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QTL IDENTIFICATION FOR CASSAVA BACTERIAL BLIGHT RESISTANCE UNDER NATURAL INFECTION CONDITIONS

Identificación de QTL de resistencia a la bacteriosis vascular en yuca bajo condiciones naturales de infección

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ABSTRACT

Cassava, *Manihot esculenta* Crantz, represents the main food source for more than one billion people. Cassava's production is affected by several diseases, one of the most serious is cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). A quantitative trait loci (QTL) analysis for CBB resistance was performed under natural infection conditions, using a mapping population of 99 full-sibs genotypes highly segregant and a SNP-based high dense genetic map. The phenotypic evaluation was carried out in Puerto López, Meta, Colombia, during the rainy season in 2015. Both resistant and susceptible transgressive segregants were detected in the mapping population. Through a non-parametric interval mapping analysis, two QTL were detected, explaining 10.9 and 12.6 % of phenotypic variance of resistance to field CBB. After a bioinformatics exploration four genes were identified in the QTL intervals. This work represents a contribution to the elucidation of the molecular bases of quantitative cassava resistance to *Xam*.

Keywords: Cassava, molecular marker, QTL, resistance, SNPs, *Xanthomonas axonopodis* pv. manihotis.

RESUMEN

La yuca, *Manihot esculenta* Crantz, representa la principal fuente de alimento para cerca de 1000 millones de personas. La producción de yuca se ve afectada por diversas enfermedades, una de las más serias es la bacteriosis vascular (CBB) causada por *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). En este estudio se realizó un análisis de loci de rasgos cuantitativos (QTL) para la resistencia a CBB en condiciones naturales de infección, usando una población de mapeo constituida por 99 genotipos de hermanos completos segregantes y un mapa genético altamente denso basado en SNPs. La evaluación fenotípica se llevó a cabo en Puerto López (Meta), Colombia, durante la época de lluvias durante el segundo semestre de 2015. En la población de mapeo fueron detectados individuos con una segregación transgresiva tanto resistentes como susceptibles. A través de un análisis no paramétrico de intervalo simple, se detectaron dos QTL que explican el 10,9 y el 12,6 % de la varianza fenotípica de la resistencia en campo a CBB. Mediante análisis bioinformáticos se identificaron cuatro genes candidatos presentes en los intervalos de los QTL. Este trabajo representa un esfuerzo por dilucidar los mecanismos moleculares implicados en la resistencia de yuca a CBB.

Palabras clave: marcador molecular, QTL, resistencia, SNPs, *Xanthomonas axonopodis* pv. *manihotis*, yuca.

INTRODUCTION

Cassava, *Manihot esculenta* Crantz, is a cross-pollinated species and belongs to the Euphorbiaceae family. It is a perennial shrub and its origin is the Amazon Basin (Olsen and Schaal, 1999). The cassava diploid genome is 2n = 36 and has sexual reproduction but for agro-economical purposes farmers use vegetative propagation (Carvalho and War, 2002; Raji *et al*., 2009). Cassava is one of the most important crops worldwide. It is the third most important source of calories in the tropics, after rice and maize (FAO, 2008). Cassava has been considered essential in protecting food security, especially for developing countries in Africa, Asia and Latin America (FAO, 2008). Due to the high adaptability to drought and acid and poor soils, cassava has been considered as an excellent alternative for an eventual world food crisis (FAO, 2008; FAO, 2013).

Brazil, Thailand, Indonesia, Angola and Ghana are the countries with the largest cassava planted area. Colombia was ranked fifteenth in world cassava production and third in Latin America after Brazil and Paraguay (Aguilera, 2012). In Colombia, Departments such as Bolívar, Córdoba, Sucre, Magdalena and Meta are those with the largest cassava planted area and production. The total production in these Departments was more than 500 thousand tons in 2014 (MINAGRO, 2016).

Cassava, as any other crop, is affected by several diseases produced by virus, fungus, oomycetes and bacteria (FAO, 2013). The most important bacterial disease affecting cassava is Cassava Bacterial Blight (CBB), which is caused by the vascular and foliar pathogen *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). Recently, *Xam* was ranked as one of the top 10 most important bacterial phytopathogens (Mansfield *et al*., 2012). CBB is a devastating disease, generating significant losses, which can reach between 12 and 100 % in infected fields (Lozano, 1986; López and Bernal, 2012). In Colombia, the *Xam* populations are highly dynamic and diverse (Restrepo *et al*., 2004; Trujillo *et al*., 2014).

Conventional breeding strategies have been used to address CBB but with limited success. The most efficient strategy to manage CBB is planting resistant cultivars. However, the knowledge of the molecular mechanisms which governs the resistance in cassava is scarce. Nevertheless, histology and cytochemistry studies of the resistance to CBB shows that callose depositions (Kpémoua, 1996; Sandino *et al*., 2105), cell wall fortification, lignification and suberization associated with callose deposition and production of flavonoids and polysaccharides are important mechanisms of resistant in response to *Xam* (Kpémoua *et al*., 1996). Also, different molecular approaches have conducted to identify resistance and defense genes (López *et al*., 2003; López *et al*., 2005).

Resistance to CBB is a quantitative trait, with polygenic and additive inheritance (; Jorge *et al*., 2000; Jorge *et al*., 2001). A number of quantitative trait loci (QTL) for resistance to CBB, with major and minor effects as well as stable and unstable have been detected. In 2000, Jorge and coworkers reported twelve QTL explaining 9 % to 27 % of the phenotypic variance. These QTL were detected under greenhouse conditions to five *Xam* strains (CIO-84, CIO-1, CIO-136, CIO-295 and ORST X-27). Eight novel QTL, explain between 7.2 % and 18.2 % of the resistance, were identified under field conditions of natural disease pressure and during two consecutive crop cycles (Jorge *et al*., 2001). Nine QTL explaining 16 % to 33 % of the phenotypic variance to four African *Xam* strains were also reported (Wydra *et al*., 2004). More recently, two new QTL explaining 62 % and 21 % of the CBB resistance were identified to the *Xam* strains CIO151 and CIO121 (López *et al*., 2007).

Undoubtedly the environmental conditions plays a key role in traits governed quantitatively and even more in plant pathogen interactions (Weinig and Schmitt, 2004; Anderson *et al*., 2014). In fact, several studies had shown that the environment conditions are a key factor in the cassava–*Xam* interaction. In particular, the humidity favors the dispersion and proliferation of the bacteria and favors the disease (Banito *et al*., 2000; Wydra and Verdier, 2002; Restrepo, 2004; Banito *et al*., 2008). Thus, is essential to perform field evaluations with high disease pressure in order to detect genetic determinants involved in CBB resistance under natural conditions where cassava grows.

The objective of this work was to identify novel QTL for CBB field resistance, based on the evaluation of a biparental population of 99 F1 segregating full sib progeny, during a rainy season in 2015 in Meta, Colombia. A bioinformatics research for genes present in the QTL regions was carried out finding some candidate genes.

MATERIALS AND METHODS

Mapping population and field design experiment

The mapping population is a full sib F1 segregating population of 99 individuals obtained by a cross between the Nigerian cultivar TMS30572 and CIAT's (International Center for Tropical Agriculture) elite cultivar CM2177-2 (Fregene, 1997). Due to cassava is a highly heterozygous species, the parental TMS30572 and CM2177-2 are not pure lines, instead are heterozygous, given arise a first progeny (F1) segregating population. This population has been used for several mapping studies (Fregene *et al*., 1997; Jorge *et al*., 2000; Jorge *et al*., 2001; Mba *et al*., 2001; Lopez *et al*., 2007), and for the construction of a high-density cassava genetic map (Soto *et al*., 2015). Each genotype was grown from stem cuttings (stakes) at the research institute "La Libertad" Corpoica, located in Puerto López, Meta, Colombia (4°03'40.3"N 73°27'22.5"W). This region belongs to ecozone 2 (ECZ2): a lowland tropical region in the Colombian eastern plains (Restrepo *et al*., 1999; Jorge *et al*., 2001; Trujillo *et al*., 2014). Ten plants of each parent and each genotype were planted in the field with a density of

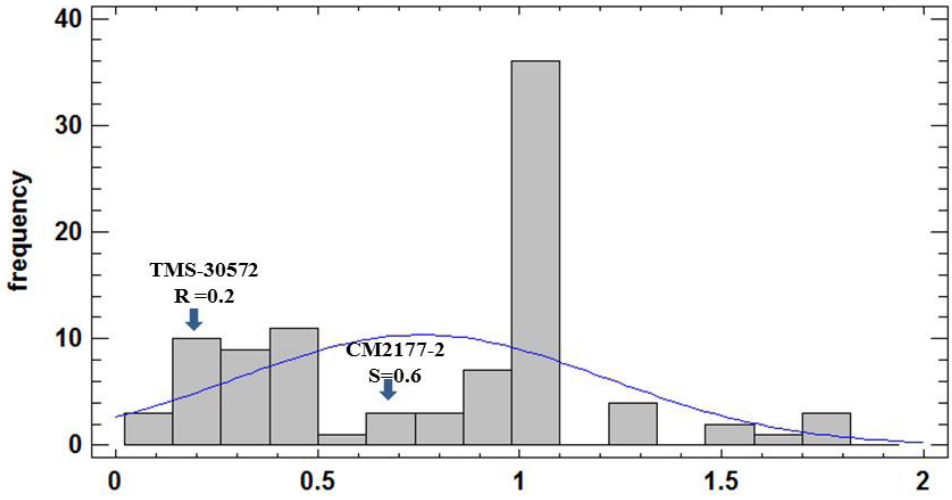

Figure 1. Histogram of the distribution of the disease index values obtained in the field evaluation of the response to cassava bacteria blight. The X-axis represents the classes of the distribution of ID values for the 99 genotypes evaluated. The Y-axis shows the frequency of genotypes in each category. ID values of the parents are shown by the arrows.

$1m^2$, in an area of 1.9 ha under a complete random design. The F1 population was planted in August, 2014. The plants were cultivated according to the agronomical practices employed by the farmers. No control to diseases was conducted. During the evaluation period of the response to natural disease pressure of *Xam* in Meta, Colombia, in July 2015, corresponding to rainy season (IDEAM, 2015). The maximum and minimal temperatures were 32 °C and 22 °C, respectively, 87 % of relative humidity and a mean precipitation of 400 mm (IDEAM, 2015). Insects or other diseases did not attack the plants, which grew as expected.

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Field evaluation of the response to CBB

Under a natural pressure of *Xam,* the disease severity was scored in ten plants by genotype and parental at ten months after planting, using a rating from 0 to 5, using the symptoms scale described by Jorge *et al*. (2001). Symptom scale: 1=healthy plant (no symptoms); 2=angular leaf spots; 3=wilting of leaves; 4=dieback of one or several apices; 5=dieback of whole plan. The average of the symptoms at the observation time was calculated for each genotype and taken as a disease index (DI). The DI of each genotype was used for QTL analysis. The transgressive segregants for resistance and susceptibility were also evaluated. The distribution of frequency of the DI was tested for normal distribution by the Shapiro-Wilk test. An analysis of variance (ANOVA) and its non-parametric test (Kruskal-Wallis) were also performed.

QTL mapping

Interval Mapping (IM) analysis with the "np" model was used for QTL detection through R/qtl V1.37-11 (Broman, 2015). The high dense genetic map of cassava with a density or distance between markers of 1,26 cM (Soto *et al*., 2015) was employed plus a set of 2,236 GBS-SNP markers with unknown genetic position but with known physical position in the current cassava genome v6.1. To declare the presence of a QTL a LOD score higher than 2.5 was used as criteria. The QTL interval was established by a LOD decrease of 0.5 from the marker peak position. Phenotypic variation explained by each QTL was determined with the R package calc.Rsq. Physical positions of the genes identified within the QTL intervals (candidate genes) were established based on the SNP-based genetic map. A BLAST (Basic Local Alignment Search Tool) analysis were performed to the current cassava genome (v6.1) for genes identified within the QTLs intervals.

RESULTS

Field evaluation of the response to cassava bacterial blight

At the end of the productive cycle (ten months after planting) and before harvest the roots, the cassava plants were scored for the presence of symptoms. Five genotypes (5 %) were symptomless, while 94 genotypes (95 %) exhibit at least one of the typical symptoms related to CBB, being the angular

leaf spots the most common. Taking into account these observations it is possible to conclude that CBB was present in the field and in consequence it was possible to evaluate the differential responses among the genotypes. The plants were categorized according to the presence of symptoms using a field scale previously established (Jorge *et al*., 2001) and a disease index (DI) was calculated for each genotype as the number of symptomatic plants over the total of evaluated plants. The DI in the mapping population did not exhibit a normal distribution, (p<0.05) (Fig. 1). However, the Kruskal-Wallis test showed significant differences (p<0.05) for the DI values obtained for the genotypes tested, showing that the response to CBB is genotype-dependent (Supplementary data). Both parents exhibited DIs statistically different (significant p<0.05), indicating contrasting responses to CBB (Supplementary data). The resistant parental TMS30572 had a DI value of 0.2 while the DI for the susceptible parent was 0.6. The DI in the mapping population ranged from 0 to 2, with a mean of 0.75 and a standard deviation of 0.45. Most DIs values were found near the average of the sample (34 genotypes with DI=1) and very few values near the upper (four genotypes with DI >1.8) and lower extremes (five genotypes with DI=0) (Fig. 1). From the 99 genotypes evaluated, 38 have equal or lower DI values than 0.2 (DI value of the resistant parent TMS30572), these were considered as resistant genotypes. On the other hand, 61 genotypes exhibited equal or higher DI values than 0.6 (DI value for the susceptible parent CM772-14) and those were considered as susceptible.

Transgressive segregants with DIs higher or lower than the parents were identified in the mapping population. The total trasngressive segregants were eight for DIs lower than 0.2 (ID value of the parent TMS30572) and 60 higher than 0.6 (ID value of the parent CM2177-2). The genotypes g52 and g135 were the extreme genotypes for susceptibility with ID values of 1.83 and 2, respectively. For resistance, the extreme genotypes were g23, g89, g92, g93 and g104, which did not exhibit any symptom related to CBB (Supplementary data).

QTL mapping

Due to the ID values in the mapping population did not exhibit a normal distribution, a non-parametric QTL interval mapping approach using the model "np" of R/qtl, was applied. Based on the phenotyping evaluation of the response to CBB in the F1 population and the previous cassava genetic map developed (Soto *et al*., 2015). This analysis allowed the identification of two QTL explaining CBB field resistance. These QTL were located in linkage groups 4 and 8 with LOD 2.5 and 2.9 respectively (Fig. 2). The QTL in the linkage group 4 was named as *QLB-4* and explains the 12.6% of the field resistance to CBB. It covers an interval length of 2.4 cM. The interval flanking markers of *QLB-4* were MB_21980 and MB_25367. The physical distance from the peak (MB_21974) to the flanking marker MB_21980 was 317bp. While the distance from the peak marker to the flanking marker MB_24367 could not be established due to these two markers belong to different scaffolds in the cassava genome v4.1.

The second QTL was located on the linkage group 8, explained 10.9 % of CBB resistance and was named as *QLB-8*. The *QLB-8* covers an interval of 1.8 cM, whit a peak marker matching to the SNP MB_8500 and interval flanking

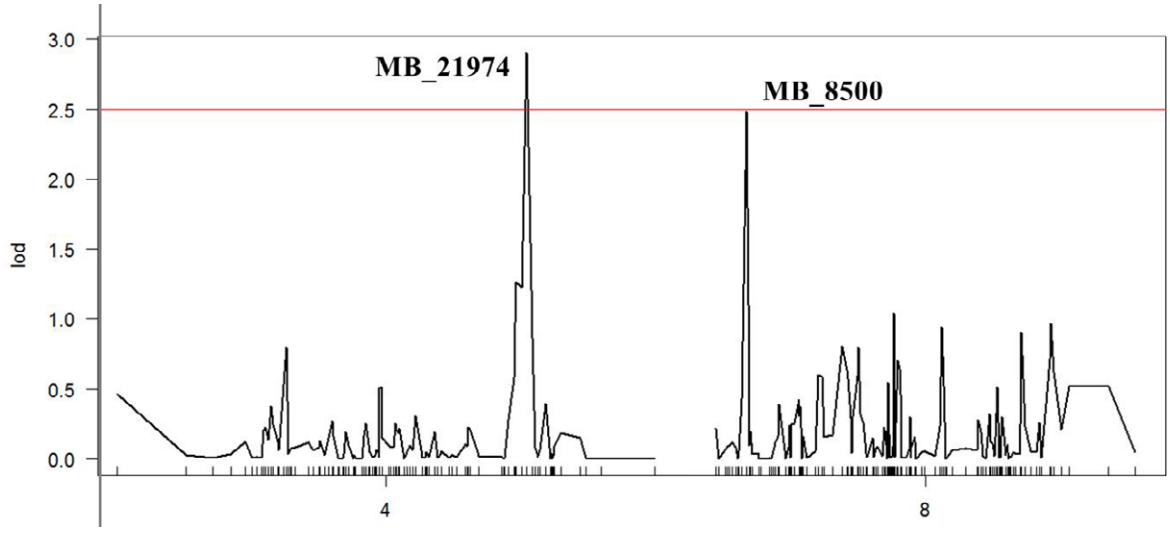

Figure 2. QTL detection for field resistance to cassava bacteria blight in linkage groups 4 and 8 by non-parametric interval mapping The Y-axis indicates the LOD values and the X-axis the linkage group with the corresponding molecular markers. The QTL peak is shown with the SNP peak marker. The red line indicates the LOD 2.5 threshold.

markers MB_2801 and MB_14991 (Table 2). The physical distance from the peak marker to the flanking markers could not be established due to the two flanking markers belonging to different scaffolds in the cassava genome v4.1.

For each QTL interval, the corresponding genomic regions were searched for the presence of coding regions based on the new cassava genome version v6.1 (phytozome.com). The positions of the SNPs markers MB_21974 (peak marker) and MB_21980 (flanking marker) of *QLB-4* match with the gene *Manes.07G062100*. According to PFAM (Protein Family) the annotation, this gene coded for a protein related to the vacuolar-sorting receptor 3. The position of the other flanking marker of *QLB-4* (MB_25367) matches with the gene *Manes.07G053100* which following the PFAM annotation corresponds to a serine protease carboxypeptidase. While in the interval of *QLB-8* two genes were detected: *Manes.S010100a* and *Manes.03G002800*, which code for a C2HC zinc finger-containing protein and for a core-2/i-branching beta-1, 6-n-acetylglucosaminyltransferase protein, respectively.

DISCUSSION

The present study evaluated the phenotypic response of 99 full-sib segregant genotypes to CBB in field during the rainy season at Meta (Eastern Plains), one of the most productive areas of cassava in Colombia (Agronet-MINAGRO, 2016). The Colombian Eastern plains belong to the ECZ2 which is characterized by savannas of acid soils, with mean temperature of 26.1 °C and mean precipitation of 400 mm per month (Jorge *et al*., 2001; Ospina *et al*., 2002; Restrepo *et al*., 2004). This ECZ2 has been described as an area with one of the highest incidence of CBB in Colombia (Jorge, 2001). Several studies have shown that *Xam* populations present ecozone-differentiation as well as pathogenic specialization to the local adapted cassava material (Restrepo and Verdier 1997; Restrepo, 1999). Thus, the QTL reported here could be useful for further breeding strategies whose interest will be developing new CBB-resistant cassava varieties highly adapted to this ECZ. It will be important to carry out studies on the pathogen in this area in order to dissect the current status of the presence of different *Xam* strains and its dynamics. The last information available on *Xam* in this particular ECZ was obtained almost two decades ago (Restrepo, 1999).

An adequate high disease pressure was observed during the field evaluation. Differences in the severity of the disease between parental genotypes, as well as differences within the individuals of the mapping population, could be detected. Based on the phenotype data obtained it was possible the identification of two QTL explaining 10.9 and 12.6 % of cassava resistance to *Xam*. In each of these QTL regions were found two coding genes, representing novel candidate genes for CBB resistance.

A higher number of susceptible genotypes (61.6 %) compared to the susceptible genotypes were identified in the mapping population. Due to the phenotypic evaluation was performed during a rainy year, it is expected that the high humidity had contributed to this finding. This is consistent with several studies showing that high humidity favored the development and speed of symptoms as well *Xam* growing and dispersion (Leu, 1978; Banito *et al*., 2000; Banito *et al*., 2001; Wydra and Verdier, 2002; Restrepo, 2004).

Both, resistant and susceptible transgressive segregants were identified in the phenotypic evaluation of the mapping population. Ten resistant transgressive genotypes were identified. This type of segregation has been described for several crops (Akinwale *et al*., 2010; Whankaew *et al*., 2011; Thanyasiriwat *et al*., 2013; Njenga *et al*., 2014), as well for cassava to CBB resistance in the same mapping population used in this study (Jorge et al., 2000; Jorge et al., 2001). The finding of these segregants suggests the action of additive and dominant genes for CBB resistance in the TMS30571 x CM2177-2 cross. The transgressive genotypes and especially the extreme resistant individuals g23, g89, g92, g93 and g104, became an important source of CBB resistance to be employed in different cassava breeding programs.

In this study two QTL were identified. A previous QTL detection study for CBB conducted also at ECZ2 (Jorge *et al*., 2001) revealed the presence of eight QTL from which six were stable. On greenhouse and controlled conditions with particular *Xam* strains more than twenty QTL have been identified (Jorge *et al*., 2000; Wydra *et al*., 2004; Lopez *et al*., 2007). Several of these QTL were strain-specific QTL. The identification of several resistance QTLs have been achieved through QTL mapping in important pathosystems, some of the most recently reports include QTL the resistance to virus diseases affecting maize (Wang *et al*., 2016), leaf rust in

Table 1. Summary of QTL detected for field resistance to CBB. The QTL name (*Q*=QTL; *LB*=La Libertad; number of linkage group), LOD score, percentage of phenotypic variance explicated (R^2), peak marker and its position in the genetic map, QTL interval, interval length in cM and number of genes within the intervals. ND= Not determinate.

QTL name	LOD score	R^2	Peak SNP Marker	Pos. cM	QTL Interval	Interval length cM	Genes in interval
QLB-4	2,5	12.6 %	MB_21974	98.34	MB_25367- MB_21980	2.4	2
QLB-8	2.9	10.9 %	MB_8500	7.21	MB_2801- MB_14991	1.8	2

wheat (Soriano and Royo, 2015) and *Xanthomonas oryzae* pv. oryzae in rice (Dejedatin *et al*., 2016) within others.

In order to evaluate the stability of the QTL reported here, it will be necessary to perform additional field evaluations during different years and dry seasons. Another important aspect will be to determinate the *Xam* strain (s) present on the infected plants to know if these QTL are strain specific.

The two QTL identified in this study cover a genetic region of 4.2 cM, 2.4 cM for *QLB-4* and 1.8 cM for *QLB-8*, respectively. The QTL cover a short interval length given the high marker density exhibited for this genetic map (Soto *et al*., 2015). In addition, this genetic map was anchored to the cassava genome which allowed the identification of the genes present in the QTL intervals. Even though some associations of candidate genes with QTL have been reported (Faris *et al*., 1999; Ramalingam *et al*., 2003; Liu *et al*., 2004), these types of studies are scarce. Here it was possible to identify four candidate genes in a relative short interval length. These genes are represented by the molecular markers located within the QTL interval. Due to those interval markers belong to different cassava genome scaffolds a physical map region was not established, in consequence, it is possible that more than four genes were located within the physical interval. Nevertheless, the presence of two genes in each of these QTL will facilitate the number of genes to be functionally validated.

The functional annotation of the four genes present within the QTL intervals are not directly related to known plant immunity related genes. Other studies have reported the presence of genes coding for proteins related in plant immunity process as pathogen perception or in signal pathways (Ramalingam *et al*., 2003; St. Clair, 2010; Lopez, 2011). However, some studies have established that the typical immunity-related genes are only a small part of the whole genes related to plant resistance (Corwin *et al*., 2016). Recently, several genes have been cloned from QTL and none of them correspond to classical *R* genes, but have different functions not directly related with pathogen recognition or defense (Poland *et al*., 2009; Bryant *et al*., 2014; Roux *et al*., 2014). Thus, the four genes here detected become new genetic factors that may be playing an important role in CBB resistance. The functional validation of these genes should be addressed in order to deepen the understanding of the cassava response to *Xam*.

CONCLUSIONS

In this study, the phenotypic evaluation of the response of 99 full-sib segregant genotypes to *Xam* infection in field conditions in Meta, Colombia, allowed the detection of two novel QTL associated to CBB resistance. These QTL explained 10.9 and 12.6 % of the field resistance to the disease. Four genes were identified in the QTL intervals. The genes coding for a protein related to the vacuolar-sorting receptor, a serine protease carboxypeptidase, a C2HC zinc finger-containing protein and for a core-2/i-branching beta-1,6-n-acetylglucosaminyltransferase protein. These genes become novel genetic factors which can be playing a role in plant resistance to *Xam*. The QTL and the genes involved in CBB field resistance detected in this study can contribute in local marker assisted-breeding strategies and further map-based studies focus on dissect the major responsible genes governing CBB resistance.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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