MAIZE STREAK VIRUS (MSV) DIVERSITY IN UGANDA

AND THE ASSESSMENT OF GENE SILENCING AS A TOOL FOR DEVELOPMENT OF RESISTANCE TO MSV

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Diversity of Maize streak virus in Uganda and gene silencing studies

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To

J.E.Owor (R.I.P)

and

My loving mother Juliana and siblings Florence, William,
Hellen, Martina, Joshua, Moses and Oscar

Declaration

I declare that the work presented in this thesis is my original work. I also affirm that this work has not been presented in this, or any other university for examination, or for any other purposes. I also declare that published work has been included with permission from the other authors.

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Abstract

Maize streak virus (MSV: Family *Geminiviridae*, Genus *Mastrevirus*) is the causal agent of maize streak disease (MSD) that contributes significantly to low maize yields in Africa, thereby threatening food security of sub-Saharan Africa's poorest people.

In Uganda, MSD has been identified as one of the most important constraints to maize production. In order to have a better understanding of the disease in that country, this thesis set out to establish MSD levels in farmers' fields; develop a new sampling and virus isolation method; assess the diversity of MSVs throughout Uganda; and, through the cloning of sampled virus genomes, to determine the genetic characteristics of different isolates. In addition, this study also included an assessment of RNA silencing as a resistance strategy against MSV.

Through an extensive survey conducted in all maize-growing regions of Uganda to assess MSD epidemiology, it was found that incidence and severity varied greatly between the different regions sampled. Average MSD incidences ranged from 1.9% in Kapchorwa District in eastern Uganda to 43% in Luwero District in the central region, with a country-wide average of 30%. Average disease severity (on a six-point scale from 0 to 5) was lowest in Kapchorwa (1.3) and highest in Bushenyi (western Uganda) (2.9). Most importantly, countrywide, there was a positive correlation between MSD incidence and severity ($P = 2.23 \times 10^{-6}$).

During the survey, an assessment of a new sampling and room-temperature storage technique demonstrated the ease with which a Whatman-based FTA Classic Card technology could be used for large-scale sampling of MSV-infected plants. This reduces the effort normally required to sample geminiviruses, which generally involves collection and transport of leaf samples, storage of leaves at −80°C, and extraction of DNA using laborious methods that require leaf-grinding in liquid nitrogen. While reducing time and costs involved, the FTA-based technique was comparable to using conventional methods such as CTAB in terms of quality and quantity of DNA extracted for downstream applications. Similarly, a DNA amplification technique which utilizes Phi29 DNA polymerase was used on DNA extracted from FTA cards, and was found to be comparable in efficiency to a PCR-based amplification method. Combined, FTA cards and Phi29 DNA polymerase were extremely useful and efficient for the cloning of full-length MSV genomes.

From infected samples collected during the survey, restriction fragment length polymorphisms (RFLPs) generated on PCR-amplified MSV fragments were used to type 391 Ugandan isolates which were grouped into 49 variants. In addition, full genomes from 68 isolates were amplified using Phi29 DNA polymerase, cloned and sequenced. Sequence data indicated that they all belong to the maize-infecting MSV-A_1 subtype that is predominant in many sampled areas in East Africa. Both inter- and intra-subtype recombination was found to be common in Ugandan MSVs, with 52 of the 68 isolates being recombinant. Based on detected recombination patterns, the Ugandan MSV sequences were classified into eight haplotypes named MSV-A_1UgI to MSV-A_1UgVIII. One haplotype designated MSV-A_1UgIII, was a recombinant and accounted for more than 60% of all sampled MSV infections in Uganda. This recombinant virus is widely distributed through-out the sampled areas in the country and causes severe MSD symptoms.

Furthermore, 15 of the cloned isolates representing the entire breadth of diversity in Uganda were made agroinfectious, and symptom characteristics caused by each isolate were assessed by agro-inoculating differentially resistant maize genotypes. Results indicated that, even in a commercially available MSV-resistant maize genotype, all but two of the isolates cause moderate to severe MSD symptoms.

Having established the epidemiology, diversity, and genetic characteristics of MSVs in the largest, most thorough MSD survey ever undertaken throughout Uganda, an attempt was made to establish a resistance mechanism that could protect against the severest, most widespread and diverse isolates identified in the survey. To investigate the efficacy of an RNA silencing mechanism in hindering MSV replication, the replication associated protein gene (*REP*) and the promoter sequences in the long intergenic region (LIR) of MSV were targeted using inverted repeats of sequences homologous to each target. Using a transient assay, it was established that MSV replication was abolished or greatly inhibited by an inverted repeat of a 678-bp portion of an intronless MSV *REP* gene. The repΔI^{678} hairpin construct significantly reduced replication of three southern African isolates: MSV-Kom (from which the repΔI^{678} hairpin was derived), MSV-MatA and MSV-Gat; a Réunion isolate: MSV-Réu, which differs from the cognate MSV-Kom by 4.62%, and three Ugandan isolates identified and cloned in this study: MSV-A[UG:Mpi11:05], MSV-A[UG:Kas75:05] and MSV-A[UG:Kab82:05]. These represent the three most common haplotypes found in Uganda, two of which (MSV-A[UG:Mpi11:05] and MSV-A[UG:Kab82:05]) cause severe MSD symptoms. The LIR hairpin construct on the other hand, had no effect on MSV replication.

Although transgenic *Digitaria sanguinalis* and Hi-II maize was produced containing the repΔI^{678} hairpin, transgenic offspring were not obtained and therefore the resistance mechanism could not be tested in transgenic plants. However, the fact that the repΔI^{678} hairpin significantly reduces replication of the most diverse maize-infecting MSV-A isolates currently identified, highlights its potential in protecting transgenic maize plants throughout sub-Saharan Africa from MSD.

Abbreviations

ACMV	African cassava mosaic virus
AGO	Argonuate
bp	Base pair
BCTV	Beat curly top virus
BGMV	Bean golden mosaic virus
BLAST	Basic Local Alignment Search Tool
BMS	Black Mexican Sweetcorn
CGVs	Cassava-infecting geminiviruses
CMD	Cassava mosaic disease
CP	Coat protein
CTAB	Cetyltrimethylammonium bromide
d.p.i	Days post inoculation
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
EACMV	East African cassava mosaic virus
EACMKV	East African cassava mosaic Kenya virus
EACMV-UG	East African cassava mosaic virus-Uganda
FTA	Flinders Technology Associates
GPS	Global positioning system
IR	Inverted repeat
Kb	Kilo bases

KBq	Kilo Becquerels
LIR	Long intergenic region
miRNAs	Micro RNAs
MP	Movement protein
MSD	Maize streak disease
MSV	Maize streak virus
ORF	Open reading frame
PCR	Polymerase chain reaction
PDR	Pathogen derived resistance
PTGS	Post transcriptional gene silencing
RBR	Retinoblastoma related protein
RCR	Rolling circle replication
RDR	RNA directed RNA polymerase
RdRp	RNA-dependent RNA polymerase
Rep	Replication associated protein
RepA	Replication associated protein A
RFLP	Restriction fragment length polymorphism
RISC	RNA-induced silencing complex
RNAi	RNA interference
r.p.m	Revolutions per minute
SDS	Sodium dodecyl sulphate
siRNA	Small/short interfering RNA
SIR	Small intergenic region
SSC	Salt Sodium Citrate
TGS	Transcriptional gene silencing
TYLCV	Tomato yellow leaf curl virus
TPCTV	Tomato pseudo-curly top virus
VIGS	Virus-induced gene silencing
VMYMV	Vigna mungo yellow mosaic virus

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Chapter 1

LITERATURE REVIEW

1.1 Introduction

World population continues to rise, and with that, demand for food. The Food and Agricultural organization (FAO 2007) predicts that despite there being enough food to feed the increasing population by 2030, much of the population in developing countries will remain hungry and food insecure, leading to failure to achieve the Millennium Development Goals (MDG) of halving world hunger by 2015 (UNDP 2008). If this goal is to be achieved, factors that affect food production need to be addressed.

In Africa, key factors that reduce the productivity of the land such as poor soils, drought, climate change, insect pests and diseases remain major constraints to food production. Productivity of major crops such as maize, cassava, sweet potato, yam, millet, sorghum and rice therefore remains low. One of the key staples on the African continent, second only to cassava, is maize. Southern and eastern Africans form the bulk of the maize consumers with over 85% of the production consumed by people as food (Aquino et al. 2001) and the rest used for animal feed.

Small and medium scale farmers are the major maize producers in sub Saharan Africa (SSA) with only a small percentage of total yields coming from commercial farms. This therefore suggests that when yields are low, it is small scale farmers, who are often also consumers, that are most affected. Addressing issues that contribute to low crop yields and food insecurity remains a priority for many African nations, and necessitates a holistic approach that will require use of old technologies, improvement of existing ones and development of novel techniques to overcome some of the production constraints.

1.2 Maize: production and importance

Maize (*Zea mays* L.) evolved from wild genotypes through modification by selective breeding in Central America and was introduced to the African continent by Portuguese traders in the 16th Century. It has subsequently become the most important cereal crop in sub-Saharan Africa, accounting for about 40% of cereal production on the African continent (FAOSTAT 2008). Despite the maize crop producing the highest food yield per hectare in comparison to other cereals, African maize yields are the lowest in the world. Average maize yields worldwide are 4.6 tonnes per hectare (t/ha) but are generally greater than 9 t/ha in developed countries such as the USA, where effective use of improved maize cultivars, fertilizers and pesticides is common practice. Maize yields in sub-Saharan Africa are, however, only 1.2 t/ha on average, with those in Uganda (1.8 t/ha), for example, being approximately half those of Africa's largest maize producer, South Africa (3.5 t/ha)(FAOSTAT 2008).

Maize, considered one of the three mega crops in the world together with wheat and rice, is widely grown in many parts of the world in a wide range of environments. It is an important dietary source of carbohydrate, protein, iron, vitamin B, and minerals in addition to use for animal feeds and raw materials for industrial products. In Africa maize is consumed as a starchy base in a wide variety of porridges, pastes, grits, and beer. Fresh on the cob, maize is eaten parched, baked, roasted or boiled. New uses of the crop, such as biofuels and production of pharmaceutical proteins (pharming) have increased demand for the crop, further expanding its use.

1.3 Constraints to maize production

Although sub-Saharan Africa's maize production has increased in the last 5 years (FAOSTAT 2008), optimal yields per unit area have not yet been achieved. Maize yields vary from country to country and whilst yields per hectare continue to rise in some countries such as South Africa where genetically engineered varieties are grown, a drop in yield has been experienced in countries such as Uganda.

Both biotic and abiotic constraints contribute to maximum maize yields not being realized. Some of the biotic constraints include damaging pests such as the stem borer *Chilo partellus* (Swinhoe), and diseases. Viruses including Maize streak virus (MSV), Maize mottle chlorotic stunt virus (MMCSV), Maize stripe virus (MSpV) and Maize dwarf mosaic virus (MDMV) (Rossel & Thottappilly 1985) cause severe disease in maize. Diseases caused by fungi such as spots, rusts, blights and rots as well as bacterial diseases also affect the crop (CIMMYT 2004). Other factors such as drought, poor soils, termites and weeds (especially the parasitic weed, Striga) are some of the other constraints to crop production.

In Uganda, three major diseases responsible for yield losses in the country are leaf blight, maize streak disease (MSD) and gray leaf spot (GLS) with GLS identified as the most important, followed by MSD (Bigirwa et al. 2001). Caused by MSV (a species in the family *Geminivirdae*), MSD is capable of causing total crop losses in susceptible maize varieties, particularly if infected early.

1.4 The family *Geminiviridae*

1.4.1 General features of geminiviruses

Geminiviruses have become pathogens of major economic importance because they pose a widespread threat to world agriculture (Fauquet & Stanley 2005), causing devastating diseases of a large number of monocot and dicot crop plants worldwide.

Although initially impacting on crop production in all tropical and sub-tropical regions of the world, geminiviruses are now a problem in temperate regions as well (Mansoor et al. 2003a). This family of plant viruses, already the best described after the family *Potyviridae*, continues to increase in importance as new species are continually discovered (Varma & Malathi 2003). Some members of this family occur together in mixed infections whilst others are even known to synergistically interact with one another. Although geminiviruses have small single stranded DNA genomes and encode only a few proteins, (typically four to six), they are apparently extremely flexible with respect to their evolution of novel pathogenic genotypes.

Specifically the viruses are characterized by recombination (Padidam et al. 1999) which may accelerate the evolution of more virulent virus strains. An important example of this is the cassava mosaic virus (Genus: *Begomovirus*) recombinant variant, East African cassava mosaic virus - Uganda (EACMV-UG) (Harrison et al. 1997, Zhou et al. 1997) that has been associated with the rapid spread of unusually severe cassava mosaic disease through most of the cassava-growing regions of nine countries of East and Central Africa. This recombinant has had a devastating impact on production of the crop in the region (Legg et al. 2006).

Geminiviruses cause distinctive symptoms such as leaf curling, vein yellowing, bright yellow mosaics, chlorosis, streaking and severe stunting, but the diagnostic utility of symptom evaluation is limited by the fact that very closely related viruses can produce vastly different symptoms whereas distantly related viruses can produce very similar symptoms.

The advancements in diagnostic techniques in recent years have accelerated the rate at which geminivirus sequences are accumulating and has resulted in the identification of new and ever increasing number of species and strains. This poses a challenge to classification, demarcation and assigning of names to new isolates.

Geminiviruses derive their name from the twinned quasi-icosohedral morphology of their particles. They have either monopartite or bipartite single stranded circular

DNA genomes (Harrison et al. 1977, Stanley & Gay 1983, Mullineaux et al. 1984) with open reading frames (ORFs) arranged on their complementary and virion sense strands.

Geminiviruses have been classed into four genera, *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*, based on their genome organizations, vector species, host ranges and sequence similarities (reviewed by Fauquet & Stanley 2003). Organization of the different components of the genomes in each of the four genera is shown in Fig. 1.1. Only one genus, *Begomovirus* has members with both monopartite and bipartite genomes (Briddon 2002) composed of DNA A and DNA B whilst the other three are all monopartite (Fig.1.1). A common feature in all four genera is a conserved nonanucleotide sequence (TAATATTAC) found in an intergenic region at their origins of virion strand replication (Lazarowitz 1987). The mastreviruses are the only genus with a distinctive small and large intergenic region whilst the other three genera have a single intergenic region. Viruses belonging to the dicot-infecting *Curtovirus* and *Begomovirus* genera express a replication enhancer protein (REn) that is unique to them, and is required for effective virus replication (Bisaro 1996). Proteins are expressed from virion and complementary sense ORFs diverging near the start site of virion strand replication.

Curtoviruses (type member: *Beet curly top virus*) are monopartite and are leafhopper transmitted to dicotyledonous plants by a single species, *Circulifer tenellus* (Briddon 2002). Five species and one tentative species are listed as belonging to this genus (Fauquet et al. 2008).

Members of the genus *Begomovirus* (type member: *Bean golden mosaic virus*) are the best described genus in the family, accounting for more than 80% of currently identified geminivirus species (Fauquet et al. 2008). Despite begomoviruses accounting for the highest proportion of viruses in the *Geminiviridae* family, mastreviruses are more diverse (Fauquet & Stanley 2003). Begomoviruses are transmitted by the whitefly, *Bemisia tabaci* (Fishpool & Burban 1994). The begomoviruses infect a wide

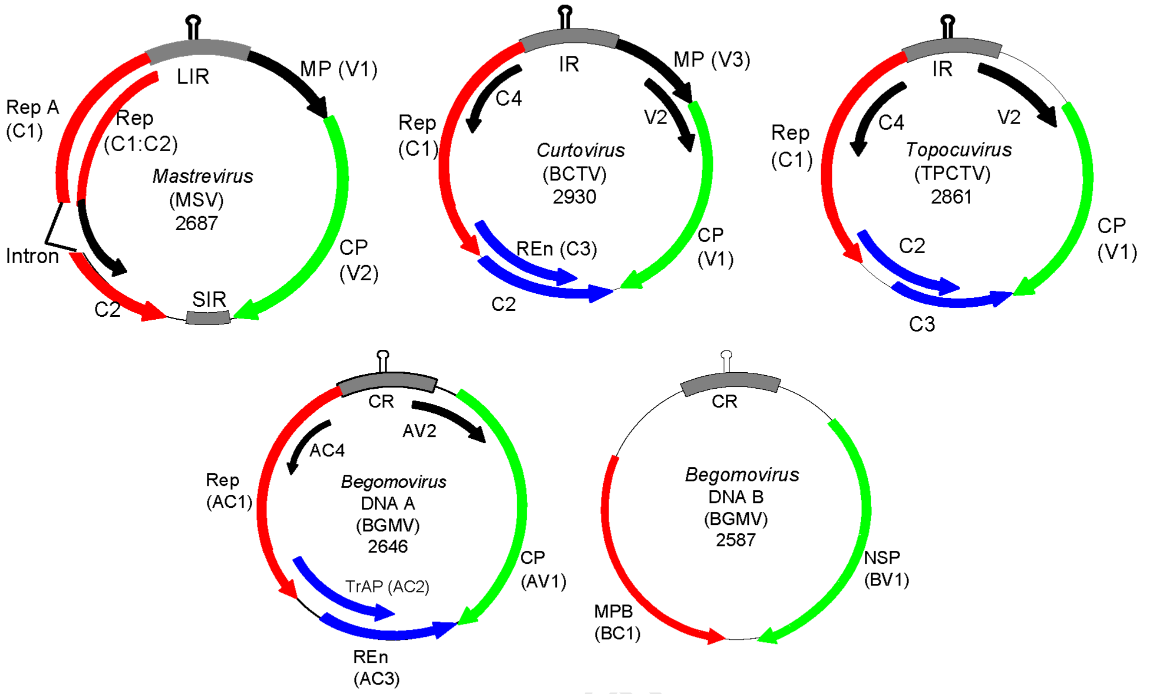


Figure 1.1: Genome arrangement within the four genera of the family *Geminiviridae* after Stanley et al. (2005). Open reading frames are represented by curved arrows and labelled according to the sense they are expressed, C for complementary and V for virion sense. Non coding regions include the LIR and SIR in *Mastrevirus*, Intergenic region (IR) in *Curtovirus* and *Topocuvirus* and a common region (CR) in Begomovirus represented here by the grey colour. Found in the IR, CR and LIR is a stem loop structure with an important role during replication. Rep: replication associated protein; RepA: replication associated protein A; For mastreviruses, the arrows representing C1 and C1:C2 are the same reading frame but produce the two different transcripts depending on presence or absence of splicing; LIR: long intergenic region; MP (V1): Movement protein; CP (V2): capsid protein; SIR: small intergenic region; IR: intergenic region; REn: replication enhancer protein; CR: common region; TrAP: transcriptional activator protein.

range of economically important hosts. Some of the important viruses in this genus which have been the focus of major disease control programmes include numerous species causing cassava mosaic and tomato yellow leaf curl diseases. A total of 178 species and 85 unassigned isolates have been identified in this genus (Fauquet et al. 2008).

The newest addition to the *Geminiviridae* is the *Topocuvirus* genus (type member: *Tomato pseudo-curly top virus*). It is believed to have arisen from recombination between a curtovirus and a member of an as yet unknown genus (Briddon et al. 1996). Topocuviruses have a monopartite genome, are treehopper transmitted and are only known to infect dicots (Fauquet & Stanley 2003). Still a relatively new genus, Tomato pseudo-curly top virus is currently the only known member.

Viruses of the genus *Mastrevirus*, of which MSV is the type member, have a single genomic component. This encodes four proteins, two on the virion sense strand (capsid and movement proteins) and two (the replication associated proteins, RepA and Rep) on the complementary sense strand (Fig. 1.1). The genome also carries two non-coding regions, the long intergenic region (LIR) and the short intergenic region (SIR) which contain the origin of plus (virion) and minus (complementary) strand replication respectively. Components of the genome interact with each other and with cellular proteins. An example of one such interaction is that between the MP and CP which has been reported in *In vitro* assays (Huanting et al. 2000). An interaction between Rep and RepA has also been reported to facilitate entry of Rep into the nucleus (Gutierrez 2000b, Hefferon et al. 2006). It has been demonstrated that the LIR of some isolates contributes to their increased pathogenicity whilst the SIR has the least effect on pathogenicity (Martin & Rybicki 2002). The Rep and the LIR are two components involved in initiation of virion strand replication.

Most described species in this genus infect monocotyledonous plants (Fauquet & Stanley 2003). Dicotyledonous-infecting mastreviruses include Tobacco yellow dwarf virus (TYDV) (Brunt et al. 1996), Bean yellow dwarf virus (BeYDV) (Liu et al. 1998) and Chickpea chlorotic dwarf Pakistan virus (Nahid et al. 2008). No new species have been added to the *Mastrevirus* genus since the Seventh ICTV report and the species listed remain 12 with five unassigned isolates within this genus (Fauquet et al. 2008) although more recently, two new species *Eragrostis streak virus* (Shepherd et al. 2008) and *Urochloa streak virus* (Oluwafemi et al. 2008) have been published. Species in this genus are distinguished based on the criteria outlined in Table 1.1. Some members of the genus include the African streak virus group [MSV, *Panicum streak virus* (Briddon et al. 1992), *Sugarcane streak virus* (Hughes et al. 1992), *Sugarcane streak*

Table 1.1: Criteria for species demarcation in the *Mastrevirus* genus

Character	Criteria
Genome features	No transcomplementation of gene products (Rep protein does not *trans*-replicate genomic components, no pseudorecombination between components
Genome sequence	<75% full sequence nucleotide identity is indicative of a different species
Protein features	Reaction of the coat protein with main antibodies may be indicative of a different species
Transmission	Different vector species (leafhoppers)
Host range	Different key natural host plant species and symptoms exhibited, for strain differentiation

After Fauquet & Stanley (2003), Fauquet et al. (2008)

Egypt virus (Bigarre et al. 1999), *Sugarcane streak Reunion virus* (Peterschmitt et al. 1991)], the Australian striate group (Pinner et al. 1992), Digitaria streak virus (Dollet et al. 1986), Wheat dwarf virus, Tobacco dwarf virus and Bean dwarf virus.

1.4.1.1 The *REP* gene

Geminivirus Rep proteins are multifunctional, are expressed early during the virus life cycle and are the only viral encoded proteins indispensable for geminivirus replication (Elmer et al. 1988, Stenger et al. 1991, Heyraud-Nitschke et al. 1995), a feature which makes them an ideal target for interference by pathogen derived resistance (PDR) strategies such as gene silencing. The different roles of the Rep protein range from recognition of virion strand origin of replication, cleavage at this site, joining of synthesized strands to terminate replication as well as interaction with host factors.

In mastreviruses, Rep and RepA are respectively produced from spliced C1:C2 transcripts (Rep), and non spliced transcripts (RepA)(Dekker et al. 1991), however only Rep is essential for virus replication (Elmer et al. 1988). RepA is unique to the mastrevirus genus (Schalk et al. 1989). RepA and Rep share a 200 amino acid sequence on the N terminus (Gutierrez 2000a) but each have unique C-termini. Whilst Rep carries a nuclear localization site in its C-terminus, the RepA does not and is found in both the nucleus and cytoplasm (Hefferon et al. 2006). In BeYDV, both Rep and RepA inhibit complementary sense gene expression and transactivate expression of virion sense genes although RepA is a stronger transactivator in comparison to the Rep (Hefferon et al. 2006). A conserved amino acid motif, LXCXE, that is involved in binding to a host protein, Retinoblastoma related protein (pRBR), is found in both Rep and RepA (Xie et al. 1995). While the RepA interacts with the host plant's pRBR, a cell cycle regulator, Rep does not (Xie et al. 1995, Horvath et al. 1998, Shepherd et al. 2005). Binding of RepA to the LXCXE binding site, leads to the release of transcription factors that promote cell division processes (Liu et al. 1999a). Horvath et al. (1998) found that the interaction of MSV Rep with pRBR can be restored through deletions of regions in the C terminus. A mutation in the LXCXE of BeYDV RepA has been shown to reduce replication of BeYDV (Hefferon & Dugdale 2003) whilst Shepherd et al. (2005) have shown that for MSV, a three nucleotide mutation in the *REP* gene, abolishing pRBR binding activity of the protein did not have any effect on viral replication in suspension cells although symptoms induced by the mutant in plants were less severe than those induced by wild type virus. In Wheat dwarf virus (WDV), a one amino acid change in the LXCXE motif abolishes Rep's ability to bind to pRBR (Xie et al. 1995). The RepA of WDV has also been shown to interact with geminivirus RepA binding proteins (GRAB), a class of proteins that regulates plant growth and development. Overexpression of GRAB in cultured wheat cells has been shown to reduce viral replication (Xie et al. 1999).

During viral replication, the Rep protein uses sequence-specific recognition to identify and interact with double stranded sequences in the LIR (Lazarowitz et al. 1992). Thereafter, it nicks the conserved TAATATTAC sequence at the virion strand origin of replication and catalyzes the fusion of virion strand sequences (Laufs et al. 1995). This interaction has been traced to the amino-terminal domain of the Rep protein (Heyraud-Nitschke et al. 1995). Binding of ATP needed for viral DNA replication also occurs via an NTP binding domain in the Rep (Desbiez et al. 1995).

1.4.1.2 The long intergenic region (LIR)

There is an intergenic region (IR) in all geminivirus genomes. Not only is the mastrevirus LIR home to the bidirectional complementary and virion sense promoters that control virus gene expression but it also contains an important conserved 9-nt sequence that is the origin of virion strand replication. This sequence, TAATATTAC, is located in the loop of a stem loop structure, a common feature of all geminiviruses, that is required for replication (Heyraud-Nitschke et al. 1995). A deletion of the stem loop structure however, does not prevent virus replication in WDV (Heyraud-Nitschke et al. 1995). This is because in WDV, there is an alternate replication initiation site,TACCC, in the LIR (Heyraud et al. 1993).

Despite containing the conserved nonanucleotide sequence that is vital for virus replication, the sequence of the LIR varies even between closely related viruses (Arguello-Astorga et al. 1994). A 67 nt sequence region in the LIR has been identified as a replication specificity determinant for MSV (Willment et al. 2007). Intra subtype recombination (intra MSV-A1) has been detected in the LIR of MSV (this study) whilst another study has reported recombination hotspots in the virion strand origin of replication in the LIR of MSV (Varsani et al. 2008).

1.4.2 Replication of geminiviruses

As geminivirus genomes are small and encode only a small number of proteins, they are dependent on host factors for their replication (Heyraud-Nitschke et al. 1995). Replication occurs mainly by a rolling circle replication mechanism (RCR) in the nucleus of infected cells via double stranded intermediates (Saunders et al. 1992). It also occurs via an alternate pathway called recombination dependent replication (Jeske et al. 2001). While some evidence suggests that recombination dependent replication may actually be the most productive route (Jeske et al. 2001), it is still unclear how much each pathway contributes to geminivirus replication.

RCR is a two step process that initially involves synthesis of a complementary strand to generate a double-stranded replicative form DNA (dsDNA) that then forms a template for the synthesis of other single-stranded DNA molecules (reviewed by Hanley-Bowdoin et al. 1999, Gutierrez et al. 2004). The Rep protein has been shown to initiate RCR by cleaving between the last T and A nucleotides inside the virion strand origin TAATATTAC sequence located in the LIR (Heyraud-Nitschke et al. 1995, Laufs et al. 1995). The cleavage results in a free 3' hydroxyl terminus which acts as a primer, and a 5' phosphorylated hydroxyl group to which Rep binds. Following RCR, virion sense genes encoding the movement protein (*mp*) and coat protein (*cp*) are expressed, and synthesized ssDNA is either encapsidated by the CP oligomers facilitating cell to cell movement, or functions as a template for synthesis of a minus strand. The *cp* and *mp* are vital for systemic infection by the virus.

1.5 Maize streak disease

MSD remains one of the most important constraints to the production of maize in sub-Saharan Africa (CIMMYT 2008). MSD occurs throughout sub-Saharan Africa and its neighbouring islands (Bock 1974, Rose 1978). First described by Fuller (1901) over a century ago in South Africa as 'mealie variegation', the disease was renamed maize streak disease by Storey (1925) who discovered it was caused by a virus. Capable of causing up to 100% yield loss if susceptible plants are infected at a very early stage (one week post emergence; Van Rensburg 1981), MSD has more serious effects on late-planted and irrigated crops (Thresh 1998). Yield losses attributed to the disease depend on the degree of susceptibility and the age at which plants are infected, with yield losses in individual fields often exceeding 70% in susceptible cultivars and only 1.5% in resistant ones (Bosque-Perez et al. 1998). For East Africa, average yield losses of 33-55% were recorded for natural infections (Cooter & Kyetere 1999), in addition to country-specific loss estimates of 68% in Uganda (Mukiibi 1976) and 33-56% in Kenya (Guthrie 1977).

Throughout sub-Saharan Africa, persistently low maize yields and erratic MSD epidemics dangerously undermine the health and social development of the world's poorest people. Despite the availability and commercial use of MSV-resistant maize genotypes for over 20 years, little progress has been made in controlling the virus in the maize fields of subsistence farmers where control is most urgently needed.

1.5.1 Symptoms of MSD

Disease symptoms are manifested initially by the development of chlorotic circular spots on the basal parts of young leaves. Subsequently, prominent white chlorotic streaking appears over and along the major veins (Storey 1925, Thottappilly et al. 1993) as shown in Fig. 1.2 B and C . The streaks, which may be broken (Fig. 1.2 B) or continuous (Fig. 1.2 C), appear only on infected and subsequent new leaves that develop (Fig. 1.3 C). Different levels of MSV infection are shown in Fig. 1.2 D, progressing from a leaf exhibiting no symptoms of MSV infection to leaves exhibiting severe chlorosis and streaking. There may be leaf necrosis, stunting and dieback if the plant is infected early or in cases of severe infection (Rossel & Thottappilly 1985), especially before six weeks in very susceptible host plants. In instances of very severe infection, affected leaves appear yellow with no or very little green area left as shown in Fig. 1.3 F. A maize field with MSV-infected plants displaying severe symptoms is shown in Figure 1.3 A while a field free of MSD is shown in Fig. 1.3 B.

1.5.2 *Maize streak virus*

MSV is transmitted in a persistent manner and has a latent period of 6-12 hours (Storey 1925, 1928). The leafhopper vector remains infective throughout its life and symptoms in infected plants appear between three days and three weeks after infection (Storey 1928). MSV is not transmitted via seeds although transmission has been

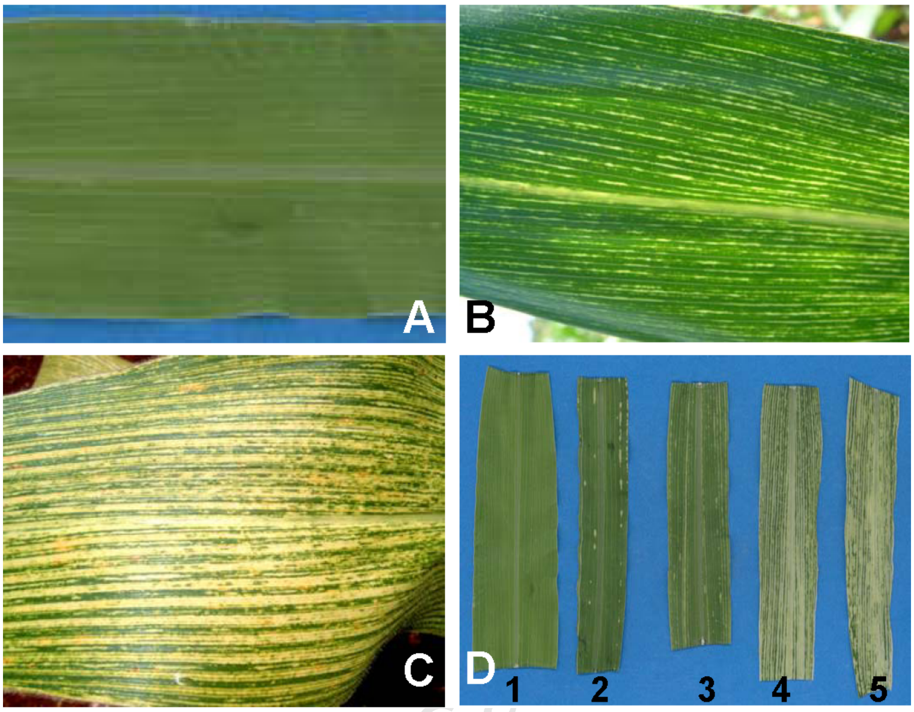


Figure 1.2: A: Section of an uninfected leaf; B: Leaf section showing moderate symptoms of MSV infection, broken streaks along the veins of the leaf; C: Severe symptoms of MSV infection showing extensive chlorosis and streaking; D: Range of symptom levels exhibited by MSV infected leaves, ranging from no symptoms (1), very mild sypmtoms (2) to severe chlorosis and streaking (5).

achieved through the vascular puncture technique (Redinbaugh et al. 2001) and experimentally through *Agrobacterium*- mediated transmission using tandem repeats of the virus genome (Grimsley et al. 1987). Apart from maize, MSV also affects other cereal crops including barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), finger millet (*Eleusine coracana*), pearl millet (*Pennisetum americanum*), oats (*Avena sativa*), sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum officinarum*). Various non-cultivated grass species including *Setaria sp.*, *Urochloa sp.*, *Digitaria sp.*, *Rottboellia sp.*, *Axonopus sp.* and *Cenchrus sp.* are also hosts of MSV (Varsani et al. 2008). In Uganda, grass species including *Eleusine indica*, *Digitaria velutina*, *Panicum maximum* and *Setaria sp.* are reported as important alternative hosts for MSV (Bigirwa 1999) whilst 11 grass species have been found to be naturally infected with MSV in Kenya (Njuguna et al. 1996). In India, viruses similar to MSV have been reported in pearl millet, wheat and weeds (Seth et al. 1972).

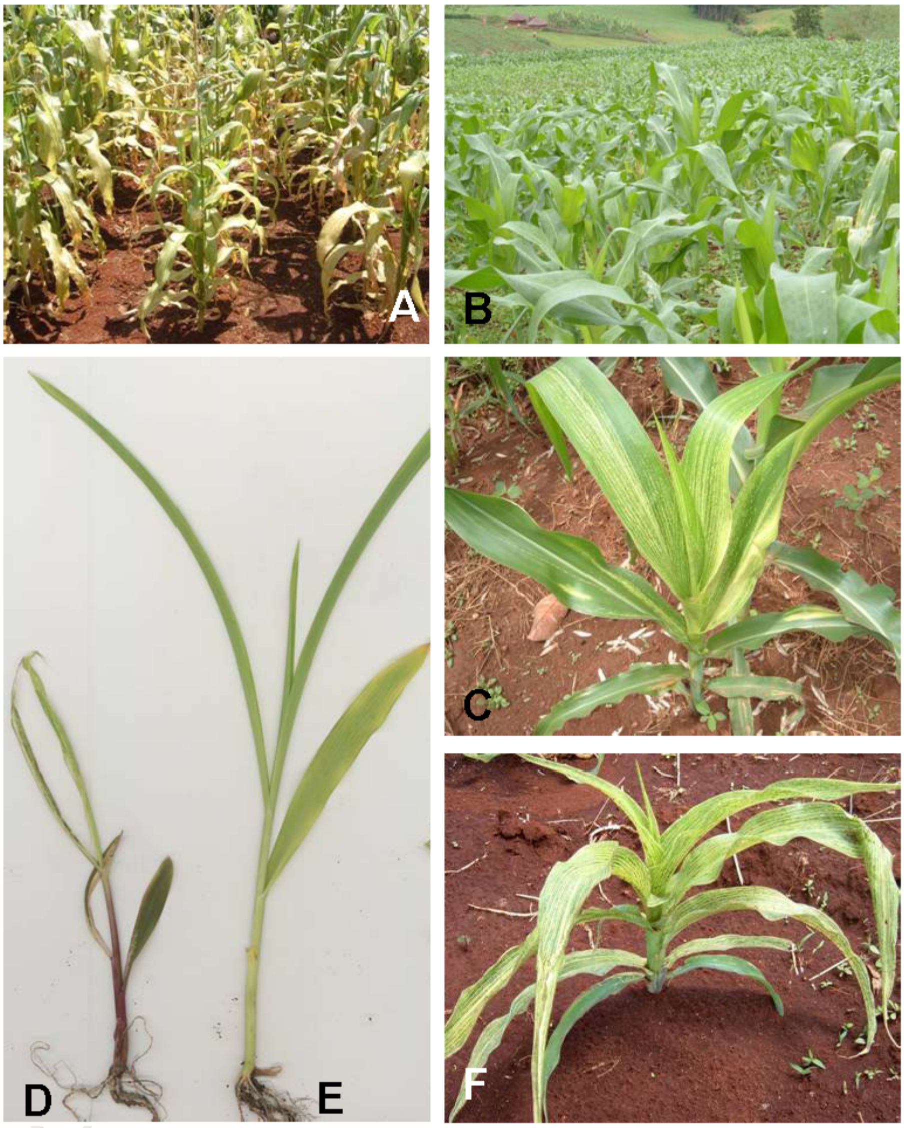


Figure 1.3: Symptoms of MSV in fields in Uganda and in agroinoculated plants. A: MSD symptoms on maize plants in the field; B: Healthy uninfected maize plants in a field free of MSV infection; C: Young maize plant exhibiting moderate MSD symptoms on top newly developed leaves; D: young maize plant at four leaf stage agroinoculated with a Ugandan MSV isolates exhibiting MSD symptoms; E: symptom-free uninoculated maize plant at four leaf stage. Note the stunting of the infected plant in plate D in comparison to the uninfected control in E; F: maize plant showing severe symptoms of MSD, with extensive chlorosis, yellowing, streaking and stunting.

1.5.3 The *Cicadulina* vector

MSV is vectored by leafhopper species (Homoptera: *Cicadellidae*, Genus: *Cicadulina Naude*) (Storey 1925), 18 of which have been reported to occur in Africa (Webb 1987) although not all 18 are vectors of MSV. Of the 18 species, nine have been reported to transmit MSV (Bosque-Perez 2000) of which *C. mbila* and *C. storeyi* are the most important (Webb 1987). Research in East Africa indicates that *C. mbila* is the most important vector of MSV in the region (Storey 1928). Furthermore, this species is mainly abundant in shade while *C. storeyi* is located mainly away from the shade (Page et al. 1999a). Transmission of geminiviruses requires several steps, the first being the acquisition of the virus from the gut into the heamocoel, followed by movement of the virus from the heamocoel into the salivary glands and subsequently injection into plant leaf tissue during feeding (Briddon et al. 1990). It has been demonstrated that the specificity of the leafhopper vector in the transmission of MSV is dependent on the heamocoel/gut barrier (Briddon et al. 1990). MSV DNA is detectable in the gut of non vector *Cicadulina* species but the gut wall interferes with passage of MSV DNA into the hemolymph thereby preventing transmission (Lett et al. 2002). Furthermore, the coat protein is the determinant of this specificity in leafhopper transmission of MSV. An interaction between the virus and GroEL protein produced by bacteria in the vector has been shown to protect Tomato yellow leaf curl virus (TYLCV) particles from degradation during their travel in the vector's heamolymph (Morin et al. 1999).

1.5.4 Diversity of MSV

Studies have suggested that maize-infecting MSVs in Africa have relatively low diversity (Briddon et al. 1994, Martin et al. 2001). Based on nucleotide sequences, less than 4% divergence has been reported although there apparently is a greater degree of diversity amongst related MSV isolates from non-maize hosts (Rybicki & Pietersen 1999). Early research in East Africa found no evidence of differences in symptom development induced by distinct isolates of MSV (Storey & Howland 1967) although their transmission efficiency varied (Bock 1980). Later on however, research based on serological data identified differences in MSV isolates from different grass species from different parts of Africa (Dekker et al. 1988) but little diversity amongst isolates from maize. Peterschmitt et al. (1991), also using serology, found no differences in maize-infecting MSV isolates from 11 African countries. Bonga (1992), however, found serological evidence suggesting variation in maize-infecting MSV isolates from Uganda, Kenya and Zimbabwe. Serology is however limited because it is better suited for showing similarities between distinct viruses, than to differentiating closely related isolates. Furthermore, the lengthy process of purifying virus particles and production of antibodies makes the method unattractive for virus detection. Despite the low diversity in nucleotide sequences of MSVs, several distinct groupings have been identified. Following recent advances in diagnostic techniques, more sophisticated PCR-generated restriction fragment length polymorphisms (RFLPs) and partial sequence analyses have been used to study diversity in African MSV isolates which enabled identification of four distinctive MSV groups, maize-infecting MSVs, wheat/grass-infecting MSV isolates, a Sugarcane streak virus group and Panicum streak virus group (Willment et al. 2001). Ever since those first two MSV isolates (Nigeria and Kenya) were sequenced in 1984 (Mullineaux et al. 1984, Howell 1984), several sequences have now been deposited in GenBank.

Some isolates of MSV such as MSV-Kom originating from South Africa, which illicits moderate symptoms in maize (Martin et al. 1999), MSV-Reu from Réunion (Briddon et al. 1994), MSV-Gat from Kenya and MSV-MatA from southern Africa, causing severe symptoms in maize (Martin et al. 1999) have been well characterized. Currently, MSV isolates have been grouped into five strains, MSV-A, B, C, D and E (Martin et al. 2001) although more recent studies have added six new strains (F-K) to this list (Varsani et al. 2008).

Of these 11 strains, MSV-A, and particularly subtype MSV-A_1 (Martin et al. 2001), is responsible for the severe disease in maize in all sampled areas across Africa. Although diversity between MSV-A viruses is low, there is strong phylogenetic support for their classification into at least six lineages or subtypes (named MSV-A_1, A_2, A_3, A_4, A_5 and MSV-A_6 that are >98% identical; Martin et al. 2001). More recently, with the availability of more sequence data, it has been found that A_5 is actually a sublineage of

A_1 (this study). There is also some evidence of variation in the subtype composition of MSV-A populations in different parts of Africa (Martin et al. 2001). In South Africa for instance, subtypes MSV-A_1 and MSV-A_4 dominate while subtype MSV-A_1 seems to prevail in other sampled areas in Africa including Uganda (this study), Nigeria, Zimbabwe and Mozambique (D.P. Martin, unpublished), making it the most widely distributed subtype.

Even small variances in diversity can have a profound effect on virus fitness. For example, Boulton et al. (1991) demonstrated that even a three nucleotide difference between mild and severe Nigerian MSV isolates resulted in a difference in severity of symptoms as well as affecting the host range of the mild isolate.

1.5.5 MSV recombination

One of the factors that plays a role in MSV evolution is recombination (Martin et al. 2001). Very frequent in occurrence amongst geminiviruses, recombination occurs between species (Zhou et al. 1997, Monci et al. 2002), within and between genera (Padidam et al. 1999) or even between virus components and satellite DNAs (Briddon et al. 2003) but it is more likely to occur between strains of a single species.

Primarily functioning as a mechanism to repair either double stranded DNA breaks or 'undo' mutations that have occurred during replication, recombination is also able to generate sequence diversity and may sometimes result in fitter or more virulent virus genotypes. An important recombination event reported from Africa is the 'Uganda variant' of East African cassava mosaic virus (EACMV-Ug), a recombinant hybrid derived from two whitefly-transmitted begomoviruses, African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV)(Deng et al. 1997). EACMV-Ug was associated with a severe disease epidemic in Uganda (Zhou et al. 1997) which subsequently spread regionally into neighbouring countries (Legg 1999, Otim-Nape et al. 2000) and now affects large areas of nine countries in East and Central Africa (Legg et al. 2006) causing great losses to the crop and continuing to pose a threat to resistant germplasm being promoted as the main control approach. Just like in begomoviruses, recombination is also rampant in the *Mastrevirus* genus (Martin et al. 2005b). Both inter and intra-species recombination have been reported in mastreviruses (Martin et al. 2001 and this study), although whilst large exchanges occur with interspecies recombination, shorter portions of nucleotides are exchanged for intra-species recombination (Martin et al. 2001). More recent data suggest that the maize-infecting MSV-A may have arisen from a recombination event between two grass viruses, MSV-B and newly identified strains MSV-F/G (Varsani et al. 2008).

1.6 Control of MSD

The development of successful resistance strategies against MSV requires knowledge of the virus, its diversity, evolution, virulence and symptoms. Various control methods have been used to reduce the impact of MSD on maize yields. The control methods that include timely planting, barriers, no planting downwind of earlier planted crops (Gorter 1953), crop rotation (Rose 1978), insecticides (Mzira 1984, Rose 1978) and varietal mixing (bean and millet) (Page et al. 1999a) reduce the leafhopper movement. While some methods reduced disease levels, others such as insecticides are costly, labour intensive and pollute the environment (Boulton 2003). Since grasses are important reservoirs for both the virus and vector (Rose 1978), weed control should therefore be considered when implementing control strategies.

Host plant resistance is one of the most effective control strategies against MSV (Rossel & Thottappilly 1988, Efron et al. 1989). Breeding for MSV resistance dates back to the 1930's in South Africa when resistance was first observed in 'Peruvian yellow', an open pollinated variety (Rose 1978). Although resistant varieties have been developed through conventional breeding, the risk of this resistance breaking down because of the quasi-species nature of MSV or cross-pollination with susceptible varieties as reported in Uganda is high (Cooter & Kyetere 1999). The use of transgenic plants to mitigate MSD is an attractive alternative approach.

1.7 Genetic engineering for the improvement of crop plants

Breeding, a lengthy and laborious procedure, has been used for centuries to improve the quality and yield of crop plants. Currently, focus has shifted to genetic engineering as an alternative or additional tool to develop virus resistant crops. The use of genetic engineering promises a shorter time and more precision for generation of disease resistance in crop plants as well as access to genes from across the species barrier previously not readily usable for crop improvement. When used to augment conventional breeding, genetic engineering offers an invaluable tool to curb debilitating diseases.

Resistance to viruses can be engineered by transforming a susceptible plant with a gene derived from the pathogen itself, a process referred to as pathogen derived resistance (PDR; Sanford & Johnson 1985). PDR in transgenic plants is protein or RNA mediated (Baulcombe 1996). PDR has been highly successful in achieving virus resistance. For example, some commercially available virus-resistant transgenic plants produced using PDR include papaya resistant to Papaya ringspot virus, squash resistant to Zucchini yellow mosaic virus and Water melon mosaic virus and tomato plants resistant to Cucumber mosaic virus (Fitch et al. 1992, Fuchs & Gonsalves 1995, Kaniewski et al. 1999). Several of the reported cases of PDR against many of these viruses occur via post transcriptional gene silencing (PTGS) (Fuchs & Gonsalves 2007). Strategies that have been used to develop virus resistance include (i) coat protein (CP)-mediated resistance (Powell Abel et al. 1986, Register & Beachy 1989) (ii) movement protein (MP)-mediated resistance (Malyshenko et al. 1993) (iii) dominant negative mutations (iv) peptide aptamers (Lopez-Ochoa et al. 2006) (v) artificial micro RNA-mediated resistance (Qu et al. 2007) (vi) antisense RNA technology and (vii) post transcriptional gene silencing (PTGS)-mediated resistance.

The CP of viruses was one of the first virus-derived genes used to generate virus-resistant transgenic plants when it was used to provide resistance to tobacco mosaic virus (Powell Abel et al. 1986, Register & Beachy 1989). Although the intended resistance was protein-mediated, in some cases this has been found to actually be RNA-mediated. For example, CP-mediated resistance developed in papaya (against Papaya ringspot virus; reviewed by Fuchs & Gonsalves 2007) is actually due to post transcriptional gene silencing (PTGS). In other cases, the resistance has been reported to be due to the inhibition of virion disassembly (Register & Beachy 1989). Regardless of the mechanism behind the resistance, CP-mediated resistance has the potential to provide resistance not only at virus level but at the level of transmission as well by exploiting the CP-virus interaction during transmission.

Another viral protein that has been used to develop virus resistance is the MP. Expression of the virus MP has been shown to provide resistance to viruses, for example TMV (Malyshenko et al. 1993, Lapidot et al. 1993, Cooper et al. 1995, Ares et al. 1998) and five potato viruses (Seppanen et al. 1997).

Modified viral proteins have also been used to develop resistance to geminiviruses. Modified rep (Anderson et al. 1992, Hong & Stanley 1996, Noris et al. 1996, Brunetti et al. 2001, Chatterji et al. 2001), *mp* (Duan et al. 1997) and the *cp* (Sinisterra et al. 1999, Schwach et al. 2004) have been used to provide virus resistance. A more recent example is transgenic maize resistant to MSV, produced by the use of dominant negative mutant and truncation of the *REP* gene (Shepherd et al. 2007a,b). Greenhouse trials so far show that resistance levels are very promising and stable in the offspring (D.N. Shepherd, personal communication).

More recently a virus resistance strategy using peptide aptamers, recombinant proteins that bind to a conserved part of the Rep protein of all known geminiviruses, therefore inactivating it, has been tested against geminiviruses and shown to reduce viral replication (Lopez-Ochoa et al. 2006).

Other than virus proteins, nucleic acids have also been used to engineer resistance to viruses. Ecker & Davis (1986) demonstrated over 20 years ago that antisense RNA is effective in controlling gene expression. For virus control, one of the early reports of antisense RNA technology in the control of Tomato golden mosaic virus in tobacco was by Day et al. (1991). Since then, plants have been successfully protected against viruses such as Tomato yellow leaf curl virus (Bendahmane & Gronenborn 1997, Yang et al. 2004, Praveen et al. 2005), Beet curly top virus (BCTV Bejarano & Lichtenstein 1994), BGMV (Aragao et al. 1998), Cotton leaf curl Kokhran virus (CLCKV; Asad et al. 2003), ACMV (Zhang et al. 2005) and Tomato leaf curl New Dehli virus (ToLCNDV; Mubin et al. 2007).

Plants produce small 21-22nt RNAs (sRNAs) referred to as micro RNAs (miRNA) from a single, long transcript that forms imperfectly base-paired hairpin structures (Carrington & Ambros 2003). These miRNAs participate in gene regulation of endogenous processes (Llave et al. 2002, Reinhart et al. 2002), by interfering with expression of mRNAs involved in the control of endogenous gene expression such as developmental timing, stem cell maintenance and other processes in plants and animals (Carrington & Ambros 2003). Expression of artificial miRNAs (amiRNAs) targeted to specific genes has been reported to induce gene silencing (Schwab et al. 2006). Similarly, resistance to viruses has been developed by designing artificial miRNAs targeted to sequences encoding a gene silencing suppressor (Qu et al. 2007).

Other strategies that have been explored for virus resistance but have not progressed beyond the laboratory are the use of antibodies (Tavladoraki et al. 1993) and defective interfering DNA (Stanley et al. 1990).

It is evident from the examples described in this section that several strategies are available and are being explored for use in the control of plant viruses. Viruses evolve quickly and these strategies represent complementary strategies to conventional breeding. While products of some of the strategies are already in use others are still at very early stages of development. Of particular interest to this study is gene silencing whose contribution to crop plant improvement has gained importance in recent years. Its potential use in controlling serious diseases is being explored for several crop plants. Whilst for some crops such as papaya and squash, progress has been tremendous with commercialization of virus-resistant plants, potential application against other viruses is still in its infancy.

1.7.1 Gene silencing

Gene silencing is an inherent form of gene regulation used by plants, animals and fungi (Bingham 1997, Fire 1999) to switch off the expression of a gene at either the transcriptional (TGS) or post-transcriptional (PTGS) levels. TGS represses transcription of the targeted gene, most likely by DNA methylation (reviewed by Razin 1998) and histone modification. PTGS prevents translation of the targeted mRNA by either translational inhibition or cleavage and subsequent degradation (reviewed by Baulcombe 2004). Collectively, this form of gene regulation is referred to as RNA silencing. The silencing phenomenon, initially observed as a hindrance during the generation of transgenic plants has gained importance and is currently a popular area of research. Fire et al. (1998) first used the term RNA interference (RNAi) to describe interference to the function of endogenous genes induced by dsRNA. This phenomenon has been reported in both animals (Fire et al. 1991) and plants (van der Krol et al. 1990) as well as fungi (Cogoni & Macino 1997).

The use of RNA to control plant gene expression was shown more than 20 years ago by Ecker & Davis (1986). Gene silencing was first observed in *Petunia hybrida* and termed co-suppression (van der Krol et al. 1990, Jorgensen 1990). Investigators attempted to enhance the colour of petunia flowers by introducing additional chalcone synthase (Chs) genes into a Chs-transgenic plant, instead they got suppression of the flower pigment. Further studies showed that the expression of both the transgene and the extra copies of Chs transgenes were suppressed (Van Blokland et al. 1994). In animals, early observations of interference with gene expression were made by Fire et al. (1991) using antisense RNA to knock down gene expression in *Caenorhabiditis elegans*. Guo & Kemphues (1995) were able to make use of antisense RNA to assess gene function by knocking down expression of the *par1* gene. However, Fire et al.

(1998) established that double stranded (ds) RNA was the substrate for RNAi and an effective inducer of silencing. In addition to demonstrating the efficacy of dsRNA in inducing silencing, the study also established that this silencing was passed on to the progeny.

The hallmark of PTGS in silenced tissue is the presence of 21-24 nt siRNAs (Hamilton & Baulcombe 1999). Whereas RNAi is triggered by direct introduction of dsRNA or siRNAs in animals, PTGS in plants, fungi and yeasts may be triggered by the over-expression of a transgene. PTGS occurs in the cytoplasm (Wassenegger 2002). The target mature mRNA produced in the nucleus is transported to the cytoplasm where instead of being translated into a corresponding protein, it is cleaved by the siRNA-directed endonucleolytic RNA-Induced Silencing Complex (RISC) of the PTGS pathway. Small/short interfering RNAs (siRNAs), ranging from 19-26nt are the effectors of RNA-mediated silencing (Hamilton & Baulcombe 1999, Hamilton et al. 2002, Voinnet 2003, Akbergenov et al. 2006). At least five classes of small RNAs are produced in plants: microRNAs (miRNAs), natural antisense transcript siRNAs (natsiRNAs), heterochromatin siRNAs (hc-siRNAs), trans-acting siRNAs (ta-siRNAs) and siRNAs from invading viruses (Vaucheret 2006).

siRNAs have 2 nt overhangs and a hydroxyl group on the 3' end as well as a phosphate group on the 5' end (Elbashir et al. 2001a). Classes of siRNAs differ in function. For example, two classes of siRNAs from transgenes (Hamilton et al. 2002), the short class, 21-22nt, and the longer class, 24-26nt, each have different roles. Whilst the longer class is involved in systemic silencing and DNA methylation, the shorter class of siRNAs is involved in mRNA degradation and translational inhibition. Precursors of siRNAs include dsRNA and hairpin RNA (hpRNA). Produced in vivo by cleavage of dsRNA precursors made up of two distinct strands of perfectly base-paired RNA from viruses, transposons or transgenes, siRNAs function to mediate gene silencing through a highly specific process. The specificity of siRNAs has been attributed to the sequence recognition involved during formation of the RNA induced silencing complex (RISC) (Matzke et al. 2004). Studies by Elbashir et al. (2001b) indicate that 21nt siRNAs are the most efficient in effecting silencing. Furthermore, the 5' end of sRNAs is important for target recognition while the nucleotides at the center of siRNAs are for cleavage. Only nucleotides 2 to 12 play an important role in specificity, with 2-8 being the recognition sequence and 9-12 the region recognized for cleavage (Haley & Zamore 2004).

Main proteins of the PTGS pathway in plants, fungi and *C. elegans* are RDRs [RNA directed RNA polymerases 1-6; RNA dependent RNA polymerases (RdRP) in RNA viruses; reviewed by Wassenegger & Krczal 2006], dicers (DCR-in animals or Dicer-like DCL in plants) and Argonaute (Ago1-10 in plants, Ago1 in *Schizosaccharomyces pombe*, 1-27 in *C. elegans*).

1.7.1.1 Dicer

One of the key components of the PTGS pathway is a conserved dsRNA-specific RNAse III-like enzyme termed dicer in animals or dicer-like (DCL) in plants (Bernstein et al. 2001b, Timmons 2002). The dicer specifically cleaves dsRNA into 21-24 nt size specific siRNAs. Both siRNAs and miRNAs are produced by a dicer (Yu & Kumar 2003) from long dsRNA and foldback or highly structured RNAs respectively. The major conserved features of this enzyme include an RNase III-like domain, two dsRNA binding domains and a protein-protein interaction domain (Lecellier & Voinnet 2004). Plants, for example Arabidopsis, have a minimum of four DCLs enzymes (Schauer et al. 2002, Margis et al. 2006). All four Arabidopsis DCLs process replicating viral RNAs into siRNAs with functional hierarchy: DCL4>DCL2>DCL3>DCL1 (Ding & Voinnet 2007, Deleris et al. 2006, Fusaro et al. 2006). Although DCL1 is capable of producing siRNAs from dsRNA (Qi et al. 2005b) and miRNAs from the stem loop of pre-microRNAs (Park et al. 2002), its major function is to produce miRNAs. DCL2 produces 22 nt siRNAs from viral dsRNA when DCL4 is suppressed (Bouche et al. 2006) and 24 nt siRNAs from dsRNA derived from stress related natural antisense transcripts (Borsani et al. 2005). DCL1 produces both endogenous siRNAs and miRNAs whilst DCL2, DCL3 and DCL4 produce only siRNAs (Bouche et al.

2006). DCL4 in particular is responsible for 21 nt siRNAs derived from virus and inverted-repeat transgenes (Dunoyer & Voinnet 2005) but in the absence of DCL4, DCL2 comes into play, producing 22 nt size class of siRNAs (Du et al. 2007). DCL3 generates the RDR2-dependent 24 nt siRNAs from transposons, repeat associated sequences and heterochromatin (Xie et al. 2004). Small RNAs derived from imperfect dsRNA duplexes or foldbacks in plants and animals mediate development processes by repressing translation (Vaucheret et al. 2004, Bartel 2004).

Virus-derived small RNAs have been reported for many plant-virus systems. For example Akbergenov et al. (2006) have characterized and found 21, 22 and 24 nt small RNAs of both sense and antisense polarities from two geminiviruses, Cabbage leaf curl virus (CLCV) and ACMV. They report that the detected RNAs are derived from both coding and non-coding regions of the virus genome. Furthermore, while 22 and 24nt siRNAs are more abundant for virus derived siRNAs, 21nt RNAs are more abundant for the transgene-derived small RNAs. Similarly, Deleris et al. (2006) have shown that DCL2 and 4 are responsible for producing 22 and 21nt siRNAs from Tobacco rattle virus (TRV) and Turnip crinkle virus (TCV). Molnar et al. (2005) have also reported virus-derived siRNAs following infection with Cymbidium ringspot tombusvirus (CymRSV) and TMV. Production of virus-derived siRNAs indicates that siRNAs play a key role in limiting virulence of viruses in susceptible plants and are a potential tool to engineer resistance.

1.7.1.2 RNA induced silencing complex (RISC)

The RISC is a vital step in the PTGS pathway where, in plants, the Argonuate1 (AGO1)-siRNA-directed slicing of target mRNA takes place. The complex contains a single short RNA strand bound to an endonuclease protein from the argonaute family (AG01) (Tuschl 2003) and exonucleolytic nucleases (Hutvagner & Zamore 2002). The Argonaute protein, a key component of the RISC, contains four domains: N-terminal, PAZ (Piwi/Argonaute/Zwille), middle and PIWI domains involved in nucleic acid binding as well as slicer activity (reviewed by Tolia & Joshua-Tor 2007).

Two of the domains, PIWI and PAZ, are RNA-binding, interacting with siRNAs to facilitate complementary base-pairing (reviewed by Song & Joshua-Tor 2006). The crescent shaped structure of the hat-like PAZ domain, with a groove at one end where the 3' 2-nt overhang of the antisense siRNA is bound, sits on PIWI. The PIWI contains the RNase H-like domain (conserved aa D-D-H) that effects the endonuclease activity. Named after the argonaute (AGO) Arabidopsis mutant phenotype, multiple argonuate proteins have been identified in a single species, many often with specialized functions. Ten (AGO1-10) argonautes have been identified in plants (reviewed by Carmell et al. 2002). Of these, *AGO1*, identified in *Arabidopsis* (Bohmert et al. 1998), is a slicer that binds 21nt sRNAs (Baumberger & Baulcombe 2005) as well. *AGO4* binds 24nt sRNAs and is involved in DNA methylation and TGS (Li et al. 2008). In *Drosophila*, Argonaute2 *(AGO2)* is part of the RISC (Hammond et al. 2001) whilst *AGO1* from the fission yeast *Schizosachromyces pombe* (Sigova et al. 2004) is involved in both PTGS and TGS in yeast.

The RISC complex ranges in size from a minimum of 160-500 kDa, although its protein composition is not yet entirely known (Hammond et al. 2001). The RISC complex is assembled on one strand of the duplex dicer-produced siRNAs and activated by cleavage of the passenger strand (Matranga et al. 2005, Leuschner et al. 2006). The type of argonaute and the complementarity of guide siRNA to target mRNA determine whether RISC activity is endonucleolytic, translational repression, deadenylation and or sequestration to P-bodies (Valencia-Sanchez et al. 2006).

In animals, it has been shown that miRNAs are preferentially loaded into RISC in comparison to siRNAs (Gregory et al. 2005). RISC is activated by the ATP-dependent unwinding of the siRNA duplex. Once the partly complementary single stranded mRNA is incorporated into the complex, it is cleaved by argonaute (Rand et al. 2005) in the activated RISC. In *Arabidopsis*, loading of miRNAs into RISC is determined by the most 5' nucleotide. Dicers Dcr-1 and Dcr-2 are both involved in loading of siRNAs into the RISC in Drosophila (Lee et al. 2004). The low internal stability at the 5' end of the duplex small RNA determines the strand that gets incorporated into the RISC in Drosophila and animals (Reynolds et al. 2004).

1.7.1.3 The silencing pathway

Gene regulation can be achieved by the control of translation or degradation of mRNAs of the target gene. The pathways through which these controls are achieved share common features (Fig. 1.4). Small RNAs such as miRNAs and siRNAs participate in effecting this gene regulation (reviewed by Baulcombe 2004). Double stranded RNAs endogenous or exogenous, produced either from invading viruses (Wassenegger 2002), pre-microRNA, transposons, transgene inverted-repeats, repeated elements or by the action of RDRs initiate the PTGS pathway when processed by dicers. In plants, the PTGS pathway is initiated by dicer-specific cleavage of dsRNA produced from; aberrant mRNAs by RNA-directed RNA polymerases (RDRs), engineered RNA foldbacks from inverted-repeats, or highly structured regions of invading RNA viruses (Fig. 1.4). The RDR6, also referred to as SDE1 and SGS2, encodes a putative RdRP in *Arabidopsis thaliana* and is required for transgene silencing (Dalmay et al. 2000). RDR6 is one of the six RDRs and amplifies the silencing signal by priming target mRNA or spliced target mRNA with primary siRNAs primers, producing more dsRNAs that are acted upon by DCLs. The duplex siRNAs are loaded onto the RISC where the sense strand is destroyed and leaving the antisense strand to act as a guide (Hammond et al. 2000, Kusaba 2004). The stability of the 5' end of the duplex determines the strand that gets incorporated into RISC (Jackson et al. 2003). The antisense strand of the duplex siRNA guides the RISC assembly, through base pairing, to a complementary region of target mRNA. The AGO component of RISC cleaves the mRNA at nucleotides 10 and 11 at the 5' end of the siRNA (reviewed by Kusaba 2004, Matzke et al. 2004, Reynolds et al. 2004). After the slicing of the mRNA, the RISC is recycled while the cut mRNA fragments are degraded by cellular exonucleases (Hutvagner & Zamore 2002).

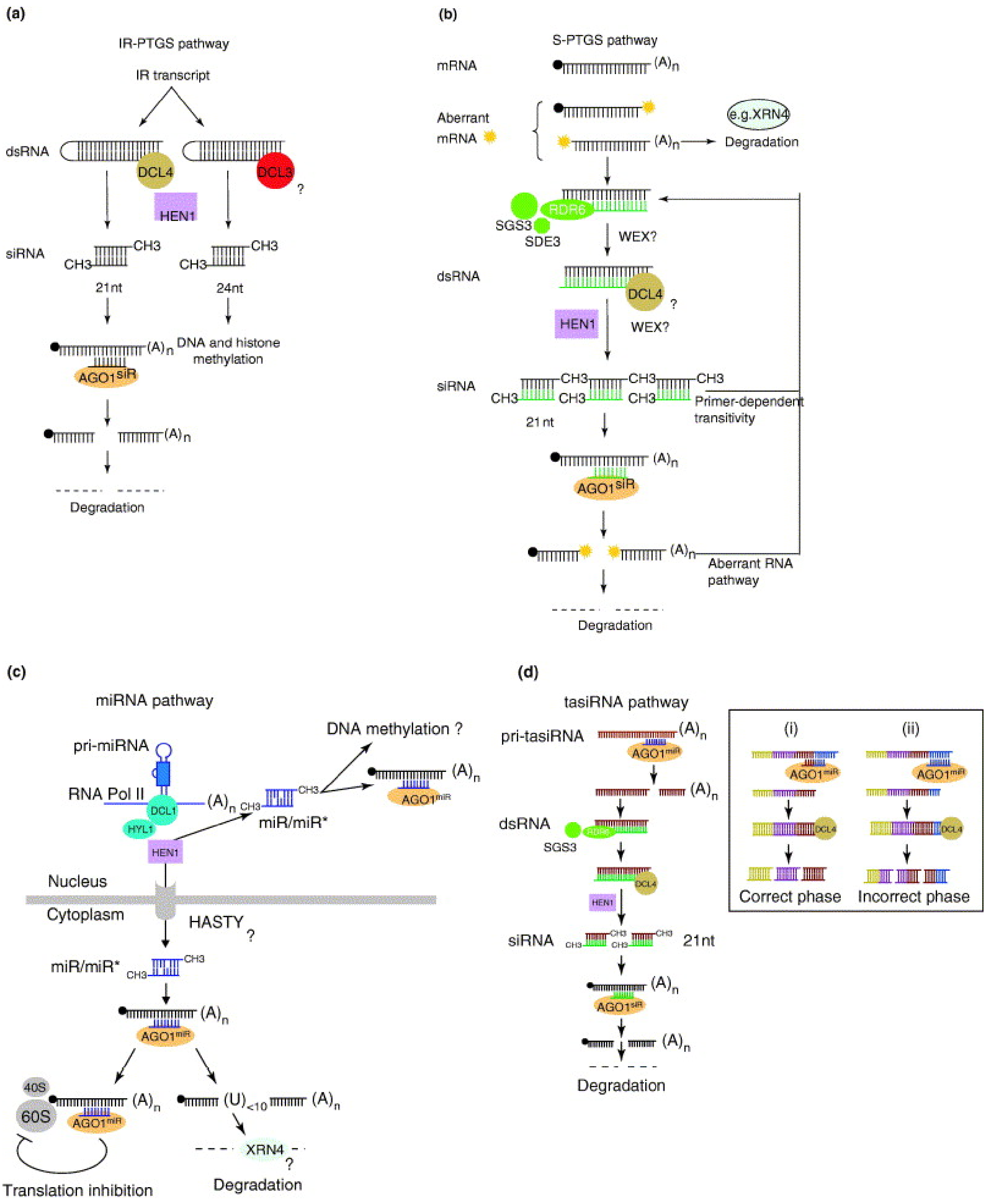


Figure 1.4: miRNA and siRNA pathway and associated proteins (Brodersen & Voinnet 2006). Double stranded RNA either from intermediates of replicating viruses, highly structured RNA viruses, trangene inverted-repeats or products of RDRs initiate the PTGS pathway when they are cleavaged by RNaseIII family dicer-like enzymes into 19-21 nt siRNAs. The duplex siRNA is incorporated into RISC. Within RISC, the duplex is unwound and the antisense RNA strand is liberated to act as a guide, through complementary base pairing with the target mRNA. The target mRNA is sliced by the Ago-dependent RNAse H-like activity. The sliced mRNA fragments are channeled into the exosome-mediated RNA decay pathway. HEN; Hua enhancer1; AGO1: Argonaute1; NRPD: Nuclear RNA polymerase IV; SDE3: Silencing defective3; WEX: Werner exonucleases; SGS3: Suppressor of gene silencing3; DCL: Dicer-like.

1.7.1.4 The silencing signal, its movement and transitivity

PTGS in plants operates at two levels, locally where silencing occurs at the cell level or systemic where the silencing spreads from the site of initiation to distal parts (Hamilton et al. 2002).

The silencing signal follows the source-sink transport system moving uni-directionally from source to sink tissues (Palauqui et al. 1997, Waterhouse et al. 2001) although it has also been suggested to move bidirectionally. This bidirectional movement was demonstrated when it was shown that even older leaves below the infiltrated leaf showed silencing though to a lesser extent compared to newly emerging leaves (Voinnet et al. 1998, Ming et al. 2006). The movement of the signal from the initiation site to distal parts of the plant occurs via the vasculature (Voinnet et al. 1998, Vance & Vaucheret 2001), with long distance movement occurring in the phloem while cell to cell movement is via the plasmodemata (Voinnet et al. 1998, Himber et al. 2003).

It has been proposed that the silencing signal is a nucleic acid (Voinnet & Baulcombe 1997) and is sequence-specific (Voinnet et al. 1998). By implication, for the signal to effect silencing, it has to be homologous to the target mRNA. Since the silencing signal is sequence specific, it is most likely an RNA species (Kalantidis 2004). siRNAs, abberrant mRNAs and double stranded RNA have been proposed as likely candidates (Mlotshwa et al. 2002) although to date no particular RNA species has been identified. The long class of siRNAs (24-26 nt) participates in systemic signaling (Waterhouse et al. 2001, Hamilton et al. 2002).

In plants, the silencing signal is amplified via an RDR-dependent pathway during transgene silencing. Silencing is boosted by the production of RDR6-dependent secondary siRNAs (Sijen et al. 2001, Himber et al. 2003) from regions not initially targeted by the primary inducer of PTGS. The concomitant amplification and spread of silencing is referred to as transivity. Key players involved in amplification of silencing include DCL-2 (Mlotshwa et al. 2008), DCL-3, RDR2, RNA IV polymerase (Brosnan et al. 2007) and RDR6 (Dalmay et al. 2000). A protein, *CLASSY1* interacts with both RDR2 and RNA-polymerase for movement of the silencing signal from cell to cell (Smith et al. 2007). Transitivity in plants is bidirectional, occurring in both the 5-3' and 3-5' direction (Braunstein et al. 2002, Petersen & Albrechtsen 2005). Although in *C. elegans*, secondary siRNAs produced target a particular transgene, they have been reported to extend their effect to cellular genes as well (Sijen et al. 2001). Small RNAs produced by transivity are exclusively 21nt long (Himber et al. 2003).

1.7.1.5 Virus-induced silencing

The majority of plant viruses contain RNA genomes while a few contain DNA. Most RNA viruses replicate via dsRNA intermediates whereas others exist as dsRNA (Murphy 1996), a substrate for PTGS. On the other hand, single stranded (ss) DNA viruses such as Geminiviruses, replicate via double stranded (ds) DNA intermediates (Abouzid et al. 1988) that are transcribed bidirectionally (Gutierrez 2000a). RdRp uses RNA as a template for the synthesis of a complementary strand, which strand anneals to the template resulting in dsRNA (Tang & Galili 2004). Virus-encoded RdRps (Dorssers et al. 1984) are used to synthesize the dsRNA replicative intermediate while transgene-induced PTGS relies on plant-encoded RDR6 for dsRNA production (Dalmay et al. 2000).

RNA viruses can trigger PTGS through their dsRNA replicative intermediates or the highly structured regions of the genome (Ratcliff et al. 1997). This mechanism referred to as virus induced gene silencing (VIGS) is an important tool for high throughput reverse or fast forward genetics to determine gene function (Ruiz et al. 1998b). Several VIGS vectors such as Potato virus X (PVX), Tobacco mosaic virus (TMV), Tomato golden mosaic virus (TGMV) and Tobacco rattle virus (TRV) (Ratcliff et al. 2001) have been developed by fusing target genes to virus backbones and used in functional genomics studies. PVX has by far been the most commonly used VIGS vector for several silencing studies.

1.7.2 Gene silencing for virus control in crop plants

The gene silencing phenomenon has found application in both plants and animals. It has been used to study gene function in plants (Fire 1999), improve nutritional quality of crops (Liu 2002, Ogita et al. 2003, Houmard et al. 2007), for suppression of undesirable phenotypes and to control tissue specific stimuli (reviewed by Kusaba 2004). PTGS, in addition to playing an important function in developmental processes, is useful in controlling debilitating viruses.

Different virus genes have been targeted for silencing (Table 1.2) although some have proved more efficient than others. While some studies have targeted the promoter region for silencing (Pooggin et al. 2003, Vanderschuren et al. 2007b), more than half have targeted genes associated with virus replication (Chellappan et al. 2004b, Akashi et al. 2001, Vanitharani et al. 2003, Zhang et al. 2005, Varma & Praveen 2006) (Table 1.2). For ACMV, for example, targeting of the *REP* gene blocked both viral and Rep accumulation in cultured plant cells (Vanitharani et al. 2003) and reduced viral replication by 98% in transgenic plants (Chellappan et al. 2004b), while Vanderschuren et al. (2007b) targeting the bidirectional promoter of ACMV DNA-A showed recovery of infected transgenic plants and attenuated disease symptoms in cassava. Zhang et al. (2005) have also shown reduced DNA viral replication in cassava by targeting the *REP* (AC1), TrAP (AC2) and REn (AC3) as have Vanitharani et al. (2003), who showed that targeting the AC1 gene particularly reduced ACMV replication by more than 60% and AC1 mRNA levels by 90%. Other studies have targeted the coat protein (Ingelbrecht et al. 1999, Kamachi et al. 2007), the pre-coat protein expressed from the AV2 gene (bipartite begomoviruses) (Mubin et al. 2007) or even suppressors of silencing (Fagoaga et al. 2006).

Even though RNAi-mediated resistance is homology-dependent, resistance to heterologous isolates of the same virus (Fermin & Gonsalves 2004) and different species of viruses (Chellappan et al. 2004b) have been reported although in the latter study, resistance against heterologous viruses was not nearly as efficient as against the cog-

Table 1.2: Selected examples of viruses targeted by RNA silencing-based resistance

Virus Species	Plant	Target for silencing	Reference
Vigna mungo yellow mosaic virus	Black gram	Promoter and common region	Pooggin et al. (2003)
African cassava mosaic virus	Cassava	Bidirectional promoter	Vanderschuren et al. (2007b)
African cassava mosaic virus	Cassava	*REP* gene (AC1)	Chellappan et al. (2004b)
African cassava mosaic virus	*Nicotiana benthamiana*	*REP* gene (AC1)	Vanitharani et al. (2003)
African cassava mosaic virus	Cassava	*REP*(AC1), TrAP (AC2), REn (AC3)	Zhang et al. (2005)
Tomato leaf curl virus	Tomato	*REP*	Praveen et al. (2006), Varma & Praveen (2006)
Potato virus Y	Potato	-	Han et al. (1999)
Sugarcane mosaic virus	*Saccharum officinarum*	Coat protein	Ingelbrecht et al. (1999)
Citrus tristeza virus	Citrus	p23 suppressor	Fagoaga et al. (2006)
Cotton leaf curl virus	Tobacco	*REP*, TrAP, REn	Asad et al. (2003)
Cucumber green mottle mosaic virus	*Nicotiana benthamiana*	Coat protein	Kamachi et al. (2007)
Rice tungro bacilliform virus	Rice	Open reading frame IV	Tyagi et al. (2008)
Bean golden mosaic virus	Beans	*REP*(AC1)	Bonfim et al. (2007)
Tomato leaf curl virus	Tomato	non coding region	Abhary et al. (2006)
Tomato leaf curl virus	Tomato	AC1 and AC4	Ramesh et al. (2007)
Tomato leaf curl virus	Tomato	AC1	Fuentes et al. (2006)
Tomato wilt spotted virus, Tomato chlorotic spot virus, Groundnut ringspot virus, Watermelon silver mottle virus	*Nicotiana benthamiana*	nucleoprotein genes	Bucher et al. (2006)
Tomato yellow leaf curl virus	*Nicotiana benthamiana*	Coat protein	Zrachya et al. (2006)
Tomato yellow leaf curl New Delhi virus	Tobacco	AV2	Mubin et al. (2007)

nate virus. However, a >90% similarity between transgene and the targeted virus guarantees a high degree of silencing (Fermin & Gonsalves 2004).

Viruses from the *Potyviridae* and other virus families have also been targeted for control using strategies that we now know involve gene silencing. For many of the studies, the coat protein was targeted to achieve resistance [Sugarcane mosaic virus (Ingelbrecht et al. 1999), Tobacco etch virus (Lindbo et al. 1993), Papaya ringspot virus (Fitch et al. 1992), Zucchini yellow mosaic virus, Watermelon mosaic virus (Fuchs & Gonsalves 1995)]. To date, transgenic papaya resistant to Papaya ring spot virus remains one of the best known commercial examples where successful virus resistance has been achieved and this resistance has now been shown to be due to RNA silencing (Fuchs & Gonsalves 2007) although at the time this resistance was developed the intended resistance strategy was coat protein-mediated resistance (Fitch et al. 1992).

Clearly, gene silencing has enormous potential for crop improvement and engineering successful virus resistance. Geminiviruses have been targeted for control using gene silencing previously and are ideal targets for this resistance strategy because, they naturally trigger gene silencing. Gene silencing has however, not been assessed for development of resistance to MSV.

1.7.3 Suppressors of gene silencing

Gene silencing is a plant's defensive response against invasion by foreign nucleic acids such as that from viruses. To counter this defense system, some pathogens produce suppressors of gene silencing (Smyth 1999). Viral suppressors interfere with one or more steps in the PTGS pathway (Waterhouse et al. 2001, McManus & Sharp 2002). Apart from acting as suppressors, these proteins often also participate in other functions such as viral replication, cell to cell movement, vector transmission (Plisson et al. 2003), genome encapsidation, virus accumulation, seed transmission and pathogenicity (Brigneti et al. 1998, Voinnet et al. 1999).

Several silencing suppressors have been reported (Table. 1.3) although detailed mechanism of action for some of them remain uncharacterized. Merai et al. (2006) have proposed that most suppressors act by binding small dsRNA and this was possibly the original mode of action of many suppressors before they diverged with time. Suppressor proteins may be distributed to all tissues of infected leaves or restricted only in the veins of newly emerging leaves (Voinnet et al. 1999).

Some suppressors may cause reversal of silencing in tissues where silencing is established, prevent initiation of silencing in new tissues or even prevent spread of systemic silencing signals in distal tissues. Others act by interfering with the action of the dicer, prevent loading of siRNA into the RISC or even block the action of RdRP in generating more dsRNA. The efficiency of some suppressors such as P0 may vary in different viruses (Pfeffer et al. 2002).

More than one suppressor may be encoded by a single virus in some cases. Merai et al. (2006) have demonstrated that suppressors may be classified depending on the how they bind to duplex siRNAs: size-dependent (P19, P21, P15 and HC-Pro) that bind only 21nt siRNAs and size-independent (P14 and the *Turnip crinkle virus* coat protein) that bind both short and long siRNAs.

Suppressors have also been shown to act together in mixed virus infections (for example ACMV and EACMV), with a result of more severe disease symptoms (Vanitharani et al. 2005). There are presently four suppressors, AC2/C2, βC1, AC4/C4 and V2 proteins, characterized from the *Geminiviridae* (Sharma & Ikegami 2007). The βC1 is a protein from satellites that have been associated with some geminivirus infections (Briddon et al. 2001). A review by Bisaro (2006) provides a comprehensive overview of suppressor activities of geminivirus proteins AC2 and AC4 and deduces that geminivirus suppressors possibly operate via transcription-dependent or independent pathways. For transcription-dependent suppression, suppressors such as AC2 from ACMV possibly act by inducing expression of host proteins that prevent an active silencing mechanism against the invading virus.

Table 1.3: Selected suppressors of gene silencing

Virus/Genus	Suppressor	Mode of action	Reference
Dianthovirus	P27/P88/viral RNA complex	DCL1 synthesis of siRNA	Takeda et al. (2005b)
Tomato bushy stunt virus	p19	binding duplex siRNAs	Wang et al. (2000), Silhavy et al. (2002), Zamore (2004)
Aureuvirus	P14	binding duplex siRNAs and long dsRNA	Merai et al. (2005)
Potato virus Y	Hc-Pro	interference with dicer activity	Llave et al. (2000)
Sobemovirus	P1	initiation and maintenance of silencing	Sarmiento et al. (2007)
Beet western yellow mosaic	P0	interference with dicer, systemic silencing , degrades *AGO1*	Pfeffer et al. (2002), Baumberger et al. (2007)
Cucumber mosaic virus	2b	movement of silencing signal, binding siRNAs and dsRNA	Brigneti et al. (1998), Guo & Ding (2002), Wassenegger (2002)
Grape vine virus	P10	binds single and ds siRNAs and miRNAs	Zhou et al. (2006)
Citrus tristeza virus	P20, P23	-	Lu et al. (2004)
Beet yellow virus	p21	-	Reed et al. (2003)

Continued…

Table 1.3 – Continued

Virus/Genus	Suppressor	Mode of action	Reference
Turnip crinkle virus	P38	interference with dicer	Qu et al. (2003), Thomas et al. (2003)
Potexviruses	p25	blocks systemic silencing	Voinnet et al. (2000)
ACMV, *Sri Lankan cassava mosaic virus*	AC2	bind siRNAs	Voinnet et al. (1999), Vanitharani et al. (2004)
Indian cassava mosaic virus	AC4	-	Vanitharani et al. (2004)
Rice yellow mottle virus	P1	-	Voinnet et al. (1999)
Tomato yellow leaf curl China virus	C2	eliminates siRNAs	Wezel et al. (2002)
Tomato golden mosaic virus	AL2	Inhibits activity of adenosine kinase	Wang et al. (2004)
Bhendi yellow vein mosaic virus	C4, βC1	-	Gopal et al. (2007)
Tomato yellow leaf curl virus	V2	-	Zrachya et al. (2007)
Tomato yellow leaf curl Java virus	C2, βC1	-	Kon et al. (2007)

The various studies described above show that suppressors of silencing act at different stages in the silencing pathway and are encoded by different proteins in different viruses. Although these suppressors have been identified for some geminiviruses, there is no published information for suppressors from other virus groups such as from mastreviruses.

1.8 Goals of this research

Viruses continue to cause great losses to agricultural products worldwide despite the efforts directed at reducing these losses. As sources of resistance remain scarce, transgenic-based approaches could offer many novel resistance options as part of a comprehensive integrated approach to disease control. There is currently widespread hesitation and skepticism when it comes to transgenic crops. This however should not undermine its potential contribution to the improvement of important crops. Pathogens in general but viruses in particular, undergo mutation and recombination events that result in the potential risk of more virulent genotypes emerging that are able to overcome existing control measures. An effective disease control strategy therefore hinges on thorough knowledge of the pathogen being targeted, its pathogenecity, diversity, recombination frequency and distribution in targeted areas. Rodier et al. (1995) have shown on the Indian Ocean Island of Réunion, for example, that maize varieties resistant to MSV in one location are susceptible to the virus in other locations. This reiterates the need to generate information on existing MSV strains, their diversity and geographical distribution to ensure that when resistance packages are designed, they will hold for all strains in all geographical regions.

Although significant progress has been made towards understanding the diversity of MSV occurring in many maize-producing areas in sub-Saharan Africa, there is currently no published information on MSV diversity virus in many maize-growing regions including Uganda.

The study described here set out firstly, to establish MSD levels in Uganda and describe the genetic characteristics of MSV isolates in this country. Secondly, it aimed to evaluate a novel technology for large scale sampling, storage and isolation of nucleic acids from MSV-infected plants in sub-Saharan Africa. Its third objective was to assess the feasibility of gene silencing as a tool for developing resistance to MSV. Through characterizing MSV strains that occur in Uganda, it was anticipated that key information would be produced that will help our understanding of factors that continue to favour MSV and cause such high losses in maize yields which occur in this country. Most importantly, knowledge gaps on the diversity of MSV strains in Uganda will be bridged.

Chapter 2

MAIZE STREAK DISEASE IN UGANDA

2.1 Summary

A survey was conducted in the main maize-growing areas of Uganda in May/June 2005 to assess the status of maize streak disease (MSD). In a total of 156 farmers' fields from three regions [western (55); central (51); eastern (50)], MSD incidence and severity were assessed, the cultivar of maize being grown was noted and field locations were recorded using a global positioning system (GPS). Average MSD incidences ranged from 1.9% in Kapchorwa District in eastern Uganda to 43% in Luwero District in the central region. Overall incidence was 30%. Average disease severity (on a six-point scale from 0 to 5) was lowest in Kapchorwa (1.3) and highest in the western Ugandan district of Bushenyi (2.9). Country-wide, there was a positive correlation between MSD incidence and severity levels ($P= 2.23 \times 10^{-6}$). Maize cultivars bred for MSV resistance among other characteristics, were recorded in 28% of the fields visited, with Longe I being the most frequent. Intercropping practices had no significant effect on MSD incidence or severity compared with sole-cropped maize. Agroinoculation of differentially resistant maize genotypes with infectious clones of 15 MSV isolates representing the entire breadth of diversity in Uganda indicated that, even in MSV resistant maize, all but two of the isolates cause moderate to

severe MSD symptoms. The results presented in this study indicate a higher disease incidence and severity since the last such survey in 2001, particularly in major maize growing areas. Associated maize yield losses will have a significant economic impact and highlights the need for updated farming practices in Uganda.

2.2 Introduction

Uganda, like many countries in sub-Saharan Africa, depends on maize as a key contributor to the daily calorific intake of its growing population. Maize is grown in almost all parts of the country, with most production concentrated in the west, east and north of the country. Iganga District (eastern Uganda) contributes 10% of the total maize crop produced in the country while the central and western districts of Luwero and Kasese are also important producers (Ntege-Nanyeenya et al. 1997).

Maize yields in Uganda and elsewhere in Africa are significantly lower than the rest of the world. An important contributor to these low yields is MSD, which is endemic to the continent and is prevalent in all maize-growing areas of sub-Saharan Africa. Three surveys in 1994, 1995 and 1996 in Uganda identified MSD as one of the most important maize diseases in the country (Bigirwa et al. 2001). A later study further confirmed MSD as one of the three most important diseases affecting maize production (Okori et al. 1999).

With reported yield losses in excess of 70% in susceptible maize cultivars (Bosque-Perez et al. 1998), MSD is more severe in younger plants and its incidence is higher in irrigated crops. In some cases of severe infection, total crop losses can occur. Like many plant virus diseases worldwide, MSD is a complex problem posing several challenges to mitigation strategies.

Published studies testing for associations between MSD incidence, agricultural practices and the use of MSV-resistant maize varieties are almost completely absent from the literature and data that could be used in meta-analyses of the problem are scarce even for the most advanced African countries such as South Africa. Such data will be absolutely necessary for development and deployment of effective control strategies. Where MSD mitigation strategies are in place, such as in South Africa, they have been largely successful, with the use of MSV resistant varieties and insecticides targeting the insect vector of MSV playing a pivotal role. Sustainable management of MSD in Africa may therefore require an approach that combines various control methods. The development of such integrated approaches and scientific evaluation of their efficacies will depend heavily on accurate country-wide MSD incidence and severity data.

Accordingly, this study sought to survey the cropping practices of Ugandan maize farmers and describe the incidence and severity of MSD in that country. In addition, infectious virus clones representing the entire range of MSV genotypes found in Uganda have been tested in highly susceptible, moderately susceptible and resistant maize genotypes to characterize their relative virulence.

2.3 Materials and Methods

2.3.1 Field survey areas

Representative districts that are high and moderate maize production areas were identified in eastern, western and central Uganda based on discussions with Uganda National Cereals Programme personnel. A survey was conducted in these regions during the first and main maize growing season in May/June 2005. In each of the central, western and eastern regions, 51, 50 and 55 fields were sampled respectively. Selected districts included Bushenyi, Kasese and Masindi for the western region, Luwero and Nakasongola for the central region and Kapchorwa and Iganga for the eastern region. In each district, 10 fields were visited with the exception of Kasese and Iganga where 11 and 12 fields were visited, respectively. A varying number of maize fields were also visited in other districts en route to and around the selected districts and included: Bugiri, Busia, Jinja, Mukono, Pallisa, Sironko, Tororo, Mbale in the eastern part of Uganda; Wakiso and Mpigi in the central region; and Hoima, Kabarole, Kiboga, Masaka, Mbarara and Mubende in western Uganda.

2.3.2 Data and sample collection

A total of 156 fields were visited during the survey. The road network in the sampled areas is limiting and for this reason, a semi-systematic sampling approach was adopted in which stops were made at 10km intervals along main roads. Additional criteria for sampling were that the maize field was >0.4 ha in size and from 1 to 3.5 months old. In each of the fields visited, 30 plants across two diagonals in the field were assessed for the presence or absence of MSD symptoms to ascertain MSD incidence. MSD symptom severity was also recorded for each of the 30 plants using a six point scale (0-5; Table 2.1; Bosque-Perez & Alam 1992). MSD incidence for each field was the proportion of symptomatic plants expressed as a percentage of the total number of plants. Mean severity for each field was the average of the severity values for symptomatic plants. Severity scores (see Table 2.1) for all infected plants in each district were used to compute average district severity for the seven selected districts. In each field visited, the farmer was asked to name the maize genotype growing in the plot. Note was made of the cropping practices as well as intercrops.

2.3.3 Survey data analyses

Incidence data for all fields were used to compute the mean MSD incidences for each district. Severity scores for all infected plants in each district were used to compute average district severity. Severity and incidences for each field were used in conjunction with the GPS coordinates to construct distribution maps. Data were analyzed using Microsoft Excel and SigmaStat (Systat Software Inc, California, United States of America). Mann-Whitney tests and Kruskal-Wallis one way ANOVA were used to perform statistical tests and correlation analysis was performed using Pearson

Table 2.1: MSD scoring scale

Rating scale	Description
0	No symptoms
1	Very few streaks on leaves
2	Light streaking on old leaves, gradually decreasing on young leaves
3	Moderate streaking on old and young leaves, slight stunting
4	Severe streaking on about 60-75% of leaf area, plant stunted
5	Severe streaking on more than 75% leaf area, plant severely stunted or dead

After Bosque-Perez & Alam (1992)

Product Moment Correlation (SigmaStat statistical package).

2.3.4 Assessment of symptom characteristics of Ugandan MSVs

To investigate the symptom characteristics of Ugandan MSV isolates, sequenced isolates (from Chapter 4) were made agro-infectious and used to infect three-day old maize seedlings. Three maize genotypes commercially obtained: Golden Bantam (Millington Seed Co. USA), Sweetcorn (cv. Star 7714) (Starke Ayres, Cape Town) and Pan77 obtained from PANNAR (pty) Ltd (Greytown, South Africa) representing susceptible, moderately susceptible and resistant genotypes were infected with Ugandan MSVs.

2.3.4.1 Construction of agro-infectious Ugandan MSV clones

Ugandan MSV isolates were made agro-infectious as described by Grimsley et al. (1987) and Martin & Rybicki (2000). Sequences of isolates used have been deposited in GenBank and are presented in Table A.1 (Appendix). Briefly, full length monomeric MSV isolates cloned into pGEMZ3f+ (described in Chapter 3) were each made into dimers by digestion of 1-2 μg DNA of each clone with *Sca*I for 2 hrs followed by a partial digest using 1 unit of *BamH*I for 15 mins (Palmer 1997). Following the digest, products were electrophoresed through a 0.8% agarose gel and DNA fragments 4.0 and 4.5 Kb in size were excised, purified from the gel using a GFX gel purification kit (GE Healthcare, United Kingdom) and ligated together using standard ligation procedures. Dimers restricted with *EcoR*I and *Xba*I were cloned into the *EcoR*I and *Xba*I sites of pBI121 (CLONTECH, Palo Alto, CA) (Appendix A.3) for isolates MSV-A[UG:Mub94:05], MSV-A[UG:Kib188:05], MSV-A[UG:Luw110:05], MSV-A[UG:Bug245:05], MSV-A[UG:Kib179:05], MSV-A[UG:Kas75:05], MSV-A[UG:Mpi11 :05], MSV-A[UG:Luw107:05], MSV-A[UG:Hoi154:05], MSV-A[UG:Bush53:05], MSV-A[UG:Kap292:05], MSV-A[UG:Kab82:05] and MSV-A[UG:Wak4:05].

2.3.4.2 Agroinoculation of seedlings

Agrobacterium C58C1 strain (Koncz & Schell 1986) was transformed with Ugandan MSV clones in pBI121 as described by An et al. (1988). Prior to agroinoculation, transformed *Agrobacterium* stocks were grown in LB with antibiotic selection for 24 hrs at 30°C. Five microlitres of this culture were used to re-inoculate fresh LB with antibiotic selection and grown at 30°C overnight. From this culture, 3 mL of culture was centrifuged for 30 secs at 7,000 r.p.m and the supernatant discarded. Fresh LB (1.5 mL) was added to the pellet and centrifuged as before to wash out the antibiotic, followed by resuspension of pellet in 200 μL of fresh LB.

The three maize genotypes were used for agroinoculation of seedlings as described by Grimsley et al. (1987) with modifications as described by Martin et al. (1999). Briefly, three-day old seedlings were randomly separated into batches of 14. A 25 μL Hamilton syringe (Hamilton, Bonaduz, Switzerland) was sterilized with 70% ethanol, rinsed out in sterile water and used to introduce *Agrobacterium* carrying infectious MSV clones into 14 seedlings for each construct being tested. Each seedling was injected 1 mm just below the coleoptile node with 2 μL of *Agrobacterium* culture. Inoculated seedlings were planted out in sterilized soil in plant rooms maintained at

16 hrs daylight and eight hours darkness. Experiments were conducted in triplicate.

2.3.5 Symptom analysis

Analysis of symptoms was conducted as described by Martin & Rybicki (1998, 2000) with the exception that no measurements of plant heights of infected plants were conducted. Briefly, plants were monitored for MSD symptoms starting three days post inoculation and each day thereafter until the end of the experiment. The percentage of inoculated plants infected was computed from these data. Fourteen days post inoculation, a leaf section from each infected plant was sampled as follows, starting with leaf two. Each leaf was folded into two, cut into half, and the top half discarded. The remaining portion was folded in half again and cut to obtain the leaf sample for image analysis. This was done for all infected plants for each isolate. Leaf sections were scanned against a blue background and high resolution images captured at a resolution of 300 dots per inch. A coin was scanned together with the leaves to provide a size standard. The procedure was repeated for leaves three, four, five and six as soon as they were fully emerged. Images captured were analyzed using a modified version of an image analysis programme developed by Martin & Rybicki (1998) that estimates the percentage of leaf area covered by chlorotic lesions in plants showing symptoms. Data for each clone were collated and from this, chlorotic area computed for leaves 2-6 for each isolate and plotted on a graph to show the progression of symptoms. Mean percentage chlorosis for leaves 2-6 was also compared using paired t-tests to determine which isolate was less severe in comparison to the others. The mean leaf area for leaves 2-6 for each isolate was also approximated using the same image analysis software.

2.4 Results

2.4.1 Maize variety diversity and cropping practices

During the survey, both “local”, (i.e probably unimproved out-bred genotypes), and improved maize cultivars were observed in the sampled fields (Table 2.2). Improved cultivars that were recorded included the Longe series: Longe I, IV, V and VI; as well as unidentified hybrids and Kawanda Composite A. Longe I, an open-pollinated and widely adopted MSD-tolerant cultivar, was the most frequently identified improved cultivar and was grown in 42% of the fields planted with improved maize (Table 2.2). Cultivars such as Longe IV and VI were only identified in one field each. While 27.6% of the 156 fields sampled during this survey were planted with improved maize cultivars, either unknown (potentially improved commercial) or “local” genotypes were found growing in the remaining 85 (55%) and 28 (18%) fields studied respectively. No improved genotypes were recorded in Bushenyi. Farmers in eastern and central Uganda were making the greatest use of improved cultivars. Whilst 19% of the fields had maize as a sole crop, the intercropped fields (81%) consisted of mixtures of maize with beans, groundnuts, coffee, cassava, tea, pumpkin, cotton, millet, soybean, sweet potato, peas, yam, sunflower, pawpaw, onions, banana, tomatoes or tobacco. There were more sole cropped maize fields recorded in eastern Uganda than in the other two regions where intercrops were more frequent (Table 2.3). Whereas more than half (55%) of fields containing only maize were planted with improved maize cultivars, only 28% of intercropped fields contained improved maize cultivars (Table 2.3). No sole maize fields were found in 8 of the 23 districts.

2.4.2 MSD incidence

MSD incidence in all three regions was moderate (Table 2.3) with the eastern region (23.7%) showing a significantly (P=0.003) lower incidence than western (32.7%) and central (33.1%) regions. In several fields, especially those in the high altitude district

Table 2.2: Maize varieties being grown by farmers in the surveyed areas of Uganda in May/June2005

Variety	Number of fields recorded	MSD incidence (%)	MSD severity
Unknown hybrids	5	28.0	2.0
Kawanda Composite	9	38.1	2.3
“Local”	24	32.4	2.3
Longe I	23	27.7	2.0
Longe IV	1	36.7	1.9
Longe V	4	34.2	2.1
Longe VI	1	46.7	2.5
Popcorn	4	30.8	2.4
Unknown	85	28.6	2.2
Average	**-**	**33.7**	**2.2**

of Kapchorwa, MSD incidence was very low, with some fields containing no diseased plants at all. Incidence was greater in areas surrounding Lake Victoria and western Uganda, and lower in eastern Uganda and some isolated localities in central and western Uganda (Fig. 2.1). There were high-incidence MSD ‘hotspots’ in the eastern and central regions, although a few scattered fields with high MSD incidence were also encountered in western districts. Overall, average MSD incidence was 30% for the surveyed areas, ranging from 1.9% in Kapchorwa to 43.3% in Luwero (Table 2.4). MSD incidence was significantly lower ($P<0.001$, Kruskal-Wallis One Way ANOVA on Ranks Test) in Kapchorwa District than elsewhere.

There was no statistically significant difference in incidence ($P=0.21$) between intercropped maize (28.1%) and sole-cropped maize (28.8%) in all surveyed areas even though district averages showed that MSD incidence was higher in intercrops, mainly in eastern districts of Iganga, Kapchorwa as well as Nakasongola in central Uganda (Table 2.3). However, eastern region had a significantly lower incidence ($P=0.04$) in intercrops than western or central region (Table 2.3), whilst incidence in sole crops was not significantly different (P=0.09) between the three regions. Incidences in improved and local/unknown genotypes were also not significantly different (P=0.56). For the local/unknown category, incidence in intercropped and sole-cropped maize did not differ significantly (P=0.10). A similar trend was observed (P=0.71) for intercropped and sole-cropped fields planted with improved cultivars. MSD incidences between the different maize genotypes (Table 2.2) were also not significantly different (P=0.44).

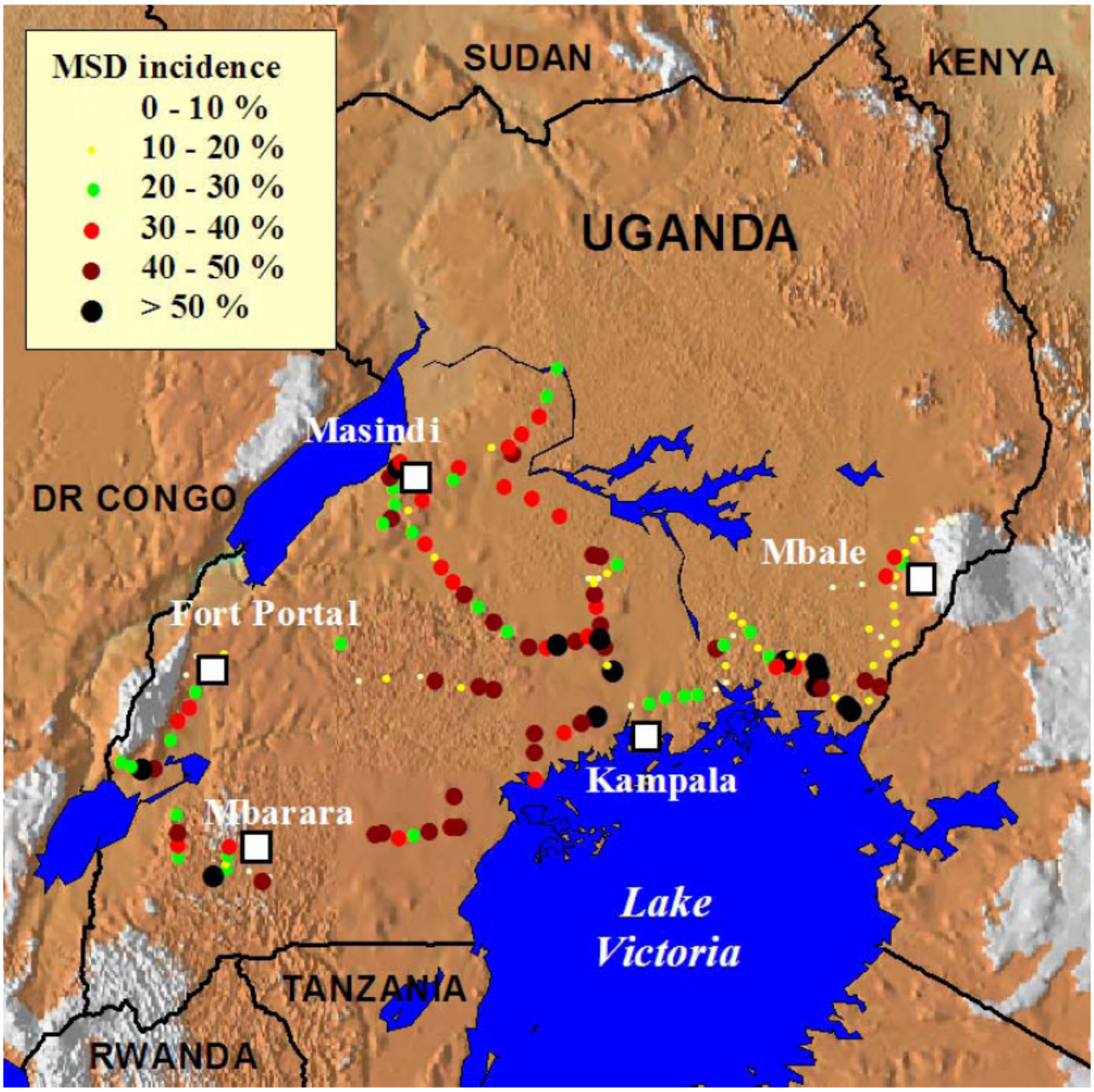


Figure 2.1: MSD Incidence for surveyed districts May/June 2005.

Table 2.3: MSD severity and incidence in surveyed districts of Uganda, May/June 2005

Region/ district	Fields of improved cultivars	Fields of local/ unknown genotypes	Incidence and severity improved cultivars	Incidence and severity local and unknown genotypes	Sole cropped fields	Inter cropped fields	Incidence and severity sole crops	Incidence and severity intercrops	Genotypes
Eastern	*17*	*33*	*28.2 (2.0)*	*21.2 (2.0)*	*17*	*33*	*21.6 (2.0)*	*24.7 (2.0)*	-
Iganga	2	9	40.0 (2.5)	24.4 (2.4)	6	5	22.8 (2.3)	32.7(2.5)	LI, Local, Unknown
Kapchorwa	4	6	1.1 (0.5)	2.5 (0.6)	2	8	0.0 (0.0)	2.7 (0.8)	LI, Hybrid, Unknown
Other	11	18	35.2 (2.3)	26.1 (2.3)	9	20	25.6 (2.3)	31.1 (2.3)	KC, LI, LV, Local, Unknown
Western	*10*	*45*	*34.0 (2.2)*	*32.3 (2.2)*	*6*	*49*	*35.0 (2.2)*	*32.4 (2.3)*	-
Bushenyi	0	10	-	29.7 (2.8)	1	9	30 .0 (2.7)	29.6 (2.8)	Popcorn, Local, Unknown
Kasese	3	8	18.9 (2.5)	32.9 (2.4)	2	9	41.7 (2.3)	26.3 (2.5)	KC, Unknown
Masindi	3	7	38.9 (2.1)	28.6 (1.9)	3	7	32.2 (2.0)	31.4 (2.0)	LI, IV, Unknown

Continued...

Table 2.3 – Continued

Region/ district	Fields of improved cultivars	Fields of local/ unknown genotypes	Incidence and severity improved cultivars	Incidence and severity local and unknown genotypes	Sole cropped fields	Inter cropped fields	Incidence and severity sole crops	Incidence and severity intercrops	Genotypes
Other	4	20	35.0 (2.2)	41.7 (2.0)	0	24	-	36.1 (2.1)	LI, LV, KC, Local, Unknown
Central	*16*	*35*	*32.5 (2.2)*	*33.4 (2.2)*	*6*	*45*	*33.3 (2.0)*	*33.1 (2.0)*	-
Luwero	4	6	39.2 (2.2)	46.1 (2.3)	1	9	56.7 (2.4)	41.9 (2.2)	LVI, Hybrid, KC
Nakasongola	1	9	10.0 (1.7)	27.4 (2.3)	2	8	23.3 (1.7)	26.3 (2.4)	LV, Popcorn, Unknown
Other	11	20	32.1 (2.3)	32.3 (2.2)	3	28	32.2 (2.1)	32.3 (2.2)	LI, Hybrid, KC, Unknown, Local
Total	43	113	-	-	29	127	-	-	
Average	-	-	33.8 (2.2)	27.2 (2.1)	-	-	28.8 (2.1)	28.1 (2.2)	-
S.E for the average	-	-	2.5 (0.1)	1.5 (0.1)	-	-	3.1 (0.1)	1.5 (0.1)	

KC = Kawanda Composite A; Hyb = Unknown hybrids; LI-Longe I; LIV=Longe IV; LV=Longe V; LVI=Longe VI; Inc=Incidence (%); sev=Severity( )

Table 2.4: MSD incidence and severity in surveyed districts of Uganda, May/June 2005

Region (District)	Fields sampled	Mean Incidence (%)	Max. Incidence (%)	Min. Incidence (%)	Mean Severity	Max. Severity	Min. Severity
Eastern	*50*	*23.7 (18.9)*	*66.7*	*0.0*	*2.0 (0.8)*	*3.7*	*0.0*
Iganga	11	27.3 (17.3)	63.3	6.7	2.6 (0.1)	3.7	1.5
Kapchorwa	10	1.9 (2.6)	6.7	0.0	1.4 (0.2)	1.5	0.0
Other	29	29.9 (20.2)	66.7	3.3	2.3 (0.5)	3.7	1.2
Central	*51*	*33.1 (14.8)*	*60.0*	*0.0*	*2.2 (0.6)*	*3.1*	*0.0*
Luwero	10	43.3 (11.3)	60.0	20.0	2.3 (0.1)	2.7	1.4
Nakasongola	10	25.7 (14.3)	43.3	10.0	2.4 (0.2)	3.1	1.6
Other	31	35.3 (14.6)	56.7	0.0	2.2 (0.6)	3.1	0.0
Western	*55*	*32.7 (12.9)*	*50.0*	*3.3*	*2.3 (0.5)*	*3.1*	*1.2*
Bushenyi	10	29.7 (13.8)	53.3	6.7	2.9 (0.1)	3.1	2.4
Masindi	10	31.7 (7.4)	43.3	16.7	2.0 (0.1)	2.3	1.2
Kasese	7	37.0 (10.4)	53.3	23.3	2.5 (0.1)	3.0	2.3
Other	28	33.1 (14.8)	53.3	3.3	2.2 (0.5)	3.0	1.3
Average	**-**	**30 (16.3)**	**66.7**	**0.0**	**2.2 (0.6)**	**3.7**	**0.0**

Standard errors are in parentheses

2.4.3 MSD severity

Patterns of variation in disease severity (Fig. 2.3) were similar to those recorded for incidence. There was a significant positive correlation between incidence and severity (Fig. 2.2; R = 0.37; $R^2 = 0.14$; P=2.23 x 10^{-6}, Pearson Product Moment Correlation). MSD severities for all surveyed areas ranged from very mild to moderate, from a low of 1.3 in Kapchorwa in the east to a high of 2.9 for Bushenyi in western Uganda (Table 2.4; Fig. 2.3). There were a few fields with no disease at all or extremely mild disease symptoms, mainly in the eastern part of the country. MSD

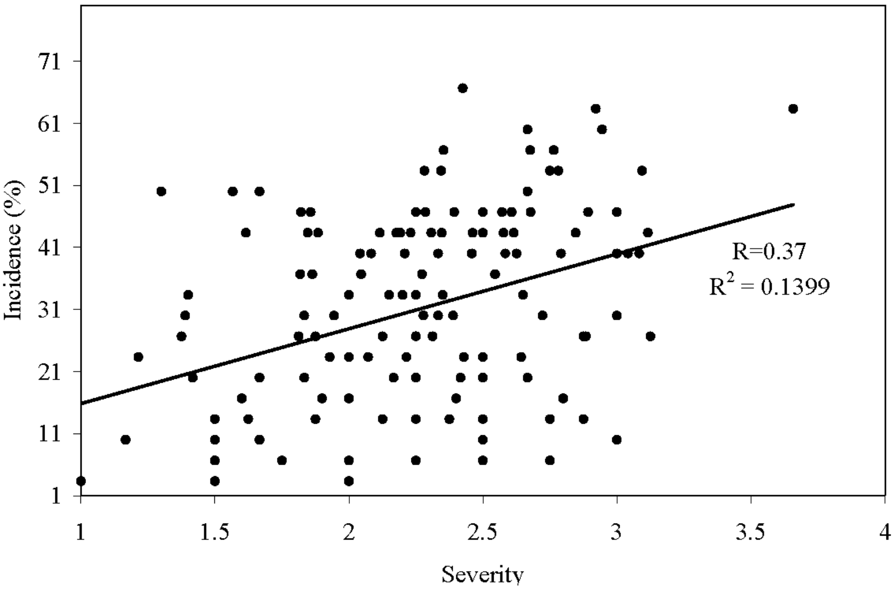


Figure 2.2: Correlation between MSV incidence and severity.

was more severe in several fields in western and central regions, as well as parts of eastern Uganda close to the shores of Lake Victoria. The rest of eastern Uganda and the central region had mild to moderate disease, and fields with no disease were recorded in Kapchorwa and Mubende (Fig. 2.3). MSD severity was not significantly different (P=0.29) for intercropped maize (mean = 2.2; sd = 0.6) and sole maize crops (mean = 2.1 sd = 0.38). Severity for the various districts, however, differed significantly (P<0.001). Kapchorwa had significantly lower (P<0.001) severity than the other six eastern Ugandan districts (Table 2.4). There was no statistically significant difference in severity (P=0.50) for local/unknown genotypes grown as intercrops or sole crops (Table 2.3). Improved cultivars grown as intercrops or sole crops also did not differ (P=0.38) in severity. Because of the high number of unidentified maize genotypes encountered, comparisons of levels of incidence and severity could not be made between improved and local/unknown genotypes.

2.4.4 Agroinoculation of maize seedlings

Fifteen agroinfectious Ugandan MSV clones were constructed and tested, representing seven of the eight haplotypes in which Ugandan isolates have been grouped (Chap-

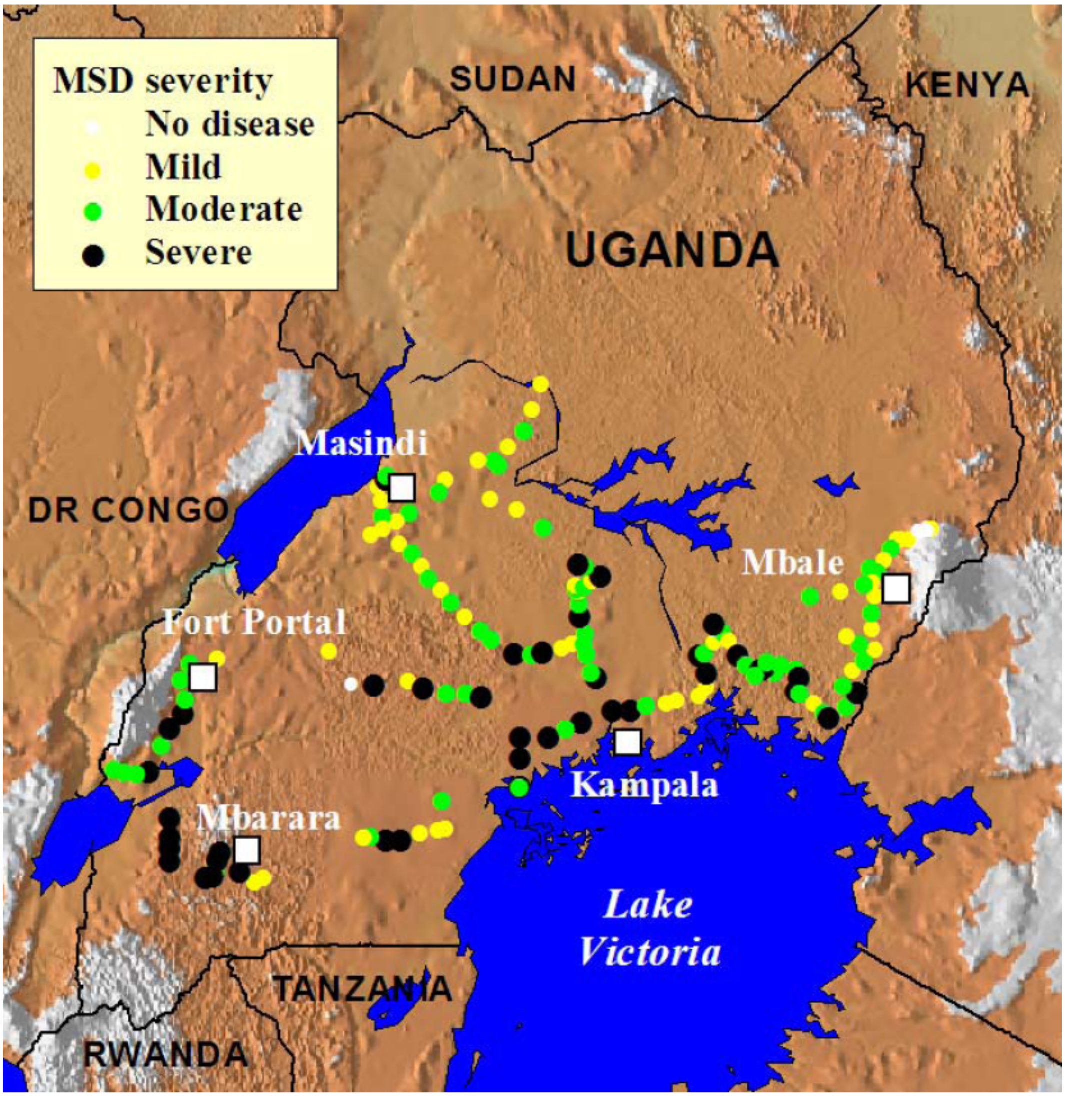


Figure 2.3: MSD severity for surveyed districts May/June 2005. Severity scores were grouped as follows: 0=no disease; 1-2=mild; 2.0-2.5=Moderate; >2.5=Severe.

ter 4). Of these, fourteen were infectious whilst one clone, MSV-A[UG:Iga235:05] belonging to the haplotype MSV-A_1Ug-VII was not infectious in any of the three maize genotypes used in the study. MSD symptoms were evident from four days post inoculation (d.p.i) for Golden Bantam plants whilst the first symptoms recorded on Sweetcorn and Pan77 were observed five d.p.i (Table 2.5). No symptoms were observed in uninoculated control plants.

Generally, more Golden Bantam plants were infected in comparison to Sweetcorn and Pan77. For nine of the isolates, the percentage of infected plants was highest in the susceptible Golden Bantam genotype, with the highest infection rate of over 90% recorded in Golden Bantam plants inoculated with MSV-A[UG:Kab82:05]. Two isolates, MSV-A[UG:Wak4:05] and MSV-A[UG:Hoi159:05], had very low infection rates in Golden Bantam and Sweetcorn (Table 2.5) and did not produce any disease symptoms in the resistant genotype Pan77. Consequently, because only one plant of Golden Bantam and Sweetcorn was available for symptom analysis for isolate MSV-A[UG:Hoi159:05], this isolate was not included in further analyses.

MSV symptoms observed following inoculation with Ugandan isolates ranged from mild to very severe in the three maize genotypes. Progression of symptom development in leaves 2-6 in the three maize genotypes is presented in Figs. 2.4 and 2.5. Isolate MSV-A[UG:Kas75:05] noticeably produced mild symptoms in all three maize genotypes tested. Patterns of progression of MSV infection in all three maize genotypes was consistent with the susceptibility levels of the genotypes tested (Figs. 2.4 and 2.5). Chlorotic leaf areas were generally higher in Golden Bantam and Sweetcorn in comparison to Pan77 which consistently showed less chlorosis for all constructs tested in the study. In particular, leaf two of Golden Bantam plants started out with a high percentage chlorotic leaf area and for many isolates, chlorosis remained higher than that of both Sweetcorn and Pan77 in subsequent leaves. Percentage chlorotic area in agro-inoculated plants and disease severity scored in the field during the survey were comparable for most isolates (Table 2.5). For example, isolate MSV-A[UG:Kas75:05] had a low field severity and infection rate as well as a corresponding low percentage chlorotic leaf area observed following agroinoculation (Table 2.5 and Fig. 2.4). Similarly, isolate MSV-A[UG:Wak4:05] had a low infection rate and correspondingly low field severity (Table 2.5). MSV-A[UG:Kab82:05], however, was an exception that showed very mild severity in the field sample but high percent infection and a corresponding high percentage chlorotic area following inoculations (Fig. 2.5).

P values for paired t-tests of percentage chlorosis between the different isolates are

Table 2.5: Agroinoculation of three maize genotypes with agroinfectious Ugandan MSV isolates

Haplotype	Isolate	Golden Bantam[a]	Sweet corn[a]	Pan77[a]	Golden Bantam[b]	Sweet corn[b]	Pan77[b]	Field severity
I	UMub94	4	6	6	61.2	54.8	50.0	3.5
II	UKib188	4	5	6	47.6	47.6	45.2	3.0
II	ULuw110	4	6	7	50.0	54.3	30.9	4.0
III	UBug245	4	5	6	59.5	54.8	28.6	3.0
III	UKib179	4	5	6	69.0	67.4	50.0	3.5
III	UKas75	5	7	8	47.6	54.3	35.7	2.5
V	UMpi11	4	5	5	50.0	60.9	52.3	4.0
V	ULuw107	4	5	6	69.0	52.2	45.2	3.0
V	UHoi154	4	5	7	59.5	65.2	35.7	4.0
VI	UBush53	4	5	7	61.9	54.3	45.2	3.0
VI	UKap292	5	6	6	61.2	40.5	28.6	4.0
VI	UKab82	4	5	6	90.5	67.4	59.5	1.5
VII	UHoi159	9	6	-	9.5	9.5	0	4.0
VII	UIga235	-	-	-	0	0	0	3.5
VIII	UWak4	5	5	-	26.3	8.7	0	2.0

[a] First appearance of symptoms (days post inoculation); [b] Percentage of Infected plants (%).I-VIII represents haplotype MSV-A$_1$UgI-VIII; UMub94 = MSV-A[UG:Mub94:05], UKib188 = MSV-A[UG:Kib188:05], ULuw110 = MSV-A[UG:Luw110:05], UBug245 = MSV-A[UG:Bug245:05], UKib179 = MSV-A[UG:Kib179:05], UKas75 = MSV-A[UG:Kas75:05], UMpi11 = MSV-A[UG:Mpi11:05], ULuw107 = MSV-A[UG:Luw107:05], UHoi154 = MSV-A[UG:Hoi154:05], UBush53 = MSV-A[UG:Bush53:05], UKap292 = MSV-A[UG:Kap292:05], UKab82 = MSV-A[UG:Kab82:05], UWak4 = MSV-A[UG:Wak4:05]

presented in Tables 2.6, 2.7 and 2.8. Whilst chlorosis for all isolates was significantly different from the control (uninoculated) plants for Golden Bantam (Table. 2.6), for both Sweetcorn and Pan77, percentage chlorosis was not significantly different between some isolates and controls (Tables 2.7 and 2.8). Isolates belonging to the same haplotype did not behave uniformly and each exhibited varying percent chlorosis in the three maize genotypes (Tables 2.6, 2.7 and 2.8).

Three isolates: MSV-A[UG:Luw110:05], MSV-A[UG:Kas75:05] and MSV-A[UG:Wak4:05] from haplotypes MSV-A_1UgII, III and VIII respectively showed the least severe chlorosis of the 13 tested isolates in the three maize genotypes. Of the haplotype MSV-A_1UgIII isolates (MSV-A[UG:Kas75:05], MSV-A[UG:Kib179:05] and MSV-A [UG:Bug245:05]), percentage chlorosis of isolate MSV-A[UG:Kas75:05] was significantly less severe than isolates MSV-A[UG:Kib179;05] and MSV-A[UG:Bug245:05] in Golden Bantam and Sweetcorn (Table 2.6, 2.7). Whilst MSV-A[UG:Kas75:05] was less severe than all tested isolates in Golden Bantam, in Sweetcorn, however, MSV-A[UG:Kas75:05] was less severe than only six of the 13 isolates. In Pan77, MSV-A[UG:Kas75:05] was significantly less severe than all but one isolate, MSV-A[UG:Luw110:05] (Table 2.8). Similarly, isolate MSV-A[UG:Wak4:05] was less severe than all but two isolates, MSV-A[UG:Kas75:05] and MSV-A[UG:Luw110:05], in Golden Bantam (Table 2.6) but only less severe than three isolates in Sweetcorn.

There were no significant differences in chlorosis between the two isolates from haplotype MSV-A_1UgII (MSV-A[UG:Kib188:05] and MSV-A[UG:Luw110:05]) nor between the three isolates (MSV-A[UG:Mpi11:05], MSV-A[UG:Luw107:05] and MSV-A[UG:Hoi154:05]) from haplotype MSV-A_1UgV in the three maize genotypes. Chlorotic leaf area in isolate MSV-A[UG:Bush53:05] was however less than that of the other two members of haplotype MSV-A_1UgVI in Sweetcorn and Pan77 but not in Golden Bantam.

Generally, the leaf area of uninfected control plants was greater than plants inoculated with the different isolates for all three maize genotypes (Fig. 2.6). There was a greater reduction in leaf growth in the highly sensitive Golden Bantam in comparison to Sweetcorn and Pan77. Isolates belonging to haplotype MSV-A_1UgII (yellow colour) caused greater stunting in Golden Bantam in comparison to the other haplotypes.

One isolate in particular, MSV-A[UG:Kib188:05] (haplotype MSV-A_1UgII), showed the greatest stunting in Golden Bantam (Fig. 2.6). Similarly, isolates from MSV-A_1UgV haplotype (blue colour) also caused severe stunting in both Golden Bantam and Sweetcorn. In contrast, isolates from haplotypes MSV-A_1UgIII and VI caused less stunting.

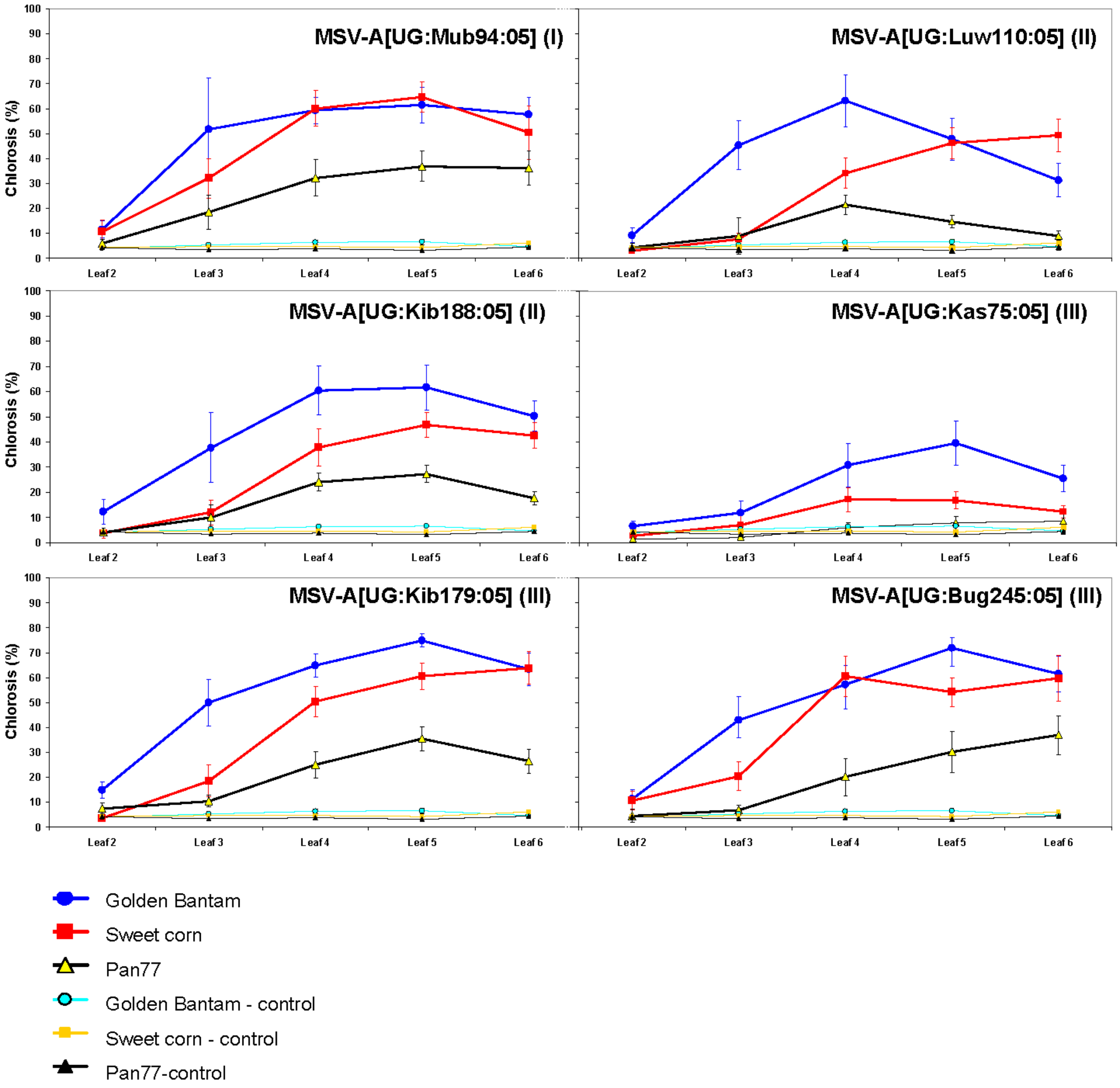


Figure 2.4: Percentage chlorotic leaf area observed on leaves 2-6 of Golden Bantam, Sweetcorn and Pan77 maize genotypes inoculated with Ugandan MSV isolates from haplotypes MSV-A_1UgI (MSV-A[UG:Mub94:05]), MSV-A_1UgII (MSV-A[UG:Kib188:05], MSV-A[UG:Luw110:05]) and MSV-A_1UgIII (MSV-A[UG:Kas75:05], MSV-A[UG:Bug245:05], MSV-A[UG:Kib179:05]). Error bars represent the 95% confidence interval of the mean. Numbers in parentheses after the isolate name refer to the haplotype to which the isolate belongs.

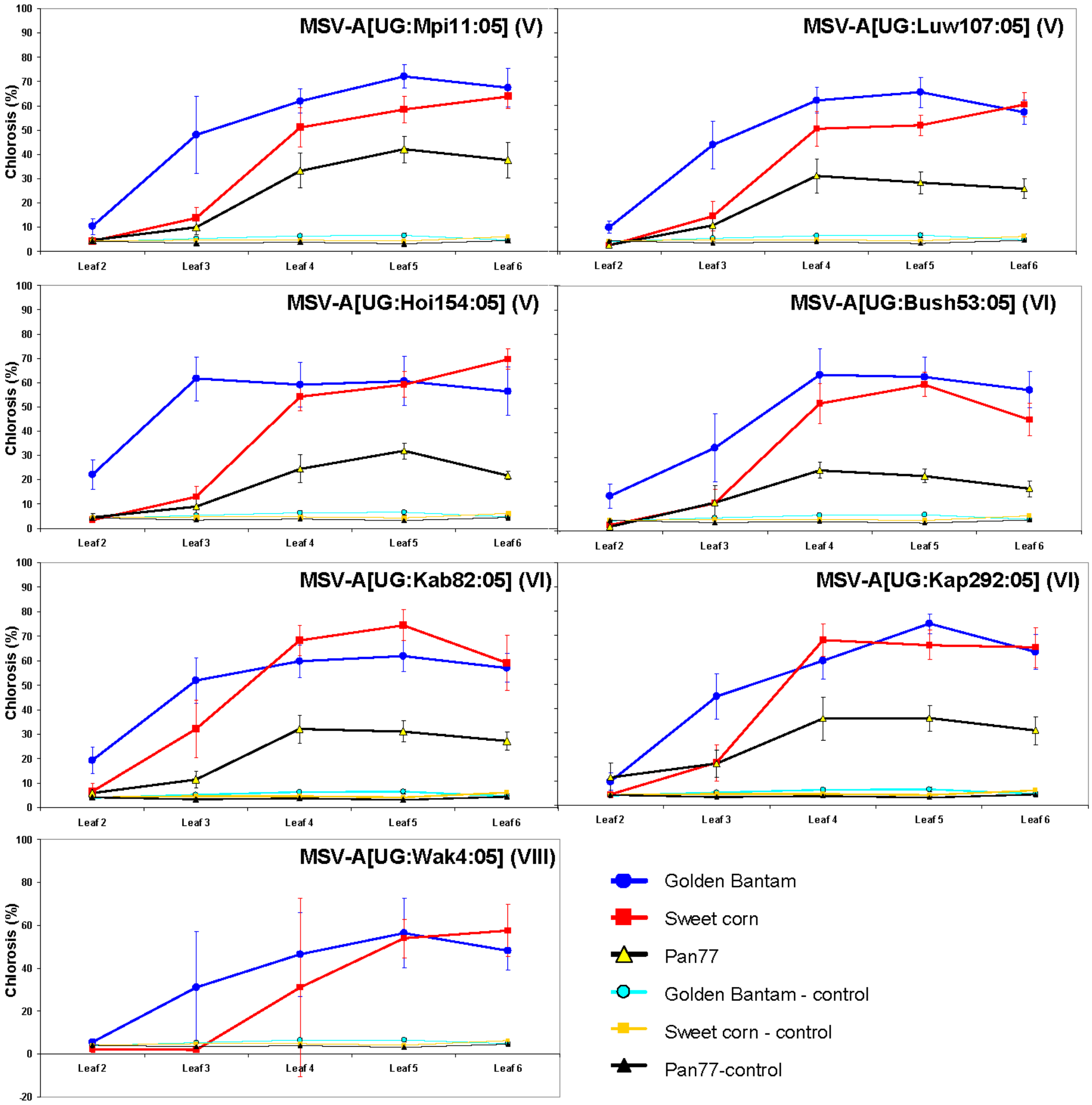


Figure 2.5: Percentage chlorotic leaf area observed on leaves 2-6 of Golden Bantam, Sweetcorn and Pan77 maize genotypes inoculated with Ugandan MSV isolates from haplotypes MSV-A_1UgV (MSV-A[UG:Mpi11:05], MSV-A[UG:Luw107:05], MSV-A[UG:Hoi154:05]), MSV-A_1UgVI(MSV-A[UG:Bush53:05], MSV-A[UG:Kab82:05], MSV-A[UG:Kap292:05]) and MSV-A_1UgVIII (MSV-A[UG:Wak4:05]). Error bars represent the 95% confidence interval of the mean. Numbers in parentheses after the isolate name refer to the haplotype to which the isolate belongs.

Table 2.6: P values for paired t-tests for percentage chlorotic leaf area of **Golden Bantam** plants inoculated with the different Ugandan MSV isolates. P values read from left to right of the table comparing which isolate is less severe than the other

-	M94	M188	M110	M75	M245	M179	M11	M107	M154	M53	M292	M82	M4
M94	-	0.265	0.159	0.987	0.850	0.101	0.271	0.159	0.772	0.813	0.572	0.313	0.981
M188	0.265	-	0.107	0.993	0.194	0.017	0.093	0.127	0.178	0.348	0.144	0.107	0.976
M110	0.159	0.365	-	0.996	0.260	0.080	0.156	0.216	0.056	0.337	0.217	0.090	0.791
M75	0.013	0.007	0.004	-	0.009	0.006	0.012	0.011	0.007	0.007	0.012	0.044	0.023
M245	0.085	0.194	0.260	0.991	-	0.015	0.105	0.547	0.606	0.452	0.131	0.791	0.998
M179	0.101	0.983	0.089	0.994	0.985	-	0.105	0.994	0.754	0.076	0.060	0.312	0.999
M11	0.271	0.093	0.156	0.988	0.105	0.343	-	0.090	0.998	0.185	0.277	0.627	0.995
M107	0.772	0.127	0.216	0.989	0.547	0.006	0.090	-	0.396	0.614	0.265	0.437	0.994
M154	0.248	0.178	0.056	0.993	0.606	0.754	0.998	0.396	-	0.397	0.805	0.395	0.967
M53	0.813	0.348	0.337	0.993	0.452	0.076	0.185	0.614	0.397	-	0.311	0.401	0.969
M292	0.572	0.144	0.217	0.998	0.131	0.060	0.277	0.265	0.805	0.311	-	0.918	0.995
M82	0.313	0.107	0.090	0.996	0.791	0.312	0.627	0.437	0.395	0.401	0.918	-	0.992
M4	0.019	0.024	0.791	0.977	0.002	0.001	0.005	0.006	0.033	0.021	0.005	0.008	-
Control	0.009	0.011	0.017	0.037	0.013	0.009	0.013	0.012	0.003	0.012	0.014	0.004	0.020

M94=MSV-A[UG:Mub94:05],M188=MSV-A[UG:Kib188:05],M110=MSV-A[UG:Luw110:05],M75=MSV-A[UG:Kas75:05],M245=MSV-A[UG:Bug245:05], M179=MSV-A[UG:Kib179:05],M11=MSV-A[UG:Mpi11:05],M107=MSV-A[UG:Luw107:05],M154=MSV-A[UG:Hoi154:05],M53=MSV-A[UG:Bush53:05],M82=MSV-A[UG:Kab82:05], M292=MSV-A[UG:Kap292:05],M4=MSV-A[UG:Wak4:05]. Underlined values represent statistically significant P values at 5% significance level indicating which isolate is significantly less severe than the other.

Table 2.7: P values for paired t-tests for percentage chlorotic leaf area of **Sweetcorn** plants inoculated with the different Ugandan MSV isolates. P values read from left to right of the table comparing which isolate is less severe than the other

-	**M94**	**M188**	**M110**	**M75**	**M245**	**M179**	**M11**	**M107**	**M154**	**M53**	**M292**	**M82**	**M4**
M94	-	0.991	0.957	0.979	0.576	0.431	0.368	0.1860	0.625	0.630	0.898	0.172	0.113
M188	0.009	-	0.797	0.952	0.016	0.040	0.065	0.104	0.090	0.176	0.046	0.016	0.882
M110	0.033	0.787	-	0.087	0.019	0.016	0.024	0.049	0.040	0.218	0.040	0.025	0.674
M75	0.011	0.048	0.087	-	0.021	0.043	0.051	0.054	0.058	0.060	0.041	0.021	0.146
M245	0.576	0.984	0.981	0.979	-	0.613	0.378	0.056	0.746	0.125	0.411	0.187	0.146
M179	0.431	0.960	0.984	0.957	0.613	-	0.336	0.076	0.824	0.214	0.225	0.105	0.959
M11	0.368	0.065	0.976	0.051	0.378	0.336	-	0.124	0.271	0.313	0.120	0.102	0.951
M107	0.180	0.104	0.951	0.054	0.056	0.076	0.124	-	0.110	0.658	0.058	0.055	0.179
M154	0.625	0.090	0.960	0.058	0.746	0.824	0.271	0.110	-	0.125	0.023	0.201	0.954
M53	0.630	0.176	0.218	0.060	0.125	0.214	0.313	0.658	0.125	-	0.037	0.007	0.431
M292	0.898	0.954	0.960	0.959	0.411	0.225	0.120	0.058	0.977	0.963	-	0.330	0.066
M82	0.172	0.984	0.975	0.979	0.187	0.105	0.102	0.055	0.201	0.993	0.330	-	0.055
M4	0.113	0.882	0.674	0.146	0.097	0.041	0.049	0.179	0.046	0.431	0.066	0.055	-
Control	0.017	0.049	0.070	0.080	0.025	0.043	0.050	0.052	0.055	0.062	0.044	0.026	0.108

M94=MSV-A[UG:Mub94:05],M188=MSV-A[UG:Kib188:05],M110=MSV-A[UG:Luw110:05],M75=MSV-A[UG:Kas75:05], M245=MSV-A[UG:Bug245:05],M179=MSV-A[UG:Kib179:05],M11=MSV-A[UG:Mpi11:05],M107=MSV-A[UG:Luw107:05], M154=MSV-A[UG:Hoi154:05],M53=MSV-A[UG:Bush53:05],M82=MSV-A[UG:Kab82:05],M292=MSV-A[UG:Kap292:05],M4=MSV-A[UG:Wak4:05]. Underlined values represent statistically significant P values at 5% significance level indicating which isolate is significantly less severe than the other.

Table 2.8: P values for paired t-tests for percentage chlorotic leaf area of **Pan77** plants inoculated with the different Ugandan MSV isolates. P values read from left to right of the table comparing which isolate is less severe than the other

-	**M94**	**M188**	**M110**	**M75**	**M245**	**M179**	**M11**	**M107**	**M154**	**M53**	**M292**	**M82**	**M4**
M94	-	0.976	0.964	9.989	0.074	0.082	0.866	0.978	0.977	0.982	0.899	0.075	-
M188	0.024	-	0.888	0.977	0.507	0.070	0.084	0.188	0.208	0.387	0.001	0.041	-
M110	0.036	0.112	-	0.065	0.247	0.084	0.086	0.084	0.045	0.124	0.009	0.045	-
M75	0.011	0.023	0.065	-	0.042	0.016	0.031	0.028	0.026	0.030	0.005	0.017	-
M245	0.074	0.507	0.247	0.958	-	0.625	0.104	0.990	0.813	0.408	0.144	0.615	-
M179	0.082	0.070	0.084	0.984	0.625	-	0.160	0.587	0.824	0.111	0.040	0.774	-
M11	0.866	0.084	0.086	0.969	0.104	0.160	-	0.111	0.975	0.084	0.837	0.230	-
M107	0.022	0.188	0.084	0.972	0.990	0.587	0.111	-	0.068	0.081	0.001	0.017	-
M154	0.023	0.208	0.955	0.974	0.813	0.824	0.025	0.068	-	0.248	0.003	0.089	-
M53	0.018	0.387	0.124	0.970	0.408	0.111	0.084	0.081	0.248	-	0.020	0.030	-
M292	0.899	0.999	0.991	0.995	0.144	0.960	0.837	0.999	0.997	0.980	-	0.999	-
M82	0.075	0.041	0.045	0.017	0.615	0.774	0.230	0.017	0.089	0.030	0.001	-	-
Control	0.021	0.044	0.062	0.065	0.032	0.046	0.006	0.048	0.051	0.012	0.047	0.393	-

M94=MSV-A[UG:Mub94:05],M188=MSV-A[UG:Kib188:05],M110=MSV-A[UG:Luw110:05],M75=MSV-A[UG:Kas75:05], M245=MSV-A[UG:Bug245:05],M179=MSV-A[UG:Kib179:05],M11=MSV-A[UG:Mpi11:05],M107=MSV-A[UG:Luw107:05],M154=MSV-A[UG:Hoi154:05],M53=MSV-A[UG:Bush53:05], M82=MSV-A[UG:Kab82:05],M292=MSV-A[UG:Kap292:05],M4=MSV-A[UG:Wak4:05]. Underlined values represent statistically significant P values at 5% significance level indicating which isolate is significantly less severe than the other.

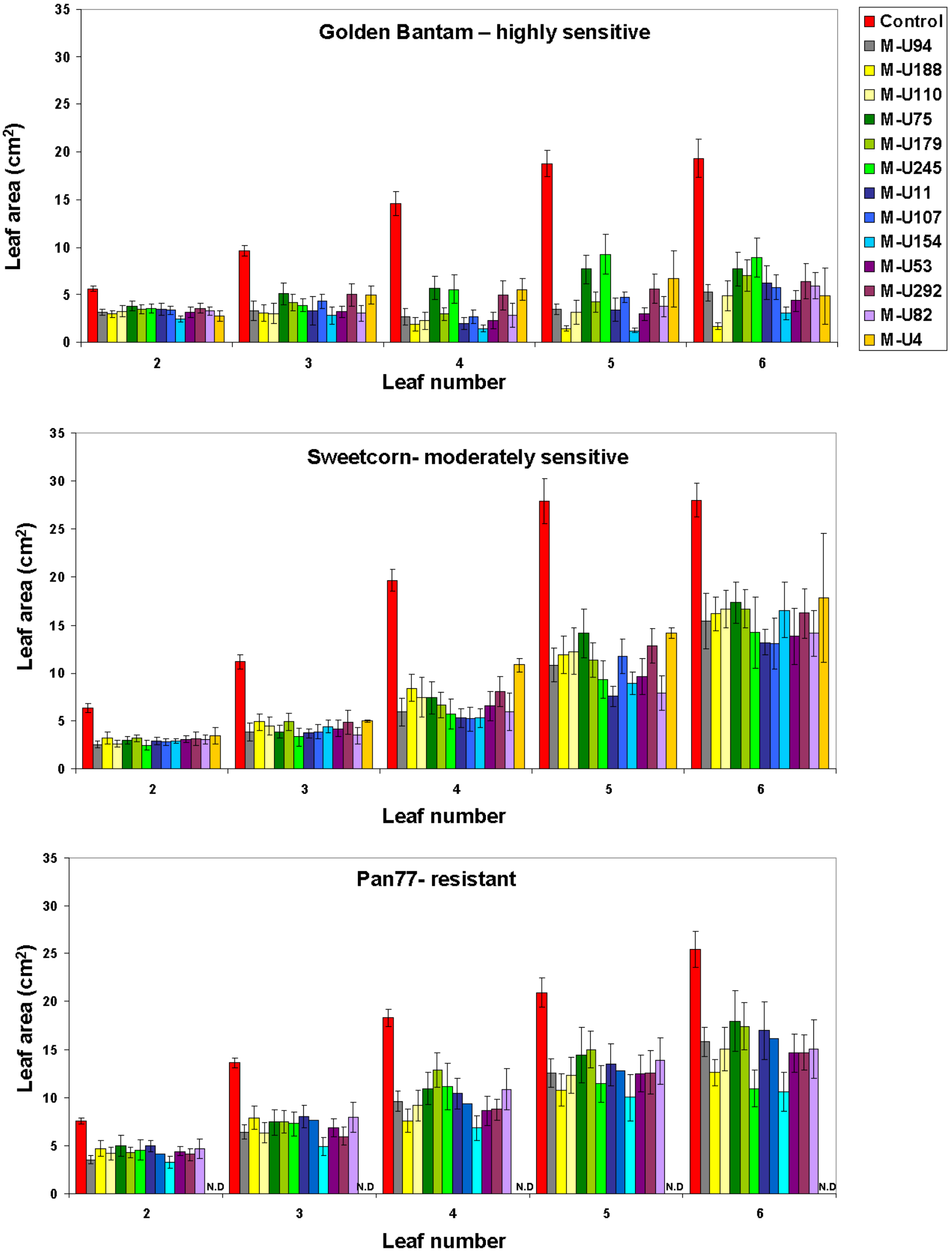


Figure 2.6: Leaf area for the three maize genotypes inoculated with different Ugandan MSV isolates. Error bars represent the 95% confidence interval of the mean. N.D = no data for Isolate MSV-A[UG:Wak4:05] that showed no symptoms in Pan77. Note Isolate names e.g M-U94=MSV-A[UG:Mub94:05]

2.5 Discussion

MSD is one of the most significant factors responsible for low maize yields throughout sub-Saharan Africa. Despite this, data on the incidence and severity of MSD in most African maize-growing countries are either scarce, outdated or completely absent. In addition, it is apparent from this study that MSD is widespread despite the availability and use of MSD-resistant/tolerant varieties. The results presented here highlight the wide distribution of MSD in Uganda, confirming its endemic status in major maize-growing areas of this country.

Both "local landraces", which were probably unimproved maize genotypes, and those specifically bred for qualities such as MSD, Gray leaf spot (GLS) and northern leaf blight resistance ("improved" genotypes), were encountered during the survey. However, because of the high number of maize genotypes that could not be identified by the farmers growing them, comparisons of levels of incidence and severity could not be made between improved and local/unknown genotypes. What could be concluded is that the adoption of improved, commercially sold maize genotypes by Ugandan farmers is still relatively restricted as apparent from their limited distribution in surveyed areas.

It was also observed that many farms (81% of those surveyed) employed intercropping, with maize grown amongst other crops such as beans, groundnuts, coffee, cassava, tea, pumpkin, cotton, millet, soybean, sweet potato, peas, yam, sunflower, pawpaw, onions, banana, tomatoes and tobacco. Rather than being a deliberate disease control strategy, intercropping is probably usually attributable to preferred land-use practices whereby farmers mix crops to optimally utilize land. The southern half of Uganda is densely populated and most farms are less than 2 ha in size. Given the widespread use of intercropping, an attempt was made to determine its potential effect on MSD. In individual fields, the data showed that intercropping had no discernable influence on either the incidence or severity of MSD (P=0.21 for incidence and P=0.29 for severity). This concurs with the findings of Page (1999b) who demonstrated that although intercropping maize decreased vector abundance and activity, it did not measurably reduce MSD incidence.

Previous surveys in Uganda reported MSD as only second to GLS in its impact on maize production with an average country-wide incidence of 14.9% and severity of 1.80 (Bigirwa et al. 2001). Results presented here show a higher incidence and severity of MSD compared with the last survey, with the mean incidence doubling to 30%. However, due to the erratic nature of MSD, a conclusion cannot be reached as to whether this represents an upward trend in disease prevalence. To do that, a survey as extensive as the one presented here would have to be undertaken in each maize growing season for several years, which was beyond the scope of this study.

No clear geographical trends were apparent in MSD incidence, other than the markedly lower incidence in the higher altitude areas of eastern Uganda in Kapchorwa District. This could be due to lack of adaptation of the leafhopper vectors to high altitudes.

An important outcome of the survey was the finding of a significant positive correlation between disease incidence and severity. A possible reason for this is that high disease incidence is associated with, or indicative of, infections having occurred early after planting when plants are most sensitive to MSD and, when infected, present with the most severe symptoms.

It was surprising to note that MSD incidence and severity were not significantly lower in improved, MSD-tolerant cultivars than they were in the local unimproved genotypes. Gibson et al. (2005) showed that although farmers may buy improved seed, they often recycle it over subsequent plantings and thereby inadvertently select out the resistant genotypes.

The practice whereby farmers save their own seed, seldom buying fresh hybrid seed from commercial sources, does not apply solely to Uganda. A study in Tanzania, for example, has shown that about 80% of farmers recycle seed of improved varieties (Nkonya et al. 1998). This highlights a need for farmer training not only in Uganda but also in other maize-growing areas on the continent on the recommended use of varieties. Furthermore, it highlights the need to develop MSV-resistant maize genotypes that will retain their resistance despite out-crossing.

An additional cultural practice that can influence MSD incidence is continuous maize cropping, which can lead to a build-up of inoculum through infection of maize crops at a young age when they are most susceptible, further increasing incidence. This is an important feature of maize cultivation in many parts of Uganda, where two crops are often grown in a year in order to make optimal use of the bimodal rainfall regime.

The apparent loss of resistance in improved maize genotypes may also provide a clue as to why not many MSD-tolerant genotypes were encountered in this survey, despite the fact they have been available for a number of years. A reason for this low adoption of resistant genotypes could be that natural MSV resistance is not usually associated with good agronomic qualities such as high yield. Because MSD is erratic, farmers generally prefer to use higher yielding MSV-sensitive genotypes despite the fact they could lose a large proportion of that yield if a MSD epidemic strikes. Farmers may also perceive that “improved” genotypes such as Longe I are no more resistant than local, non-improved genotypes for the reasons given above, and therefore choose not to adopt their use. In addition, the high proportion of unknown genotypes encountered indicates that farmers may not be paying particular attention to varieties they are growing.

During the survey, MSD symptoms were assessed for all sampled plants. Several isolates were collected and their genetic characteristics described in Chapter 4. However, there is lack of information on the virulence of these Ugandan isolates. Therefore, a previously described technique (Martin et al. 1999) using agroinoculation was used to accurately describe symptom characteristics of Ugandan MSVs. A total of 15 Ugandan infectious clones representing six of the eight haplotypes identified in Chapter 4 were constructed and tested in three maize genotypes: Golden Bantam, Sweetcorn and Pan77, representing highly sensitive, moderately sensitive and resistant genotypes respectively.

Results showed that all three maize genotypes are susceptible to Ugandan MSVs. Of the 15 clones, only MSV-A[UG:Iga235:05] was not infectious in any of the three genotypes. Furthermore, another isolate from the same haplotype, MSV-A_1UgVII (MSV-A[UG:Hoi159:05]) recorded a very low infection rate (less than 10% in Golden Bantam and Sweetcorn) and did not infect the resistant genotype, Pan77. Isolate MSV-A[UG:Wak4:05] also had low infection rates in both Golden Bantam and Sweetcorn and did not infect Pan77. For this reason, MSV-A[UG:Hoi159:05] is missing from the graphs showing percentage chlorotic areas for the different isolates. It is possible that these two isolates were unable to overcome the resistance in Pan77. Isolate MSV-A[UG:Iga235:05] not being infectious in any of the three maize genotypes tested could also possibly indicate that this virus is defective and may not be the causal virus in the plant from which it was isolated.

In previous agroinoculation studies the first appearance of symptoms was recorded at five d.p.i (Martin & Rybicki 2000). In this study, disease symptoms appeared as early as four d.p.i in Golden Bantam plants and a little later in Pan77 plants. This early appearance of symptoms may indicate that the isolates tested are very virulent and therefore observation for disease symptoms should begin earlier than 5 d.p.i. Shepherd et al. (2007b) have reported a delay in development of symptoms in MSV-resistant transgenic plants. The delay in appearance of symptoms in inoculated Pan77 plants conforms to this observation.

All three maize genotypes Golden Bantam, Sweetcorn (cv. Star 7714) and Pan77 were tested by agroinoculation for the first time in this study. Pan77 has previously been tested for MSV resistance at PANNAR (Greytown, South Africa) using leafhopper challenges. Because of the small numbers of isolates in the different haplotypes (three for haplotypes MSV-A_1UgIII, MSV-A_1UgV, MSV-A_1UgVI, two for haplotype MSV-A_1UgII, and one each for haplotypes MSV-A_1UgI and MSV-A_1UgVIII) it was not possible to directly determine whether certain of the eight haplotypes were significantly more or less virulent than the others. Both Golden Bantam and Sweetcorn exhibited higher leaf chlorosis in comparison to Pan77. These results suggest that the

Pan77 genotype exhibits some measure of resistance to Ugandan MSV clones, showing moderate symptoms whilst Golden Bantam and Sweetcorn are highly sensitive and exhibit severe disease symptoms.

Findings from Chapter 4 of this thesis have described the genetic characteristics of Ugandan MSVs and found that all Ugandan isolates belong to the maize-adapted MSV A strain. In fact all the Ugandan MSVs belong specifically to the MSV-A_1 grouping that is the most common of five different variants found throughout Africa. The MSV-A strain, and specifically MSV-A_1 is known to cause severe MSD symptoms in even the most MSV resistant maize genotypes (Martin et al. 2001) and results here of severe symptoms following agro-inoculation concur with these findings. Three of the isolates, all from different haplotypes (MSV-A[UG:Kas75:05], MSV-A[UG:Luw110:05] and MSV-A[UG:Wak4:05]), exhibited less severe symptoms than the other 10 tested in all three maize genotypes. It was interesting to note, that even when isolates belonged to the same haplotype grouping, their symptom characteristics were not uniform in terms of progression of disease symptoms. Genome-wide the isolates within each of these haplotypes differ by as few as 69 polymorphisms but it is clear that, as has been shown elsewhere (Boulton et al. 1989), even this small amount of naturally occurring genetic variation can have quite a substantial influence on the virulence of variants.

Specifically, isolate MSV-A[UG:Kas75:05] was significantly less severe than the other two analysed haplotype MSV-A_1UgIII isolates with which it shares more than 99.58% genome-wide sequence identity. Clearly one of the 12 nucleotides differentiating MSV-A[UG:Kas75:05] from the other haplotype MSV-A_1UgIII isolates is important for virulence. Another very mild isolate is MSV-A[UG:Wak4:05] which exhibited less severe symptoms than all the other isolates, excluding MSV-A[UG:Kas75:05]. However, as it is the only representative of haplotype VIII that was examined it is not possible to say whether its lack of virulence is characteristic of other haplotype MSV-A_1UgVIII viruses. Besides these two mild variants, isolate MSV-A[UG:Hoi159:05] and the one non-infectious clone (MSV-A[UG:Iga235:05]), the other Ugandan isolates all generally caused symptoms ranging from extremely severe to moderately severe in all the maize genotypes tested.

In fact all infectious viruses caused significant stunting in every maize genotype in which they produced symptoms. As expected, there was more stunting observed in the highly sensitive Golden Bantam in comparison to Sweetcorn and Pan77 that are moderately susceptible and resistant, respectively. Isolates belonging to haplotype MSV-A_1UgII and MSV-A_1UgV generally caused greater stunting in comparison to the other haplotypes although bigger samples of viruses from the different haplotypes would be needed to confirm whether this trend is significant or not. Although MSV-A_1UgIII isolates are most prevalent in Uganda (see Chapter 4), haplotype MSV-A_1UgV and MSV-A_1UgII isolates have been identified as the most prevalent MSV-A isolates in other parts of Africa (Martin et al. 2001). Demonstration that the Ugandan MSV isolates from across the diversity spectrum are capable of severely infecting even resistant maize genotypes, adds credibility to the survey finding that there was no detectable difference in MSD incidence and severity in fields of supposedly MSV-resistant and probably MSV-sensitive maize genotypes. Taken together these results indicate that it may not be possible to effectively control MSD in Uganda using currently available resistant genotypes alone.

It should be pointed out, however, that resistance was not tested here in Ugandan maize genotypes under Ugandan conditions, and therefore a remaining challenge will be to test the virulence of the Ugandan MSV isolates in this country-specific context. Nevertheless this is the first time that symptom characteristics of genetically defined Ugandan MSV isolates have been analyzed. A major contribution of this study will be that these agroinfectious constructs will be available for breeders to conduct controlled challenges of new MSV-resistant maize genotypes currently being developed and tested for release in Uganda.

In conclusion, this study reports the most up-to-date and extensive MSD epidemiology study that has ever been undertaken in Uganda, detailing the diversity of maize varieties used by farmers and their cropping practices, and relating these to country-wide and district-specific MSD incidence and severity. Furthermore, pathogenicity characteristics of six of the eight main MSV-A haplotypes that have been identified in Uganda are presented.

It has been shown that neither intercropping nor the use of MSV-resistant maize genotypes is an effective MSD control strategy. It has also been shown for the first time that Ugandan MSVs have varying pathogenicity characteristics and that a positive correlation exists between disease incidence and severity. These observations can potentially be extended to neighbouring countries where similar farming practices are followed, and should be taken into consideration in the design and implementation of effective MSD control measures in Uganda and other African maize-growing areas. Development of new cultivars suited for the different climatic regions of Uganda but with better and more stable MSV resistance than existing genotypes such as Longe I, is essential. With agro-infectious clones representing the breadth of MSV diversity found in Uganda it should be easier to test newly developed resistant varieties. Currently, both conventional breeding and transgenic methods are being pursued to enhance resistance in maize to MSV. The world's first MSV-resistant transgenic maize (Shepherd et al. 2007b), developed by an all-African team and currently at the field trial stage, is a first step in this direction.

Chapter 3

FTA® CLASSIC CARDS AND ϕ29 DNA POLYMERASE FOR LARGE-SCALE FIELD SAMPLING AND CLONING OF MAIZE STREAK VIRUS GENOMES

3.1 Summary

Leaf samples from 155 maize streak virus (MSV)-infected maize plants were collected from 155 farmers fields in 23 districts in Uganda in May/June 2005 by leaf-pressing infected samples onto FTA Classic Cards (www.whatman.com). Viral DNA was successfully extracted from cards stored at room temperature for nine months. The diversity of 127 MSV isolates was analysed by PCR-generated RFLPs. Six representative isolates having different RFLP patterns and causing either severe, moderate or mild disease symptoms, were chosen for amplification from FTA cards by bacteriophage ϕ29 DNA polymerase using the TempliPhi^{TM}.

Full-length genomes were inserted into a cloning vector using a unique restriction enzyme site, and sequenced. The 1.3-kb PCR product amplified directly from FTA-

eluted DNA and used for RFLP analysis was also cloned and sequenced. Comparison of cloned whole genome sequences with those of the original PCR products indicated that the correct virus genome had been cloned and that no errors were introduced by the ϕ29 polymerase. This is the first successful large-scale application of FTA card technology to the field, and illustrates the ease with which large numbers of infected samples can be collected and stored for downstream molecular applications such as diversity analysis and cloning of potentially new virus genomes.

3.2 Introduction

Efforts towards curbing the effects of MSV on maize production hinge on a better understanding of the disease characteristics and genetic variation of the causal pathogen. Increased knowledge of MSV, such as its epidemiology, sequence diversity, evolution, and over-wintering non-maize hosts, is vital in order to implement preventative strategies. For this reason, there is a need for rapid and easy collection and storage of MSV-infected plant material for downstream virus purification and molecular-based studies. Using Whatman FTA Classic Cards, viruses can be sampled by simply pressing infected leaves onto this paper-based technology. Use of FTA cards has the potential to reduce cost, sampling time and sample storage space. By enabling the preservation of samples at room temperature these cards also allow sampling of viruses from remote areas.

Downstream processing of DNA from conventionally sampled plant material might include application of phenol-based or chloroform-based purification as described by Sambrook & Russel (2001) or commercially available DNA purification kits. These methods, however, rely on expensive, sometimes toxic reagents, laboratory equipment and refridgerated storage facilities. Additionally, there is a processing time of between 3-4 hours for isolation of nucleic acids. This usually constrains large-scale sampling in remote areas with limited access to facilities, electricity and expensive chemicals.

Studies on the diversity of viruses and plant pathogens in general, involve extensive collection of large numbers of samples, often in remote areas. This means that a versatile, inexpensive, reliable and non-laborious method of sample collection, preservation and recovery is essential for these kinds of studies. FTA (Flinders Technology Associates) cards (Whatman Inc., Clifton, New Jersey, USA) are a technology based on Whatman paper that has been impregnated with a chemical formulation that lyses cells, captures and immobilizes nucleic acids in the paper matrix, denatures and chelates proteins and traps free radicals that might otherwise damage bound nucleic acids. FTA cards represent a simple and relatively inexpensive system for storing [dried samples are protected from degradation and remain safely stored on the card for as long as 14 years; (Whatman 2004)] and purifying viral DNA or RNA.

Although this technology has largely been applied for collection of samples from animal sources such as human blood and saliva (Whatman 2004), it has recently been used to sample DNA from both healthy and pathogen-infected plants. Other areas where the technology has been applied include forensics, molecular breeding, genotyping, and diversity studies. Initial investigations into the use of FTA cards for sampling geminivirus-diseased plants were carried out by Ndunguru et al. (2005a) using greenhouse-sampled plants and a small number of cassava and maize plants from the field (six and seven), respectively.

In the present study, the large-scale application of the method in the field is reported, where 155 maize samples were collected from three regions in Uganda, and stored on FTA cards for downstream applications in the laboratory. Rather than using a PCR-based method, as in Ndunguru et al. (2005a), to isolate geminivirus DNA from plant samples stored on FTA cards, a method employing rolling circle amplification by the bacteriophage ϕ29 DNA polymerase (TempliPhi^{TM}, GE Healthcare Life Sciences) was used. Geminivirus genomes are circular and can therefore be used as templates for ϕ29 DNA polymerase mediated amplification (Inoue-Nagata et al. 2004). This has the advantage that viral DNA can be cloned from samples containing very low concentrations of viral genomes without the need for specific primers (as are required for PCR), thus enabling the amplification and cloning of previously unknown genomes

(Haible et al. 2006). This “templiphi” method has been employed for detection and characterization of geminiviruses including Abutilon mosaic virus, African cassava mosaic virus, Sri Lankan cassava mosaic virus, Indian cassava mosaic virus, Beet curly top virus, TGMV and Tomato yellow leaf curl Sardinia virus (Haible et al. 2006).

This chapter describes a simple and rapid method of collection and storage of large numbers of MSV-infected maize samples in the field. It demonstrates that viral DNA sampled using FTA cards is suitable as a template for both PCR and TempliPhi amplification, and that full-length virus genomes can be relatively easily cloned from these cards. The protocol presented should drastically reduce the effort required to collect, store, analyse and clone geminivirus genomes sampled from natural environments.

3.3 Materials and Methods

3.3.1 Collection of samples

One hundred and fifty five maize plants displaying symptoms characteristic of maize streak disease were sampled from 155 different fields in 23 districts in the eastern, western and northcentral regions of Uganda in May-June 2005. One young symptomatic leaf removed from a potentially MSV-infected plant was placed onto a single FTA Classic Card under parafilm (American National CanTM, Chicago, Il). Each leaf was then gently pressed onto the card through the parafilm until plant sap penetrated the matrix of the paper (Fig 3.1 A). After pressing the sample onto the card, the cards were left to air dry for approximately 5 mins before being placed into plastic bags for storage at room temperature. The severity of disease symptoms on each sampled plant was scored using a 5-point scale described in Table 2.1 (Chapter 2). Fresh leaf samples were also collected, transported in a cooler box and stored first at −20°C, then −80°C prior to DNA extraction.

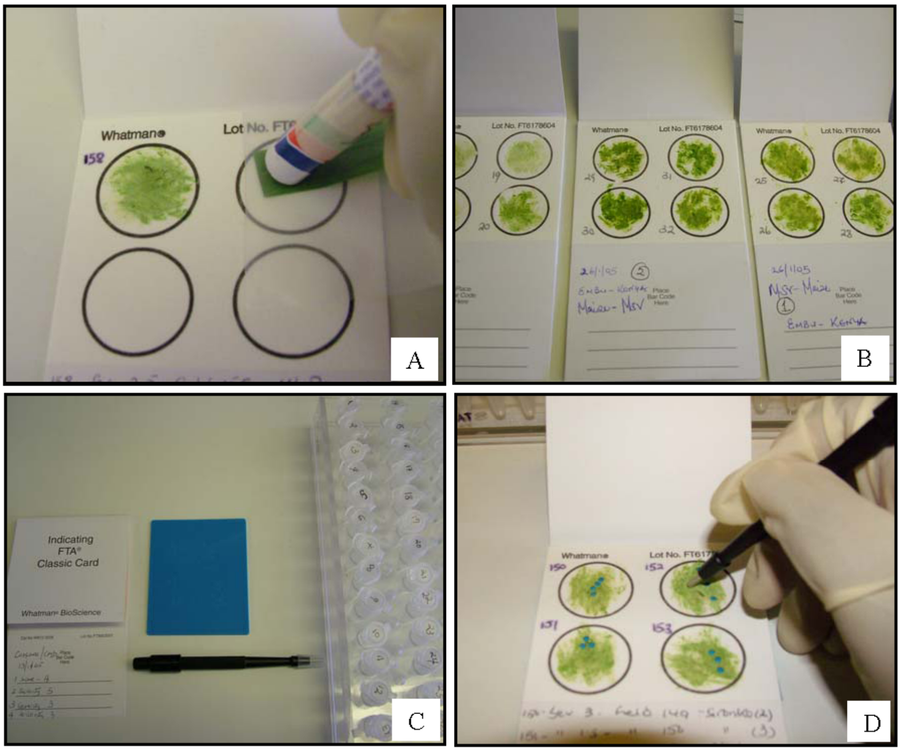


Figure 3.1: (A) A leaf sample being squashed onto an FTA card; (B) FTA cards containing samples collected from MSV-infected maize plants; (C) cards together with punch and cutting mat during recovery of DNA from card samples; (D) excision of punches for recovery of viral DNA.

3.3.2 Isolation of genomic DNA from FTA cards

DNA was recovered from diseased plant samples pressed onto FTA cards, which had been stored at room temperature for 9 months, as described by Ndunguru et al. (2005a) with minor modifications. Three discs were punched from each card (Fig. 3.1D) using a specially designed 3 mm diameter Unicore punch and cutting mat shown in Fig. 3.1C) and washed initially with 300 μL of Tris-EDTA (TE) buffer (pH 8) in an eppendorf tube, by sucking the buffer up and down vigorously for five mins using a pipette. This was followed by washes with (1) 300 μL of 70% ethanol, (2) 300 μL of TE buffer and (3) 300 μL of FTA Purification Reagent supplied with FTA cards, respectively. Discs were then transferred to fresh eppendorf tubes and air-dried for 20 mins. Viral DNA was eluted by soaking the discs in 12 μL of Tris-HCl buffer (10 mM Tris - HCl pH 8) overnight at 4°C. During this step most plant genomic DNA should remain on the disc, whereas smaller nucleic acid molecules such as those of MSV genomes should be preferentially eluted (Ndunguru et al. 2005a). In addition, as a comparison to FTA card extraction, DNA extracted from 305 leaf samples that had been stored at -80°C for three weeks using the CTAB extraction method as described by Kiprop et al. (2002) except that steps that required the addition of Protease K, Dithiothreitol (DTT), and RNAse were omitted from the protocol.

3.3.3 PCR and RFLP analyses

3.3.3.1 PCR analysis

Two previously-described degenerate primers (Willment et al. 2001); 215-234 and 1770-1792 (which bind at nucleotide co-ordinates 361-343 and 1746-1764 respectively; numbering starts from the last A of the conserved TAATATTAC sequence of the MSV-Kom isolate, in the 5'- 3' direction of the primer) were used to amplify a 1.3-kb product from each sample. PCRs were carried out in 50 μL reactions using 2 μL of DNA preparation for FTA card samples and 1 μL DNA preparation for CTAB-extracted samples as template. Amplifications were performed as described by Willment et al. (2001). Briefly, thermalcycling conditions included one initial denaturation cycle at 94°C followed by 30 cycles of 45 s at 93°C, 30 s at 54°C, 90 s at 72°C and a final extension at 72°C for 3 min.

3.3.3.2 RFLP analysis

Each PCR-amplified sample with sufficient DNA (more than 1 μg in total) was cut with a set of seven enzymes: *Rsa*I, *Hpa*II, *Hae*III, *Sau*3AI, *Hind*III, *Cfo*I and *Bam*HI, for RFLP analysis. For this analysis, 5 μL of PCR product were digested in a 20 μL volume consisting of 5 μL of PCR product, 0.5 μL restriction enzyme (1 unit/μL), 2.5 μL buffer and 12 μL water. The mix was incubated at 37°C for two hours after which digested products were electrophoresed through a 2% ethidium bromide stained gel at 100V for 2 hrs. A code letter was assigned to each restriction pattern observed with each of seven enzymes as described by Willment et al. (2001). Previously unreported restriction patterns were given a new code letter. Ultimately, each sample was represented by a string of seven letters representing a restriction profile (Table 3.2, and Figures 3.3 and 3.4). Samples with similar restriction profiles were grouped together and frequencies of occurrence as well as mean severities of disease caused by viruses with each restriction profile computed. Average symptom severities (refer to Table 2.1 in Chapter 2 for a key of symptom severities 0 through to 5) and standard deviation of the mean (wherever three or more samples were obtained) were computed.

3.3.4 Sequencing of complete Ugandan MSV genomes

3.3.4.1 Amplification of virus DNA using the TempliPhikit

After RFLP analysis, six representative samples originating from plants that had mild (1.5 on the symptom severity), moderate (severity of 2.5) and severe (severity of 4) MSD symptoms were selected for further molecular analysis to determine the efficacy of the FTA card storage system. The selected isolates were MSV-A[UG:Mba27:05](from Mbarara, western Uganda), MSV-A[UG:Kas42:05] and MSV-A[UG:Kas43:05] both from Kasese, western Uganda), MSV-A[UG:Kab48:05](from Kabarole, western Uganda), MSV-A[UG:Mub49:05] (from Mubende, western Uganda) and MSV-A[UG:Wak56:05](from Wakiso, central Uganda). DNA eluted from FTA cards was used as a template for rolling circle amplification by bacteriophage ϕ29 DNA polymerase using the Templiphi^{TM} amplification kit (GE Healthcare Life Sciences) according to the kit instructions. In brief, 2.5 μL of eluant potentially containing circular MSV genomes was added to 2.5 μL of the kit's sample buffer, and the mixture was heated to 95°C for three mins. After cooling to room temperature, 5 μL of reaction buffer and 0.2 μL of enzyme mix (provided with the kit) were added, followed by incubation at 30°C for 20 hrs.

3.3.4.2 Cloning of complete virus genomes

The TempliPhi amplification product (presumably a concatemer of linear viral genomes) was first digested with a series of restriction enzymes that are known to cut once in most MSV genomes, in order to select a unique restriction enzyme site for cloning the full-length genomes. In all cases, 1 μL of the TempliPhi product was sufficient for the digestion and cloning steps. *Bam*HI was chosen for all six samples as it was in all cases found to cut the amplification products into 2.7-kb fragments- the approximate size of complete MSV genomes. The 2.7-kb *Bam*HI-fragments were gel-purified using the Invisorb spin DNA extraction kit (Invitek, Germany) and the purified product inserted into *Bam*HI-digested pGEM-3Zf(+) (Promega Biotech).

3.3.4.3 Primer design and sequencing

The resulting insert DNA was sequenced using M13 forward and reverse primers and additional internal sequencing primers. To design internal sequencing primers, all of the available MSV sequences were aligned and primers designed based on the alignment to cover every 500 bases, with overlaps. Degeneracies were included in the primers. Four sets of primers, the positions at which they bind and degeneracies are shown in (Table 3.1).

Sequencing was outsourced to Macrogen Inc., Korea, who use ABI3730XL and ABI3700 automated sequencers. Both strands of cloned genomes were sequenced. Additionally, a 1.3-kb product was amplified from the six FTA-eluted samples representing mild, moderate and severe isolates, using proof-reading DNA polymerase (Accuzyme, Bioline, UK) and degenerate primers (Willment et al. 2001) as described section 3.3.3. The purified product was cloned into the pGENE-JET (Fermentas) vector and sequenced using pGENE-JET-specific primers supplied with the kit. This enabled a comparison between the sequences of whole MSV genomes amplified from FTA cards using Templiphi and the 1.3-kb product amplified from FTA cards by PCR. Sequences were assembled and edited using DNAMAN (version 5.2.9; Lynnon Biosoft,Quebec,

Table 3.1: Primer sequences used for PCR and sequencing of complete MSV genomes

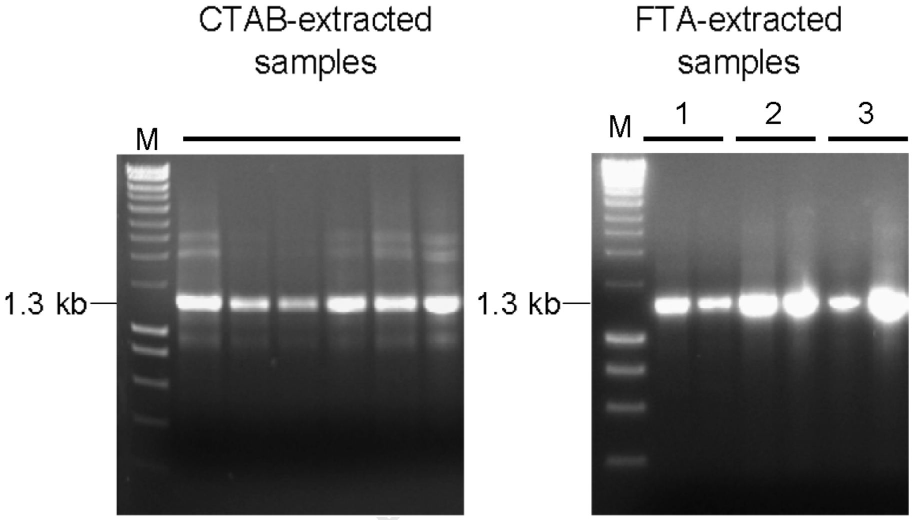
Primer name	Sequence	Binding position	Length (bp)
215-234	5'-CCA AA(GT)(AGT)TCAGCTCCTCC G-3'	361-343	20
1770-1792	5'-TTGG(CGA)CCG(AC)(ACG)GATGTA(CG)AG-3'	1746-1764	25
MSV F1	5'-GACCTCATCAAYACMTATGCCC-3'	499	22
MSV F2	5'-GTTGGGAGTGAGAACGCAGTGG-3'	899	22
MSV F3	5'-CATTTCCTTCATCCARTCTTCATCCG-3'	1333	26
MSV F4	5'-GGTGAGGAKKGYGGATGAGGATYTGRTG-3'	1811	27
MSV R1	5'-GGCCTCCTCCTCATCCAACCGTC-3'	345 RC	23
MSV R2	5'-CAYCARTCCTCATCCRCMMTCCWCACC-3'	902 RC	27
MSV R3	5'-CGGATGAAGAYTGGATGAAGGAAATG-3'	1372 RC	26
MSV R4	5'-CCACTGCGTTCTCACTCCCAAC-3'	1813 RC	22

Canada) and MEGA (version 3.1; Kumar et al. 2004). Analyses were performed in MEGA and phylogenetic trees constructed including other previously reported maize-infecting MSVs.

3.4 Results

3.4.1 PCR analysis of MSV-infected field samples collected and stored on FTA cards

Of the 155 samples processed from FTA cards, 91% (141) gave the expected amplicon of 1.3 kb and 9% (14) were PCR-negative. Similarly, of the CTAB-extracted samples, 92% (281) gave a positive PCR product and 8% (24) were PCR-negative. The strength of PCR signal for both FTA and CTAB-extracted samples was comparable (Fig. 3.2). 127 samples stored on FTA cards yielded PCR fragments of a high enough concentration (more than 1 μg in total) sufficient for RFLP analysis with seven enzymes. An analysis of the recovered samples in relation to symptoms observed on sampled plants showed that most (137/155) sampled plants had moderate to severe symptoms, and these had a higher recovery of virus in comparison with mildly diseased plants (72% of the sampled plants with mild symptoms gave a positive PCR product, compared with 93% of sampled plants with moderate/severe symptoms).


CTAB-extracted samples

FTA-extracted samples

Figure 3.2: PCR products from FTA Classic Card-extracted DNA compared with CTAB-extracted DNA. A 1.3-kb product was amplified using a MSV replicative form-specific degenerate primer pair, 215-234 and 1770-1792 (Willment al. 2001). M= HyperLadder I DNA molecular weight marker (Bioline, Germany), 1= mild severity; 2=moderate severity; 3=severe symptoms.

3.4.2 RFLP analysis

In the majority of samples, restriction enzyme analysis of PCR products yielded fragment sizes reported in previous studies employing the same PCR-RFLP analysis (Willment et al. 2001, Martin et al. 2001). Some PCR products, however, gave one or more unique restriction fragment patterns that had never been reported previously for each of the enzymes *Rsa*I (pattern L), *Hpa*II (pattern J), *Hae*III (pattern L and J) and *Hind*III (patterns F, K, H)(Fig. 3.3).

An *in silico* representation of previously reported RFLPs and new patterns identified in this study is presented in Fig. 3.4. Based on RFLPs observed with each of the seven enzymes, samples were grouped into 28 variants, each represented by a

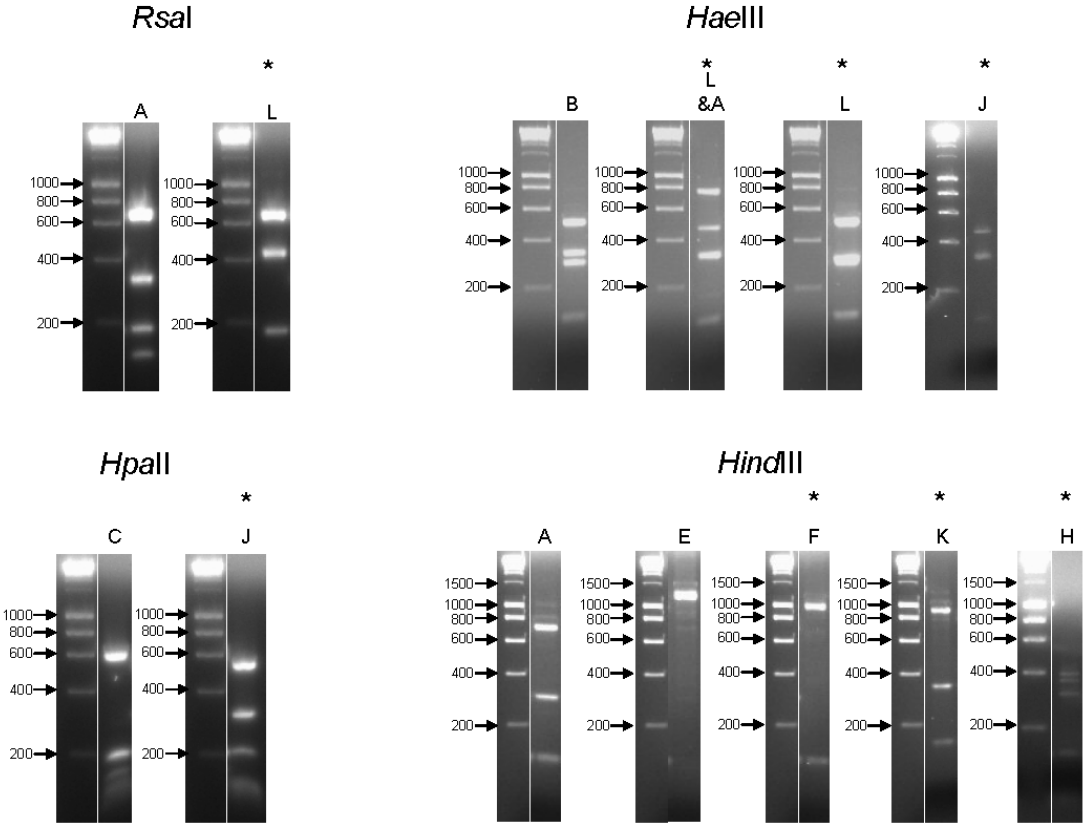


Figure 3.3: New restriction patterns identified with restriction enzymes *Rsa*I, *Hpa*II, *Hae*III and *Hind*III. Each new pattern is marked by an asterisk, and is shown in comparison with previously reported patterns for each enzyme. Each RFLP pattern is shown adjacent to a molecular weight marker (HyperLadder I, Bioline, Germany) for size comparison. Sizes of HyperLadder I fragments are given in base pairs.

different restriction profile (Table 3.2). Sixty percent of samples were grouped into one category (Isolate group 6; AABAAAD) while the second most prevalent (Isolate 1; BCAAFAA) type accounted for 8.7%. The remaining isolate groups made up the other 31.3%.

To estimate the degree of MSV diversity present within individual fields, the RFLP profiles of samples from the 96 fields for which there were two successful CTAB and one successful FTA-card derived PCR amplification products were compared. Despite 60% of samples collected country-wide sharing the same RFLP profile, in only 20.8% of fields did all three samples have the same RFLP profile. Whereas two different RFLP profiles were detected in 44.8% of fields, three different RFLP profiles were detected in 34.4 % of fields.

Table 3.2: Grouping of MSV variants based on restriction profiles of a 1.3-kb PCR product amplified from MSV-infected samples stored on FTA cards

Variant	Restriction profile	Number of isolates	Symptom severity*
1	BCAAFAA	11	3 (0.88)
2	CAAAGAK	1	3.0
5	BBAAFAA	1	2.0
6	AABAAAD	76	3.1 (0.68)
8	ABAAEAA	2	3.5
10	ACAAAAA	1	2.5
12	AALAAAD	1	2.0
13	AABAFAD	2	3.8
15	AABAKAD	3	3.5 (1.00)
16	AAAAAAD	1	3.0
17	ABAAFAA	3	2.8 (1.12)
18	ACBAAAD	4	2.6 (0.25)
25	AABAGAD	1	2.0
28	BCJAFAA	2	3.3
29	AABAAAA	2	3.5
31	CAAAGAA	1	3.0
33	BABAAAD	1	3.0
35	ACBAAAA	1	3.0
40	ACAAEAA	2	3.8
45	ACAAFAA	2	3.8
48	BCAAFAD	1	4.5
49	LCAAFAA	1	3.5
50	AJBAAAD	1	2.5
52	ACAAKAA	1	2.5

Continued. . .

Table 3.2 – Continued

Variant	Restriction profile	Number of isolates	Symptom severity*
53	AABAHAD	2	3.0
54	AAAAEAA	1	3.5
56	CAAAAAA	1	3.0
57	AABEAAD	1	3.5

*Standard deviation of the mean in parentheses

3.4.3 Cloning and sequencing of full-length virus genomes using TempliPhi

Viral DNA from each of the six selected representative MSV-infected samples was successfully amplified using TempliPhi (Fig. 3.5) and cloned into pGEM-3Zf(+) (Fig. 3.6). The identity of the six full-length virus genome clones (MSV-A[UG:Mba27:05], MSV-A[UG:Kas42:05], MSV-A[UG:Kas43:05], MSV-A[UG:Kas48:05], MSV-A[UG: Mub49:05] and MSV-A[UG:Wak56:05]), amplified and representing mild, moderate and severe isolates, was verified by sequencing the genomes in pGEM-3Zf(+). Full sequences can be accessed at the Genbank accession numbers listed in Table 3.3.

In addition, each 1.3-kb product amplified by PCR from the same FTA card-eluted samples was sequenced. That the 1300 nucleotides of sequence shared by the PCR and TempliPhi amplicons were identical for all six samples indicates both that the two amplification methods have comparable fidelity, and that neither is especially error prone. Sequences of the 1.3-kb products from the degenerate primer PCRs can be accessed at the database accession numbers listed in Table 3.3.

Phylogenetic analysis (maximum likelihood with HKY model using inference of model parameters from the data and 100 bootstrap replicates; Guindon & Gascuel 2003) of the sequences determined in this study with other publicly available MSV sequences (all aligned using the POA method of Lee et al. 2002, with default settings) indicated that they are all clearly from viruses belonging to the "maize type" or A strain of

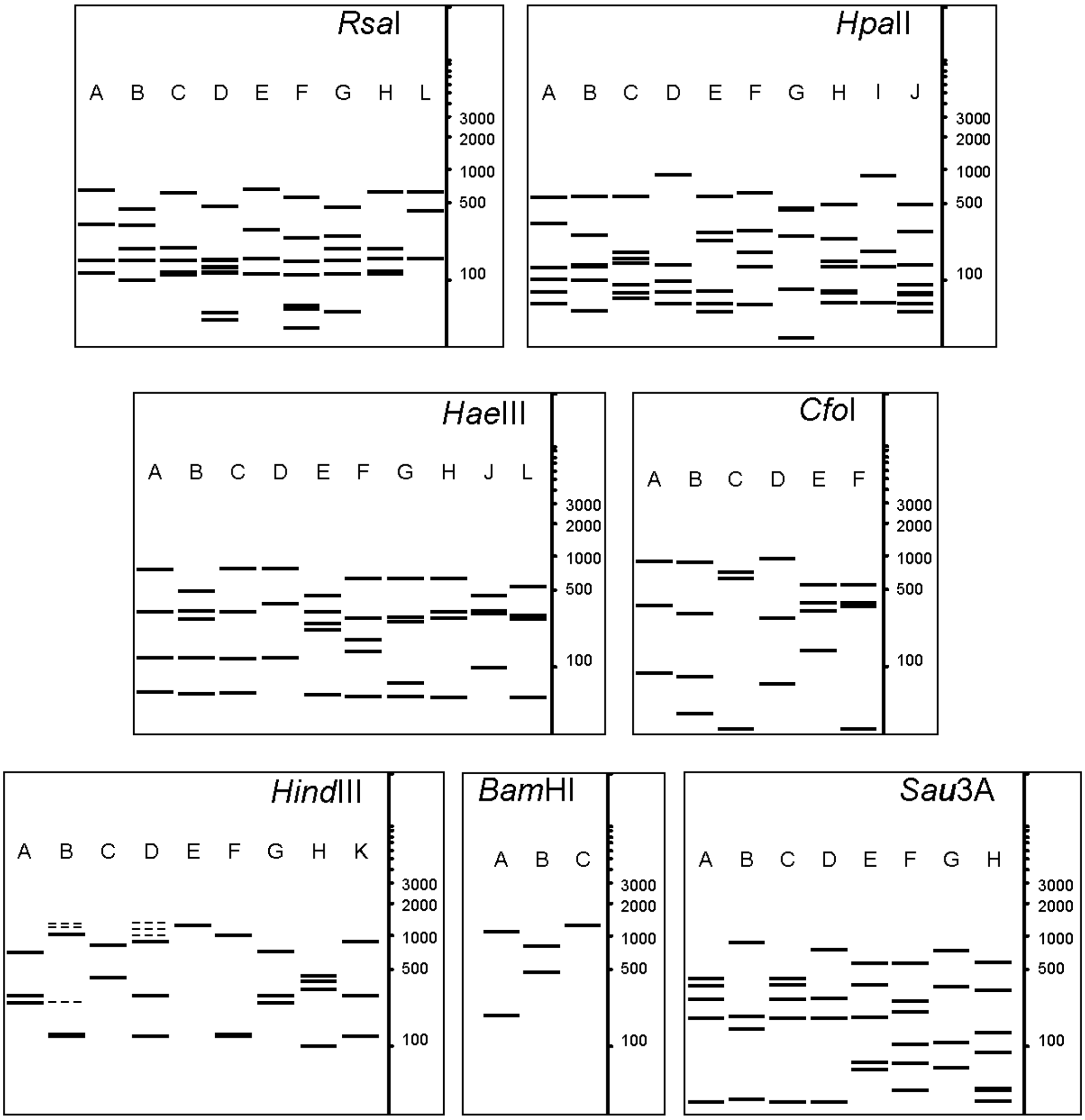


Figure 3.4: *In silico* RFLPs for the seven restriction enzymes *Rsa*I, *Hpa*II, *Hae*III, *Cfo*I, *Hind*III, *Bam*HI and *Sau*3A showing all previously reported patterns to date as well as new patterns identified in this study. A new code letter was assigned to each restriction pattern that differed from those previously reported (Willment et al. 2001) for each of these enzymes: For *Rsa*I, pattern L; *Hae*III, pattern J and L; *Hpa*II, pattern J and *Hind*III, pattern F, K and H. Numbers along the right hand side of each block indicate size of fragments in base pairs.

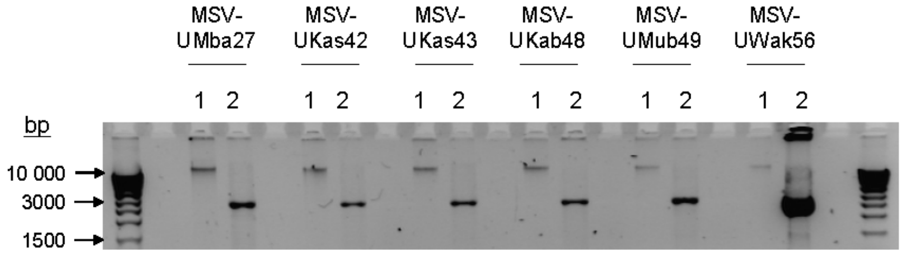


Figure 3.5: TempliPhi amplification products of MSV-A[UG:Mba27:05], MSV-A[UG:Kas42:05], MSV-A[UG:Kas43:05], MSV-A[UG:Kab48:05], MSV-A[UG:Mub49:05] and MSV-A[UG:Wak56:05] followed by restriction enzyme digestion with *Bam*HI. The 2.7-kb circular MSV genome is amplified as a concatemer (1); digestion with *Bam*HI releases linear 2.7-kb full-length genomes (2) that are then inserted into a *Bam*HI-digested cloning vector, pGEM-3Zf(+). Note that the apparent faster migration of the *Bam*HI-digested MSV-A[UG:Wak56:05] sample was due to its being overloaded and not due to a size discrepancy in comparison with the other samples.

MSV (Fig. 3.7; Martin et al. 2001). Following the naming convention proposed by Martin et al. (2001), the sequences are all of the A_1 (MSV-A[UG:Mba27:05], MSV-A[UG:Kas42:05], MSV-A[UG:Kab48:05] and MSV-A[UG:Mub49:05]) and A_5 (MSV-A[UG:Kas43:05] and MSV-A[UG:Wak56:05]) subtypes previously identified in a study of Kenyan MSV diversity as being common in East Africa (Martin et al. 2001).

3.5 Discussion

FTA card technology offers a convenient and largely unexplored method for sampling and storage of plant DNA samples. Ndunguru et al. (2005a) evaluated the efficacy of FTA Classic Card technology for the sampling, retrieval and PCR-based analyses of DNA viruses, and demonstrated that the potential number of samples that could be collected within a given time and location should significantly increase compared with other currently used sampling methods. The present study goes beyond a demonstration of potential utility to being the first practical application of the technology to large-scale sampling in the field, in which leaf samples from 155 MSV-infected maize plants were collected from 155 fields in Uganda. Following collection the samples

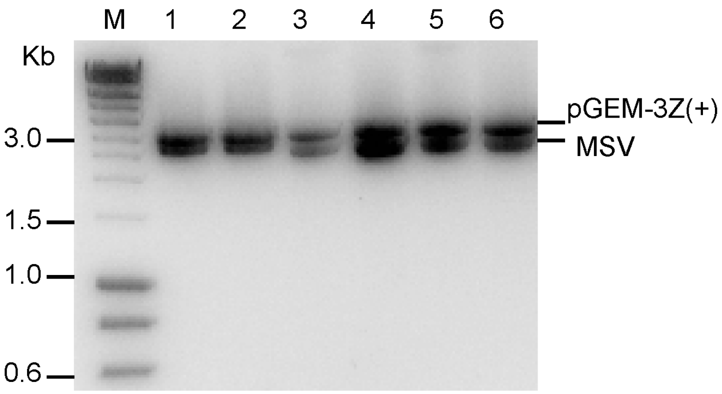


Figure 3.6: Full genome TempliPhi amplification products of MSV-A[UG:Mba27:05], MSV-A[UG:Kas42:05], MSV-A[UG:Kas43:05], MSV-A[UG:Kab48:05], MSV-A[UG:Mub49:05] and MSV-A[UG:Wak56:05] inserted into a *Bam*HI-digested cloning vector, pGEM-3Zf(+). The two bands showing linearized pGEM-3Zf(+) at 3.0kb and the 2.7Kb MSV insert.

Table 3.3: Genbank accession numbers for sequenced FTA card isolates

Sequence name	Accession number	Symptom characteristic
Full-length sequences		
MSV-A[UG:Mba27:05]	EF015781	mild
MSV-A[UG:Kas42:05]	EF015780	mild
MSV-A[UG:Kas43:05]	EF015779	moderate
MSV-A[UG:Kab48:05]	EF015782	moderate
MSV-A[UG:Mub49:05]	EF015783	severe
MSV-A[UG:Wak56:05]	EF015778	severe
1.3-kb sequences		
MSV-A[UG:Mba27:05] degen	EF015772	mild
MSV-A[UG:Kas42:05] degen	EF015777	mild
MSV-A[UG:Kas43:05] degen	EF015775	moderate
MSV-A[UG:Kab48:05] degen	EF015773	moderate
MSV-A[UG:Mub49:05] degen	EF015774	severe
MSV-A[UG:Wak56:05] degen	EF015776	severe

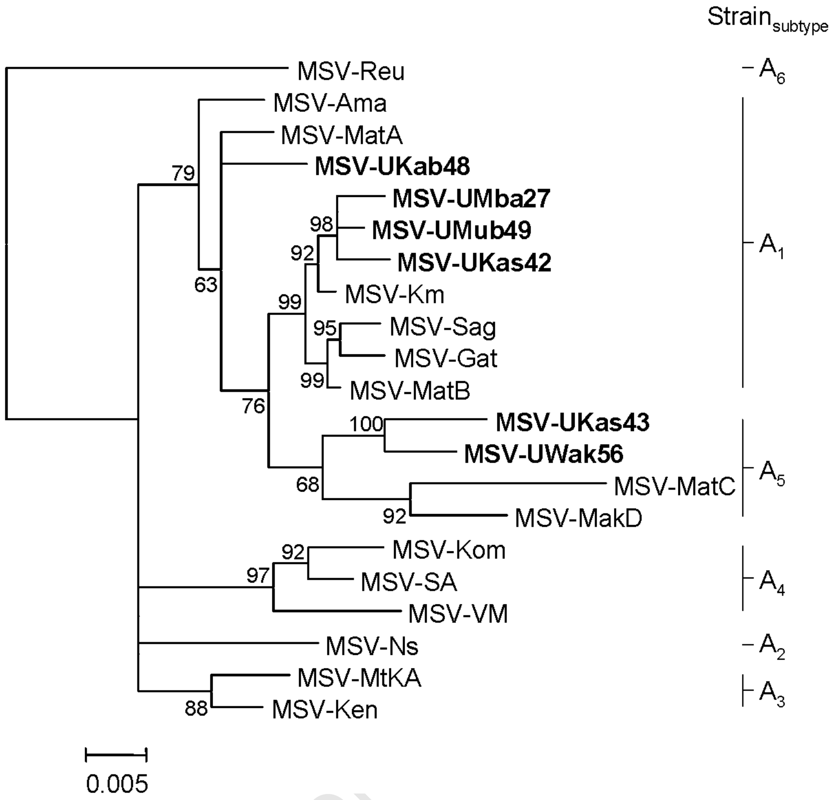


Figure 3.7: Classification of Ugandan MSV full genome sequences using the maximum likelihood method of phylogenetic reconstruction (HKY model with all parameters estimated from the data). The tree was constructed using 100 full maximum likelihood bootstrap replicates and numbers associated with branches represent the degree of bootstrap support for each branch. Branches with less than 60% support have been collapsed. All six sequences determined in this study (in bold) fall clearly within the subtype A_1 and A_5 groups. MSV-UKab48=MSV-A[UG:Kab48:05],MSV-Mba27=MSV-A[UG:Mba27:05], MSV-UMub49=MSV-A[UG:Mub49:05],MSV-UKas42=MSV-A[UG:Kas42:05],MSV-Kas43=MSV-A[UG:Kas43:05],MSV-UWak56=MSV-A[UG:Wak56:05].

were stored at room temperature for between eight and nine months before being processed. Over 90% (141/155) of the samples contained MSV DNA that was amplifiable by degenerate primer PCR. Of these, 127 (90%) yielded more than 1 μg of DNA: A quantity that is suitable for the diagnostic RFLP analysis using seven restriction enzymes previously described for MSV (Willment et al. 2001, Martin et al. 2001). This compares very well with the established sampling method for which 92% (281/305) of samples yielded PCR-amplifiable MSV DNA, 91% (257 samples) of which were suitable for diagnostic RFLP analysis. The positive PCRs indicated that viral DNA sampled, stored at room temperature, and extracted from FTA cards nine months after field sampling could be achieved at a success rate comparable to more laborious methods such as CTAB, which requires freezing of leaf samples for storage, grinding of leaf material in liquid nitrogen, the use of centrifuges and several incubation steps. Whereas it is possible that viral DNA in the PCR-negative samples had not survived storage on, and extraction from, the FTA cards, it is also possible that these samples were from plants either mistakenly identified as having MSV symptoms, or plants infected with MSV variants that were not amplifiable with the PCR primers used in this study. Compared with sampling and storage of intact MSV-infected plant material followed by CTAB extraction of viral DNA, there was a valuable saving in terms of time and resources using FTA cards. To extract DNA from, for example, 20 samples using CTAB would take at least 4-5 hrs, compared with approximately one hour from FTA cards. While FTA card extraction requires few reagents and no expensive laboratory equipment, CTAB extraction requires liquid nitrogen, numerous chemicals (including toxic reagents such as chloroform, isoamyl alcohol and mercaptoethanol), a bench top centrifuge and either a water bath or heating block (Kiprop et al. 2002). This makes FTA cards particularly useful for resource poor institutions, since it even has the added advantage that electricity is not required for its processing and use.

PCR-RFLP analyses, and PCR-cloning of virus genomes are heavily dependent on a *priori* knowledge of at least the approximate sequence of the genomes being analysed. This study therefore investigated the use of a non sequence-specific circular DNA amplification technique, for the cloning of complete MSV genomes from FTA cards. As viral genomic DNA concentrations on the cards could potentially influence the success of their amplification, an attempt was made to clone full genomes from samples obtained from plants displaying severe (disease rating = 4), moderate (disease rating = 2.5) and mild (disease rating = 1.5) MSD symptoms (two plants in each symptom category). As MSV symptom severity correlates well with viral DNA concentration (Shepherd et al. 2005), the severely infected samples should contain the most and the mildly infected samples the least viral DNA. As virus DNA was efficiently amplifiable by the Phi 29 DNA polymerase from all six samples, it is clear that the quality and quantity of DNA stored on, and elutable from, the cards is sufficient for virtually any analytical purpose. With the method described in this chapter, only 2.5 μL of FTA card-eluted DNA was used in the TempliPhi reaction in a total reaction mix of 10.2 μL. Of this reaction mix, just 1 μL of amplified product was used for the cloning of full length MSV genomes. Demonstration that the cloned and sequenced genomes had sequences that differed from one another and other sequenced MSVs, and that the sequence of each genome was identical to the homologous region amplified from FTA card-eluted DNA by degenerate primer PCR, meant that the samples had not been inadvertently cross-contaminated. This also demonstrated that neither the TempliPhi nor the PCR amplifications was inherently error prone, with both inducing false polymorphisms in less than one in every 7.8 X 10^3 nucleotides sequenced. The TempliPhi technique has since been used to clone and sequence 62 full-length virus genomes, the entire process optimized to take just three days (Chapter 4).

There are several downstream applications for FTA card archived samples. According to the manufacturers, samples have been stored for up to 14 years with no change in the integrity of the attached DNA (Ndunguru et al. 2005a). With improved vascular puncture inoculation (VPI; Louie 1995) techniques (Redinbaugh et al. 2001, Redinbaugh 2003), it may be possible to directly inoculate plants with viral DNA eluted from FTA cards using VPI. Alternatively, MSV genome concatemers generated during Phi 29 DNA polymerase amplification may also be infectious using these techniques.

In conclusion, results of this study have demonstrated that use of FTA Classic Cards facilitates large-scale and geographically extensive field sampling of viruses such as MSV, and yields DNA of sufficient stability, quantity and quality for a wide range of potential downstream analyses. The techniques that have been investigated in this study should be applicable to other plant viruses with DNA genomes. Additionally, non-specific Phi 29 DNA polymerase amplification of circular viral genomic DNA sequences from plant material sampled from FTA cards should vastly simplify the discovery of novel viruses.

Chapter 4

GENETIC ANALYSIS OF UGANDAN *MAIZE STREAK VIRUS* ISOLATES

4.1 Summary

Maize streak virus (MSV) contributes significantly to the problem of extremely low African maize yields. Previous studies on maize streak disease (MSD) in Uganda conducted over 10 years ago, presented data on disease levels but none went on further to look at MSV characteristics or attempted to ascertain whether there are different genetic variants and strains contributing to MSD in the country. Whereas a diverse range of MSV and MSV-like viruses is endemic in sub-Saharan Africa and neighbouring islands, only a single group of maize-adapted variants - MSV subtypes A_1 through A_6 - causes severe enough disease in maize to substantially influence yields. In order to assist in designing effective strategies to control MSV in maize, a large survey covering 155 locations was conducted to assess the diversity, distribution and genetic characteristics of the Ugandan MSV-A population. PCR-RFLP analyses of 391 virus isolates identified 49 genetic variants. Sixty-two full genome sequences were determined, 52 of which were detectably recombinant. All but two recombinants contained predominantly MSV-A_1-like sequences. Of the ten distinct recombination events observed, seven involved inter-MSV-A subtype recombination and three involved intra

MSV-A_1 recombination. One of the intra-MSV-A_1 recombinants, designated MSV-A_1UgIII, accounted for more than 60% of all MSV infections sampled throughout Uganda. Furthermore, this most prevalent type was widely distributed throughout the sampled regions. This chapter describes the genetic characteristics of MSV in Uganda and shows that recombination continues to play a role in the evolution and diversity of Ugandan MSVs.

4.2 Introduction

Maize streak disease significantly impacts on maize yields, vastly affecting the livelihoods of millions of people in sub-Saharan Africa who depend on this important food crop. The causal agent, MSV, belongs to a family of viruses (geminiviruses) that are very versatile, frequently mutating and recombining with other strains or species often resulting in fitter and more virulent types (Padidam et al. 1999). This often presents challenges to their management and control. MSD continues to reduce crop yields especially in fields of subsistence farmers despite the availability and use of MSV-resistant genotypes for over 20 years. Martin et al. (2001) have reported that there is very low diversity in the different maize-infecting MSVs (called MSV-A) sampled across different areas in Africa. The low continent-wide MSV diversity should vastly simplify the development of resistant maize genotypes. With cassava-infecting viruses also from the *Geminviridae* family, however, the situation is different. At least seven species of cassava-infecting geminiviruses (CGVs), each sharing <90% genome-wide sequence identity with the others, cause CMD in Africa (Fauquet & Stanley 2003, Fauquet et al. 2008). Interspecies recombinants of these viruses are also common (Ndunguru et al. 2005b), an important example being the widespread Uganda variant of East cassava mosaic virus.

Although inter-species MSV recombinants have been detected, the scale of recombination in terms of both the size of sequence tracts transferred and the genetic distances between parental viruses appears to be much smaller than that observed in the CGVs (Martin et al. 2001). It is possible that the apparently striking evolutionary and demographic differences between MSV and CGVs are because CGVs have been more intensively sampled and characterized. Alternatively, differences in the epidemiological and population genetic characteristics of the two groups could be responsible for the apparently large differences in their evolutionary trajectories.

PCR-based methods have been commonly used for cloning virus genomes prior to sequencing. This method however depends on prior knowledge of sequences for primer design. RFLPs of PCR-generated products have also provided a very useful tool for studying diversity and typing viruses. However, rapid, simple, efficient and cost effective typing of viruses together with full-genome sequence data is essential for an accurate genetic diversity study. With technologies such as the Phi 29 polymerase Templiphi system (described in Chapter 3), sequence data can now be generated with more ease. Availability of information on viruses, their genetic characteristics, variants and distribution is important for effective resistance breeding. Such information on genetic characteristics and diversity of MSV in Uganda however, remains scarce. Understanding the diversity and genetic characteristics of MSV isolates occurring in Uganda is important if effective control measures are to be implemented.

In this chapter, a study was conducted to determine the genetic characteristics of Ugandan MSVs and to identify the existing variants in the country. Furthermore, a comparison of the recombination patterns of MSV and CGVs was also made.

4.3 Materials and Methods

4.3.1 Sample collection

A survey was conducted in the main maize-growing areas of Uganda, covering 23 central, north-central, eastern and western districts of the country, during May and June 2005 (in the first cropping season). A total of 460 samples (which include the 155 analysed in Chapter 3), three from each field where possible, were collected in each of 155 fields. Each field was divided into two diagonals and the three samples were picked, two from the first diagonal and the third from the second diagonal. In fields with fewer than three diseased plants on the two diagonals up to three symptomatic plants were sampled from the remainder of the field. Samples were either fresh leaves or leaf pressings made onto FTA cards as outlined in Chapter 3. The fields were approximately 10 km or more apart and contained plants ranging from 1 to 3.5 months old. Global positioning system (GPS) coordinates were recorded for each sample.

4.3.2 DNA extraction, PCR and RFLPs analyses

Total DNA was extracted from 305 MSV-infected leaf samples following the CTAB method described by Kiprop et al. (2002) with modifications indicated in Chapter 3. Both PCR and RFLPS were conducted using a pair of degenerate primers for detection and a set of seven restriction enzymes for RFLPs as described in Chapter 3 and Willment et al. (2001). For the subsequent analyses, the 155 FTA samples described in Chapter 3 were included bringing the total number of samples to 460. Similarly, as for Chapter 3, the frequency of each restriction type was noted as well as severity types associated with the different isolate groups. In addition, *in silico* RFLP analysis was performed on sequenced genomes. Derived *in vitro* and *in silico* restriction patterns were compared and categorized according to a comparative panel of all currently published MSV restriction pattern types (Martin et al. 2001, Willment et al. 2001; Chapter 3), with newly discovered patterns being added to the comparative panel of pattern types.

4.3.3 Sequencing of full-length MSV genomes

From the 155 fields sampled, 30 fields were chosen at random using a random number generator, and for each chosen field, one sample with a positive PCR was selected for full genome sequencing. An additional 32 samples were chosen for full genome sequencing based on their unique RFLP profiles. A total of 62 samples extracted using CTAB, hereafter referred to as CTAB samples, were cloned. Cloning and sequencing were conducted as described in Chapter 3 with modifications. One microlitre of each sample was used as template for the Templiphi reaction and added to 2.5 μl of sample buffer and the rest of the protocol followed as described in Chapter 3. Concatemers generated by Phi29 DNA polymerase were digested with *Bam*HI or *Sal*I and the resulting 2.7-kb fragments (potentially containing full-length linearised MSV genomes) were cloned into pGEM-3Zf(+) (Promega Biotech) and sequenced as previously described in Chapter 3.

4.3.4 Sequence assembly and analyses

All sequences were assembled and edited using DNAMAN (version 5.2.9; Lynnon Biosoft) and MEGA (version 3.1; Kumar et al. 2004). Additionally, all complete MSV sequences available on public databases were downloaded and aligned with the new sequences. Sequence alignments were constructed using POA (Grasso & Lee 2004) and edited both by eye and using the ClustalW-based (Thompson et al. 1994) sub-sequence realignment tool implemented in MEGA. Phylogenetic trees were constructed using the neighbour joining (JC distances, 1000 bootstrap replicates) and maximum likelihood (HKY model, transition/ transversion ratio inferred from the data and 100 bootstrap replicates) methods implemented in MEGA and PHYML (Guindon & Gascuel 2003) respectively. The six sequenced FTA samples from Chapter 3 were included in the analyses. *In silico* digests were conducted using sequence data. Sequenced clones were aligned together with six Ugandan MSV genome sequences described in Chapter 3, 21 MSV genome sequences sampled elsewhere in Africa, two Panicum streak virus sequences, a Sugarcane streak virus sequence, a Sugarcane streak Reunion virus sequence and a Sugarcane streak Egypt virus sequence (94 sequences in total).

4.3.5 Classification of unsequenced isolates using RFLP data

PCR-RFLP isolates of 321 Ugandan MSV samples (197 from this study and 124 from Chapter 3) that were not analysed by full genome sequencing were classified by comparing their RFLPs to that of sequenced isolates. The sampling area in Uganda was split into seven zones and the relative proportions of the different haplotypes were determined for each of these zones. Triplet samples collected from individual farms were also examined to determine whether there were any significant differences in MSV diversity sampled on these farms at scales of hundreds of square meters relative to country-wide MSV diversity sampled on a scale of thousands of square kilometres.

4.3.6 Recombination analysis

Recombination was analyzed using the RDP (Martin & Rybicki 2000), GENECONV (Padidam et al. 1999), BOOTSCAN (Martin et al. 2005a), MAXCHI (Maynard Smith 1992), CHIMAERA (Posada & Crandall 2001), SISCAN (Gibbs et al. 2000) and LARD (Holmes et al. 1999) methods implemented in RDP3 (Martin et al. 2005c). Default settings were used throughout and only potential recombination events detected by two or more of the above methods coupled with phylogenetic evidence of recombination were considered significant. Also, the severity of Bonferroni correction during detection was minimized by only searching for recombinant signals in a single sequence within groups of sequences sharing >99.7% sequence identity. Composite likelihood estimates (CLE) of population-scaled recombination rates and estimates of population-scaled mutation rates were inferred using the PAIRWISE component of LDHAT [finite sites version of the Watterson theta inferred from the data, a minimum minor allele frequency of either 0.05 (for datasets containing 28 and 68 sequences) or 0.1 (dataset containing 14 sequences), a grid size of 100 and a maximum rho of 100, gene conversion model of recombination with an average tract length of 1000 nucleotides; McVean et al. 2002]. Site to site variation in the CLE of the population-scaled recombination rates was assessed using the INTERVAL component of LDHAT [using pre-computed likelihoods for population-scaled mutation rate = 0.01, a minimum minor allele frequency cutoff of 0.05 or 0.01 (decided as above), a gene conversion model with an average tract length of 1000 nucleotides, block penalty of 10, starting rho of 5, 107 Markov chain Monte Carlo updates with sampling every 2000 updates and the first 500 samples discarded; McVean et al. (2004)]. For variable recombination rate analysis with LDHAT analysis inaccuracies at the edges of alignments were avoided by simulating circular genome sequences. Alignments were constructed from tandem repeats of full genome sequences, with the origins of virion strand replication situated at positions 25% and 75% along the alignments. Results of analyses with these alignments were then processed to exclude the first 25% and last 25% of point recombination rate estimates. Departures from expectations of neutral evolution in full genome sequences were inferred using Tajima's D (Tajima 1989) and Fu and Li's D* (Fu & Li 1993) statistics, calculated using the CONVERT component of LDHAT with no minor allele frequency cutoff. The significance of these statistics contingent on inferred population-scaled mutation and recombination rates was tested by coalescent simulation using DnaSP (Rozas & Rozas 1999).

Having identified the most probable recombinant regions of all available MSV genomes, a recombination-free reconstruction of the MSV-A phylogeny was attempted. Firstly, the smaller recombinant fractions of identified recombinant sequences from the alignment (i.e. replacing the tracts of recombinant sequence comprising the smallest fractions of the sampled genomes with the indel symbol "-"), were removed and only those columns of the sequence alignment in which more than 80% of the sequences contained sequence data were retained. MSV-Kom, -VM and -SA are recombinants that contain MSV-B-like sequences (Martin et al. 2001). To increase statistical power during the analysis of intra MSV-A recombination, both the MSV-B-like portions of the MSV-Kom, -VM and -SA genomes and all the non-MSV-A sequences were removed from the MSV alignment before re-screening for recombination

4.3.7 Comparison between the population genetic characteristics of MSV and African cassava-infecting geminiviruses

This study provided 68 full genome sequences (including the six from Chapter 3) from samples collected at the same time point. This large number of sequence data provided by this study enabled comparisons of population-scaled recombination and mutation rates of MSV and cassava-infecting geminiviruses (CGVs), to be carried out. To compare the MSV diversity and recombination data with that of CGVs, the only other substantially sampled African geminivirus group, all 118 African cassava-infecting begomovirus DNA-A sequences available in public sequence databases on 30 November 2006 were obtained, aligned using POA, edited and subsections of the alignments re-aligned in MEGA.

To investigate whether there was any detectable population genetic evidence of MSV and the CGVs having significantly different recombination rates, firstly, CGV datasets with similar properties to that of the Ugandan MSV dataset from this study and Chapter 3 were assembled. Based on the structure of the MSV dataset, CGV populations were arbitrarily defined as groups of sequences that: (1) all either shared identical inter-species recombinant mosaic structures or were not detectably inter-species recombinants; (2) were all >96% identical to one another; (3) were all sampled over a geographical range similar to that of the Ugandan MSV sample; (4) were all sampled within two years of one another; (5) contained more than ten completely sequenced DNA-A components. Two groups, containing 28 and 14 DNA-A sequences respectively, were the only datasets that met the selection criteria. These groups contain sequences respectively classified as the East African cassava mosaic virus Uganda strain (EACMV-UG) and East African cassava mosaic Kenya virus (EACMKV). Some summary statistics indicating that these datasets are indeed very similar to the MSV dataset from a population genetic perspective are presented in Table 4.2. Only very closely related groups of sequences were used to compute recombination and mutation rate estimates for both MSV and the CGVs.

4.4 Results

4.4.1 PCR analysis and RFLP classification

A total of 305 virus DNA samples were extracted using a conventional DNA extraction method (CTAB method). Of these, 92% (281) gave a positive PCR product and 8% (24) were PCR-negative. Together with the FTA samples amplified in Chapter 3, 387 samples yielded sufficient DNA (>1 μg) for RFLP analyses.

Most of the RFLPs observed have been reported previously for MSV-A isolates (Willment et al. 2001; Chapter 3). The most frequently observed restriction patterns are presented in Fig. 4.1. For each of the seven enzymes, the most frequently recorded patterns were those previously reported whilst the new patterns identified in Chapter 3 and in this study were infrequently observed.

Whereas no new patterns were found for *Hind*III, *Cfo*I, *Bam*HI and *Hpa*II, one new pattern each for *Rsa*I (pattern M) and *Hae*III (pattern K), and five new patterns for *Sau*3AI (patterns J, L, M, N,O) (Fig. 4.2) were observed. Of the 387 samples analysed, 6.20% (24/387) had evidence of mixtures of previously described RFLP patterns. Although these samples possibly represented mixed infections, only 14 of them were included in further analyses as it was possible to unambiguously identify the RFLP profiles of the two viruses present in these (this was achievable for these 14 samples because only one of the seven restriction enzyme digests used per sample yielded evidence of multiple restriction patterns). In addition to *in vitro* RFLP analysis, cloning and sequencing of 62 full-length genomes allowed for *in silico* RFLP analysis to be performed on these sequences. This led to the identification of seven (K, L, M, N, O, P, Q) and one (I) previously unpublished RFLP patterns for *Hpa*II and *Hind*III respectively. These patterns have subtle differences that would be difficult to distinguish on an agarose gel such as that presented in Fig. 4.1 but are nonetheless different variants (Fig. 4.2). Therefore, using a combination of *in vitro* and *in silico* RFLPs, new patterns for five of the seven enzymes were observed.

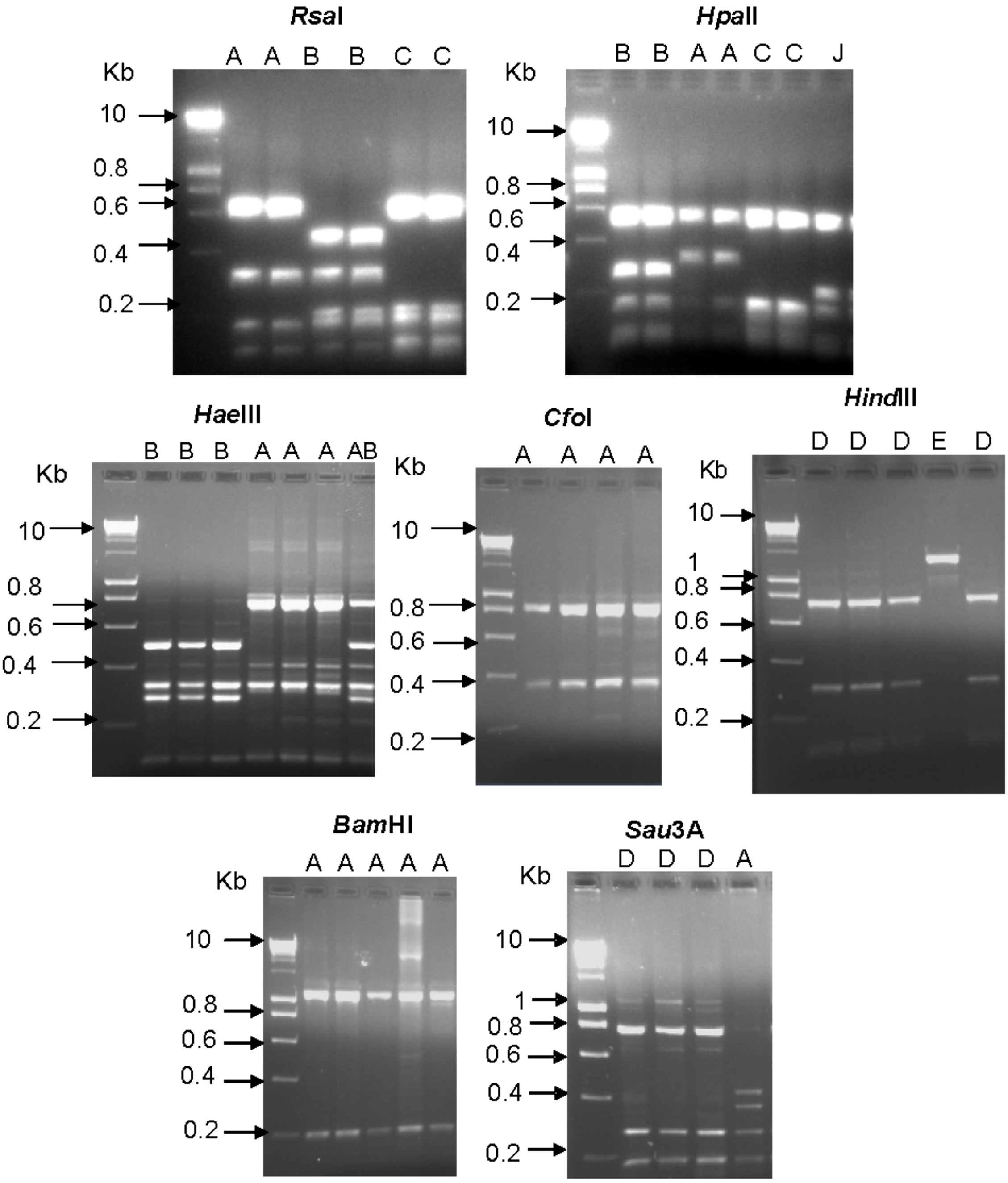


Figure 4.1: *In vitro* RFLPs showing frequent restriction patterns observed for all seven enzymes on a 2.0% agarose gel. The faint extra bands seen with samples digested with *Cfo*I and *Bam*HI are partial digests. Note the difficulty that would arise when distinguishing patterns with bands close together on an agarose gel.

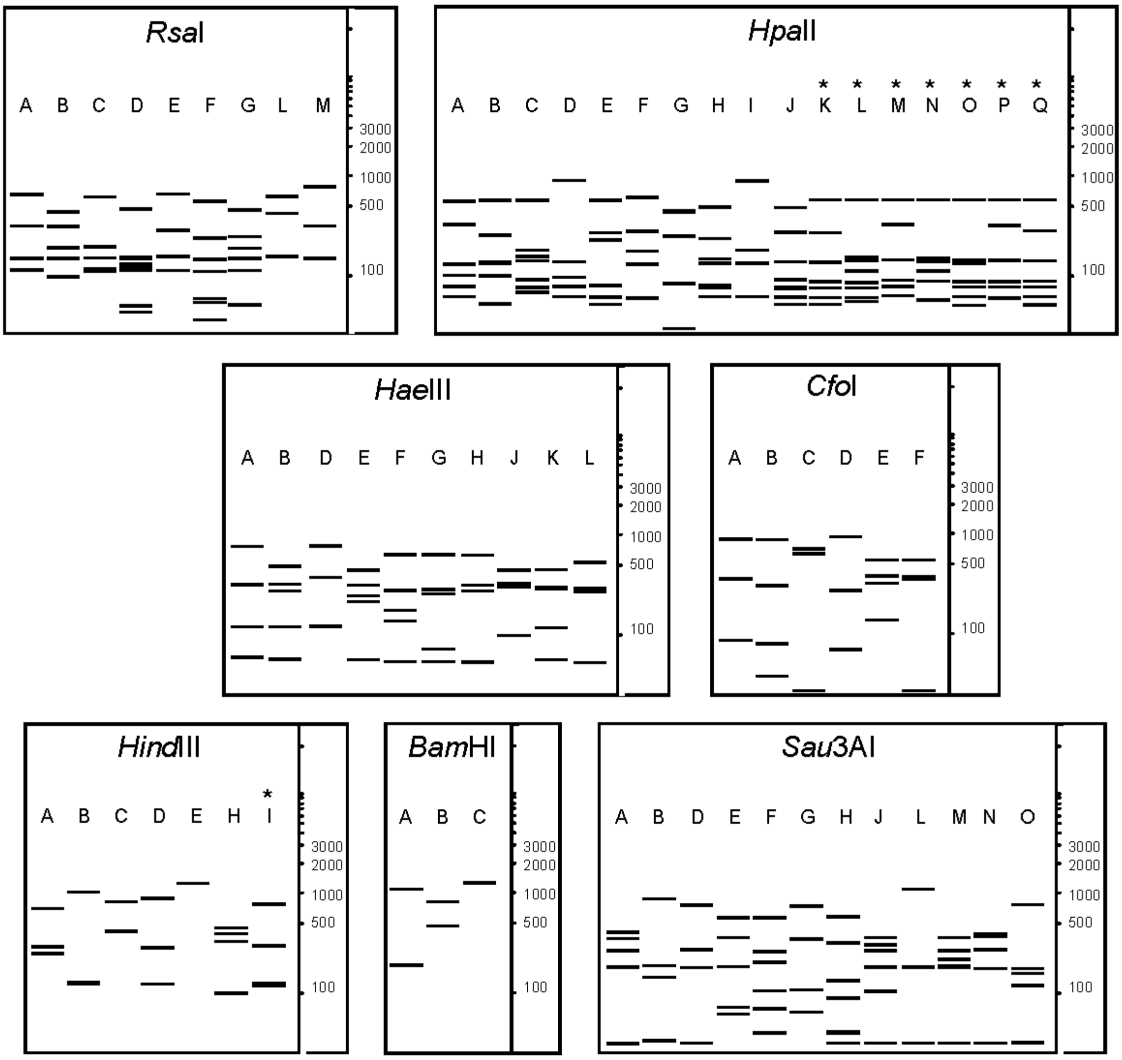


Figure 4.2: New restriction patterns identified with enzymes *Rsa*I (M), *Hpa*II (K, L, M, N, O, P, Q), *Hae*III (K), *Hind*III (I) and *Sau*3AI (J, L, M, N, O). Each new pattern is shown in comparison with previously reported patterns for each enzyme. Molecular size markers are in base pairs. Asterisks mark the patterns that were identified by *in silico*, rather than *in vitro*, digests. The following patterns would be difficult to differentiate on an agarose gel unless run side-by-side: *Hpa*II: A/J/K/Q; C/L/N/O; B/M. *Hae*III: J/K. *Hind*III: D/I.

In total, 49 different RFLP pattern combinations were identified (Table 4.1). Using the convention described by Willment et al. (2001), the four most prevalent RFLP pattern groups were AABBDAD, BCABBAA, AAABDAD and ABABBAA, respectively representing 59.34%, 11.00%, 3.07%, and 2.56% of the 391 samples analysed and accounting for 76% of the samples. The other 24% represented the remaining 45

RFLP profiles occurring at lower frequency.

Table 4.1: Grouping of MSV variants based on restriction profiles of a 1.3-kb PCR product amplified from both FTA and CTAB-extracted samples. Figures in parentheses are the standard error of mean severity.

Variant	Restriction profile	Frequency of occurrence	Symptom severity
1	BCABBAA	43	3.0 (0.9)
2	CAABAAA	5	3.3 (0.5)
3	CAABAAD	2	3.0
4	ACABDAJ	1	2.0
5	BBABBAA	3	2.5 (0.5)
6	AABBDAD	232	3.1 (0.7)
7	AABEDAD	1	2.0
8	ABABEAA	5	3.3 (0.7)
9	CAABDAA	1	3.0
10	ACABDAA	5	3.2 (0.9)
11	AJBBDAD	1	2.0
12	AALBDAD	2	2.5
13	AABBBAD	8	3.5 (0.7)
14	AABBEAD	3	3.0 (0.9)
15	AAABDAM	1	2.0
16	AAABDAD	12	3.4 (0.7)
17	ABABBAA	10	3.0 (1.0)
18	ACBBDAD	6	2.8 (0.3)
19	ABBBDAD	1	3.5
20	AAABEAA	2	2.5
21	AABBDAL	1	3.0
22	AAABDAA	2	3.0
23	ABABDAA	1	2.2

Continued. . .

Table 4.1 – Continued

Variant	Restriction profile	Frequency of occurrence	Symptom severity
24	ABABDAD	3	3.2 (1.5)
25	AABBAAD	2	2.3
26	CAABDAM	1	3.0
27	ABABBAD	2	2.8
28	BCJBBAA	2	3.5
29	AABBDAA	4	3.3 (0.6)
30	AABBDAN	2	3.8
31	AABBHAD	1	2.0
32	BAAAAAD	1	2.5
33	BABBDAD	2	3.0
34	ABABAAD	1	3.5
35	ABABCAA	2	3.5
36	AAABBAA	1	3.0
37	AEBDDAD	1	3.0
38	BCBBDAA	1	4.5
39	BCABDAA	1	4.5
40	ACABEAA	4	3.4 (0.5)
41	AABBDAO	1	3.0
42	MABBDAD	1	3.0
43	MBBBDAD	1	3.0
44	BAABBAA	1	4.0
45	ACABBAA	5	3.5 (0.4)
46	ACABCAA	1	3.5
47	BCABEAD	1	3.5
48	ACJBDAA	1	2.5
49	LCABBAA	1	3.5

4.4.2 Recombination and phylogenetic analyses of full genome sequences

A total of 62 complete MSV genomes were successfully cloned and fully sequenced (GenBank accession numbers provided in Appendix A.1). Amongst these were isolates representing 45 of the 49 unique RFLP pattern combinations observed in Table 4.1. Phylogenetic analysis of an alignment of sequenced clones including the six clones sequenced in Chapter 3 and previously reported MSVs indicated that, as expected, all the Ugandan MSV sequences were of the maize-adapted MSV-A type. More specifically, all clustered with either the MSV-A_1 or MSV-A_5 subtypes (Fig. 4.3 a) previously identified in a 1999 survey of African MSV diversity (Martin et al. 2001).

Only two previously reported recombination events between MSV-A and non-MSV-A sources involving exchange of ∼100 bp (Martin et al. 2001) were observed. These two events are, however, inter-strain recombination events whereas for Ugandan clones, seeing that they all belonged to MSV-A strain, further analyses were conducted for intra-MSV-A recombination. Following removal of MSV-B-like portions of the MSV-Kom, -VM and -SA genomes and all the non-MSV-A sequences, ten potential intra-MSV-A recombination events (Bonferroni corrected P-value <0.05 for at least two different recombination detection methods coupled with phylogenetic evidence of sequence exchange) were detected, nine of which have apparently occurred in sequences ancestral to the Ugandan MSV-A sequences determined in this study (Fig. 4.3; Fig. 4.4). Six of these nine events (events 1, 3, 4, 7, 8, and 10 in Fig. 4.4) apparently involved sequence exchanges between MSV-A_1 and viruses most closely related to those in the MSV-A_2, A_3 and A_4 subtype groups. Due to generally low sequence diversity amongst African MSV-A sequences and the comparatively poor sampling of all MSV-A subtypes other than MSV-A_1, it was not possible to convincingly identify the exact origins of the recombinant regions for three of the five inter-subtype recombination events (events 4, 7 and 10 in Fig. 4.4).

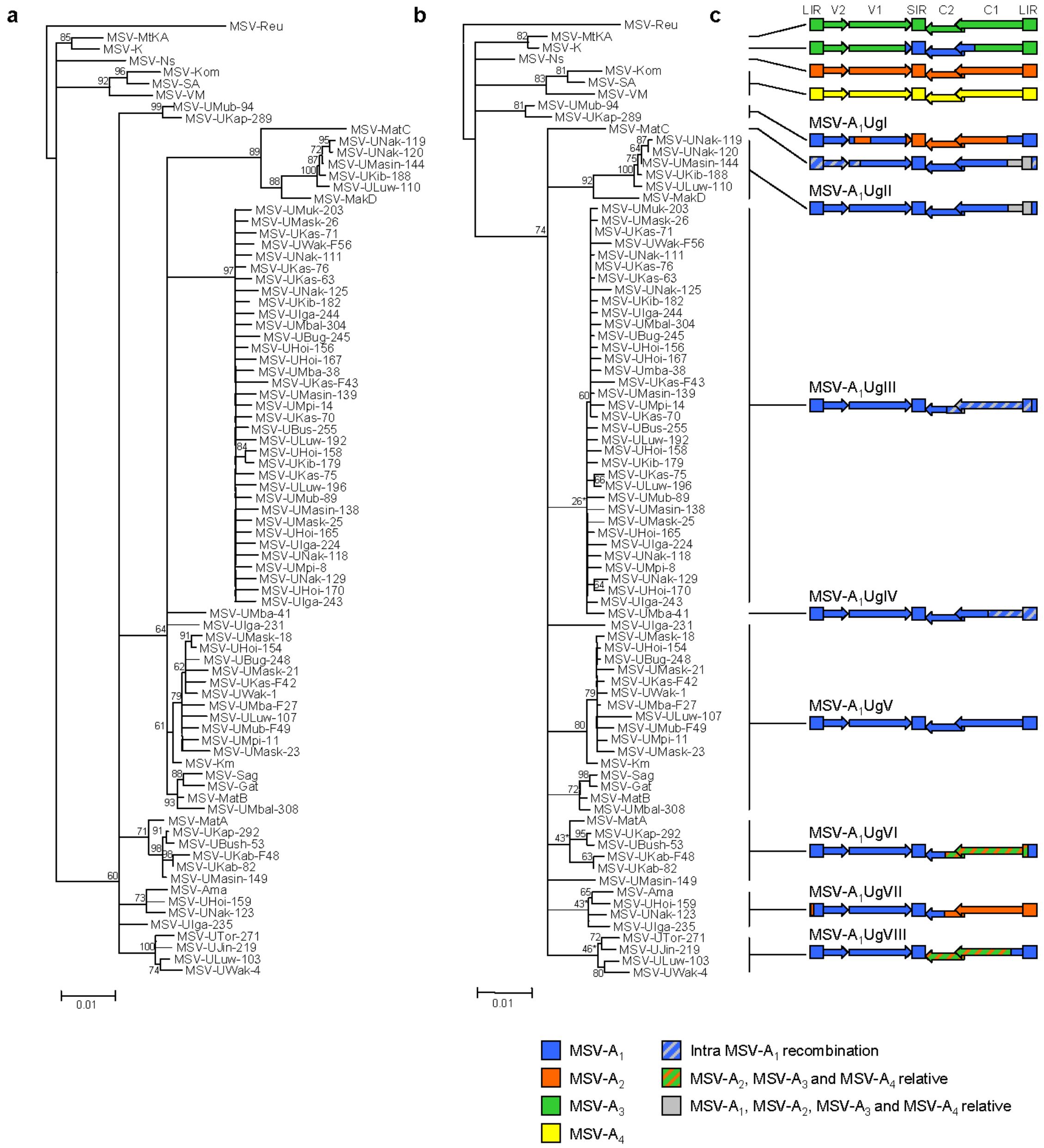
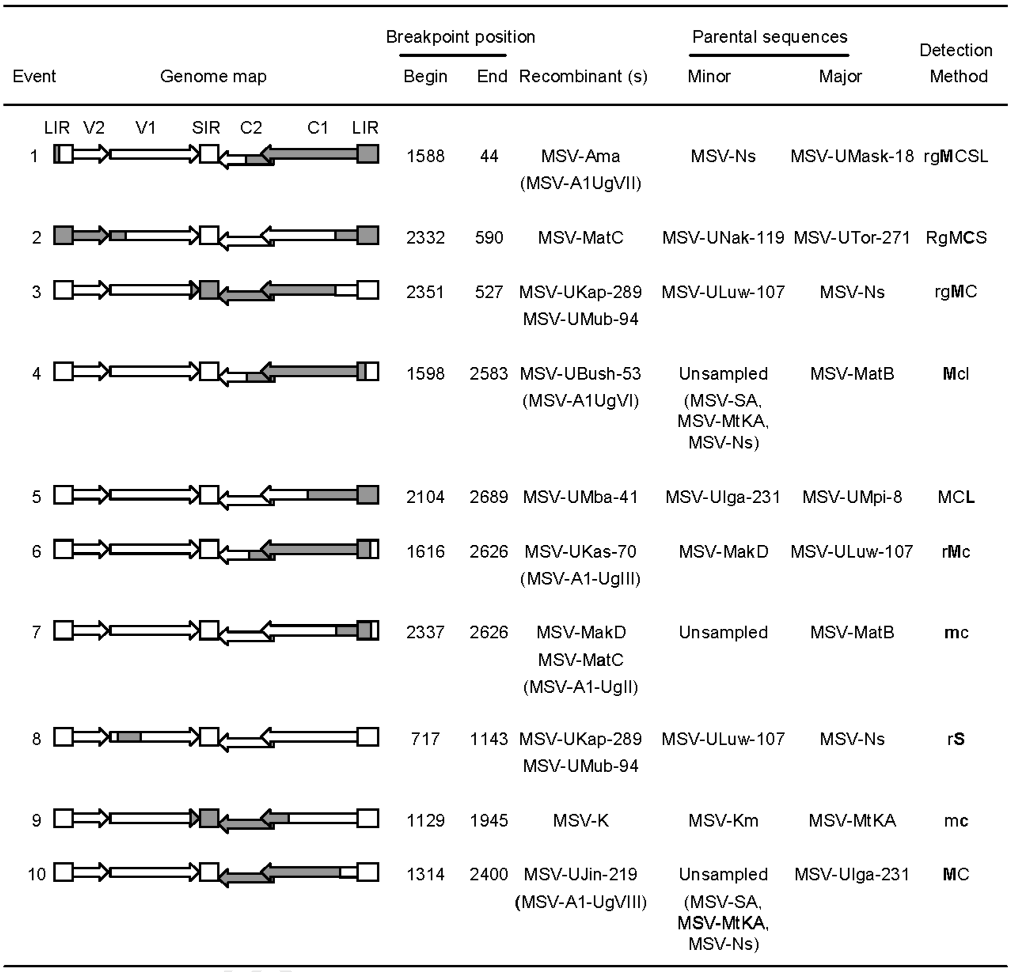


Figure 4.3: Maximum likelihood phylogenetic trees indicating possible evolutionary relationships between 84 maize-adapted maize streak virus (MSV- A) isolates. (a) Tree constructed using complete genome sequences. The names of the Ugandan sequences contain the prefix “MSV-U” followed by a three or four letter abbreviation of the sampling location name. (b) Tree constructed following removal of sequence tracts that have a probable recombinant origin (indicated by the genome cartoons in c). (c) Linearised genome cartoons depicting unique recombinant mosaics detected amongst the MSV- A sequences. Seven Ugandan mosaic sequences and one non-recombinant group are named MSV-A$_1$UgI through MSV-A$_1$UgVIII. Genome regions are indicated above the top cartoon: V2 = movement protein gene, V1 = coat protein gene, C1 and C2 = replication associated protein gene, SIR = short intergenic region, LIR = long intergenic region. Numbers associated with branches in phylogenetic trees represent the number of 100 non parametric full maximum likelihood bootstrap replicates supporting the existence of the branches. Other than branches indicated by an asterisk, those with less than 60% support have been collapsed. Branches marked with an asterisk in the tree in Fig. 4.3 b have been retained wherever both (i) groupings of the same sequences above the branches have >70% support in the tree in Fig 4.3a and (ii) where they share a common recombination mosaic. Certain mosaics contained evidence of recombination between MSV-A$_1$ and currently unsampled MSV-A subtypes. Wherever this was the case the group of most closely related currently sampled parental subtypes was given. The colour coding indicated the tentative subtype classification of four MSV-A lineages, whilst the MSV-A6 subtype was uncoded and represented by the tree outlier, MSV-Reu. Note that Ugandan isolate names in the figure have been changed in Appendix Table A1 in accordance with Fauquet et al. 2008 e.g MSV-UWak4 becomes MSV-A[UG:Wak4:05]



Event	Genome map	Breakpoint position Begin	 End	Recombinant (s)	Parental sequences Minor	 Major	Detection Method
1		1588	44	MSV-Ama (MSV-A1UgVII)	MSV-Ns	MSV-UMask-18	rg**M**CSL
2		2332	590	MSV-MatC	MSV-UNak-119	MSV-UTor-271	RgM**C**S
3		2351	527	MSV-UKap-289 MSV-UMub-94	MSV-ULuw-107	MSV-Ns	rg**M**C
4		1598	2583	MSV-UBush-53 (MSV-A1UgVI)	Unsampled (MSV-SA, MSV-MtKA, MSV-Ns)	MSV-MatB	**M**cl
5		2104	2689	MSV-UMba-41	MSV-UIga-231	MSV-UMpi-8	MC**L**
6		1616	2626	MSV-UKas-70 (MSV-A1-UgIII)	MSV-MakD	MSV-ULuw-107	r**M**c
7		2337	2626	MSV-MakD MSV-MatC (MSV-A1-UgII)	Unsampled	MSV-MatB	**m**c
8		717	1143	MSV-UKap-289 MSV-UMub-94	MSV-ULuw-107	MSV-Ns	r**S**
9		1129	1945	MSV-K	MSV-Km	MSV-MtKA	m**c**
10		1314	2400	MSV-UJin-219 (MSV-A1-UgVIII)	Unsampled (MSV-SA, MSV-MtKA, MSV-Ns)	MSV-UIga-231	**M**C

Figure 4.4: Characterization of ten recombination events detected amongst 84 maize-adapted MSV (MSV-A) full genome sequences. Sequences bounded by recombination breakpoints are shaded on the graphical representation of MSV-A genomes. "Minor" and "major" parents refer to sequences closely related to those respectively contributing the smaller and larger fractions of the recombinant's sequence. In cases where multiple parental sequences are indicated, these are all inferred to be equally close relatives of actual parental sequences. In certain cases where parental sequences are listed as "unsampled," the sequence names given in parentheses are representatives of major MSV-A lineages that are inferred to share a more recent common ancestor with the unsampled parental virus than they do with the other parental virus. Breakpoint positions represent the bounds of the strongest recombination signal but are not necessarily at, or even very close to, the breakage sites that occurred during the original recombination event. In the "detection methods" column, letters represent methods indicating the presence of recombination with greater than 95% (lower case) and 99% (upper case) confidence: R/r = RDP, G/g = GENECONV, M/m = MAXIMUM CHI SQUARE, C/c = CHIMAERA, S/s = SISCAN, L/l = LARD. Bold typeface letters indicate the method showing the clearest evidence of recombination for a particular event.

The topology of the maximum likelihood phylogeny constructed using the resulting alignment was almost completely in agreement with that constructed using the unedited alignment although the total tree length of the former phylogeny was substantially smaller than that of the latter (compare trees in Fig. 4.3a and b). Importantly, the sequences for MSV-MakD and MSV-MatC, formerly classified as belonging to the MSV-A_5 subtype by Martin et al. (2001), clearly clustered with the MSV-A_1 subtype in both of the trees. Although both maximum likelihood and neighbour joining reconstructions with only the MSV-A sequences used in the Martin et al. (2001) study confirmed the tree topology determined in that study (data not shown), both tree construction methods using this much larger MSV-A sequence dataset indicated that the MSV-A_5 subtype is in fact a sublineage of the MSV-A_1 subtype.

4.4.3 Distribution of major MSV genotypes in Uganda

Based on detected recombination patterns, the Ugandan MSV sequences were classified into eight haplotypes, named MSV-A_1UgI through MSV-A_1UgVIII (Fig. 4.3b and c), with MSV-A_1UgV representing the MSV-A_1 sequences that are not detectably recombinant. Using RFLP data for the remaining 321 Ugandan MSV samples (197 from this study and 124 from Chapter 3) that were not analysed by full genome sequencing, it was possible to classify each of these into one of the eight haplotype groupings

Mapping of the haplotypes in the sampled area of the country showed that MSV-A_1UgIV occurred only in one location in the eastern part of the country whilst MSV-A_1UgVIII was localized to only the central and eastern part of the country (Fig. 4.5). MSV-A_1UgIII was the most frequently encountered haplotype, widely distributed in most of the sampled regions. Accordingly, the majority (35) of the sequenced 68 clones were haplotype MSV-A_1UgIII. Following the split of the sampling area into seven zones, there was no significant difference in the population frequencies of different haplotypes across the seven zones (P= 0.2964; Chi square test with 8

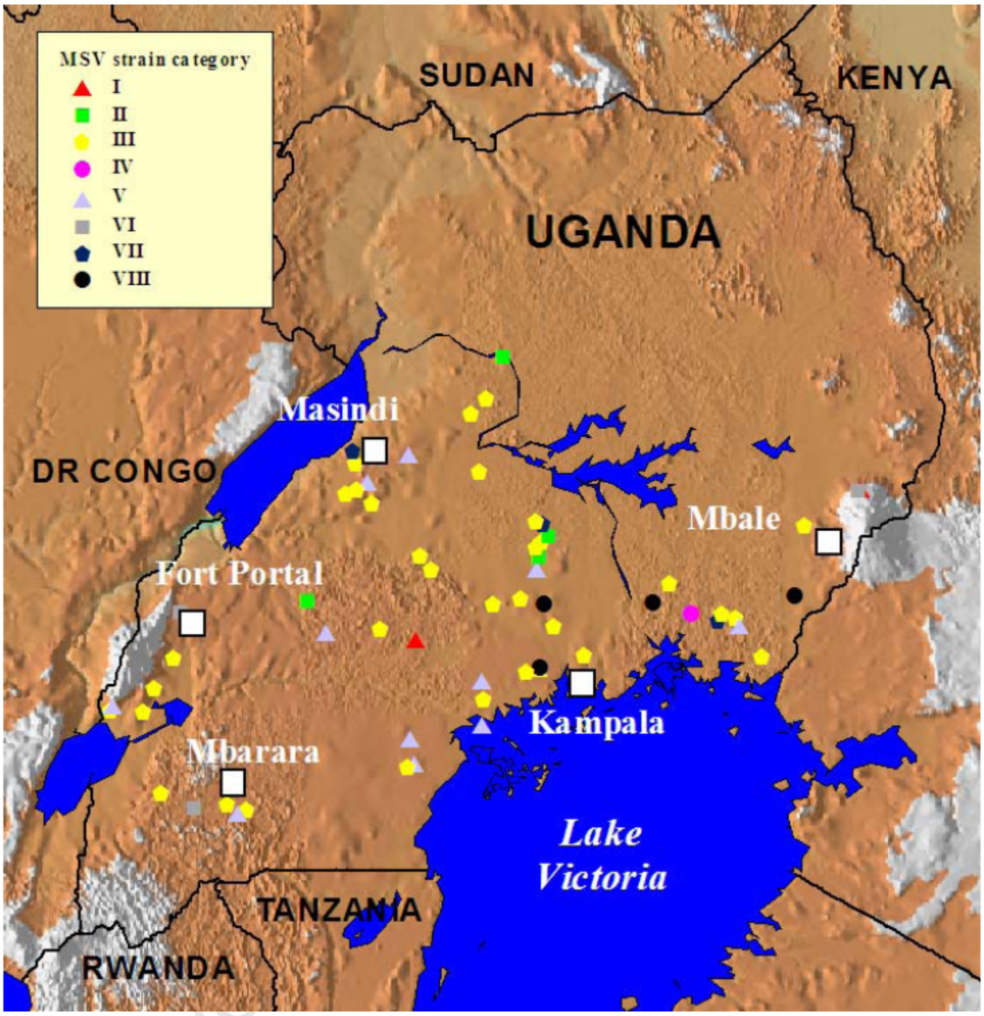


Figure 4.5: Distribution of eight identified haplotypes, MSV-A_1UgI through to MSV-A_1UgVIII in surveyed areas in Uganda.

haplotypes x 7 sampling zones) (Fig. 4.6 a) indicating that generally, the diversity of samples collected from any one of the zones was not significantly unrepresentative of country-wide MSV diversity. Triplet samples collected from individual farms showed a surprisingly high degree of intra-field diversity, with 66% of triplicate samples from individual fields containing viruses categorized as belonging to different haplotypes. There was no significant deviation in the patterns of haplotypes observed within individual farms relative to those observed country-wide ($P = 0.213$; Chi square test with 15 three-sample haplotype combinations x 7 sampling zones).

However, more careful examination of the samples collected in individual farms re-

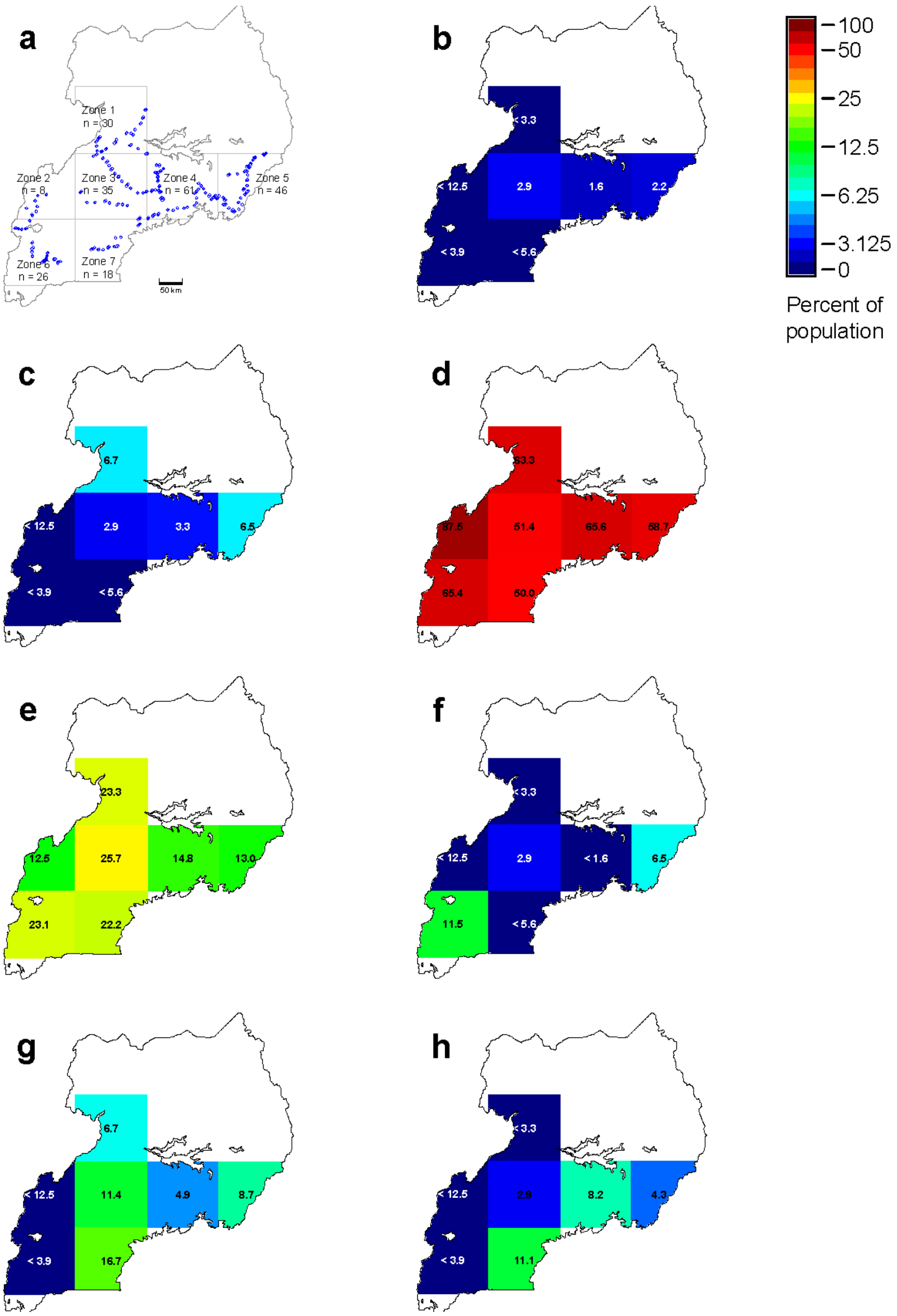


Figure 4.6: Sampling locations, distributions and population representation of seven Ugandan MSV-A haplotypes. (a) Important maize growing regions of Uganda were split into seven zones and samples were collected from 155 locations within these (blue circles). Distribution and population representation of: (b) MSV-A$_1$-UgI; (c) MSV-A$_1$-UgII; (d) MSV-A$_1$-UgIII; (e) MSV-A$_1$-UgV; (f) MSV-A$_1$-UgVI, (g) MSV-A$_1$-UgVII; and (h) MSV-A$_1$-UgVIII. The colour scale is exponential (base 2) from 3.125 - 50% and linear from both 0 - 3.125% and 50 -100%.

vealed five instances where the haplotype combinations observed were reasonably improbable (P <0.05; 2 x 2 Chi square test). Although none of these comparisons was significant following Bonferroni correction of P-values (to account for the multiple tests made), the possibility that including all three samples from each farm might introduce a sampling bias into the analysis of country-wide Ugandan MSV population structure could not be discounted. Therefore only one example of each haplotype sampled in each location was considered in subsequent analyses. While this method slightly biased estimates of population representation against the two most common haplotypes, it enabled more sensitive analysis of the distributions of the six rarer haplotypes. This selection process did not, however, grossly distort the overall representation of the respective haplotypes as illustrated in Fig. 4.7 The intra-MSV-A_1 recombinant haplotype MSV-A_1UgIII made up 50% or more of the MSV samples in all seven zones (Fig. 4.6 d) and is clearly the dominant MSV haplotype throughout Uganda. The MSV-A_1UgV haplotype, containing all of the MSV-A_1 sequences that are not obviously recombinant, is the only other haplotype that was detectable in all seven sampling zones (Fig. 4.6 e). All of the other haplotypes were either absent or present below detectable levels in two or more of the zones. As most of these were present at close to the detection limits in the zones where they were observed, it is possible that they are all present throughout the country.

There was some indication of slight variation in MSV demography in different zones. While this is particularly true for some of the rarer haplotypes such as MSV-A_1UgVI, MSV-A_1UgVII and MSV-A_1UgVIII (Fig. 4.6 f, g, and h), which display >5 fold variations in population representation in different zones, there was also evidence of variation in relative population representation of the more common haplotype, MSV-A_1UgV. This haplotype is at its highest prevalence in four of the five eastern zones and its lowest prevalence in the two western zones. Statistically significant deviation from country-wide population frequencies was, however, only evident for MSV-A_1UgVI in zones 5 and 6 (P = 0.015 and 0.042 respectively; Bonferroni corrected 2 x 2 Chi square test; Fig. 4.6 f).

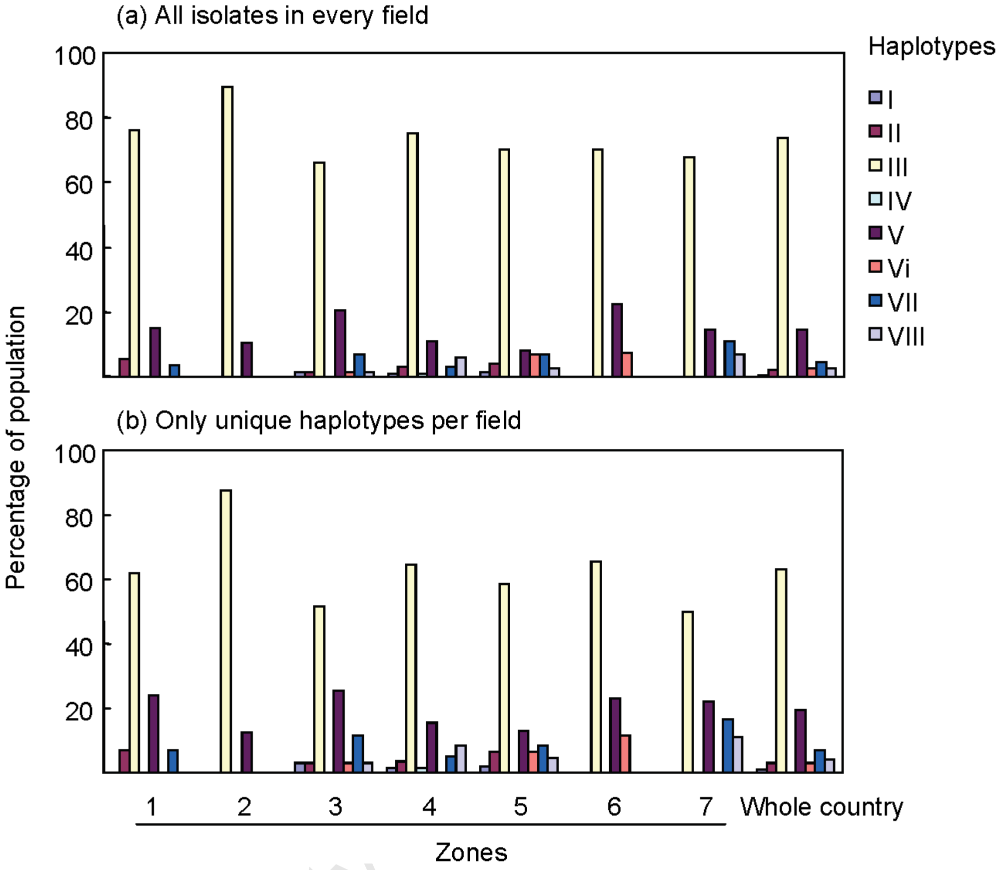


Figure 4.7: Effect of different sample-selection schemes on the estimated population representations of eight Ugandan MSV-A haplotypes sampled in seven different geographical zones. (a) Using all isolates (between one and three) sampled from every one of 155 maize fields. (b) Data presented in Fig. 4.6, using only one representative of each unique haplotype sampled from each of 155 fields. Note that whilst this selection scheme is biased against the major haplotypes (III and V), it guards against the possibility of resampling clonal isolates within individual fields.

4.4.4 MSV and CGV population genetic characteristics

Two CGV datasets, containing 28 and 14 DNA-A sequences, met the selection criteria laid out in the Methods and materials for comparing population genetic characteristics between MSV and the CGVs. These groups contain sequences respectively classified as the East African cassava mosaic virus Uganda strain (EACMV-UG) and East African cassava mosaic Kenya virus (EACMKV). Some summary statistics indicating that these datasets are indeed very similar to the MSV dataset from a population genetic perspective are presented in Table 4.2.

Having aligned all 118 African cassava-infecting begomovirus DNA-A sequences available in GenBank 30 November 2006, a total of 23 major inter-species and four intra-species recombination events were identified for CGVs using RDP3 (data not shown). Relative to the MSV dataset, the CGV sequences contained more evidence of recombination involving larger fragments of sequence between more distantly related parental viruses (Fig. 4.8). Population-scaled recombination and mutation rate estimates of

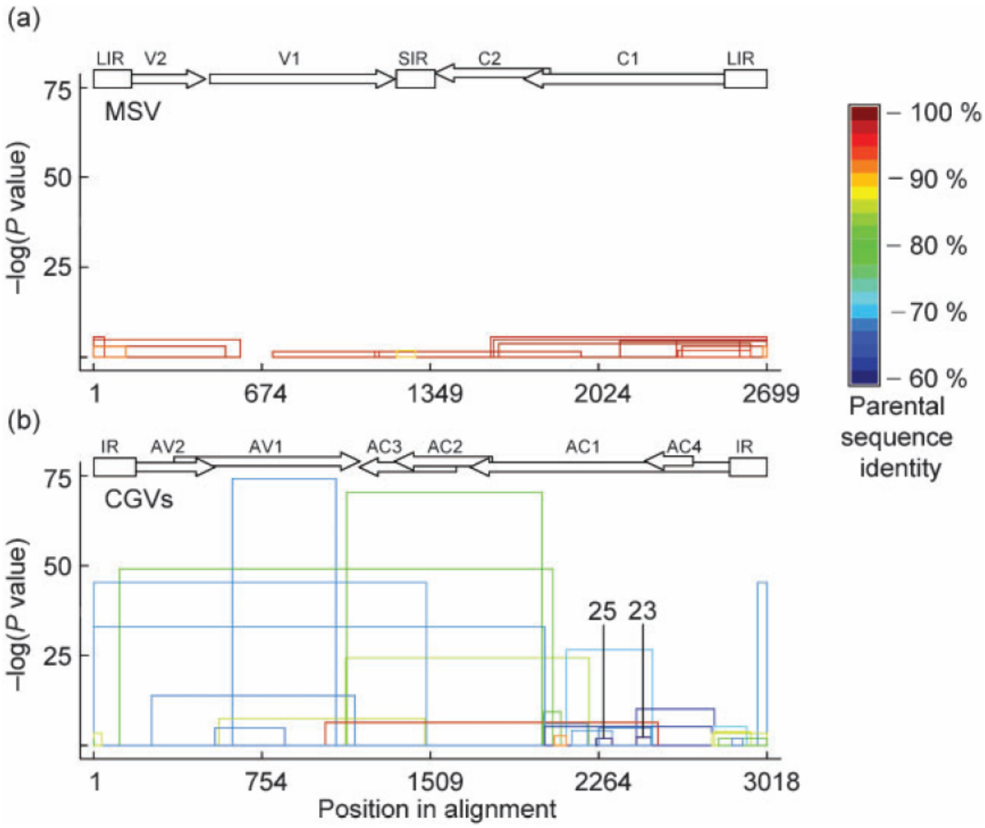


Figure 4.8: Distribution and characteristics of recombination events detected amongst (a) MSV full-genome sequences and (b) DNA-A sequences of CGVs from Africa. Each rectangle represents a recombination event, with the vertical edges indicating approximate breakpoint positions and the upper horizontal edge indicating the degree of statistical support in favour of recombination. The colour of the boxes indicates the minimum degree of sequence identity shared by parental sequences at the time of the recombination event. Evidence of two recombination events involving a non-CGV parental sequence are labelled [events 23 and 25, detailed in the supporting files Cassava.csv and Cassava.rdp (available on CD included)]. Genome cartoons above the plots indicate the starting and ending alignment positions of virion-sense genes (arrows labelled V or AV), complementary- sense genes (arrows labelled C or AC) and intergenic regions (boxes labelled IR, LIR or SIR)

Table 4.2: Population genetic statistics of matched MSV and CGV datasets

Statistic	MSV	EACMV-UG	EACMKV
No. sequences	68	28	14
Nucleotide diversity (π)	0.0162	0.0124	0.0232
Population-scaled mutation rate (θ)	77.672	83.002	85.530
Population-scaled recombination rate (ρ)	4.064††	8.13	3.704†
ρ/θ	0.052	0.098	0.043
Tajima's D	-1.880††	-2.294†††	-1.102
Fu and Li's D*	-4.291†††	-3.573†††	-1.323

† $P<0.05$; †† $P<0.01$; ††† $P<0.001$.

different MSV and CGV population datasets with similar properties are presented in Table. 4.2. Importantly, neither the recombination rates nor recombination: mutation rate ratios of the different datasets were substantially different from one another (Table. 4.2).

Despite their apparently having similar genome-wide recombination rates, it was suspected that there might be differences in regional recombination rate variation within the MSV genomes and the CGV DNA-A components. Composite likelihood estimates of regional variation in the population-scaled recombination rates of all three datasets were, however, surprisingly similar (Fig. 4.9). In all three populations it seems that recombination rates are significantly higher in genomic regions encoding complementary sense genes than they are in regions encoding virion sense genes. The similarities between the MSV and CGV datasets extended to other population genetic summary statistics. For example, both Tajima's D and Fu and Li's D* statistics calculated from complete genome sequences (and accounting for the population-scaled recombination rates calculated above), indicated statistically significant departures from neutrality in the MSV and the EACMV-UG populations (Table 4.2). The values

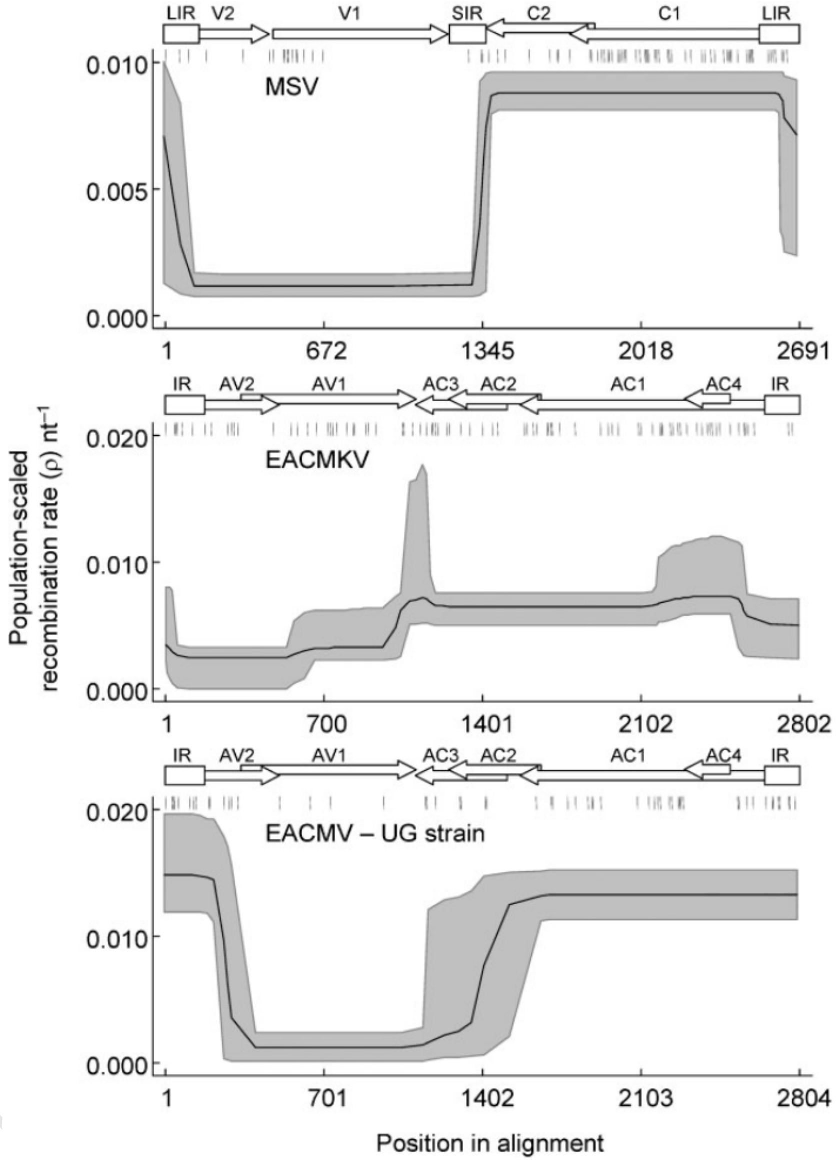


Figure 4.9: Variable recombination rates along the lengths of MSV genomes and the DNA-A genome components of the CGVs EACMKV and EACMV UG. Black lines represent mean estimates of point recombination rates determined by the reversible-jump Markov chain Monte Carlo (RJMCMC) approach implemented in the INTERVAL component of LDHAT (McVean et al. 2004). Grey regions represent the 95% credibility intervals of point recombination-rate estimates from the RJMCMC chain. Note that because of simplifying assumptions made during the approximation of likelihoods with this approach, the distribution of point recombination rates obtained from the RJMCMC chain cannot be used to infer 95% confidence intervals of the actual point recombination rate accurately. Genome cartoons above the plots indicate the starting and ending alignment positions of virion-sense genes (V or AV), complementary-sense genes (C or AC) and intergenic regions (IR, LIR or SIR). Vertical lines beneath the genome cartoons indicate the locations of polymorphic sites used for the analysis.

of these statistics were also marginally significant for the EACMKV dataset (P = 0.09 for Tajima's D and P = 0.06 for Fu and Li's D*).

4.5 Discussion

Different studies on the diversity and genetic characteristics of maize-infecting MSVs have reported that continent wide MSVs differ in sequence by no more than 3% (Briddon et al. 1992, Martin et al. 2001). This, however, does not imply that these viruses are phenotypically identical when infecting maize. Recombination plays a major role in the evolution of geminiviruses (Zhou et al. 1997, Padidam et al. 1999) and MSV is no exception. As recombination has been reported previously in MSV (Martin et al. 2001), evidence of recombination between the 84 MSV-A viruses and the other African streak viruses (both other MSV-strains and non-MSV African streak virus species) aligned in this study was analysed. Only two previously reported recombination events described by Martin et al. (2001), involving exchanges of small fragments (∼100 bp) between MSV-A and MSV-B -like viruses to yield MSV-VM in one case and, in the other, the ancestor of MSV-SA, MSV-Kom and MSV-VM was observed. The fact that no new inter-strain recombination events were observed indicates clearly, therefore, that neither inter-strain nor inter-species recombination is currently a major factor in Ugandan MSV-A diversification. This does not, however, discount the possibility that intra-MSV-A recombination might be an important feature of MSV-A evolution. Following removal of the MSV-B-like portions of the MSV-Kom, -VM and -SA genomes and all of the non-MSV-A sequences from the MSV alignment, ten potential intra-strain recombination events were detected.

Having identified the most probable recombinant regions of all available MSV genomes, a recombination-free reconstruction of the MSV-A phylogeny was attempted. Although the topology of the unedited and edited trees was the same (Fig.4.3, reduction in tree length of the tree constructed with data of alignments edited to remove recombinant tracts of sequence is exactly what would be expected following removal of the most significant recombination signals within the dataset (Schierup & Hein 2000).

RFLP analysis showed new patterns for three of the seven enzymes *Rsa*I, *Hae*III and *Sau*3A and also showed that 6.20% of the 387 samples contain mixed infections. Whilst mixed infections frequently occur for other geminiviruses such cassava mosaic geminviruses, RFLP results presented in this study suggest that for mastreviruses, mixed infections are less frequent.

In this study, Ugandan MSVs were classified into eight haplotypes. The haplotype classification scheme used here is simply a convenient and evolutionarily relevant way of splitting the Ugandan virus isolates into distinguishable groups and not intended as a taxonomic proposal. Importantly, the most prevalent and widely distributed haplotype in the country was found to be recombinant. This has important implications and should be taken into account when designing future control strategies.

Two southern African MSV strains were reclassified in this study. MSV-MakD and MSV-MakC, previously classified as belonging to the MSV-A_5 subtype, were shown to actually be sublineages of MSV-A_1. Although the reason for this discrepancy is unclear, MSV-MakD and MSV-MatC are both recombinants containing large tracts of MSV-A_1-like sequence (identified here and by Martin et al. 2001) which would be expected to compromise the correct placement of these sequences in phylogenetic trees (Schierup & Hein 2000, Posada & Crandall 2002, Awadalla 2003). Owing to the large proportion of MSV-A_1-like sequences within these two isolates and their close Ugandan relatives, MSV-A_5 was reclassified as a recombinant sublineage of MSV-A_1.

Part of this study involved division of Uganda into zones and examining the characteristics of samples collected from each of these zones. There being no difference in haplotype population frequencies distribution across the different zones indicated that, generally, the diversity of samples collected from any one of the zones was not significantly unrepresentative of country-wide MSV diversity. Furthermore, comparison of triplet samples collected from each farm showed high diversity within individual fields. Since there was no significant deviation in the patterns of haplotypes observed within individual farms relative to those observed countrywide, this indicates that MSV diversity observed within sampling areas of a few hundred square meters is also generally not significantly unrepresentative of that observable country-wide. Despite the possibility of slight geographical variations in haplotype frequencies across Uganda, the fact that MSV haplotype distributions do not differ substantially over sampling scales ranging from 0.1 through 100 000 square kilometers, strongly suggests that MSV population structure (in Uganda at least) is highly homogeneous. This in turn implies that there are no substantial impediments to the movement of viruses throughout the country.

CGV sequences contained more evidence of recombination involving larger fragments of sequence between more distantly related parental viruses than MSVs. Unsurprisingly, these quite striking differences have been noted elsewhere (Padidam et al. 1999, Martin et al. 2001, Schnippenkoetter et al. 2001). However, the reasons for these differences remain unexplored. It is probable that there are three main reasons that MSV and the CGVs might have such different patterns of recombination: (1) the biochemical recombination rate in MSV may be significantly lower than that found in CGV species; (2) although mixed infections, a prerequisite for recombination, have been observed in both MSV and CGVs (this study, Willment et al. 2001, Vanitharani et al. 2004), they may be much more common amongst the CGV's than they are amongst the MSV's; (3) whereas the CGV species are all cassava-adapted, only one MSV strain is maize-adapted and differences seen in the extents and prevalence of recombination in the CGVs and MSV may therefore be the result of purifying selection eliminating greater proportions of MSV recombinants, particularly when these contain large tracts of sequence from viruses that are not maize-adapted (Martin & Rybicki 2002, Martin et al. 2005b).

Both the recombination rates and mutation rates for MSV and CGV datasets were analysed and compared. Neither recombination rates nor recombination/mutation rates of the datasets were different. The value of the ratio for the MSV dataset falls between that determined for the two CGV datasets. Assuming that the biochemical mutation rates of MSV and the CGVs are not substantially different, these data imply that the biochemical recombination rates of the different groups of viruses are also not substantially different. This therefore indicates that the striking differences in the types of MSV and CGV recombination events detected in nature are probably not due to large differences in the biochemical recombination rates of these viruses.

Recombination rate variations within the genomes of MSV and CGV DNA-A components were also surprisingly similar between the two datasets. In all three populations, recombination rates were found to be significantly higher in genomic regions encoding complementary-sense genes than they are in regions encoding virion-sense genes. A possible mechanistic cause could be inferred from the fact that within 400 nucleotides 3' of the virion strand replication origin, recombination rate estimates are at their lowest in all three datasets. Apart from indicating that similar mechanistic processes are possibly responsible for recombination rate variation across MSV and CGV genomes, this result implies that these mechanistic processes may be features of the virion strand replication and/or complementary strand transcriptional systems. However, this conclusion is not obvious because of differences between the origins and replication proteins of mastreviruses and begomoviruses.

The negative values of the Tajima's D and Fu and Li's D* population genetic statistics as given in Table 4.2 indicate that, given an expectation of neutrality, there is an excess of low frequency nucleotide polymorphisms in viruses sampled from these populations. Such departures from neutrality might be caused by a range of population phenomena including, for example, sporadic cycles of population collapse and expansion such as those characteristic of both MSV and CGV epidemiology. It is important that both the MSV and CGV genome sequence samples bear similar marks of population genetic processes, as this indicates that differences in the patterns of recombination events detected between the two groups are possibly not due to fundamentally different evolutionary forces acting on the viruses. It is plausible therefore that, with respect to the evolutionary benefits of recombination, the primary differences between MSV and the CGVs is that the diversity of high fitness host-adapted genome constituents available for recombinational exchange is far greater for the CGVs than it is for MSV.

Whilst the survey of Ugandan maize-infecting MSV-A isolates has revealed that the vast majority of MSD infections in the country are caused by a group of very closely related viruses, this study has demonstrated that this low diversity does not necessarily equate to genetic uniformity. The study has also reported that there is substantial evidence of genetic exchange between viruses within the MSV-A group and that a recombinant is in fact the most prevalent MSV-A haplotype within the country. By using recombination patterns as a means of haplotyping MSV variants, the study has determined that the diversity of Ugandan MSVs is remarkably constant over a wide range of sampling scales, such that viral diversity within individual farms is not significantly different from that detected across the entire country. The hypothesis that recombination is an important feature of geminivirus evolution is not new, but it has been demonstrated here that its characteristics are strikingly different in MSV and the related CGVs. Some evidence that the underlying cause of these differences is probably not that CGVs have a higher biochemical recombination rate than MSV, but rather that CGVs have recombinational access to a far greater diversity of appropriately host-adapted genome constituents has also been provided.

The data provided here will be useful in future studies involving either longitudinal monitoring of Ugandan MSV population turnover or comparative genetic analyses of large MSV population samples from different parts of the African continent. This is the first comprehensive characterization of Ugandan MSVs, providing information that will be useful to development of new resistant maize varieties not only in Uganda but elsewhere as well.

Chapter 5

DEVELOPMENT OF TRANSGENIC RESISTANCE TO MAIZE STREAK VIRUS (MSV) USING GENE SILENCING

5.1 Summary

Gene silencing, a natural antiviral defense system in plants, animals and fungi, is a very attractive method for development of MSV resistance strategies since it taps into an already existing resistance mechanism. Assays were conducted to investigate the efficacy of inverted repeats of (1) the *rep*ΔI^{678} gene and (2) the promoter-containing long intergenic region (LIR) of MSV in interfering with MSV replication in black Mexican sweetcorn (BMS) suspension cells. This system was used to test the replication-inhibitory effects of both constructs against the infectious MSV isolates MSV-Kom, MSV-MatA, MSV-Gat and MSV-Reu, which respectively differ by 2.34%, 2.90% and 4.62% at the nucleotide level from the sequences used in the silencing construct. Replication of all four virus strains was greatly reduced by the repΔI^{678} hairpin construct. However, whilst the repΔI^{678} sense construct showed some level of inhibition of viral replication, there was no inhibition by either the repΔI^{678} antisense construct or the LIR hairpin construct. The repΔI^{678} hairpin

construct was also tested against three widespread and severe Ugandan MSV isolates cloned during this project: MSV-A[UG:Mpi11:05], MSV-A[UG:Kas75:05] and MSV-A[UG:Kab82:05]. As observed for the South African, Kenyan and Réunion isolates, replication of all three Ugandan strains was inhibited by the repΔI^{678} hairpin. Transgenic *Digitaria sanguinalis* and maize Hi-II, were successfully regenerated using the repΔI^{678} hairpin construct. Implications of these results for development of transgenic-based resistance to MSV are discussed.

5.2 Introduction

Resistant varieties have long proven to be the most effective and convenient control option for many plant diseases, including MSD. However, because of a multitude of factors such as farmers' selection of seeds for the next season's planting, inconsistent inoculum pressures across maize growing regions, variability in virus pathogenicity, and differences in the resistance characteristics of maize grown in different areas, apparent resistance breakdown is frequently experienced. This means that there is continually a need for novel sources of resistance to add to the already existing pool of control options.

Fortunately, genetic engineering has provided a relatively fast and precise tool for the development and implementation of new resistance strategies such as those relying on gene silencing. Activation of natural antiviral defense systems may be used to protect plants against virus infection by engineering the plant to express dsRNA to viral genes.

The use of innate antiviral defenses to protect crops against viruses is not new and dates back to 1929 when Mckinney found that tobacco plants infected by a mild strain of Tobacco mosaic virus were protected against infection by another more severe strain of the same virus (Mckinney 1929). Termed cross-protection, the use of mild virus to protect against more severe ones has been used to control some serious virus problems including Citrus tristeza virus (Muller & Costa 1968), Tomato mosaic virus (Fletcher 1978), and Papaya ringspot virus (Yeh et al. 1988). Such a strategy has also been evaluated as a control option for Cassava mosaic virus (Owor et al. 2004).

By directly activating antiviral gene silencing pathways it has been possible to provide engineered plants with resistance phenotypes ranging from delayed symptom development and reduced/delayed DNA/RNA accumulation to complete resistance to symptomatic infection (Angell & Baulcombe 1997, Chellappan et al. 2004b, Ratcliff & Baulcombe 1999).

The geminivirus *REP* gene is an ideal target for silencing because of its indispensable role in viral replication (Hanley-Bowdoin et al. 1999) and its highly conserved nature (Vanderschuren et al. 2007a). Choice of the *REP* gene as a target for silencing is further strengthened by studies with other geminiviruses in which silencing *REP* was shown to be effective in reducing viral replication in cassava, tomatoes and beans (Vanitharani et al. 2003, Chellapan et al. 2004a, Vanderschuren et al. 2007b, Varma & Praveen 2006, Bonfim et al. 2007).

Evaluation of new techniques often requires a fast and efficient system that will generate information in the shortest time possible. This is where the use of model systems becomes applicable. Well known model plants are *Arabidopsis thaliana* and *Nicotiana benthamiana* both of which have been extremely valuable in the evaluation of genetic engineering strategies against geminiviruses (Arguello-Astorga et al. 2007, Vanitharani et al. 2003). Similarly *Digitaria sanguinalis*, has proven to be a good species for evaluating resistance strategies against geminiviruses such as MSV that infect grain crops (Shepherd et al. 2007a). In addition to whole model plant systems, cells in culture have also provided an invaluable research tool for rapidly comparing the relative merits of different resistance strategies (Shepherd et al. 2005, 2007a).

In light of the growing need to diversify sources of MSD resistance genes, a study was set up to evaluate gene silencing as a possible means of achieving MSV resistance. Two hairpin constructs targeting a portion of the MSV *REP* gene and the promoter region in the long intergenic region (LIR) were tested in maize suspension cells, *Digitaria sanguinalis* and maize to determine their value as MSV resistance genes.

5.3 Materials and methods

5.3.1 Generation of hairpin constructs

A gene silencing vector, pHANNIBAL (Appendix A.2; Helliwell & Waterhouse 2003), incorporating the Gateway technology (www.invirogen.com) was obtained from Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australia. pHANNIBAL was designed so that a gene of interest can be inserted in both the sense and the antisense orientations using standard gene cloning approaches to achieve a construct containing an inverted repeat of the gene. Eight *REP* gene sequences (MSV-MtkA, MSV-Reu, MSV-Ken, MSV-MatC, MSV-MatA, MSV-MatB, MSV-MakD and MSV-Kom) were downloaded from GenBank and examined using the siRNA designing program, IMGENEX (http://www.imgenex.com /sirnatool.php). This analysis indicated that between nucleotide co-ordinates 78 to 768 (numbering starting from the rep ATG), at least three predicted siRNA sequences were common between the eight *REP* sequences. MSV-Ken, MSV-MatB and MSV-MakD had the highest number of predicted siRNAs (13) while MSV-Reu and MSV-MatC had the lowest (9). MSV-Kom, for which an infectious clone was available, was selected as the template for design of the hairpin construct. The 678 nt stretch of sequence in MSV-Kom *REP* chosen to form the hairpin was nucleotide co-ordinates 50-727 (co-ordinate numbering starting from the rep ATG), since this sequence encompassed predicted siRNAs from the *REP* of all eight isolates.

The repΔI^{678} (678 nt from intronless rep) and the LIR from MSV-Kom were inserted as *Xho*I-*EcoR*I fragments for the sense arm and as *Xba*I-*Cla*I fragments for the antisense arm into pHANNIBAL to make the hairpin constructs. To do this, two primer pairs incorporating *Xho*I/*EcoR*I sites for the sense arm [Rep-*Xho*I (F), Rep-

*EcoR*I (R) for repΔI^{678} and LIR-*Xho*I (F), LIR-*EcoR*I (R) for LIR] and *Xba*I/*Cla*I sites for the antisense arm [Rep-*Xba*I (F), Rep-*Cla*I (R) and LIR-*Xba*I (F), LIR-*Cla*I (R)] were designed (Table 5.1). These primers were used to amplify a 678 bp product containing the portion of *rep* predicted to form siRNAs from an intronless *rep* construct, pSKRep$^{\Delta I}$ (Shepherd et al. 2005) as template and a 325 bp product containing the LIR of MSV-Kom from pKom602 (Schnippenkoetter et al. 2001) as template for the PCR. Amplification conditions for both repΔI^{678} and LIR consisted of an initial denaturation at 95°C for 45 secs, 57°C for 30 secs, 72°C for 3 mins and a final extension at 72°C for 7 mins. Amplified products were electrophoresed through a 1% ethidium bromide-stained agarose gel and products, the 678 bp fragment for the repΔI^{678} and 325 bp for the LIR, were excised from the gel, purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) as per manufacturer's instruction, and cloned into p-GEMT-easy (Promega) using standard cloning procedures. Plasmids were isolated from transformed *Escherichia coli* (DH5α) using the Roche-Applied Science plasmid purification kit and the insert sequenced using M13 primers to ensure that there were no errors in the PCR products. The antisense and sense products or "arms" were inserted into pHANNIBAL using standard procedures to make pHPKomrepΔI^{678} and pHPKomLIR for the repΔI^{678} and LIR hairpins respectively. Sense repΔI^{678} and LIR constructs were made from fragments amplified as described for hairpin constructs with respective primer pairs in Table 5.1 and cloned into pHANNIBAL to make pSenKomrepΔI^{678} and pKomLIRS. Similarly, antisense repΔI^{678} (pAntKom repΔI^{678}) and LIR (pKomLIRAN) were made with primer pairs in Table 5.1 and cloned into pHANNIBAL. Cloning of the LIR, in the sense and antisense orientation was used here to refer to the insertion of the LIR in the virion sense and complementary sense.

Table 5.1: Primer sequences and co-ordinates

Primer name	Sequence	[1]Co-ordinates	Length (bp)
Sense primers			
Rep-*Xho*I (F)	CGCTAACTCGAGCTAACCTATCCAAAG	42-69	27
Rep-*Eco*RI (R)	CTAGAATTCCTGGCCCAAGTAGATTTTCCGG	1675-1702	30
LIR-*Xho*I (F)	GATGAGCTCGAGGCCATAGCCGAC	2512-2535	24
LIR-*Eco*RI (R)	CAGGGAATTCTGTGGATCCATGAATGAATC	141-170	30
Antisense arm primers			
Rep-*Xba*I (F)	CGTTCTAGACCTATCCAAAGTGTCC	50-74	25
Rep-*Cla*I (R)	CCCCATCGATTCTGGCCCAAGTAG	707-730	24
LIR-*Xba*I (F)	GACACTTCTAGATAGCCGACGACGGAG	2527-2541	27
LIR-*Cla*I (R)	GGATCGATGAATGAATCGCACTTGTTAGGC	128-157	30
Realtime PCR			
RepRTF	GTTGGCTGTCAGAGGGATTT	841-859	20
RepRTR	CCCTGGAGTCATTTCCTTCA	950-969	20
18S primers			
18S (F)	CAGGCGCGCAAATTACCCAATCC	-	23
18S (R)	CCTACCGTCCCGTCCCAAGGTC	-	22
Bar-specific			
Bar (F)	CGTCAACCACTACATCGA	-	18
Bar (R)	GAAACCCACGTCATGCCA	-	18
siRNA probes			
RepF1	TCCTGAAATCGCCTGTCAGATGATCTGGG AGCTCGTCGTTCGTTGGATTC	81-129	50
RepF5	AATGAGTCAATCAACGATTGGCTCCAGCC TAACATCTTCCAGTCATCAGA	601-649	50

[1] Relative positions of the primers in the MSV-Kom genome (GenBank accession no. AF003952) if applicable. Numbering starts from the penultimate nucleotide of the conserved TAATATTAC sequence of MSV-Kom. Positions of primers Rep-*Xho*I (F), Rep-*Eco*RI (R), Rep-*Xba*I (F) , Rep-*Cla*I (R), RepRTF and RepRTR in the intronless *rep* (starting with the ATG). Primer co-ordinates are given in the 5'-3' direction.

5.3.2 Transient assays using repΔI^{678} and LIR hairpin constructs

BMS cells subcultured three days prior to bombardment by growing 20 ml of BMS stock cultures in 50 mL BMS liquid media, were used to assay for MSV replication as described by Palmer et al. (1999). One microgram of a 1.1-mer pKom602 clone (reiterated LIRs for replicational release) (Schnippenkoetter et al. 2001), and one microgram of either pHPKomrepΔI^{678}, pSenKom repΔI^{678}, pAntKom repΔI^{678}, pHPKomLIR, pKomLIRS and pKomLIRAN or pHANNIBAL vector (i.e. containing no insert), were precipitated onto to 1 μm gold particles (50 μL of 60 mg/mL gold suspended in 50% glycerol) according to the protocol of Dunder et al. (1995). The PDS-1000/He Biolistic particle bombardment delivery system (Biorad / DuPont) was used to deliver DNA into BMS cells as described by Palmer et al. (1999). Approximately 600 ng of DNA was delivered into BMS cells from two shots at a pressure of 650-psi and a gap distance of 9mm between the rupture disc and macrocarrier containing the DNA. A control plate that was not bombarded was included for each experiment. MSV isolates MSV-Reu, MSV-MatA, MSV-Gat (obtained from D.P. Martin, University of Cape Town) and three Ugandan dimerized clones MSV-A[UG:Mpi11:05], MSV-A[UG:Kas75:05] and MSV-A[UG:Kab82:05] (constructed in Chapter 2 of this study), were also co-bombarded with the hairpin pHPKomrepΔI^{678} construct or pHANNIBAL. Immediately after bombardment, one plate that was co-bombarded with pKom602 and pHANNIBAL DNA was stored at −80°C for 96 hrs. This plate was a control to show that the input 1.1-mer pKom602 is not amplified by replicative-specific primers. The other bombarded plates were incubated at room temperature in the dark for 24 hrs and thereafter BMS cells transferred to BMS maintenance media [4.32 g/L MS + Vitamins, 2 mg/L 2,4-D, 100 mg/L Myoinositol and 3% Sucrose pH 5.8] and kept at 25°C in the dark for 72 hrs.

5.3.3 DNA and RNA isolation

Two 300 mg preparations of BMS cells, one for DNA and the other for RNA extraction were made from each bombarded BMS plate 96 hrs post bombardment. DNA was extracted using the CTAB protocol Kiprop et al. (2002) with minor modifications. Instead of grinding cells in liquid nitrogen using a mortar and pestle, warm CTAB extraction buffer was added to 300 mg BMS cells in 2 mL eppendorf tubes; to which two 1/4 inch ceramic spherical beads (BIO 101, Inc, USA) were added and the cells homogenized by vortexing the mixture for 10 mins. The rest of the protocol was followed as described by Kiprop et al. (2002) and in Chapter 3. Total RNA was extracted as described by White & Kaper (1989), with the same modifications for cell homogenization described above for DNA extractions. For each sample, 100 μg of extracted total RNA was separated into large and small fractions using the mirVana miRNA isolation kit (Ambion Inc) following the manufacturer's instructions. Both small RNA (<200nt) and large RNA (>200nt) fractions were isolated and 2 μL of each fraction electrophoresed through a 1.2% agarose gel to check its integrity. The small RNA fraction (<200nt) eluted in 100 μL of elution buffer was further treated with 98 μL of 100% ethanol to precipitate the RNA. The pellet was washed with 70% ethanol and redissolved in 10 μL RNAase-free water.

5.3.4 PCR analysis

Viral replicative-form specific PCR was used to assess the effect of the hairpin constructs on viral DNA replication. Total DNA samples at a starting concentration of 100 ng/μL were used as template for amplification using degenerate primers 215-234 and 1170-1792 (Willment et al. 2001), designed to amplify a 1305-kb MSV portion of only replicated circular viral DNA and not linear input plasmid DNA, hereafter referred to as replicative-specific (RS) PCR. Cycling conditions were as described in Chapter 3 for the same primer set.

5.3.5 Real-time PCR

Virus titres in bombarded BMS cells were quantified using real-time PCR. Primers for real-time PCR were designed to amplify a 129-bp portion of the MSV-Kom *REP* gene RepRTF and RepRTR (Table 5.1). This portion encompassed nucleotides 841-969 of the intronless *REP* (or nucleotides 933-1061 of the full-length rep), while repΔI^{678} encompassed nucleotides 50-727 of the intronless rep (all co-ordinates relative to the rep ATG); therefore these primers are specific for MSV-Kom *rep* and cannot amplify repΔI^{678} from the pHANNIBAL constructs. To confirm this, the primers were tested using a BMS sample that was bombarded with pHPKomrepΔI^{678} alone (no pKom602) as template. A second set of primers, 18S (F) and 18S (R) were used to amplify a 150 bp product from the 18S ribosomal RNA gene for each sample, to normalize the data. Prior to the runs, a standard curve was constructed by amplifying pKom602 plasmid DNA using primers RepRTF and RepRTR (Table 5.1). Five plasmid concentrations: 100, 10, 1, 0.1 and 0.01 pg/μL were used as template to generate the standard curve. This standard curve was subsequently imported into each run during quantification. To perform the PCR, genomic DNA samples were diluted to a concentration of 100 pg/μL. A real-time PCR reaction was set up with the 25 μL reaction mix consisting of: 0.3 μL SYBR green, 12.5 μL Sensimix (Sensimix DNA kit - Quantace); 25mM MgCl2 to final concentration of 3 mM; 10 μM RepRTF and RepRTR primer mix to a final concentration of 0.5 μM and 2 μL of 100 pg/μL template DNA. A standard of pKom602 plasmid DNA at a concentration of 100 pg/μL was included in each run. Each sample was prepared in duplicate. An identical reaction, although replacing the *rep*-specific primers with 18S primers was also set up for each sample in duplicate for normalizing using 18S gene amplification, which should be constant for each sample. Cycling was performed in a Rotorgene machine (Corbett, Australia) at the following conditions: initial hold at 95°C for 10 mins followed by 45 cycles of 95°C for 5 secs, 60°C for 45 secs and 72°C for 9 secs. Data were analyzed using Rotorgene software (Version 6) by importing the standard curve into each run to calculate virus concentrations. Calculated values were normalized by dividing each sample virus concentration by the value of the 18S housekeeping gene for the corresponding sample, then for each construct combination a mean was calculated.

5.3.6 siRNA detection

Small RNA detection was conducted as described by Molnar et al. (2007). Briefly, 10 μL of mirVana purified RNA was added to an equal volume of FDE buffer [1 mL deionized formamide, 200 μL of 0.5 M EDTA (pH=8.0), 10 mg xylene cyanol FF and 10 mg bromophenol blue] and incubated at 65°C for 10 mins. Denatured RNA samples were then loaded onto 15% denaturing polyacrylamide gels [25 g urea, 5 mL (10X) TBE, 10 mL (40%) acrylamide (38:2=acrylamide:bis-acrylamide), sterile dH2O to 50 mL, 300 μL 10% APS and 20 μL TEMED] pre-run in 1xTBE buffer at 100 V for 30 mins. A radiolabeled size ladder was loaded on the gel with the samples.

Samples were run at 150 V until the bromophenol dye was 1 cm from the bottom of the gel. The gel was equilibrated in Sodium phosphate buffer (10 mM Sodium phosphate buffer pH=7.0) for 10 mins, then transferred to 20X Salt Sodium Citrate(SSC) for a further 10 mins prior to performing the siRNA detection. To do this, capillary blotting (without a reservoir) was conducted. Briefly, a Hybond-N+ membrane (Amersham) was cut to the size of the gel, then wetted with sterile water and incubated in 20X SSC for 5 mins. Three pieces of Whatman paper cut slightly bigger than the gel were wetted with 20X SSC. The gel was placed on a clean glass plate, a few drops of 20X SSC poured on top, then the membrane overlayed onto the gel. Bubbles were removed by gently rolling a sterile tube over the assembly. A few more drops of 20X SSC were added onto the membrane, then prewetted Whatman paper placed on the membrane and air bubbles removed as before. Paper towels of 2-3 inch thickness were placed on top of the Whatman paper, and a glass plate and a weight (about 1 kg) placed on top of the setup. Transfer was allowed to occur overnight. After the overnight incubation, wet paper towels were removed and transfer allowed for another 2 hrs. The setup was disassembled, the membrane dried on Whatman paper for a few minutes, then UV crosslinked and left behind a shield. The membrane was transferred to a hybridization bottle to which 10 mL of hybridization buffer was added and prehybridization allowed to occur at 40°C for one hour. During this time, the radiolabelled probe was prepared. Oligonucleotide probes designed from the *REP* gene sequence: RepF1 and RepF5 (Table 5.1) and synthesized by SIGMA (GENOSYS) were labeled as follows: the oligonucleotide (oligo) was diluted to a concentration of 10 μM then denatured at 90°C for 1 min and snap cooled on ice. Two microlitres of denatured oligo were then added to 2 μL PNK (Polynucleotide Kinase) buffer (10X supplied with PNK- Fermentas), 13 μL distilled water, 1 μL T4 Polynucleotide Kinase (10 U/μL), 2 μL [γ32P] ATP (111 KBq/pmol; 370 KBq/μL) and incubated at 37°C for 1 hr. The mixture was snap cooled on ice to stop the reaction then 20 μL of distilled water added and everything put through a G-25 sephadex spin column and centrifuged at 7000 rcf to remove unincorporated nucleotides. Incorporation of radioactive label to the probe was checked by aliquoting 1 μL and checking that the count was between 500 and 2000 counts per second using a Geiger counter. After prehybridization, the buffer was discarded and 5 mL of fresh hybridization buffer was added to the membrane followed by the entire radiolabelled probe and left to hybridize at 40°C for 16 hrs. Following hybridization, the membrane was subjected to two 15 min washes with 2XSSC, 0.1%SDS at 40°C, dried, wrapped in Saran wrap and exposed to a phosphor screen. Images were processed using a Phosphoimager.

5.3.7 Generation of transgenic *D. sanguinalis*

Freshly generated (∼3 months after initiation from the inflorescence) *D. sanguinalis* calli were transformed using particle gun bombardment (Biorad/ DuPont PDS 1000-He) to generate transgenic plants as described by Shepherd et al. (2007a). Plasmids used to transform *D. sanguinalis* were pHPKomrepΔI^{678}, containing the MSV-Kom repΔI^{678} hairpin, and pAHC25 (Christensen & Quail 1996), containing the *bar* gene for herbicide [DL-Phosphinothricin (PPT), DUCHEFA, Haarlem, Netherlands] selection. A total of 2 μg, (one microgram each) of pAHC25 and pHPKomrepΔI^{678} DNA were co-precipitated onto 0.6 micron gold particles as described in section 5.3.2. DNA-coated gold particles were delivered into *D. sanguinalis* calli at a pressure of 900 psi, with a 6 mm gap between the macrocarrier carrying the DNA and the rupture disk. An unbombarded control plate was included in all experiments. All plates were incubated in the dark for 24 hrs at room temperature throughout the selection period until the regeneration process.

5.3.8 Selection and regeneration of transformed *D. sanguinalis* calli

Selection and regeneration of bombarded calli (Fig 5.1) was conducted as described by Shepherd et al. (2007a). Un-bombarded control calli were regenerated in a similar way, with the exception that the media on which they were maintained was not supplemented with PPT. Regenerated transgenic plants were screened by PCR using Rep-*Xba*I (F) and Rep-*Cla*I (R) primers (Table 5.1). Plants positive for the repΔI^{678} transgene were maintained to maturity and seeds collected. Seeds were vernalized at 4°C for four days then placed on BMS seed media (4.32 g/L MS; 8 g/L pure Agar; pH 5.8) to germinate. Additionally, for the positive *D. sanguinalis* lines, the inflorescence was excised from each line and used to initiate calli (Fig. 5.1 b) that were regenerated into plantlets (T1 plantlets) in case the seed did not germinate and in order to have enough plantlets to work with for virus transmission studies.

5.3.9 Generation of transgenic maize

Friable actively growing embryogenic calli of high type II (Hi-II, derived from a cross between A188 and B73 genotypes; obtained from Dr W. Gordon-Kamm, Pioneer Hi-Bred International, Inc., Johnston, IA, USA), and A188 generated by M. Bezuidenhout, (UCT) were co-bombarded with pHPKomrepΔI^{678} and pAHC25 to generate transgenic maize plants. Culturing and bombardment of maize were achieved as de-

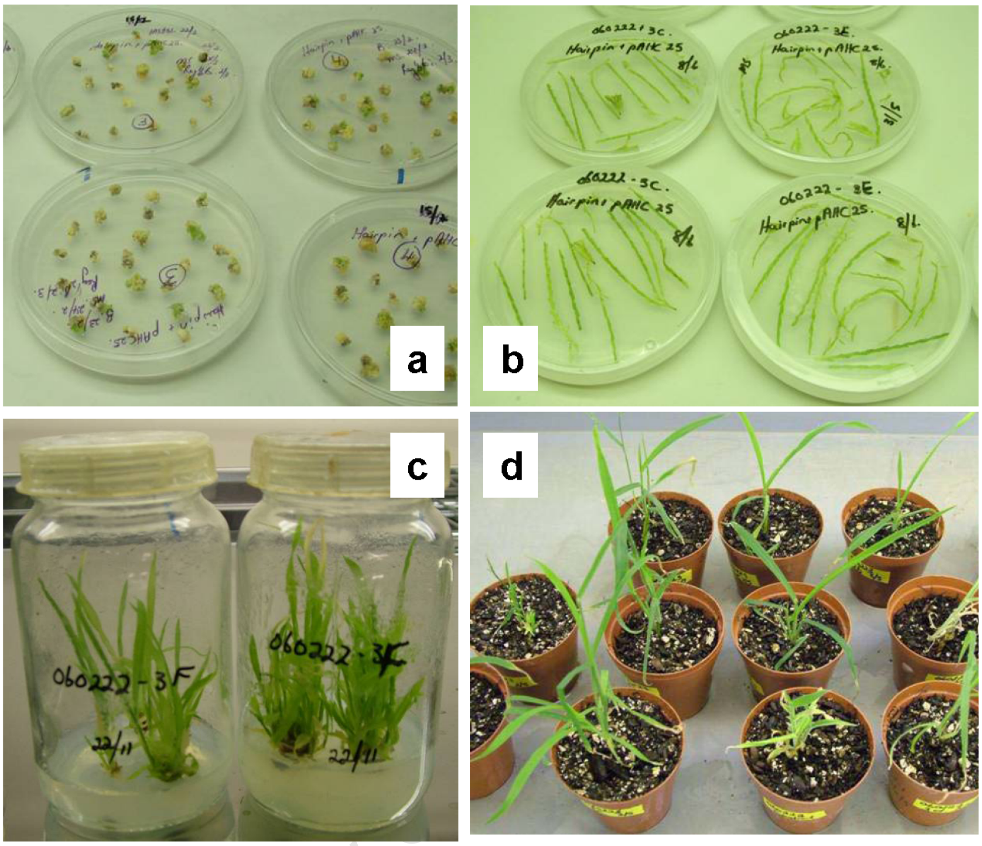


Figure 5.1: Generation of transgenic *D. sanguinalis* plantlets with the repΔI^{678}-hairpin construct. Plate a: *D. sanguinalis* calli on MS regeneration media; b: inflorescence of positive transgenic *D. sanguinalis* plantlets on regeneration media to initiate calli for generation of new plantlets; c: regenerating *D. sanguinalis* plantlets in bottles; d: hardened off putative transgenic *D. sanguinalis* plants.

scribed by Shepherd et al. (2007b). DNA precipitated onto gold particles as described in section 5.3.2 were delivered into maize calli at a pressure of 1100 psi with a gap of 9 mm between the rupture disc and the macrocarrier containing the DNA particles. Bombarded calli were incubated in the dark at room temperature throughout the selection period until the regeneration process.

5.3.10 Selection and regeneration of transformed Maize - HiII and A188 calli

Selection, maintenance and regeneration of transformed calli were conducted as described by Shepherd et al. (2007a). An unbombarded control plate was subjected to the same initiation/regeneration media containing PPT to ensure the selection was working (Fig. 5.2 d). Other unbombarded control plates were maintained on the same media but without PPT to ensure the source calli were viable and able to regenerate plantlets. Growing shoots were transferred to bottles containing MS rooting media. Plantlets were transferred to potting soil (Fig. 5.2 f) and hardened off in growth rooms maintained at 16hr light and 8hr dark 26°C.

5.3.11 Screening for transgenic lines

Total DNA was extracted from regenerated maize and *D. sanguinalis* plantlets by using the CTAB-extraction method as described in section 5.3.3 and Chapter 3. Leaf tissue was homogenized using liquid nitrogen and 300 mg of ground tissue used for the DNA extractions. Primers specific to the repΔI^{678} (Rep-*Xho*I (F) and Rep-*EcoR*I (R); Table 5.1) were used to screen both maize and *D. sanguinalis* transgenic plants. PCR conditions were as described for the primers in section 5.3. Transformants were also screened with primers specific to the *bar* gene (Table 5.1) using the same PCR conditions as for the repΔI^{678}-specific primers.

5.4 Results

5.4.1 Degenerate primer PCR and real-time PCR

5.4.1.1 Rep (repΔI^{678}) gene constructs

BMS cells were bombarded with the following plasmid combinations and virus replication assayed using quantitative PCR protocols specific for circularized virus DNA (i.e. the method will not detect input DNA): pKom602+ pHANNIBAL (wild type replication control); pKom602+pHPKomepΔI^{678}; pKom602 + pSenKomrepΔI^{678}; and pKom602+ pAntKomrepΔI^{678}. While virus was undetectable or barely detectable in BMS cells bombarded with repΔI^{678} hairpin, there was ample virus replication in

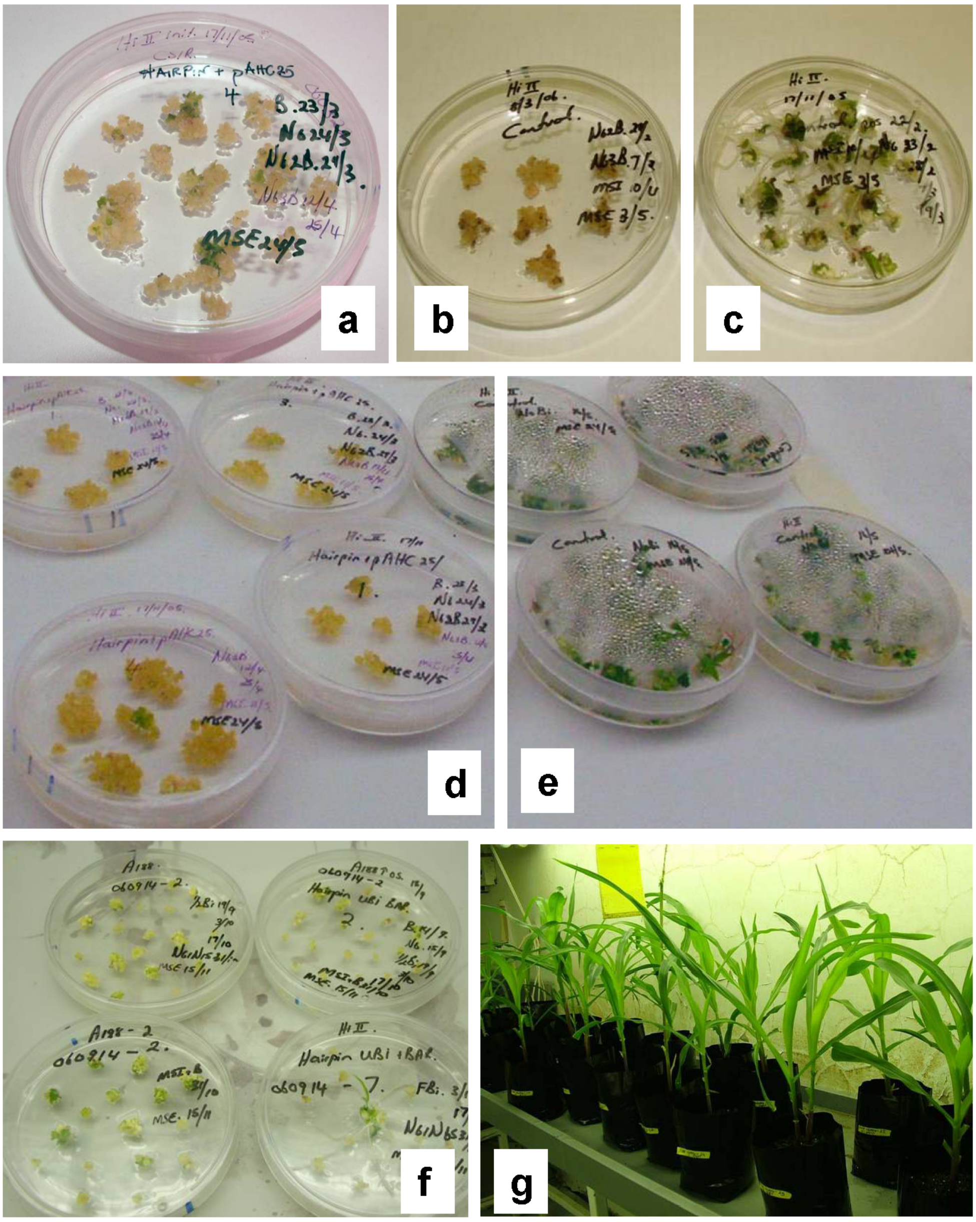


Figure 5.2: Generation of transgenic maize plantlets using the repΔI^{678}-hairpin construct. Plate a: HiII maize calli on MS elongation media; b: untransformed control HiII calli on elongation medium supplemented with PPT. Note the death of untransformed calli on the medium supplemented with PPT in comparison to control calli not undergoing PPT selection (Plate c) c: untransformed HiII calli on MS media without selection; d: transformed HiII on MS elongation medium supplemented with PPT; e: untransformed control plates on regeneration media without selection f: regenerating A188 maize calli on shoot initiation media; g: putative transgenic maize plants in soil in the plant room.

BMS cells bombarded with pKom602 + pHANNIBAL (wild type replication against which all other construct combinations were compared; Fig. 5.3A). Corresponding real-time data confirmed this observation (Fig. 5.3B).

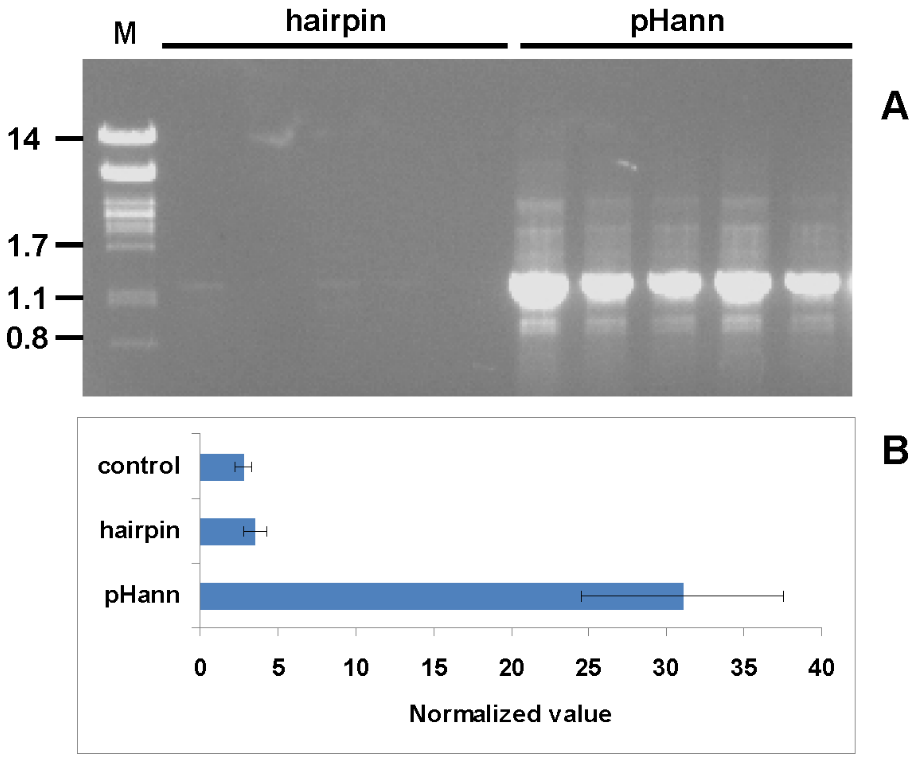


Figure 5.3: Transient assays and real-time PCR testing the effect of repΔI^{678} hairpin on MSV replication. A: replicative-specific PCR of BMS cells co-bombarded with pHPKomrepΔI^{678}+pKom602 ("hairpin") and pHANNIBAL+pKom602 ("pHann"). control: unbombarded BMS cells. Molecular marker sizes are given in kilo base pairs. B: Corresponding real-time PCR data for the samples. Normalized values for real-time PCR (n=10 for both "hairpin" and "pHan"). Error bars are the 95% confidence of the mean.

To determine if the observed virus replication inhibition was due to a dsRNA/silencing-based mechanism, two additional constructs were bombarded with pKom602: repΔI^{678} in the sense orientation alone (pSenKomrepΔI^{678}) and in the antisense orientation alone (pAntKomrepΔI^{678}). Surprisingly, while the antisense repΔI^{678} had no effect on viral replication (Fig 5.5A and 5.6A), replication in the presence of repΔI^{678} in the sense orientation was lowered in comparison to wild type replication (Figs 5.4A and 5.6A). The same trend was observed using real-time PCR (Figs 5.5B and 5.6B). When the real-time data for all four bombardments were collated (Fig 5.7), it was apparent that replication of pKom602 in the presence of the repΔI^{678} hairpin was significantly less (P=0.049) than viral replication in the presence of the sense repΔI^{678}, which was in turn significantly less (P<0.001) than viral replication in the presence of pHANNIBAL. The antisense repΔI^{678} had no significant effect on viral replication (P=0.317).

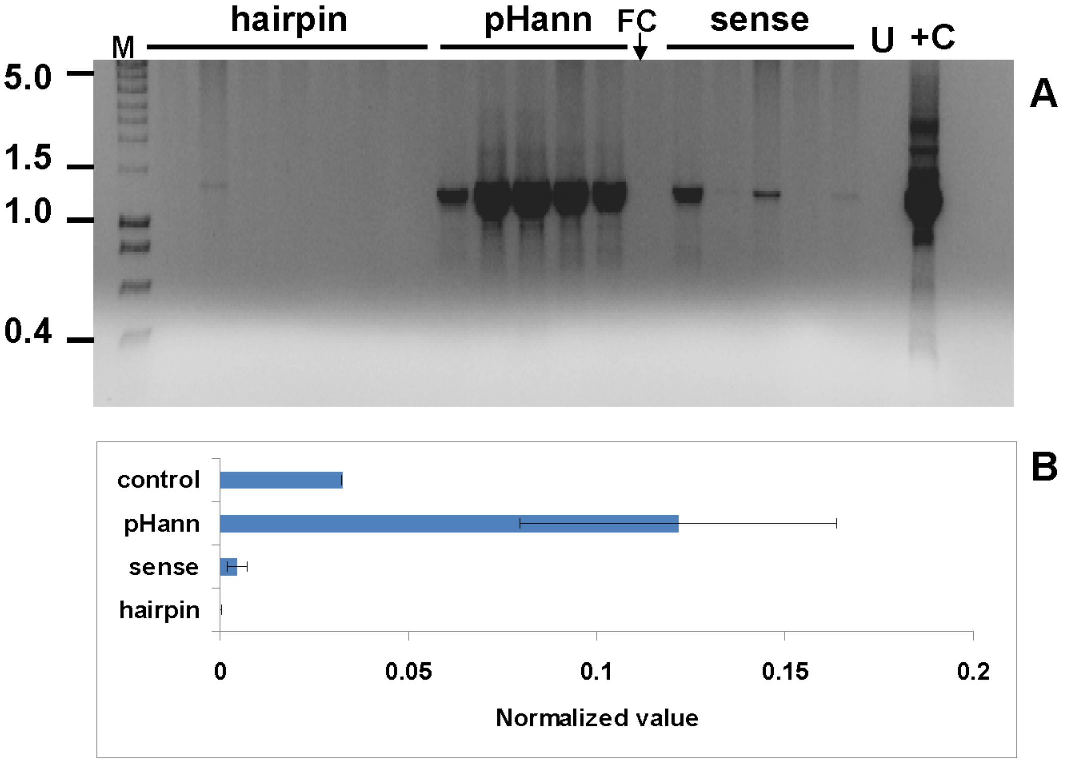


Figure 5.4: Transient assays and real-time PCR testing the effect of the hairpin and sense repΔI^{678} on MSV replication. A: Replicative-specific PCR of BMS cells co-bombarded with pHPKomrepΔI^{678}+pKom602 ("hairpin"); pHANNIBAL+pKom602 ("pHann"); pHANNIBAL+pKom602 frozen at -80C immediately after bombardment (frozen control - "FC"); pSenKomrepΔI^{678}+pKom602 ("sense"); Unbombarded control ("U"): and PCR positive control ("+C"). Molecular marker sizes are given in kilo base pairs. B: Corresponding real-time PCR data for the samples. Normalized values for real-time PCR ("pHann": n=12; "hairpin": n=12; "sense": n=8). Error bars are the 95% confidence of the mean.

Having determined that the hairpin repΔI^{678} construct inhibited cognate MSV-Kom replication (i.e. the isolate from which the *REP* gene was derived; Figs. 5.3, 5.4, 5.5 and 5.6), its effect was tested on the following virus isolates whose maximum divergence is 4.62% at the nucleotide level: the east African isolates MSV-Gat,

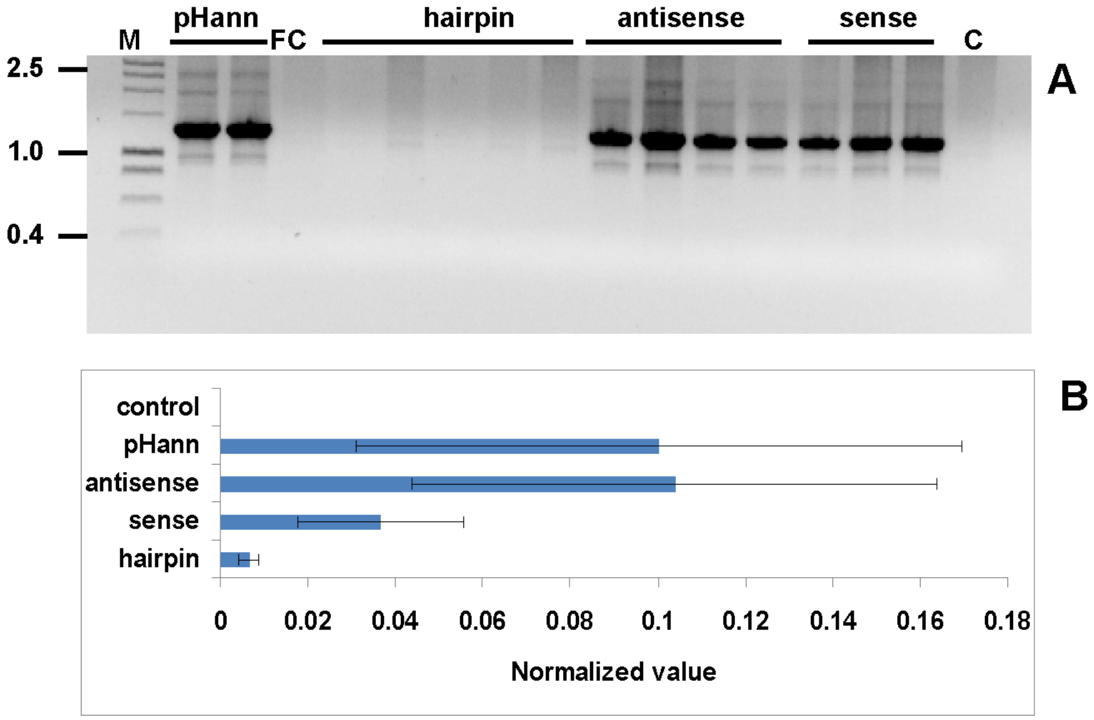


Figure 5.5: Transient assays and real-time PCR testing the effect of hairpin, sense repΔI^{678} and antisense repΔI^{678} on MSV replication. A: Replicative-specific PCR of BMS cells co-bombarded with pHANNIBAL+pKom602 ("pHann"); pHANNIBAL+pKom602 frozen at -80C immediately after bombardment (frozen control - "FC"); pHPKomrepΔI^{678}+pKom602 ("hairpin"); pAntKomrepΔI^{678}+pKom602 ("antisense"); pSenKomrepΔI^{678}+pKom602 ("sense"); and unbombarded control ("C"). Molecular marker sizes are given in kilo base pairs. B: Corresponding real-time PCR data for the samples. Normalized values for real-time PCR ("pHan": n=6; "antisense" n=8; "sense": n=6; "hairpin": n=10). Error bars are the 95% confidence of the mean.

MSV-A[UG:Mpi11:05], MSV-A[UG:Kas75:05] and MSV-A[UG:Kab82:05], the southern African isolate MSV-MatA and the Réunion isolate MSV-Reu. Replication of all six isolates was inhibited in the presence of repΔI^{678} hairpin (Figs. 5.8, 5.9), indicating a repΔI^{678}-hairpin based resistance mechanism could potentially be effective against a broad range of MSV-A isolates across sub-Saharan Africa

No 1.3 kb PCR product was amplified from unbombarded controls or a sample bombarded with pHANNIBAL and pKom602 and frozen at -80°C immediately after bombardment (Figs. 5.4 and 5.5), indicating the degenerate primers were specific for replicative form viral DNA (and did not amplify the input plasmid DNA construct).

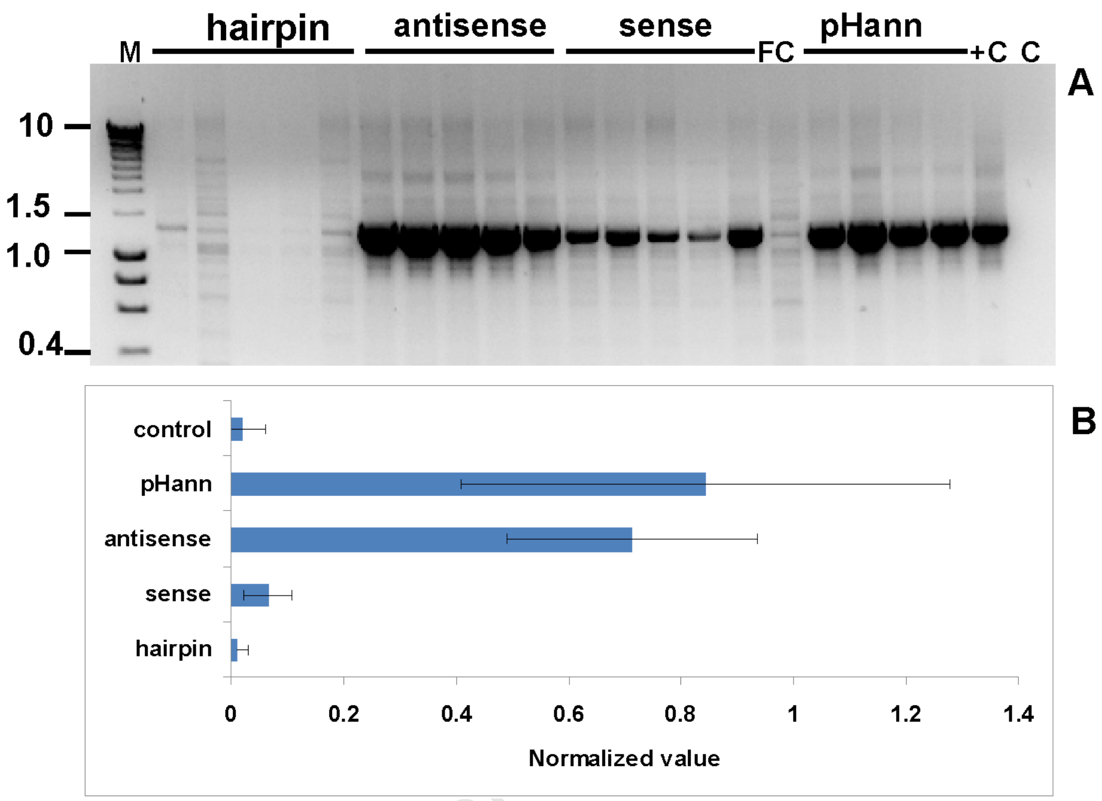


Figure 5.6: Transient assays and real-time PCR testing the effect of hairpin, sense and antisense repΔI^{678} on MSV replication. A: Replicative-specific PCR of BMS cells co-bombarded with pHPKomrepΔI^{678}+pKom602 (“hairpin”); pKom602+pAntKomrepΔI^{678} (“antisense”); pSenKomrepΔI^{678}+pKom602 (“sense”); pHANNIBAL+pKom602 frozen at -80C immediately after bombardment (frozen control - “FC”); pHANNIBAL+pKom602 (“pHann”); PCR positive control (“+C”); and unbombarded control (“C”). Molecular marker sizes are given in kilo base pairs. B: Corresponding real-time PCR data for the samples. Normalized values for real-time PCR (“pHan” n=8; “antisense” n=8; “sense” n=8; “hairpin” n=8). Error bars are the 95% confidence of the mean.

5.4.1.2 LIR constructs

MSV-Kom replication was not affected by sense, antisense or hairpin LIR. Viral replication in the presence of all LIR constructs was the same as that of pKom602+pHANNIBAL (i.e wild type replication) (Fig. 5.10). No virus was amplifiable from unbombarded control BMS cells.

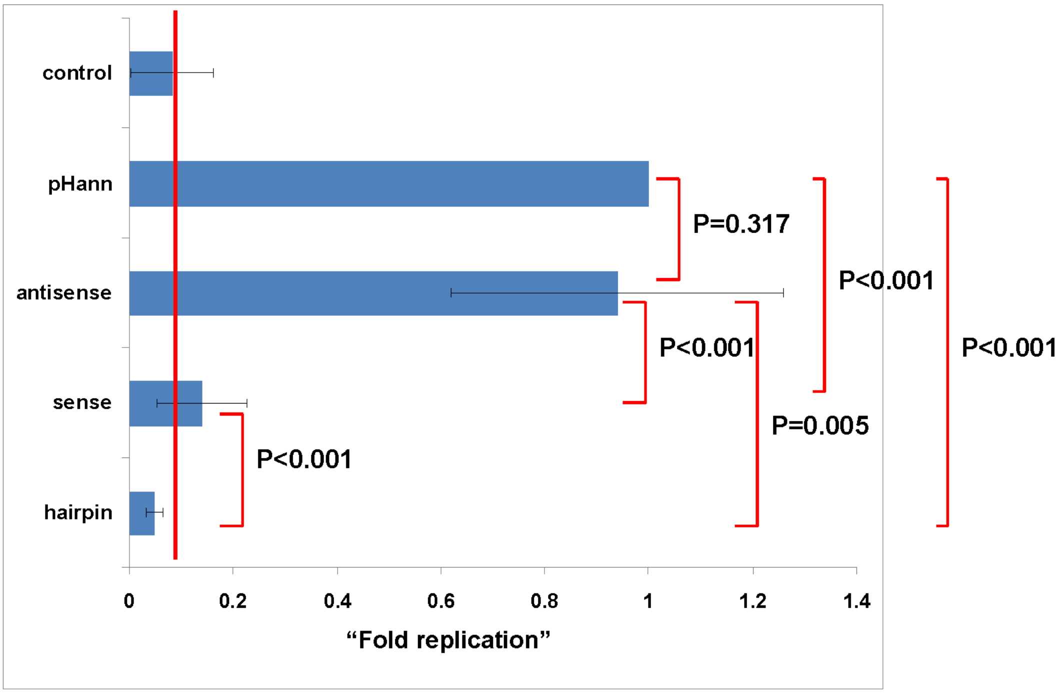


Figure 5.7: Quantification of pKom602 replication in BMS suspension cells bombarded with pKom602 and either pHANNIBAL (empty vector), sense, antisense or hairpin repΔI^{678} constructs. Normalised data from all bombardments (shown individually in figures 5.3 - 5.6) were used to find an overall mean value for each construct combination. The mean value for pKom602+pHANNIBAL was standardized as 100% replication (1-Fold), and the mean values for pKom602 + repΔI^{678} sense, antisense and hairpin constructs were expressed relative to that for pKom602+pHANNIBAL. Error bars represent the 95% confidence of the mean. The red line is the average control (background fluorescence) value. P values are for Mann-Whitney Rank sum tests between two treatments as indicated by the red bracket.

5.4.2 Detection of siRNA using northern blots

High quality total RNA was extracted from BMS cells bombarded with the different constructs (Fig. 5.11). The large and small RNA fractions were obtained following purification and separation with the mirVana kit (Fig. 5.12).

Both RepF1 and RepF5 probes showed signals following northern blots for small RNAs. A band between 20 and 30nt in size was observed in all samples including controls bombarded with pHANNIBAL alone (i.e. no virus or repΔI^{678} RNA should be present). The bands observed with the RepF1 probe were less discrete (Fig. 5.13

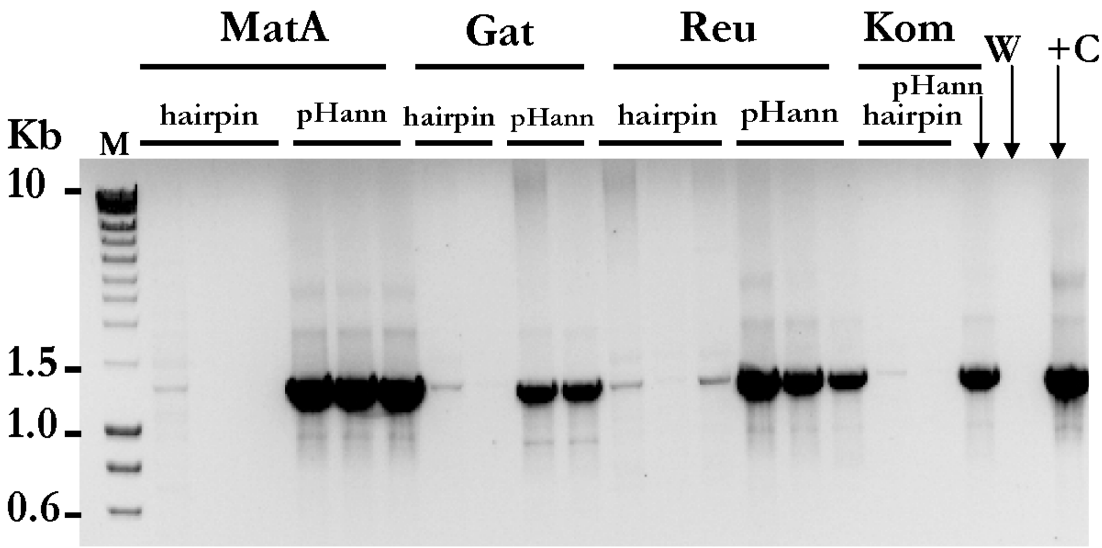


Figure 5.8: Transient assays testing the repΔI^{678} hairpin against diverse MSV isolates: Cloned dimers of MSV MatA ("MatA"); MSV-Gat ("Gat") and MSV-Reu ("Reu")- were each co-bombarded with pHPKomrepΔI^{678} ("hairpin") or pHANNIBAL ("pHann"). pKom602 ("Kom") was also bombarded for comparative purposes; W: Water (negative) control for the PCR; + C: Positive control for PCR.

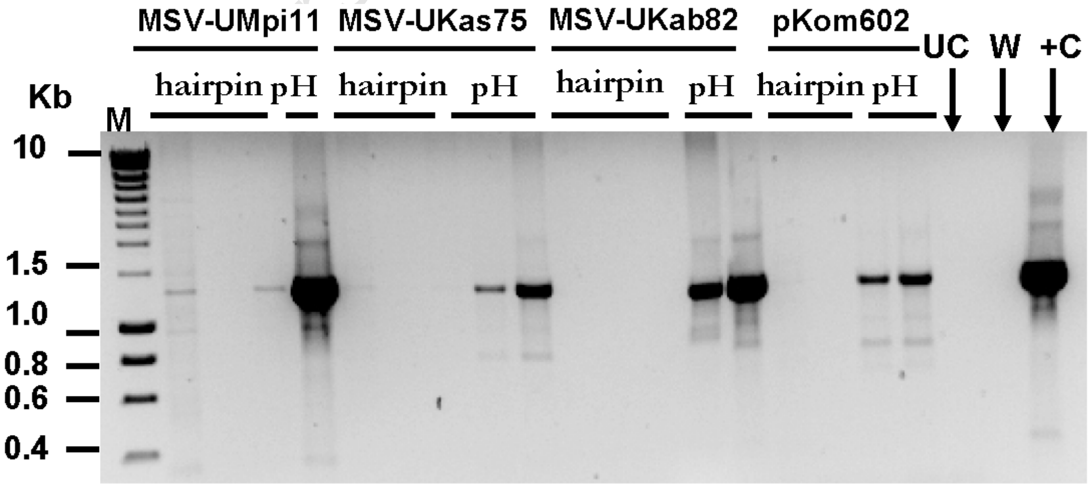


Figure 5.9: Transient assays testing the efficacy of the repΔI^{678} hairpin (pHPKomrepΔI^{678}) against Ugandan MSV isolates: MSV-A[UG:Mpi11:05], MSV-A[UG:Kas75:05]and MSV-A[UG:Kab82:05]. pKom602 was used as a control for the bombardment for comparative purposes; "pH"-Each respective virus + pHANNIBAL; "UC" - Unbombarded control; "W" - Water; "+ C" - Positive control for PCR.

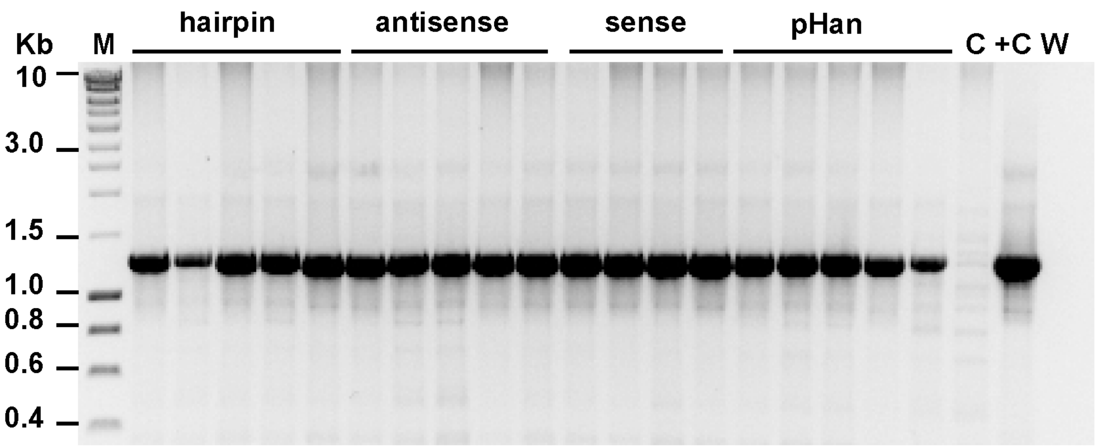


Figure 5.10: Transient assays testing the efficacy of LIR hairpin, LIR "antisense" and LIR "sense" constructs on pKom602 replication: Replicative-specific PCR of BMS cells co-bombarded with pHPKomLIR + pKom602 ("hairpin"); pKomLIRAN+pKom602 ("antisense"); pKomLIRS + pKom602 ("sense") and pHANNIBAL+pKom602 ("pHan"); Unbombarded control ("C"); Positive control for PCR ("+C"); Water ("W").

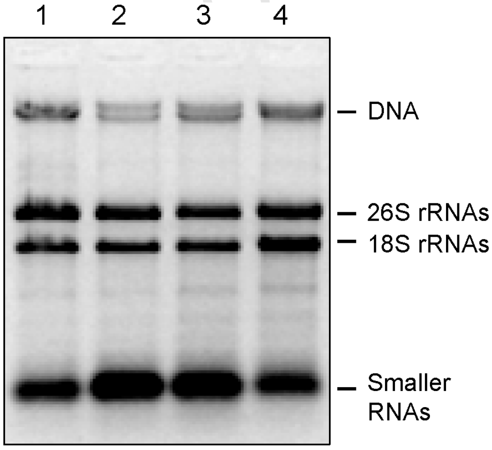


Figure 5.11: Total RNA extracted from BMS cells co-bombarded with Lane 1: pHPKomrepΔI^{678}+pKom602 ("hairpin"); 2: pKom602+pAntKomrepΔI^{678} ("antisense"); 3: pSenKomrepΔI^{678}+pKom602 ("sense");Lane 4: Unbombarded control

A) than those observed with the RepF5 probe (Fig. 5.13 B). Due to the nature of the ladder, it was not possible to work out the exact size of the bands.

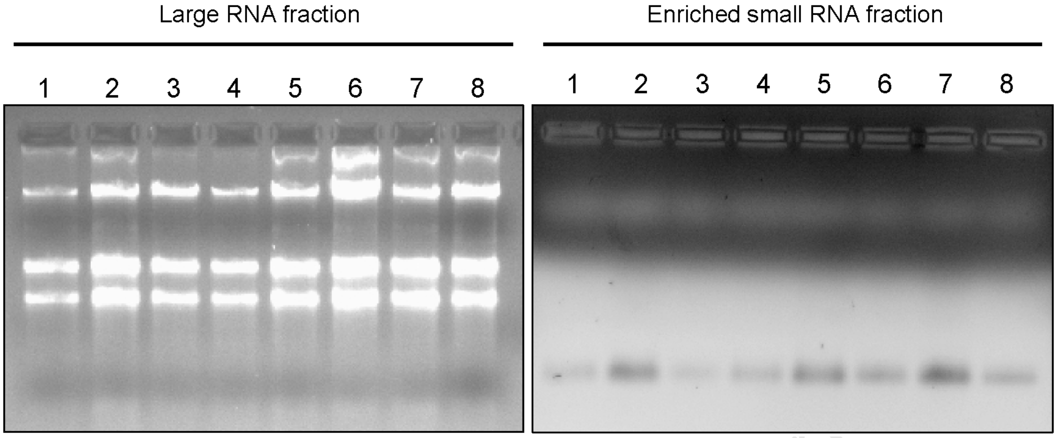


Figure 5.12: mirVana purified RNA samples showing the large and corresponding small fractions after separation and enrichment of the small RNA fraction. Lanes 1: pKom602; 2: pHANNIBAL; 3: pHPKomrepΔI^{678}+pKom602 ("hairpin"); 4: pSenKomrepΔI^{678}+pKom602 ("sense"); 5: pHPKomrepΔI^{678}; 6: pKom602+pAntKomrepΔI^{678} ("antisense"); 7: pKom602+pHANNIBAL; 8: Unbombarded control

5.4.3 Transgenic plants

5.4.3.1 Regeneration of transgenic plants

A total of 15 plates of callus were bombarded and a total of 20 potentially transgenic *D. sanguinalis* plants were regenerated(Fig. 5.1 d). *D. sanguinalis* calli from unbombarded plates did not survive in media supplemented with PPT but calli from bombarded plates survived PPT selection, growing to mature plants. The whole transformation process from calli to putative transgenic plants was accomplished in around 90 days. Transformants exhibited varying phenotypes. Whilst some plants were obviously stunted, others exhibited normal growth (data not shown). Although *D. sanguinalis* seeds collected from T_0 positive lines were sterile and failed to germinate, 32 *D. sanguinalis* plantlets were successfully regenerated from the inflorescence of positive transgenic plants.

Fifty-seven potentially transgenic maize plants were generated from two bombardments (23 plates). Non-bombarded maize calli from control plates that were subjected to PPT selection media did not survive the eight week selection (Fig. 5.2 b), while

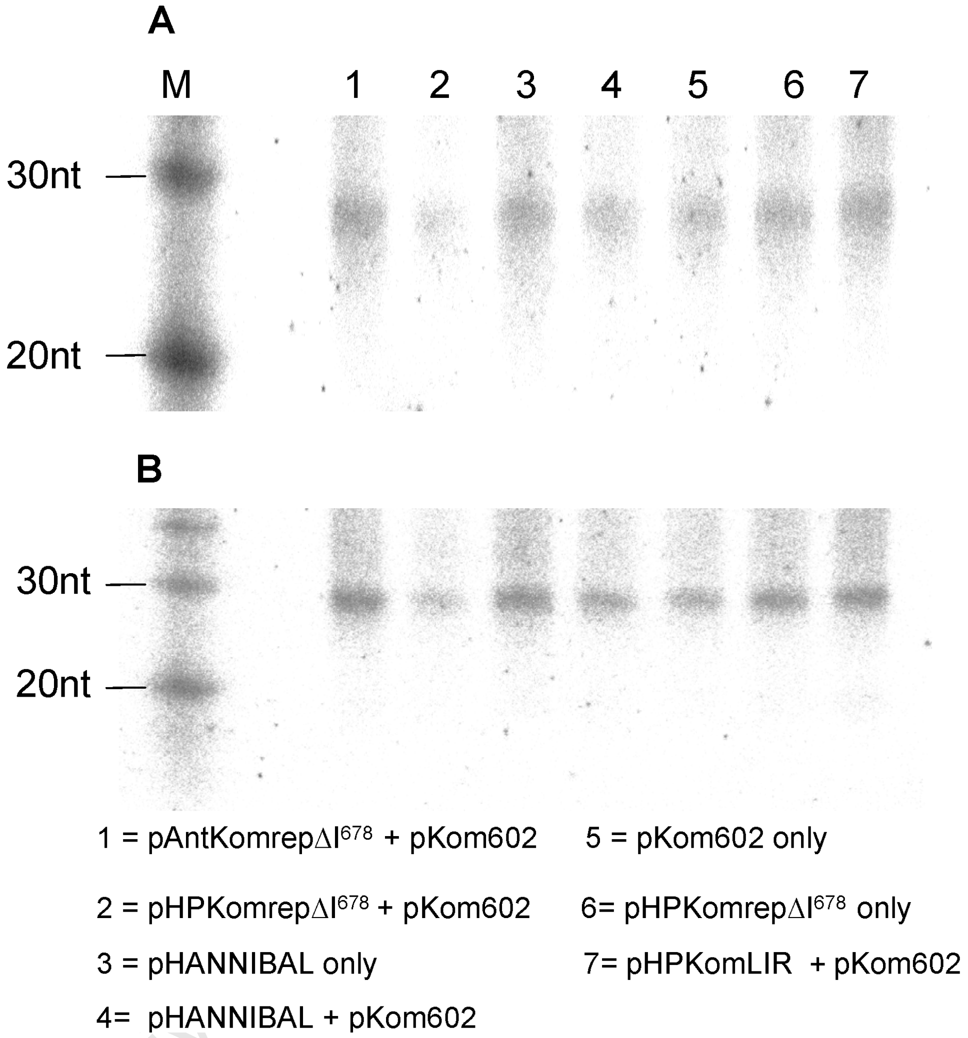


Figure 5.13: Small RNA detection using northern blots. A: blots with RepF1 probe; B: blots with Rep5F probe.

unbombarded calli cultured with no PPT selection regenerated vigorously to maturity (Fig. 5.2 e). Putative transformed calli developed into shoots (Fig. 5.2 a, c,) eventually growing into plantlets (Fig. 5.2 f).

5.4.3.2 Screening for transgenic *D. sanguinalis* plantlets

Eight plants were positive for both the repΔI^{678} transgene and the *bar* herbicide resistance gene whilst six plants were positive for only the *bar* gene. Untransformed control plants were negative for both the repΔI^{678} and *bar* transgenes (Fig. 5.14).

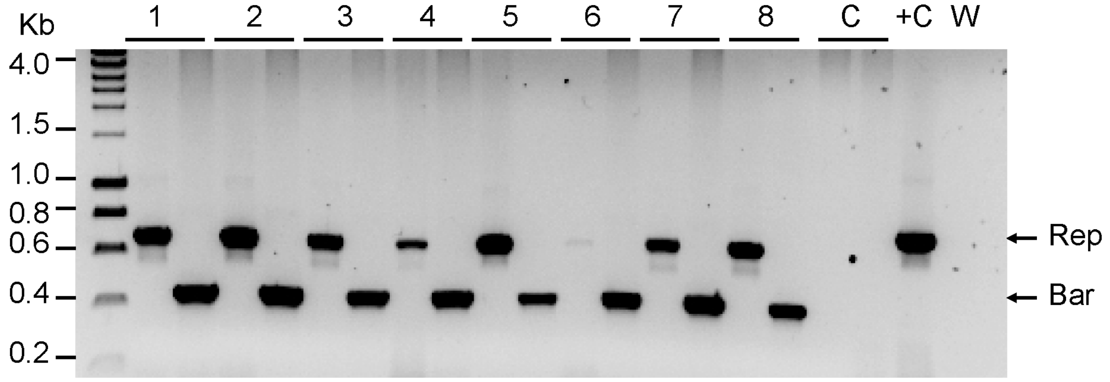


Figure 5.14: Positive *D. sanguinalis* transformants (transgenic lines 1 to 8) showing presence of the 678-bp repΔI^{678} transgene and the 400 bp *bar* transgene for herbicide resistance. C= untransformed *D. sanguinalis* control; +C = positive PCR control; W= water (negative) PCR control

After seed obtained from all eight repΔI^{678}-positive transgenic plants failed to germinate, plantlets were obtained by initiating callus from the inflorescence of each repΔI^{678}-positive T_0 line and then regenerating plantlets (which should be identical clones of the parental line) from the transgenic calli. However, none of the 25 T_1 plantlets that regenerated from the calli of positive T_0 plants shown in Fig. 5.1 d were positive for the repΔI^{678} transgene (data not shown). The calli from which the plants were regenerated, however, remained positive for the transgene indicating that the transgene had stably integrated into the genome.

5.4.3.3 Screening of putative transgenic maize plantlets

Fifty-seven potentially transgenic maize plantlets were screened for the repΔI^{678} and *bar* transgenes by PCR using repΔI^{678} and *bar* specific primers. Two plants were positive for the both the repΔI^{678} and the *bar* transgenes (Fig. 5.15) while three were positive for only the *bar* gene. The repΔI^{678} and *bar* genes were not detectably present in the remaining plants screened. There were plans to grow the two positive plants in an environment conducive for seed development (a seed company in Greytown, KwaZulu Natal) since this is not possible in the laboratory where the transformation was conducted. This was however not done in time and the positive

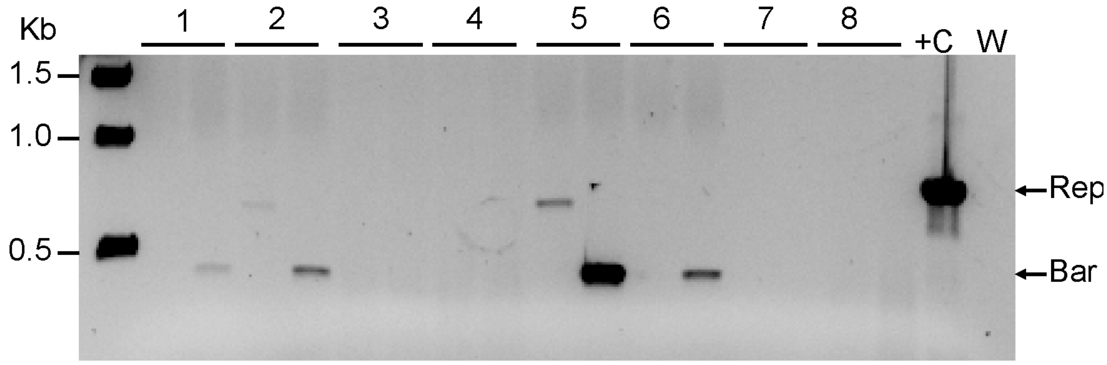


Figure 5.15: Maize T_0 regenerants screened for the 678-bp repΔI^{678} transgene and the 400bp *bar* transgene for herbicide resistance. Lines 1 and 6 are transgenic lines positive for only the *bar* transgene and negative for the repΔI^{678} transgene; Lines 2 and 5 are transgenic lines positive for both the repΔI^{678} and *bar* transgenes; lines 3,7 and 8 are negative for both repΔI^{678} and *bar* transgenes. +C = positive PCR control; W= water (negative) PCR control.

lines grew to senescence without seed and were lost.

5.5 Discussion

Gene silencing, albeit inadvertently, has been applied successfully to provide resistance to some serious plant virus problems (reviewed by Fuchs & Gonsalves 2007). Because it is a natural antivirus defense system and owing to previous studies that have demonstrated the efficacy of gene silencing in reducing replication of DNA viruses, the technology is attractive for development of MSV resistance. To rapidly assay gene silencing as a tool for development of resistance to MSV, a well established transient system combining particle bombardment of black Mexican sweetcorn suspension cultures and replicative-specific (RS) PCR that has previously been used to assay for MSV replication (Shepherd et al. 2005, 2007a) was used in this study prior to development of transgenic plants. A hairpin of 678 bp constructed from an internal portion of the intronless *REP* gene, and of the LIR of MSV cloned in pHANNIBAL were used in the assays. Previous studies of gene silencing of viruses from the *Geminiviridae* family have targeted the *REP* (Vanitharani et al. 2003, Chellappan et al. 2004b, Varma & Praveen 2006, Bonfim et al. 2007) and the bidirectional promoters (Vanderschuren et al. 2007a, Pooggin et al. 2003), and demonstrated reduction in virus replication. This study therefore, similarly, chose the *REP* and promoter-containing LIR for silencing in an attempt to interfere with MSV replication. The 678-bp portion of MSV-Kom *REP* was chose due to its containing putative siRNA-generating sequences for eight MSV isolates, MSV-MtkA, MSV-Reu, MSV-Ken, MSV-MatC, MSV-MatA, MSV-MatB, MSV-MakD and MSV-Kom, which at the time of the design of the construct, were the only available full genome sequences in GenBank. In addition, the intronless *REP* was chosen as a template because it was assumed that the RNA target for silencing would be mature mRNA; i.e. spliced transcripts.

PCR showed that the repΔI^{678} hairpin interfered with virus replication to the extent that viral DNA levels were undetectable (or in some cases only just detectable) by PCR. Although the construct with the repΔI^{678} gene in the sense orientation also reduced virus replication, virus DNA levels were significantly higher ($P < 0.001$) than in the hairpin-bombarded samples. The fact that MSV replication was not significantly reduced by the antisense repΔI^{678} construct as compared to the sense construct could possibly be because the sense orientation DNA is producing more siRNAs that the antisense construct. In the case of the repΔI^{678} hairpin, the presence of the sense and antisense versions of the gene together inhibited virus replication to a significantly greater extent than that achieved by the sense or antisense alone, indicating that virus replication inhibition most probably occurred through a dsRNA/silencing mechanism.

Real-time PCR confirmed the observations of the RS-PCR, and showed a statistically significant quantitative difference between hairpin-induced virus inhibition and inhibition by the sense repΔI^{678}. The Rep protein is expressed early during infection and has been identified as the only protein indispensable for virus replication (Stenger et al. 1991). It is probable that by using a hairpin construct to silence the *REP*, replication of MSV is interfered with.

The repΔI^{678} hairpin derived from MSV-Kom was able to reduce replication of different isolates belonging to the MSV-A group (this is the only strain known to cause severe maize streak disease in maize; Martin et al. 2001) with nt sequence identities differing by as much as 4.62%. Although the diversity of these isolates appears low, this must be put in the context that all African MSV-As differ by only 3% at the nucleotide level (Martin et al. 2001). These isolates were therefore chosen because of their relatively high diversity within the MSV-A group (partly due to the inclusion of MSV-Reu, a non-African MSV-A isolate from the island of Réunion which is more divergent than the African MSV-A types) and because they all cause severe disease in maize. The three Ugandan isolates chosen represent the most frequently occurring haplotypes (MSV-A_1UgIII, MSV-A_1UgV, and MSV-A_1UgVI) in Uganda identified in Chapter 4. The *REP* is the most conserved of the mastrevirus ORFs (Rybicki 1994) and therefore, it was not surprising that MSV-Kom repΔI^{678} could effectively reduce replication of heterologous MSV isolates. Using the IMGENEX siRNA designer programme it was predicted there were common potential siRNAs from the reps of each of the virus isolates tested in this study. This means there is good potential for this resistance mechanism to be effective against different subtypes and isolates throughout sub-Saharan Africa.

Although in some instances, an MSV-Kom-specific amplicon was detected in some samples bombarded with the repΔI^{678} hairpin, the intensity was significantly lower than that detected in pKom602+pHANNIBAL samples (wild type replication controls). In addition, real-time PCR indicated that "rep" amplicons detected in the pKom602+ repΔI^{678} hairpin samples were in fact background amplification, with fluorescence in these samples being no higher than background fluorescence from non-bombarded controls. To confirm that the rep-specific primers (which were designed to fall outside of the *rep* sequence incorporated in the repΔI^{678} hairpin) were amplifying specifically pKom602 *rep* and not the *rep* from the sense or hairpin constructs, the primers were used on a BMS sample that was bombarded with the repΔI^{678} hairpin alone (no pKom602). Accordingly, no product was amplified (data not shown).

While targeting of the bi-directional promoter has been successful for other geminiviruses such as ACMV (Vanderschuren et al. 2007b, Dogar 2006) and Mungbean mungo yellow mosaic virus (VMYMV) (Pooggin et al. 2003), contrasting findings were observed in this study. None of the MSV LIR constructs (hairpin, sense or antisense LIR) reduced replication of pKom602. Ossowski et al. (2008) propose three reasons as to why silencing would be unsuccessful: insufficient production of effective siRNAs, inaccessibility of binding sites in the target mRNA and difficulties reducing steady state mRNA levels owing to a negative feedback mechanism which causes increased levels of transcription to replace the mRNA being reduced. One or all of these three reasons may possibly hold true for the observations made when the promoter region of MSV was targeted. However, because the presence of siRNAs from the LIR inverted repeat construct was not confirmed, conclusions can not be made.

BMS samples co-bombarded with pKom602 and repΔI^{678} hairpin, antisense repΔI^{678}, pHANNIBAL and LIR hairpin; as well as samples bombarded with pHANNIBAL alone and the repΔI^{678} hairpin alone, were analysed for production of siRNAs by northern hybridization. Probes were designed to anneal to nucleotide positions between 81 and 649 of the intronless *REP* gene (numbering starts at the ATG), and the probes with the best radiolabel incorporation (RepF1 and RepF5) were used in the blot. Both probes hybridized with a band falling between 20 and 30 nt for all samples including those bombarded with pHANNIBAL alone where no signal was expected. Following these observations, a BLAST search against the maize genome was conducted to ascertain if the probes were hybridizing with maize derived small RNAs. This indeed revealed that a 13-nt portion of the *REP* sequence from which both probes were designed had a very high sequence identity to the SQUAMOSA promoter binding protein (SBP) domain protein 4 of the maize genome. In addition there was sequence homology between the *REP* and various mRNAs of maize at *REP* coordinates (starting from the ATG) 2-15; 5-18; 52-65; 295-308; 527-540; 530-543; 551-564; 652 - 665; 738-751; 745-760; 883-897; 1009-1027. A BLAST of the probe sequences also showed that they had 100% sequence identity to ORFS from the maize genome for stretches of up to 13 continuous base pairs. This may be enough sequence identity for the probes to hybridize with a small RNA from the maize genome and could possibly explain why a faint band was detected in control samples that were not bombarded with pKom602 or the repΔI^{678} hairpin. However, this is speculative and more blots have to be conducted before conclusions can be drawn.

With the availability of the maize genome sequence released this year, probes should in future be designed from regions of the *REP* that do not have homology to maize for future siRNA detections. Although it could not be determined if siRNAs were produced from the repΔI^{678} hairpin or from pKom602, the efficacy of the repΔI^{678} hairpin in limiting virus replication indicates that the inhibition most probably occurred via an siRNA-mediated mechanism. This study also shows that a rapid transient system can be used to test potential siRNA strategies.

Having determined that the repΔI^{678} hairpin worked in a transient system, a total of 52 *D. sanguinalis* plants (20 T0 plants from initial bombardments and 32 T_1 plants initiated from the inflorescence of positive transgenic lines) were regenerated. Of these, only eight lines were positive for both the repΔI^{678} transgene and the *bar* herbicide resistance gene. These positive transformants were however sterile as none of the seeds from these lines germinated. Furthermore, the repΔI^{678} transgene was not detectable in the plantlets regenerated from callus initiated from the inflorescence of positive lines, although the callus itself remained positive for the transgene. Similarly, only two maize plants of 57 regenerated T_0 plants were positive for the repΔI^{678} transgene. More *bar*-positive plants were regenerated (5 and 14 for maize and *D. saunguinalis*, respectively) in comparison to repΔI^{678} positive plants (2 and 8 for maize and *D. sanguinalis*, respectively). Efficient transformation is dependent on several factors including the transformation method and regeneration of transformants (Birch 1997). In this study however, it is probable that the repΔI^{678} hairpin had some deleterious effect, leading to the low regeneration efficiency of plants with the repΔI^{678} hairpin in both *D. sanguinalis* and maize. It is also possible that the repΔI^{678} hairpin targets an essential gene in the maize genome. However, the fact that the bar transgenics regenerated efficiently, indicates that it was not the callus or the transformation process at fault.

Transgenic *D. sanguinalis* plants were infertile and development of some of the transgenic plants was retarded. It has been proposed that transforming plants with hairpin constructs may be detrimental to the plants' development because the hairpin or dsRNA is constitutively expressed (Tang et al. 2003). It is also possible that the repΔI^{678} hairpin targets an essential gene in the maize genome. The sequence identity between the *REP* primers for probing for siRNA and the maize genome suggests that off-target effects are a possibility.

Southern blot analysis provides proof for integration of a transgene into the plant genome (Birch 1997). Because the plantlets regenerated from the eight PCR-positive lines were PCR-negative for the transgene, Southern blots were not performed. However, the calli (initiated from the inflorescence of positive T_0 lines) from which the plantlets were regenerated were positive for the repΔI^{678} indicating the transgene had integrated into the T_0 plants' genomes. It is possible that the calli were chimaeric (i.e. contained both transformed and untransformed cells) and only cells that did not contain the transgene regenerated, while those that contained the transgene did not regenerate due to possible toxicity of the hairpin repΔI^{678}. Similarly, only two maize lines were positive for the repΔI^{678} transgene following bombardment of maize. Biolistic transformation does have drawbacks associated with stability and integration of transgenes (Shrawat 2007) and generally has a low transformation efficiency in comparison to other transformation strategies (Sharma et al. 2005).

In conclusion, results presented here show that targeting the MSV *REP* and not the promoter in the LIR is effective in reducing MSV replication. Furthermore, the repΔI^{678} designed from one isolate, MSV-Kom, was effective in interfering with replication of a number of diverse virus isolates, including east African, southern African and Réunion MSV isolates. These data strongly suggest that gene silencing has a potential application in engineering resistance to a mastrevirus. Although resistance in transgenic plants could not be tested during the course of this study, results presented here have nevertheless provided some preliminary information on gene silencing of a mastrevirus and may be an effective method towards the management of MSV by genetic engineering.

Chapter 6

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Currently, there is a global food crisis that is being fueled by increasing food prices. The bulk of this crisis is mainly felt by millions of the world's poor who have become food insecure and faced with hunger. According to the International Maize and Wheat Improvement center (CIMMYT), two of the world's important staples, maize and wheat, are in short supply, partially due to increased demands from China and India. Whilst several factors are responsible for the food problem, it is clear that increasing food supplies and addressing factors that contribute to yield loss of important staples is essential and part of a holistic approach to alleviating the crisis. Proponents of the African green revolution argue that both existing and new technologies are key to achieving such a revolution. Therefore, increasing yields of main staples such as maize, for instance by controlling diseases that cause yield loss to the crop, is a step towards achieving this goal. MSD is one of the challenges to African maize breeders. Despite research efforts that have provided MSD-resistant varieties now in existence for many years, this disease remains widespread in many African maize-growing areas. Broadening the options available for the management of MSD such as the use of transgenic resistance will contribute to reducing the effects of MSD on crop yields. Already, transgenic maize resistant to MSV has been developed (Shepherd et al. 2007b) and preliminary results are promising.

The search for novel sources of resistance, however, is an on-going area of research. To aid all these efforts, continued monitoring to provide accurate information on the MSD epidemiology and the genetic characteristics of the causal virus, MSV, is important. Generation of this information requires large-scale field samplings, often in remote geographical locations. This means a convenient method of sample collection, storage as well as a simple, fast and efficient method of cloning full MSV genomes is essential.

To address some of these gaps, the studies described in this thesis set out firstly to assess the epidemiology of MSD in Uganda; secondly to evaluate a rapid sample collection, storage and processing technology; thirdly to describe the genetic characteristics of Ugandan MSVs and fourthly to assess the efficacy of gene silencing as a MSD resistance strategy. These objectives were addressed in Chapters 2, 3, 4 and 5 respectively.

MSD occurs in all maize-growing areas in sub-Saharan Africa. Chapter 2 demonstrated that MSD is widespread in sampled regions in Uganda. Furthermore, presence of MSD-resistant varieties in farmers' fields remains low. In addition, farmers do not seem to be aware of the varieties they are growing, a strong indicator that they may still be using local susceptible genotypes in preference to resistant ones. Based on this, it is evident that existing resistant varieties need to be disseminated to farmers. These findings also highlight the need to collect information on farmers' perspectives of the resistant genotypes in use and ascertain the factors underlying their low adoption. Re-assessing the true resistance levels of so-called resistant genotypes currently in use may provide useful information. This is because farmer practices such as selection of seed for the next season's planting, especially for open pollinated varieties, can contribute to loss of resistance in the genotypes. To establish resistance levels in these genotypes being grown by farmers in future studies, seed samples should be collected from farmers and subjected to controlled resistance challenges using already existing infectious clones and agro-infectious clones constructed in Chapter 2 of this study. Based on the diversity study of Ugandan virus isolates whose sampling was detailed in Chapter 2, agroinfectious clones of the major haplotypes of Ugandan MSVs were constructed in this study. Tests with three differentially resistant maize genotypes showed that Ugandan MSVs cause severe disease symptoms even in commercially available resistant genotypes. These agroinfectious clones will be available to maize breeders for use in controlled challenges during breeding for MSV-resistant genotypes. This is the most recent and comprehensive survey of MSD in Uganda by far, documenting disease levels, use of resistant genotypes and farmer practices.

Recombination was shown to be rampant in Ugandan MSVs. Given that recombination has the potential to sometimes create fitter and more virulent viruses, regular monitoring of virus diversity is important. To aid this, a simple, time-saving and convenient method of sample collection and virus DNA isolation is valuable. In Chapter 3, a Whatman paper-based technique and an elegant DNA amplification method were combined to sample and isolate virus DNA, respectively. Extraction efficiencies of virus DNA from FTA Classic Cards were comparable to conventional methods such as CTAB. Aside from the fact that FTA cards are convenient for collection and storage of samples from remote geographical locations, it also facilitates movement of samples without quarantine restrictions that are imposed on the movement of infected leaf material. A DNA polymerase from Phi29 bacteriophage has previously been used to amplify geminivirus DNA from plant samples (Inoue-Nagata et al. 2004). In this study, Phi29 DNA polymerase was used to amplify Ugandan isolates from infected maize DNA extracted from FTA cards, which were subsequently cloned, sequenced and used for a large diversity study. This resulted in the addition of sequences of 68 full-length MSV genomes to the public database. The fidelity of Phi29 DNA polymerase was also assessed. This is the first report of the successful large-scale application of FTA cards and Phi29 DNA polymerase for MSV sampling and isolation. This efficient method of cloning complete geminivirus genomes should greatly simplify geminivirus diversity studies that involve handling large numbers of samples by reducing DNA manipulation steps and shortening the time taken to sample, isolate and clone virus isolates.

MSV is one of the most studied mastreviruses. Although the genetic characteristics of MSVs have been well documented, much of the available information is for isolates from Kenya and southern Africa. It is only recently that MSV has been sampled extensively from other maize-growing areas in Africa (Varsani et al. 2008; D.P. Martin et al., unpublished). By describing the genetic characteristics of Ugandan isolates in Chapter 4, MSV diversity from a second East African country has been added to existing data on African streak viruses. As with the southern African isolates (Martin et al. 2001), recombination is noticeably widespread in Ugandan isolates. The recombination events detected involved both inter- and intra-MSV-A subtype recombination, indicating that recombination has played a major role in the diversity of Ugandan MSVs. Of eight MSV-A_1 haplotypes identified in Uganda, the most prevalent and widely distributed haplotype, MSV-A_1UgIII, was a recombinant. Generally, Ugandan isolates cause moderate to severe MSD symptoms as shown in Chapter 2. Furthermore, all isolates were found to belong to the maize-infecting MSV-A strain that has recently been shown to have resulted from a recombination event between two grass-adapted viruses (Varsani et al. 2008). Findings here that Ugandan MSVs all belong to the MSV-A1 subtype reiterate observations made by Martin et al. (2001) demonstrating geographical localizations of subtypes. This contributes significantly to the overall effort to map the genetic variants of MSV in maize-growing areas. The fact that all sampled Ugandan MSVs belong to the widely distributed MSV-A_1 and that more than half the sampled infections are by a single widely distributed haplotype has implications for disease control and should greatly ease the work of maize breeders and epidemiologists. The demonstration in Chapter 2, that a resistant genotype probably bred for resistance to southern African isolates shows some level of resistance to Ugandan isolates provides a good starting point for resistance breeding.

The search for novel sources of resistance will continue as long as plant pathogens remain constraints to crop production. In recent years, RNA silencing has taken the center stage of disease resistance research. Accordingly, the feasibility of using RNA silencing as a resistance strategy against MSV was investigated in Chapter 5. Here evidence was presented that virus replication was abolished or greatly reduced by a repΔI^{678} hairpin construct not only for the cognate pKom602, from which the rep variant was derived, but also for two southern African isolates, one Réunion isolate and three Ugandan isolates. These isolates represent the largest diversity (with a nucleotide divergence of 4.43%) of maize-infecting MSVs. Furthermore, the three Ugandan isolates tested, representing haplotypes MSV-A_1UgIII, MSV-A_1UgV, and MSV-A_1UgVI, cause severe disease symptoms as determined in Chapter 2 and are the most frequently occurring haplotypes in Uganda as shown in Chapter 4. Of particular interest is the effectiveness of the repΔI^{678} hairpin against an isolate belonging to haplotype MSV-A_1UgIII, which accounted for 60% of all MSV infections sampled throughout Uganda.

One of the set-backs to the development of resistant genotypes is the possible break down of resistance in the presence of more severe virus isolates. The observation that the repΔI^{678} hairpin is effective against virus isolates from different geographical regions clearly indicates that it can potentially provide broad-based resistance that may be effective in different maize-growing areas. Whether durable resistance to MSV can be developed using RNA silencing can only be investigated once the mechanism has been tested in maize plants. Whilst these may only be preliminary investigations, they indicate that the use of RNA silencing to develop MSV resistant genotypes has potential. There were setbacks with the recovery of transgenic plants transformed with the repΔI^{678} hairpin construct. These setbacks were attributed to the possible detrimental effect of the construct to the plant's development or the possibility that essential genes in the plant genome were targeted. For future experiments REP sequences with no homology to plant genome, tissue-specific promoters or virus inducible promoters should be used to overcome this problem.

Clearly, controlling MSD still remains a challenge to Ugandan breeders. Both FTA cards and Phi 29 DNA polymerase have greatly simplified sampling and cloning of MSV full genomes. This should see more MSV sequences being added to the database. More importantly, techniques like this will aid efforts to monitor the emergence of any new potentially more virulent virus types. Using transgenic approaches for crop improvement remains a controversial issue in the global arena. However, the contribution of the technology should not be ignored and the search for novel sources of resistance such as RNA silencing should continue.

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Appendices

Appendix A

Table A.1: Genbank accession numbers for sequenced full length Ugandan MSV genomes

Sequence name	Accession number	Symptom characteristic
CTAB-extracted samples		
MSV-A[UG:Wak1:05]	EF547063	3.0
MSV-A[UG:Wak4:05]	EF547064	2.0
MSV-A[UG:Mpi8:05]	EF547065	4.5
MSV-A[UG:Mpi11:05]	EF547066	4.0
MSV-A[UG:Mpi14:05]	EF547067	2.5
MSV-A[UG:Mask18:05]	EF547068	3.5
MSV-A[UG:Mask21:05]	EF547069	4.5
MSV-A[UG:Mask23:05]	EF547070	3.0
MSV-A[UG:Mask25:05]	EF547071	2.5
MSV-A[UG:Mask26:05]	EF547072	2.0
MSV-A[UG:Mba38:05]	EF547073	4.0
MSV-A[UG:Mba41:05]	EF547074	3.5
MSV-A[UG:Bush53:05]	EF547075	3.0
MSV-A[UG:Kas63:05]	EF547076	5.0
MSV-A[UG:Kas70:05]	EF547077	2.0
MSV-A[UG:Kas71:05]	EF547078	3.0

Continued. . .

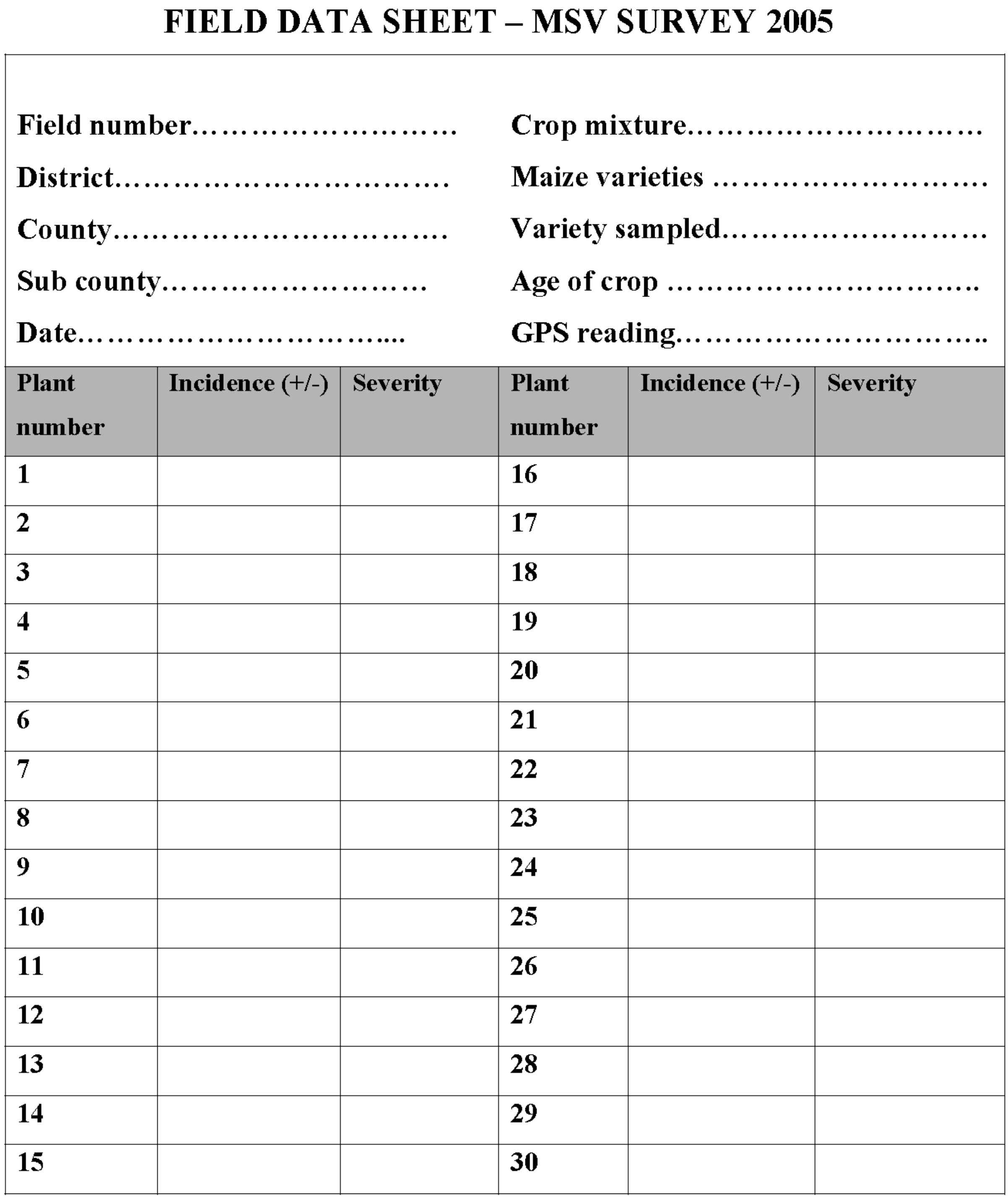
Table A.1 – Continued

Sequence name	Accession number	Symptom severity
MSV-A[UG:Kas75:05]	EF547079	2.5
MSV-A[UG:Kas76:05]	EF547080	3.0
MSV-A[UG:Kab82:05]	EF547081	1.5
MSV-A[UG:Mub89:05]	EF547082	2.5
MSV-A[UG:Mub94:05]	EF547083	3.5
MSV-A[UG:Luw103:05]	EF547084	2.0
MSV-A[UG:Luw107:05]	EF547085	3.0
MSV-A[UG:Luw110:05]	EF547087	4.0
MSV-A[UG:Nak111:05]	EF547086	2.0
MSV-A[UG:Nak118:05]	EF547088	3.0
MSV-A[UG:Nak119:05]	EF547089	2.5
MSV-A[UG:Nak120:05]	EF547090	3.0
MSV-A[UG:Nak123:05]	EF547091	3.0
MSV-A[UG:Nak125:05]	EF547092	3.0
MSV-A[UG:Nak129:05]	EF547093	2.5
MSV-A[UG:Masin138:05]	EF547094	3.0
MSV-A[UG:Masin139:05]	EF547095	3.5
MSV-A[UG:Masin144:05]	EF547097	3.0
MSV-A[UG:Masin149:05]	EF547098	3.0
MSV-A[UG:Hoi154:05]	EF547099	4.0
MSV-A[UG:Hoi156:05]	EF547100	4.0
MSV-A[UG:Hoi158:05]	EF547101	3.5
MSV-A[UG:Hoi159:05]	EF547102	4.0
MSV-A[UG:Hoi165:05]	EF547103	2.0
MSV-A[UG:Hoi167:05]	EF547104	3.0
MSV-A[UG:Hoi170:05]	EF547105	3.0
MSV-A[UG:Kib179:05]	EF547096	3.5

Continued. . .

Table A.1 – Continued

Sequence name	Accession number	Symptom severity
MSV-A[UG:Kib182:05]	EF547106	3.5
MSV-A[UG:Kib188:05]	EF547107	3.0
MSV-A[UG:Luw192:05]	EF547108	2.5
MSV-A[UG:Luw196:05]	EF547109	4.0
MSV-A[UG:Muk203:05]	EF547110	3.0
MSV-A[UG:Jin219:05]	EF547111	4.0
MSV-A[UG:Iga224:05]	EF547112	3.0
MSV-A[UG:Iga231:05]	EF547113	4.5
MSV-A[UG:Iga235:05]	EF547114	3.5
MSV-A[UG:Iga243:05]	EF547116	2.5
MSV-A[UG:Iga244:05]	EF547115	3.0
MSV-A[UG:Bug245:05]	EF547117	3.0
MSV-A[UG:Bug248:05]	EF547118	4.0
MSV-A[UG:Bus245:05]	EF547119	4.0
MSV-A[UG:Tor271:05]	EF547120	3.0
MSV-A[UG:Kap289:05]	EF547121	3.5
MSV-A[UG:Kap292:05]	EF547122	4.0
MSV-A[UG:Mbal304:05]	EF547123	3.5
MSV-A[UG:Mbal308:05]	EF547124	3.0
FTA-extracted samples		
MSV-A[UG:Mba27:05]	EF015781	4.5
MSV-A[UG:Kas42:05]	EF015780	3.5
MSV-A[UG:Kas43:05]	EF015779	2.5
MSV-A[UG:Kab48:05]	EF015782	1.5
MSV-A[UG:Mub49:05]	EF015783	1.5
MSV-A[UG:Wak56:05]	EF015778	2.5



FIELD DATA SHEET – MSV SURVEY 2005

Field number………………………

District…………………………….

County…………………………….

Sub county………………………

Date…………………………....

Crop mixture…………………………

Maize varieties ……………………….

Variety sampled…………………….…

Age of crop …………………………..

GPS reading…………………………..

Plant number	Incidence (+/-)	Severity	Plant number	Incidence (+/-)	Severity
1			16		
2			17		
3			18		
4			19		
5			20		
6			21		
7			22		
8			23		
9			24		
10			25		
11			26		
12			27		
13			28		
14			29		
15			30		

Figure A.1: Survey data sheet

Table A.2: Number of plants used for symptom analysis

Haplotype	Isolate	Golden Bantam	Sweetcorn	Pan77
MSV-A_1UgI	UMub94	19	18	23
MSV-A_1UgII	UKib188	13	23	21
MSV-A_1UgII	ULuw110	17	15	26
MSV-A_1UgIII	UBug245	23	20	15
MSV-A_1UgIII	UKib179	26	28	21
MSV-A_1UgIII	UKas75	19	22	14
MSV-A_1UgV	UMpi11	17	28	24
MSV-A_1UgV	ULuw107	12	24	12
MSV-A_1UgV	UHoi154	19	30	14
MSV-A_1UgVI	UBush53	17	22	18
MSV-A_1UgVI	UKap292	20	17	13
MSV-A_1UgVI	UKab82	23	26	18
MSV-A_1UgVII	UHoi159	1	1	0
MSV-A_1UgVII	UIga235	0	0	0
MSV-A_1UgVIII	UWak4	6	4	0

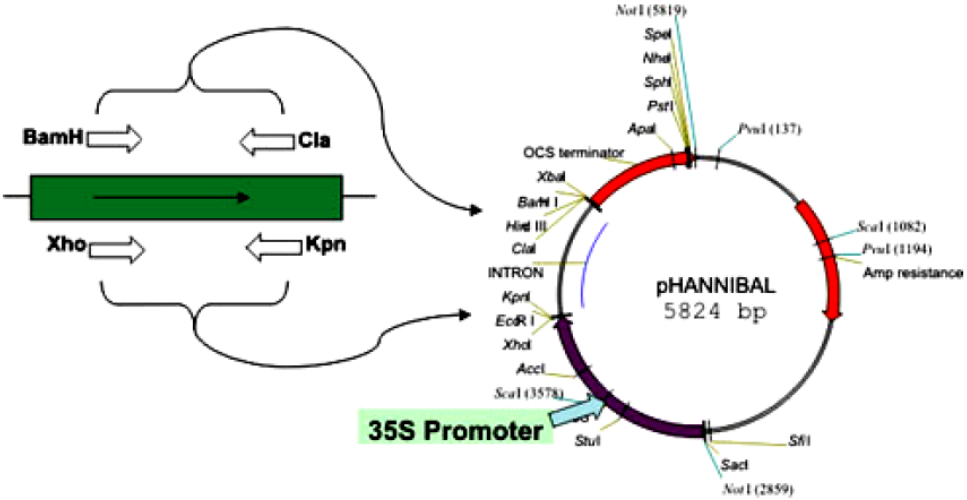


Figure A.2: Vector map showing positions of insertion of the gene of interest by making use of restriction enzymes *Bam*HI, *Hind*III, *Cla*I, to insert the antisense arm of the gene and enzymes *Xho*I, *Eco*RI, *Kpn*I to insert the sense arm of the gene (Helliwell & Waterhouse 2003)

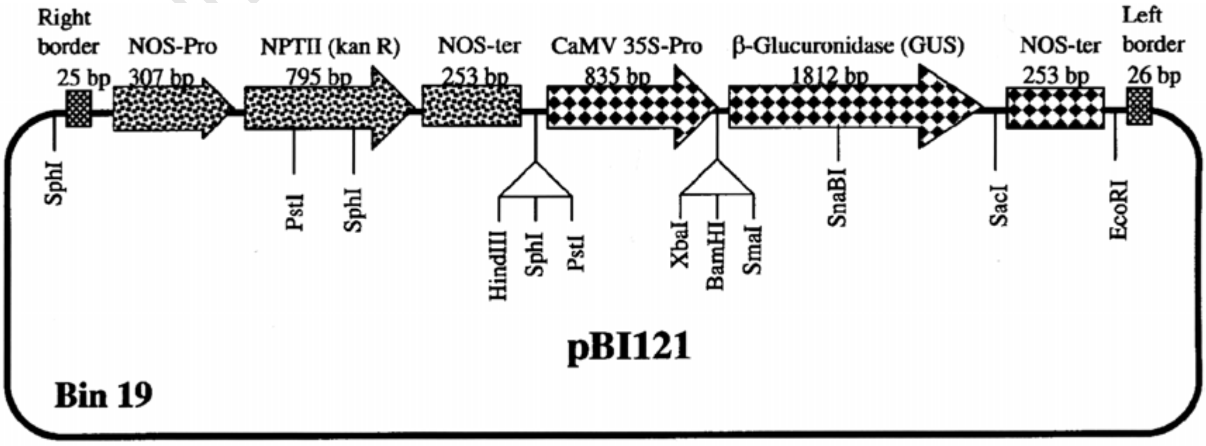


Figure A.3: Vector map of pBI121