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ORIGINAL PAPER

Development of a codominant CAPS marker linked to PRSV-P resistance in highland papaya

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Abstract Development of resistant papaya varieties is widely considered the best strategy for long-term control of the papaya ringspot virus type P (PRSV-P). Several species of "highland papaya" from the related genus *Vasconcellea* exhibit complete resistance to PRSV-P, and present a valuable source of resistance genes with potential for application in *Carica papaya*. The objectives of this study were two fold; to identify molecular markers linked to a previously characterised PRSV-P resistance gene in *V. cundinamarcensis* (*psrv-1*), and to develop codominant marker based strategies for reliable selection of PRSV-P resistant genotypes. Using a bulked segregant analysis approach, dominant randomly amplified DNA fingerprint (RAF) markers linked to *prsv-1* were revealed in the resistant DNA bulk, which comprised F2 progeny from a *V. parviflora* (susceptible) × *V. cundinamarcensis* (resistant) inter-specific cross. One marker, Opk4_1r, mapped adjacent to the *prsv-1* locus at 5.4 cM, while a second, Opa11_5r, collocated with it. Sequence characterisation of the Opk4_1r marker permitted its conversion into a codominant CAPS marker (*PsiIk4*), diagnostic for the resistant genotype based on digestion with the restriction endonuclease *Psi*I. This marker mapped within 2 cM of the *prsv-1* locus. Psilk4 was shown to correctly identify resistant genotypes 99% of the time when applied to interspecific F2 progeny segregating for the resistant character, and has potential for application in breeding programs aimed to deliver the PRSV-P resistance gene from *V. cundinamarcensis* into *C. papaya*.

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Introduction

Papaya (*Carica papaya* L.) is a dicotyledonous, herbaceous arborescent plant belonging to the *Caricaceae* family (Badillo 1993). Recently rehabilitated as a separate genus (Badillo 2000), *Vasconcellea* contains 21 of the 35 *Caricaceae* species, which are often referred to as the "highland papaya" (van Droogenbroeck et al. 2002). *Vasconcellea* is considered the most important genus of the *Caricaceae* as a consequence of its size and genetic diversity (van Droogenbroeck et al. 2004), however papaya is the only *Caricaceae* species of worldwide economic importance (Manshardt and Drew 1998).

The *Vasconcellea* species are a valuable source of disease resistances and represent an important resource for the improvement of *C. papaya*. Several species of *Vasconcellea*, namely *V. cauliflora* Jacq., *V. cundinamarcensis* (V.M. Badillo), *V. quercifolia* (St. Hil.), *and V. stipulata* (V.M. Badillo) exhibit heritable,

extreme resistance to the papaya ringspot virus (PRSV-P) (Manshardt and Wenslaff 1989b; Drew et al. 1998). This virus is considered the most significant pathogen of papaya, and losses as high as 70% of expected yield have been reported in effected areas (Manshardt and Drew 1998).

Resistance to PRSV-P has not been identified in papaya to date, and the development of resistant papaya is generally considered the best strategy for long-term virus control (Gonsalves 1998). To date, the only other resistance strategy has been transgenic, and although this approach has been successful it does not meet all the requirements of the different papaya growing regions. To elicit transgenically induced silencing of viral genes, homology between the virus and transgene must be high (>98%) (Gonsalves 1998). Since genetic divergence of the different PRSV-P strains correlate with their geographical distribution (Wang and Yeh 1997), the development of unique transgenes for different papaya growing regions is therefore required. In Australia, PRSV-P isolates have been shown to vary as much as 12% in the CP region (Bateson et al. 1994), which has been used as a transgene in the USA and Australia (Fitch et al. 1992; Lines et al. 2002).

Intergeneric hybridisation of *C. papaya* and resistant *Vasconcellea* species is one mechanism for accessing the wild germplasm. Successful intergeneric hybridisations have been reported for *C. papaya* and at least six *Vasconcellea* species (*V. parviflora* (A. DC.) (Manshardt and Wenslaff 1989b; Drew et al. 1998), *V. cundinamarcensis* (Manshardt and Wenslaff 1989b; Drew et al. 1998), *V. quercifolia* (Manshardt and Wenslaff 1989b; Drew et al. 1998), *V. gouditiana* (Tr. et Pl) (Manshardt and Wenslaff 1989a, b), *V. stipulata* (Manshardt and Wenslaff 1989b) and *V. cauliflora* (Manshardt and Wenslaff 1989b; Magdalita et al. 1997; Drew et al. 1998). Importantly, the PRSV-P resistant character has shown to be inherited by intergeneric progeny of three resistant species; *V. cauliflora* (Manshardt and Wenslaff 1987; Magdalita et al. 1997), *V. cundinamarcensis* (Manshardt and Wenslaff 1987; Drew et al. 1998) and *V. quercifolia* (Drew et al. 1998).

Instabilities such as infertility, abortion of immature embryos and poor hybrid vigour are observed to varying degrees in intergeneric hybrids of *Carica* and *Vasconcellea*. Genetic incompatibility between the distantly related genomes of *Vasconcellea* and *Carica* who's species are described as sexually incompatible has been attributed to this (Sawant 1958; Manshardt and Wenslaff 1989a; Magdalita et al. 1997; Drew et al. 1998; van Droogenbroeck et al. 2002). Hybrids of the PRSV-P resistant species *V. cundinamarcensis*, are highly sterile and *V. cauliflora* and *V. stipulata* produce weak hybrids with poor vigour of field planted trees (Manshardt and Wenslaff 1989a, b; Drew et al. 1998). This presents a significant barrier the development of PRSV-P resistant papaya via hybridisation. Heightened intergeneric compatibility between papaya and *V. parviflora* or *V. quercifolia* (Drew et al. 1998) may be exploited to bridge hybridisation between papaya and the PRSV-P resistant species, since interspecific barriers within *Vasconcellea* are less prominent.

The utility of molecular markers for assisted selection of superior genotypes has been demonstrated in a large number of crop species (Jefferies et al. 2003; Lecouls et al. 2004; Narayanan et al. 2004; Yi et al. 2004). In this study a bulked segregant analysis (BSA) approach was applied to reveal dominant DNA markers closely linked to a PRSV-P resistance gene in *V. cundinamarcensis.* Resistance conferred by *V. cundinamarcensis* was recently shown to be regulated by a single dominant gene (Dillon et al. 2005). As a result of its simple inheritance, markers linked to this gene will be ideal for marker assisted selection (MAS). A codominant cleavage amplified polymorphic sequence (CAPS) marker diagnostic for PRSV-P resistance was developed and shown to select resistant genotypes with 99% efficiency. Applied to hybrids of *V. cundinamarcensis* this marker has significant potential to enhance efficiency of artificial selection by reducing the duration of breeding cycles, and increasing the genetic gain per cycle.

Materials and methods

Plant materials

Resistant and susceptible DNA bulks, constructed from 18 and 14 individuals respectively, were used for BSA. Bulk materials were selected from a interspecific population of 246 F2 hybrids of *V. parviflora* and *V. cundinamarcensis* which had previously been evaluated for resistance and susceptibility (Dillon et al. 2005). Ninety-seven F2 individuals from the same population formed the basis of the mapping pedigree used in this study. This population was previously described by Dillon et al. (2005).

Bulked segregant analysis

Total genomic DNA was extracted from fresh or frozen leaves (−80°C). Tissue was ground in Cetyltrimethlyammonium bromide (CTAB) buffer at room temperature in a 1:10 ratio with 1% N-lauryl sarkosine and 1% polyvinylpyrrolidone (PVP) according to Delaporta

et al. (1983) with minor modifications. DNA quality and quantity was determined by agarose gel electrophoresis. Resistant and susceptible bulks were composed of pooled DNA from 18 and 14 F2 plants respectively such that the final concentration of DNA for each bulk was 25 ng/μl. Twenty-five (nanogram of genomic DNA was applied in randomly amplified DNA fingerprint (RAF) labelling reactions with α-p^{33}dATP (Easytides) as described by Waldron et al. (2002). Seventy decamer primers (Operon Technologies; kits A, B, H and primers 1–10 of kit K) were used in RAF-PCR with each bulk in duplicate (Table 1). Amplified fragments were resolved by electrophoresis on 4% polyacrylamide gels in 1× TBE buffer using a sequencing gel apparatus (Bio Rad). Labelled PCR products were visualised by exposing the dried gel to Kodak MR film for 16 h. RAF profiles were visually scored for polymorphisms between the bulks. Primers yielding polymorphic markers were applied using the same method to the resistant and susceptible bulk individuals. Polymorphic markers that were present in 100% of resistant bulk individuals were considered candidates and subsequently screened in the mapping population.

Linkage analysis of candidates

Candidates revealed by BSA were mapped into an existing linkage map of *V. cundimarcensis*, on which a putative PRSV-P resistance gene (*prsv-1*) locus had been previously assigned (Dillon et al. 2005). Dominant RAF markers were scored according to two genomic classes, presence (1) or absence (0). Co-dominant CAPS markers were scored according to genotype, homozygous (0 or 1) and heterozygous (2). Marker segregation in the F2 was tested for goodness of fit to the expected segregation ratios by χ^2 analysis. A χ^2 test statistic was calculated for each marker in comparison with the expected ratios (1:1 or 1:3) at the critical value for $P < 0.05$, 0.01 and 0.005. Segregation data was converted to the appropriate genotype codes for use with the Mapmaker program (version 3.0b, Lander et al. 1987). Groups were defined at LOD 3.5 following the 'sequence all' and 'group' commands with maximum recombination restricted to 40%. Marker orders within groups were determined at the same LOD threshold using the 'sequence' and 'compare' commands on a partial set of markers (<5), additional markers were positioned into this sequence using the 'try' command according to the 'maximum likelihood' value for each potential position. Final marker orders were confirmed using the 'ripple' test. Genomic map distances were calculated in centimorgans (Read et al. 2003) using the Kosambi correction. Maps were drawn using MapChart (Voorrips 2002).

Table 1 Operon decamer primer sequences

Primer	5′–3′ Sequence	Primer	5′–3′ Sequence
OPA01	CAGGCCCTTC	OPB01	GTTTCGCTCC
OPA02	TGCCGAGCTG	OPB02	TGATCCCTGG
OPA03	AGTCAGCCAC	OPB03	CATCCCCCTG
OPA04	AATCGGGCTG	OPB04	GGACTGGAGT
OPA05	AGGGGTCTTG	OPB05	TGCGCCCTTC
OPA06	GGTCCCTGAC	OPB06	TGCTCTGCCC
OPA07	GAAACGGGTG	OPB07	GGTGACGCAG
OPA08	GTGACGTAGG	OPB08	GTCCACACGG
OPA09	GGGTAACGCC	OPB09	TGGGGGACTC
OPA10	GTGATCGCAG	OPB10	CTGCTGGGAC
OPA11	CAATCGCCGT	OPB11	GTAGACCCGT
OPA12	TCGGCGATAG	OPB12	CCTTGACGCA
OPA13	CAGCACCCAC	OPB13	TTCCCCCGCT
OPA14	TCTGTGCTGG	OPB14	TCCGCTCTGG
OPA15	TTCCGAACCC	OPB15	GGAGGGTGTT
OPA16	AGCCAGCGAA	OPB16	TTTGCCCGGA
OPA17	GACCGCTTGT	OPB17	AGGGAACGAG
OPA18	AGGTGACCGT	OPB18	CCACAGCAGT
OPA19	CAAACGTCGG	OPB19	ACCCCCGAAG
OPA20	GTTGCGATCC	OPB20	GGACCCTTAC
OPH01	GGTCGGAGAA	OPK01	CATTCGAGCC
OPH02	TCGGACGTGA	OPK02	GTCTCCGCAA
OPH03	AGACGTCCAC	OPK03	CCAGCTTAGG
OPH04	GGAAGTCGCC	OPK04	CCGCCCAAAC
OPH05	AGTCGTCCCC	OPK05	TCTGTCGAGG
OPH06	ACGCATCGCA	OPK06	CACCTTTCCC
OPH07	CTGCATCGTG	OPK07	AGCGAGCAAG
OPH08	GAAACACCCC	OPK08	GAACACTGGG
OPH09	TGTAGCTGGG	OPK09	CCCTACCGAC
OPH10	CCTACGTCAG	OPK10	GTGCAACGTG
OPH11	CTTCCGCAGT	–	–
OPH12	ACGCGCATGT	–	–
OPH13	GACGCCACAC	–	–
OPH14	ACCAGGTTGG	–	–
OPH15	AATGGCGCAG	–	–
OPH16	TCTCAGCTGG	–	–
OPH17	CACTCTCCTC	–	–
OPH18	GAATCGGCCA	–	–
OPH19	CTGACCAGCC	–	–
OPH20	GGGAGACATC	–	–

Sequence characterised amplified region (SCAR) design and analysis

Two candidates, Opk4_1r and Opa11_5r, were converted to SCAR markers. These markers were tightly linked to prsv-1, were >200 bp in length and could be more clearly resolved than the other BSA candidates. RAF markers were isolated from the dried acrylamide gel by overlaying the developed film and excising the desired band by cutting through the film. The excised fragment was re-hydrated in a 1.5 ml centrifuge tube containing 0.2 ml of TE buffer (10 mM Tris, 1 mM EDTA) at 4°C overnight. The hydrated gel was separated from 3 mm filter paper and homogenised in the tube using a tapered plastic pestle. The mixture was

centrifuged to pellet debris and 1 μl of the supernatant used as template in PCR using the original decamer primers OPA11 and OPK04 and RAF-PCR conditions, omitting α-p^{33}dATP. Purified PCR products were cloned into pGEM-T Easy vector (Progen) following the manufacturers instructions, and sequenced using BigDye version 3.1 sequencing system (Applied Biosystems).

Sequences with homology to candidate markers were identified using the Basic Local Alignment Tool (BLAST; Altschul et al. 1990) supported on the National Centre for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov). Searches for regions of local similarity to nucleotide and protein sequences were initiated using the BLASTn and BLASTx tools respectively using default settings. Primer sets were designed, one for each marker, based on the sequenced fragment ends. Both forward and reverse primers incorporated the original decamer primer sequence plus an additional 10 or 11 bp downstream (Table 2).

SCAR primer sets were applied to genomic DNA of eight *Caricaceae* species: *V. cundinamarcensis, V. parviflora, V. cauliflora, V. quercifolia, V. gouditiana, V. stipulata* and *C. papaya* (cultivar 2.001). PCR reactions were prepared in 20 μl (25 ng genomic template or 5 μl of plasmid DNA (1/500), 1 U of Taq DNA polymerase (Fermentas), 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM forward and reverse primer). Thermal cycling was conducted at 94°C for 2 min, (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) × 25 cycles, followed by 72°C for 5 min. PCR products were resolved on 1.2% agarose gels in 1× TAE buffer, against 1 kb DNA molecular weight standard (Invitrogen) and visualised by ethidium bromide (EtBr) staining. Amplified fragments were gel purified using a QIAquick PCR Purification Kit (Qiagen), and sequenced using BigDye version 3.1 sequencing system (Applied Biosystems), according to the manufacturers instructions. The Opk4_1r SCAR from *V. cundinamarcensis* and *V. stipulata* was cloned into pGEM-T Easy vector (Progen), following the manufacturers instructions, prior to sequencing because the PCR product contained multiple sequences.

Table 2 SCAR primer sequences

Primer	Candidate marker	5′–3′ Sequence
PBK41R F	Opk4_1r	CCGCCCAAACTGCGGAACAC
PBK41R R	Opk4_1r	CCGCCCAAACCCCCAACTAG
PBA115R F	Opa11_5r	CAATCGCCGTAGGAAAATTC
PBA115R R	Opa11_5r	CAATCGCCGTAGAGGAGGAGG

SCAR allele sequences were aligned by ClustalW using MEGA version 3.0 (Kumar et al. 2004) and default gap opening and gap extension penalties. Separate alignments were constructed for the Opa11_5r and Opk4_1r sequences. Shaded multiple sequence alignment outputs were generated using BoxShade version 3.21 (Hofmann and Baron 2005).

Southern blotting

Digested genomic DNA of *V. cundinamarcensis*, *V. parviflora* and *C. papaya* was probed with ^{32}P labelled Opk4_1r via southern blotting. Genomic DNA for each species was extracted from 0.5 g of fresh tissue using a 2% CTAB buffer in a 1:10 ratio with 1% N-lauryl sarkosine (sarkosyl) and 1% PVP following the method of Delaporta et al. (1983). Twenty-five nanogram of Opk4_1r SCAR PCR product and 0.1 ng of 1 kb molecular weight standard (Invitrogen) was labelled with α^{32}P dCTP (PerkinElmer) using the Rediprime II DNA Labelling System (Amersham Biosciences) according the manufacturers instructions. Ten micro grams of genomic DNA was digested in 400 μl total volume with *Hin*dIII (New England Biolabs) for each species according to the manufacturers instructions. Restriction mapping using the ANGIS bioinformatics package indicated that the consensus site for this enzyme [A/AGCTT] was not present in the probe sequence. Digested DNA was precipitated with 20 μg glycogen (Fermentas), 2.5 × volume of 100% ethanol and 0.1 × volume of 0.3 M NaOAc for 3 h at −20°C, and centrifuged for 15 min at 4°C. Pelleted DNA was aspirated and dried at 55°C for 5 min. DNA was resuspended in 50 μl of TE buffer. To check completion of the digestion, 500 ng (2.5 μl) of sample was electrophoresed on a 0.7% agarose gel in 1× TAE buffer at 100 V for 1 h. Separated DNA was transferred to Hybond N+ membrane (Amersham Biosciences), dried and hybridised with radio labelled probe according to the method of Maniatis et al. (1982). The hybridised blot was checked with a Geiger counter and bands visualised using the FLA5000 phosphorimaging system (Fugifilm)

CAPS marker development

Codominant cleavage amplified polymorphic sequence (CAPS) markers diagnostic for the Opa11_5r and Opk4_1r *V. cundinamarcensis* SCAR allele in hybrids of *V. cundinamarcensis* and *V. parviflora* were developed. Comparative restriction mapping of the Opk4_1r and Opa11_5r SCAR from *V. cundinamarcensis* and *V. parviflora*, using the ANGIS bioinformatics package,

identified restriction enzymes with a cut site that was polymorphic in this region. Restriction digests of the Opk4_1r and Opa11_5r SCARs were conducted in 50 µl with enzymes *Psi*I and *Sna*BI (New England Biolabs) respectively for *V. cundinamarcensis*, *V. parviflora*, the resistant and susceptible bulk individuals, and 97 F2 hybrid individuals from the mapping population according to the manufacturers instructions. Twenty-five microlitre of Digest were resolved on 2% agarose gels in TAE buffer for 15 min against a 1 kb DNA molecular weight standard (Invitrogen), and visualised by ethidium bromide (EtBr) staining.

Results

Bulked segregant analysis

Approximately 4,200 dominant RAF markers were assessed in the resistant and susceptible DNA bulks. This figure was based on an estimated 50 markers amplified per primer, for 70 primers. Analysis of the bulk profiles for each primer revealed 12 discrete polymorphisms unique to the resistant bulk (Table 3). No polymorphic markers were shown to originate from the susceptible bulk. Notably, two or more markers were identified in the bulk profiles for four of the six primers that revealed polymorphisms between the bulks (Table 3).

When screened over the resistant and susceptible bulk individuals, the markers presented in Table 3 were shown to be exclusive to the resistant bulk. Markers Opa11_5R, Opa11_6R, Opk4_1r, Opk4_2R and Opb8_1R were detected in 100% of the resistant individuals (Fig. 1) indicating putative linkage to the resistant phenotype. The remaining markers segregated across the bulks and their linkage with the resistant phenotype was assumed to be weaker. Subsequent screening of the candidate RAF markers in the parents, F1, *V. cauliflora*, *V. quercifolia*, *V. gouditiana*, *V.* stipulata and *C. papaya* revealed that polymorphisms generated by primers OPK04 and OPB08 were generally specific to *V. cundinamarcensis*. However, a homologous marker was shown to co-migrate in *V. cauliflora* with the Opa11_5r marker from *V. cundinamarcensis* (Fig. 1).

Linkage mapping

All candidate RAF markers (Opa11_5R, Opa11_6R, Opk4_1r, Opk4_2R and Opb8_1R) mapped to the same linkage group as the previously characterised PRSV-P resistance gene locus (*prsv-1*) in *V. cundinamarcensis.* Four markers, Opa11_6r, Opk4_1r, Opk4_2r and Opb8_1r flanked *prsv-1* on LG7 at distances of 2.1, 5.4, 9.7 and 12.0 cM, respectively (Fig. 2a). Opa11_5r co-located with *prsv-1*. Generally, these markers mapped closer to *prsv-1* than the randomly generated markers which flanked this site in the original map (Dillon et al. 2005). Analysis of segregation ratios for these markers showed that inheritance adhered to normal Mendelian genetics at $P < 0.05$ (Table 4)

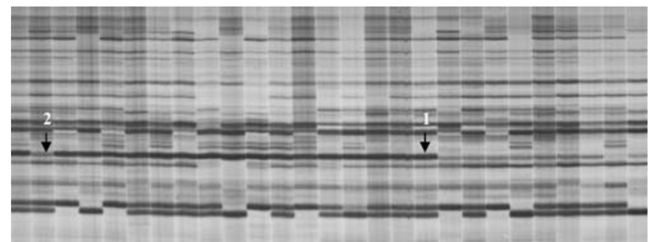

Fig. 1 RAF amplification products for primer OPK04 in *V. cundinamarcensis* (P1), *V. parviflora* (P2) and 16 resistant (R) and 14 susceptible (S) bulk individuals. Marker Opk4_1r is indicated (*1*). A faint fragment in the *V. parviflora* profile co-migrated with Opk4_1r in the hybrids (*2*)

Table 3 Markers identified as specific for the PRSV-P resistant DNA bulk

Primer	Marker	Specific for bulk	Fraction of bulk genotypes carrying marker	
			Resistant	Susceptible
OPA02	Opa2_1r	Resistant	0.28	0
	Opa2_2r	Resistant	0.38	0
OPA08	Opa8_1r	Resistant	0.31	0
OPA11	Opa11_5r	Resistant	1.0	0
	Opa11_6r	Resistant	1.0	0
OPA16	Opa16_1r	Resistant	–	0
OPB06	Opb6_1r	Resistant	0.29	0
OPB08	Opb8_1r	Resistant	1.0	0
	Opb8_2r	Resistant	0.31	0
	Opb8_3r	Resistant	0.31	0
OPK04	Opk4_1r	Resistant	1.0	0
	Opk4_2r	Resistant	1.0	0

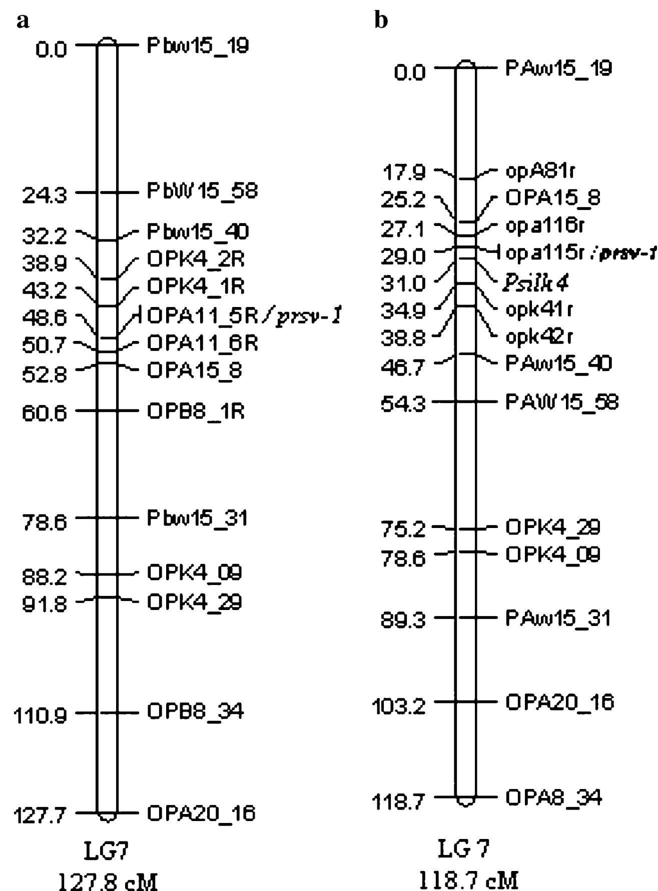

Fig. 2 **a**–**b** Genetic linage maps of *V. cundinamarcensis* chromosome 7 with RAF markers identified in the PRSV-P resistant bulk before (**a**) and after (**b**) mapping the codominant marker *PsiIk4*

Table 4 Segregation statistics for *prsv-1* and markers identified in the resistant bulk in 100 F2 progeny

Locus	Marker	Fraction in 100 F2 progeny					χ2 test statistic	Fits ratio (at $P < 0.05$)
		D	B	RR	Rr	rr		
prsv-1	Phenotypic	0.74	0.26	–	–	–	0.053	3:1
PsiIk4	Codominant	–	–	0.14	0.27	0.59	6.62	1:2:1[a]
Opa11_5r	Dominant	0.73	0.27	–	–	–	0.213	3:1
Opa11_6r	Dominant	0.75	0.25	–	–	–	0	3:1
Opk4_1r	Dominant	0.72	0.28	–	–	–	0.48	3:1
Opk4_2r	Dominant	0.70	0.30	–	–	–	1.33	3:1
Opb8_1r	Dominant	0.75	0.25	–	–	–	0	3:1
Opb8_2r	Dominant	0.01	0.99	–	–	–	0.01	0:1
Opb8_3r	Dominant	0.01	0.99	–	–	–	0.01	0:1
Opa15-8	Dominant	0.76	0.24	–	–	–	0.06	3:1
Pbw15-40	Dominant	0.76	0.24	–	–	–	0.03	3:1

Critical value for χ^2 test at $P < 0.05 = 3.84$

D present, *B* absent, *RR* homozygous for *V. cundinamarcensis* allele (B), *Rr* heterozygote (H), *rr* homozygous for *V. parviflora* allele (A)

[a] χ^2 test statistic was within the critical value (6.64) at the $P < 0.01$ level of significance

SCAR analysis

DNA sequencing of two candidate RAF markers permitted the development of primers specific for each SCAR, and amplification of the Opk4_1r (348 bp) and Opa11_5r (257 bp) SCARs from *V. cundinamarcensis* genomic DNA using standard PCR conditions. A search of the Genbank database using BLASTx (Altschul et al. 1990) revealed that the Opk4_1r and Opa11_5r SCARs were homologous to a small nuclear ribonuclear protein (snRNP) and a serine threonine protein kinase, respectively. The top hits for the

snRNP (CAA90282) and serine threonine protein kinase (BAD72247) were 86 and 96% homologous, respectively. Sequencing of PCR products amplified from genomic template of five additional *Vasconcellea* species (Table 5), the F1 and F2 interspecific hybrid progeny and *C. papaya* revealed that both SCARs were present in all the wild species, and in the case of Opa11-5r in *C. papaya* (Fig. 3). The Opk4_1r SCAR specific primer set did not detect a homologous region in *C. papaya* (cultivar 2.001), indicating that this region may be absent in this cultivar or that homology between the primer and primer binding site was low. A Southern blot of *C. papaya* genomic DNA, using Opk4_1r as a probe, indicated that a single copy of this region was present *in C. papaya* cultivar 2.001 (Fig. 4). Furthermore, single and duplicate homologous copies of the Opk4_1r SCAR were shown to be present in *V. parviflora* and *V. cundinamarcensis* respectively in the same blot.

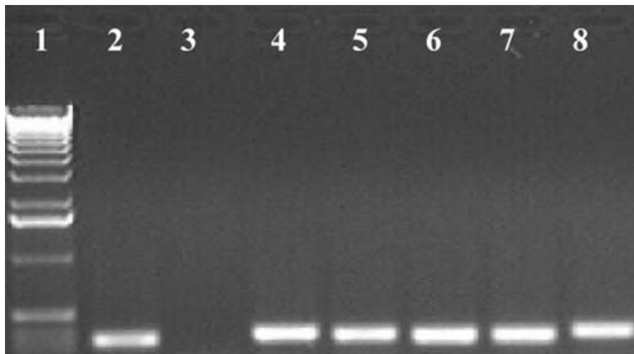

Fig. 3 Opk4_1r SCAR amplified from genomic DNA of 1 kb DNA marker (*1*), *V. cundinamarcensis* (*2*), *C. papaya* (*3*), *parviflora* (*4*), *stipulata* (*5*), *cauliflora* (*6*), *quercifolia* (*7*) and *gouditiana* (*8*)

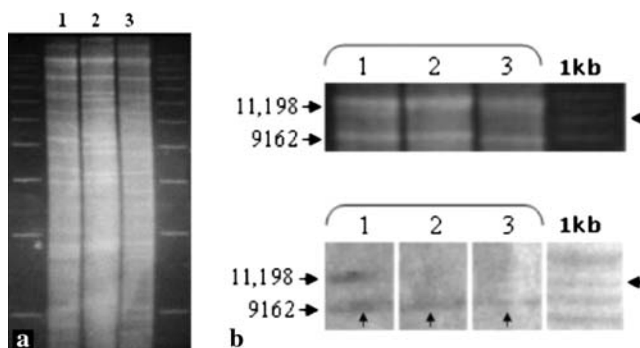

Fig. 4 **a–b** *Hin*dIII digest of *V. cundinamarcensis* (*1*), *V. parviflora* (*2*) and *C. papaya* (*3*) resolved on 0.7% agarose against 1 kb DNA standard (**a**) and result of southern hybridisation of ^{32}P labelled Opk4_1r probe to the *Hin*dIII blot for *V. cundinamarcensis* (*1*), *V. parviflora* (*2*) and *C. papaya* (*3*) (**b**). The probe hybridised to all three templates at 9,162 bp. A unique band appeared in *V. cundinamarcensis* at 11,198 bp

Table 5 Detection of SCARs in *Vasconcellea* species and *papaya*

Species	SCAR		Fragment size (bp)	
	Opk4_1r	Opa11_5r	Opk4_1r	Opa11_5r
V. cundinamarcensis	Yes[a]	Yes	360, 348	257
V. parviflora	Yes	Yes	379	257
V. quercifolia	Yes	Yes	372	257
V. cauliflora	Yes	Yes	360	257
V. stipulata	Yes[a]	Yes	379, 371	255
V. gouditiana	Yes	Yes	361	257
C. papaya	Yes	No	–	261

[a] Two unique alleles of slightly different sizes were identified in these species

DNA sequencing of the Opa11_4r and Opa11_5r PCR products from the different species and hybrids confirmed that the expected SCAR had been amplified in each case. Variation in size of the amplified products for the different species was initially apparent from the differential migration of PCR products during gel electrophoresis. Comparison of the SCAR sequences across the seven species revealed single nucleotide polymorphisms (SNPs) and indels, which resulted in variable fragment lengths (Table 5). This included a simple sequence repeat [AAG] of variable length in the Opk4_1r SCAR. The frequencies of SNP and indel polymorphisms based on the SCAR consensus sequence were 10 and 1% for Opk4_1, and 9 and 2.3% in Opa11_5r, respectively. SNP distribution along both SCAR sequences (data not shown) revealed that all SNPs with allele frequency >10%, lay within predicted non-coding DNA sequences for the snRNP and Ser/Thr Kinase, consistent with maintenance of integrity of coding regions compared to non-coding regions. In both *V. cundinamarcensis* and *V. stipulata* cloning of the Opk4_1r PCR product was required, which revealed discrete alleles with unique sequences in both species (Table 5). In *V. cundinamarcensis* the alleles varied by a 10 bp indel near the 3′ end of the SCAR, and in *V. stipulata* from variable length of the AAG repeat. For the other species and primer combinations only one SCAR allele was detected.

CAPS marker development

A SNP was identified in the Opk4_1r SCAR between *V. cundinamarcensis* and *V. parviflora*, which permitted digestion by the restriction enzyme *Psi*I [5′T(T/C)A/TAA3′] 109 bp in from the 5′ end of both *V. cundinamarcensis* alleles, but not in *parviflora*. Digestion of the Opk4_1r SCAR *V. cundinamarcensis* and *V. parviflora* afforded unique restriction profiles when resolved on agarose gels (Fig. 5). In *V. cundinamarcensis*, two bands were produced, one of 109 bp and

Table 6 Relative accuracy of markers for PRSV-P resistant genotypes

Marker	Fraction correctly predicted in F2 progeny	Accuracy for phenotype prediction (%)	Agreement between markers (%)
PsiIk4	96/97	99	96
Opk4_1r	92/97	95	
SnabIa11			
Opa11_5r	97/97	100	

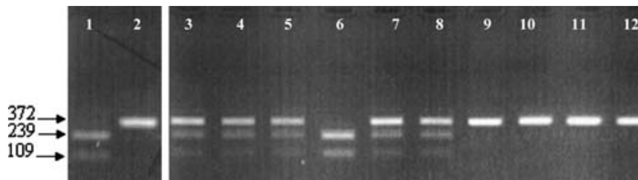

Fig. 5 *PsiIk4* CAPS marker tested in *V. cundinamarcensis* (*1*), *V. parviflora* (*2*) and known resistant [lanes *3–8* in order: L12-12 (H), L11-14 (H), L8-06 (H), L6-05 (B), L5-06 (H) and L2-02 (H)] and susceptible [lanes *9–12* in order: L15-07 (A), L17-05 (A), L5-04 (A) and L17-27(A)] F2 interspecific progeny of these species

second consisting of a mixture of two fragments, 241 and 253 bp, resulting from the alternate Opk4_1r alleles. A single band, of 372 bp, was obtained for *V. parviflora*. The Cleavage Amplified Polymorphic Sequence (CAPS) marker, named *PsiIk4*, was codominant when applied in hybrids of these two species and permitted diagnosis of homozygous PRSV-P resistant individuals. The same strategy was applied to the Opa11_5r SCAR using restriction enzyme *Sna*BI [5′TAC/GTA3′] however a smeared profile was obtained which was unsuitable for genotyping. The reason for this particular digestion pattern was not determined.

Linkage analysis of the codominant marker in the mapping population linked Psilk4 to *prsv-1* by 2 cM on linkage group 7 in the *V. cundinamarcensis* genetic map (Fig. 2b). In the mapping population the accuracy of the Psilk4 CAPS marker for predicting PRSV-P resistant genotypes was high. Resistant and susceptible phenotypes correlated with marker genotypes 99% of the time (Table 6). The χ^2 test statistic for segregation at $P < 0.01$ indicated that segregation for *PsiIk4* was close to 1:2:1 consistent with a codominant marker (Table 4).

Discussion

Experimental design

F2 populations are demonstrated to be more informative than back cross populations of similar size when conducting BSA using dominant marker systems. This arises since twice the number of markers will be expected to segregate in the F2 (Mackay and Caligari 2000). In addition, the probability of generating false positive marker in bulks of F2 individuals is also considerably less. For bulks of 10, the probability of identifying a false positive when screening a single marker is 0 and 0.3% respectively for F2 and BC populations. When larger numbers of markers are screened (~1,600), such as in this study, the probability increases to 96% for a BC population, while the probability of a false positive using an F2 population is 0.3% (Michelmore et al. 1991). To reduce the risk of identifying false positives a F2 population was employed in this study for BSA.

The power of BSA has been shown to depend on the number of markers screened as well as the size (no. individuals) of the DNA bulk. The probability of identifying a marker linked to the target gene within 1 cM, for bulks of between 10 and 20 individuals for ≥1,600 markers, has been calculated as close to 1.0 (Michelmore et al. 1991). In this study >4,000 markers were screened. Optimal bulk size is dependant on the type of population and the number of markers screened (Michelmore et al. 1991). For a dominant marker segregating in an F2 population, the probability of a bulk of n individuals revealing a marker locus which is unlinked to the target gene has been given as $2(1 - [1/4]^n)(1/4)^n$ (Michelmore et al. 1991). The probability of bulks of 14 and 18 individuals, used in this study, to reveal a false positive marker is between 7.4×10^{-9} and 2.9×10^{-11}. Therefore it is unlikely that polymorphic markers identified in the resistant bulk would represent spurious linkages.

Marker segregation and SCAR analysis

RAF has previously been reported to identify codominant simple sequence repeat (SSR) markers (Peace et al. 2003; Waldron et al. 2002). Despite this, all markers revealed by RAF in this study exhibited dominant inheritance.

All candidate RAF markers shown to be linked to *prsv-1* (Opa11_5r, Opa11_6r, Opk4_1r, Opk4_2r and

Opb8_1r) exhibited normal segregation ratios when screened over 97 F2 individuals in the mapping population (Table 4). This indicates that the *prsv-1* region on LG7 is relatively stable, and therefore genetic distances for the candidate markers in this region are not likely to be significantly distorted. The 1:2:1 segregation for the codominant marker Psilk4 at $P < 0.05$ also indicates normal Mendelian inheritance at this locus.

The specific polymorphism detected by RAF between *V. cundinamarcensis* and *V. parviflora* for each candidate could not be identified by DNA sequencing, since SCAR fragments amplified in *V. parviflora* incorporated the *V. cundinamarcensis* SCAR primer sequence. However, a SNP within one of the decamer primer binding sites could have prevented amplification of this region in *V. parviflora* using the RAF technique.

The faint band which was observed to co-migrate with Opk4_1r in *V. parviflora* and susceptible F2 progeny was assumed to be distinct from Opk4_1r. Varying levels of marker intensity were not observed for any non-polymorphic bands, which all appeared homogeneous in intensity in the parental profiles and progeny. Although not attempted in our study this could be confirmed by DNA sequencing,

The result of the southern blot indicated at least two regions of the genome with homology to Opk4_1r in *V. cundinamarcensis* (Fig. 4b). This region may not have been linked to prsv-1 in this species but verification of this was not followed. However, because psilk4 accurately detected resistant genotypes 99% of the time it is unlikely that the RAF PCR based marker described inheritance for more than one loci.

Genetic mapping

Little variation was observed in the distances between candidate RAF markers and *prsv-1* following addition of the codominant marker *PsiIk4* to linkage group 7 of the *V. cundinamarcensis* map. This can be attributed to the increasing saturation of markers in the region surrounding *prsv-1*, improving accuracy in estimations of genetic distance at this locus. Other less closely linked markers on the other hand exhibited more noticeable variations in both their position in the map and genetic distance from *prsv-1* when the candidate RAF markers and codominant marker were added to the existing map. For example, map distances with respect to *prsv-1* for markers pbw15_40 and Opa15_8 which had previously mapped close to this locus (Dillon et al. 2005) were inflated from 4 to 16.4 and 2.8 to 4.2 cM in each case. All markers consistently mapped to the same group. Mapping codominant markers in both parents of the mapping cross may permit merger of homologous parental linkage groups and construction of a single hybrid map. Comparison of homologous groups may also provide insight into chromosomal evolution during speciation.

Potential for application of CAPS to breeding

One source of heritable resistance to PRSV-P identified in *V. cundinamarcensis*, or “highland” papaya, appears to be controlled by a single locus (*prsv-1*). This has recently been demonstrated using conventional genetic analysis and molecular genetic mapping of resistance to an Australian strain of PRSV-P in *V. cundinamarcensis* (Dillon et al. 2005). Earlier studies of inheritance of an Australian strain of PRSV-P resistance in F1 intergeneric hybrids of *C. papaya* and *V. cauliflora* also suggested single gene regulation in this species (Magdalita et al. 1997). In this study the identification of several markers, via BSA, which map closely to the resistance gene locus (*prsv-1*), supports the concept that PRSV-P resistance in *V. cundinamarcensis* is a single gene trait.

Markers linked to *prsv-1* in *V. cundinamarcensis* are ideally suited to application in marker assisted breeding programs because of the dominant inheritance of this locus, and because resistance to the Australian strain of PRSV-P imparted by this gene is robust (Magdalita et al. 1997; Drew et al. 1998). The codominant CAPS marker Psilk4 develop in his study permits reliable detection of the PRSV-P resistant allele in hybrids of *V. cundinamarcensis* and *V. parviflora.* As a result this marker is a potentially powerful tool for assisted selection of homozygous resistant genotypes which may be applied in breeding programs to facilitate delivery of PRSV-P resistance from *V. cundinamarcensis* into *C. papaya.* Conversion of the Opa11_5r RAF marker into a CAPS marker would have been appealing since this marker collocated with the *prsv-1* locus on the genetic linkage map. Failure of the digested SCAR fragment to resolve clearly prevented its successful application in this study. Additional SNPs were identified between *V. parviflora* and *V. cundinamarcensis* at the Opa11_5r locus, thus alternative methods such as single nucleotide primer extension (SNuPE) or similar could be applied to genotype the F2 progeny. Further assessment of this marker and Opk4_1r in F3 and F4 populations would indicate whether the linkages to *prsv-1* are truly “tight”, and will indicate the markers reliability for breeding if linkages are not broken by generational recombination.

Direct hybridisation between resistant *Vasconcellea* species (*V. cundinamarcensis*, *V. cauliflora* and

V. quercifolia) and *C. papaya* have been reported (Manshardt and Wenslaff 1989a, b; Magdalita et al. 1997; Drew et al. 1998). However, instabilities such as infertility, abortion of immature embryos and poor hybrid vigour are observed to varying degrees in the progeny (Sawant 1958; Horovitz and Jimenez 1967; Manshardt and Wenslaff 1989a, b; Drew et al. 1998), and have impeded successful transfer of resistance genes to date. This has been attributed to Genetic incompatibility between the distantly related genomes of *Vasconcellea* and *Carica*, who's species are described as sexually incompatible (Sawant 1958; Manshardt and Wenslaff 1989a, b; Magdalita et al. 1997; Drew et al. 1998; van Droogenbroeck et al. 2002). These omnipotent genetic barriers need to be addressed before *V. cundinamarcensis* can be utilised in breeding with *C. papaya*. Some *Vasconcellea* species, such as *V. parviflora*, exhibit heightened levels of inter-fertility in interspecific crossings with *papaya* (Drew et al. 1998). Progeny of these crosses are vigorous and exhibit some fertility (Drew et al. 1998). Thus, homozygous resistant hybrids of *V. cundinamarcensis* and *V. parviflora* may provide a possible bridge to mediate stable transfer of resistance genes from *V. cundinamarcensis* into *C. papaya* using the Psilk4 marker to assist the selection process at each cycle.

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