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The *Ph1* Locus from Wheat Controls Meiotic Chromosome Pairing in Autotetraploid Rye (*Secale cereale* L.)

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Diploid-like pairing · Introgression · Meiosis · *Ph1* locus · Rye · Wheat

Abstract
The *Ph1* locus on chromosome 5B enforces strictly bivalent pairing in polyploid wheat, but the exact mechanism of its action remains unknown. Pairing restriction involves not only wheat homoeologues and all alien introgressions but also differentiated homologues. In this study we show that chromosome 5B with its *Ph1* locus also controls chromosome pairing in autotetraploid rye by apparently restricting chiasma formation between dissimilar homologues. Unlike in wheat, the effect appears to be dosage-dependent, which may be a reflection of an interaction between *Ph1* and the rye chromosome pairing control system. With 2 doses of *Ph1* present, chiasmate pairing was severely restricted resulting in a significantly higher number of univalents and bivalents per cell than in the controls. The restrictions imposed by *Ph1* virtually eliminated MI pairing of chromosome arms polymorphic for their C-band patterns and did not appear to affect arms with similar patterns. If the polymorphism for C-bands is taken as a measure of the overall chromosome similarity/divergence, such differences were recognized and acted upon by the *Ph1* locus. The fact that *Ph1* operates in rye in the same fashion as in polyploid wheats suggests that it controls some basic mechanism of chromosome recognition.

Bivalent pairing in the first metaphase of meiosis (MI) is critical for normal segregation of chromosomes in first anaphase (AI) and proper reduction of chromosome number from somatic to gametic. Any MI configuration involving a number of chromosomes other than 2 creates the potential for uneven segregation, leading to gametes with unbalanced chromosome numbers. Barring aneuploidy or heterozygosity for structural chromosome aberrations, in diploids homologues are present in pairs, hence each one has only 1 pairing partner and only bivalents are formed. In polyploids, with sets of more than 2 related/similar chromosomes, the task of matching up homologues and forming pairs is more complicated and needs to be precisely controlled.

Wheat (*Triticum aestivum* L.) is a hexaploid composed of 3 related genomes, A, B and D [Kihara, 1919]. With the exception of several ancient translocations, the homoeologues of each genome are capable of meiotic pair-

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ing. To avoid multivalent formation with its obvious consequences for uneven chromosome segregation, wheat evolved a pairing control system that enforces strictly homologous bivalent pairing. There are no observable instances of inter-genomic (homoeologous) pairing when the pairing control system is operational. The main locus of the system, *Ph1*, is located on the long arm of chromosome 5B [Sears, 1984b]. The name, *pairing homoeologous* [Wall et al., 1971], is perhaps a misnomer as it actually prevents homoeologues from pairing.

While the presence of the *Ph1* system has been known for over 50 years [Riley and Chapman, 1958; Sears and Okamoto, 1958], its mode of action still remains a mystery. Postulated hypotheses ranged from timing of meiosis [Riley, 1968], imposition of spatial separation of homologues from homoeologues [Feldman and Avivi, 1988], through enforcement of very strict stringency requirement for crossover formation [Dubcovsky et al., 1995; Luo et al., 1996] through some mode of centromere control [Martínez-Pérez et al., 2001] and changes in chromatin conformation [Mikhailova et al., 1998]. The region of chromosome 5B known to harbor the *Ph1* locus has been sequenced [Griffith et al., 2006]. Of the 36 identifiable gene sequences present, a cluster of *cdk-2*-like genes was postulated to be the *Ph1* locus itself [Griffith et al., 2006], perhaps more because of an a priori assumption of the possible function of the *Ph1* locus than an experimental proof of their actual involvement in enforcing bivalent pairing.

There is little doubt that the *Ph1* system imposes very high stringency requirement for the MI chromosome pairing (and since MI pairing is based on chiasmata, high stringency is a requirement for crossing over). While the locus was named for its effect on homoeologues in meiotic metaphase I, it is clear that it affects homologues as well. It often happens in wheat that homologues in intervarietal hybrids are unable to pair [Dvorak and McGuire, 1981; Crossway and Dvorak, 1984]. In a study of recombination in a specific segment of wheat chromosome 1B, up to a 4-fold increase in recombination frequency was observed in the absence of *Ph1*, and numerous intralocus recombinants in *Gli-B1* were recovered which were never found when *Ph1* was present [Lukaszewski and Brzezinski, 2003].

The *Ph1* locus extends its control to alien chromosomes introduced into wheat; its manipulation permits interspecific and intergeneric chromatin transfers for the benefit of agriculture [Sears, 1984a]. Schlegel et al. [1991] have shown that the *Ph1* locus, when introgressed with the entire chromosome 5B into diploid rye, dramatically reduces the overall MI pairing frequency of rye chromosomes. This suggests the effect is not limited to wheat chromosomes or to chromosomes in the genetic background of wheat, but is of a more universal system of chromosome pairing control. Given that all species with known genomic DNA sequence show clear evidence of past polyploidization events [Wendel, 2000], the issue of the enforcement of diploid-like chromosome pairing in MI of meiosis is wider than polyploid wheat or even current alloploids. In this article we present further elaboration on the effect of chromosome 5B with its *Ph1* locus on chromosome pairing in autotetraploid rye and present some evidence on the criteria in chromosome recognition by the *Ph1* locus.

Materials and Methods

In an attempt to generate single chromosome introgressions from the B genome of tetraploid wheat into rye, tetraploid triticales with a complete B genome [Lukaszewski et al., 1984] were crossed to tetraploid spring rye cv. Tetra Gator, obtained from Dr. R. Burnett, University of Florida, Gainsville, Fla., USA, via Dr. R.J. Metzger. The resulting F_1 hybrids were backcrossed several times to Tetra Gator with selection for individual B-genome chromosomes of wheat in each generation. After BC_3, plants with single chromosomes 5B and its long arm telocentrics that appeared among selected progenies, presumably by centric misdivision, were selected and intercrossed. The resulting progenies were screened by C-banding to identify plants with no wheat chromatin present, with 1 and 2 chromosomes 5B or 5BL present, as well as combinations of 5B + 5BL. Each of these combinations was intermated as populations.

Established populations were screened by C-banding and plants with desired chromosome constitutions were selected and grown. They were sampled at meiosis to collect the material for this study. From each sampled flower a portion of one anther was removed, fresh-squashed in a drop of acetocarmine, and if the desired meiotic stage was present, the remaining anthers from the flower were fixed in a mixture of absolute ethyl alcohol and glacial acetic acid at a ratio of 3:1 at 37°C for a week and stored at –20°C until used.

Chromosome pairing was analyzed on squashed preparations using fluorescent in situ hybridization (FISH). Total genomic DNA of wheat was labeled directly with rhodamine and DNA clone pTa71 [Gerlach and Bedbrook, 1979] containing a 9-kb *Eco*-RI fragment of wheat ribosomal DNA, which carries the 18S-5.8S-26S cluster of ribosomal RNA genes (here referred to as 45S rDNA) was labeled with DIG-Nick Translation Mix (Roche Applied Science, Indianapolis, Ind., USA). Total genomic DNA of rye was sheared to 200–500-bp fragments and used as a block. In all experiments, the probe to block ratio was about 1:150. FISH experiments were performed according to Kopecký et al. [2007]. Sites of probe hybridization were detected by the anti-DIG-FITC conjugate (Roche). Chromosomes were counterstained with 1.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) made in Vecta-

shield antifade solution (Vector Laboratories, Burlingame, Calif., USA). Observations were made with a Zeiss Axioscope 20 equipped with epifluorescence, recorded with a SPOT RT Color digital camera (Diagnostic Instruments Inc.), and processed using SPOT Advanced and Adobe Photoshop v. 6 software.

For each population 3 to 7 plants were analyzed with 30 meiocytes per plant scored. On each preparation, the following were scored: total pairing (numbers of chromosome arms paired in each pairing configuration), pairing of chromosome 1R and where applicable, of chromosomes 5B(L). In addition, chromosome pairing patterns were scored by C-banding in bivalents of 2 plants without 5B (controls), in 1 plant disomic for 5B and 2 plants with 5B + 5BL, with all available meiocytes analyzed. Meiotic C-banding was according to Giraldez et al. [1979].

Results and Discussion

The way the material for this study was developed (a hybrid between tetraploid triticale with BBRR genomic constitution and tetraploid rye, RRRR) resulted in some variation in the status of wheat chromosome or chromosomes 5B present. In some plants, chromosome 5B substituted rye chromosome 5R; in others it was present as an addition (fig. 1c). Also, given that the recipient was an autotetraploid, aneuploidy for rye chromosomes was expected and was present even though some care was taken to limit the number of aneuploids analyzed. Whenever aneuploids were included, the chrom some pairing indices were adjusted for the total number of chromosomes present in a given plant.

In combinations where two 5BL arms were present (2 doses of *Ph1*: three combinations: 5B″, 5BL″ and 5B + 5BL; a total of 15 plants scored) pairing of 5BL was high, averaging 93% (0.93 arms paired per chromosome, appc) and there was little variation from plant to plant. In 5B disomics, the short arms of 5B paired with the average frequency of 43% (0.43 appc). Therefore, pairing of wheat chromosome 5B in rye was normal, essentially the same as it is in wheat [Sallee and Kimber, 1978]. No instances of homoeologous pairing of 5B with any rye chromosome were observed. While this is beyond the scope of this study, all other wheat B-genome disomics isolated in this rye background had high homologous pairing but were also engaged in homoeologous pairing with rye chromosomes at levels higher than the same chromosome combination in wheat.

The NOR locus on the short arm of chromosome 1R, here labeled by the pTa71 probe, enabled monitoring of pairing behavior of this quartet in every cell. In no instance did pairing frequency of the 1R quartet deviate from the overall pairing of the remaining 6 quartets of

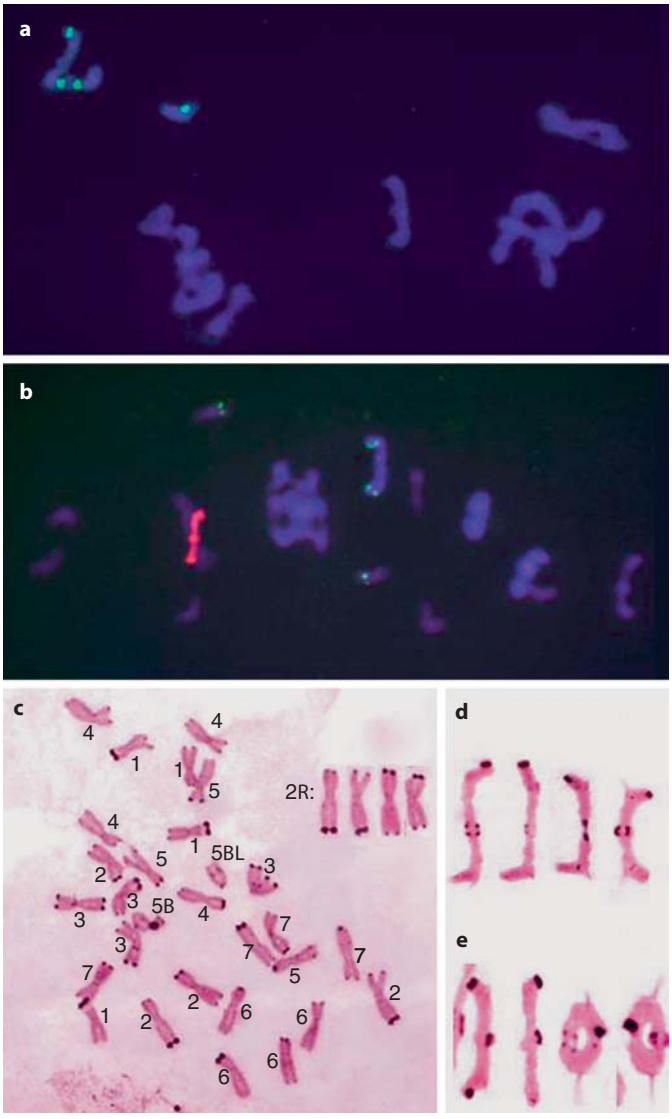

Fig. 1. Meiotic and mitotic chromosomes of autotetraploid rye with introgressions of wheat chromosome 5B. **a** Typical MI in a control plant with 5 quadrivalents, 1 trivalent, 2 bivalents and 1 univalent. Chromosome 1R (with FITC-labeled NOR region) forms the trivalent and the univalent. **b** MI pairing in a plant with a disomic introgression 5B (red; directly labeled with rhodamine): 2 quadrivalents, 6 bivalents (including 5B) and 8 univalents. Chromosome 1R forms 1 bivalent and 2 univalents. **c** Mitotic metaphase chromosomes of a 29-chromosome plant with an introgression of 5B + 5BL showing the extent of C-band polymorphism. Inset: 2R quartet with 2 distinct banding patterns. **d** Rod bivalents paired in arms with the same banding patterns; arms polymorphic for C-bands remain unpaired. **e** Rod and ring bivalents paired in arms polymorphic for terminal C-bands.

Table 1. Average metaphase I chromosome pairing frequencies per PMC in autotetraploid rye with introgressions of wheat chromosome 5B or its long arm

Combination	Plants analyzed	Univalents	Bivalents	Trivalents	Quadrivalents	Genome, appc	1R, appc
Monosomic 5B	4	2.93	9.83	0.24	1.18	1.36 ± 0.16	1.38 ± 0.42
Monotelosomic 5BL	3	1.73	9.14	0.20	1.84	1.50 ± 0.15	1.48 ± 0.42
Disomic 5B	3	4.20	9.93	0.24	0.62	1.20 ± 0.18	1.14 ± 0.44
5B + 5BL	7	3.80	9.54	0.36	0.94	1.22 ± 0.19	1.15 ± 0.44
Ditelosomic 5BL	5	3.79	9.37	0.33	1.10	1.21 ± 0.19	1.17 ± 0.43
Control (no 5BL)	7	0.77	7.62	0.29	2.78	1.68 ± 0.14	1.67 ± 0.35
Total 1 dose 5BL	7	2.41	9.53	0.22	1.46	1.42 ± 0.17	1.43 ± 0.42
Total 2 doses 5BL	15	3.88	9.56	0.32	0.93	1.22 ± 0.19	1.15 ± 0.43

appc = Arms paired per chromosome.

rye chromosomes, with appc frequency in 1R being essentially identical to that of the entire genome (table 1).

Very wide variation in pairing of rye chromosomes was evident, depending on the presence/absence and the dosage of 5BL (table 1). While the overall rye pairing in the absence of 5B (fig. 1a) was lower than expected for an autotetraploid, the average frequency of univalents was low and the frequency of quadrivalents approached 2.8 per PMC. The effect of 5BL on these indices was striking, and appeared to be dosage dependent: with 1 dose of 5BL, the average number of univalents tripled relative to controls (table 1), and the number of quadrivalents dropped by one half. With two 5BL present, there was even less pairing: the average number of univalents per PMC was 5 times higher than in normal rye while the number of quadrivalents was 3 times lower (fig. 1b). The average pairing indices for controls (no 5BL, no *Ph1*) and 2 doses of 5BL and *Ph1* were statistically significant in all categories (table 1); the indices for 1 dose of 5BL (1 copy of *Ph1*) were intermediate.

There is no ready explanation for the apparent dosage effect of 5BL *(Ph1)* in rye. In wheat, *Ph1* behaves as a dominant gene with 1 dose being sufficient to prevent all homoeologous pairing. Perhaps the difference here is in the interaction between the *Ph1* locus and rye chromosome 5R. Chromosome 5R is known to partially suppress *Ph1* in wheat, and the effect appears to be dose-sensitive in that higher doses of 5R reduce the effect of *Ph1* to a higher extent [Riley et al., 1973; Lelley, 1976]. Whether there exists any allelic variation in rye in the *Ph1* suppression mechanism is not known. Such variation is to be expected from what is known in *Aegilops speltoides* and other diploid Triticeae [Waines, 1976; Shang et al., 1989a]. On the other hand, the apparent dominant effect of the *Ph1* locus in wheat might have never been properly tested. Assuming that the locus imposes some step-wise criteria on crossing over in wheat, all alien chromosomes introduced into wheat may fall below the minimum similarity threshold level imposed by a single dose of *Ph1* and never pair; pairing of differentiated wheat homologues, such as those in intervarietal hybrids, at different dosages of *Ph1* has never been examined in detail. In higher dosages, 4 and above, the *Ph1* locus restricts MI pairing of even identical homologues [Feldman and Avivi, 1988].

The pairing data accumulated in this study leave no doubt that introgression of chromosome 5B into tetraploid rye dramatically reduces the level of chiasmate MI pairing of homologues. This is the same effect as described by Schlegel et al. [1991] for diploid rye. Unclear are the criteria by which this pairing reduction is accomplished: is there a specific key by which some chromosomes or chromosome arms are restricted from pairing or is there a general genome-wide reduction in chiasma frequency (crossover rate)? If the first is the case, some chromosome arms would retain their normal levels of pairing with *Ph1* present, while other arms would pair very little or not pair at all. If the latter is the case, the reduction would be proportional for each arm.

To test these 2 scenarios, an attempt was made to generate doubled haploids from plants disomic for chromosome 5B. This was done by androgenesis in vitro using the same method as in Kopecký et al. [2005]. A doubled

haploid would have 2 pairs of identical homologues in each of 7 chromosome quartets. How different the 2 pairs of homologues would be, would depend entirely on the level of homologue differentiation in the starting material. If pairing restriction was based on chromosome similarity/differentiation, mostly bivalent pairing would be observed. Unfortunately, all attempts at androgenesis failed; not a single embryoid was formed in ca. 10,000 anthers plated and no plants were recovered. With doubled haploids lacking, any further analysis of the reduction in the overall chromosome pairing brought about by the introgression of the long arm of 5B was by necessity coarse.

The tetraploid ryes used in this study were very polymorphic for their C-banding patterns (fig. 1c) to a point where routine chromosome identification was difficult in mitosis; in meiosis, it was virtually impossible to identify with precision each chromosome present in a cell. All further analyses of the material were based on the premise that C-band polymorphism remained in some proportion to DNA polymorphism of individual chromosomes in each quartet. If this indeed is the case, a relationship between metaphase I pairing of individual chromosome arms and the degree of their C-band polymorphism would give some indication of the nature of the overall pairing reduction in plants with 5B.

Heterozygosity for telomeric C-bands reduces MI pairing of rye chromosomes in diploid rye, in triticale and other wheat-rye hybrids [Naranjo and Lacadena, 1980; Naranjo and Orellana, 1984; Gillies and Lukaszewski, 1989]. This reduction likely is a consequence of homologue misalignment at the point of synapsis initiation [Gillies and Lukaszewski, 1989]. The level of pairing reduction also appears to depend on several factors, such as the genotype and the chromosome studied [Naranjo and Orellana, 1984]. Here, since it was virtually impossible to reliably identify every chromosome and chromosome arm in MI, especially in more complex configurations and univalents, the analysis was limited to simple scoring of all bivalents present in a cell that involved chromosomes with clear (large) telomeric C-band heteromorphism. The only answer sought was whether the homomorphic or heteromorphic arms were paired.

In 2 plants without any 5BL present, 78 bivalents were scored (most likely chromosomes 1R, 2R, 3R and 5R). Of these, there were 57 rods and 21 rings. Among rod bivalents, 44 were paired in the homomorphic arm (fig. 1d) and 13 were paired in the heteromorphic arm (fig. 1e), for the overall rate of pairing of similar arms at 65/78 (83.3%) and dissimilar arms of 34/78 (43.6%). Chromosome arms homomorphic for the telomeric C-bands were twice as likely to pair as the heteromorphic arms. Three plants disomic for the long arm of 5B were scored (one disomic 5B; two 5B + 5BL), and each plant was polymorphic for one arm in up to 3 different chromosome quartets (very likely chromosomes 1R, 2R, 3R, 5R and 7R). Among 247 bivalents observed, 234 were rods paired in similar arms (heteromorphic arms not paired), 11 were rods paired in the heteromorphic arms and there were 2 rings, for the total of 236 (95.5%) arms paired in similar arms and 13 (5.3%) paired in dissimilar arms. The proportions of paired similar to dissimilar arms in the presence and absence of 2 copies of 5B are highly significant ($\chi^2 = 50.91$, $p < 0.01$).

Both in the absence and presence of 5BL, with its *Ph1* locus, pairing of heteromorphic arms was reduced. However, 2 copies of 5BL almost completely prevented heteromorphic arms from pairing. It therefore appears justified to believe that the *Ph1* locus recognizes chromosome polymorphism, here visualized by the presence/absence of telomeric C-bands. To what degree the C-band polymorphism is an expression of chromosome divergence is not clear, but as a general rule there is more variation in banding patterns in distantly related individuals/populations than among close relatives. A relationship between C-band polymorphism and geographic distribution was noted even in wheat itself [Shang et al., 1989b]. When the overall pairing frequencies scored in the analyzed material are re-calculated using the pairing frequencies for similar versus dissimilar arms, it appears that most of the overall pairing reduction relative to that theoretically expected for an autotetraploid (2.8 quadrivalents/PMC observed vs. 4.6 quadrivalents/PMC expected) was caused by chromosome differentiation. Further reduction in the overall MI pairing produced by the introgression of chromosome arm 5BL was a consequence of a virtual elimination of crossing over in heteromorphic arms. It therefore appears safe to conclude that as in wheat, also in rye the *Ph1* locus recognizes differences among homologues present and restricts pairing to similar arms. Given a considerable differentiation of homologues in the analyzed material, this made multivalent pairing rare.

Among plants with a single copy of 5BL one stood out in that its pairing indices were directly comparable to controls, with the average of 0.77 univalents, 1.70 quadrivalents and 1.77 arms paired per chromosome. Chromosome 5B, obviously present in each PMC as shown by FISH and on the root tip squash, did not seem to have any effect on pairing. This plant may carry more similar sets of chromosomes, produced by segregation in small pop-

ulations, or be an instance of *Ph1* mutation. During the development of this material and selection of plants for the analysis, some damage to chromosome 5B was evident that closely resembled the effects of the breakage-fusion-bridge cycles in wheat-rye hybrids [Lukaszewski, 1995]. This included 1 reverse tandem duplication on the long arm and several independent cases of deficiency in the short arms. Perhaps in the process of chromosome breakage, the *Ph1* locus in this plant was inactivated or eliminated. This plant was excluded from further calculations.

Autopolyploids, with their tendency to form multivalents, suffer reduced fertility. It is for this reason that no autotetraploid grain crops are in commercial production. Doyle [1979, 1986] made a long-term effort to diploidize autotetraploid maize by gradual accumulation of minor chromosome differences such as small structural aberrations and divergent chromosomes from exotic accessions. Over time this reduced multivalent and increased bivalent MI pairing. The same process of gradual diploidization of meiotic chromosome behavior must have taken place numerous times in evolution, following each polyploidization event. The tetraploid ryes studied here have already advanced along the path of chromosome differentiation from completely random pairing; their multivalent formation was only ~60% of that expected for completely random pairing in an autotetraploid. However, the introduction of the *Ph1* locus rapidly advanced this process, underscoring the role of chromosome pairing regulation. Interestingly, there appears to be a major conflict of interest in the pairing control system. Since only diploid-like behavior in MI of meiosis assures efficient production of genetically balanced euploid gametes, it would appear that in diploid species, especially an outbreeder [see Riley and Law, 1965], a permissive system of chromosome pairing control would have an evolutionary advantage. Such a system permits regular pairing of dissimilar homologues, such as in hybrids resulting from mating of distantly related individuals, as it guarantees efficient production of chromosomally stable euploid gametes, hence increases the efficiency of reproduction. How detrimental a strict pairing control system can be in a diploid outbreeding species is illustrated by the reduction in the MI pairing indices and resultant sterility in diploid rye with a single wheat chromosome 5B [Schlegel et al., 1991]. Of course, the divergence or polymorphism of homologues would not be an issue in obligatory inbreeders.

While a permissive chromosome pairing system appears to offer a selective advantage in diploid species, in polyploids only a strict system of chromosome pairing control guarantees a similar rate of efficiency. Pairing based on strict chromosome similarity eliminates interactions of dissimilar homologues or homoeologues, thus assuring diploid-like meiosis. Most, if not all, currently known alloploids have systems enforcing diploid-like behavior in MI [Jenczewski and Alix, 2004]. Alloploids originate by hybridization of related species; they have pairs of homologues and sets of homoeologues, that is, chromosomes with the same genetic contents but differentiated by speciation [Riley, 1968]. In autopolyploids, sets of homologues are present, but depending on the breeding mode, these can be substantially differentiated. Yet, for efficient sexual reproduction, autopolyploids also need to diploidize their meiosis. All species with extensive DNA sequence information show evidence of polyploidization events in their history [Wendel, 2000]. Therefore, at some point in their evolution they might have taken advantage of a strict chromosome pairing control. In this sense, changes from permissive chromosome pairing systems in diploids to strict systems in polyploids might have been fairly regular, even if infrequent, events. Convincing genetic evidence has been presented that chromosome pairing control systems exist in diploid species such as *Triticum monococcum*, *Ae. speltoides* and *Haynaldia villosa* [Waines, 1976; Shang et al., 1989a]. That such a system must exist seems beyond doubt: in all higher eukaryotes some restrictions have to be based on crossing over lest exchanges within dispersed repetitive DNAs and segment duplications destroy a genome's integrity. If so, the *Ph1*-like systems in polyploids that impose high stringency requirements for crossing over, thereby limiting MI pairing to essentially identical chromosomes, may not be de novo appearing mutations each time a new alloploid is created, but may only represent a different state of the same general system that controls crossing over.

It will be an interesting experiment to test at which point in the evolution of a species following a polyploidization event (whether by auto- or allo-polyploidization) chromosomes become sufficiently divergent to prevent multivalent pairing even under a permissive pairing control system. The progress of directed selection in autotetraploid maize for a diploid-like behavior was slow but steady [Doyle, 1986]. Here, in autotetraploid rye, addition of the *Ph1* locus dramatically accelerated the process of diploidization. It remains to be seen if further diploidization of this rye is possible, and if so, at which point the *Ph1* locus will no longer be necessary to enforce the diploid-like chromosome behavior in meiosis.

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