Review of Literature
II. REVIEW OF LITERATURE

1. Virus Morphology

Banana bunchy top virus (BBTV) is a single stranded DNA plant virus comprising of small isometric icosahedral virions, 18-20 nm in diameter (Harding et al., 1991), (Plate 3). The viral genome consists of at least six components of circular ssDNA each of about 1 kb (Harding et al., 1993; Burns et al., 1994). Single-stranded DNA viruses of plants encompass the family Geminiviridae and the genus Nanovirus. Geminivirus DNA, one or two circular ssDNA molecules of about 2.7kb to 3.0kb (Lazarowitz et al., 1992), is individually encapsidated in a unique double icosahedral ('twinned') particle, whereas the nanovirus virion is isometric and genome comprises until now an undetermined number (at least six) of circular ssDNAs, all of about 1 kb, (Burns et al., 1994, Katul et al., 1998, Sano et al., 1998). Based on virion morphology, genome structure, and mode of transmission, BBTV is clearly not a geminivirus, the only recognized group of ssDNA plant viruses. BBTV has recently been included as member of a new group of plant viruses that potentially includes Subterranean Clover Stunt Virus (SCSV) (Boevink et al., 1995), Faba Bean Necrotic Yellows Virus (FBNYV) (Katul et al., 1995), Milk Vetch Dwarf Virus (MVDV) (Sano et al., 1998) and possibly the plant-hopper borne Coconut Foliar Decay Virus (CFDV) (Rohde et al., 1995). Six to 11 different DNA components have so far been identified in the above four assigned nanovirus species. Each DNA appears to contain only one gene and the five principal DNA components of nanoviruses code for five or six proteins which have homologues present in all nanoviruses studied so far (Beetham, Harding, 1999).
1.1 Genome Components

Banana bunchy top virus belongs to the family of single-stranded DNA viruses. Its genome comprises of multiple circular single-stranded DNA components each about 1.1 kb (Harding et al., 1991; Burns et al., 1994). Each of the six DNA components associated with BBTV encodes at least one gene (Beetham et al., 1997). Boevink et al. (1995) identified and sequenced seven ssDNA components of another nanovirus named Subterranean Clover Stunt Virus (SCSV). Each of its components had one large ORF in the virion sense, a sequence 5' of the ORF capable of forming a stable stem-loop structure and a highly conserved nanonucleotide sequence within the loop sequence. Five of the components shared a conserved common region. SCSV-component 5 encodes the SCSV coat protein (Chu et al., 1993) while two other components encode putative Reps (Boevink et al., 1995).

1.2 Rep proteins of ssDNA Viruses

Geminiviruses and nanoviruses are plant viruses with exclusively nuclear replication cycles (Bisaro et al., 1996, Boevink et al., 1995). Initiations of replication in both viruses were triggered off by virus encoded replication initiator proteins known as ‘Rep proteins’ that act as ‘sequence-specific origin recognition endonucleases’. ‘Rep’ of nanoviruses contains several sequence motifs similar to those in Reps of ssDNA plant viruses belonging to the family Geminiviridae (Boevink et al., 1995), which are essential for DNA replication (Laufs et al., 1995). The concept of a modular arrangement of specificity elements i.e., a common initiation signal - recognized and acted on by Rep proteins can thus be easily transferable from the bipartite genome of some geminiviruses to the multipartite genome of the nanoviruses.
All nanovirus DNA molecules harbour a putative stem-loop (SL) structure in their non-coding region (Palmer & Rybicki, 1998). This hairpin structure is highly conserved among the individual components of each virus, as well as among the four nanoviruses, and is very similar to that of geminiviruses. (Chu et al., 1993; Boevink et al., 1995; Burns et al., 1994; Katul et al., 1997; Wanitchakorn et al., 1997; Sano et al., 1998). The coding region present in nanoviruses is preceded by a promoter, the TATA box and is followed by a polyadenylation signal.

Harding et al., (1997), reported the sequence of BBTV component-1 from an Australian isolate, which encodes a putative replication protein based on the dNTP-binding motif ‘GGEGKT’. Karan et al. (1994) have demonstrated that BBTV DNA-1 is present in all BBTV infections from isolates tested from 11 countries and that the large ORF of BBTV component 1 (DNA-1), comprising approximately 850 nucleotides, potentially encodes the replication protein. Rep proteins generally have multifunctional roles and catalyses multiple reactions during the replicative cycle of the virus. Rep proteins have been found to be associated with a variety of plasmids of Gram- positive (Koepsel et al., 1985; te Riele et al., 1986; Gros et al., 1987) and Gram-negative bacteria (Yasukawa et al., 1991), ss and dsDNA phages and viruses (Baas & Jansz, 1988), including the geminiviruses (Laufs et al., 1995a), all of which replicate via a rolling-circle mechanism. According to the theory proposed by Wanitchakorn et al., (2000), one particular DNA component encodes the ‘master’ Rep that is responsible for replication of all other non-Rep DNAs. In Faba bean necrotic yellows virus (FBNYV), several additional Rep protein-encoding components have been associated, but only one component, FBNYV DNA2 - encoding the ‘Master Rep’,
is capable of self replication as well as initiating replication of the non-Rep protein-encoding components of this virus (Aronson et al., 2000). A similar phenomenon has been observed for the two other nanoviruses, Milk Vetch Dwarf Virus (MDV) and SCSV, giving rise to the Master Rep concept (Timchenko et al., 1999, 2000).

The Rep proteins encoded by the plasmid pT181 family members, have a modular structure and contain domains involved in their non-covalent, sequence-specific binding to the 'double stranded origin' (dso) as well as for origin nicking and subsequent ligation during replication (Dempsey et al., 1992a). Rep proteins belonging to this particular family of plasmids have conserved active sites that include the tyrosine residue involved in nicking-closing at the dso (Khan, 1997). However, these proteins show specificity in their replication of their respective genomes, (Dempsey et al., 1992b; Thomas et al., 1995) and often function as dimers, with the active tyrosine residue of each monomer involved in replication (Zhao et al., 1998). In contrast, Rep proteins of the plasmid pC194 family appear to act as monomers, while those of the pE194/pLS1 family may be present as hexamers (del Solar et al., 1998).

Rep of another ssDNA virus, Adeno-Associated Virus Type-2 (AAV-2) when isolated and studied in vitro, exhibited biochemical activities consistent with the functions of the Rep protein including DNA binding (McCarty et al., 1994), helicase activity and site-specific endonuclease activity (Muzyczka, 1992). The initiators of certain ssDNA containing bacteriophages were found to reinitiate replication on the same template DNA, such that replication of a single molecule could generate several progeny molecules (Gielow et al., 1995; Gilbert et al., 1998), additionally, the phage initiators were not subject to inactivation and could promote several initiation events.
1.3 Movement Protein and Nuclear Shuttle proteins

A particularly important requirement for plant virus infection at an early stage involves the ability of a virus to move from cell to cell. Recent findings indicate that viral proteins are involved in transcellular movement of the virus in its cell-to-cell spread (Melcher, 2000, McLean et al., 1993). All plant-infecting viruses possess one or more movement-related protein (MP) genes and they appear to derive from host plant genes for chaperonins and plasmodesmata-associated proteins (Melcher, 2000). The begomoviruses of Geminiviruses have two components, referred to as DNA A and DNA B, both of which are essential for virus proliferation. DNA A encodes proteins required for replication (REn and Rep), the control of gene expression and suppression (TrAP), and encapsidation (CP). DNA B encodes proteins required for nuclear trafficking (Nuclear Shuttle Proteins) and cell-to-cell movement (Movement proteins) while Rep and AC4 have been implicated in cell-cycle progression (Hanley, Bowdoin et al., 1999). Plant virus movement proteins generally interact with cellular macromolecules through hydrophobic interactions and several of these proteins are known to be associated with the ER membranes (McLean et al., 1995; Mushegian et al., 1993). The members of the mastrevirus group of monopartite geminiviruses such as Maize Streak Virus (MSV) encode a single movement protein (PV1) which has been shown to associate with the plasmodesmata causing conformational changes in the channel proteins (Boulton et al., 1993; Dickinson et al., 1996). All MPs of mastreviruses have a stretch of hydrophobic amino acids that are predicted to form a transmembrane structure (Boulton et al., 1993). A topological model for cell-to-cell spread, by a 30-kDa Movement Protein of the Tobacco Mosaic Virus, essential for of
the virus protein transport has been suggested by Deom et al., (1987). This protein was later found to bear two putative hydrophobic \(-\)helical transmembrane domains and a cytosolically exposed hydrophilic C terminus (Brill et al., 2000).

Experiments with a GFP reporter exhibited that the MSV MP–GFP fusion, when expressed in epidermal cells of maize leaves, demonstrated translocation of the protein from cell to cell via the Endoplasmic Reticulate network, and finally through the plasmodesmata (Kotlitzky et al., 2000). Bipartite geminiviruses encode two MPs, BR1 and BL1, which act in a cooperative fashion to facilitate cell-to-cell movement (Pascal et al., 1993). The Squash Leaf Curl geminivirus (SqLCV) BR1 protein contains localization signals that direct it to the nucleus of infected and transfected cells (Pascal et al., 1994; Sanderfoot et al., 1996), and purified SqLCV BR1 binds tightly to ssDNA (Pascal et al., 1994). However, in plant and insect cells coexpressing SqLCV BR1 and BL1, BR1 was directed away from the nucleus and toward the cell periphery (Sanderfoot and Lazarowitz, 1995). Furthermore, microinjection studies revealed that the Bean Dwarf Mosaic geminivirus (BDMV) BR1 protein redirects double-stranded (ds) DNA and ssDNA from the nucleus to the cytoplasm (Noueiry et al., 1994). The BL1 protein is associated with the cell wall fraction and facilitates transport of macromolecules through plasmodesmata. Carrington et al., (1996) later proposed a model in which BR1 provides a nuclear shuttle activity to deliver viral DNA to the cytoplasm, after which BL1 mediates trafficking of DNA to and through the plasmodesmata. Interaction of the two proteins was found to regulate the directionality of intracellular viral DNA transport (Sanderfoot et al., 1996). The BC1 protein of Bean dwarf mosaic virus was found to increase the size exclusion limit of
plasmodesmata of cells and mediated nuclear shuttling of the viral genome as well as transport from cell to cell (Noueiry et al., 1994; Rojas et al., 1998). In contrast, the BL-1 protein of *Squash leaf curl virus* (SqLCV) does not bind DNA but is present in virus-induced tubules that cross the walls of meristematic phloem tissues (Ward et al., 1997). For bipartite begomoviruses, MP is the major symptom determinant, and the expression of this protein induces disease-like symptoms (Brough, 1988; Duan, 1997, Etessami, 1988; Hou, 2000; Ingham, 1995; Pascal, 1993). Burns et al. (1995), identified a short stretch of hydrophobic residues in the N-terminus of the predicted gene product of BBTV DNA-4 and suggested that it may be a viral movement protein. Similar hydrophobic regions have been identified in the predicted gene products of FBNYV component 4 (C4) and SCSV component 1 (C1) which shared homology with the movement protein of MSV (Boulton et al., 1993). FBNYV-C4, MDV-C8 and SCSV-C1 each encode a 13-14kDa protein found to function as a movement protein, while FBNYV C8, MDV C6 and SCSV C4 encoded proteins that were very similar to nuclear shuttle proteins of begomoviruses (Katul et al., 1997).

The import of the viral DNA and/or virions into and out of the host plant cell nucleus is essential for the successful completion of their life cycle, as both replication and transcription occur in the nucleus. However, less is known about two critical viral infection processes, nuclear targeting of the viral genome, i.e. its transport into the nucleus via the nuclear pore complex (NPC), and the process of nuclear export of the virus, a prerequisite for its spread from cell to cell. Nuclear import, a fundamental aspect of cellular control is the regulation of bidirectional trafficking of macromolecules between the nucleus and cytoplasm that occurs through nuclear pore
complexes (NPCs) (Davis, 1995). Most substrates (proteins and nucleoprotein complexes) are transported across these channels by an active process that is saturable and involves the recognition of specific signal sequences on the cargo being transported (Görlich, 1999). The process is mediated by nuclear localization signals (NLSs) within the import substrate, which are recognized by soluble factors that dock the substrate to the NPC. The best characterized NLSs are the classic single and bipartite basic amino acid targeting sequences first described in simian virus 40 T-antigen and nucleoplasmin, respectively (Görlich, 1999). A third type of NLS composed of basic and hydrophobic amino acids occurs in the yeast mating factor MAT 2 and the maize transcription factor R but appears not to function in mammalian cells (Hicks and Raikhel, 1995). Despite their lack of similarity, competition binding studies using isolated plant nuclei suggest that both the MATa 2-type NLS and the classic basic NLS bind to the same nuclear site(s) (Hicks et al., 1995). Recent molecular and classical genetic studies in animal cells and yeast have identified an essential nuclear export signal (NES) in proteins that rapidly shuttle between the nucleus and cytoplasm (Wen et al., 1998). First characterized in the human immunodeficiency virus (HIV) Rev protein, transcription factor IIIA (TFIIIA) from Xenopus, and inhibitor of protein kinase A (PKI) (Fischer et al., 1995; Wen et al., 1998), this NES is a leucine-rich hydrophobic sequence of 10 to 13 amino acids. Most nucleic acid-protein complexes are exported from the nucleus via the NES sequence LSSHFQELSI (conserved hydrophobic amino acids in boldface). Mutational studies show that the leucine residues are essential for function (Fridell et al., 1996a). The export of p53 protein in mammalian cells is mediated by a highly conserved leucine-
rich nuclear export signal located in its tetramerization domain (Neil et al., 1997).

Geminiviruses manage the transport of their DNA within plants with the help of three proteins, the coat protein (CP), the nuclear shuttle protein (NSP), and the movement protein (MP) in which NSP packages DNA and MP anchors this complex to the protoplasmic leaflets of plasma membranes and microsomes for cell-to-cell movement. These proteins act cooperatively to move the viral DNA from its site of replication in the nucleus to the cytoplasm and into adjacent plant cells (Sanderfoot et al., 1995) and both proteins have been shown to determine viral host range (Ingham et al., 1995). In the monopartite Tomato yellow leaf curl virus (TYLCV), ssDNA plant virus, experiments showed that an NLS domain resides in the TYLCV CP between residues 36 and 61 (Kunik et al., 1998) which can facilitate nuclear import. A leucine-rich sequence known to serve as a nuclear export sequence was found on the BV1 protein of SqLCV, (Lazarowitz, 1999). Infectivity assays in geminiviruses by Fontes & Santos (2004) showed a positive correlation between infection rate and loss of function of NSP-Interacting Kinases (NIK) - NIK1 and NIK3 and proposed a model in which NSP acts as a virulence factor to suppress NIK-mediated antiviral responses. Hussain et al, (2004) have shown that the NSP of Tomato leaf curl New Delhi virus is a pathogenicity determinant as well as a target of host defense ‘Hypersensitive Response’.

‘NESbase’ is a database of experimentally validated Leucine-rich NESs curated from literature (La Cour et al., 2003). These signals are not annotated in databases such as SWISS-PROT, PIR or PROSITE. Each NESbase entry contains information of whether NES was shown to be necessary and/or sufficient for export, and whether the
export was shown to be mediated by the export receptor CRM1. The database is available online at http://www.cbs.dtu.dk/databases/NESbase/.

1.4 Viral Coat protein

Survival of the virus requires transport of the genetic material from an infected cell to an uninfected cell and viruses have evolved mechanisms of packaging their genomic nucleic acids, along with any other components necessary for replication, within protein coats comprising of repeated protein subunits to accomplish this. The viral capsid performs a variety of specialized functions, in different viruses, which are directed towards the goal of disseminating the viral genes to suitable hosts. Both movement protein (MP) and coat protein (CP) of *Maize streak virus* (MSV) are both required for systemic infection. Huanting *et al.*, (2000) proposed a model in which the MP diverts a CP–DNA complex from the nucleus (where viral DNA replication takes place) to the cell periphery, and in co-operation with the CP, mediates the cell-to-cell movement of the viral DNA. Coat protein of *Maize Streak virus* was found to perform multiple functions and is required for systemic movement (Morris-Krsinich *et al.*, 1985), encapsidation of MSV DNA into virions and insect transmission (Boulton *et al.*, 1989b; Lazarowitz *et al.*, 1989). The CP of the bipartite viruses is dispensable for systemic infection in an adapted host, although it can aid the movement of pathogenicity of such viruses (Qin *et al.*, 1998). In contrast, the CP of mastreviruses is required for plant infection (Boulton *et al.*, 1989b, 1993; Liu *et al.*, 1997; Woolston *et al.*, 1989). *In vitro* analysis of the interactions of the *E. coli*-expressed CPs of MSV (Liu *et al.*, 1997) and coat protein of TYLCV with viral and non-viral DNA, showed that it binds ssDNA in a highly co-operative and sequence nonspecific fashion
In the family Bromoviridae, the tripartite RNA genomes of bromo-, cucumo- and oleaviruses and the genomic RNAs of alfamo- and ilarviruses contain a homologous sequence of 145 nucleotides that represents a high-affinity binding site for CP in the 3'-termini of the viral RNAs (Pooma et al., 1996). Molecular characterization of the Coat protein of Tomato leaf curl virus showed that purified recombinant CP bound preferentially to ssDNA in a sequence non-specific manner. Deletion of 50 amino acids from the N-terminus, including a putative N-terminal alpha helix, did not result in the loss of binding to DNA. A search for motifs responsible for DNA binding indicated a conserved putative zinc finger motif in the CPs of begomoviruses was indeed involved in binding to zinc and DNA. The CP of Alfalfa mosaic virus was shown to bind to a number of AUGC-motifs separated by hairpin structures to form a viral nucleic acid-protein complex containing MP and CP and was involved in virus transport (Schoumacher et al., 1998).

1.5 Virus encoded Rb-binding Proteins

In mature plants, most cells have exited the cell cycle, undergone differentiation and no longer contain detectable levels of replicative enzymes (Coello et al., 1995; Nagar et al., 1995). To overcome this barrier, geminiviruses, the closest analogue to nanoviruses were found to reprogram their hosts to create a replication-competent environment (Nagar et al., 1995). Geminivirus Rep proteins were found to interact with a checkpoint regulator of the host's cell cycle, the retinoblastoma protein (pRB) via an LXCXE motif (Xie et al., 1995). The viral infection induces the accumulation of proliferating-cell nuclear antigen (PCNA), a processivity factor of DNA polymerase found in differentiated cells of TGMV-infected plants but not in equivalent healthy
cells (Nagar et al., 1995). Mutations in the LXCXE motif of present in the non structural proteins of two viruses, Rubella virus (causing congenital rubella syndrome) and Human Cytomegalovirus were shown to affect their respective replications (Atreya et al., 1999). Their results clearly demonstrate that the LXCXE motif is required for efficient virus replication and provide experimental evidence, that the Rb-pocket binding protein plays a role in viral replication.

In nanoviruses, a separate protein mediates such a link between cell cycle modulation and viral DNA replication (Aronson et al., 1999; Wanitchakorn et al., 2000). A 20-kDa protein encoded by FBNYV genome component-10 (C10) was found to contain an LXCXE motif which was capable of binding to members of the pRB family, and this interaction correlates with a stimulation of viral DNA replication. For it’s potential to link viral DNA replication with key regulatory pathways of the cell cycle, the FBNYV C10 protein was named Clink, for "cell cycle link." (Aronson et al., 2000). Wanitchakorn et al. (2000a) have shown that the gene product of BBTV DNA-5 is produced very early in the infection cycle as an immediate early gene product and could be an important candidate for interacting with the Rb-protein for switching the first infected cells to S-phase in preparation for virus replication. This is also supported by the results of Hafner et al. (1997a) who showed that BBTV DNA-5 is the most efficiently self-primed of the BBTV DNA components.

1.6 Phylogenetic analysis

Phylogenetic Analysis allows us to identify core sequences and protein regions that have been conserved in genomes and proteins that have a common ancestor. Comparison of the complete nucleotide sequences of DNA component 1 of banana
bunchy top virus of various isolates from 10 different countries by Karan et al., 1997 indicated two groups: the South Pacific group (isolates from Australia, Burundi, Egypt, Fiji, India, Tonga and Western Samoa) and the Asian Group (isolates from the Philippines, Taiwan and Vietnam).

2. Viral Replication

DNA viruses with small genomes do not have sufficient coding capacity to specify the DNA polymerases and accessory factors required for their replication and, instead, recruit the replication machinery of their hosts. As a consequence, these viruses can only amplify in cells that contain the requisite replication enzymes, which are typically confined to actively cycling cells. To overcome this limitation, mammalian DNA tumor viruses encode proteins that interact with components of the host transcriptional apparatus and cell cycle regulatory network (Becker et al., 1996; Nevins, 1992). They target basal transcription factors (Gruda et al., 1993), the histone transacetylase p300 (Eckner et al., 1994), and the tumor suppressors, pRb (Dyson et al., 1992) and p53 (Werness et al., 1990). These protein interactions cause quiescent animal cells to re-enter the cell division cycle and synthesize the enzymes necessary for viral DNA replication.

A key cell cycle regulator in eukaryotic cells is the Retinoblastoma tumor suppressor protein (pRB), which represses onset and progression into S phase by interacting with a wide range of cell cycle-related proteins. During the G1/S transition, pRB is progressively phosphorylated by the action of cyclin-dependent kinases, and as a result, E2F is released from the complex, and becomes available to activate the expression of S-phase-specific genes (Dyson, 1998). The pRb family members of plants
and animals display strong sequence homology across a large central highly conserved domain (Murray, 1997). This conserved pocket domain was known as the A/B pocket and enabled pRB to interact with known ‘Rb-binding proteins’ as well as plant D-type cyclins.

Viral DNA replication has long since been found to be excluded from the actively dividing regions of the plant, called meristems. It has been suggested that some plant viruses encode proteins which modify cell-cycle regulation in terminally differentiated cells of infected plants via a mechanism(s) similar to that found in animal cells (Nagar et al., 1995; Guiterrez, 1998). As a consequence, nanovirus DNA replication has been suspected for a long time to be associated somehow with a virus-induced permissive state where replication factors would be available. The initial finding that a CFDV component encoded a protein containing an LxCxE motif and interacted with human pocket proteins both in yeast and in vitro (Xie et al., 1995) was a first clue as to which mechanism could be used by nanoviruses to induce a permissive cellular state. This observation together with the identification of plant D-type cyclins (Dahl et al., 1995; Soni et al., 1995), which also contain an LxCxE motif, provides strong support for the notion that a plant RB-related (RBR) pathway might exist in plants.

Viral replication occurs at specific sites inside the cell as the replication complex is invariably found within the cell nucleus, with at least one or more viral specific proteins playing a crucial role in all these mechanisms. One of the key steps during the initiation of DNA replication is origin (ori) recognition (Xie, et al., 1995). In the genomes of both Gemini and nanovirus, the ori consists of a conserved stem–loop
and other less well-conserved sequences, including direct or inverted repeats or iterons. In geminiviruses, both components have a conserved 5' intergenic region (IR) that separates divergent open reading frames (Bisaro et al, 1996). The IR includes the plus-strand origin of replication and the promoters for leftward and rightward transcription (Sunter et al, 1991). The IR also contains a hairpin with a 9-base pair loop sequence conserved among all geminiviruses that is cleaved during initiation and termination of RCR (Orozco and Hanley-Bowdoin, L. 1997). Almost all of the nano and circovirus DNAs have the nanonucleotide sequence TAGTATTAC. As long as a nanovirus genome component contains a specific signal recognized by a given Rep protein, that particular Rep protein initiates its multiplication. This was observed when only Rep-2 of FBNYV initiated the replication of all non-rep components, in addition to its cognate DNA, in replication assays. Katul, et al.,(1998) observed that DNA sequence motifs flanking the conserved inverted repeat element are shared by rep2 and the other six genome components, whose replication depends on the action of Rep2 protein.

3. Management of BBTV

The systemic viral diseases of banana are generally controlled by the integrated measures including (1) planting of virus-free seedlings and (2) prevention of reinfestation by elimination of inoculum sources and vector control. Regular inspection, roughing of diseased plants, control of insect vectors and planting of virus-free plantlets through a system of virus-free seedling production has been recommended in countries like Australia (Colborne,1999) for the control of BBTV. The occurrence of BBTV was the lowest in plants produced by tissue culture at 0.2% followed in increasing order by plants grown from suckers and ratoons (Su et al.,1993).
Biological control of *P. nigronervosa* is being attempted in Tonga by introducing a braconid, *Aphidius colemani*, native to Australia. Cross-protection of banana plants have also been experimented in the field as a means of protection, although no mild strain of BBTV has been conclusively identified or isolated (Simmonds, 1987).

### 3.1 Recent Advances in Diagnostics of BBTV

Serological or immunological methods for detection of plant pathogens, particularly plant viruses have been available to plant pathologists for many years. With tissue culture a standard practice in banana propagation, virus diseases are often transmitted through tissue culture bearing latent infective form of BBTV. Development of rapid and sensitive techniques for the identification and detection of banana pathogens, particularly viruses, has been pursued in order to facilitate germplasm movement. Although enzyme-linked immunosorbent assays (ELISA) have been developed, these tests are often not sufficiently sensitive to detect low-titre infections and are complicated by the occurrence of serological diversity among virus isolates (Ndowora and Lockhart 1998). Immunosorbent electron microscopy is much more reliable, but its wider application for indexing large numbers of samples is limited by cost, labour, time, and equipment requirements. Specific oligonucleotide primers have been developed for use in PCR-based methods for BSV detection (Lockhart and Olszewski, 1996). Likewise, an immunocapture-PCR method has effectively amplified BSV; this assay was found to be sensitive, however not suitable for handling large volumes (Thottappilly, 2002). The PROMUSA virology working group has stressed the importance of developing reliable techniques for detection of BBTV, using appropriate
diagnostics by developing a better understanding of the genome and diversity of BBTV.

4. Analysis of nucleotide and amino acid sequences using bioinformatics tools

Computational tools such as BLAST, CLUSTALX, GeneParser, MEME, (Timothy et al.), PredictProtein (Rost, 2004), Predotar, InterProScan (Mulder et al., 2003), Modeller2 Ver 6.0 are being used to analyze nucleotide and protein sequence data in the most efficient manner to predict the biological functions of genes and proteins based on information present in databases such as GDR (http://gdbwww.gdb.org), SWISS-PROT (http://expasy.hcuge.ch/sprot/sprot-top), PDB (http://pdb.weizmann.ac.il), and GenBank (http://www.ncbi.nlm.nih.gov). ClustalW is a general purpose multiple alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences, calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Cladograms or Phylograms from (http://www.ebi.ac.uk/clustalw/) can be used to view evolutionary relationships.

5. Production of recombinant -Coat protein for Antibody generation

Serological diagnostic probes are still useful in most developing countries because PCR technology is not accessible to the great majority of the pathologists. The expression of viral coat proteins (CPs) in E.coli, followed by purification and polyclonal antiserum production has been reported for a number of different plant viruses (Vaira et al., 1996). These antisera have been used for plant virus detection in Western Blots, Immunocapture-polymerase chain reaction and Immunosorbent electron microscopy (Jelkmann and Konrad, 1997; Rubinson et al., 1997; Kai Shu Ling et al.
2000). Polyclonal Antisera prepared against the viral coat protein of four begomoviruses expressed in *Escherichia coli* were useful for the detection of begomoviruses in an array of assays (Abouzid *et al.*, 2002, Mutas-Gottgens, *et al.*, 2000). Recombinant DNA techniques permit the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest which aids in the purification of the recombinant fusion proteins by employing affinity chromatography methods. A variety of heterologous expression systems have been developed and are currently used to produce recombinant proteins. The pRSET vector from (Novagen, USA) was used in the present study as the chosen protein expression vector.

Fig. 2 Diagramatic Representation of protein expression vector pRSET-A
The pRSET-A possesses a strong phage T7 promoter, a Kozak sequence (Kozak, 1991) recognized by the *E.coli* RNA polymerase, a synthetic ribosomal binding site RBSII for high translation rates, a 6xHis tag for subsequent affinity purification, multiple cloning sites and translation stop codons in all reading frames for convenient preparation of expression constructs, transcription terminators from T7 phage, β-lactamase gene conferring ampicillin resistance at 100μg/ml (Stuber et al, 1990). The technique of protein purification using Immobilized Metal Affinity Chromatography was first demonstrated in 1975 (Porath et al, 1975). Modifications were further made to the chelating ligand iminodiacetic acid (IDA), which was charged with metal ions such as Zn, Cu, Ni and then used to purify a variety of different proteins and peptides (Sulkowski, 1985).

Fig. 3
Diagramatic Representation of the interaction between 6xHis-tagged BBTV coat protein and Ni-NTA affinity purification column from QIAGEN. (Pic. Courtesy QIAGEN Protein Expressionist Handbook).
Nitroloacetic acid (NTA) exclusively available from QIAGEN GmbH, is a tetradeutate adsorbant allowing purification of 6xHis tagged proteins from less than 1% of the total protein preparation.

6. Construction of chimeric vector for Agrobacterium mediated transformation of banana

Tremendous progress has been made in the genetic improvement of Musa in recent years, and new varieties are becoming available from breeding programs (Horry et al., 1997; Horsch et al. 1984). However, classical breeding of bananas is hampered by long-generation time, triploidy and sterility of most edible cultivars. Furthermore, certain diseases for which sources of resistance are not known, an important example being Banana Bunchy Top Virus (BBTV), genetic transformation provides an opportunity for the variety in question to retain all its original characteristics, with the simple addition of the desired trait (Haicour et al., 1993). Transformation of sterile triploid dessert banana cultivars could certainly have a significant commercial impact. Genetically resistant banana and plantain varieties are the basis upon which sustainable improved production can be developed (FAO Document Repository, 2004). Relative success in genetic engineering of bananas and plantains has been achieved recently to enable the transfer of foreign genes into plant cells (Bosque et al., 1998). Genetic transformation using microprojectile bombardment of embryogenic cell suspensions is now routine (Sagi et al., 1995, 1999, 2000) and has been applied to a range of plantain and banana cultivars and synthetic hybrids (Bosque-Pirez et al., 1998). Protocols for electroporation of protoplasts derived from embryogenic cell suspensions, particle bombardment of embryogenic cells (Sagi et al. 1995; 1999), and co-cultivation of wounded meristems
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6. Construction of chimeric vector for Agrobacterium mediated transformation of banana

Tremendous progress has been made in the genetic improvement of Musa in recent years, and new varieties are becoming available from breeding programs (Horry et al., 1997; Horsch et al.1984). However, classical breeding of bananas is hampered by long-generation time, triploidy and sterility of most edible cultivars. Furthermore, certain diseases for which sources of resistance are not known, an important example being Banana Bunchy Top Virus (BBTV), genetic transformation provides an opportunity for the variety in question to retain all its original characteristics, with the simple addition of the desired trait (Haicour et al, 1993). Transformation of sterile triploid dessert banana cultivars could certainly have a significant commercial impact. Genetically resistant banana and plantain varieties are the basis upon which sustainable improved production can be developed (FAO Document Repository, 2004). Relative success in genetic engineering of bananas and plantains has been achieved recently to enable the transfer of foreign genes into plant cells (Bosque et al,1998). Genetic transformation using microprojectile bombardment of embryogenic cell suspensions is now routine (Sagi et al., 1995,1999, 2000) and has been applied to a range of plantain and banana cultivars and synthetic hybrids (Bosque-Pirez et al.,1998). Protocols for electroporation of protoplasts derived from embryogenic cell suspensions, particle bombardment of embryogenic cells (Sagi et al. 1995;1999), and co-cultivation of wounded meristems
with Agrobacterium (May et al., 1995) are available for bananas and plantains. Genes coding for antifungal proteins that show broad antifungal activity in vitro have been introduced into a plantain landrace and the resulting transgenic plants await field testing (Remy et al., 1998).

Various protocols for Agrobacterium-mediated transformation of embryogenic cell suspensions of the banana cultivars have been studied (Ganapathi et al., 2001; Khanna et al., 2004). Application of genetic transformation techniques have already been applied in the generation of transformed plants from cvs. ‘Bluggoe’ and ‘Grand Naine’ expressing marker genes as well as antibiotic resistance genes including expression in banana and plantain of anti fungal peptides or proteins which exert fungistatic activity to Mycosphaerella and Fusarium oxysporum, the causative agents of Black Sigatoka and Panama disease respectively (Broekaert et al., 1998). The following cultivars have been transformed successfully: Williams (AAA), Three Hand Planty (AAB), Bluggoe (ABB), Cardaba (ABB), Monthan (ABB) and Tani (BB). Transient expression of the gusA reporter gene has been observed in all these cultivars following at least one of the three transformation techniques. Transgenic plants have been regenerated after particle bombardment of Bluggoe, Three Hand Planty and Williams, representing the three economically important genomic groups in MUSA.

Pathogen-Derived Resistance (PDR, Baulcombe, 1996) was the basic concept whereby the first Virus Resistant Transgenic Plants (VRTPs) were obtained more than a decade ago using the coat protein gene of TMV. Coat Protein gene is the most commonly used transgene for developing Virus Resistant Transgenic Plants (VRTPs). Jan et al. (2000b), in a recent study has shown that the complete gene is
not always necessary for inducing resistance, and genome segments as small as 110bp could induce resistance (MacFarlane et al., 1992). The mechanism of resistance induced by CP gene is either through the protein encoded by the transgene (protein-mediated) or by the transcript of the transgene (RNA-mediated) (Lomonossoff, 1995; Reimann-Philipp, 1998) or both (Yusibov et al., 1998). Coat Protein Mediated Resistance (CPMR) has been shown to work for geminiviruses (Sinisterra et al., 1999). Since the first filed test of CPMR against TMV in tomato plants in 1987 (Beachy et al., 1990), there have been increasing number of field tests in different host-virus systems and all the Virus Resistance Transgenic Plants (VRTP’s) commercialized so far are based on CPMR. Squash var. Freedom II is resistant to WMV-2 and ZYMV (Fuchs et al., 1997) while papaya var. SunUP and UH Rainbow are resistant to PRSV (Gonzalves, 1998). Using the gene for nucleocapsid protein, resistance has been introduced in crops like tomato, tobacco, lettuce, groundnut, pepper and in ornaments like Impatiens, Ageratum and Crysnathemum against tomato spotted wilt virus. Transgenic resistant plants using the CP gene have also been developed against alfalfa mosaic virus, potato virus X, Rice tungro virus, tobacco rattle virus and Papaya ring spot virus (Varma et al., 2002).

Studies of CP-MR against tobacco mosaic virus (TMV) have increased the understanding of the mechanisms that govern CP-MR (Bendahmane, 2002). As a result of such studies, it is known that transgenic CP interferes with virus disassembly early after virus entry (Bendahmane, 2002, 1997).

Another concept was the use of the replicase protein gene to confer resistance against plant viruses; this strategy is referred to as Replicase Protein Mediated Resistance (RPMR). Since it’s first demonstration in Tobacco Mosaic Virus by
Golemboski et al. (1990), it has been successfully used from 16 RNA/DNA viruses representing 11 plant virus groups (Palukitis & Zaitlin, 1997; Jones, 1998). **RPMR gives nearly immune type and highly specific resistance for the virus from which the transgene is isolated. It is more effective than CPMR and is not influenced by inoculum levels.** Molecular mechanism of RPMR is not fully understood and seems to be either protein or RNA-mediated. RPMR has shown a lot of promise in developing VRTP’s resistant to geminiviruses (Varma & Malathi, 2001). RPMR based studies on ACMV (African cassava mosaic virus) produced transient expression of AC1 (geminivirus replicase protein), and showed significant reduction in viral DNA replication. The transformed *N. benthamiana* plants remained symptomless or produced delayed and attenuated symptoms (Hong & Stanley, 1996).

Chellappan et al., (2004), developed a pathogen-derived transgenic approach to generate high levels of resistance against these pathogens in a susceptible cultivar of cassava (*Manihot esculenta*). Integration of the AC1 gene (which encodes the replication-associated protein) from African cassava mosaic virus imparted resistance against the homologous virus and provided strong cross-protection against two heterologous species of cassava-infecting geminiviruses. Short-interfering RNAs specific to the AC1 transgene were identified in the two most resistant transgenic plant lines prior to virus challenge which suppressed levels of AC1 mRNA in these plants. When challenged with geminiviruses, accumulation of viral DNA was reduced by up to 98%, providing evidence that integration of AC1 initiates protection against viral infection via a post-transcriptional gene silencing mechanism.

In CMV, replicase-mediated resistance was found to occur due to the existence
of at least two separate elements which contributed to the resistance: one resulting in a reduced level of virus replication, the other restricting virus movement (Carr et al., 1994; Nguyen et al., 1996; Wintermantel et al., 1997; Canto & Palukaitis, 1999).

Amongst the present methods used for gene insertion into plant systems, particle bombardment using a biolistic gun device has been successfully used for the introduction of the coat protein gene of Banana Bract Mosaic Virus into banana embryogenic cell suspensions (Sagi et al., 1995a). Transformed plants were ready to be established in the greenhouse six to eight months after bombardment. The method of Agrobacterium-based transformation has been used with considerable success by several research teams worldwide where banana plants were transformed with either of two selectable marker genes and a number of chimaeric genes including three different antifungal genes and various genes isolated from banana viruses such as the coat protein gene of banana bract mosaic virus (May, et al., 1995). The main attractiveness of this method is the ease and speed of transgenic plant regeneration, and the lack of requirement for specialized equipment or sophisticated in-vitro regeneration skills.

According to the FAO, though progress has been made in the development of efficient transformation systems for Musa, however, different research activities are still very much required to improve the development of genetically transformed bananas that do not cause somaclonal variation. Reliable constructs for transformation with strong promoters for transgene expression, including promoters from banana plants, are very necessary, as well as a molecular toolbox to control gene expression. One recurring problem is that standard cloning vectors for use with Agrobacterium are not available, and construction of new plasmid vectors requires optimization for
particular genes or species (McElroy et al.,1995).

The plant transformation vector, pCAMBIA 2301 selected for the construction, was tested successfully at CAMBIA for tobacco and rice (Hajdukiewicz et al.,1998) and enables direct transformation by both particle bombardment and Agrobacterium-mediated transformation, precluding the need for triparental mating. These vectors were stable in Agrobacterium even if grown under non-selective conditions because of the presence of the ‘rep’ and ‘sta’ regions from pVS1 (Deblaere et al, 1994). The maize polyubiquitin promoter (Christensen et al,1992) is one of the strongest promoters tested in banana (Sagi et al, 1995a) has been shown to be highly active in monocots. The intron present in the gusA reporter gene coding sequence ensures that the gene is not expressed in bacteria (Ohta et al,1990).

Engineering resistance to banana bunchy top virus (BBTV) is an obvious objective, since no natural resistance to this virus has been identified in the Musa genepool. One of the strategies now being tested in several laboratories is the expression of various BBTV genes in transgenic banana plants in order to interfere with the normal replication, encapsidation or movement of the virus (Sági,L., Remy S., & Swennen, R.,1999). In another study by Tripathi et al,(2005), an Agrobacterium-mediated plant transformation system was developed for the production of transgenic plantain [Musa spp. cultivar Agbagba (AAB)] using Agrobacterium strain EHA105 with the binary vector pCAMBIA 1201, having hygromycin resistance gene as a selection marker and GUS-INT as a reporter gene. Transient expression of the -glucuronidase (uid A) gene was achieved in transformed apical shoot tips. The hygromycin resistant shoots were regenerated 4 to 5 weeks after co-cultivation of
explants with Agrobacterium. The study showed the enormous potential for genetic manipulation of Musa species for disease and pest resistance, as well as abiotic factors, using a rapid and non-species specific transformation and regeneration system.