DIAGNOSIS OF COCOA SWOLLEN SHOOT VIRUS DISEASE BY POLYMERASE CHAIN REACTION (PCR).

A THESIS SUBMITTED

BY

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TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF SCIENCE, UNIVERSITY OF GHANA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY (M. PHIL.) DEGREE

DECEMBER 2000

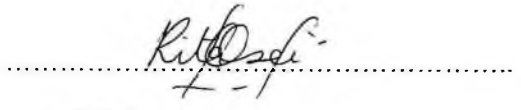
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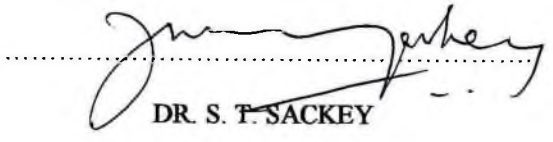
Declaration

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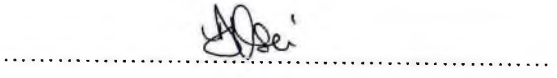
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Dedication

TO MY MUM

MISS AFUA SAFOAA BUSIA

Acknowledgements

My sincere thanks go to my supervisors Dr. Yaa Difie Osei and Dr. Sammy Tawiah Sackey for their guidance, advice and support throughout this project. I would also like to acknowledge with gratitude the assistance given to me by Professors Neil Olszweski and Ben Lockhart of the University of Minnesota.

My gratitude goes to the Ghana Cocoa Growers' Research Association (GCGRA) and Biscuits, Chocolate and Confectionery Companies Alliance (BCCCA), who jointly funded this research through Cocoa Research Institute of Ghana (CRIG).

I am sincerely grateful to the authority of CRIG, especially Dr. G. K. Owusu (Director) for making it possible for me to carry out this research at CRIG. My appreciation goes to Doctors Ollennu, Opoku-Ameyaw, Boye Frimpong, Takrama, Ackonor and Mr. Prempeh for their support. I am also grateful to all the staff of the Physiology/Biochemistry and Pathology Divisions, especially Miss Evelyn Kwame, Messrs Francis Osae-Awuku, Ben Owusu-Antwi, Fiifi Adu-Amankwa and Sammy Affram for their tremendous help.

I would also like to acknowledge all the lecturers, students and staff of the Biochemistry Department, University of Ghana especially, Prof. Gyang and my mates John, Kwamina, and Nii Kpani. I am grateful to Lynn and all working in Lab 659 (Department of Plant Biology, University of Minnesota) for their support. I am also grateful to Doctors M. Wilson, K. M. Bosompem and G Armah, Mr. C. Brown, Mr. Ayim, Michael, Harry and

Anita, and all staff of Noguchi Memorial Institute for Medical Research (NMIMR), Accra, especially members of Parasitolgy Unit Room 133, for their various contributions to this work.

To all my friends, especially Lydia and Kwame (Minnesota), Francis, Kwabena, Ato, Kojo, Peace, Christabel and Evelyn, I am very grateful for your friendship and support.

My utmost gratitude goes to my Mum, my sisters Mercy and Joyce, my brothers Ernest, Charles, Ishmael, Nat and Robert, my special cousin Afua, my nephews Paa Kwesi, Papa, Fiifi, Paa Kow and my niece Menee for their immense love and support.

I will finally thank the Lord God Almighty for His abundant love and grace, and for making things possible for me.

Abstract

Cocoa swollen shoot virus disease causes severe damage to cocoa farms leading to substantial losses in crop yield and therefore the country's revenue from cocoa. This study set out to design new PCR primers for the detection of the virus that causes the disease.

Thirty-six cocoa swollen shoot virus (CSSV) isolates, randomly selected from 5 main groups based on serological and biological properties from the CRIG museum at Tafo were used. Viral DNA extracted and purified from the infected leaves were used for PCR using two sets of universal badna primers 2+T and 3+T. The multiple amplification bands produced were cut out, gel purified and hybridised against full length cloned PCR DNA of CSSV New Juaben probes to detect the amplification products of virus origin. These were then cloned, sequenced and new primers designed based on consensus sequences derived from the alignment of the CSSV sequences with sequences from other closely related badna viruses.

The new pair of primers (badna primers 1+4) gave a single PCR amplification product of 600 base pairs. The thirty-six CSSV isolates from the CRIG museum were screened with the new primers to test the efficacy of these new primers. Out of the 36 isolates screened, 28 gave the expected amplification product and 8 did not give any amplification products. The new primers in comparison with the old primers can be said to be better at detecting CSSV.

Table of Contents

DECLARATION		**II**
DEDICATION		**III**
ACKNOWLEDGEMENTS		**IV**
ABSTRACT		**VI**
TABLE OF CONTENTS		**VII**
LIST OF FIGURES		**X**
LIST OF TABLES		**XI**
CHAPTER ONE		**1**
INTRODUCTION		**1**
1.0	Introduction	1
CHAPTER TWO		**5**
2.0	**Literature Review**	**5**
2.1	Cocoa swollen shoot virus diseasse	5
2.2	Symptoms	5
2.3	Economic impact and control of CSSVD	5
2.4	The virus morphology and genetics	6
2.5	Virus extraction and purification	9
2.6	DNA extraction and purification	10
2.7	Polymerase chain reaction	11
2.7.1	Uses of PCR	15
2.8	DNA Hybridisation analysis	16
2.9	Cloning of PCR amplification products	17
2.9.1	Ligation	17
2.9.2	Transformation, cell growth and screening of recombinants	18
2.9.3	Plasmid harvesting	18
2.9.4	Caesium chloride (CsCl) – ethidium bromide purification	19
2.10	DNA sequencing	19
2.10.1	Sanger's method	20

2.11	Design of PCR primers	22
CHAPTER THREE		**23**
3.0 MATERIALS AND METHODS		**23**
3.1	Materials	23
3.1.1	Chemical and Reagents	23
3.2	Methods	24
3.2.1	Virus isolates	24
3.2.2	Extraction and purification of virus	28
3.2.2.1	Extraction of virus	28
3.2.2.2	Sucrose cushion centrifugation	29
3.2.3	Extraction and purification of virus DNA	29
3.2.4	DNA purification for PCR	30
3.2.4.1	QIAGEN purification	30
3.2.4.2	2-butoxyethanol purification	31
3.2.5	Agarose gel electrophoresis	32
3.2.6	Polymerase chain reaction (PCR)	32
3.2.7	Gel-purification of PCR products and clones	35
3.2.8.	Hybridisation analysis	35
3.2.8.1	Southern DNA transfer	35
3.2.8.2	Dot blots	36
3.2.8.3	Synthesis of DNA probes	36
3.2.8.4	Hybridisation reaction	37
3.2.8.5	Washing and enzyme reaction	37
3.2.8.6	Removal of colour and probe	38
3.2.9	Cloning	39
3.2.9.1	Ligation reaction	39
3.2.9.2	Preparation of competent cells	40
3.2.9.3	Transformation	41
3.2.9.4	Cell culture and screening	41
3.2.10	Isolation of plasmids	42
3.2.11	Restriction digests	42

3.2.12	Large scale plasmid isolation	43
3.2.13	Caesium chloride purification	43
3.2.14	DNA sequencing	44
3.2.14.1	Sequencing gel	44
3.2.14.2	Primer radio-labelling	44
3.2.14.3	Sequencing gel electrophoresis	45
3.2.15	Primer design	46
3.2.16	PCR annealing temperature optimisation and primer testing	46
CHAPTER FOUR		**47**
4.0	**RESULTS**	**47**
4.1	Selection and grouping of isolates	47
4.2	Extraction and purification of virus and virus DNA	47
4.3	Polymerase chain reaction analysis	48
4.4	Gel purification of PCR amplification products	51
4.5	Hybridisation analysis of PCR products	51
4.6	Cloning of PCR DNA	54
4.7	Primer design	57
4.8	Results of annealing temperature optimisation	59
4.9	Testing of primers 1+4	59
4.10	Dot blot hybridisation analysis of viral DNA with 1+4 PCR DNA probes	65
4.11	CSSV sequences from clones from new primers 1+4	68
CHAPTER FIVE		**70**
5.0	**DISCUSSION AND CONCLUSION**	**70**
REFERENCES		**76**

List of Figures

Figure 1 Schematic diagram of the PCR process 12

Figure 2 Map of Ghana showing the regional distribution of the selected isolates 27

Figure 3 The CSSV Genome 34

Figure 4 PCR amplification products with primers 2+T and 3+T 49

Figure 5 Gel-purified PCR amplification bands using primers badna 3+T and 2+T 52

Figure 6 Hybridised membrane of gel-purified PCR products 53

Figure 7 Screening of PCR clones by restriction digestion of plasmids 56

Figure 8 Alignment of conserved region of CSSV sequence with that of the other badna viruses 58

Figure 9 Optimisation of PCR annealing temperature for primers 1+4 using CSSV 1A DNA 60

Figure 10 PCR with DNA from isolates from CRIG museum using primers 1+4 61

Figure 11 CSSV sequences from new primers 69

List of Tables

Table 1 Selection of isolates 25

Table 2: Analysis of CSSV CRIG museum isolates by PCR using universal badna primers 2+T and 3+T. 50

Table 3 Summary of results of experiment to identify virus-coded PCR amplification products. 55

Table 4 Analysis of CSSV CRIG museum isolates by PCR using new badna primers 1+4. 63

Table 5 Dot blot hybridisation analysis of virus DNA from CSSV isolates with probes using 1+4 PCR products 66

CHAPTER ONE

1.0 INTRODUCTION

Cocoa production in Ghana has over the years faced many challenges, but perhaps the most enduring has been cocoa swollen shoot disease. Initially, this was thought to be an agronomic problem, as the disease manifested itself by severe systemic leaf mosaic symptoms. Its name, swollen shoot disease, refers to the swelling of the stem during cancerous proliferation of phloem cells, which process contributes to the apical death of the plant. Swollen shoot disease was first reported in the Eastern region of the then Gold Coast in 1936 (Dale, 1962).

Swollen shoot disease control strategies were based on a zero tolerance philosophy and involved cutting down of infected trees and those in contact with them. Alternative control measures such as the use of barrier crops to restrict the spread of the disease, use of resistant/tolerant cocoa varieties, and cross-protection using serologically closely related mild isolates of the Cocoa Swollen Shoot Virus (CSSV) are still being investigated (Hughes *et al.*, 1995). Also, breeding programmes are pursued to develop resistant breeds, and cultural methods are used to control cocoa swollen shoot disease. For example, the Cocoa Services Division (CSD) in Ghana has a sustained program to advise farmers on methods of limiting the spread of the disease before new farms are cultivated. However, cutting down of infected trees remains the only effective method of controlling the disease in Ghana.

Studies into cocoa swollen shoot disease at the Cocoa Research Institute of Ghana have focused on the biochemical, biophysical, genomic and biological characterization of the disease causal agent. Methods have thus been developed for the extraction and

purification of the virus isolates and these in turn have facilitated the development of a host of diagnostic tools for their detection and identification in plant tissue extracts.

More recently, polymerase chain reaction (PCR) and immunocapture PCR (ICPCR) have been used for the detection of CSSV using three sets of primers (Sackey, 1995; Sackey *et al.*, 1995; Hoffman *et al.*, 1997). The first set of primers, based on nucleotide sequences derived from the severe CSSV 1A, gave single DNA products, but these were specific for CSSV 1A and closely related isolates and therefore could not detect many isolates that fell outside the 1A group (Sackey and Hull, 1991). The other two pairs of primers were designed by Lockhart and Olszewski (1993) based on the nucleotide sequence of three conserved bacilliform DNA virus (badna virus) genomic regions, the $tRNA^{met}$ binding site, reverse transcriptase and ribonuclease H genes. The nucleotide sequences used were those from the badnaviruses (commelina yellow mottle virus (CoYMV), sugar cane bacilliform virus (SCBV), Kalanchoe top spotting virus (KTSV) and rice tungro bacilliform virus (RTBV). These, however, were degenerate and most CSSV isolates gave many amplification products, most of which were found to be artefacts (Sackey *et al.*, 1995).

Breeding programs in producing countries rely on new introductions from the South American origin of the *Theobroma* species, as well as exchanges of improved materials from member countries of the Cocoa Producers' Alliance (CPA). These programs therefore rely on the establishment of quarantine facilities in non-cocoa producing countries in North America and Europe, where material from different parts of the cocoa producing world are assembled and multiplied for distribution. This requires that material received is screened for the major pests and diseases including the debilitating

witches broom disease of South America and cocoa swollen shoot disease of West Africa. Ghana as a major cocoa producing country contributes towards this international effort at conservation of cocoa germplasm. As one of the sources of the disease, Ghana also contributes towards sustaining the program by providing access to diagnostic tools developed for the detection of the various isolates in the country. Even though the tools currently available are adequate for Ghana's needs, they do not entirely address the requirements of the international quarantine programmes which have to screen for isolates from all of the West African sub-region. This initiative therefore aims at developing a new universal diagnostic system for the detection and identification of the badnaviruses (Bacilliform DNA viruses) responsible for the disease in cocoa.

The project was therefore carried out to identify and design new universal PCR primers for the detection of CSSV. The existing primers designed by Lockhart and Olszewski (1993) and previously described do not include sequences from cocoa swollen shoot virus but those from other key badnaviruses, namely BSV, CoYMV, ScBV, KTSV and RTBV. The first three are known in Ghana, as well as *Discorea alata* bacilliform virus (DaBV). Therefore, it was proposed to retain the contribution of BSV, CoYMV, ScBV particularly as these bananas, plantains, and sugarcane are often found in cocoa farms, while commelina is common as a weed. The nucleotide sequences of CSSV and DaBV were to be introduced into the new set of primers.

The general objectives of the study were to:

a. develop nucleotide sequences covering a defined segment of several CSSV isolates
b. use these sequences to design oligonucleotide primers for the rapid detection of bacilliform DNA viruses, and
c. test the specificity and sensitivity of the new primers.

Specific objectives

The specific objectives were:

i. collection of CSSV infected leaves from the Cocoa Research Institute of Ghana (CRIG) museum
ii. extraction and purification of virus isolates from infected leaves
iii. extraction and purification of virus DNA
iv. amplification of DNA by PCR using the universal badna primers 2+T and 3+T.
v. identification of virus coded PCR amplification products by hybridisation of Southern blots
vi. cloning of the virus coded DNA species
vii. sequencing of cloned PCR DNA to develop nucleotide sequence database using representatives of virus groups determined by serological methods and other badna viruses closely related to the CSSV
viii. design of new primers based on nucleotide sequence database
ix. verification of the efficacy of the new primers.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cocoa swollen shoot virus disease

Cocoa Swollen Shoot Virus Disease (CSSVD) was first reported in Ghana in 1936 (Dale, 1962) and in 1938, Posnette (1940) suggested that the disease was caused by several isolates of cocoa swollen shoot virus (CSSV) and transmitted by several species of mealy bugs in a semi- persistent manner.

2.2 Host symptoms

CSSVD is characterised by root and stem swellings, mosaic leaf chlorosis, red vein-banding of flush leaves, rounding of pods containing fewer, smaller beans in some infected trees and in some severe cases, premature death of the plant (Posnette, 1947). Various isolates show a range of combinations of these symptoms. Healthy cocoa trees that become infected may not show any symptoms for a considerable time. This latent period varies according to the type of strain of the virus, the age and condition of the tree. The virulent strains may produce severe symptoms within five months on sensitive Amelanado, but the mild strains may not express themselves for two years or more (Wood and Lass, 1985).

2.3 Economic impact and control of CSSVD

Swollen shoot disease causes severe damage to cocoa farms and therefore substantial losses in crop yield and revenue. The strategies for disease control were based on a zero tolerance philosophy and involved cutting down of infected trees and those in contact with them. At the beginning of the cutting out campaign in 1956, over a million affected and contact trees were removed. By 1982, it was estimated that 185.5 million

trees had already been removed in the Eastern Region alone and there were still 31.2 million trees to be removed (Owusu and Thresh, 1983). According to Owusu and Thresh (1983), only 23% of all infected trees in a new outbreak were identified because many of the infected trees were not noticed or were in the latent phase of infection. It was also realised that new infections were occurring faster than the removal of the infected trees (Legg, 1982). However, cutting down of infected trees still remains the only effective method of controlling the disease in Ghana. Alternative control measures such as the use of barrier crops to restrict the spread of the disease; breeding for resistant/tolerant cocoa varieties and cross-protection using serologically closely related mild isolates are still being investigated (Hughes *et al.*, 1995). There were also cultural methods where the Cocoa Services Division (CSD), Ghana, advised farmers before new farms were cultivated.

2.4 Virus morphology and genetics

Electron microscopy data have shown that cocoa swollen shoot virus is a bullet shaped particle of about 142x 27nm in size (Agrios, 1997). Lot *et al.* (1991) showed that CSSV contained a double stranded DNA genome of molecular weight 7100 base pairs. It was proposed as a member of the Bacilliform DNA (Badna) virus (Lockhart, 1990) on account of its unenveloped bacilliform particles (Brunt *et al.*, 1964) and circular double stranded DNA genome (Lot *et al.*, 1991). Hagen *et al.* (1993) also provided the complete nucleotide sequence of the CSSV Agou 1 strain from the Republic of Togo, which partially defined the badnavirus genome organisation.

Classification of the physico-chemical properties of CSSV has been hampered by a number of factors. These include the absence of a local lesion host for the biological

purification of the components of what may be mixed infections (Paine, 1945; Posnette, 1947; Posnette and Todd, 1955), and efficient methods of purifying the virus. Other difficulties were due to the fact that some of the isolates induced only stem swellings or transient leaf symptoms, and did not provide reliable sources of infected leaf material for virus purification and analysis. Consequently in Ghana, classification of viruses has been based on criteria like the location from which they were first isolated (Posnette, 1947) host range (Tinsley and Wharton, 1958; Legg and Bonney, 1967) and transmission vector (Roivainen, 1976). In some cases, classification was based on the indigenous or alternate host plants like *Adansonia digitata* (AD) and *Cola chlamydantha* (CC) from which they were isolated (Attafuah and Tinsley, 1958; Legg and Bonney, 1967).

Lesemann *et al.* (1980), used immunosorbent electron microscopy (ISEM) to detect the virus particles. This method, however, required expensive inputs. With the development of more effective methods for the purification of the CSSV from infected cocoa leaves by Adomako *et al.* (1983), monoclonal and polyclonal antisera against severe 1A were produced. These have been used for the detection of the virus in infected tissue extracts and to establish relationship between isolates.

Adomako *et al.* (1983) described the use of ISEM for the rapid detection of the CSSV in virus extract. Sagemann *et al.* (1983), used enzyme-linked immunosorbent assay (ELISA) and in 1985 used ELISA and immunoelectron microscopy (IEM) for the detection of the CSSV, where the serological activity of the virus was evaluated. Sackey *et al.* (1990), also raised polyclonal antibodies which differentiated between severe isolates, 1A and Nsaba. These methods were good for the determination of the

serological relationships between CSSV isolates in the laboratory. They could also be used in the field only on trees with the symptoms. The disadvantages here were that, symptomless or transient infections could not be detected and the assay produced high background values when the antisera had high antibody content.

In the virobacterial agglutination (VBA) method used by Hughes and Ollennu (1993), the test successfully detected CSSV in infected sap diluted 1/2560. The test was based on the affinity of protein A to the iron (Fe) region of IgG. The advantages here were that, the method was simple with rapid results and the IgG required was easy to prepare in the laboratory. However, the method was useful only in the detection of CSSV in freshly harvested leaf samples.

Further developments in the diagnosis of CSSV include DNA hybridization and amplification methods. Polymerase chain reaction (PCR) was first used for CSSV diagnosis by Sackey *et al.* (1995) and immunocapture PCR (ICPCR) by Hoffman *et al.* (1997). Two different sets of primers were used in these studies.

Sagemann *et al.* (1985) differentiated the CSSV isolates into five groups, (A-E) on the basis of leaf, stem and root symptoms, as well as serological properties by enzyme linked immunosorbent assay (ELISA) and immunosorbent electron microscopy (ISEM). When these groups were compared with those based on area of first isolation and symptoms expressed, it was found that geographical location had no apparent relationship with the serologically characterised groups Sagemann *et al.* (1985).

2.5 Virus Extraction and Purification

The extraction and concentration of CSSV have been made difficult by the presence of large amounts of tannins and mucilage in the cocoa leaves. Purification is however achieved by the addition of pectinase, polyethylene glycol (PEG), and standard sodium citrate (SSC), alternating high speed centrifugation and low speed centrifugation to remove cellular debris and filtration through celite and sepharose. The high speed centrifugation is carried out to pellet the virus while the low speed centrifugation is carried out to clarify the virus extract (Lot *et al.*, 1991).

More effective extraction, concentration and purification of CSSV from the infected leaves to give higher yields, was facilitated by the addition of the enzyme pectinase to the extraction buffer to degrade mucilage in the cocoa leaf extracts (Adomako *et al.*, 1974). The mucilage and particulate host plant materials were effectively removed by the pectinase and the release of the virus particles from the leaves was enhanced. Polyethylene glycol (PEG 6000) in the extraction buffer enhanced precipitation of the virus particles. Once freed from the mucilage and plant materials, filtration and concentration were possible without losing much of the virus.

Further purification of the extracts was necessary to get rid of more of the host plant and other unwanted materials. Filtration of crude preparations through Celite 545 (Adomako, 1974) was also found to be very beneficial but the yield of virus was compromised by the filtration step.

2.6 DNA Extraction and Purification

The quality of the virus DNA extracted is very crucial for PCR and depends on the source material (tissue), presence or absence of contaminants, method of extraction and purification and the precautions taken to maintain the integrity of the DNA. The plant tissues are known to contain large quantities of proteins, cell wall materials, and phenolic compounds which tend to contaminate the virus extract. The extraction buffer contains ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), Tris-HCl and $MgCl_2$. The EDTA inhibits the activities of naturally occurring DNase, which will degrade the virus DNA once it is released from its protective walls. The SDS is an ionic detergent that dissolves away lipid membranes to free the DNA (Roe *et al.*, 1996). Proteinase K is also added to break down proteins.

Extraction with either Tris-EDTA (TE) saturated phenol and phenol: chloroform solution removes the SDS, proteins and other related substances from the nucleic acid-buffer mixture (Newton and Graham, 1997). Addition of sodium acetate and ethanol or NaCl and isopropanol to the aqueous upper phase precipitate the virus DNA.

To further purify the virus DNA, the enzyme RNase may be used to digest contaminating RNA. Other purification methods like the use of 2- butoxyethanol can be used to remove more polysaccharides and phenolic compounds. There are also spin column chromatography methods that can be used to clean up the DNA sample and to get rid of very small single stranded and double stranded molecules.

2.7 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an *in-vitro* process used for amplifying specific regions of the genome of DNA by a factor of 10^6 (Mullis *et al.*, 1986; Mullis and Faloona, 1987). The amplification is achieved in the presence of suitable primers, deoxynucleoside tri-phosphates (dNTPs), magnesium chloride ($MgCl_2$), the DNA template to be amplified, the enzyme Taq DNA Polymerase and the enzyme buffer.

The double stranded template DNA is first heat denatured to obtain single strands. The primer anneals to the strands and the *Taq* polymerase uses the dNTPs to extend the strand thus, producing another complementary strand (figure 1).

PCR involves the repetitive cycles of heat denaturation of the double stranded DNA to be amplified at a high temperature (92-95°C), annealing of primers to template (complementary) DNA strands at a lower temperature (37-55°C) and extension of the new strands at 72°C. According to Saiki *et al.* (1988), the temperature chosen for annealing of the primer is a compromise. This is because at lower temperatures (37°C), annealing is more efficient but there is a significantly increased amount of mis-priming. At higher temperatures (55°C) however, there is increased amplification specificity but the overall efficiency is decreased.

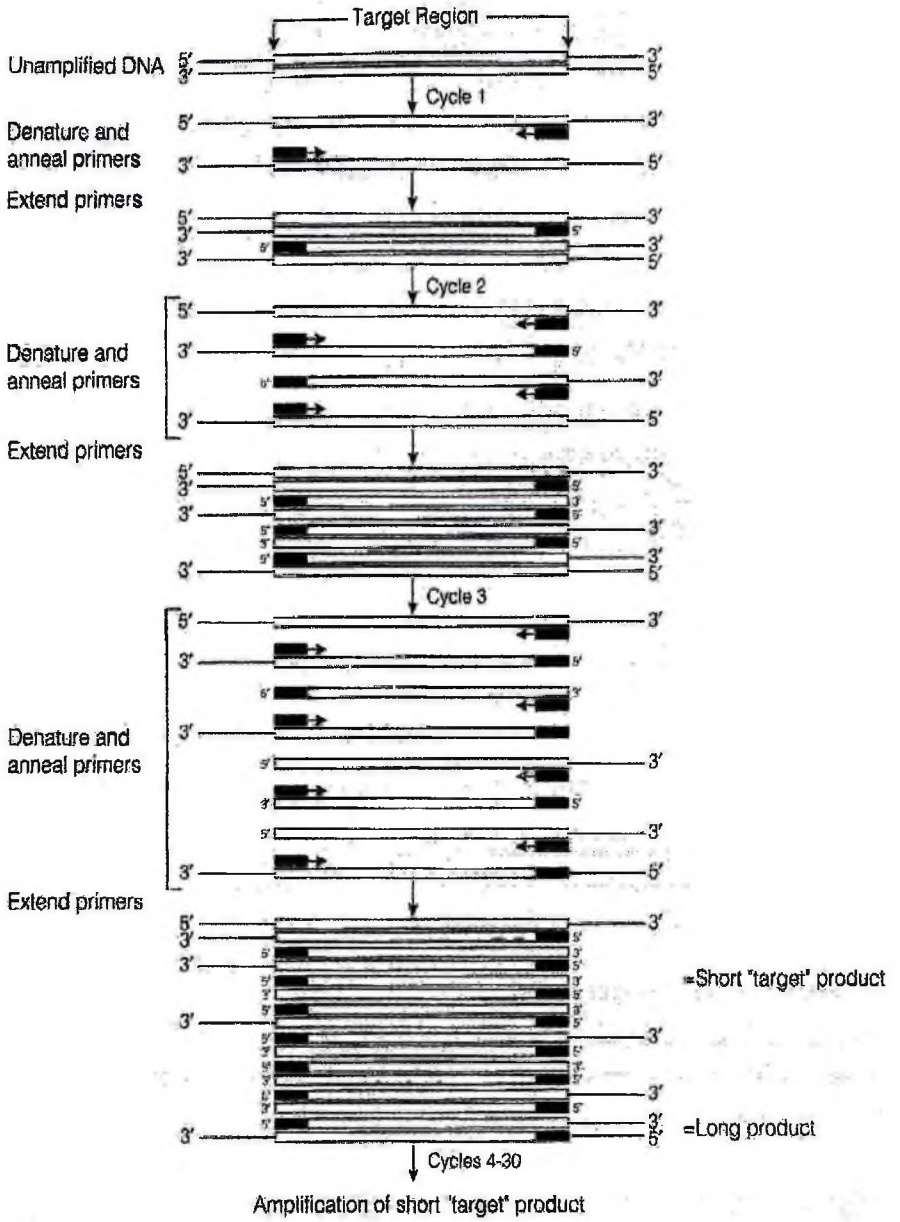


Figure 1. Schematic diagram of the PCR process

Normally, a series of reactions are set up to determine the optimal annealing temperature. Extension of the annealed primers by the *Taq* DNA polymerase uses the dNTPs at an increased temperature (72°C) for 3 minutes. The cycle then restarts. The result is an exponential increase of the DNA with the generation of over a million copies of the DNA of interest in several hours.

The PCR process is directed by sequence-specific oligonucleotide primers of between 20-24 nucleotides in length. The primers are designed with the knowledge of the sequence to be amplified. The primers thus locate and anneal to complementary sequence on the template DNA. The annealing step is an important parameter in optimising the specificity of a PCR.

The starting material, the template DNA may be single stranded or double stranded DNA. The DNA to be amplified should be free of contaminants or non-target materials to avoid their amplification rather than the target DNA. Normally, sub-nanogram quantities of the template DNA are used for PCR.

Taq DNA Polymerase, the enzyme used in PCR is a thermostable polymerase isolated from the thermophilic bacterium *Thermus aquaticus* (*Taq*). Earlier reports on PCR by Saiki *et al.* (1985) and Mullis *et al.* (1987) involved the use of Klenow fragment of *E. coli* polymerase I. This enzyme is heat labile, therefore, fresh enzyme had to be added at the beginning of each cycle of the PCR. The results were the generation of a heterogeneous set of products in addition to the DNA of interest. This is due to the non-specific binding of primers and non-processivity of the enzyme under those conditions. The discovery of *Taq* DNA polymerase therefore solved these problems because the

enzyme was added only once at the beginning of the reaction (Erlich, 1989). Apart from *Taq* DNA polymerase, its cloned and modified version, *Tth* DNA polymerase from *Thermus thermophilus*, *Pfu* DNA polymerase from *Pyrococcus furiosus* and a few others can also be used for PCR (Newton and Graham, 1997).

Taq DNA polymerase has an optimal extension rate (polymerisation rate) of 35-100 nucleotides per second at between 70-80°C (Newton and Graham, 1997). Synthesis of DNA at higher temperatures was advantageous because non-specific binding was greatly reduced (Horn, 1988).

The dNTPs are the building blocks of the DNA. They are made up of the four bases of the DNA (purine, pyrimidine, thymine and adenine) that are used by the Taq DNA polymerase to synthesize the new DNA strand. High purity dNTPs are used, normally at concentrations between 200-400μM. However, *Taq* DNA polymerase has a higher fidelity even at lower dNTP concentrations (10-100μM). An optimal concentration here depends on the concentrations of $MgCl_2$ and the primer, the number of PCR cycles, the length of the amplified product and the stringency of the reaction (Newton and Graham, 1997).

The presence of divalent cations is critical in PCR. According to Chein *et al.* (1976), Mg^{2+} ions form a soluble complex with dNTPs, which is essential for dNTP incorporation. They also stimulate the polymerase activity and increase the T_m of the double stranded DNA and primer/template interaction. The optimal concentration of magnesium ions is quite low (1.0-1.5mM) thus, the template DNA should not contain high concentrations of chelating agents like ethylene diamine tetraacetic acid (EDTA).

Any change in the volumes of any of the components of PCR will affect the concentration of the magnesium ions available. A change in the concentration of the $MgCl_2$ can have a dramatic effect on the specificity and yield of the PCR products. Generally, the insufficiency of the ions will lead to low yields whiles excess will result in the accumulation of non-specific products (Newton and Graham, 1997).

There are several buffers available for PCR. Most common buffers used with Taq DNA polymerase and its modified versions come in a 10x concentration and contain 100mM Tris-HCl, pH 8.3 at room temperature; 500mM KCl; 15mM $MgCl_2$ and 0.1% (w/v) gelatin.

2.7.1 Uses of PCR

PCR has been used in the diagnosis of genetic disorders (Saiki *et al.*, 1985; Saiki *et al.*, 1988), the analysis of allelic sequence variation (Sambrook *et al.*, 1989), and analysis of mutation or any research that involves the rapid cloning and sequencing of homologous DNA fragments (Howe and Ward, 1989). The PCR has also been used for the analysis of individual identity in forensic samples by the amplification of highly polymorphic DNA regions (Paabo *et al.*, 1988; Higuchi *et al.*, 1988), and the examination of nucleotide sequences from ancient preserved specimens (Paabo, 1990). Recently, PCR has been applied to difficult problems in developmental biology. For example, PCR of cDNA has been used to study V-J region combinations in the T-cell receptor α-chain (Loh *et al.*, 1989) and the examination of the mRNAs for growth factors in small numbers of macrophages isolated from wounds actively undergoing healing (Rappolee *et al.*, 1988).

2.8 DNA Hybridisation analysis.

Hybridisation involves the use of a radioactively- or fluorescently-labelled probe made up of a piece of single stranded DNA to detect and bind to its complementary base pairs to form duplexes on a solid phase. A cloned virus DNA probe was used to identify virus coded PCR products. The DNA species to be hybridised are first separated according to size by agarose gel electrophoresis and then transferred onto a solid support/phase (normally a nitrocellulose filter or nylon membrane) through capillary action by the technique known as Southern transfer (Southern, 1975). Before the transfer, the DNA is denatured *in situ* to separate the duplex strand by causing breakage of the hydrogen bonds linking the complementary base pairs (Sambrook *et al.*, 1989). The separation is achieved by either heating to a temperature (Tm) between 60-80°C or by soaking the gel in high salt concentrations. The nucleic acid species are neutralized *in situ* to prevent the separated strands from re-annealing.

The relative positions of the nucleic acids fragments are maintained during the transfer (Sambrook *et al.*, 1989). The DNA fragments are then immobilized onto the membrane by baking in a vacuum oven or cross-linking in an Ultra Violet (UV) cross-linker before the hybridisation can take place (Khandjian, 1986).

After hybridisation, the membrane is washed. A radioactively-labelled probe bound to its complementary sequence can be detected by autoradiography. The non-radioactively labelled probe can be detected by enzyme-linked immunoassay using antibody conjugate. A subsequent colour reaction is initiated at basic pH by the addition of a phosphate and a salt. A blue colour precipitate starts to form from within a few minutes up to three days. After the reaction, the membrane is washed in water and the

hybridised fragments can be seen visibly as blue bands. For re-use, the colour of the membrane can be removed with dimethyl formamide (DMF) and the probe removed with NaCl and SDS.

2.9 Cloning of PCR amplification products

Cloning of PCR products allows generation of relatively large amounts of the amplified region to be available whenever needed without having to repeat the reaction. PCR products are used in various other studies, such as for sequencing and hybridisation, thus the need to clone the amplicon (Newton and Graham, 1997).

The cloning process involves the use of ligase to ligate the amplified DNA into a suitable enzyme digested vector to form a recombinant molecule. This is followed by transformation of the recombinant molecule into a suitable bacterial cell line, growth and screening of colonies by isolation of the plasmid DNA, enzyme digest and hybridisation with cloned virus DNA probe.

2.9.1 Ligation

Ligation reaction involves the use of DNA ligase to introduce the amplified DNA template into the genome of a plasmid vector to form the recombinant molecule. The DNA ligase catalyses the reaction, where there is a formation of a 3', 5' phosphodiester bond that links two adjacent nucleotides of the amplified DNA and the plasmid vector.

There is a wide range of plasmid vectors commercially available. They can be acquired already digested or they can be developed in the laboratory for cloning. There are the pUC series, the pGEM series, the pBluescript (Mezei and Storts, 1994) series and a lot

more For PCR cloning, dT overhang vectors can be used due to the dA-overhang in the PCR products. There are also commercially available blunt-ended cloning vectors like pCR-Script™ from Stratagene. To use the blunt-ended vectors for PCR, the products will have to be polished to remove the dA- overhang.

2.9.2 Transformation, cell growth and screening for recombinants.

Transformation involves the introduction of the recombinant DNA molecule into suitable competent bacteria cells for replication and multiplication. This can be achieved either by electroporation method where the competent cells are prepared and stored in glycerol. There is also the heat shock method where the cells are stored in calcium chloride. Here, the transformation mixture is given conditions of consecutive cold, hot and cold to enhance the uptake of the recombinant DNA molecule into the bacteria cells.

The transformed cells are grown on agar plates containing the appropriate antibiotic, 5-bromo-4- chloro-3- indolyl-β- D- galactoside (X-gal) and isopropylthio-β-D-galactoside (IPTG). IPTG induces the amino-terminal fragment of β- galactosidase and is capable of intra-allelic complementation with a defective form of β- galactosidase encoded by the bacterial cells. Bacteria exposed to IPTG thus form blue colonies in the presence of X-gal (Horwitz *et al.*, 1964).

2.9.3 Plasmid harvesting

Plasmids are isolated from single bacterial cell cultures. The plasmids can be harvested either by alkaline lysis, a modification of two methods (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981) or by the heat lysis method adopted from Holmes and

Quigley (1981). In both cases, the cells are exposed to detergents and then lysed by boiling or treatment with alkali to release the recombinant molecule. The boiling method is however not normally recommended because endonuclease A is not completely inactivated and the bacteria release relatively large amounts of carbohydrate in this method (Sambrook *et al.*, 1989).

2.9.4 Caesium chloride (CsCl) - ethidium bromide purification

Equilibrium centrifugation in CsCl - ethidium bromide gradients of the plasmids has for many years been the method for the purification of plasmids, especially for large-scale preparations. According to Cantor and Schimmel (1980), the ethidium bromide causes the double helix of the DNA to unwind and bind to the dye at the rate of 1 ethidium bromide molecule to 2 base pairs. This differential binding of dye gives different buoyant densities to the linear and closed circular DNA molecules in CsCl- ethidium bromide gradients.

2.10 DNA Sequencing

DNA sequencing enables the determination of the base sequence in a nucleic acid. The object of DNA sequencing in this project was to develop a sequence database from which a primer could be designed. There are two main methods, the chemical reactions of Maxam and Gilbert (1977) and the enzymatic reactions of Sanger *et al.* (1977). Both methods produce a series of DNA molecules differing in length by a single nucleotide. These molecules can then be separated by gel electrophoresis (Maxam and Gilbert 1977; Sanger *et al.*, 1977).

Sequencing can be done using both single and double stranded DNA as well as cloned DNA. DNA species subjected to PCR can also be used. There are however, considerable advantages in sequencing double stranded cloned DNA than in single stranded and uncloned DNA (Howe and Ward, 1989). In the earlier years of the introduction of sequencing, single stranded and or circular DNA were mostly used (Gronenborn and Messing, 1978; Sanger *et al.*, 1980; Messing *et al.*, 1981). However, sequencing of plasmid DNA has become a powerful technique being used increasingly as the methods were improved. This is because very long inserts have been found to be unstable in M13 phages but stable in plasmid vectors. Short inserts were sequenced completely and easily (Hong, 1981). Double stranded DNA has also been found to be amenable to directed deletion sequencing methods, which allowed easy and accurate screening for size and deletions.

2.10.1 Sanger's method

Sanger's method, also known as the chain termination method, was evolved from the positive/negative (+/-) sequencing technique by Sanger and Coulson in 1975. This technique first described the use of a specific primer for extension by DNA polymerase base specific chain termination and the use of polyacrylamide gel electrophoresis (PAGE) to separate single stranded DNA differing by a single nucleotide. The technique was however inaccurate and clumsy hence, the introduction of the chain terminating dideoxynucleoside triphosphates (ddNTPs) method (Sanger *et al.*, 1977).

The ddNTPs involved in this method lack their 3'-hydroxyl group on the deoxyribose and thus prevent the formation of phosphodiester bond with the subsequent dNTP.

These ddNTPs, which are randomly selected, when present in the reaction mixture get incorporated into the growing chain and terminate the chain.

The reaction products are a mixture of oligonucleotide chains and their lengths are determined by the distance between the terminus of the primer used and the point of premature termination (Sambrook *et al.*, 1989). Oligonucleotide populations are generated from small amounts of four different ddNTPs (ddATP, ddCTP, ddGTP and ddTTP) in four separate enzymatic reactions. The sequence reaction mixture includes the template DNA, labelled primer, the ddNTPs, dNTPs, sequencing buffer, DNA polymerase and sterile distilled water. The primer anneals to its complementary sequence on the denatured template DNA strands. The DNA polymerase then extends the chain from the 3' end of the primer using dNTPs as precursors (Bankier and Barrell, 1983).

Universal primers are normally used, where they anneal to vector sequences that flank the target DNA. The primer is first either radio-labelled or fluorescently labelled. The radio-labelling can be done using either [γ or α-^{32}P] dATPs as well as [^{35}S] dATPs (Biggin *et al.*, 1983) with the enzyme polynucleotide kinase. The β-particles emitted by the ^{32}P were found to cause problems including the fact that the bands on the autoradiograph were far larger and more diffused than the bands in the DNA gel. Also, the decay of ^{32}P caused hydrolysis of the DNA. These make reading of the sequences, especially from the top part of the autoradiograph particularly difficult resulting in very few nucleotides being read (Sambrook *et al.*, 1989). The ^{35}S dATP is safer to use hence, preferred.

Two types of DNA templates could be used in Sanger's method, either single or denatured double-stranded DNA. The quality of the template DNA and the type of DNA polymerase used are very critical for sequencing. Plasmid minipreparations are found to be always contaminated, thus, they are not recommended for sequencing. DNA purified by CsCl gradient is recommended for sequencing.

Several different enzymes can be used for the chain termination method of sequencing. These include the Klenow fragment of *E. coli* DNA polymerase I (Sanger *et al.*, 1977), reverse transcriptase (Mierendorf and Pfeffer, 1987), modified bacteriophage T7 DNA polymerases (Tabor and Richardson, 1987) and *Taq* DNA polymerase.

2.11 Design of PCR primers

A prime consideration when designing a primer is that it should be complex enough to reduce the likelihood of annealing to sequences other than the chosen target (Saiki, 1989). The length of the probe depends on the A+T content. The annealing temperature (Tm) for a PCR depends directly on the length and G+C composition of the primers. The higher these parameters are the higher the Tm and therefore the stringency. The possibility of 3' overlaps in primer design should be avoided to reduce the incidence of primer dimer formation. Stretches of polypurines and/ or polypyrimidines should also be avoided.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical and Reagents

Disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen orthophosphate (NaH_2PO_4), bacto-tryptone, yeast extract, agar granules, chloroform, isopropanol and dimethylformamide (DMF) were obtained from Fisher Biotech (Pittsburg, USA).

Sodium chloride (NaCl), polyethylene glycol (PEG), sodium citrate, sucrose, Tris base, sodium dodecyl sulphate (SDS), ethylene diamine tetraacetic acid (EDTA), agarose powder, sodium acetate (NaOAc), maleic acid, lauroyl sarcosine, ampicillin, glycerol, sodium hydroxide (NaOH), phenol and 2-butoxyethanol were obtained from Sigma Chemical Company (St. Louis, Missouri, USA).

Blocking powder, 5-bromo-4-chloro-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Boehringer Mannheim™ (Mannheim, Germany).

Sodium diethyldithiocarbamate (DIECA), magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), glucose, β-mercaptoethanol, glacial acetic acid and hydrochloric acid were obtained from BDH Limited, UK.

Ethanol was obtained from Hayman Limited (UK).

Mineral (paraffin) oil was obtained from Fluka Company (Sigma-Aldrich), St. Louis, Missouri, USA.

The restriction and modifying enzymes used were obtained from various manufacturers and suppliers including Promega Corporation (Madison, USA), Stratagene™ (Austin, Texas, USA) and Boehringer Mannheim™.

3.2 METHODS

3.2.1 Virus Isolates

Thirty-six (36) CSSV isolates from the CRIG virus museum were randomly selected from each of the 5 groups (A, B. C, D and E) in the report by Sagemann *et al.* (1985) and Hughes and Ollennu (1993) (Table 1). The main group A had 4 subgroups. The selection was such that all the cocoa growing regions in Ghana were represented (Figure 2). The geographical distribution of the isolates thus indicates the various locations of the isolates in the cocoa growing regions in Ghana since the isolates were named according to the locations from which they were first isolated.

Table 1: Selection of Isolates (Modified after Sagemann *et al.* (1985) and Hughes and Ollennu (1993).

Isolate name and number	Geographic location (Region)	Type of leave symptoms	ELISA reactions	ISEM	Decoration at antiserum dilution 1:1600
Group A					
Group A_1: Mild Strains					
1. SS 167	Eastern	vm, t	+	++	+
2. SS 365B	Eastern	vm, t	++	++	+
3. Worawora	Volta	vm, t	++++		+
4. Koben	Ashanti	m, t	+	++	+
Group B: Mild Strains					
5. Bisa	Eastern	vm, t	+	+	
6.Onyimso-Agogo	Ashanti	s, t	+++	++	-
7. Donkokrom	Brong	vm, t	+		-
Group C: Severe Strains					
8. AD 14	Eastern	s, t, mo	+++		+(-)
9. AD 75	Eastern	s, mo	+++		+
10. Mampong	Eastern	s, mo	++++		+(-)
Group D: Severe Strains					
11. AD 191	Eastern	s, mo	+	+	-
12. AD 7	Eastern	s, mo	+	+	-
13. AD 135	Eastern	s, mo	++	+	
14. Kpeve	Volta	s, mo	+	+	-
Group E: Mild Strains					
15. Peki	Volta	vm, t	+	-	nt
16. Bobiriso	Ashanti	-	-	-	nt
Group A_2: Severe Strains					
17. Bechem	Brong	s, t	++++		+
18. Anibil	Western	s, t	++++		+
19. Nkawkaw	Eastern	s, t	++++		+
20. Nkrankwanta	Brong	s, t	++++		+
21. Techimantia	Brong	s, t	++++		+
22. Amafie	Western	s, t	++++		+
Group A_3: Severe Strains					
23.New Juabeng-1A	Eastern	s, t	++++		+
24. Kofi Pare – 1A	Eastern	s, t	++++		nt
25. Tafo Yellows	Eastern	s, t	++++		nt
26. Agyapomaa	Eastern	s, t	++++		nt
27.Bosomtwe	Ashanti	s, t	++++		nt
28. Bosomtwe	Ashanti	s, t	++++		nt
Group A_4: Severe Strains					
29. Onyimso-Agogo	Ashanti	s, t	++++		nt
30. Sankore	Brong	s, t	++++		nt
31. Miaso	Ashanti	s, t	++++		nt
32. Nsaba	Central	s. t	++++		nt
33. Okerikrom	Brong	s. t	++++		nt
34. Diinii	Volta	s. t	+++		nt
35.Kwakoko-Juansa	Ashanti	s. t	++++		nt
36. Enchi	Western	s. t	++++		nt

Key:

s = severe; m = mild; vm = very mild; mo = mottled leaf symptom; – = without leaf symptom; t = symptoms resemble those induced by strain 1A; nt = not tested.

Reactivity in ELISA indicate values obtained after 18 hours substrate incubation:

++++ = A 405nm>1.0; +++ = 0.300 to 1.0; ++ = 0.150 to 0.300; + = <0.150;

– = values not distinguishable from the controls.

Reference: Sagemann *et al.*, 1985 and Hughes and Ollennu, 1993.

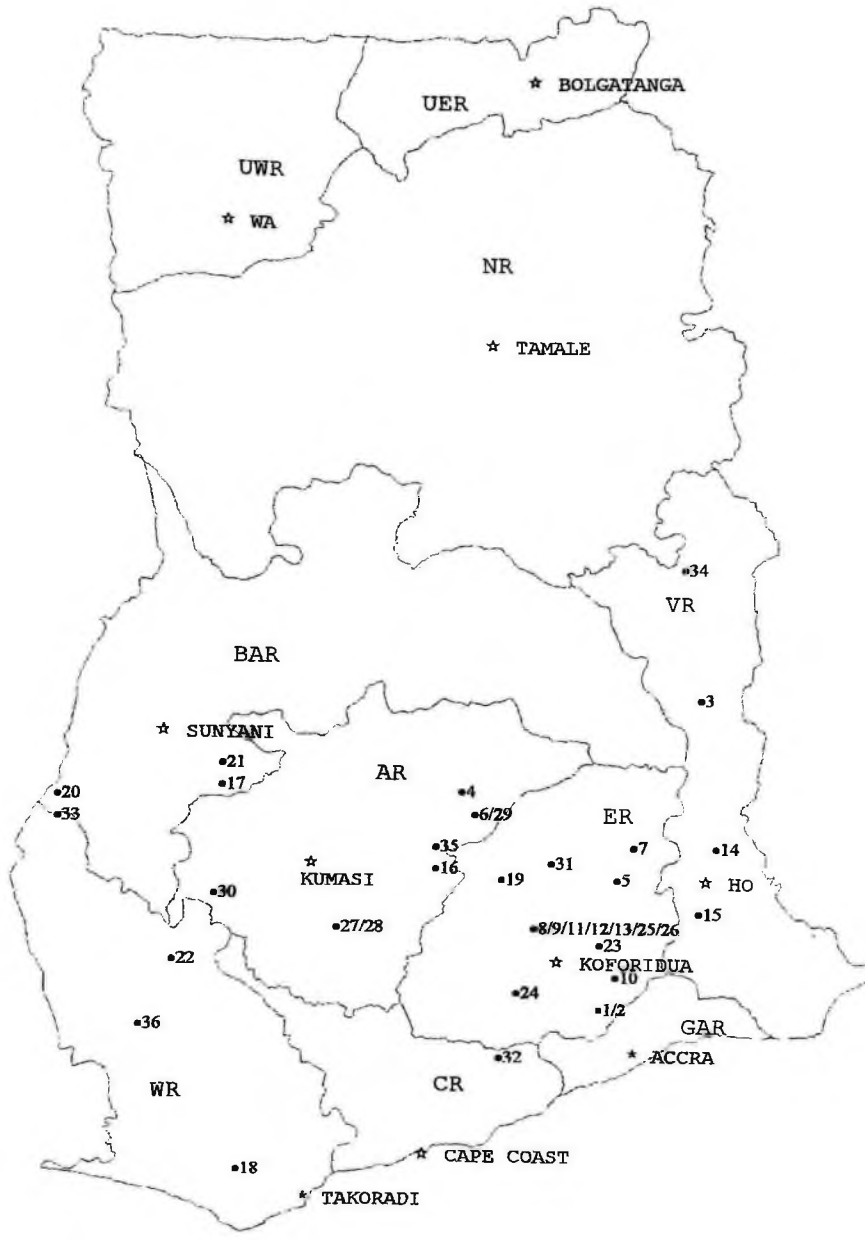


Figure 2. Map of Ghana showing the regional distribution of the selected isolates. (Modified after Thorold, 1975).

Numbers refer to isolate name and number as in Table 1.

3.2.2 Extraction and purification of Virus:

3.2.2.1 Extraction of Virus: -

The virus was isolated and purified from the leaves by the method described by Adomako *et al.*, (1983) and modified by Sackey (1998, unpublished)

Infected cocoa leaves of the selected isolates were harvested from the CRIG museum. Twenty grams each of these were weighed and washed thoroughly in mild detergent and running tap water to get rid of surface micro-organisms. The leaves were homogenised in 200ml of extraction buffer A consisting of 0.5M PO_4^{2-} buffer (0.5M NaH_2PO_4, 0.5M Na_2HPO_4) pH 6.1, 5mM sodium diethyldithiocarbamate (DIECA), 10% polyethylene glycol (PEG) 10,000, 0.2% pectinase, 5mM β-mercaptoethanol). The homogenised leaves were transferred into a beaker and shaken for 3 hours at room temperature (25°C) and sieved through a cotton cloth. The cellular debris was discarded and the extract was then clarified by centrifugation at 15,000g for 20 minutes in a Sorvall centrifuge. Sodium chloride (0.2M) and 10% (w/v) of PEG 6000 were added to the supernatant in small amounts with 20 minutes of stirring and left at room temperature for 30 minutes.

The extract was centrifuged at 25,000g for 20 minutes to pellet the virus. The pellet was then resuspended in 30ml of extraction buffer B consisting of 0.05M PO_4^{2-} buffer, pH 6.8, 5mM ethylene diamine tetraacetic acid (EDTA), 0.2M NaCl and 5mM mercaptoethanol. The extract was clarified by centrifugation at 10,000g for 20 minutes and the supernatant transferred into a fresh tube. The pellet was resuspended in another 30ml of buffer B and re-centrifuged at the same speed and time. The two supernatants of the extract were pooled and clarified by re-centrifuged as before.

3.2.2.2 Sucrose Cushion Centrifugation

A solution of 30% sucrose in extraction buffer B was prepared and 5ml transferred into type 65 centrifuge tubes. The virus extract was then layered onto the sucrose and the virus concentrated by centrifugation at 65,000g for 3 hours at 4°C in a Beckman L2-65 ultra centrifuge. The supernatant was thus discarded and the pellet resuspended in 3ml of buffer B. The extract was then centrifuged again at 4,000g for 10 minutes to clarify. The supernatant was transferred into 3ml centrifuge tubes and centrifuged at 90,000g for 45 minutes at 4°C in a Beckman L2-65 ultra centrifuge to pellet the virus. The final pellet was then resuspended in 1ml of buffer B and aliquoted into eppendorf tubes and stored at –20°C until needed.

3.2.3 Extraction and Purification of Virus DNA

To 500µl aliquot of the virus extract was added Tris-HCl, EDTA, sodium dodecyl sulphate (SDS) and Proteinase K to the final concentrations of 25mM, 10mM, 1% and 5 µg/ml, respectively. This was made up to a final reaction volume of 700µl with sterile distilled water, mixed by vortexing and incubated at 37°C for 30 minutes. The mixture was extracted once with an equal volume of phenol by vortexing for 5 minutes. The buffer phase was separated by centrifugation and transferred into a fresh sterile tube. The extraction was repeated with 1:1 phenol: chloroform. Sodium chloride (5M) to a final concentration of 500mM and an equal volume of buffered cold isopropanol were added to the aqueous phase, vortexed to mix thoroughly and incubated at room temperature for 1 hour.

The mixture was centrifuged to pellet the DNA at top speed of microcentrifuge for 10 minutes. The pellet was then washed with 70% ethanol by adding the ethanol and

centrifuging at top speed of microcentrifuge for 10 minutes. This was then drained and dried *in-vacuo*. The dried pellet was then dissolved in 80µl of TE buffer, pH 8.0. A 1% agarose gel electrophoresis was used to determine the amount and approximate molecular weight of DNA present using 10µl of the DNA extract. The rest of the DNA was frozen for future use.

3.2.4 DNA Purification for PCR

The DNA samples to be used as templates for PCR were further purified by passing them through columns from QIAGEN™ or prepared in microcentrifuge tubes using a modification of the 2-butoxyethanol purification method described by Hagen *et al.*, (1993).

3.2.4.1 QIAGEN Purification

The DNA was purified following the manufacturer's protocol as described below

Two hundred microlitres of ethanol (96–100%) was added to 400ml of DNA sample, and mixed thoroughly by vortexing. The mixture was pipetted into the DNeasy spin column, placed in the 2 ml collection tube provided and centrifuge at 6000g for 1 min. The flow-through and collection tube were discarded. The DNeasy spin column was place in a new 2 ml collection tube, 500 µl Buffer AW1 added, and centrifuged for 1 min at 6000*g*. The flow-through and collection tube were discarded. Next, the DNeasy spin column was placed in another 2 ml collection tube, 500 µl Buffer AW2 added, and centrifuged for 3 min at full speed to dry the DNeasy membrane. The flow-through and collection tube were discarded. Following the centrifugation step, the DNeasy spin column was carefully removed, placed in a clean 1.5 ml microcentrifuge tube, and 200 µl Buffer AE pipetted directly onto the DNeasy membrane. The Dneasy spin column

was incubated at room temperature for 1 min, and then centrifuge for 1 min at 6000*g* to collect the purified DNA solution.

3.2.4.2 2- butoxyethanol purification

In this method, 500µl of the DNA extract were transferred into fresh 1.5ml microcentrifuge tube. If the extract available was less than 500µl, the volume was made up to 500µl with TE buffer. Sodium acetate (3M) was added to a final concentration of 0.3M followed by 200µl of 2-butoxyethanol and mixed thoroughly. The mixture was kept on ice for 1 hour to precipitate the polysaccharides and centrifuged at top speed of microcentrifuge for 10 minutes to form a gelatinous pellet. The supernatant was carefully transferred into fresh tube and the gelatinous pellets discarded. To the supernatant, a further 300µl of 2-butoxyethanol were added, mixed thoroughly and kept on ice for another hour. It was again centrifuged at the same speed and time to pellet the DNA. The pellet was washed in 80% ethanol, drained completely and dried *in-vacuo* for 20 minutes.

The pellet was resuspended in 100µl of TE buffer and sodium chloride (5M) was added to a final concentration of 500mM. An equal volume of cold isopropanol was also added, mixed thoroughly and kept in the freezer for 1 hour to precipitate the DNA. The mixture was then centrifuged at 12,000g for 10 minutes to pellet the DNA. The pellet was washed in 80% ethanol and dried *in-vacuo*. The pellet was re-dissolved in a final volume of 50 to 100µl TE buffer pH 8.0. This was kept in the freezer for gel electrophoresis, PCR and other future use.

3.2.5 Agarose Gel Electrophoresis:

Depending on the number of samples to be run, the appropriate gel tray was chosen. Agarose gels were used at 1% and 0.8% concentration. The appropriate amount of agarose powder was weighed into a beaker and the appropriate volume of 1X TAE (8mM Tris-Acetate, pH 8.3; 0.4mM EDTA) containing ethidium bromide to a final concentration of 0.5 μg/ml was added. The agar was melted by heating in a microwave oven and allowed to cool to approximately 60°C before pouring into the tray with sealed ends and well-forming comb inserted. This was allowed to set and transferred into the gel tank containing 1X TAE after taking out the comb and the seals. Between 5-10μl of the DNA samples with appropriate amount of running dye (consisting of glycerol, TE and bromophenol blue (BPB) were then loaded and run at 65 volts. The first well was always filled with DNA kilobase marker (*Hind* III lambda marker) to help determine the approximate molecular weight of the DNAs. The gel stained with ethidium bromide was visualized under ultra violet (UV) light. A photograph of the gel was then taken with a Polaroid camera or a gel documentation system.

3.2.6 Polymerase Chain Reaction (PCR):

Two pairs of primers namely Universal Badna Primers 2+T and 3+T derived from the nucleotide sequences of three (3) conserved regions of some badna viruses were used for the amplification of the DNA. The primers had sequences as follows:

Badna T: 5'-CACCCCCGGG(AC)(CT)(AC)(AT)(AGCT)GCTCTGATACCA-3'

Badna 2: 5'-AAATGCGGCCGCTA(CT)AT(ACT)GA(CT)AT(ACT)(CT)T-3'

Badna 3: 5'-AATAGCCGCAT(ACT)AT(ACT)AT(ACT)GA(AG)AC(AGCT)GA-3'

The primers Badna 2+T were expected to give amplification bands of molecular weights 1700 and 500 base pairs, while the Badna 3+T were expected to give a single

1000 base pairs amplification band (Lockhart and Olszewski, 1983; Sackey, 1995) (Figure 3).

The amplification was carried out using a PHC-3 programmable dry block thermal cycler from Techne™ The reaction mix of 50 µl contained 1-10ng/µl of QIA or 2-butoxyethanol purified template DNA, 1X PCR buffer supplied by the manufacturer (Promega, USA), 200µM of each of the 4 deoxyribonucleoside triphosphates (dNTPs), 1.5 mM of $MgCl_2$; 2.0µM of each oligonucleotide primer (2 + T or 3 + T), 2.5 units of *Taq* polymerase enzyme (Promega, USA). Sterile double distilled water (sddw) was used to make the volume up to 50µl. The reaction mix was spun down briefly and overlaid with 50 µl of mineral oil to avoid evaporation and refluxing during thermocycling.

Amplification conditions were three cycles of 94°C for 30 seconds (template denaturation), 37°C for 30 seconds for primer to anneal and 72°C for 180 seconds for primer extension. This was followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 180 seconds for 35 cycles.

After the amplification reaction, the paraffin oil was removed by extraction with chloroform. The reaction product was analysed by using 10µl aliquot of it in a 1% agarose gel electrophoresis and viewed under UV light. A photograph was then taken if required.

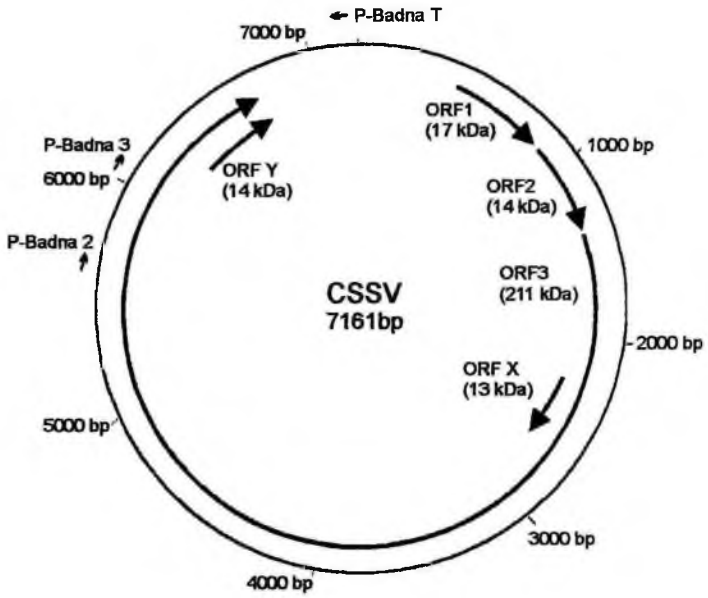


Figure 3 CSSV genome showing the location of universal Badna primers 2,3 and T

3.2.7 Gel purification of PCR products and clones:

When required, the remainder of the PCR product obtained from section 3.2.6, was applied to a 0.8% preparative agarose gel electrophoresis, and the various DNA bands of interest cut out into separate fresh tubes and 100µl of TE buffer pH 8.0 added. The tubes were centrifuged briefly and the contents frozen.

The PCR product bands were gel purified by centrifuging the frozen bands at top speed for 10 minutes. The aqueous phase was transferred into a fresh tube and phenol extracted. The aqueous phase was transferred into fresh tube and precipitated by addition of 5M NaCl to a final concentration of 500mM and cold isopropanol. This was mixed thoroughly, kept in the freezer for 2 hours and centrifuged at top speed of microcentrifuge for 10 minutes to pellet the DNA. The pellet was washed in 70% ethanol, drained, dried and the final pellet dissolved in 10µl of TE buffer pH 8.0. Aliquot of 5µl was analysed by gel electrophoresis and photograph taken. The bands in the gel were transferred onto nitrocellulose membrane for hybridisation. The rest of the samples were kept in the freezer for future use.

3.2.8 Hybridisation analysis

3.2.8.1 Southern DNA transfer

For Southern transfer, the gel was trimmed after photography and transferred into a container, 0.5M NaOH/1.5M NaCl buffer added enough to just cover the gel and left on a shaker at room temperature for about 45 minutes to denature the DNA in the gel. The gel was neutralized then by adding 1.5M NaCl/ 1.0M Tris pH 7.5 buffer to the gel and shaken for 30 minutes. The DNA in the gel was transferred onto the nitrocellulose membrane by a modification of the Southern transfer method to give two mirror images

of the gel on two membranes labelled top and bottom. Two sheets of membranes (Hybond-N) and four (4) filter papers were cut to the size of the gel, labelled and soaked in 20X SSC. The gel was placed in between the two nitrocellulose membranes and these were in turn sandwiched by two filter papers on either side of the membranes, followed by a stack of paper towels. A weight was placed on the set-up and left for a minimum of 2 hours. The membranes were removed and the DNA immobilized onto the membrane by cross-linking in an UV Stratalinker™ chamber. The membrane was either used immediately or kept for future use.

3.2.8.2 Dot Blots

The DNA samples were diluted with the manufacturer's (Boehringer Mannheim™) dilution buffer for dot blots and sterile distilled water in the ratio 2.5 to 1.5 respectively and heat denatured at 95°C for 10 minutes and immediately cooled on ice. The samples were then spotted onto ruled and labelled nitrocellulose membrane using up to 1.5µl of DNA per spot depending on the DNA concentration. The membrane was made damp by placing on wet filter paper and the DNA fixed onto the membrane by cross-linking in an UV chamber. The membranes were then kept for future use.

3.2.8.3 Synthesis of DNA hybridisation probes

The probe to be used for hybridisation was prepared from a cloned virus DNA and labelled with non-radioactive digoxigenin (DIG) labelling kit from Boehringer Mannheim™. The hapten DIG was linked through a spacer arm to the corresponding nucleotide and the probe was generated with a Klenow polymerase. The template DNA was first gel purified, denatured by heating for 10 minutes at 95°C and cooled immediately on ice for another 10 minutes. This was used in a 10µl reaction mix

containing 10ng/µl of the template DNA, 0.1µM of dNTPs, 1µM of hexanucleotide and 0.5µM of Klenow fragment. These were mixed thoroughly and incubated at 37°C for a minimum of 6 hours. The reaction was stopped by addition of 2µl of 0.2M EDTA. The synthesized DNA was precipitated by adding 2.5µl of 4M LiCl and 75µl of pre-chilled absolute ethanol and mixed thoroughly. The mixture was then kept in the freezer at –20°C for two hours before centrifuging to pellet the DNA at top speed of microcentrifuge for 10 minutes. The pellet was washed in 70% ethanol, dried *in-vacuo* and dissolved in 20µl of TE pH 8.0. Prior to use, the probe DNA was denatured by heating in boiling water for 10 minutes and rapidly cooled on ice. Ten microlitres of hybridisation solution were added to the probe before adding to the membrane for hybridisation. For subsequent hybridisations, the probe was heat-denatured and cooled rapidly before using for every hybridisation.

3.2.8.4 Hybridization reaction

Hybridisation was carried out according to the manufacturer's (Boehringer Mannheim™) protocol. The membrane was transferred into a hybridisation bottle and 10-15 ml of hybridisation solution were added to the content of the bottle and incubated in the Hybaid™ mini oven at 60°C for 1-2 hours. The hybridisation solution was poured off and the denatured, appropriately diluted probe added. The bottle was again incubated in the oven for not less than 6 hours (normally overnight).

3.2.8.5 Washing and enzyme reaction

Hybrid detection was done by the protocol provided by the manufacturer of the labelling and detection kit. After hybridization, the probe was poured from the hybridization tube into an eppendorf tube and stored in the freezer at -40°C. The

membrane was washed twice for 5 minutes each with hybridisation wash buffer 1 (2X SSC, 0.1% SDS) at room temperature. The membrane was washed further with wash buffer 2 (0.2X SSC, 0.1% SDS) twice at 60°C for 15 minutes each. The membrane was then transferred into a container and 1X maleic buffer (0.1M maleic acid, 0.14M NaCl pH 7.5) added to wash the membrane in preparation for the enzyme reaction.

This was washed on a shaker for about 3-5 minutes after which the buffer was decanted. Anti-DIG alkaline phosphatase conjugate was diluted (4µl in 10) with 1X maleic buffer and added to the membrane. This buffer was decanted and the membrane washed twice with 1X maleic buffer, first rapidly and then slowly for about 20 minutes. The maleic buffer was decanted and digoxigenin substrate buffer (0.1M Tris, 0.1M NaCl, 50mM $MgCl_2$, pH 9.5) added to wash the membrane. This was also decanted and 10ml of the same digoxigenin buffer containing 45µl of nitroblue tetrazolium salt (NTB) in dimethyl formamide (DMF) and 35µl of 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) in DMF was added to the membrane to initiate the colour formation. The container was then covered and kept in a dark place for 3 or more hours. The membrane was washed, dried, analysed and photographed.

3.2.8.6 Removal of colour and probe

About 150-200ml of DMF was poured into a 2,000ml conical flask and the hybridised membrane added. The membrane in the DMF was heated to about 60°C with periodic shaking till the colour of the precipitate just disappeared. The membrane was removed from the DMF into a bowl containing 100-200ml of 0.2M NaOH and 0.1% SDS to remove the probe. This was then incubated for 30 minutes at 37°C. After this, the membrane was removed, rinsed with 2X SSC and dried for re-use.

3.2.9 Cloning

3.2.9.1 Ligation Reaction

Three types of vectors were used for this reaction. Two (pGEM-T and pCR-Script) were commercially acquired from Promega and Strategene™ respectively and the third one (KS^+dt), was produced in the laboratory with a 3'dt overhang. The KS^+dt was used for only three of the isolates (Worawora, Donkokrom, and Kofi Pare). pGEM-T was acquired with a dT overhang and was used for almost all the other DNA samples. pCR-Script was commercially acquired from Strategene without an overhang and was used for 2 isolates selected from each of the 8 groups. In this case, the PCR products used were first purified by column chromatography and then polished to remove the dA overhang before being used for the ligation reaction.

For the pGEM-T and pCR-Script vectors, the manufacturers' protocols were used. For the KS^+dt vector, a protocol used at the CRIG Molecular Biology Laboratory was used. Each ligation reaction mix consisted of 50-150ng of PCR amplicon, 0.5-1unit of vector, 1X of ligase buffer, and 1-5units of T4 DNA ligase. For the pCR-Script, 1mM of rATP and 1unit of the enzyme *Srf* I were added. Each reaction mix was then made up to 10µl with sterile distilled water, vortexed and centrifuged briefly.

The KS^+dt reactions were kept in a water bath at 14°C overnight, the pGEM-T reactions were kept in the fridge at 4°C overnight or room temperature for 1 hour and the pCR-Script reactions were kept at room temperature for 1 hour. After the ligation reaction with the KS^+dt and the pGEM-T vectors, one tenth (1/10) of the ligation reaction volume of 5M NaCl was added to the ligation reaction mix to a final concentration of 500mM. An equal ligation reaction volume of cold isopropanol was also added. These

were vortexed to mix, centrifuged briefly and kept in the freezer for 2-3 hours to precipitate the DNA. The mixture was then centrifuged at top speed of microcentrifuge for 10 minutes to pellet the DNA. The supernatant was discarded and the pellet washed in 70% ethanol. The pellet was then dried *in-vacuo* and dissolved in 10mM TE buffer (pH 6.8). This was used for transformation or kept in the fridge to be used later. The pCR-Script reactions after the incubation at room temperature were heat denatured at 65°C, cooled rapidly on ice and used for transformation or kept in the fridge for future use.

3.2.9.2 Preparation of competent cells

The cell lines used were XL1 blue and JM83. A sterile tooth pick or pipette tip was dipped into a frozen cell culture in glycerol and transferred into a culture tube containing 10ml of sterile liquid Luria Bertani (LB) medium (10g Bacto-tryptone; 5g yeast extract; 170mM NaCl). This was incubated in a shaker at 37°C overnight. A fresh LB medium was re-inoculated with the bacteria culture in the ratio 100:1 and again incubated with shaking for $4^1/_2$ to 5 hours. The culture was then chilled briefly on ice, transferred into a 250ml centrifuge tube and centrifuged at 4,000g for 10 minutes. The supernatant was discarded and the pellet resuspended in cold sterile distilled water of equal original culture volume. Centrifugation at the same time and speed was done to re-pellet the cells. The pellet was again resuspended in distilled water but half (0.5x) of the initial volume of cold sterile distilled water used. This was followed by repeated centrifugations and re-suspension of the pellet each time in decreasing volumes (0.2, 0.02 and then 0.003 times the initial volume of cold sterile distilled water used) of cold 10% glycerol The final pellet was resuspended in 500µl of the 10% glycerol and 60µl

which was incubated at 50°C for 1 hour. The digested clones were analysed by a 1% agarose gel electrophoresis and a photograph of the gel was taken.

3.2.12 Large scale Plasmid Isolation

From the results of the mini-plasmid isolation, the two best clones based on the insert, from each ligation reaction were selected. A 2ml aliquot of one of the two selected clones was used to re-inoculate 200ml of LB medium containing ampicillin for large-scale plasmid isolation which was done in the same way as the mini-plasmid isolation except that 200ml of culture was used and the DNA was re-suspended in 8ml of TE buffer. The culture of the other one was used to re-inoculate 5ml LB medium and the plasmid DNA compared with those from the large scale. A 500µl aliquot of both cultures was stored each, separately, in 700µl of glycerol at –35°C for future use.

3.2.13 Caesium Chloride Purification

Solid CsCl was dissolved in the plasmid DNA solution at 1g CsCl/1ml of plasmid DNA suspension. The solution was transferred into a centrifuge tube of the Beckman L2-65 ultra-centrifuge and ethidium bromide added. The tube was balanced with TE and heat-sealed. The tube was turned upside down a few times to mix the contents and to check for any leakage. The sample was centrifuged at 45,000g for 16 hours at 20°C in a Beckman L2-65 ultra centrifuge. First, a hypodermic needle was used to pierce the top of the tube. The upper band, which contained linearized and nicked plasmid DNA was ignored. The lower band, which contained the closed circular plasmid DNA, was carefully taken with another hypodermic needle and transferred into fresh microcentrifuge tubes. The ethidium bromide was removed by extraction with water-saturated butanol. The CsCl was removed by dialysing against very dilute TE buffer.

3.2.10 Isolation of Plasmids

The cultures were taken out of the shaker and 1.4ml transferred into sterile 1.5ml microcentrifuge tubes. These were centrifuged at top speed of microcentrifuge for 2 minutes to pellet the cells. The pellets were thoroughly drained and resuspended in 200µl of solution A (50mM glucose; 25mM Tris-HCl pH 8.0; 25mM EDTA). After incubation at room temperature for 5 minutes in 300µl of solution B (0.2M NaOH; 1% SDS) were added and the tube inverted gently a couple of times to mix and kept at room temperature for 10 minutes. Then 400µl of solution C (cold 3M sodium acetate (NaOAc)) were added, mixed thoroughly and kept on ice for 10 minutes. The samples were centrifuged at top speed of microcentrifuge for 10 minutes to pellet the cell debris. The supernatants were transferred into fresh tubes and 600µl of cold isopropanol added. These were vortexed to mix and left at room temperature for 10 minutes to precipitate the recombinant DNA. The recombinant DNA was centrifuged at top speed of microcentrifuge for 10 minutes to pellet. The pellets were rinsed in 70% ethanol, re-centrifuged and dried *in vacuo* and dissolved in 70-100µl of TE-RNase buffer pH 8.0.

3.2.11 Restriction Digest

The 20µl reaction mix included 10 µg/µl of the recombinant DNA, 1X restriction enzyme buffer, sterile distilled water and 1unit each of the enzymes in the double-enzyme reaction. For the pGEM-T, the enzymes used were *Apa*I and *Pst*I together while 2 units of *Bst*ZI were used for the single enzyme digests. For the KS^{+}dt, the enzymes *Eco*R1 and *Hind*III were used together and for the pCR-Script, *Sac*I and *Kpn*I were used together. Each reaction was mixed thoroughly by vortexing and incubated at 37°C for a minimum of 1 hour for all the enzymes with the exception of the *Bst*ZI,

aliquots were transferred into 500µl microcentrifuge tubes and frozen at –70°C in liquid nitrogen before storage in a freezer at –35°C.

3.2.9.3 Transformation

Transformation was done using an electroporator. The competent cells and the DNA sample of CSSV isolate were mixed thoroughly and transferred into a pre-chilled cuvette. The cuvette with the content was then kept on an aluminium foil on ice for 45 seconds. The cuvette was then wiped dry with a tissue paper, loaded into the transporator, which was set at 0.82KV. At this voltage, the DNA was transformed into the bacterial cells. The transformed cells were then washed out of the cuvette into a 1.5ml tube with the LB medium containing glucose, $MgCl_2$ and $MgSO_4$ to final concentrations of 0.04mM, 0.01mM and 0.01mM respectively. This was kept in the incubator at 37°C for about 20 minutes to initiate cell growth.

3.2.9.4 Cell culture and screening

Sterile LB agar plates containing 100µg/ml isopropyl B-D-thiogalactopyranoside (IPTG), 100µg/ml 5-bromo-4-chloro-B-D-galactopyranoside (X-gal) and 50µg/ml ampicillin were prepared. A sterile bent glass rod was used to spread 100µl of the transformed cells on the LB agar plates. The plates were then labelled, inverted and incubated at 37°C overnight to allow cells to grow.

The plates were removed from the incubator and sterile tooth picks were used to transfer single white colonies (transformed cells) into culture tubes containing 3ml of sterile LB medium with ampicillin to a final concentration of 20 µg/ml. These were incubated at 37°C overnight with vigorous shaking.

The contents of bag were transferred into fresh microcentrifuge tube and the DNA precipitated with 500mM NaCl and cold isopropanol. The mixture was centrifuged at top speed of microcentrifuge for 10 minutes to pellet the DNA, which was later washed in 70% ethanol, drained and dried *in vacuo*. The DNA pellets were then dissolved in TE buffer pH 8.0 and used for sequencing.

3.2.14 DNA Sequencing

3.2.14.1 Sequencing gel

The glass slabs for the gel were thoroughly washed with mild detergent, rinsed and wiped thoroughly. They were cleaned with 95% ethanol and again wiped dry. The smaller slab supposed to be the upper was silanised with 750µl of Sigmacote from Sigma Chemical™ to make it non-sticky. The glass slabs for the gel were prepared and clamped together. The 75ml of 5% Long Ranger gel solution containing 7M urea, 1.2X TBE from 10X Tris –Borate-EDTA (TBE-890mM Tris-base, 890mM boric acid, 20mM EDTA), 5% Long Ranger from 50% Long Ranger from HydroLink™, 10% (w/v) ammonium persulphate (APS), 0.5mM N,N,N',N' tetramethylethylenediamine (TEMED) and distilled water was prepared and filtered. The gel solution was poured into the slabs gently and carefully to avoid formation of air bubbles. Sharktooth combs were inserted into the gel and clamped. The gel was allowed to set at room temperature overnight.

3.2.14.2 Primer Radio-labelling

The pUC/M13 forward primer was end labelled with [γ ^{32}P] ATP. A 10µl labelling reaction mix was prepared containing 80µg of the primer (dried in vacuum to reduce volume), 10pmol of [γ ^{32}P] ATP, 10X T4 polynucleotide kinase buffer to a 1X final

concentration, 4units of T4 polynucleotide kinase and sterile distilled water. The mix was incubated at 37°C for 30 minutes and the kinase later inactivated at 90°C for 2 minutes in a thermal cycler.

Two micro litres each of the four different d/ddNTPs (d/ddATP, d/ddGTP, d/ddCTP and d/ddTTP) were placed into four correspondingly labelled 500µl microcentrifuge tubes. Seventeen micro litres of 6µg of template DNA, 5X sequencing buffer, 1.2µg of the labelled primer, 0.3 unit of sequencing grade *Taq* DNA polymerase and sterile distilled water were prepared. This mixture was briefly vortexed and distributed equally into the tubes containing the d/ddNTPs and mixed thoroughly by pipetting. A drop of mineral oil was layered on, centrifuged briefly and put into a thermal cycler pre-heated at 95°C for 2-3 minutes. Sequencing was carried out for 30 cycles at 95°C for 30 seconds for denaturation and 70°C for 30 seconds for both annealing and extension. Two micro litres of stop solution containing bromophenol blue and xylene cyanol dye was added to stop the reactions and the tubes were then stored at 4°C.

3.2.14.3 Sequencing gel electrophoresis

The electrophoresis system was set up with 0.6X TBE. The system was pre-run at 80watts for 10 minutes. The gel wells were washed to remove excess urea and the sequencing reaction heated at 70°C for 2 minutes to denature the DNA before loading into gel. The gel was run at 65 watts for 2 hours. The glass slabs were taken out, washed and the smaller upper one removed carefully. The sequencing gel was transferred onto Whatman 3mm filter paper cut to the size of the gel. The sequencing gel was then covered with saran wrap avoiding air bubbles and dried at 80°C for 2-3 hours. The sequencing gel was transferred onto a film in dark room and put into an

envelope. A weight was placed on the envelope in a cabinet and left overnight. The X-ray film was processed and the sequence read on an illuminator.

3.2.15 Primer design

The design of the new primers was based on consensus nucleotide sequences from CSSV (severe 1A New Juaben and Nsaba), commelina yellow mottle virus (CoYMV), sugar cane bacilliform virus (SCBV), banana streak virus (BSV), *Dioscorea alata* bacilliform virus (DaBV), and kalanchoe top spotting virus (KTSV). The sequences from rice tungro bacilliform virus (RTBV) were excluded in part because though a badnavirus, its genome size and organization differs significantly from the others.

3.2.16 PCR annealing temperature optimisation and primer testing

The annealing temperature for the new PCR primers (Badna 1A and Badna 4) was optimised using a temperature range between 50°C and 65°C. The PCR was carried out for 35 cycles at 94°C for 30 seconds, 55°C to 60°C for 30 seconds and 72°C for 3 minutes. The primers were optimised using DNA from the New Juaben isolate from source (mechanically inoculated and quarantined) as a standard. After the optimisation, the primers were used to screen all the 36 isolates in Table 3.1 as well as 24 others from the CRIG museum. Uninfected cocoa leaf DNA was used as negative control. Five of the 1+4 PCR amplification products (Kofi Pare-1A, Enchi, SS 167) were randomly selected, cloned and sequenced.

CHAPTER FOUR

4.0 RESULTS

4.1 Selection and grouping of isolates

Thirty-six (36) CSSV isolates randomly selected from 5 main groups based on serological and biological properties are shown in Table 1. The largest group (A) comprised 24 isolates that are closely related to CSSV 1A. Isolates of groups A_1, B and E were mild strains whilst those in the other groups, A_2, A_3, A_4 C and D were severe strains.

Figure 2 shows the geographical distribution of the isolates selected. As illustrated, all the cocoa growing areas (Brong Ahafo, Ashanti, Volta, Western, Eastern and Central regions) in Ghana were represented. Most of the isolates (41.7%) are located in the Eastern Region of Ghana.

4.2 Extraction and purification of virus and virus DNA

The PEG precipitation method used for the extraction and purification of the CSSV from the infected leaves successfully yielded virus for extraction of DNA and development of DNA sequences. In all, DNA was extracted and purified from 36 CSSV isolates from the CRIG museum. Cleaner DNA preparations were obtained by column purification compared to the 2-butoxyethanol method of purification.

4.3 Polymerase chain reaction analysis

Figure 4 shows a typical preparative gel photograph of PCR amplification products obtained from DNA of three CSSV isolates (AD7, Peki and Anibil) using badna primers 2+T and 3+T. Each isolate was analysed in adjacent lanes using the two different sets of primers. The odd numbered lanes (1, 3, 5) show PCR amplification products using primers 2+T while the even numbered lanes (2, 4, 6) show products from primers 3+T.

For each viral DNA, the PCR reaction yielded between 2 to 5 amplification products irrespective of the primer pair used. For the primers 2+T the amplification products ranged between 1,700 and 400 base pairs whiles that for primers 3+T, the range was from 1,000 to 400 base pairs. As illustrated in Figure 4, the 500 and 400 base pair products were common bands appearing in all isolates with the 500 base pair being the most prominent. Table 2 summarizes the results of PCR analysis of all the 36 CSSV isolates using universal badna primers. As shown, the minimum number of amplification bands obtained with badna 2+T primers was one as in CSSV SS365B and the maximum was five as in Kwakoko Juansa. On the other hand, badna 3+T gave a minimum of two amplification bands for isolates from Bisa and Amafie and a maximum of 5 for those from Nkawkaw and Kwakoko Juansa. Generally, most of the CSSV isolates gave 3 amplification bands for both 2+T and 3+T primers (Table 2).

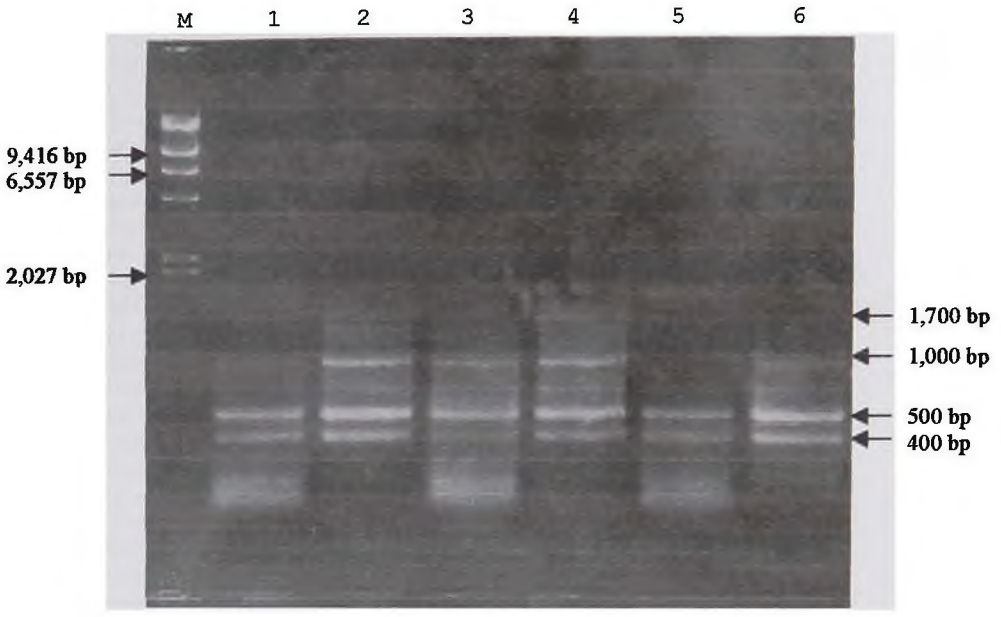


Figure. 4: PCR amplification products with primers 2+T and 3+T

Lane M = λ–*Hind* III molecular weight marker

Lane 1 = AD7 with 2+T;

Lane 2 = AD7 with 3+T;

Lane 3 = Peki with 2+T;

Lane 4 = Peki with 3+T;

Lane 5 = Anibil with 2+T;

Lane 6 = Anibil with 3+T.

Table 2: Analysis of CSSV CRIG museum isolates by PCR using universal badna primers 2+T and 3+T.

		PCR bands using different primers	
Isolate number	**Isolate Name**	**Badna 2+T**	**Badna 3+T**
1	SS167	++++	+++
2	SS365B	+	+++
3	Worawora	++++	+++
4	Koben	+++	+++
5	Bisa	+++	++
6	Onyimso Agogo	+++	+++
7	Donkorkrom	+++	+++
8	AD14	+++	+++
9	AD75	+++	+++
10	Mampong	++++	++++
11	AD191	++	+++
12	AD7	+++	+++
13	AD135	+++	+++
14	Kpeve	++++	+++
15	Peki	+++	+++
16	Bobiriso	+++	+++
17	Bechem	++++	+++
18	Anibil	++++	+++
19	Nkawkaw	++++	+++++
20	Nkrankwanta	++++	+++
21	Techimantia	++++	++++
22	Amafie	+++	++
23	New Juaben	+++	+++
24	Kofi Pare	++++	+++
25	Tafo yellows	+++	+++
26	Agyapomaa	++++	+++
27	Bosomtwe Amakom	+++	+++
28	Bosomtwe	+++	+++
29	Agogo	+++	+++
30	Sankore	+++	+++
31	Miaso	++++	++++
32	Nsaba	+++	+++
33	Okerikrom	++++	+++
34	Djinji	++++	++++
35	Kwakoko Juansa	+++++	+++++
36	Enchi	++++	++++
	Healthy Amelonado DNA	-	-

Key

+ = Number of amplification products obtained;

- = no amplification product

4.4 Gel-purified PCR amplification products

Figure 5 shows a photograph of agarose gel-purified PCR amplification products from 3 CSSV (Mampong, AD7 and Bobiriso) isolates using badna primers 2+T and 3+T. The individual amplification products with molecular weights ranging between 1700 and 400 base pairs were cut out, gel purified and electrophoresed on agarose gel. The bands appeared in ladders as shown in the figure (lanes 2 and 3 and 4 to 6). Bands in lanes 1, and 7 to 10, were too faint for photographic reproduction. The largest product had a molecular weight of 1700 base pairs whilst the smallest band was 400 base pairs.

4.5 Hybridisation analysis of PCR products

Figure 6 shows results of Southern hybridisation analysis of gel-purified PCR amplification products with DNA probe made from full-length infectious clone of severe CSSV 1A (New Juaben) to detect the virus coded DNA species. The molecular weights of the PCR amplification products that were found to be virus coded ranged between 1700 and 400 base pairs. Amplification products in lanes 3, 4 and 6 did not hybridise with the probe whilst the rest all did hybridise.



Figure. 5: Gel-purified PCR amplification products using universal badna primers.

Lane M =. λ–*Hind* III molecular weight marker;

Lanes 1 to 3 = Mampong with 3+T, bands 1-3 * respectively.

Lane 4 = AD 7 with 2+T, band 1

Lanes 5 and 7 = AD 7 with 3+T, bands 1 to3 respectively

Lanes 8 to 10 = Bobiriso with 2+T, bands 1 and 3 respectively

* bands were named 1, 2, 3, etc in decreasing molecular weights.

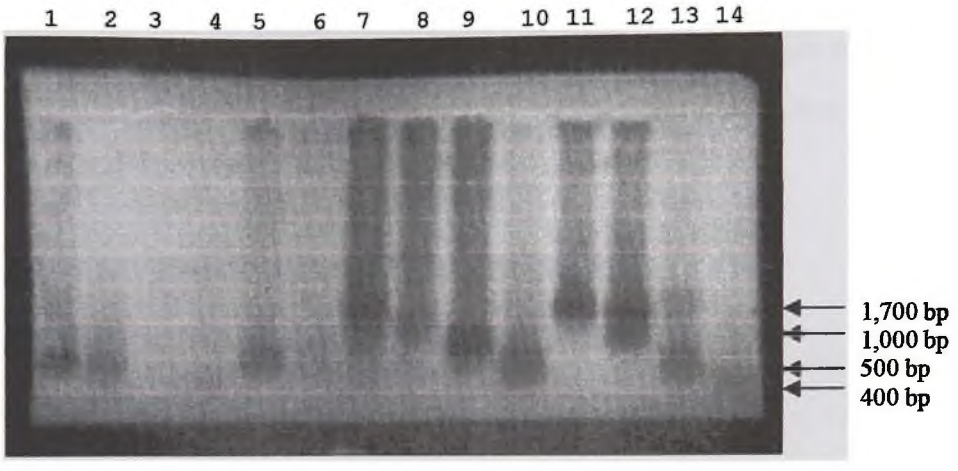


Figure. 6: Hybridised membrane of gel purified PCR products

Lanes 1 and 2 = PCR DNAs from AD 14 with 2+T, bands 1and 2 respectively.

Lanes 3 and 4 = PCR DNAs from AD 14 with 3+T, bands 1 and 2 respectively.

Lane 5 = PCR DNAs from AD 75 with 2+T, band 1.

Lane 6 = PCR DNAs from AD 75 with 3+T, band 1.

Lanes 7 to 10 = PCR DNAs from Mampong with 2+T, bands 1 to 4 respectively.

Lanes 11 to 14 = PCR DNAs from Mampong with 3+T, bands 1 to 4 respectively.

Table 3 summarises results of such hybridisation experiments to identify the virus-coded PCR amplification products. Out of the 240 PCR amplified DNA species from all the 36 isolates, 140 hybridised with the probe (virus coded). One hundred of the PCR amplification products did not hybridise with the probe, indicating that they are not of virus origin.

4.6 Cloning of PCR DNA

Figure 7 shows a sample gel photograph of the *Pvu II* digested 2+T and 3+T PCR clones. The molecular weights of the inserts were compared with that of the PCR amplification products before they were cloned to confirm their correct sizes. The sizes of the inserts ranged between 1700 and 100 base pairs.

Table 3: Summary of results of experiment to identify virus-coded PCR amplification products from 36 isolates

Total number of DNA species from PCR	240
Total number of PCR products analysed by hybridisation	240
Number of virus coded PCR products	140
Number of PCR products tested but were negative	100
Number of virus coded DNA species cloned	68

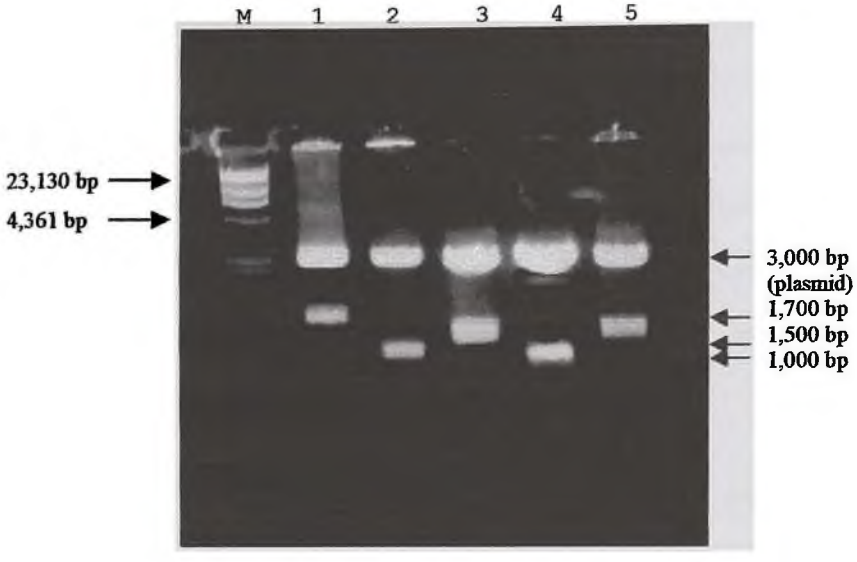


Figure 7: Screening of 2+T and 3+T PCR clones by *Pvu* II digestion.

Lane M = λ–*Hind* III molecular weight marker;

Lane 1 = AD14 clone (2+T);

Lane 2 = AD14 clone (3+T);

Lane 3 = AD135 clone (2+T);

Lane 4 = Bechem clone (3+T);

Lane 5 = Kpeve clone (2+T).

4.7 Primer design

Nucleotide sequences from PCR DNA clones using the primer pairs badna 2+T and 3+T were used along with those from the same region of other badna viruses in the design of new primers. From the several CSSV isolates used, only sequences from CSSV 1A (2T) and CSSV Nsaba (Sackey 2000) were selected for inclusion in the new primers because hybridisation analysis along with serological and host effect had shown that they were representative of the Ghanaian isolates of CSSV (Sackey, 2000). The sequences were compared with those published and deposited in DNA databanks such as the GENEBANK to check if they were CSSV.

A new pair of primers was designed based on consensus sequence from sequences of these two CSSV isolates (1A-Kofi Pare and Nsaba) and some selected closely related badna viruses (commelina yellow mottle virus (CoYMV); banana streak virus (BSV); kalanchoe top spot virus (KTSV); sugar cane bacilliform virus (ScBV); *Dioscorea alata* bacilliform virus (DaBV).

Figure 8 shows the alignment of the sequences of the viruses used in the design of the primers. Two oligonucleotide primers (below) were then designed, and named badna primers 1 and 4. Badna primer 1 has 29 nucleotides as follows

Sequence: 5'- **CTN TAY GAR TGG YTN GTN ATG CCN TTY GG** – 3'

Badna primer 4 has 25 nucleotides as follows Sequence: 5'- **TCC AYT TRC ANA YNA CNC CCC ANC C** – 3'

where **N – A, T, G or C; Y – C or T; R – A or G**

BADNA 1 PRIMER

DaBV	CTC TAC GAA TGG CTT GTA ATG CCG
CSSV	CTT TAT GAA TGG TTA GTG ATG CCC
CoYMV	CTT TAT GAA TGG CTT GTA ATG CCA
BSV	CTG TAT GAG TGG CTA GTT ATG CCC
KTSV	CTC TAC GAA TGG TTA GTA ATG CCA
DaBV	TTC GGA CTG AAG AAT GCT CCT GCC
CSSV	TTT GGG CTC AAA AAC GCT CCT GCA
CoYMV	TTT GGT CTC AAA AAT GCG CCC GCT
BSV	TTT GGA CTT AAA AAT GCT CCT GCC
KTSV	TTC GGT CTT AAA AAT GCC CCT GCT

BADNA 4 PRIMER

CSSV IA	GAA GGA TGG GGG GCC GTA TGT AAG TGG AAA
CoYMV	ACT GGC TGG GGA GCC GTC TGC AAA TGG AAA
BSV	GAA GGC TGG GGA GGG GTA TGT AAG TGG AAA
ScBV	ACT GGA TGG GGA GCA GTA TGC AAG TGG AAG
CSSV NS	GAA GGA TGG GGA GCC ATC TGC AAG TGG AAG
KTSV	GAA GGT TGG GGT GGC ATC TGC AAA TGG AAG
DaBV	GAA GGC TGG GGT GGT GTA TGC AAA TGG AAA

Figure 8: Alignment of conserved region of CSSV sequence with that of the other badna viruses.

CSSV: cocoa swollen shoot virus; CoYMV: commelina yellow mottle virus; BSV: banana streak virus; KTSV: kalanchoe top spot virus; ScBV: sugar cane bacilliform virus; DaBV: *Dioscorea alata* bacilliform virus

4.8 Results of annealing temperature optimisation

Figure 9 shows results of amplification of CSSV 1A DNA under different cycling conditions. Denaturation in all cases was at 94 for 30 seconds, and primer extension at 72°C for 3 minutes. Annealing temperatures were 50, 55, 58, 60, 62 and 65°C. There were no amplification products at temperatures 50°C, 62°C and 65°C whilst amplification products were obtained at 55, 58 and 60°C. At these annealing temperatures the expected PCR product of 600 base pairs was obtained. The PCR product at 58°C was however of lower intensity than the others. The annealing temperature of 60°C was chosen for the screening of isolates with the new primers because it had the highest intensity.

4.9 Testing of primers 1+4

Using the annealing temperature of 60°C, 36 isolates from the CRIG museum were screened in PCR reactions. DNA from 28 isolates yielded 600 base pair amplification products and 8 isolates gave no products.

Figure 10 shows a sample gel photograph of PCR amplification products using the badna primers 1+4. The uninfected Amelonado DNA in lane 1 served as a negative control. The isolate in lane 3 (Onyimso-Agogo) had a 600 base pair band with low intensity as compared to the others which all had relatively high intensities.

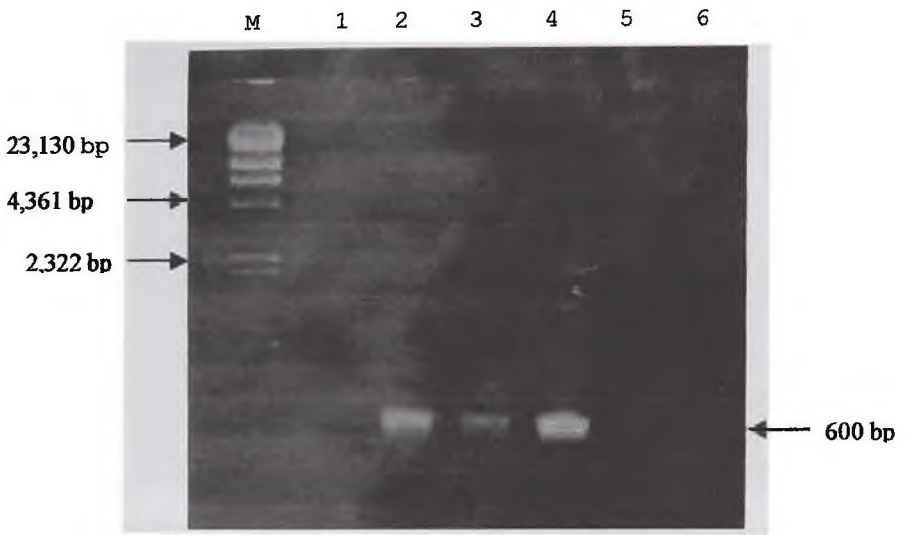


Figure 9: Optimisation of PCR annealing temperature for primers 1+4 using CSSV 1A DNA.

Lane M = λ–*Hind* III molecular weight marker;

Lane 1 = annealing temperature at 50°C;

Lane 2 = annealing temperature at 55°C;

Lane 3 = annealing temperature at 58°C;

Lane 4 = annealing temperature at 60°C;

Lane 5= annealing temperature at 62°C;

Lane 6 = annealing temperature at 65°C.

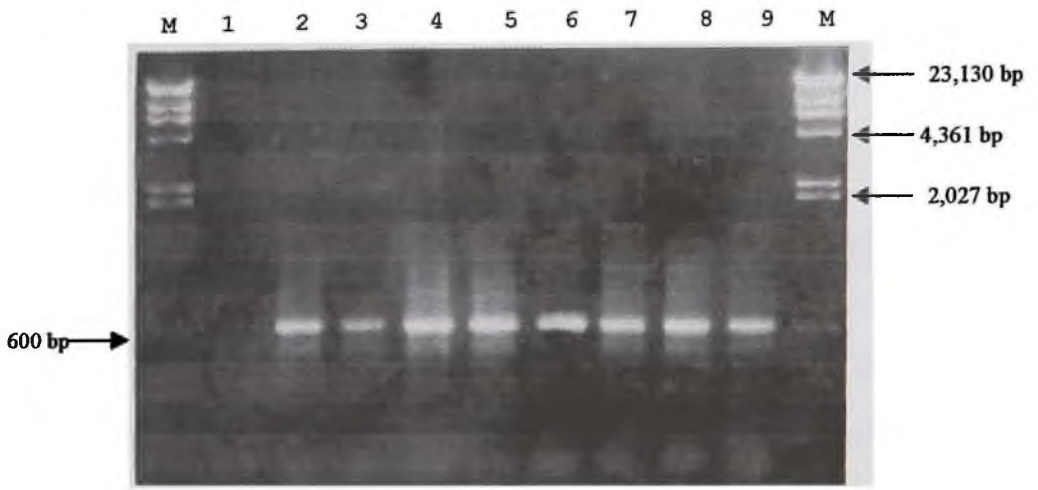


Figure 10: PCR with DNA from isolates from CRIG museum using primers 1+4

Lanes M = λ *Hind* III molecular weight marker;

Lane 1 = uninfected Amelonado DNA;

Lane 2 = Worawora;

Lane 3 = Onyimso- Agogo;

Lane 4 = Donkokrom;

Lane 5 = AD 14;

Lane 6 = Mampong;

Lane 7 = AD 7;

Lane 8 = AD 135;

Lane 9 = Peki.

Table 4 shows the summary of comparison of the PCR results for primers 2+T, 3+T and 1+4 for all the isolates used. PCR with badna primers 2+T and 3+T for all the initial 36 isolates gave 2 to 5 amplification bands with the exception of SS 365B which gave only one amplification band. PCR with the new badna primers 1+4 however, gave only one amplification band for all the 28 isolates that tested positive. Eight of the 36 isolates that tested positive with both primers 2+T and 3+T, for example the AD191 and AD75 isolates, tested negative with primers 1+4. The Enchi isolate was not hybridised even though both the 2+T and the 3+T had 4 amplification bands each. PCR amplification products from primers 2+T and 3+T for AD 191 tested negative in the hybridisation reactions. AD 191 also tested negative with the new primers 1+4.

Table:4: Analysis of CSSV CRIG museum isolates by PCR using new badna primers 1+4.

Isolate number	Isolate Name	PCR bands using different primers		
		Badna 2+T	Badna 3+T	Badna 1+4
1	SS167	++++[a]	+++ (1)[b]	+
2	SS365B	+	+++ (3)	+
3	Worawora	++++ (3)	+++(1)	+
4	Koben	+++(2)	+++(3)	-
5	Bisa	+++	++(1)	-
6	Onyimso Agogo	+++(2)	+++(3)	+
7	Donkorkrom	+++(3)	+++(3)	+
8	AD14	+++(2)	+++(2)	+
9	AD75	+++(1)	+++(1)	-
10	Mampong	++++(4)	++++(4)	+
11	AD191	++	+++	-
12	AD7	+++(1)	+++(2)	+
13	AD135	+++(1)	+++(1)	+
14	Kpeve	++++(2)	+++	+
15	Peki	+++(1)	+++(1)	+
16	Bobiriso	+++(3)	+++(2)	+
17	Bechem	++++(2)	+++(3)	-
18	Anibil	++++(2)	+++(2)	+
19	Nkawkaw	++++(4)	+++++(5)	+
20	Nkrankwanta	++++(1)	+++(1)	+
21	Techimantia	++++(1)	++++(1)	-
22	Amafie	+++	++(1)	+
23	New Juaben	+++(2)	+++(2)	+
24	Kofi Pare	++++(4)	+++(2)	+
25	Tafo yellows	+++(2)	+++(2)	-
26	Agyapomaa	++++(2)	+++(1)	-
27	Bosomtwe Amakom	+++(2)	+++(2)	+
28	Bosomtwe	+++(3)	+++(3)	+
29	Agogo	+++(3)	+++(3)	+
30	Sankore	+++(2)	+++(1)	+
31	Miaso	++++(3)	+++	+
32	Nsaba	+++(2)	+++(2)	+
33	Okerikrom	++++(3)	+++(3)	+
34	Djinji	++++(4)	++++(4)	+
35	Kwakoko Juansa	+++++(5)	+++++(5)	+
36	Enchi	++++(3)	++++(3)	-
	Healthy Amelonado DNA	-ve	-ve	-ve

Key

Expected molecular weights of amplification products

Badna 2T =1700bp; Badna 3T =1000bp; Badna 1+4 =600bp;

+ = Number of amplification products obtained;

- = no amplification product

Numbers in parenthesis are those shown by hybridisation analysis to be virus coded, using cloned DNA from infectious full length clone New Juaben as hybridisation probe.

4.10 Dot blot hybridisation analysis of viral DNA with 1+4 PCR probes

Table 5 shows summary of the results of dot blot hybridisation of the native virus DNA templates of the initial 36 CSSV isolates, using probes made from PCR DNA from the new primers 1+4. The isolates have been grouped on the basis of serology. The SS167 and Nkawkaw probes hybridised very strongly with almost all the DNA of the CSSV isolates used whilst AD14 and AD135 probes hybridized weakly with only a few of the DNA of the isolates used. The Worawora probe also hybridized mildly with some of the isolates whilst the Enchi probe hybridised strongly but with a few of the isolates used. The healthy Amelonado DNA as expected did not hybridise with any of the probes. The Kofi Pare and Bosomtwe Amakom, both of which are of group A_3 isolates hybridised with all the probes. All the isolates hybridised with at least one of the probes.

Table 5: Dot blot hybridisation analysis of virus DNA from CSSV isolates with probes using 1+4 PCR products.

Test DNA	Group	Geographical distribution (region)	Probes					
			SS 167	Wora-wora	AD 14	AD 135	Nkaw-kaw	Enchi
1. SS 167	A_1	Eastern	+	–	–	–	+	–
2. SS 365B	A_1	Eastern	–	–	–	–	+	–
3. Worawora	A_1	Volta	+	+	–	–	+	–
4. Koben	A_1	Ashanti	+	+	–	–	++	–
5. Bisa	B	Eastern	–	–	–	–	+	–
6. Onyimso-Agogo	B	Ashanti	++	–	–	–	+	–
7. Donkokrom	B	Brong Ahafo	++	–	–	–	++	–
8. AD 14	C	Eastern	++	–	+	–	++	–
9. AD 75	C	Eastern	+	+	–	–	+++	+
10. Mampong	C	Eastern	++	–	–	–	++	++
11. AD 191	D	Eastern	++	+	–	–	+++	+
12. AD 7	D	Eastern	++	–	–	–	++	–
13. AD 135	D	Eastern	+	–	–	–	++	–
14. Kpeve	D	Volta	–	–	–	–	++	–
15. Peki	E	Volta	+	–	–	–	+	–
16. Bobiriso	E	Ashanti	+++	–	–	–	+++	–
17. Bechem	A_2	Brong Ahafo	+	–	–	–	++	–
18. Anibil	A_2	Western	++++	+	–	–	+++	–
19. Nkawkaw	A_2	Eastern	–	–	–	–	+	–
20. Nkrankwanta	A_2	Brong Ahafo	+	–	–	–	+++	–
21.Techimantia	A_2	Brong Ahafo	++	+	–	–	++	++
22. Amafie	A_2	Western	++	–	–	+	++	–
23. New Juaben	A_3	Eastern	++++	+	–	–	+++	++
24. Kofi Pare	A_3	Eastern	++++	+	++	+	++	+++
25. Tafo Yellows	A_3	Eastern	++	+	–	–	++++	++
26. Agyapomaa	A_3	Eastern	++	+	–	–	++++	+
27. Bosotwe Amakom	A_3	Ashanti	+++	+	+	+	++	++
28. Bosomtwe	A_3	Ashanti	+++	–	+	+	++	+
29. Onyimso-Agogo	A_4	Ashanti	++++	+	–	–	+++	+
30. Sankore	A_4	Brong Ahafo	+++	+	++	–	+++	+++
31. Miaso	A_4	Ashanti	+++	+	–	+	+++	++
32. Nsaba	A_4	Central	++	–	–	–	++	–
33. Okenkrom	A_4	Western	+++	–	+	–	+++	–
34. Djinji	A_4	Volta	+++	+	+	–	+++	–
35. Kwakoko-Juansa	A_4	Ashanti	+++	+	+	–	+++	++
36. Enchi	A_4	Western	++	–	–	–	++	++
Uninfected Amelonado	–	–	–	–	–	–	–	–

Key: + = weak hybridisation reaction with probe; ++ = strong hybridisation reaction with probe; +++ = very strong hybridisation reaction with probe; ++++ = very, very strong hybridisation reaction with probe; – = no reaction.

4.11 CSSV sequences from clones from new primers 1+4

Figure 11 shows sequences of CSSV isolates Kofi Pare (A) and Enchi (B), derived from clones using the new primers 1+4. Samples A and B had 853 and 597 base pairs, respectively. These sequences were compared with those deposited in DNA databanks and were shown to have significant alignment with the CSSV polyprotein gene. Two out of the 5 that were sequenced were shown to be virus sequences.

A

ccacttacaa	atagcacccc	agcccacacc	aaaaacgttg	aagcgtaaga	catacataag	60
acgacgtaac	tccctctaac	tctctctctc	attctttcca	tttagttatc	tctcaagtga	120
tcctgtgggg	caggcacaat	atacttctgt	atcattactt	cattatattc	accattgctc	180
aaacttagta	aaacagcaac	aaaactagtg	acctaatctc	ttcttcaaaa	actactgacc	240
tagtttattc	ttcattacgt	ggctggggtg	gtatgtgcaa	atggactcaa	aaatgctcct	300
gcagtatttc	aaagaaaaat	ggaccaatgt	ttcaaaggca	cagaagaatt	catagctgtg	360
tatattgatg	acatcttggt	cttcagcgag	actatggcgg	aacacaccaa	gcatattgga	420
atcatgctaa	caatctgcca	agaaaatggg	ctggtcctaa	gcccaaataa	aatatgtctt	480
gctcaacgag	agattgaatt	tttgggcaca	atcatctctc	aaggtcaaat	gaagcttcag	540
cctcatgtca	taaagaagat	agtcaacaag	gcagatatgg	agctcgaaac	aactaggggc	600
ctaagatcat	ttttgggcct	cctgaactat	gcccgaatct	acatacccaa	tctggggaag	660
aagctaagtc	cactatatgc	caaaaccagt	cccaccggag	aaaagaagtt	taatcgacag	720
gattggcatc	tgataaaaga	aattaaagat	atggtccaaa	agctcccaaa	cctcgctatc	780
ccaccagcaa	gatgctgcat	tatcatagaa	agcgatggtt	gtatggaagg	gtggggtgca	840
gtttgcaagt	gga					853

B

ctatacgagt	ggttagtaat	gccgttcggg	ctcaaaaatg	cccctgcagt	atttcaaaga	60
aaaatggacc	aatgtttcaa	aggcacagaa	gaattcatag	ctgtgtatat	tgatgacatc	120
ttggtcttca	gcgagactat	ggcggaacac	accaagcata	ttggaatcat	gctaacaatc	180
tgccaagaaa	atgggctggt	cctaagccca	aacaaaatat	gtcttgctca	acgagagatt	240
gaatttttgg	gcacaatcat	ccctcaaggt	caaatgaagc	ttcagcctca	tgtcataaag	300
aagatagtca	acaaggcaga	aatggagctc	gaaacaacta	agggcctaag	atcatttttg	360
ggcctcctga	actatgcccg	aatctacata	cctaatttgg	ggaagaagct	aagtccacta	420
tatgccaaaa	ccagtcccac	cggagaaaag	aagtttaatc	gacaggattg	gcatctgata	480
aaggagatta	aaaatatggt	ccaaaagctc	ccaaacctcg	ctatcccacc	agcaagatgc	540
tgcattatca	tagaaagtga	tggttgtatg	gaaggctggg	gagccgtttg	caagtgg	597

Figure 11: Sequences of PCR products generated using the new primers badna 1+4.

A: sequence from PCR DNA (badna 1+4) obtained for CSSV 1A (Kofi Pare).

B: sequence from PCR DNA (badna 1+4) obtained for CSSV Enchi.

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

This project set out first, to develop a sequence database from as many viral DNA clones as possible and second, to identify or design new oligonucleotide primers based on a conserved sequence of the viruses for the rapid detection of bacilliform DNA virus infection in cocoa.

To achieve these objectives, 36 virus isolates were collected from the CRIG museum, based on their serological and biological characteristics (Sagemann *et al.*, 1985; Hughes and Ollennu, 1993).

Isolates in groups A_2, A_3 and A_4 had similar leaf symptoms that resembled those induced by the 1A strain. They also had similar ELISA and ISEM reaction results. All the members in groups A_1, C and A_2 had decoration at antiserum dilution 1:1600 results less than 0.150. Isolates in groups B and D did not react whilst groups A_3, A_4 and E were not tested. The similarities could be due to serological resemblance of virus isolates (Table 1). The five main isolate groups covered all the cocoa growing regions in Ghana (Brong Ahafo, Ashanti, Volta, Western, Eastern and Central regions), (Figure 2). Since the primers to be designed had to be able to detect a wide range of the isolates there was need for representation of all the regions in the cocoa growing belt of Ghana. The largest number of the isolates (41.7%) were located in the Eastern Region of Ghana where the highest incidence of the CSSV has been reported.

DNA was extracted and purified by passing them through columns from QIAGEN™ and or the use of 2-butoxyethanol method. The column purification gave cleaner DNA preparations than the 2-butoxyethanol for PCR.

The universal badna primers 2+T and 3+T were based on the nucleotide sequences of three conserved regions of five badna viruses and were expected to give 1700 base pairs for badna 2+T and 1000 base pairs for badna 3+T.

PCR carried out on the virus DNAs extracted from all the 36 isolates gave multiple amplification bands using universal badna primers 2+T and 3+T, with the exception of isolate SS 365B which had only one amplification band with primers 2+T (Figure 4 and Table 2). The multiple amplification bands obtained included the expected 1700 base pair product for the primers 2+T and 1000 base pair product for 3+T (Sackey *et al*, 1995). Seventy percent (70%) of the expected base pairs for 2+T and 83.3% of those for 3+T were found to be virus coded. The multiple amplification bands obtained were due to the degeneracy of the primers used. The single amplification band for SS 365B using primers 2+T was not of virus origin because it did not react with the probe in the hybridisation reaction. A total of 240 amplification products were obtained from the 36 CSSV isolates.

The results of the agarose gel purification of the PCR amplification products indicated that not all the bands were visible on the agarose gel electrophoresis analysis, but hybridisation reactions showed that most of them were actually present. This shows that the hybridisation method used was more sensitive than visualisation of ethidium bromide stained DNA under ultra violet light.

The expected PCR amplification products are 1,700 base pairs for 2+T and 1,000 base pairs for 3+T but the 500 base pair bands were persistent in both the 3+T and especially the 2+T hybridisation reactions. This confirms the findings of Sackey *et al.* (1995), where the presence of the 500 base pair band and also the realisation that not all the multiple amplification bands were of virus origin was first reported. Hybridisation reactions showed that the 500 base pair band was virus coded in some of the isolates. These 500 base pair fragments may be due to priming at non-specific sites and hybridising for the same reason.

Some of the isolates also yielded 1500 and 400 base pair DNA products during PCR. The 400 base pair band also hybridised with the cloned virus DNA probe but may have been a primer multimer. The origin of the 1500 amplification bands, however, is not clear. The number of "virus-coded" PCR DNA species (140) was in excess of the number of expected amplification products from the 36 isolates (i.e 72), due to the fact that some 400, 500 and 1500 base pair products also hybridised with the probe. Eighty eight percent (32/36) of the PCR amplification products for 2+T and 83% (30/36) for the 3+T were found to be virus coded.

Cloning of the virus coded PCR amplification products gave inserts of the expected sizes after the restriction digest (Figure 7). Comparison of the sequencing results with those deposited in DNA databanks showed that the sequences of all the 14/68 clones successfully sequenced were actually retro-transposones. The reason for this occurrence is not yet known. This led to the selection of two isolates representative of the Ghanaian CSSV.

Nucleotide sequences from PCR DNA clones using the primer pairs badna 2+T and 3+T were to be used along with those from the same region of other badna viruses in the design of new primers. From the several CSSV isolates used, sequences from CSSV 1A (2T) and CSSV Nsaba were selected for inclusion in the new primers. This was because hybridisation analysis along with serological and host effect suggested that they were representative of the Ghanaian isolates of CSSV (Sackey, 2000).

The new Badna primer 1 was made up of 29 nucleotides and Badna primer 4 was ``made up of 25 nucleotides. Together, they were expected to yield a 600 base pair amplification product. For amplification of cognate sequences from different strains of the same organism, or for "evolutionary PCR", the chances of getting product was increased by making the primers moderately "degenerate" (Compton, 1990). This was done by having a number of options at a few places in the primer sequences so as to allow annealing to and amplification of a variety of related sequences, hence the N (any of the nucleic acid bases), R (a purine) and Y (a pyrimidine). Primers 1 and 4 thus, had 8 and 7 degeneracies, respectively. Although degeneracies reduce the specificity of primers, meaning greater mismatch opportunities and background noise increases, such primers nonetheless can be used for the successful amplification (Rybicki and Hughes, 1990).

To test the new PCR primers (Badna 1 and Badna 4), the annealing temperature was optimised using a temperature range of between 50°C and 65°C. Amplified PCR DNAs were obtained at 55, 58 and 60°C (Figure 10). At these annealing

temperatures the expected PCR product of 600 base pairs was obtained. Because the PCR product at annealing temperature 58°C produced less DNA than the others, the choice of optimal annealing temperature was thus at either 55 or 60°C. The annealing temperature of 60°C was however chosen for the PCR because reaction at a higher temperature reduces non-specific primer annealing, increasing the amount of specific product produced and reducing the amount of primer-dimer formation (Innis and Gelfand, 1990).

PCR using the badna 1+4 primers gave the expected single 600 base pair amplification band (Figure 10) as compared to the multiple amplification bands obtained using both universal badna primers 2+T and 3+T.

Comparing these new primers (badna 1+4) with the universal badna primers, 2+T and 3+T after screening of the 36 isolates from the CRIG museum (Table 2) showed that the primers 1+4 were more specific than the 2+T and the 3+T. PCR carried out on the virus DNAs extracted from all the 36 isolates gave multiple amplification bands using universal badna primers 2+T and 3+T, with the exception of isolate SS 365B which had only one amplification band with primers 2+T (Table 4). The results of the screening of the 36 isolates with the new primers yielded 28 of the isolates giving the 600 base pair amplification product and 8, with no products. PCR is sensitive to template DNA concentration, and virus concentration from CSSV isolates is often quite low. Low virus DNA concentration could therefore account for the 8 isolates that produced no amplification products. Under these circumstances, increasing the amount of DNA template used in the amplification can result in production of amplification products. Indeed some of the 28 isolates that

gave amplification products initially tested negative but when the amount of DNA was increased the expected amplification products were obtained.

Dot blot hybridisation analysis of the native virus DNA templates of the initial 36 CSSV was done with 6 randomly selected 1+4 (AD14, AD135, Worawora, SS167, Nkawkaw and Enchi) PCR DNA probes. The results showed that the CSSV SS167 and CSSV Nkawkaw probes hybridised very strongly with almost all the DNA of the CSSV isolates used whilst CSSV isolates AD14, Worawora and AD135 probes hybridised mildly with only a few of the DNA of the isolates used. CSSV Enchi probe hybridised moderately with some of the isolates. This observation could be attributed to the integrity of the CSSV isolates SS 167 and Nkawkaw probes being better than that of the other probes. It also might have been due to the sequences of the SS 167 and Nkawkaw probes being more homologous to the sequences of the 36 isolates. The same reason could be attributed to why more of the A_3 and A_4 group isolates showed hybridisation with the other probes. CSSV Kofi Pare and CSSV Bosomtwe Amakom, both of which are of group A_3 isolates, were the only two that hybridised with all the probes. This could also be due to these two isolates having more sequence homology to the probes.

Serological groupings of the virus isolates did not affect the hybridisation pattern because SS 167 probe for instance did not hybridise with all the members of its group. The Worawora hybridised with itself and only one other (Koben) from its group. AD 14 hybridised with only itself in its group whilst AD 135 did not hybridise with itself or with any other member from its group. The Enchi on the other hand hybridised fairly well with 5 of the 8 members of its group.

The primary reason for the need of a routine method for the detection of the CSSV is to use a more specific, relatively cheaper and less labour intensive technique to detect the virus for field, laboratory and quarantine purposes. Swollen shoot disease causes severe damage to cocoa farms and therefore substantial losses in crop yield and revenue in Ghana. Since the strategies for disease control are based on a zero tolerance philosophy and involved cutting down of infected trees and those in contact with them, early detection of all infected trees in a new outbreak including those in the latent phase of infection is important. This has become possible because the new primers designed are better in the detection of CSSV using PCR. They have been found to be more specific with a single amplification product of 600 base pairs as compared to the multiple amplification products from the 2+T and 3+T primers.

The new primers were effective with 28 giving amplification products out of the 36 CRIG museum isolates screened. It is recommended that more of the isolates be screened including the rest of the CRIG museum isolates and samples from the field.

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