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The maize *rp1* rust resistance gene identifies homologues in barley that have been subjected to diversifying selection

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Abstract A number of agronomically important grasses (sorghum, wheat, panicum, sugar cane, oats, rice and barley) are shown to contain sequences homologous to *rp1*, a maize gene that confers race-specific resistance to the rust fungus *Puccinia sorghi*. Mapping of *rp1*-related sequences in barley identified three unlinked loci on chromosomes 1HL, 3HL and 7HS. The locus located on chromosome 7HS comprises a small gene family of at least four members, two of which were isolated and are predicted to encode nucleotide binding site-leucine-rich repeat (NBS-LRR) proteins that are respectively 58% and 60% identical to the maize *rp1* protein. Evidence of positive selection for sequence diversification acting upon these two barley genes was observed; however, diversifying selection was restricted to the carboxy terminal half of the LRR domain. One of these *rp1* homologous genes cosegregated with the barley *Rpg1* stem rust resistance gene amongst 148 members of the Steptoe × Morex double haploid mapping family. Three other unrelated resistance gene-like sequences, potentially encoding NBS-LRR proteins, are also shown to be linked to the *Rpg1* locus but not cosegregating with the gene.

Key words Rust resistance · *rp1* · Barley · Maize · *Rpg1* · *Puccinia*

Introduction

Three plant genes have been isolated which confer race-specific resistance against fungal rust pathogens. Two of these, *L* and *M*, have been isolated from the flax plant (*Linum usitatissimum*) and confer resistance to the flax rust fungus (*Melampsora lini*) (Lawrence et al. 1995; Anderson et al. 1997), while the *rp1* gene isolated from maize confers resistance to common maize rust (*Puccinia sorghi*) (Collins et al. 1999). The maize *rp1* locus, located on chromosome 10S, comprises a small family of related genes. The number of family members differs for different rust resistance specificities which map at the *rp1* locus. In the case of *Rp1-D*, nine family members are present, one of which encodes the *Rp1-D* specificity. The *Rp1-D* resistance gene specifies an intron-less open reading frame (ORF) of 3876 bp which encodes a 1292 amino acid protein (Collins et al. 1999).

All three cloned rust resistance genes are members of a plant disease resistance gene class which encode proteins containing an ATP or GTP nucleotide binding site (NBS) and C-terminal leucine-rich repeat region (LRR). The LRR domain comprises a series of tandem repeat units containing a consensus sequence of leucine residues and other aliphatic amino acids which form the backbone of the repeat (Kobe and Deisenhofer, 1993; 1994; Jones and Jones 1997). NBS-LRR-encoding disease resistance genes have been shown to confer race-specific resistance to a range of viral, bacterial, fungal, nematode and insect pathogens (Staskawicz et al. 1995).

Several other classes of plant disease resistance genes also encode proteins containing LRR domains. The *Cf* genes from tomato which confer resistance to the fungal pathogen *Cladosporium fulvum* are predicted to encode membrane-anchored, extracellular proteins containing an LRR domain (reviewed in Jones and Jones 1997; Parniske et al. 1997). Another class of resistance gene, represented by the *Xa21* gene family in rice, is predicted to encode receptor-kinase proteins containing an extracellular LRR domain coupled via a transmembrane domain to an intracellular protein kinase (Song et al. 1995). Contrastingly, a member of the *Xa21* gene family, *Xa21D*, encodes only a predicted extracellular LRR domain which provides an identical spectrum, but less effective form, of resistance as the *Xa21* gene (Wang et al. 1998). The structural similarity of the *Xa21D* gene product to that of the tomato *Cf* genes suggests that an evolutionary relationship exists between these two families.

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The LRR domain is therefore common to a number of plant disease resistance genes. By structural analogy to the porcine ribonuclease inhibitor protein, another LRR-containing protein (Kobe and Deisenhofer 1993, 1994), the LRR domain of plant disease resistance genes is predicted to form a β-sheet structure that has been suggested to interact with a pathogen ligand (Thomas et al. 1997; Parniske et al. 1997; Jones and Jones 1997). Contained within the LRR repeat unit is a subrepeat, xx(A)x(A)xx, which is predicted to form a β-strand/β-turn with the conserved aliphatic residues (A) of the peptide sequence directed into the hydrophobic core of the protein. The remaining residues of the subrepeat (x) are predicted to remain solvent exposed (Kobe and Deisenhofer 1993, 1994; Jones and Jones 1997).

Sequence analysis of several LRR-containing resistance genes has shown that the solvent-exposed residues of the β-strand/β-turn region have been subjected to diversifying selection. The type of selection acting upon a gene can be predicted by a comparison of nonsynomous (Ka) and synomous (Ks) nucleotide substitution rates. A Ka/Ks ratio of less than 1 suggests that selection favours sequence conservation, while a ratio greater than 1 suggests that positive selection for sequence diversification has occurred (Hughes and Nei 1988; Parniske et al. 1997). A Ka/Ks ratio of 1 occurs under neutral selection pressure (Hughes 1995). Diversifying selection is rarely observed and is frequently associated with genes which encode either antigens or antigen recognition proteins (Hughes and Nei 1988; Tanaka and Nei 1989; Endo et al. 1996). In a study of 3595 gene families, only 17 families showed evidence of positive selection for sequence diversification (Endo et al. 1996).

Sequence analysis has identified diversifying selection having acted upon the solvent-exposed residues of the β-strand/β-turn region of extracellular LRR domains in the tomato *Cf* genes (Parniske et al. 1997) and the rice *Xa21* gene family (Wang et al. 1998). Positive selection for sequence diversification has also been identified amongst the solvent-exposed β-strand/β-turn residues of NBS-LRR genes. The *RPP8* and *RPP1* gene families from *Arabidopsis*, which confer resistance to the fungal pathogen *Peronospora parasitica* (McDowell et al. 1998; Botella et al. 1998), and the *RGC2* gene family in lettuce, which confers resistance to the fungal pathogen *Bremia lactucae* (Meyers et al. 1998), all encode NBS-LRR genes which show diversifying selection acting upon the xx(A)x(A)xx motif of the LRR domain.

In the study reported here we demonstrated that the maize *rp1* gene identifies homologous gene families in a number of agronomically important grass species. Isolation and sequence analysis of two of these sequences from barley identified genes encoding NBS-LRR proteins that are highly homologous to the maize Rp1-D protein. Analysis of the nucleotide substitution patterns between these two barley sequences showed evidence of diversifying selection having acted upon the solvent-exposed residues present within the β-strand/β-turn region of the LRR of these genes. This selection, however, is confined to the carboxy terminal half of the LRR domain. Mapping of barley sequences homologous to the maize *rp1* gene identified a gene family linked to the barley *Rpg1* stem rust resistance locus. Other NBS-LRR-like genes were also shown to map within the vicinity of the *Rpg1* stem rust resistance gene.

Materials and methods

Isolation of barley genomic DNA and DNA blot analysis

Barley genomic DNA was isolated by the CTAB method of Saghai-Maroof et al. (1984) and restricted with endonucleases under conditions recommended by the manufacturer (New England Biolabs). DNA blot analysis of genomic DNA was performed as previously described (Collins et al. 1998). DNA probes used for hybridisation were labelled with [P^{32}]-dCTP using a Megaprime DNA labelling system (Amersham).

Barley genomic DNA library construction and screening

A genomic DNA library constructed from barley var. Morex was obtained from Dr. T.J. Close (University of California, Riverside, Calif.) under the auspices of the North American Genome Mapping Project. This library was constructed from genomic DNA that was partially digested with the restriction enzyme *Mbo*I, size-selected on a glycerol gradient and ligated to *Eco*RI/*Bam*HI-double-digested lambda EMBL3*cos* cloning vector (Whittaker et al. 1988). A primary library titre of 5×10^6 pfu was obtained, equivalent to approximately 13 haploid barley genomes. This library was screened under standard hybridisation conditions ($4 \times$ SSC, 65°C) essentially as described by Sambrook et al. (1989).

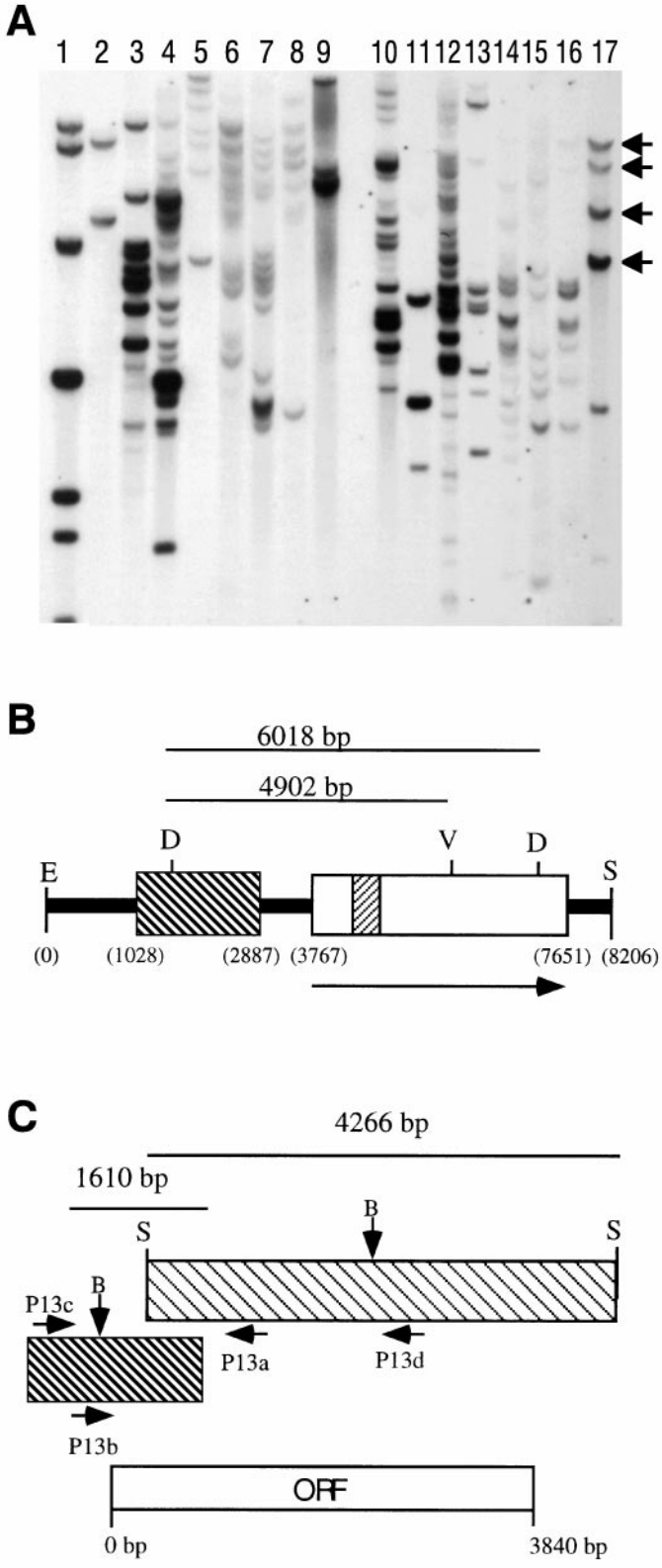
Genomic polymerase chain reaction (PCR) amplification of regions flanking a sequence contained by lambda clone M1–13 (PCR walking)

Genomic DNA which flanked a known sequence contained by lambda clone M1–13 was PCR-amplified essentially as described by Siebert et al. (1995). Total Morex genomic DNA was digested with restriction enzymes *Dra*I, *Eco*RV, *Pvu*II, *Sma*I and *Ssp*I, respectively, and a blunt-ended DNA adaptor ligated to the restriction products. Adaptor-ligated DNA template was then PCR-amplified using an adaptor-specific primer and a primer specific for the sequence present in lambda clone M1–13. The resultant amplification products were then diluted 100-fold and reamplified using the same adaptor-specific primer in conjunction with a second PCR primer specific for the sequence contained on clone M1–13. A 1.6-kbp genomic amplification product was obtained from *Dra*I-restricted Morex DNA, cloned into a T-easy cloning vector (Promega) and subsequently sequenced (see Fig. 1C).

DNA identity between a 635-bp overlap existing between lambda clone M1–13 and the cloned genomic PCR product was 100%, demonstrating that this amplified fragment was derived from the same gene contained by lambda clone M1–13. Subsequently, a pair of PCR primers were designed, one based on the sequence from clone M1–13 (called P13a) and the other based on the sequence of the PCR-amplified 5' flanking region (called P13b) (Fig. 1C). PCR amplification of Morex genomic DNA using these primers generated a product of 1095 bp, consistent with the DNA sequence of clone M1–13 being co-linear with the sequence of the previously PCR-amplified 5' flanking region. This amplified product was then sequenced directly, thereby ruling out any potential sequencing errors arising from PCR, and shown to be identical to the overlapping region of clone M1–13 and differing by three base pairs to the cloned 5' flanking region. These three base differences were presumed to represent PCR errors contained within the cloned PCR product.

Cloning and sequencing

Cloning was carried out essentially as described in Sambrook et al. (1989). DNA sequence was obtained using an ABI dye-primer sequencing kit (Applied Biosystems) and ABI Prism 377 model DNA sequencer.



Mapping and linkage analysis

Barley homolgues of the maize *rp1* gene were mapped in a Steptoe X Morex double haploid family constructed using *Hordeum bulbosum* (Chen and Hayes 1989) and obtained from Dr. P. Hayes, Oregon State University, Corvallis, Oregon, USA. Disease phenotyping of the *Rpg1* resistance gene segregating in this family was conducted by Dr. B. Steffenson using *Puccinia graminis* race Pgt-MCC (Steffenson et al. 1993; Kleinhofs et al. 1993). Restriction fragment length polymorphism (RFLP) marker data of the Steptoe × Morex mapping family (Kleinhofs et al. 1993) was downloaded from the Grain Genes database, available online. Linkage analysis was performed using the MAPMAKER version II program (Lander et al. 1987).

DNA and protein sequence comparisons

Database searches and DNA and protein sequence comparisons were performed using programs from the University of Wisconsin Genetics Computer Group (GCG) package (Devereux et al. 1984).

Results

The maize *rp1* rust resistance gene probe detects related genes in other grasses

DNA isolated from a number of grass species was analysed by DNA blot hybridisation using a probe homologous to the maize *Rp-1D* rust resistance gene (Fig. 1A). A cloned 480-bp fragment, called PIC20 (Collins et al. 1998), which is 98% homologous to the NBS region of the maize *Rp1-D* gene, was used as a DNA probe for hybridisation analysis. This *rp1* probe identifies gene families of varying complexity in all species examined,

Fig. 1A-C Identification of *rp1* gene homologues in grass species and characterisation of two homologues from barley. **A** DNA blot hybridisation of PIC20 to genomic DNA isolated from; sorghum (*lanes 1, 10*), *Panicum miliaceum* (Indian millet, *lanes 2, 11*), maize (*lane 3*), sugar cane (*lanes 4, 12*), barley (*lanes 5, 13*), wheat (*lanes 6, 14*), oats (*lanes 7, 15*), *Triticum tauschii*, which is the D-genome progenitor of wheat (*lanes 8, 16*) and rice (*lanes 9, 17*). DNAs in *lanes 1–9* and *10–17* were restricted with *Nco*I and *Bgl*II, respectively. *Arrows* indicate molecular-weight markers of 8.5, 7.4, 6.1 and 4.8 kbp. **B** Schematic diagram of an 8.2-kbp *Eco*RI/*Sal*I fragment subcloned from lambda clone M1–2 and sequenced. The *rp1* homologous ORF is indicated as a *white box* with the PIC20 homologous region of this sequence shown as a *hatched box*. The predicted direction of transcription of this sequence is indicated as an *arrow*. The *obtusely hatched box* represents an ORF with homology to retroviral related RNA polymerases. *Numbers in brackets* represent nucleotide positions and *letters E, D, V* and *S* indicate the position *of EcoRI, Dra*I, *Eco*RV and *Sal*I restriction sites, respectively. *Lines above the figure* delineate a PIC20 homologous 6018-bp *Dra*I restriction fragment and a 4902-bp *Eco*RV/*Dra*I restriction fragment that are described in the text in relation to Figs. 4B and 4D. **C** Schematic diagram showing the 4.3-kbp *Sal*I restriction fragment subcloned from lambda clone M1–13 and sequenced (represented as a *cross hatched box*) and the 1.6-kbp fragment PCR amplified from adaptor-ligated Morex genomic DNA (shown *beneath* as a *smaller hatched box*). Annealing sites of primers P13a, b, c and d, which are described in the text, are shown as *horizontal arrows*. The position of *Bgl*II restriction sites that give rise to a 3.8-kbp restriction fragment are indicated with *vertical arrows*. The full length ORF of the *rp1* homologous gene is indicated as a *white box*.

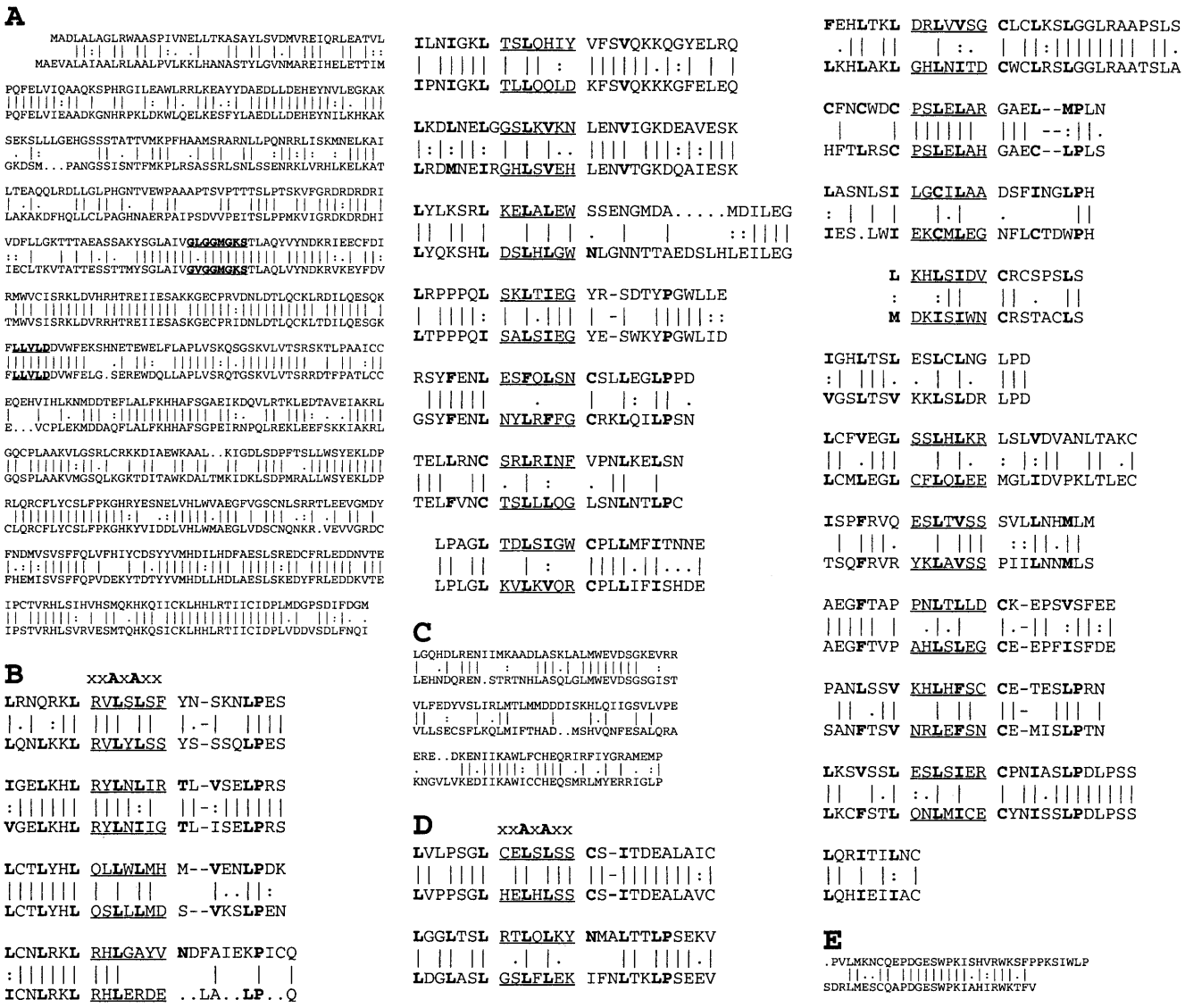


Fig. 2 Bestfit protein sequence comparison between the Rp1-D protein and the related protein partially encoded by lambda M1–13. The Rp1-D protein shown as the upper sequence is arranged into domains A to E as described by Collins et al. (1999), although with minor modification. Domain A of the protein contains the nucleotide binding site of the protein with the P-loop and kinase 2a domains *underlined* and shown in bold. Domains B and D are arranged into leucine-rich repeat units, with conserved aliphatic amino acids that form the repeat unit shown in *bold*. The xx(**A**)x(**A**)xx motifs present in domains B and D are separated from the rest of the sequence by a gap and underlined. Sections C and E can not be arranged into this repeat structure. The full-length protein partially encoded by clone M1–13 is shown as the lower sequence. Identical amino acids are indicated as a *vertical line*, while similar amino acids are indicated as either *one or two dots*, depending upon the degree of similarity.

including barley, under standard hybridisation conditions, (Fig. 1A, lanes 5 and 13). DNA blot analysis with a probe derived from the LRR of the *Rp1-D* gene identifies similar hybridisation patterns in the above grass species, including fragments that are homologous to both probes, suggesting the presence of full-length, homologous genes in these species (not shown). These data indicate that families of NBS-LRR genes, closely related to the maize *rp1* gene, occur in a number of grass species. Barley sequences related to the maize *rp1* gene were subsequently mapped and characterised.

Characterisation of barley *rp1* homologues

A Morex genomic DNA library was screened with the PIC20 probe to isolate barley homologues of the maize *rp1* rust resistance gene. Five haploid genome equivalents of a lambda genomic library were screened, resulting in the isolation of 17 lambda clones (called M1–1 to M1–17). Two lambda clones, M1–2 and M1–13, were subsequently analysed in more detail.

An 8. 2 kb *Eco*RI/*Sal*I fragment was isolated from clone M1–2 and sequenced. Contained within this 8.2-kb fragment is a contiguous ORF of 3885 bp which encodes a protein of 1294 amino acids (Fig. 1B). A bestfit (GCG) sequence comparision between the protein encoded by this ORF and the maize Rp1-D protein identified 64%

and 58% protein similarity and identity, respectively, while 70% DNA identity was observed between the ORFs of these two genes. From this comparison it is apparent that, like the maize *Rp1-D* gene, the ORF of lambda M1–2 encodes an NBS-LRR protein and that no introns are contained within the ORF.

A second ORF is located 879 bp upstream of the translation initiation site of the *rp1* homologous ORF contained upon the 8.2-kb fragment subcloned from lambda M1–2 (Fig. 1B). This second ORF encodes a 619 amino acid protein which shows homology to a large number of retroviral-like RNA polymerases and is most related to a mouse retroviral RNA polymerase (30% identity, 44% similarity, over 399 amino acids; Swissprot accession number p11369). Presumably this sequence is derived from a retrotransposable element present within the barley genome and suggests that the promoter of the M1–2 ORF lies within the 879-bp intervening stretch between these two ORFs.

A 4.3-kbp *SalI* restriction fragment was isolated from lambda clone M1–13 and also sequenced (Fig. 1C). This fragment contains a 3057-bp ORF encoding for a 1019 amino acid polypeptide that is 66% similar to amino acids 263 to 1292 of the Rp1-D protein. As this ORF is juxtaposed next to an arm of the lambda cloning vector and encodes a protein that is smaller than that encoded by both the *Rp1-D* gene and lambda clone M1–2, it was concluded that lambda clone M1–13 does not contain the entire gene. A 1.6-kbp fragment of genomic DNA that flanks the partially encoded gene on clone M1–13 was PCR amplified from Morex genomic DNA (Fig. 1C) as described in the Materials and methods. By combining the sequence data obtained from clone M1–13 with that of the genomic DNA PCR amplification product it is apparent that the full-length gene contains an 3840 bp ORF (Fig. 1C) which encodes a 1279 amino acid protein that is 66% similar and 60% identical to the Rp1-D protein (Fig. 2) and 78% similar and 74% identical to the *rp1* homologous protein encoded by lambda clone M1–2 (not shown).

Comparison between the maize Rp1-D protein and predicted proteins encoded by lambda clones M1–2 and M1–13

The Rp1-D protein has previously been arranged into five separate domains, A to E (Fig. 2) (Collins et al. 1999). The NBS region of the protein is defined by domain A, while the remainder of the protein is subdivided into domains B to E (Fig. 2). Domains B and D of the Rp1-D protein consist of a number of degenerate repeat units which contain conserved leucine-residues in addition to other aliphatic amino acids which form a consensus leucine-rich repeat backbone (Collins et al. 1999). Domains C and E of the protein do not readily fit the consensus leucine rich repeat structure, with domain C essentially bisecting the LRR region of the protein.

Table 1 Amino acid identities (similarities) between *Rp1-D* and predicted barley proteins encoded by clones M1–2 and M1–13

Domain	*Rp1-D* vs. M1–2	*Rp1-D* vs. M1–13	M1–2 vs. M1–13
A	60 (67)	65 (71)	81 (85)
B	60 (66)	59 (67)	73 (77)
C	37 (43)	43 (48)	60 (62)
D	58 (64)	55 (61)	68 (72)
E	63 (67)	75 (79)	78 (81)

The predicted proteins encoded by clones M1–2 (not shown) and M1–13 (Fig. 2) can also be arranged into these five domains. Domain C of these two barley proteins also does not readily fit the consensus LRR repeat structure, and sequence comparisons between the maize and barley proteins show that domain C is the most variable region of all three proteins (Table 1). BLAST searches with domain C from each protein do not identify any homologies common to these sequences on the database. The terminal half of the LRR region (domain D) is the second most variable between the three proteins (Table 1). Domains, B, A and E, in that order generally show increasing levels of amino acid identity within a protein comparison (Table 1).

Regions B and D of the Rp1-D protein contain a total of 169 aliphatic amino acids (shown in bold in Fig. 2) which form the backbone of the leucine-rich repeat units. Sequence comparison between the Rp1-D protein and predicted barley proteins encoded by clones M1–2 and M1–13 for these aliphatic amino acids show 75% identity (84% similarity) and 76% identity (87% similarity), respectively. The level of homology existing between these aliphatic amino acids is higher than that observed between all other regions of these proteins, suggesting that the leucine-rich repeat backbone is structurally conserved.

Diversifying selection has acted upon the β-strand/β-turn motif of domain D of clones M1–2 and M1–13

The GCG programme Diverge was used to analyse the pattern of nucleotide substitutions existing between the genes encoded by clones M1–2 and M1–13. A similar comparison between these barley genes and the maize Rp1-D gene was also attempted, however the levels of nucleotide substitutions existing between the barley and maize sequences were too high to give a meaningful analysis. The type of selection having acted upon these genes was analysed by a comparison of nonsynomous (Ka) and synomous (Ks) nucleotide substitution rates. Table 2 shows the Ka/Ks ratios calculated for the ORFs of clones M1–2 and M1–13 and individual subregions encoding domains A to E of the predicted proteins. Evidence for diversifying selection (Ka/Ks > 1) was observed only for domain D of these two genes, where a Ka/Ks ratio of 1.206 was obtained. The remainder of the genes were subjected to purifying selection (Ka/Ks<1) (Table 2).

Sequence encoding the β-strand/β-turn motifs [xx(A)x(A)xx] of the LRR domains were also analysed for nucleotide substitution patterns; however, codons specifying the conserved aliphatic core (A) of this consensus sequence were not included in the analysis. Strong evidence for diversifying selection acting upon these predicted solvent-exposed residues (x) in domain D was provided by a Ka/Ks ratio of 3.4 (Table 2). The remainder of domain D showed little evidence of positive selection for sequence diversification when codons encoding the β-strand/β-turn region were excluded from the analysis (Table 2). A similar analysis of domain B did not provide any strong indication that this region has been subjected to diversifying selection, including the solvent exposed residues of the xx(A)x(A)xx motif alone (Table 2).

Table 2 Comparison of nonsynomous (Ka) and synomous (Ks) nucleotide substitution rates between the ORFs of lambda clones M1–2 and M1–13 and subregions encoding domains A to E of the predicted proteins

Domain	Ka/KS
Entire ORF	0.670
A-entire	0.423
B-entire	0.644
B[a]-entire minus xx(A)x(A)xx	0.563
B[b]-xx(A)x(A)xx	1.038
C-entire	0.887
D-entire	1.206
D[a]-entire minus xx(A)x(A)xx	1.035
D[b]- xx(A)x(A)xx	3.400
E-entire	0.214

[a] Codons encoding the β-strand/β-turn motif were excluded from the analysis
[b] Only codons encoding the β-strand/β-turn motifs were analysed

rp1 homologues map to three loci in barley

Barley sequences identified by the maize resistance gene probe were mapped in 148 doubled haploid barley progeny derived from a cross between vs. Steptoe and Morex (Kleinhofs et al. 1993). The maize *rp1* probe identified nine polymorphic and four monomorphic restriction fragments between the two barley parental DNAs when digested with the restriction enzyme *Nco*I (Fig. 3A, lanes 1 and 2). Segregation analysis of these RFLPs amongst 148 doubled haploid progeny mapped *rp1* homologous sequences to three loci – *pic20a*, *pic20b* and *pic20c*. One RFLP fragment inherited from the Morex parent and two fragments derived from the Steptoe parent segregated as alleles (marked by * in Fig. 3A) and were mapped to

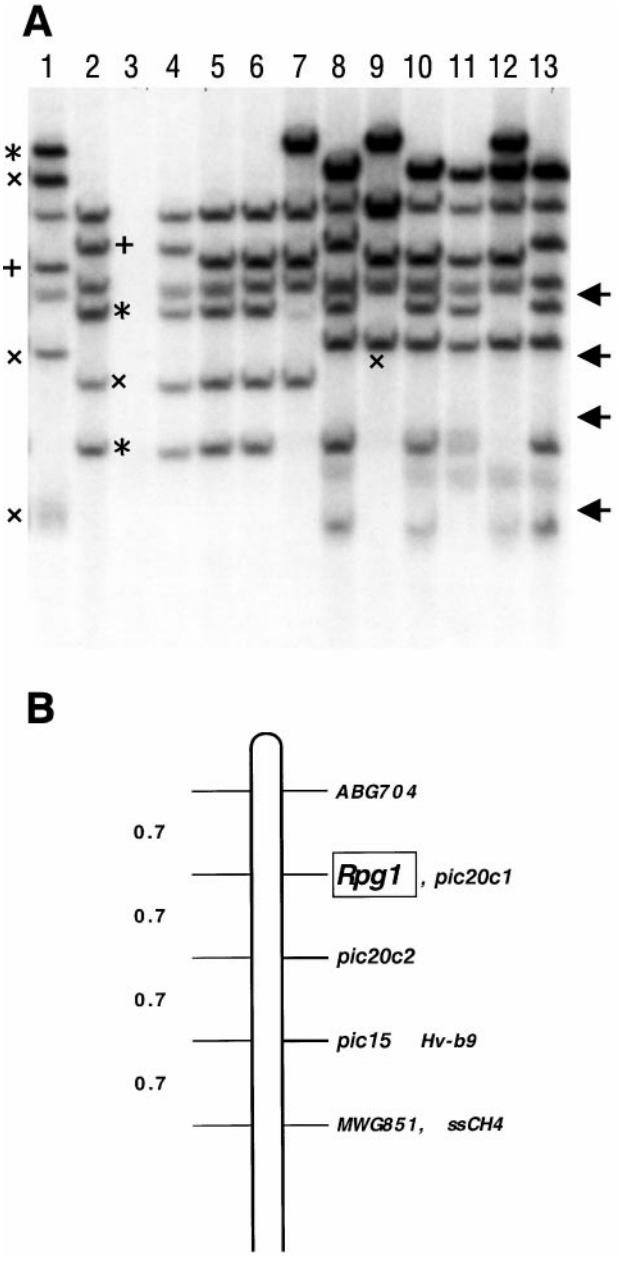


Fig. 3A, B Mapping of barley sequences homologous to the maize *rp1* gene. **A** Mapping of barley *rp1* gene homologues in the Steptoe X Morex doubled haploid family. *Lanes 1, 2* contain Morex and Steptoe genomic DNA, respectively, lanes 4–7 contain genomic DNA isolated from F2 dihaploid individuals SM-199, 179, 164 and 99 which do not contain the *Rpg1* resistance gene, *lanes 8–13* DNAs isolated from the *Rpg1* resistant dihaploid progeny SM-152, 135, 57, 55, 30 and 11, respectively. DNAs were restricted with *Nco*I and hybridised with the PIC20 probe. Bands marked with *, + and *x* in *lanes 1*and *2* are derived from loci *pic20a*, *pic20b* and *pic20c*, respectively, and map to chromosome 1HL, 3HL and 7HS, respectively. A band marked with *x* in *lane 9* also maps to the *pic20c* locus and has been inherited from the Morex parent. *Arrows* indicate molecular-weight markers of 8.5, 7.4, 6.1 and 4.8 kbp. A 4.5-kbp *Morex derived* band is difficult to observe in *lane 11* of this figure due to a lower DNA loading. **B** Map positions of loci identified by NBS encoding RFLP probes in relation to the barley *Rpg1* resistance gene (*boxed*). RFLP probes PIC20, PIC15, Hv-b9 and ssCH4 are derived from the NBS region of predicted NBS-LRR genes, and the loci on chromosome 7HS detected by these probes are indicated. Both the PIC20 and PIC15 probes were isolated from maize genomic DNA, while the Hv-b9 and ssCH4 probes were derived from barley and wheat, respectively. *Numbers* on the *left-hand side* of the figure indicate map distances in centiMorgans

chromosome 1HL (locus *pic20a*), between RFLP markers cMWG733 and ABG702, at 6.4 and 2.1 map units, respectively. One pair of RFLP fragments (marked by + in Fig. 3A) also behaved as alleles and were mapped to chromosome 3HL (locus *pic20b*), between RFLP markers Rrn5S2 and ABG453, at 2.8 and 8.6 map units, respectively. Although difficult to score in an *Nco*I digest this latter map location was subsequently confirmed using an *Nsi*I digest (not shown). No known resistance genes are located at these two map locations. Three RFLP fragments inherited from the Morex parent and one fragment from the Steptoe parent (marked by x in Fig. 3A) were mapped to chromosome 7HS between RFLP markers ABG704 and MWG851 (locus *pic20c*) (Fig. 3B).

rp1 related genes are linked to the barley *Rpg1* stem rust resistance locus

No known barley disease resistance phenotypes map to loci *pic20a* and *pic20b*; however, the *pic20c* locus shows linkage to the *Rpg1* resistance gene, which confers race-specific resistance to stem rust (*Puccinia graminis* f.sp. *tritici*) (Steffenson 1992; Kleinhofs et al. 1993). The *rp1* homologous barley RFLP fragments mapping at the *pic20c* locus (Fig. 3A, marked with an x) cosegregate with the barley *Rpg1* gene in all individual DNAs examined, except for progeny 135 (SM-135) of the doubled haploid family. SM-135 is phenotypically *Rpg1*-resistant but contains only one out of the three Morex-derived bands mapping to the *pic20c* locus (Fig. 3A, marked with an x in lane 9). A single *Nco*I fragment derived from the Steptoe parent mapped to the *pic20c* locus and this was also absent in individual SM-135 (Fig. 3A, lane 9). From the Steptoe × Morex genetic mapping data available on the Grain Genes database it is also apparent that in SM-135 a recombination event has occurred between RFLP markers ABG704 and MWG851, which flank the *Rpg1* locus (Fig. 3B) (Killian et al. 1995). These data suggest that the RFLP pattern of SM-135 has arisen from a recombination event within the *rp1* homologous gene cluster that cosegregates with the *Rpg1* gene, resulting in the loss of some, but not all, bands derived from the Morex parent. An alternative interpretation of this data, shown in Fig. 3B, is that two *rp1* homologous loci are located on chromosome 7HS, with one locus (*pic20c1*) cosegregating with the *Rpg1* gene amongst 148 dihaploid progeny, while the second locus (*pic20c2*) is located 0.7 cM proximal to the *Rpg1* gene.

The segregation of the *rp1* homologous gene family was further characterised by co-restriction with *Eco*RV and *Dra*I endonucleases. A subset of the Steptoe × Mo-

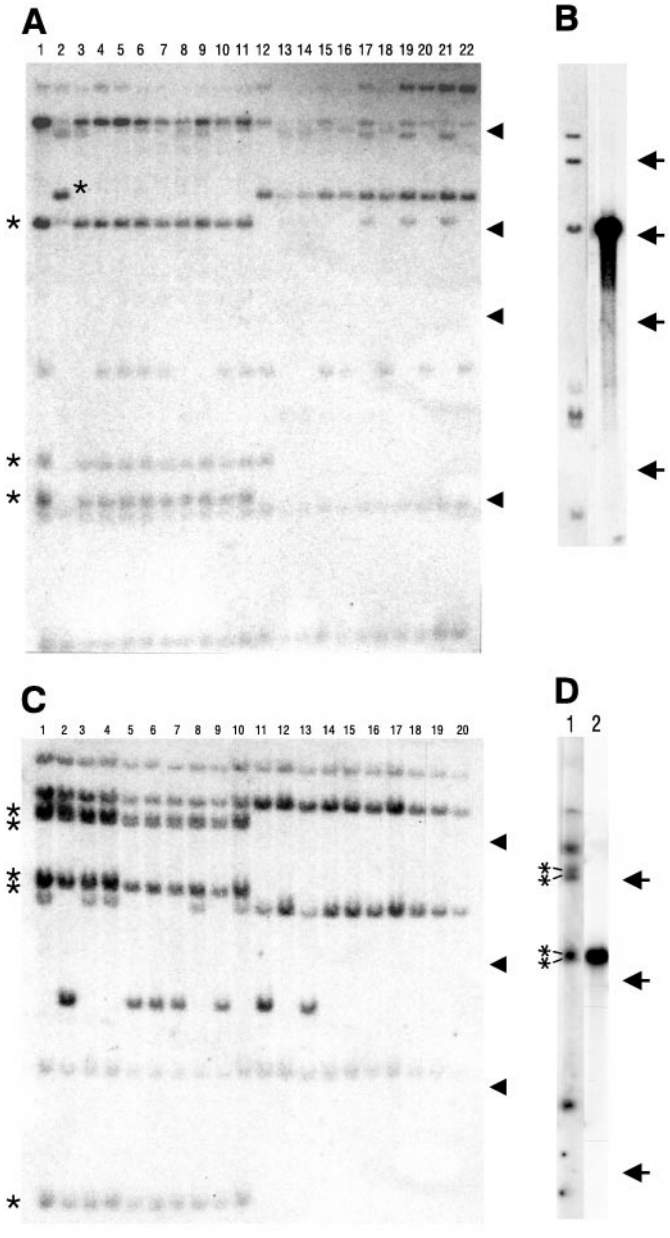


Fig. 4A-D Additional mapping of *rp1* homologues and map locations of clones M1–2 and M1–13. **A** Hybridisation of the *rp1* probe to *Eco*RV- and *DraI* restricted barley DNAs. *Lanes 1*, *2* Morex and Steptoe genomic DNA, respectively, *lanes 3–22* DNA isolated from doubled haploid progeny. DNAs in *lanes 1* and *3–12* were isolated from plants containing the *Rpg1* resistance gene, while the remaining DNAs were isolated from susceptible plants. Bands marked with an *asterix* in *lanes 1* and *2* map to the *Rpg1* locus. *Arrows* indicate molecular-weight markers of 8.5, 4.8, 2.8 and 1.2 kbp. **B** Hybridisation of PIC20 to Morex genomic DNA (*lane 1*) and lambda clone M1–2 DNA (*lane 2*) after restriction with *Eco*RV and *Dra*I. The 4.9-kbp band present in *lanes 1* and *2* was shown to be linked to the *Rpg1* gene in **Fig. 4A**. *Arrows* indicate molecular weights of 8.5, 4.8, 2.8 and 1.2 kbp. **C** Hybridisation of the *rp1* gene probe to *Dra*I-restricted barley DNA isolated from 20 doubled haploid progeny of the Steptoe × Morex mapping family. *Lanes 1–10* DNAs isolated from individuals containing the *Rpg1* resistance gene, *lanes 11–20* contain DNAs isolated from susceptible individuals. Bands marked with an *asterix* in *lane 1* are linked to the *Rpg1* locus. *Arrows* indicate molecular weights of 8.5, 4.8 and 2.8 kbp. **D** Hybridisation of the PIC20 probe to Morex genomic DNA (*lane 1*) and lambda clone M1–2 (*lane 2*) after restriction with *Dra*I. *Arrows* indicate molecular weights of 8.5, 4.8 and 2.8 kbp. A 6-kbp *Dra*I restriction fragment in *lane 2* can be seen to comigrate with a Morex *DraI* restriction fragment (*lane 1*) that is linked to the *Rpg1* locus (Fig. 4C)

rex dihaploid mapping family is shown in Fig. 4A. In this digest, four polymorphic restriction fragments were identified that are linked to the *Rpg1* locus (marked by * in Fig. 4A). A 4.9-kb band derived from the Steptoe parent and mapping to chromosome 1HL (*pic20a*) had an electrophoretic mobility similar to that of a Morex band which is linked to the *Rpg1* locus (Fig. 4A). However, this 4.9-kb Steptoe fragment can be readily distinguished from the similar-sized Morex band, in susceptible individuals, by its reduced hybridisation intensity and its alternative segregation pattern, with a 2-kb Morex allele mapping to chromosome 1HL (*pic20a*). Analysis of the RFLP pattern of SM-135 (Fig. 4A, lane 12) demonstrated that this individual has only one out of the three polymorphic Morex bands mapping to the *Rpg1* locus but also possesses an RFLP fragment derived from the Steptoe parent which maps to the *Rpg1* locus (Fig. 4A). Again this data suggests that if the PIC20 hybridising bands that are linked to the *Rpg1* locus are members of the same gene family, then the RFLP pattern of SM-135 has arisen by a recombination event within the family cluster.

Map location of barley clones M1–2 and M1–13

Sequence analysis of lambda clone M1–2 identified a 6-kbp *Dra*I restriction fragment contained within the clone (Figs. 1B, 4D). Two *Dra*I restriction fragments of 6-kbp can be seen in Fig. 4C which map to the *pic20c* locus, suggesting that the 6-kbp *Dra*I fragment of clone M1–2 (Fig. 4D) is derived from this locus. To verify this map location we subsequently restricted lambda clone M1–2 with both *Eco*RV and *Dra*I, where a 4.9-kbp fragment homologous to the maize probe was predicted from sequence analysis (Fig. 1B). A 4.9-kb band homologous to the *rp1* probe was identified in this lambda clones that comigrated with a *Eco*RV/*Dra*I Morex genomic DNA fragment (Fig. 4B) mapping to the *pic20c* locus (Fig. 4A). From this analysis it was apparent that lambda clone M1–2 encodes a member of the *rp1* homologous gene family mapping to the *pic20c2* locus and linked to the *Rpg1* gene. However, as this 4.9-kbp *Eco*RV/*Dra*I restriction fragment was not present in DNA isolated from individual SM-135 (Fig. 4A, lane 12), presumably the gene encoded on this fragment is not responsible for the *Rpg1* resistance phenotype.

Sequence analysis of clone M1–13 and the 5' flanking region identified by PCR showed that this Morex genomic sequence contains a 3.8-kbp *Bgl*II restriction fragment which is homologous to the PIC20 probe (Fig. 1C). Morex genomic DNA was PCR-amplified with primers P13c and P13d (Fig. 1C), and the resultant 4-kbp product restricted with *Bgl*II to generate a 3.8-kbp restriction fragment (Fig. 5, lane1). This fragment co-migrates with a 3.8-kbp, *rp1* homologous, *Bgl*II restriction fragment of Morex genomic DNA (Fig. 5, lane 2). The *Bgl*II restriction fragment was polymorphic between vs. Morex and Steptoe (Fig. 5, lanes 2 and 3) and shown to map to the

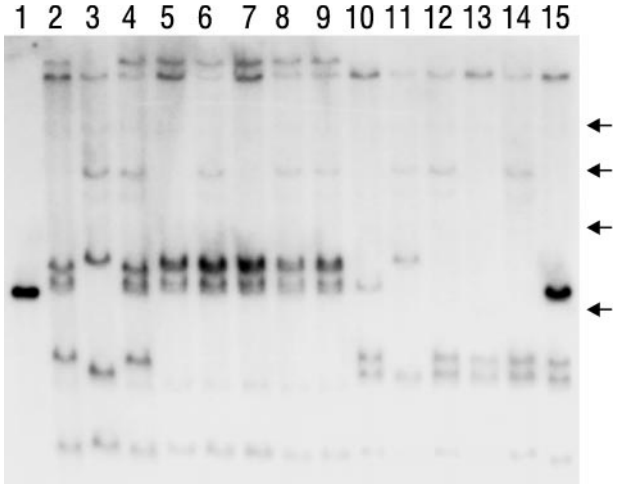


Fig. 5 The *rp1*-related sequence contained on lambda clone M1–13 cosegregates with the *Rpg1* resistance gene amongst 148 dihaploid progeny. Hybridisation of the PIC20 probe to *Bgl*II restricted DNAs. *Lanes 2, 3* Genomic DNAs of barley vs. Morex and Steptoe, respectively, *lanes 4–10* DNAs isolated from *Rpg1* resistant dihaploid progeny SM-150, 151, 152, 155, 156, 157 and 135, respectively. *Lanes 11–14* Genomic DNA isolated from *Rpg*1 susceptible dihaploid progeny, SM-158, 164, 168 and 169. *Lane 1* a *Bgl*II restricted PCR product amplified from Morex genomic DNA using PCR primers P13c and P13d (see Fig. 1C) and loaded with 5 μg of *Bgl*II-restricted human genomic DNA. *Lane 15* Genomic DNA isolated from SM-135 that has been spiked with an equivalent amount of the PCR product contained in *lane 1*. *Arrows* indicate molecular-weight mobilities of 8.5, 6.1, 4.3 and 3.6 kbp, respectively.

Rpg1 locus amongst 30 SM doubled haploid progeny, 11 of which are shown in Fig. 5. DNA blot analysis of SM-135 showed that this individual contains the 3.8-kbp *Bgl*II fragment inherited from the Morex parent (Fig. 5, lane 10). Therefore, the gene partially contained on lambda clone M1–13 is derived from the *pic20c1* locus and cosegregates with the *Rpg1* locus amongst 148 dihaploid progeny and may potentially encode the *Rpg1* resistance phenotype.

Other resistance gene analogues in the *Rpg1* region

In a previous study, NBS-LRR-like genes were PCR-amplified from maize genomic DNA using primer sequences based upon conserved regions present within the NBS domain of known NBS-LRR resistance proteins (Collins et al. 1998). Preliminary RFLP mapping in barley of one these maize resistance gene-like sequences (PIC15) identified a gene family also located at the *Rpg1* region. Of the 148 Steptoe × Morex doubled haploid progeny examined with the PIC15 probe, 2 individuals (SM-135 and SM-194) possessed a recombinant phenotype with respect to the *Rpg1* locus and PIC15 homologous gene family, thereby placing this gene family 1.4 cM proximal to the *Rpg1* gene (Fig. 3B). A BLAST search identified 77% DNA identity between the PIC15 probe and the barley *Hv-b9* resistance gene-like sequence (GenBank AF032687), which has been previously mapped near the

Rpg1 locus (Leister et al. 1998), suggesting that PIC15 probably is a maize orthologue of *Hv-b9*.

A resistance gene-like sequence PCR amplified from wheat genomic DNA (called ssCH4/Rlcs4) also identifies homologous sequences in barley which map in the vicinity of the *Rpg1* locus (Seah et al. 1998). Three Steptoe × Morex doubled haploid progeny (SM-135, SM-194 and SM-185) were recombinant for the ssCH4 locus and *Rpg1* gene, placing the *ssCH4* locus 2.1 cM proximal to the *Rpg1* gene (Fig. 3B). No significant DNA identity exists between the PIC15, ssCH4 and *rp1* probes, although each is derived from an NBS-encoding sequence. Thus, three different resistance gene-like families are located within 2.1 cM of the *Rpg1* gene, and a member of one family, which is related to the maize *rp1* rust resistance gene, cosegregates with this barley stem rust resistance gene in a Steptoe × Morex dihaploid family of 148 individuals.

Discussion

The PIC20 probe, derived from the maize *rp1* gene family located on chromosome 10S, identifies homologous sequences which map to chromosomes 1HL, 3HL and 7HS of barley. Comparative mapping between cereal species has identified gene and marker colinearity over much of the genome, with comparative studies identifying 92% marker colinearity between the maize and *Triticeae* maps (Van Deynze et al. 1995). In contrast to much of the maize genome, chromosome 10 S shows little syntenic relationship to other cereal chromosomes. For example, two RFLP markers, cdo127B and bcd1072B, which are located 13 cM proximal to the maize *rp1* gene, map to *Triticeae* chromosome 1L, while marker cdo504, which is 23 cM proximal to *rp1,* is located on chromosome 5L (Van Deynze et al. 1995). Therefore, while the maize *rp1* gene identifies a related family of sequences on chromosome 7HS of barley, these genes are probably not orthologous to the maize gene.

Cosegregation of at least one member of the *rp1* homologous barley gene family and the *Rpg1* resistance phenotype, located on chromosome 7HS, suggests that a member of this family may encode the *Rpg1* resistance specificity. However, cosegregation does not demonstrate gene function. Mapping of resistance gene analogues in potato, soybean, maize and wheat has shown that NBS-LRR genes (of unknown function) tend to cluster (Leister et al. 1996, 1998; Yu et al. 1996; Collins et al. 1998; Spielmeyer et al. 1998). From our study it can be seen that three different families of NBS-LRR resistance gene-like sequences map within a 2.1-cM interval surrounding the *Rpg1* gene. Likewise, functional resistance genes can also cluster. Within a 1- to 3-cM region of the maize genome four genes conferring rust resistance have been identified – *rp1, rp5, rp6* and *rpp9* (Saxena and Hooker 1968; Wilkinson and Hooker 1968; Hulbert and Bennetzen 1991) – while embedded in the *Pto* resistance gene cluster of tomato, which encodes serine-threonine protein kinases, is an NBS-LRR gene, *Prf*, which is essential for *Pto*-mediated resistance (Salmeron et al. 1996). Our future aim is to determine if a member of the PIC20 homologous gene family located on chromosome 7HS can confer the *Rpg1* rust resistance specificity. Two transgenic strategies have been adopted utilising complementation and cosuppression.

The two barley sequences characterised in this study, which are derived from chromosome 7HS, show evidence of positive selection for sequence diversification. Diversifying selection is rarely observed amongst gene families (Endo et al. 1996); however, analysis of a number of disease resistance gene families shows evidence of just such selection (Parniske et al. 1997; Wang et al. 1998, McDowell et al. 1998; Meyers et al. 1998; Botella et al. 1998). This selection appears to act exclusively upon the predicted solvent-exposed residues of the β-strand/β-turn motif of the LRR domain of these genes, while the remainder of the gene shows evidence of conservative selection (Meyers et al. 1998). Structural analysis of the porcine ribonuclease inhibitor suggests that the β sheet and βα loops of this molecule interact directly with the ribonuclease protein ligand (Kobe and Deisenhofer 1993, 1994). By analogy, the predicted solvent-exposed residues of the β-strand/β-turn motif of plant disease resistance genes have also been suggested to be involved in ligand binding; specifically binding of the corresponding pathogen avirulence gene ligand (Jones and Jones 1997; Thomas et al. 1997; Parniske et al. 1997).

Positive selection for sequence diversification does not always encompass all of the xx(A)x(A)xx motifs present within the LRR domain of disease resistance genes. The *Cf4/Cf9* genes of tomato show evidence of diversifying selection acting upon the first 16 amino terminal LRR repeat units but not the remaining 11 carboxy terminal repeat units (Parniske et al. 1997). Similarly, the lettuce *RC2* gene family shows a high frequency of nonsynomous substitutions in sequence encoding the xx(A)x(A)xx motif of the carboxy terminal half of the LRR domain and not over the rest of the gene (Meyers et al. 1998).

The pattern of diversifying selection observed for the two barley genes analysed in this study most closely resembles that observed for the lettuce *RC2* gene family. Some interesting parallels exist between these two gene families. Both genes encode NBS-LRR proteins that have had diversifying selection acting upon the solvent-exposed residues of the β-strand/β-turn motif of the LRR domain, but this selection is localised exclusively to the carboxy terminus of the LRR domain. The LRR encoding region of the *RC2* genes is bisected by an intron, while the LRR domain encoded by clones M1–2 and M1–13 is bisected by domain C, a region encoding an amino acid sequence that does not readily fit the LRR consensus. This analysis suggests that it is the carboxy terminus of the LRR domain of these two families of resistance proteins that is potentially involved in ligand binding.

No isolated LRR encoding resistance gene has yet been shown to interact directly with a pathogen ligand, even though the corresponding ligand has been identified in a number of cases. Potentially, a second host component may also be required to mediate such a recognition event (Jones and Jones 1997). However, evidence of diversifying selection acting upon the LRR domain of these molecules is consistent with their postulated role in ligand recognition.

In summary, the maize *rp1* rust resistance gene identifies homologous sequences in barley, some of which are located on chromosome 7HS and are linked to the barley *Rpg1* stem rust resistance gene. Several other resistance gene-like sequences have also been mapped within the vicinity of the *Rpg1* gene; however, members of the *rp1*-related gene family identified in this study are located nearest this barley stem rust resistance gene. Future work will determine if barley sequences homologous to the maize *rp1* gene play a role in barley disease resistance.

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References

Anderson PA, Lawrence GJ, Morrish BC, Ayliffe MA, Finnegan EJ, Ellis JG (1997) Inactivation of the flax rust resistance gene *M* associated with loss of a repeated unit within the leucine-rich repeat coding region. Plant Cell 9: 641–651

Botella MA, Parker JE, Frost LN, Bittner-Eddy PD, Beynon JL, Daniels MJ, Holub EB, Jones JDG (1998) Three genes of the Arabidopsis *RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. Plant Cell 10: 1847–1860

Chen FQ, Hayes PM (1989) A comparison of *Horedeum bulbosum*-mediated haploid production efficiency in barley using in vitro floret and tiller culture. Theor Appl Genet 77: 701–704

Collins N, Webb CA, Seah S, Ellis JG, Hulbert SH, Pryor AJ (1998) The isolation and mapping of disease resistance gene analogs in maize. Mol Plant Microbe Interact 11: 968–978

Collins N, Drake J, Ayliffe M, Qing S, Ellis J, Hulbert S, Pryor T (1999) Molecular characterisation of the maize *Rp1D* rust resistance haplotype and its mutants. Plant Cell 11: 1–13

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12: 387–395

Endo T, Ikeo K, Gojobori T (1996) Large-scale search for genes on which positive selection may operate. Mol Biol Evol 13: 685–690

Hughes AL (1995) Origin and evolution of HLA class-I psuedogenes. Mol Biol Evol 12: 247–258

Hughes AL, Nei M (1988) Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. Nature 335:167–170

Hulbert SH, Bennetzen JL (1991) Recombination at the *Rp1* locus in maize. Mol Gen Genet 226: 377–382

Jones DA, Jones JDG (1997) The role of leucine-rich repeat proteins in plant defenses. In: Andrews JH, Tommerup IC, Callow JA (eds) Advances in botanical research vol. 24. Academic Press, New York, pp 89–167

Killian A, Kudrna DA, Kleinhofs A, Yano M, Kurata N, Steffenson B, Sasaki T (1995) Rice-barley synteny and its application to saturation mapping of the barley *Rpg1* region. Nucleic Acids Res 23: 2719–2733

Kleinhofs A, Killian A, Saghai-Laroof MA, Biyashev RM, Hayes P, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86: 705–712

Kobe B, Deisenhofer J (1993) Crystal structure of a porcine ribonuclease inhibitor, a protein with leucine-rich repeats. Nature 366: 751–756

Kobe B, Deisenhofer J (1994) The leucine-rich repeat: a versatile binding motif. Trends Biol Sci 19: 415–421

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newberg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181

Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG (1995) The *L6* gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell 7: 1195–1206

Leister D, Ballvora A, Salamini F, Gebhardt C (1996) A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nat Genet 14: 4221–429

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefert (1998) Rapid reorganisation of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95: 370–375

McDowell JM, Dhandaydham M, Long TA, Aarts MGM, Goff S, Holub EB, Dangl JL (1998) Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the RPP8 locus of Arabidopsis. Plant Cell 10: 1861–1874

Meyers BC, Shen KA, Rohani P, Gaut BS, Michelmore RW (1998) Receptor-like genes in the major resistance locus of lettuce are subject to diversifying selection. Plant Cell 11: 1833–1846

Parniske M, Hammond-Kosack KE, Golstein C, Thomas CM, Jones DA, Harrison K, Wulff BBH, Jones JDG (1997) Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. Cell 91: 821–832

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard R (1984) Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritence, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81: 8014–8018

Salmeron JM, Oldroyd GED, Rommens CMT, Scofield SR, Kim H-S, Lavelle DT, Dahlbeck D, Staskawicz BJ (1996) Tomato *Prf* is a member of the leucine -rich repeat class of disease resistance gene and lies embedded within the *Pto* kinase gene cluster. Cell 86: 123–133

Sambrook J, Fritsch EF, Maniatis TA (1989) Molecular cloning: a laboratory manual., 2nd edition, Cold Spring Harbour Laboratory Press, New York

Saxena KMS, Hooker AL (1968) On the structure of a gene for disease resistance in maize. Proc Natl Acad Sci USA 61: 1300–1305

Seah S, Sivasithamparam K, Lagudah ES (1998) Cloning and characterisation of a family of disease resistance gene analogs from wheat and barley. Theor Appl Genet 97: 937–945

Siebert PD, Chenchik A, Kellogg DE, Lukyanov A, Lukyanov SA (1995) An improved method for walking in uncloned genomic DNA. Nucleic Acids Res 23: 1087–1088

Song W-Y, Wang G-L, Chen L, Kim H-S, Pi L-Y, Gardner J, Wang B, Holsten T, Zhai W-X, Zhu L-H, Fauquet C, Ronald PC (1995) A receptor kinase-like protein encoded by the rice disease resistance gene *Xa21*. Science 270: 1804–1806

Spielmeyer W, Robertson M, Collins N, Leister D, Schulze-Lefert P, Seah S, Moullet O, Lagudah ES (1998) A superfamily of disease resistance gene analogs are located on all homoeologous chromosome groups of wheat (*Triticum aestivum*). Genome 41: 782–785

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JDG (1995) Molecular genetics of plant disease resistance. Science 268: 661–667

Steffenson BJ (1992) Analysis of durable resistance to stem rust in barley. Euphytica 63: 153–167

Steffenson BJ, Miller JD, Jin Y (1993) Detection of the stem rust resistance gene *Rpg1* in barley seedlings. Plant Dis 77: 626–629

Tanaka T, Nei M (1989) Positive Darwinian selection observed at the variable regions of immunoglobulins. Mol Biol Evol 6: 447–459

Thomas CM, Jones DA, Parniske M, Harrison K, Balint-Kurti PJ, Hatzixanthis K, Jones JDG (1997) Characterisation of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in *Cf-4* and *Cf-9*. Plant Cell 9: 2209–2224

Van Deynze AE, Nelson JC, Yglesias ES, Harrington SE, Braga DP, McCouch SR, Sorrells M (1995) Comparative mapping in grasses. Wheat relationships. Mol Gen Genet 248: 744–754

Wang G-L, Ruan D-L, Song W-Y, Sideris S, Chen L, Pi L-Y, Zhang S, Zhang Z, Fauquet C, Gaut BS, Whalen MC, Ronald PC (1998) *Xa21D* encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution. Plant Cell 10: 765–779

Wilkinson DR, Hooker AL (1968) Genetics of reaction to *Puccinia sorghi* in ten inbred corn lines from Africa and Europe. Phytopathology 58: 605–608

Whittaker PA, Campbell AJB, Southern E, Murray NE (1988) Enhanced recovery and restriction mapping of DNA fragments cloned into a new lambda vector. Nucleic Acids Res 16: 6725–6736

Yu YG, Buss GR, Saghai Maroof MA (1996) Isolation of a superfamily of candidate disease resistance genes from soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93: 11751—11756