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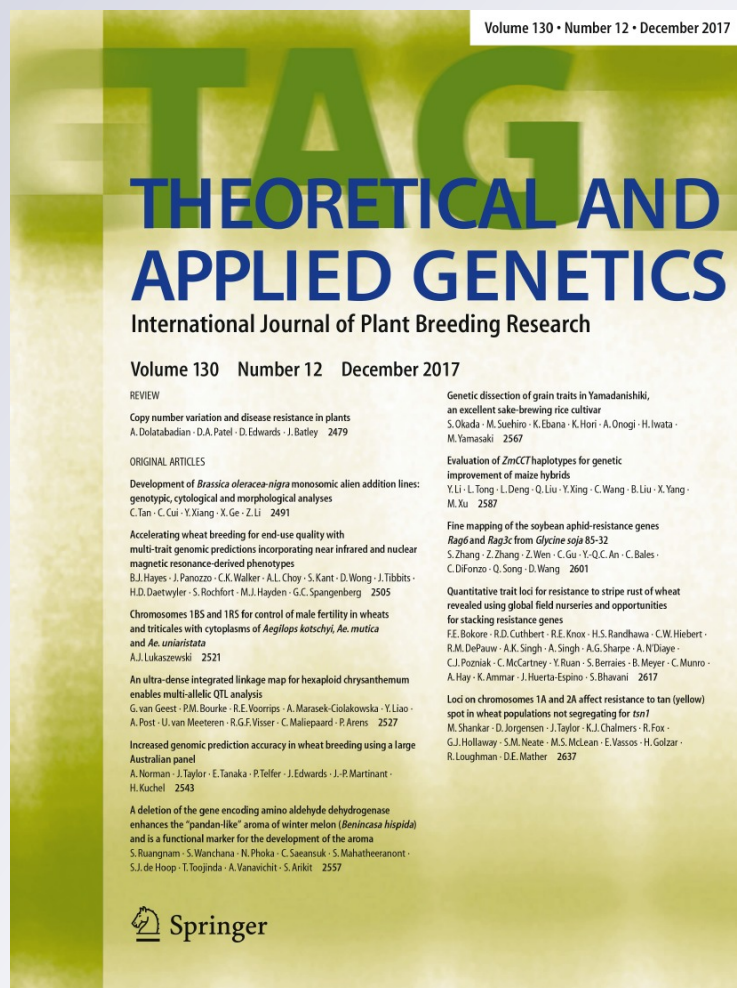
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Chromosomes 1BS and 1RS for control of male fertility in wheats and triticales with cytoplasm of *Aegilops kotschyi*, *Ae. mutica* and *Ae. uniaristata*

 Adam J. Lukaszewski¹ 

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Abstract

Key message Engineered chromosomes 1BS and 1RS offer a new alternative in the development of hybrid systems in bread wheat and triticales.

Abstract In the cytoplasmic male sterility system for hybrid wheat based on the cytoplasm of *Triticum timopheevi* fertility restoration is difficult, with few good restorer genes available. In the system based on the cytoplasm of *Aegilops kotschyi*, *Ae. uniaristata* and *Ae. mutica*, essentially all chromosomes 1B carry locus Rf^{multi} that restores male fertility; male sterility manifests itself in wheats with the 1RS.1BL translocation where 1BS chromosome arm is missing. To generate male sterile wheats without the 1RS.1BL translocation, the 1BS arm was cytogenetically engineered to replace the segment with Rf^{multi} with two short inserts of rye chromatin. Conversely, to enhance fertility restoration by doubling the number of restorers present for eventual use in wheat and triticales, a region of 1BS with Rf^{multi} was inserted into 1RS. Alloplasmic wheats with Rf^{multi} removed were completely male sterile; alloplasmic wheats with engineered 1RS carrying Rf^{multi} and without normal 1B were male fertile. An exception to the ubiquitous presence of Rf^{multi} is *T. spelta* var. *duhamelianum*; four accessions tested in this study gave inconsistent results but some did not restore male fertility. Engineered chromosomes 1BS and 1RS and chromosomes 1B of *T. spelta* offer a new alternative for practical application of a cytoplasmic male sterility system in the development of hybrid wheat and hexaploid triticales.

Introduction

Cytoplasmic male sterility (cms) is a useful tool in the development of hybrid cultivars. In wheat (*Triticum aestivum* L.), the standard cytoplasm for cms is that of *T. timopheevi* Zhuk. (Wilson and Ross 1962). Its presence in bread and durum wheats generates complete male sterility; female fertility is not affected. One of the major problems with hybrid wheat production based on timopheevi cytoplasm is in fertility restoration. There are few good male fertility restorer genes in wheat, and usually a combination of genes is required for complete fertility restoration (for review, see Edwards 2001).

Apart from the cytoplasm of *T. timopheevi*, several other cytoplasm also create male sterility in wheat (Tsunewaki 1988). Among them are the cytoplasm of three *Aegilops* species: *Ae. kotschyi* Boiss., *Ae. mutica* Boiss. and *Ae. uniaristata* Vis. In these three cytoplasm, male sterility manifests itself only in the absence of the short arm of wheat chromosome 1BS, such as in 1RS.1BL translocation lines, indicating that 1BS carries a fertility restorer. Using a set of recombinant chromosomes 1BS–1RS, Tsunewaki (2015) mapped the restoring ability to a 2.9 cM region of 1BS and as the same locus appeared to be involved in interactions with all three cytoplasm, he named it Rf^{multi} . Based on the data obtained by Tsunewaki (2015), Hohn and Lukaszewski (2016) created a chromosome 1B_{1,6}, with ca. 2.8 cM long insert from rye arm 1RS that removes the Rf^{multi} locus, thus creating the potential for the development of hybrid wheat without quality defects associated with the presence of the entire 1RS arm in wheat. Homozygosity or hemizyosity for the 1B_{1,6} chromosome in cytoplasm of the three *Aegilops* species creates complete male sterility.

As pointed out by Tsunewaki (2015), there are exceptions to the general presence of the Rf^{multi} locus on 1BS in wheat. *T. spelta* var. *duhamelianum*, carrying $Rf3$, a good restorer

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 ✉ Adam J. Lukaszewski
 adam.lukaszewski@ucr.edu

¹ Department of Botany and Plant Sci., University of California, Riverside, CA 92521, USA

for the *T. timopheevi* cytoplasm (Tahir and Tsunewaki 1969), does not appear to be able to restore male fertility to wheats with the kotschy, mutica and uniaristata cytoplasms. The critical locus is located on 1BS (Mukai and Tsunewaki 1979). It is possible that the same locus on 1BS is involved in interactions with the four cytoplasms and different allelic variants interact differently with various cytoplasms. To test this concept, chromosomes 1B from four accessions of *T. spelta* were tested for their effect on male fertility in the cytoplasms of *T. timopheevi* and *Ae. kotschy*.

The first engineered chromosome 1B for male sterility in kotschy cytoplasm (Hohn and Lukaszewski 2016) was based on preliminary results communicated by Tsunewaki (2015) and the primary recombinant chromosomes selected for the operation were chosen with extra caution. As a consequence, the length of the rye insert was not optimal; it spans a distance covered by nine DNA markers on a reasonably well saturated map (Fig. 1). Based on a Diversity Array Technology (DArT) marker map of 1BS–1RS recombinants, fertility data of Tsunewaki (2015) and observations made in this study, the location of *Rf^{multi}* can now be narrowed to a region occupied by a single marker. This article describes a series of engineered chromosomes 1BS and 1RS created to control male fertility in the three Aegilops cytoplasms: two 1BS arms with smaller rye inserts than that in 1B_{1.6}, and two rye chromosome arms 1RS with inserts of the *Rf^{multi}*. In theory, such rye chromosome arms, when translocated to wheat arms 1AL or 1DL, would enhance fertility restoration

by doubling the number of *Rf^{multi}*, but at the cost of bread-making quality problems associated with the presence of 1RS. In triticale, however, such a chromosome 1R, in its natural location and in conjunction with 1B, would provide a second dose of *Rf^{multi}* without adverse effects as bread-making quality is not an issue at the present time.

Materials and methods

The primary recombinant chromosomes 1BS–1RS used here were created by Lukaszewski (2000). With two known exceptions, all are single breakpoint translocations, in two configurations: those labeled 1B+ followed by a number have short arms composed of terminal rye segments and proximal wheat segments; those labeled T—have terminal wheat segments and proximal rye segments. The positions of translocation breakpoints in the vicinity of the *Rf^{multi}* locus are shown in Fig. 1. All lines except for 1B + 35 were tested for the presence of *Rf^{multi}* by Tsunewaki (2015). 1B + 35 was tested in this study and found not to carry *Rf^{multi}* (no seed set when homozygous or hemizygous in the cytoplasm of *Ae. kotschy*). *Rf^{multi}* is, therefore, located between breakpoints of 1B + 33 and 1B + 35. On the genetic map of DArT markers this region is represented by a single maker rPt508039. No clear reading of marker rPt-505874 was obtained for T-33 and so the position of the breakpoint is uncertain, within one DNA marker.

Locus	Position	1B+2	1B+49	1B+33	1B+35	1B+25	1B+30	1B+1	T-17	T-6	T-34	T-35
Sec-1	29.713	b	b	b	b	b	b	b	a	a	a	a
rPt-39030	34.934	b	b	b	b	b	b	b	a	a	a	a
rPt-41076	36.487	b	b	b	b	b	b	b	a	a	a	a
1B_wPt-15	37.637	b	b	b	b	b	b	b	a	a	a	a
rPt-50870	41.099	b	b	b	b	b	b	b	a	a	a	a
rPt-50526	45.797	b	b	b	b	b	b	b	a	a	a	a
rPt-40223	51.779	b	b	b	b	b	b	b	a	a	a	a
rPt-39965	57.847	b	b	b	b	b	b	b	a	a	a	a
S-6	69.855	a	b	b	b	b	b	b	b	a	a	a
wPt-11975	71.407	a	a	b	b	b	b	b	b	b	a	a
rPt-39879	72.439	a	a	b	b	b	b	b	b	b	a	a
rPt-50803	73.571	a	a	a	b	b	b	b	b	b	a	a
1B_wPt-0	74.633	a	a	a	a	b	b	b	b	b	a	a
rPt-50590	76.891	a	a	a	a	a	b	b	b	b	b	a
rPt-50582	77.391	a	a	a	a	a	b	b	b	b	b	-
1B_wPt-0	77.829	a	a	a	a	a	b	b	b	b	b	b
wPt-2657	78.923	a	a	a	a	a	a	b	b	b	b	b
Xucr-5	81.962	a	a	a	a	a	-	b	b	b	b	b
1B_wPt-1	83.645	a	a	a	a	a	a	a	b	b	b	b

Fig. 1 Genetic map positions of the 1RS–1BS translocation breakpoints in the vicinity of the *Rf^{multi}* locus on 1BS. Light shaded areas marked by letter “a” are wheat segments; dark shaded areas marked

by letter “b” are rye segments. Non-shaded areas: no clear reading of a marker. Genetic map positions are expressed in relative values (see Lukaszewski 2000)

Intercalary inserts were created as before (Lukaszewski 2000; Hohn and Lukaszewski 2016). For introgression of rye segments into 1BS, the breakpoint closest to Rf^{multi} in the T-configuration is in T-6; T-6 was matched with breakpoints in recombinants 1B + 35 and 1B + 25. Positions of breakpoints in these two pairs create the potential of generating inserts spanning three and four DNA markers, respectively. For wheat inserts into 1RS, 1BS + 49 was matched with T-35 and 1BS + 33 with T-34, creating the potential of inserts spanning five-six and two DNA markers, respectively.

Homozygotes for selected primary recombinants were crossed pairwise, their F_1 hybrids grown and self-pollinated in isolation. F_2 progenies were screened by C-banding for the presence of recombined chromosomes: normal-appearing 1B and 1RS.1BL. Preparations from plants with such chromosomes were de-stained and probed with DIG-labeled total genomic DNA: of rye for introgressions of rye segments into 1BS, and of wheat for suspected introgressions into 1RS. Blocking was by sheared total genomic DNA of the other parent, applied in ca. 1:150 ratio (probe to block). All probing with labeled DNA was done using the protocol of Massoudi-Nejad et al. (2002).

Five accessions of *T. spelta* var. *duhamelianum* were obtained from the Institute of Plant Breeding (IHAR), Radzikow, Poland. Four of those, numbered 22862, 1155, 1165 and 1167 were tested for their ability to restore male fertility to wheats with cytoplasms of *T. timopheevi* and *Ae. kotschy*. The last one is so late that it could not be crossed with any alloplasmic tester. A *cms* line of wheat cv. Pavon 76 was used here, developed by backcrosses from the tim-Chris stock originating from the collection of alloplasmic wheat lines created by S. S. Maan and provided by Dr. S. Kianian, then at North Dakota State University, Fargo, ND, USA. Male fertility was expressed as the average number of seed set per spikelet in the first three heads of a plant. Alloplasmic lines of cvs. Pavon 76 and Hahn with cytoplasms of *Ae. kotschy*, *Ae. mutica* and *Ae. uniaristata* are generated by backcrosses from stocks of cv. Chinese Spring kindly provided by K. Tsunewaki. Pavon 76 originates from the International Center for Maize and Wheat Improvement (CIMMYT), Mexico; cv. Hahn was provided by Dr. J. Dubcovsky, University of California, Davis, USA.

Three different versions of a reverse tandem duplication (rtd) involving the satellite of 1BS were recovered by the author during routine screening of various wheats. One of them, involving the entire satellite (called 1B^{SAT}, Fig. 2) was tested for its level of fertility restoration in the cytoplasm of *Ae. kotschy*. The distal breakpoint that generated this duplication appears to be located in the terminal region of the satellite; the second one appears to be quite proximal, duplicating the distal ca. 60% of the arm. For the origin and behavior of reverse tandem duplications in wheat, see

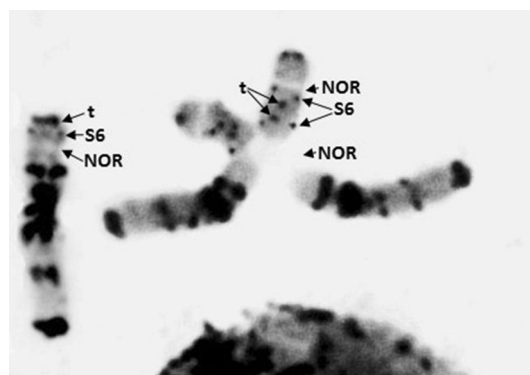


Fig. 2 Two chromosomes 1B^{SAT} with reverse tandem duplication of ca. 60% of the short arm, tangled in their NOR regions. On the left, normal chromosome 1B. Landmarks are arrowed: C-band S6, normally in the proximal part of the satellite, remnants of the telomeric C-band (labeled “t”), and two NOR regions. Locus Rf^{multi} is proximal and directly adjacent to the S6 C-band

Lukaszewski (1995). Since Rf^{multi} is located directly proximal to C-band S-6 on the satellite of 1BS (Lukaszewski 2000) and this band is present on the duplicated segment, it is believed that 1B^{SAT} may carry two copies of Rf^{multi} on each chromosome.

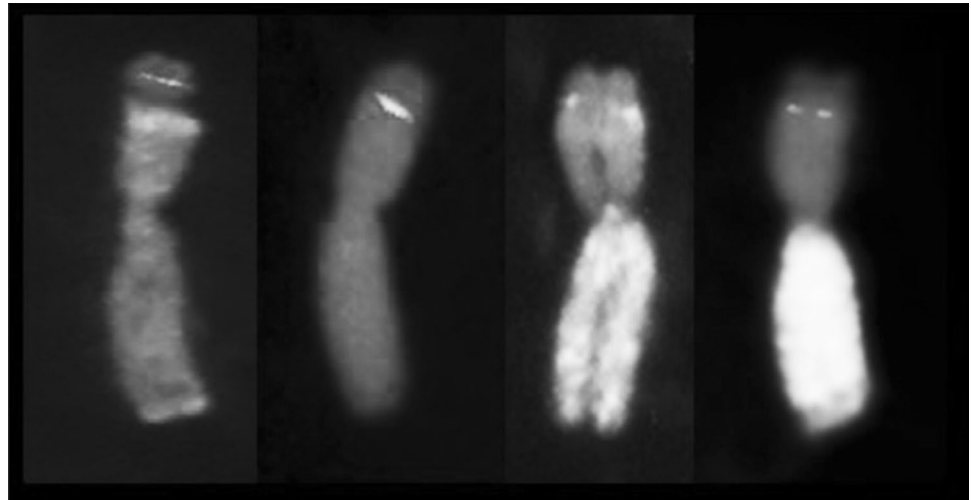
Results

Four pairs of primary recombinant chromosomes were combined to generate chromosomes 1B with two different lengths intercalary inserts from 1RS on the short arm, and chromosomes 1RS.1BL with two different length intercalary inserts from 1BS on the short arm. In both configurations, the best possible combination was chosen from among available primary recombinant chromosomes, expected to produce the shortest possible inserts (Fig. 1) and a safer one, expected to generate larger inserts.

Combination 1B + 25 × T-6 spans four markers on the DNA marker map (Fig. 1); 134 progeny were screened and four recombinant chromosomes were recovered: two normal 1RS arms and two 1BS arms with rye inserts, for the recombination frequency of 1.49% on the per chromosome basis. Recombinant chromosomes are labeled 1B_{25;6}; both were verified by in situ probing with labeled total genomic DNA of rye (Fig. 3). In combination 1B + 35 × T-6, 275 progeny were screened and four recombinant chromosomes recovered for the recombination frequency of 0.73% on the per chromosome basis. All recombinant chromosomes were in the 1B configuration; they are labeled 1B_{35;6}. The presence of the rye insert was verified by in situ probing; it spans the segment with three DNA markers.

Both chromosomes were tested in the cytoplasm of *Ae. kotschy*. Average seed set for standard Pavon 76 in Riverside

Fig. 3 Secondary recombinant chromosomes, from left to right 1B_{25;6}, 1B_{35;6}, 1RS_{49;35}.1BL and 1RS_{33;34}.1BL. In chromosomes 1B *white* signals of the total genomic rye DNA probe on the short arms show the positions and lengths of rye inserts; in chromosomes 1RS.1BL light signals of the total wheat genomic DNA probe show the positions of wheat chromatin: short inserts on the short arms and entire long arms



CA ranges between 3.0 and 3.1 grains per spikelet. Alloplasmic lines of Pavon 76 with *kotschyi* cytoplasm and a single dose of *Rf^{multi}* produce about 2.0 grains per spikelet, or about 65% of normal (Tsunewaki 2015; Hohn and Lukaszewski 2016). In test crosses, 1B_{25;6} and 1B_{35;6} deviated somewhat from the standard pattern: while both produced male sterile plants when heterozygous with 1B_{1;6} or 1RS.1BL, seed set in heterozygotes with normal 1B was different: one plant with 1B_{35;6} + 1B set only 1.25 seeds per spikelet; another plant with 1B_{25;6} + 1B set 2.91 seeds per spikelet. At this point it is not clear what might have caused these deviations; both cases require additional tests.

In the reciprocal configuration, with wheat inserts into 1RS, combination 1B + 49 × T-35 spans five or six DNA markers; 399 progeny were screened and seven recombinants recovered. Of these, five were normal-appearing arms 1RS and two 1BS arms, for recombination frequency of 0.88%. These secondary recombinant chromosomes are labeled 1RS_{49;35}.1BL; four of the five were verified by in situ probing (Fig. 3). Combination 1BS + 33 × T-34 spans two DNA markers; 325 progeny were screened and one recombinant chromosome was recovered, for the crossover frequency of 0.15% on the per-chromosome basis. Fortuitously, this recombinant chromosome was in the desired configuration: rye chromosome arm with a small insert of wheat chromatin. This chromosome is labeled 1RS_{33;34}.1BL and it was verified by in situ probing (Fig. 3). Both chromosomes were tested for their ability to restore male fertility in *Ae. kotschyi* cytoplasm. When present in a single copy (with translocation 1RS.1BL) the average seed set per spikelet was 2.0, typical for a single dose of *Rf^{multi}*. When either of the two chromosomes was present with normal 1B, seed set per spikelet averaged 2.95, ranging from 2.38 to 3.20 for individual plants. This shows that indeed *Rf^{multi}* is present in both chromosomes; some fertility reduction in individual

plants may be a consequence of early generation hybridity. Alloplasmic lines of Pavon 76 are now in BC₃.

Tests of four accessions of *T. spelta* var. *duhamelianum* produced inconsistent results. Accession #1167 produced fertile F₁ hybrids in *kotschyi* cytoplasm, and did not restore fertility to the *timopheevi* cytoplasm. In this sense, this accession does not fit the previously described characteristics of *T. spelta* var. *duhamelianum* (Tsunewaki 2015) even though morphologically it is clearly *T. spelta*. F₁ hybrids of the remaining three accessions: #1155, #1165 and #22862 with various *kotschyi* cytoplasm lines were male sterile; F₁ hybrids of #1165 and #22862 with *timopheevi* cytoplasm were also male sterile while the F₁ hybrids of #1155 were male fertile. However, in the subsequent generation, BC₁ hybrids of #1165 and #22862 to nullisomic 1B of Pavon crossed to various lines with *kotschyi* cytoplasm segregated for male sterile and male fertile individuals, with very low seed set (ca. 0.40 seeds per spikelet) among the male fertile lines. These lines require further tests; it is possible that original accessions are heterogeneous (crosses to alloplasmic lines and crosses to Pavon N1B were not done using the same single plants).

The level of fertility restoration by the reverse tandem duplication on 1BS in cv. Pavon 76 in the cytoplasm of *Ae. kotschyi* varied from plant to plant. When the chromosome was present with normal 1B, or normal-appearing 1B, seed set was normal (ca. 3.0 seed per spikelet). When the chromosome was present with 1BL, with duplication present in a single copy, in most cases seed set was at ca. 67% of normal suggesting that chromosome 1B^{SAT} carried only one copy of *Rf^{multi}*. However, in two plants the seed set was close to normal (2.93 and 2.87 seeds per spikelet). This suggests that the duplication is unstable and the segment undergoes structural changes, some of which carry two copies of *Rf^{multi}* while most others do not. An optimistic assumption here is

that a stable chromosome with two copies of Rf^{multi} can be selected from among unstable progenies.

Discussion

All three engineered chromosomes 1B with inserts of rye chromatin in the region harboring Rf^{multi} ($1B_{1;6}$, $1B_{25;6}$ and $1B_{35;6}$) generate complete male sterility in wheats with the cytoplasm of *Ae. kotschyi*, and presumably *Ae. uniaristata* and *Ae. mutica* as well. The three chromosomes differ in size of the rye insert; $1B_{25;6}$ is the shortest, $1B_{1;6}$ is the longest. Given the sizes of the inserts, no transmission problems are expected, but tests in large populations are in order. These chromosomes can now be used to generate cms lines. In addition, it appears that after careful screening, some chromosomes of the *T. spelta* accessions tested here can also be used to generate male sterility. Of the four accessions tested one restores male fertility to the three *Aegilops* cytoplasm, as do all other chromosomes 1B tested so far. Presumably, this accession is misclassified even though morphologically it is *T. spelta*.

Restoration by a single dose of Rf^{multi} (single 1B present) is incomplete in this system; two doses are required for full seed set under greenhouse conditions. If incomplete restoration manifests itself in commercial production, additional copies of Rf^{multi} can be generated either by duplication of the locus, such as on the derivatives of the chromosome 1B with duplication tested here, or by placing a second copy somewhere else in the genome. Chromosomes 1RS with Rf^{multi} inserts are being converted to normal 1R, at which point the 1RS will be translocated to 1AL and 1DL. In conjunction with standard 1B, they will offer two doses of Rf^{multi} . However, this brings back two negative aspects of the 1RS presence in such alloplasmic wheat: the general bread making quality defects and generation of haploids in the three *Aegilops* cytoplasm (Kobayashi and Tsunewaki 1980; Tsunewaki 1988). Both problems could be alleviated by making use of engineered 1RS arm (Lukaszewski 2000) and by removal of the 1RS region involved in generation of haploids. Haploid production in this system does not appear to be associated with the r_f^{multi} status, as suggested by Kobayashi and Tsunewaki (1980) but rather with the presence of 1RS. Moreover, the effect appears to be dosage dependent as the frequency of haploids is greater in alloplasmic disomics 1RS than when a single copy of 1RS is present. No haploids have been observed in the discussed alloplasmics with 1RS absent, such as in ditelosomics 1BL, or among homozygotes $1B_{1;6}$, but can be quite frequent in heterozygotes 1RS.1BL + 1B (the author avoids homozygotes 1RS.1BL in the development of alloplasmic wheats). This suggests that there is a locus on 1RS involved in haploid production, and that it is not located in the immediate vicinity of Rf^{multi} . So far, no systematic screening of the full range of

1RS–1BL recombinants has been undertaken to identify the location of the responsible locus.

The *kotschyi/uniaristata* cytoplasm system may also work in hexaploid triticale (the cytoplasm of *Ae. mutica*, similarly to wheat, causes serious delay in heading and maturity which disqualifies it from serious consideration). While many different cytoplasm systems have been tested in triticale (Nalepa 1991; Goral 2013) the list does not include the three cytoplasm systems tested here. Early indications are that alloplasmic lines of triticale cv. Presto with cytoplasm of *Ae. kotschyi*, *Ae. uniaristata* and *Ae. mutica* and chromosome 1B absent (as in 1D (1B) substitution) are male sterile and that unlike for the timopheevi cytoplasm (Curtis and Lukaszewski 1993), rye chromosomes of cv. Presto do not carry restorer genes to the three cytoplasm systems. However, haploids were observed in early generations of backcrosses, with frequencies that appear to be much higher than those in wheats growing alongside. No precise counts are yet available but it is possible that in some cross combination their frequency may reach the level of 80% reported in wheat with 1RS.1BL translocation (Kobayashi and Tsunewaki 1980). On the other hand, bread-making quality issues associated with 1RS at present are of no consequence in triticale. Triticale naturally carries chromosome 1RS and at present it is not being viewed as a bread making crop. For cms, any of the three engineered chromosomes 1B can be used, eliminating the Rf^{multi} locus. Chromosome $1B_{1;6}$ is already advanced in backcrosses to a winter triticale. For fertility restoration, a pollinator with two doses of Rf^{multi} can easily be generated: one on chromosome 1B and the other on engineered chromosomes 1R (such as 1RS_{33;34}; 1RS_{49;35}). For this purpose, engineered arms 1RS now in translocations to 1BL are being converted to chromosomes 1R. 1RS_{33;34}; 1RS_{49;35} in combination with normal chromosome 1B provide two copies of Rf^{multi} and this should guarantee complete seed set in hybrids.

Author contribution statement The author came up with the idea and did it.

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Compliance with ethical standards

Conflict of interest The author declares that they have no competing interests.

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