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A linkage map of rye

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Abstract A linkage map of rye (*Secale cereale* L.) is presented which comprises 60 loci including RFLPs, RAPDs, isozyme, morphological and physiological markers. The genetics and linkage relationships of these markers were investigated in several inbred lines of rye. For the RFLP mapping a genomic library of *Pst*I-digested DNA was constructed from which 50 size-selected clones were analysed. The portion of single-copy and multi-copy DNA and the frequency of polymorphic DNA was determined. The markers are unequally distributed over the seven chromosomes of rye. Many of them exhibit a distorted segregation. The main region of deviating segregation ratios could be localized near the self-incompatibility loci.

Key words *Secale cereale* L. · Linkage map · Molecular markers

Introduction

The importance of rye (*Secale cereale* L.) is based on its use as one of the major crop species in regions of adverse environmental conditions and its application as a source of alien chromatin for wheat. Furthermore, it is a parent of the synthetic cereal Triticale.

Although a considerable number of loci have been chromosomically localized (Melz et al. 1992), the genetic map of rye is less developed than the maps of other diploid cereals like barley or maize. There is a linkage map comprising 30 isozyme and physiological loci (Wehling 1986), and Wricke (1991) recently presented a rye linkage map that includes restriction fragment length polymorphisms (RFLPs). For chromosome 1R, 2R and 3R more precise linkage maps have been published that consist of mainly isozyme and RFLP markers (Wang et al. 1992; Devos et al. 1992a, b). Linkage maps of all seven rye chromosomes using wheat clones have been published by Devos et al. (1993), who investigated chromosomal rearrangements in the rye genome relative to that of wheat. Yet the genetic linkage relationships of other chromosomes which have been studied by isozyme and morphological markers are poorly investigated (Vaquero et al. 1990). Therefore, many gaps have still to be filled to obtain a complete map of the large and complex rye genome.

Linkage maps are subjects of great interest in genetic research and breeding programmes. For example, high-density maps with map distances of 2–5 cM are necessary for studying quantitative trait loci. In the isolation of genes, as has been done for the nematode resistance gene of tomato (Messeguer et al. 1991), high-resolution maps of the region of interest have to be constructed.

RFLPs are the most common markers used to obtain detailed linkage maps. The advantages of RFLPs over morphological markers, the codominance and the absence of pleiotropic effects are shared by isozymes. In contrast to the latter, however, the number of RFLPs are theoretically unlimited and their detection is independent of the plants' developmental stage. The main disadvantage of RFLPs is their high consumption of time and money. An alterative method introduced only recently by Williams et al. (1990), is based on the polymerase chain reaction (PCR) technique (Saiki et al. 1988). This method, called RAPD (random amplified polymorphic DNA), uses short, single random primers for the PCR. Polymorphisms are monitored by the presence or absence of an amplified DNA fragment. While this technique is fast and convenient, its main drawback is the dominant-recessive mode of inheritance of most RAPD markers. The most convenient strategy in constructing a linkage map, therefore, is to use the different classes of markers in concert. For these reasons it would appear that the best technique for constructing linkage map would be to use several kinds of methods.

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In the present paper we present our investigation of the linkage relationships of several markers, including morphological, isozyme, RFLP and RAPD markers, to obtain a broad base for constructing the map.

Materials and methods

Segregating inbred lines were produced by selfing non-inbred, self-incompatible plants of the varieties 'Danko' and 'Halo' using pseudo-compatibility at high temperature (Wricke 1978). Five of these $I_{0,1}$ progenies comprising 25–48 plants were used for segregation analysis.

Thirteen isozyme systems (aconitase, ACO; acid phosphatase, ACP; diaphorase, DIA; esterase, EST; β-galactosidase, β-GAL; β-glucosidase, β-GLU; glutamate oxaloacetate transaminase, GOT; leucine aminopeptidase, LAP; malate dehydrogenase, MDH; 6-phosphogluconate dehydrogenase, 6-PGDH; peroxidase, PRX; phosphoglucoisomerase, PGI; triosephosphate isomerase, TPI), including 26 loci, were analysed following leaf extraction and electrophoresis (Schmidt-Stohn 1979 and Wehling 1985, 1986, 1991, respectively). In addition, the morphological 'character hairy leaf sheath' (*Hs*) and a locus contributing to vernalisation (*Vrn*) were also studied.

Plant DNA was extracted with the CTAB method (Saghai-Maroof et al. 1984). For electrophoresis, 15 µg DNA was digested either with *Bam*HI, *Hin*d III or *Eco*RV and separated in 0.75% agarose gels. The gels were alkali-blotted under vacuum on Hybond N^+ membranes (Pharmacia). Probes were labeled with [α-^{32}P] dCTP with a random labeling kit (Pharmacia), and hybridization was performed following a modified protocol of Church and Gilbert (1984) under stringent conditions. Filters were exposed between 5 and 14 days to X-ray films (Fuji). Membranes were reused after stripping with 0.5% boiling SDS. Nuclear DNA was prepared from plants of the variety 'Danko' following the procedure described by Steinmüller and Apel (1986), completely digested with *Pst*I and then cloned in dephosphorylated plasmid vector pUC 19. The preparation of competent cells (*E. coli* XL1 blue and D5HA served as hosts) and transformation followed protocols given by Hanahan (1986). Plasmid DNA was isolated according to Holmes and Quigley (1983) and electrophoresed. Plasmids carrying inserts larger than 500 bp were used for the preparation of probes. Repetitive clones were identified by dot-blot hybridization. Plasmid DNA of 1.5 ml bacteria cultures was denatured and dotted on Hybond N membranes using a dot-blot apparatus (Schleicher und Schüll). The blots were hybridized with [^{32}P]-labeled nuclear DNA.

Undigested genomic DNA from leaves was used for RAPD analysis. The protocol followed Williams et al. (1990) with slight modifications. Briefly, amplifications were performed in 25 µl containing 25 ng DNA, 2.5 nmol dNTPs, 5 pmol primers and 0.5 u *Taq* polymerase (Perkin Elmer or Boehringer) in 10 m*M* Tris, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatine and 5% DMSO, RAPD oligonucleotide kit A (Operon, Alameda, Calif.) were used as primers. These primers were ten nucleotides in length with a minimum GC content of 50%. PCR was performed with 45 cycles (denaturation: 1 min at 94 °C, annealing: 1 min at 36 °C, extension: 2 min at 72 °C) using a 'Trioblock'-temperature cycler (Biometra).

Goodness-of-fit to expected segregation ratios was calculated for each locus by chi-square analysis. Codominant segregation was expected for most DNA and isozyme markers, i.e. a 1:2:1 ratio in F_2 populations. A dominant 3:1 segregation was assumed for RAPDs, DNA and isozyme loci segregating with null alleles and the morpholocigal loci, respectively. Segregations showing a significant deviation from the expected ratios were tested for gametic and zygotic selection. In these cases of selection the data can still be used for estimating the recombination values as long as one locus shows codominace. For a detailed discussion see Wagner et al. (1992).

Linkage analysis was done using the computer program Linkage-1 (Suiter et al. 1983). Linkage between markers was assumed when $P < 0.05$. Because of the limited numbers of plants, recombination frequencies greater than 30% between adjacent loci were not used for the arrangement of new loci into the linkage map with exception of the values for *β-Glu/Est-4* on chromosome 2 and *Got-1/Est-10* on chromosome 4, for which a larger number of plants was investigated.

Results

The use of the GC methylation-sensitive restriction enzyme *Pst*I to produce the random library should avoid high-copy sequences. Nevertheless, hybridization showed three classes of probes: 40% of the probes behaving like single-copies, 50% of the low-copy probes hybridizing to up to eight bands and 10% of the high repetitive probes detecting either a smear or distinguishable polymorphic bands. For genetic analysis, only the first and second classes could be used. The library was screened before Southern hybridization using the dot-blot technique, and although this selection to exclude high-copy probes was not complete, it did minimize the number of high-copy sequences.

When the three restriction endonucleases *Bam*HI, *Eco*RV and *Hin*dIII were used 50% of the probes detected polymorphisms. The average size of the fragments produced by the enzymes and the percentage polymorphism detected by the restriction enzymes were then determined. *Hin*dIII generated the smallest fragments detecting the lowest number of polymorphisms; the other endonucleases produced fragments of similar sizes and also detected polymorphisms of the same quantity.

Various RFLP segregation patterns could be observed (Fig. 1). On the basis of the sum of all three

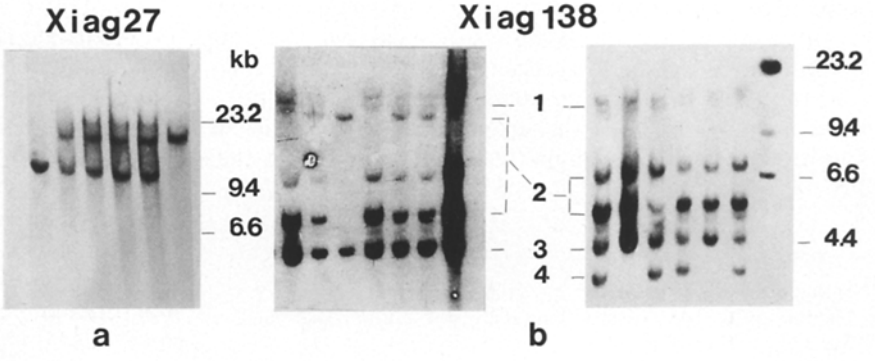

Fig. 1a, b Autoradiogram of two segregating lines hybridized with two different clones; **a** condominant at one locus; **b** segregation at four loci; locus 1 and 4 segregating with null allele, locus 3 is invariant and locus 2 shows codominance

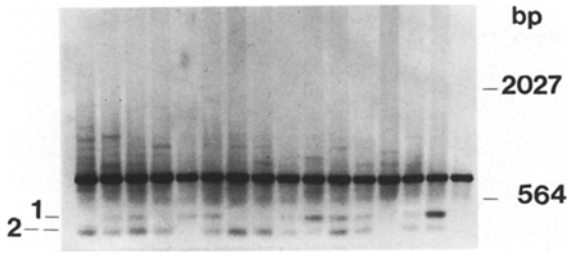

Fig. 2 Dominant-recessive inheritance of two RAPD loci (*1* and *2*) independently segregating in an inbred line

restriction enzymes, the probes were divided into two classes. According to this classification, 50% of the polymorphic probes showed a single-copy segregation (Fig. 1a). Probes of the other class detected more than 1 locus, including many patterns like aditional monomorphic bands, loci with codominant or dominant-recessive segregation or a combination of both (Fig. 1b). In this second category null alleles also were often observed while in the first class only one probe detected a null allele.

Ten of the random primers were tested, and most of these failed to produce any bands: 4 yielded bands, but only 3 detected a polymorphism, showing the expected dominant-recessive segregation. Figure 2 shows one of the RAPDs. Reproducibility of the banding patterns was increased using 5% DMSO in the incubation mix.

Segregation data were analysed for all loci showing polymorphisms. Of the loci 34.3% (22) showed a distorted segregation pattern ($P < 0.05$). For markers located on chromosomes 1R or 2R, the deviation could be explained by gametic selection caused by one of the self-incompatibility loci. For the other loci a linkage with a lethal or sublethal gene is assumed. This may also be the case for some of the segregations of loci on chromosomes 1R and 2R. Distorted segregations were the most frequently observed on chromosomes 1R and 6R.

Based on recombination frequencies, the loci including 28 of the 32 DNA markers were arranged into eight linkage groups (Fig. 3). For the localization of RFLPs in Fig. 3 information obtained from five segregational inbred lines is used. In addition, the map contains 24 isozyme loci as well as a loci contributing to vernalisation (*Vrn*) and a morphological character, hairy leaf sheath (*Hs*). For linkage analysis different selfed progenies were used, as were data obtained previously (Wehling 1986, 1991). For these reasons we prefer to give the original data in percentage recombination frequencies instead of transforming the data into map distances (cM). If more than one set of data was available for a given interval an average estimate was calculated. On the basis of all this information together, we estimated the most probable sequence of loci (Fig. 3). One linkage group comprising 6 DNA loci could not be associated with any of the seven rye chromosomes.

The total linkage distance is about 350 recombination units. The markers are not equally distributed over the seven chromosomes, and most loci are mapped on chromosomes 1R and 5R.

As described above, many probes were of the 'multicopy' type and frequently detected more than 1 segregating locus. A total of 18 duplicated loci detected by six probes are mapped. Four pairs of duplicated loci were mapped within a linkage group but partly non-adjacent; the others were mapped as independent loci distributed to different linkage groups.

Discussion

The rye genome contains 92% repetitive sequences (Flavell et al. 1974). Therefore, the cloning strategy was to reduce repeated sequences in the library using the methylation-sensitive enzyme *Pst*I. However, the genomic library still contained about 60% repetitive sequences including high- and low-copy DNA, and when this result is compared with the high frequency of single-copy probes in tomato or sugar beet *Pst*I libraries of 92% and 93% respectively (Miller and Tanksley 1990; Pillen et al. 1992), the selection does not seem to be as successful in rye as is other species. One reason might be the extraordinary high content of repeated sequences in this genome.

The efficiency of probes in detecting polymorphisms was 50% on average and thereby smaller than expected. From *Brassica*, another outbreeding crop with a strong self-incompatibility system, the degree of polymorphic DNA was greater than 80% using genomic clones. An improvement in detecting polymorphisms might be expected by the use of cDNA probes, which have been successfully applied in many plant species, e.g. in lettuce (Landry et al. 1987), lentil (Havey and Muehlbauer 1989), potato (Gebhardt et al. 1989) and tomato (Miller and Tanksley 1990). A confirmation of this presumption might be the result of Wang et al. (1992) in which 50–75% polymorphisms per probe and restriction enzyme was obtained in rye using only cDNA probes, which is significantly higher than the detected variations in this study, which ranged from 30% to 41% per enzyme and probe. In accordance with other authors (Landry et al. 1987; McCouch et al. 1988; Miller and Tanksley 1990), we found a positive correlation between average genomic fragment size produced by different endonucleases and the level of detected polymorphism. This might be explained by the assumption that DNA insertions/deletions and other DNA rearrangements primarily cause the RFLPs.

The high number of loci segregating with null alleles is remarkable. From other plant species null alleles are known only as exceptions (Landry et al. 1987; McCouch et al. 1988; Gebhardt et al. 1989; Song et al. 1991). In rye, most of the probes detecting duplicated loci also detected null alleles. This supports the idea that deletion/insertions in the rye genome are very frequent and constitute the main cause for RFLPs. Only in *Brassicacee* have more duplicated loci been identified than in

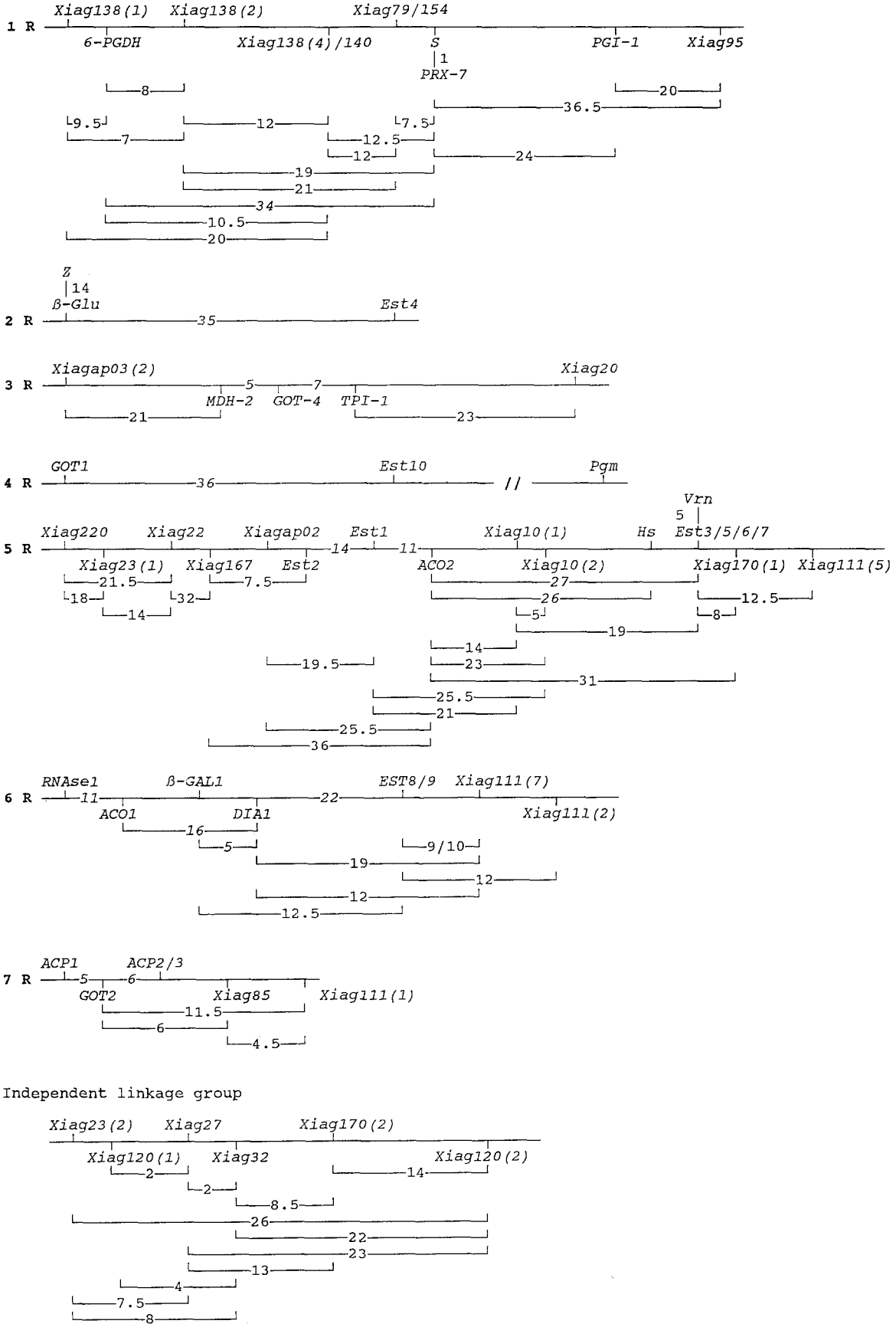
1 R
Xiag138(1)
Xiag138(2)
Xiag79/154
6-PGDH
Xiag138(4)/140
S
PGI-1
Xiag95
1
PRX-7
8
20
36.5
9.5
12
7.5
7
12.5
12
24
19
21
34
10.5
20
Z
14
ß-Glu
Est4
2 R
35
Xiagap03(2)
Xiag20
3 R
5
7
MDH-2
GOT-4
TPI-1
21
23
GOT1
Est10
Pgm
4 R
36
//
Vrn
5
Xiag220
Xiag22
Xiagap02
Est1
Xiag10(1)
Hs
Est3/5/6/7
5 R
14
11
Xiag23(1)
Xiag167
Est2
ACO2
Xiag10(2)
Xiag170(1)
Xiag111(5)
21.5
7.5
27
18
32
26
12.5
14
5
8
19
14
19.5
23
31
25.5
21
25.5
36
RNAse1
ß-GAL1
EST8/9
Xiag111(7)
6 R
11
22
ACO1
DIA1
Xiag111(2)
16
5
9/10
19
12
12
12.5
ACP1
ACP2/3
7 R
5
6
GOT2
Xiag85
Xiag111(1)
11.5
6
4.5
Independent linkage group
Xiag23(2)
Xiag27
Xiag170(2)
Xiag120(1)
Xiag32
Xiag120(2)
2
14
2
8.5
26
22
23
13
4
7.5
8

Fig. 3 Preliminary linkage map of rye. The incompatibility loci *S* and *Z* as well as *Vrn* on chromosomes 1R, 2R and 5R, respectively, could not be put in order unequivocally. Recombination values between isozymes (Wehling 1986, 1991) are written in *italics*

rye (Slocum et al. 1990; Song et al. 1991). In other plant species like tomato, *Arabidopsis* and lentil sequence duplication in the genome seems to be less frequent (Bernatzky and Tanksley 1986; Bonierbale et al. 1988; Havey and Muehlbauer 1989).

The efficiency of the random PCR primers in generating polymorphic bands was not as high what has been reported for *Brassica oleracea* and tomato (Quiros et al. 1991; Klein-Lankhorst et al. 1991). Most primers failed to give any amplification product from rye DNA. A similar negative result with random primers of 15 to 20 nucleotides in length for PCR in wheat, rye and barley was reported by Weining and Langridge (1991). Nevertheless, these authors could produce bands using primers derived from intron-splice junction sequences. An explanation for this observation might be that because of the large genome size the distances of the random-selected sequences are too large to produce any band. In these species possibly only primers based on sequence information will yield better results.

Distorted segregation ratios of isozyme or RFLP loci are observed in many plant species, e.g. in potato, lens, sugar beet and, to a lesser extent, *Brassica* (Bonierbale et al. 1988; Gebhardt et al. 1991; Muehlbauer et al. 1988; Wagner et al. 1992; Slocum et al. 1990; Song et al. 1991). In only a few cases is the cause of the deviation known, e.g. when self-incompatibility loci are localized. This has been described for the *Solanaceae* potato and tomato (Gebhardt et al. 1991) and also for rye (Wricke and Wehling 1985; Gertz and Wricke 1989). Thus, it was not unexpected to find deviations on chromosomes 1R and 2R.

The linkage analysis confirmed the previously constructed genetic map (Wehling 1986). While 22 DNA markers could be integrated into this linkage map, 8 DNA loci could not be linked to any marker and a linkage group of 6 loci could not be assigned to any chromosome. The unequal distribution of the loci over the seven rye chromosomes might be explained by the fact that chromosomes 1R and 5R have already been mapped by several isozyme loci. Therefore, it was easier to assign new loci to these chromosomes by linkage analysis than to other more scarcely mapped chromosomes. Furthermore, markers of chromosomes 1R and 5R segregated in all lines, but only few do so for the other chromosomes.

The linkage arrangement of duplicated loci varies. For loci *Xiag10(1,2)*, a tandem duplication could be assumed, while the loci of probe *Xiag138* are non-adjacently linked on chromosome 1R. A conservation of the linkage arrangement can be assumed for 2 duplicated loci (*Xiag23-Xiag170*), which were mapped to chromosome 5R and the unrelated linkage group. Different linkage arrangements of duplicated loci were also observed in *Brassicaceae* (Slocum et al. 1990; Song et al. 1991). In tomato, mainly unlinked loci have been revealed (Bernatzky and Tanksley 1986). In confirmation with previous studies using cytological and isozyme techniques (Koller and Zeller 1976, Rao and Rao 1984; Hsam et al. 1982; Wehling 1991), these results suggest that some rearrangements and duplications occurred in the complex rye genome during evolution. Further studies and comparisons with linkage maps and molecular markers of other cereals will provide more information on chromosome structures within the *Triticeae*.

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