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ORIGINAL ARTICLE

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Characterization of a knock-out mutation at the *Gc2* locus in wheat

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Abstract Gametocidal (Gc) genes, introduced into common wheat from related *Aegilops* species, are selfish genetic elements that ensure their preferential transmission by inducing chromosomal breaks. Here we report the production and characterization of a knock-out mutation of the *Gc2* gene transferred to wheat as a wheat-*Aegilops sharonensis* T4B-4S^{sh}#1 translocation chromosome. In hemizygous *Gc2*/– condition, gametophytes lacking *Gc2* suffer chromosomal fragmentation and produce nonfunctional gametes, which leads to sporophytic semisterility and exclusive transmission of the *Gc2*-carrier chromosome. We have identified one putative ethyl methylsulfonate (EMS)-induced *Gc2* mutant that restores spike fertility and shows Mendelian segregation. Progeny screening mapped the mutation to the *Gc2*-carrier chromosome T4B-4S^{sh}#1. C-banding and fluorescence in situ hybridization analyses showed that the loss of *Gc2* function in the mutant is not due to a terminal deficiency. Analysis of first and second pollen mitoses in *Gc2*^{mut}/– plants and C-banding analysis of testcross progenies showed that no chromosomal breakage occurs in the mutant. No gametophytic chromosomal breakage was observed in heterozygous *Gc2*^{mut}/*Gc2* plants, which had fully fertile spikes. These results suggest that *Gc2* encodes two agents, one causing chromosomal breaks in gametophytes lacking *Gc2* and another that protects the *Gc2* carrier from breakage. The EMS-induced *Gc2* mutant appears to be a knock-out of the gene encoding the 'breaking' agent. These data are a first crucial step toward the molecular understanding of *Gc2* action.

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Introduction

Gametocidal genes are selfish genetic elements that act as segregation distorters. Segregation distortion was defined by Sandler and coworkers (Sandler and Novitsky 1957; Sandler et al. 1959) as the phenomenon where one of the alleles at a heterozygous locus transmits at a higher frequency than the expected Mendelian ratio. Segregation distortion is observed widely in diverse eukaryotic organisms including plants, animals, and fungi.

In plants, genes that cause abortion of gametes by allelic interactions are known in wheat (Loegering and Sears 1963), maize (Maguire 1963), tomato (Rick 1966), and rice (Sano 1990). These genes induce the abortion of gametes carrying the opposite allele, although homozygous plants show no adverse effects on gamete formation. The underlying molecular mechanism(s) leading to the abortion of the gametes are largely unknown.

In animals, the Segregation Distorter (*SD*) system of *Drosophila melanogaster* (Lyttle 1991, 1993) and *t*-haplotypes in mouse (Silver 1993) are the best studied. In *Drosophila*, the *SD* chromosomes are transmitted from *SD*/*SD*$^{+}$ males to more than 90% of the progenies, whereas female transmission is normal. The major gene required for distortion is *Sd*, which interacts with a Responder (*Rsp*) locus consisting of an array of repeated satellite DNA sequences. The copy number of these repeats is related to the sensitivity of the *Rsp* locus. Spermatids receiving a copy of the *SD* chromosome with a sensitive *Rsp* locus fail to reach maturity. Recently, the *Sd* locus was cloned and shown to encode a truncated RanGAGP nuclear transport protein (Merrill et al. 1999). It was postulated that the defective RanGAP encoded by *Sd* interferes with nuclear transport in spermatids carrying the sensitive *Rsp* locus, but the underlying molecular

mechanism causing selective sperm dysfunction remains unknown.

In mice, males heterozygous for the *t*-haplotype complex (*t*/+) located on chromosome 17 transmit the *t*-haplotype chromosome to 99% of their progeny. Several distorter/sterility loci (*Tcd*'s) in the *t*-haplotype complex impair flagellar function in all spermatozoa, whereas the responder locus (*Tcr*), normally also located in the *t*-haplotype complex, rescues *t*-sperm but not wild-type sperm, leading to the preferential transmission of the *t*-haplotype complex. Recombination between the *t*-haplotype complex and the corresponding wild-type region is suppressed by several inversions, which ensure the stability of the complex. Cloning of the *Tcr* locus revealed that it is a member of a new family of protein kinases (*Smok*) (Hermann et al. 1999). The *Tcr* gene is a fusion of two kinase genes (*Rsk3* and *Smok*) and has a reduced kinase activity. *Tcr* and *Smok* may be components of a single cascade that affects phosphorylation and dephosphorylation of the axonemal components essential for flagellar movement. Transgenic mice, in which *Tcr* was inserted into the Y chromosome, produced twice as many males as females.

In common wheat (*Triticum aestivum* L.), alien chromosomes that behave as selfish genetic elements were identified during the production of alloplasmic (Tsunewaki 1980, 1993; Endo 1990) or chromosomal addition lines (Endo and Katayama 1978; Miller et al. 1982; Kota and Dvorak 1988; Friebe et al. 1993, 1999). The selfish chromosomes were not eliminated although the lines were backcrossed to wheat several times (Endo and Tsunewaki 1975). The selectively retained chromosomes cause sterility of both male and female gametes that are lacking the alien chromosome and thereby ensure their preferential transmission (Endo 1978). These chromosomes were designated as gametocidal (Gc) chromosomes and the genes that cause these effects, Gc genes (Endo 1978).

Gc genes are known to be present on chromosomes 3C of *Aegilops caudata* L. (Endo and Katayama 1978), $3C^t$ of *Ae. triuncialis* L. (Endo and Tsunewaki 1975), $2C^c$ of *Ae. cylindrica* Host (Endo 1979, 1988, 1996), 2S and 6S of *Ae. speltoides* Tausch (Tsujimoto and Tsunewaki 1983, 1985a; Kota and Dvorak 1988), $2S^l$ and $4S^l$ of *Ae. longissima* Schweinf. & Muschl. (Endo 1985; Friebe et al. 1993; Tsujimoto 1994, 1995), $2S^{sh}$ and $4S^{sh}$ of *Ae. sharonensis* Eig (Maan 1975; Endo 1982; Miller et al. 1982), and $4M^g$ of *Ae. geniculata* Roth (Friebe et al. 1999).

The mode of Gc action is different from other segregation distorter systems including one reported in wheat (Faris et al. 1998) because it affects both male and female gametogenesis. Previous studies have indicated that Gc genes ensure their preferential transmission by causing chromosomal breaks in gametophytes lacking Gc genes (Finch et al. 1984; King and Laurie 1993; Nasuda et al. 1998). The action of the *Gc2* gene located on the *Ae. sharonensis* 'cuckoo' chromosome $4S^{sh}$ (Miller et al. 1982; Nasuda et al. 1998) is very strong and results in extensive chromosomal breakage prior to the S-phase of the first postmeiotic interphase in gametophytes lacking *Gc2*. As a result, only gametes with the *Gc2* gene are functional, which causes semisterility and leads to 100% transmission of the *Gc2* carrier chromosome to the offspring. Weaker Gc genes such as the one located on *Ae. cylindrica* chromosome $2C^c$ only induce moderate breakage, and the Gc chromosome is not selectively retained. In the offspring of such plants the recovery of chromosomal rearrangements is possible and allows for the production of deletion stocks in wheat (Endo and Gill 1996), barley, *Hordeum vulgare* L. (Shi and Endo 1999, 2000), and rye, *Secale cereale* L. (Friebe et al. 2000a).

In order to unravel the molecular mechanisms underlying Gc action, Gc knock-out mutations are needed. Ethyl methanesulfonate (EMS) is known to induce mainly point mutations in the form of G/C to A/T transitions (Krieg 1963). EMS-induced knock-out mutations can be easily assayed by TILLING (targeting induced local lesions in genomes) (McCallum et al. 2000a, 2000b). Here we report an efficient production scheme for identifying EMS-induced mutations that inhibit Gc function. One knockout mutation at the *Gc2* locus was identified and characterized cytologically. This study is the first step toward an understanding of Gc function at the molecular level.

Material and methods

Plant material

The material analyzed consisted of the wheat-*Ae. sharonensis* cuckoo disomic chromosome addition (DA) line $DA4S^{sh}$#1 (to distinguish it from other $4S^{sh}$ chromosomes of different origin), a derived disomic substitution (DS) line $DS4S^{sh}$#1(4B) where wheat chromosome 4B is missing and replaced by chromosome $4S^{sh}$#1, and a spontaneous wheat-*Ae. sharonensis* translocation (T) line where the distal region of the long arm of chromosome $4S^{sh}$#1 is translocated to the long arm of wheat chromosome 4B. The translocation chromosome can be described as T4BS·4BL-$4S^{sh}$#1L and is referred to hereafter as T4B-$4S^{sh}$#1. $DA4S^{sh}$#1 and $DS4S^{sh}$#1(4B) were produced by Miller et al. (1982), and the T4B-$4S^{sh}$#1 translocation line was identified by Endo (personal communication). All lines were kindly provided by Dr. T.R. Endo, Laboratory of Plant Genetics, Kyoto University, Kyoto, Japan.

Cytological procedures

The chromosomal constitution of the wild-type and mutant *Gc2* lines was analyzed by fluorescence in situ hybridization (FISH) and C-banding. FISH has been described previously by Zhang et al. (2001). *Ae. sharonensis*-derived chromatin in T4B-$4S^{sh}$#1 was identified using clone pGc1R-1. Clone pGc1R-1 is a 258 bp fragment of a tandem repetitive element cloned from a wheat-*Ae. speltoides* T2B-2S translocation line (Nasuda 1999). It hybridizes to telomeric and subtelomeric regions of *Ae. speltoides*, *Ae. sharonensis*, and *Ae. longissima* chromosomes but not to any other diploid *Aegilops* species or *T. aestivum* (Nasuda 1999; Friebe et al. 2000b; Zhang et al., unpublished data). The repeat fragment was inserted into the EcoRI/MseI restriction site of the pT-Adv plasmid vector and has 98% sequence homology to the 5' end of the S-genome-specific repetitive element pAesKB52 isolated by Anamthawat-Jonsson and Heslop-Harrison (1993). One microgram

of pGc1R-1 plasmid DNA was labeled with fluorescein-11-dUTP (Amersham Biosciences, Piscataway, N.J., USA) using nick translation according to the manufacturer's protocol. Chromosomes were counterstained with propidium iodide in Vectashield (Vector Laboratories, Burlingame, Calif., USA). Slides were analyzed with an epifluorescence Zeiss Axioplan 2 microscope. Images were captured using a SPOT CCD (charge-coupled device) camera operated with SPOT 2.1 software (Diagnostic Instruments, Sterling Heights, Mich., USA) and processed with Adobe Photoshop v5.5 (Adobe Systems, San Jose, Calif., USA).

C-banding and chromosome identification were according to Gill et al. (1991). Microphotographs of C-banded chromosomes were taken with a Zeiss photomicroscope III using Kodak Imagelink HQ 1461 microfilm. The first and second pollen mitoses were analyzed following the protocol of Nasuda et al. (1998). Briefly, the first pollen mitosis occurs when the spike is emerging from the flag leaf. Approximately 1 week later, the generative nucleus undergoes the second pollen mitosis and produces a pair of crescent-shaped sperm nuclei. In wheat the three anthers within a floret are approximately at the same developmental stage. The developmental stage is determined in one anther by aceto-carmine staining and the remaining two anthers undergoing the first and second pollen mitoses are fixed in a mixture of one part glacial acetic acid and three parts of ethanol for further analyses. Because the male gametophytes are surrounded by thick cell walls that appear transparent after aceto-carmine staining but have a strong autofluorescence, FISH analysis at first and second pollen mitosis is difficult. We tried several enzymatic treatments to remove the cell walls, but the best results were obtained without enzyme treatment. After transferring the anthers from the fixative for 30 s into 45% acetic acid, followed by squash preparation in aceto-carmine, the cell walls of a few gametophytes break and release their nuclei, which then are accessible for FISH analysis.

Mutagenesis

Homozygous *Gc2/Gc2* plants were crossed with euploid Chinese Spring wheat to produce 2200 hemizygous *Gc2/–* seeds that were treated for 24 h with 0.4% EMS in phosphate buffer according to Williams et al. (1992). After washing in tap water, the seeds were planted into root-trainers and grown in a greenhouse. The germination frequency was determined 3 weeks after planting and selfed-seed set was determined 4 weeks after flowering. In *Gc2/–* plants only *Gc2*-carrier gametes function, and thus, the spikes are semisterile and all self-progenies are homozygous for T4B-4S^{sh}#1 (*Gc2/Gc2*). Any mutation at the *Gc2* locus or another locus that inhibits *Gc2* action will produce functional gametes, whether or not the *Gc2*-carrier chromosome is present, which results in fertile spikes that can be scored visually. Fertile M_2 families can be analyzed by FISH using clone pGc1R-1 from *Ae. speltoides* to determine the transmission of the *Gc2*-carrier chromosome T4B-4S^{sh}#1. Segregating M_2 families for putative *Gc2* mutants can be further characterized cytologically and by progeny screening.

Results

Cytological markers for the *Gc2* carrier chromosome

Cytological markers were identified on the *Gc2* carrier chromosome to follow its behavior in cell divisions and to assay its integrity following mutagenic treatment. The C-banding pattern of chromosome 4S^{sh}#1 present in the DA4S^{sh}#1 addition line and the DS4S^{sh}#1(4B) substitution line are similar to those reported earlier for chromosome 4S^{sh} of *Ae. sharonensis* (Friebe and Gill 1995). Several C-band markers distinguish 4S^{sh}#1 from chromosome 4B of wheat. Chromosome 4S^{sh}#1 has prominent telomeric C-bands in both arms, whereas 4B of wheat has large proximal C-bands in both arms and a diagnostic subtelomeric C-band in the long arm (Fig. 1a). The C-banding pattern of the short arm and most of the long arm of the translocation chromosome T4B-4S^{sh}#1 is similar to that of wheat chromosome 4B. The long arm of T4B-4S^{sh}#1 has a subtelomeric C-band derived from 4B but the more prominent telomeric C-band is of 4S^{sh}#1 origin. These data indicate that the distal 15% of the translocated chromosomal arm was derived from 4S^{sh}#1L of *Ae. sharonensis*.

Clone pGc1R-1 does not hybridize to any wheat chromosome but hybridizes to the telomeric region of the long arm of T4B-4S^{sh}#1 (Fig. 1a, b). This clone is an excellent marker that allows the detection of the T4B-4S^{sh}#1 translocation in a wheat background.

Chromosomal breakage occurs only in gametophytes lacking the *Gc2*-carrier chromosome

Although there is strong genetic evidence that only *Gc2*-carrier gametes function in hemizygous *Gc2/–* plants, direct cytological evidence has been lacking. FISH analysis with clone pGc1R-1 of ana-/telophases of the first pollen mitosis in *Gc2/–* plants will provide direct evidence that chromosomal breakage only occurs in gametophytes lacking the *Gc2*-carrier chromosome T4B-4S^{sh}#1. In Gc2/– plants 50% of the gametophytes will have the *Gc2* marker chromosome and are expected to undergo normal first and second pollen mitoses and the remaining 50% without the *Gc2*-carrier chromosome are expected to suffer from breakage. In 17 ana-/telophases, the Gc1R-1 FISH site was present, indicating the presence of T4B-4S^{sh}#1, and all were normal with no chromosomal breakage (Fig. 1c). In 14 ana-/telophases, the Gc1R-1 FISH site was absent, hence they were lacking chromosome T4B-4S^{sh}#1, and all of them suffered from extensive chromosomal fragmentation (Fig. 1d).

Identification of a *Gc2* knock-out mutant

Of the 2200 mutagenized hemizygous *Gc2–* seeds, 1950 (88.6%) germinated and were grown to maturity. 1940 M_1 plants were scored as semisterile. Ten plants were scored as fertile and their M_2 families were analyzed by FISH using clone pGc1R-1 as a probe to determine the transmission of the *Gc2*-carrier chromosome. In nine M_2 families, all progenies (10 plants per family and a total of 90 plants) analyzed were *Gc2/Gc2*, indicating 100% transmission of the *Gc2*-carrier through both the male and female gametes and, thus, were non-mutant. However, one M_2 family was segregating, indicating that also *Gc2*-noncarrier gametes were functioning as the result of a mutation that affects *Gc2* action. Among seven M_2 plants analyzed, one plant had two copies of the *Gc2*-carrier chromosome T4B-4S^{sh}#1 ($Gc2^{mut}/Gc2^{mut}$), four plants had

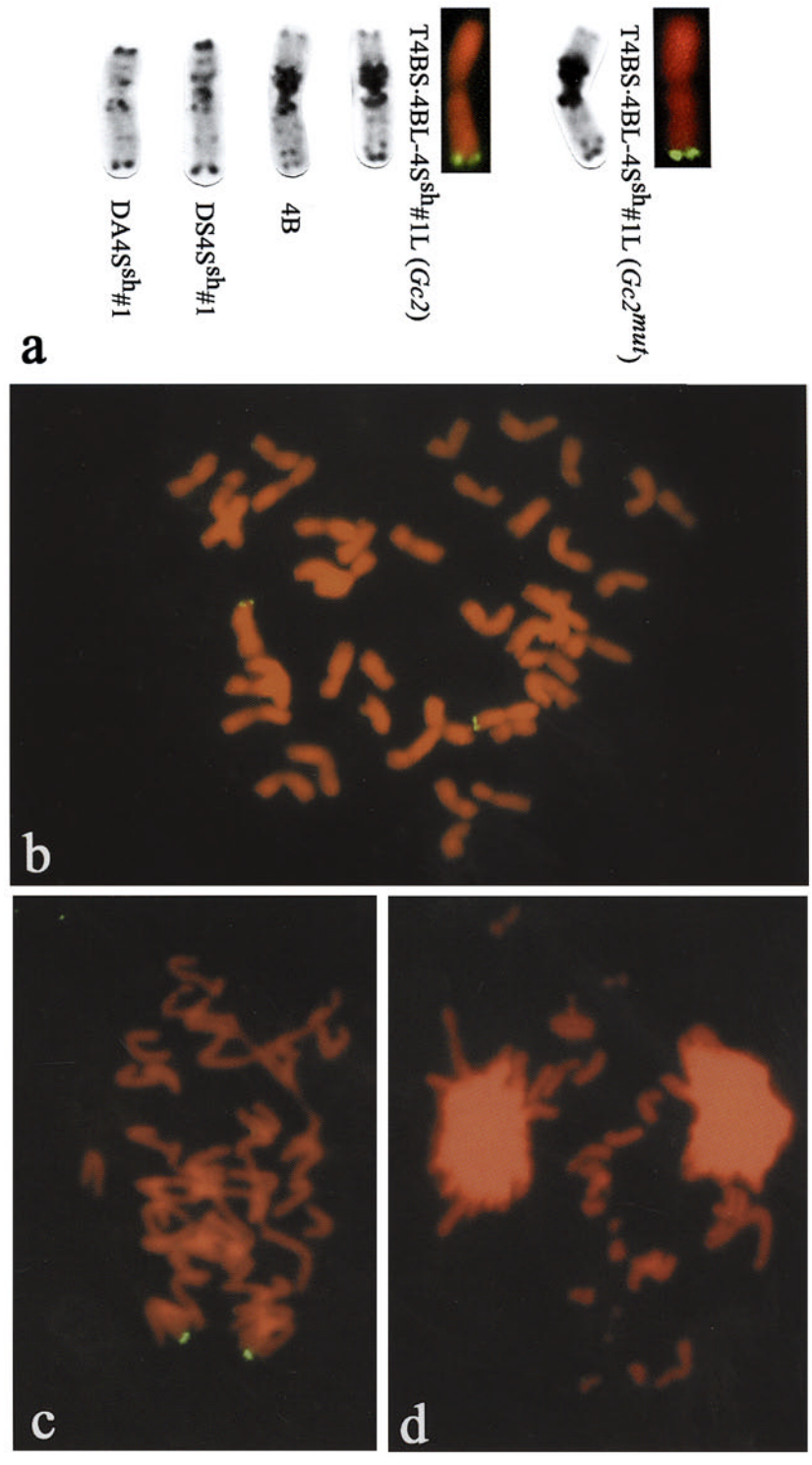

Fig. 1a–d C-banding and Gc1R-1 fluorescence in situ hybridization (FISH) patterns of the original *Gc2* and the derived *Gc2^{mut}*-carrier chromosomes T4BS·4BL-4S^{sh}#1L (**a**), Gc1R-1 FISH patterns of a mitotic metaphase of a homozygous *Gc2^{mut}/Gc2^{mut}* plant (**b**), and Gc1R-1 FISH patterns of ana-/telophases of the first pollen mitosis of a hemizygous *Gc2*/– plant (**c**, **d**). Note that the C-banding and FISH pattern of the *Gc2*-carrier chromosome is identical to the *Gc2^{mut}*-carrier chromosome, indicating that the loss of *Gc2* function is not caused by a terminal deficiency. Note also that the ana-/telophase in **c** has the Gc1R-1 FISH site indicating the presence of T4BS·4BL-4S^{sh}#1L and is normal, whereas the ana-/telophase in **d** is lacking the Gc1R-1 FISH site and is suffering from chromosomal fragmentation

one copy (*Gc2^{mut}*/–), and two plants were euploid (–/–) (Fig. 1b).

Cytogenetic characterization of the *Gc2* mutant

Chromosome T4B-4S^{sh}#1 has a telomeric Gc1R-1 FISH site in the translocated long arm. This FISH site is also present in the *Gc2^{mut}*-carrier chromosome (Fig. 1a), indicating that the loss of *Gc2* function did not result from the loss of the segment carrying the *Gc2* gene, but is likely the result of a point mutation or a deletion beyond the resolution of the microscope.

Plants hemizygous for the *Gc2^{mut}*-carrier chromosome (*Gc2^{mut}*/–) undergo normal first and second pollen mitoses (Fig. 2). We analyzed 164 ana-/telophases of

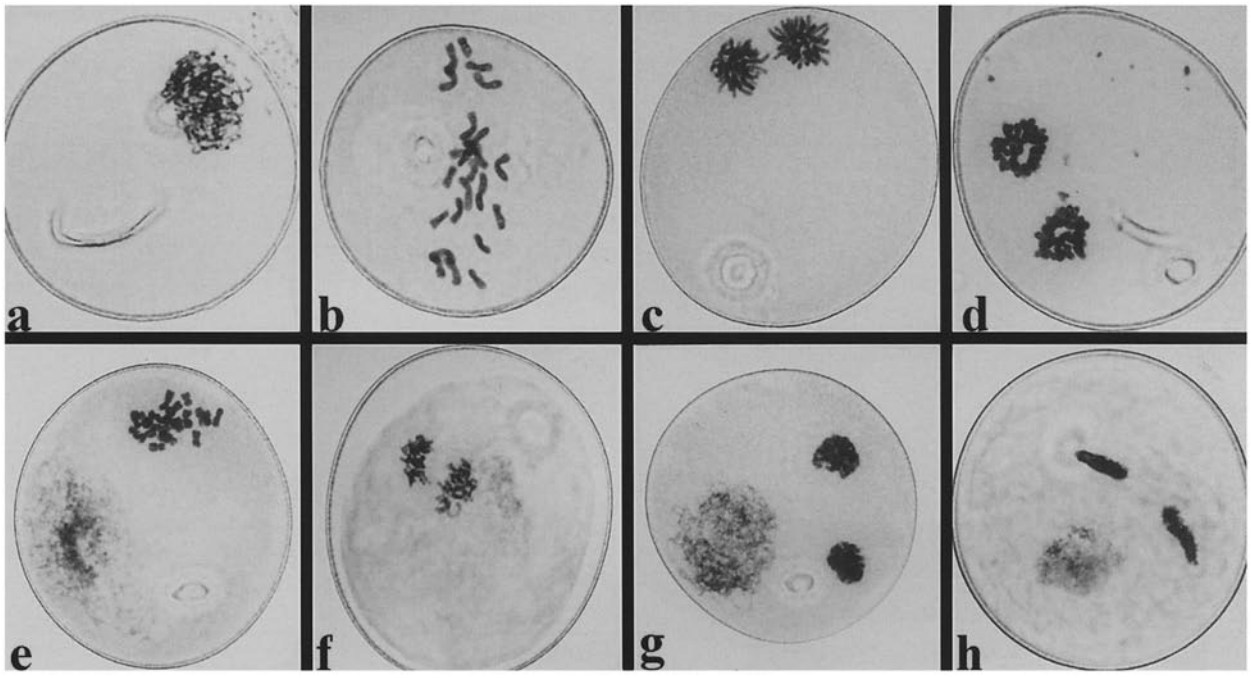

Fig. 2a–h First and second pollen mitoses in hemizygous $Gc2^{mut}$/– wheat plants. Prophase (**a**), metaphase (**b**), anaphase (**c**), and telophase (**d**) of the first pollen mitosis, and metaphase (**e**), anaphase (**f**), telophase (**g**) of the second pollen mitosis that produces mature pollen (**h**) with one vegetative and two crescent-shaped sperm nuclei. Note that in the mutant both pollen mitoses are normal with no chromosomal breakage and bridge formation

the first pollen mitosis and 149 ana-/telophases of the second pollen mitosis; all were normal with no chromosomal fragments or bridge formation. To verify that there is no leakage of chromosomal breaks, mitotic metaphase chromosomes in root-tip samples of 30 progeny plants of the testcross $Gc2^{mut}$/–×–/– were screened by C-banding analysis. No chromosomal rearrangements were observed, indicating that no breakage occurred in the mutant.

Chromosomal mapping of the *Gc2* mutation

The mutation restoring fertility and leading to Mendelian segregation of the $Gc2^{mut}$-carrier chromosome may have occurred in either T4B-$4S^{sh}$#1 or in a wheat chromosome. Progeny testing of the M_2 plants was used to distinguish between these possibilities.

Hypothesis 1: mutation is at the Gc2 locus on chromosome T4B-$4S^{sh}$#1

If the mutation occurred at the *Gc2* locus, the M_1 plant will produce two types of gametes ($Gc2^{mut}$ and –), and thus, 25% of the M_2 plants are expected to be homozygous $Gc2^{mut}/Gc2^{mut}$, 50% will be hemizygous $Gc2^{mut}$/–, and 25% will be euploid –/–. Two types of testcrosses were examined. The two euploid –/– M_2 plants were testcrossed with homozygous *Gc2/Gc2* plants. If the mutation has occurred at the *Gc2* locus (absent), all testcross progenies have the chromosomal constitution *Gc2*/–, about half of the ana-/telophases of the first pollen mitosis are expected to suffer from chromosomal breakage, and all testcross progenies are expected to be semisterile. We observed chromosomal breakage at ana-/telophase of the first pollen mitosis in both testcrosses, and all progenies (17 and 16 plants, respectively) were semisterile (Table 1, Fig. 3). The four $Gc2^{mut}$/– M_2 plants were testcrossed with euploid –/– Chinese Spring. If the mutation has occurred at the *Gc2* locus, all testcross progenies, irrespective of their chromosomal constitution, are expected to be fertile. We did not observe chromosomal breakage at ana-/telophase of the first pollen mitosis in all four testcrosses and all progenies (17, 15, 20, and 13 plants, respectively) were fertile (Table 1, Fig. 3). These data indicate that the mutation occurred at the *Gc2* locus.

Hypothesis 2: mutation is on a wheat chromosome and inhibits Gc2 function

If the mutation that inhibits *Gc2* function occurred in a wheat chromosome and acts as a dominant inhibitor of *Gc2* function (I), the M_1 plant will produce four types of gametes (*IGc2, I–, iGc2, I–)* and 75% of the M_2 plants are expected to be either homo- or heterozygous for the dominant inhibitor. In this situation 75% of the M_2 plants of the testcross combination –/–×*Gc2/Gc2* are expected to be either *I/I* or *I/i* and some testcross progenies are

Table 1 Chromosomal constitution, chromosomal breakage at ana/telophase of the first pollen mitosis, and fertility of testcross progenies of a putative *Gc2* mutant

M_2 constitution	Testcross	Testcross constitution	1st pollen mitosis		Fertility
			Normal	Fragments	
–/–	×*Gc2/Gc2*	*Gc2/–*	109[a]	84[a]	17 plants, all semisterile
–/–	×*Gc2/Gc2*	*Gc2/–*	79[a]	58[a]	16 plants, all semisterile
$Gc2^{mut}$/–	×–/–	$Gc2^{mut}$/– and –/–			17 plants, all fertile
$Gc2^{mut}$/–	×–/–	$Gc2^{mut}$/– and –/–			15 plants, all fertile
$Gc2^{mut}$/–	×–/–	$Gc2^{mut}$/– and –/–	391[b]		20 plants, all fertile
$Gc2^{mut}$/–	×–/–	$Gc2^{mut}$/– and –/–			13 plants, all fertile
$Gc2^{mut}/Gc2^{mut}$	×*Gc2/Gc2*	$Gc2^{mut}/Gc2$	529[c]		17 plants, all fertile

[a] Data taken on two plants
[b] Data taken on six plants
[c] Data taken on eight plants.

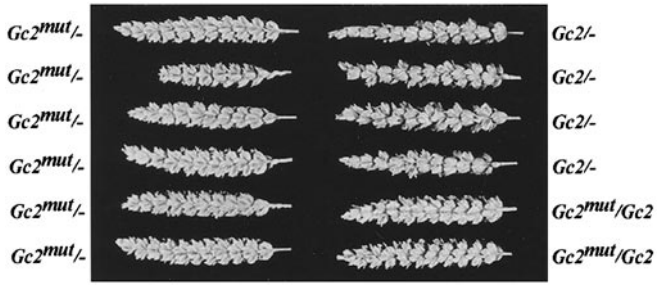

Fig. 3 Spike morphologies of testcross progenies of the $Gc2^{mut}$ M_2 family. *Left*: $Gc2^{mut}$/–×–/–, fertile (the presence of the $Gc2^{mut}$-carrier chromosome in these plants was confirmed by FISH using clone pGc1R-1 as a probe); *upper four spikes on the right*: –/–×*Gc2/Gc2*, semisterile; *lower two spikes on the right*: $Gc2^{mut}/Gc2^{mut}$×*Gc2/Gc2*, fertile

expected to be fertile. However, all testcross progenies were semisterile (Table 1, Fig. 3). Similarly, in the testcross combination $Gc2^{mut}$/–×–/–, 25% of the M_2 plants are expected to be *i/i* and some testcross progenies are expected to be semisterile, but all progenies were fertile.

Both testcross data suggest that the mutation is not a dominant inhibitor in a wheat chromosome, but maps on the *Gc2*-carrier chromosome T4B-4S^{sh}#1, and most likely is a knockout mutation at the *Gc2* locus.

The *Gc2* mutation is dominant and suppresses *Gc2* function in $Gc2^{mut}/Gc2$ heterozygotes

One homozygous $Gc2^{mut}/Gc2^{mut}$ M_2 plant was obtained and testcrossed with a *Gc2/Gc2* homozygous plant. Pollen mitosis of the $Gc2^{mut}/Gc2$ testcross progenies was normal with no chromosomal breakage and all progenies (17 plants) were fertile (Table 1, Fig. 3), indicating that the mutation is dominant. To verify the chromosomal constitution of the testcross progenies, they were crossed with euploid –/– Chinese Spring wheat. 98 plants were screened for their spike fertility; 50 plants were semisterile and 48 plants were fertile, confirming that the testcross progeny had the chromosomal constitution $Gc2^{mut}/Gc2$, and that the M_1 plant was not chimeric.

Discussion

Induced knock-out mutations are valuable sources for gene identification and cloning. The success of mutagenesis in wheat, as has been outlined by Morris and Sears (1967) and Sears (1972), depends on the nature of the target genes involved. These have been classified into three groups. The first group consists of genes that are triplicated and have orthologs in the A, B and D genomes. The majority of genes belong to this group and loss-of-function mutations of these genes are difficult to identify because they are masked by the four doses present on the remaining two genomes. The second group consists of triplicated genes that have a greater effect at six compared with four dosages. This small group of genes is responsible for the phenotypes of the nullisomic stocks where the loss of one locus can be compensated by additional copies on the homoeologous chromosomes, as has been shown in the nullisomic-tetrasomic combinations. Mutations at these genes can be readily identified by their altered phenotypes. The third class consists of major genes that behave as if they are not triplicated. These genes may have become diploidized through either loss or mutations of the orthologs. Mutations of such genes can be easy identified and usually restore the primitive phenotype.

Recently Koebner and Hadfield (2001) reported an efficient protocol that allowed large-scale mutagenesis directed at target genes in wheat whose chromosomal location is known. Plants hemizygous for the chromosome harboring the target gene are produced by crossing with the appropriate monosomic stock. The F_1 plants are then self-pollinated and the derived F_2 plants are mutagenized. The advantage of this approach is that M_1 plants can be screened directly for the loss-of-function mutation, compared with screening the derived M_2 families, which greatly reduces the number of plants that need to be scored.

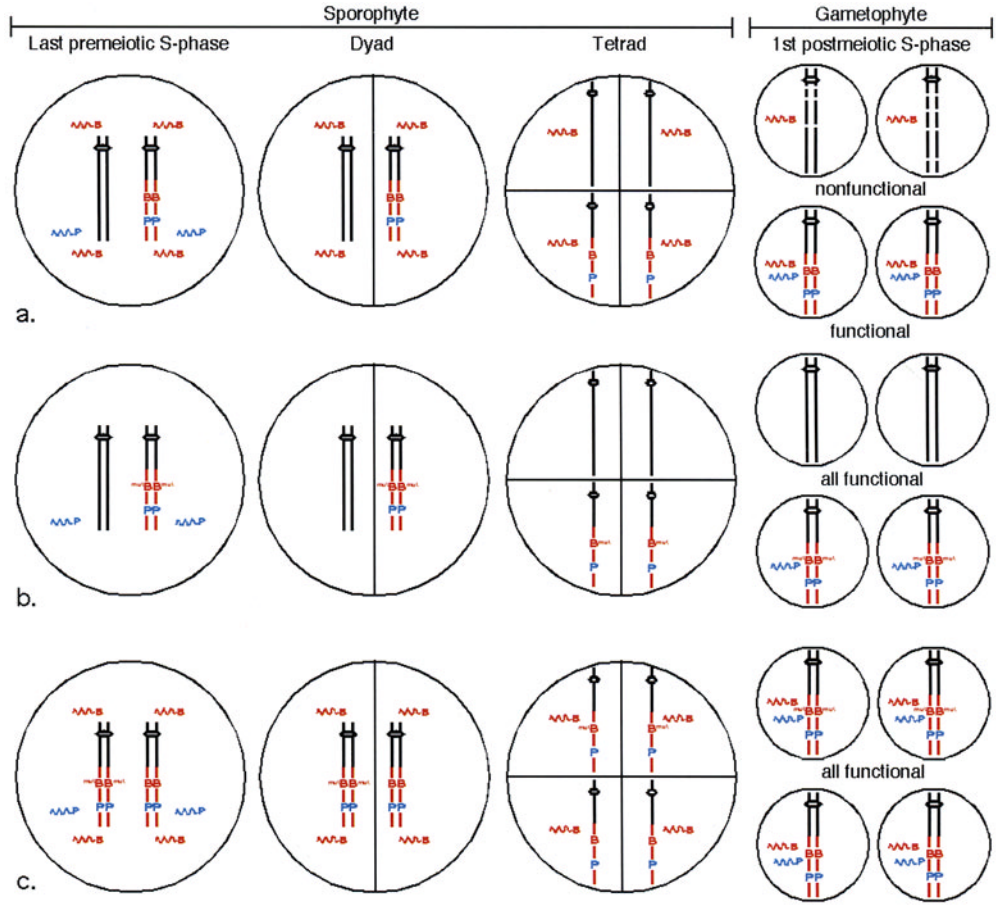

Fig. 4a–c Model of *Gc2* function. We postulate that the *Gc2* gene encodes a 'breaking' (*B*) and a 'protecting' (*P*) agent. In hemizygous *Gc2/–* plants both agents are produced during the premeiotic interphase (**a**). The protecting agent is either diluted out as the cells pass through meiosis or has a faster turn over than the breaking agent. Therefore, gametophytes without *Gc2* only receive the breaking agent, which results in chromosomal breakage. Gametophytes with *Gc2* can produce the protecting agent during the first postmeiotic interphase, which protects them from chromosomal breakage. In hemizygous *Gc2mut/–* plants the breaking agent is no longer produced and as a result all gametes are functional (**b**). In heterozygous $Gc2^{mut}/Gc2$ plants all gametophytes can produce both the breaking and protecting agents during the postmeiotic interphase and as a result all gametes are functional (**c**)

We used a similar approach and mutagenized plants that were hemizygous *Gc2/–*. Thus, any mutation that inhibits *Gc2* function can be scored directly in the mutagenized M_1 plants by screening for spike fertility. We obtained one *Gc2* mutant out of 1940 mutagenized seeds, which corresponds to a mutation frequency of 0.05%. Koebner and Hadfield (2001) used fast neutrons, γ-irradiation, and EMS treatment for inducing loss-of-function mutations in yellow rust and powdery mildew resistance genes and reported mutation frequencies ranging from 0.5% to 6.7% (4% for EMS-induced knock-outs at the *Pm3b* locus). We observed a much lower mutation frequency, which is likely caused by our screening scheme. In order to be able to screen large numbers of mutagenized M_1 plants we planted them in small root-trainers and as a result these plants produced only one tiller. Under these growing condition the seed set is low and we selected only ten plants with the best fertility for further analyses. Thus, additional mutants with low fertility caused by the environment may have been missed.

The C-banding and FISH pattern of the *Gc2*-carrier chromosome T4B-$4S^{sh}$#1 in the mutant is identical to those observed in non-mutants, indicating that the loss of *Gc2* function is not caused by a terminal deficiency. No chromosomal breakage was observed in $Gc2^{mut}/–$ plants and in derived progenies. Thus, all gametes produced in $Gc2^{mut}/–$ plants are functional, which results in fertile spikes and in the Mendelian segregation of the $Gc2^{mut}$-carrier chromosome T4B-$4S^{sh}$#1. Progeny screening mapped the mutation to the *Gc2*-carrier chromosome T4B-$4S^{sh}$#1 and suggested that the mutation is most likely a knock-out at the *Gc2* locus.

One homozygous $Gc2^{mut}/Gc2^{mut}$ M_2 plant was obtained and testcrossed with a homozygous *Gc2/Gc2* plant. The derived $Gc2^{mut}/Gc2$ progeny underwent normal first pollen mitosis with no chromosomal breakage and all plants had fully fertile spikes. This result suggests that something on the $Gc2^{mut}$-carrier chromosome protects $Gc2^{mut}$ gametophytes from chromosomal breakage and supports the dual-function model of Gc action proposed by Endo (1990). According to this model, Gc genes consist of two elements, one encoding a 'breaking' agent that induces chromosomal breakage in gametophytes that are lacking the Gc gene and another element that encodes a 'protecting' agent that protects from chromosomal breakage those gametophytes that carry the Gc gene.

If this hypothesis is correct the *Gc2* gene should encode both a breaking and a protecting agent that is expressed during premeiotic and postmeiotic interphase. Our data suggest that the EMS-induced *Gc2* mutant is a knock-out mutation at the locus that encodes the breaking agent. In hemizygous *Gc2/–* plants, both agents are produced during premeiotic interphase (Fig. 4a). The

protecting agent is either diluted out as the cells pass through meiosis or has a faster turnover than the breaking agent. Therefore, the derived gametophytes lacking the *Gc2* gene only receive the breaking agent, which results in chromosomal breakage prior to the S-phase of the first postmeiotic interphase. On the other hand, gametophytes with the *Gc2* gene can produce the protecting agent during the first postmeiotic interphase, which protects them from chromosomal breakage.

In hemizygous $Gc2^{mut}/-$ plants, the breaking agent is no longer produced and as a result all gametes are normal and functional (Fig. 4b).

In heterozygous $Gc2^{mut}/Gc2$ plants both the breaking and protecting agent are produced during premeiotic interphase. At the end of meiosis, half of the gametophytes receive a functional *Gc2* copy and half receive the $Gc2^{mut}$ copy. During the first postmeiotic interphase, both *Gc2* and $Gc2^{mut}$ gametophytes can produce the protecting agent and as a result all gametes are normal and functional (Fig. 4c).

This model is analogous to the restriction-modification system of bacteria (Wilson and Murrey 1991) as has been suggested previously by Tsujimoto and Noda (1989). Type-II restriction endonucleases (R) cleave double-stranded DNA at or near specific recognition sequences. Cognate modification enzymes (M) are methylases that modify the restriction sites and prevent them from cleavage. The tight association of a cognate enzyme and methylase is termed a (type II) restriction-modification system. The evolution and maintenance of RM systems have long been explained by their role in protecting the host bacteria from infection of foreign DNA. An alternative hypothesis was proposed by Naito et al. (1995). The loss of an RM system by segregation leads to the death of the host as a result of cleavage of the host DNA, which ensures the maintenance of the RM system in the surviving host bacteria. Thus, RM systems can be regarded as a selfish gene complex (Naito et al. 1995).

Modified Gc genes that have lost the breaking agent function may exist. One example might be the gene *Igc1-B1* located on wheat chromosome 3B of the wheat cultivar Norin 26 (Tsujimoto and Tsunewaki 1985b). *Igc1-B1* acts as a dominant suppressor of the Gc gene present in the *Ae. triuncialis* chromosome $3C^t$. In the monosomic $3C^t$ addition plants with *Igc1-B1* only a low amount of chromosomal breakage occurs, all gametes are functional and spikes are fully fertile, whereas in the absence of *Igc1-B1,* gametophytes without chromosome $3C^t$ suffer from chromosomal breakage, resulting in non-functional gametes, semisterile spikes, and exclusive transmission of chromosome $3C^t$.

Recently, De Las Heras et al. (2001) reported that differential methylation may be involved in *Gc2* function. Treatment of *Gc2/–* and *Gc2/Gc2* plants with the DNA hypomethylating agent 5-azacytidine resulted in a 9% and 16% increase in chromosomal breakage and bridge formation in root-tip meristem compared with the untreated controls. Further molecular data are needed to verify the involvement of differential methylation in the mechanism of *Gc2* action.

The presented data are a first crucial step toward the molecular understanding of *Gc2* action. We are presently performing expression studies and are developing mapping populations, which might eventually allow the cloning of this gene.

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