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Identification of a sequence characterized amplified region (SCAR) marker linked to the *Puccinia psidii* resistance gene 1 (*Ppr1*) in *Eucalyptus grandis*

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While random amplification of polymorphic DNA (RAPD) markers linked to disease resistance genes have been widely used in plant breeding programs, they generally lack reproducibility. To overcome this major disadvantage and other drawbacks, RAPD markers can be converted into sequence characterized amplified region (SCAR) markers, which are genetically defined loci amplified by polymerase chain reaction (PCR) using specific primers. Thus, SCAR markers are typically more reproducible than RAPD markers, due to specific amplification of genomic regions. In this study, a previously identified RAPD marker AT9/917 that is linked to the *Puccinia psidii* Winter (rust) resistance gene 1 (*Ppr1*) in *Eucalyptus grandis* was successfully converted into a specific SCAR marker. Seven specific SCAR primers were designed based on cloning and sequencing of the RAPD marker AT9/917. Different pairs of SCAR primers were tested in an *E. grandis* family from a crossing between a resistant and a susceptible *E. grandis*. Prime pair SCAR AT99151L and AT9915914R produced amplicons of expected size. Restriction enzyme digestion of the amplicon revealed polymorphisms between the resistant and susceptible parents. Association analysis between phenotype (rust resistance) and SCAR genotypes in the *E. grandis* family suggests that this specific SCAR is useful for marker-assisted selection of *E. grandis* trees resistance to *P. psidii* Winter.

Key words: Plant breeding, molecular markers, random amplified polymorphic DNA (RAPD), Mark-assisted selection, sequence characterized amplified region (SCAR).

INTRODUCTION

From as early as the 1970's, eucalyptus rust, caused by *Puccinia psidii* Winter, has posed great threats to eucalyptus trees in Brazil. The biotrophic pathogen *P. psidii* is a parasitic fungus that infects young leaves and the terminal branches of trees, causing deformations, death, hypertrophy, minicancer and meristematic death in

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susceptible genotypes (Alfenas et al., 1989, 2004). The incidence of rust in shoots of susceptible trees is often very severe, requiring the reformation of various settlements after coppicing (Ruiz et al., 1987). The causative fungus *P. psidii* is native to South America (Di Stefano et al., 1998) and is widely distributed in the American continents, being found in Brazil, Argentina, Colombia, Venezuela, Ecuador, Paraguay, Uruguay, Jamaica, Cuba, the Dominican Republic, Puerto Rico, Trinidad and Tobago and Southern Florida in the USA (Coutinho et al., 1998). Recently, *P. psidii* has also been identified in Hawaii (Uchida et al., 2006), California (Mellano 2006), Japan (Kawanishi et al., 2009), Australia (Carnegie et al., 2010; Carnegie and Cooper, 2011) and China (Zhuang and Wei, 2011).

Control of *P. psidii* rust has been successfully accomplished by planting resistant genotypes of trees obtained by intra-and interspecific breeding or by selection of genotypes with fast initial growth. In the latter approach, fast-growing plants experience less time exposed to the *P. psidii* pathogen in the field (Alfenas et al., 2004; Krugner and Auer, 2005). In addition, emergency applications of fungicides have been used sporadically to control the *P. psidii* rust (Alfenas et al., 2004).

The selection of superior matrices for commercial plantations or for use in genetic breeding programs is based on volumetric growth, stem form, wood quality and disease resistance. Under conditions of natural infection, disease susceptible materials may be mistakenly selected as resistant materials, due to inadvertently escaping the disease. Therefore, molecular and genetic detection tools that are independent of infection occurrence are valuable in the selection of disease resistant genotypes. In this context, identification of the molecular markers linked to the disease resistance genes has emerged as an important tool for the selection of disease resistant genotypes. These markers allow us to identify disease resistant characteristics, even in the absence of causative pathogens (Benet et al., 1995).

Investigation of genetic mapping and inheritance of rust resistance in *E. grandis* Hill ex Maiden has been conducted by Junghans et al. (2003). The authors found that rust resistance in *E. grandis* is controlled by the dominant locus *Ppr1*. Based on co-segregation analysis between rust resistance and Random Amplified Polymorphic DNA (RAPD) markers (Williams et al., 1990), they found six markers linked to *Ppr1*. The RAPD marker AT9/917 exhibited complete co-segregation with *Ppr1* in 994 analyzed plants. The AT9/917 marker was then cloned and sequenced, but no significant homology has been found in the GenBank database (Junghans et al., 2003). In addition, few studies have focused on the inheritance of resistance to leaf rust (Zamprogno et al., 2008; Teixeira et al., 2009; Alves et al., 2012).

Molecular markers have been increasingly used as a tool in plant breeding, including genetic mapping of traits of interest and marker-assisted selection of resistant genotypes of plants. RAPD markers are useful for genetic analysis and characterization of the genomes of cultivated species, however, the results obtained with RAPD markers are less reproducible, which may limit its application in marker-assisted selection (Junghans et al., 2003).

To improve the specificity and in order to better assess segregation of markers linked to the characteristics of interest, the less-specific RAPD markers can be converted into highly specific sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993). Briefly, the RAPD markers are cloned and sequenced and the obtained DNA sequences are used to design specific primers for amplification of particular polymorphic regions (Paran and Michelmore, 1993). The SCAR markers have been applied in different studies for a variety of plant species and exhibited highly specific amplification and high reproducibility (Martins Filho et al., 2002; Milla et al., 2005; Masuzaki et al., 2008; Sen et al., 2010; Truong et al., 2011). For example, SCAR markers linked to the resistance gene, *Rpf1*, were identified and characterized to select strawberry plants that are resistant to red stele root rot caused by *Phytophthora fragariae* (Haymes et al., 2000). In this study, we converted a previously identified RAPD marker into a SCAR marker, and evaluated its usefulness in selection of rust resistant genotypes.

MATERIALS AND METHODS

Plant materials

Forty-one F1 individuals from a cross between an array of *E. grandis* rust-resistant (G21) and susceptible (G38) (Junghans et al., 1999, 2003) plants were used in this study. Previous studies have proved that recombination events occurred in these 41 individuals between markers AC8/1180 and AV10/765 that flank the rust resistant gene *Ppr1* (Junghans et al., 1999, 2003).

DNA extraction and RAPD assay

DNA extraction and RAPD assay were conducted according to the protocol described by Grattapaglia and Sederoff (1994) using RAPD primer AT9.

Cloning and sequencing of the RAPD fragment

Based on the results from Junghans et al. (2003), a fragment of 917 bp, generated by RAPD primer AT9, was able to discriminate rust susceptible and resistant genotypes. However, the authors did not identify more than one type of DNA sequence in the 917 bp fragment. Thus, we started a new cloning with this 917 bp fragment. The DNA band of 917 bp linked to the resistant gene *Ppr1* was extracted from agarose gel and purified using the Concert kit™ Rapid Gel Extraction System (Life Technologies). The purified DNA was then cloned into the pGEM-T Easy vector (Promega), according to the manufacturer's recommendations. The cloned fragments were transformed to competent cells of *Escherichia coli*

Table 1. PCR primers used for the development of SCAR markers.

Primer name	Primer sequence (5'->3')	Length (bp)	Direction
AT9 R	TAGCGTCATCAGTAGGTCACCAGG	24	Reverse
AT9 F	CGAGATTTTGTGGAAGCGAAGCATTG	26	Forward
SCAT9 L	CCCTCACGTACGAAGTGGTT	20	Forward
SCAT9 R	GCGTCATCAGTAGGTCACCA	20	Reverse
AT9 915 1 L	CCGTTAGCGTGAGTAGATGTAGAG	24	Forward
AT9 915 914 R	CGTTAGCGTCATCAGTAGGTCA	22	Reverse
AT9 915 71 L	GAAGCGAAGCATTGCATGTC	20	Forward

DH5α, using the heat shock transformation method that has been previously described (Sambrook et al., 1989). The transformed cells were plated on LB medium containing ampicillin (0.1 mg / ml), IPTG (200 mg / ml) and X-GAL (20 mg / ml) and incubated at 37°C for 12 h. Colonies containing recombinant plasmids were identified by white color and were transferred to tubes containing 3 ml of LB medium with ampicillin (0.1 mg / ml) and incubated at 37°C for 12 h, under constant agitation (250 rpm). Plasmid DNA was isolated by the previously described alkaline lysis method (Sambrook et al., 1989) and quantified. Next, to confirm successful transformation, plasmid DNA was amplified via PCR, using primers M13F and M13R (Life Technologies) or digested with the enzyme *EcoRI*, which has cleavage sites in the ends of the vector cloning sites. The nucleotide sequence of the insert was determined in a Perkin-Elmer automated sequencer ABI model 310, using the Thermo Sequenase kit Dye Terminator Cycle Sequencing (Amersham), according to manufacturer's instructions.

Design of SCAR primers and SCAR amplification

The nucleotide sequence of the 917 bp RAPD fragment was used as a template to design the SCAR primers longer than those used in RAPD assay. The computer program DNAMAN was used for primer design, including the amount of bases, Tm (melting temperature), and the GC content. Finally, seven primers, including four forward and three reverse primers, were designed. These are listed in Table 1. Primers AT99151L and AT9915914R share ten and nine nucleotides with the RAPD primer AT9, respectively.

A maximal combination of 12 pairs of primer was tested by PCR for the parental *E. grandis*, G38 (rust susceptible) and G21 (rust resistant). The PCR reaction was standardized to 25 µl, containing 30 ng of genomic DNA, 0.2 mM of each dNTP (dATP, dTTP, dCTP and dGTP), 0.25 mM of each primer, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C) and one unit of Taq DNA polymerase. The reactions were then submitted to amplification in a PTC-100 thermal cycler (MJ Research Inc.). The PCR program ran for 3 min at 94°C for initial denaturation, followed by 40 cycles, each consisting of 30 s at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension of 5 min at 72 °C. The PCR reaction was kept at 4°C after completion. Confirmation of amplification was conducted by visual observation of DNA bands on agar. The PCR productions were separated on 1.4% agarose gel in TBE buffer, containing 0.2 mM bromide ethidium by electrophoresis (Sambrook et al., 1989). A DNA ladder of known size was used to identify the fragment of interest.

Each of the three units of restriction enzymes, *HinfI*, *TaqI*, *HaeIII* *PstI* or *CfoI* (Promega) was added into 20 µl of the PCR reaction to digest DNA fragments. The enzyme digestion solution was changed to 50 µl by adding an appropriate volume of 10X restriction buffer and water. The enzyme digestion solution was kept separately at the optimum temperature for 5 h for each of the enzymes. After digestion, the products were separated on agarose gel by electrophoresis, stained with ethidium bromide and visualized under an ultraviolet light transilluminator. The gel image was captured and digitized in the computer.

RESULTS AND DISCUSSION

Genetic markers represent an excellent tool in plant breeding, since the presence of genes of interest can be detected at any stage of plant development. RAPD is widely used in plant breeding, because it is easy to conduct, inexpensive and quick. However, RAPD cannot be applied to DNA samples of contamination that may generate non-specific amplification. In addition, RAPD generally has low reproducibility and results from different laboratories are difficult to compare with each other, limiting its application. To overcome the disadvantages, including low reproducibility, RAPD molecular markers have been converted into highly specific SCAR markers (Paran and Michelmore, 1993). The SCAR markers have been widely used in genotyping, marker-assisted selection, and high-resolution genetic mapping of plants (Paran and Michelmore, 1993; Xu et al., 1995; Rameau et al., 1998; Nietsche et al., 2000; Guo et al., 2003; Asif et al., 2005; Shi et al., 2009; Srivastava et al., 2012). In this study, we converted a previously identified RAPD marker linked to rust resistance gene *Ppr1* to a SCAR marker for genetic identification of rust resistance in *E. grandis*.

Nine recombinant clones were randomly selected in follow-up analyses after the RAPD fragment of 917 bp was cloned into the pGEM-T Easy vector. Since all clones showed the same pattern of enzyme digestion of four restriction enzymes, only one was sequenced and compared with the sequence previously obtained by Junghans et al. (2003). No difference has been identified between DNA sequences obtained by Junghans et al. (2003) and the one obtained in this study. Based on the DANA sequence of the 917 bp fragment, amplified by RAPD primer AT9, seven oligonucleotide primers were designed, including four forward and three reverse primers. The location of these primers on fragment AT9 (917 bp) is shown in Figure 1 and the primers sequences are listed in Table 1.

Twelve pairs of SCAR primers were tested on parental

Figure 1. Nucleotide sequence (917 bp) fragment linked to *Ppr1* and SCAR primers anneling. Regions where RAPD AT9 primers are located are underlined.

G21 (resistant) and G38 (susceptible) *E. grandis*. However, only some of the tested primer pairs generated amplicons of expected size. Positive amplifications occurred on both resistant and susceptible *E. grandis,* likely suggesting that the polymorphism obtained with the original primer AT9 must be due to one or a few unpaired nucleotides (mismatches) at the site of primer complementary regions, similar to that found by Xu et al. (2001) in tomatoes. It was noted that six primer pairs did not generate expected band patterns at 56°C of extension temperature, even in positive controls. By increasing extension temperature to 58°C, we observed that these reactions generated amplification patterns different from expected patterns or no bands were produced at all. In addition, it was observed that some primer pairs generated only one band in the region of 917 bp. However, none of the possible combinations of primers, at all temperatures tested, revealed polymorphisms between resistant and susceptible *E. grandis*. Thus, the restriction enzyme was used to identify sequence polymorphisms of PCR products generated by SCAR primers.

The AT99151L and AT9915914R pair of SCAR primers, which showed an amplification pattern of expected size, was selected for enzyme digestion analysis. The PCR product was then digested with restriction enzymes to check for the presence of polymorphisms between the two parental *E. grandis* (Figure 2). Enzymes *Hinf*I, *Taq*I, *Hae*III, *Cfo*I and *Pst*I showed the existence of several polymorphic bands between the resistant and susceptible parents on the amplified region. To verify whether or not these markers were linked to *Ppr1*, segregation was evaluated in individuals with recombination events between markers AC8/1180 and AV10/765, flanking *Ppr1* (Junghans et al., 1999, 2003).

Most polymorphic bands do not co-segregate with *Ppr1*. However, the products of the digestion of PCR products, using the enzyme *CfoI,* showed a band at lower intensity, of approximately 800 bp, which co-segregated with the *Ppr1* gene (Figure 3). Among all F1 progeny tested, only in four cases the marker $SCAR_{CfoI}$ did not

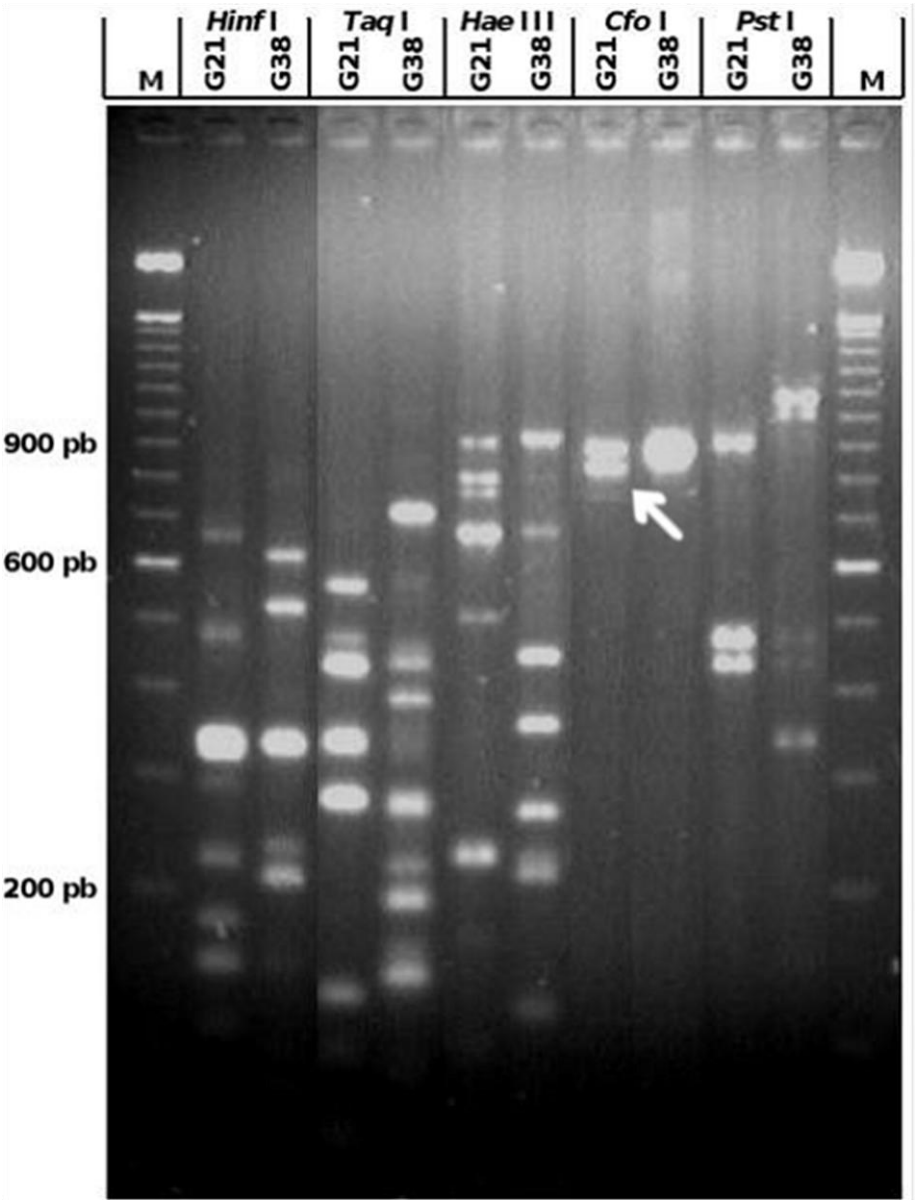

Figure 2. Digestion patterns from PCR products generated by the SCAR primers AT99151L and AT9915914R. M = 100 bp DNA Ladder; G21, the resistant genotype, G38, susceptible genotype. bp = base pairs. Arrow inficates polymorphic band between G21 and G38 genotypes.

correlate with the phenotypes of resistance or susceptibility (Table 2). In the first case (plant 196) the results lead to the assumption that there had been an error in the classification of resistance phenotypic as the resistant phenotype S1 can be confused with the S2 susceptible phenotype.

Moreover, the distance between *Ppr1* and the marker AV10/765 is 0.9 cm, but the distance between the marker AC8/1180 and gene *Ppr1* is 3.4 cM. Thus, genetic recombination between *Ppr1* and the marker AC8/1180 is easier than recombination events between *Ppr1* and the marker AV10/765. As for plant 414 that was properly characterized as the resistance phenotype and genotype, three recombination events would be required to occur: one between the RAPD marker AC8/1180 and *Ppr1*, another between *Ppr1* and RAPD marker AT9/917 and the third recombination event between SCAR marker $SCAR_{CfoI}$ and RAPD marker AV10/765. Although this is possible, the probability is very low. Therefore, this case is more likely to be an error of classification of the resistance phenotype. The third recombination event between *Ppr1* and the marker $SCAR_{CfoI}$ was detected in

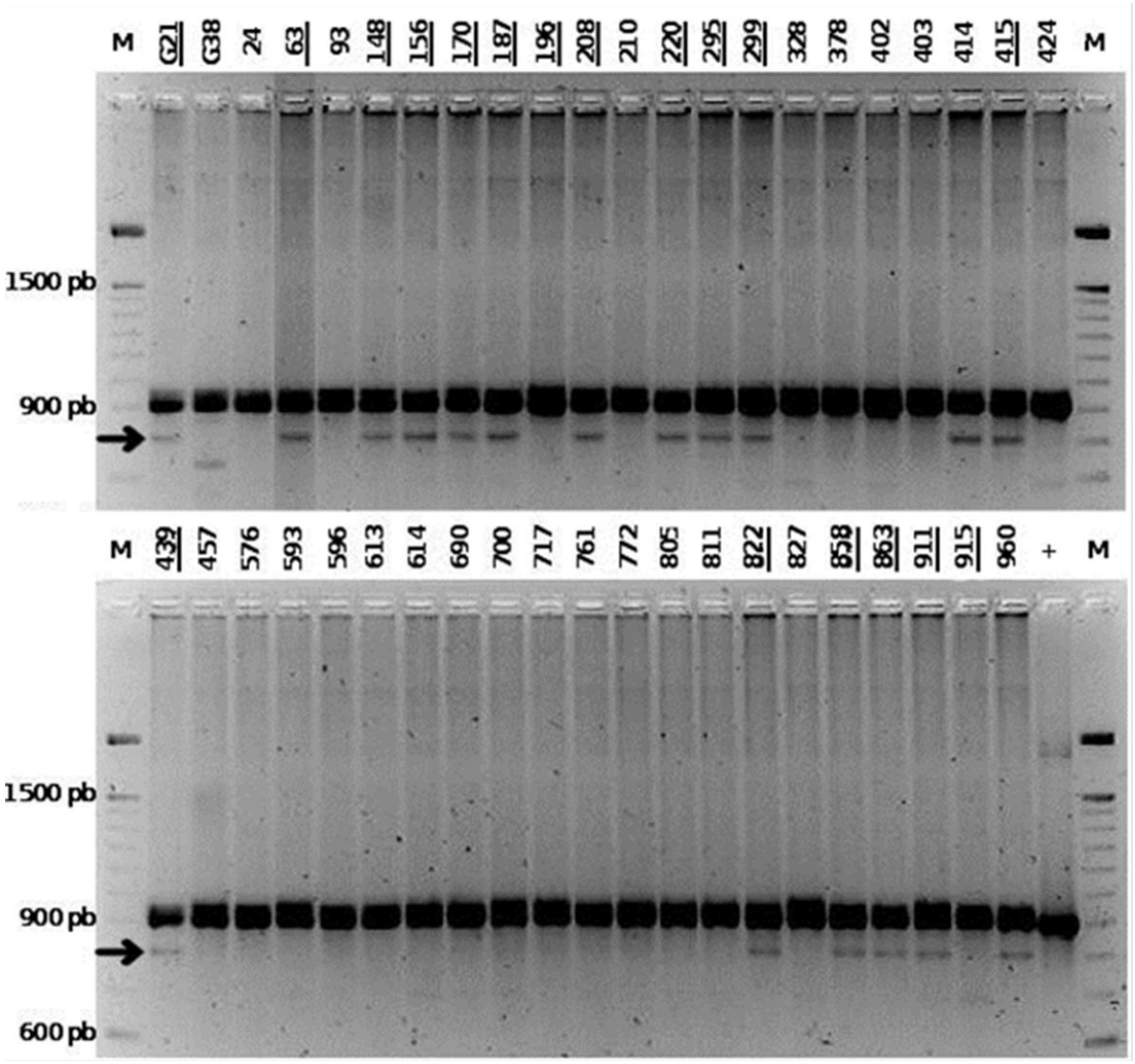

Figure 3. Co-segregation analysis of Ppr1 and $SCAR_{CfoI}$ marker in F1 *E. grandis* progeny, with recombination events near Ppr1. The PCR products generated by SCAR primers pair AT99151L and AT9915914R were digested with restriction enzyme CfoI. The digestion pattern was compared between the two parental, resistant (G21) and susceptible (G38), and F1 progeny. bp = base pairs. M = 100 bp DNA ladder. Underline = rust resistant genotype. Not underscore = eucalyptus susceptible to rust. "+" = Positive control (plasmid DNA containing the 917 bp fragment).

plant 915. In this case, there was a possible recombination between the RAPD marker AT9/917 and $SCAR_{CfoI}$. This allows us to assume that the RAPD marker AT9/917 is between the gene *Ppr1* and marker $SCAR_{CfoI}$. While the results obtained by Junghans et al. (2003) suggested that RAPD marker AT9/917 was linked to the gene *Ppr1*, they are unable to locate AT9/917. The last case of possible recombination between the marker $SCAR_{CfoI}$ and *Ppr1* occurred in plant 960. As in plant 196, these results may be due to an error in phenotypical characterization of disease resistance, since S1, which is considered resistant, can be confused with S2, which is considered susceptible, or vice versa. In addition, as the distance between the gene *Ppr1* and the marker AV10/765 is 0.9 cm, which is beyond the markers AT9/917 and $SCAR_{CfoI}$, the distance between the marker AC8/1180 and the gene *Ppr1* is 3.4 cM, indicating that recombination between *Ppr1* and AC8/1180 is easier than recombination between gene *Ppr1* is and the marker AV10/765.

Despite not having a linkage test, it can be assumed that the RAPD marker AT9/917 and SCAR marker, $SCAR_{CfoI}$, are very close to each other, and AT9/917 is between gene *Ppr1* and $SCAR_{CfoI}$. This can be very useful in positional cloning of the gene *Ppr1* is, because the $SCAR_{CfoI}$ marker can give the direction of traversal chromosomal targeting for cloning *Ppr1*. Moreover, the RAPD AT9/917 was unable to differentiate resistant from

Table 2. Co-segregation analysis of SCAR marker SCAR$_{Cfol}$ and *Ppr1* gene (Junghans et al., 2003) in F1 *E. grandis* progeny. The color breaking indicates a probable recombination.

Plant	K1	AE9	AC8	*Ppr*1		AT9/917	SCAR$_{Cfo\ l}$	AV10	AM6
G21	+	+	+	R	(S0)	+	+	+	+
G38	-	-	-	S	(S3)	-	-	-	-
24	-	-	-	S	(S2)	-	-	+	+
63	-	-	-	R	(S0)	+	+	+	+
93	+	+	+	S	(S2)	-	-	-	-
148	+	+	+	R	(S0)	+	+	-	-
156	+	+	+	R	(S1)	+	+	-	-
170	-	-	-	R	(S0)	+	+	+	+
187	-	-	-	R	(S0)	+	+	+	+
196	+	+	+	R	(S1)	-	-		+
208	+	+	+	R	(S1)	+	+	-	-
210	-	-	-	S	(S3)	-	-	+	+
220	+	+	+	R	(S1)	+	+	-	-
295	-	-	-	R	(S0)	+	+	+	+
299	+	+	+	R	(S0)	+	+	-	-
328	+	+	+	S	(S3)	-	-	-	-
378	-	-	-	S	(S3)	-	-	+	+
402	-	-	-	S	(S3)	-	-	+	+
403	-	-	-	S	(S3)	-	-	+	+
414	+	+	+	S	(S3)	+	+	-	-
415	+	+	+	R	(S0)	+	+	-	-
424	+	+	+	S	(S3)	-	-	-	-
439	+	+	+	R	(S1)	+	+	-	-
457	-	-	-	S	(S3)	-	-	+	+
576	+	+	+	S	(S3)	-	-	-	-
593	-	-	-	S	(S2)	-	-	+	-
596	-	-	-	S	(S3)	-	-	-	-
613	-	-	-	S	(S2)	-	-	+	+
614	-	-	-	S	(S2)	-	-	+	+
690	+	+	+	S	(S3)	-	-	-	-
700	-	-	-	S	(S3)	-	-	+	+
717	-	-	-	S	(S3)	-	-	+	+
761	-	-	-	S	(S3)	-	-	+	+
772	+	+	+	S	(S3)	-	-	-	-
805	-	-	-	S	(S3)	-	-	+	+
811	+	+	+	S	(S2)	-	-	-	-
822	+	+	+	R	(S0)	+	+	-	-
827	-	-	-	S	(S2)	-	-	+	+
858	+	+	+	R	(S0)	+	+	-	-
863	+	+	+	R	(S0)	+	+	-	-
911	-	-	-	R	(S0)	+	+	+	+
915	+	+	+	R	(S0)	+	-	-	-
960	-	-	-	S	(S2)	+	+	+	+

1. R = resistant genotype, S = susceptible genotype; S0 or S1 = resistant individual, S2 or S3 = susceptible individual. 2. "+" = presence; "-" = absence. 3. Green color = genomic region inherited from genotype G21 (resistant to rust). 4. Red color = Genotypes recombinant to SCAR$_{Cfol}$ marker.

susceptible individuals. Therefore, SCAR$_{Cfol}$ is useful in selection of resistant individuals, or those with increased chance of being resistant to *P. psidii*.

Conflict of Interest

The authors declared that they have no conflict of interest.

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