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R. van Blokland · M. ten Lohuis · P. Meyer

Condensation of chromatin in transcriptional regions of an inactivated plant transgene: evidence for an active role of transcription in gene silencing

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Abstract The chromatin structures of two epigenetic alleles of a transgene were investigated by measuring the local accessibility of transgene chromatin to endonucleases. The two epialleles represented the active, hypomethylated state of a transgene in line 17-I of *Petunia hybrida*, and a transcriptionally inactive, hypermethylated derivative of the same transgene in line 17-IV. In nuclear preparations the inactive epiallele was significantly less sensitive to DNaseI digestion and nuclease S7 digestion than the transcriptionally active epiallele, whereas no significant differences in accessibility were observed between naked DNA samples of the two epialleles. Our data suggest that a condensed chromatin structure is specifically imposed on transcribed regions of the construct in line 17-IV. In contrast, in both epialleles the plasmid region of the transgene, which is not transcriptionally active in plants, retains the same accessibility to endonucleases as the chromosomal integration site. These data suggest that transcriptional inactivation is linked to the process of transcription, and imply that control of transgene expression via the use of inducible or tissue-specific promoters might prevent transgene silencing and conserve the active state of transgenes during sexual propagation.

Key words Gene silencing · Chromatin structure · Transgene stability · DNA methylation

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R. van Blokland · P. Meyer (✉)
University of Leeds, Department of Biology,
Leeds LS2 9JT, UK

M. ten Lohuis
James Cook University of Northern Queensland,
Department of Molecular Sciences, Townsville,
Queensland 4811, Australia

Introduction

Inactivation of transgenes can be linked to two different molecular mechanisms: transcriptional silencing, often associated with DNA methylation, and inactivation at the post-transcriptional level (Flavell 1994; Matzke and Matzke 1995; Meyer 1995). In recent years, our group has studied expression of the maize *A1* gene in *Petunia hybrida* to monitor transcriptional inactivation (Meyer 1995a). This work led to the identification of epigenetic variants of a transgenic line, Rl01-17, that contains a single copy of the *A1* transgene (Meyer and Heidmann 1994). In the two epigenetic variants 17-I and 17-IV, the transgene has acquired different states of DNA methylation and transcriptional activity. In line 17-I, the transgene is hypomethylated and expressed, while in line 17-IV the same transgene is trancriptionally silent and hypermethylated.

Two unusual characteristics of the hypermethylated line 17-IV have led to the proposal that transcriptional inactivation in this line involves a local modification of the chromatin. Firstly, the hypermethylation pattern of the transgene in line 17-IV is not restricted to methylation of cytosine residues in symmetrical CG or CNG sequences, but also affects C residues in a non-symmetrical sequence context (Meyer et al. 1994). Symmetrical methylation patterns guarantee faithful propagation of methylation during cell division. After semi-conservative replication, maintenance methylase will restore the methylation pattern in the newly synthesised strand, if it finds a methylated C residue at a symmetrical position on the template strand (Holliday and Pugh 1975). Conservation of non-symmetrical methylation patterns associated with the inactive transgene in line 17-IV must be mediated by features other than sequence symmetry; one possible mechanism involves a modification of chromatin structure that can be maintained during replication and can provide a signal for maintenance methylation.

A second feature of line 17-IV that suggests a modification of the transgene chromatin structure, is its paramutagenic potential. Paramutation is an interaction between a paramutagenic and a paramutable allele, with the paramutagenic allele inducing a heritable change in the paramutable allele (Brink 1960). When crossed with the hypomethylated, paramutable 17-I allele, the hypermethylated, paramutagenic 17-IV allele induces a heritable change in the 17-I allele, which becomes hypermethylated and partly or completely inactive (Meyer et al. 1993). In view of the resemblance between this phenomenon and chromatin-mediated silencing processes in *Drosophila* (Dreesen et al. 1991), it is tempting to speculate that paramutation involves homologous pairing of the two alleles and an exchange of proteins that regulate chromatin repression.

The nature of chromatin modification in plants has not been clarified so far, but it has been proposed that changes in epigenetic states represent intrinsic mechanisms of plant gene regulation (Jorgenson 1994). Early electron microscopic studies seemed to contradict this concept, as they demonstrated that the gross organisation of chromatin texture is species-specific, but unaltered during cell differentiation (Nagl 1979). Subsequent studies, however, found a close correlation between chromatin structure and transcriptional activity, and characteristic differences in chromatin structure among different tissues (Baluska 1990). DNase I accessibility studies showed that DNase I-sensitive regions of plant chromatin showed markedly reduced DNA methylation (Klaas and Amasino 1989), and demonstrated a generally open chromatin structure for transgenes (Weising et al. 1990) and endogenous genes (Conconi and Ryan 1993) that was independent of the transcriptional activity of the genes.

While these data argue against the concept of chromatin-mediated modifications of gene expression, other studies support such an effect. Transition of pea *rbcS* genes from an inactive to an active state was accompanied by changes in chromatin structure, while no changes in methylation could be found (Goerz et al. 1988). Also, no changes in methylation states were observed in the 5′region of the *Adh1* gene of *Zea mays*, which contains two DNase I-sensitive regions, one being present constitutively, while the other only becomes detectable after anaerobic induction of the gene (Nick et al. 1986; Paul et al. 1987). Derepression of the *P*-locus of *Zea mays* coincides with the opening of eight DNase I-sensitive sites which are spread over more than 25 kb of chromosomal DNA. At least for some of these sites, increased sensitivity to DNase I is accompanied by hypomethylation (Lund et al. 1995).

In view of these partly conflicting data, we were interested to compare the accessibility to endonuclease of the two epialleles of the *A1* transgene and their chromosomal integration region. Our data show that the inactive 17-IV transgene is significantly less accessible than the expressed epiallele in line 17-I. A comparison of individual regions of the two epialleles suggests that transcribed regions are specific targets for chromatin condensation. These data argue in favour of participation of the transcriptional process in chromatin condensation, which may be based on the accessibility of transcribed regions to repressive chromatin factors during transcription, the induction of repressed chromatin states by DNA-RNA hybrid molecules, or an active role for the transcription complex in chromatin condensation.

Materials and methods

Isolation of nuclei

Nuclei were isolated essentially as described by Van den Broeck et al. (1994). Five grams of fresh young leaves were ground in 20 ml of Hamilton buffer (10 mM TRIS-HCl pH 7.6, 1.14 M sucrose, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) using pestle and mortar. The slurry was gently stirred for 1 h and than filtered through a nylon mesh, followed by centrifugation for 10 min at $1000 \times g$. The pellet was resuspended in Hamilton buffer containing 0.15% Triton X-100, incubated for 30 min, and centrifuged for 10 min at $1000 \times g$. The pellet was washed once in Hamilton buffer containing 0.15 Triton X-100, resuspended in nuclease digestion buffer (50 mM TRIS-HCl pH 8.0, 0.3 M sucrose, 5 mM $MgCl_2$, 1.5 mM NaCl, 0.1 mM $CaCl_2$, 5 mM β-mercaptoethanol) and centrifuged for 10 min at $1000 \times g$. For the S7 and DNase I digestions the crude nuclear pellet was resuspended in 1.2 ml of nuclease digestion buffer. In the case of *Kpn*I digestion the pellet was resuspended in 2.2 ml of *Kpn*I digestion buffer (10 mM TRIS-HCl pH 7.5, 7 mM $MgCl_2$, 7 mM β-mercaptoethanol. All manipulations were performed at 4° C.

Nuclease digestions

For *Kpn*I digestions 200 U of enzyme (USB) was added, 200-µl aliquots were removed from the reaction at various time points and digestion was stopped with 40 µl of stop buffer (0.25 M EGTA, 0.25 M EDTA). Before addition of DNase I or S7 nuclease 200-µl aliquots were removed for the T_0 and –DNase I time points. To digest DNA in preparations of nuclei, 100 U of S7 nuclease (Boehringer) or 0.5 U of DNase I (Boehringer) was added to the remaining 800 µl. At various time points 200-µl aliquots were removed and added to 40 µl of stop buffer. Aliquots (10 µg) of naked DNA were digested with 1–2 U of S7 nuclease or 0.01–0.02 U of DNase I in 160 µl of nuclease digestion buffer. The 40-µl aliquots were taken at different time points and added to 8 µl of stop buffer.

Extraction of DNA

To extract DNA from the nuclei 120 µl of H_2O and 40 µl of 10% SDS was added, followed by a 15-min incubation at 50° C. Then 132 µl of 5 M potassium acetate was added and mixed well; vials were then incubated for 15 min on ice and centrifuged for 15 min at 15 000 rpm at 4° C. The supernatant was extracted once with phenol/chloroform and DNA was precipitated by addition of 10 µg of yeast tRNA and 1 ml of 96% ethanol, incubation for 1 h at −20° C and centrifugation at 13 000 rpm at 4° C. Pellets were dissolved in 50 µl of TE (10 mM TRIS-HCl pH 8.0, 1 mM EDTA) and 10-µl aliquots were loaded on a gel to check the extent of digestion. Naked DNA was isolated as described by Dellaporta et al. (1983). Digested naked DNA was extracted with phenol/chloroform, and precipitated by addition of 10 µg yeast tRNA, 20 µl of 3 M sodium acetate and 500 µl of 96% ethanol, incubation for 1 h at −20° C and centrifugation at 13 000 rpm at 4° C. The pellet was resuspended in 50 µl TE.

PCR reactions

DNA isolated from nuclei was diluted 1:5 and 2 µl (corresponding to about 25 ng) was taken for each PCR. For PCR analysis of the digested naked DNA 2-µl aliquots was taken directly, without prior dilution. PCRs were performed in a reaction volume of 25 µl containing 10 mM TRIS-HCl pH 8.3, 50 mM KCl, 2 mM MgCl, 0.1% w\v gelatin, 0.2 mM of each nucleotide, 0.4 µM of each primer and 0.5 U of Taq polymerase (Promega). Reactions were overlaid with mineral oil. The PCR program was: 1 min at 94° C, 25 cycles of 45 s at 94° C, 1 min at 55° C, 2 min at 72° C, followed by 10 min at 72° C. The primers used for the PCRs were as follows.
GAPDH5′: 5′-AAGATGCTCCCATGTTTGTTGTTGG-3′;
GAPDH3′: 5′-CCTGCCTTGGCATCAAAGATAGTT-3′;
17L: 5′-CT TTATCTCAAATTGTTAATATC-3′;
AMP1: 5′-ACGTTGTTGC CATTGCTGCAG-3′;
AMP2: 5′-GTATTCAACATTTCCGTGT CG-3′;
35S1: 5-AGAGGACCTAACAGAACTCGCC-3′;
35S2: 5′-GATCCCCGGGTACCCTGTCC-3′;
A1–1: 5′-CATTTGGAGAGGACAGGGTACCCGGGGATCCTC-3′;
A1–2: 5′-GAAGTA CATCCATCCTGTCATC-3′;
NPT1: 5′-CCATGATCATGTCGATTGAACAAGATGG-3′;
NPT2: 5′-CCATTTTCCACCATGATATTCGGCAAGC-3′;
17R: 5′-GAATTCTATCTACCAAA GTACTC-3′.

DNA blotting and hybridisation

PCR fragments were separated in 1.2% agarose gels, blotted on Hybond N filters, crosslinked by UV irradiation. and hybridised to ^{32}P probes labelled by random priming. Filters were washed with 2 × SSC/0.1% SDS. Signals were quantified using a Fuji phosphoimager. Each PCR experiment was performed at least twice to ensure accuracy and reproducibility of the assays.

Results

Sensitivity of integrated DNA to restriction endonucleases

Two transgenic Petunia lines were used to study the relationship between chromatin structure and transcriptional gene silencing. Lines 17-I and 17-IV both derive from a single plant, Rl01-17 (Meyer and Heidmann 1994), which contained a single transgene copy. In 17-I the *A1* gene is transcribed, whereas in 17-IV it is hypermethylated and silent. We first assessed the accessibility of DNA to *Kpn*I. *Kpn*I was chosen because it is not inhibited by cytosine methylation. There are three *Kpn*I sites in the integrated DNA (Fig. 1a). *Kpn*I^a is located between the 35S promoter and the *A1* cDNA, *Kpn*I^b is located 0.9 kb 3′ of *Kpn* I^a in the *A1* cDNA, and *Kpn*I^c lies between the 35S polyadenylation signal and the *nos* promoter of the *nptII* gene. Nuclei were isolated from fresh leaves of the two lines and incubated for increasing lengths of time with *Kpn*I. DNA was then isolated from the nuclei and digested to completion with *Ssp*I. There are two *Ssp*I sites in the integrated DNA; *Ssp*I^a is located in the ampicillin resistance gene (*amp*), and *Ssp*I^b in the *nos* promoter. After digestion with *Ssp*I, DNA was fractionated on agarose gels, blotted onto nylon membranes and hybridized to a labelled 35S probe and, as an internal control, to a rRNA probe. A 35S promoter probe could in principle detect four different fragments (A–D, Fig. 1a), depending on which *Kpn*I site is cut. The results are shown in Fig. 1b. *Kpn*I^a and *Kpn*I^b were readily digested in line 17-I, as shown by the almost immediate appearance of fragments A and B. Cleavage of *Kpn*I^c is less evident, but it must be taken into account that cutting at the *Kpn*I^a or *Kpn*I^b site will result in the disappearance of fragment C. Therefore *Kpn*I^c could be readily accessible but this would not be apparent from hybridization to a 35S probe. In contrast to 17-I, hardly any digestion of transgene *Kpn*I sites was observed for line 17-IV, as shown by the virtual absence of fragments A, B or C (Fig. 1b). It was only after prolonged digestion that fragments B and C were faintly seen. Hybridization of the same filters to an rRNA probe shows that digestion of DNA was in fact more extensive in the line 17-IV, because the rRNA-specific fragment 1 disappears upon lengthy digestion in line 17-IV, while in 17-I this fragment is still detectable after prolonged digestion. These results suggest that the three *Kpn*I sites of the transgene are more accessible in the line 17-I than in the line 17-IV.

Sensitivity of the *A1* gene to DNase I

The data obtained by *Kpn*I digestion only give information about the accessibility of three particular sites in expressing and silent forms of the *A1* transgene. Therefore, an observed difference in accessibility does not necessarily reflect a more condensed chromatin structure, but might also be caused by a nucleosome shift. To obtain more information about the overall accessibility of the transgenes, nuclei were incubated with DNase I, and regions of interest were amplified by PCR. A PCR strategy was chosen because this requires only small amounts of material, and any region of interest, however small, can be investigated in this way.

As a control, we first tested whether digestion by DNase I is influenced by cytosine methylation. The *A1* transgene is more heavily methylated in the repressed mode, and it is possible that unmethylated DNA is more readily digested than methylated DNA. Therefore, any observed differences in sensitivity could reflect differences in the extent of methylation rather than in chromatin structure. DNA was isolated from leaves of both lines and incubated for increasing time periods with DNase I. 35S promoter sequences were then amplified using the primer combination 35S1–35S2 (Fig. 2a), which yields a fragment of 511 bp. As an internal control for the extent of DNase I digestion and for amplification efficiency, primers specific for the *gapdh* coding region were also added to the PCR. PCR products were then separated on agarose gels, blotted onto nylon membranes and hybridised to 35S promoter or *gapdh* probes, respectively, followed by quantification with a phosphoimager. Degradation of DNA regions located between two primers is monitored by the rate at which a particular band disappears. The *gapdh* primers amplify two fragments, of 1.2 kb and 0.5 kb. DNA of line 17-IV was

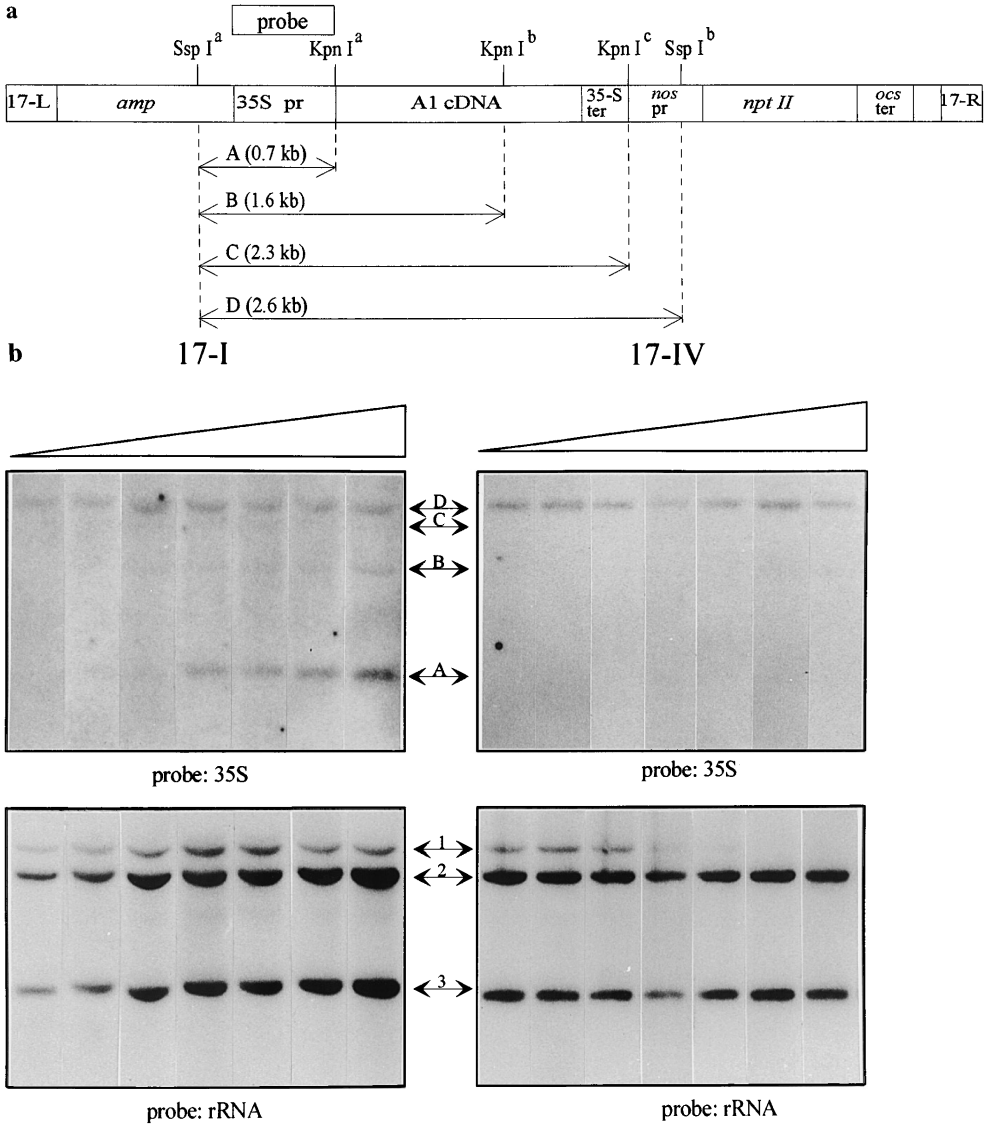

Fig. 1a, b Accessibility of the *A1* gene to *Kpn*I in isolated nuclei. **a** Schematic representation of the integrated transgene DNA. 17-L and 17-R refer to flanking plant DNA. Fragment sizes generated by *Ssp*I/*Kpn*I digestion are drawn below the map. They are marked A–D in order of decreasing mobility in the Southern blots in **b**. **b** Southern analysis of accessibility of *Kpn*I sites. Nuclei isolated from leaves of lines 17-I and 17-IV were incubated for increasing lengths of time with *Kpn*I, after which DNA was isolated and digested to completion with *Ssp*I. Filters were hybridized to a 35S promoter probe (*upper panels*) or an rRNA probe (*lower panels*)

digested slightly more extensively than that of 17-I, and consequently the *gapdh* bands disappeared more rapidly in 17-IV (Fig. 2b, autoradiograph and panel on the right). The 1.2-kb band disappeared more rapidly than the 0.51-kb band, presumably because the chance that DNase I cuts within the region between two primers increases with the distance between the primers. Like the 0.5-kb *gapdh* band, the 35S promoter fragment also disappeared more rapidly in the line 17-IV and the pattern of band intensities reflected that of the 0.5-kb *gapdh* fragment (Fig. 2b). These results suggest that digestion by DNase I is not significantly influenced by the level of cytosine methylation.

Incubation of DNA without DNase I did not result in any obvious degradation of the 0.5-kb *gapdh* fragment or the 0.51-kb 35S promoter fragment (Fig. 1b, –DNase I lanes). A comparison of these lanes with the t_0 lanes gives an estimate of the variation between individual PCR reactions. In the analysis of individual time points for DNase I digestion, we only considered two values to be significantly different, if the difference between two values exceeded the natural variability of PCR reactions, as determined from a comparison of the –DNase I lanes with the t_0 lanes.

The accessibility of the *A1* gene to DNase I in chromatin preparations of the two *Petunia* lines was tested using the primer combinations 35S1/35S2, and *A1*–1/*A1*–2, which amplify the 35S promoter region and part of the *A1* coding sequence, respectively (Fig. 2a). In each case *gapdh* primers were included as an internal control, a representative example of which is shown in Fig. 3 (upper panel). Line IV DNA was slightly more extensively digested than DNA of line I, as the two *gapdh* bands diminish more rapidly in the line IV. A control reaction in which nuclei were incubated without any added DNase I showed that DNA was not detectably degraded by plant DNases during the period of the experiment (20 min). However, overnight incubation of

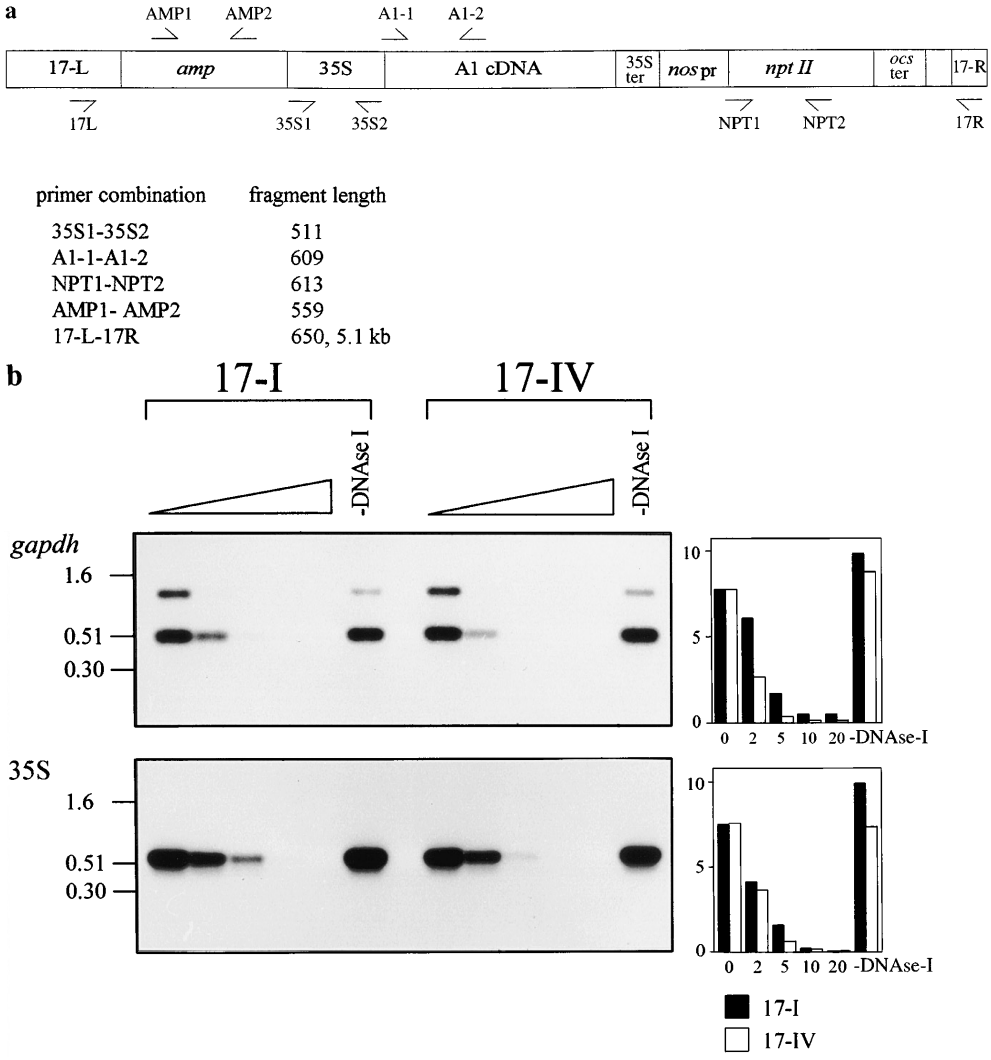

Fig. 2a, b Digestion of naked 17-I and 17-IV DNA with DNase I. **a** Schematic drawing of integrated transgene DNA and locations of primers. Primers are not to scale. The sizes of the fragments generated by the primer combinations are shown underneath the drawing. **b** Sensitivity to DNase I of naked *gapdh* and 35S promoter DNA. DNA isolated from 17-I and 17-IV leaves was incubated for 2, 5, 10 and 20 min with DNase I, or for 20 min without DNase I. *Gapdh* and 35S sequences were then amplified, PCR fragments were run on agarose gels and transferred to nylon membranes. Filters were hybridised to *gapdh* and 35S probes. Signals were quantified with a phosphoimager; the results are shown graphically represented in the panels on the right. Signal strength was expressed in arbitrary units. The T_0 values for the two lines were equalised, and the values for the other time points were proportionally adjusted

nuclei resulted in the appearance of nucleosomal ladders (data not shown), indicating the presence of minor amounts of plant nucleases in the nuclear preparations.

Figure 3 shows that the amount of the 35S promoter fragment decreased rapidly on digestion of nuclear DNA with DNase I in line 17-I. In contrast, in line 17-IV the signal increased twofold after 2 min, and then decreased. For lines I and IV, –DNase I values differed by 14%, while the t_2 timepoint in the line 17-IV exceeded that of line 17-I by a factor of 5 (Fig. 3, right panel). Interestingly, the t_2 value even increased for the line 17-IV, when compared to the t_0 value. We assume that this increase is due to an increase in the efficiency of the PCR reaction for DNA which is moderately digested by DNase I, as this might improve strand separation and primer annealing. As the *gapdh* signal in the line IV decreased immediately upon incubation, and considering the fact that digestion was more extensive in line IV, we conclude that the 35S promoter is markedly more resistant to digestion by DNase I in line IV.

Similar results were obtained using the *A1* coding region primers. In line 17-I, the *A1* signal decreases immediately upon incubation with DNase I, while in the line 17-IV the signal first increases after 2 min, and then decreases (Fig. 3). The t_2 value in line 17-IV is five times that of the line 17-I, while the –DNase I values differed by only 27% (Fig. 3, right panel). Thus, like the 35S promoter, the *A1* cDNA, at least in the region tested, is more resistant to DNase I in line 17-IV.

In the case of the *A1* coding sequence there are two bands, instead of the expected one. The lower one corresponds to single-stranded DNA, because blotting of the gels without prior denaturation resulted in detection of only the lower bands (data not shown). Sometimes amplification of 35S or *gapdh* sequences also yielded single-stranded DNA.

Sensitivity of the *A1* gene to S7 nuclease

We next examined the accessibility of chromatin to S7 nuclease. DNase I readily cuts DNA that is wrapped around the nucleosome. Micrococcal (S7) nuclease preferentially cuts in the linker region between nucleosomes,

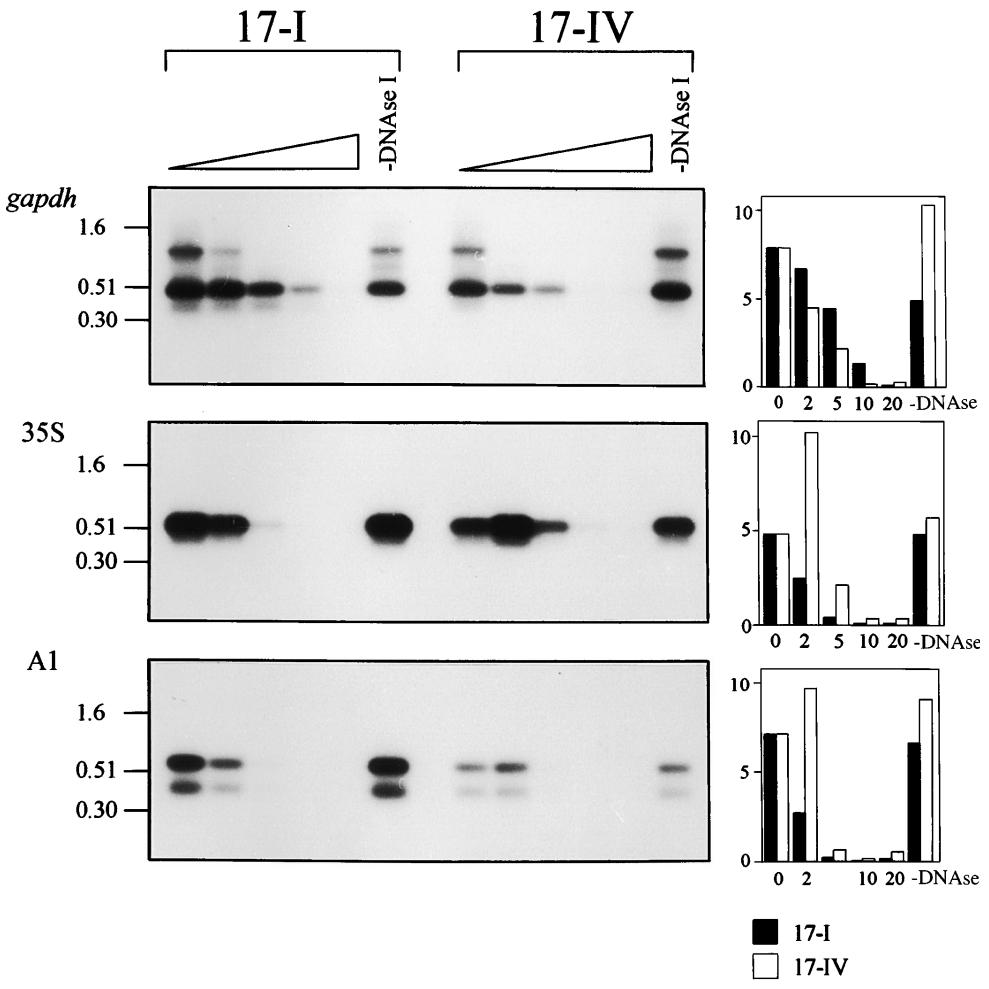

Fig. 3 Accessibility of the *A1* gene to DNase I in chromatin preparations. Nuclei were isolated from leaves of 17-I and 17-IV plants and incubated with DNase I for 2, 5, 10 and 20 min, or without enzyme for 20 min. *Gapdh*, 35S promoter and *A1* coding sequences were amplified by PCR, and hybridised to DNA probes. The results are represented graphically in the panels on the right

but linker DNA flanking the nucleosome is protected against S7 nuclease digestion when occupied by histone H1 (Allan et al. 1980). S7 nuclease accessibility studies should therefore yield not only information about higher-order chromatin structure, but also about the number of nucleosomes on a given region and thus be at least be partially complementary to the DNase I digestion data.

As with the DNAse I digestions, we first tested whether digestion by S7 nuclease was influenced by the level of cytosine methylation. Naked DNA isolated from the lines 17-I and 17-IV was incubated for various time points in the presence of S7 nuclease. The results are shown in Fig. 4. DNA from the line 17-I was slightly more extensively digested than DNA from line 17-IV, as the *gapdh* fragments disappeared more rapidly in the case of line 17-I DNA than from 17-IV DNA. Degradation of 35S promoter sequences closely followed that of the 0.5-kb *gapdh* fragment for both lines; therefore, S7 nuclease digestion is not detectably influenced by the level of DNA methylation.

Isolated nuclei were incubated for increasing periods with S7 nuclease. Digestion by S7 nuclease resulted in a typical nucleosomal ladder (Fig. 5 upper panel), visualized by hybridization to the repetitive RPS element (Ten Lohuis et al. 1995), where the bands represent DNA protected by from 1 to 7 nucleosomes. Nucleosomal ladders were not obtained when S7 nuclease was omitted (−S7), or after digestion of naked DNA with S7 nuclease for 20 min (data not shown). The extent of digestion was comparable between the two lines, which is reflected by comparable rates of degradation of *gapdh* DNA (Fig. 5). In both lines the *gapdh* signal first increased, and then decreased, again presumably because limited digestion enhances PCR efficiency. In contrast to the *gapdh* fragment, the 35S promoter fragment disappeared much more rapidly in line 17-I than in 17-IV. The −S7 values differed by 27%, while the t_5 value of line 17-IV was 2.3-fold higher than that of 17-I, and t_{10} and t_{20} values were fivefold higher for line 17-IV. The t_5 and t_{10} values of the *gapdh* signal were also higher for 17-IV, but the differences are much less pronounced (t_5: 1.2; t_{10}: 1.1). These results suggest that the 35S promoter is more resistant to S7 nuclease in the 17-IV line than in 17-I.

The *A1*–1 and the *A1*–2 primers were used to amplify *A1* coding sequences. The -S7 values for both lines differed by 15%, while the t_5 value was 2.5-fold higher in line 17-IV, and the t_{10} and t_{20} values were 1.6- and

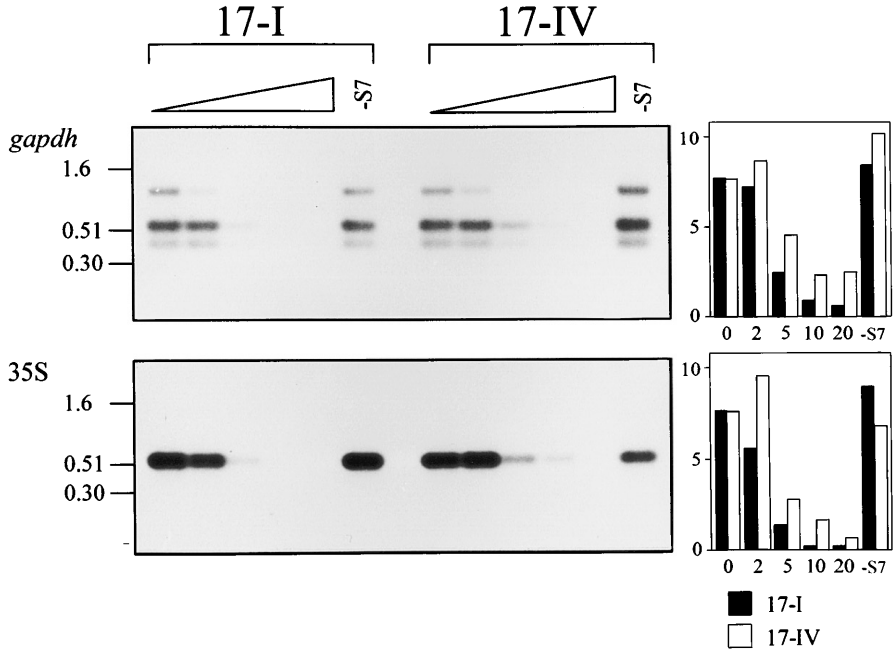

Fig. 4 Digestion of naked 17-I and 17-IV DNA by S7 nuclease. DNA was isolated from leaves of 17-I and 17-IV plants, and incubated for 2, 5, 10 and 20 min with S7 nuclease, or for 20 min without enzyme (−S7). *Gapdh* and 35S promoter sequences were amplified by PCR, and hybridised to 35S and *gapdh* probes. Quantification of the signals is shown on the right

1.7-fold higher, respectively. These results suggest that the *A1* coding sequence was also more resistant to digestion by S7 nuclease in the line 17-IV.

Sensitivity of other transgene regions

The DNase I and S7 nuclease studies suggest that the 35S promoter and the *A1* coding region are more resistant to these endonucleases in line 17-IV. It was unclear whether this difference in accessibility was limited to these regions, or whether it extended to other regions. To answer this question we tested the accessibility to DNase I of two other regions: a part of the ampicillin resistance gene (*amp*), located 5′ of the *A1* gene, and a part of the *nptII* coding sequence, which is located 3′ of the *A1* gene.

amp sequences were amplified with the primers AMP1 and AMP2, which yield a fragment of 559 bp (Fig. 2a). Figure 6 shows that there was essentially no difference between the two lines in accessibility of the *amp* region to DNase I. The *amp* fragment disappeared faster in line 17-IV, but DNase I digestion in this line was more extensive, and therefore the 0.5-kb *gapdh* fragment also disappeared faster in this line, at a comparable rate to the *amp* fragment. In contrast to the *amp* fragment, the *nptII* region was more resistant to DNase I in line 17-IV. The –DNase I values differed by 28%, while the t_2 value was 2.3 times higher in line 17-IV than in line 17-I (Fig. 6).

Sensitivity of the integration region to DNase I

Our data suggest that the CaMV 35S promoter, the *A1* and *npt II* coding regions are markedly more resistant to DNase I in line 17-IV compared to the line 17-I, while accessibility of the *amp* region is comparable between the two lines. Two models can be invoked to explain these results. In both lines the integrated DNA may acquire the chromatin conformation of the neighbouring plant DNA and the enhanced sensitivity of some regions in line 17-I reflects opening of chromatin regions that are transcribed. Alternatively, the integrated DNA may acquires the 'natural' condensation state of the neighbouring plant DNA only in line 17-I, while there is an additional condensation in line 17-IV.

To distinguish between these models we analysed the endonuclease accessibility of the genomic integration region of the *A1* transgene using primers which anneal to the endogenous plant DNA (termed 17L and 17R) next to the integrated DNA (Fig. 2a). Figure 7a shows the DNAse I digestion kinetics for chromatin preparations from lines 17-I and 17-IV amplified with *gapdh*-specific primers, and with primers 17L and 17R. Lines 17-I and 17-IV are both heterozygous for the transgene; therefore, in theory, the primers 17L and 17R should amplify a 650-bp fragment of the original integration region, and a 5.1-kb fragment comprising the integration region and 4.6 kb of the inserted transgene. However, a fragment 5.1 kb long is unlikely to be amplified in a PCR which has an extension time of 2 min. Therefore, the major product of the PCR using primers 17L and 17R is a 650-bp fragment (Fig. 7a). We also observe small amounts of single-stranded products in the chromatin kinetics and an additional ~1-kb product produced by primers 17L and 17R, the identity of which remains unclear. Figure 7a shows that, in comparison to the *gapdh* control fragment, the 17-L + R region disappeared very rapidly in chromatin preparations from both lines 17-I and 17-IV, and that the 17L + R integration region shows similar accessibility kinetics in both lines.

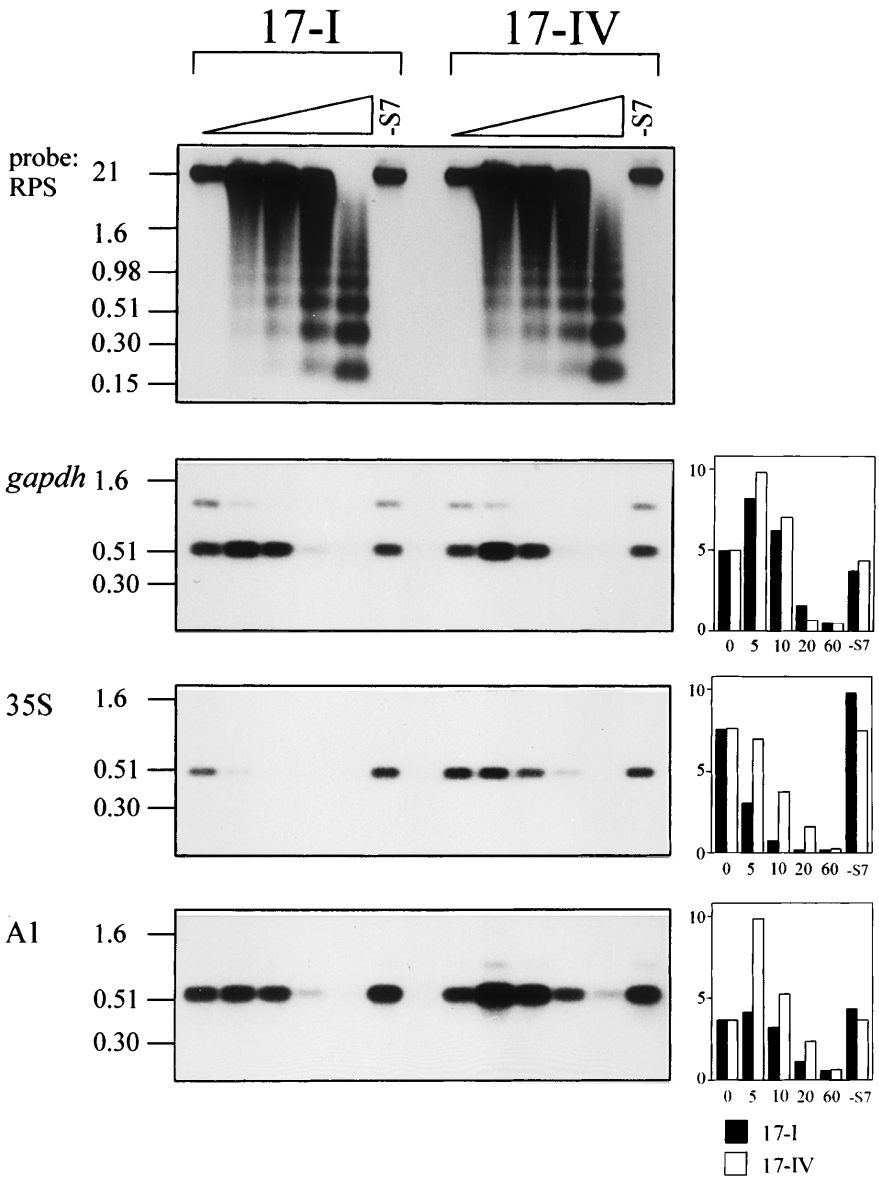

Fig. 5 Accessibility of the *A1* gene to S7 nuclease in chromatin preparations. Isolated nuclei from leaves of lines 17-I and 17-IV were incubated for 5, 10, 20 and 60 min with S7 nuclease or for 60 min without enzyme. Aliquots of S7-digested DNA were fractionated on an agarose gel and blotted onto a nylon membrane. The filter was hybridised to the RPS element (*upper panel*). *gapdh* , 35S and *A1* sequences generated by PCR amplification of S7-digested DNA were hybridised to DNA appropriate probes as indicated. Quantification of the signals is shown in the panels on the right

We can estimate the endonuclease sensitivities of individual regions if we determine the accessibility of individual regions by comparison to the accessibility of the *gapdh* control region. Figure 8a shows a comparison of endonuclease sensitivity for the five regions that were examined in lines 17-I and 17-IV. The data show the T_2/T_0 signal ratio for a given region, divided by the T_2/T_0 signal ratio for the *gapdh* internal control, with T_2 and T_0 being the scanned intensity values for PCR products at 5 min and 0 min of incubation, respectively.

A comparison of the open bars that represent the values for line 17-IV shows increased endonuclease resistance of the 35S, the *A1* and the *nptII* regions in these lines, compared to the *amp* region and the 17 L + R target region. The filled bars that indicate the relative accessibility of the regions in line 17-I, show comparable values for the four transgene-specific regions *amp*, 35S, *A1* and *nptII*. These data suggest a specific increase in chromatin condensation in the three regions 35S, *A1* and *nptII* in line 17 IV, while in line 17-I all transgene regions tested have comparable states of condensation.

A second question we wanted to answer was whether the accessibility of the transgene in line 17-I was comparable to the accessibility of the region 17 L + R, into which it had integrated. While the four transgenic regions *amp*, 35S, *A1* and *nptII* had comparable sensitivity values, the 17 L + R region was degraded about four times faster than the transgene regions. Possible explanations for this observation are that the chromatin state of the 17 L + R region is more accessible to endonucleases than the integrated transgene in line 17-I, or that the DNA of the 17 L + R region DNA is a better substrate for DNase I. To differentiate between these alternatives, we measured the digestion kinetics for

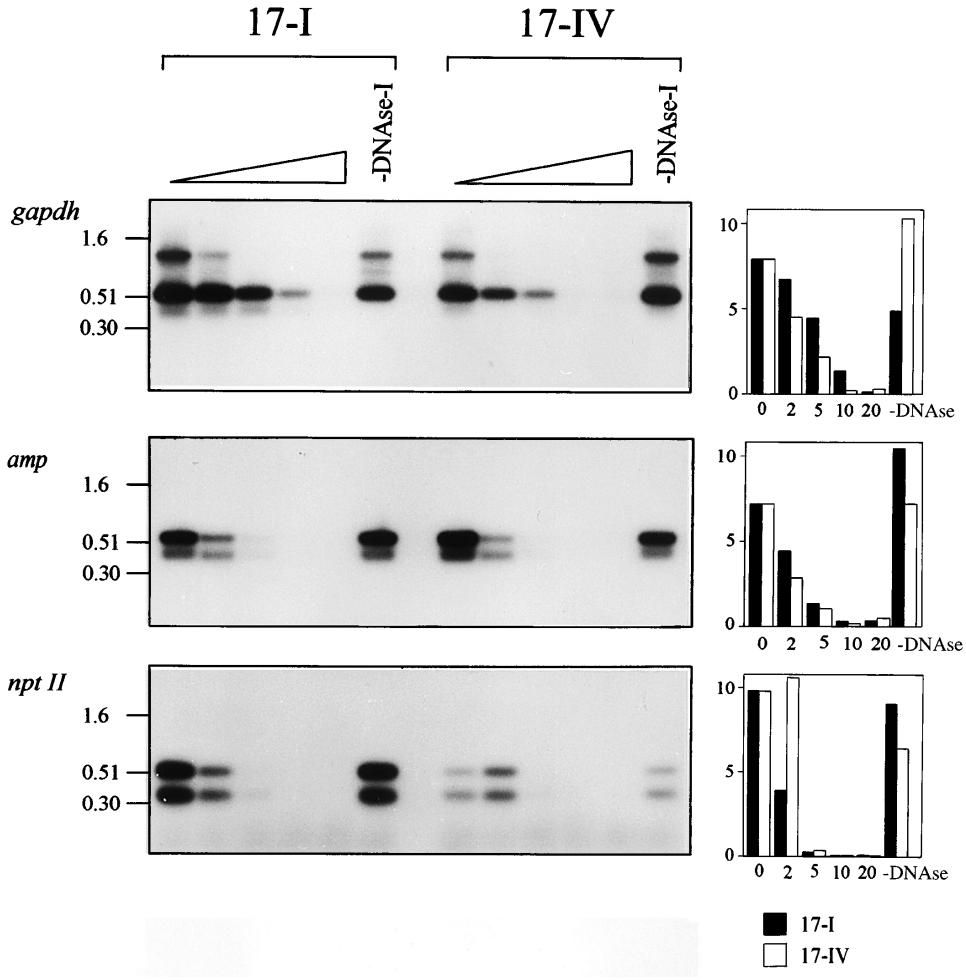

Fig. 6 Accessibility of the *amp* and *nptII* regions to DNase I in chromatin preparations. Nuclei isolated from leaves of lines 17-I and 17-IV were incubated with DNase I for 2, 5, 10 and 20 min with DNase I or for 20 min without enzyme. *Gapdh*, *amp* and *nptII* sequences were amplified by PCR, and fragments were hybridised to the indicated DNA probes. Quantification of the signals is shown on the right

naked DNA amplified by the 17L and 17R primers (Fig. 7b). This experiment shows that the 17 L + R fragment also decreases more rapidly than the *gapdh* control fragment when naked DNA is used as a substrate for DNAse I digestion. The sensitivity value of the 17 L + R region in naked DNA is about three times higher compared to the 35S region (Fig. 8b), suggesting that the observed fourfold increase in DNAse I sensitivity of this region is largely due to sequence-specific differences in sensitivity to DNase I. Our data therefore indicate that the transgene is, if at all, only slightly less accessible to DNase I than the integration site, and that the trangene DNA in line 17-I has acquired a state of chromatin condensation similar to that of the integration site in line 17-I. In summary, our data argue for an additional condensation of chromatin within the transcribed parts of the transgene in the transcriptionally silenced and hypermethylated line 17-IV.

Discussion

Modification of gene expression by alteration of chromatin packaging has been described in organisms as diverse as *Saccharomyces cerevisiae* (Rivier and Pillus 1994; Palladino and Gasser 1994; Roth 1995), *Caenorhabditis elegans* (Ryner and Swain 1995) and *Drosophila melanogaster* (Henikoff 1990; Paro 1993). In filamentous fungi (Selker 1990), mammals (Bird 1992; Bestor and Tycko 1996) and plants (Matzke and Matzke 1993; Meyer and Saedler 1996), alterations in epigenetic states are frequently accompanied by changes in DNA methylation, which might act as an auxiliary mechanism to preserve and reinforce the heritability of epigenetic changes mediated by chromatin modification. Endonucleases have been widely employed to detect alterations in chromosomal states, based on the accessibility of chromatin to restriction enzymes, DNase I or micrococcal nuclease (Gottesfeld et al. 1975; Bloom and Anderson 1978). The increased endonuclease resistance of the hypermethylated, inactive state of the transgene in line 17-IV can be explained by two alternative models. As active genes are often more prone to DNase I digestion than inactive genes (Weintraub 1985), it was conceivable that the observed differences reflect the opening of chromatin in the active 17-I allele during transgene expression, while the inactive 17-IV allele retains the chromosomal state characteristic for untranscribed

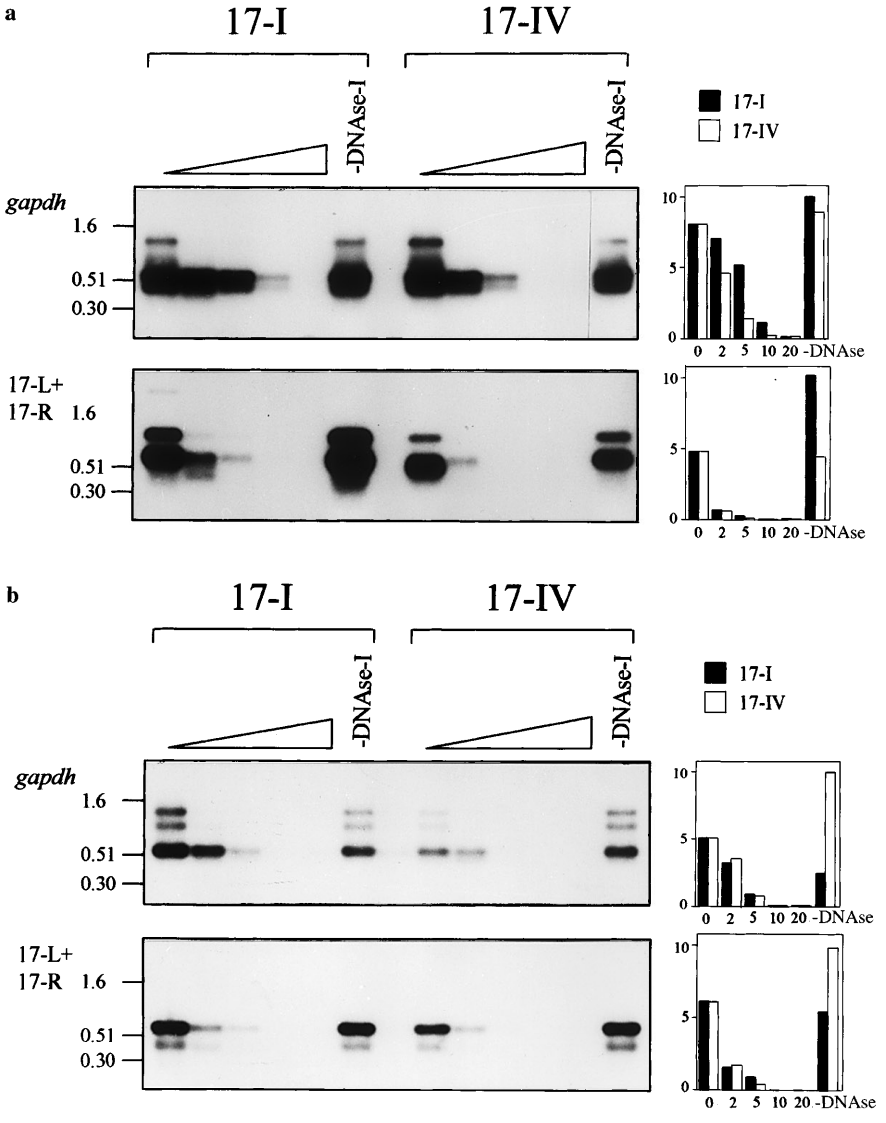

Fig. 7a, b Accessibility of the genomic integration region to DNase I. **a** DNase I digestion of the integration region (17L-17R) in chromatin preparations. Nuclei isolated from leaves of lines 17-I and 17-IV were incubated with DNase I for 2, 5, 10 and 20 min or for 20 min without enzyme. *Gapdh*-specific primers were used in a control reaction, primers which anneal to DNA flanking the integrated DNA were used to amplify the integration region. Filters containing PCR fragments were hybridised to *gapdh*, and to a petunia DNA fragment flanking the integrated plasmid DNA 5′ of the *amp* region (17-L region). The results are shown graphically in the diagrams on the right. **b** DNase I digestion of the integration region in naked DNA preparations. DNA was isolated from leaves of lines17-I and 17-IV, and incubated with DNase I for 2, 5, 10 and 20 min or without enzyme for 20 min. Filters containing PCR fragments were hybridised to *gapdh* and 17-L DNA probes

genomic regions. As an alternative to this "transcription model", a "condensation model" would predict that the chromatin structure remains unaltered in the inactive 17-I allele, but becomes specifically condensed in the silenced 17-IV allele. Both models are in agreement with our observation that significant differences in chromatin accessibility are detectable in the transcribed regions of the transgene, but not in the bacterial *amp* region.

A comparison of the transgene chromatin structure with the chromosomal integration site, however, argues in favour of the "condensation model", since the non-transcribed parts of both epialleles show a endonuclease sensitivity comparable to that of the integration site. These data are in agreement with the "permissive domain" hypothesis, which suggests that the chromatin surrounding the integration region determines the chromatin structure of integrated foreign genes (Weising et al. 1990). A similar dominance of the integration region has been observed with respect to the methylation states that were imposed on integrated transgenes (Pröls and Meyer 1992). The condensation model also matches the history of the two epigenetic lines. The inactive epiallele in line 17-IV was isolated as a derivative of the active epiallele in line 17-I (Meyer et al. 1993). Transcriptional inactivation of the 17-IV allele is accompanied by hypermethylation, which is limited to the transgenic region, while the chromosomal integration region retains its characteristic hypomethylation pattern (Meyer and Heidmann 1994). Upon integration into the genome, the *A1* transgene apparently acquired the open, hypomethylated chromatin structure of its integration site, which was a prerequisite for stable expression of the transgene. Both the *A1* gene and the *nptII* gene were constitutively expressed, but could become inactivated in some somatic cells. Inactivation was more frequently observed in older tissue, and was highly favoured by environmental stress (Meyer et al. 1992; Ten Lohuis et al. 1995). These data and the results of this study suggest that transcriptional inactivation is a stochastic, transgene-specific process that is dependent on, or at

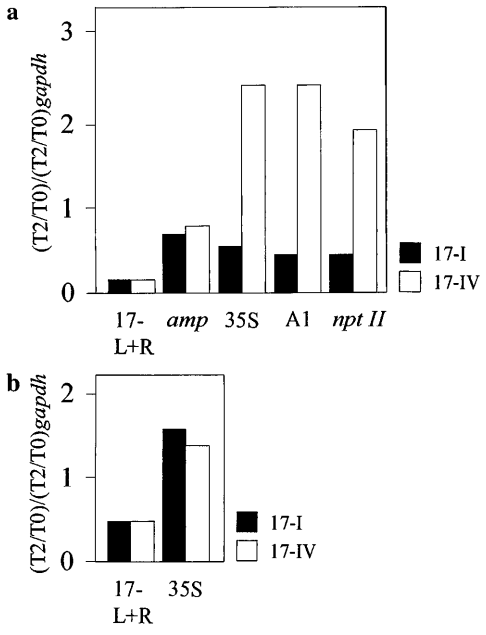

Fig. 8a, b Comparison of accessibility to endonucleases for individual regions of the transgene and its integration region. **a** Accessibility in chromatin preparations. To standardise the DNase I accessibility values for each region, the T_2/T_0 ratio for a given region was calculated and divided by the corresponding ratio obtained for the *gapdh* control. **b** A comparison of the sensitivity of the 17 L + R region and the 35S region to DNase I digestion of naked DNA. T_2/T_0 ratios were calculated for both regions and divided by *gapdh* control values determined for naked DNA

least strongly enhanced by, transcriptional activity and environmental stress. Inactivation results in a significant local change in chromatin structure that most probably prevents access of the transcription complex to the region.

Changes in accessibility of chromatin could either reflect a sequestering of the silenced region into a particular nuclear compartment or its packaging into a higher-order DNA-protein complex. Sequestering of inactivated regions into nuclear compartments has been suggested for position-effect variegation and Polycomb-dependent gene inactivation in *Drosophila*, based on the assumption that the nuclear fibre remains unaltered, but its relocation to a nuclear substructure prevents access of particular transcription factors (Schlossherr et al. 1994). However, it is difficult to imagine how a relocalised genetic region could still interact with its paramutable allele. It is more plausible to assume that the increased resistance of the transgene in 17-IV reflects the association of repressive chromatin components that can be exchanged between the paramutagenic and the paramutable allele during allelic pairing.

Modification of chromosomal states can involve displacement of nucleosomes or alteration of nucleosome mobility (Ura et al. 1995), modification of core histone acetylation states (O'Neill and Turner 1995), the substitution of core histones by histone variants (Wolffe and Pruss 1996), competitive binding of linker regions by histone H5 variants or HMG proteins (Zhao et al. 1993), or the formation of heterochromatin complexes (Locke et al. 1988; Hecht et al. 1995). Our data do not allow us to identify the molecular components involved in the induction of repressed chromatin, but the preferential condensation of transcriptional regions implies a direct role for transcripts or the transcription complex in the induction of chromatin condensation, or an indirect role in making the transgene accessible to cellular factors.

High RNA levels have been shown to induce methylation in homologous DNA sequences (Wassenegger et al. 1994), which might be due to the formation of DNA/RNA hybrids. Such a mechanism would be highly detrimental if it affected endogenous genes, but it is conceivable that the transgene provides an exceptional target for DNA/RNA formation, if high transcript levels accumulate locally due to inefficient export out of the nucleus (Meyer 1995b). However, chromatin condensation clearly extends into the promoter region, which indicates that, if induced by DNA/RNA formation, changes in chromatin structure are not precisely restricted to coding regions.

An alternative explanation for the induction of chromatin condensation during transcription is that transcription renders the promoter and the transcribed sequences accessible to nuclear factors that displace, modify or interact with core histones or linker histones. The efficiency of condensation would depend on the local concentration of repressive and activating chromosomal factors competing for the transgenic region, and on the stability of the repressed complex once it has been established. Certain factors involved in the remodelling of chromatin structure are most probably part of the transcription complex. A complete picture of the RNA polymerase II complex in plants is still lacking, but analysis of the yeast transcriptional machinery shows that the basal transcription complex is associated with enzyme complexes involved in DNA repair and in remodelling of chromatin structure, forming a "transcriptosome" complex containing approximately 50 components with a combined molecular mass of more than 3 MDa (Halle and Meisterernst 1996).

The hypermethylation patterns that coincide with the local condensation of chromatin may play a role in the conservation of the repressed mode during replication. It has been proposed that DNA methylation can induce condensation of chromatin into an inactive form (Adams 1994). Hypermethylated DNA is rendered inactive only after the formation of chromatin (Buschhausen et al. 1987), suggesting that methylated DNA induces repressed chromatin. This may be achieved by the shift in replication timing frequently reported for methylated DNA or by the methylation-specific binding of nuclear factors. In line 17, a stretch of only 4 kb of the transgene is embedded in a hypomethylated genomic integration region (Meyer and Heidmann 1994); it does not seem probable that significant differences in replication timing can be imposed on such a small region. A more likely explanation is therefore the targeting of

methylation-specific binding factors, which have been identified in animals (Meehan et al. 1989; Pawlak et al. 1991) and plants (Zhang et al. 1990). Indirect evidence for a role of methylation-specific factors in chromatin condensation comes from the analysis of two mammalian factors, MDBP-2 and MeCP-2. MDBP-2 shows sequence homology with histone H1 (Jost and Hofsteenge 1992), which is predominantly found in condensed chromatin (Kamakaka and Thomas 1990), and MeCP-2 is localised in heterochromatin (Lewis et al. 1992).

As mentioned before, it is inconceivable that a transcription-dependent inactivation process such as that discussed for the transgene could affect endogenous genes with the same efficiency. Foreign DNA may therefore be a preferential target for chromatin condensation and DNA methylation. Transgenes may be recognisable because their DNA sequence deviates from the isochore composition of the host genome or from the chromosomal region into which they have integrated randomly. This assumption is supported by the observation that an *A1* transgene that derived from a dicotyledonous species was much less sensitive to inactivation in the dicotyledonous host *P. hybrida* than an *A1* transgene from the monocotyledonous maize genome (Elomaa et al. 1995). Alternatively, endogenous genes may escape inactivation because they are located in defined chromosomal regions, which protect them from repressive chromatin factors during transcription.

A more detailed understanding of plant chromatin structure and the factors that regulate chromatin condensation is required before we can hope to control stable expression of transgenes. Our data, however, suggest that the trancriptional activity plays an important role in the induction of chromatin condensation. This implies that, in order to reduce the probability of transcriptional inactivation, transcription of transgenes should be limited to those tissues where transgene activity is required. Such a strategy will not always be applicable, but the control of transgenes by tissue-specific or inducible promoters should be preferred over constitutive expression wherever possible. Special care should be taken to ensure that the transgene is inactive in all cells that contribute to the germline, to prevent the establishment of repressed cell lineages. The necessity for early selection of transgenic tissue after transformation is a major obstacle to this strategy. To circumvent this problem, it may be advisable to remove constitutively expressed marker genes after identification of the transformed tissue, using a transposon approach (Yoder and Goldsbrough 1994). Although this strategy may not be a final solution to the problem of transgene silencing, it should provide an important step toward stabilisation of transgene expression in transgenic plants.

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