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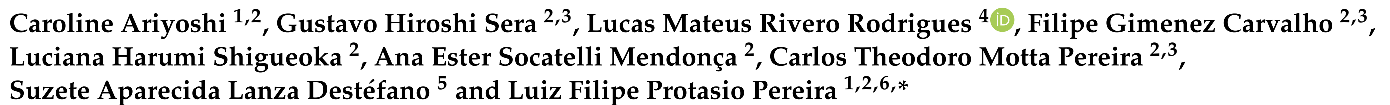
Development and Validation of an Allele-Specific Marker for Resistance to Bacterial Halo Blight in *Coffea arabica*



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Abstract: Bacterial halo blight (BHB) is a bacterial disease, caused by *Pseudomonas syringae* pv. *garcae*, which has been gaining prominence in the main coffee-producing regions. Chemical control of this disease increases production costs and is environmentally undesirable. In this scenario, the development of new cultivars resistant to BHB is the most economical and sustainable alternative. Marker-Assisted Selection (MAS) is an appropriate strategy to assist breeding programs for resistant genotype selection. In a previous Genome-Wide Association Study (GWAS) for *C. arabica* and *P. syringae* pv. *garcae* interaction, we identified a locus, probably linked to qualitative resistance to the pathogen. In this work, we developed and validated a pair of Allele-Specific-Polymerase Chain Reaction (AS-PCR) primers for this locus in *C. arabica* breeding populations. This pair of AS-PCR primers, called Psg_QL1, was tested both in a backcross (BC) (n = 38) and in an F2 population (n = 138) segregating for resistance to BHB. The linkage between the Psg_QL1 marker and qualitative resistance showed an accuracy of 93.75%. Our results demonstrated that the Psg_QL1 marker can be applied in MAS in a robust, simple, fast, and low-cost way.

Keywords: AS-PCR primers; coffee; Marker Assisted Selection; coffee breeding

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1. Introduction

Coffee is one of the most popular beverages in the world, with an annual growth in consumer demand of around 2% [1]. Among the coffee species (*Coffea* sp.) only two have economic importance as a commodity, *C. arabica* L. and *C. canephora* P., which correspond to 59.96% and 40.04% of world production, respectively [2]. *C. arabica* is an autogamous and tetraploid species ($2n = 4x = 44$) with two diploid genitors: *C. canephora* and *C. eugenioides* [3]. Originating in the highlands of southwestern Ethiopia, *C. arabica* is known for its beverage quality.

Most *C. arabica* cultivars are susceptible to the main diseases and pests of coffee [4]. This can be attributed to a bottleneck during the formation of the cultivars. The origin of most *C. arabica* cultivars is from only two varieties: Typica and Bourbon. In addition, the first breeding programs probably prioritized production over other traits such as disease resistance [5–7]. Furthermore, low genetic diversity is characteristic of the species, due to its reproductive biology and a probable relatively recent origin resulting from a single polyploidization event [8,9].

Among the diseases that affect *C. arabica*, the bacterial halo blight (BHB) has been gaining prominence in the main producing regions. This disease is caused by *Pseudomonas syringae* pv. *garcae* [10] and presents the following characteristics: brown colored lesions on leaves, accompanied by a yellow halo, and branches with dark lesions that can reach the leaf petiole, causing defoliation. Lesions are also found on inflorescences and fruits [11]. The development of BHB is favored by mild temperatures, high winds, rain, and hail, which cause plant injuries and facilitate bacterial penetration. BHB is also a major problem in nurseries, where the high density of plants and moisture on the surface of the leaves favor the disease. Under these conditions, the damage can reach 70% [12]. Controlling the disease using chemicals increases production costs, is environmentally undesirable, and can be harmful to human health. Furthermore, it should be considered that in a climate change scenario, disease or insect pest outbreak can expand to areas where they were not previously prevalent [13]. Thus, in order to maintain the market share, the main coffee-producing countries need to invest heavily in research and in new technologies able to get around this context.

For the development of cultivars resistant to diseases, Marker-Assisted Selection (MAS) is an appropriate strategy in breeding programs. Molecular markers are able to characterize individuals independent of environmental effects, allowing the selection of resistant genotypes in the absence of the pathogen at any stage of plant development. Some methodologies have been used to identify SNPs in breeding populations, such as TaqMan® and Kompetitive Allele-Specific PCR (KASP) [14,15]. However, these techniques require fluorescent probes and specialized equipment, which makes their applicability more expensive. Allele-Specific-Polymerase Chain Reaction (AS-PCR) is a technique that can reduce costs and facilitate the use of SNPs in genotype selection [16]. The design of an AS-PCR primer is based on the SNP site genomic region, and to match perfectly with the specific allele and to mismatch with the non-specific allele. The nucleotide corresponding to perfect complementarity between the primer and the SNP site is positioned within the last three nucleotides of the primer 3′ termini [17]. Normally, a mismatched 3′ termini result in lower efficiency of DNA polymerase [18]. Thus, the DNA polymerase preferentially amplifies the correctly matched 3′ termini [19].

High resistance to BHB was identified in the *Coffea arabica* varieties Harar, Dilla and Alghe, S12 Kaffa, and in wild genotypes from Ethiopia, indicating that these genotypes have qualitative resistance to this disease [20,21]. Among *C. arabica* Brazilian cultivars, only IPR 102 has high resistance, probably of the qualitative type [22], while in IPR 106, Japiam, Catiguá MG 01, Catiguá MG 02, IBC Palma 2, IPR 103, Catucaiam 2015479, IAPAR 59, IPR 104, and IPR 108, intermediate resistance was identified [21,23]. However, the *C. arabica* cultivars Mundo Novo, Catuaí, Bourbon, Acaiá Cerrado MG 1474, Topázio MG 1190, IPR 98, IPR 99, IPR 100, IPR 107, IAC 125 RN, Tupi IAC 1669–33, Arara, Catucaí Amarelo 2SL, Sabiá, Acauã, Japy and Catucaiam 24137 are very susceptible and should be avoided in places favorable to BHB incidence [12,20,21,23–25].

In a previous Genome-Wide Association Study (GWAS), using the genetic diversity found in *C. arabica* wild Ethiopian genotypes, we identified 11 quantitative trait nucleotides (QTNs) associated with resistance to BHB [26]. Among these, two QTNs located on chromosome two of eugenioides subgenome are linked to a gene predicted to encode a CC-NB-LRR protein which may act in the specific recognition of pathogen effectors and trigger defense response [26,27]. In that same work, through gene expression, we validated the participation of this gene, probably specific to the qualitative resistance response to BHB.

In view of the above, the aim of this study was to develop and validate a pair of AS-PCR primers specific for alleles associated with qualitative resistance to BHB. In addition, an inheritance study for this feature was performed.

2. Materials and Methods

2.1. Plant Material

The *C. arabica* genotypes E287 Ethiopian accession (P1-homozygous resistant to BHB), Sarchimor (P2-homozygous susceptible to BHB), F1 [P1 × P2] (heterozygous resistant to BHB), IPR102 (resistant to BHB cultivar), Catuaí Vermelho (susceptible to BHB cultivar), and Mundo Novo (susceptible to BHB cultivar) were used in this work.

To validate the marker in breeding populations, we used a backcross population (BC), n = 38, originated from controlled fertilization of an F1 plant with the susceptible genitor Sarchimor [(P1 × P2) × P2]. An F2 population, n = 138, originated from controlled self-fertilization (F1 × F1), was also used.

Plant DNA was extracted separately from leaves using the CTAB method [28]. The DNA quality and quantification were verified using agarose gel (1.2%) and spectrophotometry NanoDrop (Thermo Fisher Scientific, Waltham, MS, USA).

2.2. AS-PCR Primer Development

From the position of the QTNs associated with resistance to BHB (Chr_2_sg_E_32049720_G and Chr_2_sg_E_32049728_G), it was possible to access, in the reference *C. arabica* genome Et39 [29], the upstream and downstream sequences. With this information a pair of AS-PCR primers was designed. The pair of primers and their amplification product were called Psg_QL1 primer and Psg_QL1 marker, respectively.

The pair of Psg_QL1 primers was designed with software Primer 3 [30] and the forward primer prioritized the QTN (Chr_2_sg_E_32049728_G) position in the penultimate nucleotide of the 3′ end, as indicated by Wangkumhang et al. (2007) [31]. The forward primer was designed to match the DNA samples with the QTNs (Chr_2_sg_E_32049720_G) and (Chr_2_sg_E_32049728_G) and to mismatch the corresponding standard alleles (Chr_2_sg_E_32049720_A) and (Chr_2_sg_E_32049728_A) (Figure 1). The reverse primer was designed for a complementary genomic region common between resistant and susceptible genotypes, with no mismatches (Figure 1).

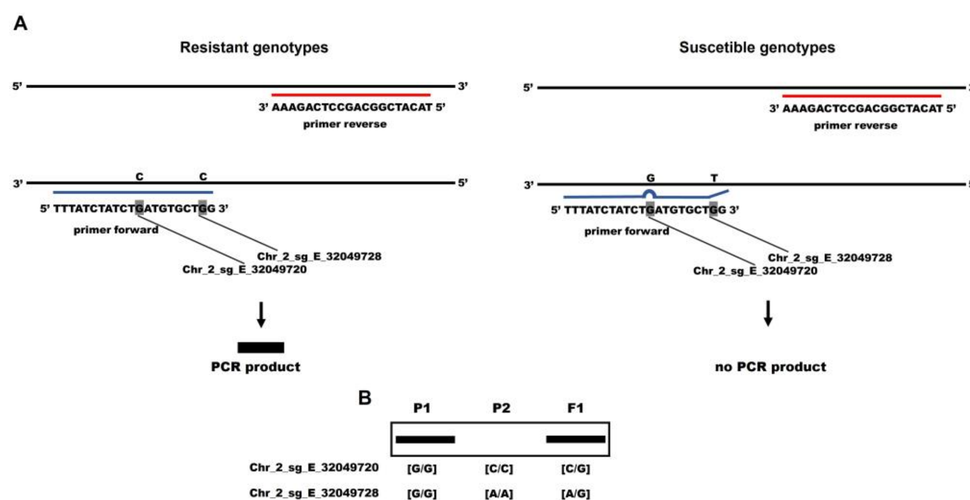


Figure 1. Schematic representation of the AS-PCR strategy. (**A**) The Psg_QL1 forward primer matches with the DNA samples of resistant to BHB coffee plants, and mismatches with susceptible plants. (**B**) Schematic representation of agarose gel showing the expected amplification result for homozygous resistant genotype (P1), homozygous susceptible genotype (P2) and heterozygous resistant genotype (F1). The alternative alleles associated with resistance to BHB are represented by the letter G; the standard alleles are represented by the letters A and C.

The following parameters were also considered in the primer design: CG content (36% to 66%), primer size (18 to 22 bp), amplicon size (200 to 600 bp), and annealing temperature (55 °C to 60 °C) [32]. To identify secondary intramolecular (hairpin and self-

dimer) and intermolecular (cross-dimer) structures, the OligoAnalyzer Tool was used [33]. The primers specificity for a unique genomic region was verified in the *C. arabica* Et39 genome using the local BLAST tool-BioEdit Sequence Alignment Editor software [34].

The PCR was performed with 1 cycle of 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and final extension of 72 °C for 8 min, in thermocycler Veriti (Applied Biosystem, Waltham, MS, USA). The final volume of reactions was 10 µL, 1.0 µL of DNA (100 ng/µL), 2.0 µL of buffer (Go Taq® Reaction Buffer 5x—Promega, Madison, WI, USA), 1.0 µL of forward primer (10 µM—Invitrogen, Waltham, MS, USA), 1.0 µL of reverse primer (10 µM—Invitrogen, Waltham, MS, USA), and 1U of Taq polymerase (Go Taq® G2 DNA 5U/µL—Promega, Madison, WI, USA). After electrophoresis in the SB buffer 1.0×, the amplified genomic fragments were visualized in agarose gel (1.2%) stained with ethidium bromide (0.5 µg/mL) under UV light.

2.3. The Psg_QL1 Marker Validation for MAS and Inheritance Study

2.3.1. Molecular Analyses

For molecular analyses, the PCR was performed with the Psg_QL1 primer and DNA samples from each *C. arabica* plant of BC and F2 populations. DNA samples from IPR 102 (highly resistant to BHB), and Catuaí Vermelho and Mundo Novo (susceptible to BHB) cultivars were also used as positive and negative controls, respectively.

The PCR and the amplified genomic fragments visualization were performed as described in the topic AS-PCR primer development. From the PCR amplification results, the Chi-square hypothesis test (χ^2) was also carried out. In this test, the Psg_QL1 marker was considered as a dominant marker, with the following expected genotypic ratio: 1:1 (PCR^+: PCR^-) for the BC population and 3:1 (PCR^+:PCR^-) for the F2 population. The Chi-square hypothesis test (χ^2) was performed using RStudio (RStudio Team 2020) [35].

2.3.2. Phenotypic Evaluations

The 5-month-old plants from the BC and F2 populations were inoculated with *P. syringae* pv. *garcae* and classified as resistance or susceptibility to BHB, according to the methodology proposed by Rodrigues et al. (2017) [36]. Inoculum was prepared from pure colonies of *P. syringae* pv. *garcae* IBSBF 1197 strain grown on agar medium (0.5% peptone, 0.3% meat extract, 0.1% NaCl, 1.8% agar, pH 7.0) for 48 h at 28 °C and suspended in saline (0.85% NaCl). Bacterial suspension was standardized, using a UV–visible spectrophotometer (Shimadzu UV-1650 PC, Tokyo, Japan), (A600 = 0.25) to contain approximately 10^8 UFC/mL.

The first two pairs of expanded leaves of the plants were inoculated with multiple needles attached in a circular bulkhead, previously dipped in the inoculum, at two points on each side of the central rib in the abaxial side of the leaves. After inoculation, the plants were kept in a humid chamber for 48 h and then transferred to greenhouse with controlled irrigation (10 cycles of 1 min/day). The relative humidity was maintained at approximately 63% and the average temperature at approximately 19 °C.

Resistance to BHB was evaluated 21 days after inoculation (dai) using a disease rating scale (DRS) with 0 to 5 points: 0 = absence of chlorosis and/or water-soaked lesions, or hypersensitivity reaction around lesions; 1 = beginning of colonization by bacterium with or without yellowing around punctures, up to 10% of inoculated surface area showing symptoms of BHB; 2 = 11–25% of inoculated area with characteristic water-soaked lesions surrounded or not by a yellow halo; 3 = 26–50% of inoculated area showing water-soaked lesions and a pronounced yellowish halo around punctures; 4 = 51–75% of inoculated area necrotic with a yellowish halo throughout the area; and 5 = necrosis on 100% of inoculated area.

Only plants that received a zero score on the DRS were classified as qualitative resistant. A Chi-square hypothesis test (χ^2) was performed based on the proportion of the qualitative resistance phenotype, expected and observed, in BC and F2 populations. In this test, the alternative hypothesis (H_1) considered that the qualitative resistance to BHB is conditioned

by a dominant locus. Thus, the expected phenotypic proportion was 1:1 (R_:rr) for the BC population and 3:1 (R_:rr) for F2. The letter R stands for highly susceptible phenotype.

2.3.3. Genetic Linkage between Psg_QL1 Marker and Qualitative Resistance to BHB

From the phenotypic and molecular data obtained in the previous steps, the plants of BC and F2 populations were divided into the following groups: (a) R_, PCR^+; (b) R_, PCR^-; (c) rr, PCR^+; (d) rr, PCR^-; (n) total number of plants. Next, the following statistical tests were performed: Accuracy of method (AM) = $(a + d)/n$; False negative rate (FNR) = $b/(b + d)$; False positive rate (FPR) = $c/(a + c)$; Total error rate (TER) = $(b + c)/n$; χ^2 McNemar with Yates continuity (χ^2 McNemar) = $([bc] - 0.5)^2/b + c$. The χ^2 McNemar test is able to estimate the significance between two assessments and was used to validate the genetic linkage between the Psg_QL1 marker and qualitative resistance to BHB.

3. Results

3.1. AS-PCR Primer Development

The Psg_QL1 forward primer was effective for amplification only the P1 genitor (*C. arabica* Ethiopian accession homozygous resistant to BHB) and F1 (heterozygous resistant to BHB) DNA samples. As expected, an amplification fragment of 224 bp was observed. The P2 genitor (Sarchimor homozygous susceptible to BHB) did not show PCR amplification activity product (Figure 2).

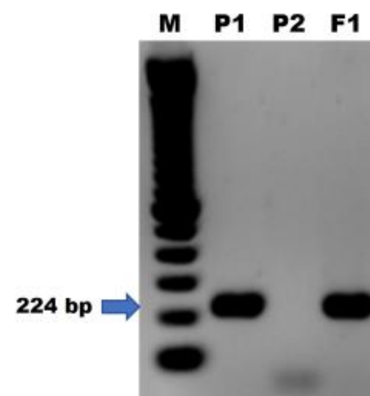


Figure 2. Amplification of the resistant genomic region for BHB employing Psg_QL1 primers; M, 100 bp ladder; P1: *Coffea arabica* Ethiopian accession E287 homozygous resistant to BHB; P2: *C. arabica* Sarchimor homozygous susceptible to BHB; F1 (P1 × P2), heterozygous resistant to BHB.

Tables 1 and 2 show the pair of Psg_QL1 primers characteristics and the Gibbs energy ΔG required to break the secondary structure of these, respectively.

Table 1. The pair of Psg_QL1 primers characteristics. The underlined nucleotides correspond to alleles associated with resistance to BHB.

	Sequence	TA °C	CG%	Size	Amplicon
Psg_QL1 forward	5′TTTATCTATCTGATGTGCTGG 3′	55 °C	38.09%	21 bp	224 bp
Psg_QL1 reverse	5′TACATCGGCAGCCTCAGAAA 3′	56 °C	50.00%	20 bp	

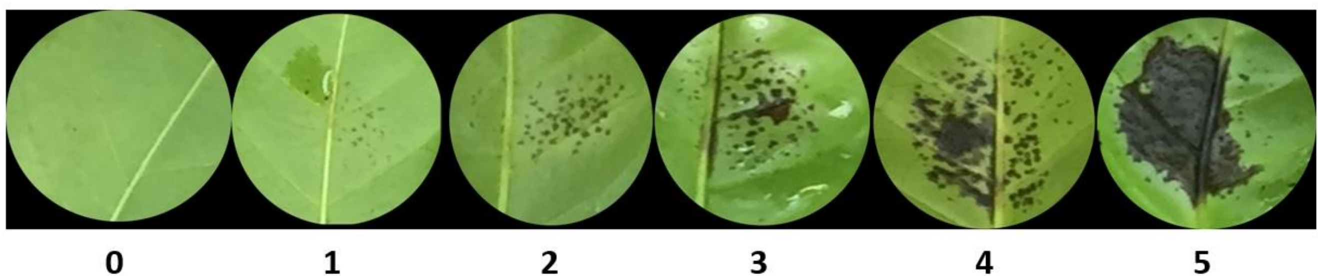
Table 2. The Gibbs energy (ΔG) values required to break the secondary structure of the pair of Psg_QL1 primers. The ΔG values are expressed in kcal/mol.

	Hairpin	**Self-Dimer**	**Cross-Dimer**
Psg_QL1 forward	0.96	−3.14	−5.61
Psg_QL1 reverse	0.76	−6.21	

3.2. The Psg_QL1 Marker Validation for MAS and Inheritance Study

3.2.1. Phenotypic Evaluations

For the BC population, n = 38, 20 plants were phenotyped with the score 0 (R_) and 18 with scores between 3 and 5 (rr). For the F2 population, n = 138, 114 plants were phenotyped with the score 0 (R_) and 24 with scores between 1 and 5 (rr). The lesion pattern used to classify the resistance to BHB is represented in Figure 3.



0 1 2 3 4 5

Figure 3. Lesion pattern caused by *Pseudomonas syringae* pv. *garcae* in *Coffea arabica* used to classify the plants for resistance to BHB.

Table 3 shows the χ^2 test results for BC and F2 populations. For both populations, the phenotypic results support H_1 (qualitative resistance to BHB conditioned by a monogenic and dominant genetic factor).

Table 3. Number of plants classified, phenotypically, as resistant (R_) and susceptible (rr) and χ^2 test results, for the segregation hypotheses: 1:1 for BC and 3:1 for F2.

R_		**rr**		**GL**	χ^2	**P(1)**
exp	obs	exp	obs			
			BC Population			
19	20	19	18	1	0^{ns}	100%
			F2 Population			
103.5	114	34.5	24	1	1.95^{ns}	16.18%

Exp = expected; obs = observed. ns = non-significant values at the 5% probability level. $P^{(1)}$ = probability (%) obtained in the χ^2 test.

3.2.2. Molecular Analyses

For the BC population, n = 38, 20 plants showed the Psg_QL1 marker amplification and 18 did not show (Figure 4). For the F2 population, n = 138, 109 plants showed amplification and 29 did not show (Figure 5).

For the χ^2 test of BC and F2 populations, the molecular characterization results support H_1 (qualitative resistance to BHB conditioned by a monogenic and dominant genetic factor), with a probability of 100% and 51.98%, respectively (Table 4).

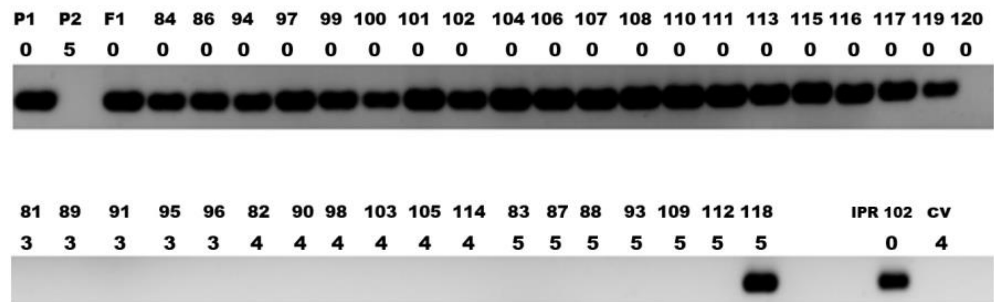


Figure 4. Molecular characterization of BC *C. arabica* population employing Psg_QL1 marker. The amplified fragments of 224 bp are specific for the Psg_QL1 marker. P1: *Coffea arabica* Ethiopian accession E287 resistant to BHB; P2: Sarchimor susceptible to BHB; F1 (P1 × P2); 81−120 [(P1 × P2) × P2]; IPR 102: cultivar with qualitative resistance to BHB; Catuaí Vermelho (CV), cultivar susceptible to BHB. Numbers below the plant identification represent their score for resistance to BHB. Only plants phenotyped with the zero score were classified as qualitative resistance.

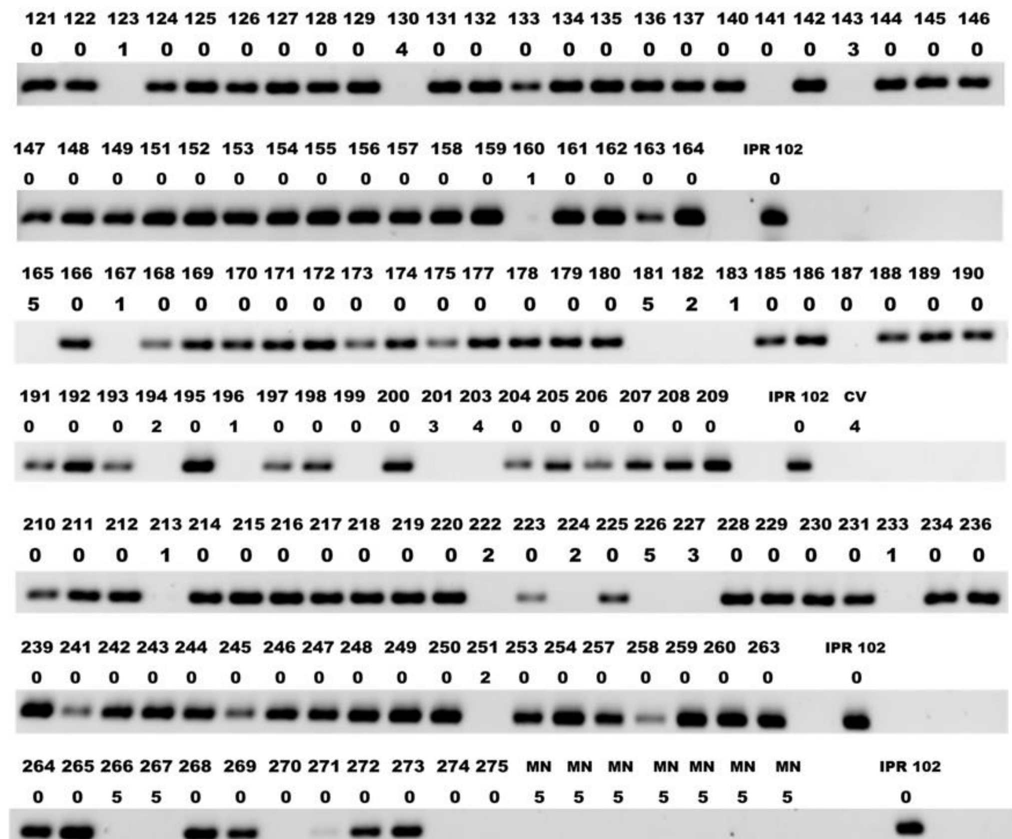


Figure 5. Molecular characterization of F2 *C. arabica* population employing Psg_QL1 marker. The amplified fragments of 224 bp are specific for the Psg_QL1 marker. 121−273 (F1 × F1), F1 (P1 × P2), P1: *Coffea arabica Ethiopian* accession E287 resistant to BHB; P2: Sarchimor susceptible to BHB; IPR 102: cultivar with qualitative resistance to BHB; Catuaí Vermelho (CV) and Mundo Novo (MN): cultivars susceptible to BHB. Numbers below the plant identification represent their score for resistance to BHB. Only plants phenotyped with the zero score were classified as qualitative resistance.

Table 4. Number of plants classified as resistant (Psg_QL1 marker PCR^+) and susceptible (Psg_QL1 marker PCR^-) and χ^2 test results for the segregation hypotheses: 1:1 for BC and 3:1 for F2.

PCR^+		PCR^-		GL	χ^2	$P^{(1)}$
exp	obs	exp	obs			
BC Population						
19	20	19	18	1	0^{ns}	100%
F2 Population						
103.5	109	34.5	29	1	0.41^{ns}	51.98%

Exp = expected; obs = observed. ^{ns} = non-significant values at the 5% probability level. $P^{(1)}$ = probability (%) obtained in the χ^2 test.

3.2.3. Genetic Linkage between Psg_QL1 Marker and Qualitative Resistance to BHB

We observed an accuracy of 93.75% between phenotyping and molecular evaluations. Through the χ^2 McNemar test at a 0.1% probability level, the null hypothesis was accepted, there is no difference between phenotypic and molecular evaluations. In addition, 17.02% of false negatives, 2.33% of false positives, and 6.25% of errors were identified (Table 5).

Table 5. Estimation of parameters related to the genetic linkage between the Psg_QL1 marker and the qualitative resistance to BHB.

Parameter	Total
Accuracy of method (AM) (%)	93.75
False negative rate (FNR) (%)	17.02
False positive rate (FPR) (%)	2.33
Total error rate (TER) (%)	6.25
χ^2 McNemar	1.45 *

* significant at the 0.1% probability level.

4. Discussion

4.1. AS-PCR Primer Development

Since the advent of the high-throughput sequencing technique, it has been possible to identify a large number of SNPs and apply them in association studies in the most diverse crops, including *C. arabica* [26,37–40]. Once associated with a trait of interest, SNPs markers can be used in MAS.

Here, we report an efficient marker for resistance to BHB in *C. arabica*, using 3′ termini match or mismatch, in a simple PCR and electrophoresis. A point to consider for Psg_QL1 forward primer performance is that the SNP (Chr_2_sg_E_32049728) is a transition A/G. The A/G transition has a high potential to destabilize primer annealing due to a mismatch, significantly increasing primer specificity [41]. The exchange of purine for a purine alters the double helix geometry and consequently decreases the hydrogen bonds [42]. Another point is that the ΔG, which represents the secondary structure strength such as hairpin, self-dimer, and cross-dimer of the pair of Psg_QL1 primers, showed no influence on the PCR efficiency (Table 2). The more negative the ΔG values, the more stable the secondary structures, and the smaller amount of primers are available to bind to the DNA template. The number of cycles employed in the PCR and the DNA concentration have also been shown to be efficient, since a high number of cycles and a high DNA concentration can lead to false positive amplification [43,44].

4.2. The Psg_QL1 Marker Validation for MAS and Inheritance Study

The hypersensitive response (HR) or cell death is considered as qualitative resistance, while the remainder of the reduced susceptibility is considered as quantitative resistance [45]. The NBS-LRR proteins are polymorphic intracellular receptors that intercept the effectors (avirulence proteins - avr) of the pathogen and induce a robust resistance called

effector-triggered immunity (ETI). ETI inhibits pathogen growth and is often associated with localized plant cell death, referred to as the HR [46].

In a previous GWAS, we identified that the QTNs Chr_2_sg_E_32049720_G and Chr_2_sg_E_32049728_G, used for the Psg_QL1 forward primer design, are inserted in a genomic region probably involved in qualitative resistance to BHB [26]. Near to these QTNs, the g010741 gene predicted by encoding an NBS-LRR with N-terminal coiled-coil (CC) domain was identified. This gene displayed a similar transcriptional downregulation profile in *C. arabica* Catuaí Vermelho IAC 99 cultivar (susceptible to BHB) and in *C. arabica* IAPAR 59 cultivar (quantitative resistance to BHB), when infected with *P. syringae* pv. *garcae*. However, peaks of upregulation were observed in *C. arabica* IPR 102 cultivar (qualitative resistance to BHB) [26].

Through the statistical test χ^2 McNemar, it was possible to validate the linkage between the qualitative resistance to BHB and the Psg_QL1 marker. This result reinforces the hypothesis about the involvement of this genomic region in qualitative resistance to BHB in *C. arabica*. Furthermore, the false positive rate (plants with the Psg_QL1 marker amplification and absence of qualitative resistance phenotype) was 2.33%, thus demonstrating the Psg_QL1 marker robustness. Based on PCR, the Psg_QL1 marker is a low-cost method that can be easily implemented in *C. arabica* breeding programs. The false negative rate (17.02%) corresponds to plants that showed qualitative resistance phenotype but did not show Psg_QL1 marker amplification. An explanation for this may be a pathogen reduction effectiveness.

Qualitative resistance to diseases in plants produces discrete classes of resistant and susceptible individuals. This is because the genetic architecture of qualitative traits is conferred by a few genes with a great effect on the phenotype. Thus, these genes are amenable to analyses in segregating populations for the feature of interest, according to Mendelian proportions and using chi-square tests [47]. In this study, the qualitative resistance to BHB phenotypic proportions observed in segregating populations validated the possibility of the trait controlled by monogenic and dominant genetic factor. The results observed in segregating populations, using the Psg_QL1 marker, also validated this possibility. Overall, the findings of this work provide information regarding the genetic basis of resistance to BHB, from the Ethiopian accession E287, which may support *C. arabica* breeding programs.

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