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Original Paper

Distinct DNase I hypersensitive sites are absent from promoters of transcriptionally incompetent genes in *Arabidopsis*

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Abstract Specifically accessible chromatin sites, detected as DNase I hypersensitive sites (DNase I HSs), have been found at promoters of most genes in yeast, human as well as in *Arabidopsis* chromatin. The DNase I HSs are constitutively present at promoters of most genes both at inactive and activated states. On the other hand, appearance and disappearance of DNase I HSs have been observed at several developmentally regulated genes in different tissues of animals. Patterns of appearance and disappearance of DNase I HSs were correlated with transcriptional competency (potential of expression) of the associated genes, suggesting that DNase I HSs are absent from the promoters of transcriptionally incompetent (unexpressed nor inducible) genes. To test this hypothesis in plants, we analyzed DNase I hypersensitivity of several well-characterized genes showing distinct patterns of tissue-specific expression in *Arabidopsis* leaves, roots and suspension cells. Distinct DNase I HSs were present at transcriptionally competent gene promoters, but absent from incompetent gene promoters. The absence of DNase I HS suggests that nucleosomes are randomly positioned on incompetent promoters, and may contribute to the transcriptional incompetency by blocking the binding of activators in tissues where expression of these genes is unnecessary.

Key words: *Arabidopsis thaliana*, chromatin accessibility, DNase I hypersensitive site, tissue-specific gene expression, transcriptional competency.

Eukaryotic transcription is not only regulated by interactions between transcription regulators and *cis*-regulatory DNA elements but also by chromatin structure, which affects these interactions. Chromatin is composed of repeating units of nucleosomes, which affect transcription because the packaging of DNA into nucleosomes generally inhibits access of transcription regulators to the underlying DNA. The specifically nucleosome-free, accessible chromatin sites typically detected as DNase I hypersensitive sites (DNase I HSs) are frequently observed at gene regulatory regions, including promoters, enhancers and locus control regions (LCRs) in eukaryotic chromatin (Gross and Garrard 1988). These DNase I HSs are known to play a critical role in transcriptional regulation of stress-inducible and constitutively expressed genes (Gross and Garrard 1988). For example, DNase I HSs at promoters regulate transcription by allowing rapid binding of activators to their target sites in yeast and *Drosophila* stress-inducible genes (Gross et al. 1993; Lu et al. 1993; Svaren et al. 1994; Venter et al. 1994), or ensuring constitutive binding of activators and general transcription machinery in yeast housekeeping genes (Angermayr et al. 2002, 2003). The DNase I HSs are constitutively present at these gene promoters both at inactive and activated states, and are thought to confer transcriptional competency (potential of expression) on associated genes by allowing access of transcription factors (Wallrath et al. 1994). For example, in the *Drosophila hsp26* gene promoter, pre-bound GAGA factor, TFIID complex and paused RNA polymerase established DNase I HS prior to heat activation, and this HS confers transcriptional competency (heat inducibility) on the *hsp26* gene by allowing the access of activator heat shock factor to its binding sites upon heat shock (Leibovitch et al. 2002).

Recently, genome-wide mapping of DNase I HSs in yeast and human revealed the presence of DNase I HSs at the promoters of most genes, whether they are expressed or not (Crawford et al. 2004, 2006; Ercan and Simpson 2004; Sabo et al. 2004, 2006; Yuan et al. 2005). In an 80 kb region of the *Arabidopsis* genome, we reported DNase I HSs at the 5′ ends of 28 out of 30 genes, irrespective of their expression levels (Kodama et al. 2007). These findings suggest that most (not all)

Abbreviations: ABA, abscisic acid; ABRE, abscisic acid responsive element; *ADH*, *Alcohol dehydrogenase*; *AG*, *AGAMOUS*; ChIP, Chromatin immunoprecipitation; DNase I HS, DNase I hypersensitive site; LCR, locus control region; MIPS, Munich Information Center for Protein Sequences; *SUP*, *SUPERMAN*.

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genes are in a transcriptionally competent (i.e., expressed, or unexpressed but inducible) state in yeast, human as well as in plants. On the other hand, the 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 lead to the hypothesis that DNase I HSs are absent from the promoters of transcriptionally incompetent (i.e., neither expressed nor inducible) genes. In support of this hypothesis in plants, the presence and absence of DNase I HSs have also been observed in *Arabidopsis*. In the *Alcohol dehydrogenase* (ADH) gene promoter, a DNase I HS is detected in suspension cells where this gene is expressed, but it is not detected in leaves where this gene is neither expressed nor induced by various stresses (de Bruxelles et al. 1996; Dolferus et al. 1994; Vega-Palas and Ferl 1995). In this study, to further test this hypothesis in plants, we 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 thought to be in transcriptionally incompetent, inducible or activated states.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia Col-0 and suspension cell strain T87 (Axelos et al. 1992) were grown as described (Kodama et al. 2007). To obtain root tissues, *Arabidopsis* plants were grown hydroponically as described previously (Naito et al. 1994).

Quantitative RT-PCR

Total RNA was isolated from *Arabidopsis* leaves, suspension cells, flowers, siliques and roots using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany). For 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, Indianapolis, IN). The generated cDNA was measured in triplicates by quantitative PCR using the LightCycler (Roche). 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 pmol gene-specific primers (Table 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.

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, purification of the DNA after DNase I digestion and DNase I sensitivity assay by Southern blotting were performed as described (Kodama et al. 2007). DNase I digestion of hydroponically grown roots was performed as described for suspension cells (Kodama et al. 2007).

Table 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

Preparation of DNA probes

All DNA probes used for DNase I sensitivity assays were 500 bp long and were prepared as described (Kodama et al. 2007). For indirect-end labeling analysis (Kodama et al. 2007), probe F (49034-49533 on M7J2 bacterial artificial 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), and probe U (63612-64111 on T17J13, abutting the *Pst*I site) were used for the *FWA*, *At2S3*, *AGAMOUS*, *SUPERMAN*, 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. All information about gene position and gene annotation is based on the MIPS *A. thaliana* database (MAtDB; http://mips.gsf.de/proj/thal/db).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay using suspension cells was performed as previously described (Kodama et al. 2007). *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 filtration and ground into a fine powder in the presence of liquid nitrogen with a mortar and pestle and processed as described for suspension cells. 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 2. All ChIP experiments were performed in triplicate.

Table 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	

Results

Gene expression levels of five target genes showing several patterns of tissue-specific expression in Arabidopsis

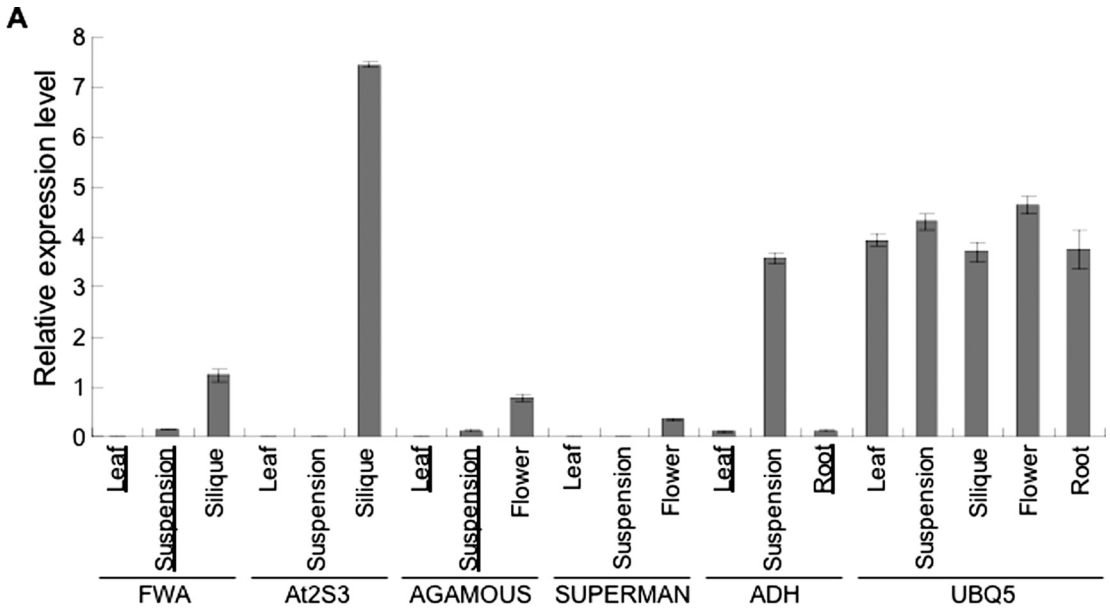
To analyze the relationship between presence of DNase I HS and transcriptional competency in *Arabidopsis*, we selected four well-documented tissue-specific genes, *FWA* (At4g25530), *At2S3* (At4g27160), *AGAMOUS* (*AG*, At4g18960), *SUPERMAN* (*SUP*, At3g23130), and one ubiquitously expressed *UBQ5* (At3g62250) gene as a control. To investigate the transcriptional competency of these 5 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 and roots by quantitative RT-PCR (qRT-PCR) assay (Figure 1A, B). 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 (Pischke et al. 2006). The *At2S3* gene encoding a seed-storage protein having one abscisic acid (ABA) responsive element (ABRE) in its promoter (indicated in Figure 2B) is specifically expressed in maturing seeds in an ABA-dependent manner, but is not expressed nor induced 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 not in leaves, while the *At2S3* gene was expressed in siliques but neither in leaves nor suspension cells (Figure 1A, B). 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 (Figure 1C).

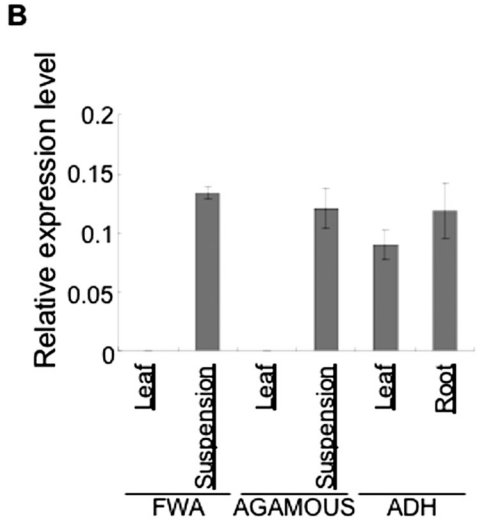
The *AG* gene encodes a MADS domain transcription factor that is important in specifying identity of floral meristem, carpel and stamen (Yanofsky et al. 1990). The *AG* gene is specifically expressed in the third and fourth whorls, carpels and stamens of flowers (Bowman et al. 1991). The *SUP* gene encodes 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 Figure 2D is shown to function as a positive element for SUP expression (Ito et al. 2003). The qRT-PCR assay confirmed these expression patterns: both the *AG* and *SUP* genes were expressed in flowers but not in leaves (Figure 1A, B). In suspension cells, the *AG* gene was expressed; in contrast, the *SUP* gene was not expressed (Figure 1A, B). These results 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 (Figure 1C).

The *UBQ5* gene encoding an ubiquitin extension protein has one CCAAT box and one TATA box in its promoter (Figure 2E) (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 our qRT-PCR assay (Figure 1A). Therefore, the *UBQ5* gene is competent and expressed in these tissues including leaves and suspension cells (Figure 1C).

DNase I HSs are absent from promoters of transcriptionally incompetent genes in Arabidopsis leaves and suspension cells

The DNase I hypersensitivity of five 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, which requires relatively large numbers 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 a single





C

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

Figure 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 each tissue. Relative qRT-PCR signals are shown in arbitrary units. (B) Relatively small qRT-PCR signals of the *FWA* and *AGAMOUS* genes in leaves and suspension cells, and of the *ADH* gene in leaves and roots (marked with underline) are shown with an enlarged Y-axis. Data are expressed as means±SD (standard deviation). (C) Transcriptional competency of target genes in leaf, suspension cell and root. The genes in a 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 leaf cells.

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 genomic DNA digested with restriction enzymes was also included in the membranes to confirm specific hybridization of a probe to its target region and to serve as a size standard.

In the *FWA* and *AG* genes, a distinct DNase I HS was detected at the 5′ region in suspension cells but not in leaves (Figure 2A, C). In the *At2S3* and *SUP* genes, a

DNase I HS was not detected at the 5′ region in either tissue (Figure 2B, D). These results suggest that the DNase I HS is absent from the 5′ region of transcriptionally incompetent genes (Figures 1C, 2A–D).

In the ubiquitously expressed *UBQ5* gene, the distinct DNase I HS was detected at the 5′ region of the *UBQ5* gene in both suspension cells and leaves (Figure 2E). This result confirms that the absence of the DNase I HS observed at the incompetent genes is not due to either inefficient digestion or over-digestion by DNase I. The DNase I digestion profiles of naked DNA gave smears or faint bands (Figure 2A–F, lanes naked), confirming that bands observed *in vivo* are dependent on chromatin structure. The absence of DNase I HS observed above may result not simply from a lack of active transcription but rather from the transcriptional incompetency of genes, because distinct DNase I HSs were found in several unexpressed (and inducible) genes in *Arabidopsis* leaves and suspension cells (Kodama et al. 2007).

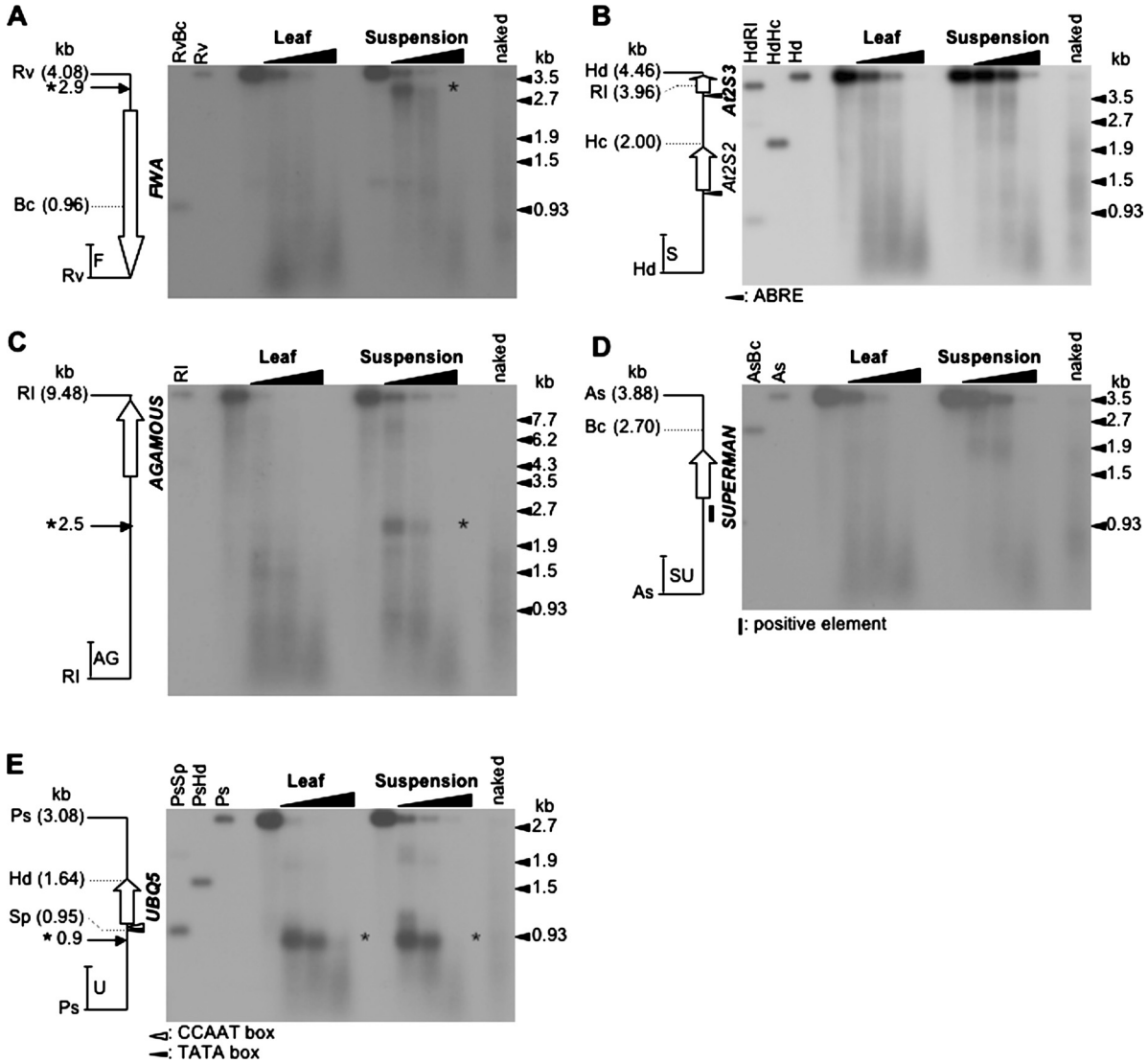


Figure 2. DNase I hypersensitivity of the *FWA*, *At2S3*, *AGAMOUS*, *SUPERMAN* and *UBQ5* genes in leaves and suspension cells. (A–E) Chromatin of leaves or suspension cells were digested with DNase I at 0, 0.25, 0.5 and 2 U ml^{-1} (leaves) or at 0, 10, 20 and 50 U ml^{-1} (suspension cells) at 30°C 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 EcoRV, *Hin*dIII, *Eco*RI, *Ase*I or *Pst*I-treated DNA was hybridized with probe F, S, AG, SU or U to detect the 5′-region of genes: *FWA* (A), *At2S3* (B), *AGAMOUS* (C), *SUPERMAN* (D), and *UBQ5* (E). The positions of the genes are indicated by open arrows. DNase I HSs are indicated by horizontal arrows, with sizes in kb and asterisks at right. *Ase*I, *Bcl*I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Sph*I, and *Pst*I restriction sites are shown by As, Bc, RI, Rv, Hc, Hd, Sp, and Ps, respectively, with bands sizes in kb. Probes F, S, AG, SU, and U are indicated by vertical bars along the left side. The positions of two ABREs (B), one positive element (D) and one TATA box (E) are indicated by horizontal closed arrowheads, and one CCAAT box (E) are indicated by horizontal open arrowheads at left. The lanes Rv, RvBc (A), Hd, HdHc, HdRI (B), RI (C), As, AsBc (D), and Ps, PsHd, PsSp (E) 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), and *Pst*I, *Pst*I-*Hin*dIII, *Pst*I-*Sph*I (E) digests of intact genome included as size standards. Molecular weight markers are indicated by arrowheads on the right sides.

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

The *ADH* gene encoding an alcohol dehydrogenase has two G-boxes and one ABRE in its promoter (Figure 3B) (Dolferus et al. 1994). In leaves, the *ADH* gene is neither expressed nor induced by ABA, hypoxia, and cold treatments, but it is inducible in roots (Dolferus et al. 1994), and constitutively expressed at high levels in suspension cells (de Bruxelles et al. 1996; Vega-Palas and Ferl 1995). A DNase I HS is found in the *ADH* promoter in suspension cells, but not in leaves (Vega-Palas and Ferl 1995). Based on this result, it is likely that the presence of the DNase I HS is correlated with *ADH* expression levels (Vega-Palas and Ferl 1995). To examine whether the presence of DNase I HS is correlated with expression levels or with competency of the *ADH* gene, we performed sensitivity assays in roots as well as leaves and suspension cells. In our qRT-PCR assay, the *ADH* gene was very weakly expressed both in leaves and roots and was highly expressed in suspension cells (Figure 1A, B). In mature plants, the *ADH* gene expression is not found in most of the green parts but is found in the base of the rosette leaves (Dolferus et al. 1994). Therefore, the weak expression levels of the *ADH* gene observed in leaves may result from the small fraction of these cells, not excluded in our 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 cells of the leaf (Figure 1C). For hypersensitivity assays, *Arabidopsis* roots were grown in 5 liters of hydroponic culture in two months; only small amounts of DNase I-digested DNA ($\sim$3 μg) was recovered, however, due to the small mass and rigidity of these roots. We mapped the DNase I HS by hybridizing continuous 500 bp DNA probes covering the *ADH* gene promoter on a set of membranes prepared with DNase I-digested DNA from leaves, suspension cells and roots. 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 (Figure 3A, 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 (Figure 3A). This pattern indicates that a DNase I HS is located in the ADH2 segment containing two G-boxes and one ABRE, and that four more sites exist 2.7 and 4.5 kb 5′ of the *ADH* gene and 3.5 and 6.2 kb 3′ to this site in suspension cells (where this gene is highly expressed) as well as in roots (where it is weakly expressed) (Figure 3). In contrast, these distinct bands were not observed in leaves where this gene is neither expressed nor induced (Figure 3A) as previously reported (Vega-Palas and Ferl 1995). Taken together, these results suggest that the presence of DNase I HS is correlated with transcriptional competency rather than with expression levels of the *ADH* gene (Figures 1, 3). The DNase I HS was located near two G boxes and one ABRE in the *ADH* gene promoter in suspension cells (Figure 3A) by indirect end labeling analysis (data not shown). 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 (Figure 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.

Active and repressive histone H3 methylation states at the ADH and At2S3 gene promoters in leaves and suspension cells

In addition to the presence of DNase I HS, histone methylation at specific lysine residues has been correlated with transcriptional competency of several developmentally regulated genes in *Drosophila* and mammals (Lachner and Jenuwein 2002). For example, at the chicken *β-globin* locus, an active epigenetic mark (H3 dimethylated at lysine 4, H3K4me2) and an inactive mark (H3 dimethylated at lysine 9, H3K9me2) are correlated with developmentally active and inactive *globin* genes, respectively (Litt et al. 2001). In *Arabidopsis*, at the incompetent *FWA*, *SUP* and *AG* genes, the repressive mark H3K9me2 is high, whereas the active mark H3K4me2 is low in leaves (Jacobsen and Meyerowitz 1997, Lindroth et al. 2004; Schubert et al. 2006, Soppe et al. 2000). To characterize the relationship between histone methylation states and absence of DNase I HS at the incompetent genes in addition to the well-characterized *FWA*, *SUP* and *AG* genes, we analyzed the active mark H3K4me2 and repressive mark H3K9me2 at the *ADH* and *At2S3* gene promoters by ChIP assay in leaves and suspension cells. As a control, the competent but unexpressed heat-inducible *HSP18.2* gene (Kodama et al. 2007) and a genomic region within dispersed cinful-type transposons located in the heterochromatic knob of chromosome IV (115509-115575 on T5L23 clone) (Gendrel et al. 2002) were also analyzed.

The competent *HSP18.2* gene exhibited high active H3K4me2 signal; in contrast the incompetent *ADH*, *At2S3* genes and the transposon exhibited low levels of the active mark (Figure 4A). Similarly, in leaves the transposon showed high signal for the repressive H3K9me2 signal, whereas the *HSP18.2*, *ADH* and *At2S3* genes showed very low signals (Figure 4A). In suspension cells, the H3K4me2 signals of the competent *HSP18.2* and *ADH* genes were similarly high, in

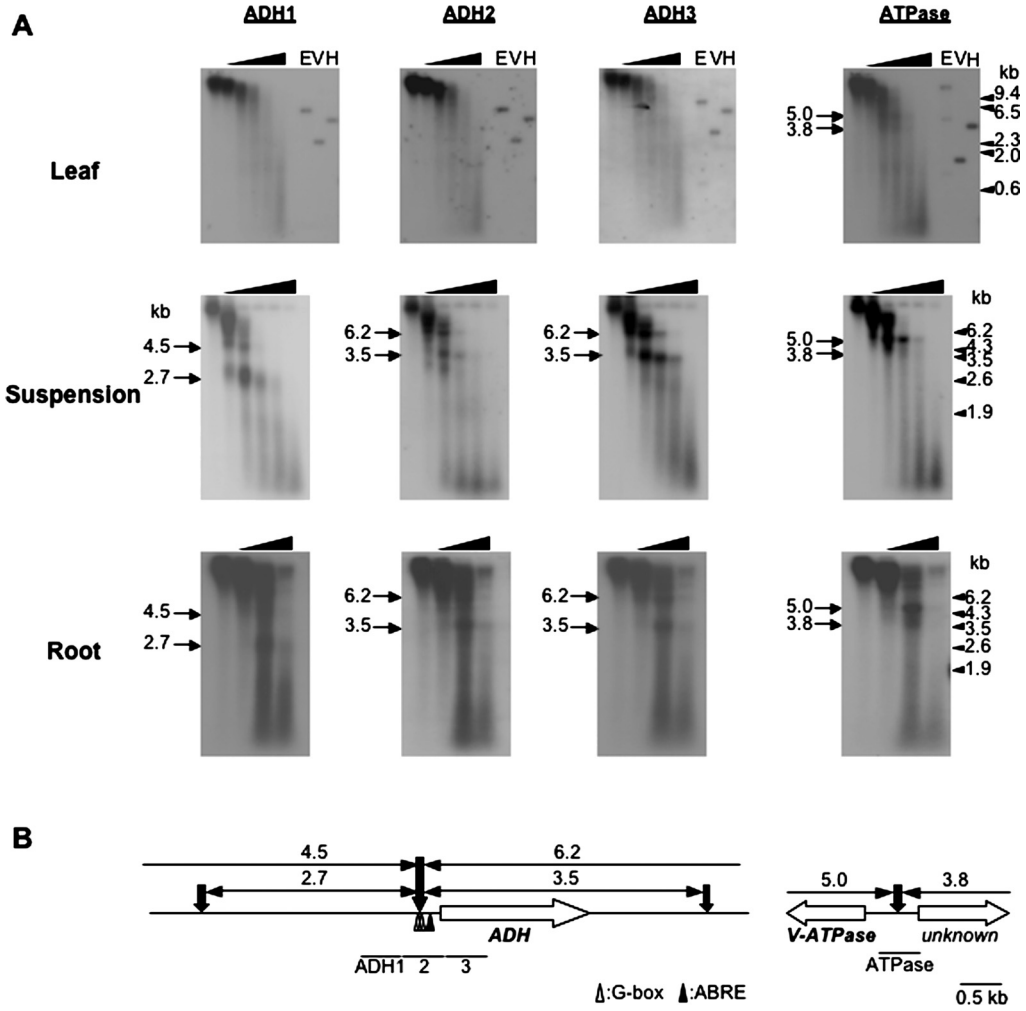


Figure 3. Mapping of the DNase I HSs at the *ADH* and *V-ATPase* gene promoters in leaves, suspension cells and roots. (A) Chromatin of mature leaves, suspension cells and roots was digested with increasing amounts of DNase I (0, 0.025, 0.25, 0.5, 1, and 2 U ml^{-1} for leaves; 0, 5, 10, 20, 30, and 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 and 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 the 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.

contrast, those of the incompetent *At2S3* gene and the transposon were similarly low (Figure 4B). In suspension cells, the incompetent *At2S3* gene and transposon showed high H3K9me2 signals, while the competent HSP18.2 and *ADH* genes showed very low signals (Figure 4B). ChIP assays without using antibodies gave undetectable signals (data not shown). Low levels of active mark H4K4me2 may not result from the lack of active transcription, because the mark is present at high levels in the competent *HSP18.2* gene even when the gene is not expressed.

Discussion

In this study, we have shown that DNase I HSs are absent from the promoters of genes in transcriptionally incompetent states. The absence of DNase I HSs suggests that the nucleosomes are randomly positioned on incompetent promoters. These randomly-positioned nucleosomes at the 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 cannot be

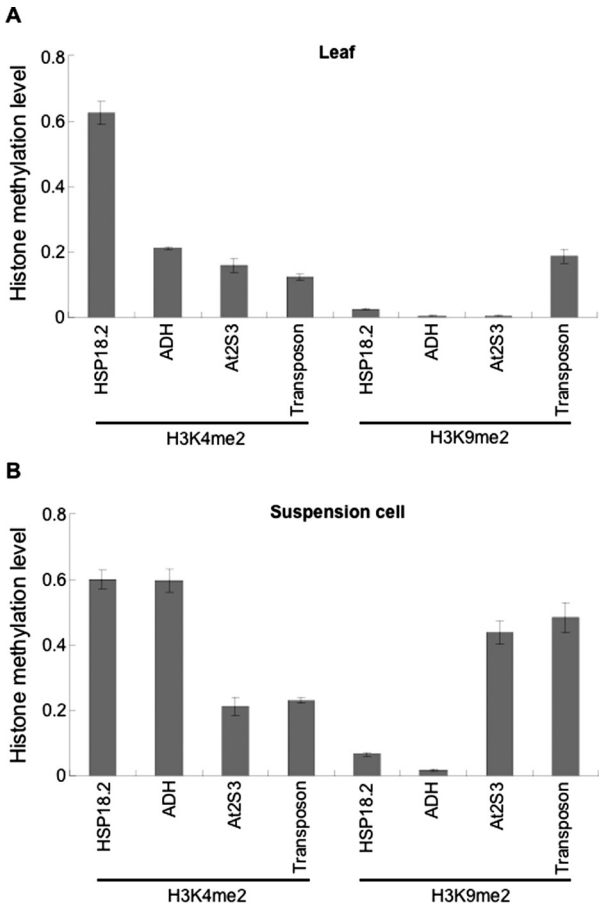


Figure 4. Histone H3 dimethylation at lysine 4 and 9 in the *HSP18.2*, *ADH* and *At2S3* genes and at the transposon. *Arabidopsis* leaves. (A) or suspension cells (B) were fixed with formaldehyde. Chromatin fragments were precipitated with antibodies specific for H3K4me2 or H3K9me2. The amounts of immunoprecipitated DNA were determined by quantitative PCR with specific primers and Taqman probes for the promoter regions of the *HSP18.2*, *ADH*, *At2S3* genes and coding region of the transposon using the input DNA from leaves or suspension cells as standard. Histone H3 methylation levels are shown in arbitrary units. The data are expressed as the means±SD (standard deviation).

activated in leaves overexpressing ABI5 even in the presence of exogenous ABA (Brocard et al. 2002). In these transgenic leaves, ABA-induction of competent genes having the ABRE in their promoters was enhanced (Brocard et al. 2002); therefore, the ABI5 binding to the ABRE may be prevented by a closed nucleosome configuration at the *At2S3* gene promoter. 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 inducible. In contrast, in leaves, the *ADH* gene is uninducible. In this case, the absence of the DNase I HS may block the binding of activators even if activators are present. 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 resulted from the active transcription of the *ADH* gene (Vega-Palas and Ferl 1995). However, the DNase I HS is also present in roots where the *ADH* gene is only weakly expressed (Figures 1, 3). In addition, we previously reported the presence of DNase I HS at several unexpressed genes in *Arabidopsis* (Kodama et al. 2007). These suggest that presence of the DNase I HS results from transcriptional competency, rather than active transcription, of the *ADH* gene. Our results emphasize the importance of three states of genes: transcriptionally incompetent (unexpressed); competent but unexpressed; and competent and expressed. This model has more explanatory power than one in which genes have only two states, unexpressed and expressed, and demonstrates the value of considering chromatin structure.

The 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, in *Arabidopsis*, the absence of the DNase I HS at incompetent gene promoters may be due to the loss of such DNA binding factors creating the HS in specific tissues.

In leaves, the *FWA*, *SUP* and *AG* genes are silenced by repressive epigenetic marks H3K9me2 (Jacobsen and Meyerowitz 1997; Lindroth et al. 2004; Soppe et al. 2000), which is enriched in constitutive heterochromatin (Houben et al. 2003). Therefore, another possible explanation for the absence of DNase I HSs is that repressive epigenetic marks induce the chromatin of incompetent genes to form highly condensed, heterochromatic structures. However, this possibility seems unlikely, because the condensation state of the *AG* gene silenced by repressive H3K9me2, H3K27me2 and H3K27me3 in addition to the repression by the 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 those of expressed genes in euchromatin as previously measured by general DNase I sensitivity assay in leaves (Kodama et al. 2006).

The active epigenetic mark H3K4me2 is low at the incompetent *ADH*, *At2S3* (Figure 4), *FWA*, *SUP* and *AG* genes (Jacobsen and Meyerowitz 1997; Lindroth et al. 2004; Soppe et al. 2000), however, H3K4me2 is high at the competent *HSP18.2* and *ADH* genes (Figure 4). In contrast, H3K9me2 is observed at the incompetent *FWA*, *SUP* and *AG* genes (Jacobsen and Meyerowitz 1997; Lindroth et al. 2004; Soppe et al. 2000) but not observed at the incompetent *ADH* and *At2S3* genes in leaves (Figure 4A). Therefore, at incompetent gene promoters, the active mark H3K4me2 is generally low, whereas

H3K9me2 can be either high or low. The *FWA*, *SUP* and *AG* genes may be further silenced by the repressive H3K9me2 in addition to the absence of DNase I HS; in contrast, the *ADH* and *At2S3* genes are not silenced by these repressive marks in leaves (Figure 4). The *At2S3* gene may be further silenced by repressive H3K9me2 in suspension cells similar to the case for *FWA*, *SUP* and *AG* genes (Figure 4).

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. In future work, it will be of interest to identify protein factors creating DNase I HSs in *Arabidopsis*, and to study the effects of eliminating HSs from a competent promoter or creating HSs at an incompetent promoter.

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