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ORIGINAL PAPER

Rdr3, a novel locus conferring black spot disease resistance in tetraploid rose: genetic analysis, LRR profiling, and SCAR marker development

Vance M. Whitaker · James M. Bradeen · Thomas Debener · Anja Biber · Stan C. Hokanson

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Abstract Black spot disease of rose, incited by the fungus *Diplocarpon rosae*, is found worldwide and is the most important disease of garden roses. A gene-for-gene interaction in this pathosystem is evidenced by the presence of pathogenic races of *D. rosae* and the previous discovery of a dominant resistance allele at the *Rdr1* locus in the diploid *Rosa multiflora*. The objective of the present study was to genetically analyze resistances to North American black spot races 3, 8, and 9 previously reported in tetraploid roses. Resistance to North American races 3 and 8 segregated 1:1 in multiple F_1 populations, indicating that both are conferred by dominant alleles at single loci and are present in simplex (Rrrr) configuration. Gene pyramiding was demonstrated by combining both resistances into single genotypes. Resistance to race 9 was partial and segregated in a quantitative fashion. Analysis of these populations with microsatellite markers previously developed for *Rdr1* revealed that the gene conferring race 3 resistance resides within the same R gene cluster as *Rdr1*. Race 8 resistance segregated independently and is, therefore, a novel locus for black spot resistance in rose which we have named *Rdr3*. NBS and LRR profiling were used in a bulked segregant analysis to identify a marker 9.1 cM from *Rdr3*, which was converted to a SCAR marker form for marker-assisted breeding.

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V. M. Whitaker
Department of Horticultural Science, Gulf Coast Research and Education Center, University of Florida, 14625 CR 672, Wimauma, FL 33598, USA
e-mail: vwhitaker@ufl.edu

J. M. Bradeen
Department of Plant Pathology, University of Minnesota, 495 Borlaug Hall, 1991 Upper Buford Circle, St. Paul, MN 55108, USA

T. Debener · A. Biber
Institute of Plant Genetics, Leibniz University, Herrenhaeuser Strasse 2, 30419 Hannover, Germany

S. C. Hokanson (✉)
Department of Horticultural Science, University of Minnesota, 1970 Folwell Ave., St. Paul, MN 55108, USA
e-mail: hokan017@umn.edu

Introduction

Black spot is considered to be the most serious disease of roses (*Rosa hybrida* L.) grown in the outdoor landscape (Dobbs 1984). It is incited by *Diplocarpon rosae* Wolf, a hemibiotrophic fungus that infects members of the genus *Rosa* (Blechert and Debener 2005). Under humid conditions it is rapidly spread via water-borne asexual spores (conidia). Symptoms include lesion development, leaf chlorosis, and ultimately defoliation (Horst and Cloyd 2007). Lesions may appear to the naked eye 4 days after infection, with spore-bearing acervuli rupturing the leaf surface in as little as 7 days (Blechert and Debener 2005). Considering the economic importance of rose to the nursery and floral industries as well as environmental concerns over pesticide applications, genetic resistance to black spot is an oft-mentioned breeding goal (Whitaker and Hokanson 2009).

A complicating factor in breeding for resistance to black spot is the well-documented presence of physiological races of *D. rosae*. The first race differentiation test, performed in Ontario, Canada, revealed the presence of three unique races (Svejda and Bolton 1980). Subsequently, five races were discovered in Germany (Debener et al. 1998),

four races were described in the United Kingdom (Yokoya et al. 2000), and four races were differentiated in Belgium (Leus 2005). Three races were differentiated from among 14 isolates collected in eastern North America (Whitaker et al. 2007). The races from Germany, the United Kingdom, and North America as well as six uncharacterized isolates were inoculated to a common set of rose cultivars (Whitaker et al. 2009). Eleven unique reaction patterns were discovered among the isolates, indicating the presence of 11 races. A unified race nomenclature using Arabic numerals was proposed (*D. rosae* races 1–11).

Most garden roses are tetrasomic tetraploids ($2n = 4x = 28$) originating from 10 to 15 mostly diploid ($2n = 2x = 14$) species (De Vries and Dubois 2001). Various fertility barriers and poor seed germination limit crossability and progeny sizes (Gudin 2000). Selfing and backcrossing typically result in inbreeding depression because of the heterozygous, outbreeding nature of this species complex. Polyploidization methods, both chemical and sexual, can be used to transfer resistance (R) genes from diploid wild species into tetraploid roses (Zlesak et al. 2005). Indeed, this strategy has been used to transmit black spot resistance into cultivated forms (Byrne et al. 1996; Von Malek and Debener 1998). Since some tetraploid roses already possess resistance to races of *D. rosae* (Debener et al. 1998; Yokoya et al. 2000; Whitaker et al. 2007), these resistances could be utilized in breeding without the need for ploidy manipulations. However, no resistance genes from tetraploid roses have been described thus far.

To date, one black spot R locus has been described in diploid rose. The gene *Rdr1* was derived from a *R. multiflora* species selection that was chromosome-doubled with colchicine and hybridized with tetraploid tea roses (Von Malek and Debener 1998). Resistance screens with *D. rosae* isolate Dort E4 (Debener et al. 1998) demonstrated the presence of a single locus carrying a dominant resistance allele. Discovery of *Rdr1* was the first direct evidence of a gene-for-gene interaction in the rose black spot pathosystem. Ongoing investigations using the German isolate Dü A3 (Debener et al. 1998) indicate that a second gene, *Rdr2*, may be tightly linked to *Rdr1* (Hattendorf et al. 2004).

Recent sequence analysis of a bacterial artificial chromosome (BAC) contig spanning the *Rdr1* locus (Kaufmann et al. 2003) revealed a cluster of nine resistance gene analogs (RGAs) encoding nucleotide binding site (NBS) and leucine-rich repeat (LRR) amino acid domains (Terefe and Debener 2009). This R gene cluster is located on linkage group 1 of the rose genetic map (Von Malek et al. 2000). Additional NBS-LRR RGAs have been cloned from rose and grouped into 40 different families (Hattendorf and Debener 2007). Some of these RGA families have been used to create markers for the latest genetic map of rose (Yan et al. 2005).

Resistance gene analog DNA sequence information is useful for developing markers linked to known resistance genes. Chen et al. (1998) utilized primer pairs targeting protein kinase, NBS, and LRR domains based on various crop species to amplify RGA sequences and detected size polymorphisms using polyacrylamide gels. Others have pursued a modified AFLP approach, substituting an RGA primer for one of the AFLP adapter primers during the selective amplification in apricot (Soriano et al. 2005) and cotton (Zhang et al. 2007). Most efforts have focused on the NBS region, since it is present in the majority of known resistance genes and encodes multiple amino acid motifs that are highly conserved (Meyers et al. 1999). The NBS profiling marker system was developed to target the NBS region while simultaneously generating polymorphisms (Van der Linden et al. 2004). It is comparable to AFLP in that it involves restriction digestion and adapter ligation. However, the PCR amplification step utilizes both an adapter primer and an NBS-specific degenerate primer. This method was demonstrated to efficiently target RGA sequences and has been used in a variety of crop species with little modification (Calenge et al. 2005; Mantovani et al. 2006; Syed et al. 2006).

The objectives of the present study are to genetically characterize the resistance of tetraploid rose cultivars to North American isolates of *D. rosae* and to develop molecular markers linked to resistance genes using RGA targeting approaches. This work is intended to support the utilization of R genes from cultivated roses in breeding, a complementary approach to the introgression of resistance from wild species.

Materials and methods

Plant materials

The tetraploid roses 'Applejack', 'Folksinger', 'George Vancouver', and Love and Peace™, were chosen as black spot resistant parents for crossing. The cultivar George Vancouver was only resistant to race 8 in a race test inoculation of 14 North American isolates of *D. rosae* (Whitaker et al. 2007). The rose Love and Peace™ was resistant to race 3 only, and 'Applejack' was resistant to races 8 and 9. Races 3, 8, and 9 were also inoculated to 'Folksinger', which was resistant to race 8 only, and to 'Chorale', 'Dorcas', and 'Morden Blush', which were susceptible to all three races (Table 1). Cultivars with race-specific resistance were crossed to susceptible cultivars to create F_1 seedling populations. The cultivar George Vancouver was crossed as a male to the susceptible genotypes 'Chorale' ($n = 79$) and 'Morden Blush' ($n = 78$) (Table 3) in order to study the inheritance of race 8

Table 1 Resistance and susceptibility of tetraploid rose cultivars used as parents in controlled crosses to three North American races of *Diplocarpon rosae*

Rose genotype (4×)	Race		
	3	8	9
'Applejack'	S[a]	R	R
'Chorale'	S	S	S
'Dorcas'	S	S	S
'Folksinger'	S	R	S
'George Vancouver'	S	R	S
Love and Peace™	R[b]	S	S
'Morden Blush'	S	S	S

[a] Susceptible

[b] Resistant

resistance. The cultivar Folksinger was crossed both as a male and female to 'Chorale' ($n = 92$) and 'Dorcas' ($n = 90$), also in order to study the inheritance of race 8 resistance. Love and Peace™ was crossed as a male to 'Chorale' ($n = 41$) and 'Morden Blush' ($n = 63$) in order to study the segregation of race 3 resistance. The 'Folksinger' × Love and Peace™ cross ($n = 32$) was performed to simultaneously study the segregation of race 3 and race 8 resistance. Finally, 'Applejack' was crossed as a male to 'Chorale' ($n = 58$) in order to study the segregation of race 9 resistance.

Inoculations and disease ratings

Race 3 (formerly race C) of *D. rosae* was represented by isolate GVH (originally collected from Hastings, MN, USA), Race 8 (formerly race A) was represented by the isolate ACT (originally collected from Brenham, TX, USA), and Race 9 (formerly race B) was represented by isolate IGWA (originally collected from Appleton, WI, USA) (Whitaker et al. 2007). Each single-spore isolate was retrieved from cryogenic storage and inoculated to detached leaves of susceptible varieties according to the methods of Debener et al. (1998) in order to increase inoculum.

Parental genotypes and seedlings were placed in a common greenhouse environment, cut back to 15 cm in height, and grown for 5–8 weeks ensuring vigorously growing and uninfected leaves. Plants were maintained in 3.8 or 7.6 L pots containing a growth media of composted pine bark amended with 6.5 kg m^{-3} Nutricote 20N–2.6P–6.6K (18-6-8) slow-release fertilizer (Chisso-Asahi Fertilizer Co. Ltd., Tokyo Japan). Sulfur was burned 4–6 h nightly to prevent powdery mildew infection. Two to five leaves were detached from each plant and placed on a moistened paper towel inside a transparent Clearpac® deli container (Dart Corporation, Mason, MI, USA). Aqueous spore suspensions were applied to each container with a handheld spray bottle. Leaves of all seedlings of a population were inoculated at one time, with the same inoculum concentration and the same number of sprays applied to each container within a population. Inoculum concentrations ranged from 3×10^4 to 1×10^5 conidia/ml among inoculations, depending on availability of spores, and one to three sprays were applied to each container (~0.75 ml/spray) depending on the number of leaves. Containers were sealed and incubated on the lab bench at room temperature. After 2 or 3 days, remaining inoculum droplets were blotted from the leaves with a paper towel.

All seedlings of a population were rated on the same day post inoculation (dpi) using a dissecting microscope as either resistant or susceptible, except those of population T06-002 ('Chorale' × 'Applejack') (see below). Populations were rated between 9 and 16 dpi depending on the rate of disease progression. Ratings were performed at the latest time point possible before secondary fungal infection began and degradation of the detached leaves obscured the ratings. Susceptible reactions were characterized by radiating mycelia and the production of spore-bearing acervuli. Leaves with no spore-bearing acervuli were rated as resistant. Three to ten inoculations were performed over time for each seedling population. Genotypes that produced large, spore-bearing lesions >3 mm in diameter in each of the first three inoculations were not replicated further. Genotypes that were initially rated resistant and those exhibiting low levels of susceptibility (lesions <3 mm in diameter and few or no acervuli) were replicated up to ten times. A chi-square analysis of segregation ratios (resistant:susceptible) was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Screening of the T06-002 ('Chorale' × 'Applejack') population was performed using an adjusted rating scale. Four independent inoculations were performed, with each genotype being replicated exactly four times. The first two inoculations were rated as either susceptible or resistant. In order to more accurately assess levels of partial resistance, a modified rating scale was used in the third and fourth inoculations: 0 = no visual symptoms, 1 = lesions present but not containing acervuli, 2 = lesions containing acervuli but not spreading from the area of the original inoculum droplet, 3 = lesions containing acervuli and spreading across <10% of the total leaf surface, 4 = lesions containing acervuli and spreading across >10% of the total leaf surface. Ratings from the third and fourth inoculations were averaged to obtain a disease score for each genotype.

DNA extraction

Young, newly expanded leaf tissue (20–40 mg) was harvested from greenhouse grown roses and frozen at −80°C

prior to DNA extraction. Extractions were performed using a CTAB method (Haymes 1996) with some modifications. The leaf tissue was added to 0.5 ml extraction buffer and pulverized using a mechanical shaker (25/s) and 2 mm stainless steel beads (2 per sample). Purifications were performed twice using 200 µl chloroform/isoamyl alcohol (24:1). After precipitation with ethanol–acetate, the pellet was washed in 70% ethanol, dried, and resuspended in TE buffer (pH 8.0). Quantifications were performed using a Qubit™ fluorometer (Invitrogen, Carlsbad, CA).

Microsatellite marker analysis

A microsatellite marker analysis was performed to analyze the positions of the race 3 and race 8 resistance genes relative to the *Rdr1* locus. Three markers were selected based on their segregation with *Rdr1* in multiple diploid populations comprising 793 plants (T. Debener unpublished). The 155 microsatellite marker is linked to *Rdr1* with no recombination [0 centimorgans (cM)] while two others, RMS015 and 69E25, flank *Rdr1* at distances of 2.26 and 0.1 cM, respectively (Table 2). In the present study, these markers were analyzed in the T06-010, T06-017, T06-035, and T06-036 populations.

Microsatellite marker PCRs were performed using tailed M13 PCR. A 19-bp labeled (IRD 700 or 800) M13 primer (5′ CAC GAC GTT GTA AAA CGA C 3′) was added to the reaction mix along with the forward primer (with the M13 tail added at the 5′ end) and the reverse primer (Table 2). Ten nanograms of genomic DNA was added to a 10-µl reaction containing 0.15 µM M13-tailed forward primer, 0.15 µM reverse primer, 0.1 µM labeled M13 primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 0.5 U *Taq* polymerase. The PCR program consisted of a 10-min hot start at 94°C followed by 30 cycles (94°C for 30 s, 55°C for 45 s, 72°C for 45 s) and a final extension at 72°C for 10 min.

RGA analysis and NBS profiling primer design

Genomic DNA samples from 'Folksinger' and Love and Peace™ were utilized as templates for PCR amplification of RGAs. Degenerate primer pairs were specific to codons encoding conserved amino acid sequences within the NBS domain. Primer pairs 3, 11, and DERES were selected based on successful amplification of rose RGAs in previous experiments (Hattendorf and Debener 2007). Primer pairs 3 and 11 were originally obtained from Pan et al. (2000), and the DERES primer pair originated from Kanazin et al. (1996) where they were shown to amplify toll-interleukin receptor (TIR) and non-TIR RGAs. PCR amplifications were performed under the reaction conditions reported by Hattendorf and Debener (2007). The PCR products were electrophoresed on 1.5% agarose gels and fragments (~500 bp for DERES primer pair, ~700 bp for primer pairs 3 and 11) were extracted and purified. The RGAs were cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and transformed into *E. coli* DH10B (Invitrogen) by electroporation. Plasmid DNA was purified using the Wizard® Plus SV Miniprep kit (Promega) following manufacturer's instructions. Seventy-four clones were sequenced using IRD700- and IRD800-labeled M13 universal primers.

Putative RGA sequences were edited and aligned using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and were searched against nucleotide sequences of all higher plants using the NCBI BLASTn algorithm. Sequences exhibiting less than 80% sequence similarity to known RGAs from rose were further analyzed by comparing them to representative sequences from each of the 40 rose RGA families discovered by Hattendorf and Debener (2007). To determine sequence similarity to the closest-matching representative sequence, percent nucleotide identities were calculated after aligning and trimming the sequences to the same length. Sequences that encompassed NBS conserved regions but having less than 80% sequence similarity to any known RGA family were classified as new families. Nucleotide and deduced amino acid sequences of the cloned RGAs were deposited into the GenBank Database under accession numbers FJ789913–FJ789985.

Degenerate primers for NBS profiling were constructed by aligning the cloned RGAs from 'Folksinger' and Love and Peace™ as well as 40 sequences selected to represent each of the RGA families previously discovered by Hattendorf and Debener (2007). Primers were constructed based on conservation within the kinase-2a and GLPL motifs. The TIR and non-TIR RGAs were aligned separately, and degenerate primers were designed independently by hand for each class (Table 2).

Previously published non-degenerate primers constructed from known resistance genes were used in LRR profiling. Eight different LRR primers described by Chen et al. (1998) were originally designed from LRR sequence of cloned R genes from rice and tomato: XLRRfor and XLRRrev (rice *Xa21*); RLRRfor and RLRRrev (rice *RPS2*), NLRRfor and NLRRrev (tomato *N*), and CLRRfor and CLRR rev (tomato *Cf9*) (Table 2).

Bulked segregant analysis

Bulked segregant analysis (Michelmore et al. 1991) was performed in the T06-010 ('Chorale' × 'George Vancouver') population. DNA of the parents and seedlings was diluted in water to a concentration of 30 ng/µl. Two independent resistant bulks and two independent

Table 2 Primers utilized in NBS profiling, LRR profiling, microsatellite marker, and SCAR marker PCR amplifications

Primer name	AA domain/motif	Nucleotide sequence (5′–3′)	Source
Primers for NBS profiling			
NBS2	NBS/P-loop	GTW GTY TTI CCY RAI CCI SSC AT	Van der Linden et al. (2004)
NBS3	NBS/P-loop	GTW GTY TTI CCY RAI CCI SSC ATI CC	Calenge et al. (2005)
NBS7	NBS/P-loop	ATT GTT GGR ATG GGM GGI MTI GG	Van der Linden et al. (2004)
NBS5	NBS/Kinase-2	YYT KRT HGT MIT KGA TGA TGT ITG G	Van der Linden et al. (2004)
NBS5a	NBS/Kinase-2	YYT KRT HGT MIT KGA TGA YGT ITG G	Syed et al. (2006)
NBS-R1	NBS/Kinase-2	RTI CTI ITI RTH MTK GAT GAT GT	TIR rose RGAs
NBS-R2	NBS/Kinase-2	TII TDI TYY TRG AYG AYV TIT GG	NonTIR rose RGAs
NBS-R3	NBS/Kinase-2	RTI CTI ITI RTH MTK GAT GAY GT	TIR rose RGAs
NBS-R4	NBS/GLPL	RIG GIM TDC CIT TRG CHC T	TIR rose RGAs
NBS-R5	NBS/GLPL	RIG GIM TDC CIT TRG CHT T	TIR rose RGAs
NBS-R6	NBS/GLPL	TGY RRI GGI BTK CCK YTI GC	NonTIR rose RGAs
NBS-R7	NBS/GLPL	TGY RRI GGI BTK CCK YTI GT	NonTIR rose RGAs
Primers for LRR profiling			
CLRRfor	LRR	TTT TCG TGT TCA ACG ACG	Chen et al. (1998)
CLRRrev	LRR	TAA CGT CTA TCG ACT TCT	
NLRRfor	LRR	TAG GGC CTC TTG CAT CGT	
NLRRrev	LRR	TAT AAA AAG TGC CGG ACT	
RLRRfor	LRR	CGC AAC CAC TAG AGT AAC	
RLRRrev	LRR	ACA CTG GTC CAT GAG GTT	
XLRRfor	LRR	CCG TTG GAC AGG AAG GAG	
XLRRrev	LRR	CCC ATA GAC CGG ACT GTT	
Primers for microsatellite markers linked to *Rdr1* and race 3 resistance			
155 forward		GAA AAG AAC GAG GGG TTT CC	
155 reverse		ACG GTC GGT AAT CAA GAT GC	
69E24 forward		TCA GGT GGG TGA GCT TCA AT	
69E24 reverse		TGA TTA GCT TGC CGG TTC TT	
RMS015 forward		TAA TGT AGG CAG ATA TAA AGG AGT	Concipio, GmbH
RMS015 reverse		GCA GCT GCA CAA CAA GGA A	Concipio, GmbH
Primers for SCAR PCR amplifications			
ND5ESCAR forward		GTT TTG AGT ATC TCC TTG ATC	
ND5ESCAR reverse		AAT GAA GTA GGA GAA AGA AAG A	
Rose actin forward		CGA GGA AGA TCT GGC ATC A	
Rose actin reverse		AGG AGC TGC TCT TGG CAG T	

susceptible bulks were created. Each bulk was comprised of DNA from seven resistant or seven susceptible progeny plants in equimolar amounts.

The NBS profiling (Van der Linden et al. 2004) and LRR profiling techniques were used for the bulked segregant analysis with some modifications. Restriction digestions were performed by mixing 360 ng DNA with 3 U restriction enzyme (*Alu*I, *Hae*III, *Rsa*I, or *Mse*I) (New England BioLabs, Ipswich, MA, USA) and 1× reaction buffer in a total volume of 25 µl and incubated at 37°C for 2 h, followed by heat inactivation for 15 min at 70°C. Digestions were diluted 1:1 in distilled, deionized water prior to adapter ligation. The adapter was synthesized via an annealing reaction in which two complementary adapter primers (10 µM each) (Van der Linden et al. 2004) were incubated in annealing buffer (10 mM Tris–HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA) for 5 min at 95°C and slowly cooled to 20°C over a period of 2 h. Ten U T4 ligase (Invitrogen) and 30 pmol adapter were added to 12.5 µl diluted digestion in a final volume of 25 µl. Ligations were performed at 20°C for 16 h and diluted 1:5 in TE buffer (pH 8.0).

Asymmetric PCR amplifications were performed with an NBS or LRR primer (Table 2) only, at a concentration of 0.25 µM along with 184 µM of each dNTP, 1.5 mM $MgCl_2$, and 0.2 U *Taq* polymerase (AmpliTaq Gold®,

Applied Biosystems, Foster City, CA, USA) in a final volume of 9.8 µl. The PCR program was performed on a GeneAmp® 2700 Thermocycler (Applied Biosystems) and consisted of a 10-min hot start at 94°C followed by 30 cycles (94°C for 30 s, 55 or 60°C depending on the degenerate primer for 1 min 40 s, 72°C for 2 min) and a final extension at 72°C for 10 min. The entire volume of the asymmetric PCR was used as a template for the final exponential PCR. Component concentrations were adjusted to 0.47 µM NBS primer, 184 µM of each dNTP, and 1.5 mM $MgCl_2$ in a final volume of 24 µl. Also added to the exponential PCR was 0.8 µM IRD700 labeled adapter primer (MWG Biotech, Ebersberg, Germany) and 0.5 U *Taq* polymerase.

NBS and LRR profiling PCRs were then diluted 1:4 in loading buffer (LI-COR Biosciences, Lincoln, NE, USA), electrophoresed on a 6.5% polyacrylamide gel using a LI-COR 4300 DNA Analyzer and scored visually from gel images produced with SAGA^{MX} Software (LI-COR Biosciences). A 50–700 bp size ladder (LI-COR Biosciences) was used to estimate band size.

Band extraction, cloning, and SCAR marker development

End-labeling of the NBS/LRR profiling adapter primer was performed with 5 µl γ^{33}P, 50 pmol adapter primer, and 20 U T4 Kinase (New England Biolabs) in a final volume of 30 µl. The reaction was incubated at 37°C for 1 h and heat-inactivated at 70°C for 10 min. Previous exponential PCRs containing LRR profiling bands of interest were diluted 20-fold in TE buffer. New exponential PCRs were performed with 2 µl of diluted exponential PCR as a template in a final volume of 10 µl containing 0.5 pmol γ^{33}P end-labeled adapter primer, 1.5 pmol NLRRrev primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 0.25 U *Taq* polymerase. The PCR conditions were the same as previously described for exponential PCRs. Samples were then electrophoresed on a 6.5% polyacrylamide gel for 2.5 h. The gel was vacuum dried and exposed to X-ray film for 48 h. Fragments were extracted from the dried gel with a sterile scalpel and incubated in 200 µl elution buffer (0.5 M NH_4Ac, 10 mM $MgAc_2$, 1 mM EDTA, and 0.1% SDS) for 45 min at 50°C. In addition to a polymorphic band identified via bulked segregant analysis, 12 other LRR profiling bands were randomly selected for extraction and re-amplification. A volume of 1 µl eluted DNA was used as a template for two rounds of re-amplification using 1.5 pmol non-labeled adapter primer (Van der Linden et al. 2004) and 1.5 pmol NLRRrev primer (Chen et al. 1998) (Table 2) using the same reaction components and conditions described above. Labeled re-amplified products were run side-by-side with the original LRR profiling PCRs on a polyacrylamide gel to confirm that only the bands of expected size were recovered. The PCR products were then cloned into the pCR®2.1-TOPO® vector and transformed into OneShot® Mach1™-T1® competent cells via the TOPO TA Cloning® kit (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was purified using the Wizard® Plus SV Miniprep kit (Promega). Sequencing was performed with T7 and M13 universal primers at the BioMedical Genomics Center, University of Minnesota.

LRR profiling fragment sequences were searched against higher plant sequences using the BLASTn algorithm. For a polymorphic fragment identified via bulked segregant analysis, sequence characterized amplified region (SCAR) primers were designed from three recovered sequences using web-based Primer3 software (http://primer3.sourceforge.net). Primers for one sequence (ND5E) were utilized to screen the parents, bulks, and individual seedlings of the 'Chorale' × 'George Vancouver' population. SCAR PCRs were performed using tailed M13 PCR. Ten ng genomic DNA was added to a 10-µl reaction containing 0.15 µM M13-tailed forward primer, 0.15 µM reverse primer (Table 2), 0.1 µM labeled M13 primer, 0.2 mM of of each dNTP, 2 mM $MgCl_2$, and 0.25 U *Taq* polymerase. Forward and reverse rose actin primers (0.15 µM each) (Table 2), which amplify a fragment of ~500 bp in all genotypes, were added to each reaction to provide confirmation of a successful PCR amplification. Negative control reactions were performed with water as a template. The PCR program consisted of a 10-min hot start at 94°C followed by 30 cycles (94°C for 30 s, 55°C for 45 s, 72°C for 45 s) and a final extension at 72°C for 10 min.

Results

Segregation of resistance

In populations with 'George Vancouver', 'Folksinger', and Love and Peace™ as parents, resistance phenotypes of seedlings segregated clearly into resistant and susceptible classes within families when challenged with *D. rosae* races (Fig. 1). In the T06-010 ('Chorale' × 'George Vancouver') and T06-008 ('Morden Blush' × 'George Vancouver') populations, segregation ratios of 1:1 (resistant:susceptible) were strongly supported by the chi-square analysis ($P = 0.57$ and $P = 0.65$, respectively) (Table 3). This ratio is consistent with a single locus conferring resistance in 'George Vancouver', at which a single dominant allele for race 8 resistance is present in simplex (Rrrr) configuration. In populations with 'Folksinger' as a parent (H04-01, H04-02, T06-035) there were fewer seedlings in the race 8 resistant class, most markedly in the T06-035 ('Folksinger' × Love

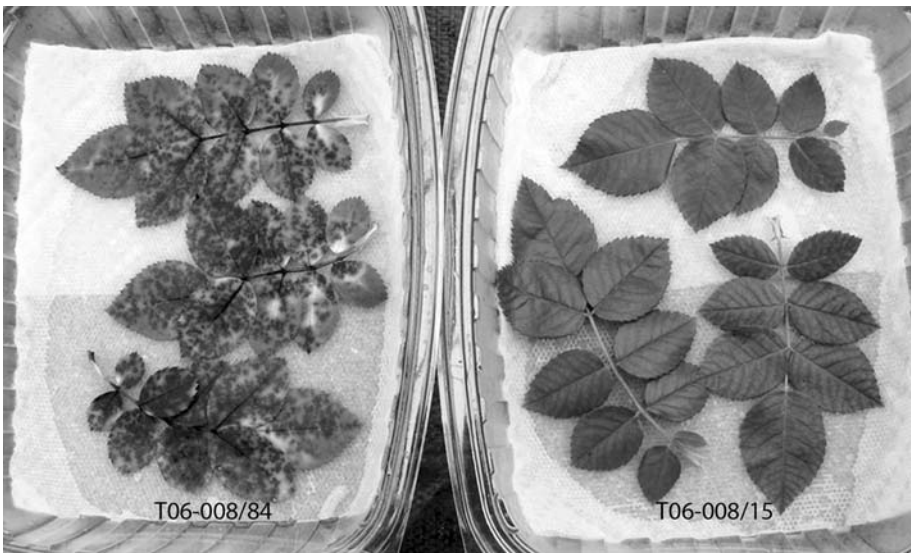

Fig. 1 Detached leaves of two seedlings of the T06-008 ('Morden Blush' × 'George Vancouver') population 12 days after inoculation with *Diplocarpon rosae* isolate ACT (Race 8) exhibiting susceptible (T06-008/84) and resistant (T06-008/15) phenotypes

Table 3 Phenotypic segregation of resistance to races 8 and 3 of *Diplocarpon rosae* in F_1 populations resulting from crosses between tetraploid rose cultivars

Cross #	F_1 Cross	*n*	Resistant	Susceptible	χ^2	*P*
Race 8						
T06-010	'Chorale' × 'George Vancouver'	79	42	37	0.32[a]	0.57
T06-008	'Morden Blush' × 'George Vancouver'	78	41	37	0.21	0.65
H04-02	'Folksinger' × 'Chorale'[b]	90	36	54	3.60	0.06
H04-01	'Folksinger' × 'Dorcas'[c]	92	38	54	2.78	0.10
T06-035	'Folksinger' × Love and Peace™	32	11	21	3.13	0.08
Race 3						
T06-036	'Chorale' × Love and Peace™	41	22	19	0.22	0.64
T06-035	'Folksinger' × Love and Peace™	32	17	15	0.13	0.72
T06-017	'Morden Blush' × Love and Peace™	63	31	32	0.02	0.90

[a] Chi-square statistics indicate goodness of fit for 1:1 segregation ratios

[b] Includes 73 seedlings from the 'Folksinger' × 'Chorale' cross and 17 seedlings from the reciprocal cross

[c] Includes 86 seedlings from the 'Folksinger' × 'Dorcas' cross and 6 seedlings from the reciprocal cross

and Peace™) population in which 11 of 32 plants were resistant. A segregation ratio of 1:1 was not supported by the chi-square analysis (Table 3).

In the T06-017 ('Morden Blush' × Love and Peace™), T06-035 ('Folksinger' × Love and Peace™), and T06-036 ('Chorale' × Love and Peace™) populations, the chi-square analysis strongly supported a segregation ratio of 1:1 ($P = 0.90$, $P = 0.72$ and $P = 0.64$, respectively), which is consistent with a single locus conferring resistance in Love and Peace™, at which a single dominant allele for race 3 resistance is present in simplex (Rrrr) configuration. In the T06-035 population, four plants were resistant to both race 3 and race 8. This indicates that resistance to race 3 and race 8 are controlled by different genes and, therefore, demonstrates a gene pyramid of race-specific black spot resistances.

In the first two of four independent inoculations, the T06-002 ('Chorale' × 'Applejack') population did not clearly segregate for resistance to race 9 according to a qualitative rating scheme (resistant or susceptible) since sporulation was observed on all seedlings; therefore, the third and fourth inoculations were rated using an alternative disease scale (0–4) to reflect quantitative differences in resistance. Ratings for the third and fourth inoculations were averaged to obtain mean disease scores, which had a theoretical range of 0–4. Disease scores in T06-002 ranged from 1.5 to 3.5 (Table 4). Some sporulating lesions were observed on each of the 58 seedlings, which is reflected by a minimum disease score of 1.5. Therefore, the resistance conferred from 'Applejack' is partial and may be conferred by more than one locus. The disease score distribution was skewed toward lower disease scores, indicative of some

Table 4 Frequencies of mean disease scores compiled from two separate inoculations of *Diplocarpon rosae* race 9 to the 58 seedlings of the T06-002 ('Chorale' × 'Applejack') population

Mean Disease Score	# Seedlings
1.5	28
2.0	16
2.5	1
3.0	0
3.5	13
Total	58

Mean disease scores for the parents 'Applejack' and 'Chorale' were 1.5 and 3.5, respectively

level of dominance. No transgressive segregation was observed as 'Applejack' and 'Chorale' received mean disease scores of 1.5 and 3.5, respectively.

Microsatellite marker analysis

Three microsatellite markers, previously shown to be linked to the *Rdr1* locus (T. Debener unpublished, Fig. 2), were analyzed in 133 plants comprising the T06-017, T06-035, and T06-036 populations segregating for race 3 resistance. Up to four alleles per locus were amplified in each of the parents. An allele of the RMS015 microsatellite marker was polymorphic in the T06-017 population and segregated with race 3 resistance with two recombinants out of 63 plants (3.2%). A 155 microsatellite marker allele was polymorphic in all three populations and segregated perfectly (0 recombinants out of 133 progeny) with race 3 resistance.

The 79 plants of the T06-010 population segregating for race 8 resistance were also analyzed with the 155, RMS015, and 69E24 microsatellite markers. Except for one marker allele of 155, all alleles from the resistant parent 'George Vancouver' were polymorphic compared to those of 'Chorale'. None of the marker alleles segregated with race 8 resistance in the T06-010 population (Fig. 2). Recombination between marker alleles and the resistant phenotype ranged from 36 to 63%, showing that race 8 resistance segregates independently of the *Rdr1* locus. Therefore, race 8 resistance is conferred by a novel locus for black spot resistance, which we have named *Rdr3*. Consequently, RGA discovery and bulked segregant analysis of the T06-010 population were undertaken in order to develop a marker linked to *Rdr3*.

RGA discovery and primer design

Out of 74 cloned sequences amplified from 'Folksinger' and Love and Peace™ using degenerate primer pairs, 73 were determined to be RGAs based on the presence of NBS conserved motifs, yielding an RGA recovery rate of greater than 98%. Of these 73 RGAs, ten had less than 80% DNA sequence similarity to representatives of the 40 RGA families previously described by Hattendorf and Debener (2007). Of these ten RGA sequences, two showed 94% sequence identity with RGAs from *R. roxburghii* (Xu et al. 2005). Therefore, of 73 RGAs cloned from 'Folksinger' and 'Love and Peace'™, eight new RGA families were discovered. Based on alignment and comparison of these 73 RGAs as well as representatives of the 40 previously discovered families, seven new degenerate primers (NBSR1–NBSR7) for NBS profiling were designed, three from the kinase-2 motif and four from the GLPL motif. Each was designed either for the TIR or non-TIR class of RGAs, thereby reducing the degeneracy of the primers (Table 2).

Bulked segregant analysis and SCAR analysis

Each primer/enzyme combination of the NBS profiling and LRR profiling methods yielded repeatable polymorphisms between the parents 'Chorale' (susceptible to races 3, 8, and 9) and 'George Vancouver' (resistant to race 8) (Fig. 3). Using five previously published NBS primers in combination with four restriction enzymes, 58 polymorphisms were observed between the parents. Seven NBS primers designed from rose RGAs yielded an additional 92 polymorphisms. By using eight LRR primers (Chen et al. 1998), 145 polymorphisms were obtained between the parents.

The NLRRrev/*Alu*I combination yielded a fragment that was polymorphic between the parents as well as between the resistant and susceptible bulks of T06-010 (Fig. 4). After band extraction, cloning, and sequencing, three 153 bp sequences were recovered from the fragment. Multiple SCAR primer pairs were designed from each sequence. All primer pairs amplified products from both parents and both bulks. However, for a primer pair designed from one of the sequences (ND5E), greater band intensity was observed for the resistant parent and resistant bulks in comparison with the susceptible parent and susceptible bulks. The PCR products from the parents were re-sequenced, and a new reverse primer was designed to take advantage of a single nucleotide polymorphism (SNP). The resulting ND5ESCAR primer pair amplified a 108-bp fragment from the resistant parent only. The presence of the marker segregated with resistance in the T06-010 population, with six recombinations among 79 plants (7.6%). The marker was then validated using 75 plants of the T06-008 population (Fig. 5), among which there were eight recombinant genotypes (10.7%). In total, 14 of the 154 plants comprising these two populations were

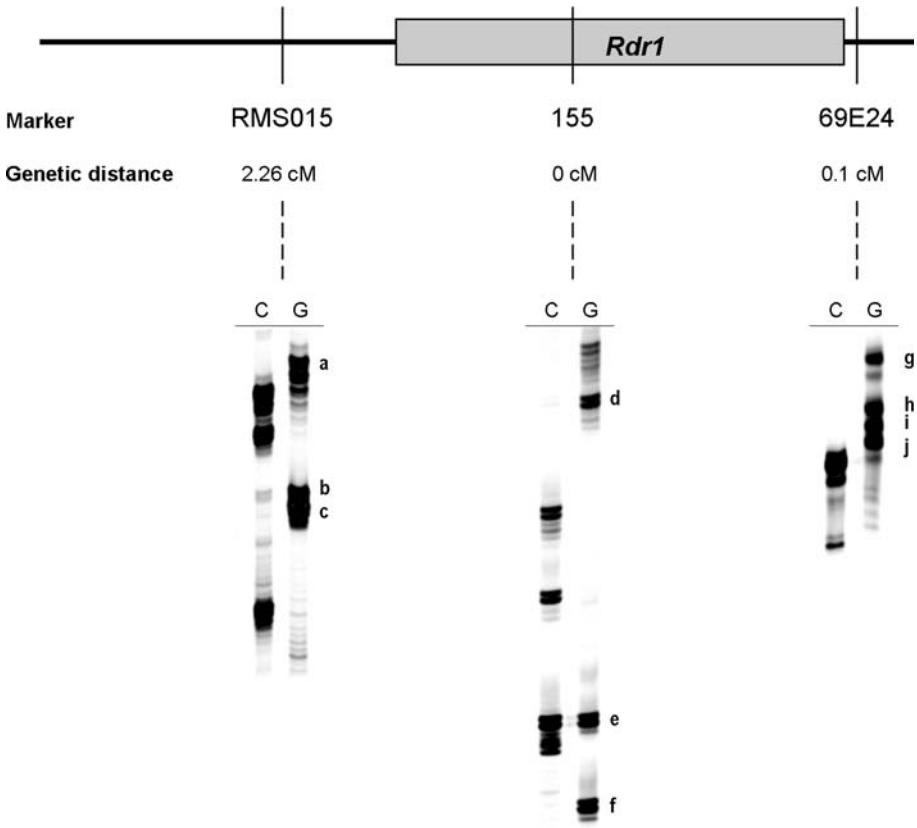

Fig. 2 Three microsatellite markers within and flanking the *Rdr1* locus developed by the Debener lab (155, 69E24) (Leibniz University, Hannover, Germany) and Concipio GmbH (RMS015) (Sangerhausen, Germany) were analyzed in the T06-010 population ($n = 79$). Amplified fragments from the susceptible parent 'Chorale' (*C*) and the race 8 resistant parent 'George Vancouver' (*G*) are shown. Marker alleles a, b, d, f, g, h, i, and j segregated 1:1 in T06-010. Allele c segregated ~5:1, indicating two copies of the allele in the resistant parent. Allele e was not polymorphic between the parents. None of the polymorphic alleles segregated with the resistance phenotype

recombinant, indicating that the ND5E marker is linked to *Rdr3* at an approximate genetic distance of 9.1 cM.

Discussion

The recognition of physiological races of the black spot pathogen and the discovery of a dominant resistance allele at the *Rdr1* locus provided the first evidence for a gene-for-gene interaction in this pathosystem (Von Malek and Debener 1998). In the present study, the discovery of two dominant genes conferring race-specific black spot resistances in roses further supports this conclusion. Additionally, combining race 3 and race 8 resistance into individual genotypes of the T06-035 population represents, to our knowledge, the first documented instance of gene pyramiding for black spot resistance in rose.

The 1:1 segregation ratios observed for race 3 and race 8 resistance indicate that both are conferred by dominant alleles present in simplex (Rrrr) configuration at single loci. This ratio is predicted for tetrasomic (auto) tetraploids under a model of random chromosome assortment. However, other segregation models exist which take into account double reduction, the phenomenon in which a gamete receives two alleles from the same chromosome (from sister chromatids). The frequency of double reduction (α) may vary for each locus, depending on the nature of quadrivalent pairing at meiosis I and the frequency of crossing over between the gene and centromere (Mather 1936). Therefore, segregation ratios for a single dominant allele in simplex could achieve 13:15 (resistant:susceptible) under random chromatid assortment ($\alpha = 1/7$) or a theoretical extreme of 11:13 for maximum equational segregation ($\alpha = 1/6$) (Burnham 1962). Based on chi-square analyses, these alternative models do not fit the observed segregation data as well as a 1:1 ratio. Indeed, for populations with 'George Vancouver' and Love and Peace™ as parents, there is a slight excess of resistant plants in reference to a 1:1 ratio, with the exception of T06-017 (Table 3). Therefore, though the progeny sizes are smaller than desired, the segregation of the *Rdr1* resistance allele from Love and Peace™ and the *Rdr3* resistance allele from 'George Vancouver' are best described by a model of random chromosome assortment. This is consistent with the previous results of Von Malek and Debener (1998), who proposed random chromosome assortment for the *Rdr1* locus based on 5:1 F_1 segregation ratios for an *Rdr1* allele in duplex (RRrr).

The skewed segregation (excess of susceptibles) in populations with 'Folksinger' as the resistant parent (Table 3) does not fit any known model of tetraploid inheritance. This result cannot be fully interpreted with the available data, but

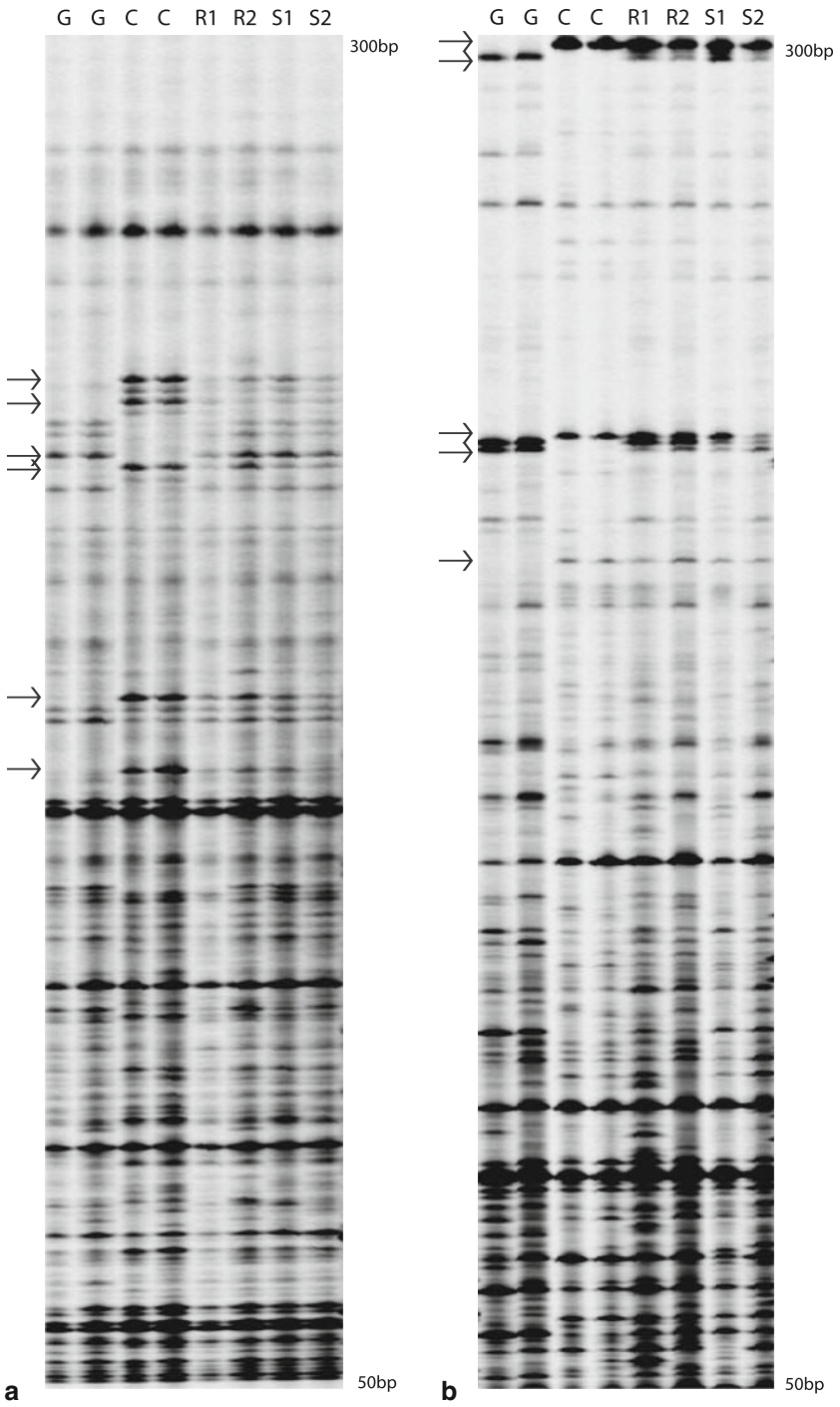

Fig. 3 Gel images of NBS profiling (**a**) and LRR profiling (**b**) used in a bulked segregant analysis of the T06-010 population. Representative repeatable polymorphisms (*arrows*) between the resistant parent 'George Vancouver' (*G*) and the susceptible parent 'Chorale' (*C*) are present. No polymorphisms occur among the resistant (*R1*, *R2*) and susceptible (*S1*, *S2*) bulks

possible explanations can be proposed. The skewed segregation could reflect a chromosomal segregation distortion, possibly due to linkage of a single dominant resistance allele in simplex (Rrrr) with a gene or genes causing differential viability in the progeny. Alternatively, the race 8 resistance allele may have decreased penetrance and/or expressivity due to minor gene effects. This was suggested as the cause for skewed segregation ratios for resistance to *Puccinia psidii* in *Eucalyptus*, which resulted in an excess of susceptibles for some genetic backgrounds (Junghans et al. 2003). A single gene may also exhibit variable expressivity based on environmental factors, which can influence disease ratings. Single genes that confer reduced or delayed sporulation, rather than complete resistance, in certain environments have been characterized in the lettuce down mildew pathosystem (Crute and Norwood 1986).

The German *D. rosae* isolate Dort E4 was originally used to identify and analyze *Rdr1*. The race 3 resistance

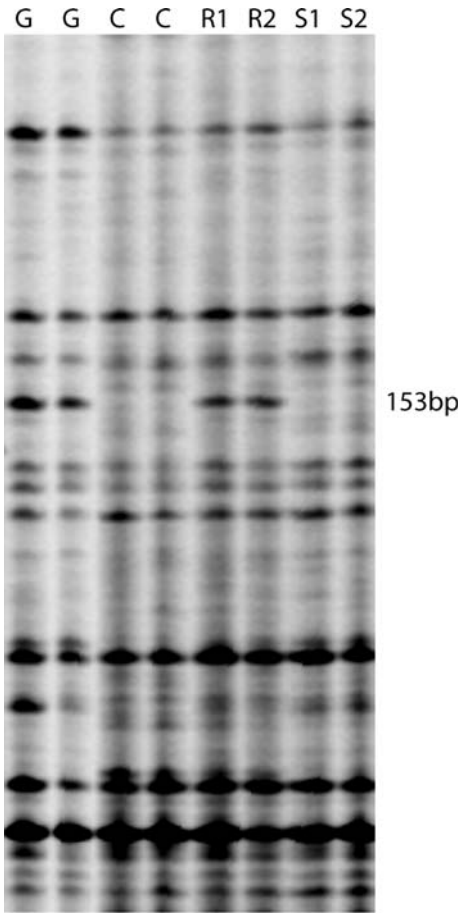

Fig. 4 Gel image of LRR profiling, performed with the *Alu*I restriction enzyme and the NLRR reverse primer in a bulked segregant analysis of the T06-010 population. A 153-bp fragment was polymorphic between the race 8 resistant parent 'George Vancouver' (*G*) and the susceptible parent 'Chorale' (*C*) as well as between two independent bulks of resistant seedlings (*R1*, *R2*) and two independent susceptible bulks (*S1*, *S2*)

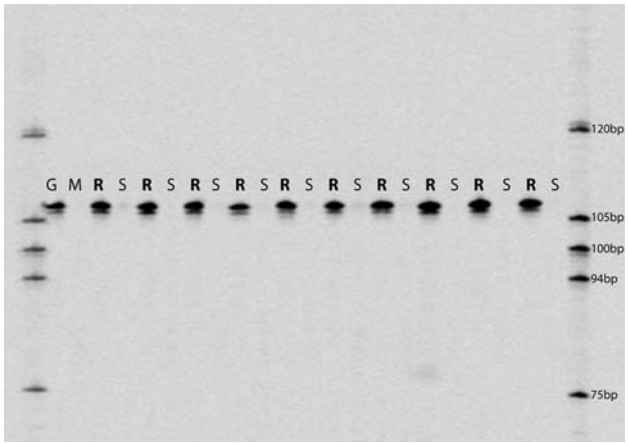

Fig. 5 The ND5E SCAR marker amplifies a 108-bp fragment in the resistant parent 'George Vancouver' (*G*) and resistant seedlings (*R*) of the T06-008 population but does not amplify in the susceptible parent 'Morden Blush' (*M*) or susceptible seedlings (*S*). Larger (~500 bp) fragments amplified from the rose actin gene as positive controls are not shown. Sizing standard ladders are on each side of the gel

gene we describe cannot currently be distinguished from *Rdr1*, since Love and Peace™ is resistant to Dort E4 and race 3 resistance segregates perfectly with a microsatellite marker within the *Rdr1* locus. Therefore, the gene conferring race 3 resistance is either closely linked to, allelic with, or the same as *Rdr1*. More specific conclusions cannot be obtained with the populations currently at our disposal. Regardless, our results confirm that the R gene cluster surrounding the *Rdr1* locus is important for black spot resistance in rose. Analysis with three microsatellite markers in the T06-010 population showed that no marker alleles from the resistant parent 'George Vancouver' segregate with resistance to race 8 of *D. rosae*. Therefore, the gene responsible for resistance to race 8 does not reside within the *Rdr1* R gene cluster but instead occurs at a novel locus which we have named *Rdr3*.

Attempts were made to determine the map position of *Rdr3* by analyzing the ND5ESCAR marker in the 94/1 diploid F_1 mapping population (Yan et al. 2005). However, the marker did not amplify in either parent. Other SCAR primer pairs designed from the ND5E sequence were used to amplify fragments from both parents. These fragments were sequenced and new primers were designed in an attempt to create a polymorphic marker, but without success. In the future, the *R. rugosa* (Kaufmann et al. 2003) or *R. chinensis* (Hess et al. 2007) BAC libraries could be screened with the ND5E fragment in order to derive new markers from BAC end sequence that can be mapped in 94/1.

The discovery of eight new RGA families from 73 cloned RGAs from cultivated roses suggests that there is still untapped RGA diversity in the genus that could be utilized for genomics applications. Indeed, the present RGA analysis allowed the construction of four NBS profiling primers from a previously unutilized motif (GLPL) and with reduced degeneracy based on specificity to the TIR and non-TIR RGAs. A total of seven new primers allowed for 28 new primer/enzyme combinations. Each of the primers produced repeatable polymorphisms among the parents.

Utilizing previously published LRR primers (Chen et al. 1998) with the NBS profiling protocol (Van der Linden et al. 2004) proved to be a straightforward method for generating additional polymorphisms, with eight primers yielding 145 polymorphisms in total. This method was also successfully used to generate a marker for race 8 resistance. However, it is not clear from the present study whether this method preferentially targets the LRR domain of RGAs over other genomic regions. As a first step toward answering this question, an additional 12 LRR profiling

bands were extracted from the gel, from which 22 unique sequences were recovered. Searches using the BLASTn algorithm did not reveal any matches to known LRR-related sequences. Similar searches with translated sequences yielded the same results. Poorer conservation in general within LRR domains compared to NBS regions (Bent 1996) may have decreased the efficacy of these primers in targeting the LRR and/or decreased the efficacy of BLAST searches. Nevertheless, this method was successfully used to identify a genomic region linked to the *Rdr3* resistance gene.

Marker assisted breeding for black spot resistance in roses could now be achieved using the 155 microsatellite marker segregating with the *Rdr1*/race 3 resistance locus (0 cM) and the ND5ESCAR marker segregating with the *Rdr3* locus (9.1 cM). These markers could be used to screen prospective parents as well as select within seedling populations of cultivated roses. This represents a complementary approach to the introgression of genes from wild donor species that avoids the complications of repeated backcrossing and purging of undesirable traits. Further experiments are planned to evaluate the efficacy of these markers for gene pyramiding and to determine their applicability to a wider range of rose germplasm.

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