Developing DNA Markers for a Wild Source of Resistance to Papaya Ringspot Virus

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Abstract

Production of papaya (*Carica papaya*) is limited throughout its growing regions by the devastating disease of Papaya Ringspot Virus type P (PRSV-P). Papaya breeding programmes for PRSV-P resistance target two sources of resistance from crop wild relatives. The resistance loci from *V. pubescens* were identified in a segregating population resulting from an interspecific cross between *V. pubescens* and *V. parviflora*. The resistance loci in *V. pubescens* were located on supercontig (SC) 28 of the papaya whole genome sequence although SC28 is not yet anchored to any linkage group (LG). Recent investigations indicate that SC28 could be located at the end of LG5. Another species *V. quercifolia* also carries PRSV-P resistance, although more quantitative in action, and this species produces some fertile hybrids when crossed to *C. papaya*. The aim of this study was to target polymorphism at the end of LG5 and on SC28 in *C. papaya* and *V. quercifolia*. Five candidate resistant genes were used, corresponding to three nucleotide binding sites (NBS; NBS9.305, NBS9.308 and NBS18.235) at the end of LG5 and one NBS (NBS28.12) and one kinase gene (STK105) on SC28. Primers were designed for each gene and used to amplify DNA of papaya variety '2.001' and an accession of *V. quercifolia*. Resultant DNA sequences were aligned by BLASTN to the papaya whole genome sequence, indicating 98-100% and 89-99% identities for the '2.001' and *V. quercifolia* amplicons, respectively, and 82-93% identity between these two species.

INTRODUCTION

Papaya Ringspot Virus type P (PRSV-P) has been the most destructive disease of papaya production worldwide. To date, no resistance to the virus has been found in *Carica papaya*. The methods to control the disease include transgenic plants and conventional breeding to develop virus resistant genotypes. However, the use of transgenic plants in many countries is limited to specific strains of virus (Tennant et al., 1994). Development of resistant varieties is likely to be a long term method to prevent and control the disease for papaya production.

Natural sources of resistance to PRSV-P have been found in crop wild relatives. Two sources of resistance, from *Vasconcellea pubescens* and *V. quercifolia*, have been used in papaya breeding programs for PRSV-P resistance. The former species, *V. pubescens*, is immune to PRSV-P (Badillo, 1993, 2001), however, it does not produce fertile hybrids when crossed with papaya (Drew et al., 1998). Thus, *V. parviflora* was introduced to the breeding programme as a bridging species to introgress resistance genes to papaya (Drew et al., 1998) and to study resistant genes controlling PRSV-P in *V. pubescens* (Dillon et al., 2005, 2006).

DNA markers linked to PRSV-P resistance alleles were identified in progeny of an interspecific cross between *V. pubescens* × *V. parviflora*. One of the markers, Opa11 5r, revealed 100% linkage to the *prsv-1* locus controlling PRSV-P resistance in *V. pubescens*. DNA sequence analysis of the marker showed similarity to a protein kinase gene (Dillon et al., 2006). One year later, the papaya genome was successfully sequenced, analysed and published by Chen et al. (2007). The sequences of alleles linked to *prsv-1* locus have been

investigated further and found to be located on supercontig (SC) 28, which has not yet been located on any linkage group (LG) of the papaya genetic map (J. Zhu, pers. commun.). Another two kinase genes and a nucleotide binding site (NBS) gene on SC28 which could be related to PRSV-P resistance have been discovered recently (M.R. Razean, pers. commun.).

Unlike *V. pubescens*, *V. quercifolia* can produce fertile hybrids when crossed to *C. papaya* (Drew et al., 1998) thus allowing transferability of the resistance alleles to papaya. Therefore, a papaya breeding program to develop PRSV-P resistant genotypes has been developed using this source of resistance and backcross populations have been achieved (Drew et al., 2006). This research aimed to develop DNA markers linked to PRSV-P resistance genes in *V. quercifolia* based on information on resistance alleles controlling PRSV-P resistance in *V. pubescens*. However, to date there is no solid evidence showing the location of the alleles controlling PRSV-P resistance in *V. pubescens* on the papaya genetic map. Therefore, this study also aimed to reveal the location of alleles specific to *prsv-1* on the published papaya genetic map using sequences of DNA markers linked to PRSV-P resistance. These markers/genes will assist the selection of genotypes carrying PRSV-P resistance genes in papaya breeding programs in the future.

MATERIALS AND METHODS

The sequences of DNA markers linked to PRSV-P resistance which were identified in *V. pubescens* were obtained from Dillon et al. (2006) and BLASTed to the papaya genome database on NCBI. The results showed sequence similarity to genes at the end of LG5 of the papaya genome, therefore, another three NBS genes (NBS9.305, NBS9.308 and NBS18.235) located near the end of LG5 (Porter et al., 2009) were also included in this study. DNA primers were designed from information of papaya DNA sequences from Razean (pers. commun.) and Porter et al. (2009) using Primer3 software (Untergasser et al., 2012) with default setting at GC content more than 40%.

Genomic DNAs were extracted from *C. papaya* var. 2.001 and *Vasconcellea quercifolia* using CTAB method (Delaporta et al., 1983) with minor modification. Quantity and quality of DNA were assessed by a NanoDrop spectrophotometer (Thermoscientific). The DNA samples were amplified using primer pairs in the reaction of 1X MyTaq PCR buffer (1 mM dNTPs, 3 mM MgCl₂, stabilizers and enhancers; Bioline), 0.15 unit MyTaq DNA polymerase (Bioline), 0.45 µM each of forward and reverse primer, 50 ng genomic DNA in a final volume of 15 µL. The PCR reactions were performed in a thermal cycler (BioRad) with the cycle of 95°C 1 min; 35 cycles of 95°C 1 min, annealing temperatures were optimized between 45-60°C for 1 min and 2 mins of 72°C; and the final extension at 72°C for 5 mins. The PCR reactions were held at 4°C until fragment analysis was performed on an agarose gel. The amplicons from papaya and *V. quercifolia* were prepared for DNA sequence analysis using a Big Dye terminator reaction and their sequences were analysed using ABI3130 (Applied Biosystems) by the Griffith University DNA Sequencing Facility at Griffith University, Nathan, Australia.

Raw DNA sequences were analysed and trimmed at Phred quality score of 30 using 4Peaks software version 1.7.1 (4Peaks by A. Griekspoor and Tom Groothuis, mekentosj.com). The sequences were then aligned and optimized for megablast (Zhang et al., 2000) and searched for similarity to the papaya genome on NCBI database and optimised for megablast using BLASTN2.2.28+ program (Altschul et al., 1997) with E-value cut off at 1E-04.

RESULTS AND DISCUSSION

Localization of *prsv-1* Locus on the Papaya Genetic Map

The sequences of two alleles, Opa11_5r and Opk4_1r (marker named *Psi1k4*), which were identified as having 100% and 99% linkage to *prsv-1* in *V. pubescens* (Dillon et al., 2006) were used to search for sequence homology on the papaya genome database. The sequence of Opa11_5r in *C. papaya* '2.001', *V. pubescens* and *V. quercifolia* was linked

to papaya unctig_7858 at 98%; and the sequence of *V. parviflora* was linked to unctig_7858 and LG5 contig_3254, at 49% and 32% query cover (Table 1). The sequences of Opa11_5r on papaya unctig_7858 and superctig_28 (SG28) were then aligned using BLASTN to search for similarity. It revealed the three sequences have 99-100% identity and indicated this part of the genome could be located on SC28 of the papaya genome (M.R. Razean, pers. commun.) in an opposite orientation (plus to minus, Table 2).

The allele of Opk4_1r was absent in *C. papaya* '2.001' genome, therefore, only the sequences on three *Vasconcellea* species were available to search for their similarities on the papaya database. The three sequences covered 73-77% of *C. papaya* chromosome LG5 contig_3254, which is the same contig to which Opa11_5r of *V. parviflora* showed similarity (Table 1). The locus Opk4_1r of *V. pubescens* showed 85% identity to *C. papaya* chromosome LG5 contig_3254 and SC9 with 4% gaps in the opposite orientation (plus to minus strand; Table 3).

The genetic linkage map of an interspecific cross *V. cundinamarcensis* (synonym *V. pubescens*) × *V. parviflora* from reported Opa11_5r was co-located to *prsv-1* locus, and marker *Psilk4*, which was a CAPS marker developed from the Opk4_1r sequence, was located 2 cM away from *prsv-1* locus (Fig. 1a). The evidence from this research revealed the location of Opk4_1r on LG5, SC9 which was anchored at the end of LG5 (Fig. 1b-d; Chen et al., 2007; Ming et al., 2008; Porter et al., 2009). The close link between Opa11_5r and *Psilk4* of 2 cM suggested an estimated physical distance of approximately 0.5 Mbp from the estimation of 269.15 Mbp of physical map to 1068.6 cM of the genetic map (Yu et al., 2009). Both Opa11_5r and Opk4_1r were aligned to the papaya genome in the opposite orientation. This could mean that if Opk4_1r was located on SC9, Opa11_5r and *prsv-1* should be located between SC9 and SC18 at the end of LG5 (Fig. 1d).

The Development of DNA Markers Related to PRSV-P Resistance in *V. quercifolia*

DNA primers were designed on known DNA sequences from SC28 on STK105 and NBS28.12, and three NBS genes at the end of LG5, NBS9.305, NBS9.308 and NBS18.235. Of 24 primer pairs, 30% successfully amplified both *C. papaya* '2.001' and *V. quercifolia* DNAs. These amplicons were then purified and their sequences aligned using align sequence nucleotides BLAST. Partial DNA sequences of five genes in this study in *C. papaya* '2.001' showed 98-100% identity to DNA sequence of papaya on the database which contains the genome sequence of *C. papaya* 'SunUp', while *V. quercifolia* had 89-99% sequence homology to 'SunUp' and 82-93% homology to '2.001'. The information of partial sequences of these five genes, potentially related to PRSV-P resistance, was used to design primers to identify gene expression in each of the target genes. The sequences of candidate alleles will be further tested for their functions by quantitative real time PCR.

CONCLUSIONS

The evidence of sequence alignment between the resistant locus, *prsv-1*, in *Vasconcellea pubescens* and papaya sequence on NCBI database showed that the locus is located on superctig 28 and could be anchored at the end of linkage group 5 of the papaya genetic map (Chen et al., 2007). DNA sequences of *V. quercifolia* and *Carica papaya* '2.001' showed 89 to 99% sequence identity in the region of the end of linkage group 5. This region could be related to PRSV-P resistance in *Vasconcellea* spp.

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Tables

Table 1. BLAST result of Opa11_5r DNA marker sequences from *C. papaya* ‘2.001’, *V. pubescens*, *V. quercifolia* and *V. parviflora*; and Opk4_1r DNA marker sequences from *V. pubescens*, *V. quercifolia* and *V. parviflora* to papaya database on NCBI using BLASTN.

Marker-DNA	BLAST hit description	Total score	Query cover	E value	Max ident	Accession
Opa11_5r-‘2.001’		453	98%	1e-126	99%	ABIM01007849.1
Opa11_5r- <i>pubescens</i>	<i>C. papaya</i> chromosome	365	98%	6e-100	92%	ABIM01007849.1
Opa11_5r- <i>quercifolia</i>	Un contig_7858, whole	381	98%	7e-105	93%	ABIM01007849.1
Opa11_5r- <i>parviflora</i>	genome shotgun sequence	354	49%	2e-96	90%	ABIM01007849.1
Opa11_5r- <i>parviflora</i>		208	32%	2e-52	88%	ABIM01003251.1
Opk4_1r- <i>pubescens</i>	<i>C. papaya</i> chromosome	288	77%	2e-76	84%	ABIM01003251.1
Opk4_1r- <i>quercifolia</i>	LG5 contig_3254, whole	302	73%	9e-81	84%	ABIM01003251.1
Opk4_1r- <i>parviflora</i>	genome shotgun sequence	279	75%	9e-74	82%	ABIM01003251.1

Table 2. Alignment result of Opa11_5r DNA marker sequence of *C. papaya* ‘2.001’ to uncontig_7858 and supercontig_28 of *C. papaya*, to the whole genome shotgun sequence using BLASTN.

Sources	Score	Expect	Identities	Gaps	Strand
Opa11_5r-‘2.001’ VS uncontig_7858	462 bits (250)	2e-133	254/256 (99%)	0/256 (0%)	Plus/Minus
Opa11_5r-‘2.001’ VS supercontig_28	462 bits (250)	3e-131	254/256 (99%)	0/256 (0%)	Plus/Minus
Uncontig_7858 VS supercontig_28	32529 bits (17615)	0.0	17615/17615 (100%)	0/17615 (0%)	Plus/Plus

Table 3. Alignment result of Opk4_1r DNA marker sequence of *V. pubescens* to linkage group 5 contig_3254 and supercontig_9 of *C. papaya*, whole genome shotgun sequence using BLASTN.

Sources	Score	Expect	Identities	Gaps	Strand
Opk4_1r- <i>pubescens</i> VS LG 5 contig_3254	263 bits (142)	3e-74	228/268 (85%)	11/268 (4%)	Plus/Minus
Opk4_1r- <i>pubescens</i> VS supercontig_9	263 bits (142)	5e-71	228/268 (85%)	11/268 (4%)	Plus/Minus
LG 5 contig_3254 VS supercontig_9	2745 bits (1486)	0.0	1486/1486 (100%)	0/1486 (0%)	Plus/Plus

Figures

Fig. 1. a) A genetic linkage map of *V. cundinamaricensis* using RAF and CAPS markers that identified *prsv-1* locus; b) SSR genetic map of *C. papaya* 'SunUp', linkage group 5 (Chen et al., 2007); c) Assembled papaya genome anchored to linkage group 5 (solid red bar on the left) using SSR markers (blue lines) to each scaffold [purple bars; (Ming et al., 2008)]; d) Distribution of predicted *C. papaya* encoding NBS genes on linkage group 5 (Porter et al., 2009).