Theor Appl Genet (1996) 93: 1180–1184

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Engineering of interstitial foreign chromosome segments containing the K^+/Na^+ selectivity gene *Kna1* by sequential homoeologous recombination in durum wheat

Received: 11 March 1996 / Accepted: 26 July 1996

Abstract Targeted homoeologous recombination mediated by the absence of the *Ph1* locus is currently the most efficient technique by which foreign genes can be introgressed into polyploid wheat species. Because intra-arm homoeologous double cross-overs are rare, introgressed foreign genes are usually on terminal foreign chromosome segments. Since the minimum length of such a segment is determined by the position of a gene in the chromosome, large chromosome segments with undesirable genetic effects are often introgressed. Introgression of foreign genes on short interstitial segments based on two cycles of homoeologous recombination is described here. The utility of the technique is demonstrated by the introgression of the *Kna1* locus, which controls K^+/Na^+ selectivity in *T. aestivum* L., on short interstitial segments of chromosome 4D into chromosome 4B of *Triticum turgidum* L. The level of recombination in a homoeologous segment is not significantly affected by a juxtaposed proximal homologous segment in the absence of the *Ph1* locus.

Key words Salt tolerance · *Triticum* · Gene transfer · RFLP · Genetic map · Linkage · Gene introgression

Introduction

Relatives of durum wheat, *Triticum turgidum* L. (2n=4x=28, genomes AABB), and bread wheat, *T. aestivum* L. (2n=6x=42, genomes AABBDD), represent a valuable source of germ plasm for wheat breeding. Agronomically important traits can be easily introgressed into polyploid wheats, from the donors of the wheat A and D genomes. The chromosomes of the remaining relatives of polyploid wheats pair poorly, or not at all, with wheat chromosomes in the presence of the wheat suppressor of homoeologous chromosome pairing, *Ph1*. Introgression of genes from those species can be achieved by irradiation-induced translocations (Sears 1956) or suppression/absence of the activity of the *Ph1* locus (Riley et al. 1968; Sears 1972). The latter principle has been successfully used in the introgression of genes from other species of *Triticum* (Riley et al. 1968; Dvořák 1977; Ceoloni et al. 1988; Dvořák and Gorham 1992; Dubcovsky et al. 1995; Lukaszewski 1995; Luo and Dvořák 1996), rye (Koebner and Shepherd 1986; Lukaszewski 1995; Rogowsky et al. 1991), and *Lophopyrum* (Sears 1972; Kibirige-Sebunya and Knott 1983).

In gene-introgression mediated by the absence of *Ph1*, it is preferable to construct plants in which the foreign chromosome and the wheat chromosome targeted for homoeologous recombination are monosomes (Sears 1972). The two monosomes tend to pair preferentially, and, as a result, a majority of recombinants recovered in the progeny usually have cross-overs between the targeted chromosomes (Sears 1977; Dvořák and Gorham 1992; Dubcovsky et al. 1996).

An asset of targeted homoeologous recombination is that a large number of recombinant chromosomes involving two specific homoeologues can be produced, and those with the most desirable characteristics can be selected. An important requirement is that a foreign segment does not involve genes with effects detrimental to the development of the gametophyte or sporophyte or any other agronomically important trait. Since the likelihood of such undesirable effects increases with the length of the segment (Dvořák et al. 1995), the most desirable recombinant chromosomes are those that have the shortest possible foreign segment still involving a gene of interest. Because virtually all recombinant chromosomes have only a single homoeologous cross-over within an arm (Rogowsky et al. 1991; Donini et al. 1995; Lukaszewski 1995; Dubcovsky et al. 1996), the position of a gene in a chromosome ultimately limits the minimum length of a foreign segment on which a gene can be introgressed into a wheat chromosome.

Communicated by G. E. Hart

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This limitation can be overcome by recombining two chromosomes that each had acquired a selected foreign gene but which differ in the origin of the centromere and the position of a cross-over relative to that gene (Sears 1981). While one chromosome must have a wheat centromere and a cross-over at the proximal side of the gene, the other chromosome must have a foreign centromere and a cross-over at the distal side of the gene. Such chromosomes can be recombined via a cross-over within the overlapping foreign region of homology, thereby generating a wheat chromosome with an interstitial foreign segment including the gene (Sears 1981). A prerequisite for this strategy is the isolation of a suitable pair of recombined chromosomes. Since the length of the interstitial foreign segment produced by this procedure is determined by the position of the cross-overs relative to the gene, the proximity of the cross-overs to the gene is of paramount importance for the development of short interstitial foreign segments.

A population of 133 chromosomes from homoeologous recombination between chromosome 4D of *T. aestivum* and chromosome 4B of *T. turgidum* was produced and analyzed in a project aimed at the transfer of enhanced K^+/Na^+ selectivity, and with it associated salt-stress tolerance, from *T. aestivum* to *T. turgidum* by targeted homoeologous recombination (Dvořák and Gorham 1992; Dvořák et al. 1994; Dubcovsky et al. 1996). Since the gene controlling K^+/Na^+ selectivity on chromosome 4D, *Kna1*, is within the distal half of the map of the 4DL arm, all recombinant chromosomes that had *Kna1* and the 4B centromere had relatively long 4DL terminal segments (Dubcovsky et al. 1996) with potentially detrimental effects on yield (Dvořák et al. 1994). Although a large number of recombinant chromosomes were analyzed, none had a cross-over distal to *Kna1* and the 4D centromere (Dubcovsky et al. 1996). The failure to obtain such a chromosome precluded the development of a 4B chromosome with *Kna1* on an interstitial 4D segment by homologous recombination between chromosomes with overlapping foreign segments.

In view of this apparent limitation of the strategy for engineering interstitial foreign chromosome segments in wheat by homologous recombination of chromosomes with overlapping foreign segments, a strategy based on two cycles of homoeologous recombination induced by the absence of *Ph1* was employed. Cross-overs generated in each cycle of homoeologous recombination were mapped utilizing restriction fragment length polymorphism (RFLP). The utility of the technique is demonstrated by engineering *T. turgidum* 4B chromosomes with interstitial segments of the *T. aestivum* chromosome 4D with the *Kna1* gene.

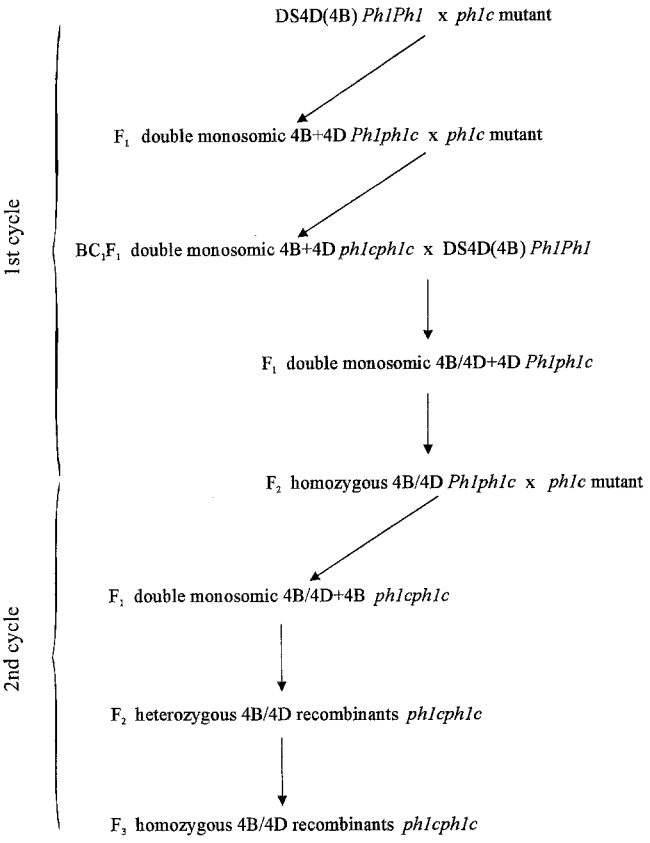

Fig. 1 A procedure for the engineering of interstitial 4D segments in chromosome 4B of *T. turgidum* by two cycles of homoeologous recombination. The first cycle has been described by Dvořák and Gorham (1992) and the second cycle is described in this paper. The generation designations refer to the genotypes under the *arrows*

Materials and methods

Homoeologous recombination

The source of chromosome 4D was a disomic substitution line in which chromosome 4D of *T. aestivum* cv Chinese Spring was substituted for *T. turgidum* cv Langdon chromosome 4B (Joppa and Williams 1988), henceforth DS4D(4B), and the source of chromosome 4B was the *T. turgidum ph1c* mutant (Giorgi and Cuozzo 1980). The development of the first-cycle 4B/4D recombinant chromosomes was described by Dvořák and Gorham (1992). In that cycle, targeted homoeologous recombination between 4B and 4D was induced by homozygosity for *ph1c* BC_1F_1 plants double-monosomic for 4B and 4D (Fig. 1). These BC_1F_1 plants were crossed with the Langdon disomic substitution line 4D(4B), and the F_1 plants were characterized by meiotic chromosome pairing, C-banding, and RFLP analysis (Dvořák and Gorham 1992; Dubcovsky et al. 1996). Following self-pollination of the F_1 plants, homozygous 4B/4D plants were selected in the F_2 generation, and K^+/Na^+ selectivity was determined in the F_3 generation (Dvořák and Gorham 1992; Dubcovsky et al. 1996).

Two F_2 families, nos. 3 and 46, were selected for the second cycle of homoeologous recombination because they had one of the shortest 4D segments still involving *Kna1* (Dubcovsky et al. 1996). Their 4B/4D recombinant chromosomes had the 4B centromere and a cross-over between *Xbcd1302* and *Xmwg2112* (see Fig. 2; for graphical genotypes of these chromosomes see Dubcovsky et al. 1996). For the second-cycle homoeologous recombination, first-cycle F_2 *Ph1ph1c* 4B/4D plants were selected by C-banding and were crossed with the *ph1c* mutant line (Fig. 1). Plants homozygous for *ph1c* (indicated by homozygosity for a deletion in 5BL, Dvořák et al. 1984) and having a 4B/4D+4B chromosome pair (indicated by heterozygosity for the 4BL terminal C-band, Dvořák and Gorham 1992) were selected by C-banding (see Fig. 2). Homozygosity for *ph1c* was verified by the absence of the *Xpsr128-5B* marker which is in the deleted region of the *ph1c* chromosome (Gill et al. 1993).

The second-cycle F_1 plants were self-pollinated, and F_2 plants were subjected to a RFLP analysis to identify those harboring 4B chromosomes with interstitial 4D segments. Some of the F_2 plants with 4B chromosomes with interstitial 4D segments were self-pollinated and second-cycle F_3 plants homozygous for a recombined 4B/4D chromosome were selected by RFLP analysis.

RFLP and map construction

Nuclear DNAs were isolated from individual plants according to Dvořák et al. (1988). Southern blotting and DNA hybridization were performed as described earlier (Dubcovsky et al. 1994). A RFLP map was constructed with the computer program Mapmaker/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992) using the Kosambi function (Kosambi 1943). LOD scores for each interval were computed with the Mapmaker program. To test the statistical significance of differences in the lengths of individual intervals between maps, interval lengths in cM were converted into % recombination, variances of the estimates were calculated according to Allard (1956), and the differences in the interval lengths between maps were tested by the *z*-test.

K^+/Na^+ selectivity

The accumulation of K^+ and Na^+ in the youngest expanded leaves was determined in solution-culture-grown plants. Seeds were surface-sterilized with 0.5 × commercial bleach and germinated in a vertical position between two sheets of blotting paper. Seedlings were transferred in a completely randomized design into a solution culture tank holding 300 l of aerated nutrient solution (Huang et al. 1992) in a greenhouse. After 1 week, the solution was renewed and adjusted to 50 mM NaCl; pH was adjusted every 3 days and the solution was changed once a week. The youngest fully expanded leaves were harvested after the plants had been in the salinized solution for 14 days. The fresh weight and dry weight (48 h at 60°C) of leaves was recorded. The concentrations of K^+ and Na^+ were determined as described earlier (Dubcovsky et al. 1996).

Results

A total of 174 F_2 progeny derived from second-cycle F_1 plants homozygous for *ph1c* and heterozygous for a 4B/4D+4B chromosome pair, involving 4B/4D chromosomes from first-cycle families no. 3 (124 second-cycle F_2 plants) and no. 46 (50 second-cycle F_2 plants), were subjected to RFLP analysis. In both second-cycle F_2 populations, the 4D segment recombined with the 4B segment in all investigated intervals, except for a 0.8-cM interval between *Xmwg2112* and a group of markers completely linked to *Kna1* (*Xwg199*, *Xabc305*, *Xpsr567*, and *Xbcd402*) (Figs. 2 and 3). A total of 11 interstitial 4D segments which included *Kna1* could be unequivocally identified in the second-cycle F_2 populations. In the progeny involving 4B/4D chromosome no. 3, a cross-over was detected within the group of markers that were completely linked to *Kna1* in the first cycle of homoeologous recombination (Fig. 3). The homoeologous cross-over in that plant (plant 3*5–4, Fig. 2) generated the shortest interstitial 4D segment recovered. This cross-over placed *Xpsr375* distal to *Xwg199*, *Xabc305*, *Xpsr567*, and *Xbcd402*. Five other cross-overs terminating in the interval *Xpsr375–XksuH11*, and generating slightly longer interstitial 4D segments involving *Kna1*, were identified.

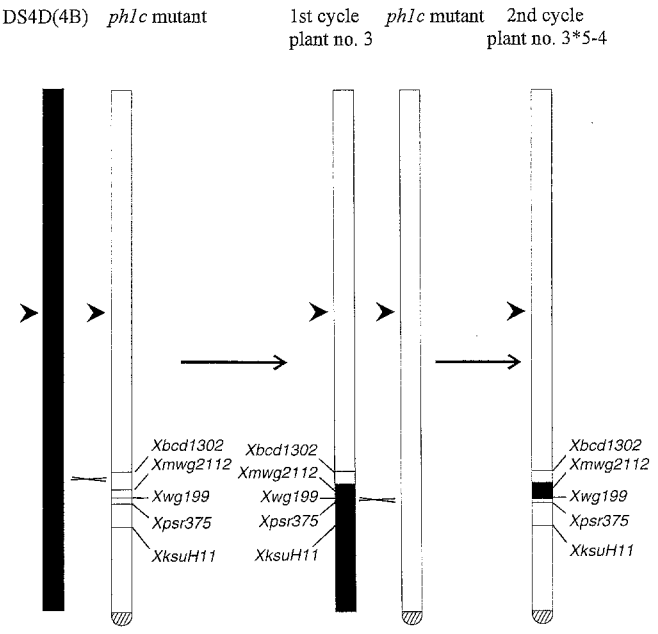

Fig. 2 The origin of an interstitial chromosome segment of chromosome 4D of *T. aestivum* (*solid rectangle*) in chromosome 4B of *T. turgidum* (*open rectangle*) in the second-cycle F_2 plant 3*5–4. The source of each chromosome is indicated above the chromosome. Only relevant molecular markers are indicated in the maps. The sources of the probes used to detect the loci have been described by Dubcovsky et al. (1996). Centromeres are indicated by *arrowheads* and homoeologous cross-overs by *crossed lines* between chromosomes. The *stippled semicircles* represent the terminal C-band of chromosome 4B

The genetic length of interval *Xmwg2112–Xabg601.2*, the latter being the most distal marker thus far mapped on the long arm, was 9.5 cM on a genetic map based on homoeologous recombination between 4B and 4D in the first cycle (Fig. 3). The same interval was 6.4 cM long on the map based on homoeologous recombination between 4B/4D and 4B in the second cycle. The difference between the two values was not statistically significant. Neither were any of the internal intervals on the two maps significantly different (Fig. 3).

Second-cycle F_2 plant 3*5–4 was heterozygous for the interstitial 4D segment. To decide whether *Kna1* is on the interstitial segment, K^+/Na^+ selectivity was determined in five salt-stressed homozygous second-cycle F_4 plants derived from plant 3*5–4 (Table 1). The plants had leaf

Table 1 Ratios of accumulated K^+/Na^+ in the youngest expanded leaves of F_4 homozygous progeny of F_2 plant 3*5–4 and three parental lines: disomic substitution line 4D(4B), Langdon, and the *ph1c* mutant line

Line	No. plants	K^+/Na^+ ratio[a]
DS4D(4B)	6	13.2a
Langdon	9	1.3b
ph1c	6	1.1b
3*5–4	5	10.3a

[a] Values followed by the same letter are not significantly different at the 1% probability level (LSD)

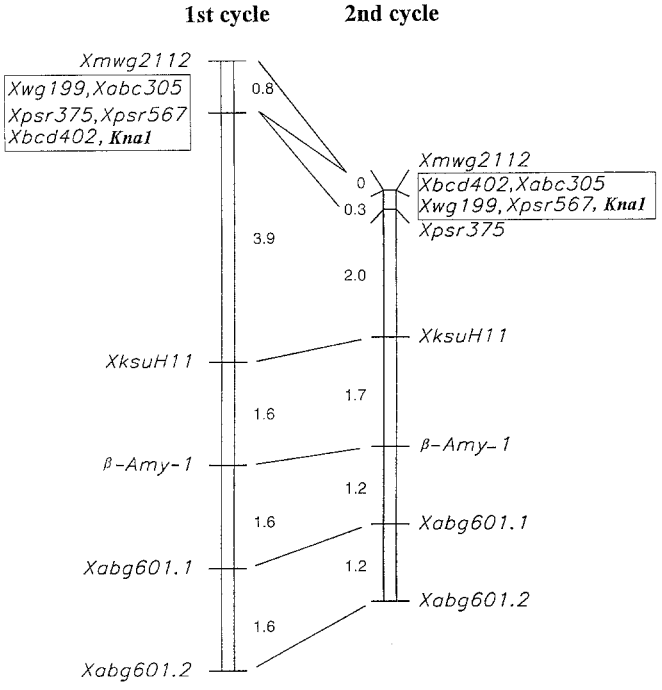

Fig. 3 Comparison of the maps of the distal region of chromosome 4 in the first cycle of homoeologous recombination and the second cycle of homoeologous recombination induced by homozygosity for *ph1c*. The sources of the probes used to detect the indicated loci have been described by Dubcovsky et al. (1996). Markers completely linked to *Kna1* are *boxed*. The lengths of intervals are in cM. All intervals had LOD > 3.0. The first-cycle recombination map is not adjusted for chromosome pairing failure and the interval lengths are, consequently, overestimated (see Discussion)

K^+/Na^+ ratios significantly higher than the parental Langdon and the *ph1c* mutant line (both *kna1*) but similar to DS4D(4B), the source of the *Kna1* allele. The interstitial 4D segment in the 4B/4D chromosome of plant 3*5–4 must, therefore, involve *Kna1* and, hence, *Kna1* must be proximal to *Xpsr375*.

Discussion

Eleven interstitial 4D segments with *Kna1* could be unequivocally identified in the population of 348 chromosomes investigated in the second cycle of homoeologous recombination. The shortest interstitial 4D segment is in plant 3*5–4. This segment is delimited by cross-overs that are distal to *Xbcd1302* and proximal to *Xpsr375* (Fig. 2). The length of this interstitial segment is less than 6.5 cM (the distance between *Xbcd1302* and *Xpsr375*) in terms of homoeologous recombination (Dubcovsky et al. 1996). In terms of homologous recombination, the segment is less than 13 cM long since the relationship between the 4B/4D map, based on homoeologous recombination, and the 4B-4D consensus map, based on homologous recombination, is 1:2 in the *Kna1* region (Dvořák et al. 1995).

A cross-over between *Xpsr375* and markers *Xwg199*, *Xabc305*, *Xpsr567* and *Xbcd402* in plant 3*5–4 facilitates more precise mapping of *Kna1*. Since *Kna1* is proximal to this cross-over and distal to *Xmwg2112* (Dubcovsky et al. 1996), *Kna1* must be within the 1.1-cM *Xmwg2112–Xpsr375* interval (Fig. 3). Using the above 1:2 relationship between homoeologous recombination and homologous recombination in the *Kna1* region, the interval including *Kna1* is approximately 2.2 cM long in terms of the homologous recombination of wheat chromosome 4D.

Lukaszewski (1995) hypothesized that preferential allocation of cross-overs into a homologous segment and positive interference affecting the position of the second chiasma would make it difficult to engineer short interstitial foreign segments by a second cycle of homoeologous recombination. Therefore, he advocated the engineering of interstitial foreign chromosome segments by homologous recombination of chromosomes with overlapping foreign segments (Sears 1981). There was a close agreement in the distances between markers for the maps based on homoeologous recombination in the first and second cycles. Since homoeologous recombination in the first cycle was measured in a male backcross progeny, recombination was overestimated due to selection against nullisomic male gametophytes resulting from the pollen mother cells (PMCs) in which chromosomes 4B and 4D had failed to form a chiasma (Dvořák and Appels 1986). In the first cycle, chromosomes 4B and 4D failed to pair in an average of 57.2% of PMCs (Dvořák and Gorham 1992). The estimate of recombination between 4B and 4D can be adjusted for this chromosome pairing failure by a formula reported by Dvořák and Appels (1986) for recombination-estimate adjustments in the progeny of a male monotelodisomic. No recombination-value adjustment was needed for the second cycle recombination because 4B/4D chromosomes 3 and 46 paired regularly with the 4B chromosome (Dvořák and Gorham 1992). The adjusted length of the *Xmwg2112–Xabg601.2* interval in the first cycle of homoeologous recombination is 5.7 cM which is similar (P = 0.5) to the 6.4 cM observed in the second-cycle of homoeologous recombination. The presence of a proximal homologous segment did not, therefore, reduce recombination within a terminal homoeologous segment in the absence of *Ph1*. Although this result failed to substantiate Lukaszewski's (1995) hypothesis, it is possible that if the positions of the segments were reversed, i.e., the homologous segment was terminal and the homoeologous segment was proximal, the cross-over allocation might conform to Lukaszewski's (1995) hypothesis because the first cross-over tends to be located distally and so may tend to occur in the homologous segment if the segment was distal. That the relative positions of homologous and homoeologous segments might matter is indicated by the observation that, in a chromosome pair composed of a normal wheat chromosome 1A and a recombined chromosome with a terminal segment of wheat chromosome arm 1AS and a proximal segment of *T. monococcum* chromosome arm $1A^mS$, recombination was increased in the terminal homologous

segment compared to recombination between normal 1A chromosomes (Luo et al. 1996).

Engineering interstitial foreign chromosome segments by sequential cycles of homoeologous recombination is clearly a viable alternative to the engineering of such segments by the homologous recombination of chromosomes with overlapping foreign segments (Sears 1981) since the former technique may in some instances be faster than the latter technique and is generally more versatile than it. The latter procedure requires three generations to develop a foreign interstitial segment. The sequential homoeologous recombination technique requires only two generations if the recombined chromosome is identified by RFLP in the F_2 generation (all F_2 progeny are homozygous for *ph1*) and a plant carrying the recombined chromosome is heterozygous for it. If the plant has a pair of recombined chromosomes, or if the chromosome was identified in a backcross generation heterozygous for *Ph1*, three generations are needed, because the plant must be crossed with a *ph1* mutant stock. If gametophytic selection operates against a foreign chromosome, most recombined chromosomes will have a wheat centromere and a distal position of a homoeologous cross-over (Dvořák et al. 1995). This will be particularly true if the male backcross is used in the first cycle of homoeologous recombination since gametophytic selection operates largely in the male. As a result, a search for recombined chromosomes with overlapping foreign segments may fail, as was the case in the present project.

Acknowledgments The authors thank to Emanuel Epstein for advice on cultural conditions and mineral analyses of the plants and gratefully acknowledge technical assistance from Ramsey Zeidan. They also acknowledge financial support for this work from the USDA-NRI program by grant 94–37100–0822 to J. Dvořák. J. Dubcovsky thanks the Argentinian Research Council (CONICET) for a fellowship.

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