Study of inheritance and identification of QTLs linked to Ceratocystis wilt resistance in cacao

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ABSTRACT

Ceratocystis wilt (CW) in cacao, caused by *Ceratocystis cacaofunesta*, is a drastic disease that results in plant death. The pathogen was recently identified in the major cacao-producing region of Brazil-Bahia. There are still many unanswered questions regarding the sources and mode of inheritance to CW. Phenotypic evaluation of CW resistance has been based on qualitative measures of the disease, therefore hindering the determination of the type of inheritance involved in this pathosystem. The identification of genetic markers tightly-linked to disease resistance loci are valuable tools for the development of resistant cultivars using marker-assisted selection (MAS). Branches of 143 six year old progenies of the F_2 Sca 6 x ICS 1 population were wounded by making a 3 mm deep cut with a sterile scalpel, and inoculated with a 20 ul drop of a spore suspension of 3 x 10^4 CFU/ml. Fifteen days after the inoculation (DAI) stems were collected and transported to the laboratory for evaluation. Stems were sliced open vertically above and below the point of inoculation and the length of the xylem discoloration (LXD) recorded. Associations between molecular markers and resistance to CW were evaluated by a simple interval mapping (SIM) and composite interval mapping (CIM) using the software MapQTL v. 5.0. Mean parental disease scores were 12.14 ± 3.5 cm for Sca 6 and 29.57 ± 6.7 cm for ICS 1. The mean disease score for the entire population was 14.22 ± 3.5 cm. Individual progenies varied from 4.28 to 33.75 cm. The length of the LXD followed a continuous distribution. The inoculation method used allowed to quantitatively phenotype the population. QTL analysis using the SIM and CIM revealed two genomic regions located in linkage groups 3 (LXD-LG3) and 9 (LXD-LG9) associated with the expression of the CW resistance with a LODmax of 2.57 and 3.1, respectively. The QTLs explained individually from 6.9 to 8.6% of the phenotypic variation. The identification of two QTLs involved in resistance to CW offers the possibility to improve the durability of resistance in cocoa by a possible accumulation of many different resistance genes located in different chromosome regions using marker-aided selection. The marker alleles used for the introgression survey on MAS can be also used for characterization of unrelated germplasm and finding new sources of resistance.

INTRODUCTION

Ceratocystis wilt (CW) is caused by the fungus *Ceratocystis cacaofunesta* (Engelbrecht and Harrington 2005); a specialized pathogen in cacao (*Theobroma cacao* L.). The fungus generally enters cacao plants through fresh wounds, such as those caused by pruning, pod harvesting (Malaguti 1952) or insect wounds, and moves through the host in the secondary xylem, causing wilting and death. Management practices can reduce the severity of the disease, the use of resistant cultivars would be the most effective way for to control this disease (Swan et al. 2000).

There are still many unanswered questions regarding the sources and mode of inheritance to CW. Phenotypic evaluation of CW resistance has been based on qualitative measures of the disease, therefore hindering the determination of the type of inheritance involved in this pathosystem. The identification of genetic markers that are tightly-linked to disease resistance loci is a valuable tool for the development of resistant cultivars through marker-assisted selection (MAS) that could be applied in the CW breeding program.

The objectives of this study were to: i) test the F_2 Sca 6 x ICS 1 population for CW segregation; ii) understand the genetic control for CW resistance and iii) detect the cacao regions of the genome involved with the expressions of CW resistance using segregating F_2 Sca 6 x ICS 1individuals.

MATERIALS E METHODS

Plant Material

One-hundred and forty three of the F_2 Sca 6 x ICS 1 individuals (eight years old) randomly distributed in three blocks in an experimental field of the Cacao Research Center (CEPEC) of CEPLAC in Bahia, Brazil, were used in this study.

Inoculation and phenotyping

Inoculum was prepared from self-fertile, single-peritecia progeny of the isolate Cf 20 grown on PDA medium (2% potato, 2% agar and 0.1% dextrose) at room temperature for seven days. Each culture was flooded with 10 ml of sterile deionized water and scraped with a sterile spatula. The spore concentration was estimated with a hemacytometer (Orbeco Inc., Farmingdale, NY), and diluted to 3.0 x 10^4 CFU/ml.

Stem inoculations were performed in the field. Stem segments of 1.5 to 2 cm in diameter were wounded by a sterile dissection scalpel used to make an incision in the middle of the branches. Ten microliters of a spore suspension (3.0 x 10^4 CFU/ml) were deposited in each incision (inoculation site). Moistened cotton was placed in the inoculation site and wrapped in parafilm. Plants were observed weekly for development of symptoms. Fifteen days after the inoculation (DAI) stems were collected and transported to the laboratory for evaluation. Stems were sliced open vertically above and below the point of inoculation, and the length of the xylem discoloration (LXD) recorded. Controls were inoculated with distilled water.

The experiment was established under a completely randomized block design, with 149 genotypes (2 parents and 143 F_2 progeny plants), with three replications of single-three plots. For each plant, four branches were inoculated and the values averaged out for the analysis. The analysis of variance, to test the effect of genotype, was done using Proc GLM, in SAS version 9.1.3 (SAS Institute, Cary, NC).

Genetic linkage map

We used a genotyping matrix used by Brown et al. (2005), with addition of markers from Faleiro et al. (2006), Lima et al. (2010) and Santos et al. (2012). Briefly, the map was constructed with the Joinmap 4.0 program (Van Ooijen 2004) using 190 molecular markers.

QTL mapping

Associations between molecular markers and resistance to CW were initially evaluated by a simple interval mapping (SIM) using the software MapQTL v. 5.0 (Van Ooijen 2006). Significance thresholds were determined by data permutation of 10.000 permutations including 5% chromosome-wide level (LODc, $P<0.05$), 7% genome-wide level (LODg, $P<0.07$) and 5% genome-wide (LOD g, $P<0.05$). The 5% chromosome-wide threshold corresponds approximately to the “suggestive linkage" threshold proposed by Lander (1999). In the second step, a composite interval mapping (CIM) referred as MQM (Mutilple QTL Models) was used after an automatic cofactor selection. The proportion of phenotypic variation explained by each QTL was calculated as the R^2 value, and the degree of dominance of a QTL, estimated as the ratio of the dominance to the additive effects.

RESULTS

Phenotypic distribution of cacao resistance to CW

There was significant variation among the genotypes (parents and progenies) in the analysis of variance (p-value < 0.0001, not shown). The phenotypic distribution of LXD was continuous suggesting that resistance was quantitatively inherited. Mean parental disease scores were 12.14 ± 3.5 cm for Sca 6 and 29.57 ± 6.7 cm for ICS 1.

QTLs analyses

QTL analysis using the Simple Interval Mapping (SIM) revealed two genomic regions located in linkage groups 3 (LXD-LG3) and 9 (LXD-LG9) associated with the expression of the CW resistance (Fig. 1) with a LODmax of 2.73 (*** $P \leq 0.01$ by KW analysis) and 3.27 (**** $P \leq 0.005$ by KW analysis), respectively. The QTLs in LG3 and LG9 explained 6.9 to 8.6% of the phenotypic variation of the LXD, respectively.

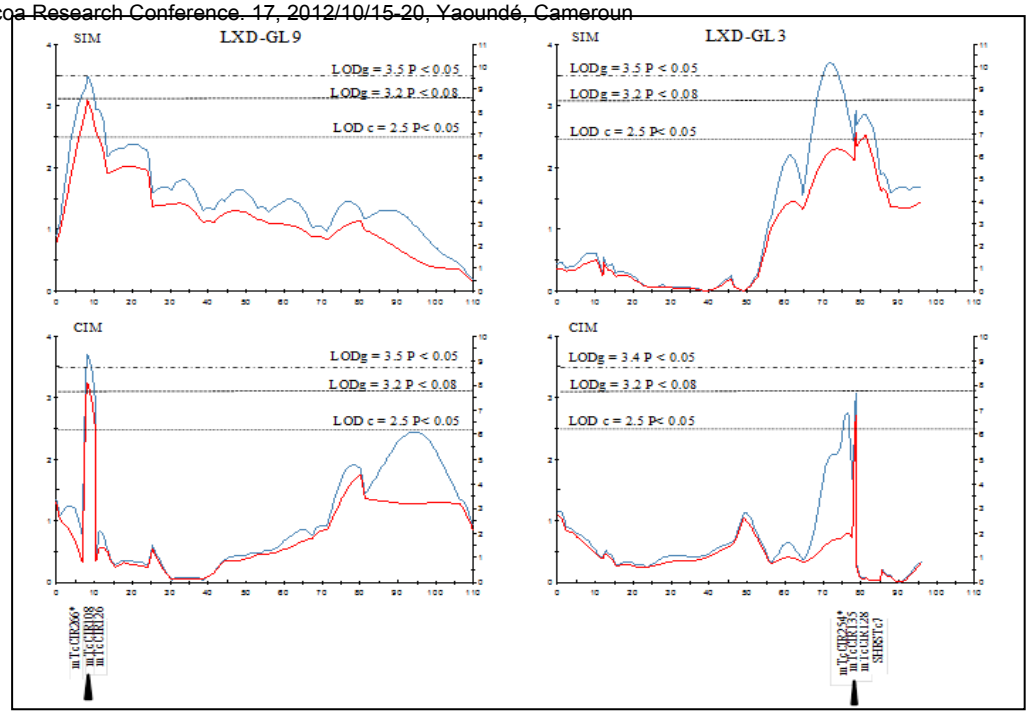

Fig. 1 Graphic display of QTLs related to resistance to Ceratocystis wilt in cacao by MapQTL 5.0 based on the F_2 population of Sca 6 x ICS1. The x-axis indicates the relative position in the linkage map. Red line indicates the LOD and blue line indicates the proportion of the phenotypic variation explained (R^2). LODg = genome wide significance threshold and LODc = chromosome wide significance threshold.

DISCUSSION

The dissemination of *C. cacaofunesta* in the tropical cacao producing region of southeastern Bahia is currently a major concern, requiring efforts for breeding elite cultivars resistant to this plant pathogen. Due to the characteristics of CW of cacao, genetically-based resistance is the most appropriate strategy for adequate control of this disease. Therefore, breeding programs need to assess and develop cacao genotypes that are resistant to CW as fast and reliable as possible.

We observe that the population segregated for resistance to CW. The segregation pattern of the trait in the F_2 population presented a continuous distribution, suggesting that the CW resistance is controlled by multiple genes rather than a single major gene. Transgressive segregation was also observed in this study, whereas individuals outside the range of parents regarding the trait and statistically different from those were observed. The transgressive segregation is of interest to the breeder because it allows the selection of individual which segregates with a number of favorable genes higher than the parents (Ramalho et al. 2000).

We attributed this result mainly to the inoculation method used herein which allowed to quantitatively phenotype the population. Previously, the selection of resistant genotypes to CW (Silva et al. 2005) has been based on: qualitative binary trait based on the mortality and evaluation of young cacao plants; therefore not allowing quantitatively evaluation of the disease. We showed that the inoculation of adult tree branches in the field plus the evaluation of the LXD as proposed by Baker et al. (2003) allowed reducing the variation due to plant age, size of the portion of the inoculated plant and number of replications.

The results of QTL mapping analyses are indicative of two suggestive regions of the genome, LXD-GL3 (LODmax = 2.73, R^2 = 7.7) and LXD-GL9 (LODmax = 3.27, R^2 = 9.3), with a positive additive effect of 0.48 and 1.56, respectively, increasing resistance to CW (Fig. 2). Lander and Kruglyak (1995) propose the term "suggestive linkage" to allow for the publication of results that are not significant but point to a certain level of association between markers and trait. This term has been adopted to consider suggestive QTLs based on chromosome-wide significance, and although, the LOD scores of the QTLs were lower than genome wide significant threshold (3.5 at P<0.05) we believe that the identified QTLs are suggestive of significant QTLs considering: (i) chromosome-wide thresholds were already used in several published works; (ii) using the genome-wide method, the LOD score of LXD-GL 9 is 3.27 at P<0.08, which is very close to the critical threshold limit; (iii) according to Van Ooijen (1999), in the case of F_2 segregation data, the threshold for significant QTL is fixed on 2.7. According to this criterion, both QTLs GL9 and GL3 could be considered as significant; (iv) we also applied to the data a Kruskal-

Wallis (KW) test, which is a single marker analysis based on ANOVA. Using this method we observed that the identified QTLs LG9 showed significance by KW analyses at $P \leq 0.005$; and (v) the phenotype means indicated that one allele combination was markedly better at each location.

The exact number of loci involved in the expression of this quantitative characteristic is still unknown. The assumption that the quantitative inheritance is involved in the control of resistance to CW is also supported by the identification of QTLs explaining a low portion of the phenotypic variation (7.7 and 9.3%). The variance not explained by the QTLs in this study may be due to an undetected small effect QTL or to epistatic interactions between QTLs.

In conclusion, the present study presents novel approaches to study cacao-*C. cacaofunesta* interaction, certainly of great value, which allowed to: (i) determine the quantitative nature of CW inheritance, (ii) identify suggestive regions linked to CW resistance with the aid of functional markers developed by Santos et al. (2012), and (iii) develop a methodology that allowed the identification of resistant genotypes in field conditions without killing valuable phenotypes. The identification of two QTLs involved in resistance to CW offers the possibility to improve the durability of resistance in cocoa by a possible accumulation of many different resistance genes located in different chromosome regions using marker-aided selection. The marker alleles used for the introgression survey on MAS can be also used for characterization of unrelated germplasm and finding new sources of resistance.

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