Table 12.13. Percent protein content of 49 inter-specific lines selected for SDS-PAGE analysis

	Name	Genotype	Maternal parent	Paternal parent	P.C. (%)
1	1	CW 178-1	OW 132-2	CW 48-1	6.39
2	2	CW 178-2	OW 132-2	CW 48-1	6.44
3	3	CW 179-1	OW 132-2	MTAI 8	7.02
4	4	CW 179-2	OW 132-2	MTAI 8	7.86
5	8	CW 179-6	OW 132-2	MTAI 8	6.72
6	14	CW 179-12	OW 132-2	MTAI 8	7.8
7	23	CW 205-4	OW 231-3	MTAI 8	7.62
8	24	CW 205-5	OW 231-4	MTAI 9	7.25
9	28	WW 3-1	OW 132-2	OW 240-6	7.31
10	31	WW 3-4	OW 132-2	OW 240-6	6.33
11	33	WW 3-6	OW 132-2	OW 240-6	7.32
12	34	CW 177-1	OW 132-2	CM 1585-13	7.19
13	35	CW 177-2	OW 132-2	CM 1585-13	6.55
14	40	CW 177-7	OW 132-2	CM 1585-13	6.84
15	42	CW 177-9	OW 132-2	CM 1585-13	6.81
16	43	CW 177-10	OW 132-2	CM 1585-13	7.43
17	59	CW 177-26	OW 132-2	CM 1585-13	8.52
18	61	CW 177-28	OW 132-2	CM 1585-13	6.7
19	70	CW 177-37	OW 132-2	CM 1585-13	7.73
20	84	CW 177-51	OW 132-2	CM 1585-13	7.05
21	87	CW 177-54	OW 132-2	CM 1585-13	7.17
22	89	CW 177-56	OW 132-3	CM 1585-14	6.77
23	97	OW 253-3	OW 132-2	CM 1585-13	8.65
24	29	CW 99-30	CW 30-29	OW 280-1	7.66
25	32	CW 160-3	CW 56-5	OW 181-2	7.86
26	33*	CW 161-1	CW 56-5	OW 189-1	8.03
27	35*	CW 161-3	CW 56-5	OW 189-1	7.42
28	37	CW 183-1	OW 180-1	CW 48-1	6.05
29	39	CW 185-1	OW 180-1	MTAI 8	7.58
30	65	CW 198-23	OW 230-3	CW 30-65	6.77
31	81	CW 201-1	OW 230-3	CW 56-5	7.1
32	92*	CW 203-3	OW 230-4	CW 48-1	6.37
33	93	CW 204-1	OW 231-3	AM 244-31	6.25
34	104	WW 14-5	OW 181-2	OW 280-2	9.18
35	134	WW 14-37	OW 181-2	OW 280-2	9.23
36	249	WW 22-76	OW 231-3	OW 240-8	7.73
37	256	WW 24-1	OW 231-3	OW 280-2	7.8
38	280	WW 24-25	OW 231-3	OW 280-2	7.66
39	290	WW 39-1	OW 280-1	OW 280-2	7.27
40	324	WW 40-12	OW 284-1	OW 280-2	8.78
41	382	WW 40-74	OW 284-1	OW 280-2	9.28
42	377	WW 40-68	OW 284-1	OW 280-2	10.46
43	409	WW 41-25	OW 284-1	OW 146-1	8.7
44	410	WW 41-26	OW 284-1	OW 146-1	8.41
45	442	CW 184-5	OW 180-1	MCOL 1734	6.27
46	447	CW 212-1	OW 284-1	MCOL 1734	6.53
47	455	CW 251-2	MCOL 1734	OW 189-1	6.61
48	457	CW 251-4	MCOL 1734	OW 189-1	6.46
49	459	CW 256-1	MCOL 1734	OW 280-1	7.2

Methodology
High protein inter-specific lines with between 6 and 10% of crude protein in the roots, from evaluations conducted in June 2002 and repeated August 2003, were established in a crossing block in September 2003 for genetic crosses to the elite line MTAI 8, a yellow variety. It has been observed that earlier crosses of wild relatives with high protein content in the roots to MTAI 8 and other yellow varieties always gave families with a high average of root proteins, confirming earlier observation that beta-carotene in cassava roots is associated with some storage proteins (Carvalho 2003, personal communications harvested in). Thirty three varieties of cassava identified earlier to have high proteins in the roots (CIAT 2003) were shipped as *in vitro* plantlets to Ghana for genetic crosses to local varieties, part of the Ph.D. degree of Ms Elizabeth Okai. The plants were held for one week at the tissue culture facility of the Botany Department of the University of Legon, Accra, then hardened and transferred to a crossing block at Wenchi. Between 5-10 plants per variety were transferred to the field at Wenchi.

Root flour from 49 inter-specific hybrids with 6-10% of crude protein in their roots from evaluations conducted in August 2003 and May 2004 were selected for SDS-PAGE gel analysis of protein content (Table 12.13.). Root flour from two cassava varieties, a high (MGUA 10) and low (CM 523-7) protein content, were used as controls. Starch granules were isolated as described by Jasso (1986). The protein compositions of starch granules were analyzed on SDS-PAGE. Dry starch granules (the pellet) was diluted to a 1:1 ratio with denaturation buffer (2% SDS and 5% ß-mercaptoethanol) and extracted by boiling for 10 minutes (Vos-Scheperkeuter, et al. 1986) The samples were centrifuged at 14 000 rpm for 20 minutes and the resulting supernatant (crude extract) was subjected to SDS-PAGE denaturing electrophoresis as described by Laemmli (1970) with some modifications (CIAT 2003). Ten µl of each sample was loaded per lane. Constant voltage of 100V was applied for 1 h at 10 °C and increased to 150V for the remaining duration of the run until the tracking dye reached the gel mold. A *Phaseolus vulgaris* protein sample (Phaseolin type I) was used as a check in the protocol assay and some cassava varieties with high (MGUA 10) and low (CM 523-7) protein contents were used as controls. A Bio-Rad low molecular weight (20 to 113 KD) (Bio-Rad´s Prestained SDS-PAGE Standards, low range) protein marker was used to determine approximate protein size.

The protein molecular weight markers in Bio-Rad´s Standard were: Phosphorylase b (103.000 Da.), albumin serum (BSA) (77.000 Da.), Ovalbumin (50.000 Da.), Carbonic anhydrase (34.300 Da.), Soybean trypsin inhibitor (28.800 Da.), and Lysozyme (20.700 Da.).

After electrophoresis, gels were stained in a solution containing 50% Methanol, 10% Acetic Acid and 0.25% Coomassie Brilliant Blue R, over night at RT. Gels were washed with water and distained by two changes of distaining solution first with 20% methanol and 10% Acetic Acid, and second with 5% methanol and 7% Acetic Acid.

Results
Back crosses were started in April 2004, a total of 5,980 crosses have been made so far and will continue until the first week of November. Table 12.14 shows the back cross families and the available or expected seeds from this year's crosses. Currently, 433 seeds have been sent to the tissue culture lab for the embryo rescue and establishment of the BC_1 populations. After embryo rescue the plantlets will be multiplied and 10 plantlets sent to the

screen house for hardening and transfer to the field. The crosses will be established next year in single row plots of 8 plants with one replication.

Table 12.14. BC_1 families for QTL mapping of high protein content generated in 2004

	BC_1 donor parent	Mother	Father	%Protein Content	BC_1 cassava parent	Seeds available or expected
1	CW 73 - 2	CM 1585 - 13	OW 284 - 1	15.94	MTAI 8	300
2	CW 187 - 222	OW 181 - 2	CW 48 - 1	12.71	MTAI 8	200
3	CW 198 - 11	OW 230 - 3	CW 30 - 65	11.28	MTAI 8	340
4	CW 201 - 2	OW 230 - 3	CW 56 - 5	10.2	MTAI 8	105
5	CW 205 - 2	OW 231 - 3	MTAI 8	10.54	MTAI 8	255
6	CW 188 - 4	OW 181 - 2	MCOL 1734	9.62	MTAI 8	145
7	CW 151 - 38	CW 48 - 1	OW 280 - 1	8.18	MTAI 8	170
8	CW 198 - 28	OW 230 - 3	CW 30 - 65	8.37	MTAI 8	200
9	CW 201 - 3	OW 230 - 3	CW 56 - 5	8.25	MTAI 8	50
10	CW 205 - 4	OW 231 - 3	MTAI 8	8.4	MTAI 8	28
11	CW 179 - 2	OW 132 - 2	MTAI 8	7.92	MTAI 8	406
12	CW 198 - 10	OW 230 - 3	CW 30 - 65	7.3	MTAI 8	124
13	CW 198 - 17	OW 230 - 3	CW 30 - 65	7.07	MTAI 8	21
14	CW 203 - 3	OW 230 - 4	CW 48 - 1	7.05	MTAI 8	200
15	CW 251 - 4	MCOL 1734	OW 189 - 1	7.99	MTAI 8	120

Crude protein content for the high protein varieties evaluated in Ghana revealed a range of 5-7% and a 0.9 correlation with previous evaluations at CIAT. These results confirm earlier results for these varieties and suggest that protein content in cassava roots is a stable trait. Based on this, genetic crosses have been started this year between these varieties and elite CIAT parental lines, more than 500 crosses have been made this year.

The SDS-PAGE profiles of the root proteins in the crude extracts from the 49 cassava genotypes, showed different protein bands (Figure 12.17). The difference among polypeptide profiles is in both the number and relative intensity of the bands. The highest molecular weight protein group (2 bands) observed was between 103,000 and 77,000 Da and the lowest band 20,700 Da. There are reports of cassava proteins showing bands at 14,000-80,000 Da of molecular mass (Glaucia, 2001), using two-dimensional gel electrophoresis. Souza *et al*, 1998 reported the isolation of a major protein of MW of about 22,000, which was restricted to the parenchyma rather than the peel of the tuber. These results show that the patterns observed in our study are between the reported ranks (22,000-80,000 Da.).

Proteins in cassava roots showed six main groups of bands between 103,000 and 20,700 Da. Group one contained two bands between 103,000 and 77,000 Da (Table 12.15). Group 1a contained one band of 77,000 Da, present in the sample 2C (control). Group two contained one band of 50,000 Da, which coincided with the ovalbumin pattern and the band of I Phaseolin (*Phaseolus vulgaris* control). Group three contained two bands of 50,000 present only in the sample 4C (control), that coincided with the bands of I phaseolin.

Table 12.15. Groups of bands found SDS-PAGE protein electrophoresis of 49 high protein lines.

Sample Number	Genotype	Group 1	Group 1a	Group 2	Group 3	Group 4	Group 5	Group 6	Sample Number	Genotype	Group 1	Group 1a	Group 2	Group 3	Group 4	Group 5	Group 6
1	CW 178-1	+		+		+			35*	CW 161-3*	-	-	-	-	-	-	-
2	CW 178-2	+		+		+			37	CW 183-1*	-	-	-	-	-	-	-
3	CW 179-1	+		+	+	+	+	+	39	CW 185-1			+		+		+
4	CW 179-2*	-		-	-	-	-	-	65	CW 198-23					+	+	
8	CW 179-6*	-		-	-	-	-	-	81	CW 201-1			+		+	+	
14	CW 179-12*	-		-	-	-	-	-	92*	CW 203-3			+		+	+	
23	CW 205-4		+						93	CW 204-1	+		+		+		
24	CW 205-5		+			+			104	WW 14-5	+		+				
28	WW 3-1		+			+			134	WW 14-37*	-	-	-	-	-	-	-
31	WW 3-4	+		+				+	249	WW 22-76			+				+
33	WW 3-6	+		+		+		+	256	WW 24-1						+	+
34	CW 177-1	+	+						280	WW 24-25*	-	-	-	-	-	-	-
35	CW 177-2		+			+			290	WW 39-1*	-	-	-	-	-	-	-
40	CW 177-7	+		+		+			324	WW 40-12	+		+		+	+	+
42	CW 177-9	+	+						382	WW 40-74	+		+			+	+
43	CW 177-10	+		+		+	+	+	377	WW 40-68	+		+		+	+	+
59	CW 177-26	+		+	+	+	+	+	409	WW 41-25*	-	-	-	-	-	-	-
61	CW 177-28	+		+		+	+	+	410	WW 41-26	+		+		+	+	+
70	CW 177-37	+	+						442	CW184-5	+		+		+	+	
84	CW 177-51		+			+			447	CW 212-1						+	+
87	CW 177-54		+			+			455	CW 251-2*	-	-	-	-	-	-	-
89	CW 177-56*	-		-	-	-	-	-	457	CW 251-4		+			+		
97	OW 253-3	+		+	+	+	+	+	459	CW 256-1		+			+		
29	CW 99-30			+				+	C4	MGUA 10				+			
32	CW 160-3			+		+			C2	CM 523-7	+	+					
33*	CW 161-1			+		+	+	+									

Group four contained two bands between 34,300 and 50,000 Da. Group five contained one band of 28,800 Da with the same weight as the soybean trypsin inhibitor. Group six contained one band between 28,800 and 20,000 Da. (Table 12.15, Figure 12.17).

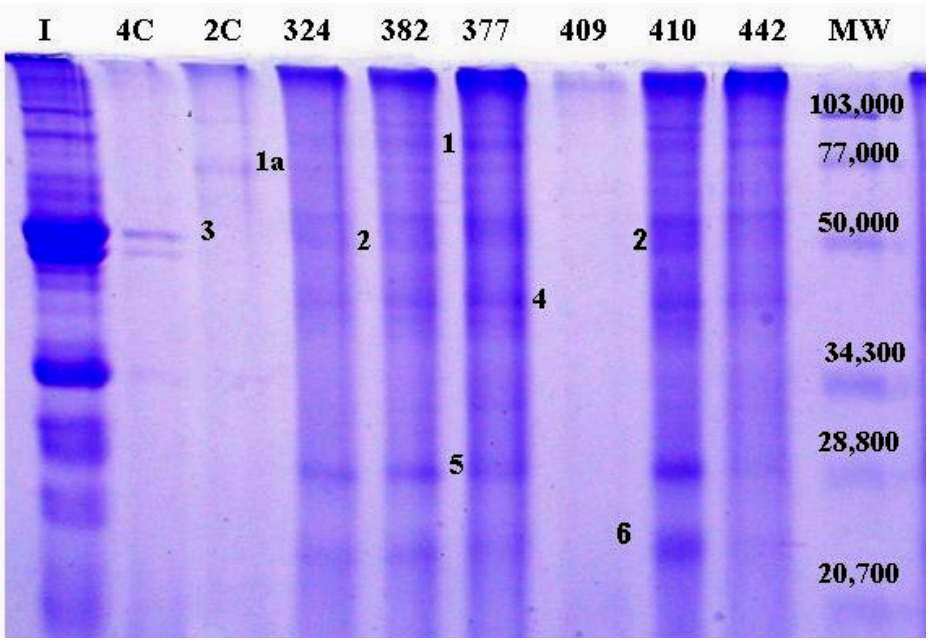

Figure 12.17. One-dimensional, SDS-PAGE gel electrophoresis of total proteins from root of eight cassava cultivars. The samples 4C and 2C are cassava varieties with high and low protein content respectively, the first late (I) is a protein extraction from *Phaseolus vulgaris* used as reference. Bands 1a and 3 only seen in the cassava controls.

Conclusions and perspectives

Generation of backcross families for QTL mapping and development of improved varieties with high protein content were initiated this year. Evaluation of protein content in Ghana of varieties from Central America conducted this year confirmed the high protein content observed earlier at CIAT. SDS-PAGE protein analysis made possible identification of polypeptides that could be isolated in future for more detailed characterization. Future perspectives include the establishment and evaluation of the BC_1 QTL mapping populations and genetic crosses between the high protein cassava varieties and elite parents of CIAT's cassava gene pools.

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Activity 12.13 Mining the Primary Gene Pool: Green Mites (CGM) Resistance Genes from Manihot tristis.

Collaborators:

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Important outputs

1. Identification of additional sources of resistance to green mites in an F_1 inter-specific hybrid between cassava and *Manihot tristis*
2. Development of BC_1 families to introgress the high level of green mites resistance into cassava

Rationale

A high level of resistance to the cassava green mites (CGM) was found 2 years ago in 4 F_1 inter-specific hybrids between cassava and *Manihot esculenta sub spp Fabellifolia.* An attempt was made to identify SSR markers linked to CGM resistance genes as well as introgress the resistance into cassava by the generation of 45 BC_1 families (CIAT 2003). This year another inter-specific family CW208, a cross between MTAI8 and OW132-2 *Manihot tristis*, was also observed to have a wide segregation for resistance/susceptibility to CGM. This same inter-specific cross also has a wide segregation for dry matter content. We describe here evaluation of this cross for resistance to CGM and efforts to develop gene tagging and breeding populations for increased resistance to CMD.

Methodology

The CW208 family is an F_1 inter-specific hybrid cross with a total of 120 genotypes. A single row trial (SRT) of the family was planted August 2003 at CIAT, Palmira. It was evaluated in April this year for resistance to mites according to a 3 class score of 1: means no visible mite damage, 3: moderate damage to mites, and a score of 5: severe damage and loss of all apical meristems due to mite damage. Trait score data was entered into Microsoft Excel and a frequency distribution of score classes derived.

Results

Frequency distribution of 3 score classes revealed a close to normal distribution pattern for resistance to green mites found in the family CW208 (Figure 12.18). The large number of highly resistant and highly susceptible genotypes makes this family an ideal one for bulked segregant analysis (BSA) to identify genes for green mite resistance. Bulks have been made with genotypes from both resistant and susceptible extremes and will be analyzed shortly with over 600 SSR markers. Backcrosses are also being made using selected progenies with resistance to CGM to MTAI 8, an elite cassava genotype that is highly susceptible to green mites.

Conclusions and Future Perspectives

An additional source of resistance to green mites has been identified in an inter-specific hybrid family between cassava and *Manihot tristis.* The family is currently being used for BSA for genes controlling resistance to CGM and for the development of BC_1 families to introgress the high level of green mites resistance into cassava

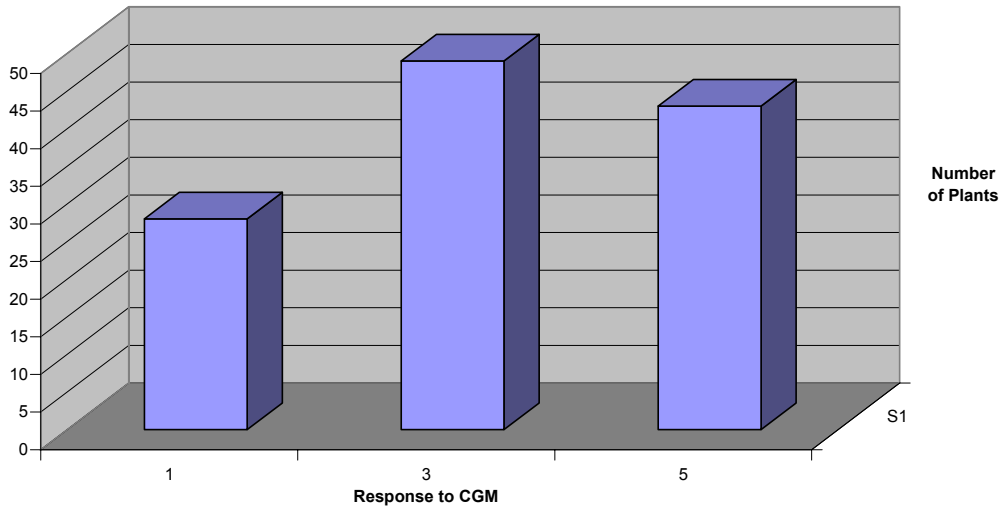

Figure 12.18. Frequency distribution of response to the cassava green mites in the inter-specific family CW208

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Activity 12.14 Identification of Naturally Occurring and Irradiation-Induced Mutant GBSSI Alleles of Cassava in a Heterozygous Genetic Background

Collaborators:

W. Castelblanco, N. Morante, H.Ceballos, A. Rosero, M. Fregene (CIAT), C. Mba (IAEA, Viena, Austria)

Funding:

IAEA, CIAT

Important Outputs:

1) Development of allele specific markers for mutant waxy alleles (GBSSI alleles) from an accession each of the *Manihot* species *M. crassisepala* (MCRA013) and *M. chlorostica* (CW14-11); initiation of genetic crosses

2) Discovery of a possible duplication for the GBSSI locus in cassava and efforts to map the gene in the cassava genome.

Rationale

A change in starch quality, such as the elimination of amylose (waxy starch), via a natural, GMO or irradiation-assisted knockout of the GBSS I gene, implies access to new markets for cassava growers. For most rural communities, a better standard of living depends on increasing income from their crop harvest. The GBSSI gene is directly implicated in the synthesis of amylose, and the identification of naturally occurring or irradiation-induced mutant alleles has great importance for the cassava-breeding program. The waxy trait is a recessive one, the function of all GBSS alleles have to be eliminated for the trait to be expressed. The elimination of the GBSSI genes has been attempted via genetic engineering, but a search for natural or irradiation-induced mutant alleles of the GBSSI gene has also been initiated (CIAT 2003). An accession each of the wild *Manihot* species *M. crassisepala* and *M. chlo*rosticta are the only genotypes from the primary and secondary gene pool of the crop discovered to possess the waxy phenotype (CIAT 1995, 2002, unpublished data). Irradiation-induced mutation of the GBSS gene has also been attempted using both gamma rays and fast neutrons irradiation of sexual seeds (This report). The heterozygous nature and possibilities of a duplicated GBSSI locus in cassava implies that mutant alleles implies that the identification of a waxy phenotype requires selfing thousands of plants or development of molecular markers for identification of mutant alleles in a heterozygous background. We describe here the development of molecular markers for the mutant allele in the wild waxy relatives and the initiation of genetic crosses to transfer this trait into cultivated gene pools around. The marker tools developed will be critical to track down these mutant alleles genes to efficiently move these genes around the different cassava gene pools defined by agro-ecologies. We also describe preliminary molecular work on the search for mutant alleles in the irradiated seeds.

Methodology

Plant materials for the development of markers for mutant GBSSI alleles were two wild *Manihot* accessions MCRA-013 and CW14-11 identified earlier with amylose content between 3-5%. Two cultivated cassava MCOL 1508 and MCOL 2269 were included as reference for normal alleles (Table 12.16). The Codon Optimized to Discover Deleterious Lesions (CODDLE) program was used to identify regions of the GBSS genes where mutations are most likely to lead a loss of function in the protein.

Table 12.16. Wild *Manihot* and cassava accessions used for the development of markers for mutant GBSS alleles.

Cassava accessions	Origin	Species	% Amylose
CRA-013 (Wild)	Mexico	*M. Crassisepala*	3.08
CW14-11	CIAT	Hybrid of *M. Clorostrictha* with unknown parent	4.97
MCOL-1508	CIAT	*M. esculenta*	23.29
MCOL-2269	CIAT	*M. esculenta*	10.55

Table 12.17 Primer sequences designed to amplify the 3' (GT1) and 5' (GT2) end of the Glycosyl Transferase regions of the GBBSSI gene**.**

GBSSI region	**Primer sequence**
GBSSI(GT1) Forward Primer	5' CAGCTATTTCCCAATTGGTTG 3'
GBSSI(GT1) Reverse Primer	5' GCGGTAGCATAAGTGCCAAG 3'
GBSSI(GT2) Forward Primer	5' GCAGGTTTGGATTACCAGGA 3'
GBSSI(GT2) Reverse Primer	5' CAGCAATGCCAGTTTTACGA 3'

Primers were designed to amplify the 3' and 5' of the Glycosyl Transferase region (GT1 and GT2) of the GBSSI gene, between 300-400bp, primer sequences are shown below (Table 12.17). PCR template was both genomic DNA and cDNA (RT-PCR) from the wild and cassava accessions and amplification was as described earlier by Salehuzzaman *et al* (1993). A full-length GBSS cDNA clone, Clone 3, isolated from a cassava root cDNA library was used as positive control.

PCR product was cloned into pGMT (Promega, Madison WI) and 5 clones each from each PCR fragment and each clone were sequenced by big dye (Perkin Elmer, Alamo, CA) cycle sequencing according to the manufacturer's instruction. Sequence information was aligned using the CLUSTAL software program and point mutations identified. An attempt was also made to map the GBSSI gene, as an RFLP marker, on to a molecular genetic map of cassava being drawn in a S_1 family (AM320) from the cassava variety MTAI8. Parental survey filters were made using the following restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *HaeIII*, and *DraI*

using the following DNA samples: MTAI8, 4 S_1 progenies, MCRA-013, CW14-11, MCOL 1508, and MCOL 2269. RFLP probe was a full length GBSSI cDNA clone (clone-3) from a root cDNA library from TMS30572. Preparation and Southern hybridization of the filters were as described by Fregene et al. (1997).

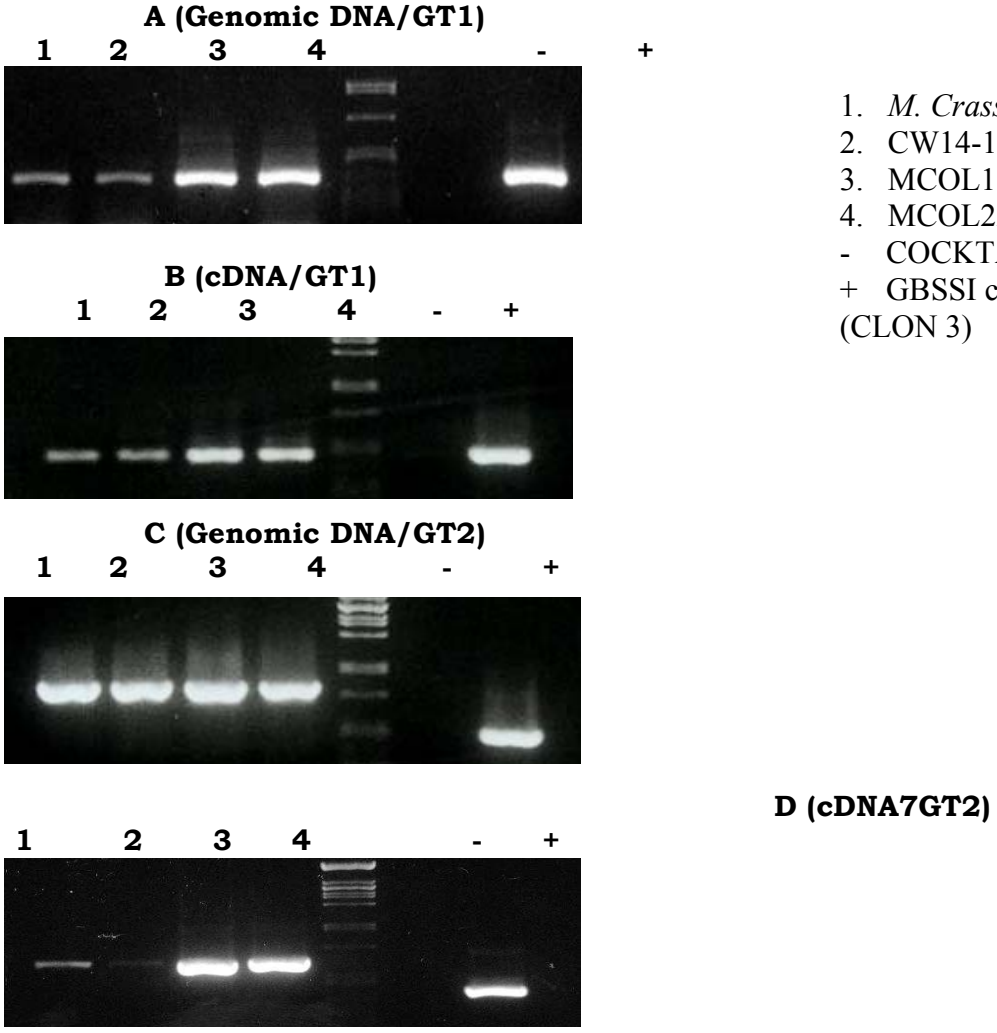

Figure 12.19. PCR amplification of genomic DNA and cDNA from the 2 wild accessions MCRA013 and CW14-11 and 2 cassava varieties MCOL1508 and MCOL2269 with primers from the GT (A and B) and from the SS region (C and D) of the GBSS gene

About 2000 sexual seeds from cassava genotypes tolerant to inbreeding shipped to the International Agency of Energy Atomic (IAEA), Vienna, Austria, for irradiation using gamma rays (a Cobalt-60 source) and fast neutrons, 1000 seeds each for both irradiation methods. The level of irradiation with gamma rays was 200Gy. They were sent back to CIAT and germinated in seedling nursery and transferred to the field, about the 1600 seeds could be

transferred to the field, the other seeds suffered from lethal mutations that affected their ability to germinate.

Results

Amplification of primers flanking the GT1 and GT2 regions from genomic DNA and cDNA (RT-PCR) revealed amplification of similar size fragments for all accessions per primer pair although the wild genotypes had a lower intensity of PCR products with the exception of PCR products from genomic DNA using the GT2 primer pair (Figure 12.19). The lack of size polymorphisms in the PCR products suggests that there are no INDELs (insertions-deletions) type polymorphisms in the wild accessions for this region of the GBSSI gene. The sequence data from DNA clones of the different accessions were aligned and four single point polymorphisms (SNPs) mutations were found that differentiated the wild accessions from the cultivated, two were common to both wild species, while two mutations distinguished *M. chlorosticta* from *M. crassisepala*. The SNPs were sense mutations that led to amino acid changes in the GBSS protein. Allele specific primers were designed for all 4 SNPs as described by Hayashi et al (2004) and have been ordered (Table 12.18). This PCR-based molecular marker could be used to follow the alleles in back-crosses with elite cassava parents.

Southern analysis of MTAI8, 4 S_1 progenies, the 2 wild accessions, and 2 cassava genotypes revealed 2 or more fragment of different intensities for all 5 restriction enzymes (Figure 12.20). This result suggests that there is more than one copy of the GBSSI gene in cassava. Previous studies have revealed 2 GBSS cDNA clones in cassava with <80% homology (Baguma et al. 2003).

The Southern hybridization also revealed polymorphism between the wild and the cultivated confirming previous results of SNPs in the wild species. These SNPs are no doubt associated with the low content of amylose in the two wild genotypes.

Table 12.18. Allele specific primers designed to amplify mutant alleles from MCRA013 and CW14-11

Source of mutant allele	**Primer sequence**
MCRA013 and CW14-11 mutant allele (1)	5'AGAAATTTGAGAAGCAGATTGAGCA**G** 3'
MCRA013 and CW14-11 mutant allele (2)	5' TCAATTTTGTCACATTCAACGAG**C** 3'
CW14-11 mutant allele (1)	5' CTGACAAGGCAAGAGGAGTTGT**T** 3'
CW14-11 mutant allele (2)	5' ACAGCTGGTGCAGACTTTATGCTT**A** 3'

Polymorphisms in a fragment of lower intensity were found with the restriction enzymes DraI and Hae111 in progenies of an S_1 mapping population (Figure.12.20). These enzymes will be used to prepare progeny filters for mapping the gene GBSSI in the S_1 mapping population derived from MTAI 8.

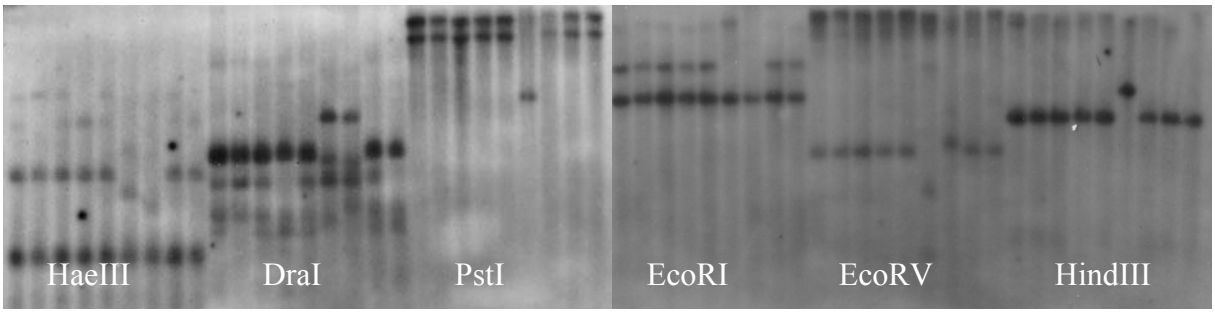

Figure 12.20. Southern blot of restriction enzyme digested cassava DNA from MTAI-8 and 4 of its S_1 progenies (lanes 1-5), MCRA013, CW14-11, MCOL1508, and MCOL2269 (lanes 6-9) probed with a ^{32}P labeled full-length GBSSI cDNA clone (clone 3). Ten microgram of digested DNA was loaded per lane.

There are plants of MCRA013 and CW14-11 growing in the field for genetic crosses to cassava. So far no flowering has been obtained with MCRA013, but several male and female flowers have been obtained with CW14-11. More than 30 crosses were made to elite parents of CIAT cassava gene pools but only 2 of these crosses have been successful to date. Additional crosses are continuing while the 2 fruits obtained till date will be harvested at the end of the crossing season.

The effect of fast neutrons on irradiated seeds was quite severe and less than 200 plants could be established in the field. At the moment there are 943 surviving plants from both gamma and fast neutron irradiation in the field. Morphological characterization of these plants has been initiated. Molecular analysis of these plants to identify mutant GBSS alleles has also been started. Briefly, 8 young shoots were harvested from all over individual plants and bulked for DNA isolation. Plants from irradiated sexual seeds are expected to contain chimeras and bulking the leaf tissue from all over the plant eliminates this problem. All DNA samples will be amplified with primers from the glycosyl transferase region and analyzed by gel electrophoresis to identify any insertions/deletions (INDELS). Amplified PCR products will then be bulked, at least, 20 genotypes per bulked, and used as template for sequencing to identify SNPs. Phenotypic evaluations for amylose content will also be conducted to identify mutants, although improbable, at all possible 4 GBSSI alleles.

Conclusion and future perspectives

Sequence and Southern hybridization analysis have identified SNPs in an accession each of *M.crassisepala* and *M.chlorosticta*. The SNPs from sequence data are sense mutations that are most likely associated with the waxy phenotype found in these genotypes. Allele specific primers have been designed from the SNPs for use in following the alleles in introgression of the waxy trait into elite gene pools. Southern analysis also revealed a high probability of duplication of the GBSSI loci in cassava. Preliminary molecular analysis of plants obtained from irradiation of cassava seeds with gamma and fast neutrons have also been initiated. Future perspectives include:

- Evaluations of allele specific primers in the wild and cassava accessions.
- Genetic mapping of the gene GBSSI in the S_1 population from MTAI8
- Crosses of the wild genotypes to elite cassava cultivars to transfer the mutant alleles.
- Molecular characterization of the irradiated population to identify GBSSI mutants

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Activity 12.15 Molecular Characterization of A Putative Waxy Cassava Starch GMO Obtained by Anti-sense Mediated Silencing of the Granule Bound Starch Synthase I (GBSSI) gene

Collaborators:
Yina J. Puentes, Edgar Barrera, Paúl Chavarriaga, Chikelu Mba,
Martin Fregene (CIAT)

Funding:
Ministerio de Agricultura y Desarrollo Rural (MADR), Colombia, CIAT.

Important Outputs

1) PCR and Southern hybridization analysis of transgenic cassava plants expressing a full-length GBSSI gene in the sense orientation revealed stable expression of the gene.
2) Application for permission to transfer biochemical evaluation of the waxy trait.
3) Preparation of 6 additional transgenic events expressing the full-length GBSSI gene in the anti-sense sense orientation for transfer to the screen house

Rationale

With funds from the Colombian Ministry of Agriculture and Rural Development, a project was initiated in 2002 to genetically engineer industrial cassava varieties to produce waxy starch via the sense- and anti-sense down-regulation of the GBSSI gene, the predominant starch synthase gene that catalyses the conversion of ADP-glucose to amylose. Preliminary transformation work was carried out with the model transformation cultivar, MNig11 (60444). One independent transgenic event was produced last year for the sense construct and plantlets were transferred to the screen house (CIAT 2003). Six independent events were produced for the anti-sense constructs and about 5 plants per event have been transferred to the screen house. We describe here molecular characterization via PCR and Southern hybridization of the sense transgenic line, Permission to transfer these plants to the field has also been requested from the CIAT biosafety committee for biochemical evaluation of the waxy phenotype.

Methodology

The isolation of a full length GBSSI cDNA clone, construction of sense- and anti-sense transformation cassettes and their insertion into the genome of the variety TMS60444 via *Agrobacterium tumefaciens* have been described earlier (CIAT 2003). Molecular characterization of the sense transgenic event was using PCR primers designed from the 3'and 5'ends of the GBSSI gene and primers for the *GUS* reporter gene, the PCR primers and conditions for PCR have also been described earlier (CIAT 2003). Southern analysis was using ten micrograms of total genomic DNA from 4 transgenic plants of the single event and 2 non-transgenic cassava genotypes using the following restriction enzymes: *EcoRI*, *EcoRV*, *HindIII*, *HaeIII*, and *DraI* . The RFLP probe was a full length GBSSI cDNA clone (clone-3) from a root cDNA library from TMS30572. Preparation and Southern hybridization of the filters were as described by Fregene et al. (1997).

Friable embryogenic callus (FECs) of the cassava genotypes 60444 and MCol2215 were transformed via *Agrobacterium tumefaciens* with the GBSSI gene in anti-sense orientation as described earlier (CIAT 2003). Plants could be regenerated for a single transgenic event and 5 events for the genotypes 60444 and MCol22 respectively.

Results

PCR amplification with the GBSSI and *GUS* gene primers yielded positive amplification in the transgenic plants of the expected size of a 2.1 kb and 700 bp fragment respectively (Figure 12.21). Southern hybridization of the GBSSI to genomic DNA digested with *PstI* also gave the expected size of 2.1kb in the transgenic plants but not in the control non-transgenic plants suggesting stable integration in the genome (Figure 12.22). Large molecular weight bands in the non-transgenic plants are indigenous GBSSI genes found in cassava containing introns.

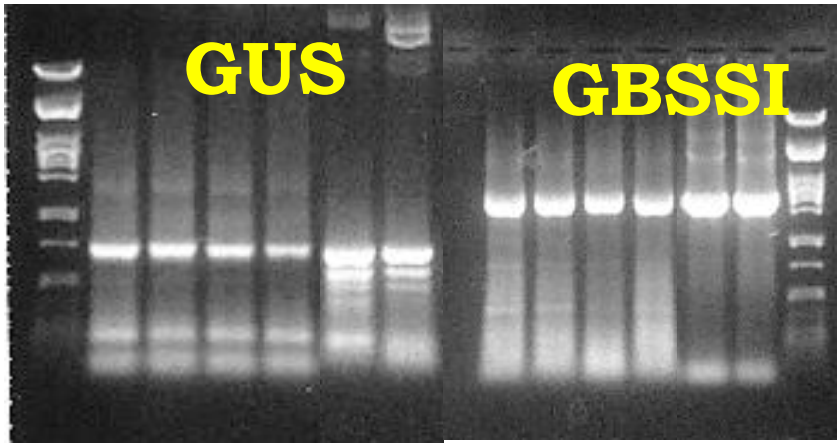

Figure 12.21. (A) PCR amplification of the *gus* using primers specific for the gene. The first and last lanes is molecular weight marker Lambda DNA, digested with *PstI*, the next four lanes from the left (section GUS) are PCR amplification of the *GUS* gene in regenerated transgenic plants transformed with the GBSSI sense construct, the next two lanes are control PCR amplification of the construct in the plasmid pCAMBIA 1305. (B) PCR amplification using primers specific for the GBSSI gene. The first four lanes in the section on the right (labeled GBSSI) are PCR amplification of the gene in regenerated transgenic plants transformed with the GBSSI sense and the last two lanes are control PCR staring of a cDNA GBSS clone (clone 3).

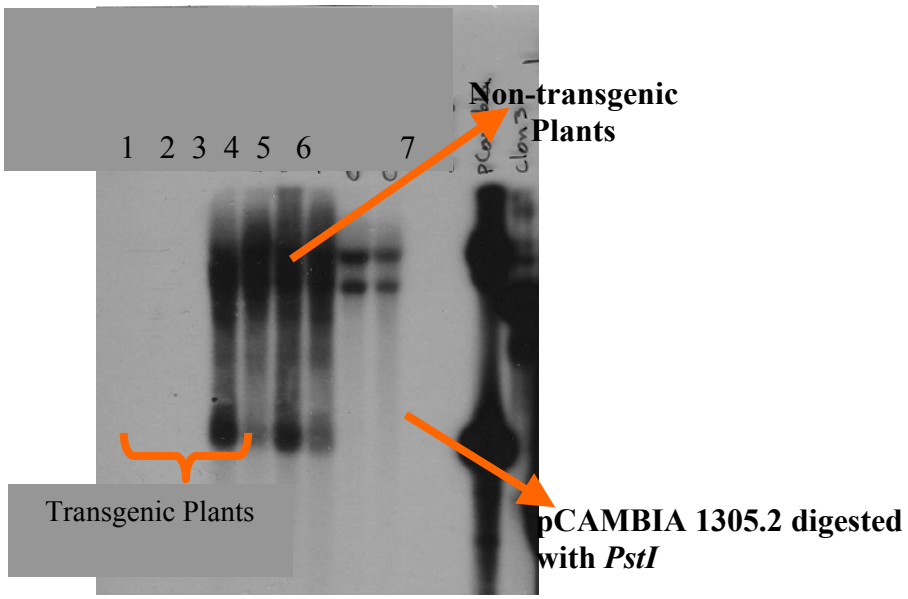

Figure 12.22 Southern hybridization of a GBSS full-length clone hybridized to genomic DNA digested with *Pst I* of transgenic, non-trangenic and the transformation construct. Lane 1-4 are transgenic plants transformed with a sense GBSSI construct, lanes 5-6 are non-transgenic control DNA, lane 7 is the construct in pCAMBIA1305.2. Large molecular weight bands in the non-transgenic plants are indigenous GBSSI genes in cassava containing introns.

Following the confirmation of successful and stable incorporation of the sense GBSSI construct into the genome of the cassava genotype 60444 as revealed by molecular characterization of transgenic plants, the stage is set for biochemical evaluation of roots in field experiments. Application for field trials has been prepared for submission to the Colombian plant quarantine authorities through the CIAT Biosafety committee.

Transgenic plants are at the moment maintained in the CIAT biosafety green house. At the same time, regenerated plants representing 6 transgenic events of the anti-sense constructs from the genotypes 60444 and MCol22 have been transferred to the screen house in preparation for molecular characterization.

Conclusion and Perspective

We have successfully transformed a full-length sense construct of the GBSSI gene into the model cassava transformation variety 60444. Molecular characterization by PCR and Southern hybridization revealed stable incorporation of the gene, biochemical tests to confirm the waxy phenotype will be conducted on field grown plants once a permit has been obtained to transfer these plants to the field. Additional transgenic events using an anti-sense construct have also been obtained and transferred to the biosafety green house in preparation for molecular characterization.

Figure 12.23. Transgenic plant in biosafety green house.

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Activity 12.16 Modification of Flowering in Cassava

Collaborators:

Yina J. Puentes P, Zaida Lentini, Joe Tohme, Paul Chavariagga, Martin Fregene (CIAT) Sarah Adeyemo, Seth Davies (Max Planck Institute for Plant Breeding Research, Cologne)

Funding:

Rockefeller Foundation

Important outputs

1) Acquisition of the APETALA and ethanol inducible promoter ***AlcR*** genes identified in other species for modification to control flowering for use in Cassava

2) Preparation of friable embryogenic callus (FECs) from the model cassava transformation variety 60444 (MNig 11)

Rationale

Flowering and its control is one of the most important challenges of cassava breeding. Shy and non-synchronized flowering can result in an inability to use a very valuable genotype in breeding. On the other hand, profuse early flowering is normally an undesired characteristic as it is associated with heavy branching and tends to lead to low harvest index and yield (Cock and El Sharkaway 1988). Conversely high yielding genotypes either do not branch or branch late and the first branches formed often do not produce fertile flowers. This leads to a dilemma for breeders who must produce shy flowering types for high yield, but require profuse early flowering types for making genetic crosses. In cassava an approach that will increase flower number and specifically target the timing of flowering would be a great asset to the field breeder.

There have been major advances in the understanding and control of flowering. For example the discovery of the LEAFY gene and its action has allowed researchers in the United States of America to induce flowering in three month old aspen trees, which normally flower earliest when they are eight years old (Salk Plant Biology). It is probable that homologous genes control flowering in cassava and much of the knowledge gained in other temperate species could be used to rapidly control flowering in cassava. Furthemore, the amount and timing of flowering can be manipulated through controlled expression of flowering-time genes under the control of an ethanol-inducible promoter (AlcR), ethanol is a common and readily available/storable compound that could be used to regulate the expression of a transgene in the field. This AlcR gene-expression system functions in many plants and works in controlled breeding environments (Sweetman et al. 2002), suggesting it will be useful in cassava.

With funding from the Rockefeller foundation and in collaboration with Dr Seth Davies group at the Max Planck Institute for Plant Breeding Research, Cologne, Germany, a project has been initiated this year to modify the control of flowering in cassava via genetic transformation using the flowering gene APETALA and LEAFY driven by an ethanol inducible promoter (AlcR). Immediate benefits of the project include making accessible to conventional breeding the many excellent cassava genotypes that are recalcitrant to flowering, and easing the difficulties of synchronizing flowering between cassava genotypes which currently flower at different times in the breeding cycle.

Methodology

The first step in the project is the acquisition of the flowering genes and ethanol inducible promoters (*AlcR*) via negotiated agreements with the intellectual owners of the LEAFY, APETALA genes and the AlcR promoter for their use in cassava. The ethanol-inducible promoter AlcR is owned by Syngenta, the APETALA gene (*AP1*) is owned by the University of California San Diego, and the LEAFY (gene) *LFY* is owned by the Salk Institute. The LFY gene is still being negotiated with the Salk Institute. The ethanol-inducible promoter will be separately fused to the two flowering-time genes and then cloned into a non-antibiotic selection marker; in this project we will be using a binary vector containing LUC PLUS and the NPT II gene. These constructs will be fully selectable and/or screenable in Arabidopsis, cassava and GUS PLUS will be used for cassava. These constructs will be generated at the Max Planck Institute in Cologne and tested in Arabidopsis genotypes that mimic "shy"

flowering. *LFY* and *AP1* are genetically both necessary and sufficient for conversion of vegetative meristems to reproductive meristems (Mouradov *et al.* 2002). The choice of these two genes lies in the one that allows the bypass of the genetic blocks of multiple pathways.

The above constructs will be then be transferred to CIAT and inserted into the cassava varieties CM3306-4 and 60444 that flower moderately. Constructs with the flowering gene will also be made with other inducible promoter systems, for example promoter systems based on sterol or tet, provided agreements for their use can be negotiated with the owners. The transformation protocol to be followed is that described by Schopke et al. (1996) with some modifications made at CIAT and has been used with success to produce several independent transgenic events (CIAT 2002). At least seven independent transformation events will be generated for each of the flowering gene constructs. The genetic pathway blocks of flowering are unknown in cassava therefore the choice of 2 genes that lie far downstream of floral initiation will facilitate the production of flowering in widely differing genotypes of cassava, irrespective of the molecular mechanisms that inhibit flowering in this plant. The effects of the flowering genes will be assessed by spraying ethanol on the transgenic plants at 4 months after planting to induce flowering.

Results

Syngenta and the University of San Diego California have made available the *AlcR* and *AP1* genes to CIAT under a limited 'for research only' MTA, another MTA will be negotiated for permission to use these genes in new cassava varieties. Over 300 apical meristem and nodal cuttings of the model cassava transformation plants 60444 and another variety CM3306-4 have been cultured in preparation for induction of Friable Embryogenic Callus (FECs).

Conclusions and perspectives

A project to modulate the control of flowering in cassava using the flowering genes LEAFY and APETALA under the ethanol inducible promoter *Alcr* have been initiated at CIAT in collaboration with Max Planck Institute for Plant Breeding Research, Cologne, Germany, and funding from the Rockefeller foundation. Constructs for the 2 flowering genes are being carried out at the moment and will be ready by the end of the year for introduction into cassava by agrobacterium transformation.

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Activity 12.17 Construction of a TME-3 Bacterial Artificial Chromosome (BAC) Library and Development of a BAC Contig around a CMD Resistance Gene

Collaborators:
Isabel Moreno, Paola Alfonso, Martin Fregene (CIAT); Jeff Tomkins, Michael Atkins, D. Main (Clemson University Genomic Institute)

Funding:
CIAT, CUGI

Important output:
1) Construction of a bacterial artificial chromosome (BAC) library of cassava from the CMD resistant genotype TME3
2) Construction of 2 BAC contigs around the CMD resistance gene, CMD2, and initiation of chromosome walk

Rationale
High resolution mapping around the cassava genome region bearing a dominant CMD resistance gene, *CMD2*, has lead to the identification of 2 markers, RME1 and NS158 that flank the gene at 1 and 2 cM respectively (Moreno 2004, unpublished data). This year, positional cloning of *CMD2* made progress with the construction of a bacterial artificial chromosome (BAC) library and BAC contigs around *CMD2*. The BAC library construction was carried out at the Clemson University Genome Institute with the participation of CIAT via a graduate student, Ms Isabel Moreno. The BAC library was brought back to CIAT and BAC plate, column and plate pools were created. PCR amplification, using primers of the SSR marker NS158 and the SCAR marker RME1, of the 'BAC Pools' were employed to identify BAC clones containing the markers closest to *CMD2* for BAC contig construction. The BAC contigs will be the start-off point for chromosome walking to the gene of interest.

Methodology
Plant material for BAC library construction was the African local variety TME3 that has resistance to CMD mediated by the dominant gene *CMD2*. Construction of the library was as described earlier by Tomkins et al. (1999a; 1999b; 2004). To estimate the distribution and average size of the clones, a total of 370 clones from the TME3 library were picked at random and grown overnight in 3ml of liquid LB medium + 12.5μg /μl Chlorampenicol. Plasmid DNA was isolated, digested with *Not* I restriction enzymes and inserts separated from the vector by pulsed-field electrophoresis.

Contig construction was by PCR amplification of 'BAC pools', namely 'plate pools' (PP), 'column pools' (CP) and 'row pools' (RP). Briefly, all 192 384-well plates were duplicated using a 384-pin replicator and allowed to grow in LB/ Cloramfenicol (12.5 ug/ml) medium at 37^{o} C over night. For BAC plate pools, all the bacteria culture in a 384-well plate was combined into an omnitray and 200ul of this transferred into a single well in a 96-well plate to yield 2 'BAC pool' plates. Simultaneously, every 10 plates of the library were inoculated into a single 384-well plate using a 384-pin replicator to give 20 384-well plates. Each row of

each of the 20 plates was inoculated, using a sterile tooth pick, into a single well containing LB/ Cloramfenicol (12.5 ug/ml) medium to form 'Row plates (RP)' 5 RPs of 96-well plates in all (Figure 12.24). The same was done for each column of the 20 384-well plates combined into a single well to form 4 96-well 'Column plates (CP)' (Figure 12.25).

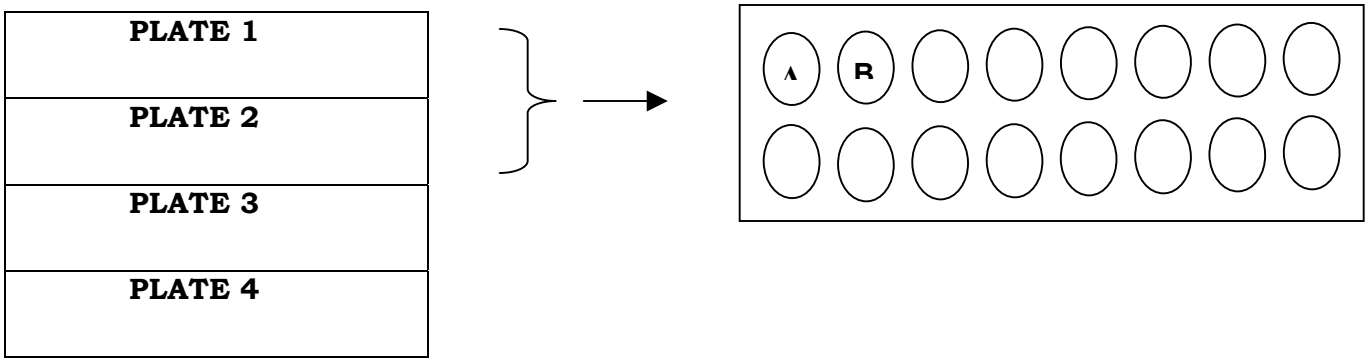

Figure 12.24. Schematic diagram of how the 'Row plates' were formed, sixteen rows from each of the plates (on the right) were combined into a single well (on the right).

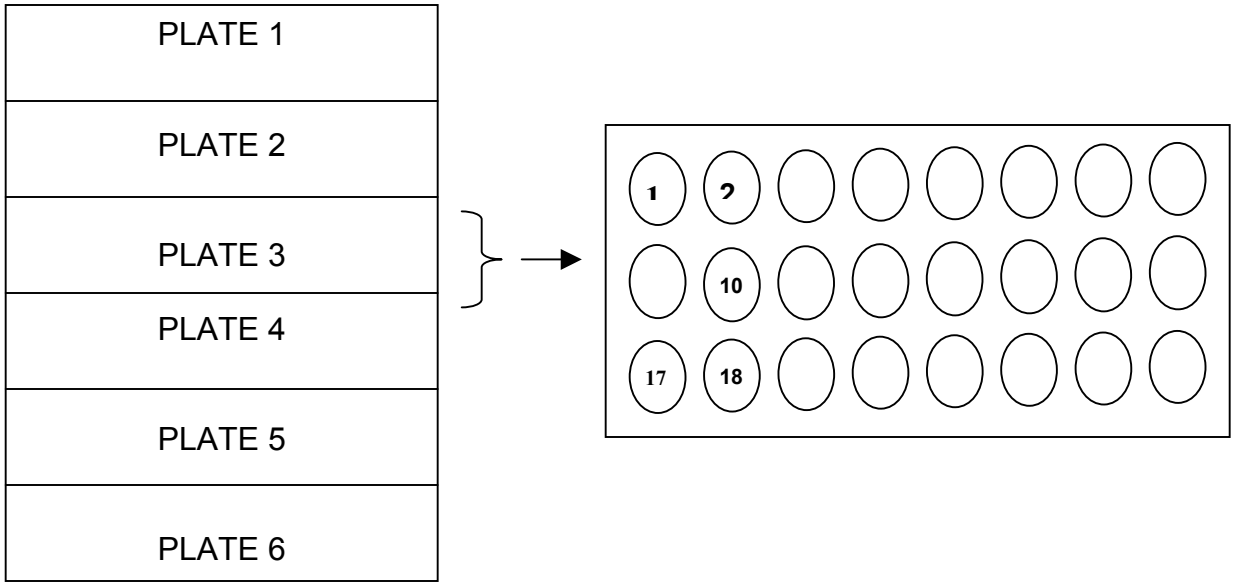

Figure 12.25. Schematic diagram of how the 'column plates' were formed, 24 column from each of the plates (on the right) were combined into a single well (on the right).

The 4 column and 5 row plates were incubated at 37^{o}C overnight. A total of 11 plates PP, RP and CP were obtained. For PCR amplification, 5ul of the bacteria culture was transferred using a multi-pipette to a clean 96 well plate and the bacteria pelleted at 4500rpm for 10

minutes in a Sorvall centrifuge. The supernatant was discarded and the pellet re-suspended in 5ul of sterile water and used as template for PCR amplification. PCR amplification condition for the SSR marker was 2 mM of $MgCl_2$, 0.2 mM of DNTPs, 0.2 uM of each primer, 1 U of taq-polimerasa, in a final volume of 50ul. Thermal cycle profile was an initial denaturation step at 95ºC 2 min, 30 cycles 94º 30s, 55ºC 1 min, 72ºC 1 min, and a final extension step of 72ºC for 5 min. For the SCAR marker, $MgCl_2$ and DNTP concentrations were increased to 2.5 mM and 0.4 mM respectively. PCR cycling conditions were: 95ºC 2 min, 30 cycles: 94ºC 30 seg, 52ºC 1 min, 72ºC 1 min; and a final extension of 72ºC for 5 min. PCR products were visualized in a 1.5% agarose gel stained with ethidium bromide.

Results

The TME3 BAC library is made up of 73 728 clones in 192 384-well plates. Insert size ranged from 20kb to 130kb with an average insert size of 100kb (Figure 12.26). The BAC library has a 10X coverage of the cassava genome.

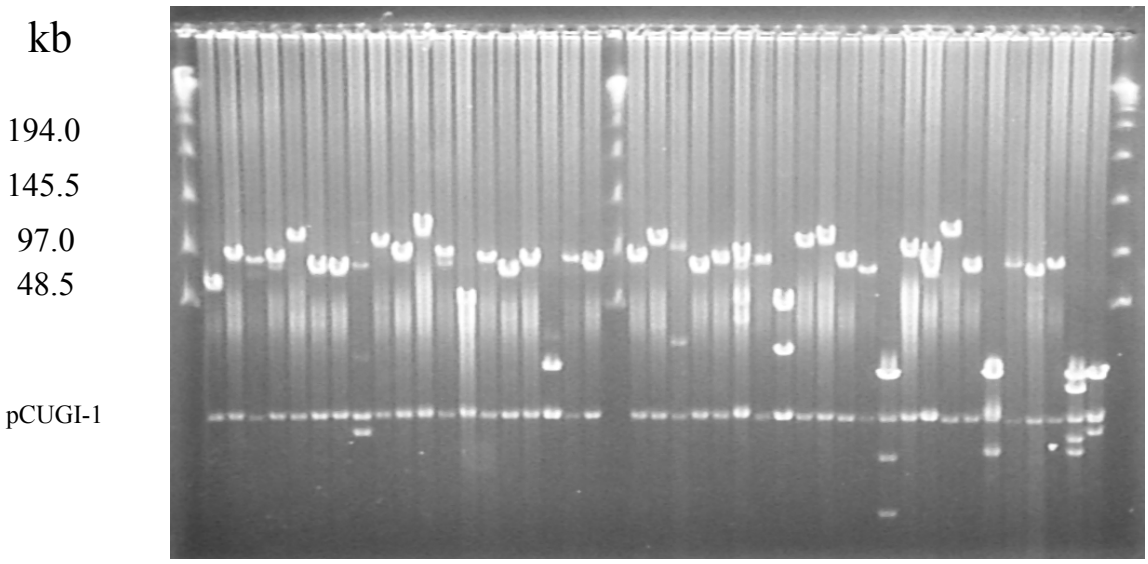

Figure 12.26. Random BAC clones from the TME3 library digested with *Not 1* and run in a 1% agarose gel in 0.5X TBE at 14ºC at 6V/cm with a switch time of 5s-15s pulse time for 14 hours.

Results of screening the 'BAC pools' with NS158 yielded 2 positive clones while screening with RME1 yielded 34 positive clones. NS158 is a single copy SSR marker while RME1 was developed from a multiple copy RAPD marker. The clones were digested with 20U of HindIII overnight and run for 24 hours on 1.2% agarose gel to obtain a BAC clone fingerprint (Figure 12.27.)

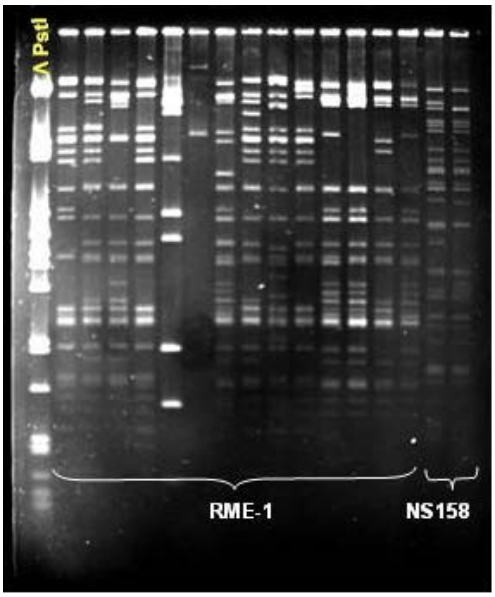

Figure 12.27. Fingerprinting of positive BAC clones.

The positive clones are listed below (Fig.8.28)

"PP" ("Pool Plate")	"RP"("Row Plate")	"CP"(Column Plate")
90	N	18
189	M	19

"PP" ("Pool Plate")	"RP"("Row Plate")	"CP"(Column Plate")
12	N	14
17	M	21
34	L	16
47	O	3
51	I	1
52	A	23
54		
85	J	23
130	D	18
136	J	8
139	G	22
155	K	24
173	F	1

Figure 12.28. List of positive clones from the 'BAC pool' screening using the SSR marker NS158 and the SCAR marker RME1.

Conclusion and Perspectives
A BAC library has been constructed from the CMD resistant genotype TME3. The BAC library was screened with markers closest to the resistance gene CMD 2 and a total of 36 BAC clones obtained. A Hind III digest of the clones yielded a fingerprint, which is being used in BAC contig construction. Primers will be designed from the end of the BAC clones that make up the extreme of the contigs and mapped in a set of recombinants from the fine mapping population. The new markers will be used in another round of screening of the BAC library and a 'walk' to the CMD2 initiated.

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Activity 12.18 Isolation of Full-Length cDNA Clones of Transcripts Differentially Expressed Between Full-Sib Genotyped Resistant and Susceptible to the Cassava Mosaic Disease (CMD).

Collaborators:
Isabel Moreno, Martin Fregene (CIAT), Ryohei Terauchi (IBRC, Iwate, Japan)

Funding:
CIAT

Important Outputs
1) Dot blot screening of cDNA library constructed from a group of CMD resistant full-sib genotypes for identification of full-length cDNA clones representing SAGE tags
2) Identification of full-length cDNA for 7 SAGE tags

Rationale
Serial analysis of gene expression (SAGE) revealed many genes differentially expressed between CMD resistant and susceptible genotypes from a full-sib family (Fregene et al. 2004). To elucidate the role above genes in host-plant resistance to CMD mediated by the dominant resistance gene CMD2, genetic complementation experiments have been planned in cooperation with the Iwate Biotech Research Center (IBRC), Kitakami, Japan. Full-length cDNA clones are required for these experiments. We describe here screening of a cassava cDNA library of cassava was obtained using as template mRNA from a group of 40 CMD resistant genotypes and short PCR amplification product of the SAGE tags using as probe.

Methodology
cDNA clones from a cDNA library developed from CMD resistant clones were hand picked and arrayed on small sized nylon membrane (Hybond-N Amersham Biosciences) filters placed on

solid LB agar media having ampicillin (100mg/ml). The filters were incubated at 37 ºC for 16 hours and the lysis of the colonies were done following the protocol described by Sambrook *et al.* (1989). The filters were stored at 4ºC until use.

SAGE tags had earlier been amplified from the cDNA library (described above) using the tag sequence as forward primer and a primer from the plasmid vector in which the cDNA was cloned (pYES2, invitrogen) as reverse (CIAT 2001). The PCR amplification products are small in size, 200 and 400bp, because the SAGE tag was obtained from the 5'end of cDNA transcripts. These PCR amplification products were cloned into the pGEMT-easy (Promega) vector. To generate probes for dot blot screening of the cDNA library, plasmid containing the SAGE tags was PCR amplified using SP6 and T7 primers. PCR amplification conditions included 3mM $MgCl_2$, 0.16mM de DNTPs, 0.10 uM of each primer and 1U of taq in final volume of 25ul, using 5ul of a 50X dilution of the plasmid DNA template. The thermal profile program was an initial denaturation step of 94°C for 1minute, 30 cycles of: denaturation 94°C for 30seconds, annealing 50°C for 30 seg, extension 72°C for 2min, follow by a final extension step of 72°C for 5 min. Five microliter of the PCR reaction was used as probe for dot blot hybridization as described by Sambrook *et al.* (1989).

Because only a single set of filters was prepared, the SAGE tags were combined after PCR amplification and only 8 probes were prepared from 25SAGE tags available

Probes for screening dot blots	**Combination of SAGE Tags**
C1	Tags 2,3,8,9
C2	Tags 12,18,19,21
C3	Tags 25,7,15,16
C4	Tags 17,26,28,1
C5	Tags 4,5,7,13
C6	Tags 14,11,13,20
C7	Tags 4,11,13,14
C8	Tags 15,16,25

The probes were labelled radioactively with P^{32} using the kit Mega prime DNA Labelling System RPN 1605 (Amersham Biosciences) following the instructions of the supplier. Post hybridization washes were 2XSSC, 0.1% SDS for 10 minutes at 65°C and 0.5XSSC, 0.1% SDS for 5 minutes at 65°C. In some cases an additional high stringency wash, 0.1XSSC 0.1% SDS, for 5 minutes at 65°C was added. Filters were exposed to X-ray films for 2-4 days at –80°C with an intensifying screens film. Positive cDNA clones were identified and verified by PCR using T7 and pYES primers and PCR conditions as described above. Sequencing of the cDNA clones was using the big dye cycle sequencing kit (APPLIED BIOSYSTEMS reference # 4303152) following the manufacturer's instructions on a 377 sequencing machine (PERKIN ELMER/APPLIED BIOSYSTEMS). Sequence homology was obtained using BLAST (www.ncbi.nlm.nih.gov)

Results

More than 19,960 cDNA clones were hand picked from a cDNA library developed from CMD resistant clones and arrayed on 52 small sized filters, 96 clones per filter. Dot blot screening yielded over 100 clones (Figure 12.29) of which 66 positive clones were selected for sequencing after PCR amplification to check size of the cDNA clone (Figure 12.30). Sequence

homology revealed that several of the clones had homology to the following genes related to the SAGE tags from which they were obtained homology for the next proteins:

β- Tubulina
Elongation factor Alpha1α

1. Ribosomal protein 40S
2. Histone
3. Hypothetical protein (*A. thaliana*)
4. Rubredoxine
5. Importing Protein

Analysis of the sequence data revealed that close to all the cDNA clones sequenced were full-length or almost full-length clones.

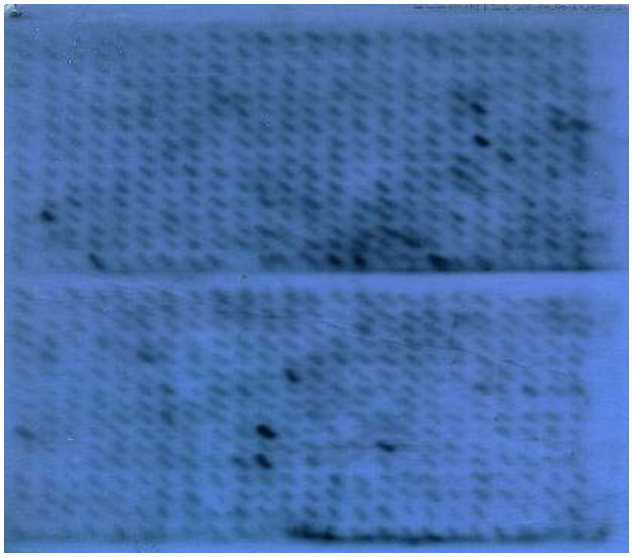

Figure 12.29. Dot blot hybridization of a cDNA library using SAGE Tags as probes, dark spots are putative cDNA clones with homology to the SAGE tag used as probe.

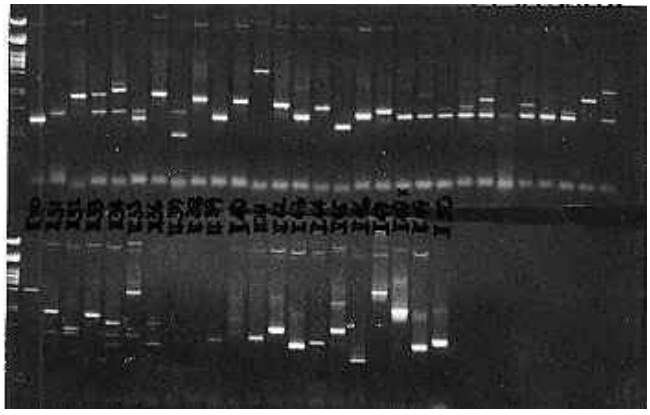

Figure 12.30. Positive clones from dot blot screening of the TME3 cDNA library amplified with the primers T7 and pYES

The full-length cDNA clones are going to be used in genetic complementation experiment to assess the function of these differentially expressed genes in relation to host-plant resistance to CMD.

Conclusions and Perspective

Dot blot screening of cDNA library constructed from a group of CMD resistant full-sib genotypes has lead to the identification of full-length cDNA clones for 7 SAGE tags. These genes will be used in genetic complementation experiments to determine the relationship of these genes of host-plant resistance to CMD mediated by *CMD2*.

References

CIAT 2001.Annual report SB-2 project. Annotation of SAGE tags (Transcripts) differentially expressed in CMD resistant and susceptible genotypes. CIAT, Cali, Colombia.

SAMBROOK, J; FRISH,E; MANIATUS, T. 1989. Lysis of Colonies and Binding of DNA to Nitrocellulose Filters : a Laboratory manual , 2nd ed. Cold Spring Harbor laboratory, Cold Spring Harbor. New York.

Activity 12.19 Embryo Rescue of Sexual Seeds from BC_2 Families for Molecular Marker-Assisted Selection (MAS) for Resistance to Cassava Green Mites (CGM) and the Cassava Mosaic Disease (CMD)

Collaborators:

Luis Guillermo Santos M., Yudi S. Moreno M., Adriana M. Alzate G., Martin Fregene (CIAT)

Funding:

Rockefeller Foundation, CIAT

Important Outputs

1) *In vitro* establishment of embryo axes of 1490 mature seeds derived from 43 BC_2 families, that combines resistance to the cassava green mites (CGM) and the cassava mosaic disease (CMD).
2) Shipment of progenies selected by MAS to Tanzania for use as parents in the RF cassava MAS project in Tanzania

Rationale

Embryo rescue of populations for molecular breeding for resistance to the cassava mosaic disease (CMD) was initiated last year for easy movement of this germplasm to partners in Africa and India. In the second phase of the MAS project at CIAT, resistance to CMD is to be combined with resistance to the cassava green mites (CGM) derived from a close wild relative of cassava, *Manihot esculenta* sub spp *flabellifolia*. This germplasm is primarily to serve as parents for a MAS project to improve CMD and CGM in Tanzania funded by the Rockefeller

Foundation, and for use in the CIAT breeding program. A total of 1490 seeds were obtained from more that 2000 controlled crosses between BC_1 progenies having excellent resistance to mites and CMD, and resistance parents at CIAT, 1291 genotypes were germinated by means of embryo rescue and propagated for MAS. Genotypes that were shown to have CMD and CGM resistance genes by MAS were further propagated and a set of 8-10 plants shipped to partners in Tanzania or transferred to the greenhouse here at CIAT for further field evaluation.

Methodology

Embryo rescue of 1490 mature seeds of cassava from 43 BC_2 progenies was done following a protocol developed earlier at CIAT (CIAT 2003). Briefly, seeds were put in a beaker of water and those that floated were discarded, viable seeds were soaked in 97% suphuric acid for 50 minutes followed by several washings with water to remove the acid. The seeds were then disinfected by soaking in 70% ethanol for 2 minutes followed by 0.5% sodium hiphoclorite for 12 minutes and three washings with sterile deionized water. The embryo axes with cotyledons were removed and placed on 17N medium. They were incubated in the dark for 5 days in the growth room and then exposed to light under photoperiod conditions of 12/12 hours night/darkness at a temperature of 27-30^{o}C. The plantlets were coded "AR" to distinguish them from the CR series having only resistance to CMD. After 3-4 weeks of growth, leaf tissue was removed from individual plants for molecular analysis with markers associated with CMD and CGM resistance. Genotypes shown to carry CMD and CGM resistance were propagated and moved to the screen house or shipped to partners in Tanzania.

This year a different method of screen house hardening was used to increase percentage of survival of tissue culture plantlets. Briefly, plantlets were transferred to polyethelene bags filled with a mixture of soil and sand (3:1) prepared by washing three times with water, drying under the sun, sieving, and sterilizing for three hours by steam. A solution of Barot (0.7 gr/1 in deionized water) was applied to the soil to prevent fungal attack especially *Phytophthora, Phytium,* and *Fusarium*. The transplanted seedlings were placed in deep plastic trays inside a big transparent plastic bag, to create an artificial high humidity chamber but still permit entrance of light, for 15 days. Water was applied as necessary. After 15 days the tray was removed from the bag and a fertilization plan started (micro and macronutrients were applied every 8 days until moved to the field). At one month the plants were watered every two days and at the three months they were carried to field. Plants at 60 and 90 days old with good foliage were transferred to a well-watered field and planted at a spacing of 0.8m between plants and 1.60m between rows.

Results

Of the 1490 seeds obtained from crosses this year 1291 were planted, 199 seeds were discarded as 'vain'. A total of 824 plantlets, or 64%, were successfully established (Table 12.19), a 10% increase over results obtained last year for genotypes having resistance to CMD, the "CR" series (CIAT, annual report 2003). Of the 824 plants evaluated by MAS, 335 genotypes were found to combine resistance to CMD and CGM the rest susceptible. Genotypes that were resistant were propagated and approximately 10 copies each per genotype was sent to partners in Tanzania, Nigeria, and transferred to the greenhouse/field at CIAT respectively. Other shipments this year were 207 CR genotypes to Tanzania, 205 CR genotypes to Nigeria and 166 CR genotypes to India, of approximately 10 plants/genotype.

Table 12.19. Embryo rescue of AR genotypes from 43 BC_2 families.

Code	Mother	Father	No. of seeds produced	No. of vain seeds	No. of seeds planted	No. of plants obtained	% recovery of plants
AR-1	C-127	CW257-12	211	20	191	181	95%
AR-2	C-4	CW236-14	102	0	102	28	27%
AR-3	C-127	CW259-43	100	8	92	67	73%
AR-4	C-19	CW234-2	1	0	1	1	100%
AR-5	C-19	CW259-3	1	0	1	0	0%
AR-6	C-4	CW235-72	48	2	46	30	65%
AR-7	C-127	CW234-2	78	24	54	46	85%
AR-8	C-243	CW234-2	11	4	7	4	57%
AR-9	C-243	CW257-12	86	6	80	70	88%
AR-10	C-243	CW259-3	5	2	3	1	33%
AR-11	C-243	CW259-43	37	7	30	20	67%
AR-12	C-33	CW234-2	154	33	121	60	50%
AR-13	C-33	CW235-2	5	0	5	0	0%
AR-14	C-33	CW257-10	25	2	23	15	65%
AR-15	C-33	CW257-12	26	5	21	10	48%
AR-16	C-33	CW259-3	55	7	48	27	56%
AR-17	C-33	CW258-17	53	2	51	28	55%
AR-18	C-377	CW257-12	17	4	13	4	31%
AR-19	C-39	CW234-2	5	3	2	0	0%
AR-20	C-39	CW257-12	22	0	22	1	5%
AR-21	C-39	CW258-17	7	0	7	2	29%
AR-22	C-39	CW259-3	3	2	1	1	100%
AR-23	C-39	CW259-43	9	2	7	1	14%
AR-24	C-413	CW257-12	12	2	10	0	0%
AR-25	C-413	CW259-3	5	2	3	1	33%
AR-26	C-413	CW259-43	5	0	5	3	60%
AR-27	C-4	CW258-17	22	3	19	16	84%
AR-28	C-243	CW258-17	2	0	2	2	100%
AR-29	C-127	CW235-72	2	0	2	0	0%
AR-30	C-413	CW258-17	5	0	5	5	100%
AR-31	C-127	CW258-17	14	6	8	4	50%
AR-32	C-33	CW259-10	8	3	5	3	60%
AR-33	C-39	CW259-10	18	2	16	2	13%
AR-34	C-19	CW259-10	6	3	3	2	67%
AR-35	C-243	CW257-10	20	5	15	3	20%
AR-36	C-127	CW259-10	37	1	36	29	81%
AR-37	C-33	CW259-42	184	27	157	111	71%
AR-38	C-377	CW259-42	21	6	15	7	47%
AR-39	C-4	CW259-42	11	1	10	7	70%
AR-40	C-39	CW259-42	37	2	35	23	66%
AR-41	C-19	CW259-42	7	2	5	2	40%
AR-42	C-413	CW259-42	9	1	8	6	75%
AR-43	C-19	CW259-43	4	0	4	1	25%
TOTAL			**1490**	**199**	**1291**	**824**	**64%**

Conclusions and Perspectives
In vitro establishment of embryo axes of mature seeds derived from 43 BC_2 families that combine resistance to cassava green mites (CGM) and to the cassava mosaic disease (CMD) were conducted this year. An increase of 10% was obtained in recovery of full plants from embryo rescue. Plants shown to be resistant to CMD and CGM by MAS were shipped to Tanzania, Nigeria and transferred to the screen house/field. Pending are the shipments of the AR and CR plants to Uganda and South Africa. Other activities planned this year are embryo rescue of 3000 seeds from the CR series and MAS for CMD resistance.

References
CIAT 2003. Annual Report IP3. Improved cassava for the developing world. Pp8-85 to 8-90

Activity 12.20 Dissemination of Improved Cassava Varieties and Management of Genetic Stocks as Tissue Culture Plantlets

Collaborators:
Luis Guillermo Santos M., Yudi Samira Moreno M., Adriana, Mercedes Alzate G., Bernardo Ospina, Hernán Ceballos, Martin Fregene (CIAT).

Funding:
CIAT

Important Outputs
1) More than 1,000 plantlets of improved cassava varieties were multiplied and shipped to partners in Meso America, South America, and Europe
2) A BC_1 mapping population and several wild and cultivated parental lines were established in vitro for safeguarding
3) One person from a Mexican National program, 2 students from the Universidad Nacional, Sede Palmira and one from CIAT partners were trained in tissue culture methods

Rationale
The vegetatively propagated nature of cassava makes tissue culture a key method for safe exchange of germplasm and for safe guarding materials in the field. Last year cassava tissue culture group received from the genetic resource unit (GRU) 37 improved varieties and partly assumed responsibility for dissemination these varieties to partners in Latin America, Asia and Africa. Other activities last year were the establishment of breeding and gene tagging populations as well as transfer of valuable genotypes in the field to *in vitro* cultures. This year the group received 2 sets of the Core collection and several hundred other genotypes for the CIAT world germplasm collection for activities in the generation challege program. These materials were multiplied and eventually transferred to the screen house. Other activities carried out this year include dissemination of improved varieties of cassava to partners in Meso America, South America, and Europe, establishment as embryo cultures of 2 BC_1 gene-tagging populations for protein content and the transfer *in vitro* of several genotypes with

valuable traits. The group also carried training activities for partners from a National program, local University and CIAT in tissue culture methods this year.

Methodology

The core collection of the world cassava germplasm collection at CIAT is to be evaluated as part of activities in the challenge program. The core collection and more than 1000 accessions are to be genotyped with SSR markers this year. Between 2-4 *in vitro* plantlets per genotype of the above germplasm was received and multiplied to give at least 6-8 plants according to standard methods at CIAT (Roca et al. 1984). Between 4-6 plants were transferred to the screen house for hardening while 2 were kept *in vitro*. A group of 37 improved varieties held at the cassava tissue culture facility was also multiplied in 4E culture media for distribution to partners using CIAT's multiplication protocol (Roca et al. 1984).

Two BC_1 populations of between 200 and 300 progenies for gene tagging of protein content were established *in vitro* from embryo axes of mature seeds. The populations were obtained from crossing the high protein inter-specific hybrids CW205-4 and CW205-7 to MTAI8. Methods used for embryo rescue of mature cassava seeds was as described earlier (CIAT 2003). Wild accessions being used for the introgression of high protein, resistance to green mites, and high dry matter content from wild species to cassava often have problems of poor vegetative propagation. This year many of these wild parents were transferred *in vitro* for safekeeping. They include: OW146-1, OW181-2, OW231-2, OW234-2, OW240-8, OW280-1, OW280-2, OW284-1, OW589-2, OW180-4, OW230-6 y CW429-1, MCra-013, OW240-6, OW181-2, OW235-3, OW230-3 and OW180-4. Shoot tips from plants growing the field or screen house were transferred to tissue culture as described earlier (CIAT 2003).

One person from a Mexican National program, 2 students from the Universidad Nacional, Sede Palmira and one from CIAT partners were trained in tissue culture methods. The training covered an introduction to equipments in the tissue culture facility and their proper use, small and large scale *in vitro* propagation, preparation of tissue culture media, necessary precautions and general safety in the laboratory, and tissue culture bibliography.

Results

Two sets of the core collection a total of 1260 genotypes and another 775 accessions were received from CIAT's GRU and successfully multiplied and 4-6 copies sent to the screen house for activities in the GCP. A total of 3506 plants from a list of 37 improved genotypes were shipped to collaborators in Nicaragua, Peru, Mexico and Austria this year (Table 12.20). A total of 160 sexual seeds out of 170 have been processed so far from more than 400 for 2 BC_1 gene tagging populations for protein content, embryo rescue of the other seeds are continuing. Of the 20 wild and inter-specific parents transferred *in vitro* this year only 12 were successfully established, the others had problems of contamination and poor growth. New attempts are being made using different media to transfer these materials into *in vitro*. An unusual development observed this year was the production *in vitro* of a female flower in the genotype AR37-15, a BC_2 progeny for breeding resistance to CMD and CGM (Figure 12.31). A Mexican national program scientist Ms Fanny Cruz from Agropecuaria Santa Genoveva, and Ms Ximena Moreno from cassava breeding were trained in tissue culture methods. Others include Milena Sepúlveda y Angie Ayala, undergraduate students from the National University of Colombia in Palmira trained in multiplication and hardening of *in vitro* plants.

Table 12.20. Summary of shipments of *in vitro* plants of cassava (*Manihot esculenta* Crantz) made to several countries from February to September 2003

ITEM	GENOTYPE	NICARAGUA	AUSTRIA	PERU	MEXICO	TOTAL
1	CG 1141-1	30	-	30	64	124
2	CG 1450-4	30	-	30	-	60
3	CM 1335-4	-	10	-	-	10
4	CM 2177-2	30	-	30	55	115
5	CM 2600-2	30	-	30	-	60
6	CM 2766-5	30	-	30	-	60
7	CM 2772-3	-	10	-	-	10
8	CM 3306-4	-	10	30	-	40
9	CM 3750-5	-	10	-	-	10
10	CM 4574-7	-	10	-	-	10
11	CM 4843-1	-	6	-	-	6
12	CM 489-1	-	10	-	-	10
13	CM 4919-1	-	10	-	-	10
14	CM 507-37	-	10	-	15	25
15	CM 523-7	-	10	-	-	10
16	CM 5306-8	-	10	-	-	10
17	CM 6119-5	-	10	-	-	10
18	CM 6438-14	-	10	-	-	10
19	CM 6740-7	-	10	-	-	10
20	CM 6754-8	-	6	-	60	66
21	CM 6921-3	-	10	-	-	10
22	CM 7033-3	-	10	-	-	10
23	CM 7073-7	-	10	-	-	10
24	CM 7514-8	-	10	-	-	10
25	CM 7951-5	-	10	-	-	10
26	CM 8027-3	-	10	-	-	10
27	HCM 1	30	10	30	39	109
28	MBRA 383	30	10	-	-	40
29	MCOL 1468	-	10	-	-	10
30	MCOL 1505	-	-	-	20	20
31	MCOL 1684	-	10	-	-	10
32	MCOL 1734	-	10	-	-	10
33	MCOL 2063	-	-	-	15	15
34	MCOL 2215	30	10	30	-	70
35	MCR 30	30	-	30	-	60
36	MCR 31	30	-	30	-	60
37	MCUB 72	-	-	-	9	9
38	MPER 183	-	10	-	-	10
39	MTAI 8	-	10	-	-	10
40	SM 1411-5	-	10	-	-	10
41	SM 1433-4	-	10	-	-	10
42	SM 1460-1	-	10	-	-	10
43	SM 1565-15	-	10	-	-	10
44	SM 1741-1	-	10	-	-	10
45	SM 1821-7	-	10	-	-	10
46	SM 667-1	-	-	-	10	10
47	SM 805-15	-	5	-	-	5
48	SM 909-25	-	10	-	-	10
	TOTAL	**300**	**357**	**300**	**287**	**1244**

Figure 12.31. A female flower formed in a 4-week old *in vitro* plantlet of the genotype AR37-15, a BC_2 progeny for breeding resistance to CMD and CGM

Conclusion and Future Perspectives

The cassava group processed more than 2000 accessions from the world germplasm collection for activities in the GCP this year. They also continued shipment of improved varieties of cassava to partners in Meso America, South America, and Europe, and tissue culture establishment of genetic stocks with valuable traits. Four partners from a Mexican national Program, the local university in Palmira and from CIAT were also trained this year.

The tissue culture facility has a growth with a capacity of around 28,000 plants at the moment it has more than 30,000 and requires additional space. Plans are ongoing to convert another of the laboratories in the cassava physiology section to a larger growth room. Future activities include the continued dissemination of improved varieties, establishment of genetic stocks and the implementation of a bar-code method for proper inventory of all plants entering the growth room.

References

ROCA, W.M., RODRÍGUEZ, J.A., MAFLA, G. Y ROA, J. (1984) Procedures for recovering cassava clones distributed *in vitro*. Centro Internacional de Agricultura Tropical (CIAT). 8 pp.

Activity 12.21 A Simple Method for the Rapid Multiplication of Clean Cassava Planting Material

Collaborators:

Ernesto Cuellar (INIVIT), Dr James George (CTCRI, Trivandrum, India), Elizabeth Okai, Emmanuel Okogbenin (CRI, Ghana), Chris Okeke (NSM, Nigeria); Dr (Mrs) Acheampong (University of Legon, Ghana), Bernardo Ospina (CLAYUCA), Luis Guillermo Santos, Samira Moreno, Martin Fregene (CIAT)

Funding: CIAT core funds and NSM

Important outputs
The establishment of a 20ha seed bank (200,000 plants) from plants obtained from 2-node cuttings of 4 improved varieties.

Rationale
The Nigerian Starch Mill (NSM) embarked upon a commercial cassava production last year to provide a reliable source of raw materials for its starch mill. Following a visit of NSM's chief executive, Dr Chris Okeke, to CIAT Colombia last year and a consultancy visit by a CIAT-CLAYUCA team, a road map was outlined for commercial scale cassava production. Two Cubans technical experts from INIVIT, the Cuban national root and tuber institute, were seconded to NSM to implement the road map. They arrived in Nigeria in February and had started work at the NSM farm in Opuoma. Their terms of reference were to set up a 200ha seed bank via a rapid 2-node multiplication of tissue culture introductions for the supply of healthy planting material to the farm amongst other things. Certified planting material has been shown to increase yield by as much as 40% (Guritno 1985). We describe here activities in the first year of the rapid multiplication of improved varieties for

Methodology
The rapid multiplication seedling nursery is supposed to establish a seed bank of high quality for NSM and it consists of 2 parts, the 2-node nursery and a screen house hardening of introduced *in vitro* germplasm. A 2-node multiplication scheme was initiated using carefully selected stems of 4 improved varieties: NR8083, TMS53, TMS4(2)1425, and TMS30572 for the establishment of a certified gene bank (stakes free of pest and disease). The micro-stakes were planted in plastic bags containing a mixture of sand and topsoil (ratio 3:1) that had previously been sterilized by placing in a dry bath and heating on an open fire for 2-4 hours. Later, the sand and topsoil mixture was replaced by topsoil alone, the soil at Opuoma is very sandy, and the sterilization step was eliminated. At 4-6 weeks after planting the plants were transferred to the field. The multiplication nursery is capable of holding > 200,000 plants at any time.

Tissue culture plantlets of 44 IITA improved germplasm were placed *in vitro* as described earlier (CIAT 2003) and multiplied at the University of Legon Ghana. This first batch of around 3000 plantlets was transferred to Nigeria at the end of January 2004. The non-completion of the NSM screen house led to their being held temporarily at the tissue culture facility at the Institute for Agricultural Research and Training (IAR&T), Ibadan. They were later transferred in April to NSM farm at Opuoma. Two additional batches of 1530 and 2,800 plantlets were shipped to Opuoma in May and August respectively. The plants were transferred to the completed screen house for hardening as described earlier (CIAT 2003).

Results
A total of 10ha have been planted in with seedling raised from carefully selected 2-node stakes from the varieties NR8083, TMS53, TME30572 and TMS4 (2)1425. There is also another 69,900 plants ready for transfer to the field, enough to plant another 7ha (Figure 12.32). A third set of 30,000 plants has been planted and will be transferred to the field

before the end of the season, a total of 20ha of cassava seed bank will be planted this year to provide clean and healthy planting material for seeding 200ha next year.

The screen house hardening section of the nursery suffered severe technical and management problems that lead to the loss of close to 90% loss of germplasm introduced as tissue culture plantlets. The 1st set of plants arrived April 15/04, they came from University of Legon Ghana via IAR&T, Ibadan, these plants were old, between 3-4 months. Of a total of more than 2000 plantlets introduced, 825 could be successfully transferred to the screen house and only 65 survived. The reason for the high failure rate was adduced to the high temperature, which is extremely high at that time of the year (April, May, June), a minimum of 24.8-26.12^{o}C and a maximum of 33.2-36.6^{o}C between 6am and 4 pm (Table1). A second set of 1053 *in vitro* plants arrived June 4, a total of 853 were transferred successfully but only 163 survived, temperature was still high but was better controlled with additional netting and misting of the screen house with the irrigation sprays.

Fig 12.32 Cassava seedling nursery raised from carefully selected 2-node stakes from the varieties NR8083, TME30572 and TMS4 (2)1425

Table 12.21 Temperature regime in the screen house at NSM Farm, Opuoma.

Month	6 am	8 am	10 am	12 am	1 pm	2 pm	4 pm	6 pm	8 pm
April	24.8	26.6	32.55	37.3	39.11	36.6	33.6	32.3	32.85
May	25.12	29.19	32.5	37.96	39.16	36.5	34.1	31.2	32.96
June	26.32	28.8	32.6	36.2	37.1	36.3	33.2	31.12	32.7
July	25.36	28.7	31.3	35.9	36.92	36.21	32.9	31.1	32.29
August	25.21	27.92	31.21	35.52	36.63	36.15	32.53	30.92	31.9

A third group of 2800 *in vitro* plants came in July, of this 2253 were be transferred to the screen house, the others were damaged or contaminated during the trip. Of this group just

72 survived, the very high mortality was blamed on lack of water over a period of 15 days. Poor water quality was also pointed out as one of the reason for the high mortality in the 3 introductions, Opuoma is a petroleum producing area and ground water from a borehole that supplies the screen house is contaminated with a reddish substance.

Conclusions

A 20ha seed bank (200,000 plants) from plants raised from 2-node cuttings of 4 improved varieties has been established at the NSM Opuoma farm. This seed bank will provide healthy planting material to plant 200ha of a seed bank next year. The screen house hardening of in vitro plants on the other hand did not succeed as expected, the Cuban agronomist handling the task blamed the high mortality on temperature, but the maximum and minimum temperatures at the screen house are comparable to those in several other places including some African countries where we have successfully shipped *in vitro* plants to (see section 8.2, this report). The water quality and the sub-optimal management of the *in vitro* plants are more plausible reasons.

Future Perspectives

A plan has been put in place to identify and resolve problems associated with hardening of *in vitro* plantlets at the NSM screen house in Opuoma. The effort consists of visit by CIAT technical personnel to the NSM farm for between 3 to 4 weeks to assist with hardening of *in vitro* plantlets

References

CIAT 2003. Annual Report. Improved cassava for the developing world. Project IP3. pp8-89 to 8-92

Guritno J.H. 1985. Influence of planting material on plant performance in cassava. Ph.d. thesis, University of Brawijaya, Malang, Indonesia. 158pp.

Activity 12.22 Training

Graduate Students

* Ms Elizabeth Okai (Ghana) Ph.D. student, University of the Free State, Bloemfontein, South Africa (expected finish date January 2007)
* Henry Ojulong (Uganda) Ph.D. student, University of the Free State, Bloemfontein, South Africa (expected finish date January 2006)
* Olalekan Akinbo (Nigeria) Ph.D. student University of the Free State, Bloemfontein, South Africa (expected finish date January 2007)
* Martha Isabel Moreno (Colombia) Universidad de Valle, Cali, Colombia (expected finish date October 2004)

Under- graduate Students

* Ana Maria Correa (Colombia) Universidad de Valle, Cali, Colombia (expected finish date October 2004)
* Milena Sepúlveda (Colombia) National University of Colombia, Palmira (expected finish date September 2005)

* Angie Ayala (Colombia) National University of Colombia, Palmira (expected finish date September 2005)

Visiting Researchers

* Ms Fanny Cruz (Mexico), Agropecuaria Santa Genoveva, Mexico
* Paola Alfonso (Colombia) Universidad Javeriana, Bogotá, Colombia (Pasantia September - December 2004)
* Jixin Zhou, (China) Chinese Academy for Tropical Agricultural Sciences, (CATAS), Guangxi, China
* Ms Claudia Ferreira (Brazil), Nacional Fruits and Cassava Research Institute (CNPMF), Cruz das Almas, Brazil

Activity 12.23 Trips

1. January 2004. Attendance of the Plant and Animal Genome Meeting, San Diego and the choice of marker system meeting of sub programme 1 of the Generation Challenge Program (GCP).
2. March/April 2004. Visit to ARI-Mikocheni, Tanzania, on the RF MAS cassava breeding project
3. May 2004. Visit to the Donald Danforth Center on the development of a proposal for the Gates Foundation challenges in global health program
4. July 2004 Visit to ARI-Mikocheni, Tanzania, on the RF MAS cassava breeding project
5. September 2004. CIAT-CLAYUCA technical back-stopping meeting to the Nigerian Starch Mill, Ihiala
6. September 2004. Attendance of the Annual Colombian Biotech meeting, Bogotá, Colombia (Jaime Marin, Wilson Castelblanco, and Yina Puentes).
7. September 2004. Attendance of the Generation Challenge Program (GCP) annual meeting in Brisbane Australia (Paula Hurtado) .
8. October 2004 Visit to ARI-Mikocheni, Tanzania on the RF MAS cassava breeding project
9. October 2004. Attendance of a conference on biotechnology, Ibague, Colombia (Anna Maria Correa,)
10. November 2004. Attendance of the ISTRC-AB Meeting Mombassa
11. November 2004. Attendance of a consultants meeting at the International Atomic Energy Agency, Vienna, on development of a research contract for vegetatively propagated crops.

Activity 12.24 Publications

Xia L., Peng K., Yang S., Wenzl P., de Vicente M.C., Fregene M., and Kilian A. (2004) DArT for high-throughput genotyping of cassava (*Manihot esculenta*) and its wild relatives Theoretical and Applied Genetics (in Press)

Fregene M. , Okogbenin E., Marin J., Moreno I., Ariyo O., Akinwale O., Barrera E., Ceballos H., and Dixon A. (2004). Molecular Marker Assisted Selection (MAS) of Resistance to the Cassava Mosaic Disease (CMD). Molecular Breeding (in press)

Kelemu, S., Mahuku, G., Fregene, M., Pachico, P., Johnson, N., Calvert, L., Rao, I., Buruchara, R., Amede, T., Kimani, P., Kirkby, P., Kaaria, S., and Ampofo, K. (2004).

Harmonizing the agricultural biotechnology debate for the benefit of African farmers. African Journal of Biotechnology 2(11):394-416.
Okogbenin E., Marin J., and Fregene M. (2004). An SSR-based molecular genetic map of cassava and QTL analysis for early yield in a Pseudo F_2 population Journal of Agronomy and Crop Science (In Press)

Activity 12.25 Project Funded and in Review with Donors

The following Project was funded this year:

1. Modification of Flowering in cassava and Mango using cloned flower gene from Arabidopsis. Rockefeller foundation US$280,000 for 4 years

The following proposals are being reviewed by donors:

1. BioCassava Plus, a project that will develop new cassava cultivars designed to improve the nutritional status of sub-Saharan Africa. Gates Foundation, US$260,000 for 5 years
2. Development of Low-Cost Technologies for Pyramiding Useful Genes From Wild Relatives of Cassava into Elite Progenitors. GCP, US$894,420 for 3 years
3. Identifying the physiological and genetic traits that make cassava one of the most drought tolerant crops. GCP, US$78,806 for 3 years.
4. Validation of Diversity Arrays Technology (DArT) as a platform for efficient discovery and utilization of molecular marker – trait associations in orphan crops. GCP, US$860,405 for 3 years
5. Capacity Building in molecular breeding and transfer of technology to improve preferred Cassava Varieties for consolidation of Food Security and generation of income for small-scale farmers in Africa. FAO, US$332,000 for 3 years
6. 'Express' Dissemination of Improved Cassava Varieties in Nigeria and Senegal Based on the Automated Temporary Immersion System (ATIS) and a Multi-stage Farmer Participatory Multiplication Program. DURAS, 139,500 Euros for 3 years, in collaboration with NRCRI, Nigeria
7. Securing the Harvest: Molecular-Assisted Introgression of Genes for Delayed Post-Harvest Physiological Deterioration (PPD), high protein and beta-carotene content into African Cassava Gene Pools. DURAS, 150,000 Euros for 3 years, in collaboration with NRCRI, Nigeria

2. *A Molecular marker-assisted, farmer-partipatory breeding project to improve local cassava varieties in Tanzania with resistance to pest and diseases*

The Tanzanian MAS project funded by the Rockefeller foundation seeks to transfer useful variability from the crop's center of diversity of cassava to Africa. This year, a total of 335 BC_2 progenies (AR lines) that combine resistance to CMD and to the cassava green mites (CGM), derived from a wild relative, and 207 genotypes (CR lines) obtained from crossing CIAT elite parents and CMD resistant lines were introduced from Colombia to Tanzania in three shipments. The plants were hardened in the screen house, evaluated for frog skin disease (FSD), and then transferred to the field. They will be evaluated later this season and no less than 60 genotypes selected based on evaluation of highly heritable agronomic traits for crosses to 90 local varieties selected from all over the country. Molecular markers associated with CMD and CGM will be used to discard much of the resulting segregating populations so that the breeder and farmers can concentrate on a small number of progeny having resistance to the principal pest and disease and farmer/end-user preferred traits. The concept of the Tanzanian MAS project is already being extended to additional NARs in

Africa, the AR and CR lines have been shipped to Uganda and Nigeria already in preparation for crosses to local varieties.

3. *Identification of sources for delayed post harvest physiological deterioration (PPD), resistance to whiteflies, and hornworms in wild Manihot species and development of populations to study genetic inheritance and for introgression of the traits into cassava.* Post harvest physiological deterioration (PPD) and anthropoid pests are severe marketing and production constraints respectively in cassava. It has been estimated that cassava farmers, typically resource-poor, lose 48 million tons of fresh root valued at US$1.4 billion every year to pests, diseases, and PPD, some 30% of total world production. Dramatically delayed PPD has been identified in inter-specific hybrids from *Manihot walkerae*. The delayed PPD trait, originally from the wild *Manihot* parent, was successfully transferred to an F_1 inter-specific hybrid suggesting a dominant or additive gene action of gene(s) involved. The only source of resistance to the cassava hornworm was also identified in 4^{th} backcross derivatives of *M. glaziovii* . Moderate to high levels of resistance to white flies have also been found in inter-specific hybrids of *M. esculenta sub spp flabellifolia*. Again, resistance was recovered easily in F_1 inter-specific hybrids, suggesting a simple inheritance of the trait. Eight mapping populations have been developed for marker-assisted study of the inheritance of these traits.