VIROLOGY

Activity 1. Resistance for cassava frogskin disease is widespread in cassava germplasm.

Introduction

In the Amazon regions of Brazil and Colombia, it was observed that there where apparent difference in the reaction of varieties to cassava frogskin disease (CFSD). Some varieties developed typical root symptoms, while other varieties that were planted in same fields did not develop symptoms. This led to the idea that some cassava landraces are resistant to CFSD. In 1995, it was decided to test the 640 accessions of the CIAT cassava core collection for resistance to cassava frogskin disease (CFSD). The results have shown that tolerance to CFSD is widespread in cassava germplasm. More than 100 tolerant lines have been identified and are potential sources of resistant to CFSD. In the last year, 42 lines were evaluated for their agronomic characteristics and resistance to other pests. All these lines, that are rated as tolerant, have remained infested with CFSD at least eight growing cycles.

Evaluation of cassava for resistance to CFSD

The plants tested were from the core collection of 640 cassava lines that are representative of the CIAT cassava collection that consists of over 6000 lines. All plants in this trial were graft inoculated using stem cuttings of the cassava line CT5460-10 infested with CFSD. Five plants from each line were inoculated by grafting with the CFSD affected stem cuttings of line 5460-10. In the last four years, these lines were grown in randomize block design of four repetitions with 10 plants per repetition and evaluated visually for root symptoms. Representative plants in these lines were assayed for CFSD by grafting stem cuttings (rootstock) to Secundina (scion), and the new leaves were examined for mosaic symptoms. All of the plants tested were positive for CFSD. The roots of each plant were rated using the following scale: 1 for no symptoms, 2 for very mild symptoms, 3 for moderate symptoms, and 4 for severe symptoms.

The ratings of 28 best lines and their yields during the last four years are summarized in **Table 1**. These are the best cassava lines in the CIAT cassava core collection that yield well in the conditions at the CIAT experiment station at Santander de Quilichao, Cauca, Colombia and are tolerant to CFSD. This year the yields are much lower than in the previous years. This was due to the lack of rain during the last year. Even in the harsh conditions of this last year, several of the lines yielded more than 10 t/ha. There was also an increase in the severity of CFSD with eight of the best lines had modest levels of disease pressure. An analysis of the temperatures, rainfall and symptoms during the last four years needs to be done. There are still twenty lines that never have developed significant symptoms over the course of this experiment.

There is ample resistance in the cassava germplasm for CFSD. It is a form of tolerance because the plants remain infected and the disease is transmitted thought the infected stem cuttings. Under the condition of mid-altitude tropics, these lines have remained tolerant year after year. After nine years of field trials, we have a solid base to state that the resistance is stable and holds up under the range of climatic variation that occurs at the screening site. From just the core collection of CIAT, landraces or varieties have been identified for most of the countries where CFSD is endemic and an important production constraint.

Although the yields were disappointing, the commercial varieties HCM-1 and CMC40 (Mcol 1468) were in the low end for yield and are moderately tolerant to CFSD as compared with the most resistant varieties. There are about 15 lines that have been identified from the CIAT core collection that are tolerant to CFSD and have yields potential that are relatively high for varieties that are directly consumed. There is also data on 100 other lines with tolerance. This means that there is a wide range of germplasm options for cassava growing areas where CFSD is a problem.

The core collection represents about 10% of the total number of cassava accession at CIAT. The process of identification of tolerant varieties is time consuming, and the climatic conditions affect the level of disease expression. If a molecular marker or set of markers were developed that could identify CFSD tolerance in cassava, the remaining germplasm could be screened rapidly. This along with the agronomic information available on the cassava germplasm collection and commercial varieties could quickly lead to the identification of germplasm for farmers in areas where CFSD is endemic. Breeding programs in Colombia, Brazil, and Costa Rica should try to incorporate CFSD resistance into their new varieties.

Table 1. The best lines in the CIAT core collection for resistance to CFSD.

	2000-2001		2001-2002		2002-2003		2003-2004		4 Years
Variety	Symptom Rating	Yield	Symptom Rating	Yield	Symptom Rating	Yield	Symptom Rating	Yield	Symptom Rating
M Per 183	1.00	3.95	1.02	5.50	1.00	3.07	1.00	0.98	1.005
M Per 438	1.00	3.95	1.00	2.69	1.00	17.9	1.02	1.28	1.005
M Chn 2	1.00	3.32	1.00	2.16	1.00	1.69	1.00	0.71	1.000
M Mex 95	1.03	2.79	1.04	2.35	1.00	2.00	1.00	0.72	1.018
M Per 213	1.00	2.70	1.00	2.16	1.00	2.11	1.00	0.61	1.000
M Bra 886	1.08	2.32	1.50	2.56	1.08	1.71	1.38	0.61	1.260
MEcu 68	1.00	1.18	1.00	1.91	1.00	3.15	1.00	0.58	1.000
MCol 634	1.00	2.54	1.19	2.04	1.29	1.56	1.07	1.22	1.140
MMal 50	1.00	3.13	1.00	1.58	1.08	1.38	1.00	0.36	1.020
MPer 431	1.00	2.12	1.00	1.86	1.00	1.98	1.00	0.91	1.000
MGua 78	1.00	1.97	1.20	1.63	1.04	2.21	1.00	0.56	1.060
MCol1468	1.03	2.21	1.30	1.83	1.33	1.52	1.13	0.10	1.200
HMC 1	1.00	1.72	1.23	1.62	1.25	1.69	1.50	0.30	1.245
MBra 325	1.00	2.22	1.00	1.68	1.25	1.12	1.33	0.20	1.145
MPer 209	1.00	1.99	1.12	1.91	1.00	1.08	1.00	1.13	1.030
MCr 59	1.13	2.00	1.06	1.59	1.20	1.43	1.24	1.17	1.160
MPer 243	1.00	1.29	1.00	1.49	1.06	1.98	1.08	0.27	1.035
MMal 24	1.00	1.91	1.04	1.66	1.08	1.65	1.70	0.64	1.220
MGua 41	1.05	1.67	1.00	1.39	1.06	1.56	1.00	0.44	1.030
MMex 80	1.00	1.40	1.07	1.82	1.06	1.04	1.28	0.57	1.100
MMal 13	1.00	1.16	1.02	2.16	1.00	1.77	1.00	0.33	1.005
MCr 79	1.23	1.71	1.23	1.22	1.21	0.50	1.31	0.55	1.245
MMal 38	1.03	0.95	1.07	1.38	1.14	1.06	1.11	0.33	1.090
MCol2157	1.00	1.13	1.00	1.14	1.04	0.77	1.65	0.53	1.200
MPer 377	1.03	1.07	1.00	0.96	1.00	0.73	1.00	0.43	1.010
MPar 163	1.03	1.47	1.09	0.48	1.23	0.58	1.00	0.26	1.090
MBol 1	1.00	0.89	1.00	0.77	1.03	0.63	1.00	0.28	1.010
MMex102	1.00	0.73	1.00	0.43	1.00	0.74	1.00	0.40	1.000

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Activity 2. The association a reolike virus in *Manihot esculenta* affected with cassava frogskin disease.

Introduction

The evidence for a reolike virus in cassava includes multiple double stranded RNA species, virus-like particles and cDNA clones that have homology with rice ragged stunt virus. The consistent association of the virus with the disease has been more difficult because of low virus titers and reoviruses tend to be extremely liable. Therefore the detection of cassava frogskin virus (CFSV) is not as consistent as it needs to be. This study used nine different isolates of cassava frogskin disease (CFSD) and used two techniques for the detection of the virus. CFSV was detected in all nine of the isolates but was not found in the healthy controls. This is further evidence of the association of CFSV with CFSD**.**

Materials and Methods

Source of host plants and isolates. The CFSD isolates were collected both in the Andean and Amazonian regions of Colombia and maintained in greenhouses by vegetative propagation. The isolates of CFSD were Secundina 5, Secundina 80, Valluna 29, CM-5460-10, SM 909-25, Regional Tolima, CMC40, Amazonas 16, and Catumare Jamundi. The first part of the name designates the name of the cassava landrace or breeding line affected by CFSD.

The healthy control plants were obtained from materials that were subjected to heat therapy and cultured *in vitro*. The *in vitro* plants were hardened and subsequently maintained in a greenhouse free of CFSD. When Secundina is affected with CFSD, it has mosaic leaf symptoms. All the test plants were grafted to Secundina to determine if the were healthy or affected with CFSD.

Extraction of dsRNA. Three grams of tissue were collected and the dsRNA was extracted (Morris and Dodds, 1979). The ds-RNAs were treated with Dnase (10µg/ml) for 40 minutes at 40°C. The samples were then subjected to an ethanol precipitation and run on agarose or polyacrylamide gels.

The synthesis of cDNA from the dsRNAs. For each sample, 5µg dsRNA, 500 ng of random primers and 500ng of 18mer-oligo(dt) (Gibco BRL) for a total volume 13µl where denatured by the addition of 13µl of 40Mm methylmercuric hydroxide (Jelkmann et al. 1989). The mixture was incubated for 10 minutes at room temperature and frozen using liquid nitrogen. The samples were the allowed to thaw out and were processed immediately.

The first strand synthesis was done in a final volume of 40µl containing 50Mm Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dATP, dCTP, dGTP, dTTP, 40 U of Rnasin (Promega) y 400 U of Superscript II RT (Gibco, BRL). The mixture was incubated for 60 min a 37° C. Then an additional 200 U of SuperScript II RT was added to the mixture and the reaction was allowed to continue for another 30 minutes. The reactions were then subjected to 70° C for 1 minute and placed in ice water for 2 minutes. To the 40µl of the first strand reaction 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10 mM$(NH_4)_2SO_4$, 0.15 mM β-NAD^+, 0.25 mM each dATP, dCTP, dGTP, dTTP, 1.2 mM DTT, 25 U *E. coli* Ligase, 40 U *E. coli* Polymerase, 4 U *E. coli* Rnase H were added and the final volume was 150µl. After the mixture

was incubated for 3 h at 16° C, 30 U of T4 DNA Polymerase was added and the reaction was continued at 16° C for 10 minutes. The reaction was stopped by the addition of 10 µl of 0.5 M EDTA, pH 8.0 and treated with phenol:chloroform:isoamyl alcohol (25:24:1). The cDNAs were precipitated with 1/10 volume of 7 M ammonium acetate and 2.5 volumes of 95% ethanol and resuspended in sterile DEPC treated water.

PCR amplification. The PCR reaction (25 µL) consisted of 0.2 µM of each primer S5 up(5'GTT AGC ATT ACC ATT CTC ACA T 3'), 2.5 mM MgCl2, 20 ng DNA, buffer 1x (100 mM Tris-HCl, 500 mM KCl, 1% Triton X-100), 0.25 mM dNTPs and 1 U Taq polymerase (Perkin Elmer). The reaction was carried out in a thermocycler using the following program: initial denaturation at 94ºC for 5 min; 39 cycles at 94ºC for 1min, 50ºC 2 min, 72ºC for 2.30 min; a final extension at 72ºC for 10 min. The PCR products were separated in agarose and capillary blotted into nylon membranes and hybridized with S5 probe labeled using DIG high prime DNA labeling kit (Roche Applied Science).

Results and Discussion

The detection of a Genomic Segments of Cassava Frogskin Virus. Nine CFSD isolates were tested using reverse transcriptase PCR for the presence of CFSV. The primers were specific for the CFSV segment S5. Multiple PCR products were amplified including a product in the controls. The products were transferred to a membrane and a CFSV S5 probe was used to detect specific PCR products. The products in the healthy controls were not specific while multiple bands in CFSD affected plants reacted with the cDNA CFSV S5 probe **(Figure 1)**. This method is highly specific but requires many steps including the purification of dsRNA, reverse transcriptase, PCR, and hybridization.

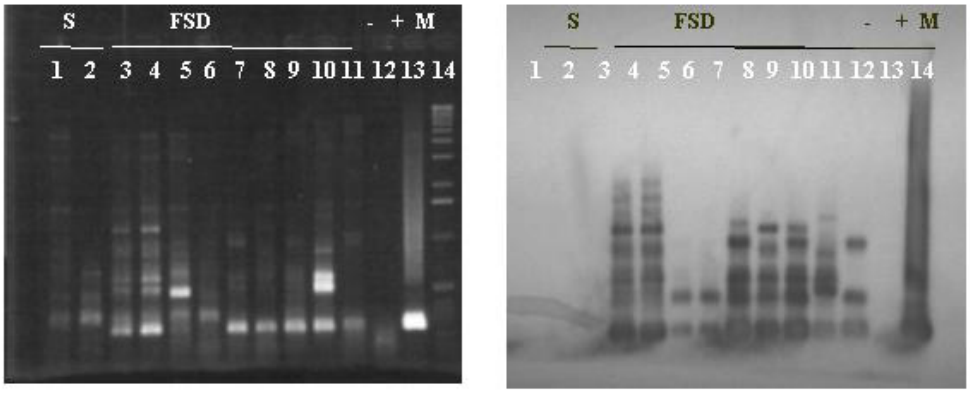

Figure 1. Detection of the S5 of cassava frogskin virus using rt-PCR. On the left is a gel showing the PCR products stained with ethidium bromide, and on the right a hybridazation using a probe specific for CFSV segment 5. The isolates are health 1: Secundina , 2: CMC40; CFSD affected 3: Secundina 5, 4: Secundina 80, 5: CFSD 29, 6: CM-5460-10, 7: SM 909-25, 8: Regional Tolima, 9: CMC40, 10: Amazonas 16, 11: Catumare Jamundi, 12: negative control, 13: positive control, 14: 1 kb molecular weight marker.

The same cassava plants affected with CFSD were tested for the presence of CFSV dsRNA genomic segment S5. The CFSV genomic segment S5 was consistent detected in the nine isolates affected with CFSD, but not in the negative controls **(Figure 2)**. This genomic segment is estimated to by 2800-3000 nucleotides in length and has homology with the rice ragged stunt virus segment S5.

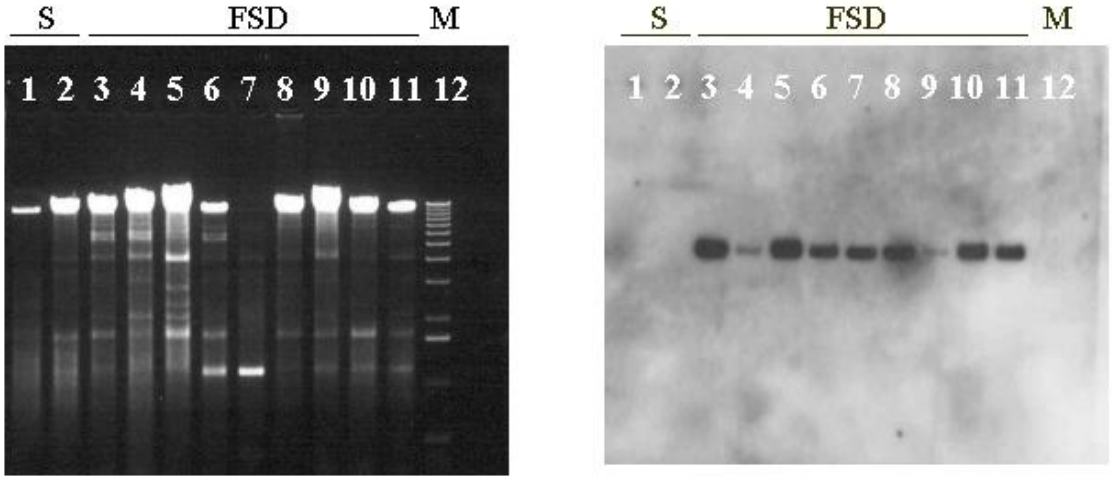

Figure 2. Detection of the dsRNA genomic segment S5 of cassava frogskin virus. On the left is a gel showing the dsRNAs stained with ethidium bromide, and on the right a hybridazation using a probe specific for CFSV segment 5. The isolates are healthy 1: Secundina , 2: CMC40. CFSD affected: 3: Secundina 5, 4: Secundina 80, 5: CDSD 29, 6: CM-5460-10, 7: SM 909-25, 8: Regional Tolima, 9: CMC40, 10: Amazonas 16, 11: Catumare Jamundi, 12: 1 kb molecular weight marker (M).

The detection of the genomic segment is not as consistent as the rt-PCR. The virus titer tends to be very low and the virus is very prone to degradation. PCR methods are more sensitive than direct methods that do not involve amplification. Nevertheless, after optimization of the conditions, we were able to detect the genomic segment S5 of CFSD.

Further characterization of CFSV. In addition to the CFSV S5 cDNA clone, there is the cDNA clone CFSV that represents a part of segment 1 (S1). Both these cDNA clones have homology at the amino acid level with rice ragged stunt virus (RRSV). A unique cDNA product was the identified by AFLP. This fragment was cloned and represents part of the genomic segment of CFSV S1. Using the same methods for CFSV S5, the full-length genomic segment S1 was detected in dsRNA gels by the clone cDNA S1. This area was purified, and cDNA clones were generated using a variety of strategies. These cDNA clones are in the process of being analyzed. The best characterized is around 1000 nucleotides and has homology at the amino acid level with segment 4 of RRSV. The genomic segments RRSV S1, S2, and S4 are 3849, 3810 and 3823 nucleotides respectively. Therefore the purification of dsRNA in the area of the CFSV S1 also should yield the S2 and S4 genomic segments. The successful cloning of the S4 segment confirms the strategy and demonstrates how previous information is helping in the

characterization of the virus. The S1 clone is 235 nucleotides and the S5 clone is 327 nucleotides. The CFSV S4 cDNA clone is the largest fragment cloned to date, and represents a significant step in the further characterization of CFSV.

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