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Genetic and physical mapping of homologues of the virus resistance gene *Rx1* and the cyst nematode resistance gene *Gpa2* in potato

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Abstract Nine resistance gene homologues (RGHs) were identified in two diploid potato clones (SH and RH), with a specific primer pair based on conserved motifs in the LRR domain of the potato cyst nematode resistance gene *Gpa2* and the potato virus X resistance gene *Rx1*. A modified AFLP method was used to facilitate the genetic mapping of the RGHs in the four haplotypes under investigation. All nine RGHs appeared to be located in the *Gpa2*/*Rx1* cluster on chromosome XII. Construction of a physical map using bacterial artificial chromosome (BAC) clones for both the *Solanum tuberosum* ssp. *tuberosum* and the *S. tuberosum* ssp. *andigena* haplotype of SH showed that the RGHs are located within a stretch of less than 200 kb. Sequence analysis of the RGHs revealed that they are highly similar (93 to 95%) to *Gpa2* and *Rx1*. The sequence identities among all RGHs range from 85 to 100%. Two pairs of RGHs are identical, or nearly so (100 and 99.9%), with each member located in a different genotype. Southern-blot analysis on genomic DNA revealed no evidence for additional homologues outside the *Gpa2*/*Rx1* cluster on chromosome XII.

Keywords *Globodera pallida* · Potato cyst nematodes · Potato virus X · Resistance gene homologues

Introduction

Plants are constantly under attack by a wide range of pathogens and pests. To defend themselves, plants have evolved an innate surveillance system consisting of a large set of resistance genes. Most resistance genes are single-dominant and confer resistance in a gene-for-gene specific manner (Flor 1971). More than 30 resistance genes (*R* genes) have been cloned from different plant species and can be divided into four classes based on common structural motives (Takken and Joosten 2000). The majority of the *R* genes are characterised by a leucine-rich repeat (LRR) and a nucleotide binding site (NBS) domain. *R* genes that belong to this superfamily confer resistance to completely unrelated taxonomic groups like bacteria, fungi, viruses and nematodes (Mindrinos et al. 1994; Whitham et al. 1994; Lawrence et al. 1995; Milligan et al. 1998; Vos et al. 1998; Bendahmane et al. 1999; Van der Vossen et al. 2000).

In potato, 19 *R* genes have been mapped to 11 chromosomal regions (Gebhardt and Valkonen 2001). Four of them, *Rx1*, *Gpa2*, *Rx2* and *R1*, have been isolated and they all belong to the NBS-LRR class. *Rx1* and *Gpa2* originate from *Solanum tuberosum* ssp. *andigena* and have been identified by map-based cloning (Bendahmane et al. 1999; Van der Vossen et al. 2000). *Rx2* has been isolated from *Solanum aucaule* using a PCR-based approach (Bendahmane et al. 2000) and *R1* has been isolated from *Solanum demissum* by using a combination of map-based cloning and the candidate gene approach (Ballvora et al. 2002). *Rx1* and *Gpa2* are highly homologous, yet they confer resistance to two completely unrelated pathogens, namely potato virus X and the potato cyst nematode *Globodera pallida*. The genes are tightly linked on chromosome XII of potato (Bendahmane et al. 1997; Rouppe van der Voort et al. 1999). Sequencing a 187-kb region revealed that *Gpa2* and *Rx1* are part of a complex locus containing at least two other closely related resistance gene homologues (RGHs): *RGC1* and the pseudogene *RGC3* (Bendahmane et al. 1997; Bendahmane et al. 1999; Rouppe van der Voort et al. 1999; Van der Vossen et al. 2000). In the LRR domain, more variation is observed between the RGHs than in the NBS domain. The mean K_a/K_s ratio for the LRR region is larger than 1, whereas in the NBS regions the ratio is smaller than 1, indicating that the LRR domain is subject

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to diversifying selection and that specificity is determined by this domain (Van der Vossen et al. 2000).

In this paper we describe the use of LRR-specific primer combinations for both the identification and mapping of nine *Gpa2*/*Rx1* homologues in two diploid potato clones (SH and RH). The value of using LRR-specific primers for the dissection of *R* gene clusters in different haplotypes will be discussed.

Materials and methods

Plant material and DNA extraction

A mapping population for *S. tuberosum* ssp. *tuberosum* was available from the diploid potato clones SH83-92-488 × RH89-039-16 (Rouppe van der Voort et al. 1997). The female parent SH83-92-488 contains an introgression segment originating from the wild accession *S. tuberosum* ssp. *andigena* CPC1673 on which the *G. pallida* resistance gene *Gpa2* and the PVX resistance gene *Rx1* are located. The male parent RH89-039-16 has been selected for its fertility and the production of vigorous offspring. SH83-92-488 will be referred to as SH, and RH89-039-16 will be referred to as RH. The mapping population F_1 SH × RH consists of 136 vigorous F1 genotypes. Genomic DNA was extracted from frozen leaf tissue of in vitro plants as described (Van der Beek et al. 1992).

BAC library and DNA extraction

The construction of the BAC library from the diploid potato clone SH83-92-488 has been described by Rouppe van der Voort et al. (1999). The library has been extended with 30,000 clones and now comprises 90,000 clones. Pooling and preparation for screening was done as described (Kanyuka et al. 1999), and resulted in 255 plate pools. DNA extracted from these BAC clones was used as a template for PCR and sequence analysis. For this, clones were cultured overnight at 37 °C in 500 ml of LB medium, supplemented with 170 μg/ml of chloramphenicol for selection. Plasmid DNA was isolated using the "very low-copy plasmid DNA purification protocol" of the plasmid midi-kit according to the manufacturer's instructions (Quiagen, Hilden, Germany). For each BAC, 1 μl of DNA was digested with *Hin*dIII to check the concentration and the purity.

PCR analysis

A cluster-specific primer pair was designed based on conserved DNA sequences in the LRR domains of *Gpa2*, *Rx1* and *SH-RGH1* [previously designated as *RGC1* (Van der Vossen et al. 2000)]. The forward primer (LRR-F: ttg gtg tcg taa cag tga gg) starts at position +1,533 of *Gpa2* and the reverse primer (LRR-R: ctg gct agt cct cag aac ac) at position +3,192 of *Gpa2* (UTR). A PCR was performed with this primer pair using the Expand High Fidelity PCR System according to the manufacturer's instructions (Roche, Mannheim, Germany). The following PCR cycle file was applied: 3 min at 94 °C followed by 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C for 10 cycles, 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C with an extension of 5 s/cycle for 25 cycles and finally a 5-min elongation at 72 °C.

Cloning and digestion analysis of the PCR products

PCR products were cloned into the PCR2.1TOPO-vector for transformation of *Escherichia coli* TOP10 cells according to the manufacturer's instructions (Invitrogen, San Diego, Calif., USA). For each PCR product, 12 positive clones were cultivated overnight at 37 °C in 2 ml of liquid LB medium with 100 μg/ml of ampicillin for selection. Each clone was re-amplified with the same LRR primer pair and the resulting PCR products were digested with *Taq*I. PCR was performed using an adjusted PCR buffer containing 100 mM of Tris HCl pH 8.3, 500 mM of KCl, 25 mM of MgCl and 10% Triton X-100 to avoid additional cleaning steps of the PCR products prior to digestion. The DNA fragments were separated on a 4% agarose gel consisting of 1% ultra-pure agarose (Life Technologies, Breda, The Netherlands) and 3% NuSieve GTG agarose (FMC, Philadelphia, Pa., USA) in 1 × TAE buffer at 120 V. Clones were selected for further analysis based on differences in the digestion patterns.

Sequence analysis

Sequence analysis of the PCR products was carried out by Greenomics, PRI, Wageningen, The Netherlands. BAC-end sequencing was performed using approximately 1 μg of template DNA in a cycle-sequencing reaction using either 100 ng of sp6 or t7 primer and 8 μl of Big Dye terminator mix (PerkinElmer, Wellesley, Mass., USA) in a total volume of 20 μl. The PCR protocol consisted of 25 cycles of 30 s at 96 °C, 15 s at 50 °C and 4 min at 60 °C. After ethanol precipitation at room temperature for 10 min and recovery, the labelled DNA was dissolved in 3 μl of formamide. DNA was then heated for 2 min at 96 °C and directly cooled on ice. Approximately 1.5 μl was then loaded on a 6% TBE (pH 8.3) polyacrylamide gel. Sequence electrophoresis was carried out on either an ABI 373XL or ABI 377 sequencer. DNA sequence analysis and comparisons were employed using DNAstar software.

CHEF-gel electrophoresis

BAC insert sizes were determined with CHEF electrophoresis on a 1% agarose gel (Seakem Gold, FMC, Philadelphia, Pa., USA) in 0.5 × TBE buffer at 4 °C using a BIO-RAD CHEF DR II system (Bio-Rad Laboratories, Hercules, Calif., USA) at 200 V, with a pulse time of 5 to 15 s for 18 h.

Southern-blot analysis

Approximately 20 ng of BAC DNA and 3 μg of genomic DNA was digested with *Hin*dIII. The digested DNA was separated on a 1% agarose (Agarose NA, Pharmacia, Peapack, N.J., USA) gel in 1 × TAE buffer at 50 V. The gels were blotted overnight on hybridisation filters using capillary forces (Sambrook et al. 1989). Southern analysis was performed based on the DIG Application Manual for filter hybridisation (Roche, Basel, Switzerland). An LRR-based probe [position +1,533 to position +1,936 (403 bp)] and an NBS- and LRR-based probe [position +1,367 to position +1,552 (185 bp)], were amplified from *Gpa2* BAC DNA. The primer pairs used are PLRR-F (ttg gtg tcg taa cag tga gg) and PLRR-R (gtt ctc tgt agg ctc atg ac) at an annealing temperature of 60 °C, and PNBSLRR-F (gtg gaa tgc atg atg tga cc) and PNBSLRR-R (ctc act gtt acg aca cca ag) at an annealing temperature of 55 °C. The results were visualised on an autoradiogram after 5 min up to 2-h exposure.

Genetic mapping

Mapping was performed using a modified method (Fig. 1) based on the AFLP technique (Vos et al. 1995). The template used was the PCR product generated from gDNA using the iPM4 primers as described (Kanyuka et al. 1999). The PCR product was digested with *Taq*I, and a *Taq*I adapter as described (Vos et al. 1995) was ligated to the digestion products. This was followed by fragment amplification on a 1:25 diluted template using a labelled *Taq*I primer [gat gag tcc tga ccg a (Vos et al. 1995)] and the original iPM4R primer. The AFLP thermal cycle conditions were applied as

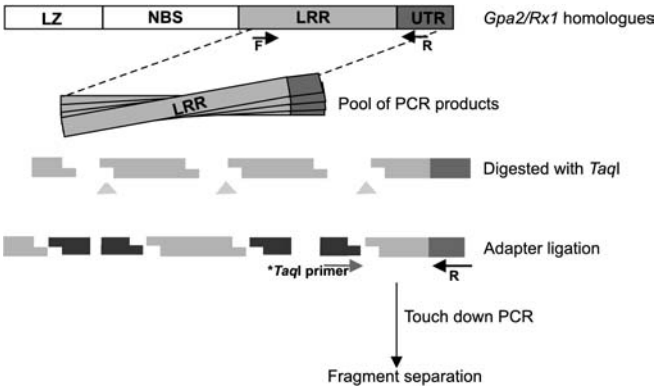

Fig. 1 Schematic depiction of the method used to genetically map the *Gpa2*/*Rx1* homologues. The LRR-specific primers iPM4F and iPM4R are used to amplify a pool of RGH sequences from either genomic DNA or BAC DNA. Subsequently, the PCR products are used as a template in a modified AFLP reaction. After digestion with the restriction enzyme *Taq*I the products are ligated to a *Taq*I adapter. A second touch-down PCR reaction is performed using the *Taq*I primer and the original iPM4R primer

Table 1 Primer sequences based on BAC end sequences to detect overlap between BAC clones

BAC end	Primers
7E16sp6	5'- cgg ggt gta atg tga tga gc-3' 5'- ggc ctg caa gtc tgt gca c-3'
7E16t7	5'- gtt cgt atg agc gag tat gg-3' 5'- tcc acg atg gtc tcc tcg-3'
25G18sp6	5'-cca att tca agc ttc ttc ata g-3' 5'-cag tca agg tgc ttt gga gg-3'
25G18t7	5'- gtt acc tgc tat gtg agc tc-3' 5'- cat cag ctg cct tgc agt tg-3'
36G3sp6	5'- gcc caa cat gat agg tcg c-3' 5'- ctt ggt atc aga gca cag ag -3'
36G3t7	5'-tgt atg aat tgg gtc att ccg-3' 5'- gcc caa tat tcc tcc atc tg-3'

described (Vos et al. 1995). Separation of labelled fragments and autoradiography was done as described by Van Eck et al. (1995).

Linkage analysis of pair-wise recombination frequencies between segregating RGHs and markers were performed using JOINMAP 1.4 (Stam 1993).

Physical mapping

BAC-end sequences were employed to design PCR primers to study overlap between BAC clones. The primers designed for each BAC-end are listed in Table 1. PCR conditions used in the amplifications are equal for all primer pairs and are as follows: 3 min at 94 °C, followed by 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C for 35 cycles and 5 min at 72 °C.

For the alignment of the contigs in SH, we used primers as described (Kanyuka et al. 1999) and 187-kb sequence information derived from four overlapping BAC clones harbouring *Gpa2*, *Rx1*, *SH-RGH1* and *SH-RGH3* (previously designated *RGC3*) (Van der Vossen et al. 2000).

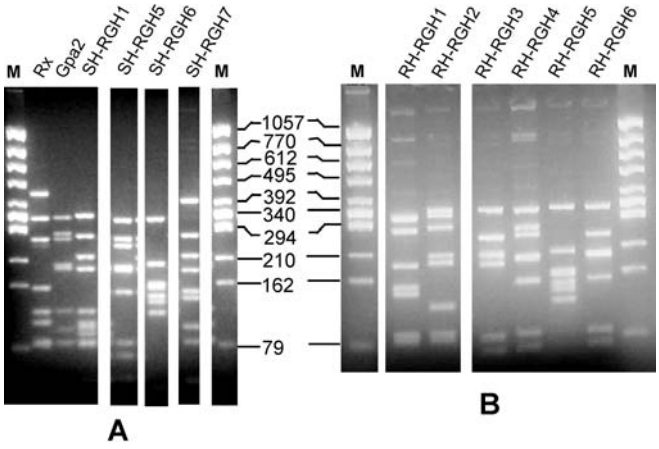

Fig. 2 Identification of *Gpa2*/*Rx1* resistance gene homologues (RGHs) in the diploid potato clone SH (**A**) and RH (**B**). RGHs were selected based on differences in their *Taq*I digestion pattern after PCR-amplification with specific LRR primers. The *Taq*I digestion patterns are related to a DNA base-pair ladder (*M*). Bands larger then 612 bp are the result of partially digested DNA

Results

Identification of *Gpa2*/*Rx1* homologues in SH and RH

To identify homologues of *Gpa2* and *Rx1*, a specific primer set was designed based on conserved regions in the LRR domain. This primer set was used to screen a BAC library of the diploid potato clone SH, which harbours the *R* genes *Gpa2* and *Rx1* on a *S. tuberosum* ssp. *andigena* introgression segment. For each positive BAC clone, amplification products were cloned and 12 transformants were used for re-amplification. After *Taq*I digestion, resistance gene homologues (RGHs) were selected based on their unique digestion pattern (Fig. 2a). This resulted in the identification of three RGHs: *SH-RGH5* (BAC clone 7E16), *SH-RGH6* and *SH-RGH 7* (both located on BAC clones 25G18 and 36G3). Also *Gpa2* (BAC 85N1), *Rx1* (BAC 43) and *SH-RGH1* (BAC 60K18) were detected based on their expected *Taq*I digestion pattern (Bendahmane et al. 1999; Van der Vossen et al. 2000). No additional RGHs were found after screening the genomic DNA of SH.

The diploid potato clone RH was studied using genomic DNA. PCR analysis resulted in three bands at 800 bp, 1,300 bp and 1,600 bp. The complete PCR product was cloned and restriction analysis of the *Taq*I patterns of the re-amplified PCR products of 25 clones resulted in the identification of six *Gpa2*/*Rx1* homologues: *RH-RGH1* to *RH-RGH6* (Fig. 2b). Interestingly, *RH-RGH4* and *RH-RGH5* resembled the digestion patterns of *SH-RGH5* and *SH-RGH6*, respectively. In total, 12 RGHs were identified in potato including *Gpa2*, *Rx1* and *SH-RGH1* using a *Gpa2*/*Rx1* cluster-specific primer set (Table 2). Another *Gpa2*/*Rx1* homologue present in the cluster, the pseudogene *SH-RGH3*, was not amplified with the cluster primer set. The annealing site of the

Table 2 Resistance gene homologues (RGHs) identified in BAC and genomic DNA of the diploid potato clones SH and RH using a primer pair based on the LRR domain of *Gpa2* and *Rx1*

RGH	PCR product (kb)	BAC clones	gDNA
SH-RGH1	1.6	85N1	+
Gpa2	1.6	85N1, 60K18	–[a]
Rx1	1.6	BAC 43	–
SH-RGH5	1.5	7E16	+
SH-RGH6	1.3	25G18, 36G3	+
SH-RGH7	1.6	25G18, 36G3	–
RH-RGH1	1.6	–	+
RH-RGH2	1.6	–	+
RH-RGH3	1.6	–	+
RH-RGH4	1.5	–	+
RH-RGH5	1.3	–	+
RH-RGH6	0.8	–	+

[a] Not detected in genomic DNA

reverse primer is not present in *SH-RGH3*, because the 3' end of its sequence is truncated.

The *Gpa2*/*Rx1* homologues of SH and RH were sequenced to determine their sequence resemblance with *Rx1* and *Gpa2*. Comparison of the nucleotide sequence of the LRR domains of the RGHs showed that they are highly similar to each other, with sequence identities ranging from 85.5% to 100% (Table 3). The sequences of *RH-RGH4* and *RH-RGH5* were indeed virtually identical to those of *SH-RGH5* and *SH-RGH6*, respectively (99.9% and 100% sequence identity).

Genetic mapping of the *Gpa2*/*Rx1* homologues in SH × RH

Gpa2/*Rx1*-specific primers were used to perform PCR on the parents SH and RH, and a progeny of 100 individuals, to determine the genetic position of the *Gpa2*/*Rx1* homologues in the potato genome. The selected BAC clones harbouring *Gpa2*/*Rx1* homologues were included as a control. On the resulting pools of PCR products, a modified AFLP analysis was performed that makes use of sequence polymorphisms in the last *Taq*I restriction sites of the PCR products. Using the sequence information of the RGHs we calculated the length of the expected fragments from the *Taq*I restriction site closest to the 3' end of the PCR products. In this way we were able to determine the positions in the gels of the RGHs. Figure 3 shows the autoradiogram with segregating bands for *Gpa2*, *Rx1*, *SH-RGH5*, *SH-RGH6* and *RH-RGH1*-*RH-RGH6* at the expected positions in the gel (summarised in Table 4). *SH-RGH1* and *SH-RGH7* cannot be linked to a segregating band, because they co-migrate with a thick band in the gel. However, *SH-RGH1* is present on the same BAC as *Gpa2*, and *SH-RGH7* on the same BAC as *SH-RGH6*. All the segregating RGHs were linked to the *Gpa2*/*Rx1* resistance-gene cluster with a logarithm of odds (LOD) score between 8.2 and 12.6. Other DNA fragments visible on the autoradiogram either co-segregate with RGHs, or do not segregate at all. They may be

Table 3 Sequence identities (%) of the LRR domains of the RGHs in SH and RH

RGH	Gpa2	Rx1	SH-RGH1	SH-RGH3*	SH-RGH5	SH-RGH6	SH-RGH7	RH-RGH1	RH-RGH2	RH-RGH3	RH-RGH4	RH-RGH5
Rx1	93.5											
SH-RGH1	93.6	93.2										
SH-RGH3*	86.5	86.8	87.3									
SH-RGH5	92.9	92.8	93.5	87.4								
SH-RGH6	94.3	93.2	93.1	92.6	92.4							
SH-RGH7	92.8	93.3	91.9	86.5	91.9	93.1						
RH-RGH1	92.9	93.8	91.8	87.0	91.6	92.8	98.5					
RH-RGH2	93.7	94.8	93.1	88.3	93.5	94.8	93.4	92.2				
RH-RGH3	94.1	93.7	93.9	87.7	93.1	94.5	91.2	93.3	93.9			
RH-RGH4	93.1	92.9	93.5	87.5	99.9	92.6	92.1	91.7	93.4	93.1		
RH-RGH5	94.3	93.2	93.1	92.6	92.4	100.0	93.0	92.8	94.7	94.5	92.5	
RH-RGH6	93.6	94.0	85.5	85.5	93.3	90.8	92.3	92.2	94.1	94.3	93.3	90.8

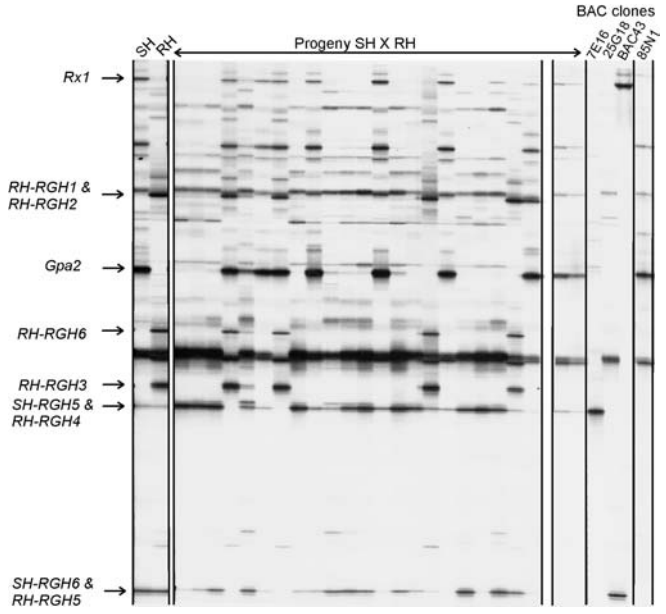

Fig. 3 Mapping of RGHs in a SH × RH cross and in a number of BAC clones using a modified AFLP method on a pool of RGH sequences. All the indicated RGHs map on chromosome XII and are linked to *Gpa2* with a LOD score between 88.2 and 12.6. *Rx1* was also identified by the co-migrating band on BAC43 from which *Rx1* was originally cloned (Bendahmane et al. 1999). Likewise, we were able to verify the expected bands for *Gpa2* and *SH-RGH1* by comparing the pattern of the mapping population with the bands in BAC 85N1. Furthermore, we could relate the bands present on BAC clones 7E16 and 25G18 with bands in the progeny. The majority of the remaining unassigned bands are artefacts of the PCR procedure

Table 4 Segregation of the RGHs in a progeny of 136 individuals. RGHs with the same mark (++ or – –) are in coupling

SH		RH	
RGH	Segregation	RGH	Segregation
Gpa2	++	*RH-RGH1*	++
Rx1	++	*RH-RGH2*	++
SH-RGH5	– –	*RH-RGH3*	++
SH-RGH6	– –	*RH-RGH6*	++
		RH-RGH4	– –
		RH-RGH5	– –

artefacts caused by the double PCR and the limited complexity of the template used.

SH-RGH5 and *SH-RGH6* are in repulsion with *Gpa2*. This implies that *SH-RGH5*, *SH-RGH6* and *SH-RGH7* are all derived from the *S. tuberosum* ssp. *tuberosum* haplotype and that no additional RGHs have been found on the *Gpa2*/*Rx1* cluster of the *S. tuberosum* ssp. *andigena* introgression segment. Furthermore, *RH-RGH1*, *2*, *3* and *6* are in coupling with each other and in repulsion with *RH-RGH4* and *5* (Table 4). From these data we can conclude that all the identified homologues are genetically linked to the *Gpa2*/*Rx1* cluster on chromosome XII in SH and in RH. Based on these and

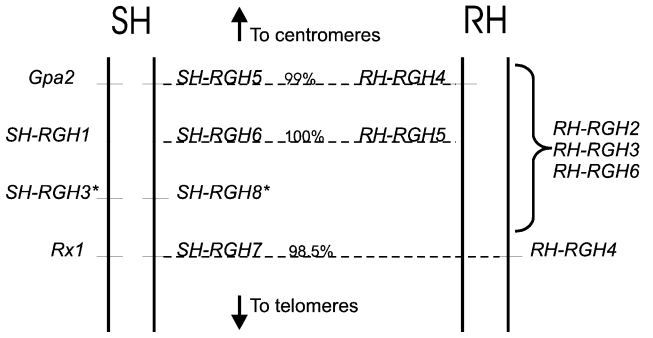

Fig. 4 Schematic drawing of the *Gpa2*/*Rx1* homologues on the four chromosomes of SH and RH. The relative order and orientation of a number of RGHs has been postulated as follows (see also Results). *SH-RGH5* is the only RGH present on a BAC clone positioned at the top of the cluster. *RH-RGH4* has a 99.9% identity to *SH-RGH5*, and therefore we assume that this RGH is also positioned at the top of the cluster in RH. Because the intron positions of *SH-RGH7* are the same as in *Rx1* we assume that this RGH, like *Rx1*, is at the bottom of the cluster. *RH-RGH1* and *SH-RGH7* have a sequence identity of 98.5 and therefore we assume that *RH-RGH1* is also at the bottom of the cluster in RH. We also assume that the two pseudogenes *SH-RGH3* and *SH-RGH8* that could not be amplified in the PCR (marked with an *asterisk*) occupy the same position in the cluster. Finally, we assume, based on their 100% sequence identity, that *RH-RGH5* has the same position in the cluster as *SH-RGH6*. No relative order for *RH-RGH2, RH-RGH3* and *RH-RGH6* could be postulated

other data (see also below), we postulated the position of the RGHs as shown in Fig. 4.

Physical mapping of the *Gpa2*/*Rx1* cluster in SH

The BAC clones containing RGHs were used to construct a physical map of the *Gpa2*/*Rx1* cluster for both the *S. tuberosum* spp. *tuberosum* and the *S. tuberosum* spp. *andigena* haplotypes of SH. Genetic mapping revealed that all these RGHs are located on chromosome XII and, hence, the BAC inserts correspond with genomic fragments of this chromosomal region of the potato genome. *SH-RGH5* and *SH-RGH6* co-migrate with markers that are in repulsion with *Gpa2*, implicating that they are located on the homologous chromosome. Based on this information we could asign the origin of the BAC clones to any of the two haplotypes: BAC 43, BAC 85N51 and BAC 34F16 were derived from the homologous chromosome in *S. tuberosum* ssp. *andigena* introgression segment, and BAC clones 25G18, 7E16 and 36G3 were derived from *S. tuberosum* ssp. *tuberosum*. The BAC clones derived from *S. tuberosum* ssp. *andigena* formed a closed contig and could be easily aligned with the original physical map based on the presence and absence of *Gpa2*, *SH-RGH1* and *Rx1*, and the markers 73L, 111R, 111L, 221R, 45L, 77R and 77L (Kanyuka et al. 1999). In order to make a contig of the *S. tuberosum* ssp. *tuberosum* haplotype, the BAC ends were sequenced and primers were designed to perform PCR on the other BAC clones of this haplotype. Additionally, BACs 25G18 and 36G3

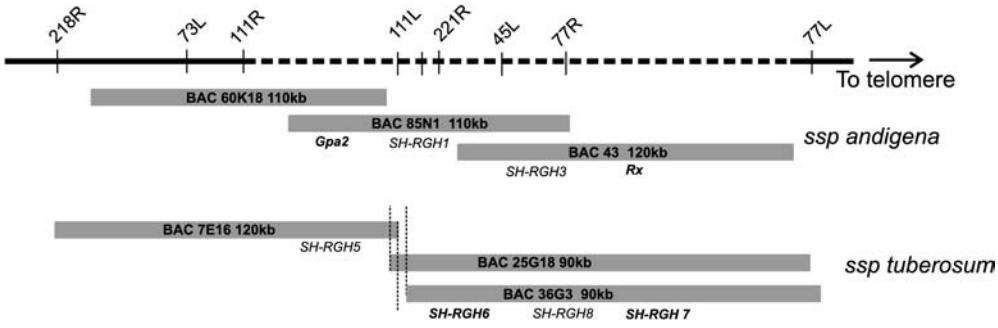

Fig. 5 Physical map of the *Gpa2*/*Rx1* cluster in the diploid potato clone SH. The region (187 kb) around the resistance genes *Gpa2*, *Rx1* and *SH-RGH1* and the pseudogene *SH-RGH3* on the haplotype derived from *S. tuberosum* ssp *andigena*, has been sequenced. On the *S. tuberosum* ssp. *tuberosum* haplotype four *Gpa2*/*Rx1* homologues are also present: *SH-RGH5*, *6, 7* and *8*. Like *SH-RGH3*, the homologue *SH-RGH8* could not be identified by PCR, but was detected by Southern-blot analysis. The BAC clones are represented in *grey rectangles* and the RGHs are indicated alongside the BACs on which they were detected. RGHs are indicated in *bold* when intron positions are known. *Vertical lines* indicate the marker positions used to align the BAC contig from the *S. tuberosum* ssp. *tuberosum* haplotype with the *S. tuberosum* ssp. *andigena* introgression segment. *Vertical dotted lines* indicate an overlap between BAC ends. The *dotted horizontal line* in the *S. tuberosum* ssp. *andigena* chromosome indicates the position of the sequenced region of 187 kb

harbour the same RGHs. These data enabled us to construct a closed contig of these BAC clones (Fig. 5). CHEF electrophoresis of the BAC inserts (data not shown) showed that BAC 7E16 is approximately 120 kb. Both 36G3 and 25G18 are approximately 90 kb in size resulting in a physical map of *S. tuberosum* ssp. *tuberosum* of about 200 kb containing *SH-RGH5*, *6* and *7*.

Moreover, we were also interested in comparing the genomic organisation of the *Gpa2*/*Rx1* cluster derived from the *S. tuberosum* spp. *tuberosum* haplotype and the one derived from the *S. tuberosum* ssp. *andigena* introgression segment. A segment of 187 kb of the *S. tuberosum* ssp. *andigena* haplotype has previously been sequenced for cloning *Rx1* and *Gpa2* (Van der Vossen et al. 2000). This sequence information was used to align the BAC end-sequences of the BAC clones derived from *S. tuberosum* ssp. *tuberosum* with the contig of the *S. tuberosum* ssp. *andigena* haplotype. The approximate 80% sequence identity for the BAC ends of the left arms of BAC clones 7E16, 25G18 and 36G3 with regions between *Gpa2* and *SH-RGH1* led to the orientation of the BAC contig as proposed in Fig. 5. The order of the homologues present on BAC clones 25G18 and 36G3 is not clear. However, a comparison of intron positions between the RGHs, showed that *SH-RGH7* has similar intron positions as *Rx1* and *SH-RGH6* has not (Bakker et al., unpublished data). Therefore we presented the order of the homologues as depicted in Fig. 5.

Southern-blot analysis

To confirm the specificity of the *Gpa2*/*Rx1*-specific primer set, Southern-blot analysis was carried out at high stringency conditions on *Hin*dIII-digested DNA of the BAC clones from SH harbouring the RGHs (data not shown). Hybridisation with a 400-bp fragment derived from the 5'-end of the LRR domain of *Gpa2* resulted in a single band for 7E16 and four bands for 25G18 and 36G3. *SH-RGH7* has a *Hin*dIII site in the probe region which accounts for one of the two additional bands detected in 25G18 and 36G3. The other additional band indicates the presence of an extra RGH in the *Gpa2*/*Rx1* cluster in the haplotype derived from *S. tuberosum* ssp. *tuberosum*. Similar to the pseudogene *SH-RGH3*, this RGH is not amplified with the cluster-specific primer pair. Based on this information together with the information on the positions of BAC 25G18 and 36G3, we assume that this additional RGH (designated *SH-RGH8*) is also a pseudogene and that it is most-likely positioned between the homologues *SH-RGH6* and *SH-RGH7* (Figs. 4 and 5). It is noted that the sequence-homologous region in the haplotype of *S. tuberosum* ssp. *andigena* contains exactly the same number of homologues. In addition, a 185-bp fragment was used to determine the number of RGHs with an NBS domain. This probe reached 73 bp into the NBS domain and 70 bp into the LRR domain, and gave identical results as the LRR probe. These data show that all *Gpa2*/*Rx1* homologues identified in the PCR-based method also possess at least part of an NBS domain similar to *Gpa2* and *Rx1*.

The total number of *Gpa2*/*Rx1* homologues in the potato genome was also determined by Southern blotting on genomic DNA of SH and RH. Hybridisation with the LRR and NBS probe confirmed our previous data and resulted in the detection of eight bands for SH and six for RH. All the bands for SH were also present in the BAC clones harbouring the *Gpa2*/*Rx1* homologues. The observation that Southern blotting resulted in the same number of RGHs as the PCR approach indicates that no additional, slightly modified, RGHs are present in RH.

Discussion

In this paper, we describe the identification and mapping of nine resistance gene homologues (RGHs) of a single complex locus in four homologous chromosomes using a cluster-specific primer combination based on the LRR domain of *Gpa2* and *Rx1*. Eight of the nine RGHs could be mapped on chromosome XII in a single-step procedure using a modified AFLP method. Physical mapping revealed that the remaining homologue (*SH-RGH7*) was also located in the *Gpa2*/*Rx1* cluster on chromosome XII. The segregation of all six RH-RGHs in the mapping population shows that the two haplotypes of RH are heterozygous at the *Gpa2*/*Rx1* locus. This is confirmed by the fact that all six RH-RGHs have different sequences. Only two pairs of RGHs are (virtually) identical: SH-RGH5 and RH-RGH4 (99.9%), and SH-RGH6 and RH-RGH5 (100%). In both cases these identical RGHs are derived from different genotypes.

Gpa2 and *Rx1* are highly homologous. At the amino-acid level, they have a homology of 88% and at the nucleotide level their identity is even 93% (Van der Vossen et al. 2000). *SH-RGH1*, an RGH present on the same haplotype as *Gpa2* and *Rx1*, has also a similar sequence identity to the LRR domains of *Gpa2* and *Rx1*, respectively 93.6 and 93.5%. A complete open reading frame was detected for this putative *R* gene based on the 187-kb sequence of this region (Van der Vossen et al. 2000). This indicates that *SH-RGH1* could be a functional homologue of *Gpa2* and *Rx1* with unknown specificity. The nine RGHs identified in this study are all closely related to *Gpa2* and *Rx1* with sequence identities ranging from 93 to 95%. Southern analysis showed that all these RGHs have at least part of an NBS domain.

Our results indicate that all homologues are located in the *Gpa2*/*Rx1* cluster on chromosome XII. Surprisingly, in *S. aucaule* a functional *Gpa2*/*Rx1* homologue with the same specificity as *Rx1* is found (Bendahmane et al. 2000). This gene (*Rx2*), however, is genetically linked to RFLP marker Gp21 on chromosome 5 (Ritter et al. 1991; Bendahmane et al. 2000). Sequence identity between *Rx1* and *Rx2* is so high (97.9% for the complete genes and 99.4% for the LRR domains) that another *Rx1*/*Gpa2*-like cluster on chromosome V, if present in SH or RH, would certainly have been identified in this study. An explanation for this remarkable phenomenon, that highly homologous genes are located on different chromosomes in two closely related species, could be a recent translocation event after the speciation of *S. tuberosum* and *S. aucaule*. However, the synteny between the more distantly related species *S. tuberosum* and *Lycopersicon esculentum* is very high (Grube et al. 2000), indicating that such translocation events are rare within the genus *Solanum*.

In this paper, a PCR-based method has been used to identify *Gpa2*/*Rx1* homologues. Remarkably, some of the amplified RGHs gave conflicting results, among others, with regard to physical mapping. For example, PCR analyses of nearly completely overlapping BAC clones (25G18 and 36G3) resulted in totally different RGHs. Sequence alignments of these RGHs with other RGHs (two by two) revealed that they were the result of a chimaeric PCR product derived from two distinct RGHs. These artificial RGHs consisted of two extraordinary stretches of several-hundred nucleotides each. One stretch was 100% identical to one RGH and the other stretch was 100% identical to another, completely different, RGH. These results were obtained with BAC DNA and genomic DNA as well. Fortunately, chimaeric PCR products were typically less-frequent than genuine RGHs and occurred in most cases only once in a series of re-amplified colonies. Although PCR techniques are commonly used to identify and map RGHs, this phenomenon has to our knowledge not been described before in the literature.

Despite the importance of potato as a food crop and its vulnerability to various pests and diseases, only few *R* genes have been cloned. To facilitate cloning genes of interest, an Ultra High Dense genetic map comprising 10,000 AFLP markers has been constructed [http://www.dpw.wageningen-ur.nl/uhd/ (Van Os et al., unpublished)]. This has been accomplished by using the mapping population of SH and RH. However, not for all potato species that harbour interesting *R* genes, a dense genetic map and BAC library will become available. The production of these tools is still laborious and costly, and the possibility to dissect *R* gene clusters with specific primer combinations in different species is a promising alternative. Comparative analysis has shown that the genomes of members of the *Solanaceae* family have a large synteny (Grube et al. 2000). The results described in this paper indicate that characterising *R* genes from other potato (sub)species with PCR-based approaches may be feasible.

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