STUDIES ON TRANSGENIC TOBACCO PLANTS CONTAINING GEMINIVIRAL DNA

M I C H E L L E B A B A Y A

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ABSTRACT

The ability to transform plant genomes with foreign genetic material by means of the *Agrobacterium tumefaciens* Ti plasmid system has made it possible to test whether plant resistance to viruses can be increased by incorporating viral nucleotide sequences into the nuclear genome of the host. As part of a model system designed to explore the feasibility of making cereals resistant to maize streak virus (MSV), tobacco plants were to be transformed with one or more genome-equivalents (multimers) of Port Elizabeth (PE) and Nigerian (N) isolates of (MSV), and analysed for evidence of expression of the viral coat protein. The constructs were mobilized into *A. tumefaciens* [C58C1Rif (pGV2260)] and introduced into tobacco by co-cultivation with leaf discs. Putatively transgenic kanamycin-resistant calli were tested for the presence of the plasmid vector marker enzyme, neomycin phosphotransferase (NPT-II). Southern hybridization analysis was used to establish the integration of MSV DNA into tobacco. Expression of the MSV coat protein was tested for by Western blotting and enzyme-linked immunosorbent assay (ELISA) techniques. Transformed calli all expressed NPT-II activity and contained integrated MSV DNA, but no coat protein could be detected serologically.

A minor part of the project involved the study of a new strain of MSV isolated from the Koedoeskop area in South Africa which produced severe disease symptoms in maize. The genomic DNA was purified, the genome cloned into pUC19 and mapped. The restriction enzyme cleavage map of the new isolate, MSV-KoeI, was compared to those of 8 other MSV isolates. Similarities and differences between these maps are discussed.

ABBREVIATIONS

Amp	ampicillin
BAP	benzylaminopurine
bp	base pair(s)
CaMV	cauliflower mosaic virus
Cb	carbenicillin
cpm	counts per minute
CsCl	caesium chloride
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
h	hours(s)
kb	kilobase(s)
kD	kilodalton(s)
Km	kanamycin
LA	Luria agar
LB	Luria broth
min	minute(s)

nos	nopaline synthase
NPT-II	neomycin phosphotransferase II
ORF	Open reading frame
p	plasmid
P35S	cauliflower mosaic virus 35S promoter
^{r}(Superscript)	resistance
Rif	rifampicin
RNA	ribonucleic acid
s	second(s)
^{s}(Superscript)	sensitivity
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
ss	single-stranded
TLC	thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
u	units of enzyme activity
v/v	volume/volume
v/w	volume/weight
λ	Lambda phage

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GENERAL INTRODUCTION

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GENERAL INTRODUCTION

The term "transgenic plants" refers to those that express a foreign gene as a result of recombinant DNA technology. At the beginning of the 1980's there were no substantiated reports of the expression of a foreign gene in a genetically engineered plant. Recently however, the situation has changed dramatically and the development of numerous vectors has facilitated detailed studies on gene expression and regulation in plants [16]. Plants have the advantage of totipotency, ie. the ability of undifferentiated somatic tissue to redifferentiate into mature plants *in vitro*. This feature provides the potential to study the expression of foreign genes in transgenic plants regenerated from genetically transformed plant cells. It also supplies the plant breeder with new techniques with which to engineer genetic improvements of agriculturally important crops, which complement rather than substitute for the more classical methods of plant breeding [86].

1.1 REQUIREMENTS FOR PLANT GENETIC ENGINEERING

The first requirement for plant genetic engineering is a system to deliver the foreign DNA to plant tissue. Delivery systems include vectors based on the tumour-inducing (Ti) plasmid of *Agrobacterium tumefaciens*, vectors based on plant viruses such as cauliflower mosaic virus (CaMV) and geminiviruses [9] and direct gene transfer. Vectors based on plant pathogens must be attenuated so that they remain capable of infection, but cause no symptoms and do not

affect plant yield. Most methods for direct gene transfer involve transformation of protoplasts and therefore these can only be applied to plant species from which viable protoplasts can be made and regenerated. However, the number of plant species which can be regenerated from protoplasts to cell and/or cell to plant is increasing.

Newly introduced genes, including a selectable marker, must be expressed in transgenic plants. They must therefore include the appropriate regulatory DNA sequences for recognition by the plant cell transcriptional and translational machinery.

A further requirement is for the foreign DNA to be inherited and reproducibly expressed in succeeding generations. Although molecular biologists are often interested in gene expression in cells, in order to be agronomically viable, applications must allow for the foreign gene(s) to be stably maintained through meiosis, so that the new gene(s) can be incorporated into plant breeding programmes.

1.1.1. Vectors based on the Ti plasmid of *Agrobacterium tumefaciens*

A. tumefaciens is a Gram negative soil bacterium that acts as a natural "genetic engineer" because during infection of a plant, it transfers a portion of the Ti plasmid DNA (known as T-DNA) to plant cells. In the infection process, *A. tumefaciens* moves towards wounded plants cells in a positive chemotatic response to attractant molecules such as acetosyringone, a signal molecule exuded from the plant tissue [37b]. Two chromosomal genes, *chv*A and *chv*B,

constitutively express gene products required for attachment of Agrobacteria to susceptible plant tissue. Acetosyringone is recognized by the chemosensory *vir*A protein located in the inner membrane of the Agrobacterial cell. *Vir*A activates the *vir*G protein which in turn activates transcription of six operons leading to translation of gene products encoded by these inducible loci. A site-specific endonuclease encoded by *vir*D nicks each of the border repeats 3 bases in from the 3′ end of the T-DNA strand. Rolling circle DNA replication is initiated from the right border nick, and in conjunction with a helicase activity extrudes the T-strand in the 5′ to 3′ direction. The left border nick signals termination of transfer.

The process is remarkably similar to bacterial plasmid conjugation. Here one strand of a double-stranded plasmid molecule is unidirectionally transferred from donor to recipient bacterium. This transfer also requires cell-cell contact and occurs through an unidentified channel between the cells. During transfer, one strand of the donor molecule is nicked by a plasmid-encoded site-specific endonuclease (analogous to *VirD*) within a *cis*-essential sequence, the origin of transfer, *ori* T. The nicked strand is unwound, also in a 5′ to 3′ direction by a helicase activity and transferred to the recipient cell as a DNA-protein complex containing a protein covalently bound to the 5′ end, single-stranded DNA binding proteins and often a primase. Transfer is associated with RNA-primed conjugal DNA synthesis of a replacement strand in the donor, and a complementary strand in the recipient. It is likely that *vir* encoded proteins also bind to the T-strand during

transfer and play a role in T-strand transfer to the plant cell [86].

The T-DNA is subsequently integrated at random sites into the plant nuclear genome where it is expressed. Genes within the integrated T-DNA encode novel enzymes resulting in the synthesis of plant hormones at elevated levels. The consequence is a neoplastic transformation of the infected tissue which proliferates to produce a tumour or gall known as a crown gall, leading to crown gall disease. Transcripts of the integrated DNA are typical eukaryotic mRNA's, synthesised by RNA Polymerase II and possessing polyadenylated 3' ends. DNA sequencing has shown that the genes have eukaryotic control signals and thus, although the T-DNA is found in bacteria, it is designed to function in plant cells [48].

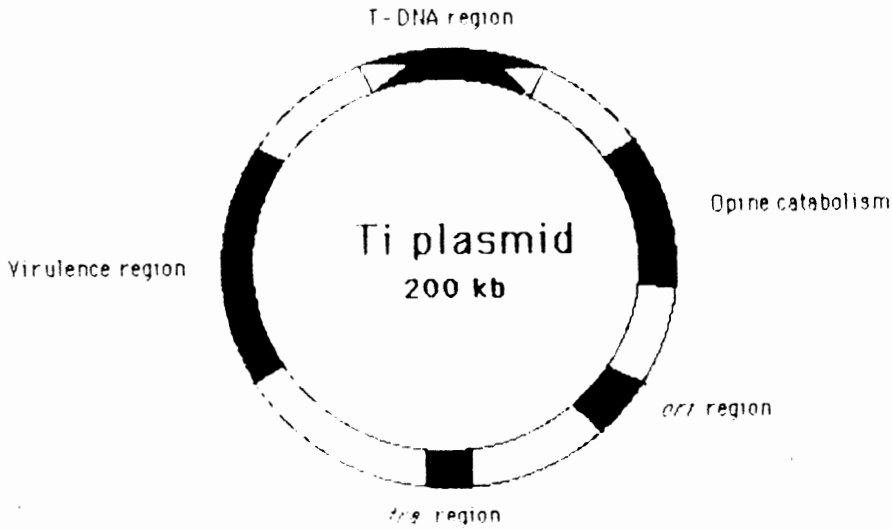

FIGURE 1:
SCHEMATIC REPRESENTATION OF THE FUNCTIONAL ORGANIZATION OF A Ti PLASMID
In the plant cell, the T-DNA expresses genes involved in the synthesis of phytohormones (cytokinin, auxin) and of opines. The 25 bp direct repeats that flank the T-DNA are marked as white triangles. Regions outside the T-DNA include those governing the catabolism of opines and genes encoding functions responsible for the conjugal transfer of the Ti plasmid [Adaptation from Reference 85].

Transposon insertion and deletion analysis studies have identified functional regions within the T-DNA. Several T-DNA genes are involved in the tumorous phenotype, and one or more in opine synthesis. The remainder of the Ti plasmid (which is not integrated into the plant genome) contains a large region that is required for gall-forming ability (virulence), as well as regions affecting host range and opine catabolism. Ti plasmid based vectors have the advantage of a broad-host-range - they are able to infect most dicotyledonous (dicot) plants and recently, within the last few years, they have been shown to infect monocotyledonous (monocot) plants as well [64].

The T-DNA is flanked by 25 bp near perfect direct repeats. The right border repeat is the only sequence absolutely required in *cis* for T-DNA transfer [12], [123]. Although the left repeat is not absolutely required for T-DNA transfer [84], it usually acts as a boundary beyond which DNA sequences are not transferred [76]. Therefore, intervening T-DNA including *onc* genes can be deleted resulting in what is termed a disarmed Ti plasmid [27]. Large pieces of DNA (up to 50kb) can be inserted between the borders and transferred in a highly efficient manner. Because gall formation cannot be used to select transformants, the vectors usually have the oncogenic genes replaced by a selectable marker.

Owing to the large size of Ti plasmids, cloning is not performed directly into them. Cloning and DNA modification

steps are done in *Escherichia coli* first and then the plasmid containing the gene construct of interest is transferred by conjugation into *A. tumefaciens*.

There are two types of Ti vector systems, cointegrate and binary. The cointegrate vector system uses a disarmed Ti plasmid in which the border sequences flank a copy of the *E. coli* plasmid vector pBR322. Any DNA sequences suitably cloned into a pBR322-like plasmid (the shuttle vector) can then be cointegrated into this Ti plasmid, between the border sequences, by homologous recombination. The shuttle vector can be mobilized from *E. coli* to *A. tumefaciens* by conjugation using a *trans*-complementing conjugative plasmid encoding *tra* genes [85]. This is depicted in Figure 2 below.

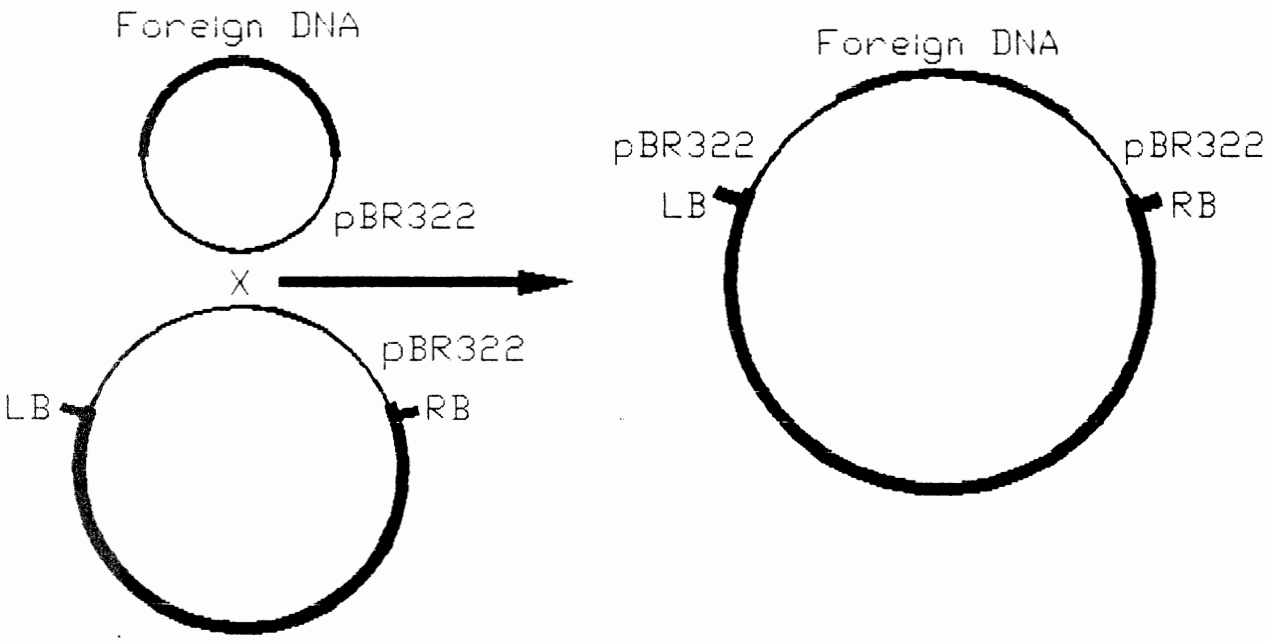

FIGURE 2:

THE COINTEGRATE VECTOR SYSTEM
Homologous recombination of pBR322 sequences allows cointegration of pBR322 plus foreign DNA into the Ti plasmid.
LB: left border
RB: right border [85]

The binary vector system uses two plasmids and makes use of the *trans*-acting properties of the *vir* regulon. Here the vector is able to replicate in *E. coli* and *A. tumefaciens* and contains the T-DNA border sequences flanking a selectable marker and suitable cloning sites. This plasmid is transferred from *E. coli* to an *A. tumefaciens* strain containing a second helper plasmid, a Ti plasmid that retains *vir* but is deleted for the T-DNA region. Complementation in *trans* then allows transfer of the DNA between the border sequences of the vector plasmid to the plant cell nuclear genome [6], [62].

A number of methods exist for the transformation of plant cells by *A. tumefaciens*. These include transformation of leaf-disks [66], co-cultivation of agrobacteria with plant cells regenerating from protoplasts [2], and in the case of viral inserts, the wounding and inoculation of whole plants [54].

Transformation and foreign gene expression using the *Agrobacterium* Ti plasmid-mediated delivery system have been reported for a wide range of plants including tobacco [25], Douglas fir [21], sunflower [38], soybean [39], tomato [42], maize [53], petunia [75], rape [104], white clover [124], carrot [49], asparagus [60], and loblolly pine [109].

1.1.2 Plant viruses as vectors

It is possible, by introducing a foreign gene into a viral genome, to enable the gene to enter plant cells as a result of the viral infection process. An advantage of this system is the fact that viruses are able to move from cell to cell

within a plant, systemically infecting the whole plant. New genes transferred to existing plants would not require the regeneration of plant cells from a single transformed cell. Moreover, as viruses replicate to a high copy number within a cell, even low levels of transcription may result in detectable foreign gene products [86].

Before genes of agronomic importance can be introduced into plants, symptomless virus strains must be developed and economic methods of introducing the vector to a crop on a large scale must be found. A practise already used is the deliberate inoculation of certain crops with a mild strain of a virus to cross-protect against severe losses caused by more severe strains: cross protection involves the protection of plants by superinfection of a related strain of the same virus or similar viruses [48], [128].

Although most plant viruses are RNA viruses, experimental work so far has mainly involved the DNA virus cauliflower mosaic virus (CaMV) [63].

The system involves inserting bacterial DNA into a nonessential site in the CaMV genome cloned into an *E. coli* plasmid. Plasmid DNA is then purified and cut with restriction endonucleases to excise the CaMV molecule from the vector. This linear DNA is then inoculated onto plants and induces typical CaMV symptoms within a couple of weeks. Disadvantages include packaging restraints limiting the amount of foreign DNA that can be incorporated within the viral capsid, host-range restrictions and the lack of transfer to sexual offspring.

1.1.3 Direct gene transfer into plants

It has recently also become possible to insert DNA directly into plant cells without the use of a biological vector: DNA can be taken up directly by cells and integrated into the plant genome. Direct methods (except for biolistics) involve transformation of protoplasts, since plants have a rigid cell wall which is an effective barrier to these methods. After treatment with foreign DNA, the protoplasts can be cultured *in vitro*. A cell wall usually regenerates within a day or two, and for a growing number of plant species, this single cell will grow and divide to form a callus, which can eventually be regenerated into a whole plant [100].

The uptake of DNA is thought to occur by endocytosis and can be enhanced by treatment of the protoplasts with agents such as polycations, polyethylene glycol (PEG), polyvinyl alcohol, calcium ions, high pH and osmotic shock. The mechanism of enhancement by these treatments is not clear, but may involve neutralizing electrostatic interactions, changing the fluidity of the membrane, protecting the DNA from nucleases, and/or stimulation of endocytosis [48].

Another "direct" technique is electroporation: this is the use of electrical pulses of high field strength to reversibly permeabilize cell membranes, which facilitates uptake of large molecules, including DNA. This technique has been used with both monocotyledons and dicotyledons. The advantages of this method include convenience, low cell toxicity, and high efficiency [45], [110], [111]. Of the

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DNA introduced into cells by direct gene transfer, not all becomes stably incorporated into the host's chromosome. If non-replicating, this extrachromosomal DNA is lost over a one to two week period as a result of dilution by cell division and susceptibility to intracellular degradation. During its transient existence in the cell, the extrachromosomal DNA is known to be transcriptionally active because typically 1 to 10% of the cells express the introduced DNA but only 0.01 to 0.1% of the cells stably integrate and maintain the introduced gene [44a]. Gene-transfer efficiency increases with increased DNA concentration and is affected by the amplitude and duration of the electric pulse as well as by the composition of the electroporation medium [45]. Electroporation is often used to monitor transient gene expression in protoplasts, which appears to be a useful indicator of gene expression in transformed callus [45a].

Another method of enhancing the uptake of nucleic acids is the use of liposomes. In a recent study an *E. coli* plasmid carrying a kanamycin resistance gene was encapsulated into negatively charged liposomes which were included to fuse with tobacco mesophyll protoplasts by PEG treatment, resulting in kanamycin-resistant plants [29].

Microinjection of DNA involves the introduction of DNA directly into the nucleus and has been successful in a number of plant and mammalian systems. Microinjection of plant cells - with and without cell walls - is being investigated; success in this direction would bypass the need for regeneration of plants from cultured tissues of

vegetative cells, and transformation could be performed at the level of fertilized embryo or even in pollen or ovum cells. Direct injection with a hypodermic needle of DNA coding for NPT-II into a developing inflorescence of rye, led to the uptake of DNA in the genome of developing germ cells, and expression of the kanamycin-resistance gene [28]. Integration of foreign DNA in callus produced from tobacco mesophyll protoplasts that had been microinjected with a bacterial plasmid has also been shown [19].

Biolistics is a new technique, first described by Klein *et al* [79] in which nucleic acids can be delivered into plant cells using high-velocity microprojectiles. After being accelerated, small tungsten particles (microprojectiles) pierce cell walls and membranes and enter intact plant cells without killing them. Microprojectiles were used to carry RNA or DNA into epidermal tissue of onion and these molecules were subsequently expressed genetically. Regeneration of transgenic soya bean following biolistic treatment of shoot meristems has also been achieved [89].

Because direct gene transfer involves the transformation of protoplasts (except in the case of biolistics), these methods can only be applied to plant species from which viable protoplasts can be made and, if required, regenerated into plants via callus tissue. The entire process can take up to 6 months from protoplasts to plant and plants derived from protoplasts can be subject to morphological and reproductive abnormalities. Screening is required to establish which transformants have the desired DNA integrated. However, direct gene transfer methods are

clearly advantageous for transient expression studies where stable transfer and integration of the DNA are not required.

1.1.4. Expression of foreign genes in plants

The transfer of genetic information by plant vectors can be considered successful only if the newly transferred gene is expressed in the new host in a manner that affects its phenotype. Although the genetic code is universal (except for eukaryotic mitochondrial DNA), control signals affecting gene expression are not : thus a protein coding region from any source will be have to be joined to appropriate plant controlling sequences, which will direct expression of RNA from the DNA and of protein from the RNA. Studies on vector construction have identified strong promoters that control the function of certain genes. Polyadenylation termination signals are also included for control.

The 35S promoter of CaMV is often used as it is known to function in plants at high efficiency. The nopaline synthase (*nos*) promoter from the T-DNA of the Ti plasmid is also used. By combining such promoters upstream of the coding sequences of bacterial antibiotic resitant genes, it is possible to produce chimeric genes that function in plants.

One widely used system is the neomycin phosphotransferase type II (NPT-II) gene from the transposon Tn5 under the control of the CaMV 35S or *nos* promoter. Expression of this gene in transformed plant tissue confers resistance to the aminoglycoside antibiotics kanamycin, neomycin, gentamycin and G418. The NPT-II enzyme reaction involves tranfer of

the γ-phosphate group of ATP to the antibiotic molecule. This addition detoxifies the antibiotic by preventing its interaction with the target site - the ribosome [16], [22], [86].

Analysis of gene expression in plants has also been monitored by using reporter genes. These are genes which are well characterized, both genetically and biochemically, and have a coding region which can be easily fused to the regulatory sequences of other genes. The enzymatic activity of the most reporter genes is not normally present in the host plant. Examples of reporter genes used in transformed plants include *E. coli* β-galactosidase [58], the firefly luciferase gene [98], β-glucuronidase (Gus) from *E. coli* [74] [77], and the chloramphenicol acetyl transferase (CAT) gene of Tn9 [26].

1.2 APPLICATIONS OF GENE TRANSFER INTO PLANTS

At present genetic engineering of plants is restricted to introducing single genes which can generate recognizeable characteristics. A number of applications will be discussed.

1.2.1 Virus resistance

The phenomenon of viral cross protection - infecting a plant with a mild non-pathogenic strain of a virus to protect it from superinfection by a more virulent strain has long been used as a biological control method [35]. However, disadvantages include a decrease in yield and a risk of a severe disease produced by an interaction between the mild strain and a second virus [3]. One way round this problem

is by the introduction of the coat protein gene (cpg) prior to challenge with virus. In a landmark study, Abel *et al* [1] introduced a cloned cDNA copy of the coat protein gene of tobacco mosaic virus (TMV) into *Nicotiana tabacum* cv. Xanthi cells on a disarmed Ti plasmid : seedlings that expressed the coat protein genes were delayed in symptom development relative to non-transgenics, or failed to be susceptible at all to TMV infection.

In a similar study [87], it was shown that the expression of alfalfa mosaic virus (AMV) coat protein gene in transgenic *Nicotiana tabacum* plants inhibited local infection by two AMV strains and prevented or delayed the development of systemic virus symptoms.

The expression of potato virus X (PVX) coat protein or its antisense RNA in transgenic tobacco has also been shown to significantly protect plants from PVX infection [59]. This was indicated by reduced lesion numbers on inoculated leaves, delay or absence of systemic symptom development and reduction in virus accumulation in both inoculated and systemic leaves. Thus, genomic insertion of virus coat protein genes seem to have great potential for the production of virus-resistant plants.

The reduction or absence of viral symptoms may also be associated with the presence of a satellite RNA. Satellite RNA molecules are generally small and are dependent on a helper virus for replication. The satellite of cucumber mosaic virus (CMV) is only replicated in CMV-infected cells and attenuates the symptoms induced by CMV. Tobacco plants

transformed with a DNA copy of this satellite RNA produced large amounts of the satellite RNA on infection with a satellite-free inoculum of CMV and, as a result, the satellite became transmissible as a component of the virus culture. CMV replication was greatly decreased and symptom development largely suppressed in transgenic plants and their sexual progeny [55], [127].

The features of protection in transgenic plants by satellite RNA are different from coat protein protection as satellite protection is observed irrespective of the concentration of the inoculum or the level of satellite transcripts in the transgenic plant. Also, protection by satellite RNA is observed with viral inoculation in the form of either virus or RNA [4].

Another method proposed for viral resistance is the use of antisense RNA. The mechanism relies on the annealing of antisense RNA to viral RNA and hence preventing its expression [108]. Due to a number of problems encountered in the past this has not proved very effective. However, successful suppression of nopaline synthase (*nos*) enzymatic activity in the leaves of tobacco plants via the overproduction of RNAs complementary to the *nos* mRNA was reported [108]. Another example of the use of antisense RNA for viral protection has been reported. RNA complementary to the 3′ end of tobacco mosaic virus (TMV) RNA conferred protection against TMV infection, although at a substantially lower level than coat protein-mediated protection for this virus [102].

1.2.2 Herbicide resistance

Most herbicides do not distinguish between weeds and crops and therefore it is important to modify plants such that they become resistant to broad-spectrum herbicides to allow their selective use for crop protection. Glyphosate (N-phosphonomethyl glycine) is a broad-spectrum herbicide of widespread use in agriculture. Tolerance to glyphosate in tobacco has been achieved by expression of a *Salmonella* gene encoding herbicide resistance [17]. Targeting a herbicide-resistant enzyme from *E. coli* to chloroplasts of *Petunia hybrida* led to the incorporation of a stable, glyphosate resistant enzyme [33]. Glyphosate tolerance was also shown in tomato [43].

Alfalfa (*Medicago sativa*) suspension cell lines 20- to 100-fold more resistant than wild-type cells to the nonselective herbicide L-phosphinothricin (PPT), an amino acid analog of glutamine synthase, were observed after amplification of glutamine synthase gene resulted in an eight-fold increase in mRNA levels, and an increased enzyme synthesis sufficient to overcome the toxic effects of the inhibitor [36]. Another approach to engineering herbicide resistance in plants is by expression of a detoxifying enzyme. The *bar* gene from *Streptomyces hygroscopicus* encodes a phosphinothricin acetyltransferase (PAT) which converts PPT into the non-toxic acetylated form [117]. The *bar* gene was placed under control of the CaMV 35S promoter and transfered to tobacco, tomato and potato plants using *Agrobacterium* - mediated transformation. Transgenic plants showed complete resistance towards the herbicide due to production of an active PAT enzyme [24].

1.2.3. Pest resistance

Modern agriculture uses a wide variety of chemical insecticide to control insect damage. Biological control of insect pests is free from chemical pollution hazards and in some instances it is more target-specific [10]. *Bacillus thuringiensis*, a Gram positive bacterium produces proteins termed Bt toxins which are specifically toxic to a variety of insect species. Spore preparations of *B. thuringiensis* have been used as a biological insecticide for many years. In a recent study, modified genes have been derived from a toxin into *Nicotiana tabacum* via a Ti plasmid delivery system. Transgenic tobacco plants expressing these genes demonstrated that the plants were able to make their own insecticide which was very effective in protecting them from damage by the tobacco hornworm. This ability was inherited in a normal Mendelian manner [120]. A similar experiment, also using *Agrobacterium*-mediated transformation showed that insect tolerance could be achieved in transgenic tomato plants [44]. In this case, truncated forms of the Bt toxin gene from another variety of *B. thuringiensis* were investigated.

A novel mechanism of insect resistance was reported in which expression of a gene encoding the cowpea trypsin inhibitor in *N. tabacum* led to enhanced resistance to the tobacco species' own herbivorous insect pests, indicating that a proteinase inhibitor can by itself, significantly reduce insect attack [61].

1.2.4. Plant gene expression

Transgenic plants can be used as tools to study the molecular organization of plant genes [113]. They can also be used to study plant gene expression by transferring plant genes, or their controlling sequences, from their native environment to heterologous hosts [125]. Generally, genes transferred into plants are expressed under the control of cotransferred *cis*-acting regluation sequences. The evolutionary distance between donor and host seems to determine whether correct expression is retained. In general, dicot-dicot transfers yield successful expression of transgenes, but monocot-dicot transfers can result in either no or inappropriate expression of the transferred gene. Presumably this is because the host plant does not contain appropriate *trans*-acting factors.

1.2.5. Future prospects

Genetic engineering offers the possibility of introducing a single trait, without altering other agronomically important characters, into a well-accepted or traditional plant variety, providing for a method of "fine-tuning" specific cultivars [73]. Potentially, any gene from any source should be able to be expressed in plants provided the necessary regulatory signals are provided. Possibilities include plant vectors transferring resistance genes between plants which cannot interbreed, and using gene transfer as a method of showing which genes make a plant sensitive to a particular pathogen [48].

In the future with an increasing number of plant species being amenable to transformation as tissue culture technology develops, it should become easier to manipulate certain plants for which the technology to date is unavailable.

Successful genetic manipulation by recombinant DNA technology will depend on how readily genotype and phenotype can be related, and the ease with which the desired gene sequence can be isolated and cloned.

Most agronomically designed improvements such as increase in yield, pest and pathogen resistance, and stress tolerance involve many genes of unknown identity. The identification, isolation and characterization of genes responsible for these traits will allow the adaptation of plants by conventional breeding techniques in conjunction with genetic transformation towards an improved agricultural product.

The Gramineae, a family that includes the world's most important cutlivated crops has, in the past, evaded genetic manipulation by recombinant DNA methodology because many species are not able to be regenerated from protoplasts and therefore are unsuitable for transformation by direct DNA uptake procedures. Also, they had previously not been regarded as hosts for *Agrobacterium* although recently Ti-mediated gene transfer has been shown to occur in a number of members of this family. Despite experiments which show that *Agrobacterium* is capable of mediating DNA transfer into cereal cells [53], no transgenic cereals have been recovered from numerous attempts to treat cereal tissues with

genetically engineered *Agrobacterium*. Because the mechanism of *Agrobacterium* gene transfer requires the plant cells to elicit a "wound response", the lack of this in cereal cells has been postulated to be responsible for the incapacity for dedifferentiation of cells adjacent to damaged tissue [101]. The few proliferations-competent cells in cereal meristems are either not accessible to infection or not very competent for transformation.

Alternatives to *Agrobacterium* as a means of stable gene transfer for the genetic manipulation of ceral do not exist. Transformation of cereal protoplasts has been possible for a number of years, although the regeneration of transgenic cereals is a recent accomplishment. Regeneration of fertile maize [106], [103], [110] and rice [111] from protoplasts have been reported. Although it is the only method by which transgenic cereals have been produced, plant regeneration from cereal protoplasts is a very delicate and often unreliable process and will take some time before the expertise has bee developed for routine gene transfer into any plant.

Other approaches with the potential to produce transgenic cereals, and which depend on systematic spread of self-replicating molecules include: pollen transformation, viral vectors, microbacter, electrophoresis, macroinjection, electroporation, liposome fusion and agroinfection. Details of these methods are discussed in a recent review by Potrykus (1989) [101].

The future offers a challenge to develop a routine gene transfer method which achieves integrative transformation with cereals and other important crops. Biolistics and microinjection into zygotic proembryos appear to be the most promising approaches [101].

1.3. AGROINFECTION OF GEMINIVIRUSES

Geminiviruses are small, DNA-containing plant viruses found to infect a variety of economically important plants including legumes, tobacco, cassava, tomato, wheat and maize. The virions are twinned icosahedral capsids, usually 18 x 30nm, and contain single molecules of covalently closed circular single-stranded DNA of approximately 2.7kb in size. This replicates via a double-stranded intermediate in the host cell nucleus. The viruses are divided into two groups according to their insect vectors: different viruses are transmitted either by a specific species of whitefly (*Bemisia tabaci*) or by several species of leafhoppers. Whitefly-transmitted geminiviruses have bipartite genomes and infect dicots only whereas the leafhopper-transmitted geminiviruses have only a single genomic component and mainly infect monocots. Whitefly-transmitted geminiviruses include tomato golden mosaic (TGMV), bean golden mosaic (BGMV) and cassava latent viruses (CLV); the leafhopper-transmitted viruses include maize streak virus (MSV), wheat dwarf (WDV) and beet curly top viruses (BCTV) [23], [81], [114].

Maize streak virus (MSV) is an obligately leafhopper-transmitted geminivirus causing stunting and formation of yellow-streaked leaves in infected maize (*Zea mays*) plants.

Maize streak disease is found in all the maize producing areas of South Africa, throughout large areas of Africa, in Mauritius and in parts of Asia [121]. The virus responsible for the disease is transmitted by various leafhopper species belonging to the genus *Cicadulina*. The MSV virus and the leafhopper vectors are limited to the host plants belonging to the grass family (*Gramineae*) such as maize, sugarcane and small grains [99].

Disease symptoms of infected plants include narrow, broken or continuous chlorotic streaks that may fuse laterally to impart a chlorotic appearance to virtually the whole leaf [121]. Plants infected at an early stage of growth become stunted and produce undersized and/or misshapen cobs. The rate of decline in maize yield falls logarithmically with increased age at time of infection. Plants infected less than a week after germination produce no yield, those infected at three weeks produce about half yield, and those infected at eight weeks produce nearly full yield of maize.

MSV replicates in the nuclei of its natural hosts where it forms characteristic arrays of virus-like particles [114]. MSV has been observed in the phloem, mesophyll and guard cells of *Zea mays*.

The MSV genome is organized in a manner similar to other leaf hopper-transmitted geminiviruses of grasses. Major open reading frames (ORF's) diverge rightward and leftward from a central or starting intergenic region (SIR) and, with the exception of two ORF's, they converge on the other side of the genome at another small intergenic region called the

terminal intergenic region (TIR). Transcription appears to be bidirectional [40].

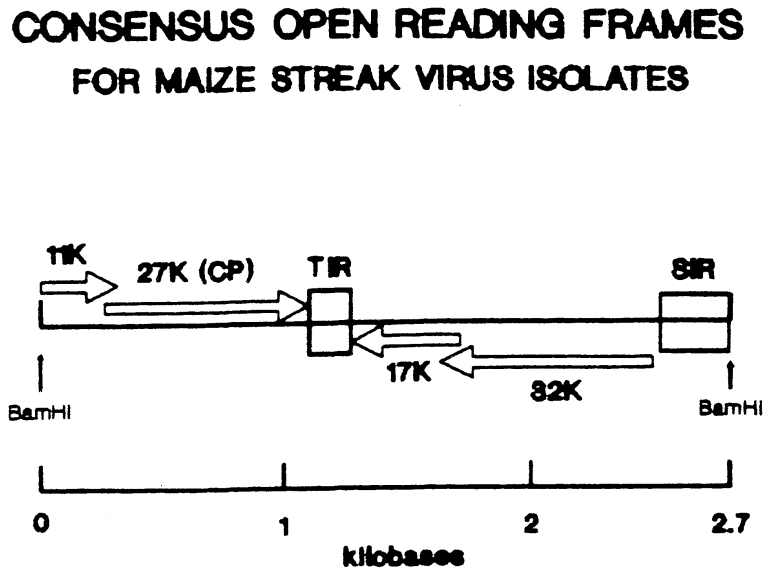

FIGURE 3:

GENOMIC ORGANIZATION OF MSV
Arrows indicate open reading frames. Size of proteins generated is indicated in kilodaltons [40].

Agroinfection, a term first used by Grimsley *et al* in 1986 [51] is used to describe the method of inoculating plants with viral genomes cloned into modified Ti plasmids of *A. tumefaciens*. This method is particularly useful when naked DNA is non-infective and the virus needs an insect vector for infection. Within the last couple of years, this technique has successfully been reported for a number of geminiviruses. Examples of geminiviruses that have been "agroinfected" include maize streak virus [7], [53], [82]; digitaria streak virus [37]; tomato golden mosaic virus [47]; [56]; wheat dwarf virus [57]; [126]; bean golden mosaic virus [92] and African cassava mosaic virus [80]; [93].

Agroinfection has been used as a rapid and sensitive marker for the transfer of DNA to plants to evaluate the efficiency of different *Agrobacterium* strains for the transfer of DNA to maize plants in order to provide a basis for the successful transformation of members of the Gramineae [7].

Although T-DNA normally integrates into the host plant genome, it is unlikely that this step is a prerequisite for the onset of viral multiplication, since the viral sequences constitute an independent replicon [52]. No nuclear integration of T-DNA into maize has been shown but the agroinfection of seedlings with MSV suggests that *vir* gene induction, the attachment of *A. tumefaciens* to the plant cell, and the process and transfer of engineered T-DNA do occur [53].

The transfer of DNA using tandem dimeric clones of MSV [7], [53], [82] to maize plant by *Agrobacterium* has been demonstrated. Plants exhibit typical systemic streak symptoms, providing evidence that *Agrobacterium* specifically interacts with maize tissues, supporting the result of Graves and Goldman [50] who reported the detection of opines in the tissue of maize seedlings following their inoculation with *A. tumefaciens*.

Viral DNA is infectious if excised from the bacterial vector at the cloning site, either as a monomer or as subgenomic fragments showing that *in vivo* ligation within the plant cell can generate circular infectious viral DNA [51]. Bacterial plasmids containing partially or completely duplicated geminivirus genomes are infectious. A small

duplication of the genome such as a 1.4mer is sufficient to induce infectivity [7]. Infection by MSV arises as a result of homologous recombination between the tandemly repeated MSV genomes to produce a circular, unit length MSV DNA molecule or a replicative intermediate is produced directly from the T-DNA [53]. Tandemly repeated genomes of tomato golden mosaic virus integrated in a plant genome have been observed to escape and given rise to infection, demonstrating that it is possible for a geminivirus to be replicated from an integrated form [107]. The released virus replicates and spreads systemically in a susceptible host plant.

Injection of maize plants with cloned MSV DNA did not result in any viral symptoms [82]. Inoculation of 8-10 day old maize seedling by direct injection into the nodal region of an *A. tumefaciens* carrying monomeric units, cloned at a number of sites in the MSV genome did not elicit an infection [7]. The lack of infectivity of the monomeric units of MSV may be due to the inability of the DNA to escape by homologous recombination. In contrast, agroinfection with monomeric units of African cassava mosaic virus have been shown to be infectious [93]. In this case intermolecular recombination between homologous sequences present in the two DNA species (DNA 1 and 2) was shown to occur.

Viral gene functions essential for systemic spread and symptom development during MSV infection have been identified. Insertion and deletion mutagenesis of the two virion-sense genes coding for the coat protein [94] and a

10.9kd non-structural protein [95] prevented symptomatic infections following *Agrobacterium*-mediated "agroinoculation" of maize seedlings [8], [83]. Mutations of the coat protein gene were able to replicate to low levels, producing dsDNA although virion ss DNA was not detected. The coat protein is however essential for systemic spread and subsequent disease development. This in in contrast to tomato golden mosaic virus (TGMV), which has a bipartite genome: the coat protein is not required for systemic spread or symptom development [47]. The sense strand open reading frame encoding the 10.9kd protein of MSV was also found to be dispensable for replication but essential for systemic spread [8], [83]. A mutant of the 10.9kd protein-encoding gene generated *in vivo* with 11 of the 14 N-terminal amino acids altered, was viable and produced symptoms typical of a wild-type infection. Infectivity assessed by replication and symptom expression, was restored by co-inoculating constructs containing single mutations in different reading frames, thus rescue can occur by trans-complementation of gene products. The experiments showed that the mutations did not affect the nucleotide sequence requirements for replication and that in all cases intermolecular recombination eventually resulted in dominant wild-type virus.

Parameters found to affect the efficiency of agroinfection of MSV include: dependency on inoculum concentration [54], the transfer of DNA from *A. tumefaciens* to maize is Ti plasmid-specific [7], DNA transfer by agroinfection has an absolute dependence on the products of the *A. tumefaciens* *vir*C genes [52], meristematic tissues of maize plants were

found to be the most susceptible to agroinfection with MSV [54] and the host variety of maize effects efficiency of infection [82]. Although *A. tumefaciens* is able to transfer MSV DNA to a wide range of plants within the Gramineae, a consideration to bear in mind is that agroinfection does not increase the host range of MSV - this is important for the breeding of virus-resistant maize and for the consideration of bacterial containment requirements [7].

Because geminiviruses infect the economically important members of the Gramineae, it is useful to determine whether the coat protein or other genes may be replaced to allow the expression of foreign genes in these crop plants. Studies on viral pathogenicity need to be made at the molecular level to investigate the potential of producing virus-resistant plants by use of coat proteins, antisense RNA or satellite RNAs. A knowledge of plant virus molecular biology has already improved virus detection procedures, identification methods and disease diagnosis [18].

1.4 AIMS OF THIS RESEARCH

The aim of this project was to clone MSV genes via *Agrobacterium* into tobacco to produce transgenic plants. This was done as a model system in our department where work of this nature had not been done previously. It was hoped that through the results obtained, further knowledge might be gained into the potential use of geminiviruses as plant vectors for the introduction of agronomically desired trait(s) into the cereals.

Two different "approaches" have been explored. As a preliminary to agroinfection a 1.5 mer of MSV-PE [14] - was constructed in a cointegrate vector and introduced into tobacco via leaf disc co-cultivation with *A. tumefaciens* harbouring the recombinant plasmid containing the MSV genomes. Because of published reports on resistance to viral infection by expression of the coat protein gene or its antisense mRNA, and recent reports into the mutational analysis and function of the 10.9 kD protein (function unknown) and the coat protein gene of MSV in systemic spread and symptom development [8], [83], a monomer of the MSV genome in both the sense and antisense orientation was cloned into a plant transformation vector. It was hoped that this approach could be used to test the potential of these constructs for the induction of MSV resistance in selected target plants. In view of the observation that one genome of MSV is not infectious, this could be done without the risk of producing infectious viral particles. All plant work was done in sterile tobacco *Nicotiana tabacum* cv. Petit Havana SR1 as a model system, although for economic feasibility, these constructs will need to be tested in maize and possibly other grasses.

CHAPTER 2

CLONING OF MSV GENES INTO *E. COLI* AND THEIR MOBILIZATION INTO *AGROBACTERIUM TUMEFACIENS*

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2.1 INTRODUCTION

This project had as its aim the cloning of the coat protein gene and its antisense from the PE isolate of MSV [14] (Figure 4) into the cointegrate vector pGSJ280 [31] (Figure 5) for eventual expression in plants. The purpose of this was to test for the induction of MSV resistance in target plants. A further aim was a construction of an agroinfectious MSV-PE clone by producing a 1.5 mer of MSV-PE in pGSJ280.

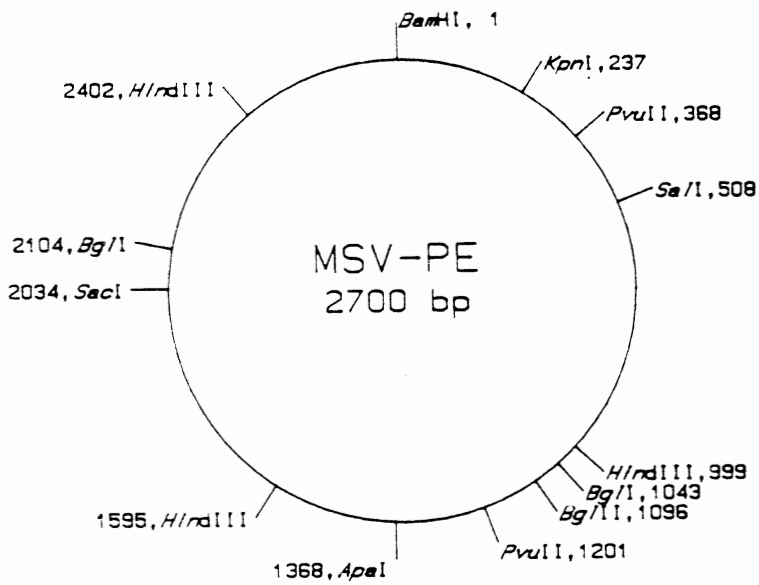


FIGURE 4:

GENETIC MAP OF MSV-PE [14]
The viral DNA is 2700 bp long.
The unique *Bam*HI site is designated position 1. The *Bgl*II site is at position 1096, approximately midway from the *Bam*HI site.

The plant expression vector pGSJ280 contains the NPT-II gene which confers kanamycin resistance to transformed plant cells, as a selectable marker. The resistance gene is under control of the nopaline synthase (*nos*) promoter. The vector also contains a *Bam*HI and *Cla*I site downstream of the very efficient CaMV35S RNA promoter, for the introduction of foreign coding sequences. The promoters used here are from constitutively expressed genes.

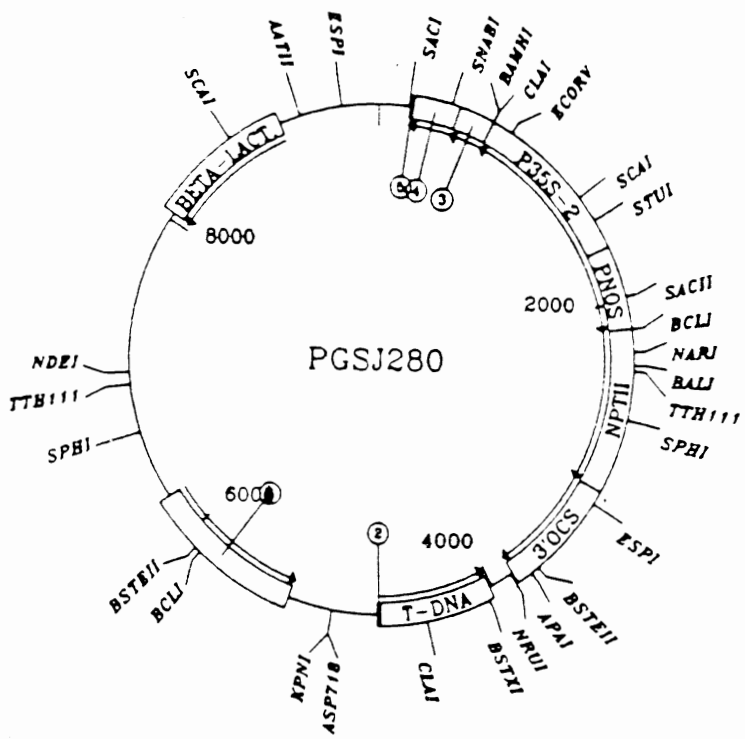


FIGURE 5:

GENETIC MAP OF PLANT EXPRESSION VECTOR pGSJ280 [31]
The vector is 9522 bp long.

1. Stremptomycin - Spectinomycin adenyl transferase
2. Left border
3. 3′ of T7
4. T-DNA
5. Right border.

β-lactamase (Amp^r) and Sp^r/Sm^r genes function in bacteria. The NPT-II gene, conferring kanamycin resistance in transformed plants, is under plant expression regulation in the form of the nopaline synthase promoter (pNOS) and the octopine synthetase polyadenylation region (3′OCS). The *Bam*HI and *Cla*I cloning sites are found between the CaMV 35S promoter (p35S) and the T7 polyadenylation region (T7 3′).

Like other co-integrate vectors, pGSJ280 is based on the common cloning vector pBR322 and is thus able to replicate in *E. coli*. It can be mobilized into *Agrobacterium as* pBR322 contains a *bom* site and mobilization as well as transfer functions can be complemented *in trans*. Since the vector cannot replicate in *Agrobacterium*, it can only be maintained if a recombination occurs between the homologous pBR322 sequences present in the vector and in the acceptor Ti plasmid pGV2260 [30]. This results in the formation of a cointegrate in which the T-DNA is flanked by directly repeated pBR322 sequences. Verification of the Ti plasmid cointegrate structure can be shown by Southern blot hybridization.

Plasmid pGV2260 has the complete T-DNA (left and right borders including the 25 bp border repeat sequences) removed from a octopine Ti plasmid (pTiB653) and replaced by pBR322 sequences, including the carbenicillin resistance gene [30]. pGV2260 contains the intact *vir* region which encodes Ti plasmid functions necessary for the T-DNA transfer and/or integration. These functions can act in *trans* to the T-DNA.

Cloned MSV-N (Nigerian isolate) tetramers in vector pBIN19 were kindly supplied by Michel Schneider of The Friedrich Miescher Institut, Basel, Switzerland to be used as a positive control. Like pGSJ280, plasmid pBIN19 utilizes the *trans*-acting function of the *vir* region of a co-resident disarmed Ti plasmid in *A. tumefaciens* (pGV2260) to transfer sequences bordered by left and right T-DNA border sequences

into the nuclear genome of plants. The plasmid pBIN19 can be transferred efficiently by the helper plasmid pRK2013 [41] into *A. tumefaciens* where it replicates autonomously. Because binary vectors do not integrate into the resident Ti plasmid derivative in *A. tumefaciens*, verification of the "recombinant" Ti plasmid by Southern blot hybridization analysis may be omitted. The T-region of pBIN19 contains the dominant selectable neomycin resistance (*neo*) gene and the *lacZ* ∝ region from the phage vector M13mp 19 that contains several unique restriction sites. The presence of *lacZ*∝ facilitates the detection of inserted DNA [6].

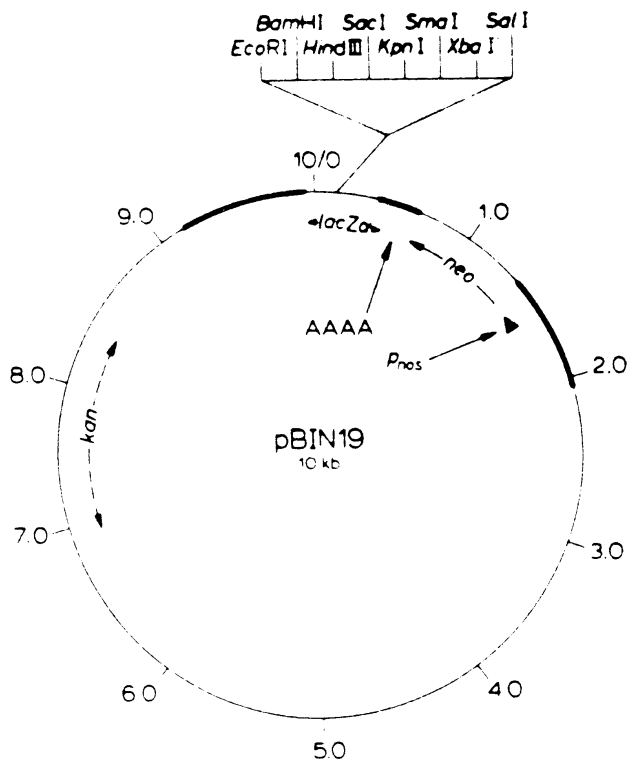


FIGURE 6:

GENETIC MAP OF VECTOR pBIN19 [6]
The 10kb vector comprises left and right T-DNA border sequences, a fragment from M13mp 19 containing the lacZ ∝ sequence, the *nos* promoter, *neo* gene, *nos* polyadenylation site, a fragment containing the RK2 origin of replication and the *kan* gene.

2.2 METHODS

2.2.1 Plasmids and Strains

A list of bacterial strains and plasmids used is presented in Table 1.

TABLE 1:

BACTERIAL STRAINS AND PLASMIDS

	Antibiotic resistance	Relevant characteristics	Origin
Strains:			
E. coli K514		*thr, leu, thi, hsd*R	16a
E. coli LK111		lacY+ derivative of K514	130
A. tumefaciens C58C1Rif	Rif	Rif^r derivative of C58 cured for pTiC58	120a
Plasmids:			
pGSJ280	Sp, Sm, Cb	Has Km^r gene gene under plant expression signals and has T-DNA borders	31
pGV2260	Cb	Disarmed derivative of pTiB653 containing pBR322 sequences	30
pRK2013	Km	Mobilizing plasmid	41

Plasmids pGSJ280 and pGV2260 were gifts from Plant Genetic Systems, Gent, Belgium. The replicative form of MSV-PE was supplied by Beverley Clarke.

All *E. coli* strains were grown in Luria broth (LB) or Luria agar (LA) (Appendix B.3). Antibiotic concentrations used are listed in Appendix B.18.

2.2.2 Construction of a monomer of MSV-PE in pGSJ280

DNA manipulation was performed essentially according to Maniatis *et al* [88]. Plasmid pGSJ280 was prepared by the alkaline lysis method described in Appendix A.1 [72]. One μg of vector pGSJ280 and 1μg of insert MSV-PE: - the double-stranded replicative form (RF) of MSV-PE prepared by B. Clarke [14] - were digested with 4u *Bam*HI (Appendix A.3) and then ligated in a vector: insert DNA ratio of 1:10 at room temperature from 4 hours to overnight (See Appendix B.2).

Aliquots of the ligation mix were transformed into *E. coli* K514 competent cells prepared by the $CaCl_2$ method (Appendix A.5) [20]. Transformation mixes with pGSJ280 were expressed for 3-4 hours at 37°C before 200μl was plated onto LA + Sp (100ug/ml) + Sm(300ug/ml) plates. Plates were incubated at 37°C overnight. Transformants containing MSV-PE cloned into pGSJ280 were selected using colony hybridization (Appendix A.8). These were verified by restriction enzyme analysis of plasmid mini preps (Appendix A.2) using *Bam*HI and *Sac*I.

2.2.3 Construction of a 1.5 mer of MSV-PE in pGSJ280

A monomer of MSV-PE in pGSJ280 was used to construct 1.5mers of the geminiviral DNA in pGSJ280 by cleaving the insert approximately mid-way (at the *Bgl*II site) and then partially (at the *Bam*HI cloning site) to release fragments of the MSV

genome (See Figure 7 overleaf). Gel slice ligation of the digested DNA according to Struhl (1985 [115] followed to allow the various digested fragments to re-ligate in a number of possible ways. The same strategy could be used to isolate the coat protein gene (cpg) independently of the rest of the MSV-PE genome (compared to a monomer of MSV-PE which has the cpg as well as all other ORF's).

FIGURE 7:

DIAGRAMMATICAL REPRESENTATION OF THE CLONING STRATEGY USED TO CONSTRUCT A 1.5 MER OF MSV-PE IN pGSJ280

A monomer of MSV-PE in pGSJ280 was cut to completion with *Bgl*II and partially with *Bam*HI to produce various fragments, which when religated can result in numerous combinations with respect to number of inserts, orientations, etc. Production of 1.5 mer and coat protein gene constructs are shown.

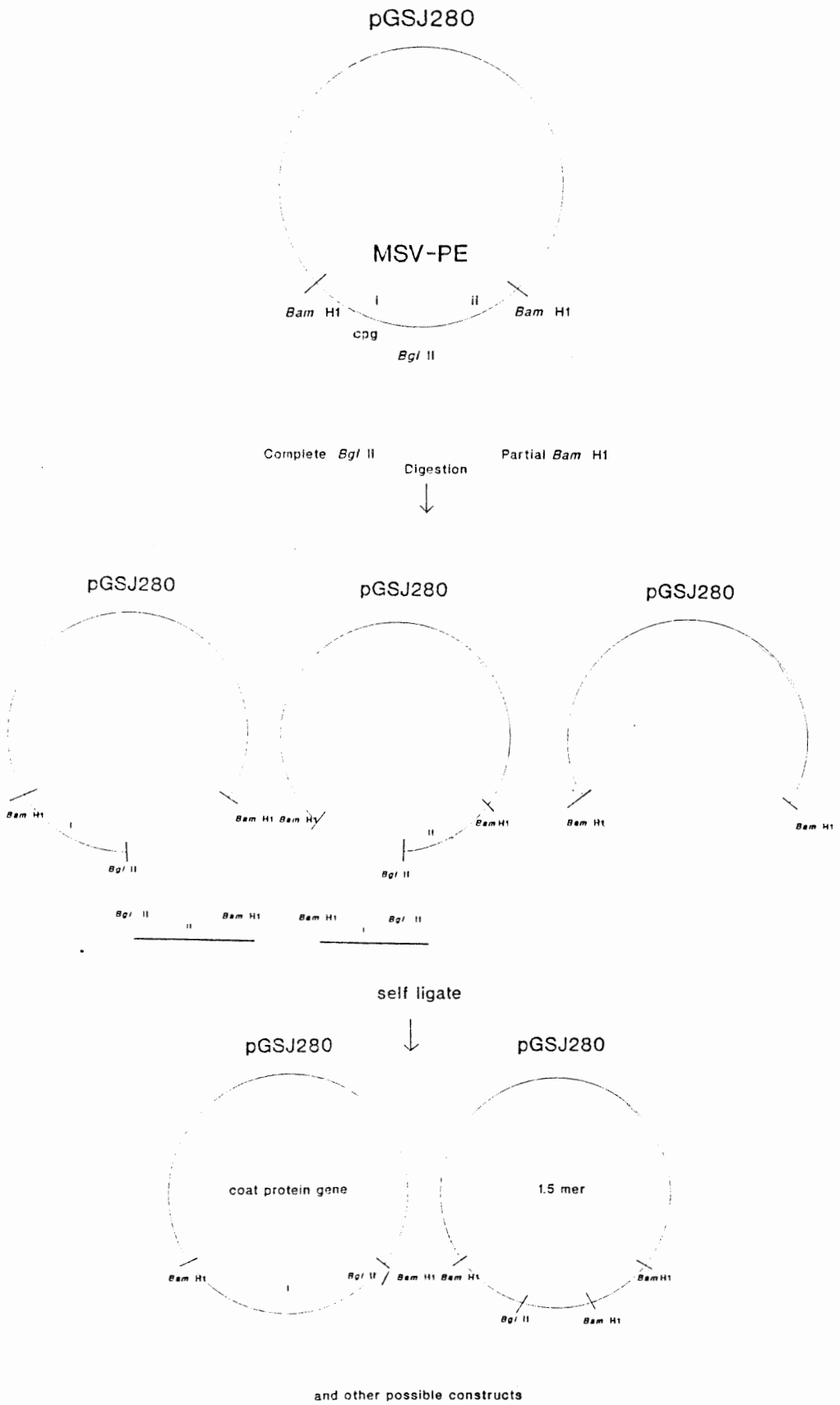


FIGURE 7:

Legend for Figure 7 on page 35

The method described in Appendix A.1 was used to prepare plasmid DNA from an overnight culture of *E. coli* K514 (pGSJ280 containing MSV-PE in the sense orientation). One ug of this DNA was digested with 1u *Bgl*II for 1 hour at 37°C. The DNA was then partially digested with 0.25u *Bam*HI at 37°C for 30 min. The digested DNA was run on a 0.7% Sea Plaque low melting point agarose gel in Tris-acetate buffer (50mM pH8.2; no EDTA). After brief staining in EtBr, a band representing a large fragment (pGSJ280 with MSV-PE insert) as opposed to smaller fragments (½ MSV genome) visualized migrating further from the wells, was excised from the gel using a blade. The gel slice was trimmed, melted at 70°C for 5 min then equilibrated at 37°c for about 20 min. Gel slice volumes of 1μl, 5μl, 7μl and 10μl were mixed with 2μl 10x ligation buffer (Appendix B.2), 1μl (1u) ligase and made up to 20μl with dH_20. The ligation mixes were held at room temperature for 2 hours. The contents of the tubes were then melted at 70°C for 5 min and diluted with 5 volumes of cold 0.1M $CaCl_2$.

Aliquots of the ligation mixes were transformed into competent *E. coli* K514 cells (Appendix A.5).

Because no direct selection method for more than one genome of inserted MSV-PE into the vector exists, plasmids prepared from transforment colonies by a "miniprep" procedure (Appendix A.2) were digested with *Bam*HI, *Bgl*II, *Sal*I and *Sac*I to orientate the clones and assess the number of sites for these restriction enzymes, thus indicating which portion

of MSV-PE was inserted into pGSJ280 and the frequency of its occurrence.

To confirm these results, *Bam*HI digested plasmids were probed with ^{32}P-labelled MSV-PE in a Southern blot [112], (Appendix A.7).

2.2.4 Transformation of MSV-N tetramers into *E. coli* cells

The supplied MSV-N tetramers in pBIN19 were transformed into competent *E. coli* LKIII cells (Appendix A.6).

2.2.5 Triparental Cross

Mobilization of *E. coli* plasmids into *A. tumefaciens* was accomplished essentially by the method described by Deblaere *et al* (1987) [31]. A single colony of acceptor *A. tumefaciens* C58Cl Rif (pGV2260) was inoculated into 5ml LB + 100μl 100mM $MgSO_4$ and incubated for 2 days at 30°C. Single colonies of helper *E. coli* HB101 (pRK2013) and donor *E. coli* strains K514 (pGSJ280) containing no MSV, one genome of MSV-PE in the sense orientation, one genome of MSV-PE in the antisense orientation and 1.5 genomes of MSV-PE as well as LKIII (pBIN19) harbouring a MSV-N tetramer, were inoculated into 5ml LB and shaken overnight at 37°C. One hundred microlitres of each donor, helper and acceptor culture was plated on a solid LA plate (300μl was spun down in a microfuge for a few seconds and resuspended in 100μl, which was spotted on the plate).

After drying, the plates were incubated for times varying from overnight to 4 days. A loopful of the conjugation mixture was collected in 1ml 100mM $MgSO_4$ and vortexed well. A serial dilution series in 100mM $MgSO_4$ was prepared and aliquots plated on LA + Rif(100ug/ml) + Sp(100ug/ml) + Sm(300ug/ml) (LA + Km [50ug/ml] in the case of the pBIN19 experiment) and onto LA + Rif (100ug/ml) to determine the titre of transconjugants per *Agrobacterium* recipient. Selection plates were incubated at 30°C for 2 days.

A number of $Rif^rSm^rSp^r$ (*A. tumefaciens* pGV2260::pGSJ280 with inserted MSV-PE genome(s) and (Rif^rKm^r) (*A. tumefaciens* pGV2260::pBIN19 (MSV-N tetramer) transconjugants, together with control *Agrobacterium* and *E. coli* cultures were then plated onto lactose agar plates (Appendix B.8) and incubated for two days. A test for the production of 3-ketolactose was carried out to verify that the transconjugants were indeed *Agrobacterium*. This involved flooding the two day old lactose plates with Benedict's reagent (Appendix B.9) [5].

2.2.6 Verification of co-integrate structure and the presence of MSV genes in *Agrobacterium*

Total *Agrobacterium* DNA was prepared for use in a Southern hybridization study according to the method of Dhaese *et al* (1979) [34]. *Agrobacterium* transconjugants, including an *A. tumefaciens* (pGV2260) control were grown overnight in 3ml LB at 30°C. Cells were harvested by centrifugation in a microfuge and resuspended in 300μl TE buffer. Sarkosyl

(100μl of a 5% solution in 1 x TE) was then added, followed by the addition of 100μl pronase (2.5mg/ml in TE). After incubation for at least one hour at 37°C, the lysate was sheared by drawing into a syringe without a needle and rapidly expelling the sheared DNA, or by vigorous vortexing. Subsequently, the lysate was extracted twice with an equal volume of phenol (equilibrated with TE). The upper aqueous phase was then extracted two to three times with chloroform (chloroform: isoamyl alcohol, 24:1). Nucleic acids in the aqueous phase were precipitated at -20°C by the addition of $^{1}/_{10}$ volume 5MNaCl and 2 volumes 96% ethanol. The pellet was eventually resuspended in 100μl dH_2O. The DNA concentration was measured at 260nm and thereafter diluted to 1ug/μl.

Between 2 and 4ug of DNA was digested to completion with *Bam*HI and electrophoresed on a 0.8% agarose gel in TAE buffer (Appendix B.6), which was blotted and probed by the method of Southern [112], with certain modifications.

DNA from the gel was transferred to Hybond N+ (Amersham International, U.K) using 0.4M NaOH in three hours. Alkali blotting allows transfer without a need to fix the DNA under UV. The membrane was rinsed briefly in 2 x SSPE (Appendix B.14) or 2 x SSC (Appendix B.13) with gentle agitation at room temperature for 10 min, before being probed with random primed MSV-PE labelled with digoxigenin-dUTP using the

Boehringer Mannheim Nonradioactive labelling and detection kit (Cat. No. 1093 657). Suppliers instructions were followed.

2.3 RESULTS

2.3.1 Monomers of MSV-PE in pGSJ280

Double-stranded RF MSV-PE was restricted with *Bam*HI and cloned into the *Bam*HI site of vector pGSJ280. Recombinant plasmids were transformed into *E. coli* K514 competent cells. Transformants with pGSJ280 were expressed for at least three hours as expression times shorter than this did not yield favourable results.

Of 100 Sp and Sm resistant colonies screened by colony hybridization with a ^{32}P-labelled MSV-PE probe for insertion of MSV-PE into pGSJ280, 5 were positive. These results were verified by restriction enzyme analysis of plasmid "minipreps" using *Bam*HI and *Sac*I (Figure 8). Both pGSJ280 and MSV-PE have only one *Bam*HI site and one *Sac*I site. *Bam*HI digests were used to show the difference between parental pGSJ280 (1 band) and clones harbouring MSV-PE insert (2 bands:1 representing linear pGSJ280 and 1 representing linear MSV-PE) on an agarose gel. *Sac*I digests were used to orientate the inserts. Of the five positive colonies identified by colony hybridization, four were found to be in the sense orientation and one in the antisense orientation.

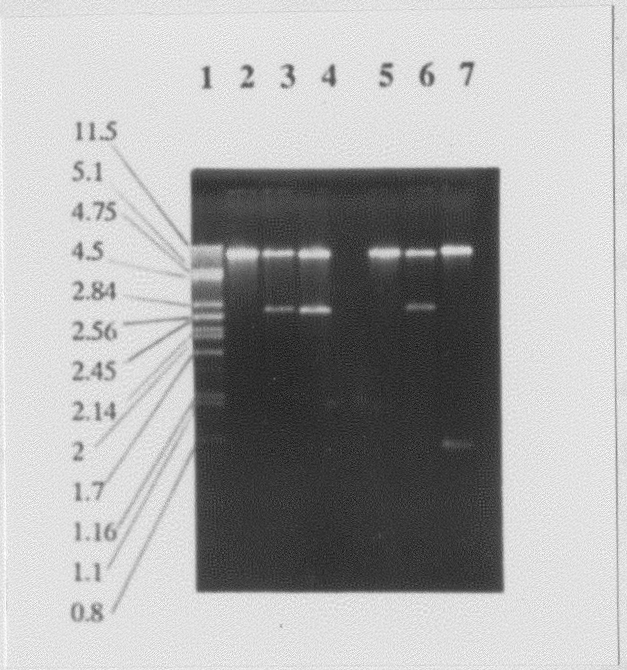


FIGURE 8:

AGAROSE GEL ELECTROPHORESIS TO SHOW THE DIFFERENT SIZE FRAGMENTS PRODUCED BY THE CLEAVAGE OF pGSJ280 (CONTROL), pGSJ280 (MSV-PE) ANTISENSE ORIENTATION AND pGSJ280 (MSV-PE) SENSE ORIENTATION BY THE RESTRICTION ENDONUCLEASES *BAM*HI AND *SAC*I.

Lane 1: λ*Pst*I
Lane 2: pGSJ280 - *Bam*HI
Lane 3: pGSJ280 (MSV-PE) antisense orientation - *Bam*HI
Lane 4: pGSJ280 (MSV-PE) sense orientation - *Bam*HI
Lane 5: pGSJ280 - *Sac*I
Lane 6: pGSJ280 (MSV-PE) antisense orientation - *Sac*I
Lane 7: pGSJ280 (MSV-PE) sense orientation - *Sac*I

In Figure 8, *Bam*HI digests show one band of 9.5kb corresponding to the size of pGSJ280 (lane 2) and a second band of 2.7kb where MSV had been incorporated (lanes 3 and 4). *Sac*I digestion of the antisense orientation clones (lane 6) yielded two bands approximately the same size as the *Bam*HI-digested fragments whereas *Sac*I cleavage of the sense orientation clone (lane 7) showed a much smaller band (0.75 kb) and a larger band (11.47 kb).

Figure 9 gives a diagrammatic representation of how *Bam*HI and *Sac*I sites were used to orientate the monomers of MSV-PE and pGSJ280. In both the sense and the antisense orientation clones, the *Bam*HI-*Bam*HI fragments are the predicted sizes for pGSJ280 and MSV-PE. However, *Sac*I digestion distinguishes sense and antisense orientation clones based on the difference in the size of fragments produced.

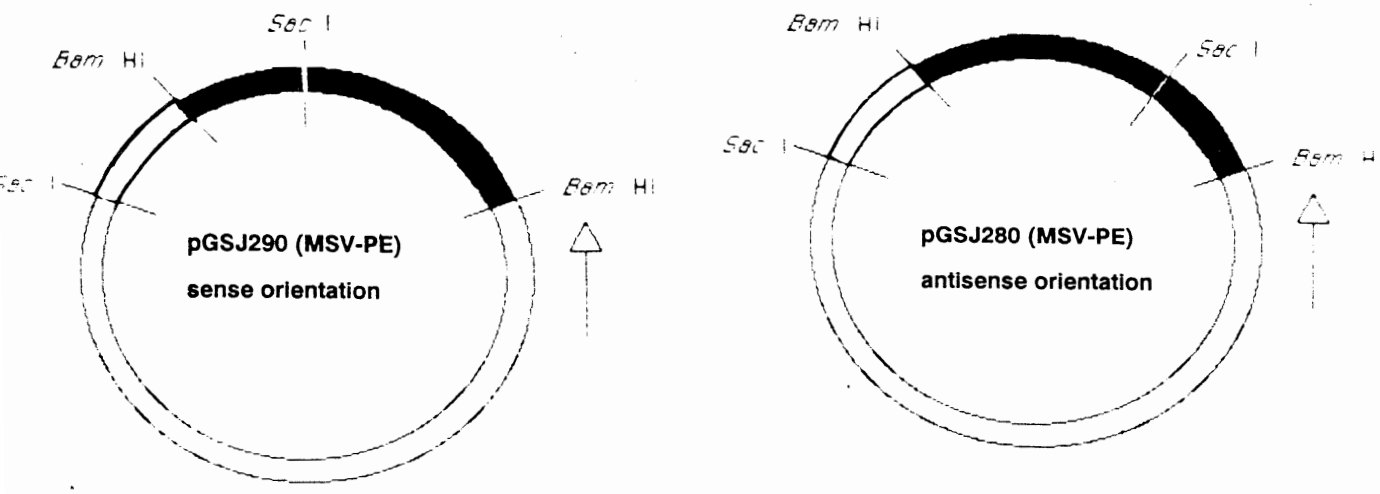


FIGURE 9:

A DIAGRAMMATIC REPRESENTATION TO SHOW MSV-PE IN pGSJ280 IN THE SENSE AND ANTISENSE ORIENTATIONS
The solid area represents MSV-PE, the white region is pGSJ280 and the arrow indicates the direction of transcription from the CaMV 35S promoter in pGSJ280.

2.3.2 1.5 mers of MSV-PE in pGSJ280

A monomer of MSV-PE in pGSJ280 in the sense orientation was used to construct 1.5mers of MSV-PE in the same vector. The insert was cleaved with *Bgl*II and then partially with *Bam*HI. Digested DNA was run through a low melting point agarose gel. The large DNA fragment (approximately 10.5 kb) seen

under UV was excised. Due to the nature of restriction digestions, this band was expected to contain: pGSJ280 only, pGSJ280 + a whole genome of MSV-PE, pGSJ280 + MSV-PE (*Bam*HI-*Bgl*II fragment i in Figure 10 below) and/or pGSJ280 + $^{1}/_{2}$ MSV-PE (*Bgl*II-*Bam*HI fragment ii in Figure 10 below).

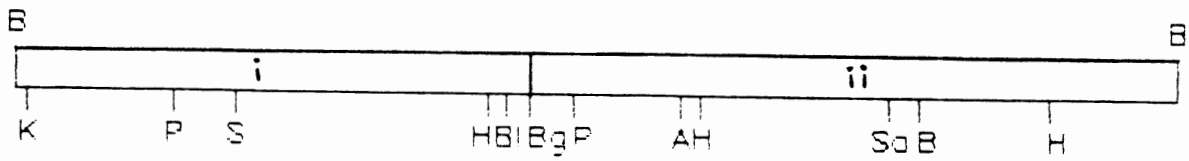


FIGURE 10:

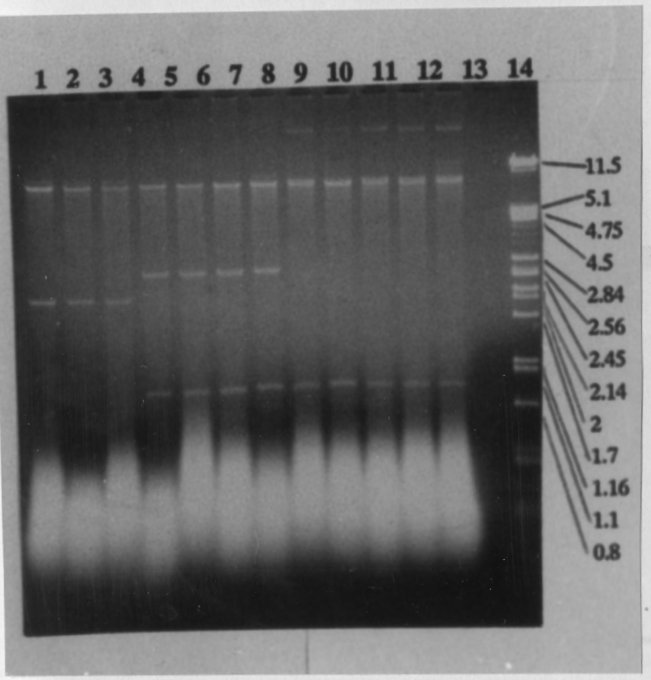
RESTRICTION MAP OF MSV-PE [14]
B: *Bam*HI; K: KpnI; P: PvuII; S: *Sal*I; H: *Hind*III; Bl: *Bgl*I; Bg: *Bgl*II; A: *Apa*I; Sa: *Sac*I
The *Bam*HI - *Bgl*II region encoding the coat protein gene has been termed (i) as opposed to the other *Bgl*II - *Bam*HI region (ii)

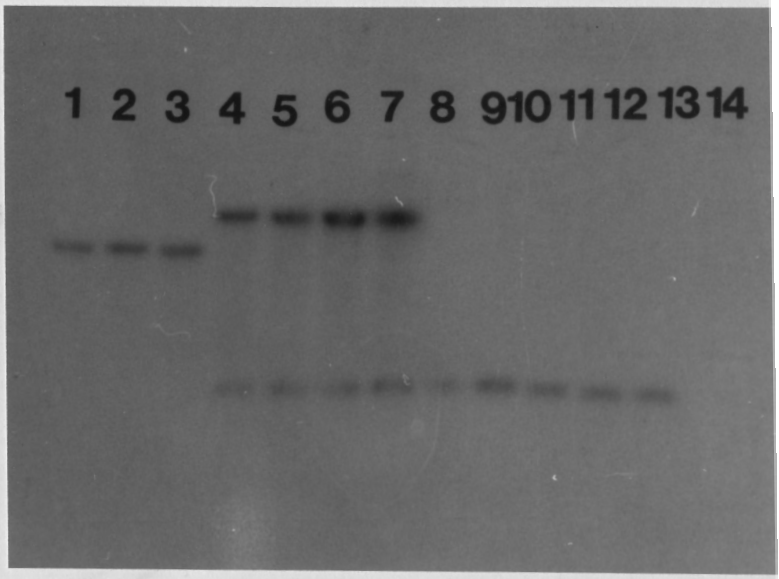
In the same way, the above strategy could be used to construct a clone containing the coat protein gene (cpg) of MSV-PE in pGSJ280. The cpg is found on the *Bam*HI - *Bgl*II fragment i in Figure 10 above and this, together with parental pGSJ280 are among the fragments predicted to be present after complete *Bgl*II and partial *Bam*HI digestion of a MSV-PE monomer in pGSJ280.

Different volumes of the gel slice were (self) ligated and the ligation mixes transformed into competent *E. coli* K514 cells. Transformation efficiency was greatest where proportionately more gel volume had been used in a ligation. *Bam*HI restriction endonuclease analysis was performed on 12

transformant colonies selected for "miniprep" alkaline hydrolysis plasmid isolation. Of the 12 transformants analyzed, 3 had a band at 9.6kb (pGSJ280) and 2.3kb (just less than full genome length of MSV-PE) (Figure 11) (lanes 1 to 3). Four clones had a band at 9.6kb (pGSJ280), a band at 2.7kb (MSV-PE) and a band at 1kb (size of approximately half a genome of MSV-PE) (lanes 4 to 7) and the remaining 5 clones (lanes 8 to 12) had a band for pGSJ280 and a band for half a MSV-PE genome (1kb) at the same position as the smallest band in lanes 4 to 7. No parental MSV-PE in pGSJ280 clones were found. The results shown in lanes 4 to 7 indicated that at least 1.4 genomes of MSV-PE had been cloned into pGSJ280, thus producing a potential clone for use in agroinfection. (See Figure 11 below).

To confirm these results, i.e. that MSV-PE had been cloned into pGSJ280, the gel with *Bam*HI-digested "miniprep" DNA samples (Figure 11A), was hybridized to a ^{32}P-labelled MSV-PE probe in a Southern blot (Figure 11B).





a b

FIGURE 11:

BAMHI-DIGESTED "MINIPREPS" ELECTROPHORESED ON A 0.8% AGAROSE GEL IN TBE BUFFER (A) AND AUTORADIOGRAPH OF SOUTHERN BLOT TO SHOW HYBRIDIZATION OF CLONES TO MSV-PE (B).

Lanes 1-3: pGSJ280 (MSV-PE) 0.9 mer
Lanes 4-7: pGSJ280 (MSV-PE) 1.5 mer
Lanes 8-12: pGSJ280 (MSV-PE) 0.5 mer
Lane 14: λ *Pst*I

Further restriction endonuclease digestions of the "1.5mers" (clones in lanes 4-7, Figure 11 above) with *Bgl*II, *Sal*I and *Sac*I revealed that all four clones were the same, consisting of a duplication of the same region of MSV-PE (region ii in Figure 10 above). The orientation of the 1.5mers is shown diagrammatically in Figure 12 below.

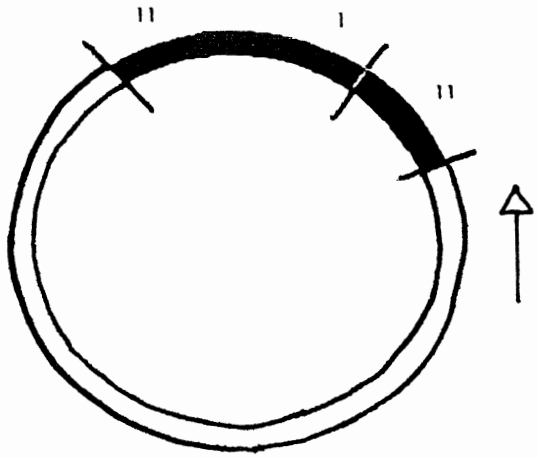


FIGURE 12:

ORIENTATION OF THE 1.5MERS OF MSV-PE IN pGSJ280

The 1.5 mer of MSV was produced after complete *Bgl*II and partial *Bam*HI digestion of a monomer of MSV-PE. Religation of digested fragments including pGSJ280, the monomer of MSV-PE (*Bam*HI-*Bgl*II fragments i and ii as described in Figure 10) and one half of MSV (*Bam*HI-*Bgl*II fragment ii as described in Figure 10) produced a 1.5 mer. The black area indicates MSV-PE, the white area is pGSJ280 and the arrow indicates the direction of transcription from pCaMV 35S in pGSJ280.

2.3.3 Transformation of MSV-N tetramers into *E. coli*

Freeze-dried DNA of MSV-N tetramers in pBIN19 were transformed into competent *E. coli* LKIII cells. To confirm presence of the insert, "mini" plasmid preparations using the alkaline hydrolysis method described in Appendix A.2 were made of a number of transformants. Plasmid pBIN19 was used as a control. Plasmid DNA was cleaved with *Eco*RI and then electrophoresed on an agarose gel.

2.3.4 Triparental cross

Clones containing MSV genes in the *E. coli* plasmids pGSJ280 and pBIN19 were mobilized into *A. tumefaciens* C58CIRif (pGV2260) by means of a triparental cross-involving *E. coli* pRK2013, supplying mobilization (*mob*) and transfer (*tra*) functions *in trans*. Transconjugants were selected for the resistance encoded by the donor plasmid (spectinomycin and streptomycin in the case of pGSJ280; kanamycin in the case of pBIN19) and resistance to rifampicin which is encoded by the *Agrobacterium* chromosome. The efficiency of transconjugate formation was 10^{-4} to 10^{-5} per recipient. The Benedict's test was used to prove that the transconjugants were *A. tumefaciens*.

2.3.5 Verification of the cointegrate structure and the presence of MSV genes in *A. tumefaciens*

Total *Agrobacterium* DNA was prepared for use in a Southern hybridization study to verify that *E. coli* pGSJ280 had been mobilized into *A. tumefaciens* and that the MSV genes carried by pGSJ280 were integrated into pGV2260.

Transconjugants of *A. tumefaciens* (pGV2260), carrying pGSJ280 (lane 3), pGSJ280 containing a monomer of MSV-PE in the antisense orientation (lane 4), pGSJ280 containing a monomer of MSV-PE in the sense orientation (lane 5) and pGSJ280 containing a 1.5mer of MSV-PE (lane 6) were digested with *Bam*HI and electrophoresed on an agarose gel, (Figure 13A). The gel was blotted onto Hybond N^+ and hybridized to non-radioactively labelled MSV-PE in a Southern blot (Figure 13B).

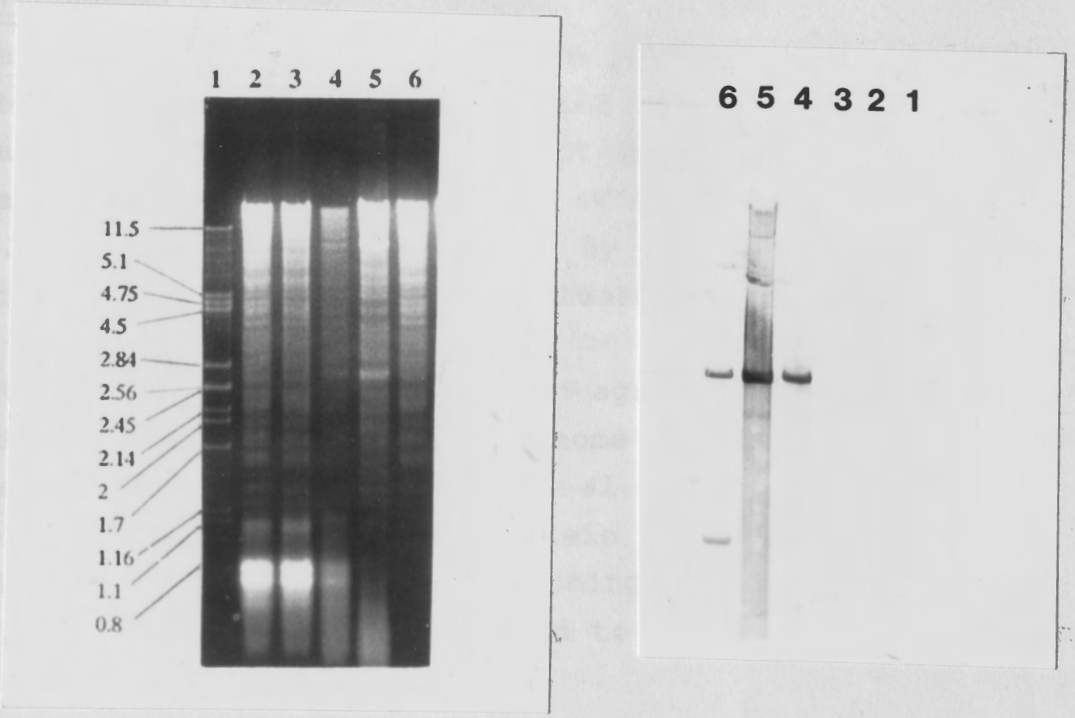


FIGURE 13:

TOTAL *AGROBACTERIUM* DNA EXTRACTS DIGESTED WITH *Bam*HI AND ELECTROPHORESED ON A 0.8% AGAROSE GEL (A) AND RESULTS OF A SOUTHERN BLOT OF THIS GEL PROBED WITH NON-RADIOACTIVELY LABELLED MSV-PE (B)

Lane 1: λ *Pst*I
Lane 2: *A. tumefaciens* C58CIRif (pGV2260)
Lane 3: *A. tumefaciens* C58CIRif (pGV2260::pGSJ280)
Lane 4: *A. tumefaciens* C58CIRif (pGV2260::pGSJ280 (MSV-PE) antisense orientation
Lane 5: *A. tumefaciens* C58CIRif (pGV2260::pGSJ280 (MSV-PE) sense orientation
Lane 6: *A. tumefaciens* C58CIRif (pGV2260::pGSJ280 (MSV-PE) 1.5 mer.

Bands of hybridization were seen for MSV in *A. tumefaciens* at the same position shown in *E. coli* (Figure 11). Thus, MSV genes were successfully cloned into *E. coli* and mobilized into *A. tumefaciens*, confirming the cointegrate structure.

2.4 DISCUSSION

Two classes of monomers of MSV-PE in pGSJ280 were obtained: the geminiviral genome was in the sense or antisense orientation in relation to the direction of transcription of the coat protein ORF, under control of the CaMV 35S promoter in pGSJ280. Clones were selected by colony hybridization and verified by restriction endonuclease digestion.

An MSV-PE monomer will not be agroinfectious as the mechanism for release of viral genome is not present [7]. Because observations by Hemenway *et al* (1988) [59] indicated that expression of viral coat protein or its antisense RNA can give some protection to incoming virus of the same strain, the clones were constructed to test their potential for conferring MSV resistance.

Construction of 1.5mers of MSV-PE in pGSJ280 was achieved using a monomer of MSV-PE in pGSJ280 (in the sense orientation), cleaving the MSV insert approximately mid-way at the *Bgl*II site and then partially digesting the monomer with *Bam*HI. Religation of this DNA using a gel slice ligation technique resulted in a number of transformant types obtained by the various fragments of digested MSV-PE in pGSJ280 re-ligating in a number of possible ways. The classes of transformants obtained indicated that this had indeed occurred. The "1.5 mers" were recovered when *Bam*HI digested plasmid DNA of transformant colonies resulting after gel slice ligation revealed three bands on an agarose gel: 1 for pGSJ280, 1 for a complete MSV genome and 1 for half an MSV genome. It should be noted that a *Bgl*II site was lost in the " MSV genome fragment". Had this not been the case, a 1.5 mer digested with *Bam*HI would be observed as a band of 9.5kb (pGSJ280) and a second band of approximately 3.9kb (1 MSV) or a band of 10.9kb (pGSJ280 + MSV) and one of 2.7kb (MSV) depending on the orientation of the MSV fragment. This would occur because ligation of a *Bam*HI and *Bgl*II site would result in loss of one *Bam*HI cleavage site. Loss of the *Bgl*II is further evidenced by the smaller than

expected size for "½ MSV". The predicted size of the *Bgl*II - *Bam*HI fragment inserted into the monomer is 1.6 kb whereas the size actually inserted is only 1 kb. Proof that DNA fragments inserted into pGSJ280 were MSV-PE was obtained from a Southern blot.

Using a similar approach it is possible to construct a clone carrying the MSV-PE cpg inserted into pGSJ280. This would come about if complete *Bgl*II and partial *Bam*HI cleavage of a monomer of MSV-PE in pGSJ280 (as described above) resulted in re-ligation of pGSJ280 and the fragment of MSV-PE coding for the cpg.

It was noted (M. Schneider, personal communication) that the tetramer of MSV-N in pBIN19 had originally been supplied as a dimer, indicating that multiple genomes of MSV are not stable. This correlates with early attempts to produce MSV-PE dimers by direct cloning (this project). The strategy used was cleaving a monomer of MSV-PE in pGSJ280 at the *Bgl*II site of MSV-PE and ligating this to a second *Bgl*II-cleaved genome of MSV-PE to produce a dimer of MSV-PE in vector pGSJ280. Results were consistently unsuccessful, with parental pGSJ280 the result after loss of the MSV genome(s), even when cipping [88] was employed. In some way, the MSV genome(s) were excised - perhaps as a result of homologous recombination of the two "MSV's" or maybe due to the increased load of insert DNA on the vector. Attempts were made to produce MSV-PE dimers in pUC19 [97] following a similar approach, ie. inserting a second MSV genome into a cleaved "MSV-PE monomer in pUC19". Results were just as unsatisfactory - parental pUC19 and monomers of MSV-PE in pUC19 being the resultant transformants, with no indication of the presence of a dimer.

The 1.5 mer of MSV-PE in pGSJ280 can be used in the future for agroinfection studies. It can possibly be compared with dimers of other MSV isolates, such as MSV-N [7], [53] and MSV-S [82].

E. coli pGSJ280 carrying MSV-PE genomes and *E. coli* pBIN19 harbouring MSV-N dimers were mobilized into *A. tumefaciens* C58Cl Rif (pGV2260) using helper strain *E. coli* pRK2013. Verification of the cointegrate structure for pGSJ280 clones was shown by Southern blot hybridization of total *Agrobacterium* DNA to an MSV-PE probe.

CHAPTER 3

PLANT TRANSFORMATION AND THE ANALYSIS OF FOREIGN GENE EXPRESSION IN TRANSGENIC PLANTS

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3.1 INTRODUCTION

Leaf disc infection has become the method of choice for inducing integration of *A. tumefaciens* Ti-DNA into leaf explants [67]. By integrating the transformation, selection and regeneration process into a simple and efficient procedure, the leaf disc transformation system permits reproducible examination of *A. tumefaciens* mediated gene transfer into cells in the disc [66], [68]. It also provides a means of quantitatively comparing differences in transformation efficiency between various vectors.

Sterile leaf discs are infected with the appropriate strain of *A. tumefaciens* carrying the vector of choice, and cocultured on regeneration medium for 2 days. During this time the virulence genes in the bacteria are induced by chemicals produced by the wounded plant tissue. The bacteria bind to the plant cells around the wounded edge of the explant and the gene transfer process occurs. After transformation has occurred, the leaf discs are transferred to regeneration/selection medium containing 500ug/ml cefotaxime to kill the bacteria, and the appropriate antibiotic (usually kanamycin) to inhibit the growth of untransformed plant cells. Hormones are added to the medium, and during the next 3 weeks the transformed cells

grow into callus or differentiate into shoots via organogenesis. Between 3 and 6 weeks after infection the shoots develop enough for them to be removed from the explant, and for induction of rooting in preparation for transfer to soil [65].

Foreign gene expression in transformed callus can be analysed in a number of ways. Because the T-DNA vector used in this work contained a chimaeric NPT-II gene to enable the selection of kanamycin resistant plant cells, the expression of this dominant selectable marker gene was monitored in transformed plant cells in two ways: (i) by the ability of transformed plant tissue to form callus on kanamycin-containing medium; and (ii) by direct assay for NPTII activity in a crude plant extract.

The NPT-II assay generally used is the one described by Reiss *et al* [105]: this allows quantitation of small amounts of NPT-II (down to 1ng) in crude cell extracts. After non-denaturing polyacrylamide gel electrophoretic separation of the NPT-II enzyme from interfering proteins, the activity of the enzyme is assayed by *in situ* phosphorylation of kanamycin using γ^{32}P-ATP. NPT-II converts kanamycin into its ^{32}P-labelled derivative, which is immobilised on phoshocellulose paper and visualised by autoradiography. The main advantages of this method are that many samples can be analysed simultaneously, the enzymatic activity can be quantified without additional purification, and biochemical alterations or inactivation of NPT-II enzymes can be

identified. Disadvantages of the assay include: it is time-consuming, complicated and involves the spreading of large amounts of radiolabelled ATP on the gel.

A dot-blot assay for the detection of NPT-II activity in transformed plant tissues was described by McDonnell *et al* [90]. It is a sensitive assay and can be used with a variety of plant species and tissue types. However, it was found in preliminary experiments that the method often produced non-specific positive results.

The method eventually adopted was the thin layer chromatography method developed by Cabanes-Bastos *et al* [11]. It is a simple, safe and rapid assay method for NPT-II based on the chromatographic separation of kanamycin phosphate from the rest of the components in a reaction mix of NPT-II with Km and γ^{32}P-ATP on polyethyleneimine (PEI) cellulose plates. The method offers greater sensitivity than assays currently in use, it is faster and easier and with less radioactive hazard than the others. The method can also be quantitative by cutting out the spots from the chromatographic plate and measuring the amount of radioactivity by scintillation counting. The one disadvantage of the TLC-NPT-II assay is that it does not allow the determination of the molecular weight of the protein, although this information is not necessary for routine assays [11].

Southern blot hybridization of total plant DNA with α-^{32}P-labelled MSV-PE was performed to demonstrate integration of MSV DNA into the plant chromosomal DNA. Although integration of pGSJ280 (and hybrid pGSJ280 carrying MSV genes) into *A. tumefaciens* was shown in Chapter 2, this could not be done for plasmid pBIN19 as it is a binary vector. Sensitive detection methods are necessary to allow for identification of what may be a single-gene copy of foreign DNA integrated into the plant nuclear genome. Multiple insertions are also likely; results under these conditions are expected to vary from the single-gene copy Southern blot results. A more intense radioactive signal would probably be observed.

Immunoelectroblotting (Western blotting) and ELISA (enzyme-linked immunosorbent assay) techniques were used to test expression of MSV genes at the translational level in transgenic tobacco: both techniques rely on enzyme amplification of immobilised and specifically-bound antibodies for the sensitive detection of proteins. Western blotting is a good means of both characterising antigens by molecular size as well as seroligical reactivity; double-antibody sandwich (DAS-) ELISA is a very sensitive means of detection of specific proteins in complex mixtures [107a] ; [107c].

3.2 METHODS

3.2.1 Bacterial strains

A. tumefaciens C58C1Rif (pGV2260) and transconjugants of this strain with pGSJ280 and pBIN19 were used in plant transformations. Table 2 gives details of the strains used.

TABLE 2:

AGROBACTERIUM STRAINS USED FOR LEAF DISC CO-CULTIVATION

STRAIN	CODE
A. tumefaciens C58C1Rif (pGV2260)	A
A. tumefaciens pGV2260 :: pGSJ280	B
A. tumefaciens pGV2260 :: pGSJ280 (MSV-PE) sense orientation	C
A. tumefaciens pGV2260 :: pGSJ280 (MSV-PE) antisense orientation	D
A. tumefaciens pGV2260 :: pGSJ280 (MSV-PE) 1.5 mer	E
A. tumefaciens pGV2260 :: pBIN19 (MSV-N) tetramer	F

3.2.2 Plant material

Leaf explants removed from *Nicotiana* tabacum cv Petit Havana SR1 were used as the starting material for plant transformation. The tobacco plants were grown as sterile shoot cultures on A1 medium (Appendix C.1), at 22°C in a 16 hours light /8 hours dark cycle with low light intensity (cool white fluorescent tubes, 65W). At approximately 6 week intervals, stems were cut into nodal sections containing an axillary bud and transferred to fresh A1 medium.

3.2.3 Leaf disc co-cultivation

The leaf disc transformation method was essentially that described by De Block et al [24]. All plant work was performed under sterile conditions in a laminar flow bench. Leaves of mid-maturity (6-8 weeks after transfer on A1 medium) were excised from sterile SR1 tobacco plants. The midrib was removed and leaves cut into segments of 0.25 to 1.0 cm^2 using a sterile scalpel. Approximately 12 segments were placed upside down in Petri dishes containing 10ml infection medium A2 (Appendix C.2). Twenty five μl of a late log culture of *A. tumefaciens* strains A-F described in Table 2, as well as an uninoculated control, grown in bacterial MinA medium (Appendix B.10) was added. The cultures were incubated in Petri dishes for 2 days in low light intensity before the medium was removed and leaf discs washed twice with infection medium A2 (Appendix C.2) containing 500mg/l cefotaxime. Each wash was for at least an hour to ensure killing of *A. tumefaciens*. The leaf discs were then blotted dry on sterile filter paper and put onto shoot-inducing medium A3 (Appendix C.3) containing kanamycin (100mg/l) to select for the growth of transformed calli.

The leaf pieces were subcultured onto fresh A3 medium weekly. After about 5 weeks, regenerating calli were transferred to A4 medium (Appendix C.4) under selection to allow for shoot induction. Two to three weeks later, shoots were transferred to rooting medium A5 without selection (Appendix C.5).

3.2.4 NPT-II assay

The detection of NPT-II gene expression in crude plant and bacterial extracts was done by the thin layer chromatography (TLC) method described by Cabanes-Bastos *et al* [11]. All extractions were carried out at 4°C. Bacterial crude extracts were prepared by centrifugation of a 1ml overnight culture; the cell pellet was resuspended in 200μl of extraction buffer (0.5M sucrose; 0.1M TrisHCl; 0.1% ascorbic acid; 0.1% cysteine HCl pH7.5) and sonicated for 4 x 10 seconds. After centrifugation for 10 min in a microfuge, the supernatant was used without further purification. Bacterial controls used included: *E. coli* containing pRK2013 [41] as a positive NPT-II control, and *E. coli* containing pGSJ280 [31] as a negative control.

Fresh untransformed *N. tabacum* cv Petit Havana SR1 leaves and transgenic tobacco calli were ground with a pointed glass rod in the extraction buffer described above (1ml buffer/1g tissue) in the presence of aluminium oxide. They were then sonicated for 4 x 10 seconds before being centrifuged in a microfuge for 10 min. The supernatant was used without further purification.

The reaction mixture contained 10μl reaction buffer (67mM Tris-malate pH7.1; 42mM $MgCl_2$; 400mM NH_4Cl; 1.67mM DTT); 10μl crude extract (1-10ug protein); 2μl Km sulphate (1mg/ml); 3μl ATP (200μM); 7.5μl H20 and 0.3μl [γ-^{32}P] ATP (20mCi/ml). The components of the reaction were mixed and

incubated for 30 min at 37°C before application to PEI-cellulose F plates (Merck).

The PEI-plates were pre-treated by developing them in 5M NaCl. Excess NaCl was removed by successive washes with dH_2O (3 x 10min), and the plates were finally air-dried.

One μl aliquots from the reaction mixture were applied to the plate, which was then developed in a 50mM Na formate/formic acid buffer pH 5.4. Once the solvent front had reached the top (+- 14cm), the PEI-plate was air-dried prior to autoradiography overnight at -70°C.

3.2.5 Total DNA extraction for Southern blot analysis

Total plant DNA extraction for the molecular analysis by Southern blot hybridization of plasmid DNA integrated into the plant genome was performed essentially according to the method described by Deblaere et al [31]. Plant material (1-2g) from control *N. tabacum* cv Petit Havana SR1 leaves and from transgenic callus was frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle and then transferred to a 50ml centrifuge tube. Extraction buffer (100mM Tris-HCl, pH8.0; 50mM EDTA pH8; 500mM NaCl; 10mM β-mercaptoethanol) (15ml) was added, followed by the addition of 10ml of 20% (w/v) SDS; the solution was mixed vigorously and then incubated for 10 min at 65°C. Five ml 5M K-acetate was added, the solution mixed vigorously and then incubated for 20 min on ice. Centrifugation for 20 min at 15 000rpm in a Sorvall SS34 rotor followed. The

supernatant was filtered through a layer of cheesecloth and collected in a new SS34 centrifuge tube. Ten ml of isopropanol was added, the preparation mixed and then incubated for 30 min at -20°C. The DNA was pelleted at 15 000rpm for 15 min and the supernatant removed. The DNA pellet was resuspended in 0.7ml TE (50mM Tris-HCl, pH8; 20mM EDTA, pH8) and transferred to a microfuge tube wherein it was centrifuged for 10 seconds to remove non-soluble debris. The supernatant was transferred to a new microfuge tube, 20µl of a 10mg/ml RNase solution was added, and incubation at 37°C for 10 min followed. One hundred µl of Tris-equilibrated phenol was added to the DNA solution which was carefully mixed and centrifuged for 10 min. The supernatant was transferred to a new tube, 200µl chloroform-isoamyl alcohol (24:1) was added, the contents of the tube mixed and then centrifuged for 5 min. The aqueous phase was transferred to a new tube and the chloroform extraction repeated. Seventy µl 3M Na-acetate was added and then 500µl isopropanol. The tube was mixed gently, the DNA centrifuged for 30 seconds, the pellet washed in 70% ethanol and then dried. The pellet was finally resuspended in 100µl TE.

Twenty µg of total plant DNA was digested with *Bam*HI and loaded on a 0.9% agarose gel. After gel electrophoresis, the DNA was transferred by blotting onto a nylon filter (Hybond N+, Amersham International) and subjected to Southern hybridization [112] using a ^{32}P-labelled MSV-PE probe, as outlined in Appendix A.7. Precautions taken to ensure high labelling efficiency for single-gene copy

detection included high probe activity (at least 10^7 cpm), the use of an intensifying screen in the X ray cassette and an exposure of three days at -70°C as compared to overnight exposures that are generally employed.

3.2.6 Plant protein extraction

Approximately 200mg of plant material was ground using a glass rod in 200µl extraction buffer (50mM Tris-HCl pH6.8; 1% β-mercaptoethanol) in an Eppendorf vial kept on ice. The preparation was vortexed briefly, spun in a microfuge at 4°C for 5 min and the supernatant then transferred to a fresh Eppendorf vial. The protein content was measured in the Bradford Bio-Rad protein assay [8a] against a standard curve obtained using a range of bovine serum albumin (BSA) concentrations diluted in extraction buffer.

For protein concentration determination, 4µl of extract (or BSA) was mixed in distilled H_2O to a final volume of 400µl. One hundred µl of Bio-Rad dye was added and the absorbance was measured on a Beckman Du-40 Spectrophotometer at 595nm.

3.2.7 Western Blotting

3.2.7.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Forty µg of plant protein extracts were mixed with an equal volume of loading buffer mix (0.1% bromophenol blue; 5mM EDTA, 200mM Tris pH8.8, 1M sucrose) and heated at 100°C for 5 min. SDS-PAGE was performed by the method of Laemmli (1970) [80a], using a Hoefer SE-600 vertical slab gel

apparatus (Hoefer Scientific Instruments). Gel sandwiches (1.5 mm thick) consisted of a 12.5% resolving gel and a 4.5% stacking gel. Electrophoresis was carried out with a constant current of 35mA. When the tracking dye front had reached the bottom of the gels, the molecular weight marker lane was excised and stained by immersion in 0.2% (w/v) Coomassie brilliant blue R-250 (BDH Chemicals) dissolved in a 45:45:10% (v/v/v) mixture of methanol, water and glacial acetic acid. Destaining of gels was by diffusion in a 25:65:10% (v/v/v) methanol / water / glacial acetic acid mixture. The rest of the gel was then immunoelectroblotted.

3.2.7.2 Electroblotting

The electroblotting procedure described by Rybicki and von Wechmar (1982) [107c], an adaptation of the method described by Towbin *et al* (1979) [118], was used with the electrodes being used horizontally instead of vertically.

An assembly consisting of two 1cm thick carbon electrodes, two wads of nappy liners, filter paper, gel and nitrocellulose sheet all soaked in blotting buffer (0.375M Tris-HCl pH 8.3, 0.192M glycine, 20% methanol) was set up as follows: a wad (1cm thick) of nappy liners was laid on the positive electrode, followed by 2 sheets of 3MM paper, a sheet of nitrocellulose membrane, the polyacrylamide gel, another layer of nappy liners and lastly the second carbon (negative electrode). Transfer was allowed to proceed for 1 hour with a current of approximately 1 ampere.

3.2.7.3 Indirect immunoassay

After electroblotting, the nitrocellulose was soaked in 5% milk powder in PBS (Appendix B.17) at room temperature overnight to saturate free protein-binding sites. The filter was rinsed for 10 min in 1% milk powder in PBS and then soaked for 2 hours at 37°C in a 1/250 dilution of rabbit anti-MSV antiserum in 1% milk powder / PBS. The antiserum used was raised in rabbits by Professor M B von Wechmar, Microbiology Department, University of Cape Town, as described by von Wechmar and Milne [122a]. The filter was washed in PBS-0.1% Triton X-100 for 3 x 15 min at room temperature before addition of a 1/3000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate in 1% milk powder in PBS. Incubation was for 2 hours at 37°C. Another wash of 3 x 15 min in PBS with 0.1% Triton X-100 followed. Freshly-made staining solution [15mg nitro blue tetrazolium chloride (NBT) in 500μl 70% N,N-dimethylformamide; 7.5mg 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP) in 500μl 100% N,N-dimethlyformamide, 49ml carbonate buffer (0.1M $NaHCO_3$ + 10mM $MgCl_2$) pH9.8] was poured over the filter. After the colour reaction appeared, the filter was rinsed in dH_20 and dried between 2 sheets of Whatman 3MM paper in the dark.

3.2.8 Enzyme-linked immunosorbent assay (ELISA)

The method followed was that of Rybicki and von Wechmar (1982a) [107b].

A standard ELISA microtitre tray (Nunc, Denmark) was used. Reaction volumes of 100μl were used for all steps, except for a 200μl substrate reaction volume. Coating antibodies (1mg/ml) were diluted 1/250 in 0.1M Tris-HCl pH 7.4. Antigens and conjugate were diluted in PBS pH 7.4 containing 0.05% Tween-20 and 0.5% milk powder. The plate was coated with IgG purified from an MSV-specific rabbit antiserum [15] and incubated for 2-3 hours at 37°C in a humid box. Three washes of 5 min each in saline-Tween (PBS containing 0.05% (v/v) Tween-20) preceded a blocking procedure for 30-60 min at 37°C. Blocking buffer was removed and four 5-fold dilutions of Ag were added in duplicate. Incubation overnight at 4°C followed. The wells were then washed three times as before and the conjugate added. The MSV-specific conjugate was made as described by Clarke *et al* [15]. A 2-3 hour incubation at 37°C followed, before another wash. Substrate (1mg/ml) 4-nitrophenyl phosphate in 10% diethanolamine pH9.8 was added and monitored at 405nm using a Titertek Multiskan 8-channel automatic read-out spectrophotometer (Type 314, Flow laboratories).

3.3 RESULTS

3.3.1 Tobacco transformation

Changes in leaf discs co-cultivated with *A. tumefaciens* were observed from about 2 weeks post-infection. Leaf discs became thicker and appeared pale green to yellow in colour progressing towards callus formation at leaf edges after 5 weekly transfers onto fresh A3 medium. Uninoculated

control leaf discs, and those inoculated with strain A (Table 2), *A. tumefaciens* C58CiRif (pGV2260), as well as leaf discs inoculated with an *E. coli* control failed to produce callus and eventually died. The rate and occurrence of callus formation varied from one leaf disc to another: some discs from one plate developed prolifically whereas others failed to regenerate at all. It was concluded that the callus produced by experimental leaf discs was transgenic due to its resistance to levels of 100mg/l kanamycin.

Callus formation was observed where leaf discs had been co-cultivated with *A. tumefaciens* strains containing pGSJ280, pGSJ280 with MSV-PE in the sense orientation, pGSJ280 with MSV-PE in the antisense orientation, pGSJ280 with a 1.5 mer of MSV-PE and pBIN19 with a MSV-N tetramer (strains B-F in Table 2). On A3 medium, callus growth consisted of an undifferentiated mass of plant tissue whereas on A4 medium, noticeable shoot development was observed. Roots began to develop on A5 medium.

Figures 14, 15, 16 and 17 depict different stages in the development of a transgenic tobacco plant from an infected leaf disc.

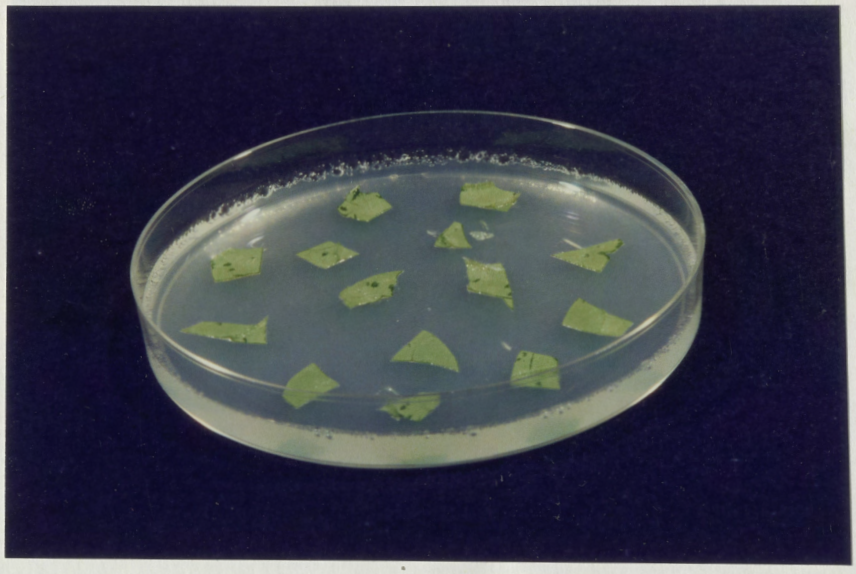

FIGURE 14:

TOBACCO LEAF DISCS ON MEDIUM A3

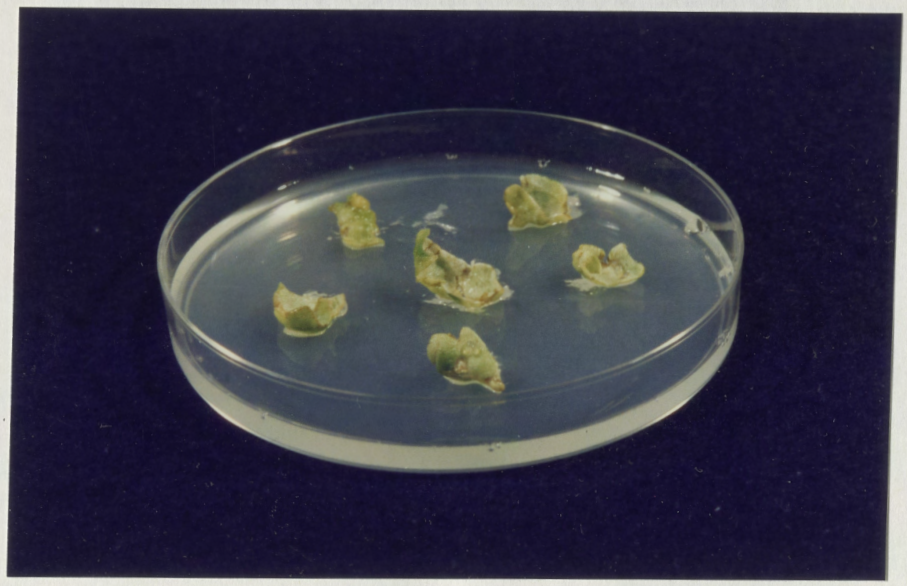

FIGURE 15:

LEAF DISCS APPEARING THICK AND PALE, SHOWING THE FIRST SIGNS OF CALLUS FORMATION

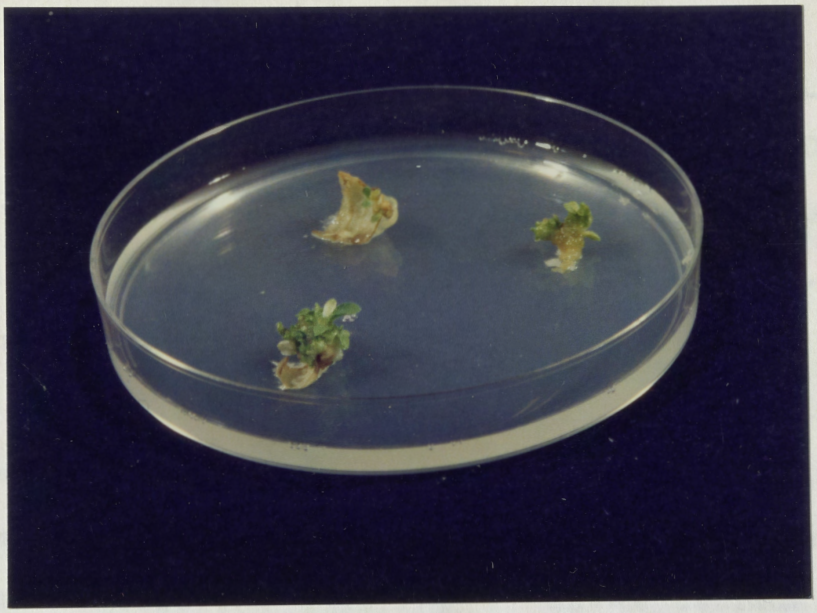

FIGURE 16:

DEVELOPING CALLI GROWING ON A3 MEDIUM SHOWING A MASS OF LEAF TISSUE

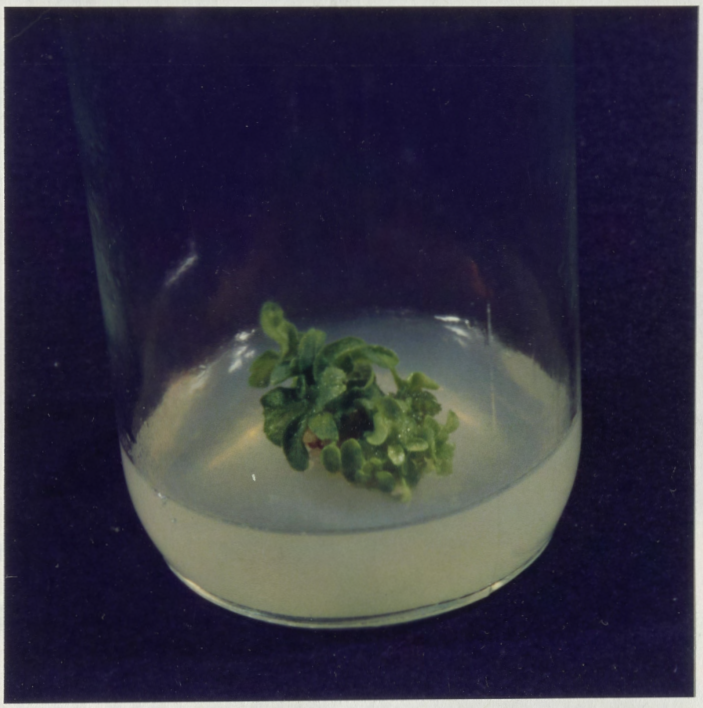

FIGURE 17:

CALLUS TRANSFERRED TO A4 SHOOTING MEDIUM

3.3.2 NPT-II gene expression in transformed tobacco

Extracts of a bacterial positive control, pRK2013 bacterial negative control, pGSJ280, leaves from sterile *N. tabacum* cv Petit Havana SR1, callus transformed with *A. tumefaciens* containing a MSV-PE monomer in the antisense orientation, callus transformed with *A. tumefaciens* containing an MSV-PE monomer in the sense orientation, callus transformed with *A. tumefaciens* carrying a MSV-PE 1.5mer, callus tranformed with *A. tumefaciens* carrying an MSV-N tetramer and callus transformed with *A. tumefaciens* pGV2260:: pGSJ280 were subjected to an NPT-II assay using the TLC method devised by Cabanes-Bastos et al [11]. Results are depicted in Figure 18.

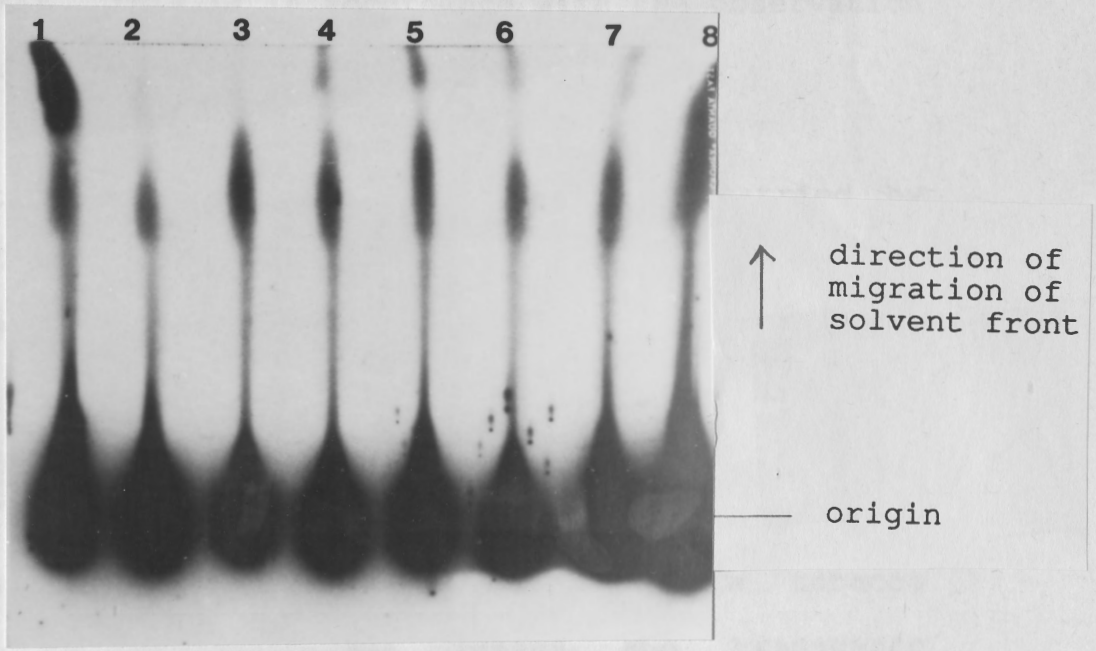

FIGURE 18:

AUTORADIOGRAPH OF TLC-NPT-II ASSAY

Lanes (1) *E. coli* pRK2013
(2) *E. coli* pGSJ280
(3) leaf tissue from sterile *N. tabacum* cv Petit Havana SR1 plant
(4) callus transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE 1mer) reverse orientation
(5) callus transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE 1mer) correct orientation
(6) callus transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE 1.5mer)
(7) callus transformed with *A. tumefaciens* pBIN19 (MSV-N tetramer)
(8) callus transformed with *A. tumefaciens* pGV2260::pGSJ280

Because γ^{32} P-ATP is used as a substreate for the phosphorylation of a variety of other compounds (eg. proteins) by other enzymes there is an accumulation of phosphorylated contaminents in the assay. However, these remain at the origin. The spot corresponding to Km phosphate appears as the solvent front. The signal for the bacterial positive control (1) is notably stronger than that for plant extracts. The bacterial negative control (2) and the plant negative control (3) are as expected, ie. no expression of NPT-II. Positive hybridizat]ion was observed for samples 4-8. This is in accordance with the observation of calli production on kanamycin-containing media.

These results indicate that the NPT II gene carried by vectors pGSJ280 amd pBIN19 containing various constructs of MSV genomes was being expressed in transgenic tobacco.

3.3.3 Southern blot hybridization of plant DNA to establish the presence of MSV:

Total plant DNA was extracted from sterile tobacco *N. tabacum* cv Petit Havana leaves and transgenic tobacco calli. Twenty micrograms of DNA from each sample was electrophoresed on an agarose gel and then blotted onto a Hybond N + filter. Hybridization to a ^{32}P-labelled MSV-PE probe with an activity of at least 10^7 cpm preceded autoradiography with

Kodak X-omat AR X-ray film, in a cassette fitted with an intensifying screen, for three days at -70°C. The results of the Southern blot are shown in Figure 19.

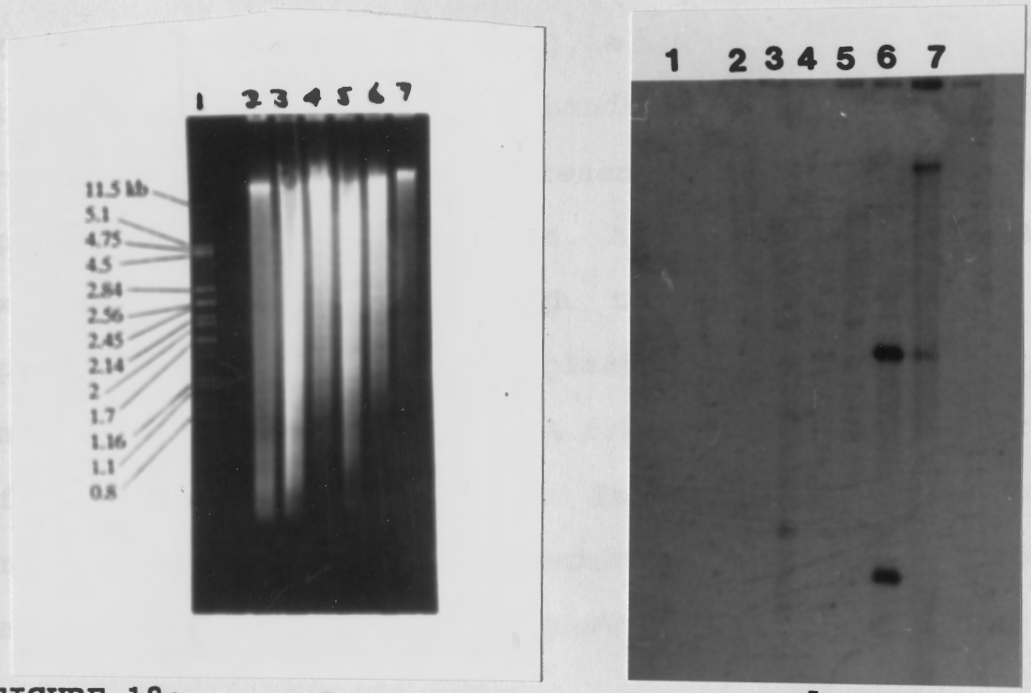

FIGURE 19:

AGAROSE GEL ELECTROPHORESIS OF TOTAL PLANT DNA (A) AND AUTORADIOGRAPH TO INDICATE INCORPORATION OF MSV INTO TOBACCO CALLI (B)

Lane 1: λ*PstI*
Lane 2: *N. tabacum* sterile leaf
Lane 3: calli transformed with *A.tumefaciens* pGV2260::pGSJ280
Lane 4: calli transformed with *A. tumefaciens* pGV2260::pGSJ280(MSV-PE) in antisense orientation
Lane 5: calli transformed with *A. tumefaciens* pGV2260::pGSJ280(MSV-PE) in sense orientation
Lane 6: calli transformed with *A. tumefaciens* pGV2260::pGSJ280(MSV-PE) 1.5 mer
Lane 7: calli transformed with *A. tumefaciens* pGV2260::pBIN19(MSV-N) tetramer

The results depicted in Figure 19 indicate that MSV DNA was successfully integrated into plant DNA in tobacco calli where *A. tumefaciens* carrying MSV-PE in the 1.5 mer form and MSV-N tetramers were co-cultivated with tobacco leaf discs. This is evidenced by the strong positive hybridization signals observed in the 2.7 kb region of the autoradiograph

in lanes 6 and 7 and at the 1 kb position for the 1.5 mer. Negative control sterile leaf tissue exhibited no hybridization to the MSV probe, as expected. Bands of hybridization can be seen for all calli samples, including the sample in which leaf discs were transformed with *A. tumefaciens* pGV2260 (pGSJ280), although these bands are not the same intensity as the bands for lanes 6 and 7. This could be explained by the presence of plant DNA in the MSV probe used for hybridization. Although the probe DNA used was MSV-PE purified through two CsCl gradients, it is possible that contaminating plant DNA sequences were present and recognized homologous DNA fragments in the DNA extracted from the transgenic calli. In this way, false-positive results for the MSV-PE monomer constructs (sense and antisense orientations) may have been attained.

3.3.4 Western Blotting

Western Blotting was used for the immunodetection of MSV coat protein in protein extracts prepared from transgenic calli, and in control *N. tabacum* cv Petit Havana leaves. Antiserum to MSV-PE coat protein was used. The antiserum was found to bind to all extracts, indicating some non-specific activity with plant proteins. To overcome this problem, the antiserum was incubated with a sheet of nitrocellulose "blocked" with healthy maize sap prior to being used for the immunoassay (Ed Rybicki, personal communication). This successfully decreased the background. Figure 20 shows the results of a Western Blot if no preabsorption of the antiserum was performed; Figure 21 shows the outcome if pre-soaking with healthy maize sap was employed.

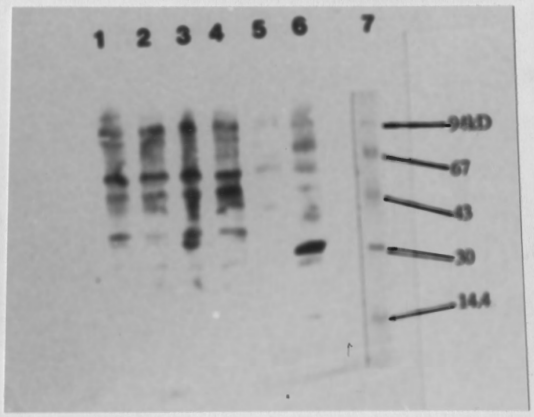

FIGURE 20:

WESTERN BLOT OF PLANT PROTEINS PROBED WITH MSV-PE ANTISERUM

(NO PRE-SOAKING WITH HEALTHY MAIZE SAP)

Lane 1: Calli transformed with *A. tumefaciens* pGV2260::pBIN19(MSV-N) tetramer
Lane 2: Calli transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE) 1.5 mer
Lane 3: Calli transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE) sense orientation
Lane 4: Calli transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE) antisense orientation
Lane 5: Sterile *N. tabacum* cv Petit Havana SR1 leaf
Lane 6: MSV-infected maize leaf
Lane 7: Protein M.W. markers (in kilodaltons, kD)

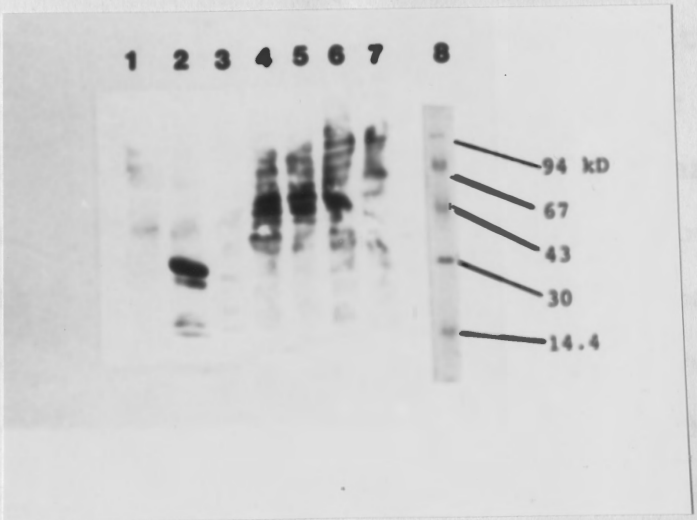

FIGURE 21:

WESTERN BLOT OF PLANT PROTEINS PROBED WITH MSV-PE ANTISERUM

(PRE-SOAKED WITH HEALTHY MAIZE SAP)

Lane 1: Healthy maize sap
Lane 2: MSV-infected maize leaf
Lane 3: Sterile *N. tabacum* cv Petit Havana SRI leaf
Lane 4: Calli transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE) antisense orientation
Lane 5: Calli transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE) sense orientation
Lane 6: Calli transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE) 1.5 mer
Lane 7: Calli transformed with *A. tumefaciens* pGV2260::pBIN19 (MSV-N) tetramer
Lane 8: Protein M.W. markers (in kD)

From Figure 20 it can be observed that antibodies in the antiserum used bind to a range of plant proteins. The effect of pre-incubating the antiserum dilution with healthy maize sap to allow for removal of non-MSV-specific antibodies, can be seen in Figure 21. In both Figures (20 and 21), a band is evident in positive control lanes in close proximity to the 30 kD molecular weight marker, in agreement with size prediction of 21 kD for the MSV coat protein. The negative control - sterile tobacco - shows the background of "non MSV-specific" antibody binding. It should be noted that the plant protein "background" levels for transgenic tobacco are higher than that for control sterile tobacco for the same amount of total protein : this could be due to different patterns of expression of reactive proteins in whole plants and in callus. For all transgenic calli tested, there was no band at the same position as the putative coat protein band in the positive control, suggesting that MSV integrated into tobacco is not translated or alternatively, is expressed but cannot be detected by this technique because of too low a concentration in the extract.

3.3.5 ELISA

After the negative results of the Western blot, DAS-ELISA tests were performed in a further attempt to detect MSV coat protein expression in transgenic tobacco: DAS-ELISA is probably a more sensitive technique for specific detection of antigens than Western blotting [107c]. Conjugated antibodies to MSV-PE were added to a 5-fold dilution series

of the various plant extracts bound to immobilized MSV coat protein antibodies, coated onto a standard microtitre tray. Figure 22 depicts the results obtained between 1 and 2 hours after the addition of the colour substrate. Readings monitored at 405 nm using a Titertek Multiskan automatic spectrophotometer were plotted onto a graph using Harvard Graphics.

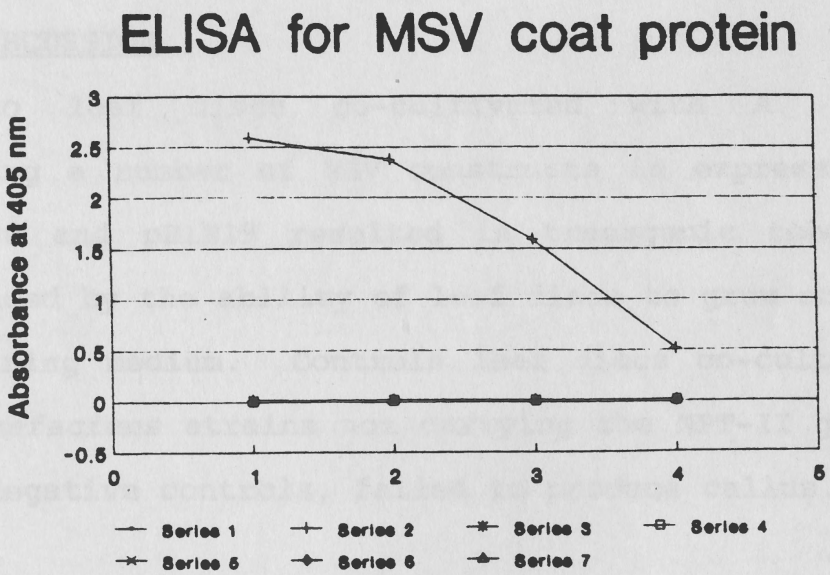

FIGURE 22:

REACTION OF PROTEINS FROM MAIZE AND TRANSGENIC TOBACCO WITH MSV-PE COAT PROTEIN ANTISERUM

Series 1: Healthy maize sap
Series 2: MSV diseased maize
Series 3: Sterile *N. tabacum*
Series 4: Tobacco Calli transformed with *A tumefaciens* pGV2260::pGSJ280 (MSV-PE) antisense orientation
Series 5: Tabacco Calli transformed with *A. Tumefaciens* pGV2260::pGSJ280 (MSV-PE) sense orientation
Series 6: Tobacco Calli transformed with *A. tumefaciens* pGV2260::pGSJ280 (MSV-PE) 1.5 mer
Series 7: Tobacco Calli transformed with *A. tumefaciens* pGV2260::pBIN19 (MSV-N) tetramer

From the results in Figure 22, it can be concluded that the positive control, namely MSV-infected maize, was the only sample exhibiting MSV coat protein expression: leaving the tray for several hours intensified the positive control wells so that they could not be read on-scale on the spectrophotometer; however, the relative absorbances of samples and negative controls did not change (not shown).

3.4 DISCUSSION

Tobacco leaf discs co-cultivated with *A. tumefaciens* carrying a number of MSV constructs in expression vectors pGSJ280 and pBIN19 resulted in transgenic tobacco calli, evidenced by the ability of leaf discs to grow on kanamycin-containing medium. Controls leaf discs co-cultivated with *A. tumefaciens* strains not carrying the NPT-II gene, and *E. coli* negative controls, failed to produce callus.

To confirm expression of the NPT-II gene, a TLC NPT-II assay was performed. The observation that NPT-II enzyme activity in transgenic calli was considerably lower than that found in the positive bacterial control is in accordance with what is expected: since plant cells contain high levels of endogenous ATPase activity, there is a strong competition for the ATP and thus, the amount of Km labelled that is detected is below the actual level of NPT-II present [11].

NPT-II activity was recorded for all transgenic calli - that is, tobacco transferred by *A. tumefaciens* carrying MSV-PE in both the sense and antisense orientations, a 1.5 mer of MSV-PE and a tetraner of MSV-N in addition to control *A. tumefaciens* pGV2260::pGSJ280.

Southern blot hybridization was performed to establish whether MSV genome(s) had integrated into the nuclear genome of transgenic tobacco calli. Results indicated that MSV constructs in which MSV-PE 1.5 mer and MSV-N tetramers were used in *A. tumefaciens* leaf disc co-cultivation with tobacco, resulted in integration of the MSV into the plant nuclear genome. The different hybridization bands produced for other constructs (monomers of MSV-PE in the sense and antisense orientations) are hard to explain, as *Bam*HI is supposed to excise monomeric MSV from the constructs; however, if one assumes that these were partial digestions, the differences in banding indicates that the different size fragments of plant DNA containing MSV were recognised by the probe as being homologous. Alternatively, the different hybridization bands maybe due to MSV integrating at a number of different sites in the tobacco genome. The false positives for the calli transformed with *A. tumefaciens* pGV2260::pGSJ280 indicate that the bands of hybridization seen for the monomers maybe due to plant DNA contamination of the MSV viral DNA probe.

It was found that due to the large size of plant genomes, at least 15 ug of plant DNA, and use of probe DNA radioactively

labelled to at least 10^7 cpm / ug, was necessary to detect the presence of inserted foreign DNA. Blots with less DNA yielded consistently negative results. Moreover, exhaustive probing with a non-radioactively labelled MSV probe failed to identify a positive signal, even when 15-20 ug of DNA was used. It is recommended for all future work in this area that only ^{32}P-labelled probes with a high specific activity (at least 10^7 cpm per ug) be used. In addition, an intensifying screen in the X-ray cassette, sensitive X-ray film and a longer exposure time than that generally used for bacteria, should be employed to obtain satisfactory results [37a].

Western blotting was used in an attempt to detect MSV expression in transgenic tobacco at the translational level. The antiserum used was found to contain antibodies to contaminating plant proteins in addition to those reacting with MSV coat protein. Use of clarified healthy maize sap in a pre-absorption procedure was found to significantly decrease the background hybridization levels. The blots (Figures 20 and 21) do not indicate any expression of MSV activity in transgenic tobacco: no MSV coat protein expression was detected. Western blotting is said to be able to detect as little as 1 ng of virus protein per lane [107c] depending on the virus in question. The results therefore may not be due to the sensitivity of the technique but rather to the very low (if any) levels of MSV coat protein being expressed.

The ELISA test also failed to detect expression of MSV coat protein in transgenic tobacco calli. The only reading above background was that observed for the positive control, MSV-infected maize. The sensitivity of this procedure is in the range of 2-20 ng/ml of sample [107a]. Approximately 20 ug of protein was used for the first dilution in 100 ul, with the protein concentration subsequently decreasing with increased dilution. The concentration of protein used in the assay therefore should be sufficient to detect MSV coat protein expression, if it exists. Thus, it can be concluded that although MSV was shown to integrate into the plant nuclear genome in tobacco calli, expression of MSV at the translational level could not be shown for any of the experimental constructs.

In similar work by Boulton *et al* [8], the MSV coat protein was detected by immunoblotting in extracts of maize plants where MSV had been shown to replicate after agroinoculation. However, immunosorbent electron microscopic examination from these plants failed to show geminate particles, and insect transmission of virus from these plants was unsuccessful.

Lazarowitz *et al* (1989) [83] produced gene replacement mutants, in which the coat protein gene of MSV was replaced by chloramphenicol acetyl transferase (CAT) as a reporter gene in a plant expression vector (MSV-CPΔ-CAT). Inoculations of maize seedlings and subsequent testing for expression of the bacterial CAT gene revealed that extracts of innoculated leaves which contained excised, freshly

replicating MSV-CPΔ-CAT DNA, exhibited CAT activity whereas inoculated leaves in which excised MSV-CPΔ-CAT DNA was not detected did not contain enzymatic activity above background levels. Activity above background was not detected in inoculated leaves containing MSV-CPΔ-CAT in which the CAT sequences were inserted in inverted orientation. The low levels of CAT activity detected are reported to be the consequence of the low number of cells initially infected. This explanation may be relevant in explaining the negative results obtained in this study for Western blot and ELISA assays - namely, no detectable expression of MSV coat protein expression may be due to a low number of cells initially infected.

Many factors may influence gene expression in transformed plants. Amongst the most important factors are the position and the number of integrations of the foreign sequence within the plant genome. The development stage of the plant, light intensities and additives such as sucrose and hormones included in the culture media may also be influencing factors [59a].

Position effects, that is, effect of the surrounding DNA or chromatin structure, result in differences in expression of the T-DNA genes in different transformants [65]. The extreme of this is complete loss of expression during differentiation of the plantlet. One possible reason for

failure to select against these escapes is that kanamycin is not a good herbicide, and shoots can continue growth in its presence once they are large enough, necessitating screening of several independent transgenic plants to identify maximal expression of the foreign gene of choice which is not always correlated with expression of the selectable marker [65].

All MSV constructs used in this project were cleaved at the *Bam*HI site of MSV and ligated to the *Bam*HI site of the vector (pGSJ280 or pBIN19). The genomic organization of MSV is such that transcription is bidirectional from the promoter region located near the *Bam*HI site. (Refer to Figure 3, Chapter 1). The 10.9 kD protein has no ATG initiation codon and since the coat protein gene is approximately 300 bases downstream, it is possible that the cpg doesn't get translated off the transcript.

Alternatively, the ribosome scanning model described by Rogers *et al* [106a] proposes that during plant gene expression, the ribosome attaches to the 5' end of the mRNA and moves along scanning for the first translational initiation signal (AUG) in the sequence. Translation is then initiated, and a protein is assembled. One prediction of this model is that AUG signals in the mRNA preceding the AUG initiator of the protein of interest will impair the expression of that protein by causing translation of a nonsense protein and decrease the frequency of correct starts. This may be a means of explaining the results obtained in this project:- no expression of MSV coat protein at the translational level.

CHAPTER 4
GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this project was to clone MSV genes via *A. tumefaciens* into tobacco to produce transgenic plants. To this end, one genome of MSV-PE in the sense and antisense orientations, and a 1.5 mer of MSV-PE, were cloned into a co-integrate plant expression vector, pGSJ280. Tetramers of MSV-N in the binary vector plasmid pBIN19, were supplied. All constructs in *E. coli* were mobilized into *A. tumefaciens* C58CIRif (pGV2260) in a triparental mating procedure using *E. coli* pRK2013 as a mobilizing strain. Southern blot hybridization verified the presence of MSV DNA in *A. tumefaciens* (for MSV-PE constructs in pGSJ280 only).

Plant transformation of *N. tabacum* cv Petit Havana leaf discs by the various *A. tumefaciens* transconjugants resulted in growth of kanamycin-resistant calli, indicating activity of the selectable marker, NPT-II. NPT-II activity was confirmed using a TLC method, by which all experimental tobacco calli yielded positive results. Southern blot hybridization of total plant DNA showed that MSV DNA had been integrated into tobacco nuclear genome for the calli transformed using the MSV constructs. Western blot and ELISA protein assays were used to investigate expression of MSV coat protein in transgenic tobacco. Results were negative for all experimental calli, indicating that MSV coat protein was not expressed in transgenic tobacco, or alternatively, could not be detected due to very low-level expression.

It had been hoped to test the potential of constructs made for the induction of MSV resistance in tobacco as a model system, with the ultimate goal of being able to produce maize (and other grasses) which would confer resistance to MSV infection. The approach considered was that used for protection against tobacco mosaic virus (TMV) in transgenic plants that express TMV coat protein (cpg) [1] or its antisense [102]. Antisense protection was found to be at a substantially lower level than cpg-mediated protection. It has been suggested that since the mechanisms of the two forms of protection are different, it may be possible to increase the number of plants that escape infection by expressing in plants both the cpg and its antisense [102] in a dual "resistance cassette".

The mechanism involved in the cpg-mediated protection observed in transgenic plants is still unkwown, although it is thought that this is achieved by prevention of uncoating of incoming virus preventing systemic spread whereas the antisense model proposes that antisense RNA binds to the 3' end of incoming viral DNA, possibly during uncoating of the virus, thereby preventing negative-strand synthesis. The **actual** mechanism(s) of cross protection need to be explored further to augment our knowledge of the initial steps of viral infection, and will facilitate the development of virus-resistant plants. To date, this has not been achieved for MSV but experimental work such as that done recently by Boulton *et al* [8] and Lazarowitz *et al* [83] to

identify the genes in MSV essential for systemic spread and symptom development, coupled with work done on other viruses with similar strategies, offers hope for producing MSV-resistant crops, albeit some time in the future.

SECTION B

CHAPTER 5

PURIFICATION AND MAPPING OF A SEVERE KOEDOESKOP ISOLATE OF MAIZE STREAK VIRUS (MSV)

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5.1 INTRODUCTION

The characterization of various maize streak virus (MSV) isolates has allowed studies on their divergence and evolutionary relationship [69], [78]. Serological studies may be used to show relatedness (similar or distinct), of different isolates [32], [99], although this is not a quantitative means of comparing genome structure or evolution. Clarke *et al* [15] and Kirby *et al* [78] have shown that evolutionary distances can be estimated from comparisons of restriction enzyme cleavage maps of isolates without it being necessary to sequence individual isolates [119].

The restriction maps of three South African isolates of MSV were compared to those of published sequences of other MSV isolates from Nigeria [96], Kenya [70], and Potchefstroom, South Africa [82]. It was found that all 6 viruses analyzed have identical *Bam*HI and *Bgl*II sites when their maps are aligned with the *Bam*HI site as map position 0. They also all have very similar core regions around position 1200, with a unique *Bgl*II site and a doublet of *Pvu*II at positions 1200 and 1300 [15]. There are other regions of similarity shared amongst some isolates but not conserved in all isolates.

A new isolate of MSV, MSV-Koe1, was collected by Professor M B von Wechmar of the Microbiology Department, UCT, from severely infected popcorn in the Koedoeskop area (north-west of Warmbaths, Transvaal, South Africa). Viral DNA was isolated from the diseased maize leaves and subjected to restriction endonuclease analysis in order to determine its relatedness to other MSV isolates from other locations in Africa. This data can be used to predict the degree of divergence of the different MSV isolates whose restriction maps are known.

Preliminary characterization of MSV-Koe1 revealed a single *Bam*HI site (conserved amongst MSV isolates, see [15]), thus allowing the virus to be cloned into the multiple cloning site of *E. coli* vector pUC19. Plasmid pUC19 is often used as the vector of choice in cloning experiments because of its numerous advantages. These include: The large number of unique cleavage sites in one polylinker region into which restricition fragments can be inserted, and the fact that vectors with inserted DNA are easily detected after transformation of *E. coli* host strains: when plated on solid media containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), *E. coli* cells containing recombinant vectors form white colonies whereas cells containing non-recombinant pUC vectors form blue colonies on X-gal plates. In addition, the small pUC plasmids can easily be introduced into *E. coli* cells by transformation even when they contain large DNA inserts, and the plasmid replicates to a high copy number [97], [122], [129].

Cloned genomic Koe1 DNA as well as the double-stranded replicative form (RF) of MSV-Koe1 were used to map the isolate. The map obtained was then compared with that of other MSV isolates.

5.2 METHODS

5.2.1 Total DNA extraction from virus-infected leaves

A modification of the method by Ikegami *et al* (1981) [71] - as described by Clarke *et al* [14] - was used. Virus-infected leaves were cut into a stainless steel bowl and frozen with liquid N_2 for about three minutes. The leaves were then ground for about 20 seconds in a coffee-grinder to a fine powder. The powder was then resuspended in enough leaf grinding buffer (0.1M Tris-HCl pH 7; 0.1M NaCl; 0.1M EDTA; 1% w/v SDS) to allow a magnetic stirrer to move fairly freely (1 volume). This was stirred for 5 minutes and then filtered through one layer of cheesecloth into an SS34 centrifuge tube. An equal volume of phenol: chloroform (1:1) was added and the tube spun at 10 000rpm for 10 min. One volume of isopropanol was added to the supernatant, which was then left at room temperature for 5 min before being centrifuged at 12 000rpm for 15 min. The pellet was resuspended in 2-3ml of TE buffer (Appendix B.4). Two volumes of 96% EtOH and $^1/_{10}$ volume 4M LiCl were added to reprecipitate the DNA. Centrifugation at 12 000rpm for 15 min followed a cooling period of at least an hour at -20°C, and the final pellet was resuspended in 1ml TE buffer, pH 8.

The crude viral double-stranded (ds) DNA extract was then purified by passing it through a Sephadex column. A 12ml column of Sephadex G-100 equilibrated in TE buffer was prepared in a 10ml syringe. The 1ml sample was loaded and allowed to migrate into the column. It was eluted with 1ml TE aliquots until 20 1ml fractions were collected in individual tubes. The fractions were then scanned at 260nm on a UV spectrophotometer to identify DNA-containing fractions.

A small aliquot from each sample was run on agarose gel to verify the presence of viral DNA. Three fractions were pooled, ethanol and salt precipitated and then resuspended in 100µl TE buffer. Because chromosomal and linear DNA was found to be contaminating the covalently closed circular (ccc) ds viral DNA, the latter (ccc) was excised from agarose by a "freeze-squeeze" method [116].

The 100µl viral DNA preparation was electrophoresed on low melting point agarose in TBE buffer with a λ-*Pst*I molecular weight marker. The ccc band was cut out from the gel (viewed briefly on a longwave UV transilluminator) and transferred to a large (1.5ml) Eppendorf tube. Ten volumes of 0.3M NaOAc; 1mM EDTA pH 7.0 was added. The tube was kept in the dark for 15-45 minutes and inverted occassionally. A small (0.5ml) Eppendorf was prepared by piercing a hole in the bottom with a wide-gauge needle, and then plugging it with a small amount of cut glass wool. A quick spin in a 1.5ml Eppendorf tube with the lid cut off, to pack the glass wool, followed.

The gel slice was placed in the small Eppendorfs, the lids were closed and the vials dropped into liquid N_2 in an ice-bucket and left for about 5 minutes to freeze solid. The vials were removed with tweezers and put directly into (drained) large Eppendorfs and spun for 10-15 minutes in a microfuge. The small vials were discarded, the volume of eluate measured and then a $^1/_{10}$ volume of 3M NaAc and 2 volumes of 96% EtOH were added. The tubes were put at -20°C for an hour and then spun for 10-15 minutes. The final pellet was resuspended in 100μl TE buffer.

5.2.2 Construction of recombinant plasmids

Plasmid pUC19 DNA was extracted by the "maxiprep" method (Appendix A.1) from a 200ml overnight culture grown in Luria Broth (Appendix B.3) supplemented with 100ug/ml ampicillin. pUC19 DNA was digested with *Bam*HI and then ligated to *Bam*HI digested double-stranded replicative form DNA isolated from MSV-Koe1 infected maize in a vector: insert ratio of 1:3 with a DNA concentration of 5pM (H. Zappe, personal communication), as described in Appendix A.3. The recombinant plasmids were transformed into *E. coli* Lk111 competent cells prepared by the DMSO method [13] (Appendix A.6). Aliquots of the expression mix were plated on X-gal media (Appendix B.16) White and blue colonies could be distinguished on the plate after overnight incubation at 37°C.

5.2.2.1 Verification of inserted DNA

"Mini-prep" DNA from white colonies and blue controls (Appendix A.2) was digested with *Eco*RI as there is only one *Eco*RI site in pUC19 and usually no sites in MSV. In this way, parentals could be distinguished from transformants on the basis of size of DNA fragment(s). Digests were electrophoresed on a 0.8% agarose gel in TBE buffer.

5.2.2.2 Confirmation of MSV insertion into pUC19

To confirm that MSV had been cloned into pUC19, a dot blot assay was performed by the method described in the (Amersham) "Membrane transfer and detection methods" manual. A number of "mini-preps", positive control MSV-PE and negative control pUC19 were probed with a ^{32}P-labelled MSV probe.

5.2.2.3 "Maxi-preps" of positive clones

"Maxi-preps" of two of the positive clones of MSV-Koe1 in pUC19 were prepared according to Appendix A.1. They were found to be identical: both inserts were in the same orientation as shown by restriction endonuclease digestions.

5.2.3 Restriction endonuclease mapping

Restriction enzyme digestions of cloned (MSV-Koe1 in pUC19) and ds replicative form (RF) MSV-Koe1 DNA were performed using restriction enzymes and buffers supplied by Boehringer Mannheim. In general, 200ng of DNA was digested with 1u of enzyme in a 20 l volume for 2 hours at 37°C. For double digests, the enzyme with the lowest salt concentration

buffer requirement was used first. At the end of incubation, the DNA was precipitated by the addition of $^{1}/_{10}$ volume 5M Na perchlorate and an equal volume of isopropanol. The reaction mixes were held on ice for 10 min, spun in a microfuge for 20 min at 4°C and the resulting pellet washed in 70% EtOH before being dried in a 42°C oven for 5 - 10 min. Seventeen µl of dH_20 was added to the pellet and the DNA allowed to resuspend during a 30 min incubation period at 37°C. Two µl of the higher salt buffer was then added, together with 1µl (1u/µl) of the second enzyme. A further 2 hours of incubation at 37°C followed. The reaction was finally stopped by the addition of 3µl sample loading buffer (Appendix B.1). The restriction endonuclease map of the Koe1 isolate of MSV was obtained using the following restriction enzymes: *Apa*I, *Bam*HI, *Bgl*I, *Bgl*II, *Cla*I, *Eco*RI, *Hind*III, *Kpn*I, *Pvu*II, *Sac*I, *Sal*I and *Xho*I: this is the panel of enzymes commonly used for mapping MSV isolates [14].

5.2.3.1 Agarose gel electrophoresis

Samples were loaded with a λ-*Pst*I molecular weight marker on 0.8% horizontal 20 x 15cm agarose gels in TBE buffer (Appendix B.5). Electrophoresis was carried out at 40V overnight as described in Appendix A.4.

5.3 RESULTS

5.3.1 Plant viral DNA extraction and purification

The Sephadex column-purified viral DNA preparation was found to consist of a mixture of plant chromosomal DNA, linear viral DNA and covalently closed circular (ccc) viral DNA. The linear band was approximately 2.7kb as observed by its migration through an agarose gel. This size is in agreement with published data on other MSV isolates [14], [70], [82], [96]. The ccc band was extracted from the gel by means of freeze-squeezing.

5.3.2 Construction of recombinant plasmids

During preliminary screening for unique restriction endonuclease sites in the MSV-Koel DNA prep, a single *Bam*HI site was found. This was chosen for cloning of the viral genome into the *Bam*HI site in the multiple cloning region of pUC19 (See Figure 23).

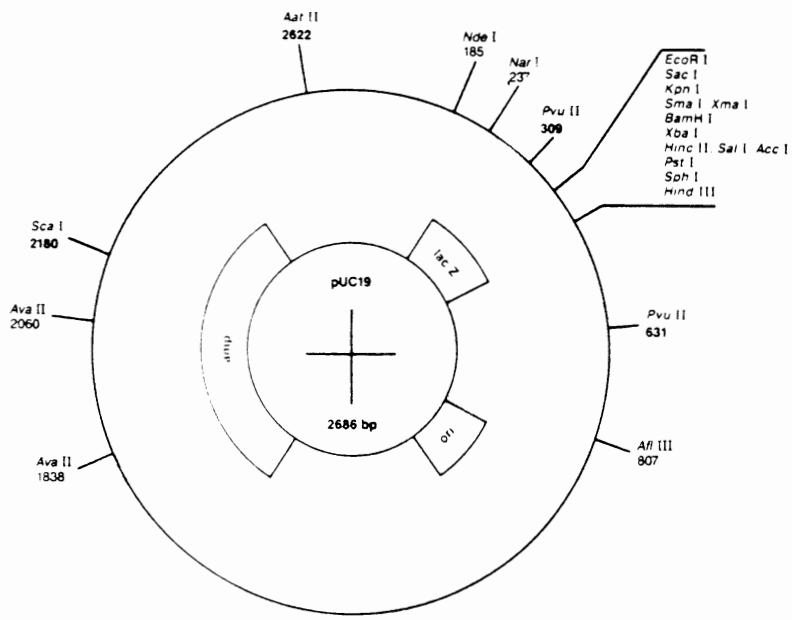

FIGURE 23:

MAP OF VECTOR pUC19 [97]

Cloning into pUC19 followed by plating on X-gal medium was found to be most advantageous as the screening of transformant colonies for inserted viral DNA is minimized due to the colour reaction. Results for the controls included in the cloning experiment: (i) uncut pUC19 to monitor transformation and (ii) cut and religated vector to assess ligation efficiency yielded expected results, viz a lawn of blue-coloured *E. coli* colonies. Of 12 white colonies selected for "mini-preps", all were found to have inserts. To confirm that the insertion of MSV accounted for the increase in band size on an agarose gel, dot blotting was done. This procedure was included because pUC19 and MSV are so similar in size and thus a doubling of the size of a fragment may not necessarily imply insertion of linearised MSV genomic DNA although this is unlikely.

5.3.3 Restriction endonuclease mapping

The map of MSV-Koe1 was obtained by digestion of plasmid or ds RF DNA with single and double digests in various combinations using the 6 base-pair restriction enzymes mentioned under "Methods". All digests were electrophoresed with a ã*Pst*I molecular weight markers in 0.8% agarose gels. A calibration curve was constructed for each gel and sizes of each fragment were accurately calculated.

MSV-Koe1 was not cut with *Cla*I or *Eco*RI. Single sites for *Apa*I, *Bam*HI, *Bgl*I, *Bgl*II, *Sac*I, *Sal*I and *Xho*I were found.

There were two *Pvu*II sites and three *Hind*III sites. The size of MSV-Koe1 is 2.68kb. For the map of MSV-Koe1, see Figure 24 below.

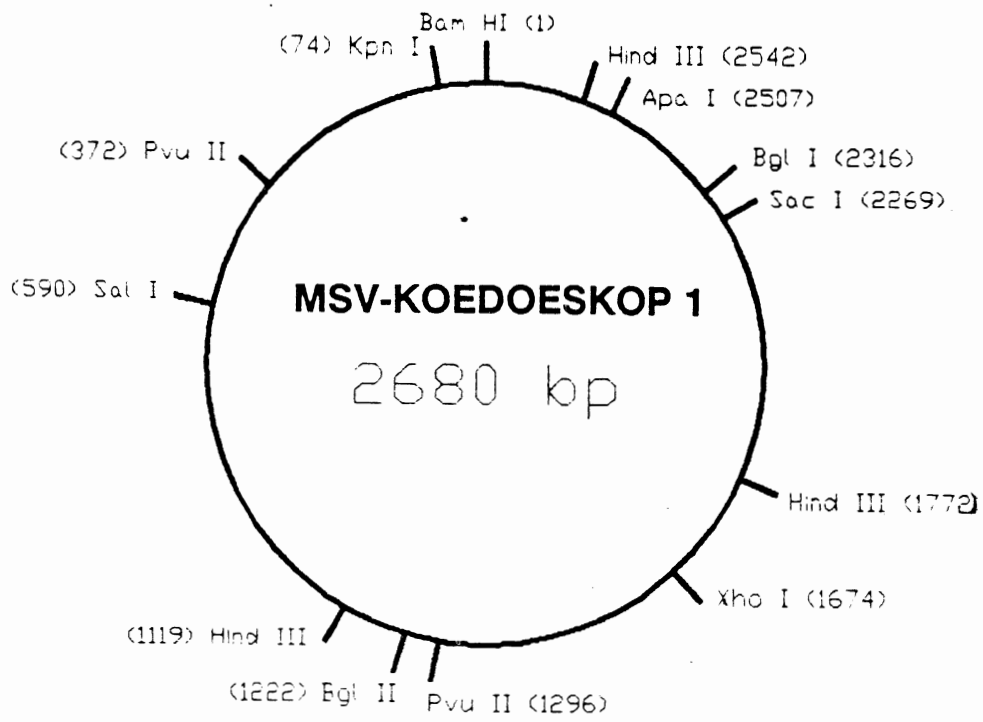

FIGURE 24:

GENETIC MAP OF MSV-KOE1.

The linear map of MSV-Koe1 is shown in Figure 25, where it is aligned with the restriction maps of other MSV isolates at the single *Bam*HI site, assigned position 0. The MSV isolates compared originate from Komatipoort (Kom) [Edge, unpublished], Koedoeskop (Koe1), [this study], Potchefstroom, (S) [82], Kenya (K) [96], Nigeria (N) [7], Port Elizabeth (PE) [14], Potchefstroom (CT) [14], South West Africa (SW) [14] and Riviersonderend (RSE1) [Hughes, unpublished].

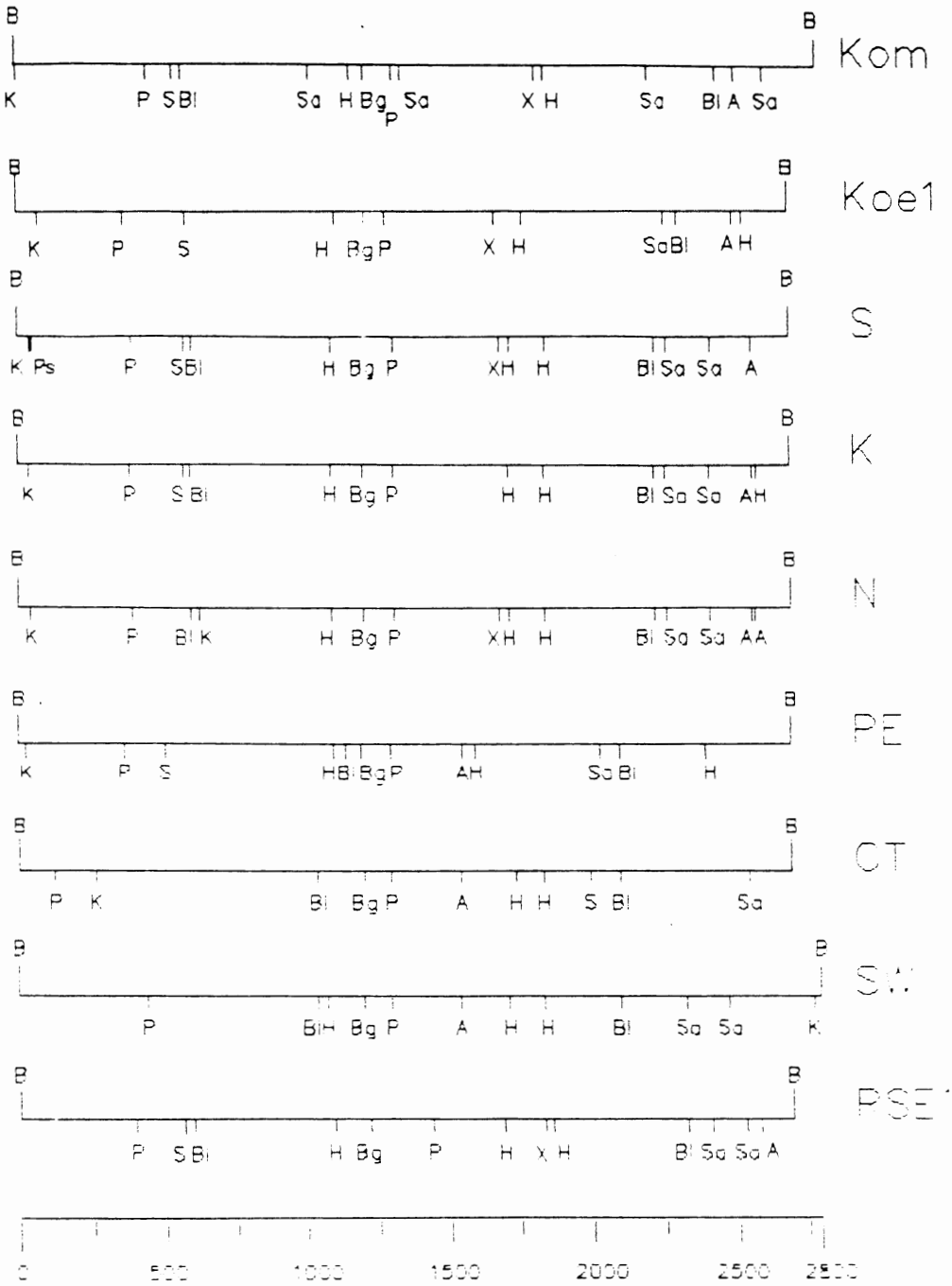

FIGURE 25:

COMPARISON OF RESTRICTION ENDONUCLEASE MAPS OF MSV ISOLATES

(by kind permission of E P Rybicki and R Kirby)

Noticeable differences as well as similarities were found between the restriction maps of the MSV isolates compared. Differences include both the position of the restriction sites on the genome as well as their frequency.

5.4 DISCUSSION

Comparison of the restriction map of MSV-Koe1 with those of other MSV isolates reveals some interesting similarities and differences. All isolates analyzed to date have identical *Bam*HI and *Bgl*II sites. The most conserved area centres around the 1000-1300 bp region with a *Hind*III and *Pvu*II site of either side of *Bgl*II. Isolate CT is an exception in this regard. The positon of *Kpn*I, *Pvu*II and *Sal*I sites in the region 0-600bp tends to vary although there is some conservation of the *Pvu*II site at position 350 in 6 of the 9 isolates. The area between positions 1700 and 1800 bp tends to have two *Hind*III sites, although in the case of the Kom and Koe1 isolates, one *Hind*III site is replaced by an *Xho*I site. Isolate PE does not follow the trend of the *Hind*III doublet. Map positions 2000-2700bp shows a great deal of variability. Restriction sites found to occur in this area include: *Sac*I, *Bgl*I, *Apa*I and *Hind*III. There is little conformity in the map position of these restriction sites.

The restriction maps shown in Figure 25 allow the direct comparison of nine MSV isolates by a method which provides individually derived sets of characters for characterization of the individual genomes and does not rely on pairwise comparisons as does serology. Estimations on evolutionary

distances by comparing restriction cleavage patterns of a number of related variant of MSV has been done [78]. The data of new isolates can be added to the pool of results for further investigation into convergence of divergence of variants. From the results obtained by Kirby *et al* [78], divergence appears to be related to geographic isolation and distance, although there are exceptional cases.

Restriction endonuclease mapping thus appears to be a convenient means of typing new isolates of MSV. The method can be used for the analysis of different isolates within: a single plant, an isolated field or a widely dispersed geographical area.

APPENDICES

APPENDIX A:

STANDARD METHODS

A.1 LARGE-SCALE *E. COLI* PLASMID DNA ISOLATION

The method described by Ish-Horowicz and Burke, 1981 [72] was used. A 500ml LB broth culture, grown overnight at 37°C in the presence of the appropriate antibiotic, was harvested by centrifugation in a Beckman JA14 rotor at 10 000rpm for 5 min. The cell pellet was resuspended in 4ml of Solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM Na_2 EDTA) and incubated at room temperature for at least 5 min. Freshly made Solution II (8ml of 0.2M NaOH, 1% (w/v) SDS) was added, mixed thoroughly and the sample put on ice for exactly 5 min. Precooled Solution III (6ml of 5M KOAc, pH 4.8) was added, mixed thoroughly and the tube returned to ice for a further 5-10 min. The lysate was centrifuged in a Beckman JA20 rotor at 10 000rpm for 10 mins at 4°C. The supernatant was removed into clean tubes. An equal volume of isopropanol was added to the fluid and the sample left to stand at room temperature for at least 2 min. The nucleic acid was precipitated by centrifugation (JA20 rotor) at 15 000rpm for 15 min. The pellet was washed with 70% ethanol and then resuspended in 5ml TE buffer. Cesium chloride (1g/ml, DNA solution) and Ethidium-bromide (EtBr) (200µl of 10mg/ml) were then added before a clearing spin (JA20 rotor) at 15 000rpm for 15 min. The refractive index of the DNA solution was adjusted (if necessary) to 1·396. The DNA preparation was sealed in Quick seal tubes and spun in a Beckman L8-70 ultracentrifuge with a VTi 65 rotor at 55 000rpm

overnight. EtBr from the resulting plasmid band was extracted with salt-saturated isopropanol (2-3 times) until no pink colour remained. The DNA was pelleted using isopropanol or ethanol and the final pellet resuspended in TE buffer. The concentration of DNA solutions were assessed spectrophotometrically by monitoring the absorbance of the solutions between 220 and 310nm. The concentration was determined using the conversion where one absorbance unit at 260nm is equivalent to 50μg DNA/ml [88].

A.2 MINI PLASMID PREP

A 5ml LB culture was grown overnight at 37°C in the presence of the appropriate antibiotic. Cells were harvested from 2ml of the culture by centrifugation in an Eppendorf microfuge for 1 min. The pellet was drained and then resuspended in 100μl Solution I. (Solutions I, II, and III are described for the large-scale plasmid isolation.) The sample was left at room temperature for 5 min, vortexed and then placed on ice for 1 min. Solution II (200μl) was added and mixed briefly on a vortex mixer. The sample was held on ice for exactly 5 min before 150μl pre-cooled Solution III was added. The tube was placed on ice for a further 5-10 min. The sample was microfuged for 5 min and the supernatant fluid was transferred to a fresh tube. Two volumes of 95% ethanol were added, the sample was allowed to stand at room temperature for 5 mins and then it was spun for 15 min in a microfuge. The pellet was dried and resuspended in 150μl TE buffer. A $^{1}/_{10}$

volume (15μl) of 3M sodium acetate and two volumes of 95% ethanol were added. The preparation was held at -20°C or on ice for 10 min and then centrifuged for 20 min. The pellet was washed in 70% ethanol, dried and resuspended in 100μl TE buffer.

A.3 RESTRICTION ENDONUCLEASE DIGESTION AND DNA LIGATION REACTIONS

The procedures followed were essentially those described by Maniatis *et al*, (1982) [88]. Digestion volumes were routinely 20μl, and 4 units of restriction enzyme was used per 1μg of DNA. Digestions were incubated at 37°C in restriction buffer, according to the salt concentration specified by the supplier, which was Boehringer Mannheim GmBH-Biochemica, West Germany. Restriction digests were stopped by heating to 68°C for 15 mins or by adding stop buffer described by Maniatis *et al*, (1982) [88] (Appendix B1). Ligations were performed in 20μl of ligation buffer (Appendix B.2) containing one unit of T4 DNA ligase at room temperature for at least 4 hours.

A.4 DNA AGAROSE GEL ELECTROPHORESIS

DNA samples in TE buffer were mixed with a small volume (3-5μl) of loading buffer (Appendix B.1). Electrophoresis of DNA was carried out using a horizontal gel system with TBE buffer (Appendix B.5) or TAE buffer (for *Agrobacterium*), (Appendix B.6), following the methods described by Maniatis et al, (1982) [88]. Agarose concentration was usually 0.75% although this was adjusted if particularly large or

small fragments needed to be resolved. EtBr (5μl of a 10mg/ml) stock was incorporated into the agarose per 100ml gel. The DNA was visualized using a 254nm wavelength (UV) transilluminator and photographed with Polaroid 667 or 665 film. Sizes of DNA fragments were calculated from standard curves using the distance travelled by known size fragments generated by the digestion of lambda DNA with *Pst*I.

A.5 COMPETENT CELL PREPARATION - $CaCl_2$ METHOD

The method used was an adaptation of that described by Dagert and Ehrlich (1979) [20]. A $^1/_{1000}$ dilution of an overnight LB broth culture was inoculated into 20ml prewarmed LB broth, and incubated at 37°C with vigorous shaking until the culture had reached early exponential phase (OD600 = 0.3). The culture was then cooled for 30 mins in a pre-cooled SS34 centrifuge tube in an ice-water slurry before being centrifuged in a Beckman JA20 rotor at 6000rpm for 6 min. The pellet was resuspended in one volume 100mM $MgCl_2$, vortexed briefly and then spun as before (6000rpm, 6 min). The pellet was then resuspended in a half volume of 100mM $CaCl_2$, vortexed briefly and then allowed to stand for between 20 min and overnight on ice (Overnight ageing was found to improve efficiency). Cells were then harvested by centrifugation at 6000rpm for 6 min and resuspended in $^1/_{10}$ original volume of 100mM $CaCl_2$. After brief vortexing and 20 min on ice, the competent cells were ready for use.

DNA in TE buffer was added to competent cells (2μl DNA solution/100μl competent cells) and the sample held on ice for 10 min. Cells were heat shocked at 42°C for 2 min and returned to ice for 10 min. Transformation mixes were routinely diluted with 900μl LB broth and incubated at 37°C for 1 hour to allow for expression of transferred DNA.

Unused cells could be stored at -70°C by adding 175μl 100% glycerol per ml of cells.

A.6 COMPETENT CELL PREPARATION - DMSO METHOD

This method was essentially that described by Chung and Miller, 1988 [13]. A $^{1}/_{50}$ dilution of an overnight culture was made in LB broth and grown to early log phase (OD600 = 0.3-0.6) at 37°C. The cells were pelleted by centrifugation in a pre-cooled sterile SS34 centrifuge tube at 5000rpm for 5 min at 4°C in a Beckman JA20 rotor. The pellet was resuspended in $^{1}/_{4}$ volume of ice-cold transformation and storage buffer (TSB): LB broth containing 10% PEG 3350 or PEG 4000, 5% DMSO and 20mM Mg++ (10mM $MgCl_2$ + 10mM $MgSO_4$) at 4°C, and incubated on ice for about 10 minutes. For transformation, 0.1ml aliquots of the cells were pipetted into cold Eppendorf tubes and mixed with 10-100ng of DNA from ligation mixtures or 0.1ng of control plasmid DNA. The cells were returned to ice for 5-30 min. Next, 0.9ml of TSB with glucose at a final concentration of 20mM (optional) was added, and incubated at 37°C for 60 min to allow expression of antibiotic resistance genes. Generally, 100μl of

expression mixture was spread on antibiotic-containing agar plates for selection of transformants. Unused cells were occassionally stored at -70°C after rapid freezing in a liquid nitrogen bath. For use, they were thawed slowly on ice.

A.7 SOUTHERN BLOTTING

Adaptations of the method of Southern (1975) [112] were used. Samples were electrophoresed in an agarose gel in TBE or TAE buffer. After electrophoresis, the gel was placed in denaturation buffer (1.5M NaCl; 0.5M NaOH) so as to completely cover the gel and shaken at room temperature for 30 min. The gel was then placed in neutralization solution (1.5M NaCl; 0.5M Tris-HCl pH7.2; 0.001M EDTA) and left for 30 min at room temperature with shaking. A capillary blot was then set up by placing the gel on a glass plate, covering it with a sheet of Hybond-N (exactly the same size as gel) avoiding air bubbles and then three sheets of 3MM paper cut to size and pre-wetted in 20 x SSC or 20 x SSPE (See Appendix B.13 and B.14). A stack of absorbent paper towels was placed on top of the 3MM paper and then a 1-1.5kg weight put on top of this. Transfer was allowed to proceed from 4-16 hours. After blotting and before removing the gel, the position of wells was marked with a pencil. The membrane was washed briefly in 2 x SSC or 2 x SSPE to remove any adhering agarose. The membrane was then allowed to air dry before being wrapped in clingwrap and placed DNA-side down on a 312nm UV transilluminator for 3-4 min. The filter was rinsed briefly in 5 X SSC or 5 X SSPE. The membrane

was then pre-hybridized at 65°C in 1MNaCl; 1% SDS, 10% PEG 6000 containing 100μg/ml sheared denatured herring sperm DNA, in a sealed plastic bag for 4 hours to overnight. The probe was denatured by boiling in water (95°C) for 5 min and then flash-cooled on wet ice for at least 5 min. It was then added to the blot and pre-hybridization mix. Incubation for hybridization was at 65°C overnight with shaking. The blot was removed from the bag and washed in large volumes in plastic boxes. Washing was performed at 65°C as follows: two low stringency washes (2 x SSPE; 1% SDS) for 30 min each, followed by 1 moderate (0.5 x SSPE; 1% SDS) or 1 high (0.1 x SSPE; 1% SDS) stringency wash for 60 min. Care was taken not to "over" wash and radioactivity was monitered during the course of washing by a Geiger counter. After washing, the blot was pressed almost dry between two pieces of Whatman 3MM paper, wrapped in plastic and then autoradiographed with Kodak X-omat or Agfa Curix X-ray film.

Hyband-N-plus (Amersham, U.K.) was used as the membrane of choice when this product became available after mid-1989. After electrophoresis, blotting was performed directly from the gel to the membrane without the need for denaturation or neutralization steps. The three sheets of Whatman's 3MM paper were soaked in 0.4M NaOH. The stacking of absorbent towels and a weight was the same as above. Transfer was complete in 2-3 hours and no fixing was required, rendering this method much quicker with equally good results.

A.8 COLONY HYBRIDIZATION

This was performed essentially as described in the Amersham manual. Master and replica plates were prepared by patching colonies onto a grid pattern on LA and antibiotic plates and incubating overnight at 37°C. A Hybond-N membrane was placed on the agar surface and the membrane and agar cut with a scalpel blade to ensure correct orientation of colonies. The membrane was removed after 1 minute and placed colony side up on a pad of absorbent filter paper soaked in denaturing solution (1.5M NaCl; 0.5M NaOH). It was left for 7 min. The membrane was then placed, colony side up, on a pad of absorbent filter paper soaked in neutralizing solution (1.5M NaCl; 0.5M Tris-HCl pH 7.2; 0.001M EDTA). The membrane was left for 3 min and then the neutralizing step was repeated with a fresh pad soaked in the same solution. Filters were washed in 2 x SSC and then transferred to dry filter paper to air dry, colony side up for 30-60 min. Filters were wrapped in clingwrap and placed colony side down on a UV transilluminator (312nm) for 3-4 min. The filters were washed in 2 x SSC + 0.5% SDS at 65°C overnight. This step was found to be vital for getting rid of all cell debris and agar. A 25ml pre-hybridization solution (7.5ml 20 x SSC, 1.25ml 100 x Denhardt's solution (Appendix B.15); 15ml sterile dH_20; 1.25ml 10% SDS) was added to the membrane, together with 0.5ml of a 1mg/ml solution of salmon sperm DNA that had been boiled for 5' (at 95-100°C) and then flash cooled on ice. Prehybridization was carried out with shaking for at least an hour at 65°C. The ^{32}P-labelled denatured

probe was added to the pre-hybridization solution at a concentration of less than 20ng/ml. Hybridization was allowed to proceed for at least 12 hours at 65°C with shaking. Filters were washed twice with 2 x SSC + 0.5% SDS at 60°C for 20 min. The wash was replaced with 3mM Tris and incubated at 60°C for 5 min before a final wash in 2 x SSC + 0.5% SDS at 60°C (time not critical). The filters were blotted to get rid of excess liquid, sealed in a plastic bag and then autoradiographed at -70°C overnight. Colonies yielding a positive signal could then be picked off the master plate.

A.9 PREPARATION OF RADIOACTIVELY-LABELLED DNA PROBES

^{32}P-labelled DNA probes were prepared by nick translation (kit N.5000) or random priming (kit 1004 760, both supplied by Amersham International, U.K. The manufacturers instructions were followed. Unincorporated 32P-labelled nucleotides were separated from the labelled probe using a Sephadex G-50 Pasteur column. This involved setting up a column in a sterile Pasteur pipette with a broken off tip and plugged with a sterile glass bead. The probe was passed through the column followed by aliquots of 0.1% SDS in STE (10mM Tris-Cl; 100mM NaCl; 1mM EDTA; pH8.0). Column eluates were collected in Eppendorf tubes and then the radioactivity monitored by a Geiger counter and/or a scintillation count. The first "peak" indicated the present of the probe. This was then heat-denatured and used for hybridization.

APPENDIX B:

BUFFERS AND MEDIA

B.1 DNA SAMPLE LOADING BUFFER

Bromophenol blue	0.25% (w/v)
Glycerol	50% (v/v)
EDTA	100mM

B.2 LIGATION BUFFER (10X)

Tris-HCl (pH 7.6)	500mM
$MgCl_2$	100mM
PEG 8000	50% (w/v)
ATP	10mM
DTT	10mM

B.3 LURIA BROTH AND AGAR (LB:LA)

Tryptone	10g
Yeast extract	5g
NaCl	5g
Agar	15g
dH_20	1l

B.4 TE BUFFER (pH 8.0)

Tris	10mM
EDTA	1mM

B.5 TBE BUFFER (pH 8.0)

Tris	89mM
Boric acid	89mM
EDTA	2.5mM

B.6 TAE BUFFER (pH 8.0)

Tris-acetate	40mM
EDTA	2mM

B.7 YEB MEDIUM (pH 7.2)

Nutrient broth	5g
Yeast extract	1g
Peptone	5g
Mannitol	5g
$MgSO_4$	0.5g
dH_20	1l

B.8 LACTOSE AGAR

Agar	20g
Lactose	10g
Yeast extract	1g
dH_20	1l

B.9 BENEDICT'S REAGENT [5]

Solution A (autoclaved)

sodium citrate	173g
sodium carbonate	100g

Dissolve in 600ml dH_20 by heating. Make up to 850ml

Solution B (filter sterilized):

copper sulphate	18g

Dissolve in 100ml dtl20. Make up to 150ml.

Slowly add Solution B to Solution A with constant stirring.

B.10 MINIMAL A BACTERIAL MEDIUM (MILLER, 1972) [91]

$K_2HPO_4.3H_20$	60mM
KH_2PO_4	33mM
$(NH_4)_2SO_4$	7.5mM
Sodiumcitrate	1.7mM
$MgSo_4$	1mM
Glucose	2g/l
Vitamin B1	50mg/l

HYBRIDIZATION SOLUTIONS

B.11 DENATURING SOLUTION

NaCl	1.5M
NaOH	0.5M

B.12 NEUTRALIZING SOLUTION

NaCl	1.5M
Tris-HCl pH 7.2	0.5M
Na_2.EDTA	0.001M

B.13 20 X SSC

NaCl	3.0M
Na_3 citrate	0.3M

B.14 20 X SSPE

NaCl	3.6M
$NaH_2PO_4.H_20$	0.2M
EDTA	0.02M

B.15 100 X DENHARDT'S SOLUTION

Bovine serum albumin	2% w/v
Ficoll	2% w/v
PVP (polyvinylpyriolidone-40)	2% w/v

B.16 X-GAL MEDIUM

250ML LA + 1ml amp (25mg/ml)
+ 0.8ml x-gal (20mg/ml)
+ 0.2ml IPTG (23.8mg/ml)

Add ampicillin, X-gal and IPTG after autoclaving luria agar

OR

To the surface of amp plates, spread 40μl 2% X-gal and 10μl IPTG.

X-GAL (5-bromo-4-chloro-3-indolyl-β-galactosidase)

Stock: 2% 20mg/ml in dimethylformamide.

Store at -70°C.

IPTG (isolpropyl-β-D-thio-galactopyranoside)

Stock: 23.8 mg/ml in dH_2O.

Store at -20°C.

B.17 Phosphate buffer saline (PBS) (10X; 1l dH_2O) (pH7.4)

NaCl	87g
$Na_2PO_4.2H_2O$	22.5g
kH_2PO_4	2g

B.18 ANTIBIOTIC CONCENTRATIONS USED (ug/ml) [88]

Antibiotic	
Amp	50 or 100
Cb	100
Km	100
Rif	100
Sm	300
Sp	100

Antibiotics were prepared according to Maniatis et al [88].

APPENDIX C:

PLANT MEDIA

C.1 A1 (STERILE SHOOT CULTURE)

½ strength Murashige and Skoog (MS) salts

1% sucrose

0.8% agar

C.2 A2 (INFECTION)

B5 medium (see Appendix C.6)

250mg/l NH_4NO_3

3% sucrose

0.5 g/l 2-(N-morpholino) ethane sulfonic acid (MES)

(pH 5.5)

C.3 A3 (CALLUS AND SHOOT INDUCTION)

B5 medium

250mg/l NH_4NO_3

2% glucose

0.5g/l MES (pH 5.7)

40 mg/l adenine

0.8% agar

0.1 mg/l indole-3-acetic acid (IAA)

1mg/l 6-benzylaminopurine (BAP)

500 mg/l cefotaxime

50-100 mg kanamycin as selective agent

C.4 A4 (SHOOT INDUCTION)

modified A3 medium : no IAA

200 mg/l cefotaxime

C.5 A5 (ROOT INDUCTION)

½ strength MS salts

3% sucrose

0.5 g/l MES (pH 5.7)

0.8% agar

100 mg/l cefotaxime

C.6 B5 MEDIUM (GAMBORG *ET AL.*, 1968) [46]

Macro-elements	NaH_2PO_4	150 mg/l
	$(NH_4)_2SO_4$	134 mg/l
	$MgSO_4.7H_20$	500 mg/l
	$CaCl_2.2H_20$	150 mg/l
	$FeSO_4.7H_20$	27.8 mg/l
	Na_2-ETDA	37.3 mg/l
	KNO_3	3000 mg/l
Organic compounds	Myo-inositol	100 mg/l
	Nicotinic acid	1 mg/l
	Thiamine-HCl	10 mg/l
	Pyridoxine	1 mg/l
	Sucrose	30000 mg/l
Micro-elements	H_3BO_3	3 mg/l
	$MnSO_4.H_20$	10 mg/l
	$ZnSO_4.7H_20$	2 mg/l
	$Na_2Mo0_4.2H_20$	0.25 mg/l
	$CaCl_2.6H_20$	0.025 mg/l
	KI	0.75 mg/l
	$CaSO_4.5H_20$	0.025 mg/l
	Agar agar	10000 mg/l

(pH 5.5)

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