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5-azacytidine induces chromosomal breakage in the root tips of wheat carrying the cuckoo chromosome $4S^L$ from *Aegilops sharonensis*

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The cuckoo chromosome $4S^L$ from *Aegilops sharonensis* is preferentially transmitted when introduced by hybridization into common wheat, *Triticum aestivum*. Gametocidal (*Gc*) factors carried in $4S^L$ induce chromosome breakage in meiospores not containing them, ensuring their transmission to the progeny. Chromosome breakage and break–fusion–bridge (BFB) cycles can also be observed during early embryo sac development of chromosome $4S^L$ addition lines to wheat, often leading to the presence of dicentric chromosomes in the subsequent progeny. However, the process responsible for inducing the primary chromosomal breaks only appears to occur during the initial divisions of the embryo and endosperm. In the presence of chromosome $4S^L$, treatment with the hypomethylating agent 5-azacytidine induces chromosome breakage in root tips. This suggests that the process of chromosome fragmentation, induced by the *Gc* factors during early seed development, is repressed at later stages by DNA methylation.

Keywords: 5-azacytidine, *Aegilops sharonensis*, BFB cycles, chromosome breakage, DNA methylation, gametocidal chromosomes.

Introduction

Gametocidal or 'cuckoo' chromosomes have been found in a number of *Aegilops* species (for a review see Endo, 1990). Cuckoo chromosomes are preferentially transmitted when introduced by hybridization into common wheat, *Triticum aestivum* ($2n = 6x = 42$, AABBDD). When present in the sporophyte, gametocidal (*Gc*) factors in the cuckoo chromosomes induce chromosomal breakage in the gametes not containing them, ensuring their own transmission to the progeny (Finch *et al*., 1984; Nasuda *et al*., 1998). Plants carrying *Gc* factors in the hemi- or heterozygous form are semi-sterile. However, plants homozygous for *Gc* factors are fully fertile. Mutations such as deletions and other rearrangements have been detected among the progeny of plants hemi- or heterozygous for the *Gc* factors. These are thought to arise from gametes that do not carry the *Gc* gene(s) and suffer chromosomal breakage at levels that are not lethal (Endo, 1988a,b, 1990). This phenomenon has been employed to produce sets of deletion stocks in the common wheat cultivar Chinese Spring (CS), using the cuckoo chromosome 2C from *Ae. cylindrica* (Endo & Gill, 1996). The mechanism inducing the chromosomal aberrations is not understood.

The cuckoo chromosome $4S^L$ from *Ae. sharonensis* ($2n = 2x = 14$, S^LS^L) has a very strong gametocidal action in CS and other wheat cultivars (King *et al*., 1991). Chromosome fragments, usually in the form of pairs of equal length single chromatid segments (SCSs), have been observed during early embryo and endosperm development of plants carrying cuckoo chromosomes (King & Laurie, 1993; de las Heras, 1999). Broken chromosome ends exhibit a tendency to fuse and form dicentric chromosomes (McClintock, 1941; Werner *et al*., 1992). These dicentrics usually give rise to chromatin bridges at anaphase and may break at telophase, thus perpetuating the break–fusion–bridge (BFB) cycle after fusion of the newly broken chromosome ends.

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The mechanism for the *Gc*-induced chromosomal damage appears to be switched off early during embryo and endosperm development (King & Laurie, 1993; de las Heras, 1999). Root tips from plants carrying the $4S^L$ cuckoo chromosome do not show chromosome fragmentation. However, dicentric chromosomes are sometimes observed, presumably resulting from chromosome fragmentation during late gametogenesis and/or early embryogenesis. In the work presented here we demonstrate that the DNA hypomethylating agent 5-azacytidine (5-AC) induces chromosome fragmentation in the root tips of plants carrying the cuckoo chromosome $4S^L$, but not in plants lacking this chromosome.

Materials and methods

Seeds from common bread wheat *Triticum aestivum* ($2n = 6x = 42$, AABBDD) var. Chinese Spring (CS), and the monosomic and disomic additions of the chromosome $4S^L$ to CS (subsequently referred to as $4S^L$mo and $4S^L$di, respectively), were placed on water-soaked filter paper in duplicated Petri dishes at 4°C for 3 days. After prechilling, the filter papers were replaced with fresh ones. Half of the filter papers in the duplicates were soaked in water, and the other half in a 100-μM 5-azacytidine (5-AC) solution. The seeds were then incubated in the dark at 27°C for 3 days to induce germination. The filters were renewed every 24 h. After germination, root tips from each dish were collected and fixed in a 3:1 ethanol:glacial acetic acid solution for 24–48 h at room temperature, and stored at 4°C. The root tips were stained with Feulgen reagent after hydrolysis with 1 M HCl at 60°C for 8–9 min, squashed in 45% acetic acid, and observed under the microscope.

Results

Observation of root tip cells at anaphase/telophase from CS wheat, germinated in either water or 5-AC, revealed the presence of only four abnormal divisions out of a total of 458 cells. These cells each contained a dicentric bridge, and chromosome fragmentation was not detected. The background level of dicentric bridge formation in euploid CS when germinated in water was less than 1% (Table 1). Treatment with 5-AC did not result in a significant increase of dicentric bridges.

Similar results were obtained for *Ae. sharonensis*, donor of the $4S^L$ cuckoo chromosome (preliminary observations, data not shown).

The root tips from plants monosomic or disomic for chromosome $4S^L$ germinated in water also contained dicentric bridges at a frequency similar to that observed in CS (Fisher's exact test, $P = 0.38$ and 0.71, respectively). Chromosome fragmentation was not observed. In contrast, treatment of root tips containing chromosome $4S^L$ with 5-AC resulted in chromosome fragmentation as well as dicentric bridges. These abnormalities appear identical to those observed during early embryo and endosperm development (King & Laurie, 1993; de las Heras, 1999), and pollen development (Finch *et al*., 1984; Nasuda *et al*., 1998). Although dicentric chromosomes and bridges were observed, chromosome fragmentation, i.e. cells containing single chromatid segments (SCSs), was by far the predominant form of aberration. In the monosomic addition, 5-AC treatment significantly increased the frequency of abnormal anaphases/telophases from less than 1% to about 9%, and in the disomic addition from 1% to about 16% (Table 1) (Fisher's exact test, $P \ll 0.001$ in both cases).

Table 1 Effect of 5-AC treatment on wheat: number of normal and abnormal cells (containing chromosome fragments and/or bridges) of $4S^L$di and $4S^L$mo root tips observed at anaphase and telophase

	Total cells observed	Normal	Abnormal	Ratio abnormal
CS	232	231	1	< 0.01
CS 5-AC	226	223	3	0.01
$4S^L$mo	399	398	1	< 0.01
$4S^L$mo 5-AC	118	107	11	0.09
$4S^L$di	590	587	3	0.01
$4S^L$di 5-AC	268	225	43	0.16

SCSs of various different sizes were observed, usually in pairs of equal length, and often associated at one end (Figs 1–3). Single and double chromatid bridges were observed, which indicates the occurrence of both chromatid and chromosome type BFB cycles, as described by McClintock (1941) and Lukaszewski (1995).

Discussion

Cytological effects of 5-AC in wheat root tips

Treatment with 5-AC resulted in the induction of chromosome fragmentation and break–fusion–bridge (BFB) cycles in wheat root tips, in the presence of the cuckoo chromosome $4S^L$. Most of the fragments are pairs of single chromatid segments (SCSs) of equal length, often associated at one end (Figs 1–3). This suggests that most of the chromosome breaks occur at the G1 phase, before DNA replication, or perhaps during replication. Similar chromosome fragments can be found during the early embryo and endosperm development in wheat plants carrying chromosome $4S^L$ (King & Laurie, 1993; de las Heras, 1999). The same type of chromosome breakage — albeit more

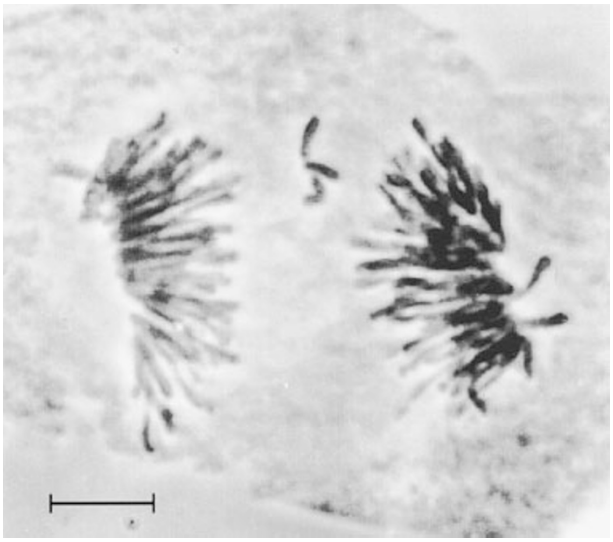

Fig. 1 A root tip anaphase from $4S^{L}$di wheat, treated with 5-AC, containing two dissimilar pairs of equal length SCSs associated at one end. Scale bar = 10 μm.

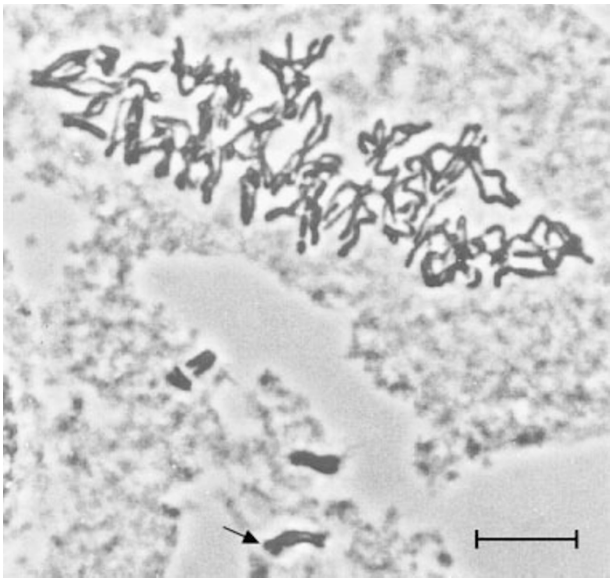

Fig. 2 A root tip cell from $4S^{L}$di wheat, treated with 5-AC, which is just starting to get into anaphase. Several chromosome fragments can be seen, including two similar pairs of equal length SCSs associated at one end, and what looks like a short dicentric chromosome (—►). Scale bar = 10 μm.

extensive — in the gametophyte is responsible for the preferential transmission of the cuckoo chromosomes (Finch *et al*., 1984; Nasuda *et al*., 1998). The fragmentation reported here in root tips seems likely to be induced by the same mechanism.

Dicentric chromosomes have been observed in euploid CS and in the $4S^{L}$ additions at low frequencies

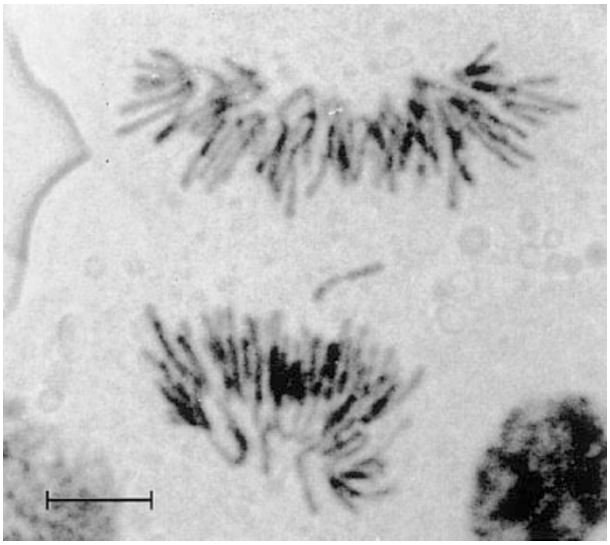

Fig. 3 A root tip anaphase from $4S^{L}$mo wheat, treated with 5-AC, containing a pair of equal length SCSs associated at one end. Scale bar = 10 μm.

(about 1%). However, chromosome fragmentation was detected only after 5-AC treatment when the cuckoo chromosome was present. Furthermore, the frequency of aberrations (i.e. chromosome fragmentation and/or dicentric chromosomes) was almost double in the root tips from the disomic addition (16%), compared to the monosomic (9%) (Table 1). This suggests that the amount of chromosome breakage in the root tips may be proportional to the number of copies of the *Gc* factors. This contrasts with the observations of the early embryo and endosperm development (King & Laurie, 1993; de las Heras, 1999). In these tissues, the number of copies of *Gc* factors does not directly correlate with the amount of chromosome damage induced by them. The frequency of chromosome aberrations in the embryo sac of disomic additions of $4S^{L}$ is intermediate between the aberration frequencies of the reciprocally obtained monosomic additions, with paternal transmission of the cuckoo having the strongest effect. This could be explained if the *Gc* factors, or the wheat target sequences upon which they act, were subjected to gametic imprinting (see below).

The primary effect of 5-AC is the nonspecific loss of methylated cytosine residues from the DNA during replication. The ability of 5-AC to induce hypomethylation and concomitantly reactivate silent genes in higher organisms is well documented (Groudine *et al*., 1981; Jaenisch *et al*., 1985; Jones, 1985; Klaas *et al*., 1989; Jones *et al*., 1990; Neves *et al*., 1995). Methylation patterns in cereals appear to change during development, as reported for wheat/rye hybrids (Neves *et al*., 1995; Amado *et al*., 1997). Thus, if target sites

for the *Gc* factors became unavailable due to methylation, this could explain the cessation of chromosome fragmentation during early embryo and endosperm development.

By generating chromosome fragmentation in root tips, using 5-AC in the presence of *Gc* factors, it should be possible to locate and clone 'freshly broken' chromosome ends which have not yet fused, using chromosome microdissection and subsequent amplification of the DNA sequences by degenerate-oligonucleotide-primed PCR (DOP-PCR), as exemplified by Guan *et al.* (1992) in the isolation of DNA from a human chromosome region frequently deleted in a type of skin cancer. The recognition of target sequences for the *Gc* system may be of key importance for the understanding of the mechanism inducing the chromosome breaks, and they may facilitate the future the development of vectors for DNA transfer into wheat.

Temporal activity of Gc factors and DNA methylation

Studies on the dominance of wheat nucleolar organisers (NORs) in wheat/rye hybrids showed that the rye NORs were suppressed by a DNA methylation-dependent mechanism (Neves *et al.*, 1995, 1996). The NOR from rye chromosome 1R was suppressed early during the seed development in embryo and endosperm, remained suppressed in the adult hybrid plant, and was reactivated prior to the first pollen grain mitosis (PGM1) during gametogenesis. This pattern interestingly coincides with the pattern of chromosomal breakage induced by the cuckoo chromosome. In addition, treatment with 5-AC is able to stably reactivate rye NORs in root tips and in the embryo sac, where they are normally inactive (Vieira *et al.*, 1990; Neves *et al.*, 1995, 1996). It was concluded that the meiotic reprogramming of the DNA methylation patterns was responsible for the rye NOR activity during pollen gametogenesis and in early embryo and endosperm development. A further change in the methylation pattern was found to take place at around day six post-fertilization which removed the gametic imprint, stably deactivating the rye NOR in both embryo and endosperm (Neves *et al.*, 1995). It is almost certain that similar changes in the methylation patterns take place in CS wheat and in the cuckoo addition lines. Similar changes in the methylation patterns have been detected in mammals, and are involved with the mechanism establishing and erasing gametic imprinting patterns (Tilghman, 1999 and references therein). Gametic imprinting has also been reported in angiosperms, especially involving endosperm factors (Kermicle & Alleman, 1990; Birchler, 1993).

Genomic imprinting

Gc factors contain two distinct functions: one induces chromosome fragmentation, whereas the other inhibits it (Endo, 1990). The phenotypic effects (i.e. chromosome fragmentation and derived aberrations) of the interactions between these two functions and the wheat genome within the embryo sac are probably determined not only by their relative dosages, but also by their parental origin. This suggests that *Gc* factors are subjected to gametic imprinting, such that the fragmentation-inducer function is partially silenced in the maternally derived cuckoo chromosomes, while the fragmentation-inhibitor function is silenced in the paternally derived cuckoos (de las Heras, 1999). This methylation-based mechanism would result in differential activity of the *Gc* factors during early embryo and endosperm development in reciprocally obtained monosomic additions, which fits the observations. An interesting implication of this idea (i.e. that *Gc* factor-induced fragmentation can be activated after hypomethylation) is that 5-AC treatment of meiocytes from a normally fertile $4S^L$ disomic addition should result in partial sterility.

Selective reactivation of genes by 5-AC has been frequently observed (Jones, 1985; Cedar, 1988), while at the same time, demethylation alone is clearly not enough to reactivate some genes, which need another secondary signal to be switched on (Doerfler, 1983; Jones, 1985). Thus, it is possible that in root tips 5-AC treatment may only be able to reactivate the fragmentation-inducer function of the *Gc* factors. This could explain the direct relationship between number of cuckoo chromosomes and amount of damage induced by them after 5-AC treatment in root tips.

Interference of epigenetic programmes in interspecific hybrids?

DNA methylation is considered to play a major role in genomic imprinting and developmental gene patterning, although the actual process is not well understood (Bartolomei & Tilghman, 1997). Genomic instability in plants and other eukaryotic organisms after treatment with methylase inhibitors has been previously reported. In particular, treatment with 5-AC can disrupt nuclear organization and may induce translocations and micronuclei in *Triticale* (Amado *et al.*, 1997; Castilho *et al.*, 1999). In addition, it can alter chromatin condensation and induce abnormal sister chromatid separation in wheat/rye hybrids (Glyn *et al.*, 1997). Although these reports do not mention either chromosome fragments of the type induced by cuckoo chromosomes or BFB cycles, they may have a common cause. It is

possible that the introduction of alien chromatin in a new genetic background disrupts the existing 'genomic programme', and this may be the cause of the observed genomic instability. This disruption could be particularly sharp if the alien chromatin contained important factors controlling this genomic programming. The strong gametocidal action of $4S^L$ might thus indicate that this chromosome contains factors that are dominant with respect to those of CS wheat. If this is the case, the study of cuckoo chromosomes might reveal important clues as to how those epigenetic processes work. It would be interesting to compare the effects of 5-AC in root tips from other chromosome addition lines, including some non-gametocidal group 4 chromosomes from other *Aegilops* species.

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