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ARTICLE

Assisted selection using molecular markers linked to rust resistance *SH3* gene in *Coffea arabica*

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Abstract: *The aim of this work was to validate markers linked to the* SH3 *gene of coffee leaf rust (CLR) resistance and use them in assisted selection. Initially, we validated the markers in genotypes already known to carry* SH3*. Next, we performed phenotype and genotype evaluation for resistance to CLR in coffee plants growing under field conditions. We used Arabica coffee progenies derived from BA-10, which carries the* SH3 *gene due to introgression of* C. liberica*. Three SCAR markers (SP-M16-SH3, BA-48-21O-f, and BA-124-12K-f) and one SSR marker (Sat244) linked to* SH3 *gene were used to amplify the coffee plants' DNA. Our assessments of markers validation in resistant genotypes, SP-M16-SH3 and BA-124-12K-f, were efficient to identify the* SH3 gene*. These two markers were used to evaluate the progenies derived from BA-10 and were significantly linked to the phenotype evaluations. The SP-M16-SH3 marker was more efficient, with the advantage of being codominant.*

Keywords: *BA-10 genotype, coffee breeding,* Hemileia vastatrix*, markers validation, SCAR markers*

INTRODUCTION

Brazil is the world's largest producer and exporter of coffee, besides being the second largest consumer of the beverage. One of the main problems that reduce Brazilian coffee production and decrease the profitability of coffee farms is the coffee leaf rust (CLR), caused by the biotrophic fungus *Hemileia vastatrix* Berk. et Br. This disease is found in all coffee producing regions and is considered the main crop disease in Brazil and in the world (Zambolim 2016). The majority of Arabica coffee cultivars cultivated in Brazil are coming from Catuaí or Mundo Novo groups (Chalfoun and Reis 2010), which are susceptible to CLR (Andreazi et al. 2015). *C. arabica* cultivars derived from the "Híbrido de Timor" (HdT) and "Icatu", coffee genotypes with *Coffea canephora* introgression, have different levels of resistance to CLR (Sera et al. 2010). However, the qualitative resistance of some of these cultivars was broken by new physiological races in Brazil, so that some cultivars became susceptible, and others now have intermediate resistance (Del Grossi et al. 2013).

The major genes that govern qualitative resistance to CLR are SH1, SH2, SH3, SH4, SH5, SH6, SH7, SH8, SH9, and SH, which can be supplanted by the

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corresponding virulence genes v1 to v? of *Hemileia vastatrix* physiological races (Bettencourt and Rodrigues 1988). The *SH3* gene has been used in several breeding programs in Brazil, since this gene has not yet been overcome by the CLR races found in this country (Sera et al. 2007). Recently, the *C. arabica* cultivar IAC Catuaí SH3, which has introgression of *C. liberica* and is highly resistant to CLR, was released by the Instituto Agronômico (IAC) (Fazuoli et al. 2019).

Molecular marker-assisted selection using markers linked to resistance to disease is an important strategy for *C. arabica* breeding programs, allowing cost and time reduction (Alkimim et al. 2017, Ariyoshi et al. 2022). Prakash et al. (2004) identified 21 amplified fragment length polymorphisms (AFLP) linked to the *SH3* gene. Subsequently, Sequence-Characterized Amplified Region (SCAR) markers were developed using these AFLP clones and a Bacterial Artificial Chromosome (BAC) Library (Mahé et al. 2008). Two small linkage groups were identified (5.7 cM and 5.9 cM), which were related to the *SH3* region, comprising markers linked to each other. In addition, from an efficiency evaluation it was possible to select four markers closest to the *SH3* gene (Sat244, SP-M16-SH3, BA-48-21O-f, and BA-124-12K-f) (Mahé et al. 2008).

Although the breeding program of the Instituto de Desenvolvimento Rural do Paraná - IAPAR-EMATER (IDR-Paraná) has several progenies of Arabica coffee with *Coffea liberica* introgression, it is not known which ones have the *SH3* gene. Thus, the aim of this study was to validate markers associated with *SH3* and use them in assisted selection in genotypes originating from the cross between *Coffea arabica* and BA-10 coffee trees belonging to the IDR-Paraná Germplasm Bank.

MATERIAL AND METHODS

Initially, we validated the four markers previously mentioned markers Sat244, SP-M16-SH3, BA-48-21O-f, and BA-124-12K-f (Mahé et al. 2008), in the following *C. arabica* genotypes carrying SH3, which are considered differentiating plants: CIFC H153/2 (SH1,3,5), CIFC H151/1 (SH3,4,5), CIFC H147/1 (SH2,3,4,5), and CIFC 33/1-S288-23 (SH3,5) (Bettencourt 1981). The cultivars IPR 100 and IPR 105 were also used, both derived from the crossing of Catuaí x (Catuaí x BA-10), which are, respectively, susceptible and resistant to CLR (Sera et al. 2010). We also used cultivar Catuaí Vermelho IAC 99 as the susceptible control (Table 1).

The DNA extraction from leaves of each *C. arabica* genotype was performed according to Pereira et al. (2009) (adapted protocol from Ferreira and Gratapaglia 1998). After extraction, the DNA quality was checked using a 1% agarose gel and the quantification was carried out in a NanoDrop 1000 (Thermo Scientific). The samples were diluted to 50 ng μL^{-1} and amplified using the SCAR corresponding to markers linked to the SH3 gene. PCR amplification was performed in a final volume of 25 µL containing 50 ng of genomic DNA, 1 × Taq DNA polymerase buffer; 2.0 mM $MgCl_2$; 0.1 mM dNTPs; 0.4 µM of each primer; and 0.5 units of Taq DNA polymerase.

The reactions were performed using a Veriti thermocycler (Applied Biosystems), with the following conditions: initial denaturation for 5 min at 95 °C; 35 cycles of 45 sec at 94 °C; annealing for 45 sec at specific temperatures for each primer; extension of 45 sec at 72 °C; and a final extension of 10 minutes at 72 °C. Polymorphisms were detected in 6% polyacrylamide gel, stained with silver nitrate, according to the protocol described by Brito et al. (2010). The markers BA-48-21O-f and Sat244 did not show the expected band pattern and therefore we did not use them in our work.

Through the BA-124-12K-f and SP-M16-SH3 SCARs markers, we performed the molecular characterization of 26 Arabica coffee plants derived from BA-10 genotype. *C. arabica* cultivars Catuaí Vermelho IAC 99, Mundo Novo IAC 376-4, IPR 100 and IPR 105 were used as control samples. The methodology used for the DNA isolation, PCR reaction and visualization of polyacrylamide gel was the same as described in the topic above. We also performed phenotypic evaluation of the severity of CLR in the field, on the same 26 Arabica coffee plants derived from BA-10, along with susceptible checks of Catuaí Vermelho IAC 99, Mundo Novo IAC 376-4, and IPR 100. CLR severity was evaluated in June 2014, under conditions of natural infection in the field, with the physiological races present at the site. A scale of scores ranging from 1 to 5 was used, where:

Table 1. Information of the molecular markers used in assisted selection, primer sequences, T (° C): annealing temperature, and cM distance (centimorgan distance) between the gene and the molecular marker estimated in the study of Mahé et al. (2008)

Marker	Primer Sequences	T (ºC)	Distance (cM)
SP-M16-SH3	R: ATCTAGCTTTGGAACATCGT F: TTAACTGGAAACTTGGCTTG	49	1.8
BA-124-12KF	R: TGCAGATTGATGGCACGTTA F: TGATTTCGCTTGTTGTCGAG	56	0

1 = plants without chlorotic lesions in the leaves; 2 = plants with lesions ranging from "flecks" to chlorosis in the infected area, but without the formation of uredospores; 3 = uredosporic pustules in small amounts (1-25% of leaves), usually in the lower third, with lower severity in the middle third; 4 = uredosporic pustules in 26 - 50% of leaves, usually in the lower and middle third, with beginning of leaf fall; 5 = uredosporic pustules in more than 50% of the leaves, from the lower third to the upper third, with high intensity of leaf fall. Plants with scores 1 and 2 were classified as resistant, and those with scores 3, 4, and 5, as susceptible (Shigueoka et al. 2014).

To identify the accuracy between molecular characterization using SCAR markers and phenotyping field evaluations, the Spearman's correlation was performed using R studio Psych package (Revelle 2016).

RESULTS AND DISCUSSION

Among the SCARs and SSR markers tested in this work (Sat244, SP-M16-SH3, BA-48-21O-f, and BA- 124-12K-f), we were successful in using SP-M16-SH3, with amplicon size of 200 bp (Figure 1a) and BA-124-12K-f, with 300 bp of amplicon (Figure 1b) The marker BA-124-12K-f showed dominance, as in the study of Alkimin et al. (2017). The susceptibility checks (Catuaí Vermelho, Mundo Novo and IPR 100) amplified susceptibility DNA fragment, as expected, while the IPR 105 resistant control had two amplified DNA fragments, as previously reported (Mahé et al. 2008, Alkimin et al. 2017). Plants T24C1, T28C2P2, T30C3, T32C2P3, T38C1, T40C4, and T40C5 were susceptible in the field evaluation, but showed DNA amplification pattern like resistant plants, indicating that BA-124-12K-f is not always associated with the high resistance to rust conferred by the *SH3*. Of 26 samples, eight did not match the field evaluation results with the marker BA-124-12K-f (Table 2). The T37C4 plant was the only one that did not have the genetic marker of resistance; however, in the phenotypic evaluation it was considered resistant, suggesting that there may have been some CLR inoculum escape in the evaluation of severity in the field.

For the SP-M16-SH3 marker, the susceptibility checks (Catuaí Vermelho, Mundo Novo and IPR 100) amplified the susceptibility DNA fragment, as expected, while the IPR 105 resistant control showed resistant fragment. It is already known that the hybrids CIFC H153/2 and CIFC H151/1 are in a heterozygous condition for *SH3*, since both were originated from the crossings between a homozygous *SH3* carrier genitor and a non-*SH3* carrier genitor (Bettencourt 1981). Thus, the codominant nature of the SP-M16-SH3 marker was confirmed, as two DNA fragments were observed in these two genotypes (Figure 1a). Among the 26 samples, only six showed different results from the field evaluation. Just like for

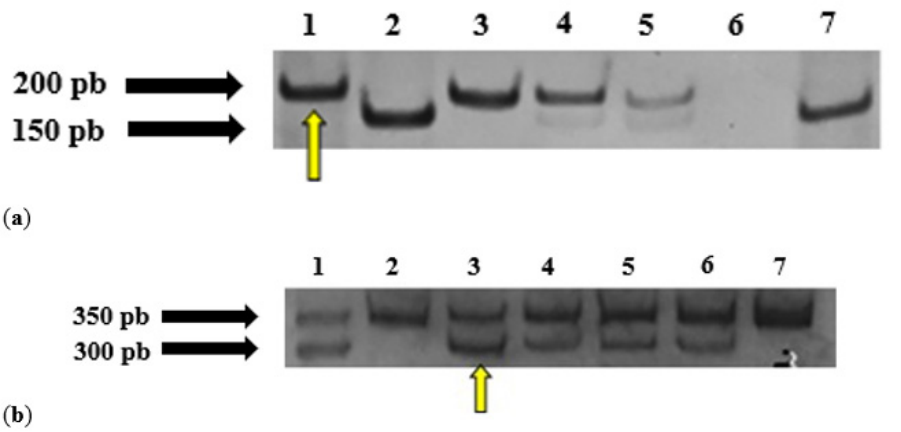

Figure 1. (**a**) Validation of the SP-M16-SH3 molecular marker linked to the *SH3* gene, in genotypes resistant and susceptible to coffee leaf rust: IPR105 (1), IPR100 (2), CIFC 33/1 S288-23 (3), CIFC H153/2 (4 – heterozygous for *SH3* gene), CIFC H151/1(5 - heterozygous for *SH3* gene), CIFC H147/1 (6 - showed no amplification for the SP-M16-SH3 marker) and Catuaí Vermelho IAC 99(7). * The yellow arrow indicates the resistance allele and the amplicon size of 200bp. (**b**) Validation of the BA-124-12K-f molecular marker linked to the *SH3* gene, in genotypes resistant and susceptible to coffee leaf rust. IPR105 (1), IPR100 (2), CIFC 33/1, S288-23 (3), CIFC H147/1 (4), CIFC H151/1 (5), CIFC H153/2 (6), and Catuaí Vermelho IAC-99 (7). * The yellow arrow indicates the resistance allele and the amplicon size of 300bp.

BA-124-12K-f marker, the T37C4 plant was resistant in the field, but showed a band for susceptibility. Plants T24C1, T28C2P2, T32C2P3, T40C4, and T40C5 were susceptible in the field, but showed amplification for resistance, as well as the results for BA-124-12K-f. Plants T30C3 and T38C1 were susceptible in the field and showed amplification for resistance for the BA-124-12K-f marker as well for susceptibility for the SP-M16-SH3 marker, indicating that the latter has a greater association with *SH3* than the former (Table 2). The chance of recombination between these markers and the *SH3* locus is higher when the distance between them is greater (Mahé et al. 2008). These same authors found that the BA-124-12K-f marker is at a distance of 0 cM, cosegregant with the SH3 gene, and theSP-M16-SH3 marker is at a distance of 1.8 cM from this gene.

In a similar work, Prakash et al. (2011) validated the markers SP-M8-SH3, Sat244, and BA-124-12K-f, all of which were closely linked to the *SH3* gene of resistance to CLR. The SP-M16-SH3 marker was more efficient, with the advantage of being codominant, as verified by Mahé et al. (2008), which would allow assisted selection to identify homozygous and heterozygous coffee plants, although a resistant genotype, CIFC H147/1, did not amplify with the marker SP-M16-SH3.

Table 2. Coffee leaf rust (CLR) resistance classification of 26 plants derived from BA-10, evaluated in June 2014 under field conditions, and with the local population of rust races at the IDR-Paraná (Londrina, PR, Brazil). Molecular characterization of resistant (BA-R) and susceptible (BA-S) plants using the BA-124-12k-F marker and resistant homozygous (SP-R), heterozygous (SP-RS), and susceptible (SP-S) using the SP-M16-SH3 marker.

Plant	Classification of resistance to CLR [1]	BA-124-12K-f	SP-M16-SH3
T23C1	R	BA-R	SP-RS
T23C3	R	BA-R	SP-RS
T24C1	S	BA-R*	SP-RS*
T24C2	S	BA-S	SP-S
T24C3	R	BA-R	SP-RS
T25C3	R	BA-R	SP-R
T27C1	R	BA-R	SP-R
T28C1	R	BA-R	SP-R
T28C2	S	BA-S	SP-S
T28C2P2	S	BA-R*	SP-RS*
T30C1	R	BA-R	SP-R
T30C3	S	BA-R*	SP-S
T31C1	S	BA-S	SP-S
T31C5	R	BA-R	SP-R
T32C2P3	S	BA-R*	SP-RS*
T32C4	R	BA-R	SP-RS
T33C1	R	BA-R	SP-RS
T35C1P3	S	BA-S	SP-S
T35C2	R	BA-R	SP-R
T37C1	S	BA-S	SP-S
T37C4	R	BA-S*	SP-S*
T38C1	S	BA-R*	SP-S
T38C3	R	BA-R	SP-RS
T40C3	R	BA-R	SP-RS
T40C4	S	BA-R*	SP-RS*
T40C5	S	BA-R*	SP-RS*
Catuaí [2]	S	BA-S	SP-S
Mundo Novo [2]	S	BA-S	SP-S
IPR 100 [2]	S	BA-S	SP-S
IPR 105 [3]	R	BA-R	SP-R

[1] Based on the severity of the CLR evaluation, plants with scores 1 and 2 were classified as resistant (R) and those with scores 3, 4, and 5 as susceptible (S) (Shigueoka et al. 2014). [2] Susceptible check. [3] Resistant check. * Markers that did not match with the CLR severity evaluation in the field.

When using BA-124-12K-f and SP-M16-SH3 markers, the percentages of correct answers in the field evaluations were, respectively, 73.33% and 80%. In another study, it was found that, for the four markers, there was identification of approximately 67% of the genotypes evaluated carrying the SH3 gene (Alkimim et al. 2017). Therefore, in the same way as BA-124-12K-f, the SP-M16-SH3 marker was not 100% associated with the presence of *SH3*.

A positive and significant association was also observed between phenotypic evaluation and BA-124-12K-f and SP-M16-SH3 markers, with correlation values of 0.60 and 0.73, respectively, confirming that these markers are associated with the presence of the *SH3* gene. The correlation between the markers was 0.86 (Figure 2). Therefore, the BA-124-12K-f and SP M16-SH3 markers proved to be efficient in the selection of CLR-resistant genotypes and demonstrate high potential to be used in coffee breeding programs.

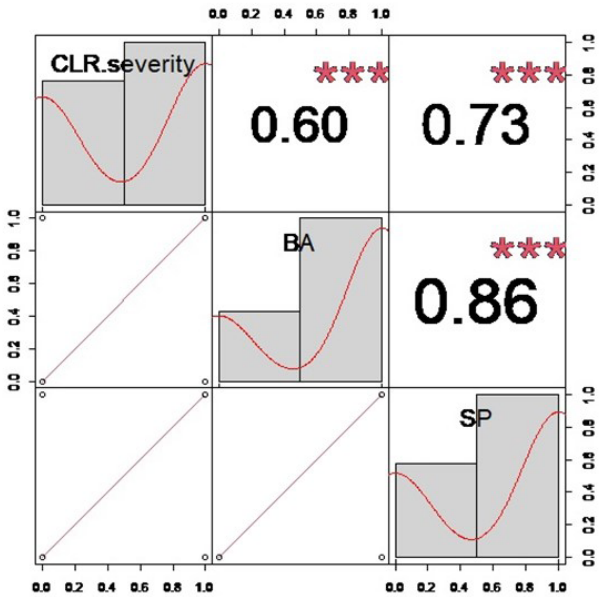

Figure 2. Spearman correlation values between phenotypic coffee leaf rust severity evaluations in the field and BA-124-12k-F (BA) and SP-M16-SH3 (SP) molecular markers.

IAC Catuaí SH3 and IPR 105 are Brazilian cultivars highly resistant to rust, derived from BA-10 (Sera et al. 2010, Fazuoli et al. 2019). However, although IPR 105 is reported as resistant to rust, probably due to the presence of *SH3* (Sera et al. 2010), until the present study it had not been confirmed that this cultivar is a carrier of alleles associated with *SH3*. In our study, the presence of alleles linked to resistance to rust were detected in validation with the differentiators and in the assisted selection, using the two markers. Furthermore, through the SP-M16-SH3 marker it was evident that the resistance to rust of IPR 105 is due to the presence of *SH3* in homozygosis.

The codominance of the SP-M16-SH3 marker and identification of heterozygous plants would be a great advantage in the development of cultivars, as it would not be necessary to advance in the generation of self-pollination to confirm whether the selected plant is heterozygous, through the observation of susceptible segregating plants. Thus, the development time of cultivars could be reduced, through early seedling selection for the trait of high resistance to CLR promoted by *SH3* in homozygosis.

CONCLUSIONS

The SP-M16-SH3 and BA-124-12K-f molecular markers were efficient to identify coffee plants with the *SH3* gene.

SP-M16-SH3 was more efficient than BA-124-12K-f for identifying plants carrying the *SH3* gene, with the advantage of being codominant, which enables the application of this marker in coffee breeding programs.

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