Map-Based Cloning of Leaf Rust Resistance Gene *Lr21* From the Large and Polyploid Genome of Bread Wheat

Li Huang,* Steven A. Brooks,* Wanlong Li,* John P. Fellers,† Harold N. Trick* and Bikram S. Gill*,[1]

**Wheat Genetics Resource Center, Department of Plant Pathology, Kansas State University, Manhattan, Kansas 66506-5502 and* †*USDA-ARS, Plant Science and Entomology Unit, Kansas State University, Manhattan, Kansas 66506-5502*

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ABSTRACT

We report the map-based cloning of the leaf rust resistance gene *Lr21*, previously mapped to a gene-rich region at the distal end of chromosome arm 1DS of bread wheat (*Triticum aestivum* L.). Molecular cloning of *Lr21* was facilitated by diploid/polyploid shuttle mapping strategy. Cloning of *Lr21* was confirmed by genetic transformation and by a stably inherited resistance phenotype in transgenic plants. *Lr21* spans 4318 bp and encodes a 1080-amino-acid protein containing a conserved nucleotide-binding site (NBS) domain, 13 imperfect leucine-rich repeats (LRRs), and a unique 151-amino-acid sequence missing from known NBS-LRR proteins at the N terminus. Fine-structure genetic analysis at the *Lr21* locus detected a noncrossover (recombination without exchange of flanking markers) within a 1415-bp region resulting from either a gene conversion tract of at least 191 bp or a double crossover. The successful map-based cloning approach as demonstrated here now opens the door for cloning of many crop-specific agronomic traits located in the gene-rich regions of bread wheat.

MAP-BASED cloning and functional genetic studies in model plant systems have become easier with the availability of whole-genome sequences and provide fundamental knowledge for understanding plant growth and environmental response. However, most agriculturally important genes, including those governing host resistance to different pathogens, are crop specific. In crops with large polyploid genomes (many crop plants are polyploid), cloning such genes and deploying them for crop improvement presents special challenges. Bread wheat (*Triticum aestivum* L., $2n = 6x = 42$, genome formula AABBDD), the most widely grown cereal crop occupying 17% of all cultivated land worldwide and a staple for 35% of the world's population providing 20% of calories consumed (http://www.CIMMYT.org/), is hexaploid and has a genome size of 16 billion bp (Arumuganathan and Earle 1991). An average ratio of 4.4 Mb/cM of physical/genetic distance (Faris and Gill 2002) presents an almost impossible task for map-based cloning of genes in wheat. However, cytogenetic and molecular mapping in the early 1990s demonstrated that the wheat genome consists of gene-rich and gene-poor compartments with recombination restricted mainly to gene-rich regions (Werner *et al.* 1992; Gill *et al.* 1996). Furthermore, most agronomic genes are located in gene-rich regions and it was hypothesized that they therefore should be amenable to map-based cloning in spite of the large genome size of bread wheat (Feuillet and Keller 1999). Conversely, traits mapped in gene-poor regions are not amenable to map-based cloning (Qi and Gill 2001).

As with all the crop systems, wheat production has been challenged constantly by diseases and pests, among which the rust diseases (leaf, stem, and stripe rust) are the most prominent. Leaf rust (causal agent *Puccinia triticina*) is the most widely distributed disease of wheat, causing an average annual yield loss of 3% worldwide (equivalent to US $2 billion; National Agricultural Statistics Service, http://www.usda.gov/nass). Host resistance is the most effective and economical method of disease control, and supplementing conventional breeding with direct gene transfer by molecular methods promises to enhance the efficiency of plant breeding.

Many disease resistance (R) genes have been cloned from diverse plant species (Hulbert *et al.* 2001). Although their protein products share several conserved domains (Dangl and Jones 2001), R genes are very diverse at the DNA level and colinearity across the grass lineages is often difficult to discern, possibly owing to their fast evolution (Leister *et al.* 1998). To date, efforts to isolate R genes from wheat on the basis of domain conservation among species or by using model plants with small genomes such as rice as surrogates in posi-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AF532105 (cosmid 69-7-1, contig 5.3), AF257240 (cosmid 69-7-1, contig 5.4, including *Lr21* gene), AY139586 (WI-*lr21*), and AY139587 (00-174-6 and 01-377 recombined *Lr21* gene).

[1]*Corresponding author:* Department of Plant Pathology, 4024 Throckmorton, Kansas State University, Manhattan, KS 66506-5502. E-mail: bsg@ksu.edu

TABLE 1

Plant materials used in molecular analysis of *Lr21*

	Pedigree/description
Cultivar	
Wichita (WI)	Hard red winter wheat cultivar
Thatcher (TC)	Spring wheat cultivar
Fielder	Spring wheat cultivar
Ae. tauschii accession	
TA1599	Collected from Iran, *Lr21* (TC) donor
TA1649	Collected from Iran, *Lr21* (WI) donor
Germplasm	
Lr21 (WI)	TA1649/3* WI (WGRC2) WI//TA1649/2*WI (WGRC7)
Lr21 (TC)	TC6*//Tetra Canthatch/TA1599
Recombinant	
97-87-43	WGRC2/WI, homozygous at *Xksud14* locus and heterozygous at *Lr21* locus
00-174-6	A homozygous-susceptible progeny of 97-87-43
00-174-23	A homozygous-resistant progeny of 97-87-43
01-377	A homozygous-susceptible progeny of 97-87-43

tional cloning have been unsuccessful (Kilian *et al.* 1997).

Two major strategies are used to clone genes encoding unknown products: map-based cloning and T-DNA/transposon-tagging. In wheat, the transposon-tagging strategy is not feasible due to the lack of an active transposon system. T-DNA tagging is impeded by the high proportion of noncoding DNA (80% repeated sequences; Smith and Flavell 1975) and by the low efficiency of Agrobacterium infection. In contrast, map-based cloning is a universal strategy to clone genes that have been finely mapped. However, gene cloning based on fine genetic and physical mapping must deal with a large genome size and a high ratio of physical to genetic distance resulting from low levels of recombination or polymorphism in wheat. These obstacles necessitate a crop-specific strategy for cloning genes of interest in wheat.

The D genome of *Aegilops tauschii Coss.* was the last integrated into polyploid wheat by chance hybridization with durum wheat ~8000 years ago (Kihara 1944; McFadden and Sears 1946; Feldman *et al.* 1995; Figure 1A). It recombines most readily with the D genome of wheat and has high levels of polymorphism (Kam-Morgan *et al.* 1989). The map-based cloning of a gene introgressed from *Ae. tauschii* offers the best chance of success as the D genome, at about one-fourth the size of the wheat genome, is the smallest among the three genome donors of hexaploid wheat.

Lr21 was first incorporated into wheat cultivar Thatcher from *Ae. tauschii* accession TA1599 via a synthetic wheat (Rowland and Kerber 1974) and has been available for breeding since the 1970s. To date, no virulent isolates have been reported for *Lr21*. Thus, *Lr21* is a potentially durable and highly effective leaf rust resistance gene in wheat. Later, an *Lr21* allele (previously designated as *Lr40*) was introgressed from a different accession (TA1649) into the wheat cultivar Wichita (WI) to develop the leaf-rust-resistant germplasm lines WGRC2 and WGRC7 (Raupp *et al.* 1983; Huang and Gill 2001; Figure 1A).

Our strategy focused on the *Lr21* genetic locus of the two introgression wheat lines WGRC2 and WGRC7. Introgression of the gene also introduced a high degree of polymorphism in the region flanking the gene and expedited mapping of markers at the genetic locus of interest. The target gene was accessed by a "shuttle genetics" strategy in which the genetic analysis was done in the recipient crop while the large-insert library was developed from the diploid donor. Here we report on the strategic map-based cloning of *Lr21* from the large genome of bread wheat, its successful transformation, and its stable expression in transgenic plants through three generations.

MATERIALS AND METHODS

The information and pedigrees of the plant materials used are listed in Table 1. The wheat cultivars WI and Fielder are susceptible to leaf rust. The mapping population consisted of 330 F_2 plants (165 each from WGRC2/WI and WGRC7/WI crosses) and 190 F_3 plants derived from 6 heterozygous F_2 plants (from the cross of WGRC7/WI). *Ae. tauschii* accession TA1649 was the donor accession of the *Lr21* allele in WGRC2 and WGRC7 and was used to construct a cosmid library for this study. The pedigree number of an F_2 plant from the cross WGRC2/WI is 97-87-43.

Plant disease inoculation and scoring: All F_2 and $F_{2:3}$ plants were inoculated with *P. triticina* culture PRTUS6 (avirulence/virulence *Lr* gene phenotype: 2a, 3ka, 9, 16, 18, 24, 26, 30/1, 2c, 3a, 10, 11, 17). Inoculations were conducted as described by Browder (1971). WGRC2 and WGRC7 were used as resistant checks and Wichita was the susceptible check. Transgenic plants were inoculated 25 days after they were transferred to soil. Fielder, at the same growth stage as the transgenic plants, was used as the susceptible control. Disease severity was assessed 8–10 days after inoculation for seedling plants and 12–15 days after inoculation for adult plants using the scale of Roelfs (1985).

Isolation of 3′- and 5′-ends of cDNA (KSU936 and KSU937): Total RNA was extracted from the leaf tissue of plants containing *Lr21* (in Thatcher background) using TRIzol reagent (Invitrogen, Carlsbad, CA). We isolated mRNA with a poly(A)-Tract mRNA isolation system (Promega, Madison, WI). Gene-specific primers GSP1 (5′-GCCTCGAGCTTCCTTCAACTTC TTATCTAGAGCCCC-3′), GSP2 (5′-GCCTCGAGCACATGA ATGCACATGATGGTGTCG-3′), and GSP3 (5′-GAAGCAGC TGGAGCTCTGGGTGCCG-3′) were designed on the basis of the KSUD14 sequence (accession no. AF257240). KSU936 and KSU937 were the 3′- and 5′-ends of cDNA isolated using primers GSP1 and GSP2 (for KSU937) and GSP3 (for KSU936) and a Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA) following the manufacturer's protocol.

RT-PCR: Total RNA and mRNA were extracted from WGRC7 using the same procedure as described above. First-strand cDNA was synthesized using the gene-specific primer B7 (5′-AGGTGGGACTAAAACCAGCC-3′). Second-strand DNA was amplified from KSUD14 primers (Huang and Gill 2001). The PCR products were cloned in a TA vector and sequenced.

Cosmid library construction and library screening: A library was constructed in the vector pHC79. Genomic DNA of the accession TA1649 was extracted as described previously (Huang and Gill 2001) and partially digested with *Eco*RI. The ligation conditions were 1:1.5 of insert:vector in 20 μl of reaction including 4 μl 5× buffer and 1 μl T4 ligase (Invitrogen) at 12° water for 8 hr. Ligated cosmids were packaged using MaxPlax Lambda packaging extract (Epicentre, Madison, WI) following the manufacturer's recommendations.

Bacteria containing cosmids were plated in a density of ~2500 colonies per plate (132 mm diameter) at 37° overnight. Petri dishes were precooled at 4° for 30 min before transferring to membranes. Colonies were transferred onto nitrocellulose membrane discs (Osmonics, Minnetonka, MN). Membranes were placed in a denaturation buffer for 5 min followed by a neutralization buffer for 3 min and a vigorous wash in 2× SSC for 15 min. Membranes were UV crosslinked after air drying.

The cosmid library was represented by 130 membranes containing $\sim 3.2 \times 10^5$ colonies. The KSUD14 probe was amplified from TA1649 genomic DNA using KSUD14 primers. The PCR products were electrophoresed twice in a 1% agarose gel (prepared with 0.5× TAE). The 1.36-kb fragment was excised from the gel, purified, and used as a probe.

Sequence annotation and open reading frame identification: Cosmid DNA preparation, subcloning, and sequencing were performed according to Brooks *et al.* (2002). Two different gene-prediction programs were used to annotate the sequences. GENSCAN 1.0 (http://genes.mit.edu/GENSCAN.html) was used to predict coding sequence (CDS) with maize, with smat as the parameter matrix. In addition, FGENSH 1.1 (http://www.softberry.com) was used for gene prediction (with monocot genomic DNA parameters). Predicted polypeptide sequences were defined by results of BLASTp searches against the National Center for Biotechnology Information nonredundant (Altshul *et al.* 1997) database (http://www.ncbi.nlm.nih.gov/BLAST/). Orientation of contig sequences was achieved by alignment to cosmid end sequences and identification of subclones spanning two gaps.

Southern hybridization, sequence-tagged site (STS) assays, and genetic mapping: A total of 20 μg of genomic DNA or 1 μg of cosmid DNA were used to make blots. Enzyme digestion, gel electrophoresis, Southern blotting, probe labeling, and hybridization were conducted as previously described (Huang and Gill 2001). All probes were prepared by PCR and purified by spin-column chromatography. The mapping data were analyzed with MAPMAKER V2.0 (Lander *et al.* 1987).

The KSUD14-STS assay, modified from the previous one of Huang and Gill (2001), used a 25-μl reaction mixture including 100- to 350-ng template DNA, 1 pmol of each primer, 1 unit Taq DNA polymerase (Bioline, Randoph, MA), 1.25 μl of 50 mm $MgCl_2$, 2.5 μl of a 10× buffer, and 2 μl of 2.5 mm dNTPs. PCR was performed in a Perkin-Elmer (Foster City, CA) PCR System 9700: 5 min at 94°, followed by 30 cycles each of 1 min at 94°, 1 min at 55°, and 2 min at 72°. The last cycle was for 5 min at 72°. PCR products were analyzed in 1% agarose gels at 50 V constant voltage.

Genetic transformation: The entire cosmid clone 69-7-1 and pHAC20 (containing the herbicide resistance gene *bar*) were cobombarded into the variety Fielder using a particle inflow gun (Finer *et al.* 1992). Immature embryos were isolated 10–14 days postanthesis and cultured as described by Altpeter *et al.* (1996). Sixteen hours after transformation, the calli were placed on a selection medium (5 mg/liter glufosinate) for 10 days and a shoot-production medium for 2 weeks and then transferred to an elongation and rooting medium for 2 weeks. Once roots formed, the plants were transferred to soil. Recovered plants were initially screened for the presence of the *bar* gene by applying a freshly prepared aqueous solution of 0.2% Liberty (AgEvo USA, Pikeville, NC) to the midlamina portion (~2.5 cm long) of the second or third youngest leaf. The painted area was marked and damage observations were recorded 5–7 days after application.

RESULTS

Fine mapping of the *Lr21* region: Previously, three restriction fragment length polymorphism (RFLP) markers were mapped near *Lr21* in the distal region of the chromosome 1D short arm (Huang and Gill 2001). *Xksud14* cosegregated with *Lr21* in all but 1 of the 520 F_2 plants at a 0.1 cM genetic distance from *Lr21* (Figure 1B). Three new markers (KSU936, KSU937, and KSU027BE590674) revealed polymorphism and were mapped in the population. KSU936 and KSU937 are 3′- and 5′-ends of cDNA isolated via rapid amplification of cDNA ends using KSUD14 specific primers (see materials and methods). *Xksu936* and *Xksu937* cosegregated with *Xksud14* in the population. KSU027BE590674 is an expressed sequence tag (EST) coding for a low-molecular-weight glutenin protein in wheat and mapped to a position 1.6 cM proximal to *Lr21*. KSUD14 is the closest marker to *Lr21*.

Isolation of cosmid clones harboring the fragment tightly linked to *Lr21*: A cosmid library was constructed from *Ae. tauschii* accession TA1649. Screening of the cosmid library by colony hybridization using KSUD14 as a probe identified 20 positive clones from 130 plates

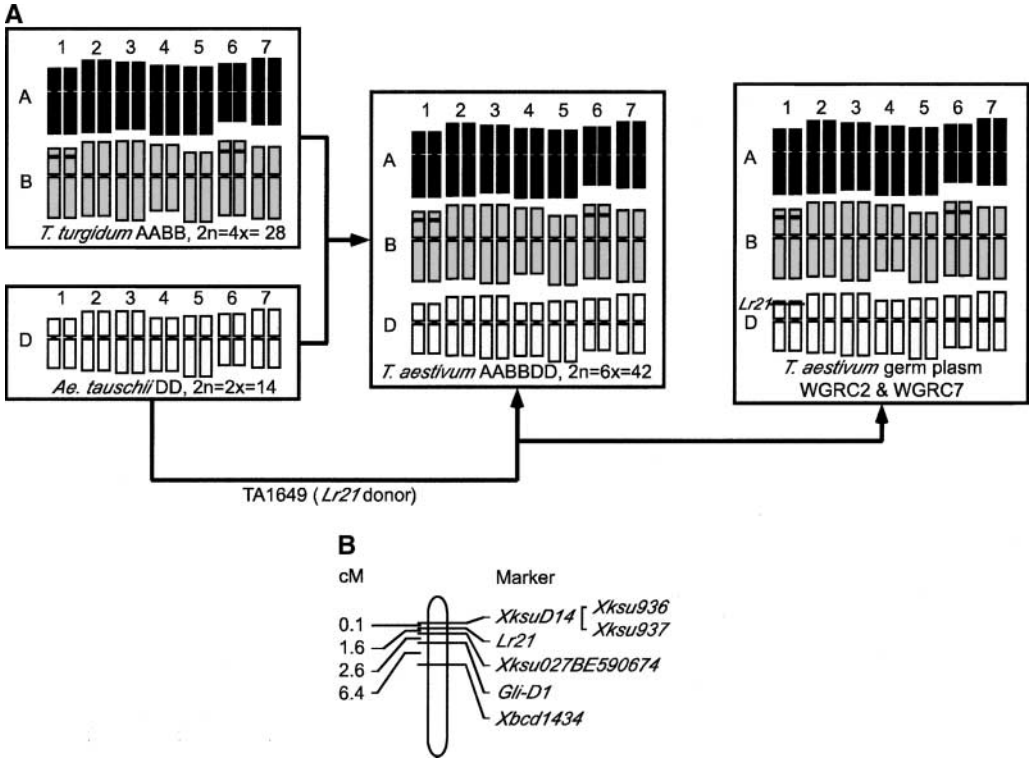

FIGURE 1.—Transfer and fine mapping of the *Lr21* gene. (A) Bread wheat (*T. aestivum*) has a narrow genetic base as it traces its origin to 8000 years ago from a rare hybrid between *T. turgidum* and *Ae. tauschii* and only one or a few gametes were sampled. In the described research, genetic variation in the D-genome diploid donor *Ae. tauschii* was exploited to introgress leaf rust resistance gene *Lr21* and polymorphism into the bread wheat genome and was strategically used to clone *Lr21*. (B) Genetic map of *Lr21* gene region in chromosome 1DS was constructed from 330 F_2 plants and 190 $F_{2:3}$ plants from the crosses of WGRC7/WI and WGRC2/WI.

($\sim 3.2 \times 10^5$ colonies, $\sim 3.2 \times$ genome coverage). Second-cycle screening via a KSUD14-STS assay and Southern hybridization using a KSUD14 probe confirmed four positive clones (32-2, 69-7-1, 75-2-1, and 75-8-6) containing different-sized fragments homologous to KSUD14. Three different size fragments—2 kb from clone 32-2, 1.36 kb from clone 69-7-1, 1.2 kb from clone 75-2-1, and 1.2 kb + 1.36 kb from clone 75-8-6—were amplified by PCR with the KSUD14 primers (HUANG and GILL 2001). These three fragments represent the three copies of KSUD14 in *Ae. tauschii* accession TA1649 (Figure 2A). Only the 1.36-kb fragment is present in WGRC2 and WGRC7 (Figure 2B). All four cosmid clones were digested with the restriction enzyme *Hin*dIII, separated by gel electrophoresis, blotted, and hybridized with the KSUD14 probe. Even though the clones 69-7-1, 75-2-1, and 75-8-6 have different KSUD14-STS patterns, they had the same size Southern hybridization band as TA1649 (Figure 2C). However, clones 69-7-1 and 75-2-1 had strong signals after a 10-min exposure, whereas clone 75-8-6 did not have a detectable signal until after a 6-hr exposure (Figure 2C), indicating that 75-8-6 has a low sequence similarity to KSUD14. On the basis of both KSUD14-STS and RFLP results, cosmid clone 69-7-1 is the clone that contains the fragment tightly linked to *Lr21*.

Characterization of cosmid clone 69-7-1: Sequencing of the cosmid clone 69-7-1 revealed a 43-kb insert that contains seven CDSs. A BLAST search revealed that only one of the CDSs homologous to KSUD14 resembled a nucleotide-binding site (NBS)-leucine-rich repeat (LRR)-type resistance gene with similarity to the ESTs of a putative nematode resistance gene in wheat (SEAH *et al.* 2000) and the rust resistance gene *Rp1-D* in maize (COLLINS *et al.* 1999). All of the other CDSs either are retrotransposon-like elements or have poor database alignment to the proteins of unknown function. On the basis of sequence analysis, the CDS homologous to KSUD14 in cosmid clone 69-7-1 is a candidate clone for *Lr21*.

Gene-complementation study: Cosmid clone 69-7-1 was used directly for a gene-complementation study. Fielder, a wheat variety susceptible to leaf rust culture PRTUS6, was cotransformed with cosmid clone 69-7-1 and pAHC20, a plasmid conferring resistance to glufosinate, the active ingredient in the herbicide Liberty. Five putative transgenic plants were obtained and inoculated with rust culture PRTUS6. All tillers of one transgenic plant (plant 1410) were resistant to the culture with a very low infection type (necrosis and a few small pustules; Figure 3A), which was similar to the reaction of WGRC2 and WGRC7. The remaining transgenic plants (1298, 1332, 1344, and 1440) and the nontransgenic control Fielder had high infection types (3+ to 4 on a scale of 0–4). All four tillers of 1410 and one tiller of each susceptible plant were assayed for the *Lr21* candidate. The cosmid clone 69-7-1 contains a 1.36-kb fragment of KSUD14-STS, and Fielder contains 1.45-kb and 1.7-kb fragments but lacks the 1.36-kb fragment. The KSUD14-STS assay showed that only the transgenic plant

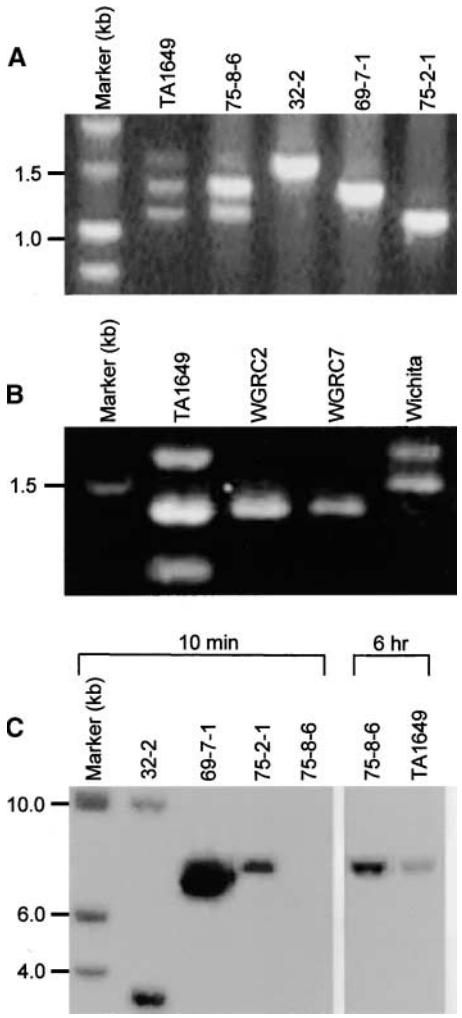

FIGURE 2.—Identification of cosmid clones harboring the fragment tightly linked to *Lr21*. (A) Gel electrophoresis pattern revealed by sequence-tagged site of KSUD14 shows that cosmid clones 32-2, 69-7-1, 75-2-1, and 75-8-6 represent three different copies of KSUD14-STS in TA1649. (B) Only one of three fragments in TA1649 is present in both WGRC2 and WGRC7. (C) RFLP pattern of TA1649 and four cosmid clones 32-2, 69-7-1, 75-2-1, and 75-8-6 revealed with KSUD14 and restriction enzyme *Hin*dIII. Cosmid clones 69-7-1 and 75-2-1 showed the same size of hybridization fragment as TA1649 after a 10-min exposure. Cosmid clone 75-8-6 did not show a hybridization signal until after a 6-hr exposure. These data indicate that only 69-7-1 contains the sequence completely homologous to KSUD14.

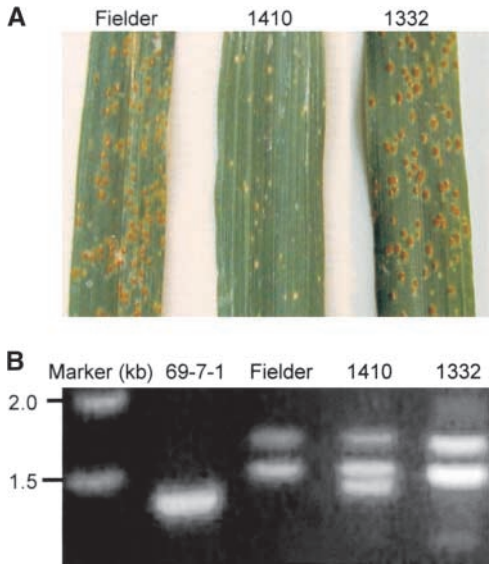

FIGURE 3.—Infection types and molecular analysis of transgenic plants. (A) Infection types of transgenic plants 1332 and 1410 compared to susceptible control Fielder 14 days after inoculation with rust culture PRTUS6. (B) Gel electrophoresis pattern of sequence tagged site of KSUD14 shows that transgenic plant 1410 amplified the same KSUD14-STS fragment as cosmid 69-7-1. Fielder and susceptible transgenic plant 1332 did not amplify the 1.36-kb KSUD14 fragment.

1410 amplified the 1.36-kb fragment (Figure 3B), indicating the presence of the cosmid clone 69-7-1 containing KSUD14 homologous CDS. None of the susceptible plants amplified the diagnostic fragment (Figure 3B). Eighteen T_1 progenies from the resistant plant 1410 were tested again with pathogen culture PRTUS6 and the molecular marker KSUD14-STS. Fifteen plants were resistant to the pathogen and showed a 1.36-kb KSUD14-STS fragment. Three plants were susceptible to the pathogen and lacked the 1.36-kb fragment (Figure 4). The genetic data indicated that the transgene for leaf rust resistance expressed stably and was inherited as a single locus. Furthermore, the pathogenic specificity of the transgenic plant 1410 was confirmed to be the same as WGRC2 and WGRC7 through multiple inoculations on homozygous T_2 progenies (third generation) of the plant 1410 with different pathogen cultures (L. HUANG and B. S. GILL, unpublished data).

We also used a subclone of the cosmid clone 69-7-1 containing only the entire CDS homologous to KSUD14 and its native promoter and terminator for a gene-complementation study. Two resistant tillers were identified from two transgenic plants. The T_1 progeny test from the two tillers confirmed that the subcloned transgene was also inherited as a single gene conferring resistance to leaf rust pathogen (data not shown).

Molecular characterization of *Lr21*: On the basis of the genetic-complementation studies, we confirmed that the KSUD14 homologous resistance gene candidate in the cosmid clone 69-7-1 is *Lr21*, which confers resistance to culture PRTUS6. *Lr21* spans 4359 bp of genomic DNA, including a 41-bp predicted promoter. Sequence comparison between the full-length cDNA and genomic DNA of the *Lr21* gene indicated that the transcribed portion of *Lr21* is 4318 bp, including a 316-bp 5′-untranslated region (UTR), a 312-bp 3′-UTR, a 3243-bp CDS, and two introns of 367 and 80 bp (Figure 5). The 3243-bp CDS of the gene encodes a 1080-amino-acid protein containing a unique 151-amino-acid sequence (from position 105 to 255) missing from known NBS-LRR proteins at the N-terminal region, a conserved

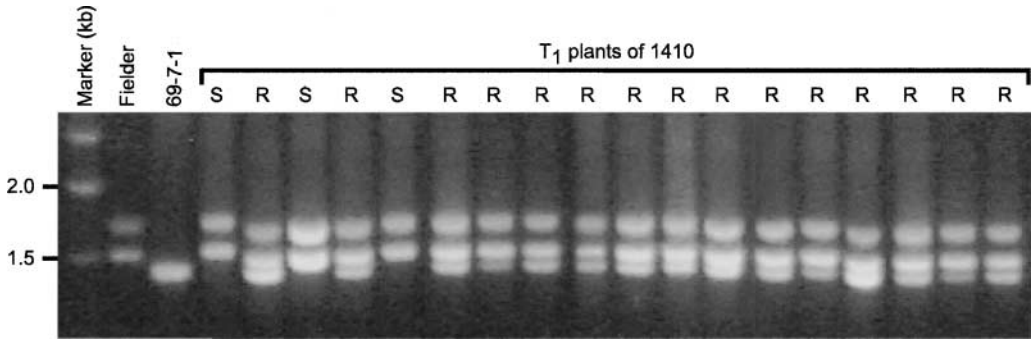

FIGURE 4.—Molecular analysis of T_1 transgenic plants. A total of 18 T_1 progenies from leaf-rust-resistant plant 1410 were tested for the pathogen cultures PRTUS6 and assayed with molecular marker KSUD14-STS. Fifteen plants were resistant to the pathogen and amplified a 1.36-kb fragment. Three plants were susceptible to the pathogen and lacked the diagnostic band.

NBS, and 13 imperfect LRRs. No other conserved domain was detected. Therefore, *Lr21* belongs to the NBS-LRR class of resistance genes.

Recombinations in *Lr21*: Plant 97-87-43 was a recombinant between the *Lr21* and *Xksud14* loci. As confirmed by RFLP and STS marker analysis, this individual was heterozygous at the *Lr21* locus but homozygous at the *Xksud14* locus. To test for intragenic recombination or gene conversion, the recombinant plant was self-fertilized to identify homozygous-resistant and homozygous-susceptible plants. Using two pairs of primers covering a region from −252 to +3935 of the *Lr21* sequence, we amplified fragments from the two parents (WI and WGRC2), two homozygous-susceptible progenies, and one homozygous-resistant progeny of the recombinant. The amplified fragments using the two pairs of primers mentioned above are expected to overlap by 664 bp from +1002 to +1666 within the region of KSUD14 (from +993 to + 2353). Sequencing the amplified fragments from WI and WGRC2 revealed three insertion/deletion (InDel) and seven single nucleotide polymorphisms (SNP). The first InDel is at −62 and is an eight-nucleotide deletion in WI or an insertion in WGRC2. The second InDel is at +761 and is a two-nucleotide deletion in WI or an insertion in WGRC2. The third InDel is at +1355 and is a 105-bp deletion in WGRC2 or an insertion in WI (Figure 6). In addition, seven SNPs were detected at positions +653, +713, +844, +1690, +1863, +1865, and +2176 between WI and WGRC2.

After fine mapping in the *Lr21* gene region using the three InDels and the seven SNPs, it was determined that two recombination events arose by a noncrossover involving either a gene conversion with tract length of at least 191 bp (from +653 to +844; Figure 6) or a double crossover spanning the promoter and first exon of the gene (from −61 to +1354). All three progenies of the recombinant plant 97-87-43 have the same sequence as WGRC2 in the regions of −252 to +652 and +845 to +3935 and lack the 105-bp insertion present in the *lr21* allele of WI. The sequence of recombined allele in plant 97-87-43 differs from WGRC2 (*Lr21*) and WI (*lr21*) in the region of +653 to +844. The second InDel at +761 in 97-87-43 is in the gene-coding region, causing a frameshift. The recombined gene can encode only a 151-amino-acid truncated protein, indicating that the deletion is the cause of susceptibility in the homozygous-susceptible progenies of 97-87-43. The analysis of recombinant allele in 97-87-43 provided further evidence that cosmid clone 69-7-1 contains *Lr21* and that molecular marker KSUD14 is a part of the resistance gene.

DISCUSSION

Strategic map-based cloning: Because of bread wheat's large genome size and extremely high mega-base-pair: centimorgan ratio of 4.4, map-based cloning of genes in bread wheat has been considered an almost impossible task. Furthermore, genetic redundancy and polyploid inheritance are thought to pose formidable obstacles. However, the vast array of cytogenetic stocks in wheat allow targeted mapping of specific chromosome regions (SEARS 1954; ENDO and GILL 1996) and genes (FARIS and GILL 2002; FARIS *et al.* 2003). Targeted mapping has shown that the wheat genome consists of gene-

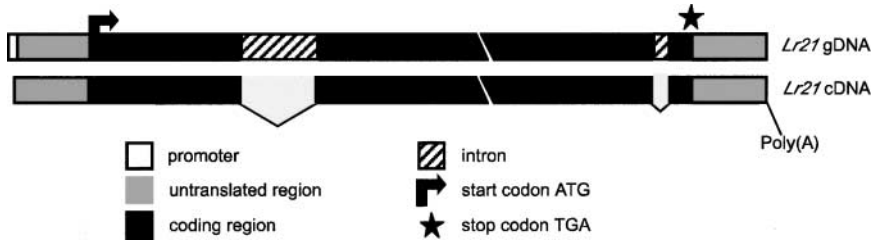

FIGURE 5.—The *Lr21* gene structure at the genomic DNA level (top) and cDNA level (bottom).

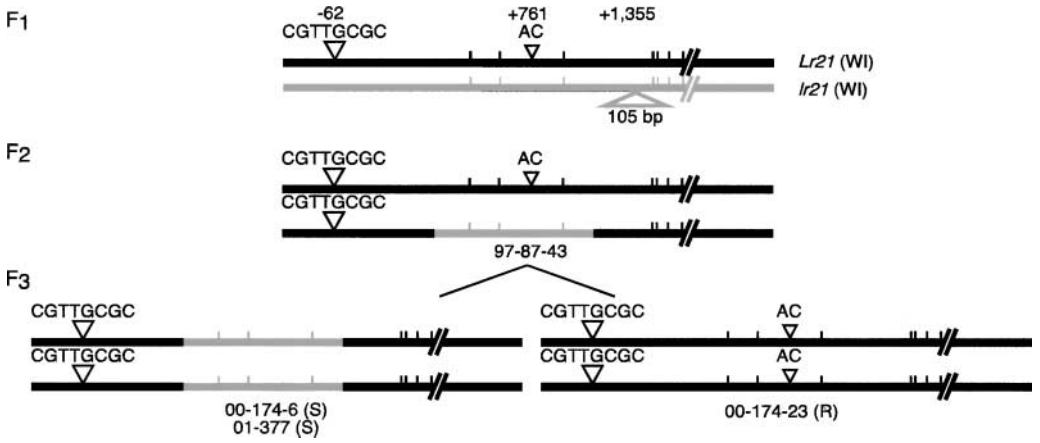

FIGURE 6.—The fine mapping of the gene region. The structure of the resistant [*Lr21*(WI)] and susceptible [*lr21*(WI)] alleles at the *Lr21* locus and the identification of two recombination events within the *Lr21* locus leading to susceptible reaction in F_3 progeny plants 00-174-6 and 01-377. The short vertical lines indicate SNPs.

rich and gene-poor regions. Recombination is restricted mainly to gene-rich regions and disease resistance genes are located mainly in gene-rich, highly recombinogenic regions (WERNER *et al.* 1992; GILL *et al.* 1996; BOYKO *et al.* 2002). Previously, *Lr21* was mapped to a gene-rich region at the distal of 1DS arm (GILL *et al.* 1996). The isolation of *Lr21* has demonstrated that genes in gene-rich, highly recombinogenic regions can be readily cloned in spite of the large genome of wheat.

The molecular cloning of *Lr21* benefited from shuttle genetic mapping between bread wheat and its D-genome diploid donor species *Ae. tauschii* (Figure 1A; KAM-MORGAN *et al.* 1989; GILL *et al.* 1991; STEIN *et al.* 2000). Introgression of *Lr21* from *Ae. tauschii* into wheat not only introduced polymorphism that flanks the gene region but also separated the other KSUD14 homologies from the *Lr21* copy (Figure 2B). We used a mapping population of only 520 F_2 plants in bread wheat to tag *Lr21* with molecular markers. We constructed and screened a cosmid library from *Ae. tauschii* TA1649 instead of common wheat to isolate the clones of interest. Favorable attributes of *Ae. tauschii*, such as high polymorphism and a relatively small genome size, can be exploited to clone genes from the large polyploid wheat genome using a map-based strategy.

Genetic transformation of large-insert constructs: The genetic complementation of cloned genes via either *Agrobacterium tumefaciens*-mediated (COLAU *et al.* 1987; GRAVES and GOLDMAN 1987) or biolistic bombardment using a particle inflow gun (FINER *et al.* 1992) is an important step for both confirming a cloned gene and further experimental manipulation of the transgene in basic and applied studies. Use of large-insert constructs has two advantages over cDNA constructs. First, large-insert genomic constructs provide genes with native regulatory elements, thereby avoiding overexpression leading to silencing of the transgenes caused by non-native promoters. Second, such constructs may provide genes with a favorable local genomic environment allowing gene expression similar to that in a native system. In general, large-insert constructs are transferred through Agrobacterium-mediated transformation to maintain the integrity of the construct. Binary-BAC (HAMILTON 1997) or binary-cosmid vectors (OLSZEWSKI *et al.* 1988) were designed for such a process and have been successfully used in Arabidopsis (BENT *et al.* 1994; GRANT *et al.* 1995) and potato (BENDAHMANE *et al.* 1999). However, Agrobacterium-mediated transformation has been difficult in cereal species because of the very low efficiency of Agrobacterium infection, most probably owing to the nonhost reaction. Recently, particle bombardment was reported to deliver large DNA fragments into the epidermal cells of barley for transient expression to confirm the cloning of *Mla1* (ZHOU *et al.* 2001) and *Mla6* (HALTERMAN *et al.* 2001). The successful genetic transformation with a large DNA clone and stably expressed resistant phenotype in the transgenic plants through three generations as documented in this study is a promising approach for use in map-based cloning as well as in deploying cloned genes in agriculture.

Gene structure of *Lr21*: Five groups of *R* genes have been identified (DANGL and JONES, 2001; HULBERT *et al.* 2001). The largest group of such genes encodes proteins with NBS and LRR domains, and the function of such proteins, so far, is associated exclusively with plant disease resistance. The NBS-LRR group can be divided further into two subgroups on the basis of whether or not the N-terminal region has homology to the Drosophila Toll protein and mammalian interleukin-1 receptor (TIR domain). The TIR-NBS-LRR subgroup of *R* genes has not been identified in cereals; instead, a non-TIR subgroup of NBS-LRR genes is common, most of which carry a coiled-coil structure or a leucine zipper (ELLIS *et al.* 2000; DANGL and JONES 2001; HULBERT *et al.* 2001). In the Triticeae, three disease resistance genes, *mlo* (BÜSCHGES *et al.* 1997), *Mla* (HALTERMAN *et al.* 2001; ZHOU *et al.* 2001), and *Rpg1* (BRUEGGEMAN *et al.* 2002), have been cloned from barley, a diploid species diverged from wheat ~12 million years ago (HUANG *et al.* 2002). As far as we are aware, *Lr21* is the first disease resistance gene cloned from wheat and belongs to the NBS-LRR

group. A 151-amino-acid sequence (from position 105 to 255) is unique in the N-terminal region of this gene, which is missing from known NBS-LRR proteins in the database. Further research will show whether this 151-amino-acid sequence has any special function.

Apart from location in a gene-rich region, we chose *Lr21* for molecular cloning because of the extensive allelic diversity at this locus in natural populations of *Ae. tauschii* (Huang and Gill 2001). Previously, we hypothesized that this allelic diversity may be due to the compound structure of the *Lr21* locus similar to the *Rp1* locus of maize in which a tandem array of genes may create new specificities through unequal crossing over and other mechanisms (Hulbert *et al.* 2001). We were surprised at the discovery of a single structural copy at the *Lr21* locus as determined by Southern analysis and sequencing of the 43-kb cosmid clone and also confirmed by large-scale allelism experiments (A. Rosa, personal communication). Using the cloned gene, we can now study molecular structure and the evolution of allelic diversity at the *Lr21* locus. Our working hypothesis is that this analysis may reveal new *Lr21* alleles with different specificities, which then may be genetically engineered for durable host resistance.

Recombination at the *Lr21* locus: On the basis of how the Holliday junction is resolved, recombination may result in either reciprocal crossovers (COs; exchange of flanking markers) or unilateral noncrossovers (NCOs; no exchange of flanking markers) (Yao *et al.* 2002) resulting from gene conversion or double crossovers (DCOs). NCOs can be easily distinguished from COs by analysis of the flanking markers. Gene conversion can be detected in yeast using tetrad analysis and in Drosophila by half-tetrad analysis (Curtis *et al.* 1989). For higher eukaryotes, tetrad or half-tetrad analysis is not available. Alternatively, linkage disequilibrium pattern analysis was used in Arabidopsis (Wiehe *et al.* 2000) and in humans (Haubold *et al.* 2002). In our study, the fine genetic analysis indicated that recombination detected at the *Lr21* locus is a NCO (Figure 6) resulting from either a gene conversion or a DCO. At present it is difficult to distinguish between these two possibilities. We believe that the NCO is possibly a gene conversion because the chance of a DCO within a 1415-bp interval should be very low on the basis of the general concept of crossover interference. The conversion tract at the *Lr21* locus is at a minimum 191 bp and no longer than 1415 bp. The average conversion tract length is 352 bp in Drosophila (Hilliker *et al.* 1994), 300 bp in Arabidopsis (Haubold *et al.* 2002), 590–789 bp in maize (Xu *et al.* 1995), and 30 bp at the human leukocyte antigen locus (Parham *et al.* 1995; Wiehe *et al.* 2000).

In yeast, recombination (or conversion) is polarized and initiated at the ends of genes (Fogel *et al.* 1981; Nicolas *et al.* 1989; White *et al.* 1991). At the *Lr21* locus, two recombination events were detected at the 5′-end of the gene and initiated near the transcription start site. Similar observations were reported in maize at the *a1* (Xu *et al.* 1995) and the *b1* loci (Patterson *et al.* 1995). Recombination hotspots in these two genes are located at the 5′-ends of the coding region near the 5′ transcription start sites. However, nonpolarized recombination was also reported in maize at the *bz* locus where meiotic recombination hotspots were distributed uniformly (Dooner and Martinez-Ferez 1997). These observations suggest that different patterns of recombination hotspots are associated with different genes in plants.

Patterns of recombination also are affected by DNA sequence homology and composition. High sequence homology promotes recombination as demonstrated in the *a1-sh2* interval of maize, in which recombination hotspots are always found in regions of high sequence identity although not all high homology regions are hotspots (Yao *et al.* 2002). Heterologies were found to reduce recombination in yeast (Borts and Haber 1987) and maize (Dooner and Martinez-Ferez 1997). At the *Lr21* locus, between the *Lr21* and *lr21* alleles recombination was most probably initiated within the region of perfect homology, an interval of 1415 bp; the migration of Holliday structures then was hindered by the sequence heterologies, namely the 8-bp and the 105-bp InDels on either side of this interval. NCOs detected in the *bz* gene of maize were found mainly in the regions flanked by two large hemizygous insertions (Dooner and Martinez-Ferez 1997). In maize, the frequency of NCOs varied from 0.007 to 0.03% in the *bz* gene region (Dooner and Martinez-Ferez 1997) to 0.4% in the *a1-sh2* interval of maize (Yao *et al.* 2002). In our study, 1 of 165 F_2 individuals from one cross was a NCO (0.6%) and a similar frequency of NCOs was observed in the *Q* gene region of wheat (Faris *et al.* 2003).

Concluding remarks: Molecular cloning of *Lr21* demonstrates that map-based cloning is a viable strategy for accessing genes from the large polyploid genome of bread wheat. Cloning of *Lr21* will facilitate studies on gene organization, evolution, and the host-pathogen interaction to elucidate the molecular mechanism of resistance in a polyploid wheat model. The molecular cloning strategy reported here, combined with fine physical mapping and soon-to-be-developed mutant resources, will greatly accelerate gene discovery in wheat. The genetic engineering of cloned genes will provide novel avenues for wheat genome manipulation and improvement to enhance world food security.

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