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Analysis of chromatin condensation states by DNase I sensitivity assay at 500-base resolution in *Arabidopsis*

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Abstract Variable levels of transgene expression are frequently observed among independent transgenic plants. Although different chromatin condensation states surrounding randomly integrated transgenes have previously been thought to be one of the causes for this variability, condensation states of plant chromatin have not been examined systematically. In this study, to analyze the condensation states of *Arabidopsis* chromatin, we evaluated the degree of chromatin condensation by measuring overall accessibility to DNase I (DNase I sensitivity) at 500-base resolution. We analyzed 30 variably expressed genes in an 80-kb genomic region, a gene repressed by polycomb group and heterochromatin protein 1-like genes, two genes near the heterochromatin, and a retrotransposon within the genetically-defined centromere in *Arabidopsis*. The centromeric region was significantly DNase I insensitive, however, sensitivity of these genes was similar irrespective of the individual gene expression levels.

Key words: *Arabidopsis thaliana*, chromatin condensation, DNase I sensitivity, position effect, variable transgene expression.

The use of transgene technology in plants is becoming increasingly important, serving multiple purposes in the fields of commerce and research. However, the level of transgene expression often varies considerably among independent plant transformants (Fagard and Vaucheret 2000), hampering the effective use of transgenic plants in both applied and basic research. Variability of transgene expression has been attributed to several factors including differences in chromosome position, sequence-specific gene silencing and copy number (Matzke and Matzke 1998; Kooter et al. 1999; Selker 1999).

The eukaryotic genome is organized as a condensed chromatin structure, is not uniform in its transcriptional activity and chromatin condensation states. In general, because chromatin condensation limits access of protein factors to the underlying DNA, it is thought that genes in relatively condensed chromatin are inactive, and active genes are contained within a decondensed chromatin. In chicken erythrocytes, for example, the active β-globin locus resides in a 30-kb decondensed chromatin domain, whereas the inactive ovalbumin gene is found in relatively condensed chromatin (Kimura et al. 1983; Fisher and Felsenfeld 1986; Caplan et al. 1987).

As a consequence of this heterogeneity of the eukaryotic genome, different chromatin condensation states surrounding integrated transgenes are thought to influence transgene expression positively or negatively; this phenomenon is referred to as the position effect (Wakimoto 1998; Wallrath 1998). For example, in the *Drosophila* and yeast genomes, inactive and condensed chromatin regions inactivate nearby transgenes by converting them into a condensed, inaccessible chromatin conformation (Gottschling 1992; Wallrath and Elgin 1995; Cryderman et al. 1999; Sun et al. 2001).

With the plant transformation methods currently used, it is not feasible to target efficiently the transgene to specific positions in the plant genome (Day et al. 2000). Differences in chromatin condensation state near the transgene integration site are also thought to be one of the causes of variable transgene expression observed in plants (Matzke and Matzke 1998). 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) or contain blocks of condensed

Abbreviations: *AG*, *AGAMOUS*; HP1, heterochromatin protein 1; MIPS, munich information center for protein sequences; PcG, polycomb group; S1, r*ibosomal protein* S1

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heterochromatic regions composed of clusters of repetitive elements in maize or tobacco (Bennetzen et al. 2000; Houben et al. 2003; Kovarik et al. 2000). However, these low-resolution microscopic analyses cannot assess the chromatin condensation states of inactive and active genes within euchromatin or heterochromatin.

In this study, to analyze the differences in chromatin condensation states at higher resolution in plants, we 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*.

Materials and methods

Plant material 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.

Digestion with DNase I 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-Aldrich, 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×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.

Preparation of DNA probes

All DNA probes used for DNase I sensitivity assays were 500 bp long and were prepared as below. Bacterial artificial chromosome (BAC) clones containing target genes were divided into continuous, non-overlapping 500-bp segments from the first nucleotide (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 BAC 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 *Athila* retrotransposon in the centromeric region of chromosome V (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 MIPS *A. thaliana* database (MAtDB; http://mips.gsf.de/proj/thal/db).

DNase I sensitivity assays by Southern blotting

The DNase I sensitivity of 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 membranes were hybridized with DNA probes randomly labeled with [α-^{32}P]dCTP, washed, and visualized by exposure to X-ray film as described previously (Nagaya et al. 2004).

Results

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*, we 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 (Yaniv and Cereghini 1986; Krebs and

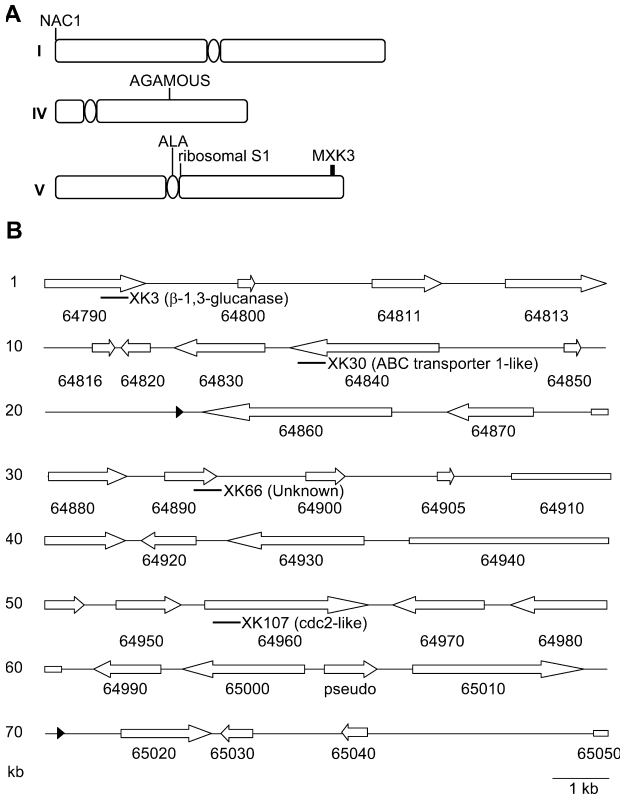

Figure 1. Map of the target regions and genes on chromosomes and in MXK3 BAC clone. (A) Chromosomal positions of the MXK3 clone, ALA region, *AGAMOUS*, *ribosomal protein S1*, and *NAC1* genes. Roman numerals indicate chromosome identities and ovals represent core centromere regions. (B) Gene organization 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 locus codes (At5gxxxxx). One pseudogene (open arrow, pseudo) and two tRNA-coding genes (closed arrowheads) 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.

Peterson 2000). We 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.

We 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) (Figure 1A). The MXK3 region lies about 1 Mb from the end of the long arm of chromosome V (Figure 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 (Figure 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 (Figure 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 Figure 2A, as representatives of the entire set of 163 autoradiographs (probe positions are indicated in Figure 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 hardly expressed in leaves. The *β-1,3-glucanase* gene gave a weak expression signal, whereas the *WRKY51*, *cdc2-like protein kinase*, *ABC transporter protein 1-like* genes are expressed at about 4-, 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 (Figure 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, Figure 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 XK66, 3, 107, and 30 segments (Figure 2A, compare lanes 5 and 6).

Because DNase I has a little sequence specificity, we 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 (Figure 2B), confirming that the signals observed in the DNase I sensitivity assays reflect the *in vivo* chromatin structure of the DNA.

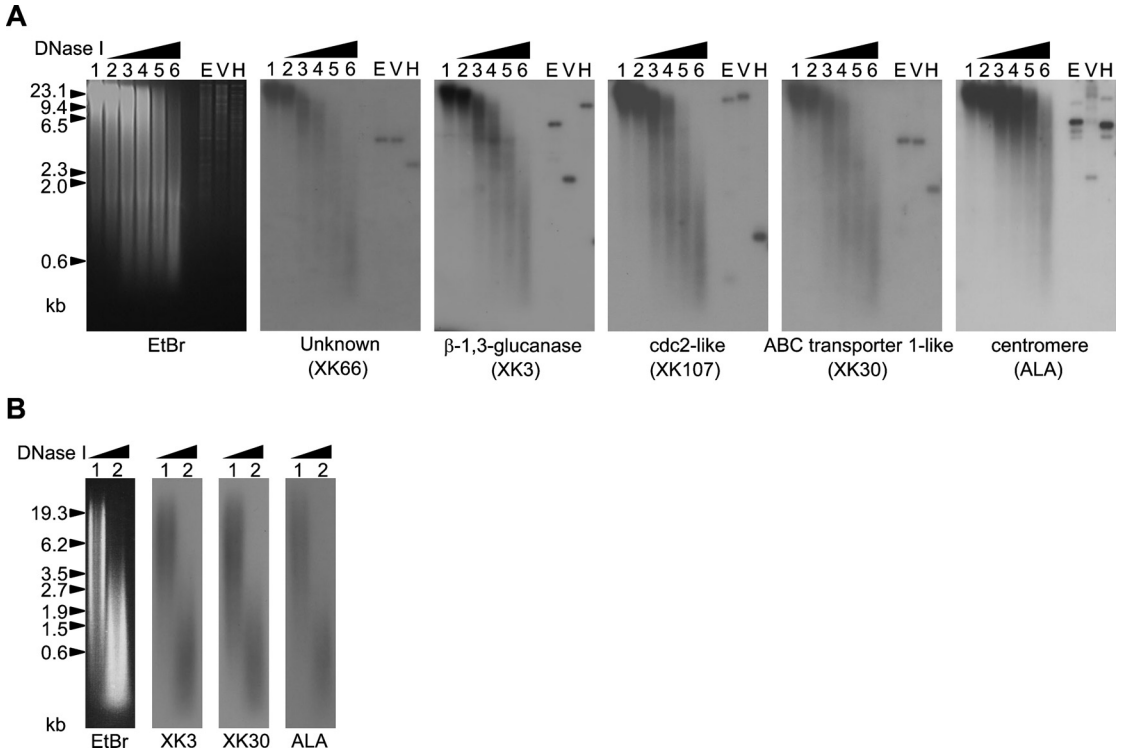

Figure 2. DNase I sensitivity of the *Unknown*, *β-1,3-glucanase*, *cdc2-like protein kinase* and *ABC transporter protein 1-like* genes, and the core centromere region of chromosome V in leaves. (A) Chromatin of leaves was digested with DNase I at 0, 0.025, 0.25, 0.5, 1, and 2 U ml^{-1} (lanes 1–6, 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 increasing amounts of DNase I (denoted by triangles above 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.

DNase I sensitivity of AGAMOUS gene and of genes near heterochromatin

In addition to the genes in the MXK3 and the ALA region, we also analyzed the floral homeotic gene *AGAMOUS* (*AG*; At4g18960; Figure 1A) on chromosome IV. In *Arabidopsis* leaf, the *AG* gene is repressed by polycomb-group (PcG) genes, *Curly leaf*, *Embryonic flower 2*, *Fertilization independent endosperm*, and heterochromatin protein 1 (HP1)-like gene *Terminal flower 2* (Goodrich et al. 1997; Kinoshita et al. 2001; Kotake et al. 2003; Moon et al. 2003; Katz et al. 2004; Nakahigashi et al. 2005). PcG proteins are known to repress homeotic genes by reducing accessibility of their chromatin in *Drosophila* (Zink and Paro 1995; Fitzgerald and Bender 2001), 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, we examined the DNase I sensitivity of the repressed *AG* in leaves. Sensitivity of the *AG* gene was comparable to that in the MXK3 region and was significantly higher than that in the ALA region (Figure 2, 3).

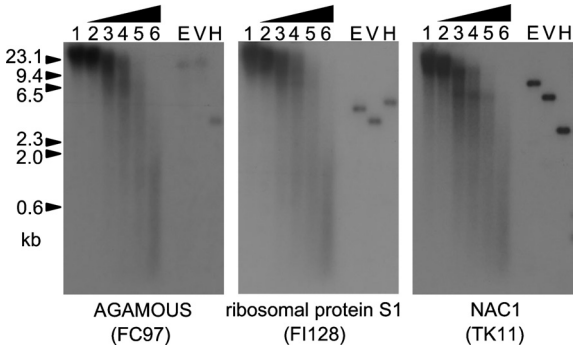

Figure 3. DNase I sensitivity of the *AGAMOUS* gene and genes near heterochromatin in leaves. The general DNase I sensitivity of the *AGAMOUS*, *ribosomal protein S1* and *NAC1* genes in leaves is shown, with the corresponding probe names below. Symbols are as in Figure 2.

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, 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), were analyzed in leaves (Figure 1A). 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 (Figure 2, 3).

Discussion

Although different chromatin condensation states near the transgene integration site are thought to be one of the causes for variable transgene expression in plants, we have shown that chromatin condensation states as measured by overall accessibility to DNase I was relatively uniform irrespective of individual gene expression levels in the 80-kb MXK3 region, at the *AGAMOUS* gene and *ribosomal protein S1* and *NAC1* genes near heterochromatin in *Arabidopsis* (Figure 2, 3). In contrast, the ALA region within genetically-defined centromere of chromosome V was significantly DNase I insensitive (Figure 2). 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 (Figure 3). 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 our sensitivity assay. In the *Arabidopsis* leaf, the upstream At4g18950 gene (located at 5.1–7.6 kb upstream from the 5′ end of the *AG* gene) and downstream At4g18965 gene (located at 1.1–2.6 kb downstream from the 3′ end of the *AG* gene) were expressed (data not shown). Furthermore, 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). Collectively, these data suggest that the expression of the two genes adjacent to the *AG* gene is not affected by the PcG and HP1-mediated heterochromatic structure of the *AG*. The *S1* and *NAC1* genes are located near heterochromatin, however, their chromatin showed similar sensitivity to that in the MXK3 in leaves (Figure 3). 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). Outside typical heterochromatin, gene density in *Arabidopsis* is uniformly high (average density of one gene/4.5 kb) (The Arabidopsis Genome Initiative 2000) and no large domains of hundreds of kilobases devoid of transcriptional activity have been found in gene expression studies at the whole genome level (Yamada et al. 2003). Although we have examined only a small portion of the *Arabidopsis* whole genome (140 Mb), the presence of genes regulated by large-scale (extending a number of genes) chromatin condensation states seems to be unlikely. Further analysis of local chromatin structure, such as nucleosome positioning or histone modifications, will reveal the principal factors controlling the accessibility of transcription factors to the *Arabidopsis* genes.

Although the DNase I sensitivity except for the centromeric region was similar in the analyzed regions in the *Arabidopsis* genome, we cannot exclude the possibility that differences in chromatin condensation states too subtle to be detected in our sensitivity assay may influence the transgene expression. However, we previously reported that 10 independent *Arabidopsis* transgenic lines, harboring a single copy of the transgene integrated at different positions in each genome, showed uniform expression levels (Nagaya et al. 2004). Consistent with that report, an extensive characterization of 132 independent transgenic lines by Schubert et al. revealed no case of variable transgene expression as a result of the site of transgene integration (Schubert et al. 2004). The uniformity of gene expression level of randomly integrated single-copy transgenes therefore makes the above possibility unlikely. Collectively, these results suggest that the differences in chromatin condensation state outside of heterochromatic regions are not a major cause of variable transgene expression in *Arabidopsis*. Interestingly, two transgenic lines harboring transgenes in the pericentromeric heterochromatin of chromosome I showed high expression levels (Schubert et al. 2004). Considering the relatively decondensed chromatin structure of the *S1* and *NAC1* genes within heterochromatin, condensed heterochromatic structure may not propagate into nearby transgenes in *Arabidopsis*. Analysis of expression level and chromatin structure of more number of transgenes integrated within heterochromatin should be undertaken to reveal this point. In barley and tobacco plants with heterochromatic regions other than typical heterochromatin in their much larger genomes (Houben et al. 2003), several transgenic lines harboring randomly-integrated single copy transgenes also showed uniform expression levels (Hobbs et al. 1990; Koprek et al. 2001). Transgenes are possibly not influenced by heterochromatic structure near the integration site in these two plants, however, studies of large number of single copy transgene expression or direct measurement of chromatin condensation states are necessary to conclude.

Variability of transgene expression is thus likely to be

caused by factors other than chromatin condensation, such as sequence-specific gene silencing mediated by small interfering RNA and/or by increasing copy number (Butaye et al. 2004; Schubert et al. 2004). Approaches to eliminating these factors intrinsic to transgenes should contribute to minimizing variability of transgene expression in plants.

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