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RESEARCH

Re-Engineering of the *Pm21* Transfer from *Haynaldia villosa* to Bread Wheat by Induced Homoeologous Recombination

Adam J. Lukaszewski* and Christina Cowger

ABSTRACT

Blumeria graminis f. sp. *tritici*, the cause of powdery mildew, can generate serious grain yield losses in wheat (*Triticum* sp.). To expand the range of resistance genes freely available to wheat breeders, a *Haynaldia villosa* (L.) Schur. (syn. *Dapsypyrum villosum* L.)-derived gene, *Pm21*, was transferred to chromosome 6AS of wheat by homoeologous recombination. The transfer showed that the genetic location of the locus was different from that suggested by an earlier transfer by irradiation. Wheat lines with two small intercalary inserts of *H. villosa* chromatin with *Pm21* were tested with a range of powdery mildew isolates and found to be completely resistant (infection score of 0).

A.J. Lukaszewski, Dep. of Botany and Plant Sciences, Univ. of California, Riverside CA 92521; C. Cowger, USDA-ARS, Dep. of Plant Pathology, North Carolina State Univ., 3409 Gardner Hall, CB 7616, Raleigh, NC 27695-7616. Received 21 Mar. 2017. Accepted 4 May 2017. *Corresponding author (adam.lukaszewski@ucr.edu). Assigned to Associate Editor Shuyu Liu.

Powdery mildew, caused by *Blumeria graminis* f. sp. *tritici,* is a serious disease of wheat (*Triticum* sp.), with grain yield losses reaching up to a third. The disease can be controlled with fungicide, but in most cases, resistance breeding is the most sensible and economical solution (Murray and Brennan, 2009). So far, some 44 distinct loci associated with resistance have been identified in wheat (McIntosh et al., 2012), with some loci carrying numerous alleles (Huang et al., 2004; Hsam et al., 2015). Many of these loci originated from related species and were transferred into wheat by various means (Friebe et al., 1996; Hsam and Zeller, 2002; Miranda et al., 2007; McIntosh et al., 2012; Petersen et al., 2015).

Notable among transfers from related species to wheat is *Pm21* originally identified as a centric translocation of the short arm of chromosome 6VS of *Haynaldia villosa* (L.) Schur. (syn. *Dapsypyrum villosum* L.) to wheat chromosome arm 6AL (Qi et al., 1993). To reduce the amount of *H. villosa* chromatin, the chromosome was later engineered by irradiation to generate wheat chromosome 6A with an intercalary segment of *H. villosa*, perhaps 20% of the physical arm length (Chen et al., 2008). After some 20 yr in cultivation, *Pm21* is said to still offer a good level of resistance to powdery mildew in China, where it is extensively used (Cao et al., 2011; M. Hua et al., personal communication, 2016). Its potential and its value in other areas of wheat cultivation are difficult to assess because of its limited circulation.

Chromosome breakage by irradiation is essentially a random process. It offers little precision, but with large enough samples, agronomically successful transfers can be produced, such as the

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transfer of leaf rust (*Puccinia recondita* Rob. ex Desm.) resistance from *Aegilops umbellulata* Zhuk. to wheat (Sears, 1956). However, that effort required screening of a large progeny sample and then careful testing of all obtained translocations to identify a satisfactory transfer. In this sense, radiation-induced transfers from wild relatives into a crops species may offer only an illusory advantage over induced homoeologous recombination. This effort was undertaken to re-engineer the 6VS.6AL translocation via induced homoeologous recombination, both as a comparison of the irradiation and recombination approaches, but also to make the *Pm21* locus more available to wheat breeders.

MATERIALS AND METHODS

The starting material was a line TA 5075 of winter wheat with centric translocation 6VS.6AL kindly provided by Dr. B. Friebe, Kansas State University, Manhattan, KS. This translocation was known to carry *Pm21*; a similar translocation in the author's possession has not been tested. TA 5075 was crossed and backcrossed to the *ph1b* line of 'Pavon 76', a spring white wheat from the International Maize and Wheat Improvement Center (CIMMYT), Mexico. The *ph1b* line of Pavon 76 was produced by backcrosses of the E. R. Sears *ph1b* mutant line of 'Chinese Spring' to Pavon Mono 5B, with seven backcrosses completed, followed by three rounds of selection from *ph1b/Ph1* heterozygotes to eliminate the winter growth habit associated with the substitution of complete chromosome 5B from Chinese Spring to Pavon 76.

After the backcross, progenies were screened by C-banding to identify plants heterozygous for the 6VS.6AL translocation and homozygous *ph1b*. Selected plants were grown and self-pollinated. Resulting progenies were again screened by C-banding to identify recombinant chromosomes 6AS-6VS (Fig. 1).

The transfer of *Pm21* undertaken here followed the two-step protocol for *ph1b*-mediated alien transfers proposed by Sears (1981). These steps included selection of primary recombinants, tests for the presence of *Pm21* and positions of translocation breakpoints, intercrossing of primary recombinants with *Pm21*, and selection of secondary recombinants.

Basic cytological screening, including that for *ph1b*, was done by C-banding using the standard protocol (Lukaszewski and Xu, 1995). Preparations with detected recombinant chromosomes were destained and probed with labeled total genomic DNA of *H. villosa* in the presence of sheared total genomic DNA

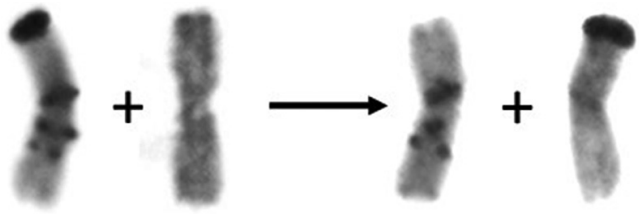

Fig. 1. C-banding patterns of chromosomes 6VS.6AL and 6A used to identify primary recombinants of chromosome arms 6VS with 6AS. From left to right: centric translocation 6VS.6AL, presumed chromosome 6A (there are no identifying marks on chromosome 6A of 'Pavon 76'), primary recombinant 6VS.6AL–, and primary recombinant 6AS+.

of wheat serving as a block, following the protocol developed by T. R. Endo (Masoudi-Nejad et al., 2002). Plants with verified recombinant chromosomes were backcrossed twice to Pavon 76. After each backcross, heterozygotes were grown and tested for powdery mildew resistance under spontaneous infections in the greenhouse. After two cycles of such screening, reproducible readings of resistance and susceptibility were obtained. Recombinants in reciprocal configurations carrying *Pm21* and with closest translocation breakpoints available were selected, intercrossed, and allowed to recombine in the presence of the *Ph1* locus. Among their progenies, chromosomes with intercalary insertions of *H. villosa* chromatin in wheat chromosome 6A were selected, grown, tested for powdery mildew resistance, and self-pollinated. From among their progenies, homozygotes of the translocations were selected.

The final two products of this operation were tested for powdery mildew resistance with each of six genetically pure (single-pustuled) isolates of *B. graminis* f. sp. *tritici*. The isolates were from the soft wheat region, where *B. graminis* f. sp. *tritici* populations have greater mean virulence complexity (i.e., are virulent to more *Pm* genes) than in the hard wheat region of the US Plains states (C. Cowger, unpublished data, 2016). The isolates were GAP-A-2-3, MSG-C-3-4, NCC-B-1-3, NCF-D-1-1, MIR(14)-D-3-3, and PAF(14)-D-1-2. They had been collected from the US states of Georgia, Michigan, Mississippi, North Carolina, and Pennsylvania and thus represented a selection of the US *B. graminis* f. sp. *tritici* population from soft wheats that was geographically and genetically diverse (Cowger et al., 2016). The isolates were collected in 2013 or 2014 and maintained in a detached-leaf culture since then.

A standard detached-leaf protocol (Parks et al., 2008) was used to screen the two final homozygote progeny and Pavon 76. Briefly, two 2-cm leaf segments from each of three plants per cultivar were inoculated with each isolate individually for a total of six leaf segments per cultivar + isolate combination. Segments of the universally susceptible cultivar Jagalene were placed at the four edges of the other leaf segments on each plate as a control for inoculation efficacy. The plates were incubated at 17°C for 9 d, and the lines were then rated using a standard 0-to-9 scale, in which scores of 0 to 3 indicate resistant, 4 to 6 indicate intermediate, and 7 to 9 indicate susceptible lines (Parks et al., 2008).

The nomenclature system for recombinant chromosomes used here follows that proposed earlier for recombinants 1RS-1BS in wheat (Lukaszewski, 2000). Primary recombinants with proximal segments of *H. villosa* chromosome 6 and terminal segments from 6AS are labeled 6VS−, followed by the number indicating the order in which they were isolated; recombinants with proximal segments of 6AS and terminal segments of 6VS were labeled 6A+ and numbered as above.

RESULTS

Among 997 progeny from plants 19" + 6VS.6AL + 6A + 5B*ph1b* screened, 31 recombinant chromosomes 6AS-6VS were discovered. Of these, 11 were in the configuration with proximal segment of 6VS and terminal segment of 6AS (6VS−), and 19 with terminal segments of 6VS and proximal segments of 6AS (Fig. 1) (6AS+). One chromosome,

with terminal 6VS segment, was lost; one appeared unstable, forming a dicentric chromosome, and was discarded. The 29 verified and retained recombinants give the crossover frequency of 2.9% calculated on the per-plant basis.

Heterozygotes of the primary recombinant chromosomes after the first and second backcrosses to 'Pavon 76' were challenged with a mixture of *Blumeria* spores virulent on Pavon 76 and all other wheats grown in the greenhouse at the time and avirulent on all 6VS.6AL translocation carriers (tested as heterozygotes after backcrosses to several different winter wheats and Pavon 76). Tests depended on natural infections; the rate of infection was moderate in the first season and heavy in the second. This test separated the recombinants into resistant and susceptible. Among 18 tested recombinants with terminal *H. villosa* segments, three were resistant and 16 were susceptible; among 11 recombinants with proximal *H. villosa* segments, eight were resistant and three were susceptible. Most crossing over occurred distal to the *Pm21* locus (23 out of 29).

Based on the hybridization patterns after in situ probing with labeled total genomic DNA of *H. villosa*, recombinant 6VS−6 was selected as having the most proximal breakpoint in this configuration and *Pm21* present; among reciprocal recombinants, 6A+10 was resistant to powdery mildew and had the most distal breakpoint. As a precaution, recombinant 6A+16 with a visibly more proximal breakpoint relative to that of 6A+10 was also selected for the second step.

Heterozygotes for chromosome 6VS−6 were crossed to heterozygotes of 6A+10 and 6A+16. Their progenies were screened by C-banding, and plants with 20" + 6VS−6 + 6A+10 and 20" + 6VS−6 + 6A+16 were selected, grown, and self-pollinated. Their progenies were screened for the presence of secondary recombinant chromosomes resulting from crossovers in overlapping *H. villosa* segments in both chromosomes. These were of two types: original translocation 6VS.6AL and chromosomes 6A with intercalary inserts of 6VS. Among 53 progeny from plants with 20" + 6VS−6 + 6A+10, one each of the two expected recombinants were recovered, 6VS.6AL and $6A_{6:10}$ for the crossover rate of 1.9% on the per-chromosome basis. Among 57 progeny also from 20" + 6VS−6 + 6A+16, two recombinants were recovered, one each of 6VS.6AL and $6A_{6:16}$, for a recombination rate of 1.7% (Fig. 2). In both screened populations, the frequency of 6A+ chromosomes was higher than 50% expected for random segregation, 59 vs. 47 for 6A+10 vs. 6VS−6 and 68 vs. 46 for 6AS+16 vs. 6VS−6, indicating lower compensating ability of chromosome 6VS−6 over the two 6AS+ chromosomes.

Progenies of plants heterozygous for the two secondary recombinants, $6A_{6:10}$ and $6A_{6:16}$, were screened by C-banding, putative homozygotes were identified and verified by in situ probing with labelled total genomic DNA of *H. villosa*. In both cases several homozygotes were recovered but the screened samples were too small to assess transmission rates of recombined chromosomes, especially that they were always present with one of the primary recombinants and not normal 6A of Pavon 76.

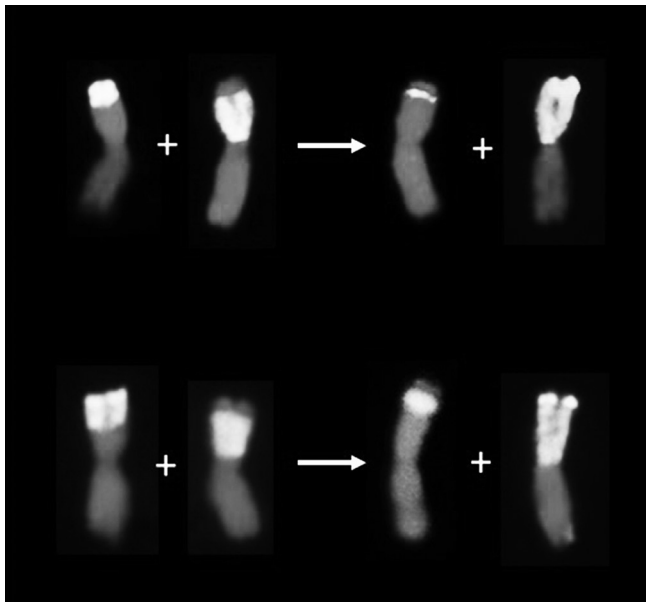

Fig. 2. Development of secondary recombinants of *H. villosa* chromosome 6VS with wheat chromosome 6AS. Top row: a crossover in the *H. villosa* segments of primary recombinants 6A+10 and 6VS.6AL–6 produces a secondary recombinant $6AS_{10:6}$ and centric translocation 6VS.6AL; bottom row: a crossover in the *H. villosa* segments of primary recombinants 6A+16 and 6VS.6AL–16 produces a secondary recombinant $6AS_{16:6}$ and centric translocation 6VS.6AL. The proximal edge of the *H. villosa* insert in chromosome $6AS_{10:6}$ shown here is ~78% of the arm length away from the centromere.

Progeny of homozygotes for the two secondary recombinants $6A_{6:10}$ and $6A_{6:16}$ were fully resistant in the detached-leaf tests (rating 0 for all leaf segments), whereas the recipient wheat Pavon 76 was uniformly susceptible (ratings 7 and 8). The control cultivar Jagalene was also fully susceptible on all plates (ratings 7 or 8, one 6), indicating that inoculation efficacy was high across the plates.

DISCUSSION

Manipulation of the centric translocation 6VS.6AL by irradiation (Chen et al., 2008) generated chromosome 6A with a relatively small insert of *H. villosa* chromatin in an intercalary position. Mapping by deletion (Qi et al., 1996, 1998) and irradiation-induced chromosome breakage (Chen et al., 2008) suggested that *Pm21* must be located no farther away from the centromere than ~58% of the 6S arm length. Even though genetic lengths of the two secondary recombinants produced here, $6A_{6:10}$ and $6A_{6:16}$, are very similar (1.9 and 1.7% recombination rate, respectively), $6A_{6:10}$ physically carries a much smaller piece of *H. villosa* chromatin than $6A_{6:16}$ with a more distal location of its proximal edge (Fig. 2) (the distal breakpoint is the same in both chromosomes). Given the mean of five measurements, this proximal edge

in $6A_{6:10}$ is located at 69.4% of the relative arm's length away from the centromere. This means that the actual position of *Pm21* in the arm is more distal, by at least 10 to 12% of the relative arm length, than the irradiation and deletion studies suggested (Qi et al., 1998; Chen et al., 2008), or that 6VS and 6AS arms are not colinear.

Whether the recombination-based approach placed the *Pm21*-containing segment in its natural (corresponding) position in 6AS is an open question. On the one hand, crossing over is symmetrical and preferentially occurs between closely related, if not outright identical, DNA sequences (Datta et al., 1997). Therefore, an approach based on crossing over is expected to place introgressions in their proper positions in recipient genomes. The unknowns in this case are the characteristics of crossing over in wheat in the absence of the *Ph1* locus. Genetic maps generated in the absence of *Ph1*, whether for homoeologues or homologues, are in general agreement with maps generated for homologues with *Ph1* present (Lukaszewski and Hohn, 2013), with some notable exceptions. Several cases of unequal crossovers were recovered by Lukaszewski and Brzezinski (2003) in homologous recombination with *Ph1* absent. At least two 1RS to 1BS recombinants obtained with *Ph1* absent (Lukaszewski 2000) appear to have resulted from unequal crossovers (J. Dubcovsky, personal communication, 2015; A.J. Lukaszewski, unpublished data, 2016).

According to experience gathered in this study, including observations during the recovery of recombinant chromosomes and their segregation ratios, centric translocation 6VS.6AL does not fully compensate for normal wheat chromosome 6A. The region responsible for reduced compensation, as measured by transmission rates to progeny, appears to be proximal, or the degree of compensation is different in the proximal and distal regions of 6VS. Primary recombinants 6AS+10 and 6AS+16, despite differences in the positions of their breakpoints, were clearly favored in transmission over 6VS−6 by a similar degree. Substantially lower recovery of recombinants in the 6VS− configuration is probably a consequence of this lower transmission rate of the proximal segment of 6VS.

The irradiation approach is often used as the ultimate step in introgression of alien chromatin into crops. It is considered simple and efficient. Although there may be some advantages to this approach, such as no requirement for chromosome pairing and little effort required in preparation of stocks, it does not appear to be any more efficient or successful than the approach based on recombination. To be done properly, it still requires very large samples. In the effort pioneering this approach, Sears (1956) sifted through 6091 progeny from irradiated stocks and identified at least 17 translocations, of which only one appeared to show no adverse effects on pollen transmission. This translocation was successfully employed in agriculture, generating considerable benefit to wheat growers (Redei, 1992). Total numbers of plants screened (originally 6091 plus all tests) were no different than the average for any *ph1b*-induced transfer, with the exception of truly recalcitrant chromosomes. In the radiation-induced transfer of *Pm21* (Chen et al., 2008), the total numbers are not given, but 534 progeny of irradiated plants and their progenies were screened and 192 chromosome aberrations recovered. Here, including the initial screening, backcrosses, testing, and selection of secondary recombinants, ~1600 plants were screened to produce two intercalary introgressions of ~1.7 and 1.9 cM in length, and it is quite likely that these inserts are in their proper positions in the genome. Whether this is fully advantageous remains to be seen. At some point, E.R. Sears suggested that the advantage of irradiation-generated introgressions is the addition of a desired fragment of a donor's genome to the genome of a recipient, as opposed to replacing it with a part of the recipient's genome, thereby possibly preserving valuable wheat loci that otherwise would be replaced by a recombination-based operation (Sears, 1956). However, in his later statements, he drifted toward the position that the task should always be to produce the shortest possible transfer in their natural positions in the genome (Sears 1993), and this can only be reliably done by crossing over.

The two homozygote progeny exhibited complete immunity to a small but geographically diverse number of *B. graminis* f. sp. *tritici* strains from across the eastern United States. There is a need for new sources of both quantitative and qualitative resistance for wheat breeding programs, as several *Pm* genes that were widely deployed in mildew-prone regions of the United States have been defeated. Given the lower virulence complexity present in *B. graminis* f. sp. *tritici* populations in the US Plains and the spring wheat state of Montana (C. Cowger, unpublished data, 2016), this germplasm is likely to be effective in those regions and thus useful to a wide range of breeding programs. Both lines ($6A_{6:10}$ and $6A_{6:16}$) are available from the senior author.

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