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Physical mapping of the barley stem rust resistance gene *rpg4*

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Abstract The barley stem rust resistance gene *rpg4* was physically and genetically localized on two overlapping BAC clones covering an estimated 300-kb region of the long arm of barley chromosome 7(5H). Initially, our target was mapped within a 6.0-cM region between the previously described flanking markers MWG740 and ABG391. This region was then saturated by integrating new markers from several existing barley and rice maps and by using BAC libraries of barley cv. Morex and rice cv. Nipponbare. Physical/genetic distances in the vicinity of *rpg4* were found to be 1.0 Mb/cM, which is lower than the average for barley (4 Mb/cM) and lower than that determined by translocation breakpoint mapping (1.8 Mb/cM). Synteny at high resolution levels has been established between the region of barley chromosome 7(5H) containing the *rpg4* locus and the subtelomeric region of rice chromosome 3 between markers S16474 and E10757. This 1.7-cM segment of the rice genome was covered by two overlapping BAC clones, about 250 kb of total length. In barley the markers S16474 and E10757 genetically delimit *rpg4*, lying 0.6 cM distal and 0.4 cM proximal to the locus, respectively.

Key words Map-based cloning · Resistance genes · Barley genome · Stem rust

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Introduction

The barley stem rust resistance gene *rpg4* confers resistance to *Puccinia graminis* f. sp. *tritici* pathotype Pgt-QCC (Jin et al. 1994b). This pathotype appeared relatively recently in the Midwestern USA, and was able to break down stem rust resistance provided by another resistance gene, *Rpg1* (Roelfs at al. 1991). The *Rpg1* gene has provided durable resistance to stem rust in barley since its discovery in 1937. In anticipation of a Pgt-QCC epidemic, mapping, isolation and characterization of *rpg4* became an important pre-requisite for efficient development of barley cultivars resistant to Pgt-QCC via molecular breeding or transgene technology. Once isolated, *rpg4* may also become a useful tool for isolation of other barley and wheat rust resistance genes using homology-based approaches.

Both *Rpg1* and *rpg4* are race-specific resistance genes. *Rpg1* is dominant, but *rpg4* is recessive under typical assay conditions (18–21 °C; Sun et al. 1996). At higher temperature (>25 °C), *Rpg1 is* functional, whereas *rpg4* is inactive (Jin et al. 1994a). The genetic map positions of these genes differ; *Rpg1* is located in the telomeric region of the short arm of chromosome 1(7H) and *rpg4* in the subtelomeric region of the long arm of chromosome 7(5H) (Kilian et al. 1994a; Borovkova et al. 1995).

Map-based cloning has been widely used in plant species where extensive genetic maps are available. Examples include not only *Arabidopsis thaliana* and rice, but also plants with large genomes, including lettuce (Meyers et al. 1998), potato (Bendahmane et al. 1997), tomato (Martin et al. 1993), apple (Patocchi et al. 1999),

pepper (Tai et al. 1999) and sugar beet (Cai et al. 1997). Three barley genes, *mlo* (Simons et al. 1997), *Rar1* (Lahaye et al. 1998) and *Mla* (Wei et al. 1999), have been cloned by the map-based approach.

The barley genome is ~5000 Mb long (Bennett and Smith 1976; Arumugamathan and Earle 1991), and the genetic map size of 1250 cM (Kleinhofs et al. 1993), indicates a physical/genetic distance ratio of about 4 Mb/cM. The most comprehensive barley map available at present consists of about 2000 markers (Kleinhofs and Graner 2000). Thus, the average marker density is about 2 per cM. This estimate discourages map-based cloning. However, recombination frequencies differ significantly across the barley genome, ranging from 0.1 Mb to 298 Mb per cM (Kunzel et al. 2000). In the vicinity of *rpg4*, the ratio of physical to genetic distance is below average, 1.8 Mb/cM, as determined by physical mapping with chromosome translocation lines (Kunzel et al. 2000). This makes map-based cloning of *rpg4* a feasible, yet challenging task, especially considering that chromosome walking in barley is inefficient owing to the abundance of repeated sequences. The physical/genetic distance ratios reported for regions flanking *rpg4* are very high i.e., 43 Mb/cM and 24 Mb/cM (Kunzel et al. 2000). The existing maps positioned *rpg4* 5 cM distal to MWG740 and 0.9 cM proximal to ABG391. One marker, R273, co-segregated with *rpg4* in a low-resolution mapping population consisting of 120 gametes (Kilian et al. 1997). Rice genetic maps and molecular markers have been successfully used to saturate the *Rpg1* region (Kilian et al. 1995; Han et al. 1999). For the *rpg4* region, barley-rice synteny has been described at a low resolution (Han et al. 1998), revealing two barley-rice rearrangements and an inversion, which could have potentially involved the putative *rpg4* homologue. Therefore, evaluation of synteny in the vicinity of *rpg4* at high resolution was necessary in order to integrate rice markers for map-based cloning in barley. In this paper we demonstrate for the first time that by using only existing barley and rice molecular markers and public BAC libraries it is possible to delimit physically and genetically a single gene in a species with a large genome, like barley.

Materials and methods

Genetic mapping

Five different barley mapping populations were used: Steptoe × Morex, consisting of 150 doubled-haploid lines (DHLs; Kleinhofs et al. 1993); Harrington × Morex (140 DHLs; Hayes et al. 1997; see http://www.ncgr.org/ag/jag/) Steptoe × Q21861 (144 DHLs; A. Kleinhofs, unpublished); Q21861 × SM89010 (129 DHLs; Steffenson et al. 1995) and Steptoe × Q21861 (750 F_2/F_3 lines; this paper). Mapping populations are available at http://www.css.orst.edu/barley/nabgmp/97/germsum.htm. A rice mapping population, consisting of 186 F_2 Nipponbare × Kasalath lines was kindly provided by Dr. T. Sasaki, Rice Genome Research Program (RGP), National Institute of Agrobiological Resources, Tsukuba, Japan. Genetic distances were calculated using Mapmaker and/or Mapmanager software or specified in terms of the number of recombination events per gamete number.

RFLP markers

The cDNA clones CDO419 from oat and BCD298 from barley were kindly provided by M. Sorrells, Cornell University. Markers E10757 and S16474 were kindly provided by T. Sasaki, RGP, Japan. Other markers were previously mapped (for details see http://www.dna.affrc.go.jp84/publicdata/physicalmap and ftp://ftp.staff.or.jp/pub/geneticmap98) or generated in this study. Technical information about mapped probes is available from the Barley Molecular Marker Database at http://barleygenomics.wsu.edu/webd/excel-search.html.

The *rpg4* phenotyping conditions and selection of recombinant lines

The *rpg4* allele carried by Q21861 was selected from a diverse collection of barley germplasm providing resistance to the stem rust pathotype Pgt-QCC (Jin et al. 1994b). Inoculation and infection phenotyping of parental and recombinant barley lines was done as described previously (Steffenson and jin 1992). Recombinant lines were developed as follows. Steptoe × Q21861 F_2 plants that had undergone recombination between flanking markers *Aga5* and ABG391 were selected and selfed to obtain the F_3 generation. The F_3 plants were phenotyped for the Pgt-QCC stem rust reaction. Flanking markers *Aga5* and ABG391 were used to select homozygous recombinants. Selected recombinants were phenotyped again for the Pgt-QCC reaction and used for high-resolution mapping.

Southern analysis of total barley genomic DNA

Isolation of total barley genomic DNA, Southern blotting and hybridization were described previously (Kleinhofs et al. 1993). Hybridization was carried out at 65 °C for homologous probes or at 62 °C for heterologous probes. Filters were washed at the hybridization temperature with 2 × SSC with 1% SDS for 20 min, 1 × SSC with 1% SDS for 20 min, 0.5 × SSC with 1% SDS for 20 min and optionally 0.2 × SSC with 1% SDS for 20 min.

BAC library analysis

The BAC library made from barley cv. Morex (average insert size 106 kb) representing six barley genome equivalents has been described (Yu et al. 2000). The rice cv. Nipponbare BAC library (average insert size 130 kb) represents five rice genome equivalents and is described at http://www.genome.clemson.edu/lib_frame.html. Both libraries were constructed in pBeloBAC11 (Wang et al. 1997) using partial *Hin*dIII digests. High-density BAC colony arrays on filters were hybridized with ^{32}P-labeled probes. Usually, several different probes were used together for one screening step. Positive colonies were inoculated into 2 ml of TB medium (1.2% Bacto Tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4) containing 25 mg/l chloramphenicol, incubated with shaking overnight at 37 °C, and BAC DNA was isolated using a standard alkaline lysis procedure (Birnboim and Doly 1979). Southern analysis for clone identification and fingerprinting was performed on *Hin*dIII-digested BAC DNA using ^{32}P-labeled probes separately.

Subcloning of BAC DNA

The *Hin*dIII-digested BAC DNA was subcloned in the pBluescript SK- vector (Stratagene). Subclones (usually 96 per BAC clone) were negatively selected using total barley DNA as a probe. Selected subclones were used for mapping or for a second round of selection based on their position in the BAC contig. For recovery of

BAC ends, BAC DNA was digested with *Nsi*I (no sites in vector), diluted, ligated and transformed into *E. coli*. The two ends, joined at the *Nsi*I site, were used to obtain end-probes by digesting with *Not*I and *Nsi*I, or subcloned into the pBluescript SK- vector as *Not*I-*Nsi*I/*Pst*I fragments (*Nsi*I and *Pst*I ends are compatible). If a *Not*I site occurred in the insert between the *Hin*dIII and *Nsi*I site, it was subcloned as a *Not*I fragment.

Pulsed-field gel electrophoresis (PFGE) of BAC clones

PFGE was performed with the CHEF-DR III system (BioRad) according to the manufacturer's instructions. Briefly, 200 ng of restriction enzyme-digested BAC DNA was loaded and PFGE performed for 15 h at 6 V with a 1–6 sec switch time. Low Range PFG Molecular Weight Marker (New England Biolabs) was used as a molecular size standard.

Results

High-resolution genetic mapping of *rpg4*

The *rpg4* region between the markers ABG391 (0.9 cM distal) and MWG740 (5 cM proximal) (Kilian et al. 1997) was more precisely mapped using the markers described below (Fig. 1A). The rice R273 probe mapped approximately 0.8 cM distal to *rpg4* in a population of 1500 gametes, and was the closest marker to *rpg4* on the telomeric side. The R273 probe did not hybridize well to barley genomic DNA and was polymorphic only with restriction endonuclease *Hpa*II. Since *Hpa*II is a methylation-sensitive enzyme, mapping based on the use of this probe may be ambiguous. To obtain a better marker for this locus, we screened a barley BAC library, subcloned positive clones and selected a polymorphic, single-copy probe, ARD032, as a R273 equivalent. Mapping of ARD032 localized this marker 0.8 cM distal to *rpg4* (Fig. 1A). On the proximal side, *Aga5* (Kilian et al. 1994b) mapped 1.8 cM from *rpg4*. We were not able to find a polymorphism for BCD298 between Steptoe and Q21861, but BCD298 and CDO419 co-segregated in a Harrington × Morex DHL mapping population and mapped 2 cM proximal to ABG391. Since Harrington and Morex are both susceptible to Pgt-QCC the location of the *rpg4* locus in these crosses is not known. In order to find other, possibly polymorphic

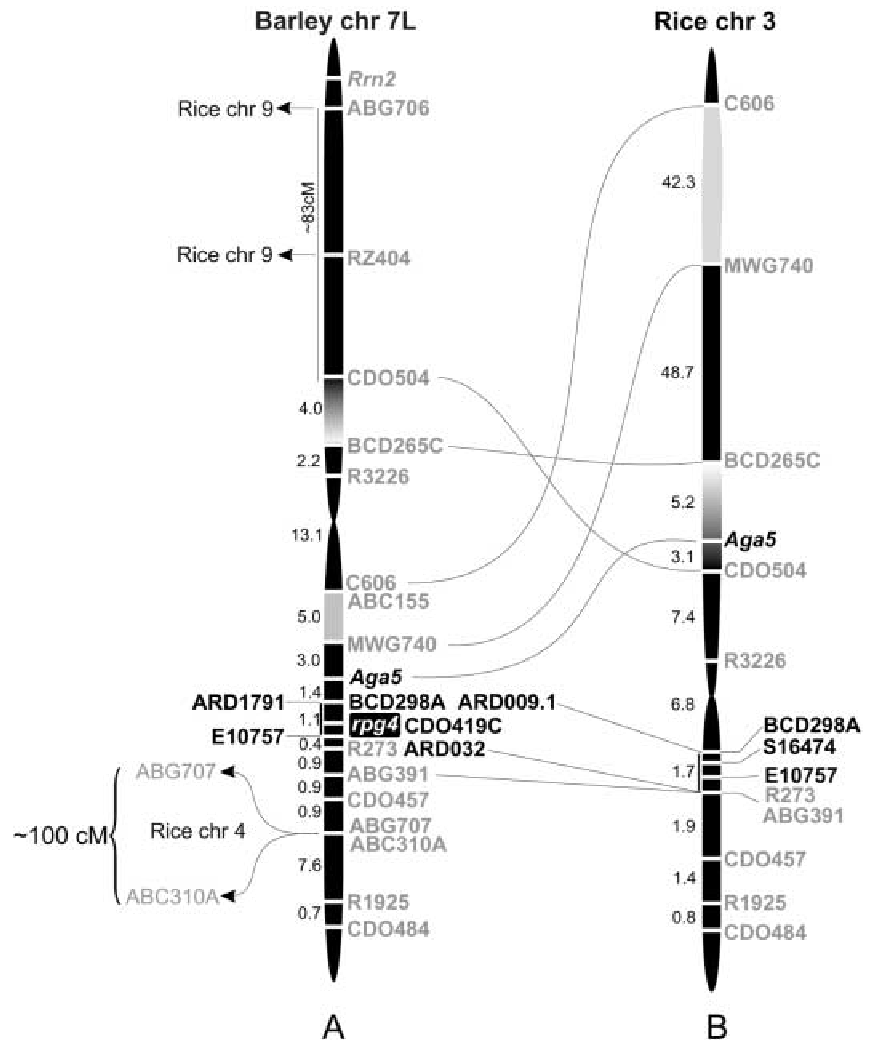

Fig. 1A, B Linkage maps of barley chromosome 7(5H) (**A**) and rice chromosome 3 (**B**). Markers shown in *grey* were mapped previously (Han et al. 1998), other markers are described in this paper. The orientations of the relative barley-rice rearrangements and putative inversions are shown by gradient fills

markers from the BCD298 region, an approach similar to that used to isolate ARD032 was used. As a result, the marker ARD009.1 was generated and mapped 0.6 cM proximal to *rpg4* based on a population of 1644 Steptoe × Q21861 gametes (Fig. 1A). One of several CDO419 loci mapped to chromosome 7(5H) in the Steptoe × Q21861 population and co-segregated with *rpg4* in 1773 gametes (Fig. 1A).

Barley-rice synteny in the vicinity of *rpg4*

Rice RFLP markers were found to be useful tools for saturating the *Rpg1* region (Kilian et al. 1995; Han et al. 1999). With regard to *rpg4*, synteny between rice chromosome 3 and barley chromosome 7(5H) was established at low resolution, demonstrating rearrangements relative to rice chromosome 3 and several regions equivalent to segments of rice chromosomes 4 and 9 (Fig. 1B) (Han et al. 1998). The rearrangement of the C606-MWG740 region was the most disquieting, because it could have included the rice *rpg4* homologue (Fig. 1B). To test this possibility, barley *Aga5* and BCD298 were mapped in a rice mapping population consisting of 186 Nipponbare × Kasalath F2 lines. The results demonstrated that the C606-MWG740 rearrangement did not extend to the *Aga5* locus. The BCD265C and CDO504 region in rice, containing the *Aga5* locus, appears to be inverted with respect to barley (Fig. 1A and B). Fortunately, BCD298 remained closely linked to R273. We were not able to map CDO419 in rice because of a lack of polymorphism. For further analysis of barley-rice synteny, two rice cDNA markers, E10757 and S16474, were selected. These probes are located, together with R273, on rice YAC clone Y3284 (T. Sasaki, personal communication). In a rice mapping population consisting of 186 F_2 plants, E10757, S16474 and R273 are closely linked, covering about 1.6 cM. The S16474 marker maps 0.3 cM distal to BCD298, and is thus the closest marker to the putative rice *rpg4* homologue (Fig. 1B). The barley equivalent of S16474, ARD1791, mapped 0.6 cM from *rpg4* and co-segregated with BCD298 in barley. On the distal side, E10757 mapped 0.5 cM from *rpg4* (Fig. 1A).

Establishment of BAC contigs in rice and barley

The Morex barley BAC library was screened with BCD298, ARD1791, CDO419, E10757 and ARD032. The Nipponbare rice BAC library was screened with S16474 and E10757. Several probes, such as E10757, BCD298 and CDO419 identified multiple unlinked loci and therefore hybridized to 30–50 BAC clones, while single-locus probes identified 2–10 BAC clones. In order to determine which BAC clones were derived from loci of interest, we mapped all possible labelled restriction fragments in mapping populations derived from crosses involving a Morex parent (Steptoe × Morex and Harrington × Morex). BAC clones were selected which contained restriction fragments identified and mapped in the vicinity of *rpg4* in Morex. Figure 2 shows one example. In this case, band *e* was mapped in the *rpg4* region in the Harrington × Morex population, and accordingly BAC clones loaded in tracks 10, 11 and 17 were selected. We did not find BAC clones that hybridized with two or more segregating probes. Even BCD298 and ARD1791, which co-segregated in a 1773-gamete population, were not located on the same BAC clones. In order to determine if barley BAC clones isolated with the probes described above overlapped, BAC end-clones were obtained and used as hybridization probes. Fingerprinting using end-clones is an ambiguous procedure in barley, since most of the end-clones contain repeated sequences. Overlaps detected by end-clones were considered genuine if presumably overlapping

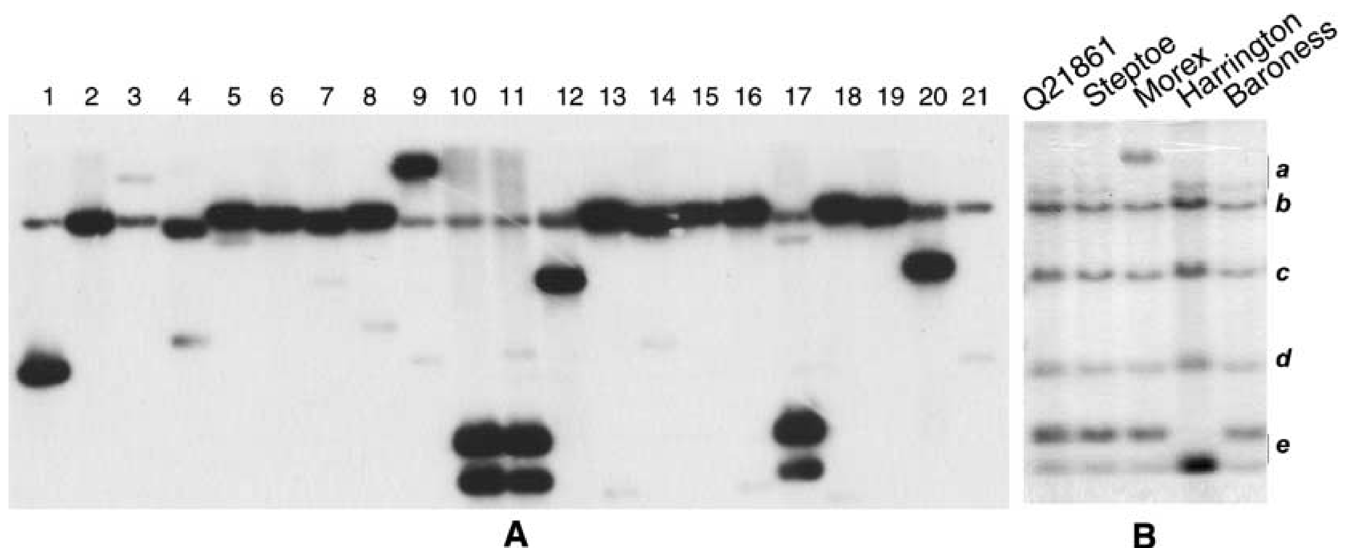

Fig. 2A, B Southern blot of CDO419-positive BAC clones (**A**) and barley genomic DNA (**B**) digested with *Eco*RI and probed with CDO419. Band *e* was mapped to the *rpg4* region and the BACs containing a band of the same size were used for further analysis

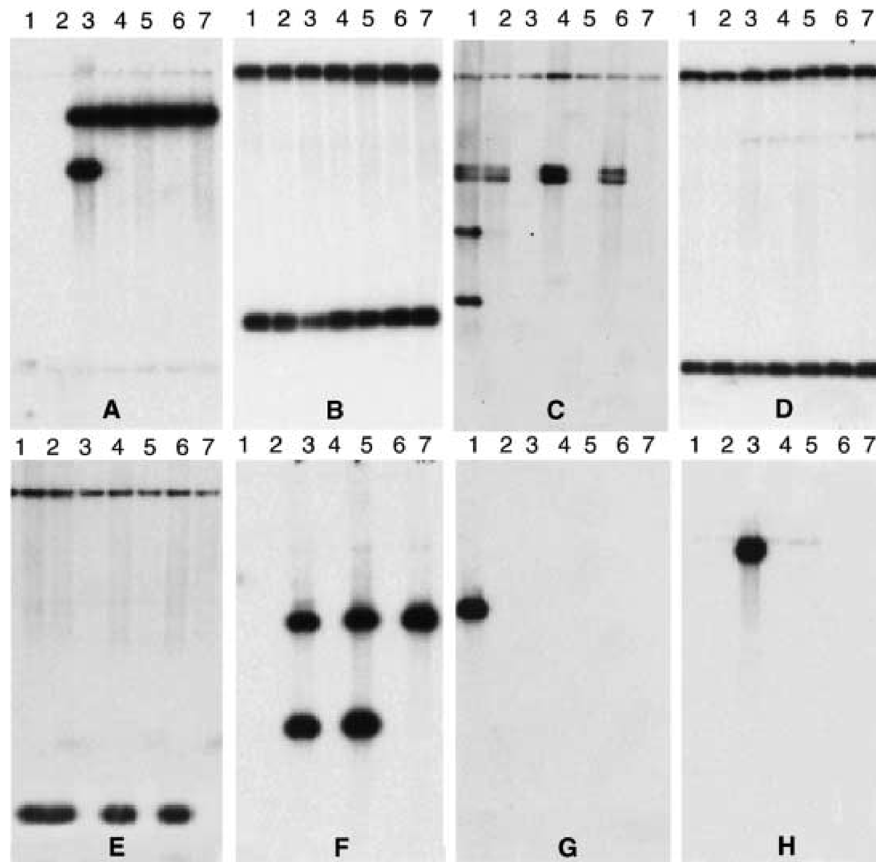

Fig. 3A–H Southern analysis of CDO419-positive BAC clones digested with *Hin*dIII and hybridized with selected probes. The BAC clones are: lanes 1, 64H24; 2, 205K7; 3, 259B20; 4, 362B9; 5, 559L23; 6, 594L22; 7, 669K4 (see also Fig. 4B). **A, B** Hybridization with the end-clones ARD1288A and ARD1288B of BAC 259B20, showing the presence of a unique band in **A,** suggesting that the 259B20 BAC clone extends the contig farthest. **C, D** Similarly, hybridization with the end-clones ARD642A and ARD642B of BAC 64H24 demonstrated that the 64H24 BAC clone extends farthest in the opposite direction. End-clones from other BACs did not yield unique bands on Southern blots, suggesting that they terminated within the region covered by 64H24 or 259B20. For example, end-clone ARD647A, derived from BAC 594L22, hybridized to 64H24, 205K7, 362B9 and 594L22, demonstrating that it extends to the 64H24 side, but terminates within 64H24, 205K7 and 362B9 (**E**). On the 259B20 side, BAC 669K4 end-clone ARD1289A hybridizes to itself and 259B20 and 559L23 BACs, demonstrating that this BAC does not extend beyond 259B20 and 559L23 (**F**). The 64H24 and 259B20 BAC clones were chosen for subcloning to generate probes for further mapping. The probe that extends furthest on the telomeric side, ARD829, hybridized only with the 64H24 BAC (**G**) and ARD2065.2 hybridized only with the 259B20 BAC (**H**). These probes were used for genetic mapping. The high-molecular-weight band present in all lines in **B–E** is due to the vector pBeloBAC11 (end-clones contained part of pBeloBAC11). The same size band or bands present in lines 3–7 (**A**), in lines 1, 2, 4, 6 (**C**) and 3, 5, 7 (**F**) are derived from repeats

BACs yielded signals of the same size and strength. We were not able to establish definite overlaps among the probes used to screen the BAC library (BCD298, ARD1791, CDO419, E10757 and ARD032).

Our next goal was to generate new markers from the BAC clones identified with the marker CDO419, which co-segregates with *rpg4*. For this purpose, contig-extending BAC clones needed to be identified. End-clones were used to order the BAC clones and identify those that extended the contig maximally in both directions. Figure 3 shows Southern blots of selected CDO419-positive BAC clones hybridized with critical end-clones. The two BAC clones that extended the contig furthest, 64H24 and 259B20, were subcloned to generate probes for further mapping. The low-copy-number probes ARD815, ARD789, ARD1111 and ARD829A, which hybridized only with the 64H24 BAC, genetically segregated from *rpg4* on the telomeric side. On the centromeric side, ARD2065.2 hybridized only with the 259B20 BAC clone and segregated away from *rpg4*. Thus, ARD829A and ARD2065.2 flank *rpg4* about 0.2 cM away (Fig. 4B). Marker ARD1111, which hybridized only to the 64H24 BAC clone was found to be two crossovers from *rpg4*. Thus, genetically, the 64H24 BAC clone covers two recombination events. BAC clones 64H24 and 259B20 were determined to be 170 kb and 150 kb long, respectively, which means that the interval between ARD1111 and ARD2065.2 is less than 320 kb. An overlap of 40 kb was determined by adding up co-migrating restriction enzyme digestion fragments, thus reducing the interval to about 280 kb. This corresponds to a genetic distance of 0.3 cM, equivalent to about 0.9 Mb/cM, two times lower than that found by translocation breakpoint mapping for the 10 cM ABG391–cMWG654 region (1.8 Mb/cM; Kunzel et al. 2000).

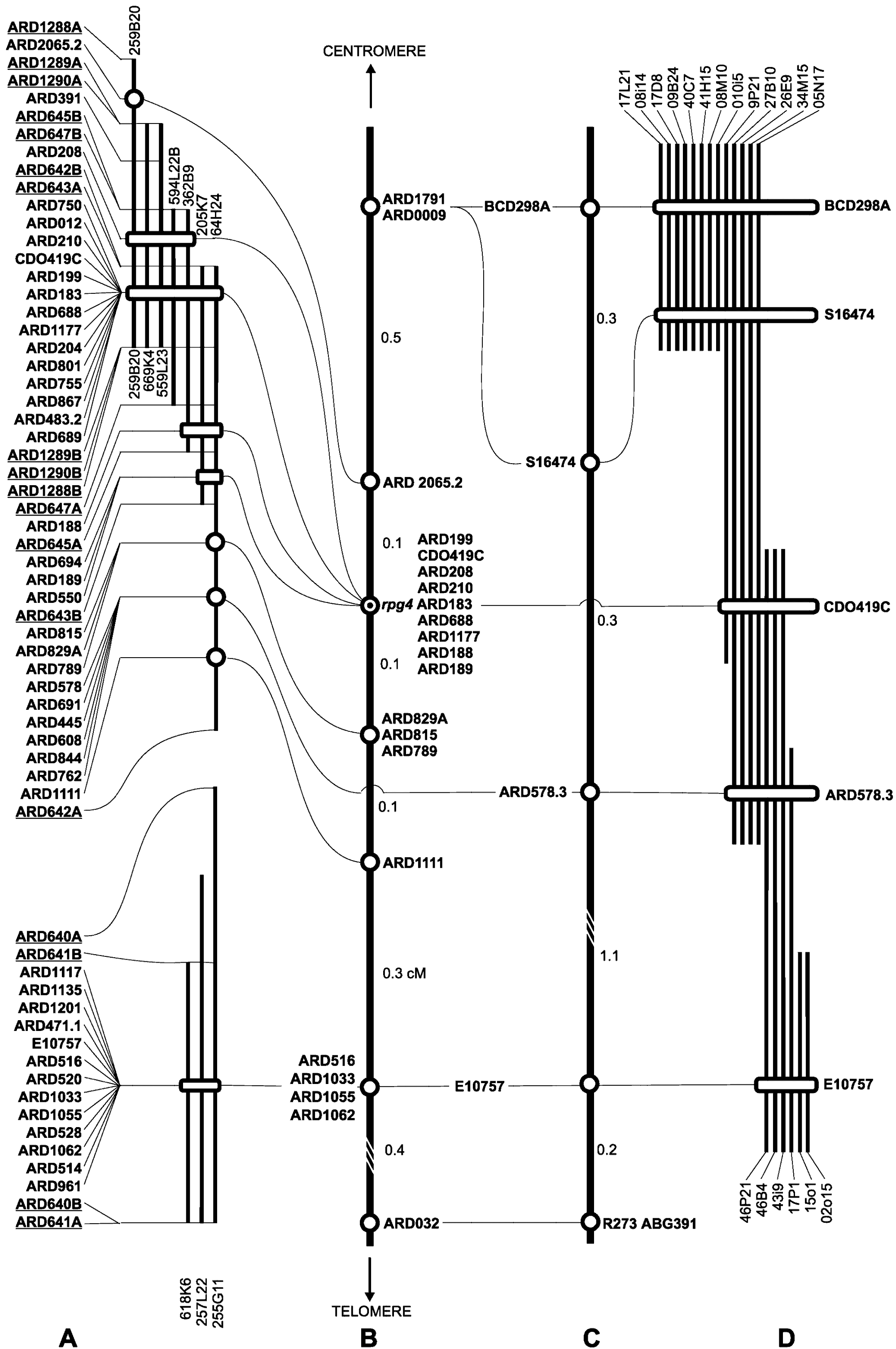

Fig. 4A–D Genetic (**B** and **C**) and physical (**A** and **D**) maps of syntenic barley and rice chromosomes, respectively, showing the physical and genetic locations of the probes used. End-clones are *underlined*. Genetic distances are show in cM. The Figure is not drawn to scale

The overlap between rice BAC clones that were positive with E10757 and S16474/BCD298 was established by hybridizing them with CDO419. Four E10757 and three S16474 rice BAC clones hybridized with CDO419, giving a single band of the same size and intensity. The contig was further refined using probe ARD578, which is a *Hin*dIII subclone of the rice BAC 43i9 (Fig. 4D). The sizes of the two overlapping rice BAC clones, 05N17 and 43i9, were 140 kb and 160 kb, respectively. The overlap size (~50 kb) was estimated using the same method as for barley BACs. Thus, the

1.7-cM interval in rice spans ~250 kb between markers E10757 and S16474, which gives a physical/genetic distance ratio of 150 kb/cM.

Discussion

An important pre-requisite for efficient and successful map-based cloning in plants with large genomes, like barley, is a favorable physical/genetic distance ratio in the region of interest. Taking into account the fact that the barley physical/genetic distance ratio reported for the *rpg4* region was 1.8 Mb/cM (Kunzel et al. 2000) and that the average marker density was less than 1 per cM, map-based cloning appeared to be a dubious task. Nevertheless, as demonstrated in this paper and in the case of the barley *Mla* gene (Wei et al. 2000), physical/genetic distance ratios may be quite variable within chromosomal regions. In the vicinity of *rpg4*, the physical/genetic distance ratio was two times lower than that estimated by translocation breakpoint mapping (Kunzel et al. 2000). We were able to generate high-resolution genetic maps using relatively small numbers of gametes and without using specialized map saturation techniques, such as AFLP. We identified three recombination events within a physical distance delimited by two BAC clones (280 kb total), and one or both of these BAC clones apparently contain(s) *rpg4*. Physical mapping of other barley resistance genes, *mlo-3* (Simons et al. 1997), *Mla* (Wei et al. 1999) and *Rar1* (Lahaye et al. 1998), involved alternative procedures, such as CAPS, RAPD and AFLP and YAC and BAC libraries. For the *Rar1* locus, located on barley chromosome 2, the physical/genetic distance ratio was 12.6 Mb/cM, although the estimate from translocation mapping was only 1.1 Mb/cM. Some differences may be due to the inherent limitations of the respective techniques, but a 10-fold difference suggests significant fluctuations within the region. Similarly, at the *Mla* locus, which is located in the terminal segment of the short arm of chromosome 5, a 10-fold variation was found in intervals adjacent to, and co-segregating with, the *Mla* cluster. In contrast, distance calculations at the *mlo* locus, located on the subtelomeric segment of chromosome 4L, gave ratios of 0.1 and 0.3 Mb/cM for two short fragments covering the gene (Simons et al. 1997), but the estimate based on translocation breakpoint mapping was 2.6 Mb/cM. In the *rpg4* region, dramatic fluctuations in recombination frequencies were not observed and the difference from that estimated by translocation mapping was not excessive. In the rice region that is syntenous to *rpg4*, which is subtelomeric on rice chromosome 3, our estimate was about 150 kb/cM – which is two times lower than the average based on YAC contig data (300 kb/cM) (Nakamura et al. 1997).

Known disease resistance genes or their analogues seem to be in non-syntenic locations in barley and rice (Leister et al. 1998, 1999). There is no known clustering of RGAs (Resistance Gene Analogues) in the vicinity of *rpg4*. This is in contrast to *Rpg1* (Leister et al. 1998; Han et al. 1999) and *Mla* (Wei et al. 1999), where several RGA families were found. In the case of *Rpg1*, the barley-rice synteny relationship breaks down in the region where the RGAs are clustered (Han et al. 1999), but in the vicinity of *rpg4* the synteny appears to be very conserved throughout the region. This may suggest that *rpg4* belongs to a different class of resistance genes and/or is a member of a gene family for which RGAs have not been found.

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