Theor Appl Genet (2002) 104:1298–1306
DOI 10.1007/s00122-002-0902-8

**N. Rostoks · J. M. Zale · J. Soule · R. Brueggeman**
**A. Druka · D. Kudrna · B. Steffenson · A. Kleinhofs**

# A barley gene family homologous to the maize rust resistance gene *Rp1-D*

Received: 28 August 2001 / Accepted: 14 November 2001 / Published online: 8 May 2002
© Springer-Verlag 2002

**Abstract** Many characterized plant disease resistance genes encode proteins which have conserved motifs such as the nucleotide binding site. Conservation extends across different species, therefore resistance genes from one species can be used to isolate homologous regions from another by employing DNA sequences encoding conserved protein motifs as probes. Here we report the isolation and characterization of a barley (*Hordeum vulgare* L.) resistance gene analog family consisting of nine members homologous to the maize rust resistance gene *Rp1-D*. Five barley *Rp1-D* homologues are clustered within approximately 400 kb on chromosome 1(7H), near, but not co-segregating with, the barley stem rust resistance gene *Rpg1*; while others are localized on chromosomes 3(3H), 5(1H), 6(6H) and 7(5H). Analyses of predicted amino-acid sequences of the barley *Rp1-D* homologues and comparison with known plant disease resistance genes are presented.

**Keywords** Resistance gene analogs · Barley · Stem rust resistance · *Rpg1* · *Rp1-D*

## Introduction

Advancement of genetic mapping and molecular cloning techniques has facilitated the characterization of many plant disease resistance genes in dicot and monocot plants (Hammond-Kosack and Jones 1997). Many of these genes encode proteins containing nucleotide binding site (NBS) and leucine rich repeat (LRR) domains (Michelmore 2000; Pan et al. 2000). The complete genome sequence of *Arabidopsis thaliana* is predicted to encode 149 NBS domain-containing proteins, 128 of which also contain a LRR domain (The Arabidopsis Genome Initiative 2000). Genes encoding NBS-LRR proteins are found in dicots and monocots, although there are some differences in gene structure. NBS-LRR proteins which have a N-terminal TIR (*Drosophila* Toll and human Interleukin Receptor-like) domain have not been found in monocot genomes, whereas NBS-LRR proteins which have an N-terminal coiled-coil domain are present in both dicots and monocots (Pan et al. 2000). Eighty five of the *A. thaliana* genes encode NBS-LRR proteins with a TIR domain and 36 encode proteins with a coiled-coil domain at their N termini, while seven genes do not encode any obvious TIR or coiled-coil domains (The Arabidopsis Genome Initiative 2000).

There are at least 20 genes and gene families encoding NBS-LRR plant disease resistance proteins that have been characterized in some detail. Several genes which confer resistance to fungal pathogens and encode NBS-LRR proteins have been cloned from dicots and monocots. Examples include the *L* and *M* genes from flax (*Linum usitatissimum*) conferring resistance to the flax rust fungus (*Melampsora lini*) (Lawrence et al. 1995; Anderson et al. 1997). The *Rp1* gene family from maize (*Zea mays*) confers resistance to the maize rust fungus *Puccinia sorghi* (Collins et al. 1999). The *I2* genes from tomato (*Lycopersicon esculentum*) confer resistance to wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Ori et al. 1997). The *Mla* genes in barley (*Hordeum vulgare*) provide resistance to powdery mildew caused by *Blumeria* (*Erysiphe*) *graminis* f. sp. *hordei* (Wei et al. 1999).

LRR domains have been shown to be involved in the recognition of specific pathogen pathotypes (Parniske et al. 1997; Dodds et al. 2001). DNA sequences encoding

Communicated by P. Langridge

N. Rostoks · J.M. Zale · J. Soule · R. Brueggeman · A. Druka
D. Kudrna · A. Kleinhofs (✉)
Department of Crop and Soil Sciences,
Washington State University, Pullman, WA 99164, USA
e-mail: andyk@wsu.edu
Tel.: +1-509-335-4389, Fax: +1-509-335-8674

B. Steffenson
Department of Plant Pathology, 495 Borlaug Hall,
1991 Upper Buford Circle, St. Paul, MN 55108-6030, USA

*Present address:*
J.M. Zale · J. Soule, USDA Wheat Genetics, Quality,
Physiology, and Disease Research Unit, 209 Johnson Hall,
Washington State University, Pullman, WA 99164, USA

LRR domains generally are not conserved. The NBS domain contains several highly conserved amino-acid motifs, e.g., P-loop, kinase 2 and GLPLAL (Traut 1994), and may be involved in activation of resistance pathways. This conservation extends across different species. It has been shown that DNA sequences encoding these conserved motifs can be used directly as probes or for the design of PCR primers to isolate homologous sequences from the same or from different species, which could potentially be involved in plant disease resistance (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). Despite sequence similarity, there often is little information on the function of such homologues, hence they are referred to as resistance gene analogs (RGAs). RGAs often map near resistance gene loci (Shen et al. 1998; Deng et al. 2000). However, resolution of genetic maps is not sufficient to conclude that an RGA is the resistance gene. For instance, three rice RGAs were isolated using homology with the *A. thaliana RPS2* gene and were mapped to a locus conferring resistance to bacterial leaf blight. Transformation of susceptible rice varieties, however, failed to confirm these RGAs as resistance genes against the pathogen (Ilag et al. 2000). Since resistance genes are often found in clusters, an RGA genetically co-segregating with a target gene needs to be further confirmed with functional assays.

Besides representing candidate genes for plant disease resistance, RGAs can also provide an insight into the evolution of gene families and plant genomes, and in the generation of disease resistance specificities (Meyers et al. 1999; Ayliffe et al. 2000; Pan et al. 2000).

The utility of RGAs for the isolation of actual disease resistance genes was demonstrated in the case of maize rust resistance gene *Rp1-D*. Collins et al. (1998) amplified 11 classes of RGAs in maize using oligonucleotide primers based on conserved motifs in the NBS domain. One of them, designated pic20, mapped to the maize rust resistance locus *Rp1* on the short arm of chromosome 10 (Collins et al. 1998; Hulbert and Bennetzen 1991). *Rp1-D* was isolated by tagging with *Mutator* and *Dissociation* transposons and the pic20 probe was used to characterize the mutants of the rust resistance gene *Rp1-D* (Collins et al. 1999). It was further used to characterize related sequences in barley, where one of the isolated barley pic20 homologues co-segregated with the barley stem rust resistance gene *Rpg1* among 148 members of the North American Barley Genome Mapping project (NABGMP) Steptoe × Morex doubled-haploid mapping population (Ayliffe et al. 2000). This mapping population has been characterized and is publicly available (http://www.css.orst.edu/barley/nabgmp/nabgmp.htm; Kleinhofs et al. 1993).

Our laboratory has been working on the map-based cloning of two barley stem rust resistance genes *Rpg1* and *rpg4* (Kilian et al. 1997; Han et al. 1999; Druka et al. 2000) which confer resistance against the MCC and QCC pathotypes of the rust fungus *Puccinia graminis* f. sp. *tritici*, respectively.

This study utilized the maize pic20 probe (kindly provided by Dr. A. Pryor), which has 97% homology with the NBS region of the *Rp1-D* gene, to isolate homologous barley sequences and determine their location in the barley genome. Hybridization of the maize pic20 probe to barley genomic DNA identified nine *Hin*dIII RFLP bands, five of which mapped very close, but proximal, to *Rpg1* in a recombinant population representing 3,072 gametes. Other RFLP bands were mapped to chromosomes 3(3H), 5(1H), 6(6H) and 7(5H). All Morex hybridizing bands were mapped genetically and none co-segregated with *Rpg1* or any other mapped barley disease resistance gene. Physical mapping and sequence analysis is also reported. A high degree of amino-acid identity among the proteins encoded by this gene family and to Rp1-D suggests conservation of function and possible involvement in disease resistance.

## Materials and methods

### Southern-blot analysis of barley genomic DNA and the BAC library

The maize pic20 probe provided by Dr. A. Pryor was labeled with [$\alpha$-$^{32}$P] dCTP (New England Nuclear) using the RTS RadPrime DNA labeling system (Gibco BRL) and hybridized either to barley genomic DNA blots or to the arrayed barley Morex BAC library (Yu et al. 2000). For genomic blots, DNA was digested with restriction enzymes following the manufacturer's recommendations (New England Biolabs, MBI Fermentas, Gibco BRL) and transferred to nylon membranes (New England Nuclear) by the alkaline transfer procedure. Hybridizations were at 62 °C and the final wash was at 62 °C with 0.5 × SSC.

### Genetic mapping

Initial genetic mapping used the Steptoe × Morex "minimapper" population consisting of 35 lines selected from the original NABGMP 150 *Hordeum bulbosum*-derived doubled-haploid line (HbDHL) population (Kleinhofs et al. 1993). The "minimapper" recombinants were selected to allow placement of probes to specific bins on the Steptoe × Morex map (http://barleygenomics.wsu.edu/Macdraw/1-150.jpg). High resolution mapping was with recombinants between the *Rpg1* flanking markers ABG704 and ABG077 isolated from the following populations: Steptoe × Morex (Hb and anther culture DHL, 370 gametes), Harrington × TR306 (HbDHL, 450 gametes), Harrington × Morex (HbDHL, 244 gametes), Steptoe × Q21861 (HbDHL, 144 gametes and F2 derived homozygotes, 1,500 gametes), Dicktoo × Morex (HbDHL, 244 gametes), Q21861 × SM89010 (anther culture DHL, 120 gametes), giving a total of 3,072 gametes. In the case of F2 populations, the lines with recombination between the flanking markers were selfed and homozygous recombinants selected by progeny analysis. Following homozygote recovery the *Rpg1* phenotype was determined.

### Disease resistance phenotyping

*Rpg1* disease resistance phenotyping was done as previously described (Kilian et al. 1994).

### Cloning and sequencing

The Morex BAC clones positive with the maize pic20 probe were isolated, DNA extracted, digested with *Hin*dIII, Southern blotted and hybridized with the pic20 probe. Representative BAC clones

**Table 1** List of plasmids, probes and GenBank accession numbers of the barley pic20 homologues

| Barley pic20 homologue | Plasmid used for mapping | BAC clone used for subcloning | Restriction enzyme/insert size | Plasmid(s) used for sequencing | GenBank accession number |
|---|---|---|---|---|---|
| A | p40-50P0.5-7 | 440K12 | *Pst*I/0.5 kb | p40-50-1 | AF414171 |
| B | pXba-HIII 1.8-3 | 326C19 | *Xba*I-*Hin*dIII/1.8 kb | p26-38[a] | AF414172 |
| C | pC_EI-P-4,5 | 326C19 | *Eco*RI-*Pst*I/0.5 kb | p26-57[a] | AF414173 |
| D | p40-50P0.5-7 | 440K12 | *Pst*I/0.5 kb | p40-29;*Pst*0.8-2[a] | AF414174 |
| E | pNRG015 | 114A03 | *Hin*dIII/2.8 kb | pNRG015 | AF414175 |
| F | p93-40 | 693F14 | *Hin*dIII/2.2 kb | p93-40 | AF414176 |
| G1 | p76-6<br>pNRG002 | 740O10 | *Hin*dIII/2.5 kb<br>*Hin*dIII/1.9 kb | pNRG002[b]<br>pNRG005[b]<br>pNRG006[b]<br>p76-6[b] | AF414177 |
| G2 | pNRG017 | 720K20 | *Hin*dIII-*Nsi*I/0.32 kb | pNRG017[c]<br>pNRG018[c] | AF414178 |
| H[d] | p323 1.4 -1 | 568G08 | *Hin*dIII/1.4 kb | p323 1.4 -1 | AF414179 |
| I[d] | p68-4 | 568G08 | *Hin*dIII/1.2 kb | p68-4 | AF414179 |

[a] Complete sequence of the region homologous to the maize pic20 was obtained by direct sequencing of the BAC clone
[b] Overlapping plasmid clones cover the 6.3-kb sequence encompassing the pic20G1 gene homologous to the maize *Rp1-D* gene
[c] Overlapping plasmid clones cover the region homologous to maize pic20
[d] The H and I bands represent a contiguous stretch of genomic DNA cleaved by the *Hin*dIII restriction enzyme as confirmed by direct sequencing of the BAC clone

containing every band detected in the Morex genomic digest were digested with *Hin*dIII and shotgun-cloned into plasmid pBluescript II KS (Stratagene) by standard techniques (Sambrook et al. 1989). Plasmid clones containing the barley pic20 homologue inserts were identified by Southern-blot analysis. These were sequenced using the BigDye terminator system on an ABI Prizm 377 DNA sequencer (PE Biosystems) at the Bioanalytical Center, Washington State University, Pullman, Wash. In some cases primers were designed and the BAC clone DNA sequenced directly. A list of plasmid clones used for sequencing is given in Table 1.

### Barley pic20 homologue cDNAs

Four arrayed barley cDNA libraries Hv_CEb (CI16151), HvSMEf, HvSMEg and HvSMEh (Morex), consisting of approximately 50,000 clones each, were hybridized with maize pic20, and barley pic20C, E, G1 and G2 probes. The arrayed cDNA library filters were obtained from Dr. R. Wing (Clemson University Genomics Institute) and are described at (http://www.genome.clemson.edu/projects/barley/).

Barley pic20 homologue cDNAs were PCR-amplified from the barley CI16155 cDNA library HV_CEa [*E. graminis* infected and uninfected seedling green leaf library prepared and provided to us by Dr. T. Close (Department of Botany and Plant Sciences, University of California, Riverside) from plant materials provided by Dr. R. Wise (Corn Insects and Crop Genetics Research, USDA-ARS, Iowa State University)] and from the barley Morex cDNA library (ovary 1 day after pollination) provided by Dr. P. Hayes (Department of Crop and Soil Sciences, Oregon State University). Degenerate primers designed from the conserved kinase 2 and GLPLAL motifs in the NBS domain were used. Primer sequences were: Kin2-F1 (5′-ARRTTYYTSMTHGTRMTNGATGAT-3′); Kin2-F2 (5′-ARRTTYYTSMTHGTRMTNGATGAC-3′); SPL-R1 (5′-CCCAYWRYWYTKGCHGCYARAGGWGA-3′).

Approximately 100 ng of DNA from cDNA library pools were used for amplification using 20 pmol of each primer and Red *Taq* polymerase according to the manufacturer's recommendations (Sigma) in a 20µl vol. Cycling parameters were: initial denaturation for 5 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min 30 s at 60 °C and 1 min at 72 °C, and a final incubation at 72 °C for 7 min.

### Rice pic20 homologue BAC clones

The rice cv Nipponbare 11 × BAC genomic clone library OSJNBa (obtained from Dr. R. Wing; Clemson University Genomics Institute) was screened with the maize pic20 probe under the same conditions as the barley BAC library. Addresses of positive BAC clones were used to determine their location in rice BAC fingerprint contigs (http://www.genome.clemson.edu/projects/rice/fpc/) which are anchored to genetic markers in the rice genome. If the rice genome sequence was available, blastn was used to identify regions homologous to the maize pic20 probe.

### DNA and deduced amino-acid sequence analysis

DNA and deduced protein sequences were initially analyzed on the NCBI WWW page (http://www.ncbi.nlm.nih.gov/). Alignment of amino-acid sequences was performed using CLUSTAL_X 1.81 (Thompson et al. 1997). The presence of a coiled-coil motif in amino-acid sequences was predicted by the PEPCOIL program (Lupas et al. 1991) from the Emboss package at the UK Human Genome Mapping Project WWW page (http://www.hgmp.mrc.ac.uk).

## Results

### Genetic and physical mapping of the barley pic20 homologues

Hybridization of the maize pic20 probe to *Hin*dIII-digested barley genomic DNA identified nine bands in Morex and a lesser number in Steptoe and Q21861. The nine Morex *Hin*dIII bands were designated A to I (Fig. 1A). All bands were mapped with the Steptoe × Morex "minimapper" population (see Materials and methods). Bands that were not polymorphic in *Hin*dIII were mapped with other restriction enzymes and their identity confirmed by hybridization with barley specific probes developed from BAC clones (Table 1). Genetic

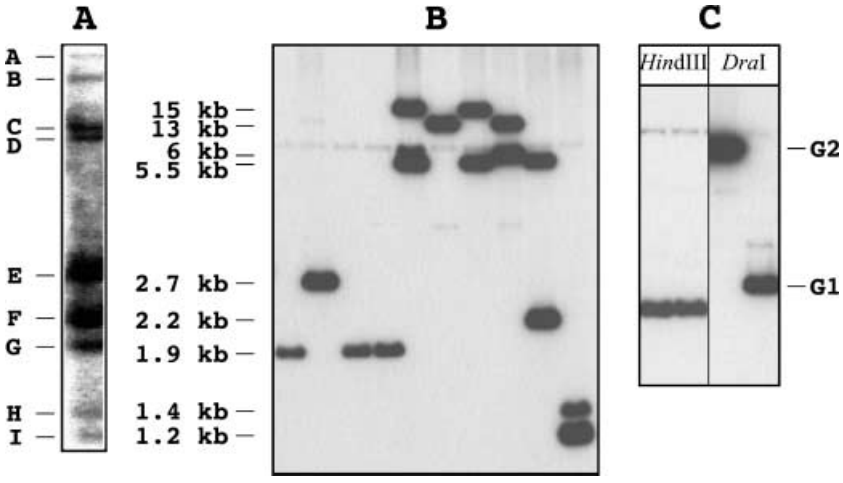


**Fig. 1A–C** The maize pic20 probe identifies ten RFLP bands in the barley Morex genomic DNA and BAC clones. **A** Hybridization of the maize pic20 probe to *Hin*dIII-digested Morex genomic DNA identified nine RFLP bands designated A to I starting from the top. **B** Hybridization of the pic20 probe to *Hin*dIII-digested BAC clones. The approximate size of the bands is indicated on the left. **C** Hybridization of the pic20 probe to *Dra*I-digested G-band BAC clones shows two different size groups designated G1 and G2

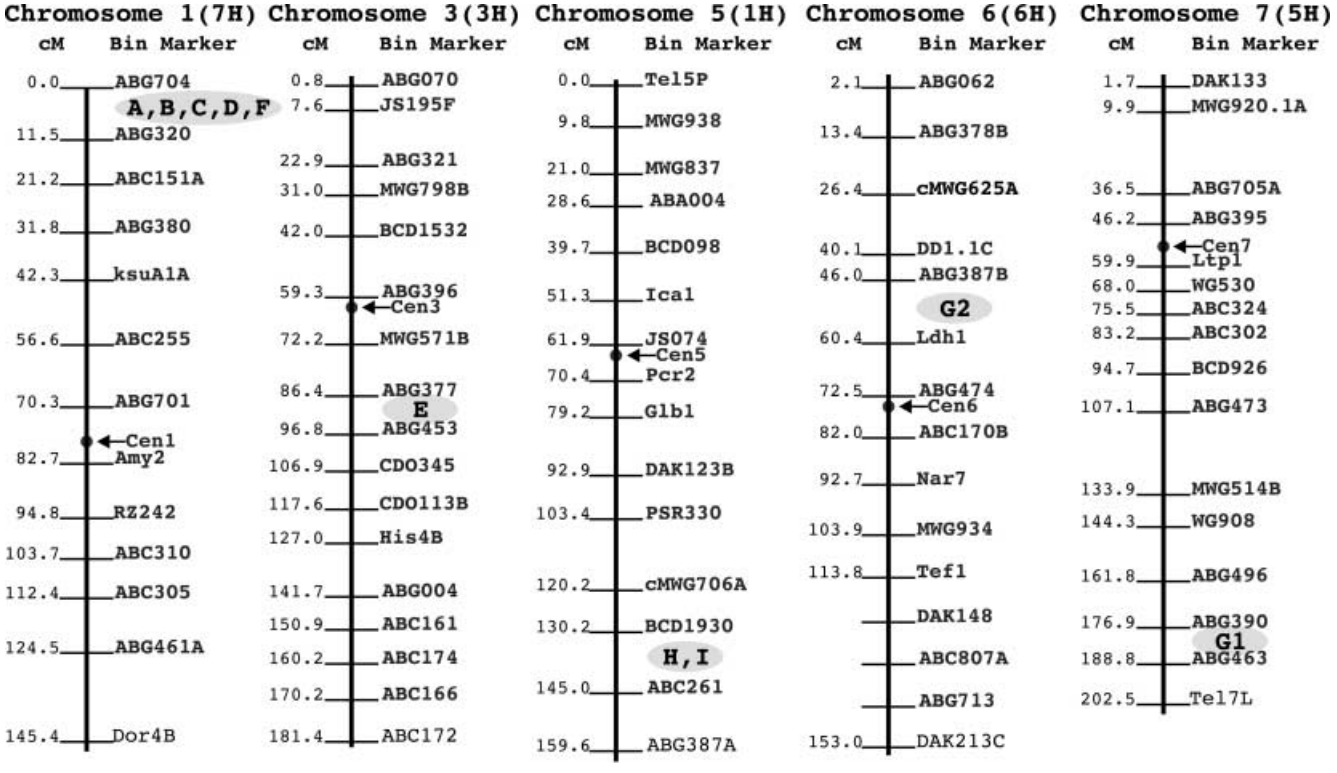


**Fig. 2** Chromosomal location of the barley pic20 homologues on the barley Steptoe × Morex bin map. The barley pic20 homologues are designated by letters A–I

map locations of the barley pic20 homologues are summarized in Fig. 2. Bands A, B, C, D and F mapped to a complex locus on chromosome 1(7H) bin 001 near the barley stem rust resistance gene *Rpg1*. In fact, the A and D bands co-segregated with *Rpg1* in this limited-resolution mapping population. The E band mapped to chromosome 3(3H) bin 008 and bands H and I co-segregated and mapped to chromosome 5(1H) bin 013. The H and I bands appeared to represent the same locus and were confirmed to be *Hin*dIII fragments of the same gene by sequencing (see below). Mapping of the G band presented difficulties until it was realized that it consisted of two different, but identical size, *Hin*dIII fragments (Fig. 1C). These were subcloned, designated G1 and G2 and mapped to chromosomes 7(5H) bin 014 and 6(6H) bin 005, respectively.

Bands A, B, C, D and F were mapped at high resolution using recombinants representing 3,072 gametes (see Materials and methods). The A and D bands co-segregated with one another and were located nearest to the *Rpg1* gene, but proximal to it by nine cross-overs out of the 3,072 gametes. The F band was proximal to the A/D locus by one cross-over and the B and C bands co-segregated with one another and were three additional cross-overs proximal to the A/D locus (Fig. 3A).

The barley Morex BAC library (Yu et al. 2000) was screened with the maize pic20 probe. Sixty BAC clones

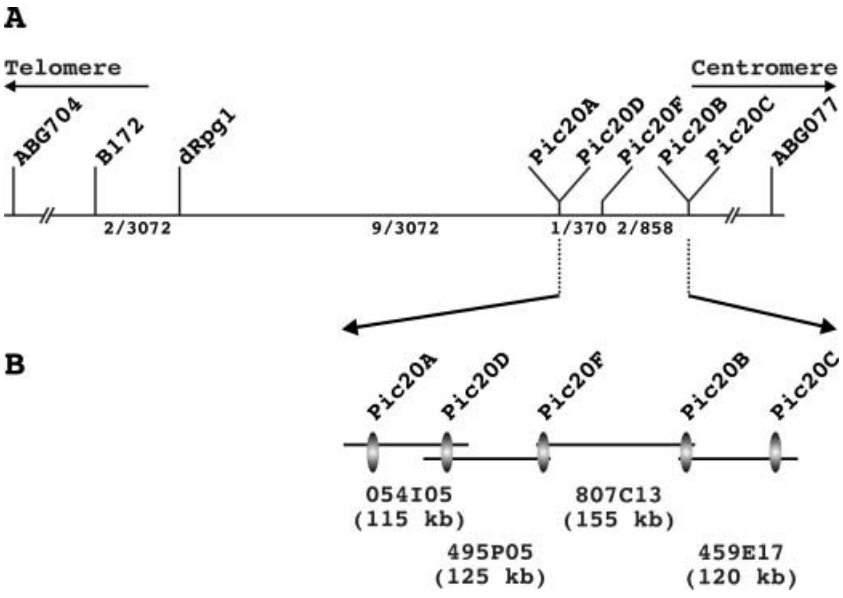


**Fig. 3A, B** Genetic and physical map of the barley chromosome 1(7H) *Rpg1* region. **A** Genetic map of the *Rpg1* region based on recombinant populations derived from up to 3,072 gametes. The genetic distances are expressed as the number of cross-overs per gametes analyzed. **B** Approximately 400-kb BAC clone contig encompassing barley pic20 homologues A, B, C, D and F

were selected and their *Hin*dIII digests confirmed that all of the RFLP bands of the Morex genomic DNA hybridizing to the maize pic20 probe were recovered (Fig. 1B). A complete list of BAC clone addresses can be found at http://barleygenomics.wsu.edu/pic20_BACs.html. Detailed analysis showed that the bands A and D, D and F, F and B, and B and C were sometimes found on the same BAC clone. Thus, it was possible to assemble these BAC clones into a contig covering roughly 400 kb (Fig. 3B). This contig was further confirmed by fingerprinting using *Hin*dIII digestion and hybridization with total barley genomic DNA (data not shown). The BAC clones specific to the bands E, G1, G2 and H/I were identified, but were not assembled into physical contigs.

## Cloning and sequence analysis of the barley pic20 homologues

BAC clones containing barley pic20 homologues were digested with *Hin*dIII and shotgun-cloned into a plasmid vector. The maize pic20 probe was used to identify clones containing barley homologues. Most of the larger *Hin*dIII clones could not be used for genetic mapping due to the presence of repetitive sequences, therefore they were further subcloned. Plasmid clones used for mapping are listed in Table 1 along with restriction enzymes used for subcloning and insert sizes.

Pic20 positive plasmid clones were sequenced in the region homologous to the maize pic20 probe and surrounding regions. Plasmids used for sequencing and GenBank accession numbers are given in Table 1.

Sequence analysis of the barley pic20 homologues indicated that most of them were uninterrupted open reading frames similar to the maize *Rp1-D* gene and thus represented functional genes. Exceptions were barley pic20C and pic20G2, which contained a nonsense mutation in the NBS region or a retrotransposon insertion upstream of the kinase 2 motif, respectively. Two barley cDNAs, identical to the pic20A and pic20C clones, were identified (GenBank accession numbers AF414181 and AF414180, respectively). The pic20A cDNA was amplified from a Morex ovary cDNA library using Kin2-F2 and SPL-R1 primers, while the pic20C cDNA was amplified from the CI16155 cDNA library HV_CEa using Kin2-F1 and SPL-R1 primers. Screening of four arrayed barley cDNA libraries (see Materials and methods), totaling approximately 200,000 clones, with the maize pic20 probe did not yield positive clones (data not shown).

Deduced amino-acid sequences (Fig. 4) covering the region homologous to the maize pic20 were aligned using Clustal_X 1.81 (Thompson et al. 1997). The kinase 2 motif of the NBS domain was perfectly conserved among the barley pic20 homologues and with the maize Rp1-D translated sequence. Similar to Rp1-D, the barley pic20 homologue amino-acid derived sequence had only imperfect kinase 3 and GLPLAL motifs.

The only published barley pic20 homologue sequence (Ayliffe et al. 2000; M1-13 clone) was identical to our pic20A amino-acid sequence and probably represented the same gene. The other barley pic20 DNA sequences appeared to be unique in a search of the non-redundant barley sequence database, indicating that we have isolated eight new barley RGAs.

## Comparison of the barley pic20G1 band with the maize *Rp1-D*

The barley pic20 homologue G1, which is located on chromosome 7(5H), possibly in a syntenous position to the maize *Rp1* locus (see Discussion), was characterized more extensively. A 6.3-kb sequence was obtained from overlapping plasmid clones. The NCBI ORF Finder indicated a 1,282 amino-acid open reading frame starting


**Fig. 4** Alignment of the deduced amino-acid sequences of the barley pic20 homologues and maize *Rp1-D*. Only regions homologous to pic20 were used in the alignment, starting with the kinase-2 motif LLVLD and encompassing kinase 3 and the GLPLAL motifs, which are *underlined*. The stop codon in the C band sequence is indicated by "!". Alignment quality is indicated below the amino-acid sequences as follows: "*" - identical residues, ":" - strongly similar residues, "." - weakly similar residues

with an ATG. The same ORF was predicted by the GENSCAN program (http://genes.mit.edu/GENSCAN.html) using *Arabidopsis* parameters (Burge and Karlin 1997). The region upstream of the ORF had no significant match in Blastn and Blastx searches. The region downstream from the ORF showed homology to *A. thaliana* putative non-LTR retrotransposon reverse transcriptase.

The best match of the 1,282 amino-acid ORF in Blastp was with the maize Rp1-D protein (56% identity, 70% similarity), therefore further comparative analysis was performed. Similar to Rp1-D, the GENSCAN-predicted pic20G1 gene did not have introns in the coding sequence. The *Rp1-D* mRNA analysis indicated the presence of introns both in 5′- and 3′-untranslated regions (Collins et al. 1999). The presence of such introns in the pic20G1 gene is possible, since there are potential splice sites upstream of and downstream from the coding region, but the cDNA sequence would be required to prove it.

Amino-acid similarity between Rp1-D and pic20G1 was not confined to the NBS domain, but extended over the entire length of both sequences. The sequences N-terminal from the P-loop motif were least homologous, showing only 45% identity (62% similarity). The sequences exhibited notable conservation in the NBS region between the P-loop and GLPLAL motifs (71% identity, 85% similarity) with the P-loop and kinase 2 motifs being identical. The LRR regions of pic20G1 and Rp1-D (as defined in Collins et al. 1999) are 56% identical (68% similar) and some parts of the barley sequence can be arranged into leucine-rich repeat units (data not shown). Characteristic of monocot NBS-LRR proteins, Rp1-D did not have a TIR domain, but it also appeared to be lacking the coiled-coil or leucine zipper motifs (Collins et al. 1999). The predicted amino-acid sequence of the barley pic20G1 also did not have the coiled-coil characteristics as determined by the PEPCOIL program (Lupas et al. 1991) from the EMBOSS package run at the default settings.

## Rice pic20 homologues

The rice cv Nipponbare 11× BAC library was screened with the maize pic20 probe and 20 clones were selected. A complete list of BAC clone addresses can be found at http://barleygenomics.wsu.edu/pic20_BACs.html.

Localization of these rice pic20 homologues in the rice genome was determined by using BAC clone fingerprinting data available from Clemson University Genomics Institute (http://www.genome.clemson.edu/projects/rice/fpc/). Fifteen BAC clones were from the fingerprint contig located on rice chromosome 1 near the genetic marker *PIB1*. One BAC clone was from a contig on chromosome 2 near the marker R3393, while another BAC clone belonged to a contig on chromosome 5 near the marker S1780. Three of the BAC clone addresses were not found in the fingerprint database. The chromosome 1 region has been sequenced by the Japanese Rice

Genome Program. Rice pic20 homologue BAC clones overlapped with sequenced rice PAC clones AP003224 and AP003368. A Blastn search of the maize pic20 sequence against PAC clone sequences identified one region homologous to pic20 on PAC clone AP003224 and three regions on PAC clone AP003368. These PAC clones overlapped in one of the pic20 homology regions; therefore, three different loci homologous to the maize pic20 probe were identified on rice chromosome 1 and at least one each on chromosomes 2 and 5.

## Discussion

The maize pic20 probe was used to identify, map and characterize barley homologues of the maize rust resistance gene *Rp1-D*. We showed that five of the barley Morex pic20 *Hin*dIII bands map to a complex locus near the barley stem rust resistance gene *Rpg1* (Fig. 3A). High-resolution genetic mapping showed that none of them co-segregated with the *Rpg1* locus, and therefore are not *Rpg1*. Ayliffe et al. (2000) mapped the barley pic20 homologues to three loci: *pic20a* on chromosome 5(1H), *pic20b* on chromosome 3(3H) and *pic20c* on chromosome 1(7H). They suggested that the *pic20c* locus might be separated into *pic20c1* and *pic20c2* loci, the first of which co-segregated with *Rpg1* in the NABGMP 148 DHL Steptoe × Morex mapping population they used (two lines missing). The *pic20c1* locus (Ayliffe et al. 2000) probably corresponded to our pic20A or D bands, which mapped closest to *Rpg1*, while the *pic20c2* may have corresponded to either of pic20F, B or C bands. Other loci, *pic20a* and *pic20b*, mapped to similar places as our pic20H, I bands and pic20E band, respectively. We have mapped two additional loci (G1 and G2) on chromosomes 6(6H) and 7(5H), respectively.

Genetic mapping of the pic20A, B, C, D and F bands in combination with the BAC clone contig encompassing these bands (Fig. 3) enabled us to estimate the ratio between genetic and physical distances in this chromosome region. The genetic distance between the pic20A/D locus and the pic20B/C locus was estimated to be 0.67 cM (three cross-overs/450 gametes), considering only the recombinants recovered from the Steptoe × Morex cross. The estimated physical distance between the pic20A and pic20C bands, based on the Morex BAC clone contig, was approximately 350 kb which yields a genetic to physical distance of about 525 kb per cM. However, in a 1,644-gamete Steptoe × Q21861 population, no recombinants were found between the markers pic20A and S1558 (found on the same BAC clone as pic20C). Southern blots of *Hin*dIII-cut DNA suggested that pic20B, C and F homologues may not be present in the Q21861 parent, or did not cross hybridize with the maize pic20 probe (data not shown). Thus, combining genetic/physical distance calculations from different crosses may not be meaningful, particularly in regions of complex disease resistance loci where different parents may contain different members of a complex gene family.

The barley chromosome 1(7H) region between RFLP markers ABG704 and ABG077 appeared to be rich in RGAs. Apart from *Rpg1*, it contained five barley pic20 homologues, the NBS-LRR type RGA named B9 (Leister et al. 1998), the pic15 locus containing RGAs pic15, pic25, pic25-1 and pic27 (Collins et al. 2001), as well as several other RGAs (Brueggeman and Kleinhofs, unpublished). Recently Tacconi et al. (2001) mapped a new barley leaf stripe resistance gene *Rdg2a* to chromosome 1(7H) 2.5 cM distal to the RFLP marker MWG2018 and 5.8 cM distal to RFLP locus ABG704. However, in the NABGMP Steptoe × Morex DHL mapping population MWG2018 mapped approximately 3.4 cM proximal to ABG704. Since both probes hybridized to several bands it is possible that one or both were mapped to a different locus by Tacconi et al. (2001). Due to the genetic proximity, it is nevertheless possible that the barley pic20 homologues could represent candidate genes for *Rdg2a*.

In maize, the *Rp1* locus mapped to the short arm of chromosome 10 which has been suggested to be syntenous to barley chromosomes 7(5H) and 2(2H) (Moore et al. 1995; Bennetzen and Freeling 1997). The barley pic20 homologue cluster on chromosome 1(7H) was out of synteny with maize and was not likely to be orthologous to the *Rp1* locus (Ayliffe et al. 2000). The barley pic20G1 band is located on the chromosome 7(5H) bin 014 approximately 50 cM distal to the marker CDO504 which is located 23 cM proximal to the *Rp1* locus (Van Deynze et al. 1995). Thus, the barley pic20G1 potentially could represent an ortholog of one of the maize *Rp1* genes, although other markers linked to maize *Rp1* do not sustain the syntenic relationship. In general, the maize chromosome 10S does not seem to have good synteny with any of the Triticeae chromosomes (Van Deynze et al. 1995).

Han et al. (1999) analyzed a rice BAC clone covering the region syntenous to barley *Rpg1* between the markers B122 and B24. Based on the B24 and B122 map locations in barley this region should also include the rice homologues of maize pic20. Fifteen putative rice genes were identified in the analyzed region, but none of them exhibited NBS-LRR characteristics. It was concluded that a *Rpg1* homologue was not present in the syntenous position in rice. Since no genes encoding NBS-LRR proteins were found by us (Han et al. 1999) or the extensive sequencing reported by the Rice Genome Program (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl), it can be concluded that this rice genome region does not contain pic20 homologues.

Ayliffe et al. (2000) showed that the maize pic20 probe hybridized to several rice genomic DNA RFLP bands. A Blastn search of the rice non-redundant database with the maize pic20 sequence revealed only one sequence on rice chromosome 1 that may have high-enough homology to hybridize under standard conditions (AP003224; average homology approximately 85% over 180 bp). This suggested that most of the rice homologues of maize pic20 remained to be discovered. Therefore we screened the rice cv Nipponbare 11× BAC library with

the maize pic20 probe. Pic20-positive BAC clone addresses were used to find their position in BAC fingerprint contigs (http://www.genome.clemson.edu/projects/rice/fpc/). We found a cluster of three rice pic20 homologues on chromosome 1 in a region which has been sequenced by the Japanese Rice Genome Program (AP003224 and AP003368), which was similar to the barley pic20 homologue cluster on chromosome 1(7H), but out of synteny with barley and maize. Fifteen rice BAC clones were found for this region in good agreement with the 11× coverage of the BAC library. In addition, one BAC clone each from chromosome 2 near genetic marker R3393 and from chromosome 5 near marker S1780 was identified with the maize pic20 probe.

Overall amino-acid identity among the barley pic20 homologues ranged between 58% to 84%, while their identity to maize pic20 remained fairly constant at 59% to 64%. Sequences of the barley pic20 homologues clustered on chromosome 1(7H) were more conserved among themselves (69% to 84% identity) and may represent gene-duplication or gene-conversion events.

The translated pic20A (M1-13; Ayliffe et al. 2000) gene amino-acid sequence exhibited strong homology and a similar arrangement of protein domains with *Rp1-D* (Ayliffe et al. 2000). More-extensive comparison with Rp1-D was performed in the case of the barley pic20 homologue, pic20G1. As in the case of pic20A, pic20G1 and Rp1-D are homologous over the entire length of both proteins, although the NBS domain exhibited the highest degree of conservation. All conserved motifs in the NBS regions of pic20G1 and Rp1-D were highly similar. Notably, the LRR regions exhibit 56% identity, although there were regions with little homology, as well as amino-acid deletions or insertions that may be important for specific function of the proteins.

In contrast, comparison of pic20G1 with the barley NBS-LRR disease resistance gene *Mla6* conferring resistance to *B.* (*E.*) *graminis* f. sp. *hordei* (Halterman et al. 2001) translated sequence showed only 27% overall identity (45% similarity). Homology was limited mostly to the conserved motifs in the NBS domain and leucine residues in the LRR domain (data not shown). No homology was detected between sequences N-terminal to the P-loop, which is consistent with lack of the coiled-coil structure in the predicted pic20G1 protein. The difference of the barley pic20 homologues from Mla6, and the remarkable homology to Rp1-D, suggested a common origin and perhaps a similar function. Although cDNAs were isolated for pic20A and C, the expression level of the barley pic20 homologues appeared to be low. No cDNA clones were found among 200,000 arrayed cDNA clones and only one cDNA of each pic20A and C were amplified by PCR from bulk libraries using degenerate primers. We can not exclude, however, the possibility that cDNA clones from an inappropriate tissue were analyzed or that the genes may need to be induced.

In summary, the barley pic20 homologues represent a gene family that has retained high homology to the maize rust resistance gene *Rp1-D*. However, they do not co-segregate genetically with the barley stem rust resistance gene *Rpg1* nor with any other well-mapped disease resistance gene.

**Acknowledgements** This is Scientific Paper No. 0208-01 from the College of Agriculture and Home Economics Research Center, Washington State University, Pullman, Wash.; Project 0196. Research was supported by USDA/NRI grant No. 9901325 to A.K.

## 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

Ayliffe MA, Collins NC, Ellis JG, Pryor A (2000) The maize *rp1* rust resistance gene identifies homologues in barley that have been subjected to diversifying selection. Theor Appl Genet 100:1144–1154

Bennetzen JL, Freeling M (1997) The unified grass genome: synergy in synteny. Genome Res 7:301–306

Burge C, Karlin S (1997) Prediction of complete gene structures in human genomic DNA. J Mol Biol 268:78–94

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

Collins NC, Drake J, Ayliffe M, Sun Q, Ellis J, Hulbert S, Pryor T (1999) Molecular characterization of the maize *Rp1-D* rust resistance haplotype and its mutants. Plant Cell 11:1365–1376

Collins N, Park R, Spielmeyer W, Ellis J, Pryor AJ (2001) Resistance gene analogs in barley and their relationship to rust resistance genes. Genome 44:375–381

Deng Z, Huang S, Ling P, Chen C, Yu C, Weber CA, Moore GA, Gmitter FG (2000) Cloning and characterization of NBS-LRR class resistance-gene candidate sequences in citrus. Theor Appl Genet 101:814–822

Dodds P, Lawrence G, Ellis J (2001) Six amino-acid changes confined to the leucine-rich repeat beta-strand/beta-turn motif determine the difference between the P and P2 rust resistance specificities in flax. Plant Cell 13:163–178

Druka A, Kudrna D, Han F, Kilian A, Steffenson B, Frisch D, Tomkins J, Wing R, Kleinhofs A (2000) Physical mapping of the barley stem rust resistance gene *rpg4*. Mol Gen Genet 264:283–290

Halterman D, Zhou F, Wei F, Wise RP, Schulze-Lefert P (2001) The MLA6 coiled-coil, NBS-LRR protein confers AvrMla6-dependent resistance specificity to *Blumeria graminis* f. sp. *Hordei* in barley and wheat. Plant J 25:335–348

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48:575–607

Han F, Kilian A, Chen J P, Kudrna D, Steffenson B, Yamamoto K, Matsumoto T, Sasaki T, Kleinhofs A (1999) Sequence analysis of a rice BAC covering the syntenous barley *Rpg1* region. Genome 42:1071–1076

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

Ilag LL, Yadav RC, Huang N, Ronald PC, Ausubel FM (2000) Isolation and characterization of disease resistance gene homologues from rice cultivar IR64. Gene 255:245–255

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11,746–11,a750

Kilian A, Steffenson BJ, Maroof MAS, Kleinhofs A (1994) RFLP markers linked to the durable stem rust resistance gene *Rpg1* in barley. Mol Plant Microbe Interact 7:298–301

Kilian A, Chen J, Han F, Steffenson B, Kleinhofs A (1997) Towards map-based cloning of the barley stem rust resistance genes *Rpg1* and *rpg4* using rice as an intergenomic cloning vehicle. Plant Mol Biol 35:187–195

Kleinhofs A, Kilian A, Saghai Maroof 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

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. Nature Genet 14:421–429

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

Lupas A, van Dyke M, Stock J (1991) Predicting coiled coils from protein sequences. Science 252:1162–1164

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20:317–332

Michelmore R (2000) Genomic approaches to plant disease resistance. Curr Opin Plant Biol 3:125–131

Moore G, Devos KM, Wang Z, Gale MD (1995) Cereal genome evolution. Grasses line up and form a circle. Curr Biol 5:737–739

Ori N, Eshed Y, Paran I, Presting G, Aviv D, Tanksley S, Zamir D, Fluhr R (1997) The *I2C* family from the wilt disease resistance locus *I2* belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9:521–532

Pan Q, Wendel J, Fluhr R (2000) Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J Mol Evol 50:203–213

Parniske M, Hammond-Kosack KE, Golstein C, Thomas CM, Jones DA, Harrison K, Wulff BB, Jones JD (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

**Note added in proof** After submitting the manuscript we screened another rice BAC clone library OSJNBb (obtained from Dr. R. Wing; Clemson University Genomics Institute) with the maize pic20 probe as described previously. This rice library was made by a different restriction enzyme (*Eco*RI) and represented 15 rice-genome equivalents. Addresses of the 36 new BAC clones hybridizing with the maize pic20 can be found at http://barleygenomics.wsu.edu/pic20_BACs.html. Twenty of the BAC clones belonged to the contig on chromosome 1 and three to the contig on chromosome 5 previously discovered. In this second screening, we did not detect any BAC clones for the chromosome 2 contig suggesting that the single BAC clone previously described may be a hybridization artifact. Thirteen of the BAC clones were not present in the fingerprint database, and may represent additional rice pic20 homologue loci.

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

Shen KA, Meyers BC, Islam-Faridi MN, Chin DB, Stelly DM, Michelmore RW (1998) Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol Plant Microbe Interact 11:815–823

Tacconi G, Cattivelli L, Faccini N, Pecchioni N, Stanca AM, Vale G (2001) Identification and mapping of a new leaf stripe resistance gene in barley (*Hordeum vulgare* L.). Theor Appl Genet 102:1286–1291

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796–815

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Traut W (1994) The functions and consensus motifs of nine types of peptide segments that form different types of nucleotide-binding sites. Eur J Biochem 222:9–19

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

Wei F, Gobelman-Werner K, Morroll SM, Kurth J, Mao L, Wing R, Leister D, Schulze-Lefert P, Wise RP (1999) The *Mla* (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosome 5S (1HS) of barley. Genetics 153:1929–1948

Yu Y, Tomkins JP, Waugh R, Frisch DA, Kudrna D, Kleinhofs A, Brueggeman RS, Muehlbauer GJ, Wise RP, Wing RA (2000) A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and the identification of clones containing putative resistance genes. Theor Appl Genet 101:1093–1099

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