Quantitative trait loci controlling cyanogenic glucoside and dry matter content in cassava (*Manihot esculenta* Crantz) roots

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Cassava (*Manihot esculenta* Crantz) is a starchy root crop grown in the tropics mainly by small-scale farmers even though agro-industrial processing is rapidly increasing. For this processing market improved varieties with high dry matter root content (DMC) is required. Potentially toxic cyanogenic glucosides are synthesized in the leaves and translocated to the roots. Selection for varieties with low cyanogenic glucoside potential (CNP) and high DMC is among the principal objectives in cassava breeding programs. However, these traits are highly influenced by the environmental conditions and the genetic control of these traits is not well understood. An S_1 population derived from a cross between two bred cassava varieties (MCOL 1684 and Rayong 1) that differ in CNP and DMC was used to study the heritability and genetic basis of these traits. A broad-sense heritability of 0.43 and 0.42 was found for CNP and DMC, respectively. The moderate heritabilities for DMC and CNP indicate that the phenotypic variation of these traits is explained by a genetic component. We found two quantitative trait loci (QTL) on two different linkage groups controlling CNP and six QTL on four different linkage groups controlling DMC. One QTL for CNP and one QTL for DMC mapped near each other, suggesting pleiotrophy and/or linkage of QTL. The two QTL for CNP showed additive effects while the six QTL for DMC showed additive effect, dominance or overdominance. This study is a first step towards developing molecular marker tools for efficient breeding of CNP and DMC in cassava.

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Cassava (*Manihot esculenta* Crantz) is a tropical root crop that is widely grown as a staple food and animal feed in countries of tropical and subtropical Africa, Asia and Latin America. It ranks fourth in production among all tropical crops, standing at 192 million tons per year in the world (FAO 2004). More than 70% of this production is in Africa and Asia from small-scale farmers by virtue of its remarkable tolerance to abiotic stresses and adverse environments. Its main value is in its storage roots though in some areas, particularly in Africa, young leaves are also harvested and processed for human consumption as a vegetable (Lancaster and Brooks 1983). The storage roots can be harvested from 6–24 months after planting depending on cultivar and growing conditions (Cock 1985). Fresh roots of cassava may differ in dry matter content (DMC, 10%–50% with an average of about 30%) depending on genotype, age and environmental condition (Kawano et al. 1987; Chavez et al. 2005). On average, about 90% of DMC is carbohydrates (Kawano et al. 1987). Cassava also produces cyanogenic glucosides, which are synthesized in the leaves and translocated to the roots (Koch et al. 1992; Siritunga and Sayre 2003; Jørgensen et al. 2005). Cassava varieties with high cyanogenic glucoside levels (>1000 mg hydrogen cyanide (HCN) equivalent kg^{-1} dry weight) are said to be toxic while cassava with low levels of cyanogenic glucosides (<200 mg HCN equivalent kg^{-1} dry weight) are considered to be safe for consumption without processing (Iglesias et al. 2002). Hydrogen cyanide (HCN) in cassava tissues has been medically proven to be a potential health hazard for consumers if the plant is inadequately processed (Mlingi et al. 1992; Tylleskär et al. 1992). Genotypes with high levels of cyanogenic glucosides must therefore be processed, for example by fermentation, to remove HCN and its toxic precursors (Essers et al. 1995). Farmers' varieties with high levels of cyanogenic glucosides have in general bitter taste and are referred to as bitter while those with low levels of cyanogenic glucosides are called sweet or cool varieties (Chiwona-Karltun et al. 2004). Although bitterness is mainly attributed to cyanogenic glucosides in the roots other compounds in the parenchyma and cortex have been detected to contribute to the taste (King and Bradbury 1995). In fact, some studies have shown a strong positive correlation between bitterness and level of cyanogenic glucosides

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in cassava roots (Sundaresan et al. 1987; Chiwona-Karltun et al. 2004), while others have found that bitterness is not always correlated with the level of cyanogenic glucosides (Gondwe 1974; Sinha and Nair 1968). The preference and proportions of the bitter and sweet varieties grown by the farmers differ in different areas (Salick et al. 1997; Elias et al. 2001; Balyejusa Kizito et al. 2006) depending on their cultures (Westby 2002). Since most of cassava production in Africa is for human consumption the farmers tend to emphasize cooking quality or starch characteristics (Ceballos et al. 2004). It has also been observed that bitter cassava improves the food security for many small-scale farmers because they are less prone to predation and theft (Essers et al. 1995; Chiwona-Karltun et al. 1998).

During the past 30 years significant progress has been made in cassava breeding and selection for the major traits such as improved yield, improved plant architecture and resistance or tolerance to pests and diseases (Kawano 2003). In Africa, due to repeated cassava mosaic disease (CMD) pandemics, breeding has tended towards development of varieties with CMD resistance (Thresh and Cooter 2005). Even though cassava is mainly grown by small-scale farmers its use for agro-industrial processing is increasing in Asia (Kawano et al. 1998). For this processing market improved varieties with higher root yield and DMC is required (Kawano et al. 1998). Selection for low levels of cyanogenic glucosides and high DMC is among principal objectives in cassava improvement program (Dixon et al. 1994). However, cyanogenic glucoside content, also known as cyanogenic glucoside potential (CNP), is one of the least understood agronomic traits in cassava due to its phenotypic plasticity (Bokanga et al. 1994). In addition, DMC in roots is significantly influenced by age at harvest, genotype, location and season of harvest (Kawano et al. 1987). Dixon et al. (1994) found that the phenotypic values of CNP and DMC in cassava were negatively correlated ranging from -0.73 to -0.55 depending on genotype and location. Given the long growth cycle of cassava and that CNP and DMC traits are probably controlled by several genes with influence from the environment, marker-assisted selection (MAS) for these traits would be an important tool in the breeding. The identification and mapping of quantitative trait loci (QTL) controlling these traits and the identification of nearby marker loci to the QTL can help improving the efficiency of the selection process. QTL analysis also gives additional information on the inheritance of the traits that could be used in cassava breeding programs.

Here we report the results of QTL mapping analyses of the genetic basis for the CNP and DMC differences in roots at a relatively early developmental stage, harvested 5 months after planting (MAP), between two cassava varieties.

MATERIAL AND METHODS

Plant materials and field experiment

The cassava variety Rayong 60, accession MTAI 8 at the Germplasm Bank at CIAT, an F_1 hybrid between a Colombian variety, accession MCOL 1684 and a Thai variety Rayong 1, accession THAI 1 was selfed to produce the S_1 mapping population AM 320 of 199 individuals used in this study. The population was developed at the Centro Internacional de Agricultura Tropical (CIAT), Cali Colombia. The MCOL 1684 and the THAI 1 accessions have been shown to differ in CNP. An average CNP of 882.9 mg HCN equivalent kg^{-1} dry weight with a 95% confidence interval of 1048.7–717.0 and 321.0 mg HCN equivalent kg^{-1} dry weight with a 95% confidence interval of 272.2–369.8, respectively, was obtained from six different field experiments. Segregation of CNP was also found within the S_1 population, where the genotypic mean for CNP ranged from 260 to 1569 mg HCN equivalent kg^{-1} dry weight (Fig. 1). Also DMC segregated within the S_1 population, where the genotypic mean for DMC ranged from 29.8% to 42.9%.

The germination of the S_1 seeds from embryo axes in vitro was according to standard procedures (Fregene et al. 1998) for safe keeping of the QTL mapping population. The plantlets were then micro-propagated in vitro, hardened in a screenhouse and thereafter transferred to the field for further cloning to obtain sufficient cuttings for the experimental set up.

The field experiment was established in August 2004 at CIAT headquarters in Palmira, Valle del Cauca department (3°31′N, 76°21′W), situated in the mid-altitude tropics of Colombia (at 1000 m a. s. l.). The mean temperature is $25+1$°C monthly and the annual rainfall is about 1000 mm. The soil has a mollisol texture (Lian and Cock 1979a, 1979b) and the water holding capacity of the soil is such that cassava rarely suffers from water stress at this site. Selected mature stem cuttings from each of the 199 S_1 clones were used in the experiment. The experimental layout was a randomized complete block design (Chahal and Gosal 2002) with eight blocks, where each block contained one plant per genotype. Borderline plants from a different variety were included. Cuttings of 199 genotypes were planted vertically on ridges at a spacing of 1×1 m. The experiment was weeded regularly and no fertilizers were applied.

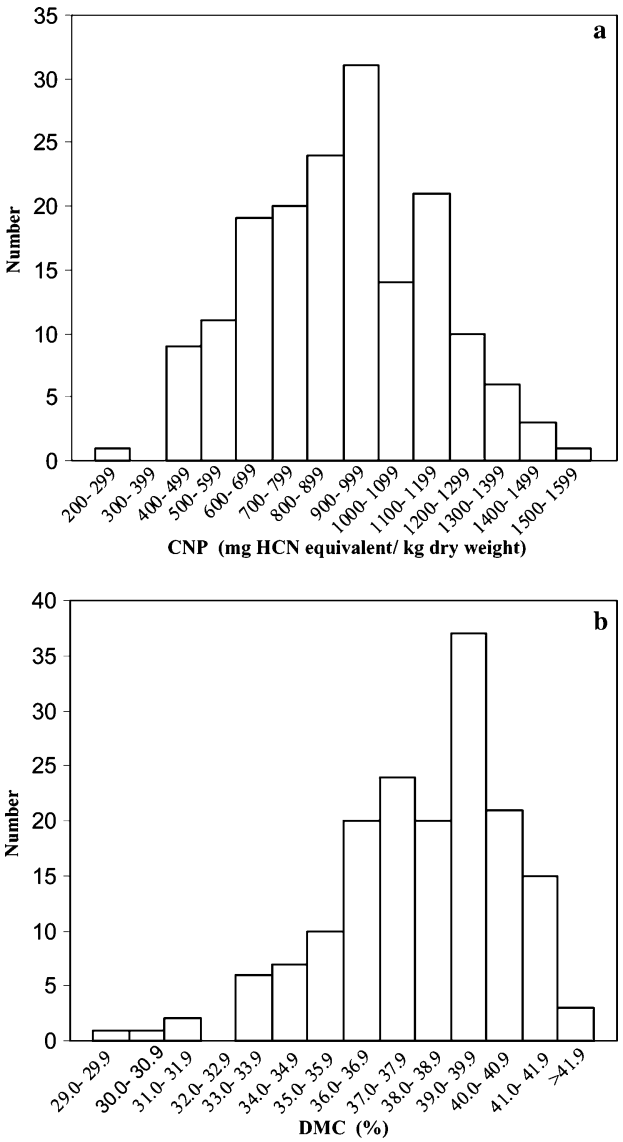

Fig. 1. Phenotypic distribution of the genotype mean (**a**) cyanogenic glucoside potential (CNP) and (**b**) dry matter content (DMC) in the S_1 population.

Phenotypic measurements

The experiment was harvested in January 2005 at 5 MAP and the roots were immediately taken to the laboratory for CNP and DMC analyses. All tuberous roots on a plant were considered. Calculations of DMC were made by measuring specific gravity by weighing roots in air and then in water (weight in air/ weight in water − weight in water). This method is based on the correlation which exists between root specific gravity, DMC and starch content (CIAT 1976). The DMC (%) was determined using the formula: DMC% = [158.3 × (weight in air/weight in water − weight in water)] (OKOGBENIN and FREGENE 2002). CNP was measured using the enzymic assay developed by COOKE (1978) and modified by O'BRIEN et al. (1991).

Marker analysis

DNA was isolated from young leaf tissue by CTAB method (DOYLE and DOYLE 1987). The S_1 cassava genotypes were analyzed at 110 simple sequence repeat (SSR) markers. PCR was carried out using 10 ng of DNA per reaction following MBA et al. (2001). The PCR product was denatured and electrophoresed on 6% polyacrylamide gels using Bio-Rad sequencing apparatus (Bio-Rad Inc., USA) and visualized by silver staining according to the Promega manufacturer's guide. The genotypes of the S_1 plants were then determined based on the genotypes of the two parents and the F_1 hybrid, which were included as controls on each gel.

Data analyses

The phenotype data was submitted to analyses of variance (ANOVA) using the JMP program version 3 (SAS Inst. 1994). Thirty-nine different genotypes had poor vigour resulting in few or none replicates in the field experiment and were therefore not included in the ANOVA and the QTL analysis. The final phenotypic data included 160 genotypes of the S_1 population with the genotype effect considered random. The ANOVA procedure was performed according to the model:

$$Y_{ij} = \mu + B_i + G_j + e_{ij}$$

where Y_{ij} is the phenotypic value for the jth genotype in the ith block, μ is the overall mean, B_i is the fixed effect of the block, G_j is the random effect of the jth genotype and e_{ij} is the residual error. The variance components were calculated using the restricted maximum likelihood method (REML) with the computer program ASReml version 2.0 (GILMOUR et al. 2006). Broad-sense heritability (H^2) was estimated using the variance components in the formula:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$$

where σ_g^2 and σ_e^2 are the variance components for the genotype effect and the residual error, respectively, based on individual plants. The phenotypic and genotypic correlations between CNP and DMC were also calculated. Standard errors ($\pm$SE) of these correlations and the heritability for CNP and DMC were estimated based on Taylor series expansion.

A linkage map of cassava was earlier drawn using 95 SSR markers and 104 individuals of the AM 320

population (CIAT 2003). We reconstructed the linkage map based on an additional 95 individuals of the AM 320 population and 15 new SSR markers using the MAPMAKER linkage analysis software, version 2.0 (LANDER et al. 1987). However, during the reconstruction of the map 12 of the previous SSR markers were excluded since they did not map to any of the linkage groups (LG) in the new map. We tested each of the markers for normal Mendelian segregation using χ^2-tests with a significance level of 0.05 corrected according to the Bonferroni-Holm sequential method (RICE 1989). The cassava genome was scanned for the presence of a QTL effect at 2.0 cM intervals using composite interval mapping (CIM) in the computer package QTL Cartographer version 1.15 (BASTEN et al. 1997). CIM combines interval mapping (which calculates the ratio of the likelihood that there is a QTL to that there is not a QTL at any position in the interval between two markers or at the markers themselves) with multiple regression so that the most significant markers outside the test interval will be included in the model (ZENG 1993, 1994). We used model 6 of BASTEN et al. (1997) with the five most significant markers as genetic background parameters and a window size of 10 cM on either side of the markers flanking the test site. All QTL above a LOD score (the strength of the data supporting a QTL) of 2.5 were presented according to LANDER and BOTSTEIN (1989). Empirical experiment-wise threshold values for significance ($P = 0.05$) were estimated from 1000 permutations of the data for each trait (CHURCHILL and DOERGE 1994). Using the dominance (d) and additive (a) values given for each QTL by the program the ratio of d/a was calculated.

RESULTS

Phenotypic analyses

We found a significant difference between S_1 genotypes for both CNP and DMC (Table 1). The distribution of the phenotypic traits in the S_1 population revealed continuous variation, typical of quantitative traits (Fig. 1). The mean CNP for the S_1 plants was 903.0 mg HCN equivalent kg^{-1} dry weight with a 95% confidence interval of 864.3–941.7 and the mean DMC was 37.7% with a 95% confidence interval of 37.3–38.1. The broad-sense heritability was 0.43 ($\pm SE = 0.04$) for CNP and 0.42 ($\pm SE = 0.04$) for DMC (Table 1). The moderate heritability observed for CNP and DMC indicates that the phenotypic variation in these traits has a genetic component thus making the population suitable for QTL mapping. Weak negative genotypic ($-0.22, \pm SE = 0.09$) and phenotypic correlations ($-0.24, \pm SE = 0.04$) were found between DMC and CNP.

Marker segregation

After checking for normal Mendelian segregation (1:2:1 for co-dominant loci, $P < 0.05$), we found distorted segregation for 17 of the 98 marker loci (17.3%) at a significance level of 0.05 corrected according to the Bonferroni-Holm sequential method (RICE 1989).

QTL for CNP and DMC

Two QTL for CNP were found on linkage groups (LG) 10 and 23, respectively (Table 2, Fig. 2). For DMC six QTL were detected, two on LG 3, two on LG 6, one on LG 10 and one on LG 17 (Table 2, Fig. 2). The QTL on LG 10 for DMC mapped close to the QTL found for CNP on the same LG. The maximum likelihood positions of the QTL for CNP and DMC varied in distance to their nearest flanking molecular marker locus (Fig. 2). One of the QTL for DMC on LG3 mapped at the marker locus SSRY9 and the QTL for CNP on LG23 mapped close to marker locus NS119. The rest of the QTL for these two traits mapped in between their flanking marker loci. All the QTL for DMC and CNP reported showed a LOD score above 2.5 (LANDER and BOTSTEIN 1989). However, only the QTL on LG 10 for CNP near marker locus SSRY105 and the QTL on LG 3 at marker locus SSRY9 and the QTL on LG 6 closest to marker locus SSRY32 for DMC were significant according to the permutation test (Table 2). The significant LOD threshold for CNP was 3.9 while for DMC it was 5.2. In some of the permuted data sets for DMC, the ECM algorithm 'bailed out' between pairs of markers due to numerical problems. This may influence the estimation of the significant threshold based on the permutation test.

Gene action and magnitudes of effect

The two QTL for CNP were both additive (Table 2). The QTL for CNP near marker locus SSRY105 had the larger additive effect (162 mg HCN equivalent kg^{-1} dry weight). The other CNP QTL at locus SSRY242 also contributed considerably to the additive effect (99 mg HCN equivalent kg^{-1} dry weight). The DMC QTL on LG6 closest to marker locus SSRY45 showed the largest additive effect (2.38%) while the other QTL on LG6 for DMC showed the largest dominance effect (2.90%). Five of the six QTL for DMC showed dominance or overdominance and one QTL showed additive gene action. The two QTL found for CNP explained 7% and 20%, respectively of the phenotypic variation (R^2) in the S_1 population (Table 2). Individual QTL for DMC explained 14% to

Table 1. *Heritability (H^2) with standard error ($\pm SE$), analysis of variance for cyanogenic glucoside potential (CNP) and dry matter content (DMC), genetic (r_{gen}) and phenotypic correlations (r_{phen}) between CNP and DMC with standard error in the S_1 population.*

	$H^2 \pm SE$	Source of variation	Degrees of freedom (df)	MS [1)]	Variance component [1)]
CNP	0.43 ± 0.04	Block	7	626083***	–
		Genotype	159	–	49250***
		Error	942	–	66225
DMC	0.42 ± 0.04	Block	7	86.2***	–
		Genotype	159	–	5.55***
		Error	942	–	7.68
r_{gen} ($\pm$SE)	-0.22 ± 0.09				
r_{phen} ($\pm$SE)	-0.24 ± 0.04				

[1)] *** significant at $P<0.001$.

40% of the variance. The relatively high R^2 values found for some QTL with lower LOD scores for DMC may be influenced by large distances between flanking markers.

DISCUSSION

Until recently the selection program in cassava have been conducted without much knowledge of the genetic architecture of the selected traits. A lot of effort has been put into breeding for major traits important for productivity such as root yield, DMC and resistance or tolerance to diseases and pests in cassava (Kawano 2003). Breeding for CNP in cassava is complicated because evaluation is considered time-consuming and is highly influenced by the environment (Dixon et al. 1994). DMC in roots is also significantly influenced by growing conditions and season of harvest (Kawano et al. 1987). MAS has the potential to make field-based breeding for CNP and DMC improvement more efficient. We have used a QTL mapping approach to study the genetic control of CNP and DMC in cassava. We have found two QTL on two different LG controlling CNP and six QTL on four different LG controlling DMC. One QTL for CNP and one QTL for DMC mapped near each other (Fig. 2, Table 2).

The translocation of carbohydrates to the roots changes with age of the cassava plant (Alves 2002) and the onset of the root bulking differs among genotypes (Okobenin and Fregene 2002). Because of the correlation which exists between DMC and starch content in cassava roots (Ciat 1976) DMC is also influenced by the age and genotype of the plant. In our study, conducted in a single environment, DMC had a broad-sense heritability of 0.42. In a study comprising five sets of trials conducted in Nigeria between 1988 and 1990 by Dixon et al. (1994) the broad-sense heritabilities of CNP varied between 0.00 to 0.50 depending on the composition of

Table 2. *Quantitative trait loci (QTL) for cyanogenic glucoside potential (CNP) and dry matter content (DMC), their locations reported by linkage group (LG), nearest flanking molecular marker locus, LOD score, additive effect (a) listed as a trait unit contribution, dominance (d) effect, dominance and additive ratio (d/a), gene action and proportion of phenotypic variance explained by a QTL (R^2).*

Trait	LG	Marker	LOD	a	d	d/a	Gene action	R^2
CNP	10	SSRY105	8.4***	161.96	4.46	0.03	additive	0.20
	23	SSRY242	3.2	98.96	10.35	0.10	additive	0.07
DMC	3	SSRY9	5.3*	1.72	0.81	0.47	additive	0.14
	3	SSRY313	3.0	0.33	2.31	7.00	overdominance	0.20
	6	SSRY32	5.9*	1.32	2.90	2.20	overdominance	0.40
	6	SSRY45	3.0	2.38	1.39	0.58	dominance	0.34
	10	SSRY223	2.8	1.86	1.45	0.78	dominance	0.21
	17	SSRY41	2.5	1.15	2.81	2.44	overdominance	0.37

*** significant at $P<0.001$, * significant at $P<0.05$ with the permutation test.

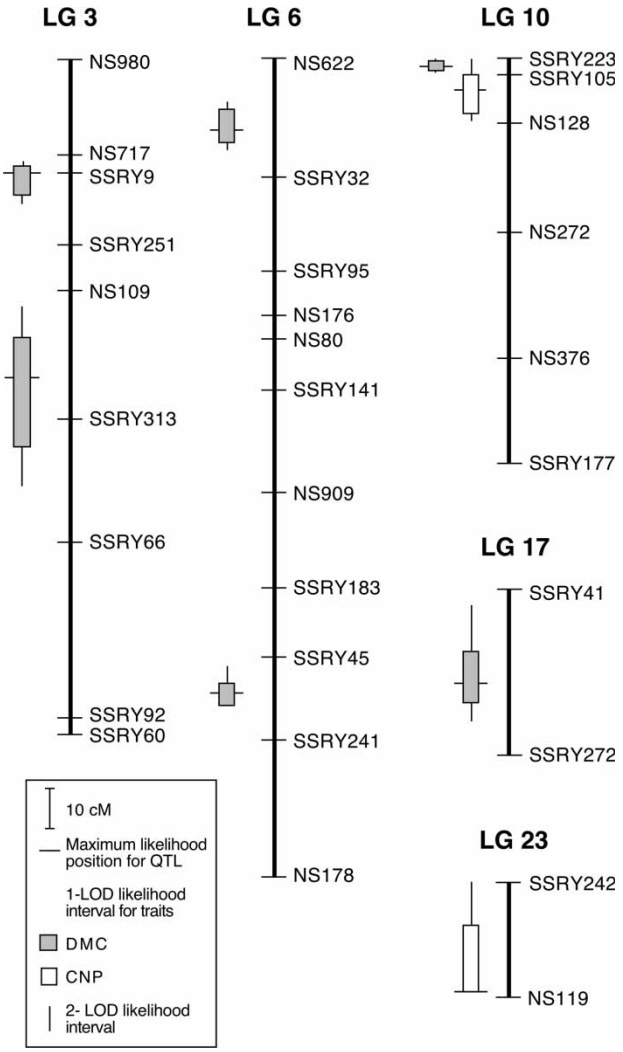

Fig. 2. Linkage groups for the S_1 population showing the positions of quantitative trait loci for cyanogenic glucoside potential (CNP) and dry matter content (DMC).

genotypes, locations, seasons and number of years evaluated. Broad-sense heritabilities ranging between 0.50 and 0.97 for DMC were estimated by Kawano et al. (1998) at different evaluation stages in a breeding program for cassava in Asia. Benesi et al. (2004) found that a large part of the total phenotypic variation in DMC was due to genetic differences in an experiment with bred and local cassava clones in Malawi. In our study a broad-sense heritability of 0.43 was found for CNP in the AM 320 population. The moderate to large heritabilities found for DMC and CNP indicate that the phenotypic variation of these traits is explained by a genetic component. However, it may be difficult to compare the heritability estimates of the different DMC studies discussed as different cassava material and plant age have been used. They have also been conducted in different number of locations, seasons and years.

The two QTL controlling CNP showed high additive effects while most QTL for DMC showed dominance or overdominance. Overdominance may indicate a heterozygote advantage. It can also be a result of two closely linked QTL for DMC both showing dominance effects. The large dominance effect that we observed for DMC is in contrast to the diallel study by Cach et al. (2005) where additive effect plays a more important role than the dominance effect for DMC in cassava. This may be due to the specificity of the cross, differences in the environmental conditions and the age of the cassava plants at harvest. In addition, we may not have a complete picture of the genetic background of DMC since it is likely that we have not been able to detect all QTL. This may also be true for CNP.

In our study we have found a weak negative phenotypic and genotypic correlation between CNP and DMC. We detected only one genomic region where a QTL for CNP and a QTL for DMC mapped together. This may partly account for the weak phenotypic correlation found between these traits. The clustering of the two QTL could either be a result of pleiotropic effect where a single QTL affects the expression of both CNP and DMC. Alternatively, it could be two closely linked QTL, each controlling one of the traits. The finding that most QTL for DMC did not map near QTL for CNP shows that these traits are at least partly controlled by different genetic backgrounds.

Since DMC is controlled by QTL showing additive effect, dominance or overdominance a simple recurrent selection program is not sufficient to capture the potential gain. A reciprocal recurrent selection program that takes into account both additive and dominance effects would therefore be a better strategy in cassava breeding. Dominance has been found in many other traits in cassava and this strategy has also been suggested by Cach et al. (2005). The fact that some improved cassava varieties are not adopted by small-scale farmers in some areas is a clear indication of differences in selection criteria between small-scale farmers and breeders. This therefore shows a need for the breeders to relate farmers' criteria to the researchers' tools. The fact that taste (which in farmers' varieties correlates with levels of CNP, Chiwona-Karltun et al. 2004) is important for small-scale farmers and a variety can be rejected on the basis of taste suggests that breeders should prioritize selection for this trait as early as possible in the breeding cycle. In addition, considering the different preferences of the small-scale farmers it would be important to breed

towards different levels of CNP for the small-scale farmers. However, if the taste is not always only determined by CNP as suggested by some studies (Gondwe 1974; Sinha and Nair 1968; King and Bradbury 1995) the genetic background for CNP and the taste may be partly different. The breeders need then to consider both traits in the breeding of cassava.

It is important to have in mind that the QTLs that we have detected for CNP and DMC at 5 MAP may not be found for cassava plants in later developmental stages, plants grown in other environments or in crosses between other varieties. The result of this study is therefore limited to this particular cross and at a relatively early plant age, and cannot be directly used in cassava breeding programs. However, this study has been a first step towards identifying QTL for CNP and DMC and contributes to the understanding of the genetic basis of two cassava important traits. For future QTL studies of these traits a saturated linkage map is needed. This will help us to find a closer linkage between the molecular marker loci and the responsible genes that will improve breeding based on MAS. It is important to verify if the same QTL for CNP and DMC will be detected at other developmental stages of cassava and in other environments using the AM 320 population. Also studies are needed to investigate the genetic correlation between the level of cyanogenic glucosides and the level of bitter tasting compounds in cassava. Furthermore, it would also be important to study the genetic basis of DMC and CNP in other populations.

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