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ORIGINAL PAPER

Fine mapping of the first multi-fertility-restoring gene, *Rf*^{multi}, of wheat for three *Aegilops* plasmons, using 1BS-1RS recombinant lines

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Abstract

Key message **Fertility-restoring genes, *Rfv1*, *Rfm1* and *Rfn1*, respectively, for the male sterile cytoplasms of *Aegilops kotschyi*, *Ae. mutica* and *Ae. uniaristata* to common wheat were located on the same locus of Pavon wheat 1BS arm.**

Abstract The male sterile cytoplasm (plasmon) and the fertility-restoring gene are essential genetic components for breeding hybrid seed crops. This article represents information on the genetic similarity of three *Aegilops* plasmons usable as the male sterile cytoplasm for hybrid wheat and provides an evidence on the possible genetic unity of three fertility-restoring genes reported for these plasmons by their genetic mapping using the 1BS-1RS recombinant lines of Pavon 76 wheat on to a single subsegment of the 1BS chromosome arm less than 2.9 cM in size: the locus is designated *Rf*^{multi}, meaning "*R*estoration of *f*ertility in *multi*ple CMS systems". Unresolved problems were discussed in the use of the present cytoplasmic male sterility-fertility restoration system for hybrid wheat breeding.

Introduction

In this article, the term 'plasmon' is used to indicate the cytoplasmic genetic system of plants, of which major constituents are mitochondrial and chloroplast genomes (after Rieger et al. 1991). Cytoplasmic male sterility (CMS) is a maternally inherited character widely recognized in angiosperm and its causal genetic element is well documented in their mitochondrial genome; two examples are *T-urf13* of maize (Wise et al. 1987) and *WA352* of rice (Luo et al. 2013). In wheat, there are many evidences for the maternal inheritance of CMS, although the location of its genetic determinant(s) in the cytoplasm is still unrevealed. Another point to be introduced before getting into the main subject is designation of the recombinants between 1B wheat and 1R rye chromosomes: 1BL.1RS is used for a recombinant chromosome consisting of the 1BL arm of wheat and 1RS arm of rye, whereas 1BS-1RS indicates a chromosome recombined between the short arms of 1B and 1R chromosomes while retaining the intact 1BL arm of wheat (Lukaszewski 2000).

Irrespective of the nature of its genetic determinant(s), CMS has been of considerable interest of wheat breeders and geneticists for hybrid wheat breeding since 1960's, stimulated by the discovery of CMS induced by the *Triticum timopheevi* cytoplasm (Wilson and Ross 1962). Despite their great effort, however, commercial cultivation of hybrid wheat has been limited and restricted to small areas and much of hybrid seed production uses chemical agents to induce male sterility rather than genetic sterility, because of the difficulty in maintenance of the male sterile (MS) line (Pickett 1993; Longin et al. 2012). In spite of this fact, CMS is still one of the more attractive possibilities because it does not rely on chemicals and we need to search for new CMS systems with better properties in relation to hybrid seed production.

Based on the results of systematic investigation on the cytoplasmic male sterility-fertility restoration (CSMFR) system in the *Triticum* (wheat)-*Aegilops* complex, Tsunewaki (1988) proposed that among 16 plasmon types identified in this complex, three more plasmon types, D^2

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of *Ae. crassa* and its relatives, N (previous designation, M^u) of *Ae. uniaristata*, and S^v of *Ae. kotschyi* and its relatives, beside the G plasmon of *T. timopheevi*, might also be usable as the MS cytoplasm for hybrid wheat breeding. In fact, hybrid wheat using the S^v plasmon of *Ae. kotschyi* (K-type plasmon) has been locally commercialized in Shaanxi Province, China (He PR personal comm.). Their CMS-RF system consists of the *Ae. kotschyi* plasmon and the 1BL.1RS translocation chromosome. This chromosome is deficient for a fertility-restoring gene, *Rfv1*, for the S^v type plasmon, that is located on chromosome 1BS arm of common wheat (Mukai and Tsunewaki 1979). This CMS-RF system, however, has a serious disadvantage because a whole rye chromosome arm, 1RS, substituted for 1BS arm of wheat is used in MS induction with the *kotschyi* plasmon, which hinders improvement of the MS parental lines in breeding hybrid wheat.

CMS-FR expression against a set of 12 tester wheat genotypes, that we call the 'fertility spectra' (Tsunewaki 1996), of three plasmons of *Ae. kotschyi, Ae. mutica* (strain T) and *Ae. uniaristata* are similar to each other, expressing complete or nearly complete MS in three common wheat testers, Salmon, Spelta and Macha, and normal or nearly normal male fertility in nine other testers, Tve, P168, Chinese Spring (CS), Norin 26, Jones Fife, Selkirk and S-615 of *T. aestivum* and single accession each of *T. sphaerococcum* and *T. compactum,* with the exceptions of Tve and S-615 which express nearly complete MS in the presence of *mutica* plasmon. In addition, CS wheat carries a fertility-restoring (*Rf*) gene to these MS plasmons on the same 1BS arm, namely, *Rfv1* gene for *Ae. kotschyi* (Mukai and Tsunewaki 1979), *Rfm1* for *Ae. mutica* (Mukai 1983) and *Rfn1* for *Ae. uniaristata* plasmon (Tsujimoto and Tsunewaki 1984). Mapping of those genes on the 1BS arm was made by the use of chromosome arm deletion or substitution stocks (Mukai and Tsunewaki 1979; Mukai 1983; Tsujimoto and Tsunewaki 1984) or by the use of chromosome terminal deletion lines (Mukai and Endo 1992).

Their fine mapping might be possible by conventional analysis, namely, by test-crossing CS × Spelta F_1 hybrid as male parent to the respective alloplasmic CS line and examining the B_1 progeny for their seed fertility and molecular markers. Unfortunately, there is one molecular map so far reported for CS/Spelta combination, in which 1B chromosome was marked only by nine molecular markers (Liu and Tsunewaki 1991), which are not sufficient for the fine mapping of those genes. Instead, we decided to use 1BS-1RS recombinant lines of Pavon 76 wheat (Pavon) produced by Lukaszewski (2000), of which the 1BS arm was marked by 14 cytological and molecular markers (Lukaszewski 2000; Sharma et al. 2009). The results suggested that the three genes may in fact represent the first case of a multifunctional *Rf* gene restoring male fertility for all of the three different plasmons of *Ae. kotschyi, Ae. mutica* and *Ae. uniaristata*.

Materials and methods

Plant material

Alloplasmic lines of CS wheat and 1BS-1RS recombinant lines of Pavon wheat: Plasmons of *Aegilops kotschyi* ($2n = 28$, nuclear genome S^vS^vUU), *Ae. mutica* ($2n = 14$, TT) and *Ae. uniaristata* ($2n = 14$, NN) were introduced into CS by repeated backcrosses for ten times or more. These CS alloplasmics, hereafter, will be denoted by (*kotschyi*)-, (*mutica*)- and (*uniaristata*)-CS, pedigrees of which are given by Tsunewaki et al. (1996).

Lukaszewski (2000) produced a large number of 1BS-1RS recombinant chromosomes in the genetic background of Pavon wheat through induced translocation (=recombination) between the chromosome arms, 1BS of Pavon and 1RS of rye, in the absence of *Ph1* gene, namely, in the *ph1b* mutant of Sears (1984). He classified them into two types, depending on the configuration: one designated T- with a proximal rye segment and terminal wheat segment, and the other 1B+ with terminal rye segment and proximal wheat segment (Lukaszewski 2000). Seed samples of these translocation lines were provided by A. J. Lukaszewski, University of California, Riverside, USA for this study.

Genetic and molecular maps of the 1BS arm of Pavon: Lukaszewski (2000) allocated the translocation breakpoints of 47 T- and 56 1B+ type recombinant stocks in the following seven chromosome arm segments marked by six genetic and cytological markers; centromere—NOR (nucleolar organizer)—S-6 (a C-band)—*Sec-1* (secaline gene)—*Lr26* (and two other leaf rust resistant genes)—*Pm8* (Powdery mildew resistant gene)—*Gli-B1* (a gliadin gene)—arm terminus. The NOR is designated *Nor-B1* (May and Apples 1988). Later, Sharma et al. (2009) mapped eight molecular markers that were incorporated into the Lukaszewski's 1BS map. They determined locations of the breakpoints of 69 1BS-1RS recombinant lines in the map (Sharma et al. 2009).

Methods

Crossing scheme and estimation of seed fertility: Three CS alloplasmic lines were manually emasculated and hand-pollinated with the pollen of Pavon recombinant lines. All F_1 hybrids from these crosses are expected to be the heterozygote of CS 1B chromosome/1BL.1BS-1RS recombinant chromosome, except the hybrids of the following five recombinant lines: 1B + 32 + 1BL

and 1B + 57 + 1BL used in the pilot experiment and T-12 + 1BS, 1B + 33 + 1BL and 1B + 35 + 1BL used in the main experiment. They produced two types of the F_1 hybrids with CS alloplasmics as female, namely, the heterozygote of CS 1B chromosome/1BL.1BS-1RS recombinant chromosome and that of CS 1B chromosome/1BS or 1BL wheat telosome. The latter heterozygote should be rarely produced because of rare chance of fertilization expected for the pollen carrying a telosome.

For each cross combination between the CS alloplasmics and a Pavon recombinant line, two plants of the same recombinant line were used in principle as the pollen parent. Generally, four to six F_1 plants were grown for each cross combination in greenhouse adjusted to 24 °C under a long day condition (14 h light period). Three ears of each F_1 plant were bagged before flowering and seed setting in the first and second florets of about 10–15 spikelets per ear was observed, from which the per cent seed setting, called seed fertility, of each F_1 plant was calculated by taking average of the three ears. Average and standard deviation of the seed fertility were calculated for each recombinant line or a group of the recombinant lines from the seed fertilities of all the F_1's in each recombinant line or its group.

Pilot experiment for mapping the *Rfv1* gene on the genetic map of the 1BS arm: The *Rfv1* gene for the *kotschyi* plasmon was mapped on the genetic map of the 1BS arm consisting of six genetic markers (Lukaszewski 2000). Number of recombinant lines employed was one for each of T- and 1B+ type as for each chromosome segment. The seed fertilities of the F_1's of both T- and 1B+ type recombinant lines indicated that the *Rfv1* gene is located in either of two adjacent segments, *Sec-1*/S-6 and S-6/*Nor-B1*.

Main experiment for mapping the *Rf* genes for the three *Aegilops* plasmons on the molecular map of the 1BS arm: Based on the result of the pilot experiment, the main experiment was designed to map three *Rf* genes, *Rfv1*, *Rfm1* and *Rfn1,* for the *kotschyi, mutica* and *uniaristata* plasmons, respectively, on the molecular map of 1BS arm, focusing on two chromosome segments between *Sec-1* and *Nor-B1* markers. Number of recombinant lines having the translocation breakpoints in these adjacent chromosome segments, *Sec-1*/S-6 and S-6/*Nor-B1*, was 22 of the T- type and 2 of the 1B+ type recombinant lines in the former segment, and 21 of the T- type and 6 of the 1B+ type recombinant lines in the latter segment. Sharma et al. (2009) mapped eight molecular markers on the 1BS arm of Pavon wheat, of which one and two markers, respectively, were located in these two segments. Thus, the arm segments between *Sec-1* and *Nor-B1* were partitioned into the following

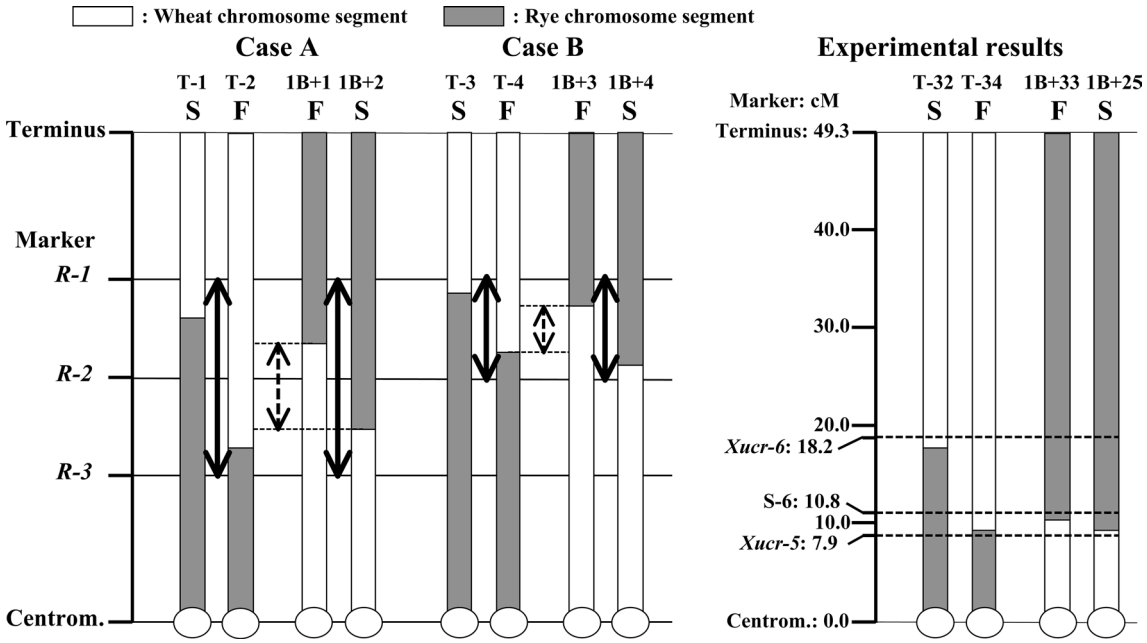

Fig. 1 Schematic models and the experiment result, indicating a possible range of the *Rf* gene location estimated from selfed seed fertilities of the F_1 hybrids between a CS alloplasmic line and 1BS-1RS recombinant lines (RLs) of Pavon wheat. T-1 to −4 and 1B + 1 to +4: 1BS-1RS recombinant chromosomes (refer to the text for detailed description) F and S: Fertile and semi-sterile hybrids, respectively, from the cross, male sterile CS alloplasmics × 1BS-1RS RLs. *Solid line* with double *arrow-heads*: range of the location of *Rf* gene assumed from the fertilities of a pair of the F_1 hybrids. *Broken line* with double *arrow-heads*: real location of *Rf* gene estimated from two pairs of the F_1 hybrids. Molecular map of the 1BS chromosome arm of Pavon, shown in "Experimental results", was cited from Sharma et al. (2009)

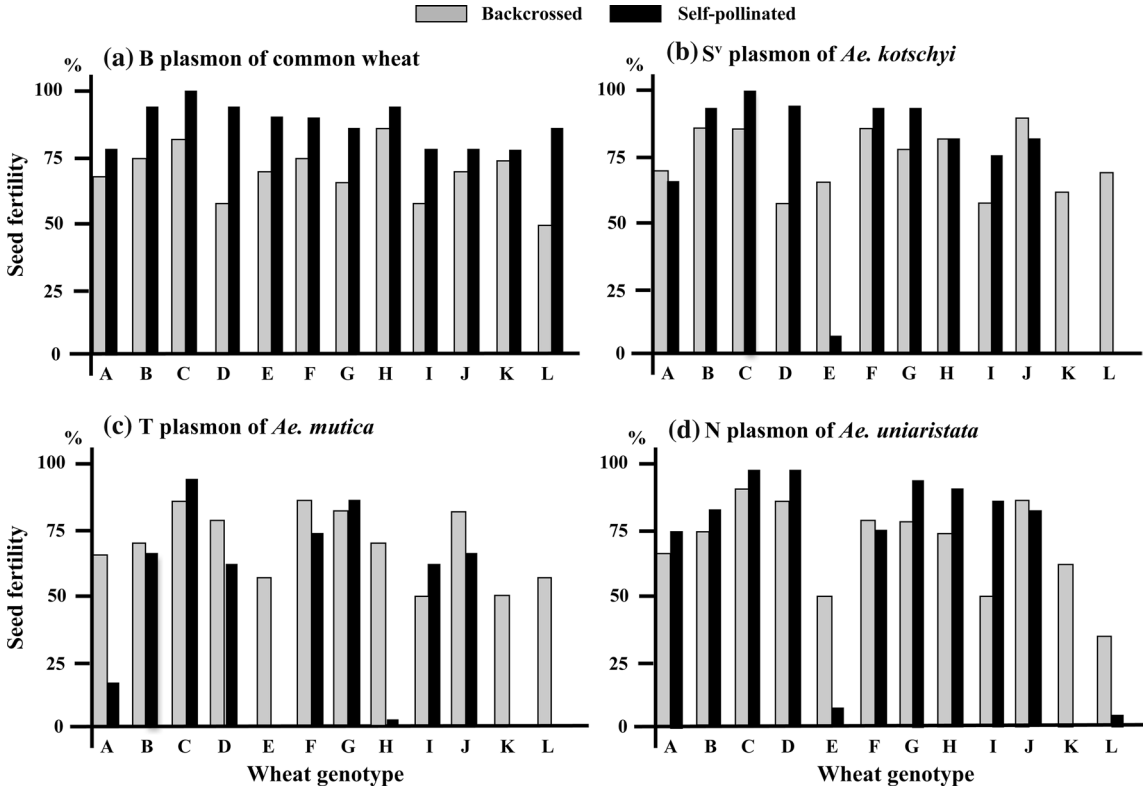

Fig. 2 Backcrossed and selfed seed fertilities (%) of 12 common wheat testers having three *Aegilops* plasmons and the respective euplasmic wheat lines as control: average of five advanced generations (mostly, SB_6 to SB_{10}) of repeated backcrosses with the respective wheat pollen parents (these fertility spectra were drawn from the data of Tables 7 and 8 of Tsunewaki et al. 1996). A–L Twelve wheat genotypes used as the tester for differentiating plasmon types in the *Triticum-Aegilops* complex. A *T. aestivum* var. *erythrospermum*, B *T. aestivum* strain P168, C *T. aestivum* cv. Chinese Spring, D *T. aestivum* cv. Norin 26, E *T. aestivum* strain Salmon, F *T. aestivum* cv. Jones Fife, G *T. aestivum* cv. Selkirk, H *T. aestivum* cv. S-615, I *T. sphaerococcum* var. *rotundatum*, J *T. compactum* strain No. 44, K *T. spelta* var. *duhamelianum*, and L *T. macha* var. *subletschchumicum*

five adjacent subsegments, *Sec-1*/*Xucr-6*, *Xucr-6*/S-6, S-6/*Xucr-5*, *Xucr-5*/*Xucr-4* and *Xucr-4*/*Nor-B1*.

The principle of mapping an *Rf* gene on the molecular map of the 1BS arm: The principle is illustrated in Fig. 1. If T-1 recombinant line produces semi-sterile F_1 hybrids with CS alloplasmics and T-2 recombinant line produces fertile ones, we may assume the *Rf* gene must be located between T-1 and T-2 breakpoints. Similarly, if 1B + 1 gives fertile and 1B + 2 does semi-sterile F_1's, we may assume the *Rf* is located between the 1B + 1 and 1B + 2 breakpoints. Combining these results together, the location of *Rf* can be narrowed between the proximal breakpoint of either T-1 or 1B + 1 and the distal one of either T-2 or 1B + 2, as shown in Fig. 1.

There are two possible cases of outcome in the scheme: in Model case A, one of the two adjacent breakpoints that occurred in neighbor segments of the 1BS arm gives fully fertile and the other semi-sterile F_1 hybrids. In this case, the location of the *Rf* gene is assumed to be between the two markers, one of which is the distal marker of the distal segment and the other is the proximal marker of the proximal segment. In Model case B, two breakpoints in the same segment give different F_1 phenotypes, one being fully fertile and the other semi-sterile. In this case, the location of the *Rf* gene is assumed between the proximal and distal markers of this segment.

Results

Similarity between the plasmons of *Ae. kotschyi, Ae. mutica* and *Ae. uniaristata* as to their CMS-RF expression in common wheat

Backcrossed seed fertility of an alloplasmic line pollinated with the pollen of its euplasmic line (=recurrent pollen parent) indicates its female fertility, whereas seed fertility after self-pollination is a product of its female and male fertilities. In case of an alloplasmic line with normal female fertility, the self-pollinated seed fertility is considered to be indicative of its male fertility.

Table 1 Correlation coefficients, *r*, between the self-pollinated seed fertility spectra of three plasmons of *Ae. kotschyi* (plasmon type, S^v), *Ae. mutica* (T) and *Ae. uniaristata* (N), as compared to that of common wheat (B)

Species	Type	Plasmon type			
		S^v	T	N	B
Ae. kotschyi	S^v	–	0.731**	0.981**	0.237^{NS}
Ae. mutica	T	–	–	0.799**	0.261^{NS}
Ae. uniaristata	N	–	–	–	0.306^{NS}
Common wheat	B	–	–	–	–

** and ^{NS}: Correlation coefficient is significant at the 1 % level of probability, and non-significant at the 5 % level of probability, respectively

Both the backcrossed and self-pollinated (hereafter, 'selfed') seed fertilities of the euplasmic and the three alloplasmic lines with the *kotschyi, mutica* and *uniaristata* plasmons of 12 common wheat testers were taken from Tables 7 and 8 of Tsunewaki et al. (1996), respectively, which are presented in the form of fertility spectrum (Fig. 2).

The average backcrossed seed fertility (grey column) of the 12 alloplasmic lines with each of the *kotschyi, mutica* and *uniaristata* plasmons, were 72.3 ± 11.5, 67.5 ± 13.0 and 67.3 ± 16.3 %, respectively, as compared to average of 69.5 ± 10.3 % of the 12 euplasmic lines. Considering a fact that those seed setting rates were obtained by hand pollination, we may conclude that the female fertilities of all alloplasmic lines have been essentially almost normal.

As to the selfed seed fertility, alloplasmic lines of Salmon (code, E), Spelta (K) and Macha (L) with all three *Aegilops* plasmons and two additional lines of S615 (H) and Tve (A) with the *mutica* plasmon showed complete or nearly complete sterilities (0–15 % seed set), comparing to high fertilities of all other alloplasmic lines (59–96 % seed set). Euplasmic lines with the common wheat plasmon invariably exhibited high selfed seed fertilities of 77–98 %.

Correlation between the fertility spectra of four plasmons was analyzed (Table 1). The selfed seed fertility spectra of the *kotschyi* plasmon (S^v type) and *uniaristata* plasmon (N type) were almost identical to each other ($r = 0.98$), whereas that of the *mutica* plasmon to the *kotschyi* and *uniaristata* plasmons was also high ($r = 0.73$–0.80), both being significant at the 1 % probability level for $df = 10$. On the contrary, the spectrum of common wheat plasmon (B type) showed little similarity to those of three *Aegilops* plasmons ($r = 0.24$–0.31), all being non-significant at the 5 % probability level. This indicated that the three *Aegilops* plasmons are genetically similar with each other in terms of their CMS-RF expression against the 12 common wheat testers.

Mapping the *Rfv1* gene on the genetic map of the 1BS arm of Pavon wheat

Based on those facts, I first carried out mapping of the *Rfv1* gene for the *kotschyi* plasmon on the Lukaszewski's 1BS genetic map, as a representative of *Rf* genes for other alloplasmons. For this purpose, a single recombinant line was selected at random for each of the two configurations, T- and 1B+ types, from each of the 1BS arm segments partitioned by the six genetic markers, except the T- type recombinant line having breakpoint in the terminus/*Gli-1* segment and the 1B+ type recombinant line having breakpoint in the *Nor-B1*/centromere segment, which were not available for this study. In total, 12 types of recombinant lines were employed in crosses to the alloplasmic CS with the *kotschyi* plasmon and their F_1 hybrids were examined for seed fertility under self-pollination, the results of which are shown in Table 2.

All the F_1's from crosses involving the T- type recombinant lines with the breakpoint distal to *Sec-1*, including complete 1RS arm, were semi-sterile, whereas all those of the same type configuration of recombinant lines with the breakpoint proximal to *Nor-B1*, including complete 1BS arm (=1BS" Pavon wheat), were fully fertile. On the other hand, all F_1's with the 1B+ type configuration recombinant lines with the breakpoint distal of *Sec-1* were fully fertile, whereas that with the 1B + type recombinant line with the breakpoint proximal to *Nor-B1*, that was represented only by the original 1BL.1RS translocation line, was semi-sterile.

These results of the F_1's both of the T- and 1B+ type recombinant lines indicated that the *Rfv1* gene locus is in either of the two adjacent segments: *Sec-1*/S-6 or S-6/*Nor-B1*, corresponding to Model case A of Fig. 1.

Mapping the *Rfv1, Rfm1* and *Rfn1* genes for the *kotschyi, mutica* and *uniaristata* plasmons, respectively, on the molecular map of the 1BS arm of Pavon wheat

Given the high genetic coincidence of the fertility spectra of the three plasmons, the assumption was made that the *Rf* genes for those plasmons were located in the same site or, if not, near sites to each other, namely perhaps, in either of two adjacent chromosome segments, *Sec-1*/S-6 and S-6/*Nor-B1,* of the 1BS arm. Genetic mapping by Sharma et al. (2009) partitioned those segments into five subsegments by three molecular and one cytological marker, as described in "Methods". The T- and 1B+ type recombinant lines that have breakpoint in these subsegments were employed in the present investigation (Table 3).

Besides those recombinant lines, one recombinant line each of the T- and 1B+ type with the breakpoint in the *Xucr-3*/*Xucr-2* subsegment was used as the reference.

Table 2 Selfed seed fertility (%) of the F_1 hybrids between (*kotschyi*)-CS and Pavon's 1BS-1RS recombinant lines

♂ parent	Site of breakpoint		No. plts.	Seed fertility (ave. ± S.D.)	Fert. class[a]
	Segment	cM			
1BL.1RS″	–	49.7	4	58.6 ± 17.3	S
(None)	Terminus/*Gli-1*	0.7	–	–	–
T-9″	*Gli-1*/*Pm8*	2.2	4	29.7 ± 8.7	S
T-8″	*Pm8*/*Lr26*	6.6	4	40.0 ± 9.6	S
T-1″	*Lr26*/*Sec-1*	6.6	4	52.3 ± 11.6	S
T-17″	*Sec-1*/S-6	22.8	4	46.5 ± 31.3	S
T-34″	S-6/*Nor-B1*	4.3	4	96.8 ± 2.3	F
T-15″	*Nor-B1*/centrom.	6.5	4	88.1 ± 11.9	F
1BS″ (Pavon)	–	–	4	96.8 ± 3.9	F
1B + 40″	Terminus/*Gli-1*	0.7	4	96.8 ± 1.8	F
1B + 14″	*Gli-1*/*Pm8*	2.2	4	97.1 ± 1.2	F
1B + 32 + 1BL[b]	*Pm8*/*Lr26*	6.6	3	95.3 ± 3.4	F
1B + 57 + 1BL[b]	*Lr26*/*Sec-1*	6.6	2	96.3 ± 5.2	F
1B + 22″	*Sec-1*/S-6	22.8	4	95.2 ± 2.3	F
1B + 25″	S-6/*Nor-B1*	4.3	4	41.7 ± 16.0	S
(None)	*Nor-B1*/centrom.	6.5	–	–	–
1BL.1RS″	–	0.0	4	58.6 ± 17.3	S

[a] S and F: Fertility of the F_1 hybrid is semi-sterile and fertile, respectively

[b] One and two sterile offtype plants were excluded in 1B + 32 + 1BL and 1B + 57 + 1BL line, respectively

Two recombinant lines, each of the T- and 1B+ type, with breakpoints in either of two adjacent subsegments, *Sec-1*/*Xucr-6* or *Xucr-6*/S-6, were also included. All of them were crossed as male to the three CS alloplasmic lines, and the F_1 hybrids produced were tested for their selfed seed fertility, of which results are given in Table 3.

All the F_1's derived from the T- type recombinant lines with breakpoints distal to S-6 were semi-sterile, under the presence of all the three *Aegilops* plasmons, whereas those with breakpoint proximal to *Xucr-5* were fully fertile. Conversely, all the F_1's derived from the 1B+ type recombinant lines with breakpoint distal to S-6 were fully fertile, whereas those with breakpoint proximal to *Xucr-5* were all semi-sterile.

As for the T- type recombinant lines, all lines with breakpoints distal to *Xucr-6* were semi-sterile, whereas those with breakpoints proximal to *Xucr-5* were fully fertile, indicating the location of *Rf* genes in two adjacent subsegments, *Xucr-6*/S-6 and S-6/*Xucr-5*, corresponding to Model case A in Fig. 1. More accurate information on the *Rf* gene location was obtained from the 1B + type recombinant lines. In this case, the F_1's of two recombinant lines, 1B + 33 + L and 1B + 25, both with breakpoint in the same subsegment, S-6/*Xucr-5*, segregated for the F_1 phenotype, namely, full fertility of the former and semi-sterility of the latter F_1's, corresponding to Model case B. As shown in the figure of "Experimental results" (Fig. 1), the combined results of the T- and 1B + type recombinant lines indicated that all three genes, *Rfv1, Rfm1* and *Rfn1*, are located within a 2.9 cM subsegment of S-6/*Xucr-5*.

Discussion

Map location of the *Rf*^{multi} gene for three *Aegilops* plasmons

The present study has shown that three *Rf* genes, namely, *Rfv1* gene for an S^v type plasmon of *Ae. kotschyi*, *Rfm1* gene for the T type plasmon of *Ae. mutica* and *Rfn1* gene for the N type plasmon of *Ae. uniaristata* are all located in the same 2.9 cM-long subsegment bordered by S-6 and *Xucr-5* markers in the 1BS arm of Pavon. Of the F_1's of three recombinant lines with their breakpoints in the above subsegment, that of 1B + 25 was semi-sterile, whereas those of T-34 and 1B + 33 were fully fertile. Although their precise breakpoints in this subsegment are not known (Sharma et al. 2009), it is certain that all three *Rf* genes must be located proximal to the breakpoint of 1B + 33, and distal to the breakpoint of T-34 or 1B + 25, of which breakpoint is distal to that of the other. Consequently, the actual location of the three *Rf* genes can eventually be allocated in a much narrower region within the 2.9 cM S-6/*Xucr-5* subsegment. In this regard, one point should be mentioned: Sharma et al. (2009) located breakpoint of the T-6 recombinant line in the above subsegment, S-6/*Xucr-5*. A recent molecular map of the 1RS chromosome arm constructed by Lukaszewski (personal communication), which consisted of 72 markers, however, revealed that the breakpoint of this recombinant line located in a distal subsegment of Sec-1/*Xucr-6* (ref. Table 3), although map positions of the breakpoints of other 12 T- type recombinant lines agreed

Table 3 Selfed seed fertility (%) and its standard deviation (%) of the F_1 hybrids between three CS alloplasmics and Pavon 1BS-1RS recombinant lines (RLs)

♂ parent (Pavon RL lines)	Location of breakpoint (subsegment)	Subseg. size (cM)[a]	♀ parent (CS alloplasmics)						Fertility class[b]
			(*kotschyi*)-CS		(*mutica* M)-CS		(*uniaristata*)-CS		
			No. plts	Fertility	No. plts	Fertility	No. plts	Fertility	
1BL.1RS″	–	49.7	6	6.3 ± 2.7	6	44.0 ± 14.6	6	27.8 ± 10.5	S
T-14″	*Sec-1*/*Xucr-6*	15.4	5	4.7 ± 4.0	6	34.7 ± 24.9	6	36.6 ± 15.0	S
T-27″			6	24.8 ± 26.3	5	33.9 ± 13.0	6	32.9 ± 17.6	S
T-12 + 1BS[c]			3	22.2 ± 4.2	3	38.0 ± 17.5	2	40.3 ± 4.0	S
T-3″			6	26.2 ± 16.2	6	26.4 ± 13.9	6	27.3 ± 15.5	S
T-10″			6	18.5 ± 11.3	5	27.2 ± 21.7	5	28.6 ± 16.0	S
T-22″			6	21.7 ± 15.3	6	32.2 ± 21.1	6	43.3 ± 13.8	S
T-25″			6	12.3 ± 19.5	6	26.6 ± 21.9	6	19.5 ± 7.3	S
T-6″			6	35.0 ± 23.2	6	22.9 ± 22.2	6	27.6 ± 15.2	S
T-29″	*Xucr-6*/S-6	7.4	6	14.8 ± 15.9	6	21.5 ± 13.2	6	36.1 ± 12.7	S
T-32″			6	12.3 ± 8.1	6	26.2 ± 13.2	6	31.7 ± 17.7	S
T-34″	S-6/*Xucr-5*	2.9	4	96.8 ± 2.3	4	93.8 ± 3.5	4	54.2 ± 13.5	F
T-33″	*Xucr-4*/*Nor-B1*	0.7	4	79.3 ± 22.3	4	98.1 ± 1.7	4	67.0 ± 14.1	F
T-15″	*Xucr-3*/*Xucr-2*	5.1	4	88.1 ± 11.9	4	97.6 ± 0.6	4	77.9 ± 11.3	F
1BS″(Pavon)	–	0.7	5	99.7 ± 0.6	6	95.6 ± 1.3	6	98.1 ± 1.9	F
1BS″ (CS)	–		4	99.0 ± 0.7	4	93.4 ± 6.6	4	92.0 ± 7.2	F
1B + 3″	*Sec-1*/*Xucr-6*	15.4	5	96.6 ± 1.9	6	91.2 ± 8.5	6	89.9 ± 0.9	F
1B + 4″			6	98.4 ± 2.1	6	95.1 ± 4.9	6	97.9 ± 1.2	F
1B + 6″			6	97.4 ± 2.4	6	96.8 ± 2.1	6	97.0 ± 2.4	F
1B + 10″			6	94.3 ± 5.7	6	95.6 ± 3.2	5	96.1 ± 2.3	F
1B + 12″			6	98.4 ± 1.6	6	98.1 ± 1.9	6	97.2 ± 2.5	F
1B + 16″			6	98.1 ± 1.4	6	95.4 ± 5.6	6	96.8 ± 2.1	F
1B + 17″			6	98.1 ± 0.7	6	91.4 ± 5.8	6	96.3 ± 3.2	F
1B + 20″			6	95.1 ± 6.6	6	93.3 ± 4.5	6	95.8 ± 1.3	F
1B + 23″			6	93.6 ± 7.3	6	94.2 ± 2.5	6	90.8 ± 6.8	F
1B + 2″	*Xucr-6*/S-6	7.4	6	98.5 ± 1.7	5	97.2 ± 2.6	6	97.2 ± 0.9	F
1B + 8″			6	95.6 ± 3.6	6	96.3 ± 3.8	3	91.7 ± 11.0	F
1B + 19″			6	96.6 ± 4.3	6	97.2 ± 2.0	5	92.2 ± 11.3	F
1B + 24″			4	94.1 ± 5.5	6	88.7 ± 11.8	6	91.0 ± 5.0	F
1B + 9″	*Sec-1*/S-6[d]	22.8	6	97.7 ± 1.7	3	93.5 ± 5.6	6	90.3 ± 7.1	F
1B + 28″			6	91.2 ± 6.0	6	88.4 ± 16.0	3	90.3 ± 5.0	F
1B + 33 + 1BL[c]	S-6/*Xucr-5*	2.9	9	87.6 ± 7.2	10	95.8 ± 2.8	6	83.2 ± 8.2	F
1B + 25″			4	41.7 ± 16.0	5	41.4 ± 8.7	6	7.3 ± 4.6	S
1B + 1″	*Xucr-5*/*Xucr-4*	0.7	6	7.6 ± 4.6	6	22.0 ± 9.8	6	39.6 ± 9.8	S
1B + 35 + 1BL[c]	*Xucr-4*/*Nor-B1*	0.7	10	17.3 ± 14.3	6	38.3 ± 3.8	12	9.0 ± 9.0	S
1B + 5″	*Xucr-3*/*Xucr-2*	5.1	4	7.5 ± 5.4	4	30.7 ± 5.0	6	13.6 ± 8.3	S

Seed fertility is shown by average ± standard deviation (%)

[a] After Sharma et al. (2009)

[b] "S" and "F": semi-sterile and fertile class, respectively

[c] Heterozygote of the 1BL.1BS-1RS recombinant chromosome and a 1BS or 1BL telosome

[d] The breakpoints of those recombinant chromosome arms are inbetween *Sec-1* and S-6 (Lukaszewski 2000) but their locating subsegment is not determined whether in the *Sec-1*/*Xucr-6* or *Xucr-6*/S-6 subsegment (Sharma et al. 2009)

Table 4 Selfed seed fertility observed in the alloplasmic F_1 hybrids with the single and double dose of *Rf multi* gene

Plasmon	Type of RLs	*Rf multi Rf multi* homozygote		*Rf multi rf multi* heterozygote	
		No. plts	Seed fert.(%)	No. plts	Seed fert.(%)
(a)					
Ae. kotschyi	T- type	12	88.1 ± 15.1	56	19.3 ± 17.4
	1B + type	96	95.5 ± 5.2	24	17.3 ± 16.3
Ae. mutica	T- type	12	96.4 ± 2.9	55	28.4 ± 17.9
	1B + type	96	94.3 ± 6.5	21	32.9 ± 10.5
Ae. uniaristata	T- type	12	66.3 ± 15.5	55	32.0 ± 14.9
	1B + type	87	93.6 ± 6.4	30	15.7 ± 14.7
Total	T- type	36	83.6 ± 17.8	166	26.5 ± 17.5
	1B + type	279	94.5 ± 6.1	75	21.1 ± 15.9

Plasmon	Fertility class[a]	Genotype[b]	No. plts.	Seed fert. (%) (aver. ± S.D.)
(b)				
Ae. kotschyi	F	*Rf Rf*	108	94.7 ± 7.3
	S	*Rf rf*	80	18.7 ± 17.0
Ae. mutica	F	*Rf Rf*	108	94.5 ± 6.3
	S	*Rf rf*	76	29.7 ± 16.3
Ae. unaristata	F	*Rf Rf*	99	90.3 ± 12.0
	S	*Rf rf*	85	26.2 ± 16.7
Total	F	*Rf Rf*	315	93.3 ± 9.0
	S	*Rf rf*	241	24.8 ± 17.2

[a] F and S: fertile and semi-sterile class, respectively

[b] The 1RS rye chromosome arm is assumed to have a nulli or recessive allele of the *Rf multi* gene

with those of Sharma et al. (2009): the present article adopted new position of the T-6 breakpoint.

From the very close location(s), if not the same, of the *Rfv1, Rfm1* and *Rfn1* genes and a high similarity of their CMS-RF expression spectra (Fig. 2), I will propose that there is a single locus for the three *Rf* genes, designating it with a symbol *Rf multi*, meaning "*R*estoration of *f*ertility in *multi*ple CMS systems". This may be the first case of a pluripotential *Rf* gene for a variety of MS plasmons in the *Triticum-Aegilops* complex. The present results on the map location and multi-function of the *Rf multi* gene will be useful for its cloning and production of a fertility-restoration transformant using the method of Ishida et al. (2015).

Fertility restoration by the *Rf multi* gene in the presence of male sterile *Aegilops* plasmons and its use for hybrid wheat breeding

Both the T- and 1B + type recombinant lines of Pavon will segregate the homo- and heterozygotes of *Rf multi* gene in the F_1 hybrid with CS alloplasmics as female, depending upon the position of breakpoint in the recombinant chromosome arms, assuming a nulli- or recessive allele of the *Rf multi* gene for the rye 1RS chromosome arm. As for the T- type recombinant line, its F_1 hybrid with alloplasmic CS will become the *Rf multi Rf multi* homozygote when the breakpoint of the recombinant chromosome arm is proximal to the *Rf multi* locus, whereas they will become the heterozygote, *Rf multi rf*, when the breakpoint is distal to it. As for the 1B + type recombinant line, on the other hand, the F_1 hybrid will become the homozygote when the breakpoint is distal to the gene locus, whereas it will become the heterozygote when it is proximal.

Table 4 shows selfed seed fertilities of the F_1 hybrids having *Rf multi* gene in the homozygous and heterozygous condition, of which Table 4a presents the F_1's of the T- and 1B + type recombinant lines separately, whereas Table 4b presents their combined data. Selfed seed fertility did not differ between of the F_1 hybrids having the T- and 1B + type recombinant chromosomes, in both the homozygous and heterozygous state of the *Rf multi* gene, except the homozygotes with the *uniaristata* plasmon, which showed evidently lower fertility of the T- type than the 1B + type F_1's. The results given in Table 4b showed that the homozygosity of *Rf multi* restored seed fertility to normal level within a range of 90–95 %, whereas its heterozygosity restored less than 30 % fertility in the presence of all three *Aegilops* plasmons.

As for the nuclear genotype, the results presented in Fig. 2 showed that three of the 12 wheat testers, namely, Salmon (code E), Spelta (K) and Macha (L), did not carry the *Rf multi* gene, becoming male sterile with the presence of the three *Aegilops* plasmons. Male sterility of alloplasmic Salmon having the above three plasmons is caused by the presence of 1RS arm, substituted for the 1BS arm (Tsunewaki 1964; Zeller 1973), similar to the 1BL.1RS

line of Pavon. Therefore, the chromosome constitution of Salmon is not desirable for hybrid wheat, because of the difficulty in genetic improvement of the 1RS arm by recombination. Macha has another serious problem as the breeding material, because it carries one of the complementary genes for the type 1 hybrid chlorosis, *Ch1*, on chromosome 2A (Hermsen and Waninge 1972; Tsunewaki 1975), whereas 96 % of more than 1,800 common wheat cultivars collected world-wide possessed its complementary gene, *Ch2* (Tsunewaki and Nakai 1973), indicating that most F_1 hybrids between Macha and common wheat cultivars suffer from this hybrid chlorosis. Thus, the source of the *rf* ^{multi} gene desirable for breeding CMS line is limited to Spelta wheat so far as the 12 common wheat testers used in this study are concerned. However, a small insert of rye chromatin could easily be produced from the 1BS-1RS recombination to remove the *Rf* locus, very much the same way as the *Sec-1* locus was removed from the 1BL.1RS translocation (Lukaszewski 2000).

As for the plasmon side, the *mutica* plasmon delayed heading of the 12 wheat testers about 12 days under field condition (after field record of Tsunewaki et al. 2002), and this is disadvantageous for hybrid wheat breeding. On the other hand, the plasmons of *Ae. uniaristata* and *Ae. kotschyi* did not show any remarkable deleterious effects on common wheat phenotype (Tsunewaki et al. 2002). Consequently, the CMS-RF expression system usable for hybrid wheat breeding is limited to the combination of the *rf* ^{multi} gene of Spelta wheat and the *kotschyi* or *uniaristata* plasmon, so far as our materials are concerned.

Fertility restoration of hybrid wheat by the *Rf* ^{multi} gene poses serious problem for its practical use. As shown in Table 4b, F_1 hybrids with a single dose of *Rf* ^{multi} gene recovered less than 30 % of seed fertility that was observed by controlled self-pollination of the heterozygote (genotype, *Rf* ^{multi} *rf* ^{multi}) under greenhouse conditions. A higher level of fertility restoration by the *Rf* ^{multi} *rf* ^{multi} heterozygote may be expected in an open field. At any rate, the present results indicated that the male fertility restoration by the *Rf* ^{multi} gene is gametophytic. Therefore, a different restorer gene recombining freely with *Rf* ^{multi} must be sought for a high level of fertility restoration in hybrid wheat by its use in combination with the *Rf* ^{multi} gene.

Author contribution statement The author carried out this research by himself, including its planning, doing experiments, analyzing the results and preparing the manuscript.

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Conflict of interest None.

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