Part II: Molecular characterisation of cassava brown streak virus

Background

Cassava is a major food staple in sub-Saharan Africa (SSA). Historically grown in West and Central Africa, production in Eastern and Southern regions has increased in recent years. Cassava is a drought hardy crop and can survive and produce a yield where cereal crops fail. As much as 85% of the cassava root dry weight is starch, making it the highest yielding calorie source per hectare of all the staple crops. It is also a very efficient crop as regards energy unit per labour input ratio, making it the cheapest supplier of calories (Puonti-Kaerlas, 1998). Cassava is a staple food for 500 million people, and 11 out of the 12 countries where consumption per capita exceeds 100kg each year, are in Africa (FAO, 1997).

Cassava brown streak disease (CBSD) was first reported in Tanzania in 1936 (Storey, 1936). The name brown streak was given to the disease because of the brown lesions which sometimes appear on the young green stem of affected plants. More seriously, most storage roots exhibit necrosis and cannot be consumed (Hillocks *et al*., 1996). In the absence of any visible parasite and the ability of the disease to be passed on through grafting, the agent of infection was suggested to be viral. Cassava is propagated through cuttings and as such is particularly prone to virus disease problems because infection tends to build up in selected clones. Natural spread of CBSD occurs only at a very low rate (Bock, 1994) and the vector is believed to be a whitefly. A recent report suggested that *Bemisia afer* and not the previously suggested *B. tabaci* was the vector responsible (Legg and Raya, 1998), although neither has been confirmed. Diagnosis of CBSD is difficult because immature leaves of infected cassava are symptomless, and symptoms of the disease vary greatly with the variety of cassava (Hillocks *et al*., 1996) and environmental conditions. Symptoms of CBSD have been observed to be more severe during the cool winter months especially in upland sites and to disappear during the hot season (Storey, 1936). During the dry season cassava plants lose their leaves and new growth often shows no sign of the disease. A recent report stated the distribution of CBSD to be Malawi, Kenya, Southern Tanzania, Southern Uganda and Northern Zambia

(IITC, 1996) although with the difficulty in diagnosis, the disease is probably more widespread than currently recorded.

The disease can be transmitted mechanically to a number of solanaceous species (Lister, 1959). Lister's studies found that extracts from mature leaves of cassava, and not the young ones, could infect a secondary host plant suggesting that the virus is concentrated in the mature cassava leaves. The most susceptible host plant was found to be *Petunia hybrida* (Lister, 1959), although later workers with this disease preferred *Nicotiana benthamiana* or *Nicotiana debneyi* (Bock, 1994; Lennon *et al*., 1986).

There is some confusion over the virus responsible for CBSD. Bock isolated two variants of the virus and subsequently maintained them on *N. debneyi* where they induced readily distinguishable symptoms (Bock, 1994). Both variants were 650nm filamentous particles. The disease has been suggested to be caused by a potyvirus, a carlavirus or a virus complex of both (Shukla *et al.,* 1994). The confusion has arisen from conflicting results of purified virus particles of carlavirus length (650-690nm) and pinwheel inclusion bodies characteristic of potyviruses found in sections of infected material (Lennon *et al.,* 1986). Attempts to separate the two suspect viruses using alternative host plants were not successful. The only reported antiserum to cross react with CBSD material had been raised to cowpea mild mottle virus, a whitefly-transmitted carlavirus (Lennon *et al.,* 1986). An antiserum was raised to the carlavirus length virus from cassava that detected the virus readily in *N. benthamiana* but only erratically in cassava (Lennon *et al*., 1986).

Project Purpose

CBSD was first reported in the 1930's, but despite the disease being assumed to be caused by a virus, its aetiology has remained obscure. The main aim of this work was to identify the virus or viruses that cause CBSD, using molecular tools.

Symptoms of the disease are known to take time to develop in infected cassava, and to vary with different cassava varieties. In some cases no above ground symptoms can be seen. A sensitive detection method was required both to support breeding programmes

for cassava, and to detect the spread of the disease in previously unaffected regions of Africa.

The variability of the virus was to be looked at by examining sequence variation with infected cassava from different regions of East Africa. The detection method could then be tested against isolates of the virus to be sure it was effective for use in all areas of East Africa.

Outputs

Output I. Molecular identification/characterisation of the causal agent of CBSD.

Serological Reactions

A range of available antiseras corresponding to *Potyviridae* and Carlavirus members were tested in Western assays against CBSV-infected material (both cassava and *Nicotiana benthamiana*) including most importantly, a peptide antiserum designed for potyviruses (designed to a highly conserved region within the coat protein). The potyvirus peptide antiserum has been proven to be 'universally' reactive against potyvirus members, but it failed to give a reaction with CBSV. Antiserum raised to the two members of the Macluraviruses (Maclura mosaic virus and Narcissus latent virus) were tried, again unsuccessfully. Apart from a slight reaction with the whitefly antiserum to the whitefly-transmitted carlavirus, cowpea mild mottle virus, with a protein of about 45kDa, no reaction was found and therefore no clear relationship was established with members of either family.

The antiserum previously raised to a carlavirus-length virus isolated from CBSD material was obtained from SCRI (Scotland). This antiserum did produce a strong reaction with a protein of about 45kDa in the CBSD-infected *N. benthamiana* and to a much lesser degree with the CBSD-infected cassava.

Antiserum	**Reaction with CBSV-infected material**	
	Cassava	***N.benthamiana***
Universal-Potyvirus	-	-
Maclura mosaic virus	-	-
Narcissus latent virus	-	-
Cowpea mild mottle	+	+
CBSV (SCRI Scotland)	+	++

The reacting protein for both cowpea and CBSV antiserum was 45kDa on the gels.
+ indicates a weak reaction, ++ indicates a strong reaction.
Protein extracted from partially purified preparations of CBSV, from *N. benthamiana* leaves was prepared and separated on SDS-PAGE gels. Coomassie blue staining of these gels revealed one major protein of about 45kDa in size. This protein cross-reacted strongly with the SCRI CBSV antiserum. This suggested that this was the same virus that had been purified by SCRI in the 1980's and identified as carlavirus in length.

RT-PCR analysis
The possibility that a Carlavirus or a Potyvirus or both were involved in CBSD was investigated using universal PCR primers designed against members of the *Potyviridae* and Carlavirus genus. PCR has proved most effective for identifying related viruses; degenerate primers are designed to conserved regions within those members already sequenced.
The universal primers Carla-Uni (Badge *et al.*, 1996) for Carlaviruses, CN48 (*Pappu et al*., 1993) for Potyviruses, NGDD (Badge *et al*., 1997) for Bymo- and Macluraviruses and PV2 (Gibbs and Machenzie, 1997) for all members of the *Potyviridae* family were tried. These primers are used with an oligo(dT) based PCR primer as the second primer, since all Carlavirus and *Potyviridae* members sequenced to date have a poly-A tail at the 3' end. However, none of these primers were found to produce a product with CBSD-infected cassava or *N. benthamiana* material. All appropriate positive and negative controls for the cDNA and primers were included.

Primer	For viruses belonging to:-	The primer is designed to:-
CN48	Potyvirus	The coat protein of the virus
Carla-Uni	Carlavirus	The 6K protein at the 3' end, before the poly A tail
NGDD	Bymo/Macluravirus	The Nib gene
PV2	Potyvirus, Bymovirus, Macluravirus, Ipomovirus, Rymovirus	The Nib gene

Positive controls

The tobacco and cassava cDNAs were tested with primers designed to amplify up host genes to ensure that the cDNAs were PCR-amplifiable. The primers were designed to tobacco and cassava sequences found in genebank databases. The tobacco control was to a gene involved in sterol biosynthesis called squalene synthetase, and the cassava-specific primers were designed to ribulose bisphosphate carboxylase. Both sets of primers were designed to produce a PCR product about 500bp in size.

Cloning part of the CBSV genome

The lack of success using the RT-PCR approach suggested that this virus was unusual and a more traditional method of identification through virus purification was tried. Virus was partially purified using a borate buffer, clarifying with chloroform and precipitating with PEG before a series of high and low centrifugation. RNA was then extracted. Double-stranded cDNA was generated using an oligo(dT) primer and ligated into *Sma*I-digested pUC18. Recombinant plasmids that contained an insert in excess of 500bp were sequenced. Three clones of 1114bp (pdT1), 911bp (pdT3) and 592bp (pdT4) were sequenced. The longest clone differed from the other two by one substitution in the untranslated region which did not affect the predicted amino acid sequence. At the 5' end of the CBSV sequence (Fig.1) a region of 200bp was sequenced but only from the longest

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     A   G   V   Q   T   S   K   R   P   C   G   E   P   D   E   G   E
1 AT GCT GGG GTA CAG ACA AGT AAA AGG CCA TGT GGA GAA CCT GAT GAA GGG GAA

     V   A   S   S   E   S   S   E   G   E   E   Q   R   T   D   K   G
54   GTT GCT AGC TCA GAG TCA AGT GAA GGT GAA GAG CAG CGA ACA GAC AAA GGA

     K   A   P   V   E   S   P   A   E   R   N   Q   S   D   K   S   T
105  AAA GCA CCT GTA GAA TCA CCT GCT GAA CGA AAT CAA TCA GAT AAG TCG ACA

     G   E   D   E   E   K   H   R   K   T   R   F   R   I   R   A   G
156  GGT GAG GAT GAG GAG AAA CAT AGA AAG ACA AGG TTC AGA ATA AGA GCT GGT

     G   G   S   E   K   R   D   D   I   D   K   I   P   T   N   T   L
207  GGT GGA AGT GAA AAG AGA GAT GAT ATA GAT AAG ATT CCA ACT AAC ACT CTA

     E   F   R   K   S   F   K   P   P   K   V   S   Q   A   A   Y   V
258  GAA TTT CGG AAG AGC TTC AAA CCA CCA AAA GTG TCA CAA GCG GCA TAT GTG

     W   I   P   R   S   Q   R   D   N   L   T   P   D   V   I   Q   N
309  TGG ATA CCG CGC TCG CAA AGA GAT AAT TTG ACA CCT GAT GTC ATA CAG AAC

     F   L   A   Y   V   P   P   S   H   A   I   D   N   Q   L   A   S
360  TTT CTA GCG TAT GTG CCT CCA TCA CAT GCT ATA GAT AAT CAA TTG GCT TCC

     G   V   E   V   E   N   W   A   I   G   V   S   K   A   Y   G   V
411  GGA GTT GAA GTT GAG AAT TGG GCC ATC GGA GTC TCA AAA GCT TAT GGA GTC

     T   I   Q   E   F   Y   R   T   I   L   P   A   W   I   V   N   C
462  ACC ATT CAA GAG TTT TAT AGA ACG ATC CTA CCT GCT TGG ATT GTC AAT TGT

     I   V   N   G   T   S   D   E   R   K   N   E   K   S   W   R   A
513  ATT GTG AAT GGG ACT AGT GAT GAA AGG AAG AAT GAG AAA TCG TGG AGA GCT

     V   E   L   N   A   Q   G   E   D   I   D   D   S   E   Y   P   M
564  GTT GAG TTA AAT GCA CAG GGT GAG GAT ATT GAT GAT TCA GAA TAC CCT ATG

     E   P   M   Y   K   F   A   L   P   T   M   R   K   I   M   R   N
615  GAA CCA ATG TAT AAA TTT GCT CTC CCG ACA ATG AGG AAA ATA ATG AGA AAT

     F   S   S   Q   A   I   L   M   Y   Q   N   S   V   T   A   G   K
666  TTT TCC AGC CAA GCA ATC TTA ATG TAT CAG AAT AGT GTG ACT GCT GGA AAA

     A   F   V   I   K   A   A   R   N   A   G   Y   T   S   I   E   N
717  GCT TTT GTA ATA AAA GCA GCC CGA AAT GCC GGG TAT ACA AGC ATT GAA AAT

     K   W   L   G   I   D   F   L   A   E   A   Q   L   S   Q   S   Q
768  AAG TGG TTG GGT ATT GAC TTC CTA GCC GAG GCA CAA TTG TCA CAA AGC CAG

     L   D   I   K   H   Q   I   L   A   A   N   V   S   R   S   K   T
819  CTT GAT ATC AAA CAT CAA ATA TTG GCT GCC AAT GTT AGT AGA AGT AAG ACT

     K   L   F   A   L   A   A   P   G   D   D   N   N   V   D   K   E
870  AAG TTG TTT GCT TTA GCT GCT CCT GGT GAC GAT AAT AAT GTG GAT AAA GAA

     R   H   T   T   R   D   V   S   A   T   R   H   S   Y   A   G   A
921  AGG CAC ACC ACG CGA GAT GTT AGT GCA ACT AGG CAT AGT TAT GCA GGT GCT

     A   I   E   STOP
972  GCT ATT GAA TAAATCAATTTGAAAGTCAAGTTTGGTGGAGTTTTAGGTAGCTTGTTATATTTT

1035 GGTTGTGAAGCCAAGATATATTCCGGAGCCCTTTCTCCATATCCTTTTATTTAGTGTGGAAACACAA

1102 TATGTAAAAGTAC(A)n
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Fig 1. Nucleotide sequence of the 3' end, 1114 nucleotides of CBSV cDNA. The predicted amino acid sequence is shown above.

clone pdT1. A PCR primer was made to the sequence at the 5' end of this sequence; CBSV9 (5'- ATGCTGGGGTACAGACAAG-3'). This primer was used with the primer CBSV8 (5'-GGCACATACGCTAGAAAG-3') to generate a PCR product of 300bp from infected cassava material. This product was cloned and sequenced, which confirmed the sequence of the pdT1 clone. Computer analysis revealed one open reading frame open at the 5' end, followed by an untranslated region (UTR) of 144 nucleotides and a poly(A) tail (Fig. 1). Submission of the predicted amino acid sequence to the GenBank data base showed sequence homology to the coat protein of known members of the *Potyviridae* family, closest homology was with the only sequenced member of the Ipomoviruses, Sweet Potato Mild Mottle Virus (SPMMV). With the discovery of a relationship between CBSV and SPMMV an antiserum raised to SPMMV was obtained. Western analysis with this antiserum against CBSV failed to give a reaction.

The coat protein sequence of the two Ipomoviruses are quite dissimilar to other members of the *Potyviridae* family so it is not surprising that they have failed to find serologically related viruses within the family. The degree of homology between the whole of the coat proteins of the two Ipomoviruses is about 34% so it is also therefore not surprising that antiserum raised to SPMMV failed to react with CBSV. The lack of sequence homology between the Ipomoviruses and other *Potyviridae* members also accounts for the failure with the 'Universal' PCR primers. The potyvirus primer is based on the sequence of the conserved amino acid motif WCIEN present in all potyviruses, the corresponding sequence in CBSV is NCIEV.

The cause of the slight antiserum reaction with cowpea mild mottle virus, the whitefly transmitted carlavirus remains unknown. This reaction has also been reported previously (Lennon *et al*., 1985). The sequence analysis (not shown) between the coat proteins of the two viruses showed no region of identity between them, indeed, no two amino acids together could be found in common.

Sequence comparison

The deduced CBSV amino acid sequence was aligned with the corresponding sequences of different Genera of the *Potyviridae* family, using Clustal W. The viruses chosen where those members of the Genera that showed closest homology to CBSV on a search

of the GenBank database. Two Bymoviruses, Barley Mild Mottle virus (BaMMV) (Kashiwazaki, 1992) and barley yellow mosaic virus (BaYMV) (Kashiwazaki, 1989), two Macluraviruses, Maclura Mosaic virus (MacMV) (Badge, 1997) and Narcissus Latent virus (NLV) (Badge, 1997), two Rymoviruses, Wheat Streak Mosaic virus (WSMV) (Niblett 1991) and Brome Streak virus (BrSV) (Gotz, 1995), one Potyvirus, Potato virus Y (PVY) (Robaglia, 1989) and one Ipomovirus, Sweet Potato Mild Mottle virus (SPMMV) (Colinet, 1996). The conserved core of the coat proteins equivalent to D^{2857} to R^{3074} in PVY, I^{80}-R^{307} in CBSV are shown in Fig 2.

Pairwise percent sequence identities between the coat protein cores of CBSV and selected members of the *Potyviridae* (equivalent to D^{2857} to R^{3074} in PVY) are shown in Table 1. CBSV shows a 42.5% sequence identity to (SPMMV) the only sequenced member of the Ipomovirus Genera. Like SPMMV, CBSV shows quite high sequence identity to the Rymoviruses, 26.7% with both BrSV and WSMV.

Comparison between the two Ipomoviruses

SPMMV and CBSV both occur in East Africa. They are both believed to be whitefly-transmitted and they both produce inclusion bodies in infected tissue of the pinwheel variety. One interesting observation is the large coat protein of the two viruses when observed on SDS-PAGE gels. The putative cleavage sites between the coat protein and NIb proteins in potyviruses are well known and the predicted coat proteins from SDS gels are found to match the sequence size. The SPMMV was predicted from gels to be 38kDa but the predicted size from the recent sequence is about 33kDa (Colinet *et al*.,1998). SDS-PAGE gels of partially purified preparations of CBSV predict the coat protein to be about 40kDa. Therefore, a characteristic of these viruses may be coat proteins that run high on SDS gels giving a false large size. This characteristic is also true of the two Maclura viruses, MacMV and NLV. They have coat proteins of 40kDa MacMV and 39.5kDa NLV but when sequenced the putative cleavage site would produce coat proteins of 34.1kDa MacMV and 32.8kDa NLV (Badge *et al.,* 1997).

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BaMMV  989  TSD--------WSLPEPKMRTLGFKSKINIET--------LANVPDGYMNT
BaYMV  164  AADGN------WSVPATKQVNAGLTLKIPLNK--------LKSVPKSVMEH
MacMV  488  DPEEK-EEEVKWVMPSINPNRGSNAIPTVNGKKLWKR-GILKHIPKQQYDA
NLV    273  DPLVD-DEVVEWVIPKMSPNIGTSPIPVINGKRLWKR-GVLKSIPKMMFNT
PVY   2857  DKDVNAGTSGTHTVPRIKAITSKMRMPTSKGATVPNL-EHLLEYAPQQIDI
BrSV   130  VADRTSGIV--FPVPTRKSTS-LYLPPKVKLRATPERIEKVRKYLPDPQQI
WSMV   310  VQDQTPGLV--FPAPKITTKA-IYMPKTVRDKIKPEMINNMIKYQPRTELI
SPMMV  353  QPDVTPAQIVTFEPPRVTGFGALWIPRQQRNYMTPSYIEKIKAYVPHSNLI
CBSV    80  IPTNTLEFRKSFKPPKVSQAAYVWIPRSQRDNLTPDVIQNFLAYVPPSHAI

BaMMV 1024  FASVATESQRRKWEEAARGDFGITDDEKWEKLLIAACIYFADNGTSPNFDE
BaYMV  201  NNSVALESELKAWTDAVRTSLGITTDEAWIDALIPFIGWCCNNGTSDKHAE
MacMV  537  STTKATSAQLAAWVEAVKKDLKIRNDDAWSIVLTAWCIWCANNGTSSEVDT
NLV    322  TSTMATQAQLTSWVEEVKQALALKTDDAWTVVITNWCIWCANNGTSSEVDT
PVY   2907  SNTRATQSQFDTWYEAVRMAYDIGETEM-PTVMNGLMVWCIENGTSPNVNG
BrSV   178  DLRYSTQQELNDWIKASADGLGQTEEAFIDNILPGWIVHCIVNTTSSENRK
WSMV   358  DNRYATTEQLNTWIKEASEGLDVTEDVFINTLLPGWVYHCIINTTSPENRA
SPMMV  404  ESGLASEAQLTSWFENTCRDYQVSMDVFMSTILPAWIVNCIINGTSQERTN
CBSV   131  DNQLASGVEVENWAIGVSKAYGVTIQEFYRTILPAWIVNCIVNGTSDERKN

BaMMV 1075  ELT-----MEVNGGLNSIKEYPVRPFVVRAKKISTLRRIFRC-YSIETKLM
BaYMV  252  NQV-----MQIDSGKGAVTEMSLSPFIVHARMNGGLRRIMRN-YSDETVLL
MacMV  588  NQD-----ME-SDSLGKVQTVRIDSFVEPAIENGGLRKIMRLLFRYHSGNL
NLV    373  SQT-----MEIRDGFGKVQAIPIEVFVNPAVENGGLRKIMRH-FSGITHEI
PVY   2957  VWV-----M--MDGNEQV-EYPLKPIVENAK--PTLRQIMAHFSDVAEAYI
BrSV   229  AGSWRCVT-NAGTADEEQVLYDIEPMYSAAN--PTMRAIMRHFSDLARLVI
WSMV   409  LGTWRVVN-NAGKDNEQQLEFKIEPMYKAAK--PSLRAIMRHFGEGARVMI
SPMMV  455  EHTWRAVIM-ANMEDQEVLYYPIKPIIINAQ--PTLRQVMRHFGEQAVAQY
CBSV   182  EKSWRAVELNAQGEDIDDSEYPMEPMYKFAL--PTMRKIMRNFSSQAILMY

BaMMV 1120  FVKLRR----VPHWAIKHGCLDE--I--VFDFMIPDQFTSRTALETLKQTK
BaYMV  297  ITNNKL----VAHWSMKHGASAN--AKYAFDFFVPRSWMNPQDIEVSKQAR
MacMV  633  GQRGKN----DSLW-NQAGFTEKAMTTLPFDFVEVTKTTPKTVKEQLAQAK
NLV    418  LKAGKR----MTAWGNKRGFTEKSMIPYAFDYYVVTNTTPKTVREQLAQSK
PVY   2998  EMRNKKE-PYMPRYGLIRNLRDMGLARYAFDFYEVTSRTPVRAREAHIQMK
BrSV   277  AESFKQGRPLIPKGYIKAGVLDASSAAAACDFVVRDRHDTATFVQVQNQVL
WSMV   457  EESVRIGKPIIPRGFDKAGVLSINNIVAACDFIMRGADDTPNFVQVQNSVA
SPMMV  503  MNSLQVGKPFTVKGAVTAGYANVQDAWLGIDFLRDTMKLTTKQMEVKHQII
CBSV   231  QNSVTAGKAFVIKAARNAGYTSIENKWLGIDFLAEA-QLSQSQLDIKHQIL

BaMMV 1163  LAAIGVGTSNSLLTSEQTNMRTTETRR
BaYMV  342  LAALGTGTYNTMLTSDTTNLRKTTNHR
MacMV  679  IAAIGHGTRRAMVTDGSVHGNKTSYER
NLV    465  AAAIGSGVTRKMVLDGNIQGSHASYER
PVY   3048  AAALKSAQPRLFGLDGGISTQEENTER
BrSV   328  VNRVSGITNRLFAQAMPSAGANEDMAR
WSMV   508  VNRLRGIQNKLFAQARLSAGTNEDNSR
SPMMV  554  AANVTRRKIRVFALAAPGDGDELDTER
CBSV   281  AANVSRSKTKLFALAAPGDDNNVDKER
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Fig 2.Multiple alignment of the C-terminal core amino acids (equivalent to D^{2857}-R^{3074} in PVY) of CBSV with corresponding protein sequences of BaMMV(GenBank D10949), BaYMV (GenBank D00544), MacMV (GenBank U58771), NLV (GenBank U58770)), PVY (GenBank D00441), WSMV, BrSV (GenBank Z48506) and SPMMV (GenBank Z48058). Residues conserved in all sequences are in bold type. Sequences were aligned using Clustal W.

The Rymoviruses also have large coat proteins which may turn out to be excessive in their estimate, for example WSMV is 47kDa.

The range of particle lengths for *Potyviridae* membership was extended to 680-900nm in 1976 (Fenner, 1976). The two Ipomoviruses are at either end of this spectum, 650-700nm for CBSV and 850-950nm for SPMMV, size appears not to be a characteristic. CBSV is clearly a most unusual virus. Tests that are available for other *Potyviridae* members have proven ineffective with this virus. It was clear that the only way forward was to design PCR or antibody tests that were specific for CBSV, using the part of the virus genome that had been cloned and sequenced.

Output II. Development of a diagnostic test for CBSV

Detection of CBSV within infected tissue

Infected and uninfected *N. benthamiana* plant sap was spotted onto nitro-cellulose membrane and probed with radioactively labelled CBSV pdT1. CBSV pdT1 only hybridised to the infected sap (results not shown). A duplicate dot blot with infected cassava sap failed to detect the virus. The virus was clearly at a much lower concentration in the cassava plant, compared to the tobacco.

With the failure of the SCRI antiserum and dot blot hybridisation to detect the virus in cassava plants, a RT-PCR test was developed.

The most important part of a RT-PCR test is the development of a good RNA extraction method. The RNA must be undegraded and of high quality to ensure that cDNA can be transcribed from it. Many RNA extraction methods have been developed for plants because a method developed for one plant seldom works for a different plant species or indeed different tissue of the same plant. With the absence of a method published for the extraction of RNA from cassava leaves a number of protocols were tried, including kits. Cassava proved to be particularly challenging, with most protocols resulting in degraded RNA or, in the case of the kits, a material that would not go down the columns. The best method was that of Lodhi *et al*., 1994) which had been adapted by Rick Mumford (NRI Chatham) for use with yams. The only kit that gave useable RNA was the Qiagen Plant RNA easy kit, the RLC and not the RLT buffer was effective, the sample must not be

heated and some of the centrifugation times were increased. Good quality RNA resulted in the development of a trouble free test. Both Promega and Stratagene first-strand cDNA kits worked well and a range of PCR primers were found to give a product. The best primer pair proved to be the one that the computer designed using the Genejockey III program. This primer pair was tested under a range of conditions to see the extremes under which the test would still work (these included different PCR machines, different PCR buffers, different magnesium concentration and different cDNA concentrations). This test has been shown to work with both infected cassava and *N. benthamiana* leaves. More importantly samples of cassava leaves from plants that were known to be infected but were not yet showing symptoms were obtained from NRI Chatham. These samples also produced a positive result with the PCR test.

The PCR test developed is written in detail in Appendix 1. This test has been transferred to Sue Seal at NRI Chatham and to Graham Thompson in Pretoria, South Africa. Dr Thompson is the head of Virology at ARC-Roodeplaat the Vegetable and Ornamental Plant Institute; he wishes to investigate whether CBSV is the poty-like particles that have been found in some cassava samples.

Preliminary *E. coli* expression of CBSV coat protein

The 1114 nucleotide clone of CBSV (pdT1) had been cloned into *Sma*I-cut pUC18. The orientation and reading frame of the insert was such that expression of the protein was possible. Clone pdT1 was induced to produce a product using IPTG and the total protein extracted and separated on a SDS-PAGE gel. Total protein was also extracted from un-induced pdT1 and *E. coli* containing unmodified pUC18, induced and un-induced with IPTG. The CBSV antiserum from SCRI reacted with a protein of about 40kDa in both the induced and un-induced pdT1, no reaction was seen with the control bacterial proteins. The cross-reaction was strongest with the induced bacteria. Therefore the virus we have cloned corresponds to the virus previously identified by SCRI as the putative carlavirus component of the disease and for which antiserum was raised.

Antiserum production

PCR is an extremely sensitive test and can cope well with transcripts that are in low abundance. An ELISA test would also be very good in some situations and as a backup to the PCR test. An ELISA test requires the production of antiserum.

The SCRI antiserum was raised in a rabbit from purified virus particles. Purifying this virus in sufficient quantities for antibody production was an achievement, others have tried and failed. However, this antiserum was not specific to CBSV, since other proteins in plants and bacteria were seen to react. This suggested that contaminating plant proteins had also been present in the virus purification. The antiserum has also proved unreliable at detecting the virus in cassava, especially infected cassava which does not show symptoms. In contrast to SCRI, we chose to express the coat protein region of the virus in bacteria and purify this protein to raise antibodies.

The virus coat protein was cut from the pdT1 clone and put into the expression vector pGEX-4T-1, to create the CBSV-CP-GST fusion protein construct. The fusion protein proved to be very insoluble, to the extent that the GST part of the fusion protein was unable to bind to appropriate columns to aid purification. High quality purification of this protein to the degree required for antiserum production was achieved using the BioRad 491 Prep-Cell. The Prep-Cell gel is quite small and a number of gels were required to purify the 2mg of protein required for antibody production. Polyclonal antiserum was produced using a rabbit. At the same time polyclonal antiserum was raised to sweet potato chlorotic stunt closterovirus using a similar construct expressed by bacteria. The sweet potato antiserum was of good quality and has been passed on to NRI Chatham; but the cassava antiserum has proved to be poor, unable to detect the virus in cassava with both western analysis and ELISAs.

A second attempt at antiserum is in progress through generation of a multiple antigenic peptide (MAP). This is when the peptide is synthesised on a branched poly-Lysine core sequence, enabling eight peptide chains to be incorporated into each molecule. These octomeric peptides give a compound of a high enough molecular weight, to stimulate antibody production without the need to couple the peptide to a carrier protein.

The MAP-peptide generated is to 15 amino acids at the 5' end of the CBSV coat protein. The amino acid sequence of CBSV coat protein was analysed to find the most

hydrophilic and antigenic regions. A region of 21 amino acids close to the 5' end appeared the most suitable. Alta Bioscience at Birmingham University made a peptide to 15 of these amino acids (ERNQSDKSTGEDEEK). The peptide was purified by dialysis and yielded 45mg. This peptide is being used presently to generate polyclonal antibodies.

Output III CBSV Isolates

CBSV Symptoms

All infected cassava plants were maintained at NRI Chatham. The virus was known to be at low concentrations in the cassava, and cassava leaves have waxy cuticles and are difficult to extract RNA, protein or virus particles from. To work with the virus effectively, the alternative host plant *N. benthamiana* was selected. Initial observations of infected *N. benthamiana* plants ranged from plants which showed strong mosaic symptoms on the systemic leaves and which stayed healthy for some weeks to plants that wilted and died very quickly with various samples of CBSD material sent from NRI. The main problem in the initial stages of the project was to get infected plants, as large numbers of plants did not show any symptoms.

1 2 3

Fig. 3 *Nicotiana benthamiana* plants infected with cassava brown streak disease, showing either no symptoms (1), mosaic symptoms (2) or wilting symptoms (3).

Closer observation of symptoms was undertaken using infected cassava material designated A, B, or C, which was collected (12.9.1996) by Mrs Kiddo Mtunda at Kibaha, Tanzania from different varieties of cassava; A = cv. Kibaha, B = cv. Vumbi, C = cv. Mukukumkuku

Figure 4. Leaves of *Nicotiana benthamiana* infected with cassava brown streak virus from infected cassava A, B or C (left to right). All attempts to produce symptoms on this tobacco with type A cassava have failed. Type B did not produce clear local lesions but areas of infection where present with some plants. Type C produced very clear local lesions.

Fig 5. *Nicotiana benthamiana* plant infected with type B, systemic symptoms are strong mosaic pattern and stunted growth (the plant remains quite healthy).

Fig 6.*Nicotiana benthamiana* plant infected with type C, systemic symptoms of petiole thinning, and wilting. Plants do not survive more than a few of days after symptoms first appear.

Mechanical inoculation of this virus involved grinding the infected leaf in a small amount of water and with some carborundum rubbing the mixture onto leaves. If the virus is very dilute infection does not occur. Mechanical inoculation of the virus to a secondary host can still be difficult even when type B or type C are involved. Many more plants should be inoculated than are required. The best inoculum comes from freshly infected tobacco. Long term storage of material was achieved by snap-freezing in liquid nitrogen and storage in a -70°C freezer.

The tobacco variety known as SR1 has been reported to be a local lesion host for this virus. This was found to be true for type C but both type A and B failed to produce local lesions.

The speed at which symptoms appear can be accelerated by increasing the temperature, at 28-30°C symptoms can be seen after 1 week. At lower temperatures, typically 23°C symptoms appear between 10 and 14 days. At higher temperatures the symptoms appear more severe.

Sequence Variation

With the cloning of the virus and development of a PCR test, types A, B and C could be sequenced. The kilobase of DNA sequence of the virus determined varied from one another by about 8%. The differences were not evenly distributed along the length of the sequence but tended to be localised in regions. Fig 7A and 7B show a region where there are a large number of differences.

Fig 7A. Nucleotide changes

```
       / //  /     / /   ///          //     / /   ///  //     /// /
Type A AGACGAAGAGCAACGAACTGACAAAGGAAAAACACCTATGGAACCGCCAACTGAATTGAT
Type B AGATGAGGAGCAACAAACAAGCAAAGGAAAGACACCTGTGGAATCACCTGCTGAACGAAA
Type C GGGTGAAGAGCAGCGAACAGACAAAGGAAAAGCACCTGTAGAATTACCTGCTGAACGAAA
```

Fig 7B. Amino Acid Changes

```
          /    /   / /   / / / / //  /  /  /    /  /
Type A    PESSEDEEQRTDKGKTPMEPPTELIQSEKSMGESEEKHKKTK
Type B    PESSEDEEQQTSKGKTPVESPAERNQSEKSIGEEEEKHKKTR
Type C    SESSEGEEQRTDKGKAPVELPAERNQSDKSTGEDEEKHRKTR
```

The dashes above the sequences indicate a difference found with at least one sequence.

A, B and C represent three isolates of the virus, all found in the same region of Tanzania.

Independent testing of the RT-PCR diagnostic test for CBSV

In conjunction with Sue Seal at NRI Chatham, we were given 17 cassava leaves from Tanzania. No further information was given about the samples and the tests were carried out independently. Two samples of cassava leaves from Mozambique (Fernando and Mululeia) were also obtained. As well as carrying out the RT-PCR test (described in Appendix 1), a larger PCR product was cloned and sequenced from some of the positive samples and sequence comparisons carried out.

Table 2. RT-PCR of cassava samples from Tanzania

Name of sample	**Positive or negative for PCR**
Nan 01	+
Nan 02	+
Alb 02	+
Alb 03	+
Alb 04	+
Kib 01	+
Kib 02-1	+
Kib 02-2	+
Kib 03	+
Kit 02	-
Kit 03	-
Kig 02	-
Kig 03	-
NRI "transmission" samples:	
Ebwa Mukino 1	-
Ebwa Mukino 2	-
TC Tan 2 1	-
TC Tan 2 1B	-

These results were confirmed at NRI (see Appendix 2). Figure 8, shows a DNA alignment of the cassava brown streak isolates A, B and C with some sequence from samples from Tanzania and two samples from Mozambique (Fernando and Mululeia).

```
                          10        20        30        40        50
                          |         |         |         |         |
ALB 02          ----------------------------------------------------------
ALB 03          ----------------------------------------------------------
ALB 04          ----------------------------------------------------------
Fernando        CAAAAGGCCATGTGGAGAACCTGATGAAGGGGAAGTTGCTAGCCCAGGGTCAAGTGA
KIB 01          CAAAAGGCCATGTGGAGAACCTGATGAAGGGGAAGTTGCTAGCCCAGAGTCAAGTGA
KIB 02-2        CAAAAGGCCATGTGGAGAACCTGATGAAGGGGAAGTTGCTAGCCCAGAGTCAAGTGA
KIB 03          ----------------------------------------------------------
mululeia        CAAAAGGCCATGTGGAGAACCTGATGAAGGGGAAGTTGCTAGCCCAGAGTCAAGTGA
TYPE A          CAAAAGGCCGTGTGGAGAACCTGATGAAGGGGAAGTTGCTAGCCCAGAGTCAAGTGA
TYPE B          TAAGAGACCATGTGGAGAACCTGATGAAGGAGAAGTTGCTAGCCCAGAGTCAAGTGA
Type C          TAAAAGGCCATGTGGAGAACCTGATGAAGGGGAAGTTGCTAGCTCAGAGTCAAGTGA

                           60        70        80        90       100       110
                           |         |         |         |         |         |
ALB 02          ----------------------------------------------------------
ALB 03          ----------------------------------------------------------
ALB 04          ----------------------------------------------------------
Fernando        AGACGAGGAGCAACGAATGAACAGAGGGAAAGCGCCTATGGAACCGCCTACTGAATT
KIB 01          AGACGAGGAGCAACGAATGAACAGAGGGAAAGCGCCTATGGAACCGCCTACTGAATT
KIB 02-2        AGACGAGGAGCAACGAACGAACAAAGGGAAAGCGCCTATGGAACCGCCCACTGAATT
KIB 03          ----------------------------------------------------------
mululeia        AGACGAGGAGCAACGAATGAACAGAGGGAAAGCGCCTATGGAANCGCCTACTGAATT
TYPE A          AGACGAAGAGCAACGAACTGACAAAGGAAAAACACCTATGGAACCGCCAACTGAATT
TYPE B          AGATGAGGAGCAACAAACAAGCAAAGGAAAGACACCTGTGGAATCACCTGCTGAACG
Type C          GGGTGAAGAGCAGCGAACAGACAAAGGAAAAGCACCTGTAGAATTACCTGCTGAACG

                             120       130       140       150       160       170
                             |         |         |         |         |         |
ALB 02          ----------------------------------------------------------
ALB 03          ----CAATCAGATAAGTCGACAGGTGAGGATGAGGAGAAACATAGAAAGACAAGGTT
ALB 04          ----CAATCAGATAAGTCGACAGGTGAGGATGAGGAGAAACAGAAAAAGACAAGATT
Fernando        GATTCAATCAGAGAAGTCTATTGGTGAGAGTGAGGAAAAACATAAGAAAACAAAGTT
KIB 01          GATTCAATCAGAGAAGTCTATTGGTGAGAGTGAGGAAAAACATAAGAAAACAAAGTT
KIB 02-2        GATTCAATCAGAGAAGTCTATTGGTGAGAGTGAGGAAAAACATAAGAAAACAAAGTT
KIB 03          ----CAATCAGATAAGTCGACAGGTGAGGATGAGGAGAAACAGAAAAAGACAAGATT
mululeia        GATTCAATCAGAGAAGTCTATTGGTGAGAGTGAGGAAAAACATAAGAAAACAAAGTT
TYPE A          GATTCAATCAGAGAAGTCTATGGGTGAGAGTGAGGAAAAACATAAAAAGACTAAGTT
TYPE B          AAATCAATCAGAGAAATCTATAGGTGAGGAGGAGGAGAAACATAAAAAGACAAGGTT
Type C          AAATCAATCAGATAAGTCGACAGGTGAGGATGAGGAGAAACATAGAAAGACAAGGTT

                                 180       190       200       210       220
                                 |         |         |         |         |
ALB 02          ----------------------------------------------------------
ALB 03          CAGAATAAGAGCTGGTGGTGGAAGTGAAAAGAGAGATGATATAGATAAGATTCCAAC
ALB 04          CAGAATAAGAGCTGGTGGTGGAAGTGAAAAGAGAGATGATATAGACAAGATTCCAAC
Fernando        CAGAATAAGAGCTGGTGGTGGAAATGAGAAGAGAGATGATATAGATAAGATTCCAAC
KIB 01          CAGAATAAGAGCTGGTGGTGGAAATGAGAAGAGAGATGATATAGATAAGATTCCAAC
KIB 02-2        CAGAATAAGAGCTGGTGGTGGAAATGAGAAGAGAGATGATATAGATAAGATTCCAAC
KIB 03          CAGAATAAGAGCTGGTGGTGGAAGTGAAAAGAGAGATGATATAGATAAGATTCCAAC
mululeia        CAGAATAAGAGCTGGTGGTGGAAATGAGAAGAGAGATGATATAGATAAGATTCCAAC
TYPE A          CAGAATAAGAGCTGGTGGTGGAAATGAGAAGAGAGATGATATAGATAAGATTCCGAC
TYPE B          CAGAATAAGAGCTGGTGGTGGAAGTGAAAAGAGAGATGATATAGATAAGATTCCGAC
Type C          CAGAATAAGAGCTGGTGGTGGAAGTGAAAAGAGAGATGATATAGATAAGATTCCAAC

                               230       240       250       260       270       280
                               |         |         |         |         |         |
ALB 02          ----------------------------------------------------------
ALB 03          TAACACTCTAGAATTTCGGAAGAGCTTCAAACCACCAAAAGTGTCACAAGCGGCATA
ALB 04          CAATGCTCTAGAATTTCGAAAGAGCTTCAAGCCACCAAAGGTGTCACAAGCAGCATA
Fernando        CAACGCTCTAGAATTTCGGAAGAGCTTCAAACCACCAAAAGTTTCACAAGCAGCATA
KIB 01          CAACGCTCTAGAATTTCGGAAGAGCTTCAAACCACCAAAAGTTTCACAAGCAGCATA
KIB 02-2        CAACGCTCTAGAATTTCGGAAGAACTTCAAACCACCAAAAGTTTCACAAGCAGCATA
KIB 03          CAATGCTCTAGAATTTCGAAAGAGCTTCAAGCCACCAAAGGTGTCACAAGCAGCATA
mululeia        CAACGCTCTAGAATTTCGGAAGAGCTTCAAACCACCAAAAGTTTCACAAGCAGCATA
TYPE A          CAACGCTCTAGAATTTCGGAAGAGCTTTAAACCACCAAAAGTGTCACAAGCAGCATA
TYPE B          TAATGCTTTAGAATTTCGAAAGAGCTTCAAGCCACCAAAAGTGTCACAAGCAGCATA
Type C          TAACGCTCTAGAATTTCGGAAGAGTTTCAAACCACCAAAAGTGTCACAAGCGGCATA
```

```
                   290        300        310        320        330        340
                     |          |          |          |          |          |
ALB 02             ------------------------------------------------------------
ALB 03             TGTGTGGATACCGCGCTCGCAAAGAGATAATTTGACACCTGATGTCATACAGAACTT
ALB 04             TGTGTGGATACCGCGTTCGCAAAGAGACAATTTGACACCTGATGTCATCCAGAACTT
Fernando           TGTGTGGATACCGCGTTCGCAAAGAGATAACTTGACACCTGATGTCATACAGAATTT
KIB 01             TGTGTGGATACCGCGTTCGCAAAGAGATAACTTGACACCTGATGTCATACAGAATTT
KIB 02-2           TGTGTGGATACCGCGTTCGCAAAGAGATAACTTGACACCTGATGTCATACAGAATTT
KIB 03             TGTGTGGATACCGCGTTCGCAAAGAGATAATTTGACACCTGATGTCATCCAGAACTT
mululeia           TGTGTGGATACCGCGTTCGCAAAGAGATAACTTGACACCTGGTGTCATACAGAATTT
TYPE A             TGTGTGGATACCACGTTCGCAAAGAGATAATCTGACACCTGATGTCATACAGAATTT
TYPE B             TGTGTGGATACCGCGTTCGCAAAGAGATAATTTGACACCTGATGTCATACAGAACTT
Type C             TGTGTGGATACCGCGCTCGCAAAGAGATAATTTGACACCTGATGTCATACAGAACTT

                        350        360        370        380        390
                          |          |          |          |          |
ALB 02             ------------------------------------------------------------
ALB 03             TCTAGCGTATGTGCCTCCATCACATGCTATAGATAATCAATTGGCTTCCGGAGTTGA
ALB 04             TCTAGCGTACGTGCCTCCGTCACATGCTATAGACAATCAATTGGCTTCTGGAGTTGA
Fernando           TCTAGCGTACGTGCCTCCATCACATGCCATAGACAATCAATTGGCTTCTGGAGTTGA
KIB 01             TCTAGCGTACGTGCCTCCATCACATGCTATAGACAATCAATTGGCTTCTGGAGTTGA
KIB 02-2           TCTAGCGTACGTGCCTCCATCACATGCCATAGACAATCAATTGGCTTCTGGAGTTGA
KIB 03             TCTAGCGTACGTGCCTCCATCACATGCTATAGATAATCAATTGGCTTCTGGAGTTGA
mululeia           TCTAGCGTACGTGCCTCCATCACATGCCATAGACAATCAATTGGCTTCTGGAGTTGA
TYPE A             TCTAGCGTACGTGCCTCCATCACATGCTATAGACAATCAATTGGCTTCTGGAGTTGA
TYPE B             TCTAGCGTACGTGCCTCCATCACATGCTATAGACAATCAATTGGCTTCCGGAGCTGA
Type C             TCTAGCGTATGTGCCTCCATCACATGCTATAGATAATCAATTGGCTTCCGGATTTGA

                   400        410        420        430        440        450
                     |          |          |          |          |          |
ALB 02             ------------------------------------------------------------
ALB 03             AGTTGAGAATTGGGCCATCGGAGTCTCAAAAGCTTATGGAGTCACCATTCAAGAGTT
ALB 04             GGTTGAAAATTGGGCTATCGAAGTCTCAAAAGCTTATGGAGTCACCATTCAAGAGTT
Fernando           AGTTGAGAATTGGGCCATTGAGGTTTCAAAAGCTTATGGAGTCACCATTCAAGAGTT
KIB 01             AGTTGAGAATTGGGCCATTGAGGTTTCAAAAGCTTATGGAGTCACCATTCAAGAGTT
KIB 02-2           AGTTGANAATTGGGCCATTGAGGGTTCAAAAGCTTATGGAGTCACCGTTCAAGAGTT
KIB 03             AGTTGAAAATTGGGCTATCGAAGTTTCAAAAGCTTATGGAGTCACCATCCAAGAGTT
mululeia           AGTTGAGAATTGGGCCATTGAGGTTTCAAAAGCTTATGGAGTCACCATTCAAGAGTT
TYPE A             AGTTGAGAATTGGGCCATTGAGGTTTCAAAAGCTTATGGAGTCACCATTCAAGAGTT
TYPE B             AGTTGAGAATTGGGCCATCGAAGTTTCAAAAGCTTATGGAGTCACCATTCAAGAGTT
Type C             AGTTGAGAATTGGGCCATCGGAGTCTCAAAAGCTTATGGAGTCACCATTCAAGAGTT

                    460        470        480        490        500        510
                      |          |          |          |          |          |
ALB 02             ------------------------------------------------------------
ALB 03             TTATAGAACGATTCTACCTGCTTGGATTGTCAATTGTATTGTGAATGGGACTAATGA
ALB 04             TTATAGAACGATCCTGCCTGCTTGGATTGTCAATTGTATTGTGAATGGGACTAGTGA
Fernando           TTATAGAACGATCCTACCTGCTTGGATTGTTAATTGTATTGTGAATGGGACTAGCGA
KIB 01             TTATAGAACGATCCTACCTGCTTGGATTGTNAATTGTATTGTGAATGGGACTAGCGA
KIB 02-2           TTATAGAACGATCCTACCTGCNTGGATTGTTA-TTGTATTGTGAATGGGACTAGCGA
KIB 03             TTATAGAACGATTCTACCTGCTTGGATTGTCAATTGTATTGTGAATGGGACTAATGA
mululeia           TTATAGAACGATCCTACCTGCTTGGATTGTTAATTGTATTGTGAATGGGACTAGCGA
TYPE A             TTATAGAACGATCCTCCCTGCTTGGATTGTTAATTGTATTGTGAATGGGACTAGTGA
TYPE B             TTATAGGACGATCCTACCTGCTTGGATTGTCAATTGTATTGTGAATGGGACTAGCGA
Type C             TTATAGAACGATCCTACCTGCTTGGATTGTCAATTGTATTGTGAATGGGACTAGTGA

                        520        530        540        550        560        570
                          |          |          |          |          |          |
ALB 02             ------------------------------------------------------------
ALB 03             TGAAAGGAAGAATGAGAAATCGTGGAGAGCTGTTGAGTTAAATGCACAAGGTGAGGA
ALB 04             TGAGAGGAAGAATGAGAAATCGTGGAGAGCTGTTGAGCTAAATGCACANGGTGAGGA
Fernando           TGAAAGGAAGAATGAGAAATCGTGGAGAGCTGTTGAGCTAAATGCGCAGGGTGAGGA
KIB 01             TGAAAGGAAGAATGAGAAATCGTGGAGAGCTGTTGAGCTAAATGCGCAGGGTGAGGA
KIB 02-2           TGAAA
KIB 03             TGAGANGAAGAATGAGAAATCGTGGAGAGCTGTTGAGCTAAATGCACAAGGTGAAGA
mululeia           TGAAAGGAAGAATGAGAAATCGTGGAGAGCTGTTGAGCTAAATGCGCAGGGTGAGGA
TYPE A             TGAAAGGAAGAACGAGAAATCGTGGAGAGCTGTTGAGCTAAATGCGCAGGGTGAGGA
TYPE B             TGAAAGGAAGAATGAGAAATCGTGGAGAGCTGTTGAGTTAAATGCACAGGGTGAGGA
Type C             TGAAAGGAAGAATGAGAAATCGTGGAGAGCTGTTGAGCTAAATGCACAGGGTGAGGA
```

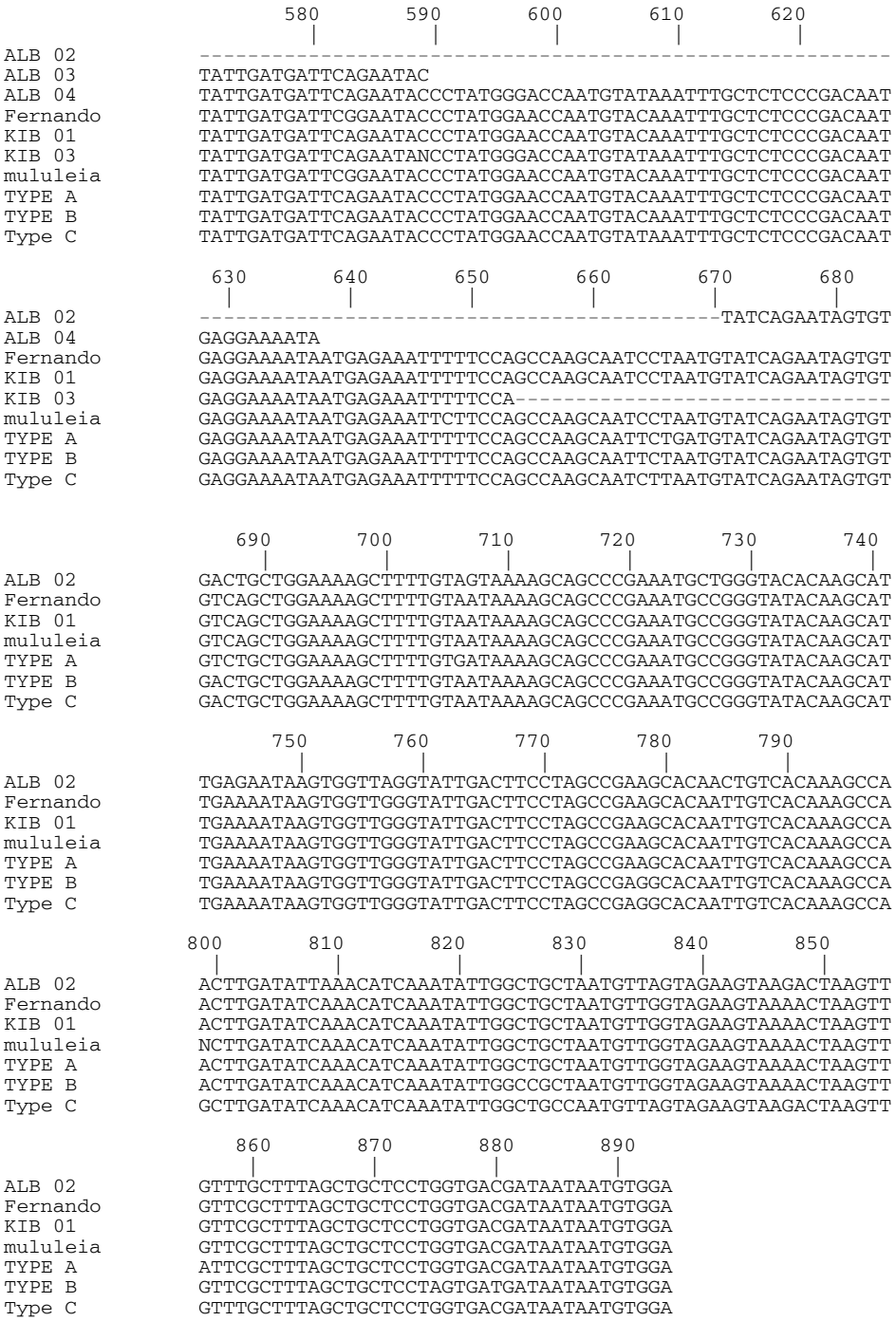

Fig 8. The DNA alignment of some Cassava brown streak virus samples from Tanzania and Mozambique.

The two Mozambique samples, Fernando and Mululeia were very similar, 99+% and can be considered to be the same isolate. The Tanzanian sample Kib 01 was found to contain an isolate of the virus very similar to the Mozambique isolates, 99+% at the DNA level. Together, these three samples were found to be closest in sequence to type A, about 97%, and have tentatively been designated as type D.

Kib 02-2 was found to be identical in sequence to Kib 02-1. They showed closest sequence identity to type D, at 97%, then to type A (94%) and have been designated tentatively as Type E.

Kib 03 contained an isolate that was closest but still distinct (94%) from type C and is tentatively designated as type F.

The sequence of the sample called Alb 02 was very short and no conclusions could be drawn from this.

Alb 03 had an isolate that showed more than 98% identity with type C

Alb 04 had an isolate that was more than 98% similar to Kib 03

Therefore my present isolate groupings are as follows:-

Type A	Type B	TypeC	TypeD?	Type E?	TypeF?
		Alb 03	Fernando	Kib 02-1	Kib 03
			Mululeia	Kib 02-2	Alb 04
			Kib 01		

Time did not allow for symptom differences to be assessed on secondary host plants. The possibility of more than one isolate in a cassava plant is also something that was not assessed.

Implications of sequence variation

CBSD is found in cassava across East Africa. The samples sequenced here come mainly from Tanzania with two from Mozambique and clearly more samples are required from other affected countries. Among the isolates identified there are regions of sequence variation, where designing a PCR primer could result in the PCR test not working with

one or more isolates. These DNA differences are not all silent changes as can be seen with the amino acid alignment (Fig 7b).

Engineering resistance to the virus

Obtaining part of the virus genome, opens the possibility of engineering resistance to the virus. A number of steps are required to achieve this.

1) Building suitable transformation constructs.
2) Transforming a test plant normally susceptible to the virus but easy to transform.
3) Evaluating the constructs by testing the transformed plants for resistance qualities.
4) Transforming cassava with the best constructs.

The pBECKS_6 range of binary vectors were obtained (McCormac *et al*., 1997). These vectors have identical T-DNA regions but are carried on slightly different backbones. pBECKS_6 400 Spec and pBECKS_6 Gen were used, these carry resistance to the antibiotics spectinomycin and gentamycin respectively.

As previously stated a clone called pdT1 had been generated containing 1114bp of the 3' end of the virus genome. The 1114bp of virus genome was cut out of its vector and cloned into the multiple cloning site within the T-DNA of the two pBECKS vectors (with *Bam*H1 and *Sac*1 restriction enzymes). This resulted in identical T-DNA's that contained the npt II gene for kanamycin resistance and the dT1 in the antisense orientation driven by a 35S promoter. These constructs have been used successfully with *Agrobacterium tumefaciens* to generate transgenic SR1 tobacco. Due to the constraints of time it was not possible to carry out further evaluation of the plants.

Dissemination outputs

Research papers

MONGER W.A., SEAL S., ISAAC A.M., FOSTER G.D. Molecular characterisation of cassava brown streak virus (in preparation).

MONGER W.A., SEAL S., FOSTER G.D. A detection method for cassava brown streak virus in symptom-less cassava plants (in preparation).

MONGER W.A., SEAL S., FOSTER G.D. Symptom differences with isolates of cassava brown streak virus (in preparation).

MONGER W.A., SPENCE N., FOSTER G.D. Tomato mild mottle virus, an aphid-transmissible ipomovirus.

Conference papers

W.A. MONGER, S. SEAL, A.M. ISAAC and G.D. FOSTER Molecular characterisation of cassava brown streak virus. Paper presented to the Virology Offered Papers Meeting in York UK on the 8-9 April 1999.

Transferred technology

A RT-PCR test for cassava brown streak virus has been developed and given to Sue Seal at NRI Chatham. This test has been repeated successfully at Chatham.

Graham Thompson the head of Virology at ARC-Roodeplaat the vegetable and ornamental plant institute in Pretoria, South Africa has also received a copy of the test. This was requested due to fears that South Africa may have the virus.

Contribution of outputs

This project has identified the virus that causes cassava brown steak disease. As a result of sequencing part of the virus genome, a RT-PCR test was developed. This test has been used to identify isolates of this virus. The extreme symptom differences with these isolates are presumably the main reason a second virus was mistakenly linked with this disease in the past. The potential uses of this test are:
1) To identify more isolates of the virus.
2) To screen for the virus in plant breeding programs and provide quarantine measures by which unaffected areas and countries can remain free of the disease.

Clearly, to effectively use this test, much more testing on cassava in East Africa is required. There is a need to obtain samples of infected cassava from across East Africa. Further research on isolate differences is required to ensure the test will work for all isolates, and cassava varieties need to be tested against all isolates of the virus if these varieties are going to be promoted under the assumption that they are resistant.

Previous work with this disease, had suggested that the cause was two viruses, a carlavirus of 650-690nm (Lister, 1959, Lennon *et al* ., 1985, Bock, 1994) and a potyvirus to account for the presence of pinwheel inclusion bodies (Lennon *et al.,* 1985). Our work has shown, through sequencing, PCR, partial purification and western analysis that the carlavirus length virus previously isolated is the Ipomovirus sequenced here. The Ipomovirus would also account for the presence of pinwheel inclusion bodies. The differences observed in symptoms of the disease especially on secondary host plants (Bock, 1994), can be accounted for by isolates of this Ipomovirus. We found no evidence for a second virus.

The following key points suggest some of the additional work required to fully exploit these results.

A diagnostic test for cassava

From the work of Lister (1959) it is known that within the cassava plant the virus is at lowest concentration within young leaves. The young leaves of infected cassava do not show symptoms and some cassava varieties are reported not to show leaf symptoms at all (Hillocks *et al*., 1996). A diagnostic test for this virus must be able to detect the virus in the new young symptom-free leaves of the cassava plant to be an effective tool. A reliable and repeatable RT-PCR test has been developed. This test has been used to detect the virus even in the young leaves of cassava that are not yet showing symptoms of the virus. This is in contrast to the SCRI antiserum that is not reliable at detecting the virus in cassava. However, a reliable antiserum would prove very useful in some circumstances as a detection method.

The production of a peptide antiserum was the next logical step in this work and one is being generated. If all goes as planned this antiserum will be available in December 1999 and will require testing.

Isolates of CBSV

This work has found a number of isolates of the virus from just a small area of the affected region. These isolates differ from one another by as much as 10% at the DNA level across their coat proteins. Cassava brown streak disease has been found over a large region of East Africa, the extent to which this virus may differ in sequence could be enormous. If so, the designed PCR test may prove ineffective for other regions with uncharacterised isolates of the virus. Further work is needed to explore the diversity of this virus throughout its range.

Resistant Cassava Varieties

Traditional breeding methods have given rise to cassava varieties that are believed to be more resistant to pests and diseases than other varieties. However, this work with CBSV isolates raises a question about so called resistant varieties. A, B, and C were samples of

CBSD on different varieties of cassava but from the same region of Tanzania. These samples were shown to give quite different symptoms on the secondary host plant *Nicotiana benthamiana* ranging from no symptoms (Type A) to stunting, leaf curling, petiole thinning and death (Type C). If isolates of the virus give rise to different severities of infection; particular cassava varieties may be selected for resistant qualities which are not, in fact reliable. The variety may have only been challenged with a 'weaker' isolate of the virus.

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Appendix 1

RT-PCR test for CBSV (cassava brown streak virus)

1) Extraction of RNA from cassava leaves
2) Generate cDNA from the RNA
3) Perform the PCR test with specific primers to the virus

RNA extraction

A variety of methods have been tried to extract RNA from cassava including kit methods. Most methods resulted in degraded or poor quality RNA. The Qiagen kit was found to be the best kit, the yield of RNA was poor but this did generate cDNA. This required the alternative buffer RLC that is supplied, longer centrifuge times than those suggested were required to filter extract through the columns. The overall best method by far is the CTAB RNA extraction method, Rick Mumford (NRI Chatham, personal communication) adaptated from Lodhi (1994) Plant Molecular Biology Report. 12, 6-13. A copy of the method is attached.

cDNA generation

The successful production of cDNA depends on the quality of the RNA previously extracted. There are a number of commercially produced kits available on the market to generate cDNA and these have always proved to be good, i.e. Stratagene. The virus has a poly(A) tail and oligo dT is used to generate the first-strand cDNA.

The PCR test

Both the MJ research Minicycler™ and the BioRad Gene Cycler™ 24 well machines have been used with this test and both work well. Most of the tests were carried out with the MJ research machine which uses the larger 0.5ml microcentrifuge tubes which are easier to handle. However, this machine only has 16 wells and may not be suitable if large numbers of reactions are intended.

A range of PCR primers were made to the sequence of CBSV. One combination proved most consistent in generating a strong product band. CBSV10 the forward primer (5'-ATC AGA ATA GTG TGA CTG CTG G-3') and CBSV11 the reverse primer (5'-CCA CAT TAT TAT CGT CAC CAG G-3'). Together these generate a 221bp product from within the coding region of the virus coat protein close to the 3' end.

The PCR reaction mix used a Lab buffer made from filter sterilised individual components. The buffer provided with the Taq was also tested and this was successful but the product band was not as strong. The 20µl reaction mix contained 80ng of each primer, 1.8µl of 11x buffer (494.4mM Tris.Cl pH8.8, 123mM $(NH_4)_2$ SO_4, 49.6mM $MgCl_2$, 75mM 2-mercaptoethanol, 50µM EDTA pH8.0, 11.1nM each dNTP, 1.26mg ml^{-1} BSA), 1µl of cDNA, 0.5U Taq polymerase (Advanced Biotechnologies). 20µl of mineral oil was over-laid onto each reaction to prevent evaporation, this was not necessary with the BioRad machines. The typical PCR program was 94°C for 1min, 50°C for 1min and 72°C for 1min; repeated for 30 cycles. Products were separated on a 1% agarose gel containing ethidium bromide and viewed under UV light.

A range of magnesium chloride concentrations have been tried with the buffer that comes with the *Taq* polymerase. The PCR was found to be successful within the range 0.5-3.0mM.

The cDNA was diluted down as far as 1 in 30. A product band was still observed although the product was greatest when the cDNA was undiluted.

CTAB RNA Extraction Method

Richard Mumford's adaptation from Lodhi *et al*., (1994) Plant Molecular Biology Report. 12: 6-13.

1. Grind in liquid nitrogen 0.1-0.3g of plant tissue.
2. Before thawing occurs add 10 volumes of grinding buffer and vortex thoroughly.
3. Take 800µl of the ground sap into a microfuge tube and incubate at 65°C for 10-15 mins.
4. After incubation, add 600µl of chloroform:IAA (24:1) and mix to emulsion by inverting the tube.
5. Centrifuge at 13,000 rpm in a microfuge for 10 min at room temperature.
6. Remove upper aqueous layer and transfer to a fresh tube. Repeat extraction with the chloroform.
7. Remove aqueous layer, taking extra care not to disturb the interphase (about 300µl) and mix with 0.5 volumes of 5M NaCl and 2 volumes of ice-cold ethanol. Mix well and incubate at -20°C for 20-30 min.
8. Centrifuge at 6,500 rpm for 10 min to pellet the nucleic acid. Remove ethanol and re-suspend the pellet in 2M LiCl. Incubate at 4°C overnight
9. Centrifuge for 30 min at 13,000 at 4°C if possible to pellet the RNA.
10. Decant off LiCl and wash pellet by adding 500µl 70% ethanol.
11. Decant off the ethanol and dry the pellet to remove residual ethanol. NB. do not dry completely as the pellet will become difficult to re-suspend.
12. Re-suspend pellet in 50-100µl of DEPC-treated SDW.

From step 7 onwards care must be taken not to contaminate with RNase

Grinding buffer	**100ml**
2% CTAB	2g
2% PVP-40	2g
100mM Tris-HCl, pH8.0	10mls of 1M stock
20mM EDTA	4mls of 0.5M stock
1.4M NaCl	8.1g

Mix and autoclave

Added fresh before use 20mM DTT (200µl of 1M stock to 10ml of buffer)

11x PCR buffer	Total 676µl
2M Tris-Cl (pH 8.8)	165.0µl
1M Ammonium sulphate	85.0µl
1M Magnesium chloride	33.5µl
2-mercaptoethanol	3.6µl
10mM EDTA	3.4µl
Each dNTP (100mM stock)	75.0µl x 4
10mg/ml BSA	85.0µl

This can be filter sterilised if not all components are sterile to begin with and stored in aliquots at -20°C.

N.B. 1.8µl of this buffer is used in a 20µl reaction, and the buffer includes the nucleotides and magnesium.

Appendix 2: Add on funding (to R6765) : Cassava brown streak stick testing

Objectives:

a) Cassava material to be maintained in greenhouse for sending infected and healthy leaves to Bristol University.

b) RT-PCR testing of one hundred cassava sticks from Tanzanian field trials to determine whether atypical symptoms are linked with CBSV, and whether asymptomatic material is virus-free or latently infected. The testing will be done at NRI rather than Bristol University primarily to ensure that the leaves are in a good state when tested. Some of the samples were sent to Bristol to confirm that the two laboratories' results agree.

Results:

Three batches of cassava sticks totalling 71 different samples were received from Tanzania as duplicate sticks and no details were provided to molecular diagnostics staff of the field symptoms by these sticks. The sticks were potted up in the greenhouse after treatment with fungicides and insecticides. The maintenance of these cassava sticks was more labour intensive than envisaged in the budget costings due to severe infestations by mealybugs as well as mites.

b) Of the 71 samples received as duplicate sticks, only 37 grew in the greenhouse. Both sticks of all of these samples were tested by RT-PCR using slightly modified RT-PCR conditions from those used at Bristol. Leaves of 12 of the samples were sent to Bristol University to test by RT-PCR, together with other odd sticks that were being maintained from Mozambique and that were being used in transmission experiments by British Council- funded PhD student W. Mark Thompson.

RT-PCR results between NRI and Bristol were in perfect agreement. Once all results were obtained, they were presented to Dr Rory Hillocks to see how the RT-PCR results compared to field symptoms recorded. For the first set of samples received, results were as expected with the following exceptions:

1. Kit (Kitumbua) and Kig (Kigoma Red) samples were recorded as showing symptoms and hence expected to be positive. Sticks of these samples were the only sticks in this batch to not show CBSV symptoms in the glasshouse at NRI.

2. Dr Hillocks' records suggested that samples of symptomless cultivars Sheria and Kibandameno had also been sent, but these were not received by NRI. This was unfortunate as the results of these samples were of particular interest.

None of the sticks grew from the second set of samples received. Details of the origin of the third set of samples (June 99) have been requested and it is hoped that the Tanzanian collaborators will be able to supply these soon.

Cassava samples from Tanzania

21 Samples (received 2/12/98)	**PCR results: NRI**	**PCR results: Bristol University**
Nan 01	+/+	+
Nan 02	+/+	+
Nan 03	Sticks not viable	Nd
Alb 01	Sticks not viable	Nd
Alb 02	+/+	+
Alb 03	+/+	+
Alb 04	+/+	+
Alb 05	Sticks not viable	Nd
Alb 06	Sticks not viable	Nd
Mre 01	Sticks not viable	Nd
Mre 02	Sticks not viable	Nd
Mre 03	Sticks not viable	Nd
Kib 01	Plantlets died	+
Kib 02	Plantlets died	+/+
Kib 03	Plantlets died	+
Kit 01	Sticks not viable	Nd
Kit 02	-/-	-
Kit 03	-/-	-
Kig 01	Sticks not viable	Nd
Kig 02	-/-	-
Kig 03	-/-	-

24 samples (received 26/2/99)	**All sticks not viable**	**All sticks not viable**

26 samples (received 14/6/99)	**PCR results: NRI**	**Comments**
Tan 01	-/-	
Tan 02	-/-	
Tan 03	-/-	
Tan 04	-/-	
Tan 05	-/-	
Tan 06	-/-	
Tan 07	-/-	
Tan 08	+/+	
Tan 09	-/-	
Tan 10	Sticks not viable	
Tan 11	+/+	
Tan 12	-/-	
Tan 13	+/+	
Tan 14	-/-	
Tan 15	+/+	
Tan 16	+/+	
Tan 17	No leaves available	Severe mealybug infection
Tan 18	-/-	
Tan 19	+/+	
Tan 20	-/-	
Tan 21	?	Inhibitors in RNA extracts
Tan 22	No leaves available	Severe mealybug infection
Tan 23	-/-	
Tan 24	-/-	
Tan 25	-/-	
Tan 26	+/+	