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### Permalink

<https://escholarship.org/uc/item/5m37893c>

### Journal

Cytogenetic and Genome Research, 142(4)

### ISSN

1424-8581

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### Publication Date

2014

### DOI

10.1159/000358848

Peer reviewed

# The *Ph1* Locus of Wheat Does Not Discriminate between Identical and Non-Identical Homologues in Rye

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## Key Words

Doubled haploids · Meiotic fidelity · *Ph* (pairing homoeologous) · Tetraploid rye

## Abstract

The main locus responsible for diploid-like behavior of polyploid wheat in meiosis, *Ph1*, is located on the long arm of chromosome 5B (5BL). It restricts metaphase I pairing to essentially identical homologues. Introduction of 5BL into outcrossing autotetraploid rye severely reduced multivalent formation and increased the frequency of bivalents and univalents, but the key by which homologues were selected for effective pairing was not clear. We created doubled haploids of autotetraploid rye with the long arm of wheat 5BL, verified their nature by DNA markers, and analyzed metaphase I chromosome pairing. The doubled haploid nature guaranteed the presence of pairs of identical and non-identical homologues in each homologous group. The metaphase I pairing patterns were essentially the same as in plants from open pollination, with frequent bivalents and univalents and rare multivalents. The level of pairing was low and depended on the dosage of 5BL. The pairing levels show that unlike in wheat, in rye the *Ph1* locus does not use homologue similarity as the criterion in selection of pairing partners. It is pos-

sible that the *Ph1* of wheat and the rye chromosome pairing system are mutually exclusive. The minimum level of chromosome differences required for effective pairing in rye may be well above the maximum difference level tolerated by the *Ph1* system of wheat. In other words, effective chromosome pairing in rye may be possible between non-identical chromosomes that might not normally pair in the *Ph1* wheat background.

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Meiosis performs best when dealing with pairs of chromosomes: eligible chromosomes form bivalents in meiotic prophase, bivalents arrange themselves on the metaphase plate in metaphase I (MI) and separate constituent chromosomes to the opposite poles in anaphase I, producing 2 haploid daughter nuclei each with 1 copy of each homologue present. Any deviation from the system of 2 chromosomes-2 poles creates potential for errors, and errors reduce the efficiency of the entire process. Polyploidy, a situation where there are more than 2 genetically identical/similar chromosomes eligible for pairing, is bound to create problems in meiosis, and in most cases it does. Polyploidization events are usually followed by prolonged periods of chromosome instability until chromo-

somes differentiate sufficiently not to recognize one another in the process of homologue pairing. This process may be assisted by the development of a system which enforces bivalent pairing. Many such systems are known or are suspected to exist among polyploid species [Jenczewski and Alix, 2004]. The most studied of these is the *Ph* (pairing homoeologous) system of wheat [Jenczewski et al., 2013]. It is composed of at least 2 loci: the main *Ph1* on chromosome 5B and *Ph2*, with a much weaker effect, on 3D. *Ph1* was first discovered by Sears and Okamoto [1958] and Riley and Chapman [1958] and is still studied today.

The difficulty in developing a coherent model of *Ph1* action is related in part to a wide range of its known effects. It prevents MI pairing of homoeologues from the constituent wheat genomes A, B, and D, making wheat's meiosis diploid-like. It also prevents MI pairing of homoeologues from related species. It is less frequently noted that *Ph1* also affects homologues: in some intervarietal wheat hybrids, it prevents entire chromosome arms from pairing [Dvorak and McGuire, 1981]; in specific segments of homologues, removal of *Ph1* increases the cross-over rates up to 2.5-fold [Lukaszewski and Hohn, unpubl. data]. Comparisons of homologous and homoeologous crossing over rates imply that the locus recognizes local differences in chromosome affinity [Luo et al., 1996].

Wheat's *Ph1* locus not only controls pairing of alien chromosomes in wheat but also of alien chromosomes in their native environment. Both in diploid [Schlegel et al., 1991] and autotetraploid rye [Lukaszewski and Kopecky, 2010], introduction of wheat chromosome 5B or its long arm with the *Ph1* locus significantly reduced the MI chromosome pairing. In tetraploid rye, the effect was dosage-dependent: pairing reduction was greater with 2 doses of *Ph1* than with 1, and 2 doses practically eliminated multivalents. However, the key by which homologues were selected for MI pairing was far from clear. Karyotype analyses suggested that structurally similar homologues were more likely to pair. However, the issue of pairing success vs. structural similarity in the presence of *Ph1* could only be resolved by creating plants with homologues of known levels of affinity/similarity, such as doubled haploids (DH). In a DH of autotetraploid rye each quartet of homologues should consist of 2 pairs of perfectly identical homologues; the differences between the pairs may vary depending on the level of differentiation of homologues in the parental material. Despite earlier failures [Lukaszewski and Kopecky, 2010], attempts to produce DHs to resolve this issue continued and eventually succeeded in regenerating several tetraploid DH

plants with 1 or 2 doses of wheat chromosome arm 5BL with the *Ph1* locus. Here, we report that the presence of pairs of identical chromosomes in such plants did not in any way affect the pattern of chromosome pairing, indicating that in rye the *Ph1* locus does not favor identical chromosomes for pairing.

## Materials and Methods

The starting material were 3 lines of autotetraploid rye (*Secale cereale* L.) cv. Tetra Gator (from now on abbreviated as TG) with introgressions of the long arm of wheat chromosome 5B either in the form of a complete 2-armed chromosome (5B) or its long arm telocentric (5BL) as described previously [Lukaszewski and Kopecky, 2010]. These lines were TG 5B, TG Dt5BL, and TG 5B + 5BL where Dt stands for ditelocentric. Plants of these lines were grown in the greenhouse at the IHAR in Radzikow, Poland, and all procedures of material collection, storage, plating, and anther culture conditions were standard as described for triticale [Warzecha et al., 2005] and often used for rye. For each line, anthers from 30 heads were plated which amounted to ca. 4,000–4,500 anthers per combination. Regenerated plants were grown in pots in a greenhouse at the campus of University of California, Riverside, Calif., USA. For chromosome counts and karyotyping, root tips were repeatedly collected to ice water for ca. 24 h and fixed in a mixture of 3:1 ethanol:glacial acetic acid. For observations of meiosis, tillers at appropriate stages were harvested and dissected. A portion of one anther from a spikelet was live-stained in acetocarmine and checked under a microscope. If the desired stage was present, the remaining anthers of the spikelet were fixed in the same mixture of ethanol and glacial acetic acid at 37°C for a week and then stored frozen at –20°C. Preparations were made and all procedures of in situ probing with DNA probes were performed according to Mas-soudi-Nejad et al. [2002].

Several combinations of probes and blocks were tested, but the interpretation of the MI pairing configurations was easiest when a rye centromeric probe [Francki, 2001] labeled with DIG-Oxygenin and conjugated with antiDIG-FITC was used in conjunction with propidium iodide counter staining (fig. 1). All reagents and conditions used were the same as in previous publications [Lukaszewski and Kopecky, 2010; Oleszczuk and Lukaszewski, in press].

To test the genetic status of regenerated plants, DNA was individually isolated from 9 androgenic plants and samples of the parental line TG Dt5BL: one bulk of 7 plants from the original line (population) and 18 individual seeds or seedlings of half-sibs of the original TG Dt5BL plants used for the production of androgenic plants. Thirteen of the 18 individual plants were used to prepare a temporary, second DNA bulk for rapid selection of informative microsatellite markers. DNA was extracted using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) or using the protocol of the Diversity Array Technology as posted at <http://www.diversityarrays.com/sample-submissions>. Finally, DNA was suspended in 50 µl of the PE buffer (5 mM Tris-HCl, pH 8.5) or 250 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

Microsatellite analyses were performed in 2 stages. First, 14 simple sequence repeat (SSR) markers targeting 6 rye chromosomes (table 1) were tested on a set of 16 DNAs: 9 individual mi-

**Table 1.** Allelic frequencies of the microsatellite markers selected for detection of heterozygosity in doubled haploids of tetraploid rye

Locus	Chromosome location	Total number of alleles	H	PIC	Allele frequencies in parental population							
					null	1	2	3	4	5	6	
<i>Xrems1132</i>	2R	1	0.5	0.375	0.500	0.500						
<i>Xrems1152</i>	6R	2	0.625	0.555	0.250	0.500	0.250					
<i>Xrems1162</i>	7R	2	0.645	0.570	0.421	0.359	0.220					
<i>Xrems1188</i>	7R	3	0.705	0.652	0.155	0.139	0.382	0.324				
<i>Xrems1194</i>	2R	2	0.635	0.561	0.208	0.458	0.334					
<i>Xrems1259</i>	6R	3	0.739	0.691	0.292	0.292	0.250	0.167				
<i>Xrems1261</i>	3R	6	0.819	0.793	0.213	0.220	0.058	0.116	0.156	0.214	0.023	
<i>Xscm0116</i>	4R	4	0.763	0.723	0.175	0.258	0.217	0.050	0.300			
<i>Xscm0126</i>	1R	3	0.675	0.616	0.346	0.422	0.122	0.110				
<i>Xscm0127</i>	1R	2	0.641	0.568	0.248	0.289	0.463					
<i>Xscm274</i>	1R	3	0.680	0.622	0.167	0.417	0.333	0.083				
<i>Xscm340</i>	1R	2	0.666	0.593	0.333	0.333	0.333					
<i>Xscm369</i>	3R	2	0.661	0.587	0.340	0.278	0.382					

H = Heterozygosity; PIC = polymorphic information contents.

crospore derived plants, 2 bulks (made of 7, and 13 half-sib plants of TG Dt5BL), and 5 individual half-sibs of TG Dt5BL. The second stage analysis tested allele distribution for preselected markers (*rems1162*, *rems1188*, *rems1261*, *scm126*, *scm127*, *scm369*) among individual plants used to create the second bulk. Information on primer sequences was kindly provided by Dr. V. Korzun, Lochow-Petkus, Germany. Allele frequencies for autotetraploid rye were estimated according to Liu et al. [2007], while the polymorphic information content was calculated according to Nagy et al. [2012].

PCR reactions were carried out in 20 µl containing: 1 × PCR buffer Fermentas (10 mM Tris-HCl, pH 8.8, at 25°C, 50 mM KCl, 0.08% Nonidet P40), 250 nM of each primer, 2.5 mM MgCl<sub>2</sub>, 250 µM dNTPs, 400 µM spermidine, 0.6 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), and 15–50 ng of DNA. The PCR regime used was as follows: 4 min at 95°C; 7 cycles of 45 s at 94°C, 45 s at 65°C, decreasing 1°C/cycle, and 45 s at 72°C; 40 cycles of 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C; with a final extension of 10 min at 72°C. The resulting PCR products were resolved on 5.5% polyacrylamide sequencing gels (350 × 450 mm) for 1.2 h and silver-stained [Chalhoub et al., 1997]. DNA markers pGEM® and SilverSTR® III Allelic Ladder Mix (Promega, Fitchburg, Wis., USA) were used to determine the sizes of PCR products.

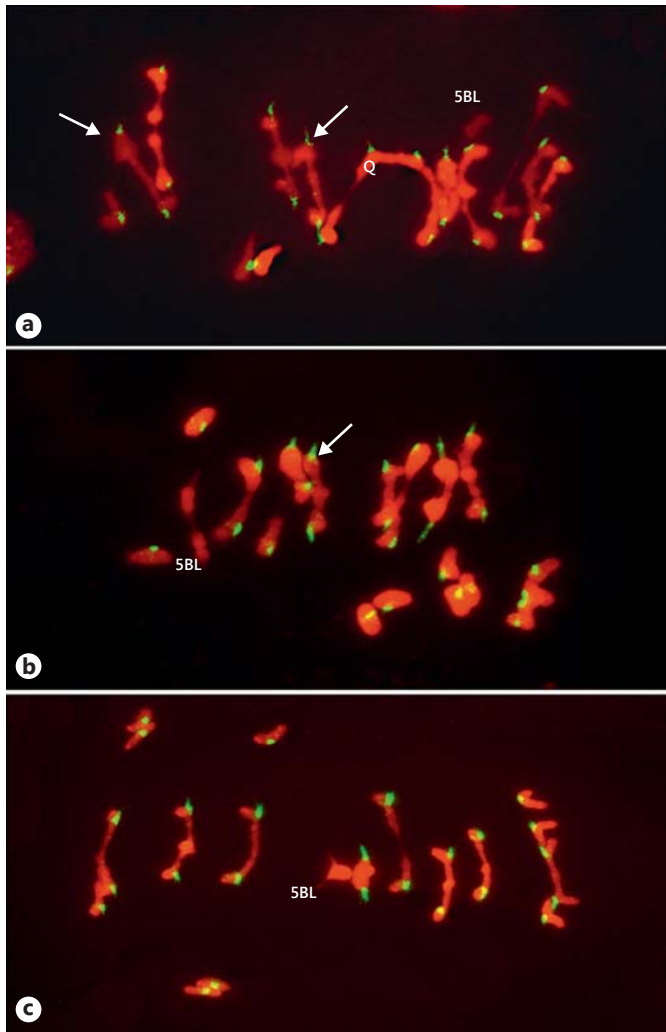
## Results and Discussion

Only the third attempt at production of androgenic plants from *S. cereale* cv. Tetra Gator was successful and only in the TG Dt5BL population. Twelve green plants were obtained, all originating from anthers of a single head. Of these, 10 plants survived to flowering and were analyzed. All regenerated plants were weak with very

poor root systems which contributed to mortality in handling and seriously complicated karyotyping. Still, each of the surviving plants had its chromosome number determined, and 7 were karyotyped using standard C-banding. While all plants tillered well, they produced few tillers with heads, and no repeated analyses of chromosome pairing were possible.

No plants with gametic chromosome numbers (here 14) were obtained; all were tetraploids. Chromosome numbers of individual plants ranged from 26–28 with either 25 or 26 rye chromosomes and 1 or 2 telocentric 5BL (table 2). Spontaneous chromosome doubling of the microspore-derived plants does occur in rye [Tenhola-Roininen et al., 2006], but it is expected to result in even chromosome numbers. Here, 4 regenerants had an odd number of rye chromosomes (25), a single dose of 5BL, or both, raising suspicions of their sporophytic origin. The nature of material necessitated tests of their genetic constitution.

Considerable variation in C-banding patterns on individual chromosomes of rye cv. Tetra Gator was noted before [Lukaszewski and Kopecky, 2010]. Here, in each of the 7 karyotyped plants, wherever polymorphism of C-banding patterns was present within a homoeologous group, 2 pairs of similar patterns were observed. However, some patterns were so unusual for rye that no positive identification of all chromosomes in all plants was possible, and the exact chromosome constitution of all plants could not be determined. It appeared that chromo-



**Fig. 1.** Metaphase I pairing in pollen mother cells of doubled haploids of autotetraploid rye with wheat chromosome arm 5BL. **a** 25 chromosomes of rye forming  $1^{IV} + 2^{III} + 7^{II} + 1^I$ , 5BL is univalent. Q = Quadrivalent. **b** 26 chromosomes of rye forming  $1^{III} + 6^{II} + 11^I$ , 5BL are paired. **c** 25 chromosomes of rye forming  $9^{II} + 7^I$ , 5BL are paired. Trivalents in **a** and **b** are arrowed. Telocentric univalent 5BL in **a** and bivalents in **b** and **c** are labeled.

some arm 5BL substituted, at least partially, for missing chromosomes 5R.

Of the 14 DNA markers tested, *scm46* was uninformative and was dropped. For the remaining 13 markers, the numbers of alleles present at any given locus in the parental population ranged from 1–6, and the respective polymorphic information content values ranged from 0.375 for *Xrems1132* to 0.819 for *Xrems1261* (table 1). Among regenerants, only in one instance (DHR6a) there ap-

peared to be more than 2 alleles present at a locus (3 alleles at *Xscm126-1R*). Given the total number of tests performed, this appears more likely to represent a mutation than heterozygosity within a pair of what should be identical chromosomes.

Marker analysis suggested that DHR2, DHR3, and DHR9b were identical. The presence of clones among the regenerated material cannot be excluded; clones are frequent among androgenic regenerants [Oleszczuk et al., in press]. While DHR2 differs from the other 2 by the chromosome number and constitution (table 2), somatic loss of a chromosome during regeneration cannot be ruled out especially given the difficulty with which the current set was recovered. To further test if the regenerated plants were indeed DH, the expected frequencies of alleles, gametes, and phenotypes were calculated using allelic frequencies in the parental population (table 1) for 2 assumptions: that the regenerants were obtained from somatic tissue of anthers hence represent a random half-sib population or that they were regenerated from a random sample of microspores. In all cases the probability ratios favored gametophytic origin of regenerants in a 2:1 ratio. The probability that the material was indeed of the gametophytic origin and indeed DH is further increased by the fact that whenever polymorphism for C-banding patterns was present among 4 chromosomes of the same homologous group (usually 2–3 homologous groups in each plant), it was always in the 2:2 ratio (2 pairs of chromosomes with identical patterns) and never in the 3:1 ratio.

Because of poor heading in most plants, the numbers of analyzed pollen mother cells (PMCs) were rather low (table 2). In all plants, the frequencies of multivalents (trivalents and quadrivalents) were greatly reduced, and the frequencies of bivalents and univalents were increased relative to the full pairing potential of an autotetraploid [Sybenga, 1992] and the previous observations of rye cv. Tetra Gator [Lukaszewski and Kopecky, 2010]. The effect of 5BL (*Ph1*) was again dosage-dependent: in 3 plants with single doses of 5BL, the average number of paired rye chromosome arms per PMC ranged from 15–17.23 and the average pairing frequency per arm from 0.30–0.34, while in plants with 2 doses of 5BL, the average number of paired arms ranged from 9.48–12.3 and the average pairing per chromosome arm from 0.18–0.23. Pairing indices of these 2 groups of plants never overlapped. Two copies of 5BL reduced multivalents formation and increased the numbers of bivalents and univalents per PMC relative to 1 copy of 5BL (fig. 1).

**Table 2.** Metaphase I pairing in doubled haploids of tetraploid rye with substitution of wheat chromosome arm 5BL

Plant ID	Chromosome number/ constitution	Number of PMCs scored	Rye chromosome pairing (average per PCM)					average rye arms paired per PMC	average pairing per arm
			univalents	bivalents	trivalents	quadrivalents			
DHR 1	25+Mt5BL	25	5.32	3.74	1.56	1.88	15.00	0.30	
DHR 7	25+Mt5BL	30	5.06	5.90	1.96	0.57	17.23	0.34	
DHR 2	26+Mt5BL	22	2.82	3.95	2.05	2.32	16.91	0.32	
DHR 6	26+Dt5BL	52	8.05	5.40	1.58	0.60	11.73	0.22	
DHR 6a	25+Dt5BL	27	6.67	6.05	1.78	0.22	11.74	0.23	
DHR 3	26+Dt5BL	67	12.90	4.66	0.88	0.28	10.48	0.20	
DHR 4	26+Dt5BL	62	11.27	5.22	0.95	0.40	9.48	0.18	
DHR 9	26+Dt5BL	65	10.17	6.52	0.92	0.01	10.03	0.19	
DHR 9b	26+Dt5BL	74	8.24	6.98	1.28	0.26	10.42	0.20	
DHR 10	26+Dt5BL	30	7.50	7.33	1.10	0.13	12.30	0.24	

Mt = Monotelosomic; Dt = ditelosomic; PMC = pollen mother cell.

The results of tests performed here appear quite convincing that the plants in this study were indeed DHs. Therefore, apart from cases of aneuploidy for individual chromosomes, these plants must have had, in each homologous group, either 4 identical chromosomes or a pair of identical and a pair of non-identical chromosomes. This change in chromosome affinity (similarity) did not have any effect on the MI pairing indices relative to the previous study where the analyzed plants were obtained after cross pollination and up to 3 different banding patterns were observed in individual quartets of chromosomes [Lukaszewski and Kopecky, 2010]. It does not appear that the presence of the *Ph1* locus, which in wheat clearly favors more similar chromosomes, favored identical pairs of rye chromosomes in rye.

Rye is a self-incompatible, cross-pollinating species. In typical outcrossing populations, rye chromosomes pair regularly, with high per-arm pairing frequencies [Rees, 1955; Rees and Thompson, 1956; Lelley, 1978]. Upon inbreeding, chromosome pairing frequencies always drop to different levels in different lineages [Lelley, 1978], but normal levels of MI pairing are restored in hybrids, even of the lowest-pairing inbred lines [Lelley, 1981], implying that homozygosity has a negative effect on the ability of rye chromosomes to pair [Rees, 1955; Rees and Thompson, 1956]. However, in triticales made from low-pairing inbred lines of rye, normal meiotic pairing of rye chromosomes is not restored in hybrids [Lelley, 1981]. The *Ph1* chromosome pairing control system triticales clearly exerts some control over rye chromosomes, and its presence prevents the restoration

of normal pairing level in heterozygotes. It is therefore possible that the system of chromosome pairing control in rye, which favors heterozygosity, and the *Ph1* system of wheat, which demands homozygosity, are mutually exclusive. *Ph1* requires high levels of similarity for effective pairing, and the level of heterozygosity acceptable for *Ph1* may be well below the minimum required for effective pairing of rye chromosomes. As a consequence, with *Ph1* present, higher levels of homozygosity result in higher pairing of wheat chromosomes but reduced pairing of rye chromosomes. Interestingly, preliminary observations suggest that wheat homoeologues introduced into rye pair and recombine with their rye homoeologues with higher frequencies than they do so in the absence of the *Ph1* locus in wheat [Lukaszewski, unpubl. data].

It appears that the system of chromosome pairing control operating in rye uses different criteria than the restrictive *Ph1* system of wheat. *Ph1* permits crossovers and chiasma formation only between essentially identical chromosomes, and this is why the crossover rates in specific segments of non-identical homologues increase when *Ph1* is removed. In a self-pollinating polyploid composed of 3 closely related genomes such as wheat, restriction of pairing to practically identical chromosomes is adaptive: it eliminates all chances of multivalents and with inbreeding, identical homologues are almost always present. This assures regular bivalent pairing and regular meiosis. On the other hand, in an outcrossing diploid species such as rye, a permissive pairing control system is more adaptive. Not only a certain level of chromosome

divergence is tolerated; it appears to be required for effective MI pairing. A pairing control system that favors non-identical homologues may be another tool to enforce outcrossing by reducing fitness of progeny resulting from inbreeding. These contradictory requirements of the

pairing systems might have affected the results of this test. Perhaps the experiment should be repeated to observe pairing behavior of identical and non-identical pairs of rye homologues in the genetic environment of wheat with the *Ph1* locus present and absent.

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