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5-Methylcytosine distribution and genome organization in Triticale before and after treatment with 5-azacytidine

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SUMMARY

Triticale (2n=6x=42) is a hybrid plant including rye (R) and wheat (A and B) genomes. Using genomic in situ hybridization with rye DNA as a probe, we found the chromosomes of the R genome were not intermixed with the wheat chromosomes in 85% of nuclei. After treatment of seedlings with low doses of the drug 5-azacytidine (5-AC), leading to hypomethylation of the DNA, the chromosomes became intermixed in 60% of nuclei; the next generation showed intermediate organization. These results correlate with previous data showing that expression of R-genome rRNA genes, normally suppressed, is activated by 5-AC treatment and remains partially activated in the next generation. The distribution of 5-methylcytosine (5-mC) was studied using an antibody to 5-mC. Methylation was detected along the lengths of all chromosomes; there were some chromosome regions with enhanced and reduced methylation, but these were not located at consistent positions, nor were there differences between R and wheat genome chromosomes. After 5-AC treatment, lower levels of methylation were detected. After 5-AC treatment, in situ hybridization with rye genomic DNA sometimes showed micronuclei of rye origin and multiple translocations between wheat and rye chromosomes. Genomic DNA was analysed using methylation-sensitive restriction enzymes and, as probes, two rDNA sequences, two tandemly organised DNA sequences from rye (pSc200 and pSc250), and *copia* and the *gypsy* group retrotransposon fragments from rye and wheat. DNA extracted immediately after 5-AC treatment was cut more by methylation-sensitive restriction enzymes than DNA from untreated seedlings. Each probe gave a characteristic restriction fragment pattern, but rye- and wheat-origin probes behaved similarly, indicating that hypomethylation was induced in both genomes. In DNA samples from leaves taken 13-41 days after treatment, RFLP (Restriction Fragment Length Polymorphism) patterns were indistinguishable from controls and 5-AC treatments with all probes. Surprising differences in hybridization patterns were seen between DNA from root tips and leaves with the *copia*-fragment probes.

Key words: Methylation, Confocal microscopy, Immunofluorescence labelling, 5-methylcytosine, 5-azacytidine, LTR-retroelement

INTRODUCTION

In plants, DNA methylation at cytosine residues to 5-methylcytosine (5-mC) is extensive in the nuclear genome (Finnegan et al., 1993). Approximately 80% of cytosines in CpG dinucleotides are modified (Gruenbaum et al., 1981) and plants, like animals, may contain unmethylated CG regions (CpG islands) related to transcriptionally active genes (Antequera and Bird, 1988). Cytosine modification may also be extensive at non-symmetrical (not CG or CNG) sites (Goubely et al., 1999). In most plant species, the majority of the DNA consists of repetitive sequences including tandemly repeated and dispersed elements (Kubis et al., 1998). Moore et al. (1993) found that most of the potential sites for CpG methylation-sensitive restriction enzymes are methylated, particularly in tandemly repeated DNA sequences. Retrotransposons, which may make up 50% of the genome (San Miguel et al., 1996), are present in all eukaryotic genomes and represent the major class of mobile elements in plants (Flavell et al., 1992). The prevalence of methylated transposons has been known for some time (White et al., 1994; San Miguel et al., 1996).

DNA methylation has been implicated in many aspects of the control of gene expression in both animals and plants (Bird et al., 1981; Flavell et al., 1988; Cedar, 1988; Barlow, 1993; Sardana et al., 1993; Razin and Cedar, 1994; Neves et al., 1995; Finnegan et al., 1998). Most experiments indicate that methylation leads to repression of genes at the level of transcription initiation (Tate and Bird, 1993), although methylation does not seem to repress the activity of all

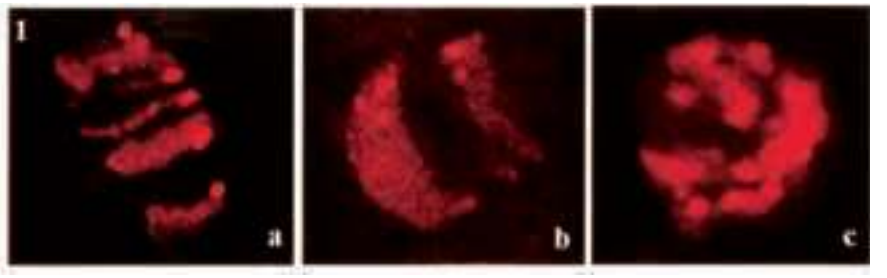

Fig. 1. Confocal sections of Triticale root-tip interphase nuclei after in situ hybridization of genomic DNA to label the R (rye) genome chromatin (red). (a,b) Nuclei from an untreated root tip showing wheat (dark) and rye (labelled red; telomeric heterochromatin seen as bright spots) chromatin in (a) interspersed slices or (b) discrete domains. (c) A nucleus of a 5-AC treated root tip (M0) showing intermixing of rye (red) and wheat (dark) chromatin: no clear wheat and rye genome domains were detected (see Table 1).

Table 1. Analysis of genomic organization in Triticale interphase cells of root tips

	Organization of rye chromatin (% of nuclei)			
	Defined slices	Defined domains	No clear domains	Nuclei scored
Control	81.4	5.1	15.3	177
M0	33.9	5.7	60.4	106
M1	54.2	3.6	42.2	225

Root tips germinated in water (control), in 5-azacytidine (M0) and root tips from the progeny of treated seeds (M1) were analysed.

The table summarises the frequency of nuclei with different dispositions of wheat and rye chromatin.

retrotransposons (Martienssen and Baron, 1994). Expression patterns of many genes have been correlated with differential methylation of alleles or promoter regions either associated with hypermethylation or hypomethylation. For example, the 'nucleolar dominance' phenomenon in sexual hybrids, where the rRNA genes from one parental species are suppressed in the presence of the other parental species, has been widely studied. In hybrids between wheat and rye, and the amphiploid Triticale, the partial inactivation of rye rRNA genes has been correlated with methylation (reviewed in Neves et al., 1997). Many studies indicate that methylation could suppress gene expression through an indirect mechanism affecting chromatin structure (Davey et al., 1997; Kass et al., 1997; Bergman and Mostoslavsky, 1998). With respect to transposon insertions, methylation may mediate interaction between the sequences and chromatin factors which 'hide' the sequences from the rest of the genome (Kass et al., 1997).

The drug 5-azacytidine (5-AC) is a structurally modified cytosine analogue that is a potent inhibitor of methyltransferases and can be incorporated into the DNA during replication leading to hypomethylation of genomic DNA. The effects of 5-AC treatment on altering methylation patterns have been studied at the molecular level both in genomic DNA and in particular sequences, using restriction enzymes sensitive to methylation (Bezdek et al., 1991; Kovarik et al., 1994; Vyskot et al., 1995; Glyn et al., 1997). 5-AC treatments are known to cause activation of silent genes (Jones and Taylor, 1980; Cedar and Razin, 1990) and can activate rRNA genes from rye origin in Triticale (Vieira et al., 1990; Neves et al., 1995). Bezdek et al. (1991) used 5-AC treatment to reduce the methylation level of DNA repeats largely clustered at subtelomeric regions in tobacco chromosomes, and these tandem repeats can maintain their hypomethylated state in the course of protoplast and plant regeneration (Koukalova et al., 1994). It has been reported that the phenotypes induced in the first generation of exposure to 5-AC can be inherited in subsequent generations (Amado et al., 1997): the progeny of 5-AC plants may show phenotypic differences from untreated Triticale plants (Heslop-Harrison, 1990), although methylation patterns change during plant development and particularly meiosis (Silva et al., 1995) and embryogenesis (Castilho et al., 1995). The rye-origin rRNA gene expression in the progeny of Triticale plants, treated at different stages with 5-AC, showed a gradual increase in expression and, in the next generation, modified expression patterns (Amado et al., 1997).

Here, we aimed to investigate genomic organization and the distribution of 5-mC residues in Triticale, and to see if hypomethylation induced by 5-AC was related to alterations in chromosome or genome organization. We also examined changes in methylation during development and in the generation following treatment, using different DNA sequences and Southern hybridization with methylation-sensitive restriction enzymes, to investigate any genome-specific effects, the occurrence of de novo methylation and the transmission of methylation patterns.

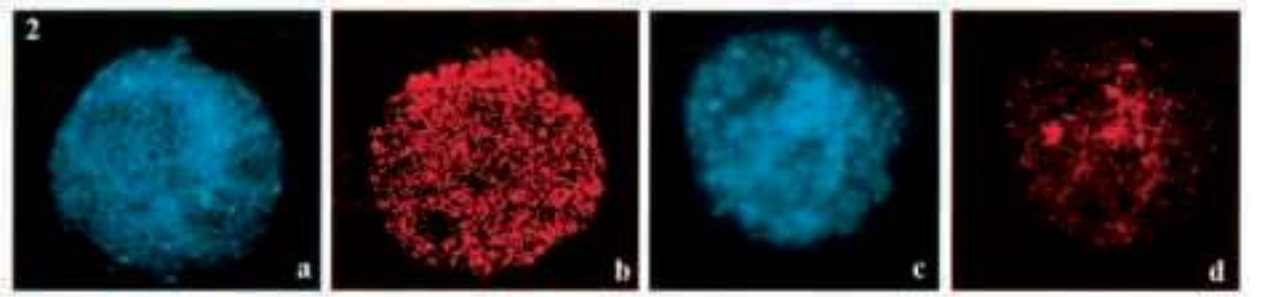

Fig. 2. In situ immunodetection of the 5-methylcytosine (5-mC) residues on Triticale squashed root-tip interphase nuclei. (a,c) DAPI (blue) staining of chromatin; (b,d) distribution of 5-mC residues (detected red) in water and 5-azacytidine germinated seeds, respectively.

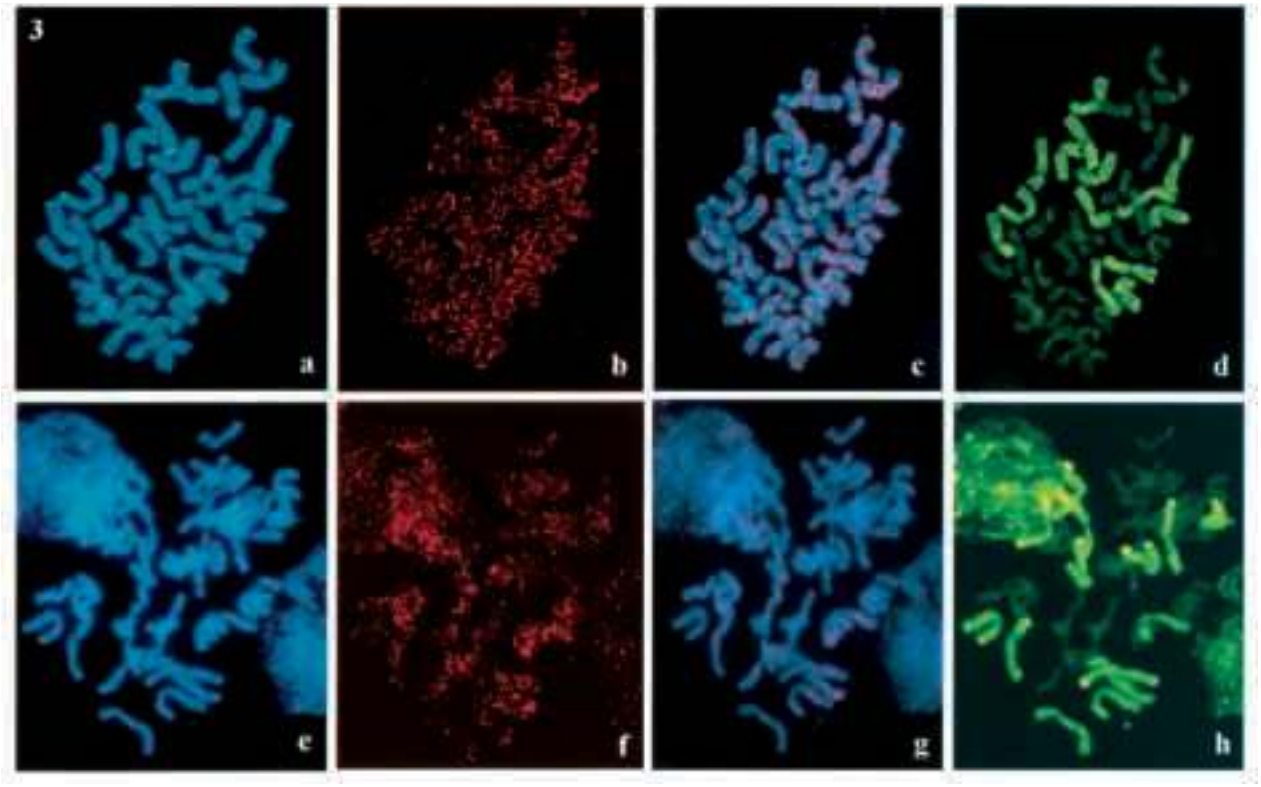

Fig. 3. In situ immunodetection of the 5-methylcytosine (5-mC) residues and sequential rye genomic in situ hybridization on Triticale squashed root-tip metaphase cell from a water germinated seed (a-d) and a 5-azacytidine treated seed (e-h). (a,e) DAPI stained chromosomes; (b,f) distribution of 5-mC residues (detected red); (c,f) superimposed images of DAPI stained chromosomes and hybridization signal of 5-mC residues; (d,h) in situ hybridization allowing detection of wheat (detected dark green) and rye-origin chromosomes (detected bright green).

MATERIALS AND METHODS

Plant material

Seeds from Triticale (× Triticosecale Wittmack) 'Lasko' were germinated for 3 days at 25°C in Petri dishes containing either water or a 10 μg/ml solution of 5-azacytidine (5-AC; prepared fresh every day); these were referred to as the M0 generation. The level of treatment was 10-10^3 times lower than that used by other authors (e.g. Heslop-Harrison 1990; Glyn et al., 1997). Seeds from plants, grown from seeds treated in the same way with 5-AC, were also germinated in water (M1 generation).

Chromosome preparation

Root tips approximately 15 mm long were collected and fixed in 1:3 (v/v) acid acetic:ethanol for at least 2 days. Mitotic preparations of squashed nuclei were made as described by Neves et al. (1995).

For confocal microscopy, root tips were collected and fixed in 4% (w/v) paraformaldehyde in PEM (50 mM Pipes, 5 mM EGTA, 5 mM $MgSO_4$, adjusted to pH 6.9 with KOH) for 1 hour at room temperature, followed by washing for 10 minutes in TBS (10 mM Tris-HCl, 140 mM NaCl, pH 7.4). Root tips were sectioned under water into 30 μm thick sections using a Vibratome Series 1000 (TAAB Laboratories, Aldermaston, UK). Sections were placed immediately on multiwell slides (INC Biomedicals Inc., Costa Mesa, CA) coated with glutaraldehyde-activated-aminopropyl triethoxy silane (APTES, Sigma), and left to air dry.

After collection of root tips, plants were potted up and allowed to grow in the greenhouse for DNA extraction from leaves.

DNA extractions

Total genomic DNA was extracted from Triticale root tips germinated in water, in 5-AC (M0) and from the progeny of 5-AC treated seedlings (M1). To follow induced hypomethylation during growth, seeds germinated in 5-AC were grown and DNA was extracted from leaves at weekly intervals, coded by 'Ext' followed by the number of days since potting. As controls, we used DNA from young leaves of rye 'Petkus' and tetraploid wheat *Triticum turgidum* ssp. *durum*. DNA extraction followed the procedures described by Sharp et al. (1988).

Sequential immunodetection of 5-methylcytosine residues and rye genomic hybridization to squashed nuclei

Chromosome slide preparations were treated in 10 μg/ml RNase solution for 1 hour at 37°C in a humid chamber. After washing in PBS (0.14 M NaCl, 8 mM Na_2HPO_4, 1.8 mM KH2PO4, 2.7 mM KCl, pH 7.4), the slides were dehydrated in 70% and 100% (v/v) ethanol and air dried. The slides were then blocked for 30 minutes in PBST (PBS supplemented with 0.5% Tween 20) containing 1% (w/v) bovine serum albumin.

Detection of methylation patterns was performed by incubating the

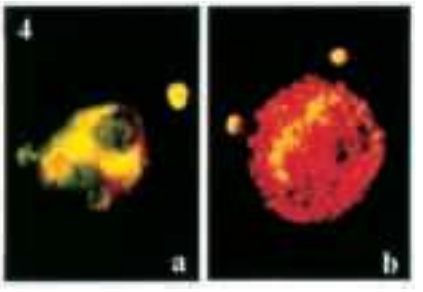

Fig. 4. Root-tip interphase cells of Triticale after rye genomic in situ hybridization from a seed (a) germinated in water showing one micronucleus of rye origin (detected yellow) and (b) germinated in 5-azacytidine showing two micronuclei from rye origin.

slides in a 1 μg/ml solution of a mouse monoclonal antibody raised against 5-methylcytosine (5-mC; Podestá et al., 1993) for 1 hour at 37°C in a humid chamber. Slides were washed in PBST and a secondary antibody, biotinylated-anti mouse IgG (H+L; Vector) was applied (1:2,000 dilution in PBST) to each slide for 1 hour at 37°C in a humid chamber. The hybridization signal was detected by incubating the slides in a 1:300 dilution in PBST of Streptavidin-Cy3-conjugate for 1 hour at 37°C in a humid chamber. Finally, the slides were washed and stained in DAPI.

The methylation patterns were analysed under an epifluorescence microscope and microphotographs were taken on Fuji Superia 400 ASA films.

Genomic in situ hybridization using as a probe total genomic DNA from rye and blocking DNA from *Triticum turgidum* ssp. *durum*, was performed to identify any differences in the methylation patterns of the two Triticale genomes. The slides were first washed in PBST overnight to remove traces of mountant and dehydrated through an ethanol series (70% and 100%). Total genomic DNA from rye was labelled with digoxigenin-11-dUTP by nick translation. The preparation of hybridization mixture and in situ hybridization procedures followed those described in Castilho et al. (1996).

Genomic in situ hybridization for optical sectioning of nuclei

Tissue sections on slides were treated with 2% (w/v) cellulase (Onozuka R-10) in TBS for 1 hour at room temperature in a humid chamber. After washing in TBS, the procedures for genomic in situ hybridization followed those for squashed nuclei except that rye total DNA was labelled with biotin-16-dUTP by nick translation. Confocal optical section stacks 1 μm thick were collected using a Leica TCS NT confocal scanning microscope. Images were transferred to a Macintosh computer and assembled into composite images using Photoshop (Adobe) and NIH image programmes.

Southern hybridization and probe preparation

Total genomic DNA from Triticale samples and control plants was digested with an excess of methylation-sensitive restriction enzymes *Hpa*II (GibcoBRL) and *Cfo*I (Roche) according to the manufacturers' instructions. DNA digests were fractionated in 1% agarose by gel electrophoresis. After alkaline DNA transfer to Hybond N+ (Amersham), the non-radioactive chemiluminescence method, ECL (Amersham) was used for probe labelling, hybridization and detection of hybridization sites following the manufacturers' instructions and methods described by Anamthawat-Jónsson et al. (1990). The blots were hybridized with different DNA sequences obtained as follows: pTa71, containing a 9 kb *Eco*RI fragment of the 18S-25S rDNA isolated from *T. aestivum* (Gerlach and Bedbrook, 1979) and recloned in pUC19. The whole clone was digested with *Hin*dIII and the linearized plasmid was used as probe. pScR4, containing a 2.4 kb *Taq*I fragment of the rye intergenic spacer (Appels et al., 1986). The clone was digested with *Taq*I for 40 minutes at 65°C and the 2.4 kb fragment was isolated, cleaned and used as probe. pSc200 and pSc250, containing 521 and 476 bp fragments, respectively, of rye repetitive DNA sequences mapped in the terminal heterochromatin (Vershinin et al., 1995). The inserts were amplified by PCR and used as probes.

Internal domains of the reverse transcriptase (RT) gene of *copia* group retrotransposons were obtained from rye (Petkus) and tetraploid wheat using PCR with flanking oligonucleotide primers corresponding to the peptide sequences TAFLHG (upstream) and YVDDML (downstream) (Flavell et al., 1992). The retrotransposon pool was used as probe. *Gypsy* group retrotransposons from rye (Petkus) and tetraploid wheat were amplified by PCR using flanking primers specifying the peptide sequences RMCVDYR (upstream) and YAKLSKC (downstream) (Dr A Brandes, personal communication). The PCR reactions were similar to those used for the *copia* group except the programme was 94°C for 3 minutes followed by 30 cycles each comprising 1 minute at 94°C, 50 seconds at 39°C and 40 seconds at 72°C, with a final elongation of 50 seconds at 30°C and 5 minutes at 72°C. The *gypsy* fragments were about 400 bp long.

RESULTS

Triticale chromatin organization

Confocal optical sections of Triticale root-tip nuclei following genomic in situ hybridization showed that wheat and rye genomes tended to occupy non-intermixed domains, seen as discrete regions of labelled and unlabelled chromatin through the nuclear volume (Fig. 1a,b; Table 1). Nuclei from root tips germinated in 5-AC showed a notably different organization, with interspersion of chromatin from both genomes (5-AC M0; Fig. 1c; Table 1). Some effects of 5-AC are known to be transmitted to the next generation (Heslop-Harrison, 1990; Koukalova et al., 1994; Amado et al., 1997) so we also analysed the organization of genomes in root tips of seedlings from the progeny of plants treated with 5-AC (5-AC M1, Table 1); these showed essentially an intermediate organization between water and M0 treatments.

In situ immunodetection of the 5-methylcytosine

Genomic methylation was studied by in situ immunodetection of 5-mC residues on squashed Triticale nuclei, followed by genomic in situ hybridization to determine the origin of each chromosome in the nuclei. In mitotic cells germinated in water, strong 5-mC antibody signal was detected at interphase (red; Fig. 2a,b) and on metaphase chromosomes (Fig. 3a-c). Interphase nuclei showed a relatively uniform signal. Chromosomes from metaphase cells showed a punctuate pattern along both arms, with some chromosome arms or segments being more strongly labelled than others (Fig. 3c), suggesting that methylation is not uniform throughout the genome. However, homologous chromosome pairs did not always show similar patterns, and no bands correlating with repetitive DNA at telomeric, centromeric or rDNA sites were evident. After 5-AC treatment, the fluorescent signal was weaker and dispersed in both interphase nuclei (Fig. 2c,d) and along metaphase chromosomes (Fig. 3e-g), indicating that the drug lead to hypomethylation of the genomic DNA.

The sequential genomic in situ hybridization identified rye-origin chromosomes, and the results obtained for cells germinated in water (Fig. 3d) and in 5-AC (Fig. 3h) showed no evidence for difference in methylation of the wheat and rye genomes. The distribution of 5-mC was similar on chromosomes from both genomes and the reduction in methylation levels induced by 5-AC treatment was not genome-specific.

The analysis of the genomic in situ hybridization results showed two other effects that were confined to the 5-AC treated roots. A significant increase in the frequency of micronucleated cells (Fig. 4) was observed after 5-AC treatment in comparison to untreated plants (from 2% to 11% in 100 cells scored in each case). The hybridization showed that the micronuclei contained chromosomes or chromosome fragments of rye origin (Fig. 4a,b), but unlabelled, wheat-origin micronuclei were not observed. 5-AC treatment was also found occasionally to induce multiple translocations between wheat and rye chromosomes (Fig. 5), which were never detected in water germinated plants. These included several types of chromosome rearrangements seen as terminal, intercalary, centromeric and near centromeric

translocations. Some individual chromosome fragments were seen but the method would not identify wheat-wheat or rye-rye translocations.

Effects of 5-AC on CG methylation

We made genomic DNA restriction digestions from root tips (water, 5-AC M0 and M1 generations) and leaves (water and various ages of 5-AC plants) to examine the methylation status of eight repetitive sequences by Southern hybridization (Figs 6 and 7). *Hpa*II recognises CCGG and is sensitive to methylation at either cytosine residue, while *Cfo*I recognises GCGC and is sensitive to methylation at the 3′ cytosine. Although no clear differences between lanes were visible in the ethidium bromide-stained gels (Figs 6a, 7a), as expected, 5-AC treatment reduced DNA methylation so that there were fewer short fragments hybridizing with all probes in DNA from the water (lane 1 in all figure parts) compared to the 5-AC treated root tips (lane 2 in all figure parts) where more, shorter, unmethylated, fragments were seen. The hybridization of all sequences to root-tip DNA from the M1 generation of treated plants (lane 3 in all figure parts) resembled that of water plants, indicating that these sequences had become remethylated.

Southern hybridization with the cloned repetitive sequences (pTa71, pScR4, pSc200 and pSc250) hybridized to leaf DNA extracted from untreated leaves, and at various times after 5-AC treatment, showed no obvious differences from untreated root-tip DNA (Figs 6, 7). The result indicates that the sequences probed were either remethylated in the period between extractions or that the 5-AC had no effect on leaf DNA.

We also investigated the status and patterns of methylation of the *gypsy* and *copia* groups of retrotransposons. The hybridization shows differences in abundance of the wheat and rye pools between the wheat and rye genomes (lanes 10, 11). For all retrotransposons, differences that could be easily ascribed to 5-AC effects on methylation were again seen between root-tip DNA from water and 5-AC treatments. The rye *gypsy* pool showed no obvious differences in hybridization pattern between water root tip and leaf DNA digests (Fig. 6d). However, a remarkable difference was detected between root-tip and leaf DNA for the organization of *copia* elements (Fig. 7), with a major difference in the banding pattern being found between the two tissues, although there was no effect of 5-AC or time seen in the different leaf patterns. Similar bands were seen in the control wheat and rye DNA. In all leaf samples four distinct bands of 2.0, 1.9, 1.2 (band of highest intensity) and 0.6 kb were detected.

DISCUSSION

Domains at interphase

Individual chromosomes of both plants and animals are not intermixed at interphase but remain in discrete domains (see Heslop-Harrison and Bennett, 1990; Lichter et al., 1988; Cremer et al., 1993). In synthetic animal cell fusion and sexual plant hybrids, parental genomes are also known to remain in discrete domains (Zelesco and Graves, 1988; Dekken et al., 1989; Leitch et al., 1990, 1996; Schwarzacher et al., 1992). The positions of parental genomes within a species had rarely been studied at interphase because of the difficulty in identifying maternal and paternal chromosomes. Using radioactively labelled sperm, Odarchenko and Keneklis (1973) have shown that rat parental genomes remain apart for several cell cycles. Gleba et al. (1987) showed that chromosomes from the genomes of *Nicotiana* cell-fusion hybrids were in separate genome domains, but treatment with colchicine caused genomes to become intermixed. In sexual diploid F_1 intergeneric hybrids (2n=2x=7+7) between cereals analysed previously (Leitch et al., 1992; Schwarzacher et al., 1992), whole genomes remained in single domains. Lima-Brito et al. (1996) found that chromosomes from each genome were grouped in complex sexual hybrids including wheat, rye and *Hordeum* genomes. Within allopolyploid plants, there are indications that genomes remain separated not only in synthetic hybrids, but also in polyploid species such as *Millium montianum* (Bennett and Bennett, 1992). The results presented here show that the rye (R) and wheat (A and B) genomes in untreated cells of the polyploid hybrid species Triticale are not intermixed, but groups of chromosomes from the rye, R, genome remain together (Fig. 1a,b; Table 1).

Treatment of plants with 5-azacytidine is well known to reduce the level of DNA methylation. Here, we find it disrupts the nuclear organization seen in untreated plants: after treatment, most nuclei did not show R genome chromosomes in clear domains (Fig. 1c; Table 1). DNA methylation and gene regulation patterns have been shown to be related in several cases, with hypomethylation of DNA being associated with active transcription (Finnegen et al., 1993); this effect might also be correlated with reorganization of the nucleus. Neves et al. (1995) have shown in Triticale that the expression of R-genome 18S-25S rRNA genes is correlated with their methylation. In root tips treated with 5-AC, 90% of cells had active genes, contrasting with only 7% of cells from untreated seeds. Amado et al. (1997) studied the activity of rRNA genes in the M1 generation and found that the 5-AC treatment effects were still detectable, with 60% of cells showing rRNA gene activity. Our results show these expression results correlate with chromosome disposition: 60% of nuclei from the M0 plants and 42% of M1 nuclei showed no clear domains, compared to only 15% in the untreated cells (Table 1). In our experiments looking at chromosome disposition, we cannot distinguish the interrelated effects of drug treatment, of 5-AC on gene expression and of any direct 5-AC effect on nuclear organization.

Distribution of 5-methylcytosine in the genome

We were able to investigate in situ the correlation between 5-azacytidine treatment and the status of DNA methylation. In normal Triticale plants, the monoclonal antibody against 5-methylcytosine showed that the R and wheat genomes were strongly methylated and the distribution of 5-mC residues was not uniform along the chromosomes: some regions are more methylated, as seen by the punctuated pattern of hybridization (Figs 2a,b, 3a-d). The particular location of 5-mC residues in different chromosomal regions has been previously reported in humans (De Capoa et al., 1995) and in plants (Frediani et al., 1996; Siroky et al., 1998). As found by Frediani et al. (1996) in *Vicia faba,* we found no preferential hybribization of the anti-methylcytosine antibody to particular chromosomal regions although there were some regions or chromosome arms with higher levels of antibody binding. We also found little equivalence in 5-mC distribution between homologous pairs, as reported by Frediani et al. (1996). The use of genomic in situ hybridization, enabling the discrimination of the R and wheat

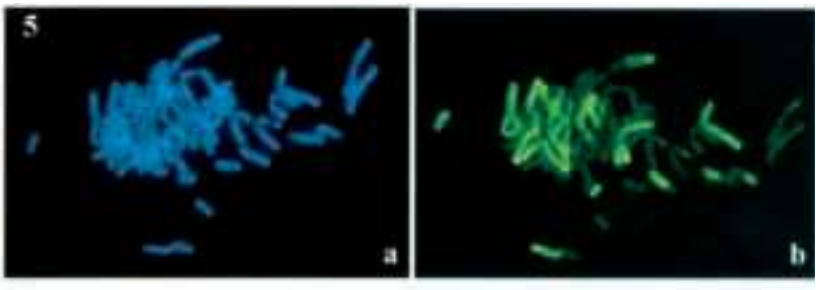

Fig. 5. Metaphase cell of Triticale from a seed germinated in 5-azacytidine. (a) DAPI stained chromosomes; (b) after rye genomic in situ hybridization showing multiple chromosomal rearrangements involving different types of wheat-rye translocations.

genomes (Fig. 3d), showed no evidence that the large-scale methylation pattern was genomic-specific since the 5-mC residues showed similar distributions in both genomes.

In plants germinated in 5-AC, the results show that the drug is effective in inducing genome-wide DNA hypomethylation, but again there is no evidence for genome-specific effects: both R and wheat genomes seem to be affected in a similar way by the 5-AC treatment (Figs 2c,d, 3e-h). The fact that the 5-AC treatment does not lead to a complete loss of DNA 5-mC is supported by other results, which indicated that some cytosine sites remain methylated (Bezdek et al., 1991).

Induction of micronuclei and translocations by 5-AC

In normal Triticale plants, micronuclei and related chromosome instabilities are a rare (but not unknown) phenomenon. After 5-AC treatment during seed germination, micronuclei were frequently seen (Fig. 4). All the micronuclei detected originated from the rye genome (small wheat introgressions, or micronuclei with both rye and wheat chromosome fragments, would not be distinguished). Multi-nucleated cells and micronuclei have been observed in rat cells cultured in the presence of 5-AC (Parrow et al., 1989). The induction of unequal segregation of B chromosomes in root tips has also been seen in rye seedlings treated with 5-AC (Neves et al., 1992).

In some 5-AC treated root tips of Triticale, rye-wheat translocations were detected by genomic in situ hybridization (Fig. 5); such translocations were never seen in our untreated lines. 5-AC treatment has been reported to cause numerous types of chromosome structural abnormality. In *Allium cepa*, Leyton et al. (1995) found translocations and broken chromosomes (particularly evident in anaphases), while chromatid aberrations were seen in *Vicia faba* (Fucik et al., 1970) and chromosome rearrangements in rice, maize and soybean (Sano et al., 1990; Katoh et al., 1993). 5-AC treatment was reported to increase recombination between human chromosomes, suggested to be caused by somatic association involving constitutive heterochromatin (Kokalj-Vokak et al., 1993). The results presented here show that 5-AC treatment induces a range of chromosome instabilities in Triticale, which may relate to DNA methylation, or shock and unbalanced gene expression induced by the drug.

Methylation in leaves and differences between roots leaves

Many studies have shown that 5-AC is an effective inducer of hypomethylation (e.g. Figs 2, 3) shortly after treatment, while higher levels of methylation are detected in later DNA extractions from the same material (Bezdek et al., 1991; Heslop-Harrison, 1990; Amado et al., 1997). We aimed to analyse methylation changes by Southern hybridization at various times after 5-AC treatment to examine when de novo methylation occurred. Digestion with methylation-sensitive restriction enzymes showed that the low-concentration 5-AC treatments used here altered methylation in root-tip DNA extracted immediately after the seeds were exposed to the drug (Figs 6, 7) with respect to sequences in LTR-retroelements and tandemly repeated DNA (sequences pSc200 and pSc250 from terminal heterochromatin of rye; Vershinin et al., 1995, 1996; and the rDNA). In general, the DNA from 5-AC-treated roots showed more short fragments and a stronger ladder of fragments, consistent with the more complete DNA digestions expected to be obtained from unmethylated DNA using methylation-sensitive restriction enzymes. No differences were seen in the behaviour of rye-origin or wheat-origin sequences, in agreement

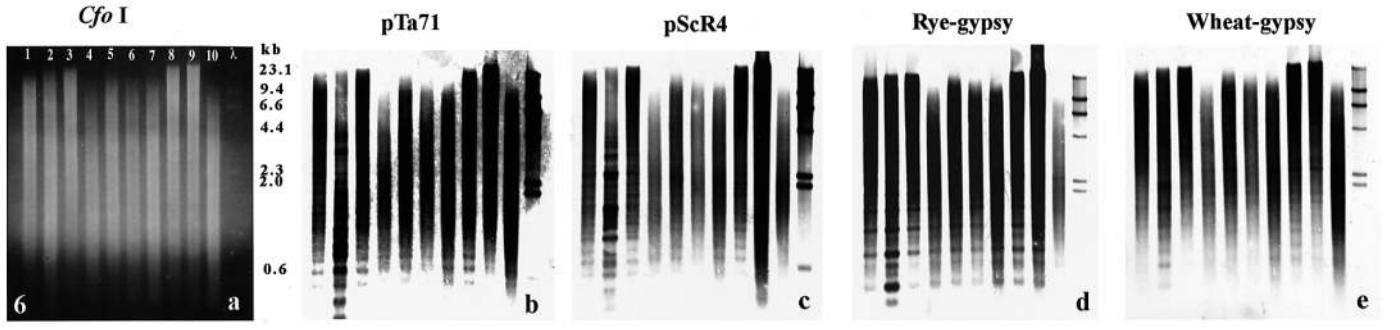

Fig. 6. CG methylation pattern analysis through Southern blot hybridization. (a) Ethidium bromide-stained gel of *Cfo*I genomic DNA digests from Triticale root tips germinated in water (lane 1); after germination in 5-AC (lane 2); M1 generation of treated root tips (lane 3); and from Triticale leaf DNA from seeds germinated in water (lane 4); treated in 5-AC during seed germination (lane 5, Ext13, extraction 13 days after 5-AC treatment ended) and from later leaf stages (lanes 6-8, Ext20-34). Leaf DNA digests were also obtained from control plants of rye Petkus (lane 9) and wheat *Triticum durum* (lane 10). Restriction fragments after hybridization to (b) total rDNA sequences (pTa71); (c) rye-origin, largely rye-specific intergenic spacers of rDNA (pScR4); (d) rye origin *gypsy* group of retroelements and (e) wheat origin *gypsy* group of retroelements. Lane 11 shows the DNA size marker λ *Hin*dIII.

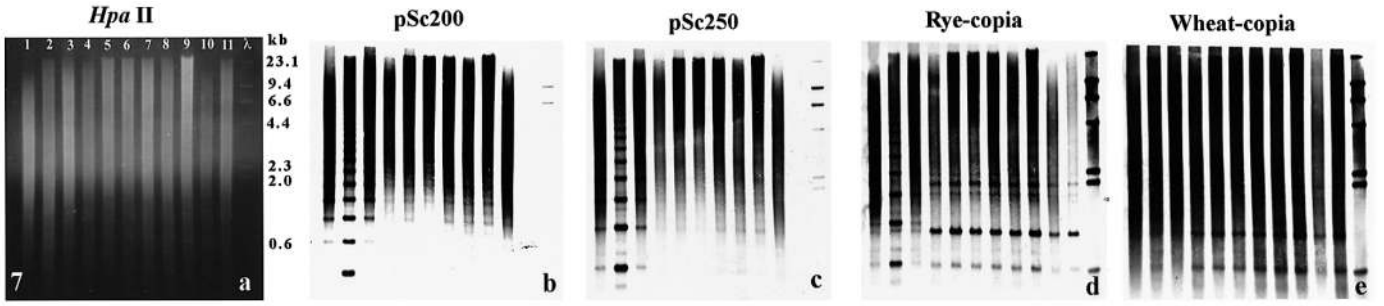

Fig. 7. CG methylation pattern analysis by Southern blot hybridization. (a) Ethidium bromide-stained gel of *Hpa*II genomic DNA digests from Triticale root tips germinated in water (lane 1); after germination in 5-AC (lane 2); M1 generation of treated root tips (lane 3); and from Triticale leaf DNA from seeds germinated in water (lane 4); treated in 5-AC during seed germination (lane 5, Ext13) and from later leaf stages (lanes 6-9, Ext20-41). Leaf DNA digests were also obtained from control plants of rye Petkus (lane 10) and wheat *Triticum durum* (lane 11). Restriction fragments after hybridization to (b) rye origin repetitive subtelomeric DNA sequence pSc200 (c) and pSc250 (d) rye origin *copia* group of retroelements and (e) wheat origin *copia* group of retroelements. Lane 12 shows the DNA size marker λ *Hin*dIII.

with the in situ hybridization results showing no genome-specific effects. In the M1 roots, methylation patterns had reverted to being similar to those in the untreated roots: the resetting of methylation patterns between generations was described during embryogenesis (Castilho et al., 1995) and, for the rDNA genes, during meiosis (Silva et al., 1995).

In leaf DNA, we found no changes in methylation patterns between control plants and extractions at different times after treatment (13-41 days). These results contrast with previous reports (Neves et al., 1995; Glyn et al., 1997) where hypomethylation was observed in leaf material. However, the dosage of 5-AC used here was lower than previous studies: Glyn et al. (1997) used two different concentrations of 5-AC and showed that higher dosages are more effective in causing DNA hypomethylation. In our experiments we have used 5-AC concentrations 10^3 times lower, so it may be the drug did not reach the leaf methylases, or had only very transient effects. Despite not finding changes in leaf methylation either in the ethidium bromide-stained digests or using a range of repetitive DNA probes (Figs 6, 7), several studies have shown that DNA methylation is modulated during plant development and aging (Klass and Amasino, 1989). Kazmierczak (1998) has shown that DNA methylation in *Vicia faba* progressively increases from the first leaves of seedlings, through roots, meristmatic buds or differentiated roots, young leaves, old leaves or cotyledons.

We found a surprising difference between root and leaf DNA regardless of the 5-AC treatment (Fig. 7). Using the rye *copia* pool of retroelements as a probe, different restriction fragments were obtained from leaf and root DNA, indicating a different genomic organization between the two tissues. The single blot was reprobed four times (Fig. 7b-e), so the differences cannot be attributed to DNA digestion or loading as no notable differences are seen between leaf and root lanes using the tandemly repeated probes pSc200 and pSc250.

The results presented here show that DNA methylation varies between tissues and changes during plant development. 5-AC-induced DNA hypomethylation affects various repetitive DNA sequences and is not specific to a particular genome or chromosome region in Triticale. Some changes induced during seedling germination are inherited, and visible not only through altered rRNA gene expression but also in the organization of the nucleus studied by confocal microscopy and in situ hybridization.

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