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DArT markers tightly linked with the *Rfc1* gene controlling restoration of male fertility in the CMS-C system in cultivated rye (*Secale cereale* L.)

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Abstract The *Rfc1* gene controls restoration of male fertility in rye (*Secale cereale* L.) with sterility-inducing cytoplasm CMS-C. Two populations of recombinant inbred lines (RIL) were used in this study to identify DArT markers located on the 4RL chromosome, in the close vicinity of the *Rfc1* gene. In the population developed from the 541×2020LM intercross, numerous markers tightly linked with the restorer gene were identified. This group contained 91 DArT markers and three SCARs additionally analyzed in the study. All these markers were mapped in the distance not exceeding 6 cM from the gene of interest. In the second mapping population (541×Ot1-3 intercross), only 9 DArT markers located closely to the *Rfc1* gene were identified. Five of these DArT markers were polymorphic in both populations.

Keywords Cytoplasmic male sterility · Genetic mapping · Restorer gene · Rye

Rye (*Secale cereale* L.) is a crop of a great importance in the north-east part of Europe due to its tolerance to low temperatures in winter, aluminum ions in the soil and other environmental stresses often occurring in this region. The breeding progress recently achieved in hybrids is significantly higher than in population cultivars of rye (Geiger 2007). This leads to a regular increase of the growing areas of hybrid cultivars in European countries.

Generally, the breeding of rye hybrids is based on the Pampa sterility-inducing cytoplasm (CMS-P). The use of an alternative type of CMS in practice is strongly recommended for reducing risks arising from plasmotype uniformity (Morgenstern and Geiger 1982; Adolf and Winkel 1985; Geiger et al. 1995). One of potentially applicable sources of CMS in rye is the C cytoplasm (CMS-C) discovered by Łapiński (1972) in the old Polish cultivar ‘Smolickie’. In consideration of the low frequency of sterility alleles in populations of cultivated rye, development of new non-restorer lines in the CMS-C system can be significantly facilitated by the application of marker assisted selection (MAS). The main gene controlling restoration of male fertility in the C cytoplasm (*Rfc1*) is located on the 4RL chromosome (Stojałowski et al. 2004) and some molecular markers linked with this locus were identified (Stojałowski et al. 2005; Hackauf et al. 2009). Recently a high density genetic map of rye was developed by Bolibok-Brągoszewska et al. (2009) with the use of Diversity Array Technology (DArT). This study was aimed at identification of new markers based on Diversity Array Technology which are tightly linked to the *Rfc1* gene.

Two mapping populations composed of recombinant inbred lines (RILs) were used in the study. First population consisted of 92 RILs of F_5 generation developed from the cross between the inbred lines: 541 (non-restorer) and 2020LM (restorer). The second population contained 144 RILs (F_9) originating from the cross between the same non-restorer line 541 and the restorer Ot1-3. The origin of

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inbred lines 541 and Ot1-3 is different and the genetic distance between them is significant (Myśków et al. 2001). The line 2020LM was kindly provided by L. Madej from the Institute of Plant Breeding and Acclimatization, Radzików. Its pedigree is unknown, however it proved to be genetically distant from line 541 (Myśków et al. 2010). Maternal line of both mapping populations (line 541) possessed normal cytoplasm, so this type of cytoplasm was present in all RILs analyzed.

All recombinant inbred lines were genotyped with the use of Diversity Array Technology essentially as described by Bolibok-Brągoszewska et al. (2009). Genomic representations of individual RILs were prepared using the *Pst*I/*Taq*I complexity reduction method, Cy3 or Cy5 labeled and hybridized to the rye genotyping array 2.0. Two differently labeled representations were hybridized to each slide. Tiff images of slides acquired with fluorescent microarray scanner (Tecan LS300,Grödig, Salzburg, Austria) were automatically analyzed with dedicated software (DArTsoft version 7.3, DArT P/L, Yarralumla, Australia, unpublished) to identify and classify polymorphic markers. Markers with Q>80% and a call rate of at least 90% were selected for subsequent analyses.

Additionally, a set of polymorphic PCR-based sequence specific markers (SCAR, STS) with known chromosomal localization were analyzed in both populations. Between them, there were markers described by Stracke et al. (2003), Milczarski et al. (2007) and two newly developed SCAR markers obtained by sequencing RAPD fragments located on the 4RL chromosome (Stojałowski et al. 2004). Sequences of primers for these markers are as follows:

Xscsz23L500 – CGAAGCAGACCCGTACACAT / CGAAGCAGACGAGGATATTT
Xscsz670L900 – GGACTGGAGTGATAAGGACG / GGACTGGAGTCTAATATATG.

Segregations of all the markers significantly deviating from the 1:1 ratio (by Chi^2 test, at $P<0.01$) were not taken under consideration.

In order to determine whether a given recombinant line carries the non-restorer or restorer allele in the *Rfc1* locus, each genotype of both mapping populations was testcrossed with the male sterile genotype. As a source of sterility-inducing cytoplasm, the male sterile single cross between two related inbred lines (CMS-C line 585/92-1-2 and non-restorer line 585/92-6-1) was used. Male fertility/sterility of plants obtained as a result of the testcrosses between the male sterile source of CMS-C and a given recombinant inbred line was assessed in the field conditions in Szczecin. The necessary amount of hybrid seeds was gained for crosses between source of CMS and 81 RILs of population 541×2020LM and 107 RILs 541×Ot1-3. From 5 to 12 plants for each of the 188 cross combinations were grown during the vegetation season 2009/2010 with 25×25cm spacing and at the flowering time all plants were assessed visually with the use of the Geiger and Morgenstern (1975) scale. Additionally, results of visual scoring were verified by evaluation of seed setting on spikes bagged on each plant before flowering. Male fertile progeny of a testcross indicated that the analyzed RIL had a restorer allele in the *Rfc1* locus, while male sterile one – a non-restorer allele. Occasionally, observed segregation for male fertile and male sterile plants indicated the heterozygous character of the RIL with respect to the *Rfc1* locus.

Identification and localization of markers tightly linked with the *Rfc1* gene were performed with the use of the JoinMap 3.0 software (Van Ooijen and Voorrips 2001). First, for both mapping populations linkage groups were constructed at the LOD=15. Then, within the group containing the *Rfc1* locus the “show strong linkages” command was applied for the selection of markers with recombination frequency to the *Rfc1* not exceeding the 0.05 level. Segregations of the selected markers as well as that of the *Rfc1* gene, were used for map construction. In order to allow for comparisons of localization of the markers present in the constructed maps to the other, formerly published genetic maps of rye, a STS marker *Xpsr119* was included into the analysis. This PCR-based marker was developed from a RFLP probe (Milczarski et al. 2007) and it was polymorphic in both studied RILs populations.

Molecular markers common for both mapping populations were applied for construction of an integrated genetic map and estimation of average frequencies of recombination per meiosis between these markers and *Rfc1* locus.

The assessment of male fertility in the testcrosses has revealed a distribution of progenies into two main phenotypic classes: male sterile and male fertile, indicating that analyzed RILs belonged to the genotypic classes of maintainers and restorers, respectively (Table 1). Partial restoration was observed occasionally in individual plants, but never in whole progenies. The proportion of both genotypic classes (maintainers: restorers) in studied hybrids remained in agreement with monogenic model of inheritance in a RIL population (1:1). There were, however, some exceptions found in both mapping populations. Five RILs in the population 541×Ot1-3 and four RILs in the 541×2020LM showed heterozygous character giving segregating progenies in the testcrosses. These nine lines were excluded from mapping analyses.

Restoration of male fertility in plants with the C cytoplasm stays under control of at least 2–3 nuclear genes (Łapiński 1990; Stojałowski et al. 2004). On the other hand, monogenic inheritance of male fertility restoration in rye with CMS-C was reported in some cross combinations (Łapiński 1990). Likely, in both mapping populations analyzed in this study, only one restorer gene showed

Table 1 Male fertility (mean scores) of analyzed testcrosses with the CMS-C cytoplasm

Phenotype	Intervals of average male fertility scores	Number of recombinant inbred lines[1]	
		541×2020LM	541×Ot1-3
Male sterile (MS)	1-1.9	0	1
	2-2.9	21	44
	3-3.9	10	5
Male fertile (MF)	4-4.9	0	0
	5-5.9	0	0
	6-6.9	0	0
	7-7.9	0	4
	8-8.9	23	32
	9	23	16
Total		77	102
Chi^2 (1:1 segregation ratio of MS:MF)		2.92	0.0004
P		0.10-0.05	>0.99

[1] RILs heterozygous in *Rfc1* locus were omitted.

polymorphism. A similar situation was observed in the research on mapping of the *Rfg1* gene controlling male fertility in rye with CMS-G cytoplasm (Börner et al. 1998). Production of fertile pollen in plants with G cytoplasm stays under control of at least three genes (Melz and Adolf 1991), but in the cross used by Börner et al. (1998) for localization of the *Rfg1* locus on the 4RL chromosome, monogenic model of inheritance was assumed.

It should be noted, that assessment of male fertility/sterility of testcrosses with C cytoplasm was performed in only one environment. This does not allow us any consideration about environmental sensitivity of the studied trait. According to observations of Geiger et al. (1995) on rye plants with Pampa sterilising cytoplasm, environmental conditions are important for expression of male fertility, but their significance is rather limited to partially male fertile genotypes. This class of genotypes was not observed in testcrosses assessed in our study (Table 1), and for this reason we assumed that classification of phenotypes reported here should be sufficient for mapping purposes.

The first linkage group (LOD=15) containing the *Rfc1* locus was constructed after the analysis of 2281 polymorphic markers in the 541×2020LM population. The group was composed of 174 loci (173 molecular markers and *Rfc1* gene). The selection of markers very tightly linked with the *Rfc1* resulted in identification of a group of 94 molecular markers (Table 2). This group consisted of three SCAR markers and 91 DArTs. Recombination frequency between *Rfc1* and molecular markers, not exceeding the level of 0.01, was observed in the case of 27 DArTs.

In the population 541×Ot1-3 2097 polymorphic markers were genotyped. In the linkage group containing *Rfc1* locus there were 238 molecular markers (mostly DArTs), but only nine of them showed recombination frequency not exceeding the 0.05 level (Table 1).

The mapping analysis was performed only with the use of markers revealing recombination frequency below 0.05 with the exception of *Xpsr119* STS marker and the SCAR marker *Xscsz23L500*. Although *Xscsz23L500* was localized not so close to the *Rfc1* locus in the 541×Ot1-3 cross, it belonged to the group of markers tightly linked to the restorer gene in the 541×2020LM population.

Seven markers (*Xpsr119*, *Xscsz23L500*, and five DArT loci) were present in both linkage maps. Considering only the order of these common markers, the genetic maps constructed for the two studied populations remain in agreement (Fig. 1). However, some significant differences between both linkage groups are easily noticed. The distances between the *Xpsr119*, *Xscsz23L500*, common DArT markers and *Rfc1* gene are longer on the map of 541×Ot1-3, in spite of significantly lower number of loci in the linkage group. The same five DArT markers, which in the population 541×2020LM revealed no recombination with the *Rfc1*, in the cross of 541×Ot1-3 belonged to the most closely located markers, but with the distance of about 5 cM.

Table 2 Number of markers within the linkage groups and their recombination frequency with the *Rfc1* locus

Recombination frequency	541×2020LM	541×Ot1-3
<0.01	27	0
0.01-0.02	9	0
0.02-0.03	42	0
0.03-0.04	7	0
0.04-0.05	9	9
Total in the linkage group[1]	173	238

[1] linkage groups containing the *Rfc1* locus were constructed at LOD=15.

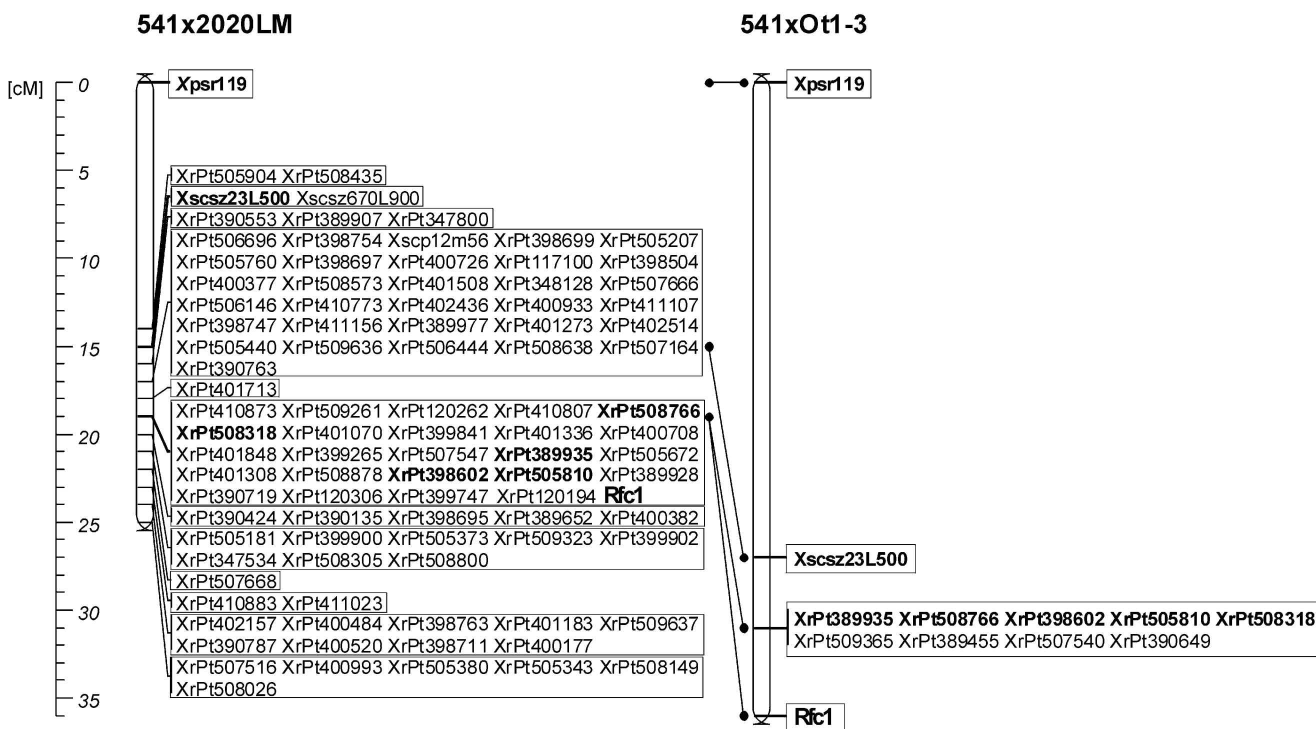

Fig. 1 Linkage groups of molecular markers from 4RL chromosome and localization of *Rfc1* gene. Positions of loci common for both mapping populations are printed in bold. Markers located in the same position on the genetic map are framed

The results of this study enable more precise localization of *Rfc1* locus than the interval mapping analyses performed before (Stojałowski et al. 2004; Hackauf et al. 2009). In the mapping population (544cms-C×Ot0-20 intercross) used during these former studies, activity of at least two restorer genes was observed resulting in problems with precise localization of the *Rfc1* locus on the 4RL chromosome. Linkage maps developed with the use of reported here mapping populations allowed for more accurate localization of this restorer gene. On the other hand, some inconsistencies occurred when both new genetic maps were compared. First of all, at the moment it is difficult to explain the unexpected differences in the results of selection of the most tightly linked markers in both related mapping populations. The source of non-restorer alleles was the same in both crosses. Naturally, it can not be absolutely excluded that the restorer locus in lines 2020LM and Ot1-3 is different, but the presence of the same markers revealing lower recombination frequency and similar order of loci on both genetic maps indicates that it is rather the same gene. The total number of polymorphic molecular markers was comparable in both crosses, as well as the number of loci within the identified linkage groups containing the *Rfc1* locus. It seems, however, that the level of recombination events on the long arm of 4R chromosome was slightly higher in the population 541×Ot1-3. Distances between loci

Table 3 Distances between *Rfc1* gene and molecular markers loci on the integrated genetic map of the 4RL chromosome and estimated frequencies of recombinants

Marker	Distance to the *Rfc1* (cM)	Frequency of recombinants between a marker and *Rfc1* locus per one generation (%)	
		Mean	Standard deviation
Xpsr119	25.04	6.05	1.09
Xscsz23L500	5.06	1.37	0.70
XrPt508318	2.52	0.65	0.75
XrPt389935	2.51	0.63	0.73
XrPt508766	2.50	0.63	0.73
XrPt398602	2.49	0.61	0.71
XrPt505810	2.47	0.57	0.66

on genetic maps can be expanded by multiple rounds of meiosis during inbreeding (Teuscher et al. 2005). The RILs population 541×Ot1-3 is more advanced than 541×2020LM as concerns the inbreeding (F_9 vs. F_5), but this fact only partly explains significant differences in recombination frequency. Average frequency of recombinants per meiosis stays with agreement with the distances between *Rfc1* locus and molecular markers on the integrated genetic map (Table 3). Five DArT markers segregating in both mapping populations are located on this map about 2.5 cM from the *Rfc1* gene, but differences between recombination frequencies in both studied populations are appreciable in high values of standard deviation. As suggested by Palmer et al. (1998), factors affecting the recombination efficiency, like genotype × environment interactions, possible differences in chromosome structure between parents and genetic background of mapping populations may partially explain observed inconsistencies.

Expressing of more general conclusions regarding the precise localization of the *Rfc1* gene is currently rather difficult. This is caused by a relatively low number of genotyped lines within mapping populations and by possible inaccuracy in genetic maps as a result of heterogeneity of some RILs not detectable when dominant molecular markers are used for genotyping. Warzecha and Salak-Warzecha (1996) suggest that sources of sterility-inducing cytoplasms CMS-C and CMS-G are genetically very similar or even identical. Localization of the *Rfc1* locus on the long arm of the 4R chromosome stays with agreement with observations done by Börner et al. (1998) about position of the *Rfg1* gene controlling restoration of male fertility in rye with G cytoplasm. In the same region of rye genome, the gene controlling pollen production of rye with Pampa cytoplasm was mapped by Miedaner et al. (2000). Unfortunately, the lack of common molecular markers (with the only exception of *Xpsr119*) and insufficient density of genetic maps reported before (Börner et al. 1998; Miedaner et al. 2000) do not allow for concluding about identity of nuclear genes regulating release of pollen by plants with different sources of CMS. It can be stated however, that the 4RL chromosome is a region of rye genome with a great importance as regards of restoration of male fertility in different CMS systems.

It should be stressed, that as a result of this study a group of DArT markers tightly linked to the *Rfc1* gene was identified. These markers may be useful as a tool for further genetic analyses of CMS-C system, especially if positional cloning of the *Rfc1* gene were performed with the use of genomic libraries of rye. Low frequency of recombination between *Rfc1* and indicated markers also opens new perspectives for effective marker-assisted selection in the development of rye hybrids based on the CMS-C system. Obviously, hybridization of selected genotypes with whole rye DArT chip is economically not recommendable but sequencing DNA of clones tightly linked with the *Rfc1* gene should allow for development of polymorphic PCR-SCAR markers applicable in commercial breeding.

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