Large-scale chromatin decondensation induced in a developmentally activated transgene locus

Eva Wegel[1], Ruben H. Vallejos[2], Paul Christou[3], Eva Stöger[4] and Peter Shaw[1,*]

[1]Department of Cell and Developmental Biology, John Innes Centre, Norwich Research Park, Colney Lane, Norwich, NR4 7UH, UK
[2]Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), CONICET-UNR-F-LILLO, Suipacha 531, S202LRK Rosario, Argentina
[3]Fraunhofer Institute for Molecular Biology and Applied Ecology, IME, Grafschaft, Auf dem Aberg 1, 57392 Schmallenberg, Germany
[4]Institute of Molecular Biotechnology, BiologieVII, RWTH Aachen, Worringerweg 1, 52074 Aachen, Germany
*Author for correspondence (e-mail: peter.shaw@bbsrc.ac.uk)

Accepted 9 December 2004
Journal of Cell Science 118, 1021-1031 Published by The Company of Biologists 2005
doi:10.1242/jcs.01685

Summary

The high molecular weight (HMW) glutenin-encoding genes in wheat are developmentally activated in the endosperm at about 8 days after anthesis. We have investigated the physical changes that occur in these genes in two transgenic lines containing about 20 and 50 copies each of the HMW glutenin genes together with their promoters. Using fluorescence in-situ hybridisation (FISH) and confocal imaging, we demonstrate that, in non-expressing tissue, each transgene locus consists of one or two highly condensed sites, which decondense into many foci upon activation of transcription in endosperm nuclei. Initiation of transcription can precede decondensation but not vice versa. We show that, in one of the lines, cytoplasmic transcript levels are high after onset of transcription but disappear by 14 days after anthesis, whereas small interfering RNAs, which indicate post-transcriptional gene silencing (PTGS), are detected at this stage. However, the transcript levels remain high at the transcription sites, most of the transgene copies are transcriptionally active and transcriptional activity in the nucleus ceases only with cell death at the end of endosperm development.

Key words: Chromatin Decondensation, PTGS, HMW glutenin, Nuclear transcript

Introduction

In recent years, several studies using mammalian cell cultures have shown that chromatin decondenses when it becomes transcriptionally active. Using the *lac* operator-repressor interaction, Tumbar et al. (Tumbar et al., 1999) showed that targeting of the acidic activation domain of the herpes simplex virus transcriptional activator VP16 to specific chromosomal sites resulted in the unfolding of the sites and their transcriptional activation. Tsukamoto et al. (Tsukamoto et al., 2000) made use of the same *lac* operator-repressor sequence in a different environment and hormonally induced chromatin decondensation at the transgene locus. Müller et al. (Müller et al., 2001) reported different degrees of decondensation depending on the level of transcription of their reporter genes after the activation of the mouse mammary tumour virus promoter by its cognate transcription factor. In plants, two studies of *Arabidopsis* have recently shown decondensation of a previously hypermethylated and silenced transgene locus in the background of a *decrease in DNA methylation1* (*ddm1*) mutant and looping out of a proportion of the ribosomal DNA (rDNA) loci correlated with an overall increase in rDNA transcript in the leaves of developing seedlings (Mathieu et al., 2003; Probst et al., 2003). In mammalian cell cultures, highly transcribed chromosomal regions have been found to extend away from chromosomal territories when they become transcriptionally active, which seems to be the result of chromatin decondensation over one to several megabases (Volpi et al., 2000; Williams et al., 2002), and looping out of genes from the chromosome territory has also recently been reported for a gene cluster of 90 kb (Chambeyron and Bickmore, 2004). Transcript at the site of transcription has been localized in various animal and human cell lines (Xing et al., 1993; Dirks et al., 1995; Custódio et al., 1999; Tsukamoto et al., 2000; Müller et al., 2001). Different patterns of the main RNA signal inside the nucleus were observed, ranging from dots to elongated dots and tracks. In plants, only ribosomal RNAs have previously been located with their gene loci (Highett et al., 1993; González-Melendi et al., 2001).

We have used the activation of storage protein expression in a transgenic bread-wheat line to study changes in the physical organization of a transgene locus upon transcriptional activation. One group of these proteins are the high molecular weight (HMW) subunits of the glutenins, which account for up to 12% of the total grain protein content and are only expressed in the endosperm of developing wheat grain (for a review, see Shewry et al., 2003). HMW-glutenin-encoding genes are located on the long arms of chromosomes 1A, 1B and 1D. Each locus contains two genes. One of the six genes is always silenced, and cultivated wheat expresses three to five genes, depending on the variety (Shewry et al., 2003). The HMW subunits are activated about 8 days post anthesis (dpa) and the transcripts disappear between 36 dpa and 38 dpa (Altenbach et al., 2002). Previously, wheat genomic clones encoding the HMW glutenin subunits 1Ax1 and 1Dx5 (GenBank accession numbers: *glu-1Ax1*, X61009; *glu-1D-1b*, X12928; the genes will be referred to hereafter as 1Ax1 and 1Dx5), together with their promoter regions, had been introduced into the commercial wheat cultivar Federal, which expresses the

endogenous subunits 1Ax2*, 1Bx7, 1By9, 1Dx5 and 1Dy10. Two of the resulting transgenic lines, containing an estimated 20 copies (line E) and 50 copies (line F) of the transgenes, were substantially silenced, resulting in a 60% (line F) to 90% (line E) reduction in total HMW glutenin protein compared with the wild type (Alvarez et al., 2000).

Two types of silencing are known: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). TGS usually occurs via methylation of promoter sequences, which causes transcription to cease. PTGS is characterized by ongoing transcription, subsequent transcript degradation and the appearance of small interfering RNAs (siRNAs) of 21-25 bp (Hamilton and Baulcombe, 1999; Zamore et al., 2000), which are produced by a double-stranded-RNA-specific endonuclease (Dicer) that was first detected in *Drosophila* (Bernstein et al., 2001). Dicer-like enzyme activity has also been found in wheat-germ extract (Tang et al., 2003). siRNAs can guide heterochromatin formation and therefore TGS through RNA-directed methylation of homologous DNA and through methylation of histone H3 at lysine 9 (Matzke et al., 2001; Finnegan and Matzke, 2003; Schramke and Allshire, 2003).

Here, we show that the transgene loci in both line E and line F are visible as condensed foci in tissues in which they are not transcriptionally active and decondense in transcriptionally active tissue. A detailed analysis of line E reveals that decondensation starts upon transcriptional activation in the endosperm at 8 dpa, although the onset of transcription can precede any visible decondensation. The great majority of the transgene copies are transcriptionally active, and transcriptional activity in the nucleus ceases only with cell death at the end of endosperm development. We also show that the transcript is absent from the cytoplasm by 14 dpa, whereas siRNAs can be detected in RNA extracts from seeds of the same age, indicating that the silencing mechanism is PTGS. Transcripts at the loci remain high throughout the silenced period.

Materials and Methods

Plant material

Triticum aestivum L., cv Pro INTA Federal and the transgenic lines E and F, which had been generated from Federal by particle bombardment (Alvarez et al., 2000), were used for the experiments. The following plasmids had been used in equimolar concentrations for the transformation: pHMW1Dx5 (Halford et al., 1989), containing the complete coding sequence of the 1Dx5 gene; pHMW1Ax1 (Halford et al., 1992), containing the complete coding sequence of the 1Ax1 gene; and pAHC25 (Christensen and Quail, 1996), containing the *BAR* resistance gene and the *GUS* marker gene. Lines E and F contain ~20 and ~50 copies, respectively, of pHMW1Ax1 and pHMW1Dx5. All plants were homozygous.

Preparation of wheat roots for FISH analysis in metaphase spreads and root sections

Seeds were germinated in a Petri dish lined with wet filter paper for 3 days and root tips approximately 5 mm long were excised. Metaphase spreads and tissue sections for the analysis of interphase nuclei were prepared according to Abranches et al. (Abranches et al., 2000), including pretreatments for in situ hybridization. The protocol included RNase A treatment to remove transcript.

Preparation of wheat seeds for sectioning

Wheat plants were grown in a controlled environment room (15°C, 16 hours light / 8 hours dark photoperiod, 70% humidity). For preparation of tissue sections, immature seeds were harvested at different time points and fixed for 6 hours in 4% (w/v) formaldehyde in PEM (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$, pH 6.9). For wax sectioning, seeds were fixed as above and embedded in wax in a Sakura Tissue-Tek Vacuum Infiltration Processor (Bayer Diagnostics, Newbury, UK) using the manufacturer's recommended protocol. For unembedded vibratome sectioning, fixed seeds were dehydrated through an ethanol:water series (10%, 20%, 40%, 60%, 80% and absolute ethanol for 4-12 hours per step) and stored in absolute ethanol at 4°C before sectioning.

Seed sections

30 μm wax sections from embedded seeds were cut on a Leica RM2055 microtome (Leica, Nussloch, Germany). Sections were allowed to dry on poly-L-lysine-coated slides (BDH). 100 μm sections from unembedded seeds were prepared under absolute ethanol using a Vibratome Series 1000plus (TAAB Laboratories Equipment, Aldermaston, UK) and rehydrated in water. Wax sections from embedded seeds for RNA in situ hybridization were pretreated according to Jackson (Jackson, 1991) with the following modifications: histoclear treatments were for 30 minutes each and no NaCl was included in the ethanol series. Also, before the pronase treatment, the sections were treated with 2% (w/v) Onozuka R10 cellulase (Yakult Pharmaceutical, Tokyo, Japan) in TBS (10 mM Tris, 140 mM NaCl, pH 7.4) for 30 minutes at room temperature. The final fixation step was omitted. After histoclear treatment and rehydration, wax sections for DNA FISH were treated by incubation with cellulase for 1 hour and washed in TBS for 10 minutes. The slides were then incubated in 0.1 mg ml^{-1} RNase A (Sigma) in 2× SSC (300 mM NaCl, 30 mM tri-sodium citrate) for 1 hour at 37°C before another wash in TBS. After dehydration in an ethanol:water series (70% and absolute ethanol), the slides were air dried. Sections from unembedded seeds were treated with cellulase for 1 hour like the wax sections, followed by RNase treatment when transcript detection was not required.

Probes and in situ hybridization

Probes were made from the same plasmids that had been used for the transformation (Alvarez et al., 2000): pHMW1Dx5 (Halford et al., 1989), containing an 8.7-kb *Eco*RI genomic fragment including the complete coding sequence of the 1Dx5 gene (Anderson et al., 1989), flanked by approximately 3.8 kb and 2.2 kb of 5′ and 3′ sequences, respectively; pHMW1Ax1 (Halford et al., 1992) containing a 7.0-kb *Eco*RI fragment including the complete coding sequence of the 1Ax1 gene, flanked by approximately 2.2 kb and 2.1 kb of 5′ and 3′ sequences, respectively. For chromosome spreads, probes were made by nick translation of the *Eco*RI fragments of both genes labelled with digoxigenin-11-dUTP (Roche) and of PUC19 labelled with biotin-16-dUTP (Roche). For labelling of the locus on its own in sections, probes were prepared by nick translation of pHMW1Dx5 and pHMW1Ax1 labelled with digoxigenin-11-dUTP or biotin-16-dUTP. For the RNA time-course experiment, digoxigenin-11-UTP labelled sense and antisense probes were prepared by in vitro transcription of the 2.5-kb coding region of the 1Ax1 gene amplified using the polymerase chain reaction (PCR; forward primer, 5′-TACGATTA-ACCCTCACTAAAGGAGATGACTAAGCGGTTGGTTC-3′; reverse primer, 5′-TACGAATACGACTCACTATAGGAGCTGCAGAGAGT-TCTATCAC-3′; both with overhanging primers for T3 polymerase and T7 polymerase, respectively). Biotin-16-UTP-labelled probes using the same primers were prepared for transcript detection in double-labelling experiments. In the same experiments, the loci were detected by digoxigenin-11-UTP-labelled sense and antisense probes prepared by in-vitro transcription of the PCR-amplified

vector and gene flanking sequences of the 1Ax1 and 1Dx5 genes, with the sizes described above. The following primer pairs were used for PCR:
5′-TACGAATACGACTCACTATAGCGTTTTACAACGTCGTGACTGGG-3′ and 5′-TACGATTAACCCTCACTAAAGCCTGTGTGAAATTGTTATCCGCT-3′ (vector PUC19);
5′-TACGAATACGACTCACTATAGGTGGACTGTCGGTGAATTGATC-3′ and 5′-TACGATTAACCCTCACTAAAGCCCAGTCACGACGTTGTAAAACG-3′ (left genomic flank of 1Ax1);
5′-TACGAATACGACTCACTATAGCTTAGGCATGCATGCACCTTAG-3′ and 5′-TACGATTAACCCTCACTAAAGAGCGGATAACAATTTCACACAGG-3′ (right genomic flank of 1Ax1);
5′-TACGAATACGACTCACTATAGCGGTGTTGTGGGTGATGATAAG-3′ and 5′-TACGATTAACCCTCACTAAAGCCCAGTCACGACGTTGTAAAACG-3′ (left genomic flank of 1Dx5);
5′-TACGAATACGACTCACTATAGGCTTAGGCATGCATGCCTTTAG-3′ and 5′-TACGATTAACCCTCACTAAAGAGCGGATAACAATTTCACACAGG-3′ (right genomic flank of 1Dx5).
Fig. 1 illustrates the positions on the plasmids of the probes used for single and double labelling of interphase nuclei. Added on to the forward and reverse primers are the primers for T3 polymerase and T7 polymerase, respectively.

0.5-1.0 μg template was used for the in-vitro transcription reactions and all in-vitro transcription probes were size reduced to an average of 75 bases using alkaline hydrolysis in a 100 mM carbonate buffer (pH 10.2) at 60°C. Approximately 2-4% of each in-vitro transcription reaction was used alone (1Ax1 coding region) or in a probe mixture (flanking sequences and vector) in a total volume of 40 μl per slide in a modified hybridization buffer according to Ingham et al. (Ingham et al., 1985). All probes were heat denatured and cooled on ice immediately before hybridization. For the detection of the HMW-glutenin-encoding genes on metaphase spreads, a mixture of the *Eco*RI fragments of the 1Ax1 and 1Dx5 genes was used with the combined concentration of 100 ng, and 100 ng PUC19 probe per slide for the identification of the transgene loci. A mixture of pHMW-1Ax1 and pHMW-1Dx5 (200 ng probe per slide) was used for all experiments that did not include transcript detection. FISH on chromosome spreads and sections without transcript detection was performed according to Abranches et al. (Abranches et al., 2000). The hybridization for the RNA expression time-course was carried out at 50°C overnight. Slides were washed in 2× SSC, 50% formamide at 50°C followed by 1× SSC, 50% formamide at 50°C. For the simultaneous detection of transgene loci and transcript, unembedded sections of wheat seeds were first treated with the avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions using a biotin blocking step, followed by avidin, followed by biotin to block biotin binding sites in the tissue.

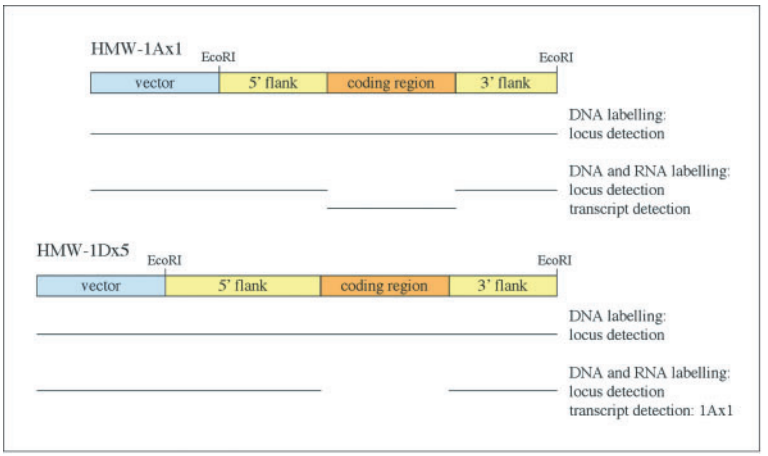

Fig. 1. Probes used for locus and transcript detection in interphase nuclei.

After a final wash in PBS, samples were immediately hybridized with the antisense or sense in-vitro-transcription probe for the coding region of the 1Ax1 gene at 37°C for 7 hours. Post-hybridization washes were carried out in 2× SSC, 50% formamide at 50°C followed by 1× SSC, 50% formamide at 50°C. After a short wash in 2× SSC the slides were fixed for 10 minutes in 4% formaldehyde in PEM followed by three short washes in 2× SSC. They were then immediately hybridized with the probe mixture for the locus detection in a modified Thermocycler (Omnislide; Hybaid, Ashford, UK) with a chromatin-denaturing step at 75°C for 8 minutes and hybridization at 37°C overnight. Post-hybridization washes were carried out using 2× SSC, 50% formamide at 50°C followed by 1× SSC, 50% formamide at 50°C.

Immunodetection of DNA and RNA probes

Biotin-labelled probe for chromosome spreads was detected with Extravidin-Cy3 (Sigma). Biotin-labelled probe for the transcript in seed sections was detected with a streptavidin Alexa Fluor® 633 conjugate (Molecular Probes, Leiden, The Netherlands) to avoid overlap with the emission spectrum of Alexa Fluor 488 used for the locus detection. For all FISH experiments, probes labelled with digoxigenin were detected with a mouse anti-digoxin antibody (Sigma), which also recognises digoxigenin, followed by a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (Molecular Probes). Antibodies were diluted in 4× SSC, 0.2% Tween-20 according to the manufacturer's instructions. Antibody incubation was performed in a humid chamber for 1 hour at 37°C followed by three 5-minute washes in 4× SSC, 0.2% Tween-20 at room temperature. Metaphase spreads were counterstained with 6 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 10 minutes. Sections were counterstained in 1 μg ml^{-1} DAPI for 4 minutes (root tips) or 10 minutes (seeds). For the RNA time-course experiments, digoxigenin-labelled probes were detected as described in the instructions for the Roche digoxigenin/nucleic-acid detection kit with modifications according to Coen et al. (Coen et al., 1990). Alternatively, they were detected with Alexa Fluor 488 and the slides counterstained with DAPI as described above. All slides were mounted in Vectashield (Vector Laboratories).

Image acquisition, analysis and measurements

Chromosome spreads and the RNA time-course experiments were analysed with a Nikon E600 microscope. Photographs were either taken on Fujicolor 400 print film and digitized with a Microtek ScanMaker 5 or they were taken on a Nikon Coolpix 990 digital camera. Vibratome tissue sections in FISH experiments were analysed on a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany) equipped with two argon lasers (351 nm, 363 nm and 457 nm, 488 nm, 514 nm) and two helium/neon lasers (543 nm and 633 nm). The confocal microscopy data were then transferred to ImageJ (W. Rasband, http://rsb.info.nih.gov/ij/). Measurements of nuclear and locus volumes were done as follows. For each section, the extent of fluorescence was determined either by thresholding and using a plug-in for ImageJ called 'voxel counter' (loci) or by tracing the edge of the fluorescent area by hand and measuring the area using one of the program tools (nuclei). Volumes were determined by multiplying the areas by the spacing of the optical sections or by multiplying the number of voxels (volume elements) counted by the voxel volume. We also made linear length measurements of the paths connecting fluorescent foci from the same locus. Although the volume measurements only took fluorescent regions into account, the locus-length measurements included the shortest distance in three dimensions between two fluorescent foci belonging to the same locus and the

length of the foci themselves. The non-expressing locus in endosperm, which is often seen as two foci in metaphase chromosomes and therefore contains a large intervening portion of genomic DNA, could be composed of one focus or two at variable distances from each other. Likewise, the decondensed loci were measured in three dimensions from the first focus through to the last within the linear path, including gaps between foci, but this measurement could only be done in nuclei where a clear linear path was discernable. Nuclei at 6 dpa were never found to express the HMW-glutenin-encoding genes and all nuclei at 10 dpa onwards did express them, as judged by transcript labelling. All images were composed using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Detection of siRNAs

RNA extraction and detection of siRNAs were done according to Hamilton and Baulcombe (Hamilton and Baulcombe, 1999). The antisense probe for the small RNA gel was prepared by in-vitro transcription of the PCR-amplified coding region of 1Ax1 using the primers described above and labelled with α^{32}P-UTP (Amersham).

Flow cytometry

Isolated endosperms were chopped with a razor blade and extracted using a two-step disaggregation and DAPI staining kit (CyStain UV Precise P; Partec, Münster, Germany) according to the manufacturer's instructions. The extracts were passed through a 50 μm filter and stored on ice until measurement. The DNA content of nuclei (C value) was measured using a Ploidy Analyser PA-II (Partec) with ultraviolet excitation provided by a mercury arc lamp. For each sample between 6000 and 16,000 nuclei were measured. Nuclei from young wheat leaves were used as a standard to determine the positions of 2C and 4C. The results were presented in histograms in a logarithmic scale to avoid missing peaks of higher C values.

Results

Transgene integration pattern on metaphase chromosomes

The integration pattern of the transgene copies in lines E and F was analysed by FISH on metaphase spreads. The genomic fragments containing 1Ax1 and 1Dx5 were labelled with digoxigenin as probes for the endogenous and transgenic HMW subunits, whereas biotin-labelled vector (pUC19) was used to identify the transgene sites. Both genomic fragments labelled the transgene loci very strongly and hybridized with the endogenous loci strongly enough to just detect them. The vector probe labelled only the transgene integration sites. The FISH results for the two homozygous lines showed two integration sites close together on one chromosome arm in line E (Figs 2, 3) and one large locus and one small locus on two chromosomes in line F (Fig. 3). Co-hybridization with a probe coding for repetitive sequences from *Aegilops squarrosa* resulted in a distinct banding pattern for each of the three chromosomes, meaning that the insertions in lines E and F are in different chromosomes (data not shown). The integration sites did not correspond to any of the group 1 chromosomes carrying the endogenous HMW-glutenin-encoding genes (shown for line E in Fig. 2, data for line F not shown).

Expression-related changes in 3D gene organization I

In initial experiments, the three-dimensional (3D) transgene

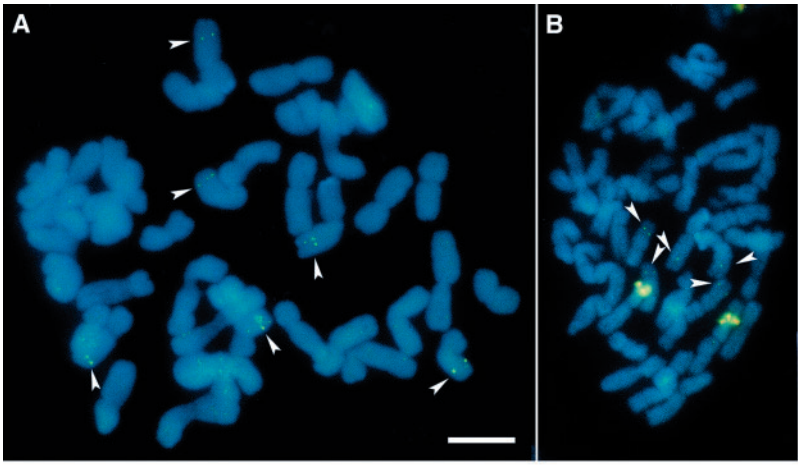

Fig. 2. FISH analysis of metaphase chromosomes. Metaphase spreads were hybridized with probes detecting the genomic fragments (green) and the vector sequences (red). (A) Loci encoding HMW-glutenin subunits in wild-type wheat (arrowheads). (B) Transgene loci (orange, hybridizing with both genomic and vector sequences) and gene loci (green, arrowheads) of loci encoding HMW-glutenin subunits in line E. Bar, 10 μm.

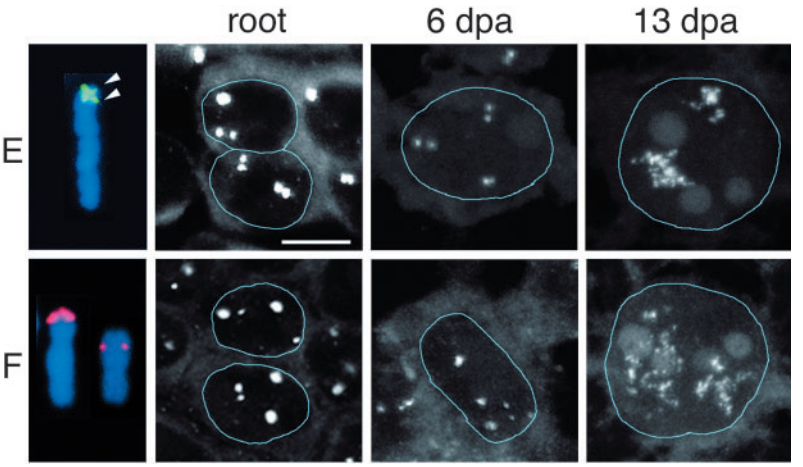

Fig. 3. Transgene locus structure in non-expressing and expressing tissue. (top) Line E. (bottom) Line F. (Left) Chromosomes with transgene loci. Two loci very close together are visible in line E (arrowheads); visualized using a probe against genomic fragments of 1Ax1 and 1Dx5. Line F contains two chromosomes with transgene loci; visualized using a probe against vector sequence. Tissue sections of line E and F were hybridized with pHMW-1Ax1 and pHMW-1Dx5 to detect the transgene loci in the diploid root (as non-expressing tissue) and in the triploid endosperm at 6 dpa (before the onset of expression) and 13 dpa (after the onset of expression). The image shows projections of serial confocal sections through one layer of nuclei. A blue line is drawn around the edge of each nucleus. The insertion sites in E are visible as one or two foci in root tips and young endosperm, whereas each locus in line F is visible as one focus. In expressing endosperm tissue, the loci in both lines are decondensed into many foci. Bar, 10 μm; section spacing is 0.6 μm.

locus structure in both lines was compared in expressing and non-expressing tissue. Root tips and endosperm at 6 dpa (before the onset of expression) and 13 dpa (after the onset of expression) were hybridized with pHMW-1Ax1 and pHMW-1Dx5 following RNase treatment. Root tips were vibratome sectioned without embedding, whereas seeds were wax embedded. In non-expressing root tips, transgene loci were detected as one or two condensed foci per chromosome in line E, whereas the loci in line F appeared as a large focus and a small focus (Fig. 3). In the triploid endosperm at 6 dpa, three large and three small foci were seen in line F and three to six foci in line E. In expressing endosperm at 13 dpa, the loci in both lines had decondensed into many foci. To quantify the data, we measured the volume occupied by locus fluorescence in nuclei of lines E and F before and well into glutenin expression. Fig. 4 shows that the average increase in locus volume from 6 dpa to 14 dpa is about fourfold in line E and threefold in line F.

When measuring fluorescence, thresholds have to be set to separate signal from background. This can introduce inaccuracies into the resulting volume calculations. In line E, decondensed loci were occasionally observed in a roughly linear path (Fig. 5B). This enabled us to reduce the measurement of chromatin compaction to one dimension with no need for thresholding. 42 non-expressing loci at 6 dpa were measured, giving an average length of 2.84 μm (±0.73) per locus; this figure is an average over loci with two separated foci and loci that show only a single, fused site. The average over loci where the locus was a single site was 2.17 μm; where there were two sites, the individual sites were correspondingly smaller (1.0-1.5 μm). We defined loci with a length of >10 μm at 14-16 dpa as linearly expanded. The average length of ten of those linear loci was 13.66±3.34 μm. In two nuclei of nearly identical volume (2426 μm^3 and 2412 μm^3), a decondensed locus at 16 dpa had a length of 16.25 μm and was 9.3 times as long as a condensed one at 6 dpa with a length of 1.75 μm (Fig. 5). The decondensed locus spanned more than half the nucleus.

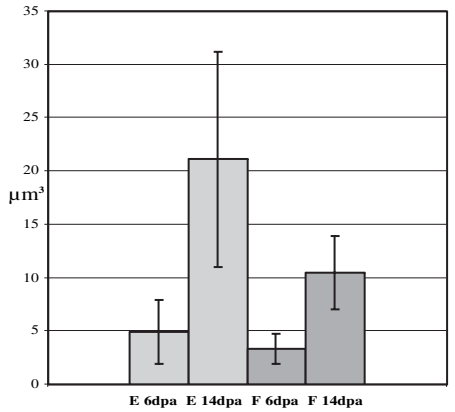

Fig. 4. Locus decondensation in lines E and F. After hybridization with pHMW-1Ax1 and pHMW-1Dx5 to detect the transgene loci the fluorescent area per optical section was measured in 79 and 18 randomly selected nuclei of lines E and F, respectively, at 6 dpa and in 59 and 18 nuclei of lines E and F, respectively, at 14 dpa. The total number of voxels in the fluorescent region per nucleus was multiplied by the voxel volume and the resulting fluorescent volume is shown on the *y*-axis.

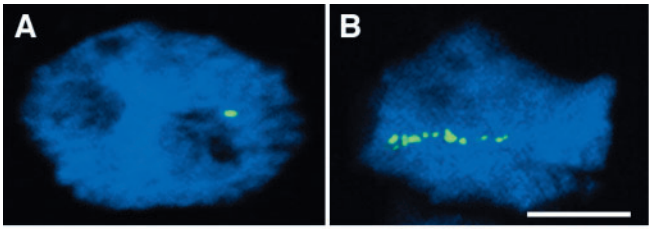

Fig. 5. DNA decondensation in transcriptionally active loci. Endosperm nuclei of line E were hybridized with probes for pHMW-1Ax1 and pHMW-1Dx5, detecting the whole plasmids (green, A) or with probes detecting the flanking sequences and the vector (green, B) and counterstained with DAPI (blue). Overlay of several confocal sections showing only one of the three loci per nucleus. An overlay of all optical sections through the nucleus is shown for the DAPI staining in A and B to illustrate the similar sizes of the two nuclei. (A) Condensed locus at 6 dpa before the start of expression. (B) Transcriptionally active locus at 16 dpa in a rarely seen linear conformation. In the straightened chromatin fibres, the complex structure of the locus, which is broken up by genomic sequences, becomes visible. Bar, 10 μm; section spacing is 0.4-0.6 μm.

For a detailed analysis of locus decondensation in relation to transcription and gene silencing, we chose line E because it contains two closely linked insertion sites, which will be referred to as the locus below to simplify discussion.

Time course of HMW subunit silencing

In a time-course RNA in-situ hybridization experiment, HMW-glutenin expression in the wild-type was compared with that of line E (Fig. 6). Seeds of line E and the wild type were wax embedded at different developmental stages. 30 μm sections were hybridized with digoxigenin-labelled 1Ax1 antisense probes. The inclusion of a probe for 1Dx5 was not necessary because, in dot blot experiments, the antisense probes for the coding regions of both 1Ax1 and 1Dx5 hybridized equally well with both plasmids (data not shown). Both line E and the wild type started expressing glutenin-encoding mRNA at 8 dpa. The level of mRNA signal seen in line E dropped dramatically at 12 dpa, whereas the level of transcript in the wild type increased steadily and was still high at 20 dpa (data not shown). The same results were obtained by detecting the transcripts with Alexa Fluor 488 instead of the alkaline-phosphatase reaction. In line E, fluorescence in and around the nuclei started in a few single cells. The cytoplasmic transcript signal spread to form sectors of fluorescence without ever comprising the entire endosperm and by 12-14 dpa was reduced again to a few isolated cells, disappearing completely at later stages (data not shown). By contrast, nuclear transcript in line E was detected in every endosperm nucleus 1 or 2 days after the onset of expression and persisted for weeks (Fig. 7). In the wild type, the entire endosperm was expressing by 10 dpa.

Detection of siRNAs as a marker for PTGS

The early disappearance of cytoplasmic RNA signal in the transgenic line in combination with continuing nuclear transcription suggested PTGS. In order to confirm this, total seed RNA was extracted at 8 dpa, the beginning of HMW-

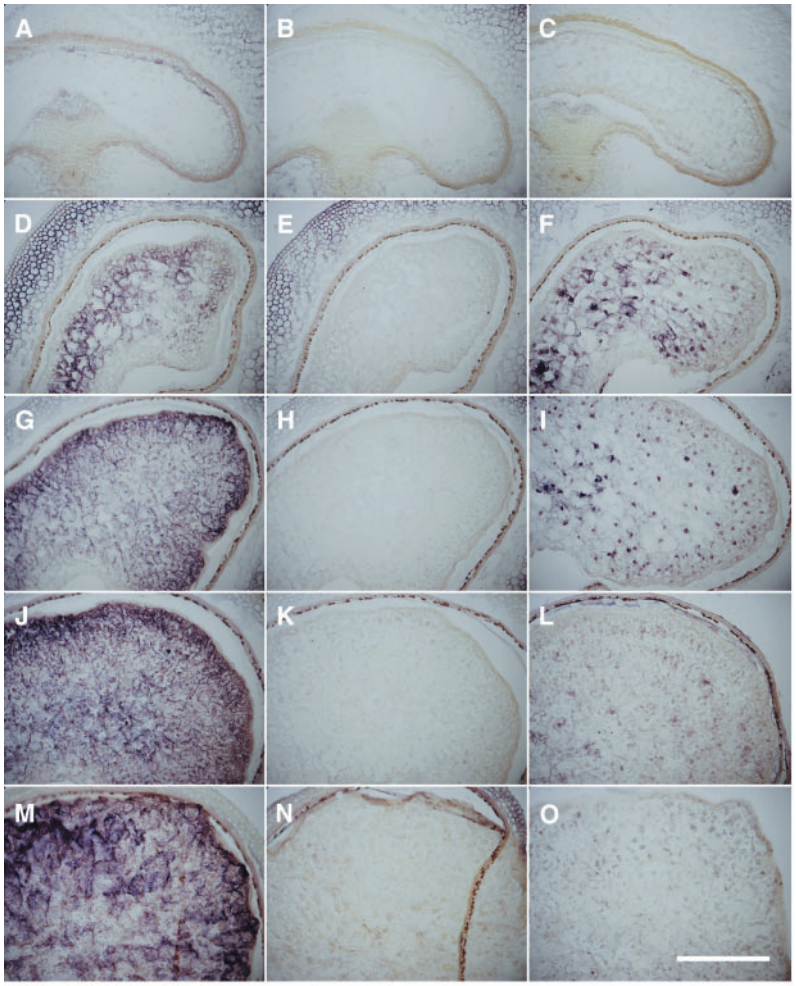

Fig. 6. Time course of HMW-glutenin-subunit expression in endosperm. HMW-glutenin-encoding transcripts in seed sections of the wild type and line E were detected with an antisense probe for the coding region of 1Ax1. The sense probe was used as control for unspecific labelling. Left column, wild-type labelled with anti-sense probe; middle column, wild-type labelled with sense probe; right column, line E labelled with anti-sense probe. (A-C) 5 dpa. Before the onset of expression, no RNA signal is visible in the endosperm. The pericarp and some aleurone cells show unspecific labelling. (D-F) 8 dpa. Onset of expression in endosperm cells, unspecific labelling in the pericarp. (G-I) 10 dpa. Strong expression in wild-type endosperm, whereas the RNA signal in line E is much weaker and mainly concentrated around the nuclei instead of the whole of the cytoplasm. (J-L) 12 dpa. Very weak expression in line E, in contrast to the wild type. (M-O) 16 dpa. The wild type is still expressing strongly, whereas expression in line E has ceased. Bar, 0.5 mm.

glutenin expression and, at 14 dpa, after the onset of silencing in line E. The RNA was separated by electrophoresis on a polyacrylamide-urea gel, blotted and hybridized with an antisense probe for the 1Ax1 coding region. At 14 dpa siRNAs indicative of PTGS were detected in the transgenic line (Fig. 8).

Expression-related changes in 3D gene organization II

In order to characterize further the changes in the 3D structure of the transgene loci caused by their activation during endosperm development seeds of line E were harvested at different developmental stages from 6 dpa, before the onset of glutenin expression, through to 37 dpa, just before seed maturation. The wild type was used as a negative control. Seeds were vibratome sectioned without embedding. For transcript detection, sections were hybridized with a biotin-labelled 2.5-kb antisense RNA probe for the coding region of 1Ax1. After fixation and washes, a second hybridization was carried out to detect the loci using the vector and sense and antisense RNA probes for the gene flanking sequences of both clones but not the coding region itself, in order to avoid cross-hybridization with the transcript (Fig. 1). Because no transcript was detected in tissue at 6 dpa and control experiments using a sense probe showed negligible labelling of the loci (data not shown), we concluded that the antisense probe for the transcript hybridized almost exclusively with the transcript and not with the locus.

Before the onset of expression, each transgene locus was detected as one or two condensed foci (Fig. 9A). At around 7 dpa, the first few nuclei in the centre of the lobes of the endosperm started showing transcript signal at the transgene loci. At 8 dpa, nuclei transcribing the HMW-glutenin subunits were found in all parts of the endosperm. Generally, all loci within a nucleus were expressing but, occasionally, that was not the case (Fig. 9B). Expressing loci were condensed or slightly decondensed and both conformations could be found in the same nucleus. No decondensed, non-expressing loci were seen (Table 1). At 10 dpa all endosperm nuclei were transcriptionally active and the majority had decondensed loci. At subsequent time points (12 dpa, 14 dpa, 16 dpa and 20 dpa), all nuclei had transcriptionally active, decondensed loci and increasing numbers of larger nuclei were found, which are indicative of endoreduplication events (Fig. 9E,F). The latest time point investigated was 37 dpa, at which stage the seeds were just about to turn from green to brown. Some nuclei were still expressing, although mostly at lower levels, and showed fully decondensed loci, whereas the chromatin of other nuclei was disintegrating (Fig. 9G,H). In interphase nuclei of both line E and the wild type, the endogenous loci (which were barely visible on metaphase spreads) were rarely detected. In some experiments, transcript was detected in a few small foci in the wild-type nuclei, which probably represented the endogenous loci (not shown). Colocalization experiments using line F at 13-16 dpa also showed transcript on the transgene loci (data not shown).

For most parts of each locus, double labelling of the transgene DNA and the transcript showed a high degree of coincidence. However, smaller regions of gene label without any or with very faint RNA label were detected right from the beginning of HMW-glutenin-subunit expression (Fig. 9B-F). This suggests that most of the transgene copies at the locus are transcriptionally active. The transcriptionally inactive regions detected might comprise concatemers of flanking sequences and vector caused by plasmid rearrangements or might contain transcriptionally inactive or truncated transgene copies.

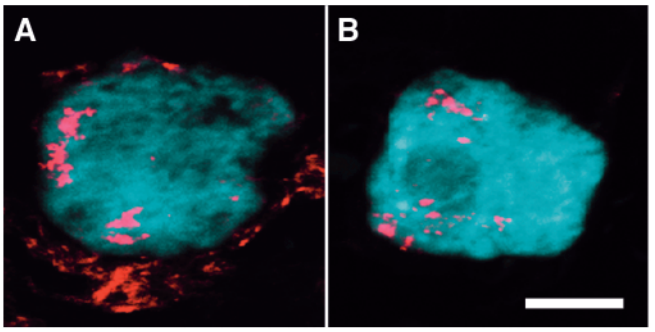

Fig. 7. Disappearance of cytoplasmic HMW-glutenin-encoding RNA at the onset of silencing. Sections from wax-embedded endosperm of line E were hybridized with an antisense probe for the coding region of 1Ax1 and detected with Cy3 (red). The nuclei were counterstained with DAPI. The images comprise only the nucleus and the cytoplasm immediately around it. (A) 10 dpa. HMW-glutenin-encoding RNA can be seen inside the nucleus and in the cytoplasm around it. (B) 14 dpa. RNA is still visible inside the nucleus, whereas it has disappeared from the cytoplasm. Bar, 10 µm.

Locus decondensation at the beginning of expression is not linked to endoreduplication in endosperm nuclei

Nuclei in the developing wheat endosperm undergo one or two cycles of endoreduplication and an analysis of 97 varieties showed that the proportion of endoreplicated nuclei ranged from 3% to 32%, the most represented class being 12C and the highest value (24C) corresponding to two rounds of endoreduplication (Brunori et al., 1989). We wanted to know at what stage endoreduplication starts, what proportion of nuclei are affected and whether the observed decondensation of the loci could be, in part, a reflection of endoreduplication. Therefore, flow-cytometric analyses of the DNA content of line E were carried out before and shortly after the onset of HMW-glutenin-subunit expression and at 16 dpa, well into glutenin expression, when the tissue showed a fair number of large nuclei (Fig. 9, Table 2). The increase in DNA content at the beginning of transcription was small compared with 2 days earlier and could not account for the increase in focus size and number observed in so many nuclei at these early stages. By 16 dpa, the balance had shifted from a majority of nuclei in G1 to a majority in G2, but still only about 12% of nuclei had completed one round of endoreduplication and none had a higher C content. In nuclei with a DNA content of 12C, one would theoretically expect a maximum of eight foci per locus compared with the maximum of two foci per locus seen in nuclei in G1 phase (3C) before the onset of glutenin expression. The observed number of foci was, however, much higher (Fig. 9). Therefore, even at 16 dpa, the increased DNA content could only partly contribute to the observed locus size.

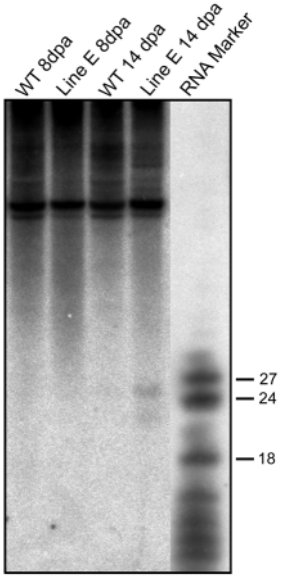

Fig. 8. Detection of siRNAs corresponding to the 1Ax1 coding region. Total seed RNA of the wild-type (WT) and line E extracted before (8 dpa) and after (14 dpa) the onset of silencing was hybridized with an antisense probe for the coding region of 1Ax1. Two RNA bands of ~22 bp and 25 bp (which are indicative of PTGS) are visible in line E at 14 dpa (lane 4).

Discussion

Transcriptional activation precedes observable locus decondensation

We have studied transcriptionally induced chromatin decondensation in two wheat lines with large, developmentally activated arrays of transgenes that could easily be detected by FISH. The loci contain endogenous genes under the control of their own promoters, which were transferred as genomic clones containing a few kilobases of flanking sequences on either side so that at least the microenvironment is a natural one, even if the chromosomal position and the tandem arrays are not. The fact that decondensation was observed in both lines with transgene insertions into different chromosomes shows that the effect is independent of the surrounding chromatin.

At the beginning of expression, 22% of the transcriptionally active loci in line E were condensed and we therefore conclude that transcriptional activation can precede any significant loosening of the chromatin structure. Similar observations have recently been published for an inducible transgene array in a mammalian cell line, in which an increase in RNA levels preceded noticeable changes in higher-order chromatin structure (Janicki et al., 2004). The endoreduplication data show that the observed chromatin decondensation upon gene activation is not caused by an increase in ploidy during endosperm development. Further evidence comes from the DNA compaction measurements, in which two nuclei of almost identical volume exhibit very different degrees of locus decondensation. In the example given, the condensed locus was nine times as linearly compacted as the transcriptionally active one. Similar decondensation ratios were reported by Müller et al. (Müller et al., 2001),who reported that the 2 Mb MMTV array decondensed from ~0.5 µm to an average of 6 µm and a maximum of 10 µm after hormonal activation. In two hormonally inducible transgene arrays, the first changes in chromatin organization were visible 30 minutes or less after the addition of the activator and the chromatin was fully extended after 2 hours or 4 hours (Tsukamoto et al., 2000; Janicki et al., 2004). Fully decondensed loci in wheat endosperm were first detected ~1-2 days after the start of transcription.

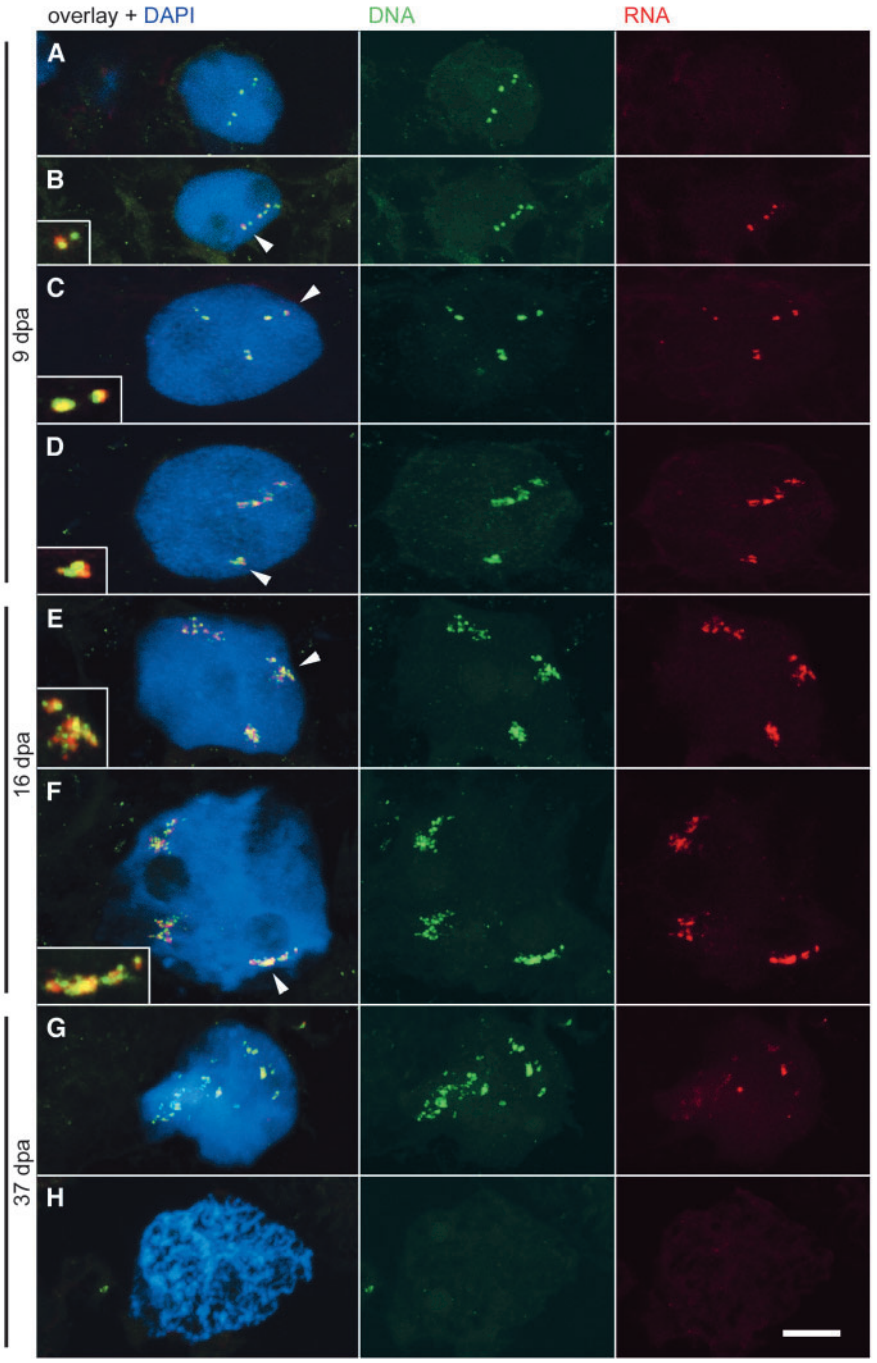

Fig. 9. Localization of transgene loci and their transcripts during endosperm development. Endosperm nuclei counterstained with DAPI (blue) were hybridized with probes for the gene flanking regions and vector sequences of pHMW-1Ax1 and pHMW-1Dx5 to detect the locus (green) and with an antisense probe for the 1Ax1 coding region to detect the transcript (red). The insets are 2× enlargements of the loci marked by an arrowhead in the overlay. The sizes of the nuclei vary considerably owing to the cell-cycle phase they are in and because of endoreduplication at later stages. A and B are probably G1 nuclei, and C-E and G are probably G2 nuclei. F shows an endoreplicated nucleus. Each image is a projection of serial confocal sections. (A) Non-expressing nucleus in tissue starting to express. One locus is seen as one focus in the centre of the nucleus, whereas the two loci above and below are seen as two foci each. (B) Nucleus starting to transcribe the transgene in the same tissue as in A. All three loci are seen as two foci. Only the left-hand part of the locus denoted by the arrowhead is transcribing. (C) Nucleus in which all three loci are transcribing and condensed. The inset shows that some areas of the transgene locus are not transcriptionally active. (D) Transcriptionally active nucleus in the centre of the endosperm starting to decondense. One region in the locus in the inset shows no transcript. (E,F) Nuclei with fully decondensed loci, which are largely but not completely transcriptionally active. (G) Dying nucleus with dispersed transgene DNA and very weak RNA labelling. (H) Nucleus with degrading chromatin and no hybridization signal for the transgenes and their transcripts. Bar, 10 μm; section spacing is 0.4-0.6 μm.

The analysis of wheat chromosome structure has so far been based on the observation of selectively labelled rye chromosome additions and introgressed rye chromosome arms in wheat roots and anthers, in which chromosomes span the width of the nucleus in flexible, rod-like structures with the two arms of each chromosome close together and centromeres and telomeres located at opposite poles in a Rabl configuration (Abranches et al., 1998; Martínez-Pérez et al., 1999). We have made essentially the same observations in endosperm (Wegel and Shaw, 2005). The shape of the active loci in line E was highly variable (Figs 5, 9) and most of the decondensed transgene loci are too big to fit into the structure predicted for wheat chromosomes based on the findings above. If the assumed chromosome shape is correct then the transgene loci must extend beyond it. Using mammalian cell cultures, several groups have produced evidence for chromatin decondensation outside the chromosome territory in regions where densely packed endogenous genes are actively transcribed (Volpi et al., 2000; Mahy et al., 2002; Williams et al., 2002; Chambeyron and Bickmore, 2004). The same is happening in our highly transcribed and gene-dense locus. A recent extensive mapping study (Erayman et al., 2004) estimated that only 1.2-2.4% of the wheat genome consists of genes and showed that wheat genes are organized into gene-rich regions with a range of one

Table 1. Locus structure and transcript detection in nuclei at the beginning of expression

	Nuclei with condensed loci	Nuclei with decondensing loci
No transcript	3.8% (2)	0.0% (0)
Transcript	20.8% (11)	75.5% (40)

Nuclei with detectable loci in endosperm sections of line E at 9 dpa were randomly selected and scored for the presence of nascent transcript.

Table 2. Ploidy levels during endosperm development in line E

DNA content	7 dpa	9 dpa	16 dpa
3C	71.5±3.7%	62.8±3.5%	40.3±1.1%
6C	26.6±1.3%	32.8±3.8%	47.9±1.0%
>6C*	1.8±2.5%	4.4±3.1%	11.8±0.7%

The proportions are averages over five experiments. For each experiment, three isolated endosperms were pooled for 7-day-old seeds and two for 9- and 16-day-old seeds.

*These nuclei have endoreplicated. For seeds at 16 dpa, this peak was always at 12C but at 7 dpa and 9 dpa, an additional peak appeared with a C value between 6C and 12C.

gene per 78-5500 kb. Based on the sequence for selected wheat and barley regions, the authors concluded that gene density might average at one per 10-20 kb when the gene-rich regions were further partitioned (Erayman et al., 2004). The latter corresponds to the gene density in our transgene locus with 10-12 kb per plasmid and, if the estimates by Erayman et al. are correct, visible decondensation should also occur in gene-rich regions in wheat provided that the genes are transcribed at the same time in the same nucleus. Our results show that, in plants, gene sequences undergo considerable decompaction upon activation. Some of the loci extended in more or less a straight line (Fig. 5). If they reflect the direction in which the chromosome spans the nucleus they are extended to at least one-third of the total chromosome length.

In summary, this demonstrates that transcription is maintained in very variable 3D chromatin structures in which the chromatin is decondensed beyond the boundaries of the chromosome territory. A chromatin territory therefore seems to be constantly remodelled with the onset and cessation of transcriptional activity. The question remains of whether the chromatin loops that extend away from the chromosomal territory intermingle with other chromosome territories or are found in the interchromatin space. The latter is supported by electron-microscopy studies suggesting that chromosomal territories are mostly separated into non-overlapping spaces surrounded by interchromatin space (Visser and Aten, 1999; Visser et al., 2000). This could be tested by looking for overlaps between the transgene loci and the rye chromosome arm present in Federal, which carries a 1RS (*Secale cereale*) chromosome arm substitution for the 1BS wheat chromosome arm ($1B^{l}/1R^{s}$).

Transcript localization within the nucleus

In our experiments, the main RNA signal in the nucleus was always restricted to the close vicinity of the locus. Dirks et al. (Dirks et al., 1995) looked at transgene loci and reported RNA tracks at the site of transcription of two highly spliced viral sequences and elongated dots at the site of transcription of the luciferase-encoding gene with an artificial intron. For one of the viral sequences and the luciferase-encoding gene, they also saw halo-like signal distribution around the main RNA signal radiating towards the nuclear periphery. Xing et al. (Xing et al., 1993) frequently found the RNA encoding the fibronectin gene in tracks emanating from the gene, whereas the transcript of the neurotensin-encoding gene was seen as a focus overlapping the gene. Both groups suggest that the shape of the RNA signal at the transcription site depends largely on how much the pre-mRNA is processed (i.e. on the number of introns that have to be removed). This view is supported by the fact that splicing factors (for a review, see Spector, 1996) and polyadenylated transcripts (Xing et al., 1993; Dirks et al., 1997) have been detected at the site of transcription. It has been shown that polyadenylation and transcription are coupled via the C-terminal domain (CTD) of the large subunit of RNA polymerase II, which forms a functional complex on the pre-mRNA with polyadenylation factors to catalyse endonucleolytic cleavage (for a review, see Hirose and Manley, 2000). HMW-glutenin-encoding genes have no introns and so are likely to leave the site of transcription soon after they have been released from the RNA-polymerase complex. Therefore, only relatively small pools of transcript are detected around the locus (red areas in the insets of Fig. 9), whereas most of the signal localizes with the locus as nascent transcript.

PTGS in wheat endosperm

So far, mainly biochemical techniques have been used to study PTGS. In this report, we have used in situ hybridization to show PTGS at the cellular level. From the start of expression until cell death, HMW-glutenin transcript was detected by FISH on top of the transgene locus. The fluorescence in the cytoplasm was strong at the beginning of HMW-subunit expression and had disappeared almost completely in line E by 14 dpa, whereas it remained strong in the wild type. At the biochemical level, PTGS was confirmed by the detection of siRNAs. The siRNA lengths seen in wheat endosperm correspond to the lengths of the long (25 nt) and short (21-22 nt) siRNAs reported by Hamilton et al. (Hamilton et al., 2002) for transgene silencing in *Nicotiana benthamiana*. Recently, Tang et al. (Tang et al., 2003) showed Dicer activity in wheat-germ extracts, which are essentially cytoplasm, and found a similar distribution of both RNA classes to ours when double-stranded RNA was digested into double-stranded siRNA. PTGS can be caused by highly transcribed transgenes or by an inverted transgene repeat, in both cases leading to the production of a double-stranded transcript that is a substrate for Dicer and causes silencing (Fagard and Vaucheret, 2000; Ketting et al., 2001; Béclin et al., 2002). We tested the presence of an inverted repeat in line E using single PCR primers at several positions in and around the coding sequences but the results were negative (data not shown). In *Arabidopsis*, sequences for four *Dicer*-like genes have been found, two with a nuclear targeting signal and two that are probably cytoplasmic (Finnegan et al., 2003). Although we have shown that the mostly nascent transcript at the locus remains throughout the life of the nucleus, the method used was not sensitive enough to detect single RNA molecules on their way

through the nucleus. It is most likely that the transcript is digested by a Dicer-like enzyme in the cytoplasm, as shown by Tang et al. (Tang et al., 2003), but we cannot exclude the possibility that a proportion is cleaved in the nucleus after it has left the proximity of the locus. Hamilton et al. (Hamilton et al., 2002) propose that the longer siRNAs could mediate nucleotide-specific methylation of DNA and thus transcriptional silencing. In the two generations of line E that were analysed, we did not find any perceptible decrease in transcriptionally active parts of the locus. Our own unpublished data also show no changes in cytosine methylation levels in the transgenes in leaves and endosperm, whether before or shortly after the onset of expression, or after the onset of PTGS.

In summary, we have followed the onset and spread of PTGS by tracking the disappearance of cytoplasmic but not nuclear mRNA and supported our findings with the biochemical detection of small siRNAs. It is clear from our results that, even after a sustained period of PTGS, there is no consequent transcriptional silencing in this system. In the future, it will be interesting to correlate the transcription-related changes in higher chromatin order seen in the transgene locus with possible changes in DNA methylation state and histone modifications (Mathieu et al., 2003; Chambeyron and Bickmore, 2004).

E. Wegel was supported by the UK Biotechnology and Biological Science Research Council (BBSRC) Gene Flow Initiative and E. Stöger was supported by the Sofia-Kovalevskaja Award of the Alexander von Humboldt Foundation. R. H. Vallejos is a member of the Research Career from CONICET, Argentina. We thank F. Vaistij for technical assistance with the detection of siRNAs. N. Halford (Rothamsted Research, Harpenden, UK) kindly provided plasmids pHMW1Ax1 and pHMW1Dx5.

References

Abranches, R., Beven, A. F., Aragón-Alcaide, L. and Shaw, P. J. (1998). Transcription sites are not correlated with chromosome territories in wheat nuclei. *J. Cell Biol.* **143**, 5-12.

Abranches, R., Santos, A. P., Wegel, E., Williams, S., Castilho, A., Christou, P., Shaw, P. and Stoger, E. (2000). Widely separated multiple transgene integration sites in wheat chromosomes are brought together at interphase. *Plant J.* **24**, 713-723.

Altenbach, S. B., Kothari, K. M. and Lieu, D. (2002). Environmental conditions during wheat grain development alter temporal regulation of major gluten protein genes. *Cereal Chem.* **79**, 279-285.

Alvarez, M. L., Guelman, S., Halford, N. G., Lustig, S., Reggiardo, M. I., Ryabushkina, N., Shewry, P., Stein, J. and Vallejos, R. H. (2000). Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. *Theor. Appl. Genet.* **100**, 319-327.

Anderson, O. D., Greene, F. C., Yip, R. E., Halford, N. G., Shewry, P. R. and Malpica-Romero, J. M. (1989). Nucleotide sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyenne. *Nucleic Acids Res.* **17**, 461-462.

Béclin, C., Boutet, S., Waterhouse, P. and Vaucheret, H. (2002). A branched pathway for transgene-induced RNA silencing in plants. *Curr. Biol.* **12**, 684-688.

Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.

Brunori, A., Forino, L. M. C. and Frediani, M. (1989). Polyploid DNA contents in the starchy endosperm nuclei and seed weight in *Triticum aestivum*. *J. Genet. Breed.* **43**, 131-134.

Chambeyron, S. and Bickmore, W. A. (2004). Chromatin decondensation and nuclear reorganization of the *HoxB* locus upon induction of transcription. *Genes Dev.* **18**, 1119-1130.

Christensen, A. H. and Quail, P. H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res.* **5**, 213-218.

Coen, E. S., Romero, J. M., Doyle, S., Elliott, R., Murphy, G. and Carpenter, R. (1990). *floricaula*: a homeotic gene required for flower development in *Antirrhinum majus*. *Cell* **63**, 1311-1322.

Custódio, N., Carmo-Fonseca, M., Geraghty, F., Pereira, H. S., Grosveld, F. and Antoniou, M. (1999). Inefficient processing impairs release of RNA from the site of transcription. *EMBO J.* **18**, 2855-2866.

Dirks, R. W., Daniël, K. C. and Raap, A. K. (1995). RNAs radiate from gene to cytoplasm as revealed by fluorescence in situ hybridization. *J. Cell Sci.* **108**, 2565-2572.

Dirks, R. W., de Pauw, E. S. and Raap, A. K. (1997). Splicing factors associate with nuclear HCMV-IE transcripts after transcriptional activation of the gene, but dissociate upon transcription inhibition: evidence for a dynamic organization of splicing factors. *J. Cell Sci.* **110**, 515-522.

Erayman, M., Sandhu, D., Sidhu, D., Dilbirligi, M., Baenziger, P. S. and Gill, K. S. (2004). Demarcating the gene-rich regions of the wheat genome. *Nucleic Acids Res.* **32**, 3546-3565.

Fagard, M. and Vaucheret, H. (2000). (Trans)gene silencing in plants: how many mechanisms? *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 167-194.

Finnegan, E. J. and Matzke, M. A. (2003). The small RNA world. *J. Cell Sci.* **116**, 4689-4693.

Finnegan, E. J., Margis, R. and Waterhouse, P. M. (2003). Posttranscriptional gene silencing is not compromised in the *Arabidopsis CARPEL FACTORY* (*DICER-LIKE1*) mutant, a homolog of *dicer-1* from *Drosophila*. *Curr. Biol.* **13**, 236-240.

González-Melendi, P., Wells, B., Beven, A. F. and Shaw, P. J. (2001). Single ribosomal transcription units are linear, compacted Christmas trees in plant nucleoli. *Plant J.* **27**, 223-233.

Halford, N. G., Forde, J., Shewry, P. R. and Kreis, M. (1989). Functional analysis of the upstream regions of a silent and an expressed member of a family of wheat seed protein genes in transgenic tobacco. *Plant Sci.* **62**, 207-216.

Halford, N. G., Field, J. M., Blair, H., Urwin, P., Moore, K., Robert, L., Thompson, R., Flavell, R. B., Tatham, A. S. and Shewry, P. R. (1992). Analysis of HMW glutenin subunits encoded by chromosome 1a of bread wheat (*Triticum aestivum* L) indicates quantitative effects on grain quality. *Theor. Appl. Genet.* **83**, 373-378.

Hamilton, A. J. and Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-952.

Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J.* **21**, 4671-4679.

Highett, M. I., Beven, A. F. and Shaw, P. J. (1993). Localization of 5S genes and transcripts in *Pisum sativum* nuclei. *J. Cell Sci.* **105**, 1151-1158.

Hirose, Y. and Manley, J. L. (2000). RNA polymerase II and the integration of nuclear events. *Genes Dev.* **14**, 1415-1429.

Ingham, P. W., Howard, K. R. and Ishhorowicz, D. (1985). Transcription pattern of the *Drosophila* segmentation gene *Hairy*. *Nature* **318**, 439-445.

Jackson, D. P. (1991). In situ hybridization in plants. In *Molecular Plant Pathology: A Practical Approach*, vol. 1 (eds S. J. Gurr, M. J. McPherson and D. J. Bowles), pp. 163-174. Oxford, UK: Oxford University Press.

Janicki, S. M., Tsukamoto, T., Salghetti, S. E., Tansey, W. P., Sachidanandam, R., Prasanth, K. V., Ried, T., Shav-Tal, Y., Bertrand, E., Singer, R. H. et al. (2004). From silencing to gene expression: real-time analysis in single cells. *Cell* **116**, 683-698.

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J. and Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* **15**, 2654-2659.

Mahy, N. L., Perry, P. E. and Bickmore, W. A. (2002). Gene density and transcription influence the localization of chromatin outside of chromosome territories detectable by FISH. *J. Cell Biol.* **159**, 753-763.

Martínez-Pérez, E., Shaw, P., Reader, S., Aragón-Alcaide, L., Miller, T. and Moore, G. (1999). Homologous chromosome pairing in wheat. *J. Cell Sci.* **112**, 1761-1769.

Mathieu, O., Jasencakova, Z., Vaillant, I., Gendrel, A. V., Colot, V., Schubert, I. and Tourmente, S. (2003). Changes in 5S rDNA chromatin organization and transcription during heterochromatin establishment in *Arabidopsis*. *Plant Cell* **15**, 2929-2939.

Matzke, M., Matzke, A. J. and Kooter, J. M. (2001). RNA: guiding gene silencing. *Science* **293**, 1080-1083.

Müller, W. G., Walker, D., Hager, G. L. and McNally, J. G. (2001). Large-scale chromatin decondensation and recondensation regulated by transcription from a natural promoter. *J. Cell Biol.* **154**, 33-48.

Probst, A. V., Fransz, P. F., Paszkowski, J. and Scheid, O. M. (2003). Two means of transcriptional reactivation within heterochromatin. *Plant J.* **33**, 743-749.

Schramke, V. and Allshire, R. (2003). Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. *Science* **301**, 1069-1074.

Shewry, P. R., Halford, N. G., Tatham, A. S., Popineau, Y., Lafiandra, D. and Belton, P. S. (2003). The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. *Adv. Food Nutr. Res.* **45**, 219-302.

Spector, D. L. (1996). Nuclear organization and gene expression. *Exp. Cell Res.* **229**, 189-197.

Tang, G., Reinhart, B. J., Bartel, D. P. and Zamore, P. D. (2003). A biochemical framework for RNA silencing in plants. *Genes Dev.* **17**, 49-63.

Tsukamoto, T., Hashiguchi, N., Janicki, S. M., Tumbar, T., Belmont, A. S. and Spector, D. L. (2000). Visualization of gene activity in living cells. *Nat. Cell Biol.* **2**, 871-878.

Tumbar, T., Sudlow, G. and Belmont, A. S. (1999). Large-scale chromatin unfolding and remodeling induced by VP16 acidic activation domain. *J. Cell Biol.* **145**, 1341-1354.

Visser, A. E. and Aten, J. A. (1999). Chromosomes as well as chromosomal subdomains constitute distinct units in interphase nuclei. *J. Cell Sci.* **112**, 3353-3360.

Visser, A. E., Jaunin, F., Fakan, S. and Aten, J. A. (2000). High resolution analysis of interphase chromosome domains. *J. Cell Sci.* **113**, 2585-2593.

Volpi, E. V., Chevret, E., Jones, T., Vatcheva, R., Williamson, J., Beck, S., Campbell, R. D., Goldsworthy, M., Powis, S. H., Ragoussis, J. et al. (2000). Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. *J. Cell Sci.* **113**, 1565-1576.

Wegel, E. and Shaw, P. (2005). Chromosome organisation in wheat endosperm and embryo. *Cytogenet. Genome Res.* (in press).

Williams, R. R., Broad, S., Sheer, D. and Ragoussis, J. (2002). Subchromosomal positioning of the epidermal differentiation complex (EDC) in keratinocyte and lymphoblast interphase nuclei. *Exp. Cell Res.* **272**, 163-175.

Xing, Y., Johnson, C. V., Dobner, P. R. and Lawrence, J. B. (1993). Higher level organization of individual gene transcription and RNA splicing. *Science* **259**, 1326-1330.

Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.