Highly Recombinogenic Regions at Seed Storage Protein Loci on Chromosome 1DS of *Aegilops tauschii*, the D-Genome Donor of Wheat

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ABSTRACT

A detailed RFLP map was constructed of the distal end of the short arm of chromosome 1D of *Aegilops tauschii*, the diploid D-genome donor species of hexaploid wheat. *Ae. tauschii* was used to overcome some of the limitations commonly associated with molecular studies of wheat such as low levels of DNA polymorphism. Detection of multiple loci by most RFLP probes suggests that gene duplication events have occurred throughout this chromosomal region. Large DNA fragments isolated from a BAC library of *Ae. tauschii* were used to determine the relationship between physical and genetic distance at seed storage protein loci located at the distal end of chromosome 1DS. Highly recombinogenic regions were identified where the ratio of physical to genetic distance was estimated to be <20 kb/cM. These results are discussed in relation to the genome-wide estimate of the relationship between physical and genetic distance.

SEVERAL agronomically important genes are located in the distal region on the short arm of group 1 chromosomes of wheat (McIntosh *et al.* 1998). Multigene loci encoding proteins such as gliadins (*Gli-1*) and low molecular weight glutenins (*Glu-3*), which are part of the prolamin class of seed storage proteins, are located near the distal ends of chromosome 1AS, 1BS, and 1DS. Segregation analysis of DNA markers and storage proteins established the tight linkage between the *Gli-1* and *Glu-3* loci with interlocus recombination being reported on all three chromosomes of hexaploid wheat (Singh and Shepherd 1988; Jones *et al.* 1990; Dubcovsky *et al.* 1997). Furthermore, intragenic crossover events have also been reported among gliadin gene members on chromosome 1AS (Felix *et al.* 1996; Dubcovsky *et al.* 1997), 1BS (Pogna *et al.* 1995), and 1DS (Metakovsky *et al.* 1986). Disease resistance genes effective against leaf, stripe, and stem rusts have been mapped in close proximity to the prolamin loci within this homoeologous region. For instance, on chromosome 1DS at least two genes that confer resistance to *Puccinia recondita* (*Lr21*, *Lr40*) as well as genes effective against races of *P. graminis* (*Sr45*, *Sr33*) are located within a genetic interval of ~15 cM flanking the seed storage protein loci (Jones *et al.* 1990; Cox *et al.* 1994).

To study this region further we used diploid *Aegilops tauschii*, the D-genome progenitor species of hexaploid wheat, for the detailed molecular analysis of the distal end of chromosome 1DS (Gill *et al.* 1991; Lagudah *et al.* 1991b). Previous studies have shown that the level of DNA polymorphism was generally higher among *Ae. tauschii* accessions than for hexaploid wheat, facilitating the construction of genetic linkage maps. Recombination frequency within a given genetic interval may also increase in the diploid compared to the hexaploid genome, enhancing the resolution of tightly linked markers (Dubcovsky *et al.* 1995). The diploid status of *Ae. tauschii* helped in the construction of a large DNA insert library [bacterial artificial chromosome (BAC)] of the D genome (Moullet *et al.* 1999). This BAC library, combined with genetic linkage maps of *Ae. tauschii*, is being used to investigate the relationship between genetic and physical distances for regions of interest. Because of the considerable variation observed in recombination frequency per unit length of DNA within plant genomes (Puchta and Hohn 1996; Schnable *et al.* 1998), genetic distance may not provide a good estimate of the physical distance between a marker and the gene of interest. To assess the feasibility of using tightly linked markers as starting points to identify candidate genes from this chromosomal region, we isolated large DNA fragments from the BAC library. On the basis of common restriction fragments between genetic markers and BAC clones, we estimated maximum physical distances between genetic loci.

MATERIALS AND METHODS

Plant materials and mapping families: *Ae. tauschii* accessions AUS 18913 and CPI 110856 were crossed to generate an F_2 family of 58 individuals. AUS 18913 was also used as the source of genomic DNA for the construction of the *Ae. tauschii* BAC library (Moullet *et al.* 1999).

RFLP clones: Restriction fragment length polymorphism (RFLP) clones previously mapped to chromosome 1 were

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kindly supplied by A. Graner, Gatersleben (Mwg938); G. Wricke, University of Hannover (Iag95); C. Feuillet, University of Zurich (LrK10, Lrr10, Mwg2245); and V. Mohler, TU Munich (Whs179). A member of the gamma-gliadin gene family was provided by O. Anderson, USDA Albany, to map *Gli-D1* loci. The coding region of the low molecular weight glutenin gene was used to map *Glu-D3* loci (Colot *et al.* 1989).

RGA clones: The majority of plant resistance genes that have been isolated from a wide range of plant species (Staskawicz *et al.* 1995) belong to the class encoding nucleotide binding sites-leucine rich repeat proteins (NBS-LRR; see review in Baker *et al.* 1997). Short peptide sequences within or adjacent to the NBS region are well conserved among gene members of this class and have been used to design primers to amplify resistance gene analogs (RGAs). The clone Rga5.2 was isolated by PCR from one of the *Ae. tauschii* BAC clones, designated A6, using degenerate primers for the kinase-2a and GLPLAL motifs (Collins *et al.* 1998). Rga5.2 clone (350 bp) is 70% identical at the nucleotide level to other previously identified RGA sequences tightly linked to the *Cre3* cereal cyst resistance gene of wheat (Spielmeyer *et al.* 1998). PCR amplification conditions and cloning of PCR products were carried out according to Collins *et al.* (1998). RgaYr10 is a cDNA clone (400 bp) containing the kinase-2, kinase-3, and GLPLAL motifs showing <50% DNA sequence homology to Rga5.2. This cDNA clone was shown to cosegregate with the stripe rust resistance gene *Yr10* located distal to the prolamin genes on chromosome 1BS and is considered a candidate gene for *Yr10* (Frick *et al.* 1998).

Isolation and characterization of BAC clones: High-density filters of BAC clones were screened with RFLP probes according to DNA hybridization and washing conditions as descibed by Lagudah *et al.* (1991b). Plasmid DNA from individual BAC clones was isolated using an alkaline lysis method (Sambrook *et al.* 1989) and DNA insert sizes estimated by pulsed field gel electrophoresis (PFGE) according to Moullet *et al.* (1999). Plasmid DNA that was used for direct sequencing was extracted using the midi prep plasmid purification system (QIAGEN, Hilden, Germany) following the company's amended protocol. Approximately 1 μg of plasmid DNA was used with Big Dye chemistry and automated DNA sequencer 377 (ABI Systems, Columbia, MO) to sequence the ends of large DNA inserts. To identify overlapping regions, BAC clones were digested with restriction enzymes *Hin*dIII and *Cla*I/*Eco*RI, which cut within the polycloning site of the vector but not within the vector itself. Digested DNA was probed with total BAC DNA that had previously been digested with *Hin*dIII (integration site of the DNA insert), phenol/chloroform extracted, and precipitated with ethanol.

DNA isolation and RFLP mapping: Genomic DNA was prepared from leaves (Lagudah *et al.* 1991a) and DNA transfer analysis carried out according to Lagudah *et al.* (1991b). RFLP markers were mapped using progeny from the above mapping families after probes were screened for DNA polymorphism between parental lines that had been digested with a set of restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Nsi*I, and *Xba*I). The marker order and genetic distances were determined using the MAPMAKER program (Lander *et al.* 1987).

RESULTS

Detailed RFLP map of the distal region of chromosome 1DS: To assess the map locations of agronomically important genes in the distal region of chromosome 1DS, a detailed RFLP linkage map was constructed using 58 individuals from an F_2 family of *Ae. tauschii* (AUS 18913 × CPI 110856; Figure 1). The linkage map contains RFLP markers identified by DNA probes derived from three sources: DNA sequences previously assigned to this region (Whs179, Mwg938, Iag95, LrK10, and Mwg2245), RGA probes Rga5.2 and RgaYr10 derived from NBS-LRR sequences, and DNA sequences corresponding to the low molecular weight glutenins (*Glu3*) and gamma-gliadins (*Gli1*; Van Deynze *et al.* 1995).

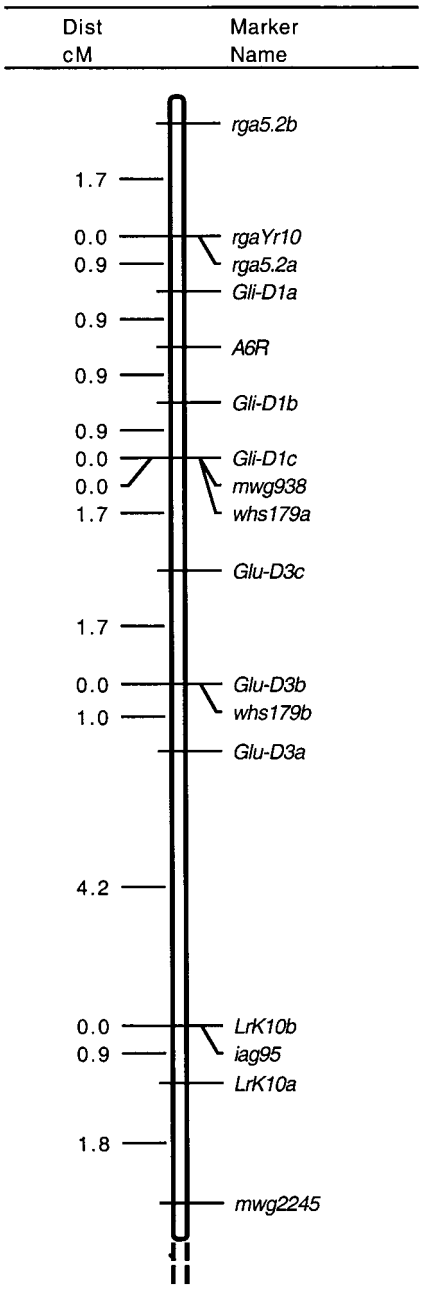

Figure 1.—RFLP map of the distal end of chromosome 1DS. The map was constructed with 58 F_2 individuals derived from the cross between *Ae. tauschii* lines AUS 18913 and CPI 110856. Genetic distance (centimorgans) is indicated on the left.

Five out of nine clones used for RFLP mapping identified multiple loci within the target region (Figure 1).

DNA probes derived from the coding region of the *Glu3* and *Gli1* genes detected approximately eight members of the *Glu-D3* and four members of the *Gli-D1* gene family on digested genomic DNA of parental lines (not shown). A subset of hybridizing bands from both gene families was mapped to six separate but tightly linked loci on chromosome 1DS (Figure 1). The number of Glu3/Gli1 bands detected on membrane filters containing DNA from *Ae. tauschii* was comparable to previous estimates from 1DS in wheat (Payne 1987; Cassidy and Dvorak 1991). However, the frequency of recombination observed between gliadin and glutenin loci was higher in *Ae. tauschii* than previously reported in hexaploid wheat (Singh and Shepherd 1988; Jones *et al.* 1990; Dubcovsky *et al.* 1997). Recombination events were also observed in *Ae. tauschii* between members within the gliadin and glutenin gene families. Previous studies in tetraploid and hexaploid wheat also identified recombination events between individual members of gliadin genes (Metakovsky *et al.* 1986; Pogna *et al.* 1995; Felix *et al.* 1996; Dubcovsky *et al.* 1997).

Two RGA clones, Rga5.2 and RgaYr10, mapped distal to the gliadins, also detecting multiple loci (Figure 1). These clones were not expected to cross-hybridize because both sequences contained insufficient DNA homology (<50%). Hybridization of Rga5.2 to digested genomic DNA of *Ae. tauschii* identified three major bands that mapped to two loci within the target interval (Figure 1). RgaYr10 also detected three major bands, one of which mapped to the *rgaYr10* position cosegregating with *rga5.2a*. The other two bands were monomorphic between parental lines and could not be mapped. The results suggest that gene duplication events have occurred within the distal end of chromosome 1D, generating multiple loci in the prolamins and adjoining regions.

Relationship between physical and genetic distance on the distal end of 1DS: Five DNA clones (KsuD14, Whs179, LrK10, Lrr10, and Iag95) that mapped to the distal end of chromosome 1DS were used to screen the BAC library. At least one BAC clone was isolated for each of the RFLP markers that were mapped using these probes. As one of the parental lines (AUS 18913) used in constructing the mapping family was also used as the source of genomic DNA to construct the BAC library, clones were assigned to genetic loci on the basis of common restriction fragments identified between BACs and RFLP markers in the genomic DNA of AUS 18913 (Figure 2). PFGE analysis revealed that the insert size of BAC clones ranged from ~65 to 125 kb. Three clones (D8, I3, and A6) were used to estimate the ratio of physical to genetic distance within the prolamin region (Figure 3). BAC clone D8 was ~110 kb in size and contained RFLP markers *LrK10b* and *whs179b*, which mapped over 5 cM apart (Figure 2). Clone D8 also contained at least two members of the low molecular weight glutenin gene family. One of the two *Glu-D3*

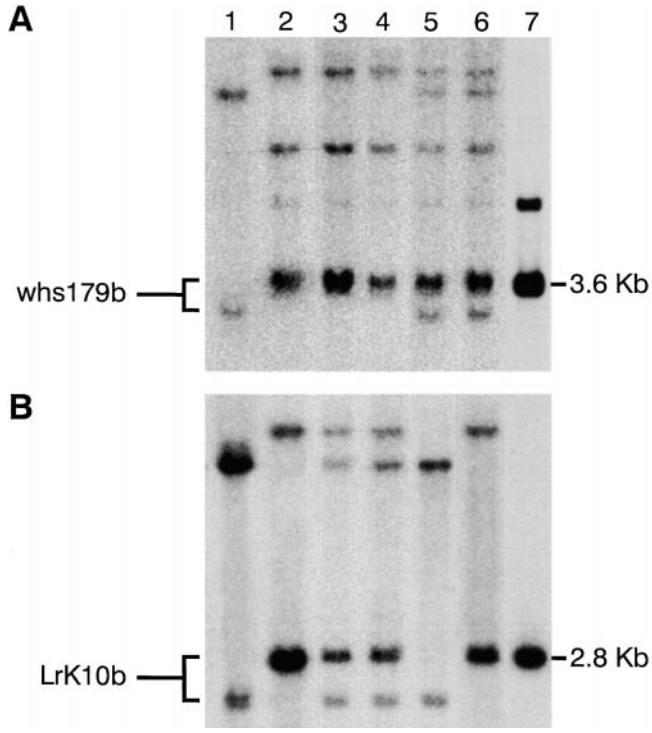

Figure 2.—BAC clone D8 spans a genetic distance of ~5 cM. (A) Genomic DNA digested with *Eco*RI restriction enzyme and probed with Whs179. Lane 1, parental line CPI 110856; lane 2, parental line AUS 18913; lanes 3–6, critical recombinant F_2 lines; lane 7, BAC clone D8. Codominant marker bands of *whs179b* are indicated (fork). (B) Genomic DNA digested with *Dra*I restriction enzyme and probed with LrK10. Codominant marker bands of *LrK10b* are indicated (fork). The critical recombinant F_2 lines contain four recombination events that occurred between *whs179b* and *LrK10b* in a family of 58 individuals. The genotypes of lines 3–6 probed with Whs179 were parent 2, parent 2, heterozygote, heterozygote. The genotypes of the same lines probed with LrK10 were heterozygote, heterozygote, parent 1, parent 2. The corresponding RFLP bands of *whs179b* and *LrK10b* were present within BAC D8 (110 kb), demonstrating that the physical DNA sequence of D8 is spanning ~5 cM of genetic distance.

bands contained within D8 recombined with *LrK10b* and *whs179b*, placing this *Glu-D3* marker (*Glu-D3a*) within the 5-cM interval (Figure 3). Two other BAC clones, I3 and A6, were similar in size to D8 and contained RFLP markers that were separated by recombination. One *Gli-D1* marker (*Gli-D1a*) was present in A6 together with the proximal member of the *rga5.2* gene family, which was separated by ~1 cM (equivalent to one recombination event). The DNA clone A6R derived from the BAC end sequence of the A6 insert also recombined with *Gli-D1a*, identifying a separate locus (see below). The BAC clone I3 contained one band that hybridized to the Whs179 probe and mapped to *whs179a*. BAC clone I3 also contained at least one glutenin marker that mapped to *Glu-D3c*; in two individuals *Glu-D3c* recombined with *whs179a* within a maximum physical interval of ~100 kb. For the chromosomal re-

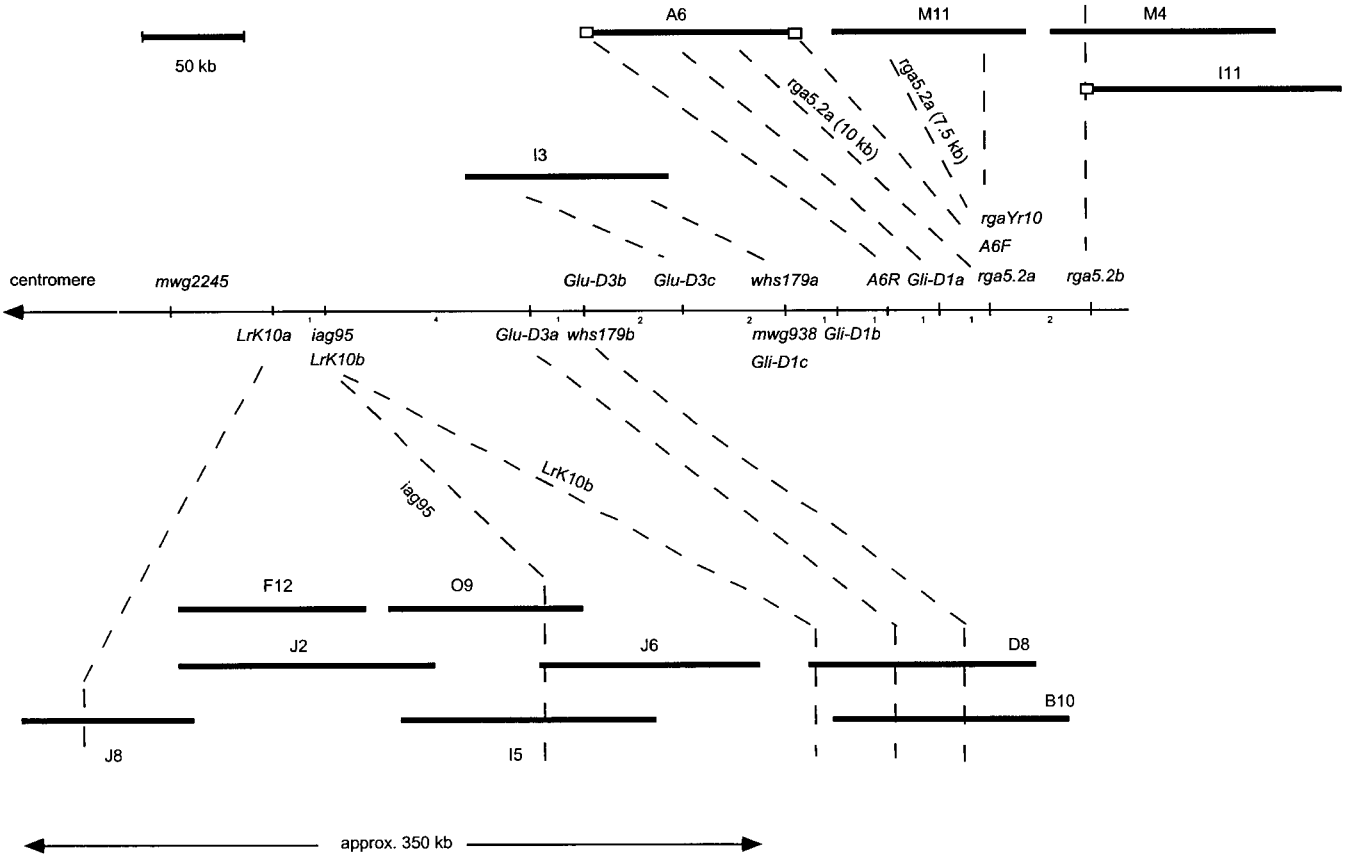

Figure 3.—Genetic and physical map of the end of chromosome 1DS. Genetic loci were assigned to BAC clones on the basis of common restriction fragments. Overlapping regions between BAC clones were identified by using BAC insert end sequences in PCR and DNA hybridization assays and DNA fingerprinting of clones by probing total BAC DNA to digested BAC clones. The numbers along the genetic map designate the crossover events that occurred within a given interval. Open squares at the end of BAC clones A6 and I11 designate that the low-copy end sequences were mapped genetically.

gions spanned by BAC clones D8 and I3/A6, we estimate the ratio of physical to genetic distance to be <20 and 50kb/cM, respectively.

The relationship of physical and genetic distances was also studied in the chromosomal region flanking the seed storage protein genes at the distal end of 1DS. To test the possibility of BAC clones overlapping and forming a contiguous sequence, ~1 kb was directly sequenced from the insert ends of BAC clones A6, I11, M4, and M11; these represent the clones containing loci that were mapped distal to the gliadins. Specific primer sequences were used to amplify fragments of ~400 to 500 bp in size. To identify additional DNA markers and orient BAC clones, these end sequences were screened for repetitive DNA using database searches and hybridization to genomic DNA. Three out of eight BAC insert ends were shown to contain low copy sequences, which were used as probes in RFLP mapping. One of these low copy ends from BAC A6 was mapped to a new genetic locus *A6R* within the gliadin gene region (Figure 3). The other low copy end from A6 (*A6F*) cosegregated with existing RFLP marker *rga5.2a*. The third end sequence was derived from I11 and contained the Rga5.2 gene member, which mapped to *rga5.2b*.

BAC clones were screened for overlapping regions by using specific primers in PCR assays and probes derived from end sequences in DNA hybridization. Using this approach, no overlapping regions were identified between BAC clones A6, M11, and I11/M4, although A6 and M11 contained RFLP markers that cosegregated (Figure 3). Given the size of the mapping family used in this study, cosegregating markers may be separated by genetic distances of up to 2.5 cM (95% confidence interval; Hanson 1959). Two Rga5.2 hybridizing bands, ~10 and 7.5 kb in size, mapped to the same locus, *rga5.2a*, but were located on separate BAC clones A6 and M11 (Figure 3). BAC clone M11 also contained a member of the RgaYr10 gene family, which cosegregated with *rga5.2a*. Likewise, cosegregating markers at other map locations that did not reside on the same BAC clone were identified: I3 contained *whs179a*, but not the markers *Gli-D1c* and *mwg938*; D8 contained *whs179b* and *LrK10b*, but not the markers *Glu-D3b* and *iag95*, respectively. The information can be used to determine the physical order of cosegregating markers

at three loci: *Glu-D3c-whs179a-mwg938 / Gli-D1c*; *Glu-D3a-whs179b-Glu-D3b*; and *LrK10a-iag95-LrK10b-Glu-D3a*. Tightly linked markers located on separate BAC clones may indicate that the corresponding physical / genetic distance was greater than what was observed for regions covered by BAC clones D8, I3, and A6. To investigate this possibility we used BAC clones to build a contiguous DNA sequence in the proximal region flanked by loci *mwg2245* and *LrK10b*. Inserts were screened for potential overlapping regions by probing digested BAC clones with total BAC DNA (see materials and methods and Figure 4). A contig was constructed spanning the genetic interval *LrK10a-iag95*, for which the minimum physical distance of 170 kb (proximal end of J2 to proximal end of J6) and a maximum distance of 270 kb were estimated (proximal end of J8 to distal end of O9; see Figure 3). For the same interval, calculations for the ratio of physical to genetic distance ranged from 56 to 270 kb / cM, which were based on a genetic distance of 1–3 cM (95% confidence interval; Hanson 1959). These values, when compared to previous estimates of 20–50 kb / cM for the regions corresponding to BAC clones D8, I3, and A6, showed that the highest ratios for clones I3 and A6 corresponded to the lowest estimate calculated for the interval between *LrK10a* and *iag95*.

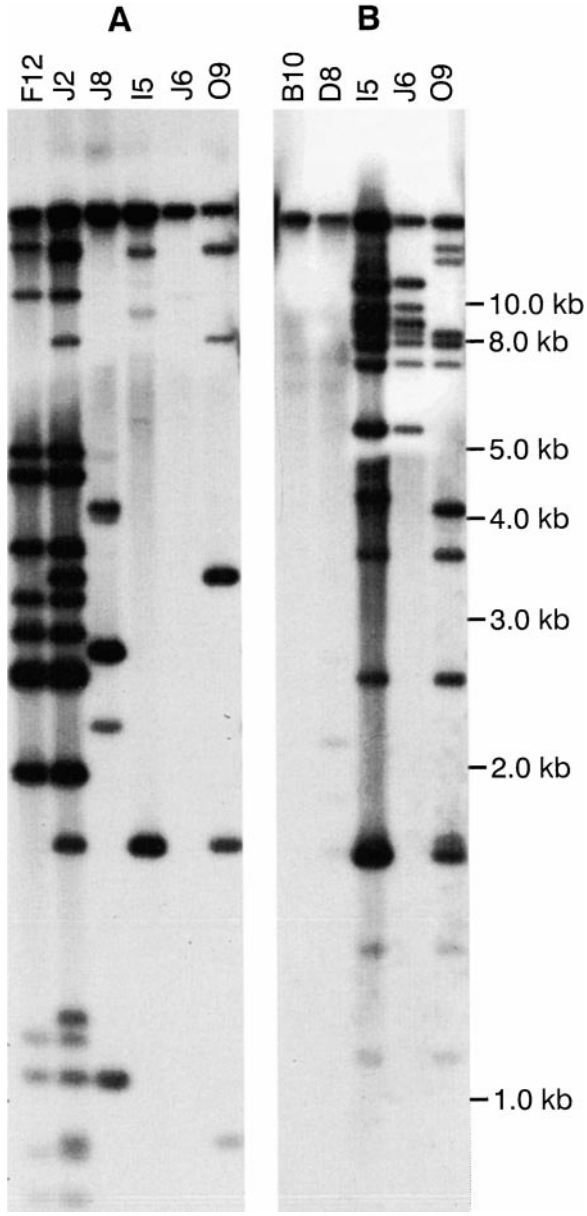

Figure 4.—DNA fingerprinting of BAC clones isolated from the genetic interval *LrK10a-iag95 / LrK10b*. BAC clones were digested with *Hin*dIII (integration site of DNA insert into the vector) and probed with (A) total BAC DNA of J2 and (B) total BAC DNA of I5. On the basis of common restriction fragments, BAC clones J8, F12, J2, O9, I5, and J6 constitute a physical contig of ~350 kb.

DISCUSSION

To develop a detailed genetic linkage map of the end of the short arm of chromosome 1D, we used the diploid species *Ae. tauschii* as a model system. Because of the lower ploidy level and less complex DNA hybridization patterns, a greater number of codominant markers with a higher degree of confidence were mapped in *Ae. tauschii* than would have been possible to map in wheat. Gene duplication events have probably generated multiple loci detected by five DNA probes mapped within this region. A family size of 58 F_2 individuals was sufficient to identify recombinants between most of the mapped RFLP markers. A higher recombination frequency was observed between and within *Glu-D3* and *Gli-D1* loci in *Ae. tauschii* as compared to previous studies in the corresponding D genome of hexaploid wheat. Variation was reported at seed storage protein loci among *Ae. tauschii* accessions from diverse geographical origins (Lagudah and Halloran 1988). Most of the 79 accessions examined were distinguishable on the basis of unique gliadin (*Gli-D1*) haplotypes. Such high levels of diagnostic haplotypes occurring in *Ae. tauschii* could be accounted for by the relatively high recombination frequency among the *Gli-D1* loci observed in our study.

To obtain an estimate for the relationship between physical and genetic distance at the end of chromosome 1DS, large DNA fragments were isolated from a BAC library of *Ae. tauschii* with RFLP probes previously mapped to the region. Three BAC clones were informative, containing members of the *Gli-D1* and *Glu-D3* gene families that recombined with other RFLP markers in an F_2 family of 58 individuals, providing estimates of 20–50 kb / cM for the physical / genetic distances. Within a chromosomal region located proximal to the seed storage protein loci (*LrK10a-iag95*), the relationship between the physical and genetic distances was estimated to range between 56 and 270 kb / cM, indicating that the ratio for this region may be significantly greater than for the *Gli-D1* and *Glu-D3* region.

This apparently high level of recombination in the

TABLE 1

The relationship of physical and genetic distance in different plant genomes

	Genome average (kb/cM)	Specific intragenic/gene region (kb/cM)	Reference
Arabidopsis	150	30–50 Chromosome 4 hot spots	Schmidt *et al.* (1995)
Tomato	200	43 I_2 resistance gene	Segal *et al.* (1992)
Rice	260	61 *Xa5* resistance gene	Yang *et al.* (1998)
Maize	1500	14 *Bronze* gene	Dooner (1986)
		217 *a1* gene	Civardi *et al.* (1994)
Barley	3000	25 *Mlo* gene	Büschges *et al.* (1997)
Ae. tauschii	3000	20 Prolamin gene region	This article

Glu-D3/*Gli-D1* region contrasts with the other prolamin members at the *Glu-1* locus that encode high molecular weight glutenin polypeptides. The *Glu-1* genes are among the most extensively studied loci in hexaploid wheat, and to date, no genetic recombination has been confirmed between the two tightly linked *Glu-D1x* and *Glu-D1y* gene members located on chromosome 1DL. Similarly the *Ae. tauschii* mapping family used in this study was previously shown to lack any recombinants between these *Glu-D1* gene members physically located on the same BAC clone of ~117 kb (Moullet *et al.* 1999). The divergent physical/genetic distances among the prolamin storage proteins reveal a consistent pattern: The relatively high recombination frequency and unique haplotypes occurring among *Gli-D1* gene members contrast with the apparent absence or relatively low recombination frequency at *Glu-D1* and a corresponding lower number of haplotypes reported among the same set of *Ae. tauschii* accessions (Lagudah and Halloran 1988). Similarly, in hexaploid wheat >90% of genotypes in a worldwide survey carried either of two *Glu-D1* haplotypes (Payne and Lawrence 1983).

These estimates of physical distances per unit of recombination from the distal 1DS region vary by one to two orders of magnitude from the genome-wide estimate of 3000 kb/cM for wheat (Bennett and Smith 1991). Detailed studies in barley, a monocot genome of similar size to *Ae. tauschii*, have shown that the rate of recombination per unit length of DNA within the *Mlo* resistance gene was similar to that obtained for the *Glu-D3*/*Gli-D1* region of *Ae. tauschii* (Büschges *et al.* 1997). In fact, in the majority of studies the relationship between physical and genetic distance revealed a remarkable similarity within genes or gene-rich regions across a wide range of plant species (Table 1). The difference between the average recombination frequency across the entire genome and the intra- or intergenic recombination frequency has largely been attributed to the nonrandom distribution of genes, which are recombinationally active, embedded within larger regions of short repeat structures that are not involved in recombination. The integration of genetic loci with a physical map of chromosome 1 using single-break deletion lines of wheat showed that 86% of markers, most of which were cDNA clones, were present in five major clusters comprising ~10% of the chromosome (Gill *et al.* 1996). This finding and results presented here are consistent with the hypothesis that genes and gene-rich regions are themselves targets for recombination (Civardi *et al.* 1994). The variation in physical/genetic distances observed in this study may indicate that certain regions within the wheat genome are amenable to positional cloning strategies.

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