Theor Appl Genet (1998) 97: 526–534

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High-resolution genetic and physical mapping of the *Rar1* locus in barley

Received: 11 February 1998 / Accepted: 3 March 1998

Abstract The *Mla-12*-mediated resistance in barley against *Erysiphe graminis* f. sp. *hordei* requires for its function the *Rar1* gene. High-resolution genetic mapping was accomplished by inspecting more than 4000 plants segregating for *Rar1* within an 0.7-cM interval containing the target gene. Marker enrichment in the target region was carried out by an amplified fragment length polymorphism (AFLP)-based search for polymorphic loci using bulked DNA templates from resistant and susceptible recombinants adjacent to *Rar1*. RFLP markers closely linked to *Rar1* were used to investigate the relationship between physical and genetical distances by PFGE Southern analysis, indicating the physical linkage of two genetically separated RFLP loci. Comparative mapping of *Rar1*-linked RFLP probes in barley and rice identified a break of collinearity in the orthologous chromosome segments.

Key words Resistance reaction · AFLP · Bulked segregants · CAPS · High-resolution map · Powdery mildew fungus

Communicated by F. Salamini

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Introduction

Genetic studies of plant-pathogen interactions has in many cases revealed that resistance to a particular pathogen race is controlled by monogenically inherited resistance (*R*) genes. The *R* gene interacts, directly or indirectly, with a race-specific avirulence determinant produced by the pathogen and encoded by the corresponding avirulence (*Avr*) gene. Absence or inactivation of either gene results in a compatible interaction. *R* genes are presumed to enable plants to detect *Avr* gene-specified pathogen molecules and to initiate plant defence reactions. The isolation of several *R* genes revealed that the corresponding proteins share structurally related domains including leucine-rich repeats, a nucleotide-binding site, and a kinase motif (Staskawicz et al. 1995; Baker et al. 1997). How *R* gene-encoded proteins activate downstream defence responses is not yet understood. Recently, mutational approaches in several plant species have been applied to identify genes that are required for the function of race-specific *R* genes (Freialdenhoven et al. 1994; Hammond-Kosack et al. 1994; Salmeron et al. 1994; Century et al. 1995; Parker et al. 1996). It seems likely that these genes encode either signalling or effector components of *R* gene-triggered resistance reactions.

Screening of a mutagenized barley line (Sultan-5) containing the powdery mildew *R* gene *Mla-12* for susceptible mutants led, in addition to numerous defective *Mla-12* alleles, to the identification of *Rar1* and *Rar2*, two loci that are necessary for *Mla-12*-specified resistance (Torp and Jørgensen 1986; Jørgensen 1988; Freialdenhoven et al. 1994). *Rar1* and *Rar2* wild-type alleles are essential for triggering a hypersensitive host-cell death reaction during an early stage of pathogen invasion and both genes control also the temporal accumulation pattern of defence-related gene transcripts upon pathogen challenge (Freialdenhoven et al. 1994). Further investigation revealed that *Rar1* and

Rar2 are not only a component of the *Mla-12*-mediated resistance but that they are also required for the function of different resistance alleles encoded at *Mla* as well as for other race-specific powdery mildew *R* genes unlinked to *Mla* (Jørgensen 1996). These features suggest that the *Rar* genes encode either common signalling or common effector components required for resistance reactions triggered by a diverse set of race-specific powdery mildew resistance genes.

A previous interval mapping procedure located *Rar1* on barley chromosome 2 and flanked by RFLP loci cMWG694 and MWG503 within a 5-cM interval (Freialdenhoven et al. 1994). Here we describe the construction of a high-resolution linkage map at the *Rar1* locus and assess physical and genetical distances at the target locus as a first step towards the map-based isolation of the gene.

Materials and methods

Plant material

Seeds of the doubled-haploid barley (*Hordeum vulgare*) line Sultan-5 and the Sultan-5-derived mutants M82 (*rar1*-1) and M100 (*rar1*-2) were generously provided by J. Helms Jørgensen, Ris, Denmark (Torp and Jørgensen 1986). Sultan-5 and the two mutants contain the macroscopically visible marker gene *ant2* (anthocyanin deficiency in the leaf sheath). The *Mla-12* backcross (BC) lines in cultivars Siri and Pallas were a gift from Lisa Munk, Royal Veterinary and Agricultural University, Copenhagen, Denmark. Their production has been described previously (Kølster et al. 1986; Kølster and Stølen 1987).

The *Mla-12* BC line in cultivar Ingrid was a gift from James McKey, University of Upsala, Upsala, Sweden. This line was generated through seven backcrosses with *H. vulgare* cv Ingrid followed by at least seven selfings. Each of the mutants M82 and M100 was pollinated with pollen derived from the *Mla-12* BC-line cultivars. F_1 plants from each cross were grown to maturity providing the various segregating F_2 populations.

A segregating F_2 population of 186 individuals derived from the cross Nipponbare × Kasalath (Kurata et al. 1994) was used for mapping in rice. The map position of locus MWG876 in rice was independently tested in a second segregating F_2 population of 123 individuals derived from the cross IR20 × 63–83 (Quarrie et al. 1997).

Tests for resistance

Tests for resistance were carried out as described previously (Freialdenhoven et al. 1994). The phenotype of the recombinants was determined after selfing and subsequent inoculation experiments in F_3 and F_4 families comprising at least 25 individuals. F_3 individuals were tested by cleavable amplifiable polymorphic sequence analysis (CAPS) to identify homozygous recombinants. These plants were again selfed and subjected to resistance tests in F_4 families. Plants were scored for resistance/susceptibility 7 days after inoculation.

Pulsed-field gel electrophoresis (PFGE) and Southern analysis

High-molecular-weight DNA of barley was isolated from leaf material of 5–7-day-old seedlings using a procedure according to Siedler and Graner (1990). DNA was digested with six rare-cutting restriction enzymes (*Cla*I, *Mlu*I, *Sal*I, *Not*I, *Sfi*I, *Sgf*I, *Sma*I) using the protocol of Ganal et al. (1989). For size fractionation a 1.2% agarose gel was run in an LKB Pulsaphor apparatus (Pharmacia Biotech, Upsala, Sweden) at 180 V with pulse times from 10 to 60 s (linear interpolation) for 25 h in 0.5 × TBE (50 mM Tris-HCl, 50 mM boric acid, 1 mM EDTA, pH 8.3) at 12°C. Capillary transfer and non-radioactive Southern hybridisation was performed as described in Lahaye et al. (1996).

AFLP/CAPS analysis

Genomic DNA for CAPS and AFLP analysis was isolated according to Stewart and Via (1993). Primer PCR conditions and the respective restriction enzymes used for CAPS marker display are shown in Table 1. CAPS analysis was performed in a volume of 20 µl [100 pmol of each primer, 200 µM dNTPs, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, 50 mM KCl_2, 0.5 U *Taq* Polymerase (Boehringer)] using 50 ng of barley genomic template DNA. The digested PCR products were subsequently size-fractionated on 2% agarose gels. AFLP analysis (Vos et al. 1995) was performed on bulked DNA samples of resistant and susceptible plants (Giovannoni et al. 1991; Michelmore et al. 1991) using *Pst*I and *Mse*I restriction enzymes, *Pst*I and *Mse*I adapters, and a set of primers corresponding to the *Pst*I and *Mse*I adapters with two or three selective nucleotides at the 3′-end, respectively. Utilising seven *Pst*I + 2 (2 selective bases)- and 56 *Mse*I + 3 (3 selective bases)-primers, 392 combinations were analysed in total.

Results

Mapping of RFLPs and establishment of CAPS

The *Rar1* mutants M82 (*rar1*-1) and M100 (*rar1*-2) were originally isolated in the context of a functionally active *Mla-12* resistance allele in the spring cultivar Sultan-5 (Torp and Jørgensen 1986). Due to the low frequency of DNA polymorphism, especially within different spring types (Russell et al. 1997), we reasoned that the availability of alternative crosses would be advantageous because it increased the likelihood of finding a polymorphism with a given probe. Therefore both mutants were crossed with three *Mla-12* BC lines [*Mla-12* BC Ingrid, *Mla-12* BC Pallas, *Mla-12* BC Siri; (Kølster et al. 1986; Kølster and Stølen 1987; Freialdenhoven et al. 1994)] representing different genetic backgrounds of barley spring types.

RFLP markers MWG503, MWG87 and cMWG694 which define an approximately 5-cM interval containing *Rar1* [Fig. 1 A; (Freialdenhoven et al. 1994)] were sequenced, oligonucleotides for amplification of the corresponding loci were derived, and polymorphisms between the susceptible (*rar1*-1, *rar1*-2) and resistant parents (*Mla-12* BC Ingrid, *Mla-12* BC Pallas, *Mla-12* BC Siri) were determined (Fig. 2 A, B, C). To increase marker density adjacent to *Rar1* we selected three further RFLP markers which map close to the above-mentioned RFLP loci from a general RFLP map [MWG876, MWG892 and MWG2123 (Graner et al. 1994)]. Each of these RFLPs was converted to a CAPS marker (Fig. 2 D, E, F) and was mapped relative to

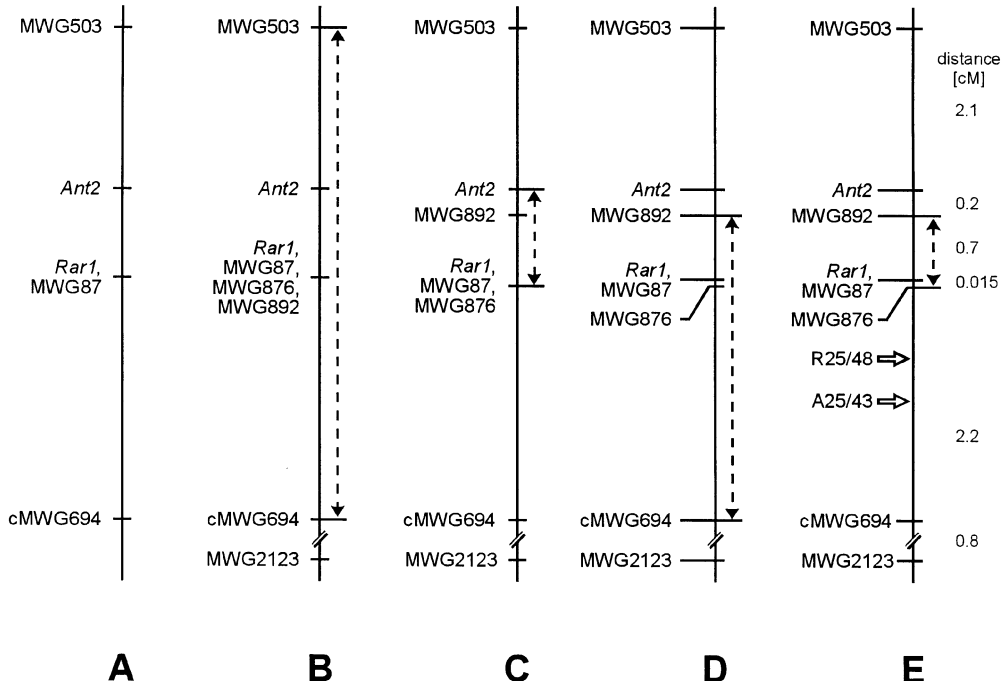

Fig. 1A–E Consecutive stages of high-resolution mapping at *Rar1*. Using the initial RFLP map (**A**), which was based on 50 plants (Freialdenhoven et al. 1994), the CAPS markers *MWG876*, *MWG892* and *MWG2123* were integrated into the genetic map (**B**). A phenotypic screen was used to analyse 1040 plants for recombination events between *Ant2* and *Rar1*. The observed recombinants were used to reveal that *MWG892* maps distal in relation to *Rar1* (**C**). A subsequent CAPS-based recombinant screen of 1063 additional plants was performed in the marker interval *MWG892–cMWG694*. Analysis of the observed recombinants positioned MWG876 proximal in relation to *Rar1* (**D**). Finally another 2207 plants were investigated in the marker interval *MWG892–MWG876* (**E**). *Vertical dashed arrows* indicate the interval which was analysed in the respective recombinant screen. The position of AFLP markers *R25/48* and *A25/43* is indicated in **E** by *arrows*. Since the AFLPs *R25/48* and *A25/43* were tested solely on the DNA of pool individuals (Fig. 4), we have only indicated their relative map position (*horizontal arrows*). The two recombinants identified with *R25/48* and *A25/43* revealed co-segregation of *Rar1* and *MWG876*, thus both AFLP loci must be located distal from *MWG876*. Genetic distances (cM) were calculated on the basis of two-point estimates

Rar1, based on a population of 50 segregants. In this population MWG876 and MWG892 showed cosegregation with *Rar1*, whereas MWG2123 was positioned distal to cMWG694 (Fig. 1 B).

Recombinant screen

The cultivar Sultan-5 (*Mla-12*, *Rar1*), from which both *Rar1* mutants (*rar1*-1, *rar1*-2) are derived, contains an anthocyanin pigmentation deficiency (*ant2*), whereas the three resistant *Mla-12* BC lines used for mapping (*Mla-12* BC Ingrid, *Mla-12* BC Pallas, *Mla-12* BC Siri) carry the *Ant2* wild type allele. The *Ant2* locus was previously shown to map at a distance of approximately 0.5 cM proximal to *Rar1* (Freialdenhoven et al. 1994). To identify rare recombinants in the small interval between *Rar1* and *Ant2* we selected 1040 susceptible F_2 individuals (*rar1*/*rar1*) and screened for the presence of the anthocyanin wild-type allele *Ant2* (Fig. 1 C). A total of 14 recombinants were found and these were tested for alleles in MWG87, MWG876, and MWG892. Analysis of the 14 recombinants showed co-segregation of *Rar1,* MWG87 and MWG876. Marker MWG892 was positioned between *Ant2* and *Rar1*, separated from the former by two recombination events (Fig. 1 C). The different crosses between the two allelic *Rar1* mutants and the three *Mla-12* BC lines did not reveal significantly different recombination frequencies. Therefore we restricted our search for further recombinants to one cross only (M100 × *Mla-12* BC Ingrid). This also enabled us to use the identified recombinants adjacent to *Rar1* for the targeted AFLP-based marker screen described below.

To increase the genetic resolution in the vicinity of the target locus another 1063 F_2 plants were screened for recombination events flanking *Rar1* by utilising CAPS markers MWG892 and cMWG694 (Fig. 1 D). Subsequent investigation of the MWG87 and MWG876 alleles revealed complete linkage for MWG87 and *Rar1* but one recombination event

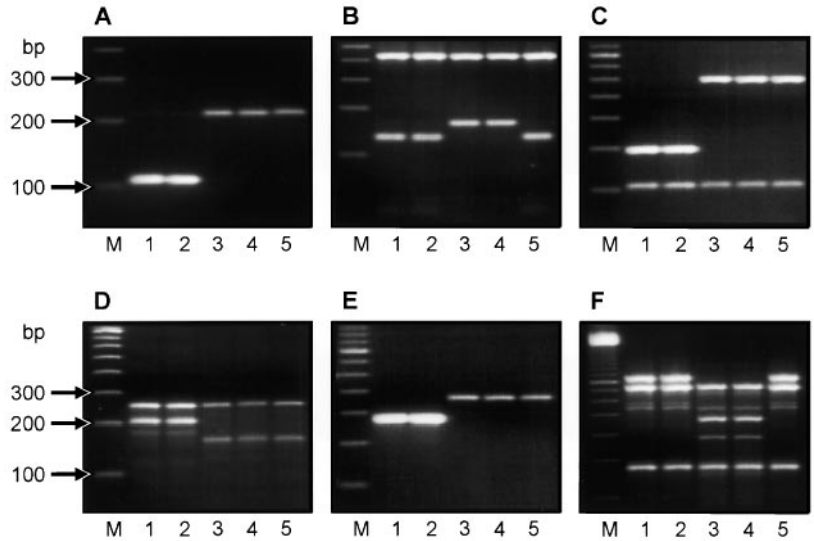

Fig. 2A–F PCR-based markers linked to *Rar1*. Ethidium bromide-stained 2.5% agarose gels displaying restriction enzyme-digested amplification products using M82 (*1*), M100 (*2*), *Mla-12* BC Ingrid (*3*), *Mla-12* BC Pallas (*4*) and *Mla-12* BC Siri (*5*) as template DNA. Amplification and digestion were carried out as described in Table 2. The displayed CAPS markers correspond to RFLP loci MWG87 (**A**), MWG503 (**B**), cMWG694 (**C**), MWG876 (**D**), and MWG2123 (**F**). PCR-primers for locus MWG892 (**E**) enabled allele-specific discrimination of PCR products without subsequent restriction digestion. The minor bands shown in (**F**) are due to incomplete *Bcl*I digests of the PCR amplicons. M = size marker

between MWG876 and the target gene, positioning this RFLP distal to *Rar1* (Fig. 1 D).

We attempted to separate MWG87 genetically from *Rar1* by testing a further 2207 F_2 plants for recombination events in the marker interval MWG876–MWG892 by CAPS analysis (Fig. 1 E). However, investigation of the observed recombinant plants still revealed co-segregation of *Rar1* and MWG87 (Fig. 1 E). The tight genetic linkage of MWG87, MWG876 and *Rar1* may indicate small physical distances between these loci, but could also be the result of a low recombination frequency in this genomic segment. To investigate these possibilities, and to enrich the interval with additional DNA markers, we initiated an AFLP-based marker search. If the tight genetic linkage of *Rar1*, MWG87 and MWG876 is caused by a suppression of recombination, then the large physical interval would be expected to reveal a large number of linked AFLP markers in the target region.

Targeted AFLP marker search

We employed the AFLP technology (Vos et al. 1995) in conjunction with a bulked segregant analysis (Giovannoni et al. 1991; Michelmore et al. 1991) by using selected F_2 progenies of the cross M100 × *Mla-12* BC Ingrid. To minimise the detection of AFLP markers which are not tightly linked to *Rar1* we used DNA marker-selected recombinants for the construction of DNA pools which we identified in the CAPS-based recombinant screen described above. Both DNA pools each comprised ten F_2 plants. At the time we initiated the AFLP marker screen we had analysed only 500 F_2 individuals in the marker interval MWG892–cMWG694, and the recombinant between MWG876 and *Rar1* had not yet been identified. In consequence, MWG876 could not be used to select suitable recombinants. The susceptible pool (*rar1*-2 /*rar1*-2) contained three individuals with a recombination between cMWG694 and *Rar1*, four individuals with a recombination between MWG892 and *Rar1* and three susceptible individuals without a recombination event in the investigated marker interval (Fig. 3). The selection of recombinants for the resistant pool was based on DNA markers only. By using plants which show the allelic pattern of the resistant parent for cMWG694 and MWG892 we could ensure homozygosity in the corresponding genetic interval. Therefore linked AFLP markers are expected in *trans* and *cis*. To narrow-down the target interval of the resistant pool we employed plants carrying a recombination event between MWG503 and MWG892 (two plants) or cMWG694 and MWG2123 (two plants). In addition to the recombinant individuals we used six plants without a detectable recombination event in the investigated marker interval.

The genome-wide frequency of AFLP polymorphisms between M100 and *Mla-12* BC Ingrid was found to be 7%. Each AFLP primer combination displayed, on average, 100 DNA fragments. Therefore, using seven *Pst*I + 2- and 56 *Mse*I + 3-primers in 392

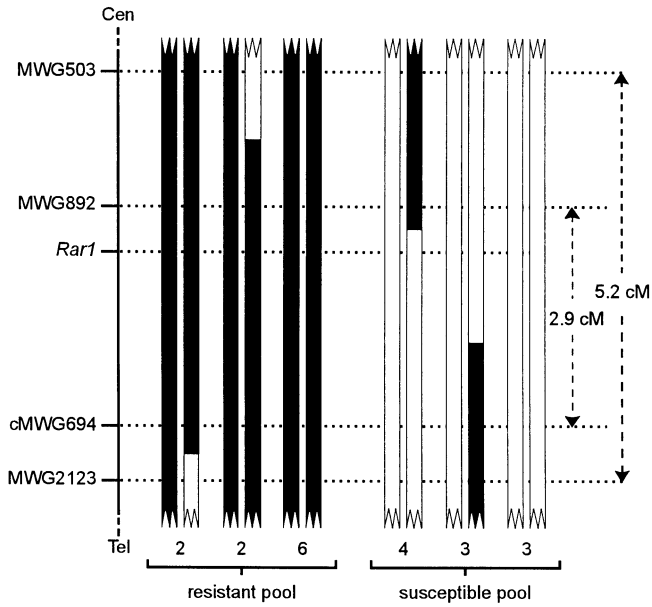

Fig. 3 Graphical genotypes of bulked segregant pools for targeted AFLP marker identification. The *resistant pool* and *susceptible pool* each consist of three different classes of individuals which are: (1) recombinant in distal orientation of *Rar1* (2) recombinant in proximal orientation of *Rar1* and (3) plants which are not recombinant in the marker interval *MWG503–MWG2123*. *Vertical bars* represent the investigated chromosome segment. *Open bars* designate chromosomal regions for the resistant parent and *black bars* designate chromosomal regions for the susceptible parent. The *number of recombinants* for each class is given below the graphical chromosome segments. *Horizontal dotted lines* represent the map positions of CAPS markers (shown on the left) which have been used to select individuals from the population segregating for *Rar1*. *Vertical dashed arrows* indicate the target interval size which was chosen for the *resistant* (5.2 cM) and *susceptible* (2.9 cM) *pool*

Fig. 4 Segregation of AFLP marker *R25/48*. *R25/48* was identified in the resistant bulked segregants. The autoradiograph shows the phenotype of DNA templates from the resistant (*Pr*; *Mla-12* BC Ingrid) and susceptible parent (*Ps*, *rar1*-1), bulked resistant (*Br*) and bulked susceptible plants (*Bs*), and the respective susceptible (*s*) and resistant (*r*) pool individuals. PCR products were analysed on a 4.5% denaturing polyacrylamide gel. The AFLP signal, linked to resistance (*Rar1*), is marked by a *horizontal arrow*. The presence of the linked AFLP signal in one of the susceptible pool individuals is marked by a *vertical arrow*, indicating a recombination event between the AFLP locus *R25/48* and *Rar1*, which is not displayed in the susceptible pool (*Bs*) ▼

combinations, approximately 40000 loci were inspected for polymorphism. Only two primer combinations identified AFLP markers linked to *Rar1* in the DNA pools. Analysis of these on individuals of each pool revealed that they are separated from the target gene by one (R25/48) and two (A25/48) recombination events and map distal relative to *Rar1* (Fig. 4 and Fig. 1 E).

The small number of identified AFLP markers linked to *Rar1* is certainly influenced by the way we assembled the DNA pools. It may also indicate that the small genetic interval in which we searched for DNA markers is physically not excessively large. To obtain more precise estimates of the relationship of genetic and physical distances in the vicinity of *Rar1* we performed PFGE Southern analysis in combination with rare-cutting restriction enzymes and RFLP probes linked to *Rar1*.

Long-range physical mapping

Fragment sizes after restriction with seven different rare-cutting restriction endonucleases were determined using the co-segregating probe MWG87 and flanking probes MWG892 and MWG876. The analysis revealed a single co-migrating *Mlu*I restriction fragment hybridising to MWG87 and MWG876 (Table 1, Fig. 5). This may indicate a maximal physical distance of 550 kb between MWG876 and MWG87. Fragments of common size were also detected using the probe /restriction enzyme combinations MWG876 /*Not*I, *Sal*I and *Sma*I (90 kb) and MWG87 /*Sfi*I and *Sma*I (100 kb). These fragments of common size obtained by using one probe and different endonucleases are possibly caused by a clustering of restriction sites, which has previously been reported in vertebrates (Bickmore and Bird 1992; Larsen et al. 1992).

Synteny adjacent to *Rar1* in barley and rice

To determine whether DNA marker loci linked to *Rar1* map in a collinear region in the rice genome, we carried out cross-hybridisation experiments with rice genomic DNA. Cross-hybridisation was observed with probes

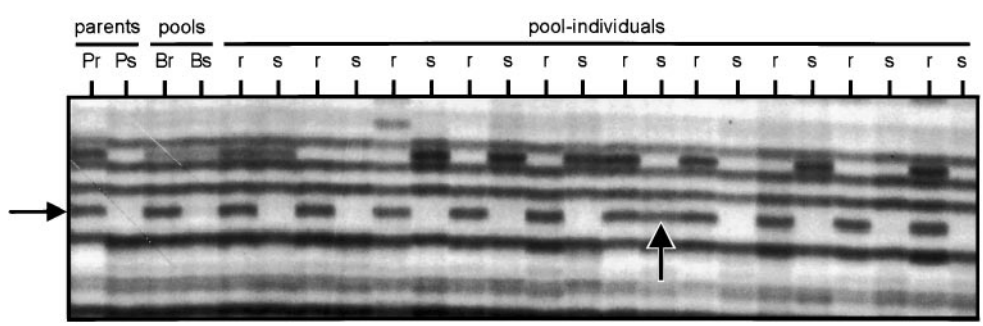

Table 1 Size of PFGE-separated restriction fragments (in kb) detected by Southern analysis in cultivar Sultan-5. ND, not determined

Fragment	Probe		
	MWG 876	MWG 87	MWG 892
*Cla*I	350	50	110
*Mlu*I	550	550	260
*Not*I	90	530	170
*Sal*I	90	240	310
*Sfi*I	90	240	310
*Sgf*I	120	100	140
*Sma*I	90	100	80

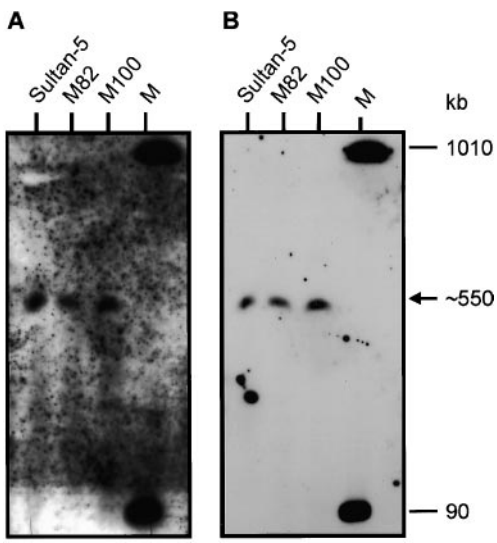

Fig. 5A, B PFGE Southern analysis of two RFLP loci tightly linked to *Rar1*. High molecular weight DNA of *Sultan-5*, *M82* and *M100* was restricted with *Mlu*I and separated on a PFGE gel for a size range of 100–1000 kb. The corresponding membrane was then probed with MWG876 (**A**) and MWG87 (**B**). The *arrow* indicates co-migrating fragments of approximately 550 kb detected with each probe. Common plasmid sequences within the probe and yeast chromosomes account for cross-hybridisation of the probe with two DNA fragments in the size marker (Lahaye et al. 1996)

MWG503, MWG876 and cMWG694. MWG503 and cMWG694, which define a 5-cM interval on barley chromosome 2, were mapped to the syntenic rice chromosome 4, enclosing a 19.3-cM interval (Fig. 6). Surprisingly MWG876, which in barley is located between MWG503 and cMWG694, was mapped to a non-syntenic region on rice chromosome 3. This map position was confirmed by using two different rice mapping populations and it indicates a break of synteny in the target interval.

Discussion

Positional cloning in complex plant genomes generally involves genetic fine mapping and DNA marker

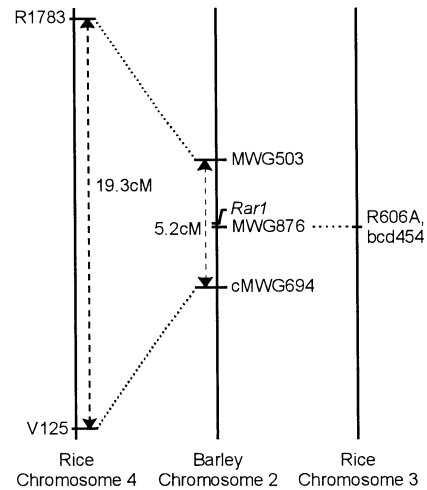

Fig. 6 Comparative mapping of barley probes linked to *Rar1* in rice. *Horizontal lines* indicate segments of *rice chromosome* 4, *rice chromosome 3*, and *barley chromosome 2*. Map positions in rice are based on 186 F_2 individuals from the Nipponbare × Kasalath cross. The barley probes *MWG503* and *cMWG694* which border a genetic interval of 5.2 cM on barley chromosome 2 enclose an interval of *19.3 cM* on the syntenic segment on *rice chromosome 4*. The probe *MWG876* maps in a non-syntenic chromosome segment on *rice chromosome 3*. Map positions of the indicated barley probes are connected via *dashed lines* with the respective rice probes [*R17830*, *V125*, *R606 A and bcd454* (Kurata et al. 1994; Quarrie et al. 1997)] which map to the same genetic position

identification in the target interval to delimit the physical size of the chromosome segment in which the target gene resides. The construction of a high-resolution genetic map at the respective target locus is, therefore, a prerequisite for the isolation of a gene based only on its map position.

In this paper, we describe the high-resolution mapping adjacent to the *Rar1* locus. Our data demonstrate that CAPS markers MWG876 and MWG892 define a 0.7-cM interval harbouring *Rar1*. We screened 4310/3270 segregants distal/proximal to *Rar1*, corresponding to a genetic resolution of 0.012/0.015 cM. The CAPS marker MWG87 showed complete linkage with *Rar1* on the basis of the inspected meiotic events. Since 1 cM corresponds to approximately 3000 kb in barley (Bennett and Smith 1991; Becker et al. 1995), *Rar1* would be expected to be located not further than 50 kb from MWG87. This distance would be small enough to use MWG87 in a chromosome landing approach (Tanksley et al. 1995) to isolate a single large-insert-size genomic clone containing *Rar1*. However, these calculations are based on genome-wide estimates of genetic and physical relationship and locus-dependent deviations from these estimates have been frequently reported. For example, a correlation of 43 kb/cM has been found in the tomato genome in the vicinity of the resistance locus I_2, (Segal et al. 1992) whereas, in the region surrounding the *Tm-2a* gene, 1 cM corresponds

Table 2 CAPS markers linked to the *Rar1* locus. For each CAPS marker the sequence of the primers, the PCR conditions, and the restriction enzyme used to detect the polymorphism are shown. Amplification products corresponding to the RFLP locus MWG892 reveal different lengths in the *Rar1* mutants and the *Mla12* BC lines Ingrid, Pallas and Siri

Marker	Primer	PCR conditions	Restriction enzyme
cMWG694	5′-AGTATCAGATGCTACCATGCCTGG 5′-CTCTGGAGGAGCCGAGTGTCAGC	94°C, 10 s 60°C, 20 s 72°C, 30 s } 35 cycles	*Hae*III
MWG87	5′-ATCAAACCAAGCAAAGGTCCCTTG 5′-CTGCAGGCGCACTTTAGGGGAAC	94°C, 10 s 58°C, 20 s 72°C, 30 s } 35 cycles	*Tru*I
MWG503	5′-CGTCAGAGCCCACGCCACACGTAG 5′-GCCGAACGTGCTCCAAGCGGCAAC	94°C, 10 s 60°C, 20 s 72°C, 40 s } 35 cycles	*Hin*6I
MWG876	5′-GTGGTCAAGGGCTTGTAGACTGGGTAC 5′-GCCCATCGGTGGTCGCCGTAGTCGCG	94°C, 10 s 60°C, 20 s 72°C, 30 s } 35 cycles	*Mva*I
MWG892	5′-GGAATCTTCCAGTGGGCTGGATGAG 5′-CAACCGGCCACTAGGCGTAAAGG	94°C, 10 s 60°C, 20 s 72°C, 30 s } 35 cycles	–
MWG2123	5′-CTGCGGCGAGAGCTTGAGAGCAGT 5′-GTGTGCATGGTCTCTTCCGCCCCG	94°C, 10 s 60°C, 20 s 72°C, 60 s } 35 cycles	*Bcl*I

to 4000 kb (Ganal et al. 1989). To analyse the relationship between physical and genetic distances adjacent to *Rar1*, we employed rare-cutting restriction enzymes in conjunction with PFGE Southern analysis.

PFGE Southern analysis revealed only one co-migrating 550-kb *Mlu*I restriction fragment hybridising to MWG876 and MWG87 (Fig. 5). This may indicate that the physical distance between the two loci is $\leqslant$ 550 kb. The genetic distance between both probes is 0.015 cM which is expected to correspond to less than 50 kb. Thus, one might expect additional common restriction fragments with other rare-cutting restriction enzymes, which we did not observe (Table 1). However, the distribution of rare-cutter recognition sites has been reported to be clustered rather than randomly distributed (Bickmore and Bird 1992; Larsen et al. 1992), complicating the interpretation of PFGE Southern analysis.

The data in Table 1 suggest that clustering of rare-cutter recognition sites may indeed be occurring in the analysed area because different restriction enzymes give rise to similar-sized fragments for a given probe. For example, using the MWG876 probe we apparently observed a common fragment size of 90 kb for *Not*I, *Sal*I and *Sma*I. Similarly, a 100-kb fragment was detected for the MWG87 probe following restriction with *Sma*I and *Sfi*I. However, the current amount of data is insufficient to prove or disprove the clustering of rare-cutter recognition sites close to *Rar1*.

Apart from the problem of clustered rare-cutter recognition sites, PFGE-based long-range physical mapping is limited due to the paucity of restriction endonucleases which generate large restriction fragments in the 500-kb size range. In this study we determined the average fragment size to be 230 kb. Similar studies at the *Hor2* locus on barley chromosome 1H identified the average size of restriction fragments as 100–300 kb (Siedler and Graner 1990), whereas the average size at the *Rpg1* locus on barley chromosome 7H was only 95 kb (Kilian et al. 1997). Estimates of physical distances based on the analysis of large insert clones circumvent the problems associated with rare-cutting endonucleases, such as methylation and a clustering of recognition sites. Screening of a barley YAC library [four genome equivalents, average insert size 480 kb; (Simons et al. 1997)] indicated that out of four DNA pools (each comprising DNA from 100 YAC clones) which are positive with MWG87, two are also positive with MWG876 (Lahaye and Schulze-Lefert; unpublished data). This observation supports our inference of a close physical linkage between MWG876 and MWG87 suggested by the common *Mlu*I restriction fragment.

Based on a genome-wide polymorphism of 7% and 40000 displayed AFLP fragments, we analysed about 3000 loci for linkage to *Rar1*. The barley genetic map covers approximately 1800 cM (Becker et al. 1995), thus we expected to spot about two AFLP markers

per cM. We identified two AFLP loci by bulked segregant analysis. This is in reasonable agreement with the predictions given the fact that the recombinants used for the targeted marker search are expected to narrow the actual interval size down to considerably less than 2.9 cM (Fig. 3). The finding of two AFLP markers may also indicate that there is no substantial suppression of recombination in the *Rar1* region.

The combination of bulked segregants and the AFLP technique is known to be an efficient approach for a targeted DNA-marker search (Cnops et al. 1996; Büschges et al. 1997; Simons et al. 1997). Nevertheless, it is still a time consuming, expensive, and laborious approach which necessitates the careful design of DNA pools optimised to detect only the most tightly linked AFLPs. Therefore the assembly of bulked segregant pools was optimised to identify markers closely linked to *Rar1*. Comparable marker-based selection schemes for pool assembly have been applied at the *Cf-9* locus in tomato (Thomas et al. 1995), the *Rps1* locus in soybean (Kasuga et al. 1997), the *TORNADO1* locus in Arabidopsis (Cnops et al. 1996), and the *R1* and *Gro1* loci in potato (Ballvora et al. 1995; Meksem et al. 1995).

The composition of DNA pools which were used for the AFLP marker screen at *Rar1* should theoretically exclude markers which are separated by one or more recombinants in the DNA pools. However, the limited detection sensitivity of X-ray films led to the identification of AFLP markers in the DNA pools which could still be separated from *Rar1* when using the DNA of pool individuals. Similar observations have been made at the *R1* locus in potato (Meksem et al. 1995). Using established DNA markers, these authors analysed plants in a target interval of about 20 cM, including *R1*, and identified recombinants suitable for a consecutive AFLP-based marker search. A comparison of different DNA pools without recombinants and with four heterozygous recombinants in a pool of eight plants revealed that the amount of detected AFLP markers in the target region was not affected. Analogous to our interpretation it was concluded that this was due to the low number of recombinants in the DNA pools.

In our AFLP marker search no marker was found that showed complete linkage to *Rar1*. One reason for this is probably the small physical size of the target interval which is defined by the recombinants (Fig. 3). To find further AFLPs more tightly linked or co-segregating with *Rar1*, we would have clearly needed to analyse more primer combinations. Since we already found one RFLP marker that showed co-segregation based on a genetical resolution of 0.015 cM we decided not to continue the AFLP marker screen. We calculated, that the analysis of 120000 polymorphic AFLP fragments (corresponding to 17000 primer combinations) would be necessary to detect an AFLP which is as tightly linked as MWG87.

Collinearity of markers among grass species has been investigated at the macro- and micro-level (Devos et al. 1993; Dunford et al. 1995; Devos and Gale 1997; Foote et al. 1997). Rice has been proposed to be the model plant for cereal genomes due to its small genome size and the low amount of repetitive DNA it contains. These features of the rice genome, combined with the conservation of gene order among grass species (synteny), could provide a basis for the isolation of genes from cereals with larger genomes. The usefulness of rice as a tool for positional cloning relies entirely on the maintenance of collinearity in a given target interval. Therefore, we investigated synteny next to *Rar1* by using the probes MWG503 and cMWG694 which enclose a 5.2-cM interval in barley. These two probes map to the expected syntenic region on rice chromosome 4. Surprisingly, MWG876, which is located between MWG503 and cMWG694, maps to a non-syntenic region on rice chromosome 3. A similar observation has been reported close to the *Rpg1* locus on barley chromosome 7H. Although collinearity was observed for several loci, one rice probe mapped to a non-syntenic region (Kilian et al. 1995). The indication of a break point adjacent to *Rar1* limits future attempts to use synteny between barley and rice for the search of candidate genes in the target region.

Future experiments towards the isolation of *Rar1* will focus on the physical delimitation of *Rar1* within a large insert clone. The successful outcome of this approach will to a large extent depend on the local relationship between genetic and physical distances.

Acknowledgements We thank our colleagues Abdelhafid Bendamane, Collin Kleanthous, and Edda von Röpenack for helpful suggestions on the manuscript. For the mapping of barley probes in rice we thank David Laurie, The John Innes Centre, Norwich, UK. This work was supported by grants from the Gatsby Foundation and from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, Germany (grant #0310687/996) to P. S.-L.

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