

Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome

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More than 50 leaf rust resistance (*Lr*) genes against the fungal pathogen *Puccinia triticina* have been identified in the wheat gene pool, and a large number of them have been extensively used in breeding. Of the 50 *Lr* genes, all are known only from their phenotype and/or map position except for *Lr21*, which was cloned recently. For many years, the problems of molecular work in the large (1.6×10^{10} bp), highly repetitive (80%), and hexaploid bread wheat (*Triticum aestivum* L.) genome have hampered map-based cloning. Here, we report the isolation of the *Lr* gene *Lr10* from hexaploid wheat by using a combination of subgenome map-based cloning and haplotype studies in the genus *Triticum*. *Lr10* is a single-copy gene on chromosome 1AS. It encodes a CC-NBS-LRR type of protein with an N-terminal domain, which is under diversifying selection. When overexpressed in transgenic wheat plants, *Lr10* confers enhanced resistance to leaf rust. *Lr10* has similarities to *RPM1* in *Arabidopsis thaliana* and to resistance gene analogs in rice and barley, but is not closely related to other wheat *Lr* genes based on Southern analysis. We conclude that map-based cloning of genes of agronomic importance in hexaploid wheat is now feasible, opening perspectives for molecular bread wheat improvement through transgenic strategies and diagnostic allele detection.

During the past decade, a number of disease resistance genes have been isolated from model plant species such as *Arabidopsis thaliana* and rice or from diploid crop plants such as tomato and barley (for recent reviews see refs. 1 and 2). In many cases, gene isolation was performed through map-based cloning. This process requires the development of high-density genetic maps and the possibility to perform chromosome walking on large genomic fragments. Until recently, such positional cloning has been limited to small genomes, and it has remained very difficult in large (>5,000 Mb) and repetitive (>80%) genomes such as those of barley and wheat. *Mlo*, the first barley disease resistance gene isolated by map-based cloning (3), was identified through the use of a yeast artificial chromosome (YAC) library and the subsequent construction of a bacterial artificial chromosome (BAC) library from the YAC clone spanning the resistance locus. More recently, the construction of a BAC library from the barley cultivar Morex (4) has greatly facilitated the map-based isolation of the powdery mildew resistance genes *Mla1* and *Mla6* (5, 6) as well as the stem rust resistance gene *Rpg1* (7). In wheat, BAC libraries have been constructed from the diploid species *Triticum monococcum* (8) and *Ae. tauschii* (9), whose genomes are related to the A and D genome of hexaploid wheat, respectively. The *T. monococcum* DV92 library has been used to isolate *VRN1*, a gene controlling vernalization response in *T. monococcum* (10), as well as a candidate gene for the *Q* gene, which confers free-threshing character to domesticated wheat (11). Very recently, Huang *et al.* (12) have isolated the *Lr21* resistance gene introgressed on chromosome 1DS by using a cosmid library from the *Aegilops tauschii* *Lr21* donor line.

Many genes of agronomic interest, including >50 leaf rust (*Lr*) disease resistance genes, have been characterized by genetic analysis in hexaploid wheat. The *Lr10* gene originates from hexaploid wheat and is located on chromosome 1AS (13). In the

absence of BAC or cosmid libraries from a wheat variety containing *Lr10*, cloning of the complete *Lr10* resistance locus was not possible from hexaploid wheat. Therefore, we have developed a subgenome chromosome walking strategy in which genetic mapping was performed in a hexaploid wheat population segregating for the *Lr10* resistance, and chromosome walking was performed by using BAC clones from the diploid *T. monococcum* DV92 (14). In one step of chromosome walking, a *T. monococcum* DV92 physical contig of 280 kb spanning the *Lr10* locus in hexaploid bread wheat was established. Sequencing of 211 kb from this contig revealed the presence of two resistance gene analogs (RGAs), *rga1* and *rga2*, in a region that showed complete linkage to *Lr10* in *Triticum aestivum* (14, 15).

Here, two genes (*T10rga1* and *T10rga2-1A*) orthologous to the *T. monococcum* genes *rga1* and *rga2* were isolated from the *Lr10* donor line Thatcher*Lr10*. Haplotype studies in the wheat gene pool showed that these two genes are the best possible candidates for *Lr10*. Mutational analysis and stable transformation of the candidate genes demonstrated that *T10rga1* is the *Lr10* resistance gene. Our data show that map-based cloning of genes of agronomic interest is feasible from hexaploid wheat by using a combination of subgenome chromosome walking and haplotype studies.

Materials and Methods

Mutant Screening. Three thousand seeds of the parental line Thatcher*Lr10* were treated with 0.35% ethyl methanesulfonate (EMS). M2 seedlings (52,000) were then artificially infected with the leaf rust (*Puccinia triticina*) isolate, (*AvrLr10*) 89–201 CBT-B(TX), (13) by using a large-scale infection procedure modified from Schachermayr *et al.* (16). Thirty-three putative mutants were grown to the next generation and were reassessed for susceptibility by artificial infection. Five susceptible fertile mutants were crossed with the susceptible cv. Frisal. Three of the mutants (EMS_19, EMS_25, and EMS_31) resulted in susceptible F1 progeny, suggesting that the mutation is in the *Lr10* gene.

PCR Amplification of the *T10rga1* and *T10rga2-1A* Genes and RT-PCR. Three overlapping fragments spanning the *T10rga1* gene were amplified by PCR on 50 ng of genomic DNA extracted from the

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Abbreviations: BAC, bacterial artificial chromosome; RGA, resistance gene analog; EMS, ethyl methanesulfonate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY270157–AY270159).

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three EMS mutants and from the hexaploid wheat line Canadian3842 by using the following primer combinations: ThLr10.T (5'-CTGAGTGAGCATGAGCAAC-3') and ThLr10.P (5'-TGGAATTGAGACAGTACAC-3'); ThLr10.E (5'-AGC CTAATATGGCAACC-3') and ThLr10.H (5'-TGTAGAACC CTGCGCTTAC-3'); and ThLr10.G (5'-GCTCTTCTA ACGGGGATC-3') and ThLr10.J (5'-CATCTCTTGAA AGCTCC-3'). Four overlapping fragments spanning *Tl0rga2-1A* were amplified by PCR using the specific primer combinations: Rga2.F (5'-GATGGAGACGACGGTGCT-3') and Rga2.U (5'-CAACTGCTTGTGATCTGGT-3'); Rga2.V (5'-GAAGCCGGATTATAGTGCA-3') and Rga2.W (5'-CTGCCAGCTAAGTTCTTG-3'); Rga2.X (5'-CAATTGT GATGAACTCCTCA-3') and Rga2.N (5'-AGGTGACA GATAGATTCAC-3'); and Rga2.K (5'-CTTCTGCGAG TGCTGGAC-3') and Rga2.E (5'-TTCATAGCTCATTG CATC-3'). A second PCR amplification was performed for the regions where point mutations were identified to confirm that they did not result from *Taq* polymerase errors. RT-PCRs were performed on 3 μ g of poly(A)⁺ RNA by using the primer combinations ThLr10.D (5'-GTCAAGATCCCGTATCAG-3')/ThLr10.H or ThLr10.G/ThLr10.H for *Tl0rga1* and the specific primers Rga2.V/Rga2.W for *Tl0rga2-1A*.

Biolistic Transformation of the Susceptible Wheat Bobwhite 5 98 56.

A 4.4-kb genomic fragment containing the entire *Tl0rga1* coding region, 21-bp upstream sequence and 380-bp downstream sequence, was cloned under the control of the maize ubiquitin promoter by using the strategy described by Clausen *et al.* (17) to generate the plasmid *pUbi-Tl0rga1*. *pUbi-Tl0rga2-1A*, which contains a 3-kb *Tl0rga2-1A* full-length cDNA with 65 bp upstream and 203 bp downstream of the coding sequence was generated by using a similar strategy. A total of 350 immature embryos of the Bobwhite accession SH 98 56 (18) were cotransformed with *pUbi-Tl0rga1*, *pUbi-Tl0rga2-1A*, and a plasmid containing the selectable phosphomannose isomerase marker (19) by using the PDS-1000/He biolistic particle delivery System (Bio-Rad). Regeneration and selection of the transformed plants were performed as described (18, 19). Eight independent T0 transgenic lines containing either the two transgenes or only one of the transgenes were obtained. T1 transgenic plants were artificially infected with the leaf rust isolate TCB/TD. The number of *Tl0rga1* transgene integration events in the T1 transgenic plants was analyzed by Southern hybridization (13). Expression of the *Tl0rga1* transgene was analyzed by Northern analysis (13) with 20 μ g of total RNA extracted from the T1 transgenic plants.

Substitution Rate Analysis. Nucleotide sequences from the *rga1* sequences of *T. aestivum* cvs. Thatcher*Lr10* and Canadian3842 as well as from *Triticum durum* cv. Langdon and *T. monococcum* DV92 were aligned by using the PILEUP program of the GCG software. The nucleotide alignment was adjusted manually with the program LINEUP by using the amino acid sequence alignment as a guide to keep a codon-by-codon alignment. The rate of nonsynonymous (K_a) vs. synonymous (K_s) nucleotide substitutions per 100 sites (K_a/K_s) was computed with the program DIVERGE, which is based on the algorithm developed by Li (20) and uses Kimura's two-parameter method (21) for analysis. The different domains (N-terminal, NBS, spacer, and LRR) were chosen according to the domains defined by Meyers *et al.* (22) for NBS-LRR-encoding genes. A Student *t* test (SAS package, SAS Institute, Cary, NC) was used to test for the significance of differences between the K_a/K_s mean values of the N-terminal and the LRR domains.

Phylogenetic Analysis. Alignment of the amino acid sequences for the genes was performed by using the CLUSTALX program (23).

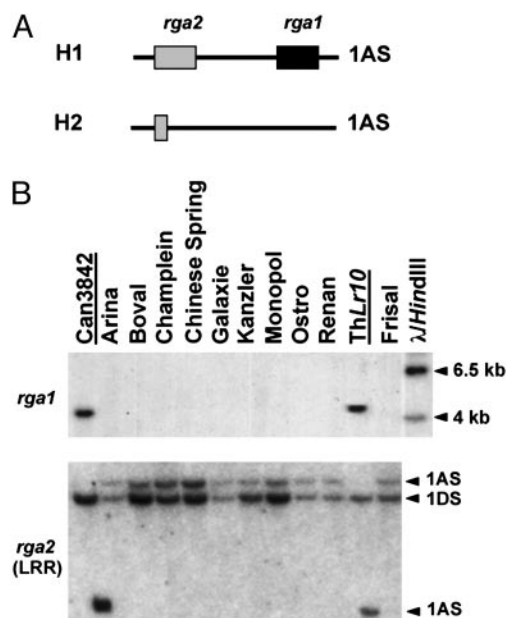


Fig. 1. Two haplotypes defined by the presence or absence of two candidates for the *Lr10* resistance gene on chromosome 1AS are present in the hexaploid wheat gene pool. (A) Schematic representation of the H1 and H2 haplotypes at the *Lr10* locus. The candidate genes *rga1* and *rga2* are present on chromosome 1AS in lines with the H1 haplotype (e.g., Thatcher*Lr10*), but not in lines with the H2 haplotype (e.g., Frisal). (B) The H2 haplotype is predominant and is very conserved in the wheat gene pool. Southern hybridization with *HindIII* (Upper)- and *DraI* (Lower)-digested genomic DNA isolated from a subset of 56 hexaploid European wheat breeding lines. Hybridizations were performed with *rga1* (Upper) and the LRR domain of *rga2* (Lower) as probes. Lines with an H1 haplotype are underlined. The fragments corresponding to *Tl0rga2-1A* on chromosome 1AS and *Tl0rga2-1D* on chromosome 1DS (25) are indicated with arrowheads.

A neighbor-joining (NJ) method was then applied to produce a phylogenetic tree. The relative degree of branch support was determined within the NJ framework by using the bootstrap procedure (24). The original data set was resampled 1,000 times. The LR21 sequence was used as an outgroup.

Results

Isolation of Two Candidate Genes for *Lr10* in Hexaploid Wheat. Two RGAs, named *rga1* and *rga2*, have been previously identified in a genomic region of *T. monococcum* DV92 spanning the *Lr10* resistance locus in *T. aestivum*, Thatcher*Lr10* (14, 15). To test whether these genes are the best candidate genes for *Lr10*, we have analyzed the *Lr10* locus at the molecular level in the wheat gene pool (25). Southern analysis of 113 wild and cultivated diploid and polyploid wheat lines has revealed the presence of two characteristic haplotypes on chromosome 1AS. Haplotype H1 is defined by the presence of the two full-length *rga1* and *rga2* genes, whereas in haplotype H2, *rga1* is absent and only a truncated LRR domain of *rga2* can be detected (ref. 25 and Fig. 1A). In a survey of 56 hexaploid European wheat breeding lines, only eight lines had the H1 haplotype, indicating that the H2 haplotype is predominant in *T. aestivum* (Fig. 1B). Moreover, all of the lines of the H2 haplotype showed identical hybridization patterns at the *Lr10* locus; i.e., they lack *rga1* and a single fragment hybridizes with the 3' end of the LRR domain of *rga2* on chromosome 1A (Fig. 1B). These data suggest that there is very little variability within the H1 and H2 haplotypes and that there are possibly no other haplotypes at the *Lr10* locus in the hexaploid wheat gene pool. Therefore, molecular analysis of

MAPCLVSASTGAMGSLT¹⁹KLETMLDDEYILLNVRDRIKFIHELAMQSE¹⁹LDVADFEPEG | CC
 QHDKSCADLVRELSYDIEDKIDNSMSLMLHACPKSGIKKHMSEKFNLLPVKIPYQIAKDI
 KDKSKQILEVSNRCERYRFEVDCLARTEFVDPRLCTVDTCAADLVGIDGPKHELKVLWRNG
 EDES²⁵VHQKQVVSIV

GCAGL²⁵KTTLAKQVYDEL²⁵RINF²⁵YRAF²⁵VSIS²⁵RSPN²⁵MATIL²⁵KCVLS²⁵QFHAQ²⁵DYSS²⁵DESE²⁵IE²⁵PK
 LVDQIRDLLQDYFVI²⁵IDD²⁵IWM²⁵KMT²⁵VDL²⁵KCAL²⁵CKNS²⁵CGSV²⁵IM²⁵TT²⁵RIY²⁵DVAK²⁵SCCS²⁵NGDL | NBS
 VYNI²⁵QPLSVAD²⁵SEEL²⁵FLNR²⁵VFG²⁵HEK²⁵GF²⁵PEL²⁵KEV²⁵SK²⁵DVLR²⁵CK²⁵GGL²⁵PLAIN²⁵AISS²⁵LAAEKI
 EE²⁵WDRV²⁵GLSN²⁵VFA²⁵QGEK²⁵SD²⁵DAM²⁵KY²⁵KL²⁵SL²⁵CF²⁵DL²⁵PL²⁵HL²⁵RS²⁵CL²⁵LY²⁵IM²⁵FP²⁵ED²⁵CL²⁵IE²⁵KER²⁵L²⁵VH
 RW²⁵ISE²⁵GF²⁵IR²⁵NED²⁵GED²⁵LV²⁵EG²⁵RY²⁵LY²⁵EL²⁵VN²⁵RS²⁵LIE²⁵SV²⁵GP²⁵YD²⁵GK²⁵AR²⁵FY²⁵RV²⁵ENV²⁵IL²⁵DF²⁵LM²⁵IKS

MEEN²⁵FCT²⁵LTS²⁵NQ²⁵SRL²⁵DY²⁵KV²⁵RR²⁵LS²⁵L²⁵FANK²⁵DPS | Spacer

CT²⁵AQ²⁵LD²⁵L²⁵SHARS²⁵IGAS²⁵GH²⁵LQ²⁵LIS²⁵SVKSN
 AL²⁵RV²⁵LD²⁵VQDC²⁵SEL²⁵GN²⁵HH²⁵VK²⁵DIG²⁵RNP
 LL²⁵RY²⁵L²⁵ISG²⁵TD²⁵-V²⁵TEL²⁵PI²⁵Q²⁵-IGDMG
 F²⁵LET²⁵L²⁵DAS²⁵FTE²⁵-L²⁵VE²⁵MP²⁵GS²⁵IT²⁵RLR
 QL²⁵QRL²⁵F²⁵VSDE²⁵T²⁵K²⁵L²⁵PDE²⁵IGN²⁵MK
 RL²⁵QEL²⁵GD²⁵INAF²⁵KQ²⁵SV²⁵NFL²⁵NEL²⁵GKLT
 GL²⁵RK²⁵L²⁵GI²⁵WDT²⁵ND²⁵IL²⁵KS²⁵GK²⁵SG²⁵SE²⁵K²⁵RL²⁵VSS²⁵L²⁵SKLDAG | LRR
 RL²⁵SN²⁵LY²⁵TF²⁵YL²⁵RE²⁵KD²⁵GF²⁵I²⁵GH²⁵P²⁵FL²⁵PALN
 S²⁵IRE²⁵V²⁵Y²⁵LR²⁵RG²⁵-M²⁵C²⁵W²⁵N²⁵K²⁵L²⁵LSLA
 N²⁵E²⁵K²⁵LY²⁵IS²⁵GG²⁵DE²⁵IE²⁵Q²⁵DDL²⁵RT²⁵VG
 S²⁵I²⁵PT²⁵LV²⁵E²⁵PK²⁵LY²⁵S²⁵G²⁵CL²⁵G²⁵PI²⁵I²⁵SS²⁵G²⁵PE
 QL²⁵ER²⁵LE²⁵L²⁵K²⁵FS²⁵FS²⁵QL²⁵T²⁵FE²⁵V²⁵GAMP
 NL²⁵KK²⁵LD²⁵LV²⁵YS²⁵K²⁵FK²⁵SAG²⁵AG²⁵DF²⁵GI²⁵Q²⁵HL²⁵S
 SL²⁵AC²⁵VS²⁵I²⁵VF²⁵CE²⁵GV²⁵SA²⁵AY²⁵VEA²⁵AE²⁵G
 AF²⁵KSM²⁵V²⁵NA²⁵HP²⁵NP²⁵NR²⁵PL²⁵EM²⁵TRES²⁵AD²⁵FS²⁵MQ²⁵DE

Fig. 2. Amino acid sequence of the *Lr10* gene. The CC, NBS, spacer, and LRR domains are indicated. Amino acids belonging to characteristic motives in each domain are bold. In the NBS domain they are in the following order: P-loop, RNBS-A, kinase2, RNBS-B, RNBS-C, GLPL, RNBS-D, and MHDV. The four domains that have been used in the K_a/K_s analysis are separated from each other by an empty line. The spacer sequence is indicated in italics and the aliphatic (a) residues in the consensus (xxaxxx) region of the LRR domain are boxed in yellow. The amino acid residues that are modified in the three EMS mutant genes are highlighted with red boxes with the number of the mutant above them.

these two haplotypes should allow the identification of the complete set of *Lr10* candidate genes.

With the exception of the variety Canadian3842 (Can3842), the H1 lines, including Thatcher*Lr10*, were resistant to leaf rust isolates avirulent on *Lr10* (*AvrLr10*) (data not shown). To identify all candidate genes possibly present at the *Lr10* locus in Thatcher*Lr10*, we have characterized the H1 and H2 haplotypes in more detail at the molecular level. Two BAC contigs were established at the *Lr10* locus in the tetraploid *T. durum* cv. Langdon (H1 haplotype) and in the *T. aestivum* cv. Renan (H2 haplotype) (unpublished data). Low-pass sequencing of the two BACs revealed the presence of the chromosome condensation factor, nodulin-like, and actin genes, which were already identified at the *Lr10* locus in *T. monococcum* DV92 (H1 haplotype) (15). It also confirmed the complete deletion of the *rga1* sequence and the presence of a truncated LRR domain of *rga2* in the Renan H2 haplotype (data not shown). These studies did not provide additional candidate genes to *rga1* and *rga2* in the physical interval between markers flanking *Lr10* in hexaploid wheat. Therefore, we conclude that *rga1* and *rga2* of haplotype H1 are the only promising candidate orthologs for the *T. aestivum* *Lr* gene *Lr10*.

The *T. monococcum* *rga1* and *rga2* genes were used as probes to isolate the orthologous genes *T10rga1* and *T10rga2* from a λ library of the resistant hexaploid wheat variety Thatcher*Lr10*. The *T10rga1* gene (GenBank accession no. AY270157) has a length of 3,935 bp and contains one intron of 1,171 bp, which, as in most cereal RGAs, is located in the NBS domain at the N terminus of the kinase-2 motif (26). The gene encodes a coiled coil (CC), nucleotide-binding site (NBS), leucine-rich repeat (LRR) (CNL) protein of 919 amino acids with 14 imperfect LRRs at the C terminus (Fig. 2). The length of the CC, NBS,

spacer, and LRR domains, as well as the type of amino acid residues contained in the different motives, indicate that T10RGA1 is related to the CNL-C type of proteins (22). By Southern hybridization, it was found that *T10rga1* is a single-copy gene on chromosome 1AS in Th*Lr10*. For *rga2*, two homoeologous genes, *T10rga2-1A* and *T10rga2-1D*, were identified on chromosomes 1A and 1D, respectively. The *T10rga2-1A* gene (GenBank accession no. AY270159) is 4,756 bp long and has two introns of 944 and 310 bp. It encodes a CC-NBS-LRR protein of 1,169 amino acids that is unrelated to T10RGA1. Linkage analysis in a population of 3,120 F2 plants showed that *T10rga1* and *T10rga2-1A* are both completely linked to *Lr10*. RT-PCR analysis demonstrated that both genes are expressed in the resistant variety Thatcher*Lr10* (see Fig. 5A, which is published as supporting information on the PNAS web site) and are not induced on leaf rust infection (Fig. 5B).

Three Independent Mutations Affecting *T10rga1* Lead to Susceptibility to Leaf Rust Carrying *AvrLr10*.

Three independent *lr10* mutants (T10.EMS19, T10.EMS25, and T10.EMS31) were identified and characterized at the molecular level. The *T10rga1* and *T10rga2-1A* genes were amplified by PCR from the mutants, and their sequences were compared with the sequences of Thatcher*Lr10*. In all mutants, point mutations were detected in *T10rga1* but not *T10rga2-1A*. In *T10rga1*.EMS19, a C-to-T transition at position 151 resulted in a change of the last leucine residue in the putative CC domain into a phenylalanine residue (Fig. 2). In *T10rga1*.EMS25, a G-to-A transition at position 608 changed a glycine into an arginine residue in the third conserved glycine of the P-loop motif in the NBS (Fig. 2). A similar transition was detected at position 3,461 in the LRR domain of the *T10rga1*.EMS31 sequence. It introduced a stop codon instead of a tryptophan residue at the end of the ninth LRR in the LRR domain (Fig. 2). Thus, three independent mutations affect the *T10rga1* gene at different positions and result in a loss of resistance against leaf rust, demonstrating that *T10rga1* is the *Lr10* gene. In addition, these data indicate that the last five LRR are required for *Lr10* function and underline the essential role of the leucine and glycine residues in the CC and P-loop motives, respectively.

Transgenic Wheat Plants Overexpressing *T10rga1* Show Increased Resistance to Leaf Rust Avirulent on *Lr10*.

To test whether *T10rga1* is sufficient to confer rust resistance to wheat plants, plasmids containing the *T10rga1* (*pUbi.T10rga1*) and *T10rga2-1A* (*pUbi.T10rga2-1A*) genes under control of the maize ubiquitin promoter were cotransformed into the susceptible wheat accession Bobwhite SH 98 56 (18), which has the H2 haplotype. Artificial infection and molecular analysis performed on T1 progeny plants identified two families expressing resistance to leaf rust avirulent on *Lr10*. In both families (T14 and T17), cosegregation was found between the resistance phenotype and the *pUbi.T10rga1* transgene (Fig. 3A). *pUbi.T10rga2-1A* was present in all of the *pUbi.T10rga1* transgenic plants of the T17 family but was not detected in plants of the T14 family. All resistant plants from the segregating T1 families expressed *pUbi.T10rga1*, as shown by Northern analysis (Fig. 3B), confirming that *T10rga1* is the *Lr10* gene. Other T1 families, which all contained multiple transgene insertions and rearrangements, were susceptible suggesting gene silencing or incomplete transgene integration (data not shown). The level of expression in the transgenic resistant plants was 8–25 times higher than in Thatcher*Lr10* (Fig. 3B). Interestingly, in the plants overexpressing *T10rga1*, the infection type differed from the moderate resistance (small to middle size uredinias surrounded by chlorosis) conferred by the endogenous *Lr10* (Fig. 3C). The resistant transgenic plants either showed hypersensitive flecks or developed strong chlorosis and necrotic spots upon leaf rust infection,

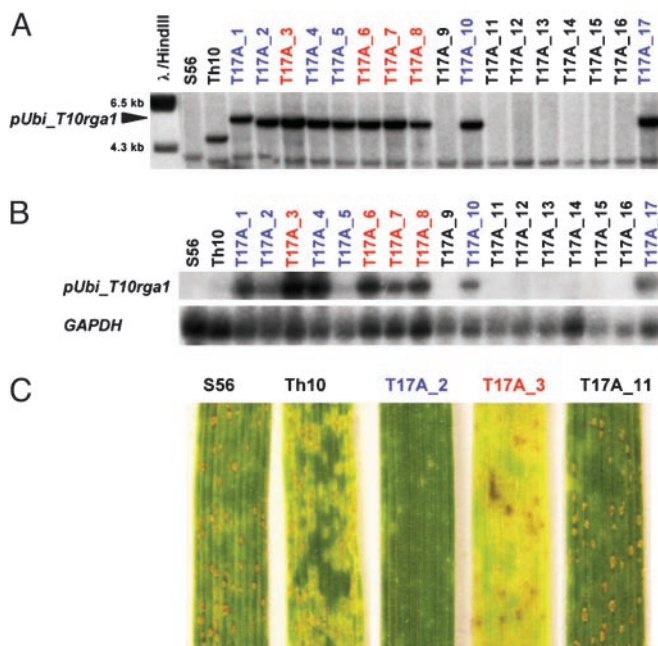


Fig. 3. Transgenic wheat seedlings overexpressing *T10rga1* show enhanced resistance to leaf rust. (A) Southern hybridization of *Hind*III-digested genomic DNA extracted from Bobwhite SH 98 56 (S56), Thatcher*Lr10* (Th10), and 17 transgenic T1 plants of the T17 family (T17A.x) with *T10rga1* as a probe. The arrowhead indicates the 5.6-kb fragment, which is expected from a *Hind*III digest of the *pUbi_T10rga1* construct. (B) Northern blot of total RNA extracted from the same seedlings hybridized with *T10rga1* as a probe. The same blot was hybridized with the housekeeping *GAPDH* gene as a control. The relative intensity of the hybridization signals in the transgenics vs. wild-type plants was estimated with the Cyclone gene array system (Perkin-Elmer, Boston). Transgenic plants showing chlorotic hypersensitive resistance reaction (i.e., T17A.2) are blue, those with a necrotic phenotype (i.e., T17A.3) are red, while susceptible T1 plants are black. (C) Phenotypes of transgenic plants overexpressing *Lr10* compared with the resistant Thatcher*Lr10* and the susceptible Bobwhite SH 98 56, 10 days after artificial infection with the leaf rust isolate TCB/TD *AvrLr10*.

and no uredinia were formed (Fig. 3C). These data suggest that overexpression of the *Lr10* gene enhances leaf rust resistance.

Diversifying Selection Acts on the N-Terminal Region of the CNL-C *Lr10* Gene Product. Comparison of allelic sequences has shown that diversifying selection acts on the LRR encoding domain of many plant disease resistance genes (27). To study whether a particular selective pressure acts on the *Lr10* gene, we have compared the sequences of the *Lr10* alleles of the two hexaploid wheat lines Thatcher*Lr10* (*T10rga1*, GenBank accession no. AY270157) and Can3842 (*Can_rga1*, GenBank accession no. AY270158), the *T. monococcum* DV92 (*rga1*, GenBank accession no. AF326781), as well as a gene amplified from the tetraploid *T. durum* var. Langdon (*Td_rga1*, unpublished data). The two latter lines are resistant to leaf rust carrying *AvrLr10*, but we do not know whether this resistance is conferred by the *Lr10* allele, because other resistance genes are possibly present. In contrast, Can3842 is the only susceptible hexaploid wheat line identified so far with an H1 haplotype. *Can_rga1* is expressed (see Fig. 6, which is published as supporting information on the PNAS web site), indicating that the susceptibility of Can3842 to *AvrLr10* is not due to a lack of gene expression but might result from a loss of recognition between the CAN_RGA1 protein and AVR*Lr10*. The protein sequences encoded by the four genes share, on average, 93% similarity over the entire sequence. Interestingly, conservation is lower in the first 426 amino acids of the LR10

Table 1. Average rates of nucleotide substitutions per 100 sites among four alleles (*T10rga1*, *Can_rga1*, *Td_rga1*, and *rga1*) of the *Lr10* gene

	Complete gene	N-terminal	NBS	Spacer	LRR
K_a	3.71	8.10	3.70	1.85	1.58
K_s	3.69	5.89	3.33	3.18	2.88
K_a/K_s	0.98	1.48	0.99	0.83	0.39

The coding sequences for the N-terminal (amino acids 1–197), NBS (208–502), spacer (503–533), and LRR (534–919) domains were analyzed separately. The K_a/K_s ratio was calculated by averaging the ratio for each comparison.

protein (89%) than in the remaining 493 amino acids (96.5%), which mostly form the LRR domain (see Fig. 7, which is published as supporting information on the PNAS web site). Estimation of the number of synonymous (K_s) and nonsynonymous (K_a) nucleotide substitution per site showed that K_s is identical to K_a , indicating selection on the gene as a whole (Table 1). Analysis of the different domains of LR10 (N-terminal, NBS, spacer, and LRR; Fig. 7) revealed that with a K_a/K_s ratio of 0.39, the LRR region is not under positive selection compared with the N-terminal region of the protein, which has a ratio >1 (1.48; Table 1). A statistical *t* test of the K_a/K_s mean values for the N-terminal and LRR domains showed that the difference is significant ($P = 0.0005$). The K_a/K_s ratio remained <1 for the intervening residues as well as for the exposed residues (xxaxxx) of the LRR (data not shown). We conclude that the 5' end of the *Lr10* gene is under diversifying selection.

***Lr10* Is Related to Other Fungal Disease Resistance Genes in Grasses.**

To analyze whether *Lr10* is similar to other plant disease resistance genes or RGAs, the *Lr10* sequence was compared against the databases and against the *Lr21* resistance gene (12). More than 50% similarity was found at the amino acid level over the entire sequence with RGAs from rice, barley, sorghum, and wheat, whereas similarity with the LR21 sequence was very low (41%) and restricted to 336 amino acids of the NBS domain. Phylogenetic analysis showed that the wheat LR10 sequence is closely related to RGAs encoded by three predicted genes (Os.RGA1a, Os.RGA1b, and Os.RGA1c) located on a single BAC (AP005158) of chromosome 8 in rice as well as with a sorghum *RPM1*-like gene product (Sb.RPM1) located on chromosome F (Fig. 4). There was more similarity with the rice and sorghum sequences than with wheat disease RGAs such as M32-NBS-LRR, which segregates with *Lr21* at the telomeric end of chromosome 1D in *Ae. tauschii* (28) or with the putative *Yr10* stripe rust resistance gene on chromosome 1BS. Significant similarity was also found with the powdery mildew resistance genes *Mla1*, *Mla6*, and *Mla13*, which are located on chromosome 1HS in barley (5, 6) as well as with two proteins (Sb.MLA1, Sb.YR10/MLA1) encoded by *Mla*-like genes on chromosome G in sorghum (ref. 29 and Fig. 4). Finally, LR10 shows significant similarity to the *A. thaliana* RPM1 sequence (43% over 875 amino acids), which also belongs to the CNL-C class of disease resistance genes and was used as an outgroup in the phylogenetic analysis (Fig. 4). We conclude from this analysis that *Lr10* belongs to an ancient class of CNL resistance genes of which members are conserved in the grass genomes. Finally, the *Lr10* gene was used as a probe on Southern blots of 24 near isogenic lines of the cultivar Thatcher containing different *Lr* genes and alleles (*Lr1* to *Lr34*; ref. 30). None of the lines including Thatcher*Lr21* showed a hybridizing fragment (data not shown), indicating that the other leaf rust genes do not belong to the same NBS-LRR family as *Lr10*.

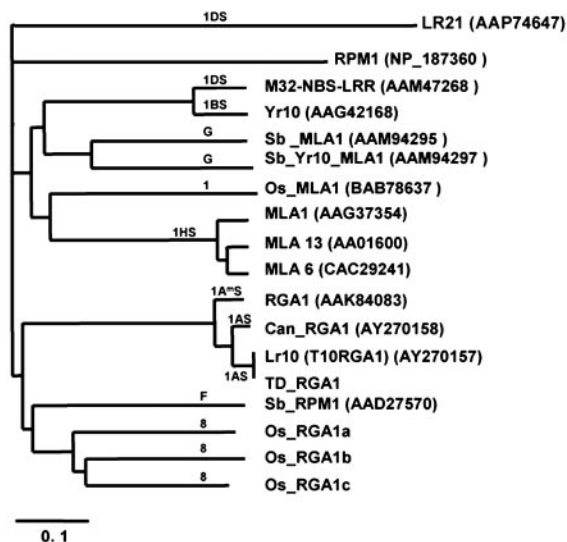


Fig. 4. NJ phylogenetic tree of LR10, LR21, and products of RGAs from different grass species. Except for Td.RGA1 (unpublished results) and the rice homologs on chromosome 8, which were annotated from a BAC sequence (GenBank accession no. AP005158) in this work, the accession numbers of the proteins are given next to the gene names. Genes that did not have names in the database were named after the species of origin (OS, rice; Sb, sorghum) and the homology defined by the authors in the database annotation. The chromosomal location of the genes is indicated on the branch of the phylogenetic tree.

Discussion

Map-Based Cloning in Hexaploid Wheat. The construction of the first BAC library from the diploid wheat *T. monococcum* DV92 (8) has allowed the recent map-based isolation of *VRN1* directly from *T. monococcum* (10) and the identification of candidate gene orthologs for the hexaploid wheat genes *Lr10* (14) and *Q* (11) by using subgenome map-based cloning. The *T. monococcum* DV92 line is not known to carry *Lr10*. Therefore, our work demonstrates that it is possible to isolate a gene from hexaploid bread wheat by using available BAC resources from diploid relatives that do not necessarily contain the target gene. Very recently, the *Lr21* resistance gene, which originates from *Ae. tauschii*, has been isolated (12). In this case, map-based isolation of the gene was performed without chromosome walking as the restriction fragment-length polymorphism probe KSUD14, which is part of the *Lr21* gene, could be used directly to screen a cosmid library from the *Ae. tauschii* *Lr21* donor line (12).

The subgenome map-based cloning of *Lr10* has succeeded, because *T. monococcum* DV92 has the same haplotype (H1) as the target hexaploid wheat variety Thatcher $Lr10$ and, therefore, colinearity between the two species was very high at the *Lr10* locus. A number of recent studies performed at the interspecific level (31–33), as well as between inbred lines (34), indicate that many rearrangements involving genes have occurred at orthologous loci during plant evolution. For this reason, the isolation of agronomically important genes from hexaploid wheat through subgenome map-based cloning strategies should be performed together with a detailed haplotype characterization of the target locus in the wheat gene pool to identify the most appropriate genomic tools.

Comparison of the *Lr10* sequence with *Lr21*, the only other *Lr* gene isolated so far (12), showed no significant similarity between the two NBS-LRR sequences, suggesting that the different wheat *Lr* genes are not very closely related to each other. Consequently, each *Lr* gene must probably be isolated independently. High-genome coverage BAC libraries have been very

recently constructed from tetraploid (35) and hexaploid bread wheat (B. Chalhoub, personal communication). However, given the amount of clones required, i.e., ≈ 1 million BAC clones for a $6\times$ time coverage of the hexaploid wheat genome, such libraries cannot be constructed for every wheat genotype of interest. In contrast, it should be possible to develop methods for rapidly constructing nonarrayed BAC libraries from a number of diploid wheat genotypes at low cost. We have shown here that libraries from diploid wheat relatives are efficient tools for the map-based isolation of genes from hexaploid wheat.

***Lr10* Encodes a CNL-C Protein with an N-Terminal Domain Under Diversifying Selection.** The nucleotide substitution pattern in *Lr10* alleles varies across the gene with a higher frequency of non-synonymous substitutions in the 5' end of the gene, compared with the region encoding the LRR domain, which is highly conserved. This pattern contrasts with the majority of LRR-containing disease resistance proteins for which allelic sequence comparisons and K_a/K_s analysis have demonstrated that the LRR domain is under diversifying selection and plays a role in resistance specificity (27). Only in few cases, such as for *L* alleles in flax, the N-terminal TIR domain has also been shown to be involved in determining resistance specificity (36). Here, we have compared four alleles of *Lr10*, and it was not possible to correlate variations observed in the amino acid sequences of the diversifying N-terminal domain with the resistance phenotypes. However, with the *Lr10* sequence information and our knowledge about the haplotype composition in the wheat gene pool (25), we have now the possibility to isolate additional *Lr10* alleles from wild relatives of wheat. Complementation tests using these alleles will provide essential information if they act as *Lr* genes and will ultimately help to define the role of the N-terminal region in LR10 resistance specificity. Finally, analysis of the EMS *Lr10* mutants has demonstrated that the last five LRRs are required for *Lr10* function and has confirmed the important role of the leucine and glycine residues in the CC and P-loop motifs, respectively. Similar loss of resistance has been observed in mutants of the flax rust resistance *M* gene lacking 426 bp encoding part of the LRR domain (37), whereas mutational analysis performed in the tobacco *N* (38) and in the *A. thaliana* *RPM1* (39) genes have highlighted the importance of an intact P-loop motif for resistance gene function.

***Lr10* Is Conserved in Grass Species, and It Shows Similarities to *RPM1* in *A. thaliana*.** LR10 has significant sequence similarity with the *Arabidopsis* *RPM1* protein. Both genes belong to the same class of CNL-C resistance genes, suggesting that they originate from a common ancestor. *Lr10* homologs have been identified in barley, sorghum, and rice. Interestingly, there was more similarity between LR10, a sorghum *RPM1*-like protein sequence, and the three protein sequences predicted from genes located on rice chromosome 8 than with any other wheat RGAs, suggesting conservation of the *RPM1/Lr10* type of resistance genes in these species. Except for the barley *Mla* genes, none of these grass homologs has been identified as a disease resistance gene or has been mapped at disease resistance loci. The positions of the barley and wheat homologs on the genetic maps of the homoeologous chromosomes 1 did not suggest orthology between these genes and *Lr10*. However, we have identified by hybridization a homolog of *Lr10* in barley, which maps at the telomeric region of chromosome 1HS (unpublished data), where the *Rph4* gene is located (40). Mapping of this gene in a population segregating for *Rph4* needs to be performed to study whether both wheat and barley rust genes are orthologous. Orthology between resistance genes in grasses is not expected from previous analyses (41), which have shown rapid reorganization of resistance gene loci between related grass species. However, it is possible that in species that are very closely related some orthologous relation-

ships are conserved, as found by Pan *et al.* (42) for four resistance gene homolog loci in tomato and potato.

Overexpression of *Lr10* Enhances Resistance to Leaf Rust Carrying *AvrLr10*. In Thatcher*Lr10*, the *Lr10* gene provides only moderate resistance, is constitutively expressed, and is not induced on leaf rust infection. Plant disease resistance genes are only rarely induced by pathogen attack and are usually expressed at low levels (2), suggesting that too high expression might have negative effects. This result is supported by the finding that in the *A. thaliana* epigenetic variant *ball*, which overexpresses an NBS-LRR gene, developmental abnormalities such as late flowering, dwarfing, and altered floral structures were described (43). Interestingly, we have observed that some resistant transgenic plants overexpressing *Lr10* are smaller and have fewer tillers compared with nontransgenic plants (unpublished data). Possible correlations between the level of *T10rgal* expression and developmental alterations will be analyzed in the next generations. The overexpression of *Lr10* resulted in enhanced resistance with a complete prevention of rust sporulation compared with Thatcher*Lr10*. In addition, a necrotic phenotype, which has not been described before for *Lr10* (30), has been observed in some transgenic resistant plants. Similar to transgenic barley plants expressing the stem rust resistance gene *Rpg1* (44), it was not possible to strictly correlate the level of *Lr10*

transgene expression with the resistance types observed in the transgenic plants. Analysis in the next generation will provide additional information about the genotype of the T1 plants and will allow the comparison of rust fungal growth in the two resistant types. Thus, our data suggest that overexpression of a disease resistance gene can improve resistance. This finding has also been shown in the cases of the *Pto* and *Prf* genes in tomato (45, 46), and more recently for the *Rpg1* resistance gene in barley (44).

In the near future, additional wheat disease resistance genes will be isolated by using similar strategies as in this work. Their characterization will provide a better understanding of the molecular basis of disease resistance in wheat and will allow, in the long term, the development of genomics-guided transgene strategies (47), such as the combination of resistance specificities, the overexpression of resistance genes, and the use of the cloned genes as “perfect” markers for molecular breeding. These strategies should ultimately lead to improved resistance of wheat against fungal diseases.

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