Activity 8.9 Construction of an SSR Map of Cassava Based upon Linkage Analysis in a F_2 Cross Derived from Non-Inbred Parents and QTL Mapping of Early Bulking.

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Funding: **The Rockefeller Foundation**

Important Outputs

1. Construction of a SSR linkage map of cassava
2. Discovery of QTLs, consistent across different generation with additive or dominant/recessive gene action for dry root yield, fresh foliage weight and harvest index.

Rationale

The QTL mapping early bulking at CIAT has identified a number of major QTLs for this important trait (Okogbenin and Fregene 2002). Last year, a new F_2 mapping population was developed from an F_1 individual to validate the authenticity, magnitude, and action of these QTLs. Quantitative trait loci (QTL) mapping in single generation, full-sib pedigrees of allogamous crops is complicated by the inability to access all information on the genetic architecture of the quantitative trait in question. Furthermore, marker genotype in these mapping populations result from the independent meioses and crossovers in the maternal and paternal parents leading to separate maps for each parent which alters QTL mapping by redefining mating type at a locus level rather than all loci in both parents. (Groover et al. 1994; Van Eck et al. 1994; Grattapaglia et al. 1994).

The new genetic map of cassava was constructed using SSR markers, a relatively easier to use marker system. The use of SSR markers have considerably cut down the time required for the development of a cassava map compared to earlier efforts, from 3 years to a little over a year. Although, 3 F_2 crosses were developed, only one, the cross with the highest number of heterozygous markers in the F_1 parents, was chosen for map development.

Methodology

The F_2 population obtained from an F_1 progeny, K150, of the cassava map population was selected as our F_2 mapping population because of the large number of markers that were heterozygous in K150, more than 60%, and the relatively large number of progeny, 372. Due to poor seedling development of certain genotypes (resulting in senescence in some few cases), only 268 plants of initial 372 in the F_2 population were used for genotyping. The F_2 seedlings were initially germinated in the screen house at CIAT headquarters in Palmira under intensive management and care in February 2000. Seedlings were later transplanted to the field in July 2000 and harvested for planting materials at 11 months after planting (MAP) the following year (2001). Of the 268 genotypes used for mapping analysis, only 207 genotypes with relatively sufficient stem cuttings (12 stakes) of about 25 cm long each could be planted for QTL mapping experiment at Santa Elena, a location 25 Km from CIAT headquarters. Individual genotypes were sown on May 18, 2001 in single row plots of 6 plants each in randomized complete block design of two replications. Plants were planted at 0.8 cm between plants and 1m between rows. All plants were harvested 7 months after planting (MAP). Traits measured include: dry root yield, fresh foliage and harvest index.

DNA was extraction from individual genotypes of the F_2 population has been described earlier (CIAT 2001). For the parental survey, 186 SSR markers developed by Mba et al. (2001), 132 SSR markers from a cassava root and leaf cDNA library (Mba et al., unpublished data), and 154 SSR markers generated by Fregene et al. (unpublished data), a total of about 500 markers, were used. PCR amplification and PAGE gel electrophoresis were as described by Mba et al (2001). For linkage analysis, individuals of the F_2 population were by the three different genotypic classes expected for a F_2 population and Chi square values were computed to test for significant deviations from expected ratios (segregation distortion). Linkage analysis was using MAPMAKER/EXP 3.0 (Lander et al., 1987). Recombination fractions were converted to map distances, centiMorgan (cM), using Kosambi mapping function..

The mean of each genotype over two replications was used for the correlation analysis and QTL mapping. Phenotypic correlations coefficients between yield and components were estimated and tested for significance ($P< 0.05$). Type III mean squares from ANOVA based on General Linear Model procedure (Proc GLM; SAS 1996) was used to calculate broad sense heritability estimates for each trait.

The locations of putative QTLs were determined by interval mapping analysis using MAPMAKER/QTL 1.1b (Patterson et al. 1988). Maximum likelihood estimates of both additive (a) and dominance (d) effects were calculated simultaneously during the genome scan for QTLs as performed by MAPMAKER/QTL. The gene action of a QTL, largely additive, dominant or recessive, can be determined by evaluating the relative likelihood of gene models. To test for additional QTLs, we fixed the position and effect of one QTL, the single QTL model (SQM), then re-scan the genome searching for other QTLs, using a two-QTL model (TQM). Two-QTL map allows each locus to control its fraction of the variance while at the same time estimating the effect of the other. The multi-locus model was used explain how much of the phenotypic variance among the F_2 population for each trait was explained by fitting in the model, QTLs identified in SQM and other additional QTLs detected in TQM.

Results

A total of 122 SSR markers, or 25%, were polymorphic in the F_1 parent and could be scored in the F_2 mapping population (Fig 1). This number is close to what is expected, that is 50% x 50% (average percent polymorphisms of SSRs in cassava x percent polymorphisms expected in a F_1 genotype). Most of the markers surveyed had the expected segregation, a ratio of 1:2:1, for homozygous for parent A, heterozygous and homozygous for parent B respectively. Deviation from the expected 1:2:1 genotype frequency was significant ($P \leq 0.05$) for 33 (27%) of the 122 markers scored. The 122 markers were employed in constructing a linkage map (Fig2). The linkage map consists of 100 markers, 22 markers remained unlinked. The presence of so many unlinked markers suggests that the available SSRs still do not cover the entire cassava genome. The number of linkage groups in this map (22) exceeds the haploid number of chromosomes for cassava, indicating that the map is also unsaturated.

Phenotypic data for DR, FF and HI showing means, standard deviation, kurtosis, skewness, and W-test are summarized in Table 8.14. Data range for each trait measured revealed wide variation in the F_2 population as equally observed in the F_1. All of the traits studied showed continuous distribution as expected for quantitative traits. The heritability (H^2) estimates in the F_2 were 66% for dry root yield (DR), 68% for Fresh foliage weight (FF) and 78% harvest index (HI). The relatively high heritability of the three traits is in agreement with high estimates obtained in the F_1 (Okogbenin and Fregene 2002). A total of nine QTLs (LOD> 2.0)

(three QTLs each) influencing FF. DR and HI were identified by interval mapping analysis on seven linkage groups (Table 8.15). Results revealed that two QTLs (*Dr3* and *Ff3*) fall within a single interval (NS 928 – SSRY 153) separated by only 4 cM (Table 8.15). The direction of the genetic effects of these two QTLs are similar, suggesting that they are probably not different QTLs, providing evidence for gene pleiotropy for DR and FF at this locus.

Seven highly significant two-QTL interactions were identified for FF, and 4 each for DR and HI (Table 8.16). Phenotypic variance (PV) explained for these interactions varied from 11 to 36% with LOD scores ranging between 2.74 and 8.97. Some of the QTLs identified in the single QTL model (SQM) significantly interacted with each other. In some instances, interactions led to highly significant increase in LOD and PV explained. For example, *Dr1* significantly interacted with *Dr13* resulting in LOD of 5.14 and explained PV of 17.2%, which were higher than the sum of LOD scores and PVE for both QTLs under the SQM. All additional QTLs identified in the two-QTL model (TQM) were fitted along with those identified in the SQM in a multi-locus model to determine total phenotypic variance explained among the F_2 progeny for each trait. The total PV explained based on multiple QTL model are 33% for foliage, 44% for DR and 37% for HI.

The gene action of individual QTLs was evaluated by comparing the fits of individual QTL models (Lander and Botstein, 1989). The three QTLs detected for FF revealed different gene actions: *Ff3* had a recessive gene model, *Ff5* dominance, while *Ff9* exhibited additive gene action. Two of the QTLs identified for DR (*Dr3* and *Dr13*) were consistent with a recessive gene model. Two other QTLs, *Hi2* and *Hi9* exactly fit a pure additive model. The additive effect, which is the measurement of the change in a population mean when an allele of a QTL is substituted, showed that *Hi9* increased harvest index while *Hi2* decreased HI. The third QTL for HI (*Hi12*) was recessive.

A comparison was made of QTLs identified in the experiments using F_1 and F_2 crosses. Results reveal that 1, 7, and 3 common QTLs were detected for fresh foliage, dry matter yield and harvest index respectively (Table 8.17).

Future Perspectives

1. Test QTLs that are stable across generations and thatexplain substantial phenoytpic variance (>20%) in a different genetic background.

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Table 8.14 Performances of three traits evaluated in the F_2 population

Trait	Range	Mean	Standard deviation	Skewness	Kurtosis	W-statistic
Fresh foliage (g)	112.50-3900.00	1152.15	583.26	1.08	2.34	0.94*
Fresh root (g)	213.24	213.24	120.62	0.27	-0.43	0.96*
Harvest Index	0.38	0.38	0.14	-0.4	-0.28	0.96*

Table 8.15. QTLs associated with dry root yield (DR),fresh foliage(FF) and harvest index (HI) in the F_2 population.

Trait	QTL	Flanking markers	Length (cM)	Linkage group	LOD	QTL position (cM)	PVE	*a*	*d*	*d/a*	Mode
FF	*Ff3*	NS 928 – SSRy 153	16.3	3	2.95	0.0	7.6	-87.84	274.90	-3.13	R
	Ff5	SSRy 35 – SSRy 284	28.1	5	2.26	10.0	31.1	-414.21	-556.36	1.34	D
	Ff9	SSRy 12 – SSRy 91	31.0	9	2.16	38.0	5.5	-211.98	-21.83	0.10	A
DR	*Dr1*	NS 911-NS 847	17.7	1	2.18	12.0	9.2	0.3	73.70	245.67	DR
	Dr3	SSRy 928 - SSRy153	16.3	3	2.14	4.0	7.3	-21.53	52.40	-2.43	R
	Dr16	NS 33 - SSRy 100	16.3	13	2.25	18.0	6.0	47.35	-64.61	-1.36	R
HI	*Hi2*	NS 149 - SSRy83	7.3	2	6.67	0.0	15.0	-0.08	0.00	0.05	A
	Hi9	SSRy52 - NS 340	3.1	9	2.25	0.0	54.3	0.05	0.00	0.00	A
	Hi12	NS 74 - NS 389	44.4	12	2.10	0.0	4.9	0.01	-0.06	-6.00	A

Individual QTL loci are named by trait (abbreviation indicated in titles) and linkage groups. The LOD score (LOD) and percent phenotypic variance explained (PVE) by the QTLs are presented from the single-QTL model with unconstrained gene action. The additive effect (*a*) dominance deviation (*d*), and ratio of dominance to additivity (*d/a*) for each QTL are presented in their original units. The possible pure modes of gene action (Mode) for each QTL are indicated based on testing of additive (A) and dominant (D, R) models as described in Materials and methods (if $d = 0$, then A, if $d = a$ then D, if $d = -a$ then R). If a model reduced likelihood by 10-fold or more, it was deemed unlikely. When two pure modes of gene action could not be deemed unlikely, the more likely mode was listed first (e.g for *Dr1*, dominance (D) was most likely but recessivity (R) could not be deemed unlikely, thus the mode for this locus is denoted DR. QTL position is position of LOD peak given as distance from the first marker listed in the interval.

Table 8.16 Two-QTL interactions affecting Dry root yield, fresh foliage and harvest index.

Trait	LG	Interval 1	QTL	QTL position (cM)	Interval 2	QTL	QTL position (cM)	LG	PVE	LOD
FF	9	SSRY 12 – SSRy 91	*Ff9*	38.0	NS 717 – SSRy 3	*Ff4a*	14.0	4	12.0	3.07
	9	SSRY 12 – SSRy 91	*Ff9*	38.0	NS 217 – NS74	*Ff12*	0.0	12	11.1	4.02
	9	SSRY 12 – SSRy 91	*Ff9*	38.0	SSRy50 – SSRy 281	*Ff15a*	40.0	15	11.9	2.74
	3	NS 928 – SSRy 153	*Ff3*	0.0	NS 717 –SSRy 3	*Ff4a*	14.0	4	16.0	3.97
	3	NS 928 – SSRy 153	*Ff3*	0.0	SSRy12 – SSRy91	*Ff9*	38.0	9	13.2	4.98
	3	NS 928 – SSRy 153	*Ff3*	0.0	SSRy 50 – SSRy281	*Ff15b*	28.0	15	15.4	3.56
	5	SSRy 35 – SSRy 284	*Ff5*	10.0	NS 717 – SSRy 3	*Ff4b*	8.0	4	36.0	3.48
DR	3	NS 928 – SSRy 153	*Dr3*	4.0	NS 717 – SSRy 3	Dr4	16.0	4	13.2	3.47
	16	NS 33 – SSRy 100	*Dr16a*	18.0	NS 74 – NS 319	*Dr12*	20.0	12	14.6	3.30
	16	NS 33 – SSRy 100	*Dr16a*	18.0	NS 33 – SSRy 100	*Dr16a*	12.0	16	15.4	3.23
	1	NS 911 – NS 847	*Dr1*	12.0	NS 33 – SSRy 100	*Dr16b*	18.0	16	17.2	5.14
HI	2	NS 149 – SSRy 83	*Hi2*	0.0	SSRy 182 – SSRy 148	*Hi8*	8.0	17	22.1	8.97
	9	SSRy 52 – NS 340	*Hi9*	0.0	NS 149 – SSRy 83	*Hi2*	0.0	2	17.0	7.64
	12	NS 74 – NS 389	*Hi12*	0.0	NS 267 – SSRy 1	*Hi18*	26.0	18	13.1	2.76
	12	NS 74 – NS 389	*Hi12*	0.0	NS 149 – SSRy 83	*Hi2*	0.0	2	18.3	8.25

In cases where multiple QTLs affecting a trait were found along the same linkage group, the QTLs are distinguished by letters indicating the temporal order in which they were discovered (e.g. *Ff15a* and *Ff15b*). The LOD score (LOD) and percent phenotypic variance explained (PVE) by the QTLs are presented from the two-QTL model with unconstrained gene

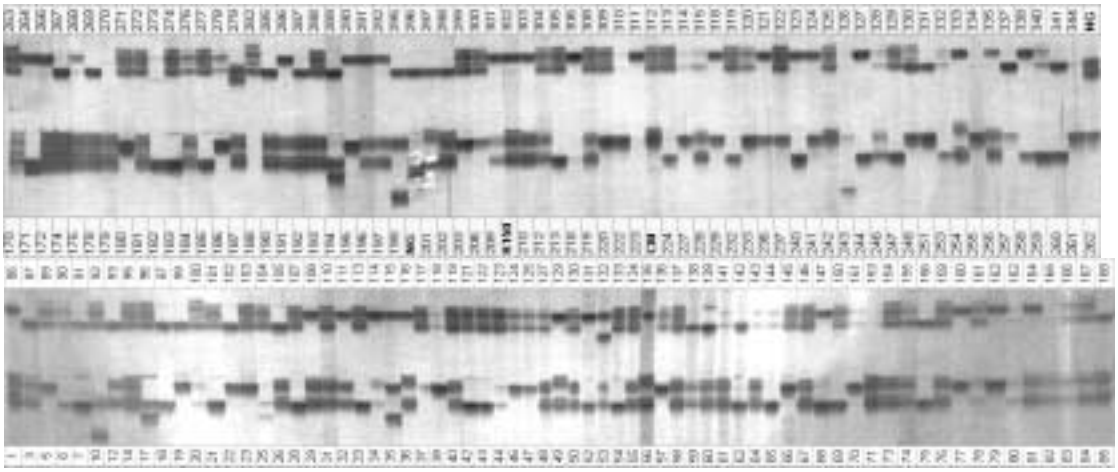

Figure 8.8. Silver stained polyacrylamide gel showing segregation of SSRY marker SSRY 105 in invididuals of the F_2 mapping population.

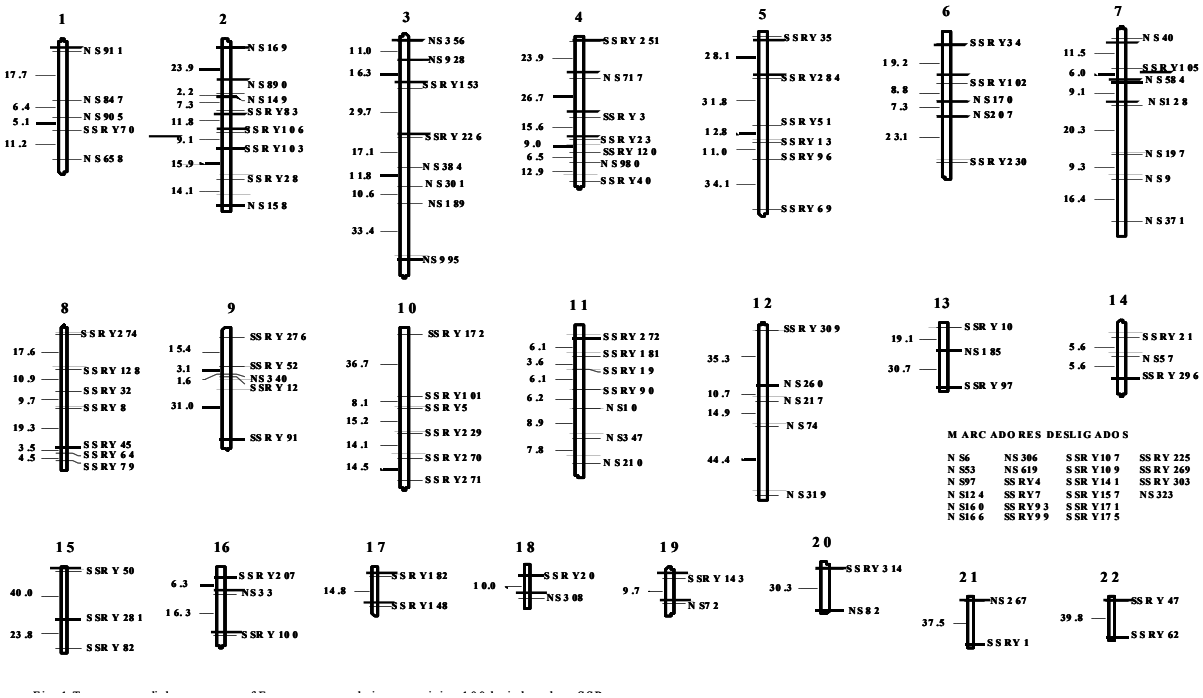

Figure 8.8. A linkage map of cassava (*Manihot esculenta* Crantz) based upon a F_2 cross and SSR markers.

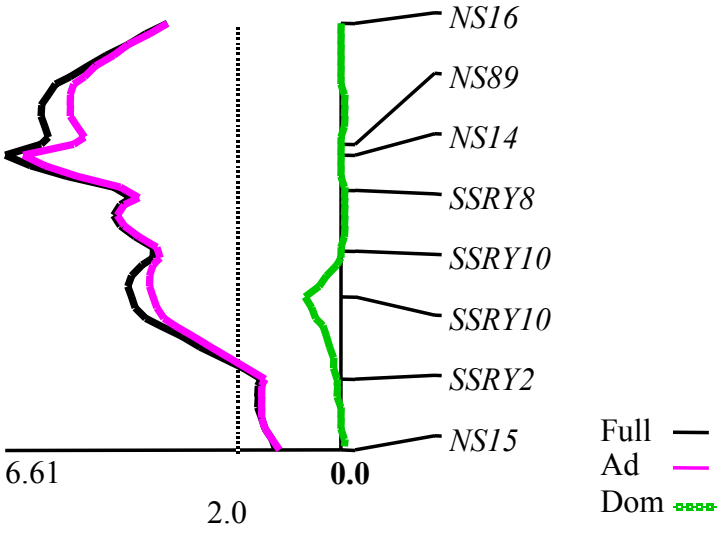

Figure 8.9. A first order QTL for harvest index detected by interval mapping.

Table 8.17. Putative QTLs that were common in the F_1 y F_2 mapping populations for fresh foliage, dry matter yield and harvest index

Trait	QTL F_1	QTL F_2
F.F	GP R (GY48)	GP 22 (SSRY47-SSRY62)*
DR	GP S (GY153-GY212)	GP 1 (NS911-NS847)
	GP G (GY6)	GP 3 (NS928-SSRY53)
	GP D (GY181-GY42)	GP 4 (NS 717-SSRY3)* (SSRY3-SSRY23)*
	GP J (K10)	GP 10 (SSRY5-SSRY229)*
	GP-L (CBB1; CDY131)	GP 18 (SSRY20-NS308)*
	GP UD (GY24)	GP 2 (NS149-SSRY83)
HI	GP E (NGY162)	GP 12 (NS74-NS319)*
	GP A (rBEST)	GP 20 (SSRY 314-NS82)*
	GP J (GY34)	GP 17 (SSRY182-SSRY148)

FF = fresh foilage yield; DR = Dry matter yield; HI = Harvest index. GP = Linkage group. The marker interval where the QTL was found is indicated in parenthesis.

Activity 8.10 QTL Mapping of Cyanogenic Potential (CNP) in Cassava

Collaborators: Elizabeth Kizito (Ph.D. Student, Swedish Agricultural University, SLU, Uppsala, Sweden) Dr Urban Gullberg (SLU, Uppsala, Sweden) Dr Thomas Egwang (Medical Biotech Laboratories MBL, Kampala, Uganda), Dr Anton Bua (NARO, Namulonge, Uganda), Martin Fregene (CIAT)

Funding: **BIOEARN, SAREC,Stockholm, Sweden**

Important Outputs

1. Establishment of a SSR marker lab at the Medical Biotech Laboratories (MBL) for the genotyping of segregating populations.
2. Generation and establishment of cyanogenic mapping populations adapted to Uganda, particularly the cassava mosaic disease (CMD).

Rationale

Cassava produces cyanogenic glucosides which is often rightly or wrongly seen as a health hazard to consumers, particularly in the very poor segment of the population that depend on cassava as a staple. Conventional breeding for low cyanogenic potential (CNP) is fraught with the confounding effect of the environment and developmental stage at which CNP is measured. A project has been initiated in Uganda to map the genes controlling cyanogenic potential (CNP) in cassava with funding from the Swedish project for Biotechnology and Biosafety Research Network in East Africa, (BIOEARN). The identification of molecular

marker associated with CNP will increase the efficiency and the cost-effectiveness of breeding low CNP cassava varieties for Uganda. The BIOEARN project is being conducted as a joint project between the MBL, Swedish University of Agricultural Sciences (SLU), Uppsala, NARO, Namulonge, and International Centre for Tropical Agriculture (CIAT). Activities in the project so far include the development of mapping populations and their establishment at National Agricultural Research Organization (NARO), Namulonge. In addition, facilities for simple sequence repeat (SSR) genotyping of the mapping population have been made available at the MBL. A meeting of partners under the project was planned for mid-may to

a) Resolve bottlenecks in the molecular marker activity
b) Appraise progress made so far and write a short report
c) Make recommendations on future activities

Methodology

Two key requirements for the genetic mapping of CNP are segregating populations with a simple pedigree and easily assayed molecular markers on a genome wide basis. For cassava two kinds of markers are currently available on a genome-wide basis: simple sequence repeat (SSR) markers and restriction fragment length polymorphism (RFLP) markers. The SSR are highly informative and polymerase-chain-reaction (PCR)-based markers making them most appropriate for genetic mapping. The RFLP markers are also informative, but tedious to use, they are therefore being converted to PCR-based markers known as single strand conformation polymorphism (SSCP) markers.

To implement the SSR marker technology at the MBL for genetic mapping of CNP, 8 SSR markers from the Cassava MapPairs were amplified by PCR in 7 parental genotypes, 2 grand parental and 5 parental genotypes, of some of the F_2 mapping progenies using a Perkin Elmer 9700 PCR machine. The PCR reaction was electrophoresed on 6% polyacrylamide gels and visualized by silver staining.

The original mapping population for the study was several F_2 families derived from selfing F_1 progeny from the Gomani x Mbundumali cross. Initial *in vitro* establishment of the sexual seeds from embryo axes was conducted at the tissue culture facility of the Namulonge station using 30 seeds from the F_2 family GMM13. Poor growth of the majority of the cultures was observed. It was then decided to continue establishment in pots filled with sterile soils. An issue raised with the use of the Gomani x Mbundumali F_2s is their poor adaptation to Uganda, particularly adaptation to the devastating cassava mosaic disease (CMD). It was therefore decided to create back-up F_2 families generated from local varieties. A very bitter local variety known as Tongolo was crossed to two CMD resistant varieties, SS4 and TME4. The F_1 progenies were planted directly in the field March 12 and are now 10 weeks old.

Results

The parental survey of parents of the mapping population with more than 500 SSR markers have begun. Results obtained so far reveal a good level of polymorphisms in the parents (Figure 8.10). A summary of families and number of plants expected are shown in Table 8.18. Not all families will be established due to their small sizes.

Table 8.18. Summary of the total number of seeds, viable seeds and expected plants from Gomani x Mbundumali F_2 families.

Code	Total No. of seeds	Total No. of viable seeds	Plants expected (80% germination)
GMM3	333	221	160
GMM96	266	173	138
GMM66	151	121	96

Table 8.19 summarizes the F1 and reciprocals of the Tongolo crosses currently growing in the field at NARO, Namulonge.

Table 8.19. Summary of the total number of plants from the Tongolo x SS4 and Tongolo x TME4 and their reciprocals.

Code	Cross	Number of plants
To be determined	Tongolo x SS4	170
To be determined	SS4 x Tongolo	73
To be determined	Tongolo x TME4	102
To be determined	TME4 x Tongolo	37

It is necessary to keep the field weed free and watered during the oncoming dry season that begins in June. The plants also will greatly benefit from some fertilizer application to ensure that woody stakes can be obtained by December when they will be cloned. At the moment, the crosses have not been given a code, neither have the individual plants been labelled, it is necessary that this be done within the next two months to avoid a mix-up later in the experiments.

Future Perspectives

1. Parental survey of Mbundumali, Gomani, Tongolo, SS4, TME 4 and selected progenies using SSR and SSCP markers. To be included are 5 parents and 7 progenies (a total of 12 samples) to be analyzed with all available markers
2. Harvest of two roots from the new CNP crosses at 7 months after planting (MAP), evaluation for CNP using the picrate method and generation of F2 families for mapping

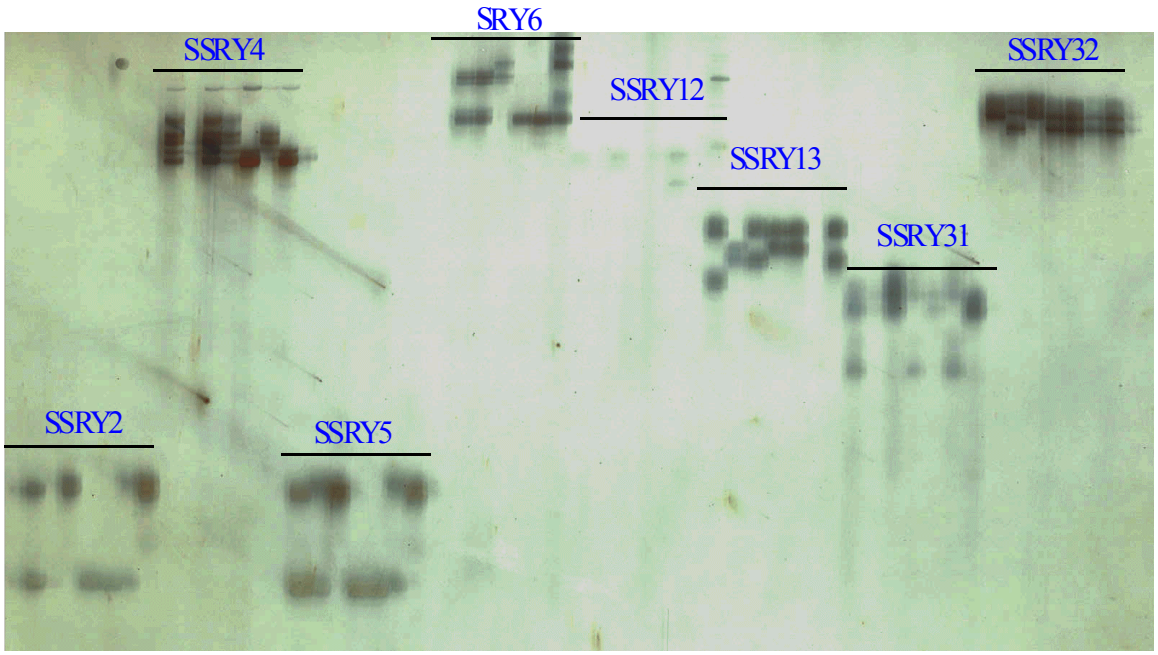

Fig 1. Polyacrylamide silver stained gel of 8 SSR markers and parental genotypes of the CNP mapping F2 populations. Orders of the parents are: Gomani, Mbundumali, GMM46, GMM66, GMM88, GMM91, and GMM96.

Activity 8.11 Gene Tagging of Beta-Carotene Content in Cassava

Collaborators: Nelson Morante, Teresa Sanchez, Alba Lucia Chavez, Hernan Ceballos, Martin Fregene (CIAT)
Funding: **CIAT**

Important Outputs

1. Identification of an ideal gene tagging population for beta-carotene
2. A scheme to increase content of beta-carotene by plant breeding and MAS.

Rationale

Occurrence of vitamin A deficiency and other nutritional problems overlaps with areas where cassava is an important staple food. Efforts have therefore been made to realize the potential

of cassava to improve the vitamin A consumption of people living in the tropical belt, where it is a predominant crop. Both roots and leaves of cassava contain considerable amounts of vitamin A precursors (β-carotene), 0.102 to 1.040 mg/100 g fresh roots and 12.05 to 96.42 mg/100 g fresh leaf weight (CIAT 2001). Several studies have highlighted the possibility of increasing available content of beta-carotene in both leaves and roots (Iglesias et al 1997, CIAT 2002, this report). Furthermore, the high β- carotene content is not often combined with high dry matter yield, resistance to pests and diseases and acceptable root quality. Crosses between selected sources of cassava with high nutritional quality and adapted local will need to be made.

Given the long growth cycle of cassava, improvement of a trait that is expressed only late in the growth cycle of the crop, such as β carotene, will benefit from marker-assisted selection. The inheritance of β- carotene in cassava has been demonstrated to be controlled by 2 genes (Iglesias et al. 1997). It is therefore a relatively simple trait to identify markers for. Molecular markers associated with β-carotene will allow for its selection early in the breeding cycle. We describe here discovery of a S_1 cross from the cassava land race MCol72 with a wide spectrum of segregation for beta-carotene from pure white to pink color. This family is appropriate for the development of markers for beta-carotene, the precursor of vitamin A.

Methodology

As part of an initiative to develop cassava populations tolerant to inbreeding, 14 genotypes commonly used as parents in the CIAT breeding were selfed and the seeds established at the experimental station of CENICAñA last year. This year, a clonal observation experiment of 10 plants per genotype, was set up at CIAT Palmira. At 10 months after planting the experiment was harvested. It was observed that in the S_1 family of 38 plants from the Colombian land race MCol72, a wide segregation was observed in root color from white to pink. The roots were scored qualitatively using the usual CIAT scale of 1 (white) to 8 (pink); pictures were also taken of a cross section of the root. Two genotypes with the deepest pink coloration were selected for quantitative determination of carotenes in the roots using high performance liquid chromatography. The parental genotype MCol72 has a root color that is normally described as cream colored or 4 on the CIAT scale.

The discovery of a wide segregation for root color in an S_1 cross from a cream colored variety provides an ideal population for bulk segregant analysis (BSA) of β carotene content and to identify markers associated with genes controlling the above trait. For DNA isolation, 1-2g of young leaves were harvested from all 38 genotypes into small paper envelopes and dried for 24h in an oven at 48^{o}C. The dried leaves were ground using a power drill and washed sand and DNA was isolated from 200mg using a miniprep version of the Dellaporta (1983) protocol. Two bulks of 6-10 DNA sample from genotypes with pure white and pink roots respectively were created. DNA from the bulks and parent will be genotyped with the 500 cassava SSR markers; markers polymorphic in the bulks will be employed to analyze the entire population. Markers associated with yellow/pink color will be determined by a simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using the computer package Q-GENE 2.30B (Nelson, 1997). The amount of phenotypic variance explained by each marker will be considered significant if the probability of observing an R^2 value is less than 0.005.

Results

The S_1 cross showed a wide spectrum of segregation for root color (Figure 8.11).

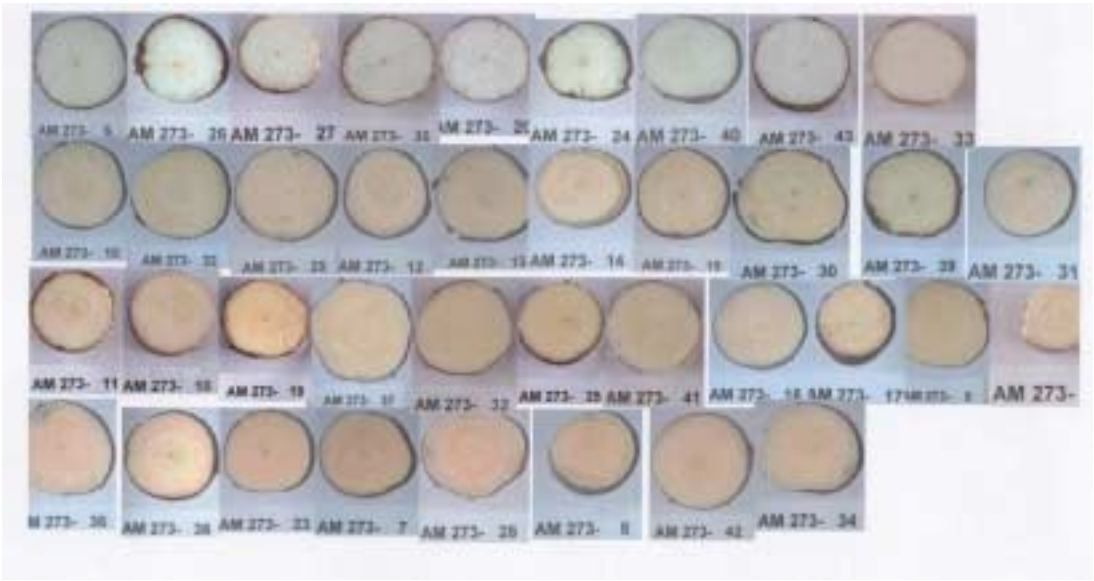

Figure 8.11. Cross section of root parenchyma of 38 genotypes of the S1 family derived from MCol72. A wide spectrum of variation in color can be seen from pure white to pink. Also to be noted is the pattern of deposition of beta-carotene.

The evaluation of two genotypes with pink roots revealed total beta-carotene content of 1.69mg/100g fresh weight (AM273-23) and 1.38100g fresh weight (AM273-7) respectively. The value obtained from AM273-23 is the highest to date from the characterization of the CIAT germplasm bank for beta-carotene. These results reveal the potential to increase beta-carotene in the roots. A series of experiments have therefore been planned to:

a) Generate a larger S_1 family from Mcol72 for gene tagging of beta-carotene.
b) Generate additional S_1 families from other cream, yellow and pink varieties and tag the genes involved
c) Determine if alleles of genes that control beta-carotene content from different varieties are complementary by making crosses between genotypes that carry them.

Genotypes for crosses to generate additional S_1 families can be observed in Table 8.20.

Table 8.20. Summary of genotypes established at CIAT Palmira this year to identify favorable alleles of genes controlling beta-carotene content.

	Genotype	No. of plants
Cream or yellow varieties		
1	CM 507-34	10
2	CM 996- 6	10
3	SM 526- 3	10
4	MCOL 144	10
5	MCOL 721	10
6	MCOL 1530	10
7	MCOL 1721	10
8	MCOL 2435	10
9	MCR 54	10
10	MGUA 29	10
11	MPER 572	10
Deep yellow or pink varieties		
1	MCR87	10
2	MBRA337	10
3	COL 2199	10
4	MCOL 2318	10
5	MPER297	10

Future Perspectives

1. Bulk segregant analysis (BSA) of beta carotene content in cassava
2. Generation of additional S_1 families from other cream, yellow and pink varieties and BSA analysis to detect additional alleles that may be of a complementary nature

References

Iglesias, C., Mayer J., Chávez A.L., Calle, F., 1997. Genetic potential and stability of carotene content in cassava roots. Euphytica 94:367-373.

Activity 8.12 Production of Waxy Cassava Starch via the Down Regulation of GBSSI Gene.

Collaborators: Gina Jazbleidi, Paul Chavariagga, Chikelu Mba, Martin Fregene (CIAT)
Funding: Ministerio de Agricultura y Desarollo Rural de Colombia.

Important Outputs

1. Isolation of a complete cDNA clone of the GBSS gene from cassava and construction of a transformation cassette with the GBSS gene in the anti-sense orientation
2. Initiation of transformation activities

3. Training of a Colombian undergraduate project student, Gina Jazbleidi, in the tools and methodology of genetic transformation in cassava

Rationale

Higher incomes from cassava in marginal areas of the developing world where the crop is generally found requires the industrialization of the crop and the development of novel industrial products for cassava with the aid of modern biotechnology. There are several novel products that can be produced from cassava. They include modified starches, such as 100% amylopectin or 100% amylose starches, from the down regulation of the granule bound starch synthethase (GBSS) gene, or the starch branching enzyme (SBE) gene. The industrial applications of either pure amylopectin or pure amylose starches, such as the production of high value biodegradable polymers from pure amylose starches or the use of 100% amylopectin in thickeners, pastes, and glues, is a market with unlimited growth potential.

With funds from the Ministerio de Agricultura y Desarollo Rural of Colombia a project has been initiated to genetically engineer industrial varieties with an anti-sense construct of the GBSSI. The granule bound synthethase (GBSS), is the predominant starch synthase gene, and catalyses the conversion of ADP-glucose to amylose through the linkage of a ADP glucose to a pre-existing glucan chain. Anti-sense disruption of the GBSSI gene has been employed to create potato transformants with 70-100% amylopectin via the down-regulation of the GBSSI gene Salehuzzaman et. al. (1993).

Methodology

Isolation of a cassava GBSS cDNA clone

More than 87, 000 clones of a cassava root and leaf cDNA library cloned in the vector pCMV SPORT (GIBCO BRL inc. USA) has been gridded onto high density filters (Mba et al. 2000, unpublished data). The library was screened using a potato GBSS cDNA clones, a kind gift of Dr Christine Gebhardt, Max Planck Institute, Cologne, Germany. The potato GBSS gene was labeled with [^{32}P] dATP by random primer labelling and hybridized overnight to the cDNA filters according to standard protocols for Southern hybridization used in cassava (Fregene et al. 1997). The filters were washed 2 times with 2XSSC + 0.1%SDS for 5 minutes at 60^{o}C and autoradiography was at –80^{o}C using 2 intensifying screens.

Construction of transformation cassettes.

Primers were designed from published sequences of a full length cassava cDNAs of the GBSSI gene (Salehuzzaman et. al. 1993) that incorporate *BamHI* and *XbaI* restriction enzyme recognition sites to enable sub-cloning of the cDNA clone in the anti-sense orientation into the multiple cloning site (MCS) of the vector pRT101.). The primers were used to amplify the cDNA clone obtained above and the PCR product was cleaned using the QIAGEN PCR clean up kit (QIAGEN Inc., Los Angeles, California), digested with the appropriate enzymes. A 2.1kb *BamHI/XbaI* fragment was subcloned in the sense and anti-sense between the 35S promoter and the 35S poly adenylated terminator region of vector pRT101, a kind gift of Dr Ryohei Terauchi, Iwate Biotechnology Research Center, Kitakami, Japan. The 35S promoter, GBSS gene, in anti-sense orientation, and the termintor region were excised using the restriction enzyme Hind *III* , separated on a special gel, symergel (Diversified Biotech Inc., USA), eluted and cloned into the Hind *III* site of the binary vector pBIG101 having the GUS-intron and *nptII* reporter genes, a gift of Dr Richard Sayre, Ohio State University, Columbus Ohio.

Genetic Transformation

Genetic transformation was by particle bombardment and *Agrobacterium* transformation of friable embryo callus (FEC) cultures. About 20µg of the pBIG101 constructs plasmid was coated onto gold particles and used in the helium gun bombardment of new FEC suspensions of the model variety for cassava transformation TMS60444 according to standard protocols established for cassava at CIAT (CIAT2001). For *Agrobacterium* transformation, the pBIG101 construct was transformed into strain EHA105 according by electroporation and transformed clones were selected on LB media plates plus Kanamycin (50ug/ml final). After 2 days, white colonies were picked and incubated in 10ml LB + Kanamycin (50ug/ml final) for another two days at 28°C. Bacteria was collected by centrifugation at 3000rpm for 20 min in a table top centrifuge and re-suspended in 500ul of solution containing 10mM MgCl2, 1omM MES, and 100uM Acetosyringone. Cassava FEC was co-cultured with the *Agrobacterium* suspension according to standard protocols at CIAT (CIAT 2001)

Results

Three GBSS cDNA clone obtained from screening the cassava library were sequenced and one clone was found to be a complete cDNA clone. The cDNA clone has the ATG start codon 81 base pairs down stream from the beginning of the cDNA sequence and a stop codon about 100 base pairs from the poly A tail. PCR amplification with the designed primers yielded a fragment about 2.1kb in size that corresponds to the full length GBSS cDNA clone (Figure 8.12).

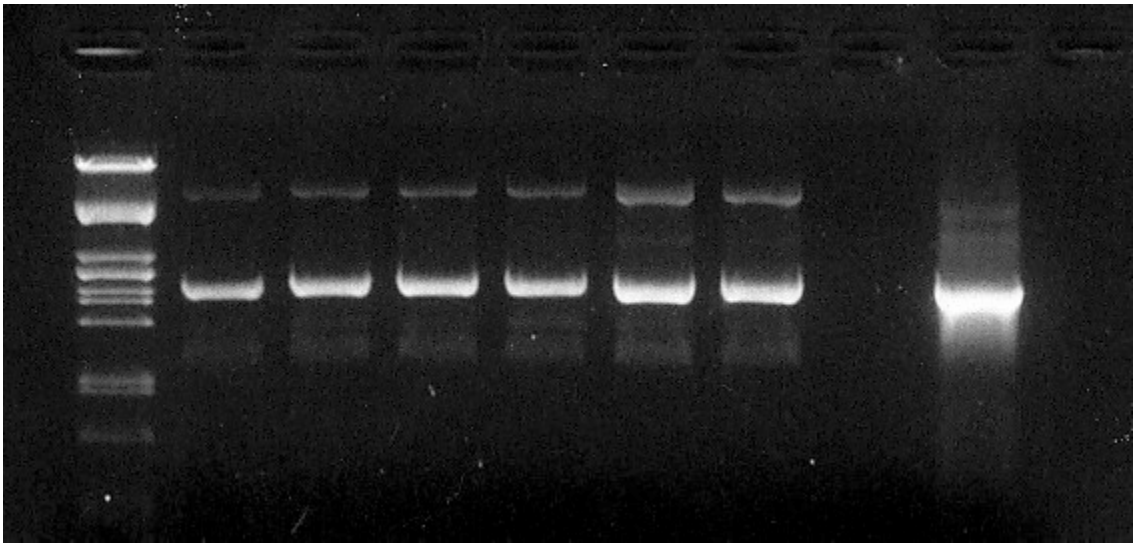

Figure 8.12. PCR amplification of the GBSS cDNA clone using primers designed to introduce restriction enzyme sites at the ends of the gene. The first lane by the right is molecular weight marker Lambda DNA digested with *HindIII* , the next six lanes are PCR amplification of the GBSS gene, the last lane is a control, PCR product of the GBSS potato gene.

The resulting PCR fragment was digested with *BamHI* and *XbaI* restriction enzyme digestion and cloned into the MCS of pRT101. The GBSS gene, promoter and terminator sequences were excised with *Hind III*, and the two resulting fragments of sizes 2.7 and 2.6 kb were separated by electrophoresis (Figure 8.13). The bigger fragment was eluted and cloned into the *HindIII site* of pBIG101. This is the construct that was used in the particle gun and *Agrobacterium* mediated transformation.

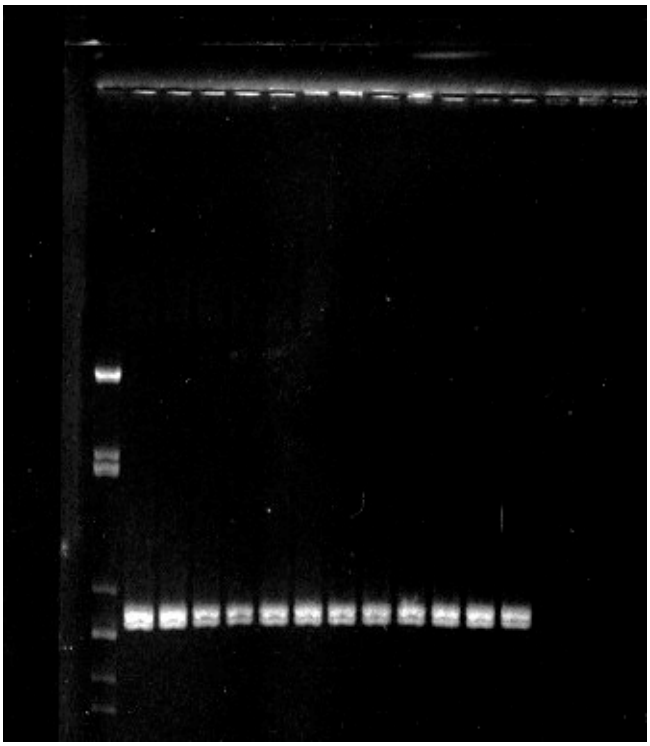

Figure 8.13. *HindIII* digested pRT101 plasmid containing the cassava GBSS gene in anti-sense orientation. The fragments of about 2.7kb and 2.6kb in size respectively represent the GBSS gene flanked by the 35S promoter and the polyadenylated terminator sequence and the rest of the pRT101 plasmid.

The transformation experiments are ongoing and conclusive results of reporter gene assays are expected at the end of December. Once the transformed calli has been revealed to have stable incorporation of the construct, regeneration of the transgenic calli will be initiated.

Future Perspectives

1. Agrobacterium transformation and regeneration of the industrial cassava variety "Reina" with the anti-sense construct of GBSSI cloned in pBIG101**.**

References

CIAT, (2001). Annual Report Project SB2, Assessing and Utilizing Agrobiodiversity through Biotechnology, CIAT, Cali, Colombia, pp 202-205.

Fregene M, Angel F, Gomez R, Rodriguez F, Chavarriaga P, Roca W, Tohme J, Bonierbale M (1997) A molecular genetic map of cassava (*Manihot esculenta Crantz*). Theor Appl Genet 95: 431-441

Salehuzzaman, S.N.I.M, Jacobsen, E., and Visser, R.G. F. (1993) Isolation and characterization of a cDNA-encoding granule-bound starch synthase in cassava (Manihot esculenta Crantz) and its antisense expression in potato. Plant Mol. Biol. 23:947-962.

8.13 Transient Transformation Assay of a Cassava Mosaic Disease (CMD) Candidate Resistance Gene Differentially Expressed During Host Plant Disease Resistance Response

Collaborators: Dr Ryohei Terauchi, Dr Hiromasa Saitoh, Ms Shizuko Fujisawa (IBRC, Kitakami, Japan), Janneth Patricia Gutierrez, Martin Fregene (CIAT)

Funding: The Rockefeller Foundation.

Important Outputs

1. Establishment of a system for the transient assay of foreign genes (luciferase) in cassava using a biolistic method.
2. Biolistic- inoculation of a CMD resistant and susceptible cassava genotypes with infectious virus DNA clones.
3. Co-transformation of a CMD susceptible genotype with a full length beta-tubulin gene and infectious virus DNA clones
4. Agro-inoculation (infiltration) of *N. tabaccum* and *N. benthamiania* with infectious virus clones and with a full length beta-tubulin gene.

Rationale

High levels of resistance to the cassava mosaic disease (CMD), the crop's most important production constraint in Africa, is mediated by a single dominant genes designated *CMD2*. The serial analysis of gene expression (SAGE) has been employed to identify many genes differentially expressed in resistant genotypes in response to heavy disease pressure of the African Cassava Mosaic Virus (ACMV) and the East African Cassava Mosaic Virus. The most differentially expressed gene was a beta-tubulin gene found to be expressed 12 times in CMD resistant genotypes compared to susceptible genotypes. Tubulin are the building block of

microtubules the main component of celluar cytoskeleton and they have been implicated in cell-to-cell progression and cytoplasm-to-nucleus movement of viruses.

To further understand the role beta-tubulin plays in the molecular basis of resistance, an experiment was designed to transiently co-transform a CMD susceptible cassava genotype and other host of the virus, for example *Nicotiana benthamiana* and *Nicotiana tabacum*, with infectious viral clones and the beta-tubulin gene. Infectious DNA clones of two strains of the virus were obtained from Drs John Stanley and Rob Briddon, John Innes Center, Norwich UK, and a license to work with them was obtained by the Iwate Biotech Research Center (IBRC). Method of transient transformation was by agro-infiltration, *N. benthamiana* and *N. tabaccum*, or biolistic inoculation, cassava. A CMD susceptible cassava variety, TMS30555, and the cassava land race that is the original source of CMD2, the single dominant CMD resistance gene, TME3, were used for the experiments.

Methodology

Plant materials were the CMD resistant variety TME3 and the susceptible variety TMS30555. Both genotypes were obtained as tissue culture plantlets from IITA and transferred to pots with soil for the experiments. A full length beta-tubulin cDNA clone was PCR amplified using primers that contain the recognition site for the appropriate restriction enzymes and cloned into PRT101 (35S promoter), for biolistic inoculation, or into the XVE inducible binary expression vector or into the binary vector pBIN m-gfp-ER, for agro-inoculation. The PCR fragment was cleaned and digested with the appropriate enzyme and eluted from a 1.5 % agarose gel. The purified fragment was then cloned into appropriate vector and transformed by electroporation into *E.Coli* strain HB101. A 1:50 dilution of plasmid preparations from an overnight culture of a single *E.Coli* colony was analyzed by PCR, using the original beta-tubulin primers, to confirm success of the cloning experiment. To that ensure that a full length beta-tubulin protein will be expressed in the transient assay, 3 clones from each cloning experiment was sequenced with 4 primers that covers the entire length of the gene.

Infectious DNA clones from two virus strains were employed in the transient assay experiment, partial repeats of the A and B genome of the Sri-Lanka Cassava Mosaic Virus (SLCMV A and SLCMVB) cloned into the binary vector pBIN PLUS, and full length clones of the African Cassava Mosaic Virus (ACMV) A and B genome cloned into pUC19 (ACMV A and ACMVB). Infectivity of cassava by virus partial repeats in a binary vector clones and agro-inoculation has not been demonstrated, therefore all agro-inoculation experiments were with *N. benthamiana* and *N. tabaccum*. But cassava has been successfully infected by biolistics, therefore the biolistic experiments were with cassava and *N. tabaccum*, as control.

A preliminary biolistic experiment was conducted to standardize the conditions for the BIO-RAD particle gun bombardment and to ensure expression of the beta-tubulin gene cloned into PRT101. Leaf discs of about $5cm^2$ from the cassava genotype TME3 and *N. tabaccum* were used for the preliminary experiment. There were three treatments: bombardment with the PRT101 plasmid containing the luciferase gene alone, the luciferase construct combined with a PRT101 plasmid in which the luciferase gene has been replaced with a beta-tubulin gene having the 11bp truncation and the non-truncated beta-tubulin gene. Three leaf discs were used for each treatment and per crop. Particle gun bombardment was according to standard procedures established at the IBRC (Terauchi, 2002, personal communication). One 1 month-old live plant each was included for both cassava and *N. tabaccum* in the luciferase treatment as control. Leaf discs were bombarded once with 2.5ug of DNA from the

respective clones, while whole plants were bombarded 3 times. After bombardment, leaves were placed on water in a petri dish an incubated at 25°C for two days.

Two days later, total proteins were isolated from leaf discs transiently transformed with the beta-tubulin gen, to confirm the expression of the beta-tubulin gene using standard procedures established at the IBRC (Saitoh, 2002 personal communication). Exactly 3.6ug of total protein lysate from all leaf discs from treatments 2 and 3, and 2 non-bombarded controls were separated on a SDS-PAGE gel. Western blot analysis, using an anti-body raised to purified rat brain beta-tubulin, was carried out as described in the manufacturer's product sheet (SIGMA Inc, St. Louis, USA).

Following the preliminary experiment, a biolistic experiment was designed to inoculate the CMD resistance genotype TME3 and the CMD susceptible variety TMS30555 with the ACMV A and B infectious clones. A second treatment was co-bombardment of the ACMV clones and the beta-tubulin gene into the CMD susceptible genotype TMS30555. Three one-month old plants of TME 3 were used in the first treatment, while 2 one-month old plants of TMS30555 were each used in the first and second treatments. Plants were bombarded twice with 2.5ug of DNA from the respective clones as described earlier. The inoculated plants were transferred to a virus containment area in a secure green house.

For the agro-inoculation by infiltration experiment, the SLCMV A and SLCMV B clones were transformed into agro-bacterium strains MOG and EHA105. Similarly the beta-tubulin gene in the binary vectors XVE and pBIN m-gfp-ER respectively was transformed into agro-bacterium strains MOG and EHA105. Agro-infiltration was according to standard methods established at the IBRC (Saitoh 2002, personal communication). There were 4 treatments: the virus alone (a mixture of SLCMV A and SLCMV B), the virus and the beta-tubulin gene (in XVE), the beta-tubulin gene alone, and a dilution of the beta-tubulin gene to reflect the concentration in the mixture with viral clones. Three plants of *N. benthamiana* and *N. tabaccum* each were used per treatment. Agro-infiltrated plants were transferred to a virus containment area in a secure green house. After two days, the beta-tubulin gene will be induced by treatment of agro-infiltrated plants with the animal steriod, estradiol. Plant tissue will be collected from all plants and stored for Western analysis of gene expression using a conjugated mouse beta-tubulin antibody or antibodies raised to the coat protein of ACMV. Agro-inoculation of cassava could not be achieved due to poor infiltration, cassava leaves are covered with a thick cuticle.

Results

The full length of the beta-tubulin gene is 1667bp, the translated portion of the gene corresponds to 1341bp or 447 amino acid motifs. The beta tubulin protein showed more than 80% homology with similar genes from rice and arabidopsis. Comparison of the complete sequence of the beta-tubulin gene with ESTs generated for tag annotation during the SAGE experiment revealed two beta-tubulin molecules, one transcript had an 11bp truncation in the 3' untranslated end of the cDNA molecule. This molecule was also the most abundant from the EST project, 4 as against 1 of the non-truncated. It is not clear if this molecule plays any role in the over-expression of beta-tubulin, therefore the molecules were separated in at least one of the transient assay experiments, the biolistic inoculation.

Results of the biolistic experiment to standardize the conditions for the BIO-RAD particle gun bombardment for expression of the beta-tubulin gene particle gun revealed good expression

of the full length beta-tubulin gene in all leaf disc by western blot (Fig 1). Similar results were also obtained with non-bombarded controls suggesting the detection of endogenous beta-tubulin. There is therefore a need to separate the confounding influence of endogenous tubulin and transient expression via the use of a non-plant secondary antibody fused to the tubulin protein. The luciferase assay was conducted on leaf discs from all treatment using a rapid enzyme substrate assay and a flourescence reader according to standard methods in use at the IBRC (Terauchi et al. 2002, personal communication). Results revealed good enzyme activity on the average for all samples, although samples from the co-inoculation (luciferase and beta-tubulin) had a 5 to 10 magnitude reduction. This may be an effect of the quantity of leaf samples, as more than half of the leaf disc had already been used for the Western blot analysis. The luciferase activity of the bombarded cassava and *N. tabaccum* whole plants were the highest for all samples.
The western blot analysis of the tobacco plants agro-infiltrated with the infectious virus clones or beta-tubulin gene or both remain to be carried out. The plant tissue is stored at –80oC pending a trip to Japan to conclude the above studies.

Future Perspectives
1. Western blots of agro-bacterium and particle gun infected plans to assess expression of the beta-tubulin gene and the infectious viral clones
2. Additional co-transformation experiments to better synchronize expression of the beta-tubulin gene and infection by viral clones.

8.14 Progress Towards a PCR-Marker Based Map of Cassava

Collaborators: **Angela Zarate, Edgar Barrera, Martin Fregene (CIAT)**
Funding: **CIAT**

Important Outputs
1. Implementation of the SSCP technique for the analysis of sequence tagged sites (STS) in cassava
2. Design of primers for another 141 SSR markers for cassava

Rationale
Progress towards a PCR-based map of cassava for gene tagging and marker-assisted selection (MAS) has crossed a significant mile stone with the report of a SSR only map of cassava (CIAT 2002, this report). However, 20% of SSR markers evaluated remained unlinked and denotes that the map is not complete and more markers need to be developed. At the same time, saturation of the RFLP genetic map of cassava has continued with 57 SSR markers from genomic sequences (Zarate et al 2002, unpublished data) and another 45 SSR markers from cDNA sequences (Garcia et al 2002, unpublished data) added to the map.

To ensure that a PCR-based map of cassava can be achieved within the near future efforts have been geared this year to converting RFLP markers on the genetic map of cassava to single sequence conformation polymorphism (SSCPs). Furthermore, additional SSR markers

have been designed from cassava genomic sequences reported in GeneBank, with particular reference to BAC end sequences developed at the Clemson University Genome Institute (CUGI).

Methodology

More than 100 RFLPs from the molecular genetic map of cassava have been sequenced. Primers were designed from 2 RFLP clones, CYP79D1 and CYP79D2 and used to amplify total genomic DNA from the two parents of the mapping population and a sub-set of 20 progenies. PCR amplifications were carried out in 12.5-µl reactions containing 100 ng of DNA, 0.5µM of each primer, 10 X of *Taq* polymerase buffer (500mM KCl, 100mM Tris-HCI (pH 8.5), and 1 mg/ml gelatin), 2mM of $MgCl_2$, 0.5mM of dNTPs and 0.25 U of *Taq* polymerase. The PCR profile was: 94^{O}C for 2 min, followed by 30cycles of 95^{0}C for 1 min, 55 ^{O}C for 2 min and 72 ^{O}C for 2min. A final extension step of 72 ^{O}C for 10 min was added at the end. The PCR amplification was cleaned with the QIAGEN PCR clean-up kit (QIAGEN Inc. Los Angeles, CA) and digested to completion with 10U of Hinf I restriction enzyme for 3h at 37 ^{0}C according to the manufacturer's instruction (New England Biolabs, Cambridge, MA). The digestion product was electrophoresed on 6% polyacrylamide-MDE gels (Cambrex Bio Science Inc, Baltimore, MD) made up of 10% glycerol, 6ml 10XTBE, 25ml MDE gel solution, 65ul TEMED, 500ul APS, and 60ml deionized water, at 40Watts for 48h. The gel was stained as described by Slaubaugh et al (1997). Briefly, the gel was fixed for 3 min in 10% ethanol, 0.5% acetic acid, stained for 5 min in fixing solution plus 0.2% silver nitrate , washed in water for 1 min and developed for approximately 10-20 min in 3% NaOH and 0.27 % formaldehyde in water. Following staining, the gel was fixed for a further 5 min and washed in water.

The construction of a bacterial artificial chromosome (BAC) library from the white fly resistance variety MOLC72 has been described earlier (Tomkins et al 2001). A total of 2301 BAC ends were sequenced and 1755 good sequences were deposited in gene bank (Tomkins et al 2002. Unpublished data). The BAC end sequences were downloaded from GeneBank and the following SSR motifs: (AT), (GA), (CA), (GC), (GT), (TCT), (GCT), (AGC), (GCC), (GGT), (ATT), (GGA), (TATG), and (GTGA) were searched for in the sequences using the DNAMAN software. The local BLAST facility at CIAT (http://gene2/BLAST/inicio.htm) was used to compare the sequences with each other to eliminate duplicated sequences. Primer design was using Primer 3.0, the primer picking software found at http://waldo.wi.mit.edu/cgi-bin/primer/primer3.

Results

SSCP analysis of the parents of the cassava mapping population and a sub-set of 20 genotypes of the same population revealed polymorphisms that segregated as single dose restriction fragment (SDRF) polymorphism. The entire mapping population is being analyzed at the moment. Primers will be designed from other sequenced RFLP markers that are currently on the map to also convert them to PCR-based markers. A draw back of the SSCP methodology is the need to always purchase costly MDE gel solutions (Cambrex Bio Science Inc, Baltimore, MD). Efforts are also being made to try normal polyacrylamide gel solutions in an effort to reduce costs. The sequenced RFLP markers being converted to SSCP markers can also be described as sequence tagged sites (STSs). These have the advantage that once they have been analyzed in any population the map location as well as the sequence is known.

A total of 141 BAC end sequences were found to contain SSR motifs. The distribution of SSR sequences was as follows: 67 dinucleotide, 46 trinucleotides, 4 tetranucleotide, and 24 other (mixture of different repeats) motifs, a mixture of two or three motifs (Table 8.21). Average length of the dinucleotide repeats was 8 repeats for the dinuleotide, 6 for the tri and 4 for the tetras. Primers designed will shortly be sent for synthesis and once available they will be placed on the existing map of cassava beginning with the parental survey.

Table 8.21. Di, tri, tetra nucleotide, and other SSR repeats found in the BAC end sequences.

Type of SSR	Number	Percentage
AT	**38**	**27**
GA	**24**	**17**
CA	**4**	**3**
GC	**1**	**0.7**
TCT	**11**	**8**
GCT	**3**	**2**
AGC	**3**	**2**
GGT	**4**	**2**
ATT	**12**	**8**
GGA	**13**	**9**
TATG	**4**	**2**
Others	**24**	**17**
Total	**141**	

Future perspectives

1. Continue the SSCP analysis for the genetic mapping of STSs in cassava
2. Parental survey of the additional 141 primers and mapping of polymorphic ones.

References

Tomkins J., Fregene M., Tohme J., and Wing R (2001). Development of BAC Library resources for cassava. In: C. Fauquet, and N.N.Taylor (Eds). Proceedings of the Fifth International Scientific Meeting of the Cassava Biotechnology Network, Danforth Plant Science Center, St. Louis, Missouri, USA

Slabaugh M. B. et al.(1997) Sequence-based genetic markers for genes and gene families: single-strand conformational polymorphisms for the fatty acid synthesis genes of Cuphea. Theor Appl Genet 94:400-408

Activity 8.15 Development of Populations Tolerant to Inbreeding Depression in Cassava

Collaborators: Nelson Morante, Teresa Sanchez, Hernan Ceballos, Martin Fregene (CIAT)
Funding: CIAT

Important Outputs
1. Development of populations tolerant to inbreeding depression.
2. Generation of S_2 families from 6 elite cassava genotypes

Rationale
Cassava is an allogamous tetraploid that accumulates a significant genetic load that is released on inbreeding. The average yield of selfed lines was observed to be about half the average yield of parental genotypes and the degree of inbreeding varied greatly amongst different genotypes (Kawano et al. 1978). Inbreeding depression primarily affects the general growth and vigor of the plant, reflected directly in lower root yield, and therefore no inbreeding is carried out at any stage of traditional cassava breeding. Cassava breeders in general strive to select progenitors and make crosses that maximize heterozygosity and minimize inbreeding

But inbreeding is desirable for cassava for the reduction of the genetic load currently carried by many elite clones which in turn permit the use of valuable breeding schemes such as back-cross breeding. But more importantly inbreeding is important because it eliminates the confounding effect of dominance process and maximizes the additive gene variance on selection (Ceballos et al 2002). Inbred lines also have the advantage that they can be shipped and stored as botanical seed, facilitating the exchange of germplasm which, at the moment can only be done via expensive tissue culture shipments. A selection program was therefore set-up for tolerance to inbreeding in 1999, at the moment more than 300 S_1 lines have been produced from 5 elite clones and S_2 lines were produced this year (see net section of this report). We describe here evaluation of the S_1 families

Methodology
Fourteen cassava genotypes were chosen for the development of populations tolerant to inbreeding. The genotypes were chosen due to their good general combining ability performance for yield, dry matter yield or root quality. They include the following lines: MCOl22, CM523-7, MCOL1684, MBRA12, MCOL2060, MVEN77, MCOL1522, MTAI1, MPAN51, MECU169, MCOL1468, MCOL72, CM849-1, HMC1. More than 300 pollinations were made per genotype and between 30-150 seeds were obtained per genotype. The seeds were planted at the CENICAñA experimental station October 2000.

At 10 months after planting, harvested August 2001, all plants were harvested and measured for fresh root yield, dry matter content, foliage weight, harvest index, culinary quality, starch content/quality, and frog skin disease according to standard CIAT procedures. Six families were selected to continue the process of inbreeding based on their average yields and flowering. The families were also planted in an observational trial, six plants in single row replication, in October 2001 at CIAT Palmira and harvested in May 2002. The traits described

above were evaluated for all plants.

Results

Inbreeding depression was severe in many of the genotypes, particularly in MBRA12, MCOL1684, MCOL1468 and MCOL1522, where inbreeding depression of more than 70% was observed. Very poor vigor and sufficient stakes could not be obtained for further experiments and were therefore eliminated. Six families showed better tolerance to inbreeding depression as observed from their yield data (Table 8.22), highlighting the great differences in cassava to inbreeding depression. Maximum yield of these families were on an average of 18 tons/ha of fresh root with the exception of family AM312 that had 35t/ha, this family also had the largest standard deviation. Average root yield was less than half of the average yield of the parents which is in agreement with earlier observations of inbreeding depression in cassava. The 2002 experiment, the clonal observation experiment agreed quite closely with data from the seedling trial (data not shown). Distribution of dry matter content and harvest index from the clonal observation revealed normal distribution of the traits in all families (Figure 8.23.

Table 8.22. Summary of fresh root yield from a seedling trial of S_1 families conducted at the CENICAñA experimental station in 2001.

Family name	Parent	Maximum fresh root yield t/ha	Minimum fresh root yield t/ha	Average root yield t/ha	Standard deviation
AM244	MCOL1505	18.5	0.00	7.58	5.46
AM266	HMC-1	18.5	0.00	8.31	4.17
AM273	MCOL72	14.7	0.00	7.0	4.38
AM277	MTAI1	18.0	0.00	6.64	5.96
AM278	MVEN77	15.0	0.00	3.86	5.38
AM312	CM849-1	35.5	0.00	6.04	6.04

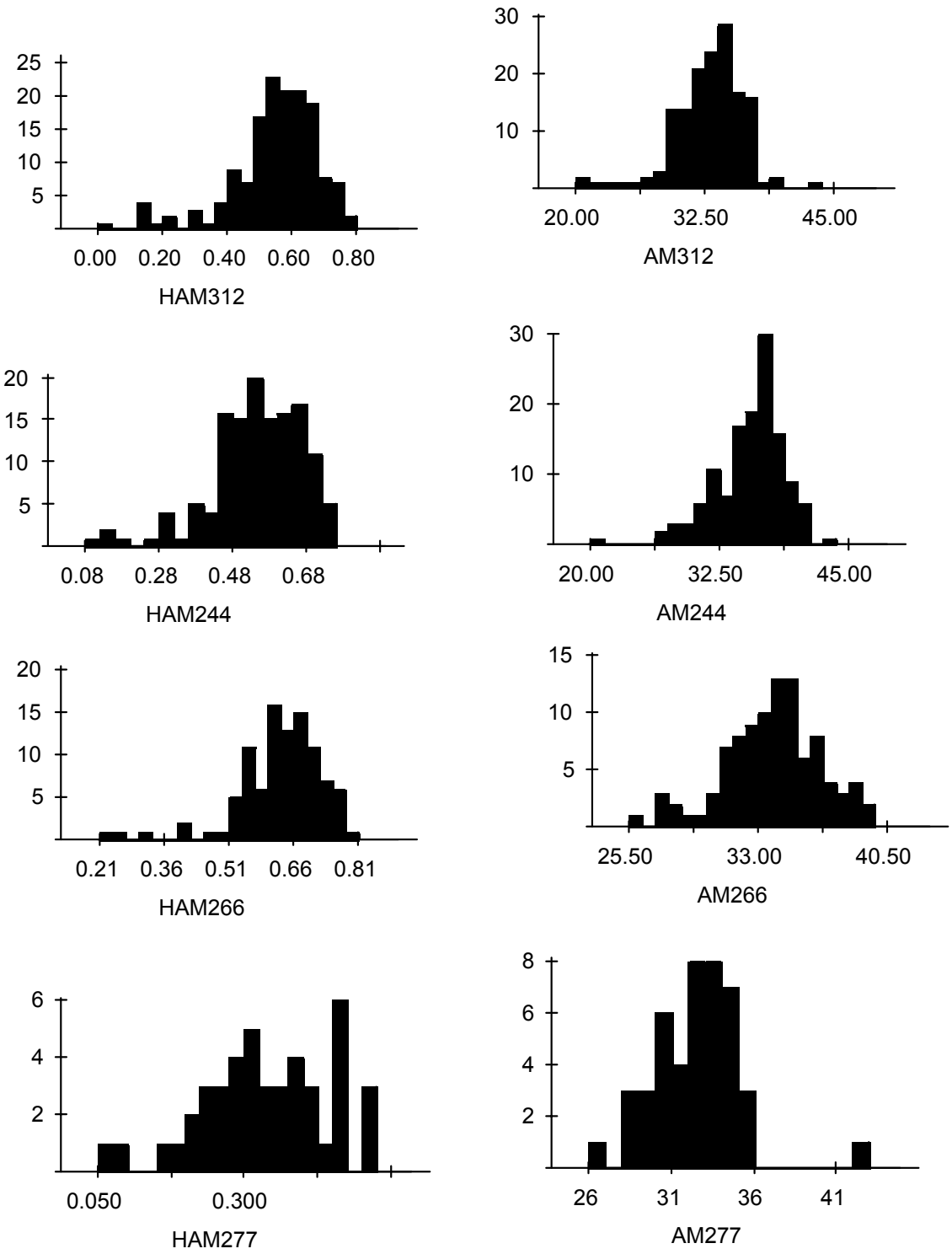

Figure 8.14. Distribution of harvest index (prefix H-) and dry matter content in 4 S_1 families

Table 8.23 Best 25 genotypes for dry matter yield and their yield components in six S_1 families.

Genotype	Parent	FY t/ha	HI	% DMC	DMY t/ha	Starch	Taste
AM 266- 108	HMC 1	98.00	0.66	33.58	32.91	1	1
AM 266- 103	HMC 1	91.20	0.69	35.73	32.59	1	1
AM 312- 18	CM 849- 1	78.25	0.56	37.32	29.20	2	3
AM 266- 21	HMC 1	77.88	0.63	38.68	30.12	1	1
AM 244- 79	MCOL 1505	75.17	0.54	37.14	27.92	2	3
AM 312- 15	CM 849- 1	72.75	0.65	35.57	25.88	2	2
AM 266- 58	HMC 1	72.08	0.53	33.34	24.03	1	2
AM 266- 24	HMC 1	71.63	0.68	37.66	26.97	1	1
AM 312- 103	CM 849- 1	68.17	0.63	37.14	25.32	2	5
AM 266- 18	HMC 1	66.80	0.60	36.40	24.31	1	1
AM 312- 32	CM 849- 1	66.17	0.62	37.48	24.80	1	2
AM 266- 113	HMC 1	65.83	0.64	34.84	22.93	1	1
AM 312- 130	CM 849- 1	62.83	0.67	34.08	21.41	3	4
AM 312- 92	CM 849- 1	60.42	0.54	36.53	22.07	3	5
AM 266- 82	HMC 1	60.00	0.64	39.47	23.68	1	1
AM 244- 39	MCOL 1505	58.75	0.69	40.03	23.52	2	2
AM 312- 49	CM 849- 1	58.38	0.73	32.10	18.74	3	3
AM 266- 87	HMC 1	58.20	0.71	35.55	20.69	1	1
AM 312- 138	CM 849- 1	58.17	0.58	30.55	17.77	2	5
AM 266- 31	HMC 1	58.13	0.62	37.00	21.51	1	1
AM 244- 53	MCOL 1505	58.10	0.58	33.76	19.62	1	3
AM 244- 129	MCOL 1505	58.00	0.71	31.64	18.35	2	2
AM 277- 29	MTAI 1	57.83	0.45	34.40	19.89	4	4
AM 266- 19	HMC 1	57.50	0.76	33.51	19.27	2	2
AM 266- 17	HMC 1	57.33	0.61	36.68	21.03	1	2
Statistics of best 25 inter-specific hybrids evaluated							
Maximum		98.00	0.76	40.03	32.91	1.00	1.00
Minimum		57.33	20.03	30.55	17.77	4.00	5.00
Average		66.70	0.63	35.61	23.78	1.68	2.32
Standard Dev.		10.90	0.07	2.41	4.29	0.85	1.38
Statistics of 343 inter-specific hybrids evaluated							
Maximum		98.00	0.80	43.69	32.90	1.00	1.00
Minimum		0.50	0.03	18.69	0.00	5.00	5.00
Average		27.37	0.53	33.42	9.45	1.96	2.78
Standard Dev.		16.68	0.15	3.63	6.14	1.08	1.35

FY: Fresh root yield; DMY: Dry matter yield; HI: Harvest Index; DMC: Dry matter content

Table 8.24. Best 25 genotypes for dry matter content in six S_1 families.

Genotype	Parent	FY t/ha	HI	% DMC	DMY t/ha	Starch	Taste
AM 312- 140	CM 849- 1	32.75	0.56	43.69	14.31	3	5
AM 244- 95	MCOL 1505	34.75	0.68	42.93	14.92	1	2
AM 277- 44	MTAI 1	37.08	0.32	42.07	15.60	5	5
AM 244- 146	MCOL 1505	37.20	0.51	41.01	15.25	1	2
AM 244- 82	MCOL 1505	25.75	0.63	40.84	10.52	1	2
AM 244- 164	MCOL 1505	36.42	0.58	40.73	14.83	1	1
AM 244- 81	MCOL 1505	45.17	0.46	40.22	18.16	1	2
AM 244- 101	MCOL 1505	39.00	0.50	40.08	15.63	1	2
AM 244- 39	MCOL 1505	58.75	0.69	40.03	23.52	2	2
AM 244- 33	MCOL 1505	52.25	0.64	39.68	20.73	1	1
AM 312- 17	CM 849- 1	54.50	0.65	39.62	21.59	2	5
AM 244- 133	MCOL 1505	22.75	0.59	39.61	9.01	1	1
AM 266- 82	HMC 1	60.00	0.64	39.47	23.68	1	1
AM 244- 62	MCOL 1505	21.92	0.46	39.47	8.65	1	2
AM 244- 154	MCOL 1505	48.08	0.52	39.43	18.96	1	2
AM 312- 33	CM 849- 1	42.42	0.66	39.33	16.68	1	2
AM 244- 75	MCOL 1505	49.67	0.62	39.21	19.48	2	2
AM 244- 56	MCOL 1505	50.00	0.46	39.16	19.58	1	2
AM 244- 96	MCOL 1505	44.40	0.58	39.12	17.37	5	5
AM 266- 92	HMC 1	39.50	0.70	39.02	15.41	1	1
AM 244- 98	MCOL 1505	28.17	0.62	38.95	10.97	1	2
AM 244- 88	MCOL 1505	30.08	0.50	38.84	11.68	1	3
AM 266- 21	HMC 1	77.88	0.63	38.68	30.12	1	1
AM 266- 64	HMC 1	35.33	0.61	38.65	13.66	1	2
AM 244- 124	MCOL 1505	29.92	0.58	38.64	11.56	1	1
Statistics of best 25 inter-specific hybrids evaluated							
Maximum		77.88	0.70	43.69	30.12	1.00	1.00
Minimum		21.92	0.32	38.64	8.65	5.00	5.00
Average		41.35	0.57	39.94	16.48	1.52	2.24
Standard Dev.		13.09	0.09	1.31	5.05	1.16	1.33
Statistics of 343 inter-specific hybrids evaluated							
Maximum		98.00	0.80	43.69	32.90	1.00	1.00
Minimum		0.50	0.03	18.69	0.00	5.00	5.00
Average		27.37	0.53	33.42	9.45	1.96	2.78
Standard Dev.		16.68	0.15	3.63	6.14	1.08	1.35

FY: Fresh yield; DMY: Dry matter yield; HI: Harvest Index; DMC: Dry matter content

Two families, AM266 and AM312 account for 83% of the best 25 genotypes for fresh yield in the clonal observation trial (Tables 8.23 and 8.24). A genotype from AM266 had maximum yield of 98 t/ha, this same family produced the genotype with the highest yield in the seedling trial albeit different genotypes, underscoring the big error that can occur when evaluation is based on a single plant and the effects of inter-genotypic competition. The family AM312 accounted for 67% of the best 25 genotypes for dry matter content. The trend

observed in the data from the second year evaluation that individuals from certain S_1 families tend to dominate the group of best genotypes for certain traits highlights the important role additive genes play in the expression of these traits.

Future Perspectives

1. Establishment and evaluation of S_2 progenies

References

Ceballos H., J.C. Pérez, C. Iglesias M. Fregene, F. Calle, G. Jarmillo, N. Morante, and J. López (2002) The use of doubled-haploids in cassava breeding. In (Howeler) Ed. Cassava's Potential in the 21st Century: Present Situation and Future Research and Development Needs. Proceedings of the Sixth Regional Workshop, held in Ho Chi Minh city, Vietnam. Feb 21-25, 2000, CIAT, Bangkok, Thailand, pp 5-15

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Activity 8.16. Culture of Embryo Axes of Immature and Mature Sexual Seeds for the Establishment of Genetic Stocks and Breeding Populations

Collaborators: Luis Guillermo Montes, Nelson Morante, Martin Fregene
Funding: The Rockefeller Foundation

Important Outputs

1. Recovery of S_2 plants from immature seeds by embryo culture
2. Optimization of protocols for embryo culture of mature and immature seeds.

Rationale

To safeguard some of the very valuable cassava genotypes infected by the frog skin disease (FSD) during the last growing season, the construction of a tissue culture facility for clean-up of the most valuable FSD infected lines via tissue culture and thermo-therapy was approved by CIAT management. The tissue culture facility, completed late August, consists of a growth room and a bio-safety hood room. It is available to the CIAT cassava community for tissue culture clean-up of FSD infected materials and propagation of clean planting materials.

Another use to which the facility has been put is embryo rescue of immature and mature sexual seeds. Interventions to control the escalating problems of white flies at the CIAT experimental station, namely a compulsory one-month "zero cassava" period at CIAT has meant that all plants other than plants in the hybridization block need to be harvested at 11 months after panting. This year, poor flowering of S_1 genotypes in the hybridization block and profuse flowering of S_1 plants in clonal observation trials (COT) implied that the COT plants had to be used for making genetic crosses to generate S_2 families. Because these

plants must be removed in less than a year, the fruits were harvested less than 90 days after pollination, in other words before maturity. The embryos of the immature seeds from the S_2 families were rescued by embryo culture according to protocols developed at CIAT (Fregene 1999).

The facility was also used for establishment, from embryo axes, of mature seeds of breeding populations for CMD resistance. A key reason for CMD breeding at CIAT is to develop Latin America cassava gene pools adapted to the disease should in case it makes its debut in the region. A second important objective is to facilitate germplasm shipment of CIAT's elite cassava germplasm to regions, such as India and Sub Saharan Africa, where CMD is endemic, through the introgression of CMD resistance into CIAT's elite germplasm. To permit marker-assisted selection (MAS) of CMD resistance at CIAT for Latin America and at the same time fulfill plant quarantine conditions for the shipment of the CMD resistant CIAT germplasm to India and Africa, it is necessary to germinate and maintain in vitro breeding populations. This year more than 3000 controlled crosses, and >4000 open pollinated crosses were made involving CMD resistant parents introduced from IITA. The seeds were harvested as mature or immature seeds and are being germinated from embryo axes.

Specific Objectives

a) To rescue embryos of immature seeds of S_2 families
b) To germinate from embryo axes of seeds from CMD breeding populations
c) To rescue embryos from crosses between high root protein content accessions of wild Manihot species and elite cassava parents

Methodology

Immature fruits, the fruits were cut open using a sharp knife and the seeds removed and tested for viability (ability to sink in water). A total of 446 viable S_2 seeds were obtained. Another 3600 matured seeds were obtained from crosses between CMD resistance and elite parents of CIAT cassava gene pools, wild accessions with high protein and high beta-carotene varieties. More than 1000 immature seeds were obtained from crosses between high root protein content accessions of wild *Manihot* species and elite cassava parents and also required embryo rescue.

Immature or mature seeds were removed and surface-sterilized by immersion in 70% alcohol for 1 min, followed by immersion in 0.5% sodium hypochlorite for 6 min, and then rinsed three times with sterile water. Under aseptic conditions, the seeds were split along the longitudinal axis utilizing sterile pliers and the embryonic axes were removed with sterile forceps and scapel. Excised embryonic axes were placed radicle down on 1/3 MS medium, supplemented with 0.01 mgl^{-1} NAA, 0.01 mgl^{-1} GA_3, 1.0 mgl^{-1} thiamine-HCL, 100 mgl^{-1} inositol, 2% sucrose, 0.7% agar (Sigma Co.) and 25 mgl^{-1} of a commercial fertilizer containing: N 10, P 52, K 10, pH 5.7-5.8 (Roca 1984). This medium is also known as 17N. The embryo cultures were incubated under an alternate temperature regime of 35°C for 16 h and 25°C for 8 h, in darkness for the first 5 days, to promote growth of the radicle, then under continuos illumination from a 40 W fluorescent bulb (5,000 µmol m^{-2} s^{-1}) for the next 5 days. The cultures were then transferred to a growth chamber with a 12h photoperiod (illumination, 5,000 µmol m^{-2} s^{-1}) at 27°C and grown for 40 to 45 days.

Over time, several modifications were made to the protocol to reduce the difficulty of opening up immature fruits, given the large number of fruits to be handled, and also to reduce damage to the embryos during removal. The first modification was to cut the fruit in half, remove the seed, disinfect and place directly on 17N media. The temperature and light regime was also modified for better germination as follows: a constant temperature regime of 28-30^{o}C and a 12/12h photo-period and the use of a piece of dark cloth for 10 days to reduce influx of light, after which the cultures were fully exposed to light.

Problems with bacterial contamination were solved by adding the antibiotic rinfampicilin to the media at a concentration of 50mg/l. Fungal contamination on the other hand was eliminated by increasing the concentration of sodium hypochlorite to 5% and extending incubation times up to 10 min. Due to continued problems with poor germination, a modification of the seed scarification and surface-sterilization was added for mature seeds. Seeds were soaked in 50% sulfuric acid for 30 minutes followed by immersion in sterile water for 30 min, then a 5 min wash in alcohol, and incubation in 5% sodium hypochlorite for 6 min, followed by 3 final rinses with sterile water. The softened testa was then scrapped off with a knife and the whole seed, cotyledons and embryos, was placed on the media.

Results

The 446 immature S_2 seeds were harvested the same day and stored at room temperature. Embryos were extracted and cultured over a period of 2 weeks, with an average of 40 seeds processed every day. During the first week, problems of fungal infection, apparently due to storage at room temperature, lead to a huge loss of seeds. This problem was eventually solved, but the 8-14 day storage of the immature fruits, reduced the viability of the seeds considerably. Previous experiences revealed that air drying excised immature seeds at room temperature drastically reduced germination rates (Fregene 1999). It appears that storage of immature fruits at room temperatures had the same debilitating effect as air drying immature seeds. Immature seeds from the inter-specific crosses were therefore harvested and embryos cultures on a daily basis. Only 67 plants or 15.02% could be recovered from the 446 embryos cultured. The S_1 genotypes from which S_1 plants cold be recovered include AM244-35, AM244-38, AM244-39, AM244-64, AM244-101, AM244-109, AM244-135, AM244-164, AM266-21, AM266-41, AM266-50, Y AM266-76. These S_1 plants have been multiplied in preparation for transfer to the green house and to the field the following year.

Results of more than 500 immature seeds from inter-specific crosses processed so far have revealed more than 80% germination rates, confirming the damaging effects of storing immature seeds before embryo culture. So far, more than 100 mature seeds from the crosses generated for marker-assisted breeding of CMD resistance were cultured from embryo axes. Germination rates were higher than 90%. Results were much better here presumably due to the maturity of the seeds and the sulphuric acid treatment. No effect on germination and growth was observed of changing the temperature regime from the previous regimes described earlier for embryo rescue (Fregene et al 1997) to a constant 28-30^{o}C day and night.

Future Perspectives

Completion of the embryo culture of the CMD resistance breeding populations and the inter-

specific hybrids for higher protein content.

References

Fregene, M.; Ospina J. A. Y Roca, W. M. (1999). Recovery of cassava (*Manihot esculenta* Crantz) plants from culture of inmature zygotic embryos. En: *Plant Cell Tissue and organ culture,* 55: 39-43

Roca, W. M. (1984). Cassava. En: Sharp W. R.; Evans,D. A.; Ammirato, P. V. Y Yamada, Y. (Eds). Handbook of Plant Cell Culture; 2: Crop Species. MacMillan, New York. p 269-301.

Activity 8.17 TRIPS

1. Visit to the Iwate Biotechnology Research Center (IBRC), Kitakami, Iwate, Japan. Transient assay of a CMD candidate gene for resistance to the African Cassava Mosaic virus. March 23-April 19, 2002
2. Visit to Uganda. Set-up of a SSR marker lab in Kampala (medical biotech labs) and visit to NARS partners. May 15-May 228 2002
3. Visit to India. Visit to CTCRI on the MoU between CIAT and CTCRI on germplasm transfer and training July 20 to July 30, 2002.
4. Attend the Meeting on Biotechnology, Breeding and Seed System s for African Crops in Entebbe Nov 3-7.

Activity 8.18 TRAINING

Visiting Researchers

1. Ms Cach, Vietnam (BSA of flowering)
2. Ms E.lizabeth Okai, Ghana (Genetic diversity)
3. Mr Luis Monte, Guatemala (Genetic diversity)
4. Ms Prapit, Thailand (BSA dry matter)
5. Ms Chommanat, Thailand (Genetic diversity)
6. Luis Guillermo, Colombia (Tissue culture)
7. Charles Buitrago, Colombia (Web-based data base management)
8. Mr Emmanuel Okogbenin, Nigeria (Fine mapping).

Students

1. Jaime Marin, undergraduate student Universidad de Tolima, Ibague (QTL mapping of early bulking)
2. Angela Zarate, undergraduate student Universidad de Tolima, Ibague (Conversion of RFLP to SSCP markers)
3. Martha Isabel Moreno, Post graduate (M.Sc.) student Universidad de Valle, Cali (Gene cloning of CMD2)
4. Gina Puentes Jazbleidi, undergraduate student Universidad Nacional, Sede Palmira (Waxy cassava starch)
5. Paula Andres, undergraduate student, Universidad Javeriana, Bogota (Gene tagging of CBB resistance)

PUBLICATIONS

Refereed journals

Akano A., Barrera E., Dixon A.G.O., Fregene M. (2002). Molecular Genetic Mapping of Resistance to the African Cassava Mosaic Disease. Theor and Appl Genet 105:521-525.

Okogbenin E. and Fregene M (2001) Genetic Analysis and QTL Mapping of Early Bulking in an F_1 Segregating Population from Non-inbred Parents in Cassava (*Manihot esculenta* Crantz) (Theor and Appl Genet published online September 10)

Book chapter

Fregene M., Tohme J., Roca W., ChavariaggaP., Escobar R., and Ceballos H. (2002) Biotechlogia para la Yuca. In: Ospina and Ceballos (eds). La Yuca en el tercer Milenio. Sistemas Modernos de produccion, Procesamiento, Utilization y Comercializacion. CIAT. Cali, Colombia