Theor Appl Genet (1999) 98: 679–689

K. Kanyuka · A. Bendahmane
J. N. A. M. Rouppe van der Voort
E. A. G. van der Vossen · D. C. Baulcombe

Mapping of intra-locus duplications and introgressed DNA: aids to map-based cloning of genes from complex genomes illustrated by physical analysis of the *Rx* locus in tetraploid potato

Received: 13 July 1998 / Accepted: 17 September 1998

Abstract We describe here novel approaches to high-resolution mapping of repeated and introgressed DNA which may prove generally useful in map-based cloning from complex genomes. These approaches were developed in order to clone the *Rx* locus in potato. First, we prepared a BAC library from a tetraploid plant carrying *Rx* in the duplex condition (*Rx*, *Rx*, *rx*, *rx*). BAC clones were then isolated with close markers on either side of *Rx*. However these clones did not extend across *Rx*: in the cloned DNA the closest markers to *Rx* were separated by single recombination events on either side of *Rx*. To bridge the gap we exploited the finding that the BAC clones on the right side of *Rx* contained resistance-gene homologues. Anticipating that there would be duplicated copies of these resistance gene homologues in the vicinity of *Rx*, we used low-stringency PCR conditions to identify additional markers. One of these markers was completely linked to *Rx* in our mapping population and was used to isolate a BAC (BAC77) that had not been previously identified by screening with *Rx*-flanking markers. Based on two criteria it was concluded that BAC77 spans *Rx*. There was a chromosomal recombination in one plant of our mapping population that separated the BAC77 right end from *Rx*. On the other side of *Rx* it was found that the BAC77 left end was outside the region of DNA carrying *Rx* that had been introgressed into potato cv Amaryl from a *Solanum tuberosum* ssp. *andigena* accession CPC1673.

Key words Map-based cloning · BAC · *Rx* · PVX · Potato

Communicated by F. Salamini

K. Kanyuka · A. Bendahmane · D. C. Baulcombe (✉)
The Sainsbury Laboratory, John Innes Centre,
Norwich Research Park, Colney, Norwich NR4 7UH, UK
Tel.: +44 (01603) 452571
Fax: +44 (01603) 250024
E-mail: baulcombe@bbsrc.ac.uk

K.K. and A.B. contributed equally to this research

J. N. A. M. Rouppe van der Voort
Department of Nematology, The Graduate School of Experimental Plant Sciences, Wageningen Agricultural University, P.O. Box 8123, 6700 ES Wageningen, The Netherlands

E. A. G. van der Vossen
Centre for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, 6700 AA Wageningen, The Netherlands

Introduction

We are currently adopting a map-based cloning approach to the molecular cloning of the *Rx* locus in potato (Cockerham 1970). *Rx* confers extreme resistance against potato virus X (PVX) and, although PVX is not a major problem in potato production, there are several reasons for the cloning and further analysis of this gene. The genetical analysis of the *Rx*-mediated resistance in both the host plant and the pathogen shows that it fits the gene-for-gene paradigm (Flor 1971; Keen 1990), indicating that the findings from *Rx*-mediated resistance will be relevant to resistance against fungi, bacteria and other pathogens. Furthermore, analysis of *Rx*-mediated resistance in protoplasts prompted the suggestion that it may be possible to produce broad spectrum virus resistance by the manipulation of *Rx*-mediated resistance. This protoplast analysis demonstrated that the specificity of *Rx*-mediated resistance is determined at the level of recognition and that there is a response phase that can suppress several viruses, and not only PVX (Köhm et al. 1993; Bendahmane et al. 1995).

Until now, no genes have been cloned from potato using map-based approaches despite the high-resolution mapping of several loci in the potato genome (Ballvora et al. 1995; Meksem et al. 1995; Brigneti et al.

1997; De Jong et al. 1997). In fact, only in *Arabidopsis thaliana* have map-based approaches been widely applied (Giraudat et al. 1992; Bent et al. 1994; Mindrinos et al. 1994; Busch et al. 1996; Lukowitz et al. 1996; Ryals et al. 1997). In other plants there are only few reports of genes that have been cloned by a map-based approach (Martin et al. 1993; Dixon et al. 1996; Büschges et al. 1997). In the present paper we describe an extension of our previous mapping of *Rx* that was designed to explore the feasibility of a map-based cloning approach. From our findings we developed novel strategies leading to identification of a bacterial artificial chromosome (BAC; Shizuya et al. 1992) clone that spans *Rx*. This approach was successful despite the suppression of recombination and the depletion of AFLP (Vos et al. 1995) and RFLP markers in the vicinity of *Rx*, and could be applied to other map-based cloning projects in plants with complex genomes.

Materials and methods

Plant material

F_1 seeds from the cross between potato cv Cara (*Rx*, *rx*, *rx*, *rx*) and cv Huinkel (*rx*, *rx*, *rx*, *rx*) were obtained from the Plant Breeding Institute, Cambridge. The progeny of this cross (370 individuals) was used in initial experiments (Bendahmane et al. 1997) to identify the chromosomal position of *Rx*, and to assign RFLP markers linked to *Rx*. The self pollinated (S_1) progeny of cv Cara (1350 plants) (Bendahmane et al. 1997) was used as a mapping population to position *Rx* with respect to RFLP, AFLP and BAC-derived PCR markers. An additional 1438 plants of S1 cv Cara were employed to ascertain whether BAC77 spans *Rx*. A diploid potato clone SH83-92-488 (Rouppe van der Voort et al. 1997) having cv Amaryl (*RxGpa2*, *rxgpa2*) in its pedigree, cv Amaryl and self-pollinated offspring of *Solanum tuberosum* ssp. *andigena* accession CPC1673 (encoded CPC3520 and obtained from the Commonwealth Potato Collection, Scottish Crop Research Institute, Dundee, UK) were used for 'introgression mapping'.

Construction of the potato BAC library

High-molecular-weight DNA was prepared in agarose plugs from potato protoplasts essentially as described in Bendahmane et al. (1997). The agarose plugs were dialysed three times for 30 min against TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA), once at room temperature and twice at 4°C. The plugs were then equilibrated in *Hin*dIII buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.9) twice on ice for 1 h. Half of each plug (about 5 μg of DNA) was transferred to a test tube containing 360 μl of *Hin*dIII buffer and 10–15 units of *Hin*dIII restriction enzyme. The enzyme was allowed to diffuse into a plug at 4°C for 1 h and the digestion was carried out at 37°C for 30 min. The reaction was stopped by adding 1 ml of 0.5 M EDTA. Plugs were washed in 0.5 × TBE (45 mM Tris-borate pH 8.0, 1 mM EDTA), loaded into a 1% low-melting-point agarose gel and subjected to contour-clamped homogeneous electric field (CHEF; Chu, 1989) electrophoresis in a CHEF DR II system (Bio-Rad Laboratories, USA) in 0.5 × TBE buffer at 150 V for 10 h at 4°C and a constant pulse time of 5 s or 8 s. Compression zones containing the DNA fragments of approximately 100 kb or approximately 150 kb were excised from the gel and dialysed against 30 ml of TE in a 15-cm Petri dish for 2 h at 4°C. Dialysed agarose slices then were transferred to a 1.5 ml test tube, melted at 65°C for 10 min, and digested with 1 unit of *Gelase* (Epicentre Technologies, USA) per 100 μg of an agarose gel for 1 h at 45°C.

The size-selected potato DNA (25–50 ng) was ligated to 25–50 ng of *Hin*dIII-digested and de-phosphorylated pBeloBAC11 vector, kindly provided by Dr. H. Shizuya (University of Southern California, Los Angeles; Shizuya et al. 1992), using 400–800 units of T4 DNA Ligase (New England BioLabs, USA) at 16°C for 24 h in a total volume of 50 μl. The ligation products were dialysed against 1 × TE using 0.025 μM MF-Millipore membrane filter (Millipore, UK) at 4°C for 2 h and 30 min at room temperature using the 'drop dialysis' method of Maruzyk and Sergeant (1980).

Transformation of *Escherichia coli* DH10B cells was carried out by electroporation using a BRL Cell-Popagator System (Life Technologies Ltd, UK). To 20 μl of electro-competent cells, 0.5–3 μl of ligation mixture was added. After electroporation, *E. coli* cells were mixed with 1 ml of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated at 37°C for 1 h with gentle shaking (80 rpm). The cells were spread on Luria broth (LB) agar plates containing chloramphenicol (12.5 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) (40 μg/ml) and isopropyl-1-thio-β-D-galactoside (IPTG) (0.12 mg/ml). Plates were incubated at 37°C for 24 h.

DNA from the compression zones of 100 and 150 kb led to clones with an average insert size of 100 kb. The 100-kb DNA produced approximately 1000 white colonies per μl of ligation mixture, whereas the 150-kb DNA produced only 150 white colonies per μl of ligation mixture. Approximately 92 000 white colonies were picked individually into 384-well microtiter plates (Genetix, UK) containing LB freezing buffer [36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% V/V glycerol, 12.5 μg/ml of chloramphenicol in LB medium], grown overnight at 37°C and stored at −80°C. We also prepared 100 bacterial pools containing about 500–1000 white colonies each (these pools also contained approximately 500–1500 blue bacterial colonies with an empty pBeloBAC11) by scraping the colonies from agar plates into the LB medium containing 18% glycerol and 12.5 μg/ml chloramphenicol using a sterile glass spreader. These pools were also stored at −80°C.

Screening of the potato BAC library

The potato BAC library was screened with IPM3, IPM4 and IPM5 CAPS markers, corresponding to the AFLP markers PM3, PM4 and PM5 flanking the *Rx* locus (Bendahmane et al. 1997), as follows.

For the first part of the library of 92 160 clones stored in 384-well microtiter plates the plasmid DNA was isolated using the standard alkaline-lysis protocol (Heilig et al. 1997) from pooled bacteria of each plate to produce 240 plate pools. Aliquots of these plate pools were combined to prepare 26 'superpools' each of which contains DNA from nine plate pools, and one superpool containing DNA from six plate pools. To identify individual BAC clones carrying the CAPS markers, the superpools and then the corresponding plate pools were screened. Once an individual plate had been identified the clones corresponding to each of the 24 columns of the positive plate were grown for 3–4 h at 37°C in LB medium and PCR was carried out on 3 μl of bacteria. After identification of a positive column a colony PCR on each of the corresponding 16 colonies of this column was carried out leading to the identification of a single positive BAC clone.

The second part of the library was stored as 100 pools of approximately 1000 clones. The plasmid DNA was isolated from each pool of clones using a standard alkaline-lysis protocol and PCR was carried out to identify the positive pool. The bacteria corresponding to the positive pool were diluted, plated on LB agar plates and then

colony hybridisation was carried out as described in Sambrook et al. (1989) using ^{32}P-labelled DNA probes corresponding to CAPS markers. PCR with the corresponding CAPS primers was used to distinguish hybridising colonies carrying the markers that had been previously mapped from the homologues located elsewhere in the genome.

Analysis of the BAC library

BACs containing potato DNA were isolated from 5-ml overnight cultures (LB medium supplemented with 12.5 mg/ml of chloramphenicol) using the standard alkaline-lysis miniprep protocol (Engebrecht et al. 1997) and re-suspended in 50 µl of TE. Plasmid DNA (10 µl) was digested with *Not*I for 3 h at 37°C to release the insert DNA from the pBeloBAC11 vector. The digested DNA was separated by CHEF electrophoresis in a 1% agarose gel in 0.5 × TBE at 4°C using a BIO-RAD CHEF DR II system (Bio-Rad Laboratories, USA) at 150 V with a constant pulse time of 14 s for 16 h.

Isolation and characterization of BAC ends

An inverse polymerase chain reaction (IPCR; Ochman et al. 1990) was used to isolate the right and left end-sequences of insert DNAs. BAC DNA was isolated and digested separately with *Nla*III, *Hpa*II, *Mse*I, *Hin*P1I, *Pvu*II, and *Hae*III (for isolation of a left end-sequence) or with *Rsa*I, *Sac*I, *Eco*RI, *Hae*III, *Mae*II, *Mse*I, *Pvu*II, and *Hin*P1I (for isolation of a right end-sequence) for 4 h at 37°C and re-circularised by self-ligation for 2 h at 20°C. Ligations were carried out using 5–50 ng of digested DNA and 5–10 units of

Table 1 PCR-based markers linked to the *Rx* locus

Marker	Primers	PCR conditions[b]	Restriction enzyme
117L	5′-CCTAGCGTAGAGCGGTGTATCCA 5′-GTAGACATTTAATAATTCGTCGATAC	94°C, 15 s × 35 cycles 57°C, 20 s × 35 cycles 72°C, 120 s × 35 cycles	*Rsa*I
191L	5′-ACAAATTGTATAATTATAGTGATACG 5′-CAAGACATTAATTAACCAAACAATGG	94°C, 15 s × 35 cycles 50°C, 15 s × 35 cycles 72°C, 120 s × 35 cycles	*Eco*RI
77L	5′-GCTTCTAAACTCTAAATTCAGATTC 5′-CATGTGCGGACTCGTTCTTTTGTAG	94°C, 15 s × 35 cycles 64°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	*Alu*I
IPM4	5′-GTACTGGAGAGCTAGTAGTGATCA 5′-GAACACCTTAACTACACGCTGCAGG	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	*Taq*I
77R[a]	5′-GAAAGACAATTCCAGTGTGATGCG 5′-CAGGTAAGCCTCCTCATAACATGC	94°C, 15 s × 35 cycles 66°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	
45L[a]	5′-GGAGTCAATGCAGGGTCTATGGAA 5′-CTCATTTGACACTTCTCGAACACA	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 50 s × 35 cycles	
221R[a]	5′-GCTTACATTTGCTCGAAGAAGCCAC 5′-CCTTAATAATCAATAGATTCAACTCG	94°C, 15 s × 35 cycles 60°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	
111L[a]	5′-CCGAGTTTGCTCGATTCCGAGTTTT 5′-CTAAGGGATCCACTAGTCTAATTTG	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	
111R[a]	5′-CCACTGTGTAAGGGTCAACTATAGTC 5′-GAGATGAAGATTTTCTTGTCTGATGG	94°C, 15 s × 35 cycles 65°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	
73L	5′-CATTTCCTGAATTGCTTCCGACTTC 5′-CCATGAAAATTGTTATCACTGAGGTC	94°C, 15 s × 35 cycles 60°C, 15 s × 35 cycles 72°C, 50 s × 35 cycles	*Alu*I
218R	5′-GATTACAGTTGTGAATTAGTTCGGTA 5′-GCAACAGATATATTCCACTTACCATTC	94°C, 15 s × 35 cycles 60°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	*Alu*I
218L[a]	5′-CTTAACAAACCTATCATATTGGCCAT 5′-AGCTTCACATTGAACCAGAGGCCT	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	

[a] These markers are allele-specific (i.e. marker primers are able to amplify only an allele linked in *cis* to *Rx*). All other markers were CAPS revealed in the *Rx*-linked form by digestion with a corresponding enzyme (right hand column)

[b] The PCR conditions are the standard PCR conditions used with these primers. Some of the primers were also used in low-stringency PCR with a primer annealing temperature at 10°C below the temperature shown

T4 DNA Ligase (Boehringer Mannheim, Germany) in a total volume of 100 µl. PCR amplification of the re-circularised DNA was carried out using 3 µl of self-ligated DNA as the template. AB1 (5′-CCTAAATAGCTTGGCGTAATCATG-3′) and AB2 (5′-TGACACTATAGAATACTCAAGCTT-3′) primers were used for PCR-amplification of the left end-sequence of insert DNA, AB3 (5′-CGACCTGCAGGCATGCAAGCTT-3′), while AB4 (5′-AC-TCTAGAGGATCCCCGGGTAC-3′) primers were used for PCR-amplification of the right end-sequence of insert DNA. PCR conditions were as follows: 94°C for 15 s, 60°C for 15 s, 72°C for 90 s – for 35 cycles. PCR products were digested simultaneously with *Hin*dIII and the restriction enzyme used in the preparation of the IPCR DNA template. The released insert ends were gel-purified and cloned into pGEM-3Z(f+) (Promega, USA). Sequences of the clones containing approximately 1–2 kb inserts were determined using a 377 or 373 DNA Sequencing System (Applied Biosystems, UK), and these sequences were employed to design PCR primers for amplification of the BAC end-region in potato genomic DNA.

The sequences of primers and the PCR conditions used in the amplification of each BAC end-sequence are summarised in Table 1. The BAC end-sequences were named according to the name of the BAC, followed by the letter L for the left end- or by the letter R for the right end-sequence. For example, the left and the right end-sequences of BAC218 are 218L and 218R, respectively.

The translated versions of BAC end-sequences and of other markers in the vicinity of *Rx* were compared with the PDB, SWISS-PROT, PIR(R), GenPept, and GenPept databases using BLAST (Altschul et al. 1990).

Results

Genome representation in a BAC library

Our previous analysis of *Rx* in potato was based on potato cultivar (cv) Cara carrying *Rx* in a simplex condition (*Rx*, *rx*, *rx*, *rx*). To increase the probability of cloning *Rx*, we prepared a BAC library of 160 000 clones from plant SC-781 which is a self-pollinated progeny of cv Cara carrying *Rx* in the duplex condition (*Rx*, *Rx*, *rx*, *rx*). Based on the average insert size of 100 kb in the BAC clones (data not shown), a haploid genome size of 900 000 kb in potato (Arumuganathan and Early 1991), and taking into account the presence of *Rx* in the duplex condition, we estimate that there is more than a 99% probability that this library carries the DNA of the *Rx* (or indeed any other) locus. This estimate is based on the formula: $N = Ln(1 - P)/Ln(1 - I/G)$, where I is the size of the average cloned fragment in base pairs, and G is the size of the genome in base pairs (Clark and Carbon 1976). According to this formula, for any gene carried in the duplex condition, there would need to be more than 80 000 BAC clones of 100 kb to have a 99% probability of representation in a library.

To confirm the genome representation in this potato BAC library we screened for clones containing single-copy *c*leaved *a*mplified *p*olymorphic *s*equence (CAPS; Konieczny and Ausubel 1993) markers (GP34, PM3, CT99 and CT129) that are linked to *Rx* (Bendahmane et al. 1997). In each instance we obtained between one and eight positive clones (data not shown). At least one clone identified with each marker tested contained an allele linked in *cis* to *Rx*. Therefore, the BAC library is representative and could be used to identify BAC clones spanning many regions of interest in the potato genome.

Screening of the potato BAC library with markers closely linked to and flanking the *Rx* locus

The CAPS markers IPM3 and IPM4 flank *Rx* and span an interval of 0.23 centiMorgans (cM) (Bendahmane et al. 1997). As the estimated physical distance between these markers is only about 100 kb (Bendahmane et al. 1997) we expected to isolate a single BAC clone or a set of clones which span the *Rx* locus.

Using the IPM3 marker to screen the BAC library we identified three BAC clones, BAC167, BAC191 and BAC117, with potato DNA inserts ranging from 100 to 120 kb (Fig. 1). *Dde*I digestion of the IPM3 DNA in these BACs and in potato DNA samples revealed that BAC117 carried the IPM3 allele that was linked in *cis* to *Rx*. The other two BACs, BAC167 and BAC191, contained alleles from a corresponding region of the *rx* chromosomes (Fig. 2a).

To identify the relative genome positions of these BACs we designed pairs of PCR primers based on the sequence of the right and left ends of the insert (see Materials and methods). PCR tests using the BAC DNAs as templates showed that these BACs overlapped in the order BAC167, BAC117, BAC191, *Rx*. The 191L marker was separated from *Rx* by only a single chromosomal recombination event (in plant #1146) in a mapping population of 1720 plants. In the same

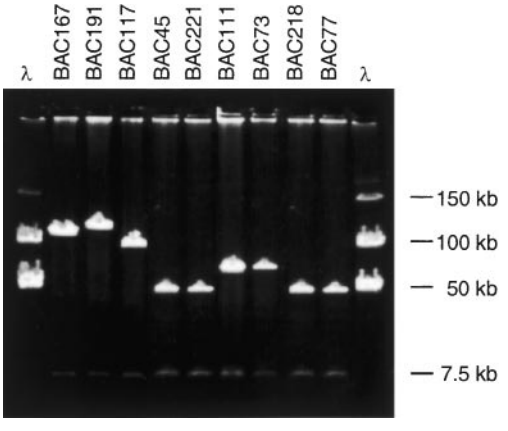

Fig. 1 CHEF electrophoresis analysis of BAC clones containing DNA from *Rx* locus BAC DNA was isolated, digested with *Not*I, and subjected to CHEF electrophoresis as described in Materials and methods. *Names* of the BAC clones are indicated at the top of each line. The high-molecular-weight *Not*I restriction fragments are potato BAC DNA inserts. The DNA fragment of about 7.5 kb present in all BAC lanes corresponds to a linear pBeloBAC11 vector DNA. Concatemers of bacteriophage lambda DNA, '*λ*', were used as size standards as indicated on the right side of the panel

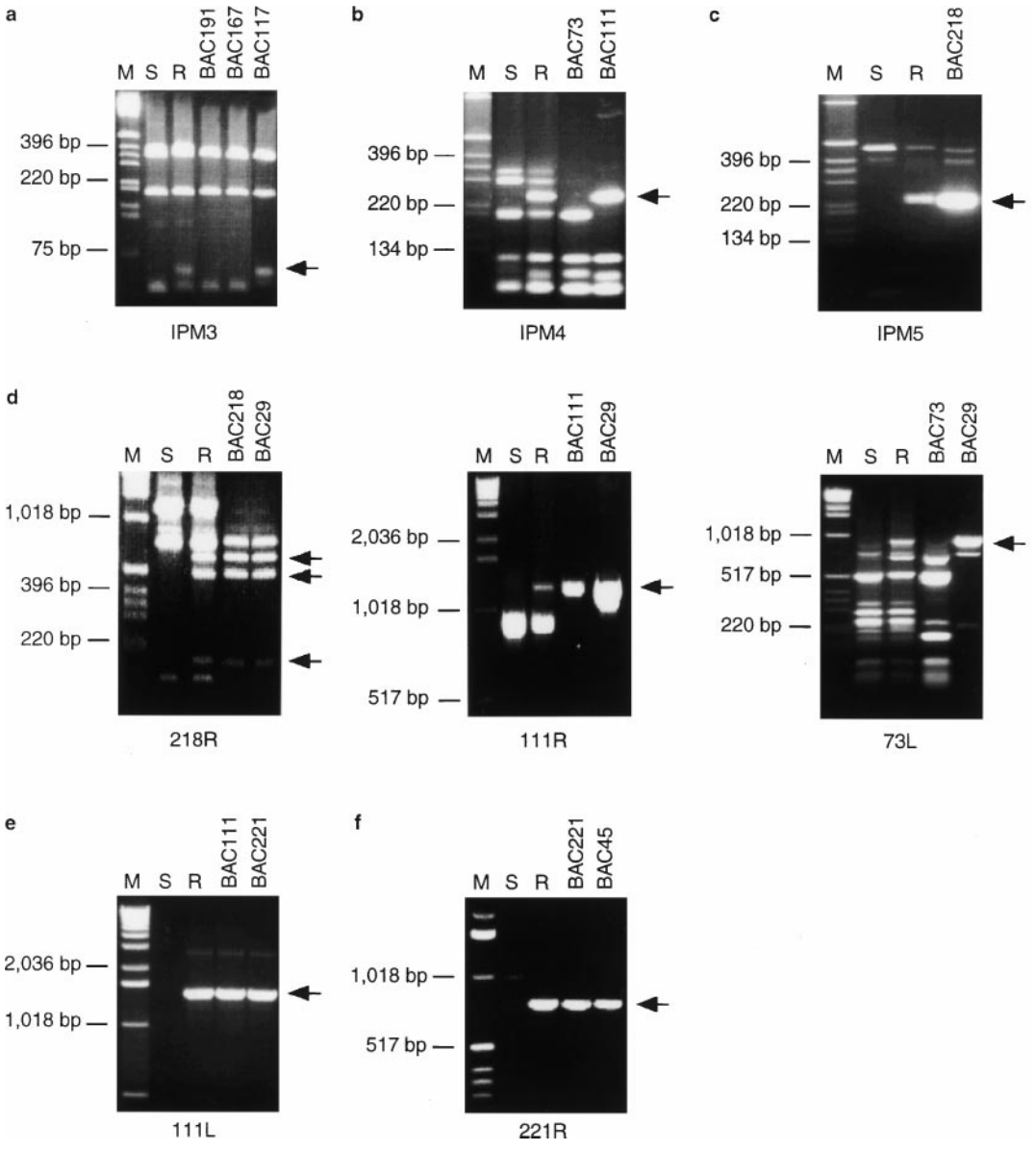

Fig. 2a–f PCR analysis of potato BAC clones identified with markers from the *Rx* locus Pooled DNA of 20 *Rx* plants (*R*), pooled DNA of 20 *rx* plants (*S*), and DNA of BAC clones was subjected to PCR using the primers indicated at the bottom of each panel. *Names* of the BAC clones are indicated at the top of each lane. PCR products obtained with CAPS marker primers (IPM3, IPM4, IPM5, 218R and 73L) were digested with corresponding restriction enzymes (see text, and Table 1) prior to gel electrophoresis. Allele-specific PCR products (111R, 111L and 221R) were subjected to an agarose-gel electrophoresis without digestion. The *arrow*(*s*) identifying the DNA fragment(s) linked in *cis* to *Rx* are indicated on the right of each panel. DNA size markers are indicated as '*M*'

population, 117L and IPM3 markers were separated from *Rx* by two and three recombination events respectively whereas the GP34 marker, present in BAC167, was separated from *Rx* by 13 recombinations (Fig. 3). The BAC library did not contain additional BACs extending further towards *Rx* from the 191L marker.

Characterization of the BAC clones linked to the IPM4 CAPS marker

The IPM4 marker has been mapped at 0.06 cM from *Rx* on the side away from IPM3 (Bendahmane et al. 1997). Screening of the BAC library with IPM4 identified two clones, BAC73 and BAC111, with inserts of approximately 70 kb each (Fig. 1). The *Taq*I digestion of the IPM4 CAPS marker in these clones indicated that BAC111 was linked in *cis* to the *Rx* locus whereas BAC73 carries a DNA insert from the *rx* chromosome (Fig. 2 b).

To determine the relative genome position of BAC111 and BAC73 we performed PCR tests using end-sequence primers of these BAC clones (Table 1). The results of these tests suggested that BAC73 overlaps with BAC111 and that 73L and 111L represent opposite ends of these BACs. Both 73L and 111L,

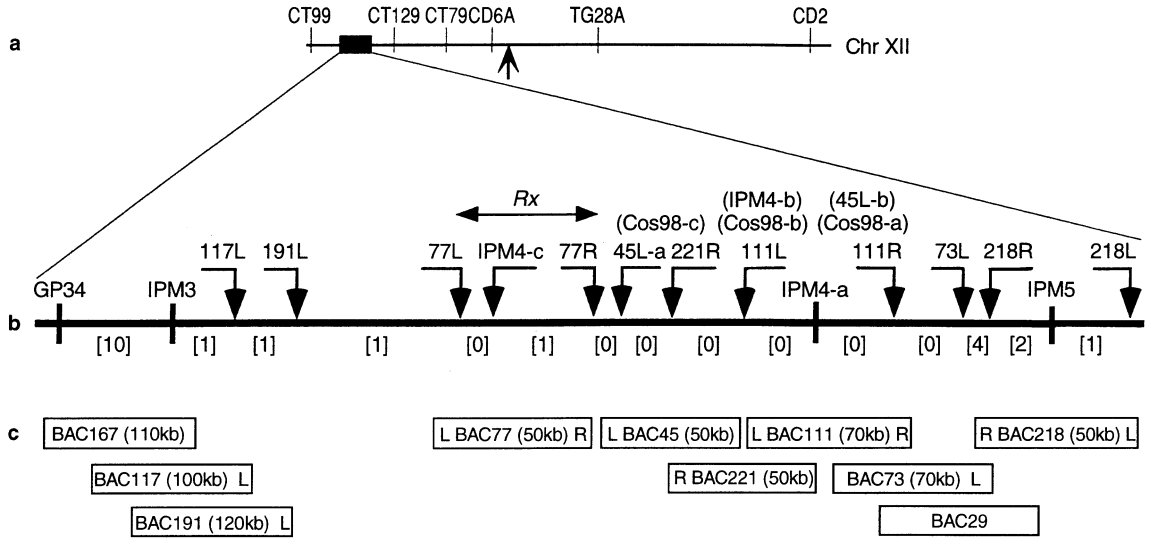

Fig. 3a–c High-resolution map of the *Rx* locus (not drawn to scale). Panel **a** shows a simplified genetic map of potato chromosome XII (denoted by a *horizontal line*) in which the area left of the *arrow* is reversed in the potato and tomato genetic maps (Tanksley et al. 1992). *Vertical lines* indicate positions of previously mapped RFLP markers (Tanksley et al. 1992; Bendahmane et al. 1997). The *filled rectangle* denotes a genetic interval between markers GP34 and 218L, which is magnified in panels **b** and **c**. Panel (**b**) shows the genetic map of the GP34–218L interval (denoted by a *horizontal line*). Positions of the RFLP marker GP34 and the AFLP markers IPM3, IPM4-a and IPM5 are indicated by *vertical lines*. The positions of BAC end-derived markers are indicated by *arrows*. The positions of markers enclosed *in parentheses* (e.g. 45L-b) have not been determined precisely. The *numbers* in square brackets indicate the numbers of S1 Cara individuals containing recombination events in each marker interval. The predicted position of *Rx* is shown by a ' ' symbol. Panel (**c**) shows the positions of BAC clones in the GP34–218L interval. Each *open rectangle* represents one BAC insert DNA. Inside of each rectangle is the name of the BAC clone, the estimated insert size in kb (except for BAC29) and a symbol '*L*' or '*R*' denoting the BAC ends that had been mapped relative to *Rx*

co-segregated with IPM4. In our mapping population of 1720 individuals these markers were both separated from *Rx* by one recombination event (in individual #761) and it was not possible to determine directly which of these markers was physically closer to *Rx*. Hence, to orientate these BACs relative to *Rx*, we screened the BAC library with CAPS markers 111L and 73L. We also screened the BAC library with the IPM5 CAPS marker, which is on the same side of *Rx* as IPM4 but further from *Rx* (Bendahmane et al. 1997). We anticipated that BACs containing IPM5 would allow us to orientate the 111L and 73L markers relative to *Rx* (Fig. 3).

These analyses identified BAC218, carrying an allele of IPM5 identified by *Pst*I digestion as being linked in *cis* to *Rx* (Fig. 1; Fig. 2c). The end-sequences of BAC218 insert DNA were converted into the CAPS markers 218L and 218R, and mapped genetically to the recombination events between GP34 and IPM5. Marker 218L was positioned at eight recombination events (0.48 cM) from *Rx*, between IPM5 and CT129. The 218R marker was positioned between IPM4 and IPM5 at five recombination events (0.30 cM) from *Rx*. We also identified a single BAC pool #29 which contains three markers: 218R, 73L and 111R. CAPS analysis revealed that each of these markers in the BAC pool #29 is represented by the allele linked in *cis* to *Rx* (Fig. 2d). As it is very unlikely ($P = 0.05$) that a single BAC pool contains two different BAC clones carrying markers 218R and 73L or 218R and 111R separately we conclude that BAC pool #29 contains a single BAC clone, BAC29, with a DNA insert linked in *cis* to *Rx*. Therefore, BAC29 provided a link between BAC218 and the IPM4 BAC contig and allowed us to orientate the markers from the IPM4 contig in the following order: *Rx*, 111L, IPM4, 73L (Fig. 3).

By screening the BAC library with 111L allele-specific primers we identified BAC221 which carries an insert DNA of 50 kb and is linked in *cis* to *Rx* (Fig. 1; Fig. 2e). The left end-sequence of BAC221 is located within BAC111 whereas the right end-sequence of BAC221 extends further towards *Rx* (Fig. 3). However the marker 221R co-segregated with IPM4 (Fig 3) in our mapping population and was separated from *Rx* by the recombination event in plant #761.

To extend the IPM4 contig further towards *Rx* we screened the BAC library with 221R allele-specific primers and identified BAC45 which has an insert DNA of 50 kb and is linked in *cis* to *Rx* (Fig. 1; Fig. 2f). The right end-sequence of BAC45 is located inside of BAC221, whereas the left end-sequence of BAC45, 45L, extends further towards *Rx* (Fig. 3). However, BAC45 does not contain *Rx* since the CAPS marker 45L is genetically separated from *Rx* by the recombination event in plant #761. Additional PCR screening of the BAC library with the 45L marker failed to identify any

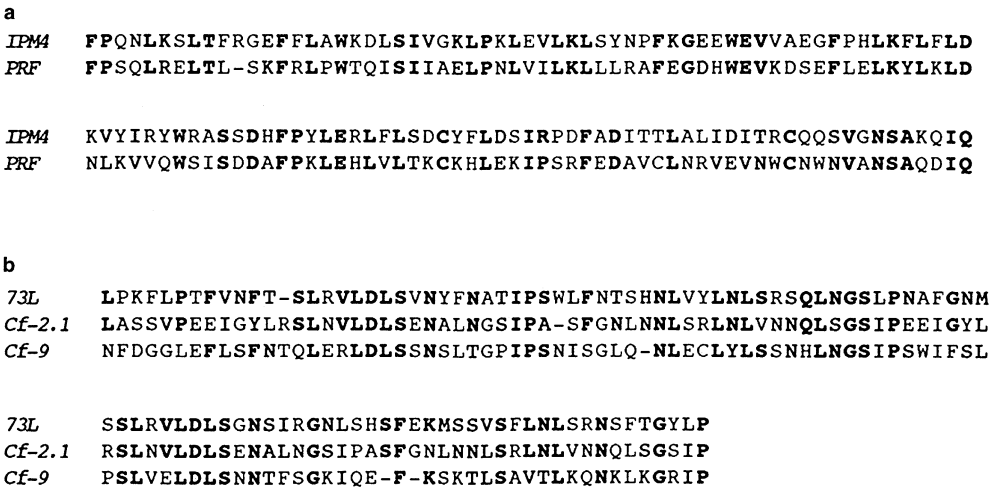

Fig. 4a, b Alignments of putative peptide sequences derived from *Rx*-linked markers. **a** The aligned putative peptide sequence of the IPM4 marker with the homologous region of Prf (Salmeron et al. 1996), and **b** the putative peptide sequences of the 73L marker aligned with homologous regions of the Cf-2 and Cf-9 proteins (Jones et al. 1994; Dixon et al. 1996). Sequences were aligned using BLAST (Altschul et al. 1990). Identical residues are indicated in *bold*, similar residues are in *black*, and different residues are in *grey*

new BAC clones, therefore leaving a gap between the IPM3 and IPM4 BAC contigs (Fig. 3).

Analysis and utilization of duplicated DNA sequences for mapping of *Rx*

The translated versions of several BAC-derived markers from IPM4 contig are similar to cloned disease resistance genes (Fig. 4, and data not shown). The marker IPM4 was similar to the tomato *Prf* gene required for the resistance to *Pseudomonas syringae* pv. *tomato* (Salmeron et al. 1996), and the marker 73L showed a significant degree of homology to the *Cf-2* and *Cf-9* resistance genes of tomato (Dixon et al. 1996; Jones et al. 1994) (Fig. 4). These data indicated that *Rx* is in a cluster of disease resistance-like genes.

Disease resistance loci in plants are often highly complex with small families of resistance genes clustered within tens of kilobases (Ellis et al. 1995; Hulbert and Bennetzen 1991; Jones et al. 1994; Martin et al. 1993; Parniske et al. 1997; Witham et al. 1994). These clusters are due to DNA duplications that may reflect a mechanism of resistance gene evolution (Anderson et al. 1996; Song et al. 1997). Taking into account these findings, we have tested for the presence of the duplicated sequences related to CAPS markers from the vicinity of *Rx* (IPM3–IPM5 interval). In these tests we used pools of DNA from 20 resistant plants (R pool) and 20 susceptible plants (S pool) and the individual BAC clones from the IPM4 contig as templates for PCR amplifications. The primer annealing temperature in PCR reactions was 5 to 10°C lower than in conditions originally developed for each CAPS primer pair (Table 1) so that there would be amplification of the original marker and related homologues. The PCR products obtained with many CAPS primer pairs were the same size as the products produced under high stringency conditions (data not shown). However in three instances digestion of the low stringency PCR products with either *Taq*I, *Alu*I or *Dde*I restriction enzymes revealed several new DNA fragments that were not identified previously. These included fragments that were nonpolymorphic as well as fragments polymorphic between the R and S pools.

The new markers revealed with 45L and cos98 primers all mapped within the BAC contig described above and were not useful for isolation of *Rx* (Fig. 3 and data not shown). However low stringency PCR with the IPM4 primers did reveal a new marker that was more tightly linked to *Rx* than any other of the previously identified markers. *Taq*I digestion of the low stringency IPM4 products from the R pool identified the original IPM4 locus (IPM4-a) in BAC111 and two other restriction fragments that had not previously been detected (Fig. 5a). One of these fragments (IPM4-b) was nonpolymorphic in the R and S pools. This fragment must have originated from BAC221 as the *Taq*I restriction fragment of similar size was also detectable after digestion of IPM4 marker derived from this BAC (Fig. 5a). However, the second new DNA fragment was polymorphic between the R and S pools and was not detected after digestion of IPM4 markers in BAC111 or BAC221 (Fig. 5 a). This fragment segregated with *Rx* in all plants of our mapping population including plant #761 and was designated IPM4-c (Fig. 5 b).

The potato BAC library was screened by PCR with IPM4 primers using conditions for the detection of IPM4-c. One new BAC clone with a DNA insert of about 50 kb, BAC77, carrying the IPM4-c locus, was identified (Fig. 5 a). The end-fragments of the BAC77 DNA insert were cloned, sequenced and converted into the CAPS markers, 77L and 77R. Marker 77L cosegregated with both IPM4-c and *Rx*, whereas 77R was separated from *Rx* by one recombination event in the recombinant individual #761 (based on an analysis of 1720 segregants; see Figs. 3 and 6).

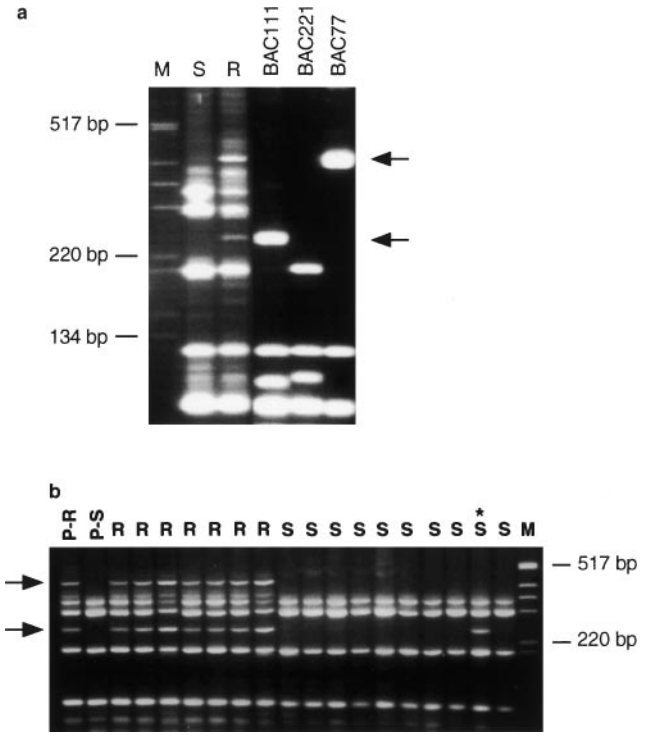

Fig. 5a, b Identification of duplicated sequences in the vicinity of *Rx*. Panel **a** displays the restriction enzyme digestions of the low-stringency PCR products (see text and Table 1) from the R DNA pool (*R*), the S DNA pool (*S*) and BAC DNAs using the IPM4 marker primers. Polymorphic DNA fragments linked in *cis* to *Rx* are indicated by *arrows*. The DNA fragments linked in *cis* to *Rx* but non-polymorphic between R and S pools in the conditions of this experiment are indicated by *double-line arrows*. '*M*', the 1-kb ladder markers (Life Technologies, UK). Panel **b** shows the analysis of individual plants [resistant to PVX (*R*) or susceptible (*S*)] with recombination events in the GP34–IPM5 interval using the IPM4 CAPS marker produced under low-stringency PCR conditions and after digestion with *Taq*I. The R pool and S pool are indicated as '*P-R*' and '*P-S*' respectively, and plant #761 with a recombination event between IPM4-a and IPM4-c/*Rx* is indicated by *a star*. *Arrows* show the positions of IPM4-a- (*lower arrow*) and IPM4-c-specific (*upper arrow*) *Taq*I fragments

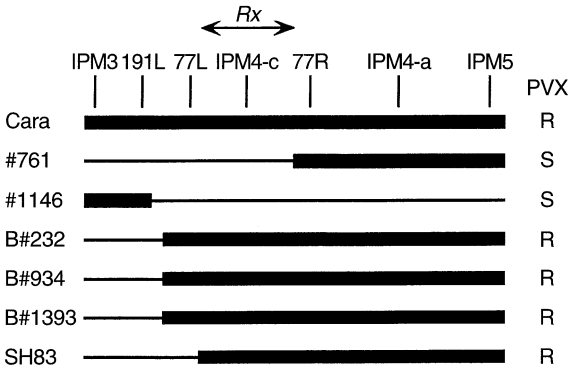

Fig. 6 Introgressed DNA in the vicinity of *Rx*. The diagrams show chromosomal DNA in the IPM3–IPM5 interval from potato cv Cara and clone SH83, and from individuals in our S_1 Cara mapping population (identified in left hand column). These plants were either *Rx* (*R*) or *rx* (*S*) genotypes and the chromosomal region introgressed from *S. tuberosum* spp. *andigena* is shown as a *closed rectangle*. The *S. tuberosum* spp. *tuberosum* DNA is shown as a *thin line*. DNA markers used to map the introgressed DNA are identified over the *vertical lines*, and the region containing *Rx* is delineated by the *horizontal arrow*

Assignment of *Rx* to BAC77 by introgression mapping

To ascertain whether BAC77 spans *Rx*, an additional 1438 individuals from the S1 Cara population were screened with markers 77L, IPM4-c and the flanking markers 191L and 77R (data not shown). There were no plants with recombination events between *Rx* and markers 77L and/or IPM4-c. However, three individuals, B#232, B#934 and B#1393, were found with recombination events in the 191L–77L interval. All of these plants contained *rx*-linked alleles of 191L but were resistant to PVX and also contained the *Rx*-linked allele of 77L (data not shown, Rouppe van der Voort et al. 1998). These data suggest a tight linkage between *Rx* and the marker 77L (Fig. 3) but did not allow *Rx* to be mapped precisely in the 191L–77R interval.

We obtained confirmation that BAC77 spans *Rx* by 'introgression mapping' experiments carried out in a parallel project on the mapping of the *Globodera pallida* resistance gene *Gpa2*. Like *Rx*, this gene resides on the short arm of the potato chromosome XII (Rouppe van der Voort et al. 1997). Both *Gpa2* and *Rx* were introgressed into cultivars Cara and Amaryl from the *S. tuberosum* ssp. *andigena* accession CPC1673 and diploid potato clone SH83-92-488 (referred to hereafter as 'SH83'). SH83 is derived from the cultivar Amaryl and carries both *Rx* and *Gpa2* (Rouppe van der Voort et al. 1998). To map the CPC1673-introgressed DNA in cv Cara, cv Amaryl and in clone SH83, the DNA of these potato genotypes was analysed with CAPS markers in the GP34–IPM5 genetic interval. This analysis provided evidence that all these potato genotypes contain DNA introgressions from the *S. tuberosum* ssp. *andigena* accession CPC1673 within the GP34–IPM5 interval. However, the size of introgressed DNA segments varied between these genotypes (Fig. 6). CPC1673 and cv Cara contain *Rx*-linked alleles of each CAPS marker within the GP34–IPM5 interval. However, in cv Amaryl and clone SH83, the alleles originated from the CPC1673 clone and, linked in *cis* to *Rx*, were confined to the IPM4-c–IPM5 interval (Figs. 6 and 3, and data not shown). The markers in the interval 77L–IPM3 were derived from the PVX-susceptible progenitors of cv Amaryl and clone SH83. Therefore, the left border of the DNA introgressed into these potatoes from CPC1673 is between 77L and IPM4-c (Fig. 3). When combined, the introgression mapping and recombination analyses show that *Rx* is located between markers 77L and 77R (Fig. 6) in the vicinity of IPM4-c.

Discussion

In this report we describe the isolation of a BAC clone which, according to genetic mapping criteria, spans the *Rx* locus in cv Cara. We encountered several issues in the course of isolating this clone that may be relevant to other map-based or marker-assisted cloning projects. One of these issues concerns the frequency of recombination in regions of introgressed DNA. Previous studies have reported a low frequency of recombination in DNA that has been introgressed from alien species (Liharska et al. 1996). Obviously such an effect could hinder map-based cloning of genes in introgressed DNA. However, in the region of DNA that has been introgressed into Cara from *S. tuberosum* ssp. *andigena* accession CPC1673 there is considerable variation in the frequency of recombination. There are certainly some regions, including the region containing *Rx* from 77L to 73R, in which recombination is rare. In our mapping populations of 3150 progeny we detected only two recombination events in that interval of more than 170 kb. However, in other regions, including those spanned by BAC167 and BAC29, the recombination frequency is much higher. In BAC167 the difference was particularly striking because there were ten recombinations within 110 kb of chromosomal DNA, corresponding to approximately 180 kb cM^{-1}. Overall the recombination frequency in potato DNA is higher than 750 kb cM^{-1} (Tanksley et al. 1992).

Initially the presence of repeated DNA sequences around the *Rx* locus complicated the characterization of BAC clones and the construction of BAC contigs. For example, we isolated several BAC clones from our library that were not from the *Rx* chromosome although they had been initially screened with allele-specific PCR or CAPS markers (A.B., K.K. and D.C.B., unpublished). We now know that this anomaly is due to differential PCR in genomic (high-complexity) and cloned (low-complexity) DNA. Repeated DNA loci that are not PCR-amplified from a genomic DNA template may get amplified from cloned DNA because the primer-complementary sequences are more abundant in the cloned DNA than in genomic DNA and because there is no potential for competition for the primers between similar loci.

The complications associated with repeated sequences were compounded by the tetraploid nature of the potato genome and the high degree of polymorphism in the constituent genomes. At some loci there were four different alleles (A.B., K.K. and D.C.B., unpublished). There may be an additional complication with the *Rx* locus which is duplicated on chromosomes V and XII (Ritter et al. 1991). However, despite these complications, we have been able to exploit the presence of repeated sequences for the cloning of the *Rx* locus in an approach which may prove more generally useful.

We reasoned that the *Rx* locus, like other resistance gene loci, may comprise duplicated blocks of resistance genes and that these resistance gene homologues and sequences adjacent to the resistance genes in *Rx*-linked BACs might be duplicated elsewhere in the vicinity of *Rx*. To reveal the other resistance gene homologues we employed low-stringency PCR. This approach is different from the identification of resistance gene homologues using highly degenerate primers based on conserved sequence motifs in resistance genes (Kanazin et al. 1996; Leister et al. 1996, 1998). In the experiments described here we deliberately chose the primers so that these conserved sequence motifs were avoided. It was intended that the low-stringency PCR would be selective for genes at the *Rx* locus rather than homologues located elsewhere in the genome. A future approach to the cloning of resistance genes could exploit both conserved sequence motifs and locus-specific motifs. The highly conserved motifs could be used for rapid identification of resistance gene homologues that are linked to the locus of interest. Low-stringency PCR with non-degenerate primers from other regions of these genes could then be used to identify markers within the resistance locus that are closer to the gene and that could be mapped in a large population.

To demonstrate that BAC77 spans *Rx* it was necessary to identify chromosomal recombinations between *Rx* and each end of this BAC. On the right side, despite the low frequency of recombination in that region, we identified a plant, #761 in our mapping population, with a chromosomal recombination between 77R and *Rx* (Fig. 3). We did not find progeny with chromosomal recombination on the left side between *Rx* and 77L. However in cv Amaryl, in which *Rx* had been introgressed from the same source as in our mapping population, there was a boundary of the introgressed DNA in the *Rx*–77L interval (Fig. 6). We are currently transforming the DNA from this interval (BAC77) into an *rx* cultivar of potato to confirm the presence of *Rx*.

Clearly, marker-assisted cloning of disease resistance genes is now feasible even from plants with complex genomes. However, based on our experience with *Rx*, we can suggest several technical points that should be considered in other similar projects. These include relatively trivial points, such as the construction of BAC libraries with several different restriction enzymes in an attempt to improve representation in the BAC library. In our present large library constructed with *Hin*dIII there were no clones from the 191L–77L region. We can also recommend the use of introgression mapping where several cultivars have been produced carrying the gene of interest introgressed from a single source. Low-stringency PCR may also be useful for complex loci as an approach to the focused identification of new markers. However, even with these combined approaches, there is always the possibility that the gene of interest will be within an interval that is physically large but impossible to dissect by recombination. In

these situations it may be necessary to combine genetical analysis with complementation using transient assays for the trait of interest (Mindrinos et al. 1994) or stable transformation with binary BAC vectors for the assay of long stretches of DNA (Hamilton 1997).

Acknowledgements We are grateful to Julie Gilbert, Cinzia Dedi and June Chapman for help with the preparation of potato protoplasts and maintaining the in vitro plants; Mike Hill for horticultural assistance; Paddy Bovill, David Baker and Alan Cavill for operating automated sequencing machines; Yvonne Pinto, Jeanmarie Verchot and Sue Angell for critical reading of the manuscript. We also thank the Gatsby Charitable Foundation for continuing support. The use of imported and genetically modified viruses was authorised by the Ministry of Agriculture, Fisheries and Food under the plant health licence no. PHL 24/2512.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Anderson PA, Okubara PA, Arroyo Garcia R, Meyers BC, Michelmore RW (1996) Molecular analysis of irradiation-induced and spontaneous deletion mutants at a disease resistance locus in *Lactuca sativa*. Mol Gen Genet 251:316–325

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208–218

Ballvora A, Hesselbach J, Niewöhner J, Leister D, Salamini F, Gebhardt C (1995) Marker enrichment and high-resolution map of the segment of potato chromosome 7 harbouring the nematode resistance gene *Gro*1. Mol Gen Genet 249: 82–90

Bendahmane A, Köhm BA, Dedi C, Baulcombe DC (1995) The coat protein of potato virus X is a strain-specific elicitor of *Rx1*-mediated virus resistance in potato. Plant J 8:933–941

Bendahmane A, Kanyuka K, Baulcombe DC (1997) High-resolution genetical and physical mapping of the *Rx* gene for extreme resistance to potato virus X in tetraploid potato.Theor Appl Genet 95:153–162

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ (1994) *RPS*2 of *Arabidopsis thaliana* – a leucine-rich repeat class of plant disease resistance genes. Science 265:1856–1860

Brigneti G, Garcia Mas J, Baulcombe DC (1997) Molecular mapping of the potato virus Y resistance gene *Ry*(*sto*) in potato. Theor Appl Genet 94:198–203

Busch M, Mayer U, Jurgens G (1996) Molecular analysis of the *Arabidopsis* pattern-formation gene *GNOM* – gene structure and intragenic complementation. Mol Gen Genet 6:681–691

Büschges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, van Daelen R, van der Lee T, Diergaarde P, Groenendijk J, Töpsch S, Vos P, Salamini F, Schulze-Lefert P (1997) The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell 88:695–705

Clark L, Carbon J (1976) A colony bank containing synthetic ColE1 hybrids representative of the entire *E. coli* genome. Cell 9:91–99

Cockerham G (1970) Genetical studies on resistance to potato viruses X and Y. Heredity 25:309–348

De Jong W, Forsyth A, Leister D, Gebhardt C, Baulcombe DC (1997) A potato hypersensitive resistance gene against potato virus X maps to a resistance gene cluster on chromosome 5. Theor Appl Genet 95:246–252

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JDG (1996) The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84:451–459

Ellis JG, Lawrence GJ, Finnegan EJ, Anderson PA (1995) Contrasting complexity of two rust resistance loci in flax. Proc Natl Acad Sci USA 92:4185–4188

Engebrecht J, Brent R, Kaderbhai MA (1997) Large-scale preparation of plasmid DNA. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. John Wiley and Sons Inc, USA, pp 1.6.1–1.6.2

Flor HH (1971) Current status of the gene-for-gene concept. Annu Rev Phytopathol 9:275–298

Giraudat J, Hauge BM, Valon C, Parcy F, Goodman HM (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. Plant Cell 4:1251–1261

Hamilton CM (1997) A binary BAC system for plant transformation with high-molecular-weight DNA. Gene 200:107–116

Heilig JS, Lech K, Brent R (1997) Large-scale preparation of plasmid DNA. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. John Wiley and Sons Inc, USA, pp 1.7.1–1.7.3

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

Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789–793

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11746–11750

Keen NT (1990) Gene-for-gene complementarity in plant-pathogen interactions. Annu Rev Genet 24:447–463

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J 4:403–410

Köhm BA, Goulden MG, Gilbert JE, Kavanagh TA, Baulcombe DC (1993) A potato virus X resistance gene mediates an induced, nonspecific resistance in protoplasts. Plant Cell 5:913–920

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 14:421–428

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

Liharska T, Koornneef M, van Wordragen M, van Kammen A, Zabel P (1996) Tomato chromosome 6 – effect of alien chromosomal segments on recombinant frequencies. Genome 39: 485–491

Lukowitz W, Mayer U, Jurgens G (1996) Cytokinesis in the *Arabidopsis* embryo involves the syntaxin-related *KNOLLE* gene-product. Cell 84:61–71

Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD (1993) Map-based cloning of protein kinase gene conferring disease resistance in tomato.Science 262:1432–1436

Maruzyk R, Sergeant A (1980) A simple method for dialysis of small volume samples. Anal Biochem 105:403–404

Meksem K, Leister D, Peleman J, Zabeau M, Salamini F, Gebhardt C (1995) A high-resolution map of the vicinity of the *R1* locus on chromosome 5 of potato based on RFLP and AFLP markers. Mol Gen Genet 249:74–81

Mindrinos M, Katagiri F, Yu GL, Ausubel FM (1994) The *A. thaliana* disease resistance gene *RPS*2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell 78:1089–1099

Ochman H, Mehdora MM, Garza D, Hartl DL (1990) Amplification of flanking sequences by inverse PCR. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. Academic Press, San Diego, pp 219–227

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

Ritter E, Debener T, Barone A, Salamini F, Gebhardt C (1991) RFLP mapping on potato chromosomes of two genes controlling extreme resistance to potato virus-X (PVX). Mol Gen Genet 227 : 81–85

Rouppe van der Voort J, Wolters P, Folkerstma R, Hutten R, van Zandvoort P, Vinke H, Kanyuka K, Bendahmane A, Jacobsen E, Janssen R, Bakker J (1997) Mapping of the cyst nematode resistance locus *Gpa2* in potato using a strategy based on comigrating AFLP markers. Theor Appl Genet 95 : 874–880

Rouppe van der Voort J, Kanyuka K, van der Vossen E, Bendahmane A, Mooijman P, Klein-Lankhorst R, Stiekema W, Baulcombe D, Bakker J (1998) Tight physical linkage of the nematode resistance gene *Gpa2* and the virus resistance gene *Rx* on a single segment introgressed from the wild species *Solanum tuberosum* ssp. *andigena* CPC 1673 into cultivated potato. Mol Plant-Microbe Interact, in press

Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, Steiner HY, Johnson J, Delaney TP, Jesse T, Vos P, Uknes S (1997) The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor IκB. Plant Cell 9 : 425–439

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

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

Song WY, Pi LY, Wang GL, Gardner J, Holsten T, Ronald PC (1997) Evolution of the rice *Xa21* disease resistance gene family. Plant Cell 9 : 1279–1287

Shizuya H, Birren B, Kim UJ, Mancino V, Slepak T, Tachiiri Y, Simon M (1992) Cloning and stable maintenance of 300-kb fragments of human DNA in *Escherichia coli* using an F′-factor-based vector. Proc Natl Acad Sci USA 89 : 8794–8797

Tanksley SD, Ganal MW, Prince JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Röder MS, Wing RA, Wu W, Young ND (1992) High-density molecular linkage maps of the tomato and potato genomes. Genetics 132 : 1141–1160

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP – a new technique for DNA fingerprinting. Nucleic Acids Res 23 : 4407–4414

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene *N* – similarity to Toll and the interleukin-1 receptor. Cell 78 : 1101–1115