by quantitative PCR with specific primers and Taqman probes for the *HSP18.2* promoter (primer and probe set 2F, 2R and 2T) and *V-ATPase* coding region (primer and probe set VF, VR and VT) using the input DNA from heat-untreated cells as standard and are shown in arbitrary units. The absolute histone H3 (B, left) and H4 occupancy (C, left), H3 (D, left) and H4 acetylation (E, left) levels at the *HSP18.2* promoter and *V-ATPase* coding region, the normalized H3 (B, right) and H4 occupancy (C, right), and doubly-normalized H3 (D, right) and H4 acetylation (E, right) at the *HSP18.2* promoter before and after heat treatment are shown as gray bars. For the method of normalization, see text. The data are expressed as the mean ± SD (standard deviation).

HSP18.2 promoter decreased by about 2-fold upon transcriptional activation (Fig. 2-4B, right). The histone H4 occupancy showed changes similar to those of the H3 (Fig. 2-4C).

I next measured the histone acetylation at the *HSP18.2* promoter in heat-untreated and treated cells by ChIP assay using antibodies against histone H3 acetylated at K9 and K14. The absolute histone H3 acetylation signals at the *HSP18.2* promoter were virtually unchanged upon activation (Fig. 2-4D, left). Because the histone H3 occupancy decreased at the *HSP18.2* promoter following activation, the absolute histone acetylation signals were normalized to the H3 occupancy at the same promoter. The normalized signal at the *HSP18.2* promoter was further normalized to the signal obtained at the *V-ATPase* coding region to correct for differences in untreated and treated samples (Fig. 2-4D, right). The doubly-normalized H3 acetylation levels increased by 1.6-fold at the *HSP18.2* promoter following heating (Fig. 2-4D, right). The doubly-normalized histone H4 acetylation levels were also increased about 2.5-fold following heating (Fig. 2-4E, right). Thus, histones H3 and H4 are acetylated and/or removed from the *HSP18.2* promoter upon transcriptional activation of the *HSP18.2* gene. The increased histone acetylation may reflect the acetylation of histones prior to their removal and/or the acetylation of histones adjacent to the removed histone(s).

ChIP assays using normal rabbit IgG as a negative control gave undetectable signals at the *HSP18.2* promoter and *V-ATPase* coding region in untreated and treated cells (data not shown).

2.3.5 The transcription activator HSF1 primarily binds HSE2 upon transcriptional activation

To analyze the relationship between the altered nuclease accessibility and transcription activator binding in the *HSP18.2* promoter, I analyzed HSF1 binding at the *HSP18.2* promoter (primer and probe set 2F, 2R and 2T in Fig. 2-4A) and *V-ATPase* coding region (primer and probe set VF, VR and VT in Fig. 2-4A) as a negative control by ChIP assay using antibodies specific for the HSF1 in heat-untreated and treated cells. HSF1 was previously identified as a transcriptional activator of the *HSP18.2* gene (Lohmann et al. 2004, Zhang et al. 2003). The HSF1 ChIP signal at the *HSP18.2* promoter was similar to that of the *V-ATPase* coding region in its inactive state, but upon activation, this signal increased by 7–fold (Fig. 2-5A). Thus, HSF1 binds the *HSP18.2* promoter upon activation as previously reported (Zhang et al. 2003).

To determine whether HSF1 binds HSE1 and/or HSE2, chromatin from heat-treated cells was digested with *Bcl*I before immunoprecipitation. Because a *Bcl*I site is located

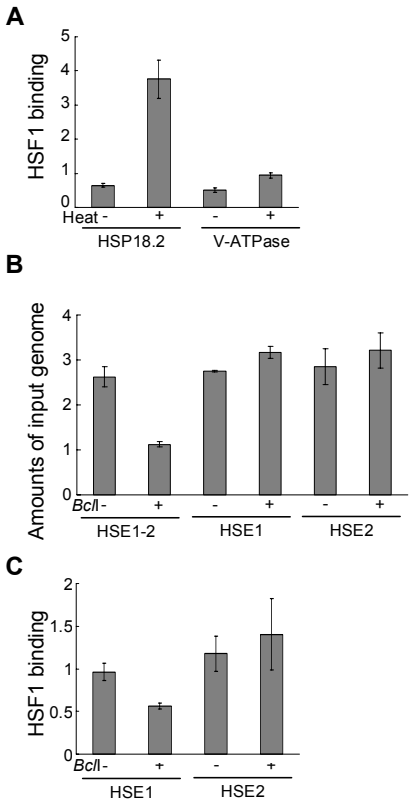


Fig. 2-5 ChIP analysis of activator HSF1 binding to the *HSP18.2* promoter in its inactive and active states. (A) Suspension cells before (Heat -) and after heat treatment (Heat +) at 30°C for 0.5 h were fixed with formaldehyde. Chromatin fragments were precipitated with antibodies specific for HSF1. The amount of coimmunoprecipitated DNA was determined by quantitative PCR with specific primers and Taqman probes for the *HSP18.2* promoter (primer and probe set 2F, 2R and 2T in Fig. 2-4A) and *V-ATPase* coding region (primer and probe set VF, VR and VT in Fig. 2-4A) using the input DNA from heat-untreated cells as a standard, and these data are shown as gray bars in arbitrary units. (B) Crosslinked chromatin of heat-treated cells was mock incubated (*Bcl*I -) or incubated with *Bcl*I (*Bcl*I +) and sonicated. The amount of the respective input DNA was determined by quantitative PCR with primer and probe set 1F, 1R and 1T (HSE1), 2F, 2R and 2T (HSE2) and 1F, 2R and 1T (HSE1-2) using the input DNA from mock incubated cells as a standard, and these data are shown as gray bars in arbitrary units. (C) The amounts of immunoprecipitated DNA with antibodies specific for HSF1 in the undigested (*Bcl*I -) and digested (*Bcl*I +) chromatin were determined by quantitative PCR with primer and Taqman probe set for the HSE1 or HSE2 using the input DNA from mock incubated cells as a standard, and these data are shown as gray bars in arbitrary units. The data are expressed as the mean ± SD (standard deviation).

between HSE1 and HSE2 (Fig. 2-4A), digestion will separate the chromatin fragments containing HSE1 or HSE2. I first estimated the degree of *Bcl*I digestion at this site by quantitative PCR using DNA from *Bcl*I-undigested and digested chromatin as templates. The input DNA was recovered before immunoprecipitation and measured by quantitative PCR with primer and probe set 1F, 2R and 1T (HSE1-2 set) to detect undigested DNA, 1F, 1R and 1T (HSE1 set) or 2F, 2R and 2T (HSE2 set) to detect total DNA (Figs. 2-4A, 2-5B). When measured with the HSE1-2 primer set, the amount of input DNA from the *Bcl*I-digested chromatin (*Bcl*I +) was reduced by approximately 60% compared with that from the *Bcl*I-undigested chromatin (*Bcl*I -) (Fig. 2-5B). In contrast, when measured with the HSE1 or HSE2 primer set, the input DNA from the *Bcl*I-digested chromatin was similar to that from the *Bcl*I-undigested chromatin (Fig. 2-5B). Thus, approximately 60% of the chromatin was digested at the *Bcl*I site between HSE1 and HSE2. I further analyzed HSF1 binding by ChIP assay using *Bcl*I-undigested and digested chromatin. When measured with the HSE1 primer set, the absolute HSF1 ChIP signal from *Bcl*I-digested chromatin was reduced by approximately 40% compared with undigested chromatin (Fig. 2-5C). In contrast, when measured with the HSE2 primer set, the absolute HSF1 ChIP signal from *Bcl*I-digested chromatin was similar to that seen from undigested chromatin (Fig. 2-5C). These data show that the activator

HSF1 primarily binds to HSE2 upon *HSP18.2* activation. ChIP assays using normal rabbit IgG as a negative control gave undetectable signals at the *HSP18.2* promoter and *V-ATPase* coding region in untreated and treated cells (data not shown).

2.4 Discussion

In this Chapter II, I identified distinct DNase I HSs at the 5' and/or 3' ends of most genes in an 80 kb genomic region in *Arabidopsis*. The presence of the DNase I HSs at the 5' ends of the *Arabidopsis* genes is similar to that seen in yeast and human chromatin where HSs are also found in the promoters of many genes (Crawford et al. 2006a, Ercan and Simpson 2004, Sabo et al. 2006, Yuan et al. 2005). The DNase I HSs were also found at the 3' ends of genes in the *Arabidopsis* MXK3 region (Fig. 2-1B), and this is similar to the data from human chromatin where HSs are located at many transcription termination sites (Crawford et al. 2006a, Sabo et al. 2006). In yeast mutants lacking chromatin remodeling factor, chromatin structural changes at 3' ends and less efficient transcriptional termination were observed in some genes (Alen et al. 2002, Morillon et al. 2003). Therefore, some of the DNase I HSs at 3' ends of *Arabidopsis* genes may function in transcriptional termination. The DNase I HSs were never found within the coding regions of the *Arabidopsis* genes analyzed here as previously seen for yeast

genes (Ercan and Simpson 2004, Yuan et al. 2005). This absence of DNase I HS contrasts with data from the human genome where about half of the HSs are located within introns (Sabo et al. 2006). This difference may reflect different methods of gene regulation in *Arabidopsis* and humans or the much shorter intron lengths of *Arabidopsis* genes (total intron length on average; 1.2 kb/*Arabidopsis* gene versus 14 kb/human gene, Deutsch and Long 1999). A DNase I HS was also found in the 5'-region of a pseudogene in the MXK3 region (Fig. 2-1B), but this site is presumably involved in the regulation of an expressed upstream gene (At5g65000) arranged in a head-to-head orientation. In contrast to the DNase I HSs located at the 5' or 3' ends of genes, two sites were not associated with protein-coding genes (marked with asterisks in Fig. 2-1B). It is possible that as-yet undiscovered genes exist adjacent to these two sites. Indeed, the At5g64811, 13 and 16 genes were formerly annotated as the single At5g64810 gene, and the presence of two DNase I HSs within this gene was exceptional. However, the recent discovery of three new genes made the position of these HSs consistent with current models (Fig. 2-1B). Other possibility is that these DNase I HSs represent locus control regions regulating their target genes at a distance (Dean 2006). The DNase I HSs are also present at the 5' regions of two tRNA genes (Fig. 2-1B). The DNase I HS may be involved in the transcriptional regulation of tRNA genes by RNA polymerase III

in *Arabidopsis* as shown in polymerase III promoter of human Alu repetitive elements (Humphrey et al. 1996).

DNase I HSs were found in differentiated quiescent leaves and undifferentiated dividing suspension cells at the same positions in well-characterized gene promoters (Fig. 2-2), suggesting that HS is a pervasive structure. The presence of DNase I HSs at the 5' ends of most genes, whether the gene is active at different levels or inactive, suggests that basically nucleosome-free regions exist at these 5' ends of genes. Some of the factors that might contribute to creation of nucleosome-free DNase I HSs are the binding of transcription factors, changes in DNA confirmation caused by protein binding, altered or remodeled nucleosomes (Gross and Garrard 1988) or DNA sequence itself (Angermayr et al. 2002, Segal et al. 2006). The identification of the factor(s) responsible for regulating DNase I HSs and their relationship with transcriptional control are essential for a complete understanding of gene regulation in *Arabidopsis*. The presence of DNase I HSs regardless of the gene expression levels raises the possibilities that some sites may function in processes other than transcription such as DNA replication (Brown et al. 1991, Ercan and Simpson 2004), structural organization of chromatin through interaction with nuclear architecture (Udvardy et al. 1985, Zhao et al. 1995) or a minor role yet to be detected.

DNase I HSs were found near the *cis*-regulatory elements of the *HSP18.2*, *RD29b*, *PCNA1* and *V-ATPase* genes (Fig. 2-2). In the *HSP18.2* promoter, the DNase I HS was pre-formed near the promoter distal HSE1, and upon transcriptional activation, this HS was extended accompanied by the histone H3, H4 acetylation and/or removal (Figs. 2-3, 2-4). In addition to these dynamic changes in nucleosome structure that occur during the activation process, I demonstrated that the activator HSF1 primarily bound the HSE2 site (Fig. 2-5). This binding of the HSF1 to the HSE2 and activated transcription of the *HSP18.2* gene (Figs. 2-3, 2-5) suggest that the DNase I HS extends to the HSE2 and adjacent TATA element (15 bp downstream of the HSE2) upon activation. I summarized these results of the *HSP18.2* gene into a conceptual model shown in Fig. 2-6. These changes and activator binding are similar to the activation process of the yeast *PHO5* gene, in which the nucleosome remodeling complex (including histone acetyltransferase and SWI/SNF) is recruited to the pre-formed DNase I HS, acetylates and removes nearby histones, thereby facilitating the binding of activator PHO4 to the newly exposed elements (Reinke and Horz 2003, Svaren et al. 1994, Venter et al. 1994). Similarly, DNase I HSs may participate in the transcriptional regulation of *Arabidopsis* genes by enhancing access to chromatin remodeling factors and/or transcription factors.

In contrast to the *Arabidopsis HSP18.2* gene, well-documented yeast *HSC82* and

Drosophila hsp26 gene promoters have pre-formed DNase I HSs containing critical HSEs and TATA box, and these HSs remain relatively unchanged upon transcriptional activation (Cartwright and Elgin 1986, Erkine et al. 1996). These DNase I HSs are pre-formed by the pre-bound activator HSF in the *HSC82* promoter (Erkine et al. 1996), and by the pre-bound activator GAGA, TFIID complex and paused RNA polymerase in the *hsp26* promoter (Leibovitch et al. 2002). Although the DNase I HS of the *HSP18.2* gene showed dynamic changes upon transcriptional activation different from the *HSC82* and *hsp26* genes, the HS observed at the *HSP18.2* promoter before activation may be similarly pre-formed by yet unknown pre-bound transcription factor(s).

In this Chapter II, I reported the widespread prevalence of DNase I HSs at the 5' and/or 3' ends of most *Arabidopsis* genes in an 80-kb genomic region, at several well-characterized genes, and I have described the changes in HS during the transcriptional activation of the *HSP18.2* gene. These results encourage a more detailed functional analysis of the DNase I HS in transcriptional regulation as well as the large-scale analysis of HS by using genomic tiling arrays (Crawford et al. 2006a, Sabo et al. 2006, Yuan et al. 2005) or sequencing analysis (Crawford et al. 2004, 2006b) pioneered in yeast and human genome to understand the regulation of gene expression in *Arabidopsis*. Mapping of the DNase I HSs in other plant species may reveal the

general and plant-specific chromatin structural features.

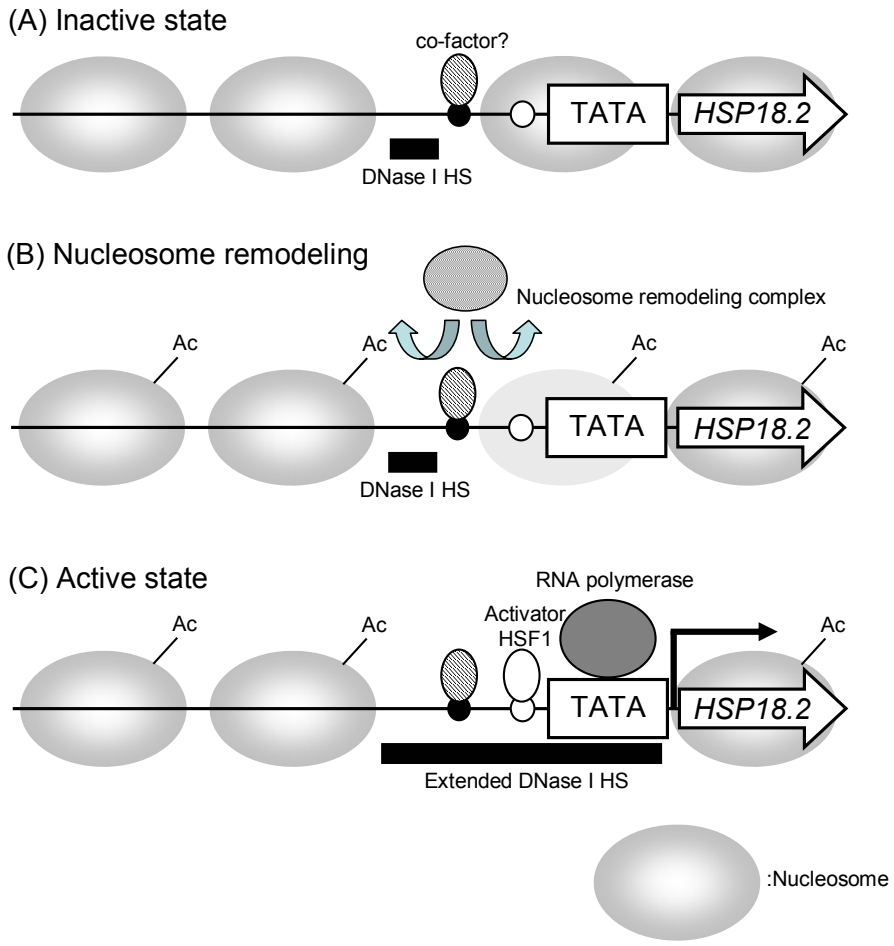


Fig. 2-6 A conceptual model of the function of DNase I HS in transcriptional activation of the *HSP18.2* gene. (A) At inactive state, the DNase I HS (black bar) is preformed by unknown DNA binding co-factor(s) (hatched oval) bound to the *cis*-element(s) (black circle). The *cis*-activating element HSE2 (open circle) and TATA box (open box with TATA) are packaged into nucleosome(s). (B) Upon activation, nucleosome remodeling complex (dotted oval) access to preformed DNase I HS, acetylate (denoted by nucleosomes with Ac) and remove neaby histone(s) (denoted by a more transparent nucleosome). (C) At activated state, the HSE2 and TATA box are exposed as detected by extended DNase I HS. Transcription activator HSF1 bind to this newly-opened HSE2, recruit RNA polymerase (gray oval) to the TATA box and initiate transcription. Genes and nucleosomes are indicated by arrows and transparent ovals, respectively.

2.5 Tables

Table 2-1

The gene specific primer sets and Taqman probes used in the ChIP assays.

Gene	Name	Sequence (5' to 3')	Position from ATG
HSP18.2	1F	AACAAAGCAAAACGGCACGT	-304
	1R	CATTTCAGGAAGTTTCGTTTGTTG	-221
	1T	TTTTAATTGTAACCAAGGATTGCATTTCGGTCTT	-282
	2F	AACTTCCCTATAAATATGTCCTTTGCTAA	-86
	2R	TTCGTTGCTTTTCGGGAGA	-3
	2T	AGATCAAATCAGCAGGAAAATCAAGAACCAAAA	-55
V-ATPase	VF	CGTAACCATACTCGCTCTCCTTC	+2
	VR	TGCCGGCGTTTTACGG	+70
	VT	CATCGTCCTCGAAGGTCGTAAGCTTTCC	+45

CHAPTER III: DNase I Hypersensitive Site and Transcriptional Competency

3.1 Introduction

In the Chapter II, the DNase I HSs are found at the 5' ends of most genes whether they are expressed or not in *Arabidopsis*. In addition, the DNase I HSs are formed near *cis*-regulatory elements at the *HSP18.2* gene promoter prior to activation. The DNase I HSs constitutively present at these gene promoters are thought to confer transcriptional competency (potential of expression) on associated genes by allowing access of transcription factors (Wallrath et al. 1994). Therefore, these findings suggest that most (not all) genes are in transcriptionally competent (expressed, or unexpressed but inducible) state in *Arabidopsis*. On the other hand, appearance and disappearance of DNase I HSs have been observed at several developmentally-regulated genes in different tissues of animals (Gross and Garrard 1988). For example, among 10 DNase I HSs identified in 50 kb of chicken lysozyme gene locus, patterns of appearance and disappearance of DNase I HSs were detected in different tissues or cell types, and these patterns were correlated with transcriptional competency of the lysozyme gene (Fritton et al. 1984). These results suggest the hypothesis that DNase I HS is absent from promoter of transcriptionally incompetent (unexpressed nor inducible) gene. The

presence and absence of DNase I HS have also been observed in plants. In *Arabidopsis Alcohol dehydrogenase* gene promoter, DNase I HS is detected in suspension cells where this gene is expressed, but it is not detected in leaves where this gene is unexpressed (Vega-Palas and Ferl 1995). This example supports the above hypothesis also in plants. In this study, to test this hypothesis in plants, I analyzed DNase I hypersensitivity of well-characterized genes showing several patterns of tissue-specific expression in *Arabidopsis* leaves, roots and suspension cells where these genes are in transcriptionally incompetent, inducible or activated states.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia Col-0 and suspension cell strain T87 (Axelos et al. 1992) were grown as described in the Chapter II. To obtain root tissues, *Arabidopsis* plants were grown hydroponically as described previously (Naito et al. 1994).

3.2.2 Quantitative RT-PCR

Total RNA was isolated from *Arabidopsis* leaves, suspension cells, flowers, siliques

and roots using an RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. For quantitative RT-PCR (qRT-PCR) analysis, total RNA was treated with DNase I and reverse transcribed using an oligo-dT primer and Transcriptor First Strand cDNA Synthesis kit (Roche). The generated cDNA was measured in triplicates by quantitative PCR using the LightCycler (Roche) according to the manufacturer's recommendations. The quantitative PCR amplification was performed using a 5 μl aliquot of cDNA (equivalent to 5 ng of total RNA) as a template with 10 pmoles gene-specific primers (sequence of primers is listed in Table 3-1) in 20 μl reaction mix using SYBR Green Master Mix (Roche). The standard curve was generated using 100, 20, 4, 0.8 and 0.16 ng of *Arabidopsis* genomic DNA.

3.2.3 DNase I sensitivity assay

Leaves of 6-week-old plants, 6-day-old suspension cells and 2-month-old hydroponically-grown roots were used for DNase I sensitivity assays. DNase I digestion of *Arabidopsis* leaves and suspension cells was performed as described in the Chapter II. DNase I digestion of hydroponically-grown roots was performed as described for suspension cells in the Chapter II. Purification of the DNA after DNase I digestion and DNase I sensitivity assay by Southern blotting were performed as

described in the Chapter I.

3.2.4 Preparation of DNA probes

All DNA probes used for DNase I sensitivity assays were 500 bp long and were prepared as described in the Chapter I. For indirect-end labeling analysis, probe F (49034-49533 on M7J2 clone, abutting the *Eco*RV site), probe S (28304-28803 on T24A18, abutting the *Hin*dIII site), probe AG (44292-44791 on F13C5, abutting the *Eco*RI site), probe SU (8-507 on K14B15, abutting the *Ase*I site), probe AD (95490-95989 on F22K20, abutting the *Hin*dIII site), and probe U (63612-64111 on T17J13, abutting the *Pst*I site) were used for the *FWA*, *At2S3*, *AGAMOUS*, *SUPERMAN*, *ADH* and *UBQ5* genes, respectively. For DNase I hypersensitivity assay without restriction enzyme digestion, probe ADH1 (-1000 to -501 relative to the ATG of the *ADH*), ADH2 (-500 to -1) and ADH3 (+1 to +500) were used for the *ADH* gene, and probe ATPase (-574 to -75 relative to the ATG of the *V-ATPase*) was used for the *V-ATPase* gene.

3.2.5 ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed using modifications

of previously described methods (Gendrel et al. 2002). *Arabidopsis* leaves were crosslinked by incubation in 1% formaldehyde solution under vacuum (-0.08 MPa) at room temperature for 15 min. Crosslinking was stopped by adding glycine to a final concentration of 0.1 M and incubating at room temperature for 5 min under vacuum (-0.08 MPa). Crosslinked cells were collected by vacuum infiltration and ground into a fine powder in the presence of liquid nitrogen with a mortar and pestle and processed as described in the Chapter II. The specific antibodies used were, H3-dimethyl-K4 (#07-030, Upstate) and H3-dimethyl-K9 (#ab7312, Abcam). The specific primers and Taqman probes used in ChIP assays are listed in Table 3-2.

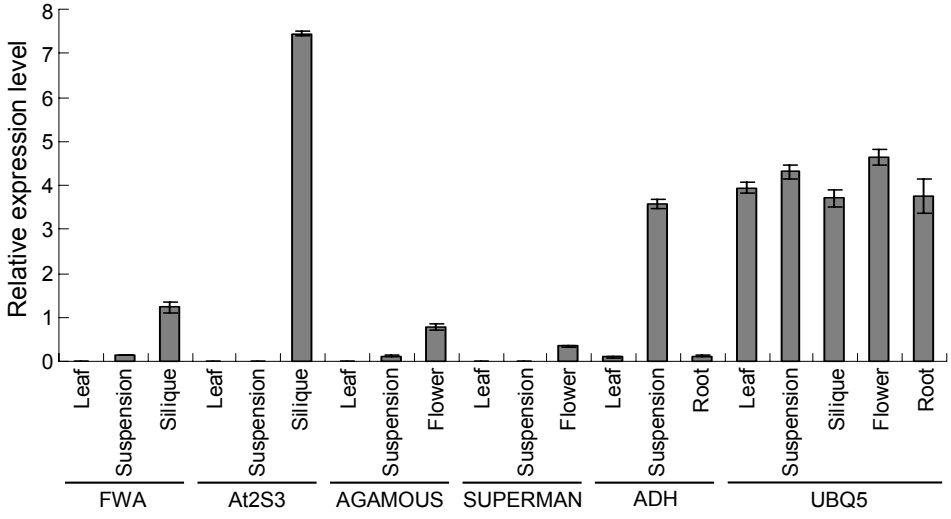
3.3 Results

3.3.1 Gene expression levels of six target genes showing several patterns of tissue-specific expression in *Arabidopsis*

To analyze the relationship between presence of DNase I HS and transcriptional competency in *Arabidopsis*, I selected 5 well-documented tissue-specific genes, *FWA* (At4g25530), *At2S3* (At4g27160), *AGAMOUS* (*AG*, At4g18960), *SUPERMAN* (*SUP*, At3g23130), and *Alcohol dehydrogenase* (*ADH*, At1g77120) genes, and one ubiquitously-expressed *UBQ5* (At3g62250) gene as a control. To investigate the

transcriptional competency of these 6 target genes in *Arabidopsis* samples used for DNase I hypersensitivity assay, expression levels of these genes were measured in *Arabidopsis* leaves, suspension cells, siliques, flowers or roots by qRT-PCR assay (Fig. 3-1A). All primer sets gave undetectable signals without cDNA templates (data not shown). The *FWA* gene encodes a homeodomain-containing transcription factor that is important for the transition to flowering, as well as for floral meristem identity (Soppe et al. 2000). The *FWA* gene is specifically expressed in the central cell of the female gametophyte and the endosperm of plants (Kinoshita et al. 2004), but this gene is expressed in suspension cells for uncertain reason (Pischke et al. 2006). The *At2S3* gene encoding a seed-storage protein having one ABA responsive element (ABRE) in its promoter (indicated in Fig. 3-2B), is specifically expressed in maturing seeds in an ABA-dependent manner, but is not expressed nor inducible by ABA in leaves (Parcy et al. 1994). The qRT-PCR assay confirmed these expression patterns, the *FWA* gene was expressed in siliques and suspension cells but was not in leaves, the *At2S3* gene was expressed in siliques but was not in leaves and suspension cells (Fig. 3-1A). These suggest that the *FWA* gene is transcriptionally incompetent in leaves and competent in suspension cells, and that the *At2S3* gene is incompetent in both tissues (summarized in Fig. 3-1B).

A



B

	Tissue		
Gene	**Leaf**	**Suspension cell**	**Root**
FWA	X	O	
At2S3	X	X	
AGAMOUS	X	O	
SUPERMAN	X	X	
ADH	X (Most cells)	O	O
UBQ5	O	O	

O: Competent

X: Incompetent

Fig. 3-1 Transcriptional states of the *FWA*, *At2S3*, *AGAMOUS*, *SUPERMAN*, *ADH* and *UBQ5* genes in leaves, suspension cells, siliques, flowers or roots. (A) Quantitative RT-PCR was performed using cDNAs prepared from leaves, suspension cells, siliques, flowers or roots as templates with gene-specific primer sets. The relative qRT-PCR signals are shown in arbitrary units. The data are expressed as the mean ± SD (standard deviation). (B) Transcriptional competency of the target genes in leaf, suspension cell and root. The genes in transcriptionally competent and incompetent state are indicated by "O" and "X", respectively. In the *ADH* gene, "X (most cells)" in leaf means that the *ADH* gene is incompetent in most of leaf cells.

The *AG* gene encoding a MADS domain transcription factor that is important in specifying identity of floral meristem, carpel and stamen (Yanofsky et al. 1990). The *AG* gene is specifically expressed in the third and fourth whorls, carpels and stamens of flowers (Bowman et al. 1991). The *SUP* gene encodes a putative zinc-finger domain transcription factor controlling the boundary of carpel and stamen and is specifically expressed in the third whorl of flowers (Sakai et al. 1995). The promoter DNA region indicated in Fig. 3-2D is shown to function as a positive element for *SUP* expression (Ito et al. 2003). The qRT-PCR assay confirmed these expression patterns, the *AG* and *SUP* genes were expressed in flowers but were not in leaves (Fig. 3-1A). In suspension cells, the *AG* gene was expressed for uncertain reason, in contrast, the *SUP* gene was not expressed (Fig. 3-1A). These suggest that the *AG* gene is transcriptionally incompetent in leaves and competent in suspension cells, and that the *SUP* gene is incompetent in both tissues (Fig. 3-1B).

The *ADH* gene encoding an alcohol dehydrogenase has two G-boxes and one ABRE in its promoter (Dolferus et al. 1994). The *ADH* gene is inducible by ABA, hypoxia, and cold treatments in roots but is not inducible in leaves (Dolferus et al. 1994), and is constitutively-expressed in suspension cells (de Bruxelles et al. 1996, Vega-Palas and Ferl 1995). In my qRT-PCR assay, the *ADH* gene was very weakly expressed both

in leaves and roots and was highly expressed in suspension cells (Fig. 3-1A). In mature plants, it is previously reported that the *ADH* gene expression is not found in most of the green parts but this gene is expressed in the base of the rosette leaves (Dolferus et al. 1994). Therefore, the weak expression levels of the *ADH* gene observed in leaves may be resulted from the small fraction of these cells (not excluded in my leaf samples) where the *ADH* gene is expressed. Therefore, the *ADH* gene is competent in suspension cells and roots and may be incompetent in most of leaf cells (Fig. 3-1B).

The *UBQ5* gene encoding an ubiquitin extension protein has one CCAAT box and one TATA box in its promoter (indicated in Fig. 3-2F; Higo et al. 1999). The *UBQ5* gene is known to be expressed ubiquitously, and this gene is highly-expressed in leaves, suspension cells, siliques, flowers and roots in my qRT-PCR assay (Fig. 3-1A). Therefore, the *UBQ5* gene is competent and expressed in these tissues including leaves and suspension cells (Fig. 3-1B).

3.3.2 DNase I HS is absent from promoters of transcriptionally incompetent genes in *Arabidopsis* leaves and suspension cells

The DNase I hypersensitivity of 6 target genes were analyzed in two types of tissues, terminally-differentiated *Arabidopsis* leaves and actively-dividing suspension

cells, because both are suitable for hypersensitivity assay requiring relatively large amounts of cells. To analyze DNase I hypersensitivity of different genomic regions in parallel, hybridization was performed using a set of membranes prepared with genomic DNA from leaves and suspension cells that had been subjected to the same DNase I digestion series. The DNase I-treated genomic DNA was further digested with single restriction enzyme, and the DNase I hypersensitivity of target genes was analyzed by indirect-end labeling analysis using a probe abutting this restriction enzyme recognition site. Intact genome digested with restriction enzymes were also included in the membranes to confirm specific hybridization of a probe to its target region and to serve as a size standard.

As a result, in the *FWA* and *AG* genes, a distinct DNase I HS was detected at the 5' region in suspension cells but was not detected in leaves (Figs. 3-2A, C). In the *At2S3* and *SUP* genes, a DNase I HS was not detected at the 5' region in both tissues (Figs. 3-2B, D). These results indicate that the DNase I HS is absent from the 5' region of transcriptionally incompetent genes (Figs. 3-1B, 3-2A-D). At the 3' region of the *SUP* gene, a less distinct DNase I HS was detected in suspension cells for unknown reason (Fig. 3-2D).

In the *ADH* gene promoter, a distinct DNase I HS was detected near the two

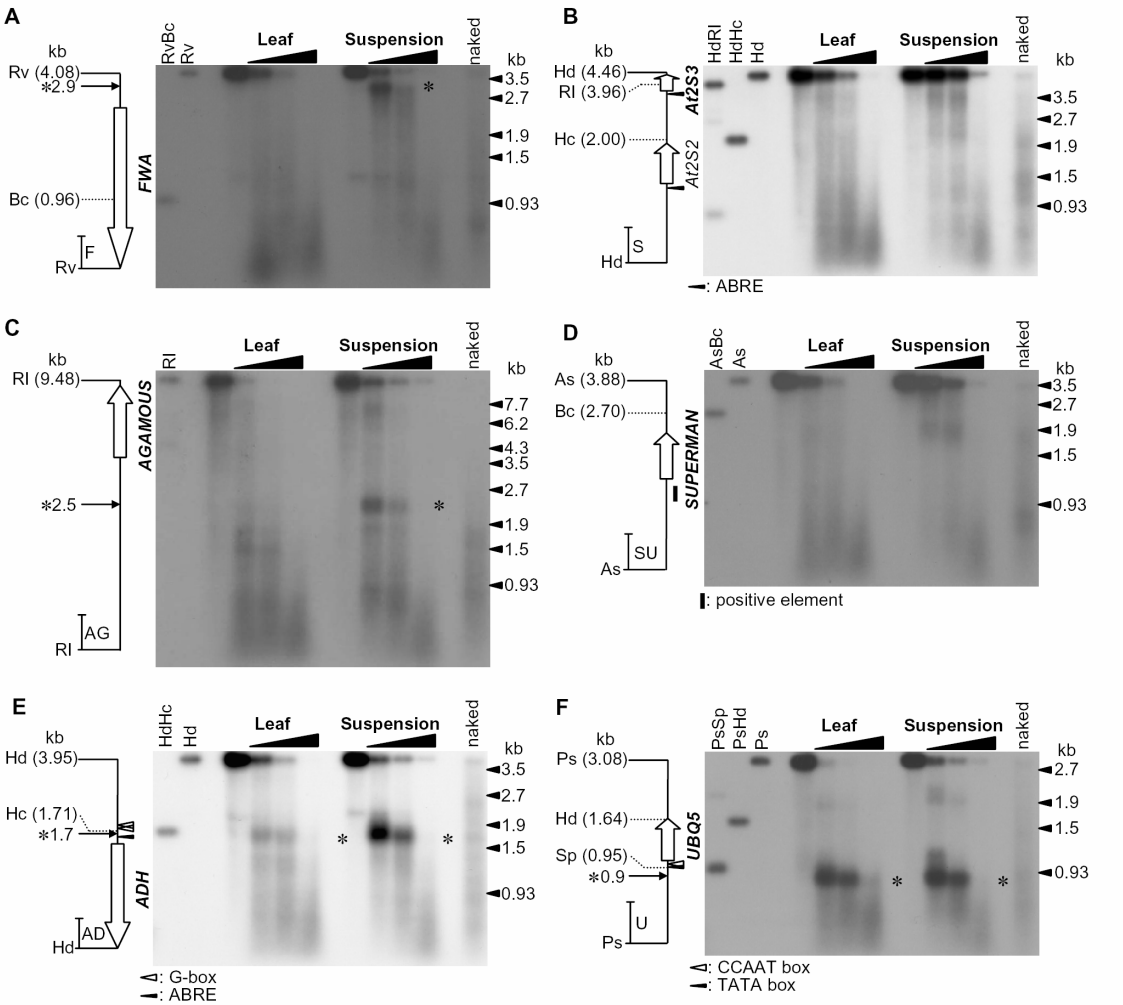


Fig. 3-2 DNase I hypersensitivity of the *FWA*, *At2S3*, *AGAMOUS*, *SUPERMAN*, *ADH* and *UBQ5* genes in leaves and suspension cells. (A-F) Chromatin of leaves or suspension cells were digested with DNase I at 0, 0.25, 0.5 and 2 U ml^{-1} (leaves) or at 0, 10, 20 and 50 U ml^{-1} (suspension cells) at 30°C for 10 min. Naked genomic DNA (6.5 μg) was digested with DNase I at 0.01 U ml^{-1} (lanes naked) for 30°C for 10 min. The isolated DNA (6.5 μg) was further digested with *Eco*RV, *Hin*dIII, *Eco*RI, *Ase*I or *Pst*I, and was separated on 1% agarose gels and transferred to nylon membranes. The blot containing *Eco*RV, *Hin*dIII, *Eco*RI, *Ase*I or *Pst*I-treated DNA was hybridized with probe F, S, AG, SU, P, AD or U to detect the 5'-region of *FWA* (A), *At2S3* (B), *AGAMOUS* (C), *SUPERMAN* (D), *ADH* (E) and *UBQ5* (F) genes. The positions of the *Ase*I, *Bcl*I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Sph*I, and *Pst*I restriction sites (As, Bc, RI, Rv, Hc, Hd, Sp, and Ps with band sizes in kb, respectively), and probes F, S, AG, SU, AD and U (vertical bars with F, S, AG, SU, AD and U, respectively) are shown at the left sides. The positions of two ABREs (B), one positive element (D), one ABRE (E), and one TATA box (F) are indicated by horizontal closed arrowheads, two G-boxes (E) and one CCAAT box (F) are indicated by horizontal open arrowheads at left. The lanes Rv, RvBc (A), Hd, HdHc, HdRI (B), RI (C), As, AsBc (D), Hd, HdHc (E), and Ps, PsHd, PsSp (F) indicate *Eco*RV, *Eco*RV-*Bcl*I (A), *Hin*dIII, *Hin*dIII-*Hin*cII, *Hin*dIII-*Eco*RI (B), *Eco*RI (C), *Ase*I, *Ase*I-*Bcl*I (D), *Hin*dIII, *Hin*dIII-*Hin*cII (E), and *Pst*I, *Pst*I-*Hin*dIII, *Pst*I-*Sph*I (F) digests of intact genome included as size standards. Other symbols are as in Fig. 2-2 in the Chapter II.

G-boxes and one ABRE in suspension cells, and weak HS was detected at the same position in leaves where the *ADH* gene is incompetent in most cells (Fig. 3-2E) similar to the previous report (Vega-Palas and Ferl 1995). The weak DNase I HS observed in leaves may be resulted from the presence of DNase I HS in small fraction of cells where the *ADH* gene is competent (Fig. 3-1).

In the ubiquitously-expressed *UBQ5* gene, the distinct DNase I HS was detected at the 5' region of the *UBQ5* gene both in leaves and suspension cells (Fig. 3-2F). The weak DNase I HS was also detected at the 3' region of the *UBQ5* gene in leaves and suspension cells (Fig. 3-2F), consistent with the results in the Chapter II showing the presence of the DNase I HS at the 3' ends of genes (Fig. 2-1B). This result confirms that the absence of the DNase I HS observed at the incompetent genes is not due to the inefficient digestion or over-digestion by DNase I. The DNase I digestion profiles of naked DNA gave smears or faint bands (Figs. 3-2A-F, lanes naked), confirming that bands observed *in vivo* are dependent on chromatin structure of the DNA. The absence of DNase I HS observed above may not simply due to the lack of gene expression but due to the transcriptional incompetency of genes, because distinct DNase I HSs were found in several unexpressed genes in *Arabidopsis* leaves and suspension cells (Figs. 2-1, 2-2 in the Chapter II).

3.3.3 Presence of the DNase I HS is correlated with transcriptional competency of the *ADH* gene in leaves, suspension cells and roots

To further confirm that DNase I HS observed at the *ADH* gene promoter is not correlated with expression levels but with transcriptional competency, I examined hypersensitivity in hydroponically-grown roots. For hypersensitivity assay, *Arabidopsis* roots were grown in 5 liter of hydroponic culture during two months, however, only small amounts of DNase I-digested DNA (~3 μg) was recovered due to the small mass and rigidity of these roots. I mapped the DNase I HS by hybridizing continuous 500 bp DNA probes covering the *ADH* gene promoter with a set of membranes prepared with DNase I-digested DNA from leaves, suspension cells and roots without restriction enzyme digestion. To confirm the specific hybridization of a probe to its target genomic region, *Eco*RI-, *Eco*RV- or *Hin*dIII-digested intact genomes were also included in the membranes prepared with leaf genomic DNA (Fig. 3-3, lanes E, V and H, respectively). As a result, distinct bands derived from the DNase I HSs were detected at 4.5 and 2.7 kb in the ADH1 segment, at 6.2 and 3.5 kb in the ADH2 and 3 segments both in roots and suspension cells (Fig. 3-3A). This pattern indicates that a DNase I HS is located in the ADH2 segment, and that four more sites exist 2.7 and 4.5

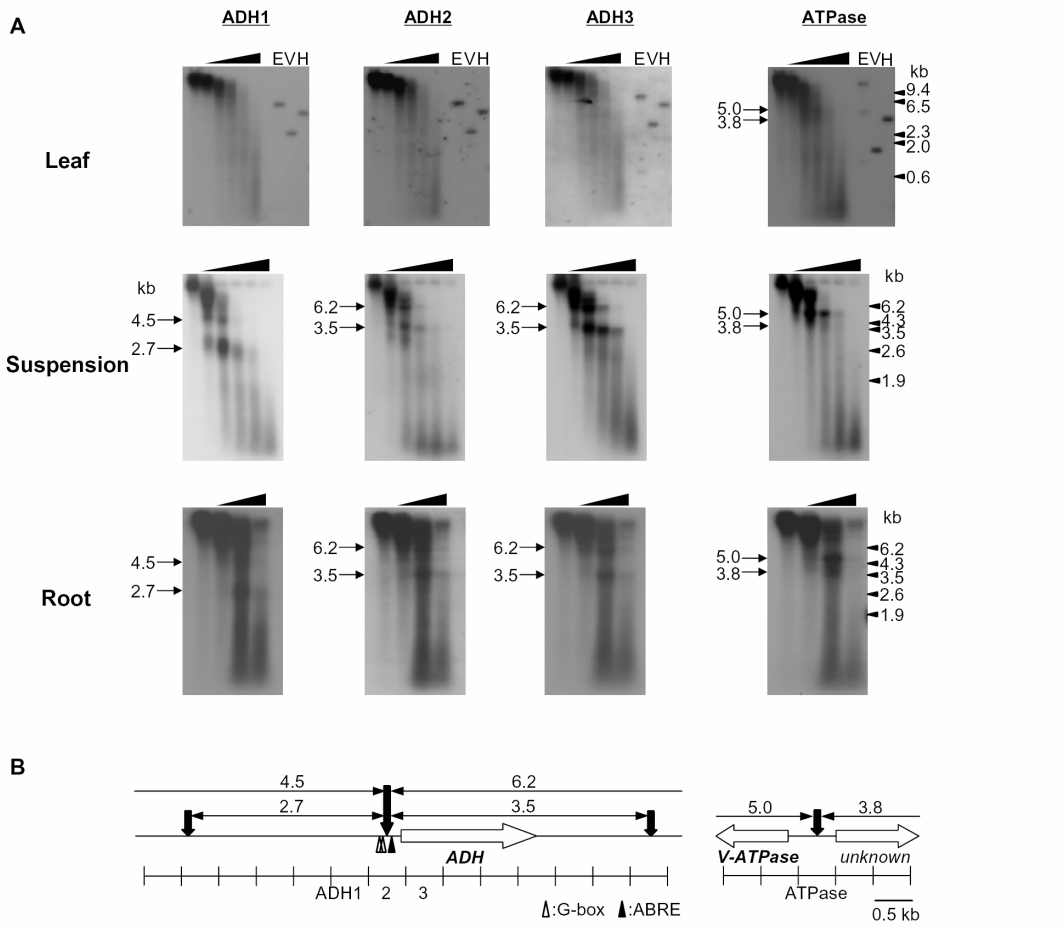


Fig. 3-3 Mapping of the DNase I HSs at the *ADH* and *V-ATPase* gene promoters in leaves, suspension cells and roots. (A) Chromatin of mature leaves, suspension cells and roots was digested with increasing amounts of DNase I (0, 0.025, 0.25, 0.5, 1, and 2 U ml^{-1} for leaves; 0, 5, 10, 20, 30, and 50 U ml^{-1} for suspension cells; 0, 0.1, 0.5, and 1 U ml^{-1} for roots) at 30°C for 10 min. The isolated DNA (20 μg from leaf; 15 μg from suspension cells; 3 μg from roots) was separated on 1% agarose gels. The intact genome (3 μg) was digested completely with *Eco*RI, *Eco*RV, or *Hin*dIII, and each digest was separated in parallel with leaf genomic DNA (lanes E, V and H, respectively). The separated DNA was transferred to nylon membranes and hybridized with probe ADH1-3 or ATPase. The DNase I sensitivity of the ADH1-3 and ATPase segments in leaves, suspension cells and roots are shown with the arrows on the left sides indicating the positions of the bands. Molecular weight markers are indicated by arrowheads on right sides. (B) A map of the DNase I HSs around the *ADH* and *V-ATPase* genes. The genes are indicated by open arrows with their names below. The two G-boxes (vertical open arrowheads) and one ABRE (a vertical closed arrowhead) are also indicated. The DNase I HSs are indicated by vertical arrows with the distance between each site in kb. The positions of the probes ADH1-3 and ATPase are indicated under the genes with their names. Bar indicates 0.5 kb.

kb 5' of the *ADH* gene and 3.5 and 6.2 kb 3' to this site both in roots and suspension cells irrespective of the gene expression levels (Fig. 3-3B). In contrast, these distinct bands were not observed in leaves (Fig. 3-3A). The weak DNase I HS was detected in Fig. 3-2E due to higher sensitivity of the indirect-end labeling analysis than this method without using restriction enzyme. Taken together, these results suggest that the intensity of DNase I HS is correlated with transcriptional competency but not with expression levels of the *ADH* gene (Figs. 3-1, 3-3). At the ubiquitously-expressed *V-ATPase* gene (Magnotta and Gogarten 2002), distinct bands (derived from the HS at the *V-ATPase* promoter and two neighboring HSs) were detected at 5.0 and 3.8 kb in the ATPase segment in leaves, suspension cells and roots (Fig. 2-2 in the Chapter II, 3-3). This result indicates that the presence and absence of distinct bands observed at the *ADH* gene are not due to differences in sample preparations.

3.3.4 Histone H3 is not methylated at lysine 4 and 9 in the incompetent *ADH* and *At2S3* gene promoters in leaves

The histone methylation states are shown to be involved in the transcriptional regulation of developmentally-regulated genes in *Arabidopsis* (Reyes et al. 2002). The H3K4me2 is an epigenetic mark of active genes, whereas, the H3K9me2, H3K27me2

and H3K27me3 are epigenetic marks of silenced genes or heterochromatin in *Arabidopsis* (Houben et al. 2003, Reyes et al. 2002). At the incompetent *FWA* and *SUP* genes, repressive H3K9me2 and H3K27me2 are high, and an active epigenetic mark H3K4me2 is low in leaves (Jacobsen and Meyerowitz 1997, Lindroth et al. 2004, Soppe et al. 2000). In the incompetent *AG* gene, repressive H3K27me2 and H3K27me3 are high, and active H3K4me2 is low in leaves (Schubert et al. 2006). To characterize the histone methylation states at the incompetent genes in addition to the *FWA*, *SUP* and *AG* genes, I analyzed H3K4me2 and H3K9me2 at the *ADH* and *At2S3* gene promoters by ChIP assay in leaves and suspension cells. As a control, the competent but unexpressed *HSP18.2* gene and a transposon located within the heterochromatic knob of chromosome IV (Gendrel et al. 2002) were also analyzed. As a result, the H3K4me2 signals of the *HSP18.2* gene was high, in contrast, those of the *ADH*, *At2S3* genes and the transposon were similarly low in leaves (Fig. 3-4A). For the H3K9me2 signals, the transposon showed high signals, in contrast, the *HSP18.2*, *ADH* and *At2S3* genes showed very low signals in leaves (Fig. 3-4A). In suspension cells, the H3K4me2 signals of the *HSP18.2* and *ADH* genes were similarly high, in contrast, those of the *At2S3* gene and the transposon were similarly low (Fig. 3-4B). For the H3K9me2 signals, the transposon and *At2S3* gene showed high signals, in contrast, the

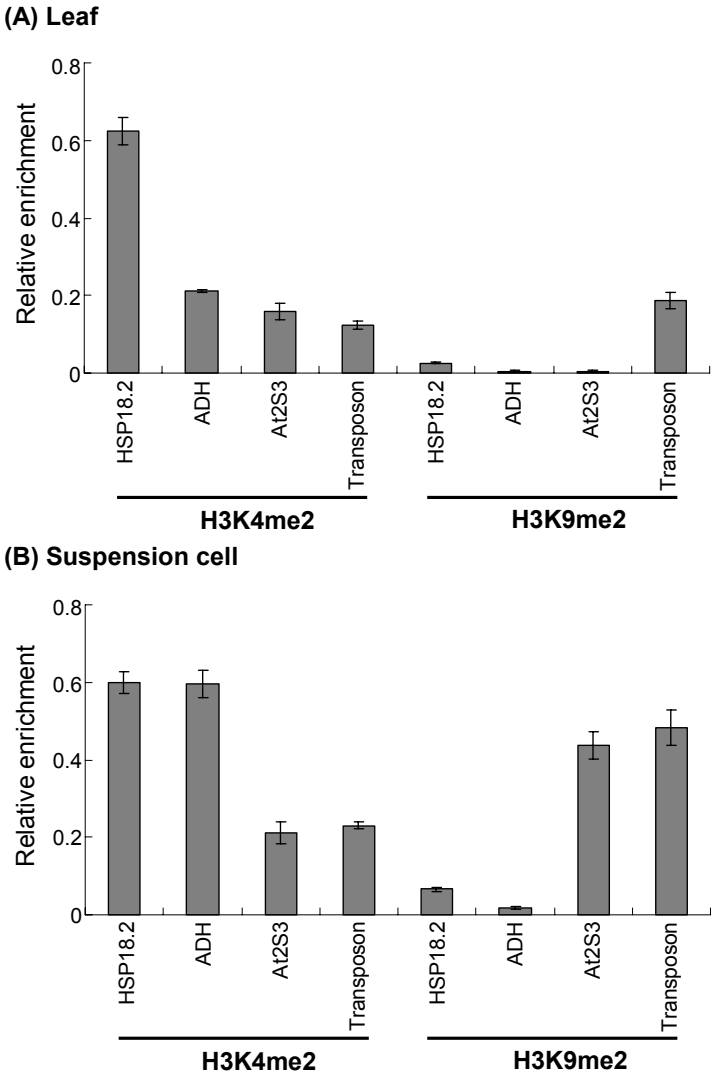


Fig. 3-4 ChIP analysis of H3K4me2 and H3K9me2 at the *HSP18.2*, *ADH* and *At2S3* genes and at the transposon in leaves (A) and suspension cells (B). *Arabidopsis* leaves or suspension cells were fixed with formaldehyde. Chromatin fragments were precipitated with antibodies specific for H3K4me2 or H3K9me2. The amounts of immunoprecipitated DNA were determined by quantitative PCR with specific primers and Taqman probes for the promoter regions of the *HSP18.2*, *ADH*, *At2S3* genes and of the transposon using the input DNA from suspension cells as standard. Histone H3 methylation levels are shown in arbitrary units. The data are expressed as the mean ±SD (standard deviation).

HSP18.2 and *ADH* genes showed very low signals in suspension cells (Fig. 3-4B). In other words, the incompetent *ADH* and *At2S3* genes showed low active and low repressive modifications in leaves. In suspension cells, the competent *ADH* gene showed high active and low repressive modifications similar to the competent *HSP18.2* gene, whereas, the incompetent *At2S3* gene showed low active and high repressive modifications similar to the transposon.

3.4 Discussion

In this Chapter III, I have shown that DNase I HS is absent from the promoters of 5 genes in transcriptionally incompetent states. The absence of the DNase I HS suggests that the nucleosomes are randomly positioned on these incompetent promoters. This closed nucleosome configuration around promoters may contribute to the transcriptional incompetency of the genes by blocking the binding of activators in tissues where expression of these genes is unnecessary. Consistent with this notion, the *At2S3* gene is activated by the binding of activator ABI5 to its ABRE in seeds (Carles et al. 2002, Lopez-Molina et al. 2002), however, this gene can not be activated in leaves over-expressing ABI5 even in the presence of exogenous ABA (Brocard et al. 2002). Perhaps, the ABI5 binding to the ABRE is prevented by closed nucleosome

configuration at the *At2S3* gene promoter in leaves. Similarly, at the *ADH* gene promoter, the presence of DNase I HS near the G-boxes and ABRE facilitates binding of activators to these elements in roots and suspension cells where this gene is stress-inducible, in contrast, the absence of this HS may block the binding of activators even if these activators exist in leaves where this gene is not stress-inducible. Previously, based on the nuclease sensitivity assay of the *ADH* gene in leaves and suspension cells, it is suggested that the formation of the DNase I HS in suspension cells is resulted from the active transcription of the *ADH* gene (Vega-Palas and Ferl 1995). However, presence of the DNase I HS in roots where the *ADH* gene is only weakly expressed (Figs. 3-1A, 3-3) and at the unexpressed *HSP18.2* gene promoter (Figs. 2-2, 2-3 in the Chapter II) suggests that presence of the DNase I HS is resulted from the transcriptional competency rather than the active transcription of the *ADH* gene. In addition to the incompetent genes analyzed in this Chapter III, DNase I HS was not found around the At5g64880 and At5g64890 genes in the 80 kb MXK3 region (Fig. 2-1B in the Chapter II). These two genes are functional genes identified as ESTs, and are not expressed or induced by cold treatment in leaves (Hanano et al. 2002), suggesting that both are incompetent in leaves. My results emphasize the importance of three states of genes, transcriptionally incompetent (unexpressed), competent but

unexpressed, competent and expressed states rather than the simple two states, unexpressed and expressed states, when considering the chromatin structure of genes showing changes in their competency.

Molecular mechanisms underlying the absence of DNase I HS are largely unknown. In the *Drosophila* heat-inducible *hsp26* gene, the nucleosome-free DNase I HSs are created at the heat shock elements by the binding of GAGA factors (Lu et al. 1993). The elimination of the GAGA binding from this promoter leads to the loss of these DNase I HSs and heat-inducibility of the *hsp26* gene (Lu et al. 1993). Analogously, the absence of the DNase I HS at incompetent gene promoter may be due to the loss of such DNA binding factors creating the HS in specific tissues of *Arabidopsis*.

In leaves, the *FWA* and *SUP* genes are silenced by repressive epigenetic marks, H3K9me2, H3K27me2 and DNA methylation (Jacobsen and Meyerowitz 1997, Lindroth et al. 2004, Soppe et al. 2000). Therefore, another possibility for the absence of DNase I HS is that chromatin of these incompetent genes forms highly-condensed, heterochromatic structure through these repressive epigenetic marks. However, this possibility seems to be unlikely, because the condensation state of the *AG* gene silenced by H3K27me2, H3K27me3, and by polycomb group proteins and heterochromatin protein 1 in leaves (Goodrich et al. 1997, Kotake et al. 2003, Nakahigashi et al. 2005,

Schubert et al. 2006), is relatively similar to that of expressed genes in euchromatin as measured in the Chapter I. In addition to this observation, H3K9me2 was not detected in the incompetent *At2S3* and *ADH* gene promoters in leaves (Fig. 3-4). One of the histone modifications characteristic to the incompetent gene promoters may be the low active H3K4me2 marks. The *FWA*, *SUP* and *AG* genes may be further silenced by the repressive H3K9me2, H3K27me2 or H3K27me3 which inhibit histone acetylation (Johnson et al. 2004), in contrast, the *ADH* gene is not silenced by these repressive marks in leaves. The *At2S3* gene is not repressed by H3K9me2 similar to the *ADH* gene in leaves, however, this gene may be further silenced by repressive H3K9me2 in suspension cells similar to the transposon for unknown reason.

To understand the importance of the absence of the DNase I HS at the incompetent *FWA*, *At2S3*, *AGAMOUS* and *SUPERMAN* gene promoters, it is necessary to analyze the presence and position of DNase I HS in flowers or siliques where these genes are normally expressed and functional. However, DNase I sensitivity assay is difficult using these tiny tissues, therefore, a novel assay system needs to be developed that can analyze chromatin accessibility in small amounts of cells. Identification of protein factors creating DNase I HSs in *Arabidopsis*, and elimination of HS from competent promoter or creation of the HS at incompetent promoter will be interesting future

research.

I summarized these results into a conceptual model in Fig. 3-5. At incompetent state, the DNase I HS is absent possibly due to the absence of unknown DNA-binding co-factor(s) creating HS (Fig. 3-5A). At incompetent promoter, active histone modifications H3K4me2 is low or absent (represented by K4me2 overlaid with X) and repressive histone modifications H3K9me2 is not always present (represented by K9me in parentheses). In these incompetent promoters, the binding of activators or remodeling factors to their binding sites is physically blocked by randomly-positioned nucleosomes. In contrast, at competent promoter, the DNase I HS was created by unknown DNA binding proteins (co-factor in Fig. 3-5B) and H3K4me2 is high allowing the access of activators and/or chromatin remodeling factors to the promoter leading to the activation of the gene. Transcriptional competency is regulated mainly by local nucleosome positioning and histone modifications and not by global higher-order chromatin structure as suggested in the Chapter I.

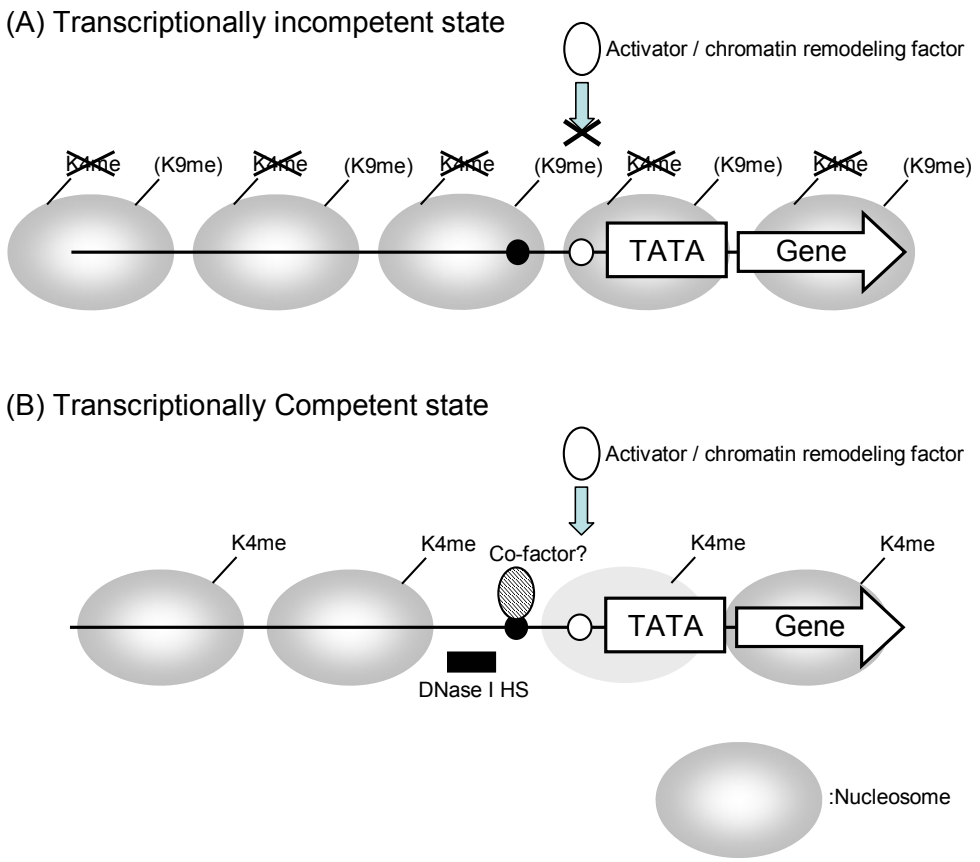


Fig. 3-5 A conceptual model of the function of DNase I HS in the establishment of transcriptional competency of the *Arabidopsis* gene. (A) At transcriptionally incompetent state, the DNase I HS is absent due to the unavailability of unknown DNA binding co-factor(s) creating the HS. At this circumstance, the access of transcription activators and/or chromatin remodeling complex to their binding sites are inhibited by randomly positioned nucleosomes. At the incompetent promoter, active histone modifications H3K4me2 is low or absent (represented by K4me overlaid with X), whereas, heterochromatic histone modifications H3K9me2 is either present or not (represented by nucleosomes with K9me in parentheses). (B) At competent gene promoter, DNase I HS is preformed both at expressed as well as unexpressed states as explained in Fig. 2-6 in the Chapter II. Histones presumably have euchromatic histone modifications (represented by nucleosomes with K4me). In this promoter, activators and/or chromatin remodeling factors can access to the promoter. Other symbols are as in Fig. 2-6 in the Chapter II.

3.5 Tables

Table 3-1

The gene specific primer sets used in the qRT-PCR assay.

Gene	Direction	Sequence (5' to 3')
FWA	F	GAGACACTGAATATGAGGTGCAA
	R	ATCTCCCGCTCCAAGTTAGC
2S3	F	AGCAAAACATGGCTAACAAGC
	R	TTGGTGAGGAGGAAGCAGAG
AGAMOUS	F	GACCAAACCGCTCTCCAGT
	R	TTATTCACTCCCGGCCATT
SUPERMAN	F	TTACTCTTACTCAACCATGGCAAA
	R	GGGTTGGAAATAGGGTTAGAGG
ADH	F	CCGTTGTTTCCACGTATCTTC
	R	TCCTTCTCCAACACTCTCAACA
UBQ5	F	CGATGGATCTGGAAAGGTTC
	R	AGCTCCACAGGTTGCGTTAG

Table 3-2

The gene specific primers and Taqman probes used in the ChIP assay.

Gene	Name	Sequence (5' to 3')	Position from ATG
HSP18.2	2F	AACTTCCCTATAAATATGTCCTTTGCTAA	-86
	2R	TTCGTTGCTTTTCGGGAGA	-3
	2T	AGATCAAATCAGCAGGAAAATCAAGAACCAAAA	-55
ADH	AF	TCGAGGAAGTGGAGGTTGCT	-201
	AR	GGTGTGACAGAGAGAAGTGAAGAGAA	-261
	AT	CACCGCAGAAACACGAAGTTCGTATCAA	-222
At2S3	SF	AAGCGGAGCTATGATGAGTGG	-234
	SR	ACGTTGTTGTCGGGATTATGTG	-178
	ST	ATTGTTTTGTTCGTCACTTGTCACTCTTTTCCA	-212
Transposon	TF	GCCTCGATTTGCTTCGGAT	
	TR	CTGGCGAGTTCCTCGGATAC	
	TT	CGATGCCGCGGTGCGTGA	

DISCUSSION

In the Chapter I, I have reported the relatively uniform chromatin condensation states irrespective of the gene expression levels within *Arabidopsis* euchromatin. This finding is contradictory to generally believed chromatin structure, active genes are open and accessible, whereas, inactive genes are closed and inaccessible. However, recent genome-wide analysis of human chromatin has revealed that the condensation state of higher-order chromatin is correlated with gene density and not with gene activity (Gilbert et al. 2004). Therefore, above belief mainly derived from analysis of limited number of model genes (tend to be atypical genes showing dynamic regulation) in insects and animals is proven not to be general (Sproul et al. 2005).

In the Chapter II, I have found the presence of DNase I HS at 5' ends of most genes irrespective of gene expression levels in *Arabidopsis*. Based on the results in the Chapter II, these DNase I HSs are suggested to function in transcriptional regulation of *Arabidopsis* genes by providing accessible sites for transcription activators and/or chromatin remodeling factors. Combined with the results of the Chapter I, these DNase I HSs preformed before activation may allow transcriptional activation of associated genes without changing higher-order chromatin structure in *Arabidopsis*. Recently, genome-wide analysis revealed that the DNase I HSs are found at 5' regions of

many genes whether they are expressed or not in yeast and human chromatin. Therefore, constitutive presence of DNase I HS at gene promoters irrespective of gene expression levels may have fundamental roles in eukaryotic transcription.

In the Chapter III, I have found the correlation between the presence of DNase I HS and transcriptional competency of *Arabidopsis* genes. This finding suggests that locally closed nucleosome configuration at the promoter may contribute transcriptional incompetency of *Arabidopsis* genes. In addition, this correlation emphasizes the importance of three transcriptional states of genes, incompetent (unexpressed), competent but unexpressed, competent and expressed states.

In *Arabidopsis*, my results suggest that transcription is mainly regulated by local nucleosome structure including nucleosome positioning and histone modifications at promoter regions. On the other hand, in animal systems, developmentally-regulated important genes, for example, *β-globin* and *homeobox* genes are strictly regulated by changes in higher-order chromatin structure (Sproul et al. 2005). In the *Arabidopsis* genome, aside from the constitutive heterochromatin, gene-density is uniformly high (gene/4.5 kb) and no large domains of hundreds of kilobases devoid of gene activity appear to exist (The Arabidopsis Genome Initiative 2000, Yamada et al. 2003). Together with my results, it seems likely that even developmentally-regulated important

Arabidopsis genes are not strictly regulated by changes in higher-order chromatin structure as seen in animals. This relatively less stringent transcriptional regulation of genes by chromatin structure possibly underlies flexible plant features including totipotency or great ability to respond to various environmental stresses.

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