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High-resolution RFLP mapping of the fertility restoration (*Rf3*) gene against *Triticum timopheevi* cytoplasm located on chromosome 1BS of common wheat

Toshio Kojima, Hisashi Tsujimoto, and Yasunari Ogihara*

Kihara Institute for Biological Research, Yokohama City University, Maioka-cho 641-12, Yokohama 244, Japan

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Near-isogenic lines of alloplasmic wheat for the *Rf3* gene controlling fertility restoration against the cytoplasm of *Triticum timopheevi* were developed by successive backcrossing of Chinese Spring to the F_1 plants between (*timopheevi*)-CS (male sterile) and *T. spelta* (carrying *Rf3*). The resultant three BC_3F_1 plants were self-pollinated so as to obtain 125 BC_3F_2 progenies. Using this population, we precisely mapped the *Rf3* gene on the short arm of wheat chromosome 1B (1BS) of the RFLP linkage map. The *Rf3* gene was localized at a position 1.2 cM and 2.6 cM distant from *Xcdo388* and *Xabc156*, respectively. The genetic distances of *Rf3* from *Nor* and *Gli-B1* were calculated to be 22.3 cM and 18.6 cM, respectively, supporting previous data. Estimation of the physical distance of the region suggets that the *Rf3* gene resides within 500 kbp from the adjacent RFLP markers. The marker order of the genetic map corresponded to that of the cytological map, except for the position of *Xbcd98*. Comparison between genetic and cytological maps clearly shows that RFLP markers are unevenly distributed throughout the chromosome arm, and recombinations took place unequally in the chromosome arm.

INTRODUCTION

Nucleus-cytoplasm (NC) hybrids among related plants sometimes display phenotypic anomalies such as cytoplasmic male sterility (CMS). CMS is caused by the disturbance of cooperative functions between nucleus and cytoplasm, and male sterility is maternally inherited (Levings, 1991). Stimulated by the pioneering work of Kihara (1951) and Fukasawa (1955), who discovered CMS and fertility restoration system in wheat, many efforts for the production of commercial hybrid varieties of common wheat have been carried out. Since Wilson and Ross (1962) discovered the induction of complete male sterility in common wheat with the cytoplasm of *Triticum timopheevi*, this cytoplasm has been used intensively for the production of hybrid wheats all over the world. Seven genes are known to control the restoration of fertility against *T. timopheevi* cytoplasm, and their chromosome locations have been determined, namely, *Rf1* (1A), *Rf2* (7D), *Rf3* (1B), *Rf4* (6B), *Rf5* (6D), *Rf6* (5D), and *Rf7* (7B) (Tahir and Tsunewaki, 1969; Yen et al., 1969; Bahl and Maan, 1973). Since the effectiveness of the *Rf3* gene derived from *T. spelta* var. *duhamelianum* for fertility restoration was discovered (Kihara and Tsunewaki, 1966), further analysis of the chromosome location of *Rf3* was carried out: *Rf3*, a single dominant gene (Tahir and Tsunewaki, 1969), was mapped at a position of the short arm of 1B (1BS) that was 23 cM distant from each of *Nor* and *Gli-B1* (Snape et al., 1985).

The recent accumulation of RFLP markers and chromosome deletion lines in common wheat allow us to determine the precise position of genes on the genetic map (Gale et al., 1995) as well as on the cytological map (Ogihara et al., 1994; Gill et al., 1996), and allow tagging of genes for breeding selection (e.g., Galiba et al., 1995). Wheat has been believed to be inadequate as a model system for molecular breeding because of its large genome size (1.6×10^{10} bp in common wheat; Bennet and Smith, 1967) and polyploidy. Although the overall genome size of wheat is about ten times as large as that of the rice genome (per each genome), physical distances of adjacent genes in specific chromosome regions, named gene-rich regions, were estimated to correspond to those of smaller genomes of rice and tomato (Gill et al., 1996). Three sets of genes are usually contained in the genomes of common wheat. However, differentiation of gene actions is sometimes observed among the three sets as in the case of *Rf3*. These findings suggest that wheat can be used for the isolation of some genes, if not all, showing special expressions on the polyploid background, which are uniquely found in the wheat system.

In order to elucidate the action of *Rf3*, we have established near-isogenic lines for *Rf3* by successive backcrosses of *Triticum aestivum* cv. Chinese Spring (abbreviated as

* Corresponding author.

CS) to the F_1 between CS with the cytoplasm of *T. timopheevi* [abbreviated as (tim)-CS] and *T. spelta* (SP). Van Deynze et al. (1995) reported the location of *Rf3* on the RFLP consensus map of wheat; *Rf3* co-segregated with *Xcdo388*. We report herein on the further mapping and tagging of the *Rf3* gene by use of RFLP markers; we were able to obtain the recombinant descendants for *Rf3* and *Xcdo388*, and map *Rf3* at a position 1.2 cM and 2.6 cM distant from *Xcdo388* and *Xabc156*, respectively. Furthermore, we traced the chromosomal location of these markers on the cytological map established by the deletion lines of chromosome segments (Gill et al., 1996). By comparing the RFLP linkage map to the cytological map, we found characteristic features of the genetic map of wheat.

MATERIALS AND METHODS

Mapping population. The cytoplasm of *T. timopheevi* has been transferred to CS by repeated backcrosses (Tsunewaki, 1980). The alloplasmic line of CS with the cytoplasm of (tim)-CS (BC9 generations) was crossed with SP, which carries the fertility restoration gene (*Rf3*; Tahir and Tsunewaki, 1969) against *timopheevi* cytoplasm. The F_1 plants were pollinated with CS. Since (tim)-CS is sterile, fertile descendants were selected and used for the next pollination. After additional pollinations, fertile plants (BC_3F_1) were self-pollinated so as to obtained the F_2 population. Three fertile plants produced 39, 40, and 46 seeds, respectively. In total, 125 plants were used for segregation analysis of fertility and RFLP mapping. The pedigree of the mapping population is illustrated in Figure 1.

Selfed seed fertility. Selfed seed fertility of individual BC_3F_2 plants as well as that of their parental lines was tested in the field in Yokohama, Japan. The materials were sown in October of 1995 and transplanted to the field. The plants were planted 20 cm apart, ears of the three main culms per plant were bagged before flowering, and their selfed seed fertilities (%) were estimated by the seed-setting rate of the first and second florets of all spikelets.

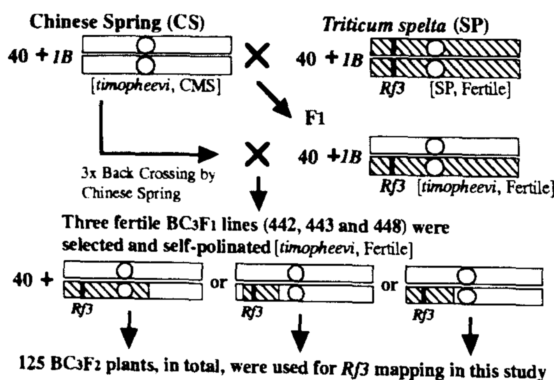

Fig. 1. Crossing scheme for generating near-isogenic lines to map the *Rf3* gene. Shaded portion of the chromosome represents the segment derived from the chromosome of *T. spelta*.

RFLP analysis. Total DNA of the 125 F_2 plants and their parental lines was extracted according to the CTAB method (Murray and Thompson, 1980). To check for polymorphisms between the parental lines, we digested 10 μg of total DNAs of CS, SP, and ditelo-1BL of CS with the following four restriction endonucleases; *Dra*I, *Eco*RI, *Eco*RV, and *Hin*dIII. When no polymorphisms between the parental lines were detected, other restriction enzymes, *Bam*HI, *Bgl*II, *Xba*I, and *Xho*I, were tested. DNAs were probed, as previously decribed (Ogihara et al., 1994), with cDNA clones (*Gli-1*, Bartels et al., 1986; rDNA clones, Gerlach and Bedbrook, 1979; BCD and ABC clones of barley, Anderson et al., 1992; Kleinhofs et al., 1993; CDO clones of oat, Anderson et al., 1992) and genomic clones (PSR clones of common wheat, Gale et al., 1995; KSU clones of *Aegilops squarrosa*, Gill et al., 1991) known from previous works to be located on the short arm of wheat chromosome 1B (1BS). Probes detecting polymorphisms between the parents were used to check the segregation pattern in the F_2 population. Genetic maps were constructed with the aid of the computer program Mapmaker/EXP 3.0 (Lander et al., 1987) using the Kosambi function (Kosambi, 1944). Multipoint analysis was carried out with an initial LOD threshold of three, and lowered LOD threshold to two to map additional markers. Marker orders were checked by the "RIPPLE" command with a window-size of 5 and LOD threshold of 2.

RESULTS

Portin of *T. spelta* segment integrated by successive backcrossing. Near-isogenic lines for the *Rf3* gene were established by successive backcrossing of CS to the F_1 plant between (tim)-CS and SP (BC_3F_1). Then, three lines were selected for production of F_2 seeds, because of their high seed fertility (95, 83, and 75%, respectively). The *Rf3* gene is known to be located on the distal part of the nucleolar organizer region of 1BS (Snape et al., 1985; Mukai and Endo, 1992). Five probes (KSUD14, *Gli*-1, KSUE18, CDO1173, and PSR161) were used for Southern hybridization so as to determine the integration points of the SP chromosome. The results are shown in Figure 2. The *XksuD14*, which is the most distal marker of 1BS so far obtained, revealed the CS type in line 443; whereas it showed the SP type in the others, indicating that recombination for integration of the SP chromosome took place at the position between *XksuD14* and *XGli-1* in line 443. The other recombination points were recognized at a position between *Xpsr161* and *Xcdo1188* in line 442, and between *Xcdo1173* and *Xcdo1188* in 443 line. Thusfar, however, the integration point in the line 448 is still unclear. Consequently, it was verified that all the other loci between *XGli-1* and *Xcdo1173* were heterologous in every BC_3F_1 lines, and so a high-

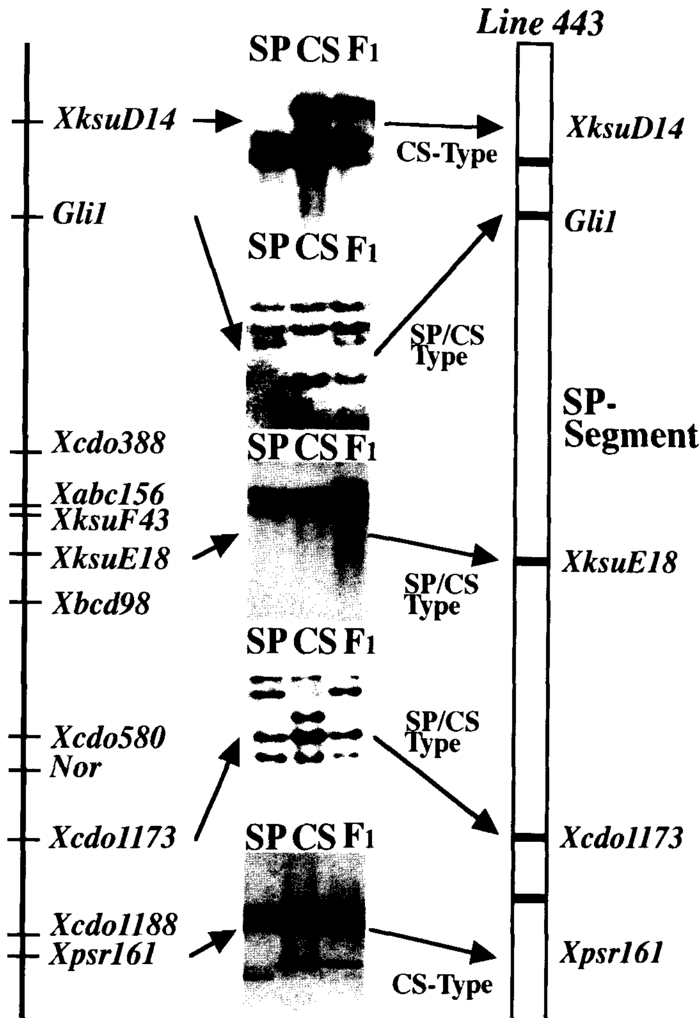

Fig. 2. Estimation of integrated *T. spelta* regions in 1BS of BC_3F_1 lines.

resoltuion genetic map would be constructed in this critical region. Addtionally, RFLP markers of KSUD14, CDO1188, and PSR161 were also mapped on the genetic map using the individuals in line 442 and 448 for the comparison between genetic and cytological map in the corresponding region.

Segregation pattern of RFLP markers in the BC_3F_2 population. Nine critical RFLP markers located on the target region of 1BS were used for construction of the genetic map. Segregation patterns of these markers in the BC_3F_2 population (125 individuals in total) showed good fitness to the ratio of 3 : 1 or 1 : 2 : 1 (Table 1). RFLP markers also revealed Mendelian segregation in each of the three BC_3F_2 subpopulations. No segregation distortion was found for these RFLP markers, indicating the normal segregation patterns of the population.

Segregation pattern of the selfed seed-fertility in the BC_3F_2 population. The selfed seed-fertility of 125 lines in the BC_3F_2 population was characterized. The segregation pattern is presented in Figure 3. Although *Rf3* was reported as a single dominant gene (Tahir and Tsunewaki, 1969), segregation patterns in this population did not fit exactly to the bimodal disbribution (Fig. 3), probably due to environmental factor(s) (see below).

Genotype determination of individual plants in the BC_3F_2 population. As the selfed seed fertility did not show essential bimodal distribution, the genotype of each individual was determined. Since the *Rf3* is known to tightly link to the *Xcdo388* and *Xabc156* (Van Deyze et al., 1995; the present investigation), the RFLP patterns of both probes were traced in the individual plants of population. Six recombinants showing two recombinant genotypes were found; one of them showed high selfed seed fertility (90%).

Table 1. Segregation patterns of ten genetic markers including *Rf3* gene in BC_3F_2 population

Marker	Total No. plants	No. plants classified into each genotype				χ^2-value
		SP/SP	SP-	SP/CS	CS/CS	
Gli-1	121	33		61	27	0.60^{ns}
CDO388	122		83		39	3.16^{ns}
Rf3	125		98		27	0.45^{ns}
ABC156	123		88		35	0.78^{ns}
KSUF43	122		97		25	1.32^{ns}
KSUE18	123	28		64	31	0.35^{ns}
BCD98	122		82		40	3.95*
CDO580	119	29		67	23	2.50^{ns}
Nor	122		91		31	0.01^{ns}
CDO1173	123	34		58	31	0.54^{ns}

ns and *: Non-significant and significant at the 5% level, respectively.

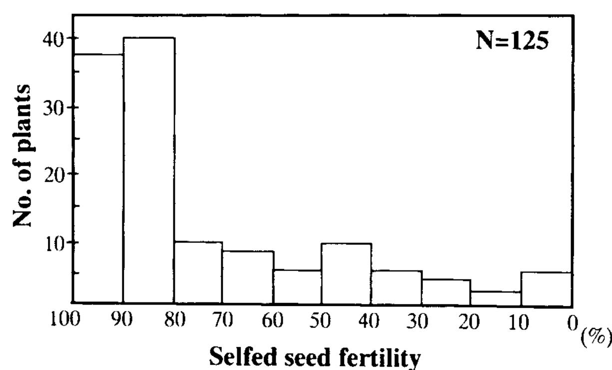

Fig. 3. Frequencies of the plants showing different levels of selfed seed fertilities in the BC_3F_2 generation.

Table 2. Number of BC_3F_2 individuals with three genotypes for the *Rf3* gene supplied for checking the seed fertilities in the next generation*

Genotype	Class of seed fertility (%) 0-20	20-40	40-60	60-80	80-100	Total
Rf3/Rf3			1	3	6	10
Rf3/rf3			2	6	2	10
rf3/rf3	2	2	6			10
	2	2	9	9	8	30

*, All recombinant types were included.

indicating that it harbored the *Rf3* gene, whereas the remaining five revealed low seed fertility. The other 119 plants were non-recombinant types, concerning to the RFLPs of CDO388 and ABC156. In order to confirm the genotypes of the plants for *Rf3* in the BC_3F_2 population, ten grains each picked up from the 30 lines showing each class of seed fertility (Table 2), in which six recombinant types were included, were sown in October of 1996. After harvesting, the selfed seed fertility of these plants were checked. The results is shown in Figure 4. Homo- and heterotypes of the estaimated individuals (Table 2) completely showed the representative segregation phenotypes in the next generation. Segregation pattern of seed fertility of the heterozygous plants displayed the essential bimodal distribution (χ^2 = 0.003; not significant). Based on these fertility check, the genotypes of all plants in the BC_3F_2

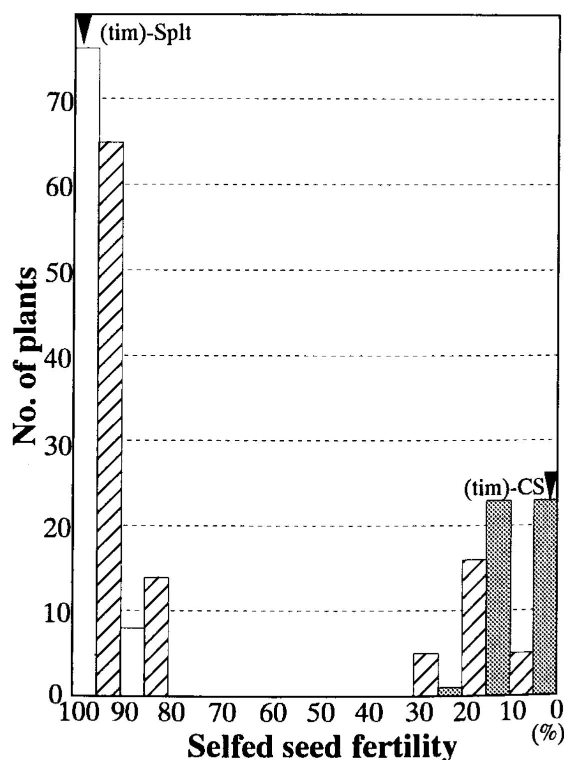

Fig. 4. Number of the plants showing segregation of selfed seed fertilities in the BC_3F_3 generation. Open bar indicates the number of the descendants of individuals with the *Rf3/Rf3* genotype. Hatched and shaded bars stand for those with the *Rf3/rf3* and *rf3/rf3* genotypes, respectively. Arrowheads indicate the seed fertilities of *T. spelta* with *timopheevi* cytoplasm [(tim)-Splt] (fertile) and (tim)-CS (sterile), respectively.

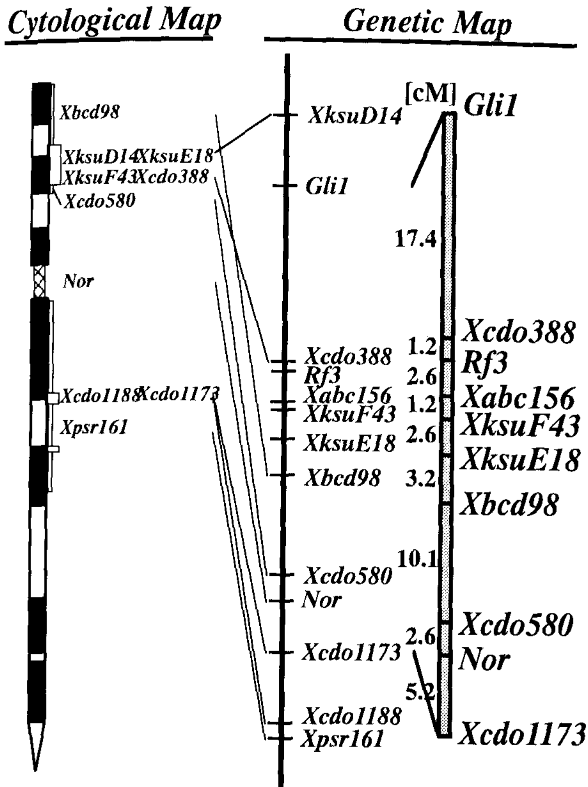

Fig. 5. Chromosome mapping of the *Rf3* gene in the BC_3F_2 generation of the cross, [(tim)-CS × SP] × CS. Numerals besides the line indicate genetic distances (cM). Marker positions on the cytological map were cited from Gill et al. (1996).

population could be determined. Consequently, out of 125 lines, 98 lines appeared to be fertile and 27 sterile (Table 1), suggesting a 3:1 segregation ratio of a single dominant gene (Tahir and Tsunewaki, 1969).

Construction of genetic map. The map of the critical region between *Gli-1* and *Xcdo1173* was constructed on the basis of the genotypic classifications for the 125 BC_3F_2 lines, as shown in Figure 5. This map covers about 85% of the genetic map, and 40% of the cytological one of 1BS (Gill et al., 1996). The total map length was 46.1 cM. As previously mapped (Snape et al., 1985), *Rf3* was located 22.3 cM and 18.6 cM distal to *Nor* and *Gli-1*, respetively. Furthermore, *Rf3* was mapped at a position 1.2 cM from *Xcdo388* and 2.6 cM from *Xabc156*. Additionally, three RFLP markers (*XksuD14*, *Xcdo1188*, and *Xpsr161*), which are located outside of the critical region (Fig. 5), were mapped by using the segregation data of the BC_3F_2 lines except those of line 443.

DISCUSSION

Although alloplasmic common wheats with the cytoplasm of *T. timopheevi* had showed male sterility (Wilson and Ross, 1962), seven genes controlling restoration of fertility against *timopheevi* cytoplasm, namely, *Rf1* to *Rf7* (Tahir and Tsunewaki, 1969; Yen et al., 1969; Bahl and Maan, 1973) were subsequently reported. Among these genes, *Rf3* derived from *T. spelta* var. *duhamelianum* was shown to be a single dominant gene that effectively restores fertility of the alloplasmic wheats (Tahir and Tsunewaki, 1969). To map the *Rf3* gene on 1BS, we established near-isogenic marker lines by successive backcrossing of *T. aestivum* cv. Chinese Spring to (tim)-CS/SP (BC_3F_1 generation). The integrated chromosome portions of two lines, out of three, were determined by examination of RFLP markers (Fig. 2). In these lines, two restorer genes are considered to be active; *Rf3* located on 1BS promotes fertility restoration, and *rf2*, located on 7D, suppressed fertility to some extent (Tahir and Tsunewaki, 1969). On this background, the BC_3F_2 population showed a segregation pattern for fertility, as shown in Figure 3. Determination of genotypes of individual plants indicates that segregation pattern of fertile and sterile plants was fitted to the 3 : 1 ratio (Table 1), showing epistasy of *Rf3*. All the other RFLP markers showed the statistical 3 : 1 or 1 : 2 : 1 segregation ratio (Table 1), suggesting no genetic distortion of the population. Based on the segregation pattern in the population, *Rf3* was localized at the precise position on the genetic map.

We have mapped ten markers on chromosome 1B of common wheat in the near-isogenic lines (Fig. 5). *Rf3* was mapped on the genetic map, 1.2 cM and 2.6 cM distant from *Xcdo388* and *Xabc156*, respectively. In our map, *Rf3* was distant 22.3 cM from *Nor*, and 18.6 cM from *Gli-B1*. These marker order and genetic distances coincided with those of Snape et al. (1985) 23 cM from both *Nor* and *Gli-B1*. Furthermore, our map corresponded to that of Van Deynze et al. (1995), although we were able to separate *Rf3* from *Xcdo388*. For the purpose of breeding hybrid wheat, both of these markers can be converted to the sequence tagged sites (STS) for polymerase chain reaction (PCR)-based diagnostic markers.

In addition to *Rf3*, a number of other fertility-restoration genes against alien cytoplasms have been reported to be located on the homeologous group 1 chromosomes. These are the following: (1) *Rf1* located on 1A, restoring fertility to *T. timpheevi* cytoplasm (Yen et al., 1969; Bahl and Maan, 1973), (2) *Rfu1* on 1B against *Ae. umbellulata* cytoplasm (Tsunewaki, 1974), (3) *Rfv1* on 1BS against *Ae. kotschyi* and *Ae. variabilis* cytoplasms (Mukai and Tsunewaki, 1979), (4) *Rfc3* on 1D against *Ae. caudata* cytoplasm (Tsunewaki, 1974), and (5) *Rfc1* on 1C of *Ae. caudata* against the cytoplasms of *Ae. caudata*, *Ae. ovata*, *Ae. umbellulata*, *Ae. triuncialis*, *Ae. biuncialis*, *Ae. columnaris*, and *Ae. triaristata* (Tahir and Tsunewaki, 1971; Tsunewaki et al., 1978). Although *Rfv1*, restoring fertility to *Ae. kotschyi* and *Ae. variabilis* cytoplasms was confirmed to be tighly linked to, but distinct from, *Rf3* on chromosome 1BS (Mukai and Endo, 1992), their allelism remains to be tested.

The present genetic map for 1BS of common wheat was compared with the cytological map, which was constructed by analysis of deletion lines of chromosome 1BS (Gill et al., 1996). Some RFLP markers between KSUD14 and CDO580 were mapped in a specific region so called "gene-rich region", showing unequal distribution of RFLP markers throughout 1BS. Gill et al. (1996) estimated the physical distance of RFLP markers; most markers were derived from the gene-rich region, and physical distance between markers in this region was calculated as 118 kbp per cM. If their calculation is true, *Rf3* should exist within 500 kbp of the adjacent RFLP markers (Fig. 5).

Although the cytological map gives us much information about the location of DNA markers, there were some discrepancies between the genetic map and the cytological map; as depicted in Figure 5, *Xbcd98* was located on the distal part of 1BS in the cytological map, whereas it was mapped on the middle part of the genetic map. Since the marker order in the genetic map was identical among the genetic maps of wheat so far reported, a deletion line(s) defined by the cytological positions of DNA markers should carry some chromosomal rearrangements instead of having a single breakage.

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