SUPPRESSION OF VIRAL AND ENDOGENOUS GENE EXPRESSION IN *N.tabacum* USING ANTISENSE RNA.

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To Jane

ABSTRACT

Antisense RNA can be used to suppress gene expression in prokaryotes and eukaryotes. In this work, DNA encoding antisense RNA targeted against genes of the plant viral pathogen, tomato golden mosaic virus, were stably transformed into *Nicotiana tabacum* by *Agrobacterium tumefaciens* mediated transformation. In order to select for expression of the antisense genes, they were encoded on the same transcriptional unit as the selectable drug resistance marker. Transgenic plant lines were produced and tested for resistance to TGMV. A number of lines were shown to be less susceptible to TGMV infection than control lines. Some of the transgenic lines transformed with anti-TGMV genes have an abnormal morphology which has an apparent correlation with the presence of these genes.

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List of Abbreviations.

6-BAP	6-benzylaminopurine
ADA	adenosine deaminase
APRT	adenine phosphoribosyl transferase
asRNA	antisense RNA
ATP	adenosine triphosphate
BCTV	beet curly top virus
BGMV	bean golden mosaic virus
bp	base pair
BSA	bovine serum albumin
CaMV 35S	cauliflower mosaic virus 35S RNA
CaMV 195S	cauliflower mosaic virus 19S RNA
CAT	chloramphenicol acetyl transferase
CHS	chalcone synthase
CIP	calf intestinal phosphatase
CLV	cassava latent virus
CSF	human granulocyte-macrophage colony stimulating factor
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytidine triphosphate
ddATP	dideoxy adenosine triphosphate
ddCTP	dideoxy cytidine triphosphate
ddGTP	dideoxy guanosine triphosphate
ddTTP	dideoxy thymidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxy guanosine triphosphate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate

DTT	dithiothreitol
dTTP	deoxy thymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSV-I	herpes simplex virus type I
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase
LMP	low melting point
MHC	myosin heavy chain
mRNA	messenger RNA
MSV	maize streak virus
NAA	naphthaleneacetic acid
NOS	nopaline synthase
NPT II	neomycin phosphotransferase II
ORF	open reading frame
PAT	phosphinothricin acetyl transferase
PG	polygalacturonase
PMSF	phenyl methyl sulphonyl fluoride
RF	replicative form
RNA	ribonucleic acid
RUBISCO	ribulose bisphosphate carboxylase
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
TEMED	N,N,N,N'-tetramethyl ethylene diamine
TGMV	tomato golden mosaic virus
TK	thymidine kinase
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
WDV	wheat dwarf virus

X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
XGPRT	xanthine-guanine phosphoribosyl transferase

1: INTRODUCTION.

The work described in this thesis involves the application of antisense RNA (asRNA) technology to the suppression of gene expression in tobacco plants. The targets for suppression were genes of the plant pathogen, tomato golden mosaic virus (TGMV). One of the viral genes chosen as a target appears serendipitously to have homology with an endogenous, developmental plant gene. Investigation of this gene is at a preliminary stage.

1.1 Tomato Golden Mosaic Virus (TGMV), A Geminivirus.

TGMV is the causative agent of the disease 'Tomato Golden Mosaic' (Mosaico Dourado) of *Lycopersicon esculentum* and is widespread in Brazil. The virus was first purified from leaves of infected *Nicotiana glutinosa*[1] and was assigned to the geminivirus group[2,3].

1.1.1 Geminivirus, An Overview.

There have been a number of reviews on geminiviruses[4,5,6,7,8] and I will not, therefore, give an extensive bibliography.

When viewed under the electron microscope, geminiviruses appear to consist of twinned (hence the name) quasi–icosohedral particles of about 20x35nm; trimers and tetramers can also be observed. Each particle contains a small, circular, single–stranded (ss) DNA molecule of between 2.5 and 3.0 kilobase (kb) in size.

Geminiviruses may be classified on the basis of the insect vector which transmits the disease to plants (whitefly or leafhopper) and host range (dicotyledonous or monocotyledonous plants). Of the twenty-one definitive and potential members of the geminivirus family listed by Harrison[5] about half are transmitted by whitefly and infect dicotyledonous plants, while the remaining members of the group are transmitted by leafhoppers (and one by a treehopper) and infect predominantly monocotyledonous plants. Virusescan persist in their insect vector for life[5]. The whitefly transmitted geminiviruses have bipartite genomes, whilst the leafhopper transmitted viruses have monopartite

genomes. TGMV and cassava latent virus (CLV, also called african cassava mosaic virus) are whitefly transmitted and both have genomes consisting of two ssDNA molecules (of 2.5-3.0kb each); there is only a limited, intergenic region of homology between the two ssDNA molecules (discussed below). Beet curly top virus (BCTV), wheat dwarf virus (WDV) and maize streak virus (MSV) have only one ssDNA component; BCTV is unusual in that although it is leafhopper transmitted and has a monopartite genome, it infects dicotyledonous plants. There has been speculation[7] that the difference between the monopartite and bipartite subgroups is evolutionarily related to the insect vector. Moreover, there is strong evidence (discussed below) that the coat protein, encoded on DNA component A (related to the single component of the monopartite group), is responsible for vector specificity and (in TGMV and CLV at least) DNA component B is responsible for cell to cell transmission.

Geminiviruses are found almost exclusively in the nuclei (where it is thought that they replicate) and most dicot-infecting geminiviruses are associated almost exclusively with the phloem, especially phloem parenchyma. However, BCTV is occasionally found outside the phloem and geminiviruses infecting *Graminacae* can infect almost all kinds of cells. When total DNA is prepared from infected tissue, single stranded circular, double stranded open circular and double stranded closed circular viral DNA forms are found.

Geminviruses are not transmitted through the germ line.

1.1.2 TGMV, Genome Organisation and Function

Figure 1.1 shows the genetic organisation of TGMV. Table 1 gives the transcriptional and translational data. It was reported[9] that the mRNA steady state level of AR1 (the coat protein gene) and BL1 were high relative to the other viral transcripts. The gene organisation of all of the bipartite genome geminiviruses is very similar; the gene organisation of the monopartite geminiviruses is related to the A genome of the bipartite viruses. When discussing the genes of the bipartite geminiviruses I shall use the nomenclature of TGMV genes , viz. AL1, BR1 etc (Figure 1.1).

All geminiviruses sequenced to date have a non-coding region of about 200 base

pairs which contains a potential stem loop structure. In the bipartite geminiviruses the 200bp region is highly conserved between the two components, but, with the exception of the stem loop structure, it is not conserved between different geminiviruses.

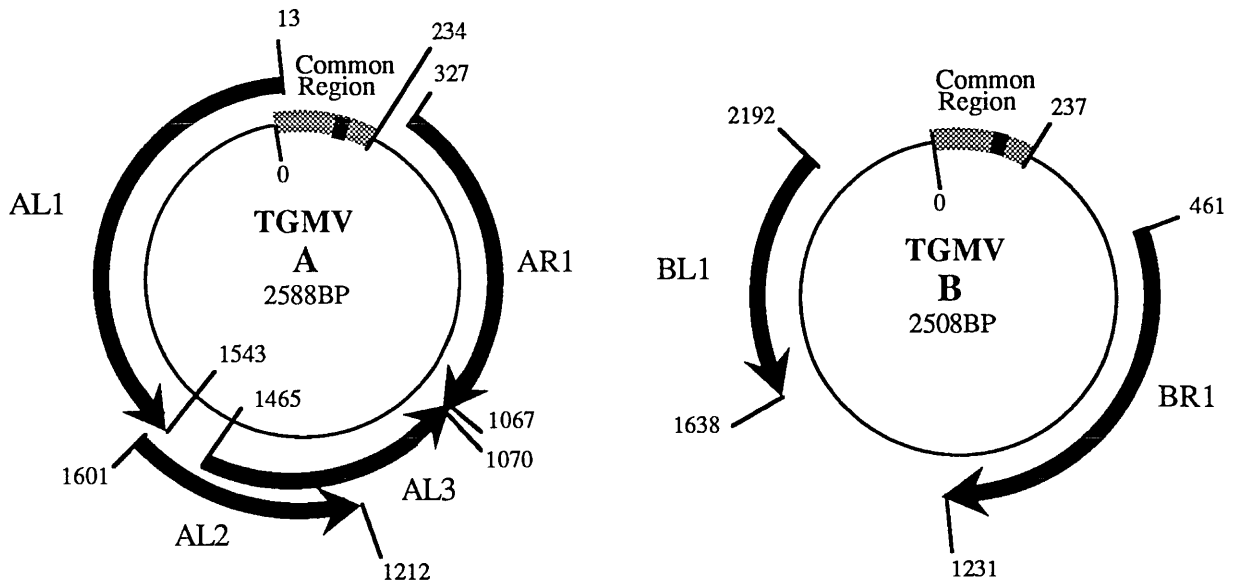


Figure 1.1. Maps of TGMV double stranded genomes. Nucleotide positions of the ORFs are marked. Genes transcribed in the rightward direction (AR1 and BR1) are encoded on the viral + strand. The 200bp[common region is shaded grey with the stem loop marked in black.

ORF	Putative promoter (TATA)	Transcription start (Cap Site)	Translation		Putative Poly A (AATAAA)	Approx. RNA Stop
			start	Stop		
AR1	287-291	319	327	1070	1069-1074	1110
AL1	65-61	30	13	1543	1088-1083	1040
AL2	?	1919?	1601	1212	1088-1084	1040
AL3	1659-1656	1679	1465	1067	1088-1083	1040
BR1	415-422	445	461	1231	1314-1319	1320
BL1	94-89	64	2192	1638	1331-1326	1300

Table 1. Transcriptional[9] and translational data for TGMV.

The B component of CLV will not complement the A component of TGMV and vice versa[10]; the non-stem loop part of the 200bp region must be important in viral 'speciation'. The stem loop structure consists of a highly GC rich, complementary region flanking an AT rich loop; this structure is fairly well conserved in all geminiviruses sequenced thus far and a nanonucleotide in the AT loop is invariant. It has also been noted that there is strong homology (7/9 nucleotides) between the AT loop and a similar loop which is the recognition and cleavage site for the bacteriophage ØX174 gene A protein[11]. Gene A protein is a site specific, nicking endonuclease essential for replication of double stranded RF I and the formation of single strands from RF I and RF II. Whether or not the stem loop structure is involved in replication, its high degree of conservation across a wide range of geminiviruses argues that it is important for viral viability.

Geminiviruses may be introduced into plants *via Agrobacterium tumefaciens*. The viral genomes, or parts thereof, may be stably integrated into the plant genome (for a fuller discussion of plant transformation see Appendix I). Alternatively, inoculation of whole plants with *A.tumefaciens* containing multimers (but not normally monomers; see however[12]), of geminivirus genomes on their T-DNA gives rise to systemic infection with high efficiency; even with viruses that can not normally be transmitted by the traditional method of mechanical inoculation[13]. This process is known as 'agroinfection'. It is presumed that infectious virus arises from agroinfection either by recombination or by replication of virus molecules from one initiation site to the next; it is unclear whether this process happens prior, during or after integration of the T-DNA into the plant genome. The combination of the two approaches of stable transformation and agroinfection, together with more traditional mutagenesis studies, have led to an increase in knowledge of the function of the TGMV genome.

When dimers of TGMV A were cloned into the chromosome of petunia plants, freely replicating DNA A components were found[11] and shown to be encapsidated in virions of apparently normal morphology[14] (in symptomless plants). Trimers of TGMV B were cloned into the genome of petunia, but no freely replicating DNA B molecules were found. However, when these A2 and B3 plants were crossed, a systemic infection

resulted in F1 heterozygotes. Furthermore, agroinfection with *A.tumefaciens* strains containing B dimers complemented A2 plants, and agroinfection with *A.tumefaciens* strains containing A dimers complemented B2 plants; systemic infection was seen in both cases[15,16] . Inoculation of naked viral DNA, mutant in either BR1 or BL1, abolishes systemic infectivity[14]. This suggests that the A particle encodes the functions necessary for replication and encapsidation, and the B particle, those necessary for cell to cell spread and symptom development, both BR1 and BL1 being necessary.

It has also been shown that deletion and/or mutation of the coat protein gene causes no loss in infectivity, although symptom severity is diminished[17,18]. The coat protein gene is well conserved at the amino acid level amongst the whitefly transmitted viruses, but not between the whitefly and leafhopper transmitted viruses[4,7]. Indeed, the 'missing link', BCTV, which is monopartite and leafhopper transmitted but infects dicotyledonous plants, has strong homology to component A of the whitefly transmitted bipartite geminiviruses, except in the coat protein gene. This suggests that the coat protein may be predominantly involved in the determination of insect vector rather than host range. Evidence in support of this comes from Davies[19]; in this work the coat protein of a leafhopper transmitted geminivirus was replaced by that from a whitefly transmitted geminivirus. This onverted its vector to a whitefly.

The conclusions that have been drawn from the work described above are:

1. Geminiviruses occur (and probably replicate) in the nuclei of infected plant cells.
2. TGMV (in common with other bipartite, white fly transmitted gemini viruses) is phloem limited.
3. The so called common region containing a stem loop structure is important to viral viability and may be involved in DNA replication and/or second strand synthesis. In light of the different common regions found in different geminiviruses, it may also be related to host range.
4. The TGMV B genome is required for cell to cell transmission and symptom development but not for replication or encapsidation. There is evidence that this is also true for CLV[20].

5. The coat protein gene is not required for symptom development or replication, although symptoms are attenuated in its absence; perhaps it is necessary for the virus to be packaged prior to transport around the vascular system of the host. The coat protein is a determinant of insect vector.
6. Viral replication is determined by the protein products of the AL1, AL2 and AL3. These genes are all on the viral minus strand, therefore second strand synthesis must be initially carried out by host encoded proteins.

Work has recently been carried out[21] to dissect the functions of the AL1, AL2 and AL3 genes, both in whole plants and in agroinfected leaf discs (looking at replication in the absence of cell to cell transmission). Infectivity in whole plants was carried out by infecting 'permissive' plants (containing chromosomally integrated dimers of TGMV B) with mutant TGMV A, either as linear DNA, or by agroinfection. In whole plant inoculations, mutations in AL1 or AL2 completely abolished symptom development while mutations in AL3 resulted in delayed and greatly attenuated symptom development. Investigation of replication after agroinfection of wild type petunia leaf discs and analysis of total plant DNA, prepared from the leaf discs (Appendix I, Section 7.4.2) yielded the following: i) inoculation by mutants in AL2 and AL3 gave rise to all the expected forms of viral DNA (open circular, closed circular and single stranded), although levels of ssDNA were diminished in AL2 mutants; ii) inoculation by mutants in AL1 produced no detectable virus in any DNA form. From these experiments and the coat protein deletion analysis it appears that AL1 and the common region is all that is required for viral replication. In support of this hypothesis, it has been shown that plants in which the AL1 gene, driven by a strong promoter, is integrated into the chromosome of transgenic tobacco plants, will support the replication of TGMV B molecules, and in leaf disc assays these TGMV B genomes may have inserts of up to 10kb[22]. AL2 is necessary for systemic infection, presumably in conjunction with TGMV B proteins. As AL2 mutants have diminished levels of ssDNA, the AL2 protein may be a ssDNA binding protein that protects the ssDNA from degradation. A model for TGMV A replication based on that of phage ØX174 has

been proposed[21] (Figure 1.2).

At this point I wish to look at some of the differences between the geminiviruses.

In MSV, it has been shown that the coat protein is essential for systemic infection[23]; in contrast, the coat protein is dispensable in the dicot infecting bipartite geminiviruses. It would be interesting to know if this is also true for BCTV and thus whether this role for the coat protein is dependent on monocot versus dicot hosts or on monopartite versus bipartite viral genome (the role of the coat protein in vector determination has already been shown[19]).

It should also be mentioned that there is homology between the coat protein gene, AR1 and BR1 in TGMV, CLV and bean golden mosaic virus (BGMV)[24] and it is possible that BR1 encodes functions necessary for systemic infection that are supplied by the coat protein in the monopartite geminiviruses. When leaf disc innoculations (Appendix I, Section 7.4.2) of AL1, AL2 transgenic plants by mutant TGMV B molecules were carried out, it was observed that mutations in BL1 did not effect the amount of virus found, whereas mutations in BR1 led to a decreased yield of viral DNAs[22]. This was interpreted as suggesting that BR1 is neccessary for cell to cell transmission (perhaps in addition to symptom development) and BL1 for symptom development alone[22]. Considerably more work will have to be done to confirm this hypothesis.

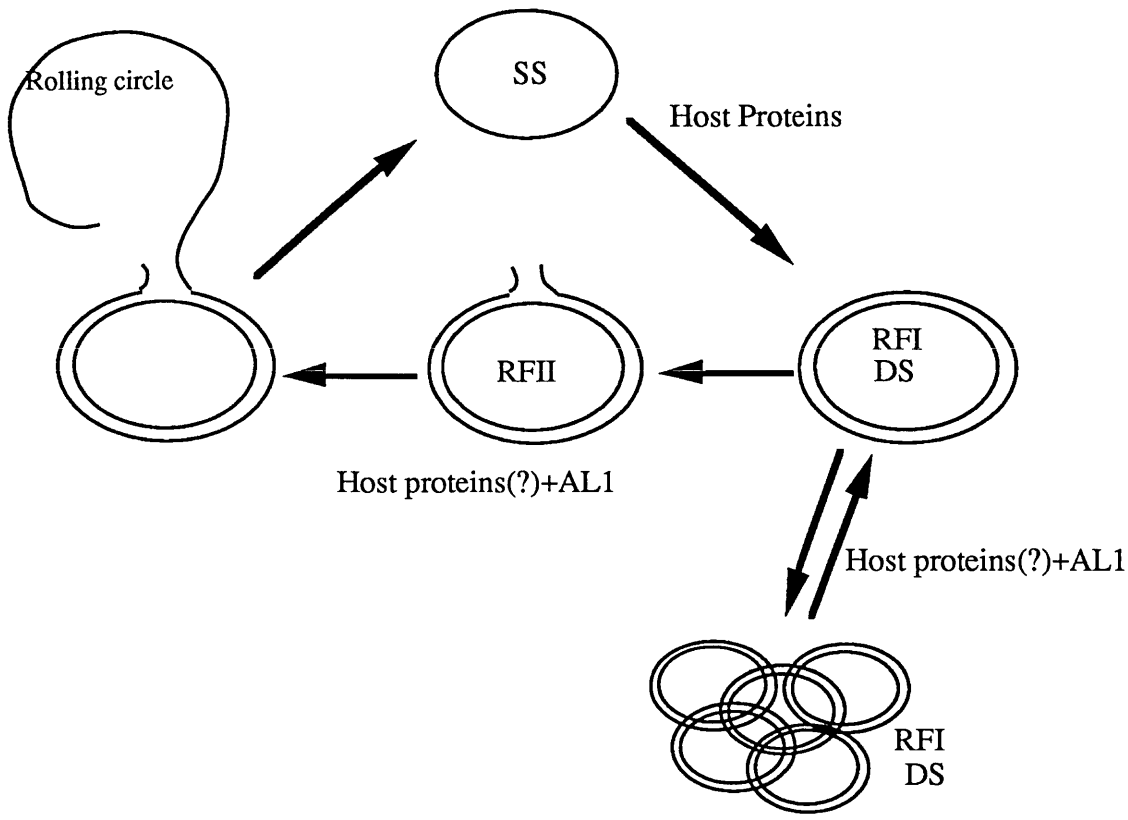


Figure 1.2. Model for TGMV A replication based on ØX174.

There is amino acid homology between the two proteins apparently encoded by adjacent ORFs in the monocot infecting geminiviruses and the single protein encoded by the replication gene AL1 in the dicot infecting geminiviruses (including the monopartite BCTV). It has been shown [25] that the two ORFs in the monocot infecting virus, WDV, are spliced by deletion of an intron and a frame shift to give a transcript encoding a single protein with homology to the product of the AL1 gene of dicot infecting geminiviruses. Mutagenesis and splicing of the two ORFs *in vitro* gave rise to a genome which would replicate. Infection of whole plants was not demonstrated. Interestingly, analysis of the sequences of other monocot infecting geminiviruses revealed splice donor and acceptor sites at positions equivalent to those in WDV, suggesting that this form of post transcriptional processing is common to monocot infecting geminiviruses. It is possible that two polypeptides are expressed, one corresponding to the fused protein and another corresponding to the product of one of the unspliced mRNAs. It is interesting to speculate

whether, if this is so, it is determined by the monocot host or by virtue of a more efficient virus in which one ORF encodes two proteins of different function; one of these functions may be encoded on the second genome in the bipartite viruses. Further work is needed to determine if this is so.

A short complementary oligonucleotide, capable of priming second strand synthesis *in vitro* has been found in the monocot infecting geminiviruses[26,27,28]; it is usually complementary to an intergenic terminating region found between the ends of the rightward and leftward ORFs, and has ribonucleotides at the 5' termini. A corresponding primer has not been found in the dicot infecting geminiviruses. The full implications of the differences between the monocot and dicot infecting geminiviruses await biochemical analysis to elucidate the functions of the proteins encoded by the various viral genes.

The analysis of the geminiviruses, which I have described here in part, has led to the development of geminivirus vectors which replicate freely in infected plants and express chimæric genes (some aspects of this work are described more fully in Appendix I, Section 7.4.2).

1.2 Antisense RNA.

When a gene is expressed the usual sequence of events is as follows. One strand of double stranded DNA is transcribed to a single stranded RNA copy, which may then be processed before the mature messenger (m)RNA is translated into the polypeptide gene product. If this sequence of events is disrupted at any stage, gene expression will be suppressed. Antisense RNA is produced by reversing the target region of a gene with respect to its regulatory signals. The antisense (as)RNA that is transcribed is not only inverted in orientation but is also complementary to normal sense or mRNA. The asRNA can bind to the sense RNA (eg. mRNA) or DNA by complementary base pairing and may thus disrupt its expression.

1.2.1 Antisense RNA control in prokaryotes.

Antisense control in prokaryotes has been extensively reviewed[29,30,35] and I will not, therefore, give an extensive bibliography.

Naturally occurring antisense control systems have been identified for regulation of Col E1 replication, R1 plasmid replication, Inc FII plasmids, pTI81 plasmids, IS 10 transposition, plasmid conjugation, the lysis/lysogeny decision of temperate phages amongst others. Antisense RNA has also been implicated in the regulation of expression of the *E.coli* chromosomal genes, OmpC, OmpF and CRP.

In essentially all of the naturally occurring antisense regulatory systems so far discovered in prokaryotes, secondary structure is thought to be important. This structure usually consists of a series of complex stem loops 5' to the target region; the loops often being of an energetically favoured size (6-7bp); the detailed secondary structure probably changes as the transcript is synthesized.

These structures are thought to be important in determining the stability of the asRNAs in addition to mediating RNA duplex formation. The interaction between sense and antisense transcripts has been studied in some detail in Col E1 plasmids, IS10 transposons and P22 *sar* phage. In all cases, the reaction is essentially the same. In Col E1, for example, the first stage is a reversible interaction between complementary base pairs on the three loops of the sense RNA (RNA I) and the three loops on the asRNA (RNA II). This reversible 'kissing' reaction is followed by the formation of a stable interaction 3' to the stem loop structures (in RNA I) and then by collapse of the stem loop secondary structures to form a stable RNA duplex.

Inhibition of gene expression is thought to take place primarily by occlusion of the ribosome binding site and/or start codon by the duplex RNA. Support for this mechanism (in addition to that discussed in reference[29]) comes from studies on artificial antisense systems in *E.coli* in which antisense directed towards the Shine-Delgarno sequence and initiation codon is much more efficient at blocking gene expression than antisense directed towards regions 3' to the initiation codon (see [35]). Alternatively duplex RNA may be more susceptible to degradation by RNAases, as is thought to be the case in eukaryotes. However, in a strain deficient in RNAase III (known to be involved in degrading RNA duplexes) antisense control occurs at 90-95% of the normal level in regulation of IS 10 transposition[29]. Antisense RNA has also been proposed, in some cases, to act at the level

of transcription; for example, by preventing secondary structure occlusion of a rho-independent termination site, and so causing premature termination of the transcript[31]. Naturally occuring antisense regulation in prokaryotes is considerably more elegant and sophisticated than anything that has been engineered. This is in part because the antisense and target genes have evolved together to optimise regulation.

Engineered asRNAs have been used in bacteria. Antisense RNA has been targeted against such things as phage, giving rise to immunity[32,33] and also against chromosomally expressed genes[29,30,35]. In almost all cases, they are much less effective than the natural systems, working efficiently only when driven by a strong promoter and resident on a high copy number plasmid[29]. In general, the constructs lack stabilizing secondary structures which are also necessary for rapid nucleation of stable binding; unfortunately, this is a consequence of artificial systems in which the target RNA does not usually have the required complementary secondary structures. However, in an attempt to stabilize artificial antisense transcripts, Green *et al* [35] constructed vectors for use in prokaryotic antisense systems in which the cloning site for insertion of the antisense gene is flanked by two stem loop structures modeled on those naturally occurring asRNAs. It is not clear how effective this strategy has been. The same workers also demonstrated[29] that duplication of the antisense region on a single transcript doubled the degree of inhibition of the target gene. This may have implications for engineered antisense technologies in eukaryotic systems.

1.2.2 Antisense RNA control in eukaryotes.

Both natural and artificial antisense control in eukaryotes have been recently reviewed[34,35]. Engineered antisense has found many more applications in eukaryotic systems than in prokaryotic, presumably because of the ease, in prokaryotes, of gene disruption by other techniques such as mutagenesis and homologous recombination.

Two approaches have been taken to artificial gene regulation by asRNA in eukaryotes. Transient expression of asRNA by exogenously introduced nucleic acids and expression of asRNA from stably integrated antisense genes. The former approach may be further subdivided into the use of chemically modified antisense oligonucleotides and non-

modified transiently expressed DNAs or RNAs.

1.2.2.1 Transient antisense systems.

The use of chemically modified oligonucleotides as antisense tools is outside the scope of this introduction; their use to date has been primarily limited to cultured cells and oocytes (see reference[36] for recent review). However they do have great therapeutic potential and I will mention one dramatic application[37]. Miller and Ts'O[37] demonstrated that an oligomer DNA methyl phosphonate directed against herpes simplex virus type I (HSV-I) was able to prevent herpetic lesions when applied to a mouse's ear as a cream. The yield of virus was reduced in both the skin and nerve tissue, and furthermore, when the application of the cream was stopped, the lesions returned.

The first demonstration of the effective use of an artificial antisense gene in an eukaryotic system was carried out by Izant and Weintraub in 1984[38]. Plasmids containing an HSV thymidine kinase (TK) antisense gene (containing a region complementary to the whole of the TK coding region) and an HSV-TK sense gene were coinjected into mouse TK^- L cells in a ratio of 100:1, this led to a 4 to 5 fold drop in the number of TK^+ cells compared to controls cells injected only with the sense TK gene and an unrelated plasmid. It was shown that the inhibition was sequence-specific and not function-specific. They later extended this work[39] by showing that a 52bp antisense sequence complementary to the TK 5' leader sequence was sufficient to inhibit TK expression when coinjected at a ratio of 200:1. It was also demonstrated that co-transfection of antisense and sense TK genes gave rise to a lower number of stably transformed TK^+ cells relative to cells co-transfected with sense genes alone. This effect was observed at a ratio of as:s of 10:1 but not 1:1.

The effects of asRNA in transient systems has been investigated by other workers by microinjection of RNA into *Xenopus* oocytes(Eg. [40,41]) and electroporation of plasmids into carrot protoplasts[42]. In all of these cases an excess of 10 fold was required for a measurable inhibition to be observed, and at least 100 fold for a reasonable degree of inhibition. The mechanism of inhibition in these cases is not clear. In the case of injected RNAs, the inhibition must presumably be taking place at the level of blockage of translation in the cytoplasm, and RNA duplexes have been detected in some cases[38]. When plasmids

are introduced into cells, the inhibition may be at any point or points along the pathway of gene expression, from transcription to translation *via* mRNA maturation and transport. In all of the transient expression systems described, vast amounts of nucleic acid, relative to the endogenous levels, are introduced into the cell; furthermore, it is not clear how much the introduction of this nucleic acid disrupts the organelle membranes. It is therefore not surprising that quantitatively different results have been obtained with stably transformed systems (discussed below). The use of transient antisense systems has however, shown its utility in identification of cloned *Drosophila* developmental genes. Injection of asRNA from cloned cDNAs into *Drosophila* embryos has given rise to phenocopies of the *Krüppel*[43], *Wingless*[44] and *Snail*[45] mutations. Injection of asRNAs also revealed the presence of an RNA duplex unwinding activity in *Xenopus* eggs[46,47,48]. This activity irreversibly suppresses the binding of asRNAs to their target by substituting inosine for adenosine in about 50% of the adenosine residues in the RNA duplex[48]. It is not clear what the function of this activity is *in vivo*, or even whether it is responsible for suppressing the antisense effect, as it would be expected that modified RNAs would be mistranslated to produce non-functional proteins[48]. A similar activity has since been identified in a number of mammalian cell lines[49].

1.2.2.2 Antisense RNA in stably transformed systems.

The mode of action of asRNA in stably transformed eukaryotic cells was first addressed by Kim and Wold[50]. Mouse L cells were transformed with DNA encoding adenine phosphoribosyl transferase (APRT) and HSV-TK. A transformed APRT$^+$ TK$^+$ clone was grown in mediumthat imposes selection for APRT. Effective antisense inhibition of TK was obtained by re-transformation with a bifunctional construct in which an antisense gene (complementary to 225 bp at the 3' end of the TK gene) was fused to the 5' end of a dihydrofolate reductase (DHFR) gene. Thus, expression of DHFR implies concomitant expression of anti TK RNA on the same transcript. DHFR confers resistance to the antibiotic methotrexate. Cells resistant to increasing levels of methotrexate were selected by gradually increasing the concentration of methotrexate in the culture media. The increased resistance occurred by gene duplication and high levels of methotrexate resistance

resulted in high levels of antisense containing transcripts. A ratio of antisense:sense of 300:1 was needed to obtain measurable levels of TK inhibition. At steady state, the asRNA was distributed such that 60% was in the cytoplasm and 40% in the nucleus. The presence of double stranded RNA in the nucleus was demonstrated and TK mRNA was shown to build up in the nucleus. The presence of these duplexes in the nucleus together with the high level of TK sense RNA relative to TK activity was taken to suggest that the duplex formation inhibits transport across the nuclear membrane. The antisense fragment was not directed against the 5' region of the TK gene, this argues against inhibition of translation. It should also be noted that constructs containing antisense to different regions were shown to be ineffective and this was correlated to decreased transcript stability. In other systems (discussed below) different results have been obtained: lower ratios of antisense:sense are needed for inhibition, duplex RNA is not found and there is no build up of sense RNA in the nucleus.

Since this work, asRNA has been used to investigate the function of a large number of genes by down regulation (reviewed in[34]). I will mention just a few of these as representative examples or where they illuminate mechanism of the asRNA effect.

The utility of stably transformed antisense systems to investigate gene function was first demonstrated in the slime mold *Dictyostelium discoidium*, which is a useful model in the study of development. *D.discoidium* grows as single cells; however, when starved the cells aggregate to form a multicellular organism which goes through several developmental stages before forming a fruiting body consisting of stalk and spore cells. Mutants in the discoidin genes are known and do not 'stream' on plastic plates, but form aggregates by random cell collision[51]. Crowley *et al*[52] produced a phenocopy of this mutant by introducing plasmids encoding asRNA targeted against the discoidin genes. The region chosen for the antisense constructs was a 320bp fragment from the centre of one of the three endogenous discoidin genes; it was driven from the same promoter as the sense gene. The antisense construct was introduced at a copy number of about 100, however it was not possible to determine the ratio of antisense to sense RNA as no antisense or mRNA, either single stranded or duplex, was detected. Nuclear run-on experiments,

however, showed that both message and antimessage were actively transcribed. This suggests a model where the antisense and mRNA form duplexes in the nucleus which are rapidly turned over.

No mutants in myosin heavy chain (MHC) have been isolated in *D.discoidium.* However, Knecht and Loomis[53,54] suppressed MHC synthesis by the use of asRNA and demonstrated that *D.discoidium* lacking MHC are unable to form multicellular aggregates normally and do not undergo subsequent morphogenesis; large multinuclear progeny are produced. In this case asRNA has elucidated a role for a gene for which it had not been possible to find a natural mutant.

In *D.melanogaster* stable transformation with antisense to the rpA1 gene[55] driven by a heat shock promoter gave rise to 'small egg' phenocopies and severely disrupted oogenesis upon induction of asRNA synthesis.

Antisense has also been useful in elucidating the function of a number of proto-onco genes. For example, Amini *et al* [56] used antisense to suppress the expression of $pp60^{c\text{-}src}$ protein in polyomavirus transformed rat cells. The antisense transcript was driven by a metallothionein-I promoter. Upon induction by cadmium ions the cells grew more slowly in monolayer culture, formed fewer foci on monolayers of normal rat cells and formed fewer colonies in soft agar. See reference [34] for other examples.

1.2.2.3 Engineered antisense in plants.

The core of this project is based on the application of antisense technologies to plants, and at this time (November, 1989) the volume of literature on the subject is not yet large. Thus, in this section I am able to summarise most of the reported results in this field to date.

Suppression of introduced genes

The first report on the antisense inhibition of genes in plant cells was by Ecker and Davis in 1986[42]. They used a transient expression system whereby chloramphenicol acetyl transferase (CAT) and anti-CAT encoding plasmids were electroporated into carrot protoplasts. Inhibition was only observed at very high ratios of antisense plasmid:sense plasmid. RNA levels were not investigated.

In 1987 Rothstein *et al*[57] retransformed transgenic tobacco plants expressing *nos* (nopaline synthase) with an anti *nos* gene. The *nos* gene was driven by its own weak constitutive promoter; the anti *nos* gene, containing about 50% of the 5' coding region and 10bp 5' leader sequence, by the strong constitutive CaMV 35S promoter. The decrease in NOS enzyme levels was between 8 and 50 fold compared to control plants; steady state mRNA levels were similarly decreased by a factor of between 8 and 10 fold. The *nos* gene was also used by Sandler *et al*[58] in a similar experiment.

Delauney *et al*[59] took an approach similar to that taken in my work. A bifunctional construct was used to overcome the so called position effect. It has been observed that a non-selected gene adjacent to a selected gene (eg. a drug resistance marker) may be expressed at a very much lower level than the selected gene; this is presumed to be due to flanking sequences at the site of integration of the foreign DNA in the plant genome (see Appendix I, Section 7.2.5). Transgenic tobacco plants expressing CAT from a weak constitutive CaMV 19S promoter were retransformed with a bifunctional construct in which an anti *cat* gene was fused 3' to the coding region of a hygromycin resistance gene; transcription of this bifunctional construct was driven by the strong, constitutive CaMV 35S promoter. In the absence of DNA rearrangements, selection for high levels of hygromycin resistance co-selects for expression of the anti *cat* gene since they are on the same transcriptional unit (a similar approach to that taken by Kim and Wold[50]). Between 30 and 50% of retransformed plants had decreased levels of CAT, in some cases to background level. A construct containing the whole of the CAT coding region appeared to be more efficient than one containing only the 5' terminal 172bp. There was a strong correlation between mRNA levels and CAT levels. In the plants which showed no decrease in CAT activity, no asRNA was observed. Levels of expression of the hygromycin resistance gene were not measured. It was suggested that in those plants in which there was no decrease in CAT activity the transcript level was enough to confer resistance to hygromycin but insufficient for inhibition of *cat* expression and that CAT mRNA titrated out the anti CAT RNA by formation of duplexes which where rapidly turned over and thus not seen by northern blotting.

Cornelissen and Vandewiele[60] investigated the mechanism of inhibition of gene expression by asRNA in tobacco protoplasts. Tobacco plants expressing the *bar* gene, encoding phosphinothricin acetyl transferase (PAT), and the hygromycin resistance gene (*hpt*) under the control of the TR dual promoter, were retransformed with an anti-*bar* gene. PAT synthesis in protoplasts from these plants was diminished to between 3% and 78% in 5 independent antisense transformants (compared to protoplasts from singly transformed control plants). Steady state mRNA levels and*de novo* protein synthesis were measured in protoplasts prepared from one of these transformants and in control plants. Comparable levels of *hpt* mRNA were found in both, but *bar* mRNA was decreased 4 fold and PAT protein by 13 fold in the antisense protoplasts. This suggested that the asRNA decreases mRNA levels and in addition inhibits translation in the cytoplasm.

Further experiments were directed at dissecting these two antisense mechanisms[61]. It was demonstrated that although the absolute level of *bar* mRNA was decreased, the ratio of cytoplasmic to total *bar* mRNA was unchanged in the presence of anti bar RNA. Since mRNA accumulates in the cytoplasm irrespective of the presence of asRNA, translation of *bar* mRNA in the cytoplasm must be inhibited by asRNA; no RNA duplexes were found and so the inhibition must be the result of unstable interactions. Furthermore, it implies that inhibition is not mediated just by blocking transport across the nuclear membrane; in this case we would expect a build up of mRNA in the nucleus relative to the cytoplasm. Increasing the anti *bar* gene dosage by transforming again resulted in an increase of antisense gene copy number from 2 to 4 copies per genome. This resulted in a negligible increase in steady state levels of asRNA but a 4 fold decrease in *bar* mRNA, implying that inhibition of mRNA levels is dependent not on steady state asRNA levels but on the rate of asRNA transcription. The only *caveat* on these results is that only one transformant was looked at and it is possible that it was atypical. However, these result are consistent with those of Sheehey *et al*[64] (see next section).

Suppression of endogenous and viral genes

Antisense RNA has been used to suppress the expression of both viral and endogenous plant genes[62,63,64,65,66,67,68].

The reports of protection against viral infectivity in plants by asRNA to date[66,67,68] have focused on RNA viruses which replicate in the cytoplasm (unlike TGMV which is thought to replicate in the nucleus). This limits the points at which the virus can be blocked; if the most efficient antisense mechanisms rely on nuclear interactions, then suppression of viral infection by asRNA will be difficult. In two cases, the RNA viruses cucumber mosaic virus[66] and potato virus X[67], the gene targeted was the coat protein gene; this was essentially a control for a cross protection experiment, in which constitutive expression of viral coat protein by a plant suppresses viral symptom development. Coat protein genes will not, in general, be good targets for suppression of viral infectivity by antisense technology as they tend to be expressed at higher levels than other viral genes[69] and may sometimes be removed without abolishing replication and symptom development (see 1.1.2 and Appendix I). In both of the above cases protection was only observed at low levels of viral inoculum. Protection was assayed by symptom development and not replication at the cellular level.

In a third case[68] asRNA was directed against different regions of the cucumber mosaic virus genome. Little or no protection was observed. However, there is little symptom development with the host used and infection was assayed by dot blot analysis, generally a more stringent test for infection than symptom development (see 5.3).

The endogenous ribulose bisphosphate carboxylase (RUBISCO) small subunit multigene family has been suppressed to about 30% of the wild type using antisense from a cDNA clone driven by the CaMV 35S promoter[62]. Northern blots show decreased levels of mRNA and negligible levels of asRNA; a result consistent with the model of duplex formation and rapid turnover.

Expression of the polygalacturonase (PG) gene is required for the softening of tomato fruits during ripening. This gene has been suppressed to 20% of the wild type[63] by transformation with an antisense construct derived from a cDNA clone, again using the strong constitutive CaMV 35S promoter.

Similar work has been carried out by Sheehy *et al*[64]. They achieved reductions in PG levels to between 10 and 30% of wild type. The steady state level of asRNA in

transformed plants was somewhat lower than that of mRNA in untransformed plants. However, nuclear run-on experiments demonstrated that the asRNA was transcribed at a much higher rate than the mRNA. This implies that the asRNA is turned over more rapidly and/or processed less efficiently than the mRNA. Inhibition presumably takes place soon after transcription (before the levels of asRNA have decreased or mRNA has built up) but not at the level of transcription, since transcription of endogenous PG genes does not appear to be inhibited by the transcription of the asRNA[64].

van der Krol *et al*[65,70] used antisense techniques to suppress the developmentally regulated, endogenous chalcone synthase (CHS) genes (members of a small multi-gene family). Expression of some of these genes is required for colour development in flower anthers and petals. The CaMV 35S promoter was used to drive an antisense transcript complementary to the whole of the petunia CHS gene A coding sequence. A number of petunia, tobacco and potato transformants which exhibited a variety of phenotypes were obtained. A wild type phenotype (red flower pigmentation) and a white flower phenotype, expected if the CHS gene is suppressed by the asRNA, were obtained. Various patterned flower phenotypes were also obtained, with segmented or radially distributed bands of colour on a white background. White areas of petal were correlated with an absence of CHS and the corresponding mRNA. There was no correlation between phenotype and leaf steady state asRNA level; some transformants expressing high levels of asRNA had a wild type phenotype and conversely some transformants expressing low levels of asRNA had a pronounced reduction in flower pigmentation. The variegated flower patterns were also shown to vary with the physiological state of the plants (Eg.. light intensity and developmental condition of the plants) in some transformants. The interpretation placed on the differential expression of the antisense genes (i.e. in the variegated flower patterns) transcribed from a supposedly constitutive promoter is that flanking sequences at the site of integration (the 'position effect') are responsible for modifying the promoter specificity. This is a salutary lesson for workers intending to use antisense in plants, as most genes do not give rise to such easily visible effects and in most of the cases discussed so far, differential expression of antisense genes, as observed in this case, would not have been

noticed.

The observation that the closely related, but not identical, *chv* genes of potato and tobacco and the petunia *chs J* gene (86% homology to gene A) were inhibited indicates that complete homology is not required for an effective antisense effect. However, in other eukaryotic systems[71] this is not so; inhibition of the *hsp26* gene in *Drosophila* by asRNA did not result in inhibition of the 72% homologous *hsp28* gene. This difference in specificity may be related to organism/cell type and/or the particular antisense sequence. If this looseness in requirement for total homology turns out to be the norm rather than the exception it may prove useful in antiviral applications where some genes are highly conserved between different, but related viruses (see 1.1 and discussion).

In an extension of the *chs* work[70], the effects of the antisense gene were dissected by making four antisense constructs directed at various parts of the coding region. Different levels of phenotypic effect were observed with different constructs; the most effective was directed against the 3' end of the coding region and the least effective against the 5' end of the coding region. There was a correlation between steady state levels of asRNA in leaf tissue and degree of phenotypic effect for the four constructs. However, antisense directed against the whole coding region had a relatively low steady state asRNA level but was just as effective as the best of the four partial constructs. This suggests that there is a correlation between steady state asRNA levels and inhibition; moreover, there may also be a correlation between length of antisense transcript and degree of inhibition.

In another experiment asRNA was indirectly shown to be much less stable than the corresponding mRNA; in this case the same promoter was used to transcribe both the sense and antisense genes. The steady state level of asRNA was much lower than the steady state level of mRNA in RNA pooled from ten independent transformants. Transformation with additional copies of a **sense** *chs* gene also led to a reduction in CHS levels! These observations, together with the absence of detectable levels of RNA duplex, led to the suggestion that suppression of gene expression was taking place by interference with transcription via the formation of RNA:DNA duplexes (RNA binding to either the plus or minus strand of a transcribing section of DNA must be both assumed to interfere with

transcription). As the cells expressing CHS are no longer actively dividing, it was suggested that DNA replication can not act to disrupt the putative RNA:DNA duplex. There is some evidence to support this hypothesis; it was shown by nuclear run-on experiments[76] that antisense directed against the *myc* gene in mammalian cell culture dramatically inhibits the rate of transcription. However, this is not a typical example; the *myc* gene is under complex regulation by differential transcription involving transcription of both strands in a short region upstream of exon-1. I think that control at transcriptional level is not necessarily taking place in the *chv* case. There are many examples of genes which are expressed at a high level in non-dividing cells, Eg.. the RUBISCO and PG genes, therefore the suppression of transcription by high levels of mRNA is not likely to be commonplace or these genes would be self suppressing. Furthermore, inhibition of PG by asRNA does not take effect at the level oftranscription[64]. In this case while the steady state level of asRNA is low, the rate of asRNA transcription is high; this implies that the rate of transcription has a more important effect on the efficacy of antisense inhibition than steady state asRNA levels. This does not explain the suppression of *chs* gene expression by sense constructs and nuclear run off experiments need to be carried out.

1.2.2.4 Naturally occurring asRNA in eukaryotes.

A number of transcripts have been identified in eukaryotes that are complementary to known gene transcripts (see references[34,35] for reviews). However, their role *in vivo* remains unclear; some small (100bp) naturally occurring antisense transcripts, having complementarity to both the 3' and 5' of their putative target genes, have been shown to be capable of inhibiting translation *in vitro* [72,73]. Most of these transcripts have only relatively short stretches of complementarity to their proposed target, however an antisense to alpha-amylase in barley covers the whole length of both type A and type B barley alpha-amylase transcripts[74]. The homology is not perfect and so the antisense gene must be transcribed from a different location to the sense genes. It is hoped that mutational analysis will be able to elucidate whether these genes have a role or are just 'junk' transcripts arising by gene duplication, inversion and mutation.

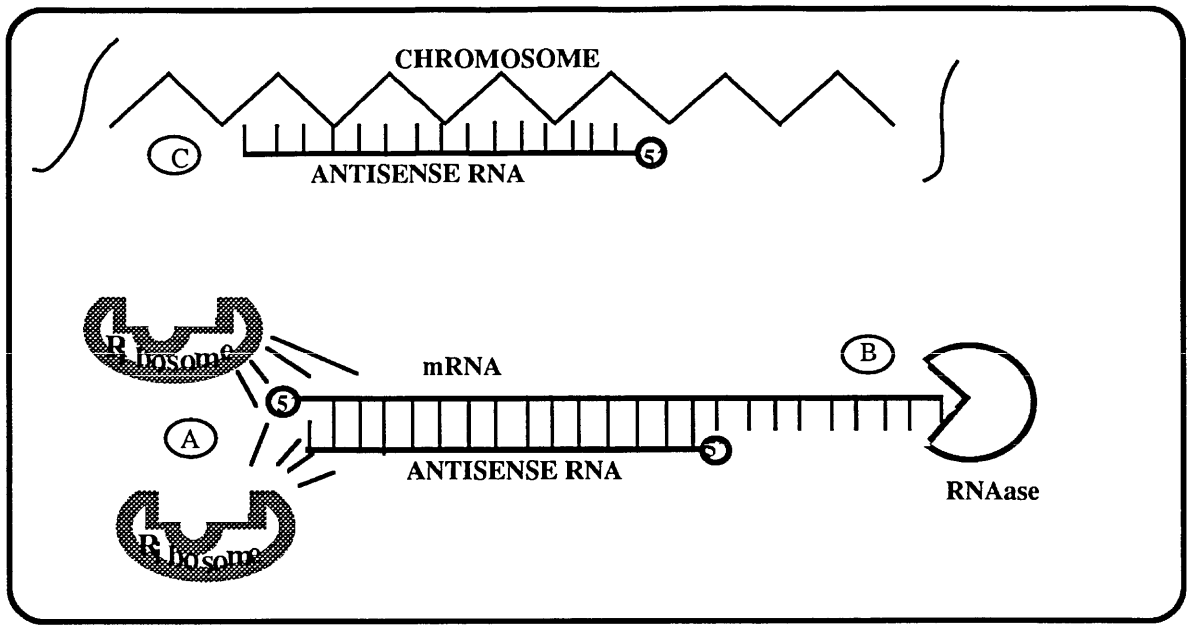
An interesting case has been described of a mutation giving rise to altered

pigmentation in snap dragon flowers[75]. A transposon induced rearrangement gave rise to an inversion and duplication in the CHS gene promoter which might lead to synthesis of an anti CHS transcript.

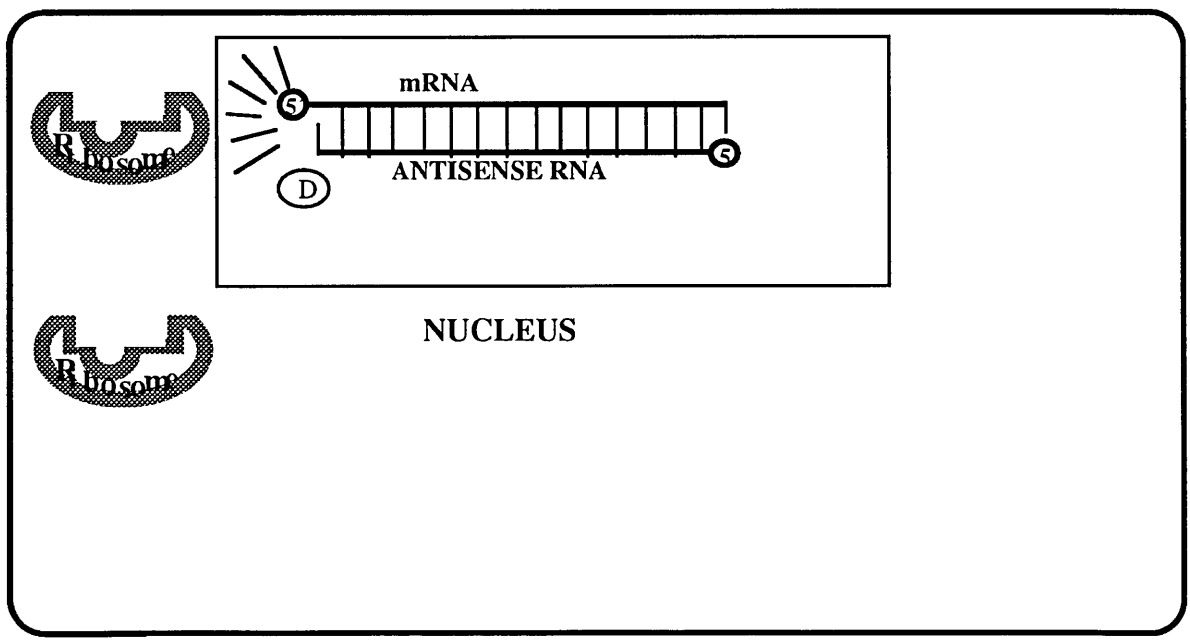
1.2.3 Antisense RNA mechanisms.

There are a number of potential mechanisms by which asRNA can disrupt gene expression (Figure 1.3). Binding at the region of translation initiation may block ribosome binding and thus block translation. Double stranded RNA may be turned over much more rapidly than ssRNA. In eukaryotes, transport across the nuclear membrane may be blocked and post transcriptional modifications such as capping and splicing out of introns may be blocked. It is also possible that transcription may be inhibited by binding of the asRNA to the sense DNA.

There is evidence that all of these mechanisms may come into play, albeit with differing degrees of importance in different systems. In prokaryotes, interference with translation is important, and in naturally occurring cases, the efficiency appears to be mediated by complex secondary structure present on the asRNA and its target.



PROKARYOTE or EUKARYOTE



EUKARYOTE

Figure 1.3. Potential modes of action of asRNA. A: Ribosome binding blocked by presence of RNA duplex at cap or initiation site. B: Rapid turnover of duplex RNA. C: RNA:DNA duplex formation inhibits transcription. D: RNA duplex blocks transport across the nuclear membrane and/or post-transcriptional modifications.

In eukaryotes a variety of different factors come into play. In the transient systems there appears to be a requirement for a large excess of antisense over sense RNA and it may be that this is a requirement if expression is being inhibited predominantly at the level of translation. The appearance of stable RNA duplexes does not appear to be the norm. RNA duplexes have been detected in only three cases[50,54,76]. In all three cases, the duplex RNA was found to be localised in the nucleus. In one case[50] at least, there was a build up of mRNA in the nucleus suggesting that transport across the nuclear membrane was blocked. In other cases where duplex RNA has not been detected, it has been suggested that RNA duplexes are rapidly turned over. Cornelissen[61] found no build up of mRNA in the nucleus, the cytoplasmic: nuclear mRNA ratio was identical in antisense and control plants and only the absolute amount of mRNA was decreased in antisense plants. These two mechanisms are not mutually exclusive, and may be sequence dependent. For some RNA sequences, the duplex may be stable but blocked from active transport across the nuclear membrane, while other sequences may be rapidly turned over on duplex formation in the nucleus. In the later case, the mRNA would be degraded before transport could be blocked. Once the mRNA had titrated out the asRNA, leading to degradation of both, the remaining mRNA would be transported as normal leading to a cytoplasmic:nuclear mRNA ratio no different to control plants.

There is some correlation between steady state asRNA levels and inhibition of expression[50], but it is by no means perfect[65]. Where nuclear run-on experiments have been performed[52,64] and asRNA is effective, it has been shown to be more rapidly transcribed than the corresponding mRNA even when the asRNA steady state levels are low. This suggests that the rate of transcription is more important than the steady state RNA level in determining degree of inhibition in some cases. This hypothesis is supported indirectly by the observation[61] that an increase in antisense gene dosage leads to increased inhibition of the target gene without a significant increase in asRNA steady state levels. There is direct evidence of antisense interference with transcription in only one case[76] and this is a special case (see 1.2.2.3); indirect evidence[70] based on steady state RNA levels and inhibition by increased sense RNA dosage also supports the idea of control at the

transcriptional level. In other cases interference with transcription has been specifically ruled out[64] and, as already discussed (1.2.2.3), this is unlikely to be a general mechanism for suppression of gene expression.

Presumably asRNA forms duplexes with its target soon after transcription and this duplex is usually rapidly degraded. If asRNA is sufficiently unstable it might be turned over before it can interact with its target and this may be the reason for the correlation observed in some cases between asRNA steady state levels and efficiency of inhibition[eg. 70]. Consider the following (Figure 1.4):

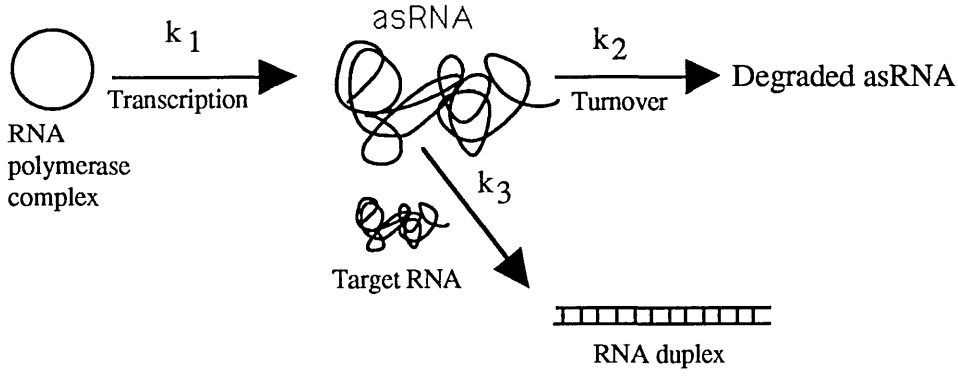


Figure 1.4. asRNA kinetics.

Steady state asRNA level is dependent on k_1 and k_2.

If $k_3 >> k_2$ then inhibition will be dependent only on k_1 (rate of transcription) and hence inhibition is independent of steady state asRNA level .

If $k_3 << k_2$ then inhibition will be dependent on k_1 and k_2 and hence inhibition is dependent on steady state asRNA level .

Similarly if $k_3 \approx k_2$ then inhibition will be dependent on k_1 and k_2 and hence inhibition is dependent on steady state asRNA level.

It has also been shown that unstable asRNA:mRNA interactions in the cytoplasm can decrease the efficiency of translation[61] in stably transformed eukaryotic cells. Whether asRNA directed at the 5' region of the target gene is necessary for inhibition at the level of translation, or even whether inhibition at this level is a general phenomenon in eukaryotes, is not clear.

Introns have been targeted by asRNA[77,78], and while introns are effective targets

for the suppression of gene expression[78] by asRNA, there was no difference in the degree of inhibition attained by intron containing and intronless asRNAs[77]. This suggests that suppression of intron processing is not the main mode of action of asRNA.

The efficiency of asRNA in suppressing gene expression is dependent on a number of factors. These include stability, secondary structure, length of antisense sequence, binding kinetics, transcriptional efficiency, and ratio of asRNA to sense RNA. The individual contribution of these factors is likely to be different for any given RNA sequence. However, based on the evidence, we might propose the following general mechanism for suppression of gene expression by asRNA in eukaryotes:

asRNA is transcribed and rapidly interacts with its target RNA. The RNA duplex formed is usually rapidly degraded; if it is not degraded, it is blocked from transport into the cytoplasm and may accumulate or be degraded more slowly. mRNA and asRNA which do not sequester each other find their way into the cytoplasm where they may interact to inhibit mRNA translation. In rare cases asRNA may interact directly with the DNA to inhibit transcription.

1.3 Polyfunctional transcripts in eukaryotes.

In this work bifunctional constructs (in which the antibiotic resistance gene is encoded on the same transcriptional unit as the antisense region) were used in an attempt to increase the level of antisense transcript and minimise position effects. It is useful, therefore, to review what is known about polycistronic transcripts in eukaryotic systems.

The occurrence of polycistronic transcripts in eukaryotes is very unusual but not unknown. For example, polycistronic transcripts are thought to be produced in *Trypanosoma brucei* and rapidly processed into individual mRNAs[79] and dicistronic transcripts have been identified in a eukaryotic algae, *Cyanophora paradoxa* encoding the alpha and beta sub-units of two light harvesting genes[80].

Chimæric polycistronic messages have been introduced into mammalian cells[81,82,83]. When a DHFR coding region was fused 3' to a xanthine-guanine phosphoribosyl transferase (XGPRT) coding region, translation was reduced by 5-20 fold

(relative to the monocistronic construct) depending on the construction[81]. There was no further reduction when an additional two short reading frames were inserted between the XGPRT and DHFR coding regions[82]. As long as the downstream start codon was less than 50 nucleotides (up or downstream) from the previous termination codon, the downstream gene would be translated. However[82], if the distance was much greater than this, translation was decreased a further 5 fold.

When different upstream genes were used the result was quantitatively different[83]. An adenosine deaminase (ADA) coding region or a human granulocyte-macrophage colony stimulating factor (CSF) coding region was fused 5' to a DHFR coding region. Following this, an additional CSF coding region was fused 5' to the ADA-DHFR construct. The downstream genes in the di or tricistronic constructs were expressed at between 100 and 300 fold less than when the genes were in monocistronic constructs or where they were the first coding region of a polycistronic construct. A set of similar results have been reported in plants[84]; in this case the bicistronic message was very much less stable than the equivalent monocistronic message and selection could not be applied for antibiotic resistance encoded on the dicistronic transcript. Clearly, this instability is not a general phenomenon in plants[59, this work].

These results are consistent with the ribosome binding to the cap site, translation starting at the first AUG and continuing to the first stop codon, at which point the ribosome pauses and binds to a nearby AUG codon (if there is one) from which it translates a second polypeptide[82,83].

1.4 Introduction to this project.

The aim of this project was the suppression of viral infection in plants. There are a number of approaches, other than asRNA, that we could have taken to address this problem, viz.: i) coat protein cross protection, ii) ribozymes, and iii) antibody protection.

i) Coat protein cross protection has already been mentioned (1.2.2.3) with reference to cucumber mosaic virus[66] and potato X virus[67]. In this technique the viral coat

protein is expressed constitutively by the trangsenic plant. Protection is thought to be conferred by the excess of coat protein in the cell preventing the virus from being uncoated[85]. TGMV is a DNA virus; all successful reports of coat protein cross protection to date have been in RNA viruses. Unlike RNA viruses (see Appendix I), TGMV infection is relatively unaltered by mutations in the coat protein (see 1.1.2) and for this reason coat protein cross protection may be less effective. It may, however, be worth trying.

ii) Ribozymes are catalytic RNAs which base pair to a target RNA (as antisense RNA does) and then cleave it. They are associated with some RNA viruses (and viroids), where they are involved in processing eg.[86]. If the recognition sequences are changed the target RNA can be altered. So far the utility of engineered ribozymes has only been demonstrated *in vitro*[87,88]. It is probably only a matter of time before their use *in vivo* is demonstrated and they will make a useful adjunct to asRNA.

iii) It has recently been shown that functional mammalian antibodies can be expressed in transgenic plants[89]. It may be possible to raise antibodies against plant viral proteins and express them in plants. How effective this approach will be in the absence of the rest of the mammalian immune system remains to be seen.

At the time we started this project there was no awareness of the latter two techniques and asRNA seems a technique of more general applicability than coat protein cross protection.

The target for this work was TGMV. It was chosen because it had been cloned and sequenced, and because it is a DNA virus which is thought to replicate in the nucleus. There are many more points on pathways of replication and gene expression at which asRNA can act on a virus that replicates in the nucleus.

There are six potential genes in TGMV which we could have chosen as targets for suppression by asRNA. At the time nothing was known about the function of these genes, except for AR1 which had been identified as a coat protein gene; all other genes had been

shown by mutagenesis to be essential for infectivity. We thought (from work on asRNA in prokaryotes) that it might be important to have an antisense gene covering at least the 5' region and preferably the whole of the coding region of the target. We chose the gene AL1 because it was the only gene large enough to be a DNA polymerase. We chose BL1 because of the existence of convenient restriction sites just before the initiation codon, and because mutagenesis had shown that both genes on TGMV B are essential for symptom development. We were lucky in 50% of our choices: AL1 has been shown to be the only gene essential for viral DNA replication, it is transcribed at low levels, and the exonuclease III deletion which we chose for our construct starts at +2 of the cap site (it also includes the 5' regions of AL2 and AL3). BL1, on the other hand, is transcribed at high levels, is not involved in replication, and has a 400bp sequence between the cap site and the initiation site for translation.

Our rationale for choosing to use bifunctional constructs to express our antisense gene was two fold. Firstly, we wanted to minimise the effects of any position effect caused by sequences flanking the site of insertion of our constructs; the variability in expression caused by this effect is particularly pronounced in the *nos* promoter (see Appendix I), the only plant promoter which had been studied in any detail at the time; problems had already arisen in our laboratory due to the position effect[90]. Secondly, we wanted to increase the levels of our antisense transcript by selecting for high levels of drug resistance, in a similar manner to Kim and Wold's[50] work with *Xenopus* oocytes and as Delauney *et al*[59] did some time later in plants. In this way we could be sure that our antisense gene would be efficiently expressed. Furthermore, we hoped that if we put the antisense gene upstream of the drug resistance gene we would decrease the translational efficiency of the drug resistance gene, as has since been shown (see 1.2.3), thus, selection for drug resistance would select for even higher transcript levels.

The promoter we used was the strongest constitutive plant promoter available at the time, and enhancer sequences were added to the constructs as soon as their effect had been reported.

Two drug resistance genes were used in this work so as to allow selection for

double transformants or hybrids expressing both antisense genes, had this been thought to be desirable.

2: MATERIALS AND METHODS.

2.1 Materials.

2.1.1 Consumables.

Bacto-agar, tryptone, and yeast extract were purchased from Difco Laboratories, West Molesey, Surrey.

Nutrient broth was purchased from Oxoid Ltd. Basingstoke, Hampshire.

Cellulases used in the preparation of protoplasts were purchased from R.W.Unwin, Welwyn Garden City, Hertfordshire.

Prepackaged plant media was purchased from Flow Laboratories Ltd. Rickmansworth, Hertfordshire.

BSA, lysozyme, salmon sperm DNA, IPTG, TLC plates, X-gal, amino acids, Ficoll, polyvinylpyrrolidone, leupeptin, hydroquinone, HEPES, PMSF, chloramphenicol, tetracycline, ampicillin, streptomycin and kanamycin, were all purchased from Sigma Chemicals Ltd., Kingston-Upon-Thames, Surrey.

Ethidium bromide, EDTA, DTT, 2-mercaptoethanol, DEPC, chromic acid, cæsium chloride, acrylamide, methylene bisacrylamide (electrophoresis grade), polyethylene glycol (6000), Triton-X 100, dimethyldichlorosilane solution, agarose "ultra-pure" and low melting point agarose were purchased from BDH Ltd., Poole, Dorset.

Urea "ultra-pure" was purchased from Swartz-Mann, Orangeburg, N.Y., USA.

Calf intestinal alkaline phosphatase, phenol (molecular biology grade), oligo-dT cellulose, glycogen , RNAse A, DNAse, DNA polymerase 1 (Klenow-fragment) and Avian Myoblastosis Virus (AMV) reverse transcriptase were purchased from Boehringer Corporation (London) Ltd., Lewes, Sussex.

All isotopes, Hybond N and Hybond C were purchased from Amersham International, Amersham, Buckinghamshire.

Restriction endonuclease enzymes, M13 universal primer, hybridization primer, Exonuclease III, and SI nuclease were purchased from New England Biolabs (CP Labs., Bishops Stortford).

Sephadex G50 and random hexanucleotides were purchased from Pharmacia Fine Chemicals, Milton Keynes, Buckinghamshire.

Miracloth was purchased from Calbiochem, La Jolla, California, USA.

Disposable pollyallomer centrifuge tubes were purchased from Sarstedt, Leicester, Leicestershire.

Sterile disposable plastic ware (Nunc and Sterilin tubes) were purchased from Sterilin Ltd., Milton Keynes, Buckinghamshire.

2.1.2 Media.

Unless otherwise stated, solid media was made by addition of 1.5% agar to the liquid media prior to autoclaving. Plant media was solidified with 0.8% agar (agarose for transformation and first rooting).

Luria Broth: 10g tryptone, 5g yeast extract, 5g sodium chloride, 1g glucose dissolved in 1 litre of distilled water.

Top agar was made by the addition of 0.7% agar and magnesium chloride (10mM final concentration) to Luria broth.

SOC Medium: 20g tryptone, 5g yeast extract, 580mg sodium chloride, 186 mg potassium chloride autoclaved in 970ml of "Milli-Q-water" and the following added from filter sterilised stocks: 1M magnesium chloride (10ml), 1M magnesium sulphate (10ml) and 2M glucose (10ml).

2xYT Medium: 16g tryptone, 10g yeast extract and 10g of sodium chloride dissolved in 1 litre of distilled water and autoclaved.

Nutrient Broth Medium: 13 g nutrient broth dissolved in 1 litre of distilled water and autoclaved.

MSO Medium: 440mg $CaCl_2$:$2H_2O$, 1650mg NH_4NO_3, 1900mg KNO_3, 0.830mg KI, 0.025mg $CoCl_2$:$6H_2O$, 170mg KH_2PO_4, 6.200mg H_3BO_3, 0.250mg Na_2MoO_4:$2H_2O$, 370mg $MgSO_4$:$7H_2O$, 22.0mg $MnSO_4$:$4H_2O$, 0.025mg $CuSO_4$:$5H_2O$, 8.60mg $ZnSO_4$:$4H_2O$, 27.85mg $FeSO_4$, 37.25mg Na_2EDTA, 2.00mg glycine, 100mg inositol, 0.500mg nicotinic acid, 0.500mg pyrodoxine:HCl, 0.100mg thiamine:HCl and

30g of sucrose dissolved in 1 litre of distilled water, adjusted to pH5.8 with NaOH and autoclaved. Thankfully, the above components, with the exception of sucrose, are sold in premixed sachets by Flow Laboratories.

MSD 4x2 Medium: As MSO with the addition of 0.1mg/l naphthaleneacetic acid (NAA) and 1.0mg/l 6-BAP.

MSP1 9M Medium: As MSO with the addition of 2.0mg/l naphthaleneacetic acid (NAA), 0.5mg/l 6-BAP and 90g/l mannitol.

CPW 9M Medium: 27.20mg KH_2PO_4, 101.00mg KNO_3, 246.00mg $MgSO_4{:}7H_2O$, 0.160mg KI, 0.025mg $CuSO_4{:}5H_2O$, 1480mg $CaCl_2{:}2H_2O$ and 90g mannitol dissolved in 1 litre of distilled water, adjusted to pH5.8 with NaOH and autoclaved.

2.1.3 Bacterial strains.

Strain	Genotype	Source
E.coli TG2	lac$^-$, pro$^-$, supE$^+$, thi$^-$,recA$^-$, Srl::Tn10(Tcr), hsd deletion 5, EcoK, (rk$^-$, mk), F, tra, 36, proAB, lacIQ,lacZ deletion M15.	Gift of D.Glover, Imperial College.
E.coli CB36	SupE, SupF, (rk$^-$, mk$^-$), met$^-$, dam^{-3}, rpsl.	Gift of C.Boyd, Beatson Institute, Glascow.
E.coli DH5 alpha	F',endA1,hsdR17, (rk$^-$, mk$^-$), supE44,thi$^-$1, recA1, gyrA96, relA (lacZYA$^-$argF), v19, 80, lacZ deletion M15.	Bethesda Research Labs.
E.coli HB101 (RK2013)	F', hsdS20 (r$^-$Bm$^-$B), recA13, Km^r, ara$^-$14, proA2, lacY1, galK2, rspL20(Sm^r), xyl-5, mtl-1, supE44, lambda$^-$.	91, 92
A.tumefaciens LBA4404	Ach5 Chromosomal background, Plasmid pAB4404, onc$^-$, Rf^r.	93

Table 2.1. Bacterial strains.

2.2 Methods.

2.2.1 Microbiological techniques.

2.2.1(i) Antibiotic selection.

Throughout this work the standard antibiotic concentrations given in Table 2.2 were used in both liquid and plate selections unless otherwise stated. The appropriate volume of the antibiotic stock solution was mixed with the molten medium which had been pre-cooled to 45°C.

Antibiotic	Stock Solution (mg/ml)	Final Concentration (μg/ml)
Ampicillin	100 in water	100
Kanamycin	50 in water	100 (plants and bacteria)
Tetracyclin	12.5 in 50% ethanol	12.5
Rifampicin	50 in methanol	100
Carbanicillin	100 in water	100
Hygromycin	50 in water	25-50 (plants, see text)

Table 2.2. Antibiotic concentrations.

2.2.1(ii) Transformation of E.coli with plasmid DNA.

The general procedure for transforming bacteria with plasmid DNA was modified from Maniatis[94]. 2.0ml of an overnight bacterial culture (DH5-alpha or TG2) were diluted in 100ml of 2xYT liquid media and shaken at 250 rpm on a platform shaker at 37°C until the optical density at 590nm (OD_{590}) was 0.4-0.6 units. The cells were centrifuged at 4°C for 5 minutes at 8000 rpm in a Sorvall RC-5 superspeed refrigerated centrifuge using a GSA rotor, then resuspended in 20ml of ice cold 0.1M magnesium chloride and placed on ice for 10 -15 minutes. After recentrifugation at 3000 rpm for 5 minutes in a Sorvall RC-5 superspeed refrigerated centrifuge using a SS34 rotor, the cells were resuspended in 2ml ice cold 0.1M calcium chloride and kept on ice for 1 hour. For optimal transformation efficiency the cells were used immediately. To 0.2ml of this suspension held on ice in a

1.5 ml eppendorf tube was added 1-100ng of DNA and the mixture incubated at 0°C for 0.5-2 hours.

The cells were heat shocked at 42°C for 2 minutes and chilled on ice for a further few minutes. Either SOC or 2xYT broth (0.8ml) was added to the mixture at room temperature (use of SOC broth gives an increase in transformation efficiency of 4-5 fold over 2xYT). Following incubation at 37°C for 1 hour to allow cell wall recovery and expression of the transforming plasmid, 10-100µl of the mixture was plated onto LB-medium plates containing antibiotics at the appropriate concentration (see Table 2.2). When it was required to plate the total transformation mixture, the cells were pelleted for 5 minutes in a microfuge at room temperature, resuspended in 100µl of media and plated as above. Using this procedure, transformation frequencies of 10^6 per µg of DNA were routinely obtained. In order to ensure that the transformation was working properly, controls with no plasmid DNA and with 1ng of pBlueScribe+ were carried out.

2.2.1(iii) Conjugation of recombinant plasmids into *Agrobacterium* by triparental mating[95].

Overnight cultures of the *E.coli* strain containing the recombinant plasmid and the *E.coli* helper strain HB101 (pRK2013) were grown at 37°C overnight in Luria broth. The recipient *Agrobacterium* strain (LBA4404) was grown for two days in nutrient broth at 30°C; if grown at temperatures above 32°C the Ti plasmid is lost[95]. The three cultures (100µl of each) were mixed on an upturned nutrient agar plate and incubated at 30°C overnight. A streak was taken from the resultant bacterial lawn and transferred to nutrient agar plates containing Rifampicin (to select against *E.coli*) and the appropriate selection for the recombinant plasmid (to select for the transformed *Agrobacterium*); the plates were incubated for 2 days at 30°C. The resultant colonies (typically 10-100) were streaked onto fresh plates and the integrity of the conjugated plasmid checked by Southern blotting of total *Agrobacterium* DNA (see 2.2.3G(i)). Appropriate controls for transformation were also carried out.

2.2.1(iv) Sterility.

Solutions and media were sterilised by autoclaving for 15-60 minutes, depending

on the volume being used, at 15 p.s.i. (120°C) using a domestic pressure cooker or the industrial autoclave facilities in the Imperial College Pilot Plant. In addition, glass and plastic ware were put through the drying cycle in the industrial autoclaves.
Heat labile solutions were filter sterilised by passage through a pre-sterilised 0.2μm pore sterilising unit ("Acrodisc" Gelman Sciences).

2.2.2 Plant tissue culture techniques.

All plant tissue culture was carried out using sterile techniques in a sterile laminar flow hood.

2.2.2(i) Plant leaf disc transformation mediated by *Agrobacterium*[95].

Two slightly different methods were used; there was no discernible difference in transformation efficiency although one method was less work than the other.

Transformation was carried out on *N.tabacum* cultivar Samson N. N or cultivar Petit Havana SR1. Leaves were either from healthy greenhouse grown plants, sterilised for 10 minutes in 10% Domestos and rinsed 4 times in sterile distilled water, or from plants grown axenically in tissue culture. The mid-vein was removed from the leaves and they were cut into approximately 0.5cm squares.

In the first method the leaf discs were put onto MSD 4x2 solid media in petri dishes in the tissue culture room at 24-28°C under constant light for 48 hours. They were then dipped into a 50 fold dilution of fully grown *Agrobacterium* culture in MSO liquid media, and returned to the plates for a further 48 hours. Following this, the leaf discs were transferred to fresh plates containing solid MSD 4x2 media + Carbenicillin (to kill the *Agrobacterium*) and the appropriate selective antibiotic. The plates were sealed with Parafilm to prevent evaporation.

In the alternative procedure the freshly cut leaf discs were floated upside down on 100-fold *Agrobacterium* dilution in liquid MSO media for 48 hours prior to transfer to solid selective MSD 4x2 media as above.

After 10 days, the first signs of callus or green spots start to appear on the edges of the leaf discs. After 20 to 30 days shootlets and callus appear on the edge of the leaf discs.

The shoots are cut away and transferred to solid rooting media (MSO) plus the appropriate antibiotics. If the shoot roots in the presence of selection, it is almost certainly transformed. This plantlet may be grown up and micro–propagated in tissue culture or transferred to soil and hardened off before going to flower and producing F1 seeds.

2.2.2(ii) Sterile micro-propagation of *N.tabacum*.

Each plant was cut away from its roots and the stem cut into sections, each having one leaf. The leaf was removed and the stem piece placed in solid MSO media containing appropriate selection in a 500ml screw cap jar. A new plant developed from the axillary bud which lies between the leaf and stem; roots normally developed from the lower end of the stem piece. One to five stem pieces were typically put into each jar and subculturing was normally carried out every month, and no less frequently than every two months. Each line was propagated in more than one jar due to the ever present possibility of fungal contamination.

2.2.2(iii) Transfer to soil and hardening off of micro–propagated plants.

When micro–propagated plants had reached a size of 2.5cm they could be transferred to compost if required. After careful removal from the jar, the excess media was gently removed using distilled water, taking care not to damage the roots. The plant was then placed in a hole in loosely packed compost (Fisons No. 1) in a 5 inch pot (or seedling tray). The pot was covered with a plastic bag to keep the humidity at 100%. The humidity was reduced over 3 or 4 days by the simple expedient of cutting one corner off the bag every day or so before finally removing the bag completely. If this procedure is not carried out there is a chance that the young plants will die from dehydration before developing a waxy cuticle on the upper surface of their leaves.

2.2.2(iv) Selfing of plants to generate F1 seeds and selection of drug resistant F1 plants.

At the first sign of flowering, the developing buds were covered with a bag in order that the plants would be self pollinated. The bags made from grease proof paper, were sealed with masking tape; if plastic bags were used the flowers tended to rot. About a month after the plants had set seed, the mature seeds were collected and stored in 10ml

Sterilin tubes at room temperature in a dry place. Under such conditions the seeds remain viable indefinitely.

Seeds to be germinated on drug selection were sterilised for 10 minutes in 10% Domestos and rinsed 4 times in sterile distilled water. They were then placed on solid MSO media containing the selective antibiotic. Germination took place after 5-10 days. After a further 7-21 days the non-resistant seedlings could be distinguished from the resistant seedlings (see Figure 2.1). If the seedlings were not resistant, no more leaves were formed after the initial cotyledons and the root did not develop. After a longer time, the non-resistant plants became bleached and died. Resistant plants may be micropropagated or transferred to soil and hardened off as described above (2.2.2(iii)).

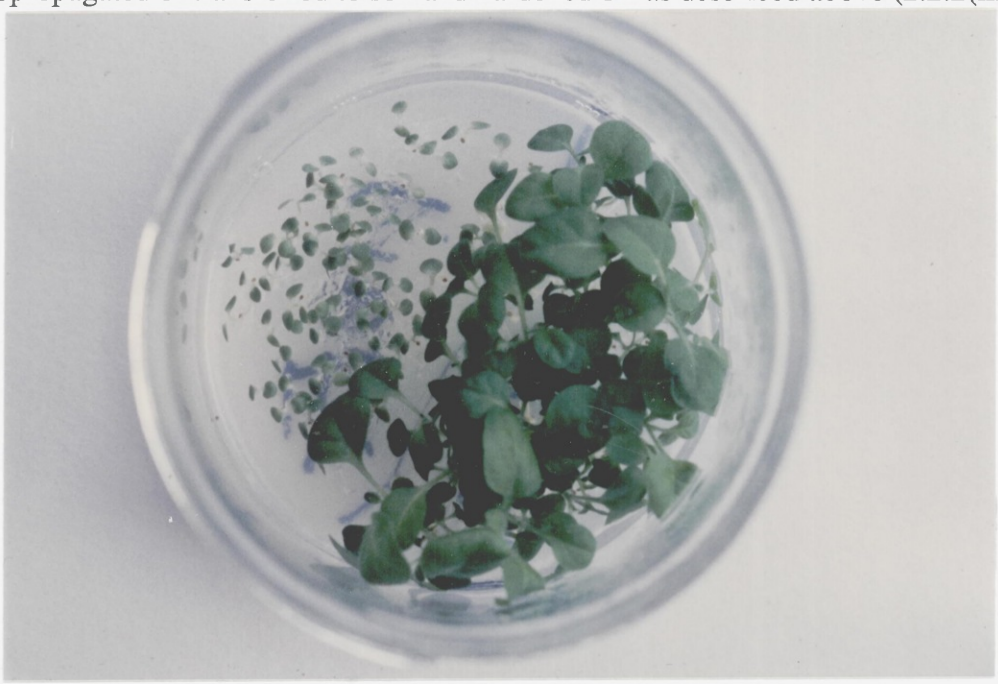


Figure 2.1: Kanamycin sensitive and resistant seedlings on MSO + kanamycin (3 weeks after germination).

2.2.2(v) Protoplast isolation from cultured suspension cells of Albino *Petunia hybrida*, Var. "Blue Lace"[95].

A four day old suspension culture of *P. hybrida*, Var. "Blue Lace" (25ml) (supplied by John Draper, Botany Dept., University of Leicester) was allowed to settle for 30 minutes and the medium drawn off with a sterile Pasteur pipette. To the cells were added 40mls of an enzyme mixture (0.2% w/v cellulase "Onozuka" R10, 0.05% w/v Macerozyme R10 in CPW 13M medium); the cells were then incubated for 16 hours at 25°C to allow the enzymes to dissolve the cell wall. The protoplasts were filtered through a

64μm mesh sterile sieve into a 'Nunc' tube and centrifuged at 400rpm in an MSE centaur 2 centrifuge. The pellet was gently resuspended in CPW 13 M medium, pelleted as before and washed twice in MSP1 9M medium before being resuspended in 10ml of the same medium. The protoplasts were examined under a microscope using a haemocytometer to determine the number of viable protoplasts (which should be spherical and have chloroplasts spread evenly around the surface).

It should be noted that the concentration of enzyme and the amount of time that cells are in contact with the solution will vary with the enzyme batch and the condition of the culture, and should be determined empirically.

2.2.2(vi) Electroporation of plasmid into *P. hybrida* protoplasts for transient expression[95].

Plasmid DNA (between 3 and 6μg of in 20μl sterile distilled water) was added to ~5 x 10^5 protoplasts in 0.5ml of electroporation buffer (MSP1 9M, 15mM magnesium chloride). The mixture was heat shocked at 45°C for 5 minutes and cooled on ice. An equal volume of a 30% PEG solution was added and the mixture was incubated at room temperature for 10 minutes. The mixture was electroporated at 2000V/cm with a square wave pulse for 3 x 30μs in the chamber of a Kruss model TA 750 electroporator. The protoplasts were expelled into a 5cm Petri dish containing 2ml of solid MSP1 9M medium. After 10 minutes at room temperature, 10 ml of liquid MSP1 9M medium was added and the plates were sealed with Parafilm and incubated in the dark at 28°C for 48 hours before harvesting and performing the CAT assay as described (2.2.4A(v)).

All protoplast manipulations were carried out during the Plant Genetic Transformation Course run by John Draper at the University of Leicester in December 1986.

2.2.2(vii) Infection of *N.tabacum* and *N.benthamiana* with TGMV.

Two methods were routinely used for infecting *Nicotiana* with TGMV: the mechanical inoculation method and the more efficient 'agroinfection' method.

N.benthamiana is a more permissive host for TGMV than *N.tabacum* and 100% of the inoculated plants would routinely produce symptoms. In the case of *N.tabacum* , it

was harder to obtain symptomatic plants. Rates of infection could be increased by keeping the plants in the dark for 36 hours prior to inoculation[96] and by removing the lower leaves.

In mechanical inoculation, after removal of the lower leaves, the plants (typically 2-5 inches high) were sprayed with a fine mist of carborundum powder; 5µl of a viral pellet (see 2.2.2(viii)) was put on the upper developing leaves and gently rubbed in with a gloved finger; the plants were then watered. If no symptoms had developed after 10 days, lower leaves were again removed and the process repeated. If the pellet was infectious, symptoms were usually observed 7-14 days after the second infection. Control plants were treated in the same way, with distilled water used in place of the viral pellet.

Agroinfection[97] (see Appendix I, Section 7.2.7) is a technique in which infection is mediated by *Agrobacterium* strains containing a tandem repeat of the geminivirus DNA within T-DNA borders. This method of infection is much more efficient than mechanical inoculation and, in some cases, allows plants to be infected with viruses which are not otherwise transmissible in the absence of the natural insect vector[98]. Overnight cultures of the *Agrobacterium* strains containing the dimers of TGMV A and TGMV B, cloned in both orientations in the binary vector polylinker (4 strains) were mixed. Between 5 and 10µl of the mixed culture was injected into the plant stem just below the developing leaves (plants were treated as for mechanical inoculation). Symptoms normally developed 7-10 days after inoculation. Control plants were treated in the same way, with distilled water used in place of the agroinfectious strains.

2.2.2(viii) Preparation of TGMV viral inoculum[99].

It was observed that the most infectious inoculum was obtained from infected *N.benthamiana* . All solutions were precooled to 4°C and the preparation carried out in the cold room where possible. Leaves were collected from mature, infected, symptomatic plants and either frozen at -70°C or used straight away for preparation of the viral inoculum; 20 to 100g of leaf material was typically used. Frozen leaves were ground up in a precooled Braun domestic coffee grinder before addition of 2 volumes of extraction buffer (100mM sodium citrate, 0.75% sodium sulphite, 5mM EDTA, 1% 2-mercaptoethanol, 0.32% ascorbic acid, adjusted to pH 7.0 with NaOH/HCl), fresh leaves were homogenised

in the presence of the buffer in a Waring blender. The mixture was transferred to a glass beaker and Triton-X100 was added slowly with stirring to a final concentration of 2.5%. The mixture was stirred overnight at 4°C and filtered through 2 layers of muslin before being centrifuged at 10,000 rpm for 10 minutes in a SS-34 rotor. The supernatant was again filtered through 2 layers of muslin and ultra centrifuged at 40,000rpm in VTi-60 rotor for 45 minutes. The pellets were resuspended in TE buffer (10mM Tris:HCl, pH 7.6;1mM EDTA) (1ml/g of leaf tissue) and stored at -20°C. Repeated freezing and thawing was found to inactivate the inoculum. Infectivity was tested by infecting *N.benthamiana* as described above (2.2.2(vii)).

2.2.3 Biochemical techniques.

2.2.3A Miscellaneous biochemical techniques.

2.2.3A(i) Extraction of DNA with phenol and chloroform.

This was carried out to purify the DNA from contaminating proteins and other molecules such as SDS or high salt, which may inhibit enzymic modification.

Phenol was made 0.1% with 8-hydroxyquinoline and extracted several times with an equal volume of 1M Tris pH 8.0, followed by 0.1M Tris pH 8.0, until the pH of the aqueous phase was >pH7.6. The phenol was stored under the equilibration buffer at -20°C. Chloroform was a mixture (24:1 v/v) of chloroform and isoamyl alcohol, the latter reduces foaming and facilitates phase separation.

To the crude DNA sample, an equal volume of phenol was added, vortexed vigorously (except in the case of chromosomal DNA where mixing was carried out by careful inversion) and centrifuged in a microfuge for 5 minutes. The aqueous phase was transferred to a clean eppendorf tube and similarly extracted with an equal volume of a 1:1 mixture of phenol/chloroform. The upper aqueous phase was finally extracted with an equal volume of chloroform before transfer to a fresh eppendorf tube and alcohol precipitation .

2.2.3A(ii) Precipitation of DNA with ethanol.

The DNA solution was adjusted to a final concentration of 0.3M sodium acetate,

pH 4.8, or 2.5M ammonium acetate, pH7.5 and either 2.5 volumes of absolute ethanol or 0.8 volumes of isopropanol were added. The solution was kept at 4°C for 10-30 minutes and the DNA was pelleted by centrifugation in a microfuge for 15 minutes. The DNA pellet was washed in 70% ethanol . After decanting the ethanol, the last drops were removed from the DNA pellet by the capillary action of the corner of a Kimwipe tissue. The DNA was dissolved in TE buffer to the desired concentration.

2.2.3A(iii) Spectrophotometry.

DNA and RNA concentration and purity were estimated by measuring the optical density (O.D.) at 260nm and 280nm in a Car 210 dual beam recording spectrophotometer, using a path length of 1cm. An O.D. of 1.0 at 260nm indicates a DNA concentration of 50μg/ml and a RNA concentration of 40μg/ml; an O.D. ratio of 1.8 for 260nm:280nm wavelengths indicates that a DNA preparation is free from contaminating RNA and proteins, a ratio of 2.0 indicates that RNA is free from contaminating DNA and proteins[94]. The O.D. of bacterial cultures was measured at OD_{590} (this measurement is related to scattering) in order to estimate the number of cells/ml. An O.D. of 1.0 at 590nm indicates a cell concentration of approximately 10^8 cells/ml.

2.2.3A(iv) Autoradiography.

Southern blots, Northern blots, and DNA sequencing gels were autoradiographed on Fuji or Kodak X-ray film in Harmer cassettes containing 2 intensifier screens at -70°C. Length of exposure was dependent on the intensity of the signal. Films were developed in a Kodak industrial X-OMAT processor-model 3.

2.2.4A(v) CAT assay in protoplasts[95].

The electroporated protoplasts were collected by centrifugation in a 1.5ml Eppendorf tube at 1000rpm, resuspended in 100 μl extraction buffer (0.25M Tris:HCl, pH7.8; 0.50mM leupeptine) and sonicated on ice for 30 seconds at high power using a MSE sonicator with a microlip probe. The extracts were incubated at 65°C for 10 minutes and then allowed to cool to room temperature. Following this, 5 μl of ^{14}C-chloramphenicol (Amersham CFA.515) and 3.0μl of a freshly prepared 20% w/v Acetyl-CoA solution were added. The mixture was incubated for one hour at 37°C and

extracted twice with 150µl of ethyl acetate. The combined organic phases were evaporated to dryness in a vacuum desiccator and the residue dissolved in 20µl of ethyl acetate prior to application to a silica TLC plate. The TLC plate was developed in chloroform:methanol 95:5, air dried and autoradiographed overnight. CAT activity is indicated by the presence of high Rf chloramphenicol acetates (3 forms). The assay may be quantified by cutting out the radioactive spots and carrying out a scintillation count.

2.2.3B Nucleic acid preparation.

2.2.3B(i) Large scale preparation of covalently closed supercoiled plasmid DNA[100].

Cells were grown to stationary phase overnight at 37°C in 500ml of 2xYT with shaking. The cells were then centrifuged in 500ml Sorvall bottles at 6000 rpm for 10-15 minutes at 4°C in a GSA rotor and the cell pellet resuspended in 4ml of TSE (25% w/v sucrose; 50mM Tris:HCl, pH 8.0; and 50mM EDTA); 0.5ml of a lysozyme solution (10mg/ml) was added. The mixture was incubated on ice for 10 minutes and the cells were lysed by the addition of 5ml of lytic mixture (2% Triton X-100; 50mM Tris:HCl, pH 8.0; and 50mM EDTA). Successful lysis was indicated by the clearing of the solution which also becomes very mucilaginous and usually occurred within two or three minutes at room temperature. The lysate was cleared by centrifugation for 40 minutes at 18,000 rpm at 4°C in a Sorvall RC-5 superspeed refrigerated centrifuge using a SS-34 rotor. The bacterial debris and the chromosomal DNA were pelleted, leaving mostly plasmid DNA in the supernatant. The supernatant was transferred gently to a 25ml test tube containing 0.5ml of ethidium bromide (10mg/ml) and cæsium chloride was added until the refractive index was 1.3957 (corresponding to a density of 1.66g/ml). This mixture was centrifuged for 15 minutes at 15,000 rpm in 40ml Sorvall tubes. The lipid layer, which rises to the top, was easily removed. The preparation was centrifuged in a VTi 65.2 rotor using 'supa seal' tubes at 45,000 rpm and at 15°C for 14-18 hours. This generated an equilibrium gradient in which different DNA species band at different heights corresponding to their buoyant density. These can be visualised by short exposure to long wave UV light or, quite often, in natural light. The denser, lower band (covalently closed circular plasmid DNA) was

collected using a 19 gauge needle connected to a 2ml syringe. The top of the tube was punctured before the needle was gently introduced into the lower band. The ethidium bromide bound to the DNA was removed by repeated extractions with isopropanol saturated with 20xSSC (3.0M sodium chloride, 0.3M trisodium citrate). The extractions were repeated until the upper, organic layer was colourless. The DNA solution was diluted three-fold with distilled water and precipitated by the addition of 0.8 volumes of isopropanol or 2.5 volumes of ethanol, washed in 70% ethanol and redissolved in TE. Alternatively, the DNA was dialysed against 4 litres of TE at 4°C, with three changes over 24 hours to remove the cæsium chloride. Occasionally, when buffer was added prior to enzymic modification, the DNA was found to be degraded. This was presumably due to a nuclease which had bound to the DNA during the purification procedure and been activated by the buffer. This problem could be alleviated by inserting a phenol/chloroform extraction step at this stage; a white layer at the interface being evidence of protein contamination.

The concentration of the DNA was measured as described in section 2.2.3A(iii).

2.2.3B(ii) Quick plasmid DNA preparation.

This method is a modification of that described by Birnboim and Doly[101]. The three solutions required in this procedure are as follows. Solution I : 10mM EDTA, 25mM Tris:HCl, pH 8.0. Solution II : 0.2M sodium hydroxide, 1% SDS (kept for no more than 1 week at room temperature). Solution III : 3M sodium acetate, pH 4.8.

The cells from a 2ml overnight culture were pelleted in a microfuge for 1 minute and the supernatant was removed by aspiration. The pellet was resuspended in 0.1ml of solution I and kept on ice for 5 minutes. 0.2ml of solution II was gently mixed with the cell suspension and followed by 0.15ml of solution III; the mixture was left on ice for 10 minutes. Insoluble materials, including chromosomal DNA, were pelleted by centrifugation in a microfuge for 5 minutes at 4°C. The supernatant was carefully transferred to a clean tube and extracted with 1 volume of phenol/chloroform. Two volumes of absolute ethanol were added and the DNA precipitated (at 4°C for 10 minutes) and pelleted by centrifugation in a microfuge for 10 minutes. The pellet was washed twice with 70% ethanol and resuspended in 0.1ml of TE. 5µl of DNA solution were commonly

used for analysis on an agarose gel or for restriction endonuclease analysis.

2.2.3B(iii) Preparation of total DNA from *A.tumefaciens*.

This method was adapted from Garfinkel *et al.*[102]. One bacterial colony was grown overnight with shaking at 30°C in 5ml of nutrient broth. After harvesting, the cells were spun in a microfuge for 30 seconds and resuspended in 0.3ml of buffer (50mM Tris:HCl, pH 8.0; 20mM EDTA). 0.05ml lysozyme (10mg/ml) was added and the cells were incubated on ice for 10 minutes. This was followed by the addition of 0.1ml of 10% SDS solution and proteinase K to a final concentration of 50μg/ml. The mixture was incubated at 65°C for 10 minutes before gently extracting with phenol. The preparation was then centrifuged for 5 minutes in a microfuge to separate the layers. The upper aqueous layer was collected using a wide bore pipette (to minimize shearing of the chromosomal DNA). The DNA was extracted twice with phenol/chloroform and twice with chloroform as described in section 2.2.3A(i). The DNA was then precipitated with 2 volumes of absolute ethanol and spun down at 10000 rpm for 20 seconds in a microfuge. The pellet was washed in 70% ethanol; the last drops of ethanol were removed from the nucleic acid pellet by the capillary action of the corner of a Kimwipe tissue. The pellet was dissolved in 100μl of TE by gentle heating in a water bath at 55°C. The DNA solution was stored at -20°C.

2.2.3B(iv) Preparation of total DNA from *N.tabacum*[103].

Tobacco leaves or seedlings were washed in tap water, drained, frozen on dry ice and stored at -70°C before use. Leaves had the midveins removed before freezing as these contain polysaccharide and little DNA. A Braun, domestic coffee grinder was prechilled for 10 minutes with dry ice and 1-20g of frozen plant material was added together with about 0.1g diethyl thiocarbamic acid to inhibit polyphenol oxidases; if less than 5g of material was used, the bulk was made up with dry ice pellets to ensure efficient grinding. After grinding for between 30 seconds and 1 minute, the still frozen powder was transferred to 12 ml of extraction buffer (100mM Tris:HCl, pH 8.0; 50mM EDTA, pH 8.0; 500mM NaCl; 10mM 2-mercaptoethanol) in a 30ml Sarstedt disposable pollyallomer centrifuge tube. After gentle mixing to ensure that all dry ice had evaporated,

8ml of 20% SDS was added and the tube capped and mixed vigorously by inversion. The mixture was then frozen on dry ice until all samples had been processed.

Following this, the tubes were put in a water bath at 65°C (with caps removed) for 25 minutes. Following this 4ml of a 5M potassium acetate were added and, after vigorous mixing, the tubes were kept on ice for 20 minutes. The protein/SDS complex was removed by centrifugation for 20 minutes at 10,000rpm at 4°C in a Sorvall RC-5 superspeed refrigerated centrifuge using a SS34 rotor. The supernatant was filtered through Miracloth into a fresh tube containing 8ml of isopropanol. After 5 minutes at room temperature, the crude nucleic acids were spun down as above, the supernatant discarded and the pellet dissolved in 0.7ml high salt TE (50mM Tris:HCl, pH 8.0; 20 mM EDTA, pH 8.0). There was insoluble material present at this stage and great care had to be taken so as not to shear the high molecular weight DNA in attempting to dissolve the pellet; dissolution could be aided by gentle heating at 55°C, inversion on an overhead mixer or gentle pipetting with a 1ml Gilson pipette with the tip cut off. The solution was transferred to a 1.5ml eppendorf tube and treated with 20µl of DNAase free RNAase solution (10mg/ml) for 10 minutes at 37°C. The solution was then carefully extracted with phenol and chloroform (as described in section 2.2.3A(i)) and the DNA was precipitated by addition of 70µl 3M sodium acetate and 500µl isopropanol. The pellet was washed in 70% ethanol and redissolved in 1ml of sterile distilled water, ethidium bromide was added to a final concentration of 200µg/ml; cæsium chloride was then added until the refractive index was 1.39± 0.02. This was made up to volume with a solution of cæsium chloride in water of the same refractive index (~1g/ml) and the DNA purified by 'banding' as described for large scale plasmid preparations in section 2.2.3B(i). Yields were typically 10-30µg DNA per gram of wet weight plant tissue.

If the quality of the DNA was not critical (eg for use in slot blots), the following modifications were made to the above described method. The leaf material was thawed in the presence of extraction buffer and rapidly disrupted using a Polytron homogenizer on high speed for 30 seconds. The preparation was carried on as above until the chloroform extraction stage and, after which the DNA was ethanol precipitated and dissolved in sterile

distilled water as above. The DNA was quantitated by agarose gel electrophoresis or using a Hoefer DNA fluorimeter model TKO 100 according to the manufacturers instructions; it was used without further purification. DNA prepared in this way is badly sheared by the action of endogenous nucleases released on thawing prior to disruption and by the violent action of the Polytron itself; it is also contaminated with protein and polysaccharides. However, the preparation is rapid and adequate for slot blot analysis.

2.2.3B(v) Isolation of M13 single-stranded DNA.

The method used for the preparation of M13 single-stranded DNA was according to Schreier and Cortese[104], with the following modifications. *E.coli* strain TG2 was grown to an OD_{590} of 0.2 units in 2xYT medium. Using a Pasteur pipette, an isolated colourless, M13 plaque was picked into 2ml of the growing culture. The culture was vigorously shaken at 37°C for 6-7 hours. The cells were then spun down in a 1.5ml Eppendorf tube at 10,000 rpm for 2 minutes. 1ml of the supernatant fraction was transferred to another eppendorf tube containing 0.2ml of 2.5M sodium chloride, 20% polyethylene glycol 6000 and the mixture was left on ice for 10 minutes. Following centrifugation in a microfuge 5 minutes, all of the supernatant fraction was drained from the tube. Following a short centrifugation, the last few drops of PEG-6000 were removed by using a drawn-out Pasteur pipette (formed by melting the tip of the pipette over a Bunsen burner). The phage pellet was then dissolved in 0.1ml of TE and extracted with phenol/chloroform as described in section 2.2.3A(i). Single-stranded DNA was precipitated in 0.3M sodium acetate, pH 6.0, with 2.5 volumes of absolute ethanol; the rest of the procedure is as described in section 2.2.3B(ii). The DNA pellet was resuspended in 25µl of distilled water and stored at -20°C until needed.

2.2.3B(vi) Isolation of M13 replicative form DNA.

The following method was used for the preparation of replicative form (RF) DNA of M13, derived cloning vectors and M13 derived recombinant phages. A single purified plaque of M13 phage was picked into 1.5ml of a TG2 culture grown at 37°C in 2xYT to an OD_{590} of 0.2-0.4 units. After 6-7 hours of vigorous shaking at 37°C the M13 infected cells were centrifuged and the supernatant used to infect 500ml of growing TG2 (OD_{590} =

0.2). These cells were grown at 37°C for 4 hours and then centrifuged. The M13 RF DNA was prepared from the cell pellet as described for the preparation of the covalently closed supercoiled plasmid DNA (section 2.2.3B(i)).

2.2.3B(vii) Purification of DNA restriction fragments from agarose gels.

A special electro-elution box (Figure 2.2) for eluting the DNA from agarose gels was made by the Imperial College Biochemistry workshop. An agarose gel slice containing the DNA was cut into small pieces (2mm x 2mm) and placed in the sample wells at the centre of the electro-elution tank. The tank was filled with 500ml of 1x electro-elution buffer (10mM Tris:HCl, pH 8.0; 5mM NaCl; 1mM EDTA). All air bubbles were carefully removed using a 0.1ml micropipette connected to a 1ml disposable syringe and 60µl of dye mix (3M sodium acetate, pH7.8; 0.025% bromophenol blue) was carefully added to the extraction holes. The DNA was eluted at 150V for 30 minutes and, at the end of the run, 0.4ml of the sample was withdrawn from the extraction hole via a 0.1ml micropipette connected to a 1ml syringe. This procedure was repeated. The fractions were combined and 0.1µg of glycogen (as an inert carrier) was added to the sample followed by two volumes of absolute ethanol. After incubation at 0°C, the precipitated DNA (and carrier glycogen) was centrifuged in a microfuge for 15 minutes. The DNA pellet was washed with 70% ethanol and dried under vacuum in a desiccator. The DNA was resuspended in a small amount of distilled water and the concentration determined by running an aliquot on a 0.8% agarose gel with known concentrations of DNA and visually comparing the fluorescent intensities of the DNA bands.

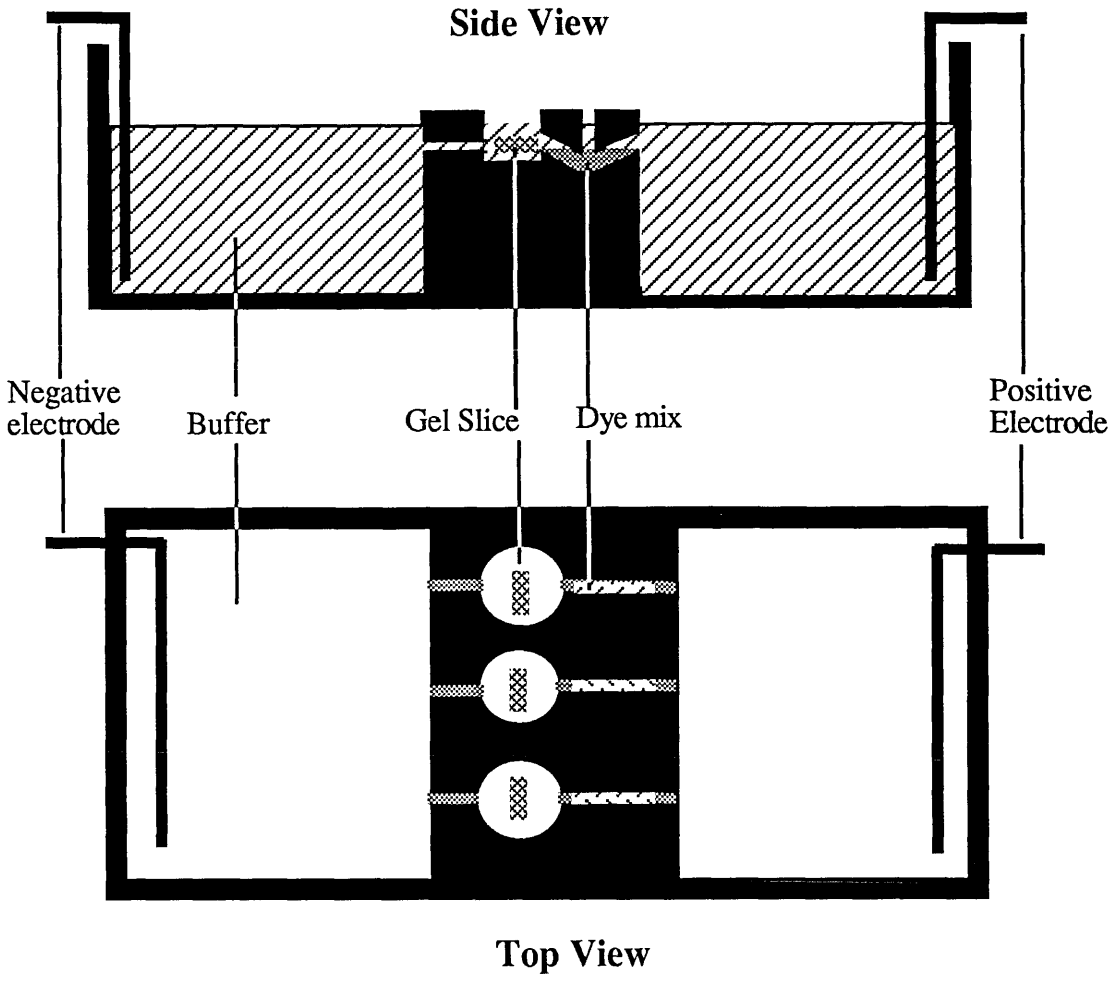


Figure 2.2. Electro-elution Box.

2.2.3B(viii) Preparation of total RNA from plant tissue.

A number of methods for the isolation of total plant RNA were tried[105,106,107] and the method used was adapted from these.

The following precautions were taken to minimise the risk of RNAase contamination. Disposable rubber gloves were worn at all times. All water was double distilled and treated with 0.1% DEPC overnight at 37°C prior to autoclaving. All solutions (with the exception of the extraction buffer) were made up in DEPC treated water and re-treated with DEPC prior to use. All glassware was either soaked overnight in 0.1% DEPC prior to autoclaving or soaked for 15 minutes in chromic acid and rinsed in DEPC treated water. Disposable plastic ware was used without further treatment only if taken from an unopened bag or one which had only been used with precautions appropriate to RNA extraction.

A Braun domestic coffee grinder was prechilled for 10 minutes with dry ice and 15-30g of frozen plant material (deveined leaf or seedling) was added together with about 0.1g diethyl thiocarbamic acid to inhibit polyphenol oxidases. After grinding for between 30 seconds and 1 minute, the still frozen powder was transferred to 2 volumes of a 1:1 mixture of extraction buffer (100mM LiCl; 1% SDS, 100mM Tris, pH 9.0; and 10mM EDTA) tris equilibrated phenol, pH9.0 in a 500ml conical flask at 85°C, and swirled vigorously in a water bath at 85°C. At this high temperature, the phenol and aqueous solution form one phase in which the RNAases should be denatured as the plant material thaws. The mixture was transferred to 50ml sterile 'Nunc' tubes and shaken on a rotary shaker at 200 rpm at 30°C for 5 minutes. One volume of chloroform was added and the shaking continued for a further 30-45 minutes. After transfer to 30ml Sarstedt disposable pollyallomer centrifuge tubes, the mixture was centrifuged at 10,000 rpm in a SS-34 rotor for 15 minutes and the upper aqueous layer was transferred to fresh tubes and shaken with 1 volume of chloroform for 15 minutes as before. After recentrifugation, the aqueous layer was transferred to fresh tubes and total nucleic acids were precipitated by the addition of 0.1 volume of 3M sodium acetate, pH 4.8 and 0.8 volumes of isopropanol. Following incubation on ice for 30 minutes, the nucleic acids were centrifuged at 10,000rpm in a SS-34 rotor for 10 minutes. The pellet was washed in 80% ethanol and dissolved in 4ml water at 55°C for 10-30 minutes. Any undissolved material (polysaccharide and protein) could be centrifuged out at this stage. Three volumes of 4M sodium acetate, pH 5.2 or pH 6.0, were added to the nucleic acid solution and it was incubated on ice for one hour to preferentially precipitate RNA. The pellet was washed with 3M sodium acetate, pH5.2 or pH 6.0, and 80% ethanol before being dissolved in 1ml of water and stored at -20°C until needed. The integrity of the RNA was checked by observation of the ribosomal RNAs as described in 2.2.3G(ii). The RNA was quantitated spectrophotometrically (2.2.3A(iii)). The RNA obtained at this stage was often contaminated with DNA and, if prepared from seedlings grown in soil, with a brown pigment. These contaminants were removed during the preparation of PolyA$^+$ RNA.

2.2.3B(ix) Preparation of Poly A$^+$ RNA.

Polyadenylated RNA (PolyA$^+$ RNA) could be separated from structural RNAs (which can be over 99% of the total RNA) by affinity chromatography with oligo-dT bound to a solid support matrix[94]. The polyadenylated mRNA binds to the oligo-dT by complementary base pairing.

Oligo-dT cellulose (0.25g) was suspended in 5ml 1x binding buffer (10mM Tris:HCl, pH7.6; 0.4M NaCl; 1mM EDTA; and 1%SDS) in a 10ml Sterilin tube. The slurry was poured in a 5ml column and washed with 10ml 1x binding buffer. Total RNA (100-1000µg in 0.11-th) was mixed with 1 volume of 2x binding buffer and heated to 65°C for 5 minutes; it was applied to the column. The eluent was reapplied to the column once more and reheated at 65°C before reapplying twice more. This process was repeated one more time (a total of 6 applications). The column was then washed with 100ml binding buffer and the PolyA$^+$ RNA was eluted with 5ml of elution buffer (10mM Tris:HCl, pH7.6; 1mM EDTA; and 0.05% SDS) at 42°C; the PolyA$^+$RNA was precipitated with isopropanol and quantitated as described above (2.2.3B(viii)). The yield was between 0.8 and 1.5% of the total RNA and was 50-80% PolyA$^+$ RNA[108]. The integrity of the RNA was confirmed during northern blotting (2.2.3G(ii)).

2.2.3C Gel electrophoresis.

All DNA gel electrophoresis was carried out in 1xTBE buffer (1.08% Tris:HCl 0.55% boric acid; and 0.093% EDTA). Gel loading dye (6x) was prepared with 0.25% bromophenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in distilled water.

2.2.3C(i) Useful molecular markers.

Bacteriophage lambda DNA, digested with Hind III or with BstE II, and ØX 174 DNA, digested with Hae III, were commonly used molecular markers to estimate the molecular weights of unknown DNA restriction fragments. The fragment sizes shown below are given in base pairs.

Lambda Hind III: 23130, 9416, 6557, 4361, 2322, 2027, 564, 125.

Lambda Bst EII: 8454, 7242, 6396, 5686, 4822, 4324, 3647, 2323, 1929, 1374, 1264,

702, 224.

ØX174 Hae III: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

2.2.3C(ii) DNA electrophoresis on horizontal agarose gels.

This was carried out essentially according to Maniatis *et al.*[94]. Electrophoresis was carried out in BRL gel boxes (35 x 11.5 cm) at constant voltage between 20 and 100 V. Up to 40µl per track could be loaded. Agarose powder (0.7-1.1% w/v) was added to 1x TBE buffer. 100ml of the mixture was heated for 2 minutes at 1000W in a Phillips domestic microwave oven to dissolve the agarose; ethidium bromide was added to a final concentration 0.5µg/ml and the gel was poured and allowed to set.

After electrophoresis, the gels were viewed on a 260nm transluminator and photographed using a Polaroid MP4 land camera through an orange Kodak wratten filter. If the bands were to be cut out and purified, the gel was viewed on a long wave (365nm) transluminator.

2.2.3C(iii) Denaturing thin acrylamide gels for DNA sequencing.

The gel plates were first cleaned with ethanol and then treated with dimethyl-dichloro-silane solution. 0.35mm thick strips of "plasticard" (Raven Scientific Ltd.) were placed along the longitudinal edge of one of the plates and the two plates taped together. The gel mixture (50% "ultra pure" urea; 0.07% ammonium persulphate; 6% acrylamide) was made up in 1x TBE. The solution (50ml) was filtered through a Nalgene 20µm filter unit and degassed prior to use. Polymerisation was initiated by the addition of 70µl of TEMED. The gel was poured between two plates, and a plasticard comb inserted before polymerisation was complete. An aluminium plate (3mm thick and of the same dimensions as the gel plate) was always placed on the back of the glass plate and the gel pre-run at 40W (in order to obtain even heating) for 45-60 minutes before loading. 6% denaturing acrylamide gels were used routinely and the order of loading was always A, G, C, T. Before loading, the samples were heated to 95°C for 2 minutes. Whilst the samples were still hot, 4ul of sample per track were loaded via a fine drawn-out Pasteur pipette. After electrophoresis at 40W constant power (until 20 minutes after the first dye had run off the end of the plate), the gel was transferred to 3MM Whatman paper and dried on an ATTO

gel drying processor (AE-3700) at 80°C for 40-60 minutes and then set up for autoradiography as described in section 2.2.3A(vi).

2.2.3D Enzymatic modification of DNA.

2.2.3D(i) Restriction endonuclease digestion and partial digestion of DNA.

Restriction endonuclease digestion of DNA was carried out in the manufacturer's recommended buffer, or the closest equivalent supplied by Amersham International, at the manufacturer's recommended temperature. Typically, 0.5-1.0 μg of plasmid DNA was incubated with 2-10units of enzyme for 1-2 hours in a total volume of 20μl prior to agarose gel electrophoresis. If the plasmid DNA samples were contaminated with RNA (eg. from small scale DNA preparations), then 1-2μl DNAase free RNAase (2.2.3D(viii)) was added to the reaction mixture at the same time as the restriction endonuclease.

In the case of chromosomal digests for plant Southern blot analysis, 10μg of DNA was digested with 30-40 units of enzyme in a total volume of 300μl for 3-18 hours. The mixture was ethanol precipitated, washed with 70% ethanol and the pellet redissolved in 20μl TE for electrophoresis.

If it was necessary to carry out a partial digest (eg. only one of two identical sites required to be cut), the reaction was carried out with 0.5 enzyme units per μg DNA for 30 minutes. The DNA was frozen at -20°C and a small aliquot was run out on a horizontal agarose gel to assess the proportion cut. If the digest had not proceeded far enough the sample was thawed and a further 0.5 enzyme units per μg DNA was added and the mixture incubated for 15 minutes longer.

2.2.3D(ii) *In vitro* ligation of DNA using T4 DNA ligase.

Ligation of DNA fragments were performed according to Maniatis *et al*[94]. Cohesive end ligations were carried out in ligation buffer (66mM Tris:HCl, pH 7.5; 1mM ATP; 1mM spermidine; 10mM $MgCl_2$; 15mM DTT; and 0.2mg/ml BSA) for 12-16 hours at 14°C with 2-3 units of T4 DNA ligase per 100ng of DNA in a total volume of 15μl. Blunt end ligations were performed under the same conditions but in the presence of 15% PEG 6000. In order that the success of the ligation could be assessed after transformation

(2.2.1(ii)), reactions were set up with no ligase and, if phosphorylated vector was used (2.2.3D(v)), with no insert.

2.2.3D(iii) Random prime labelling of DNA.

DNA was labelled by the random primed synthesis method of Feinberg and Vogelstein[109]. This method is, compared to nick-translation, relatively insensitive to impurities present in DNA fragments isolated from agarose and when required DNA from a low melting point (LMP) agarose gel was used directly.

After visualisation using long wave transluminator, the band containing the required DNA fragment was excised and assuming a density of 1g/ml the DNA concentration calculated. The gel slice was boiled for 5 minutes and diluted 5 fold in distilled water before being labeled as described below.

Up to 50ng of DNA was boiled for 5 minutes in 30µl of water and then cooled rapidly by placing it in an ice/water bath for 5 minutes (only to room temperature if LMP agarose was used). To start the reaction, the following were added to the DNA in order: 10µl of oligo-labelling buffer, 2µl of BSA (10mg/ml), 5µl of [α–^{32}P] dCTP (Amersham PB10205) and 2-4 units Klenow polymerase.

The reaction was carried out at 37°C for 2-3 hours and then stopped by freezing at -20°C or by boiling prior to addition to the hybridization solution.

Incorporation of up to 5 x 10^9 counts per minute per µg of DNA were routinely obtained.

Oligo-labelling buffer (OLB):

Solution O: 1.25M Tris-HCl, pH 8.0; 0.125M $MgCl_2$.

Solution A: 5µM each dATP, dTTP, dGTP and 1.8% 2-mercaptoethanol in solution O.

Solution B: 2M HEPES, pH 6.6.

Solution C: Hexadeoxyribonucleotides (P-L No.2166) suspended in TE at 90 O.D. units/ml.

To make OLB, solutions A, B and C were mixed in a ratio of 1:2.5:1.5 respectively. All solutions were stored at -20°C.

2.2.3D(iv) Separation of unincorporated nucleotides from radiolabelled DNA[94].

Unincorporated radioactive nucleotides can give rise to background in Southern and northern blots and so should be separated before use, in addition separation gives a rough guide to incorporation of radioactivity in the probe.

The labelled DNA was separated from unincorporated radionucleotide by centrifugation at 2000 rpm for 4 minutes through a Sephadex G-50 (Pharmacia) spin column (0.9ml bed volume) equilibrated with 10mM Tris:HCl, pH 8.0; 100mM NaCl; 1mM EDTA as described[94]. Monitoring of the eluent, containing the radiolabelled oligonucleotides, and the column, containing the unincorporated nucleotides, with a beta radiation monitor gave an indication of the ratio of incorporated to unincorporated radioactivity.

2.2.3D(v) Dephosphorylation of the 5' terminus of DNA.

This is done to prevent a cut vector ligating back to itself; 5' dephosphorylated termini can only be ligated to phosphorylated termini.

Calf intestinal phosphatase (CIP) was used. After digestion with the appropriate restriction enzymes, 1μg of DNA (blunt ended or with 5' protruding termini) was incubated with 20 units of CIP in 20ul reaction volume containing 1X CIP buffer (50mM Tris:HCl, pH 9.0; 1mM $MgCl_2$; 0.1mM $ZnCl_2$; and 1mM spermidine) at 37°C for 15 minutes, then at 55°C for a further 15 minutes. An additional 20 units of enzyme was added and the process repeated. The sample was then treated with 20mM EGTA (to sequester the Zn^{2+} CIP cofactor) for 1 hour at 65°C, phenol extracted (2.2.3A(i)), ethanol precipitated, washed with 70% ethanol and finally resuspended in 10μl of TE. To ensure that the dephosphorylation was successful, a ligation reaction using the vector alone was carried out (no ligation should occur).

2.2.3D(vi) Preparation of labelled DNA restriction fragments.

The 5' overhanging end of the restriction endonuclease digested DNA was filled in using [α-^{32}P] dCTP and Klenow polymerase. 100ng of DNA were incubated in a 20μl reaction volume (50mM Tris:HCl, pH 7.2; 10mM MgSO4; 0.1mM DTT; 50μg/ml BSA;

0.2mM of dATP, dTTP and dGTP; 2μCi of [α-^{32}P] dCTP (Amersham PB10205) and 1 unit of Klenow polymerase) at room temperature for 30 minutes. The reaction was stopped by the addition of EDTA to a final concentration of 5mM.

2.2.3D(vii) Unidirectional deletion of double stranded AL1 DNA using exonuclease III and SI nuclease.

5μg of pBSALAS DNA were cleaved with BamHI (to yield a 3' recessed end) and Sph I (to yield a 3' overhanging end which is not digested by exonuclease III) until cut to completion. The DNA was extracted with phenol (2.2.3A(i)), ethanol precipitated, washed with 70% ethanol and dissolved in 100ul of distilled water. To 10μl (~500ng) were added 40 μl of distilled water, 10μl of 10x exonuclease buffer (500mM Tris:HCl, pH8.0; 50mM $MgCl_2$; and 100mM DTT) and 100 units of Exonuclease III. This solution was incubated at room temperature and 5μl aliquots were taken at 30 second intervals and transferred to eppendorf tubes containing 45μl of 1x SI nuclease buffer (30mM sodium acetate, pH4.6; 50mM NaCl; and 1mM $ZnSO_4$) and kept on ice until the last aliquot was collected (5 minutes). 1 unit of SI nuclease was added to each tube and incubated on ice for 20 minutes, followed by 10 minutes at room temperature in order to delete single stranded DNA and render the ends blunt. The DNA was phenol extracted, ethanol precipitated and resuspended in 30μl of distilled water. 10μl of each sample was cut with Eco RI to liberate the AL1 fragment and loaded onto a 0.8% agarose gel (Figure 2.3). The remaining 20μl of DNA was ligated to recircularise and transformed to bacterial strain TG2. The plates containing the transformants from each time point (50-100 colonies/plate) were washed with 4ml 2xYT+ ampicillin and grown overnight to prepare mixed cultures. Plasmid DNA was prepared from each culture, cut with Hind III and SacI and ligated into phosphatased, Hind III, SacI cut M13 vector mp10 as described in section 2.2.3F. Single stranded DNA was prepared from three plaques from each time point. This DNA was sequenced as described in section 2.2.3E in order to identify the required deletions (see 3.2.7).

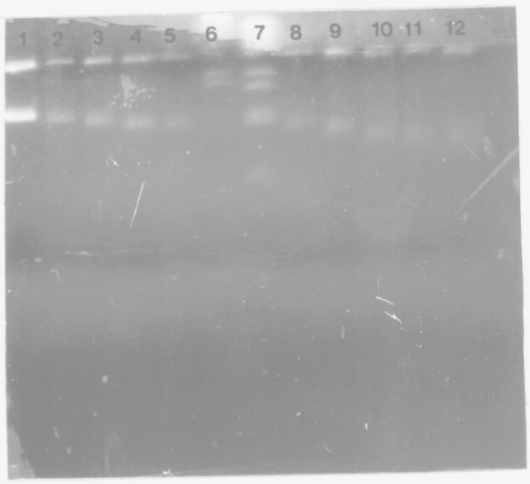


Figure 2.3: Exonuclease III deletions of pBSALAS taken at 30 second intervals. Hind III and Bst EII cut lamda marker DNA are in lanes 6 and 7 respectively .

2.2.3D(viii) Preparation of DNAase free RNAase.

This was carried out according to Maniatis *et al* [94]. RNAase A from Sigma was dissolved in sterile distilled water (10mg/ml) and incubated in a boiling water bath for 10 minutes and allowed to cool to room temperature; the process was repeated. 1ml aliquots were stored frozen at -20°C.

2.2.3D(ix) Conversion of 3' and 5' protruding DNA termini to 'blunt ends'[94].

5' protruding termini were rendered blunt ended by filling in with Klenow polymerase. A solution containing 1µg of DNA was suspended in 'nick translation buffer' (10mM Tris:HCl, pH 7.6; 50mM NaCl: 10mM $MgCl_2$; 1mM EDTA; and 0.2mM dNTPs) and 1 unit of Klenow polymerase added. After incubation at room temperature for 30 minutes, 100µl of water was added and the reaction was stopped by extraction with phenol/chloroform. The DNA was ethanol precipitated and dissolved in TE buffer to the required concentration.

3' protruding termini were rendered blunt ended by digestion of the single stranded region with T4 DNA polymerase. A solution containing 1µg of DNA was suspended in T4 DNA polymerase buffer (33mM Tris:acetate, pH 7.9; 66mM KCl: 10mM magnesium acetate; 5mM DTT; 100µg/ml BSA; and 0.2mM dNTPs) and 1 unit of T4 DNA polymerase

added. After incubation at room temperature for 5 minutes, 100µl of water was added and the reaction was stopped by extraction with phenol/chloroform. The DNA was ethanol precipitated and dissolved in nick translation buffer before treatment with Klenow polymerase as described above.

2.2.3E DNA sequencing.

The method employed was that of Sanger *et al.*[110] with the following modifications. DNA fragments were first cloned in M13 vector mp10[111] which contains multiple cloning sites in the alpha-peptide of the beta-galactosidase gene (section 2.2.3F). Single-stranded DNA was prepared from mature phage particles and used directly in the sequencing reaction.

2.2.3E(i) DNA sequencing reaction mixture.

The sequencing reaction consisted of: a working solution, chase solution, M13 universal primer, ^{35}S–dATP (2000 Ci/mM) (Amersham SJ304)and Klenow polymerase. The components and concentrations used in the preparation of the working reaction mixture for use with ^{35}S-dATP are indicated in Table 2.3. Chase solution is 0.25mM dNTPs in distilled water.

	T	G	A	C
0.5mM dTTP (µl)	6.25	125.00	125.00	125.00
0.5mM dCTP (µl)	125.00	6.25	125.00	125.00
0.5mM dGTP (µl)	125.00	125.00	6.25	125.00
10.0mM ddTTP (µl)	12.50	-	-	-
1.0mM ddCTP (µl)	-	18.00	-	-
10.0mM ddGTP (µl)	-	-	5.00	-
1.0mM ddATP (µl)	-	-	-	5.00
dds water (µl)	230.00	225.00	240.00	120.00

Table 2.3. Sequencing solutions.

2.2.3E(ii) Annealing and polymerisation.

3µl of DNA template, 0.5µl (2.5pmol) M13 universal primer and 0.75µl of 10x sequencing buffer (50mM $MgCl_2$; 100mM Tris:HCl, pH7.6; and 75mM DTT) were mixed in a 0.5ml eppendorf tube and covered with a thick layer of aluminium foil. The template-primer mixture was placed in a 65°C water bath for 20 minutes, then removed along with 1l of water and allowed to cool slowly to room temperature (about 1 hour). 1.5µl of ^{35}S-dATP (Amersham SJ304) and 2 units of Klenow polymerase were added to the annealed template and spun down in a microfuge for a few seconds. After mixing, 2.5µl aliquots of this mixture were placed in each of 4 eppendorf tubes labelled A, G, C, and T containing 2.5µl of the appropriate working sequencing solution. The reaction was allowed to proceed for 20 minutes at 37°C, at which time 2µl of chase solution were added to each tube and the incubation continued for a further 15 minutes at 37°C. The reactions were stopped by freezing at -20°C. Running dye (4µl) was added to each sample before running on a 6% acrylamide gel as described in 2.2.3C(iii). Running dye is 0.25% bromophenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in formamide.

2.2.3F Cloning into M13 phage vectors.

2.2.3F(i) Identification of the insert in M13 phage vectors.

After ligation of the restriction fragments into the appropriate sites of the M13 phage vectors, 100ng of the ligated DNA were used to transform bacterial strain TG2. Transformants were then added to 3ml of molten 2x YT top agar at 45°C, together with 200µl of an exponentially growing culture of TG2 and 50µl of IPTG (20mg/ml in distilled water) and 50µl of X-gal (25mg/ml in dimethylformamide). This mixture was vortexed and quickly poured onto 2xYT plates which were allowed to set and incubated at 37°C. White plaques were picked for further analysis.

2.2.3G Nucleic acid hybridisation analysis.

2.2.3G(i) Southern blotting.

The localization of particular DNA fragments in the total DNA of plants or *Agrobacterium* was carried out by the transfer technique described by Southern[112] with

modifications based on Church et al[113].

Plant DNA (10μg) or *Agrobacterium* DNA (~1-2μg) was cut with the appropriate restriction endonuclease and run on an agarose gel (see section 2.2.3C). After photographing the gel under shortwave ultraviolet (UV) light, 250ml (for 110 x 140mm gels) of depurinating solution (0.25M HCl) were added and the gel soaked with gentle agitation for 15 minutes in order to break up large DNA fragments. Following this the gel was rinsed in distilled water and soaked in 250 ml of denaturing solution (0.5M NaOH; 1.5M NaCl) with gentle shaking for 30-60 minutes (until the colour of the tracking dye had changed). The liquid was poured off and the gel rinsed in distilled water. Neutralising solution (0.5M Tris:HCl, pH 7.2; 1.5M NaCl) was then added and the gel shaken for a further 30 minutes. The DNA was transferred to a nylon (Hybond N) filter, either by capillary blotting as described by Maniatis *et al.*[94] or by vacuum blotting for 2 hours using a Hybaid Vacublot apparatus according to the manufacturer's instructions. The transfer medium in both cases was 20xSSC (3.0M NaCl; 0.3M sodium citrate). When using the vacublot apparatus the gel was sealed to the apparatus with 100ml of 4% agarose in 1x TBE before addition of 20x SSC. When blotting was completed the filter was rinsed in 2x SSC and baked between 2 sheets of Whatman 3MM paper for 2 hours at 80°C to fix the DNA to the membrane. UV cross-linking was not used to fix the DNA to the membrane because without careful calibration of the dosage the signal obtained can be very poor[114]. The membrane was either stored until needed or used immediately for hybridisation.

2.2.3G(ii) Northern blotting.

The method described is based on glyoxal as a denaturant[94].

The gel apparatus was soaked overnight in 0.1% DEPC and rinsed in running buffer prior to use. All solutions were treated with DEPC (see 2.2.3B(viii)). PolyA$^+$ RNA (1-5μg in 5-10μl of water) was added to 5μl of deionised glyoxal, 3μl 80mM sodium phosphate, pH 6.5 and 12μl DMSO and incubated at 55°C for 45 minutes to denature the RNA by binding of the glyoxal. The sample was cooled on ice and 4μl of loading buffer (0.05% bromophenol blue, 50% sucrose in DEPC treated water) was added. The sample was applied to a horizontal 1% agarose gel and run in 10mM sodium phosphate, pH6.5.

The gel was run at 100V constant voltage and the buffer was recirculated with a peristaltic pump. After the tracking dye had run to about 3cm from the end, the gel was transferred to a sandwich box and stained with ethidium bromide (1μg/ml in running buffer) for 30 minutes. After destaining with 1mM $MgCl_2$ (in running buffer) for 30 minutes the gel was photographed under short wave UV illumination. The integrity of RNA could be judged by the presence of ribosomal RNA bands. The gel was then transferred to Hybond N by capillary action as for Southern blotting (2.2.3G(i)). The membrane was removed from the gel and, without prior washing, baked at 80°C for 3 hours to deglyoxalate and fix the RNA. The membrane was then probed with a radiolabelled probe as described for Southern blotting (2.2.3G(iv)) except that the final, high stringency wash was with 0.2x SSC, 0.1% SDS. There was no difference in the strength of signal observed between unstained and ethidium bromide stained gels (data not shown).

2.2.3G(iii) Slot/Dot blotting.

1-2μg of DNA was rendered single stranded by treatment with denaturing solution (2.2.3G (i)) and applied directly to the Hybond N membrane using Gilson pipette or a Schleicher and Schuell Minifold II slot blot apparatus according to the manufacturer's instructions. The membrane was neutralised by immersion in neutralising solution (2.2.3G(i)), washed in 2x SSC and the DNA was fixed to the membrane as described for Southern blotting (2.2.3G (i)), before probing with a radiolabelled probe (2.2.3G (iv)).

2.2.3G(iv) Hybridisation of radiolabelled DNA probes to nylon membranes.

40ml church hybridisation buffer[113] (0.5M Sodium phosphate, pH 7.2; 1mM EDTA; and 7% SDS) was preheated to 65°C in a sandwich box and the filter added.

The double stranded probe (10-100ng in 50-100μl) (described in section 2.2.3D(iii)) was adjusted to 0.4m NaOH and allowed to stand at room temperature for 5 minutes to denature before being added to the hybridisation buffer and filter. These were then incubated with shaking at 65°C for 16-48 hours. The filter was then washed twice at low stringency (2xSSC, 1%SDS) for 15 minutes and twice at high stringency (0.1SSC, 0,1%SDS) for 30 minutes. Following this the damp filter was wrapped in cling film and

set up for autoradiography for a suitable time according to the intensity of the bound radioactivity as described in section 2.2.3A(vi).

2.2.3G(v) Stripping and reprobing of nylon membranes.

Stripping of DNA from Hybond N was carried out according to the manufacturer's instructions. It was essential that the membrane had not dried out prior to stripping off the old probe. The membrane was heated to 45°C in 0.4M NaOH for 15 minutes and then neutralised in 0.2M Tris HCl pH7.6, 0.1 x SSC, 0.1% SDS for 30 minutes at 45°C.

RNA blots cannot be stripped by the action of alkali as this will hydrolyse the bound RNA. Northern blots were instead stripped by boiling for 10 minutes in 0.1% SDS, 10mM Tris-HCl pH 7.6.

Removal of the probe was checked by autoradiography for a minimum of 48 hours to ensure complete removal of the probe. Southern blots could then be reprobed with a minimal loss of signal. In my hands, however, the signal obtained on reprobing northern blots decreased by between 5 and 20 fold after stripping, and it was not possible to reprobe an RNA blot for a third time. This problem has been ascribed to variability between different batches of Hybond N membrane[115].

3: PLASMID CONSTRUCTION.

The methods used for DNA modifications are described in detail in chapter 2. Because some Polaroid photographs of analytical gels have degraded with time some photographs are not shown. All precursor plasmids from which the constructs were made had their integrity checked by restriction mapping. This mapping is not discussed.

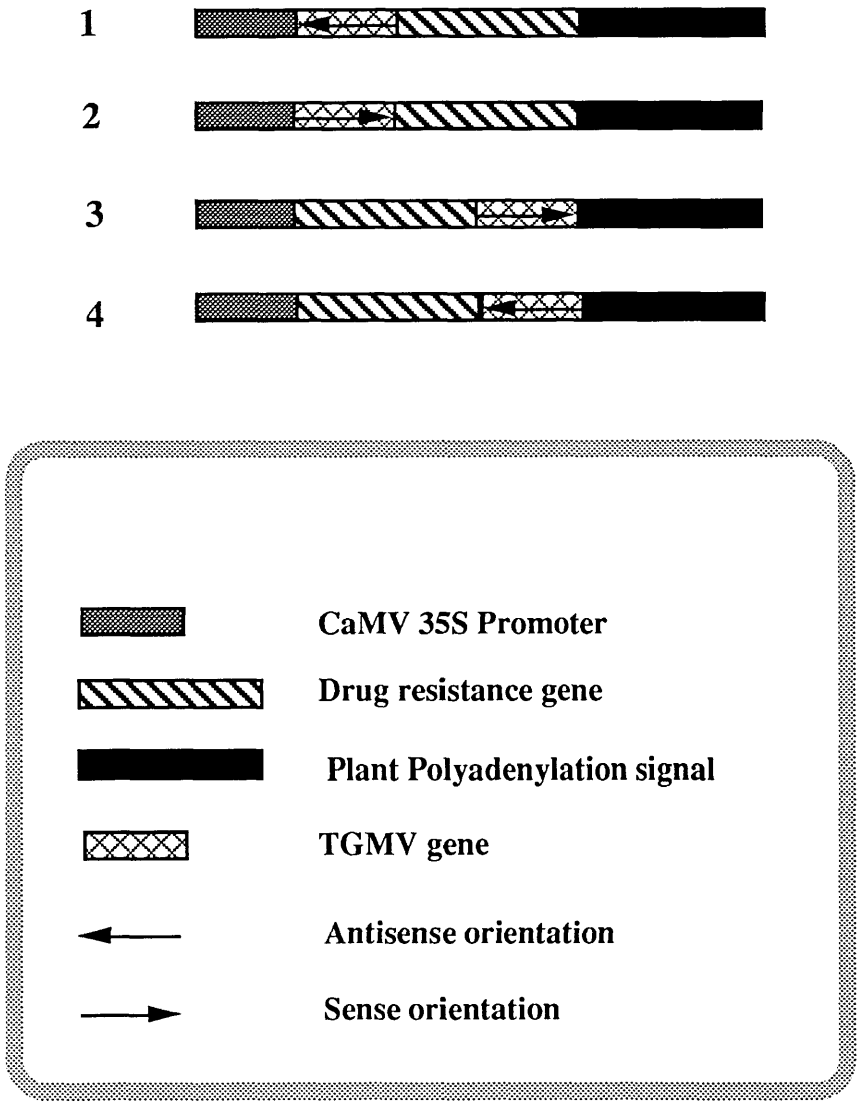


Figure 3.1: Bifunctional constructs used to express TGMV antisense genes.

The final constructs used to transform tobacco plants are bifunctional; the selectable drug marker is on the same transcriptional unit as the antisense TGMV gene. The transcript

is driven by the strong constitutive viral plant promoter, pCaMV 35S. These constructs are summarized in Figure 3.1; a lineage and list of plasmids constructed is shown in Appendix II

I decided to make the constructions in the small, high copy number cloning vector pBlueScribe plus (pBS+)[116] because it is easier to manipulate, and gives higher yields of DNA than most plant cloning vectors, which are large. Following this, the completed genetic units (or cassettes) were transferred to the plant binary vector, pDLT201 (see 3.3); for an explanation of binary vectors see Appendix I, section 7.2.3.2.

3.1 The pJCD Series of Constructs (and Related Plasmids).

The pJCD series of plasmids represent the first attempt to construct the bifunctional genetic units prior to cloning them in a plant binary vector.

3.1.1 pJCD1.

The first stage of this construction was the (attempted) cloning of a polyadenylation signal into a polylinker site of pBS+.

The plasmid, pBS+ was cut with Eco RI, the 5' protruding termini were filled in using Klenow polymerase and the 5' ends were dephosphorylated using calf intestinal phosphatase (CIP). pN19[117] was digested with Stu I and Bam HI to liberate two 1kb fragments, one of which carried the polyadenylation signal from the nopaline synthase gene of plasmid pTiC58[118]. It was not possible to separate these two fragments by gel electrophoresis. The 5' protruding termini were filled in with Klenow polymerase, and the DNA was run out on an agarose gel. The band containing the two 1kb fragments was cut out and the DNA electroeluted. The two fragments were ligated to Eco R1 cut pBS+ vector and transformed into *E.coli* strain DH5 alpha. DNA was prepared from 10 colonies and cut with Eco RI. There is an Eco RI site, which was thought to be unique, at the end of the fragment containing the polyadenylation signal. Four transformants had a unique Eco RI site. DNA was digested to check the orientation of the insert (Figure 3.2, Table 3.1); the Nae I digests have only cut partially. A strain containing the insert in the correct orientation was designated pJCD1.

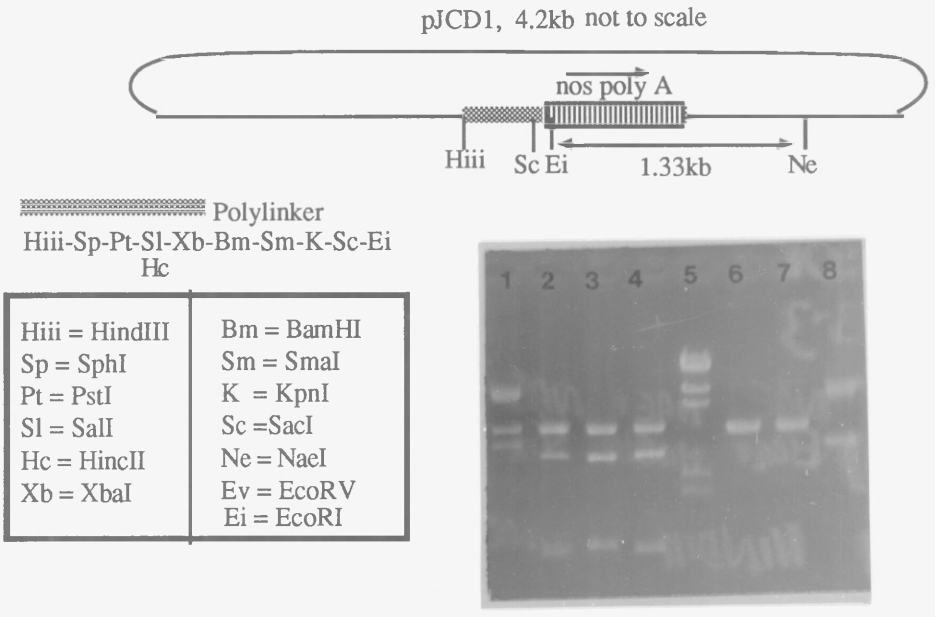


Figure 3.2. Map of pJCD1 and restriction digests. Lane 1, Nae I (partial); lane 2, Nae I (partial)/Eco RI; lane 3, Nae I (partial)/Hind III; lane 4, Nae I (partial)/Sac I; lane 5, lambda Hind III markers; lane 6, Sac I; lane 7, Hind III; lane 8, uncut.

Digest	Predicted sizes (kb)
Nae I	4.2
Nae I + Eco RI	2.87, 1.33
Nae I + Hind II	2.82, 1.38
Nae I + SacI	2.83, 1.33
SacI	4.2
Hind III	4.2

Table 3.1. pJCD1 digests.

3.1.2 pJCD2.

To complete the cloning of the plant regulatory signals it was next necessary to clone the CaMV 35S promoter.

The plasmid, pJEA9[119] was cut with Bam HI to release a 491bp fragment containing the CaMV 35S promoter. The fragment contained an ATG downstream of the transcriptional start site; deletion of this spurious ATG was necessary in order to ensure

efficient translation of any gene under the control of the promoter (see 1.3). In order to accomplish this, the 491bp fragment was gel purified, electroeluted and recut with Hph I. This yielded a 360bp fragment containing the CaMV 35S promoter which extends 4bp downstream from the transcriptional start site[119]. This fragment was treated with T4 DNA polymerase and klenow polymerase to remove the 3' protruding termini and to fill in the 5' protruding termini.

pJCD1 was cut with Pst I, treated with T4 DNA polymerase and klenow polymerase, as above, to remove the 3' protruding termini and dephosphorylated with CIP. The two fragments were ligated together and transformed into *E.coli* strain DH5 alpha. DNA from 24 transformants were cut with Hind III + Eco RV and run in a 2% agarose gel against ØX174 Hae III markers in order to identify a recombinant with the promoter cloned in the correct orientation. DNA from those recombinants which appeared to contain the correct DNA insert were digested to check the orientation (Figure 3.3, Table 3.2). Unexpected Eco RV sites were found in the polyadenylation sequence. A recombinant with the insert in the correct orientation was called pJCD2. (A recombinant with the promoter in the incorrect orientation was called pCamPaWR and stored in case it was needed for future cloning).

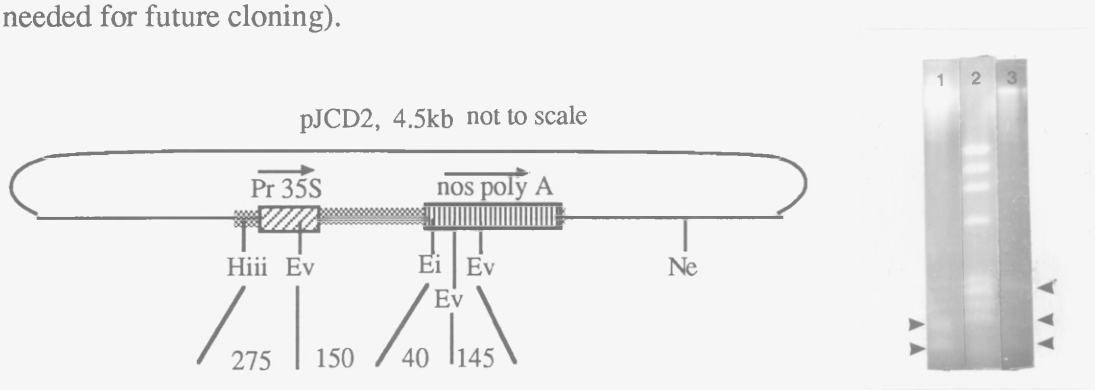


Figure 3.3. Map of pJCD2 and restriction digests. Lane 1, Eco RV; lane 2, ØX174 Hae III markers lane 3, Eco RV/Hind III. Small bands arrowed.

Digest	Predicted sizes (kb) (incorporating observed Eco RV sites)
Eco RV	0.180, 0.145, 4.2
Eco RV + Eco RI	0.149, 0.145, 0.036, 4.2
Eco RV + Hind II	0.180, 0.145, 0.275, 3.9
Eco RV + Eco RI + Hind III	0.149, 0.145, 0.036, 0.275, 3.9

Table 3.2. pJCD2 digests.

3.1.3 pCamBS.

Before carrying on with the construction of the bifunctional constructs, I wanted to have a readily accessible source of the CaMV 35S promoter, flanked by convenient restriction sites, for use in future constructs.

To obtain this, the blunt ended, 360bp Bam HI/Hph I CaMV 35S promoter fragment (3.1.2) was cloned into the Sma I site of pBS+. The orientation of the promoter with respect to the polylinker was determined by digests with Eco RI + Eco RV and Eco RV + Hind III (data not shown). The promoter was cloned such that transcription is from the Eco RI polylinker site towards the Hind III site.

3.1.4 pBSPA.

I next wanted to have a readily accessible source of the nopaline synthase polyadenylation signal fragment, flanked by convenient restriction sites, for use in future constructs.

To accomplish this, the blunt ended, Bam HI/Stu I fragment containing the nopaline synthase polyadenylation signal (3.1.1) was cloned into the Sma I site of pBS+. The orientation of the promoter with respect to the polylinker was determined by digests with Eco RI (data not shown). The orientation was such that digestion with Eco RI liberated a 1kb fragment indicating that the polyadenylation signal would terminate transcription driven from the direction of the polylinker Hind III site.

3.1.5 pJCD3.

The next stage in the construction of the bifunctional plant cassettes was the cloning

of the hygromycin resistance coding region between the plant regulatory signals.

pJCD2, containing the CaMV 35S promoter and putative polyadenylation signal, was cut with Bam HI and the 5' protruding termini were filled in with Klenow polymerase. The cut plasmid was dephosphorylated with CIP.

The 1322bp Hind III/Bgl II fragment from pSVhyg (a gift from A.Smith, Cambridge University), contained the coding region of the hygromycin resistance gene of the naturally occurring *E.coli* plasmid pKC203[120]. This fragment was gel purified and the 5' overhanging termini filled in, as above, by the action of Klenow polymerase. This fragment goes from 11bp upstream of the start of translation to 286 bp downstream of the termination codon.

The hygromycin coding region was ligated into the now blunt, formerBam HI site of pJCD2. The ligated mixture was then transformed into *E.coli* strain DH5 alpha. DNA from 12 transformants were cut with Eco RI to determine the presence and orientation of the insert. Plasmids containing the insert in both orientations were cut with a variety of restriction enzymes (Figure 3.4, Table 3.3); bands of the expected size were seen.

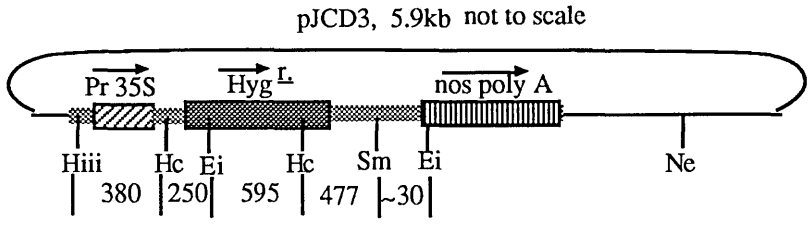


Figure 3.4. Map of pJCD3.

Digest	Predicted sizes (kb) (sense)	Predicted sizes (kb) (antisense)
Eco RI	1.1, 4.8	0.29, 5.6
Eco RI + Hind III	0.63, 1.1, 4.2	1.45, 0.29, 4.2
Hinc II	0.85, 5.0	0.46, 5.4

Table 3.3. pJCD3 digests.

A recombinant with the hygromycin resistance gene in the sense orientation was designated pJCD3 (and one with the hygromycin resistance gene in the antisense orientation was designated pJCDIII).

3.2 The pJC series (and related plasmids):

At this point a sequence for the nopaline synthase gene became available (from the IBM program, Microgenie) and the predicted restriction map was not consistent with the fragment cloned into pJCD1. There were no Eco RV sites and no silent mutations that could give rise to them (the fragment contained 435bp of nos coding region). Nor was there an Eco RI site in this sequence; however, a silent mutation (a mutation in a degenerate third base pair which does not effect the amino acid encoded) could give rise to an Eco RI site in the position marked on the pN19 map[117]. Clearly, the wrong fragment had been cloned, the observed Eco RI site was regenerated by cloning the blunt Bam HI fragment into a now blunt, former Eco RI site. Thus pJCD1, 2 and 3 have the wrong fragment, pBSPA, on the other hand, contains the correct fragment as neither blunt Bam HI or Stu I sequences cloned into a Sma I site regenerate an Eco RI site.

3.2.1 pJC2 and pJC3.

In order to salvage pJCD2 and pJCD3 they were digested with Nae I (a blunt cutter) and Kpn I, which release a fragment containing 332bp of pBS+ sequence and the wrong fragment from pN19. The larger fragment was gel purified and treated with CIP.

I decided to use the polyadenylation signal from the the octopine synthase gene. As there was (and is) little known about the effect of different polyadenylation signals on the stability of plant RNA transcripts, the choice of the octopine synthase polyadenylation signal was dictated by its availability on a plasmid in which it is flanked by convenient restriction sites. This plasmid, pOCS^{-}, was supplied by J.Tovar (Imperial College). An Apa I/Eco RI fragment containing the termination of transcription and polyadenylation signal from the octopine synthase gene had been rendered blunt ended and cloned into the Sma I site of pBS+. This plasmid was digested with Hinc II (a blunt cutter) and Kpn I, and the 930bp fragment released was gel purified and ligated into the Kpn I/Nae I, double digested pJCD2 and pJCD3. These ligated mixtures transformed into *E.coli* strain, DH5 alpha. DNA from transformants were cut with Xba I to identify recombinants containing the expected insert. A recombinant of the pJCD2 derivative was cut with a variety of

restriction enzymes to check the integrity of the construct (Figure 3.5, Table 3.4).

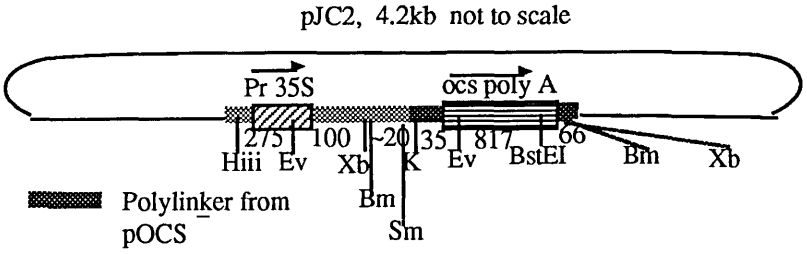


Figure 3.5. Map of pJC2.

Digest	Predicted sizes (kb)
Sma I	4.16
Xba I	0.94, 3.23
Bam HI	0.94, 3.23
Eco RV	0.16, 4.0
Bst EII	4.16
Bst EII + Xba I	0.08, 0.90, 3.2

Table 3.4. pJC2 digests.

A recombinant having the expected bands (0.08kb band not observed) was designated pJC2.

A recombinant of the pJCD3 derivative was cut with a variety of restriction enzymes to check the integrity of the construct (Figure 3.6, Table 3.5).

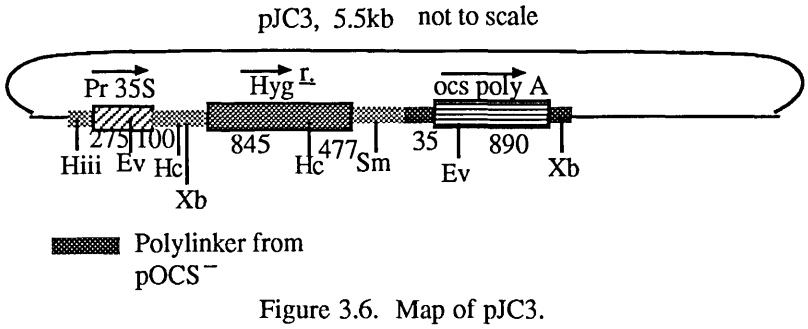


Figure 3.6. Map of pJC3.

Digest	Predicted sizes (kb)
Sma I	5.48
Xba I	2.25, 3.23
Xba I + Hinc II	0.85, 1.4, 3.23
Eco RV + Sma I	0.04, 1.42, 4.0
Eco RV + Xba I	0.10, 2.25, 3.1

Table 3.5. pJC3 digests.

A recombinant having the expected bands (0.10 and 0.04kb bands not observed) was designated pJC3.

3.2.2 pBSBS and pBSBA.

Before cloning the BL1 gene from TGMV B into the plant expression cassettes already constructed (see above), it was convenient to first clone it into the Sma I site of pBS^+.

In order to do this, the plasmid, pBH604[121], which contains TGMV B cloned into the Cla I site of pAT 153, was transformed into the dam^- *E.coli* strain, CB36, and the unmethylated DNA from this transformant was cut with Fnu 4HI and the dam methylase sensitive enzyme, Bcl I. This liberated a number of fragments less than 500bp in size (there are 9 Fnu 4HI sites in TGMV B), a 978 bp fragment and a 585bp fragment containing the coding region from the viral gene BL1. This fragment extends from 27bp upstream of the translational start site to 4bp beyond the stop codon. This fragment was gel purified and the 5' overhanging termini filled in by the action of Klenow polymerase. Following this, the fragment was ligated into the Sma I site of pBS+ and transformed into *E.coli* strain, DH5 alpha. DNA from 12 transformants were cut with Eco RI + Eco RV and Hind III + Eco RV to identify clones containing the expected insert, and to determine the orientation of that insert with respect to the polylinker. The Eco RV site is 104bp from the 3' end of BL1. Recombinants in which the Eco RV site was closest to the polylinker Eco RI site were designated pBSBA; those in which it was closest to the polylinker Hind III site were designated pBSBS.

3.2.3 pJC4S and pJC4AS.

The BL1 gene was next cloned between the plant regulatory signals in pJC2. A Kpn I/Xba I fragment from pBSBS, containing BL1, was gel purified and treated with T4 polymerase and Klenow polymerase to render it blunt ended. This fragment was ligated into Sma I cut, CIP treated pJC2 and transformed into *E.coli* strain, TG2. DNA was prepared from 12 transformants and digested with Eco RV to identify recombinants containing the expected insert, and the orientation of that insert. DNA from recombinants containing the insert were digested with a variety of restriction enzymes to check the orientation of that insert (Figure 3.7, Table 3.6).

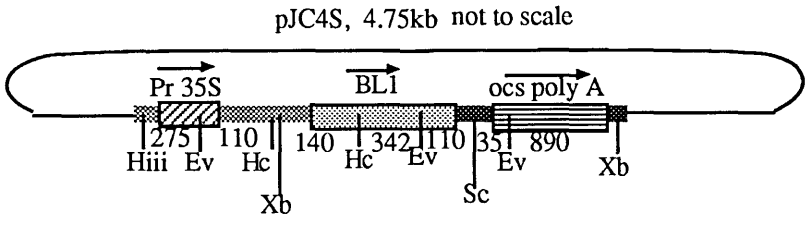


Figure 3.7. Map of pJC4S.

Digest	Predicted sizes (kb) (sense)	Predicted sizes (kb) (antisense)
Eco RV	0.59, 0.14, 4.0	0.22, 0.52, 4.0
Hinc II	0.15, 4.6	0.46, 4.3
Hind III + Xba I	0.38, 1.51, 2.85	0.38, 1.51, 2.85

Table 3.6. pJC4 digests.

Gels of 0.8% and 1.5% agarose were run in order to check the sizes of the large and small fragments. Fragments of the expected sizes were seen (Table 3.6). Recombinants carrying the insert in the sense orientation were called pJC4S and those carrying it in the antisense orientation were called pJC4AS.

3.2.4 pEXP, expression of a Tn*5* neomycin phosphotransferase gene.

The plasmid pNeo was supplied by J.Tovar (Imperial College). This plasmid had the neomycin phosphotransferase (NPTII) coding region from $Tn5^{122}$ on a blunt ended 1016bp fragment cloned into the Sal I site of an Xba I - Sal I - Xba I polylinker. The

fragment goes from a Sau 3A site 11bp 5' of the first ATG of the coding region to an Mst II site 208 bp 3' of the termination codon. To construct this coding region without spurious upstream ATGs, it was necessary to cut and religate sequences inside the coding region[123]. For this reason I decided to check that the gene still encoded a functional protein.

The expression vector, pKK223-3[124] was used. This vector contains an ampicillin resistance gene which is expressed in prokaryotes, and a polylinker sequence flanked by an inducible prokaryotic promoter and a prokaryotic termination of transcription signal. By cloning the *npt II* coding region into the polylinker site it was possible to select for the expression of this coding region by growing bacteria, transformed with this plasmid, on kanamycin plates.

The *npt II* coding region was released on an Xba I fragment, rendered blunt ended with Klenow polymerase and ligated into the Sma I site of the (un-phosphatased) expression vector, pKK223-3. The vector was also religated back to itself as a control. Both ligation mixtures were transformed into *E.coli* strain, TG2 and 1/50th plated onto kanamycin and ampicillin plates containing IPTG (which is necessary for expression with this vector system). DNA from kanamycin resistant colonies were cut with a variety of restriction enzymes (Table 3.7).

Antibiotic selection	Number of colonies
amp + kan (neo)	60
amp (neo)	~5000
amp + kan (control)	0
amp (control)	~5000

Table 3.7. Antibiotic resistant colonies arising from pEXP transformations. The ligation mixture used to transform the bacteria is shown in brackets; 'neo' indicates the mixture with the *npt II* coding region and 'control' indicates the vector religated back to itself.

The presence of 60 colonies on kanamycin selection when the ligation mixture contained the *npt II* coding region, and no colonies when the ligation mixture contained vector only, demonstrates that this *npt II* gene encodes a functional protein. DNA prepared

from four of the colonies which grew on amp + kan were cut with Pst I and Eco RI + Hind III; the expected fragments were released. These recombinants were designated pEXP. It was noted that there was some limited growth on kanamycin plates in the absence of IPTG suggesting that the promoter in this vector is leaky.

3.2.5. pJC5.

Having assured myself that the *npt II* coding region was functional, the next stage was to clone it between the plant regulatory signals of pJC2.

The plasmid, pJC2 was cut with Sma I and treated with CIP; the 1016bp Xba I fragment, containing the *npt II* gene, (described above, 3.2.4) was ligated into the Sma I site of pJC2 and the mixture was transformed into *E.coli* strain, TG2. DNA from transformants were cut with Pst I to identify recombinants containing the insert. These were then digested with the following enzymes to check the integrity of the constructs (Figure 3.8, Table 3.8;).

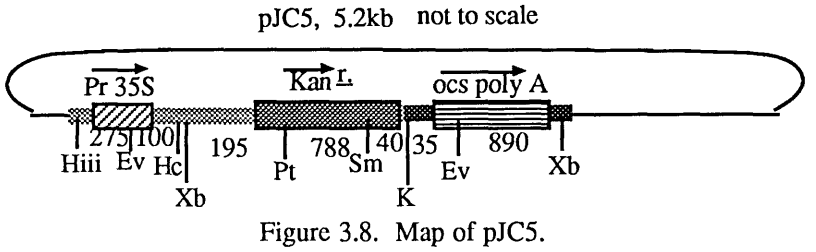


Figure 3.8. Map of pJC5.

Digest	Predicted sizes (kb)
Pst I + Kpn I	0.83, 4.53
Sma I + Hinc II	0.99, 4.17
Eco RV	1.16, 4.39
Kpn I	5.16
Hinc II	5.16

Table 3.8. pJC5 digests.

Gels of 0.8% and 1.5% agarose were run in order to check the sizes of the large and small fragments. Bands of the expected sizes were seen. Recombinants carrying the

insert in the correct orientation were called pJC5.

3.2.6 pJCVIS and pJCVIAS.

The next stage was the cloning of the BL1 gene downstream of the *npt II* gene in pJC5, in both orientations, to make the first bifunctional genetic units for expression in plants.

The plasmid, pJC5 was digested with Kpn I, rendered blunt ended by treatment with T4 DNA polymerase and klenow polymerase, and dephoshorylated by treatment with CIP.

A Xba I/Kpn I fragment from pBSBA, containing the BL1 coding region, was rendered blunt ended with T4 polymerase and klenow polymerase, ligated into the now blunt, former Kpn I site of pJC5 and transformed into *E.coli* strain, TG2. DNA from 12 transformants were cut with Bam HI to identify recombinants containing the insert. These were then digested with the following enzymes to check the integrity of the constructs (Figure 3.9, Table 3.9).

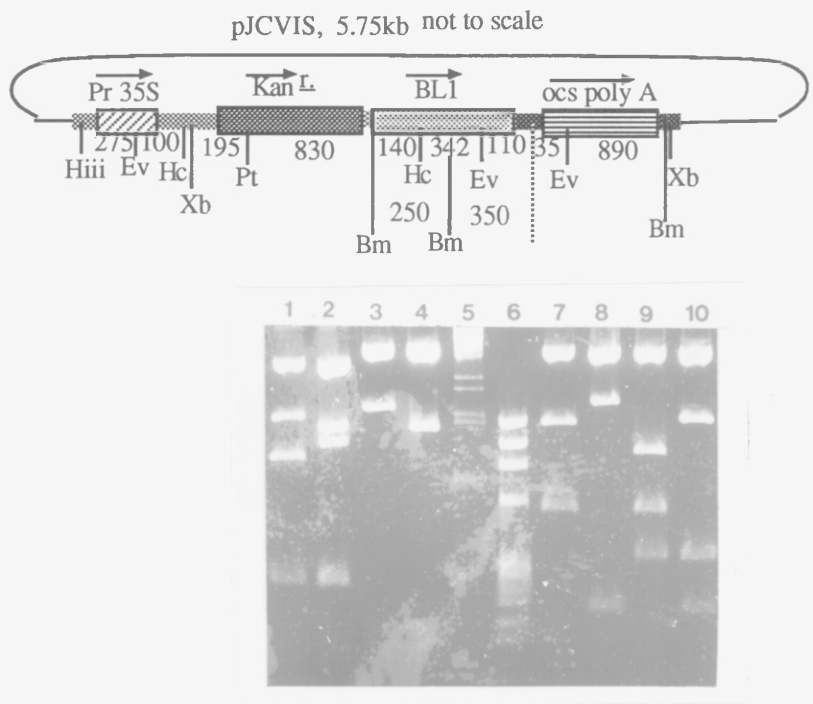


Figure 3.9. Map of pJCVIS and restriction digests. Lane1, AS-Bam HI; lane 2, S-Bam HI; lane 3, S-Hinc II; lane 4, AS-Hinc II; lane 5, lambde Bst EII markers; lane 6, ØX 174 Hae III markers; lane 7, AS-Eco RV; lane 8, S-Eco RV; lane 9, AS-Pst I/Eco RV; lane 10, S-Pst I/Eco RV.

Digest	Predicted sizes (kb) (sense)	Predicted sizes (kb) (antisense)
Eco RV	1.60, 0.14, 4.0	1.23, 0.52, 4.0
Pst I + Eco RV	0.3, 1.31, 0.14, 4.0	0.3, 0.95, 0.52, 4.0
Hinc II	1.16, 4.6	1.48, 4.3
Bam HI	1.27, 0.25, 1.02, 3.2	0.92, 0.25, 1.37, 3.2

Table 3.9. pJCVI digests.

The digests were run in 1.5% agarose gels (Figure 3.9). Bands of the expected sizes were seen. Recombinants carrying the insert in the sense orientation were called pJCVIS and those carrying it in the antisense orientation were called pJCVIAS.

3.2.7 pBSALS and pBSALAS.

Before proceeding with the construction of the bifunctional plant constructs, it was necessary to isolate the AL1 gene from TGMV A. There were two stages to this process. TGMV A had been cut in the coding region of AL1 prior to cloning into the Eco RI site of pAT153 to generate plasmid pBH401[118]. In order to be able to liberate the whole of the AL1 gene, it was necessary to recreate the circular virus DNA. Following this, the promoter had to be deleted from the AL1 gene by exonuclease III digestion. This is described in the next section, 3.2.8.

12µg of pBH401 were cut with Eco RI and the lower (2.6kb) TGMV A DNA band was gel purified and electroeluted. 5µg was dissolved in 1ml of T4 DNA ligase buffer and treated with 15units of T4 DNA ligase overnight at 15°C. After ethanol precipitation, the DNA was dissolved in a 100µl of TE buffer and a small amount was run on a gel. The DNA appeared to have completely recircularized. The DNA was digested with Nde I and Bam HI to release a 1448bp fragment containing the AL1 promoter and coding region from 203bp 5' of the initiation codon to 193bp 3' of translation termination (including the 5' regions of AL2 and AL3). After gel purification, the DNA was rendered blunt ended by treatment with Klenow polymerase. Following this, the fragment was ligated into the Sma I site of pBS+ and transformed into *E.coli* strain, TG2. DNA from 12 transformants were

cut with Eco RI to identify clones containing the expected insert, and the orientation of that insert with respect to the polylinker. These were then digested with the following enzymes to check the integrity of the constructs (Figure 3.10, Table 3.10).

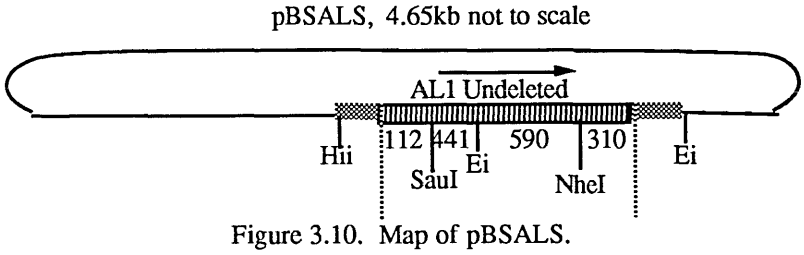


Figure 3.10. Map of pBSALS.

Digest	Predicted sizes (kb) (Sense)	Predicted sizes (kb) (antisense)
Eco RI	0.9, 3.75	0.55, 4.1
Hind III + Eco RI	0.55, 0.9, 3.2	0.55, 0.9, 3.2
Nhe I	4.65	4.65
Sau I	4.65	4.65

Table 3.10. pBSALS and pBSALAS digests.

The digests were run in 1.5% agarose gels against Hae III cut ØX 174 DNA and Bst EII cut lambda DNA. Bands of the expected sizes were seen. Recombinants carrying the insert in the sense orientation (coding region from Hind III polylinker site towards Eco RI polylinker site) were called pBSALS and those carrying it in the reverse orientation were called pBSALAS.

3.2.8 pBSAL1.

It was necessary to delete part of the AL1 gene cloned in pBSALAS in order to remove the promoter. This was done using Exonuclease III as described (2.2.3D(vii)). The clones in mp10 were labelled ∂1-∂5 depending on the exonuclease time point. According to the T-tracks, the clones were deleted as follows (Figure 3.11, Table 3.11).

Clone	Deletion point (relative to 1st ATG of AL1)
∂2.1-∂2.4	all at -119
∂3.1-∂3.4, ∂3b, ∂3d	all at 1
∂4.1-∂4.3	all at -15
∂2d,∂2e	all at -84
∂3a	+51

Table 3.11. AL1 deletions.

Single stranded DNA from ∂4.1 and ∂4.2 was sequenced fully to confirm the deletion (data not shown). Double stranded RF DNA was prepared from ∂4.1 and cut with Kpn I and Hind III to release a 1258bp fragment containing the entire AL1 coding region from -15 of translation (+2 of transcription) to 185bp 3' of the coding region; this includes 5' regions from AL2 and AL3. This fragment was gel purified, electroeluted and rendered blunt ended by the action of T4 DNA polymerase and Klenow polymerase. Following this, the fragment was ligated into the Sma I site of pBS+ and transformed into *E.coli* strain DH5 alpha. Recombinants were picked by blue white selection (as described for selecting recombinants in M13, section 2.2.3F). DNA from 24 transformants were cut with Eco RI to identify clones containing the expected insert, and the orientation of that insert with respect to the polylinker. Seventeen transformants appeared to have the insert. In all cases the insert was in only one orientation. The odds against this happening by chance, assuming an equal probability for both orientations, is $\sim 2^{17}:1 = 131{,}072:1$. The reason for this is unclear; it is possible that the clone in the second orientation was not observed due to production of a toxic fusion protein or DNA secondary structure. DNA from these clones were then digested with a variety of enzymes to check the integrity of the constructs (Figure 3.12, Table 3.12).

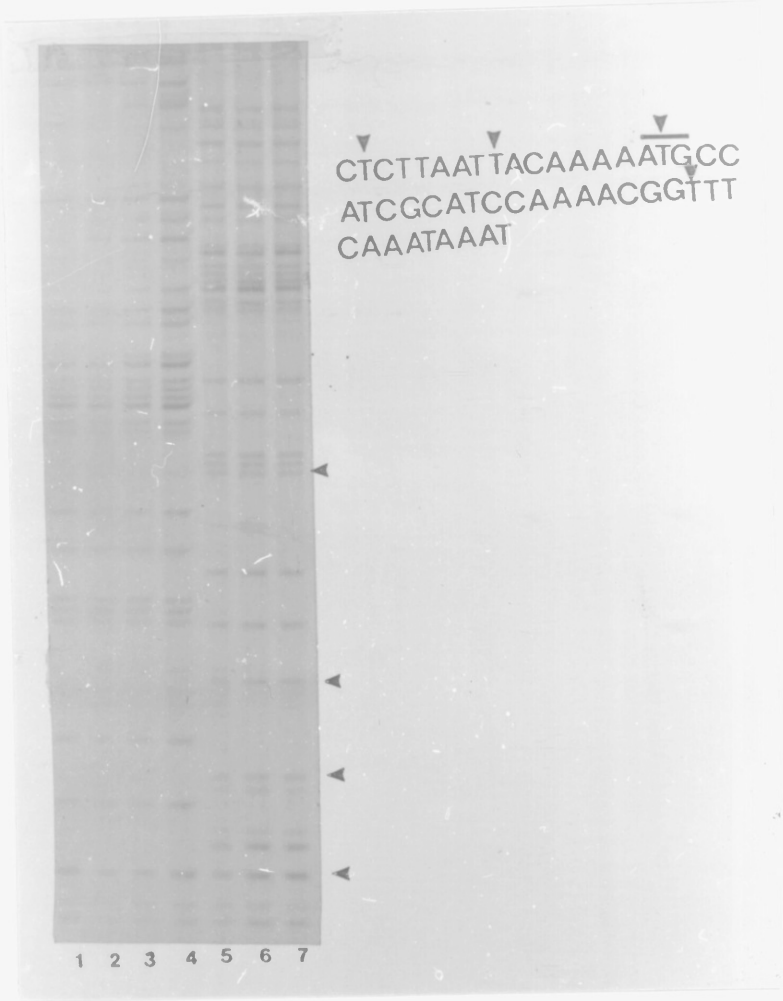


Figure 3.11. T-Tracks. Lanes 1-4, ∂3.1-∂3.4; lanes 5-7, ∂4.1-∂4.3. AL1 sequence is shown from -15 to +33. Start codon has a bar over it. Ts arrowed are also arrowed on autoradiograph. First two Ts on autoradiograph are from M13 polylinker.

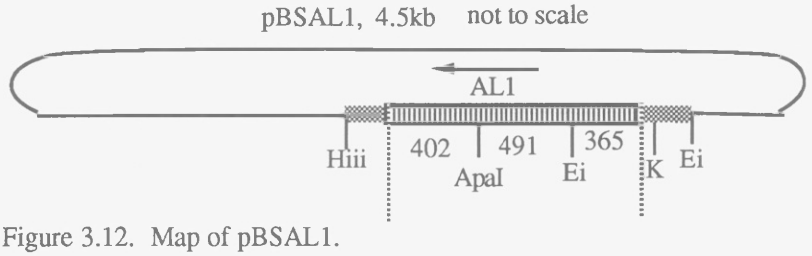


Figure 3.12. Map of pBSAL1.

Digest	Predicted sizes (kb)
Eco RI	0.37, 4.1
Apa I + Kpn I	0.86, 3.6

Table 3.12. pBSAL1 digests.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis. Recombinants carrying the insert in the antisense orientation (the coding region reading from the Eco RI polylinker site towards the Hind III polylinker site) were called pBSAL1.

3.2.9 pJC7S and pJC7AS.

Having isolated, and deleted the promoter from AL1, it was now possible to clone it upstream of the *npt II* gene to generate the next of the bifunctional genetic units for expression in plants.

The plasmid, pJC5 was cut with Sal I, rendered blunt ended by the action of Klenow polymerase, and treated with CIP. A Kpn I/Bam HI fragment from pBSAL1 was rendered blunt ended by the action of T4 DNA polymerase and Klenow polymerase, and was then ligated into the Sal I site of pJC5; the mixture was transformed into *E.coli* strain, TG2. DNA from 12 transformants were cut with Eco RI + Hind III to identify recombinants containing the insert. These were then digested with a variety of enzymes to check the integrity of the constructs (Figure 3.13, Table 3.13).

Digest	Predicted sizes (kb) (sense)	Predicted sizes (kb) (antisense)
Hind III	6.4	6.4
Hind III + Eco RI	0.75, 5.8	1.27, 5.2
Bam HI + Hind III	1.93, 1.64, 2.87	1.93, 1.64, 2.87
Apa I + Sma I	1.38, 5.0	1.85, 4.6

Table 3.13. pJC7 digests.

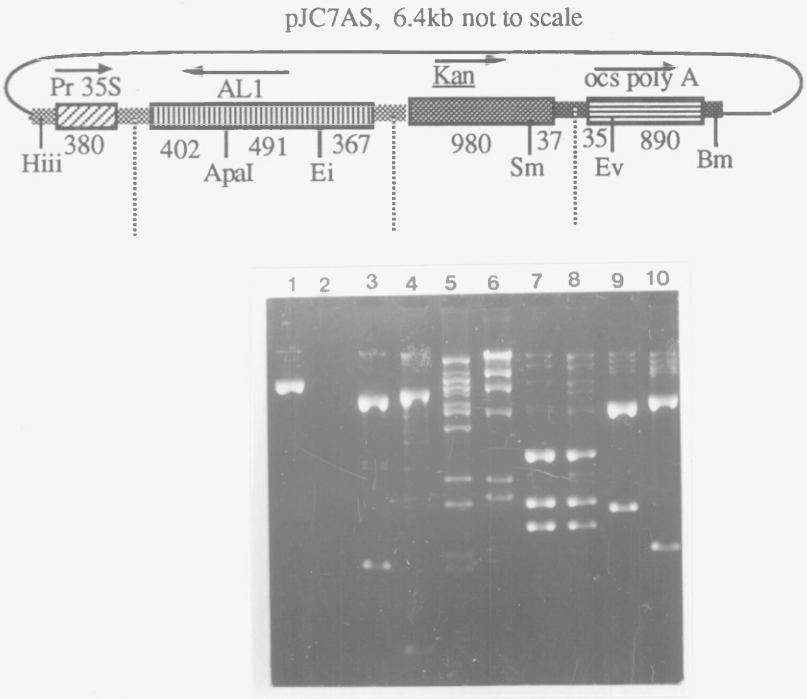


Figure 3.13. Map of pJC7AS and restriction digests. Lane 1, AS-Hind III; lane 2, blank; lane 3, AS-Hind III/Eco RI; lane 4, S-Hind III/Eco RI; lane 5, lambda Bst EII markers; lane 6, lambda Hind III markers; lane 7, AS-Bam HI/ Hind III; lane 8, S-Bam HI/ Hind III; lane 9, AS-Apa I/Sma I; lane 10, S-Apa I/Sma I.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis. Recombinants carrying the insert in the sense orientation were called pJC7S and those carrying it in the antisense orientation were called pJC7AS.

3.2.10 pJC8S and pJC8AS.

The AL1 gene was also cloned upstream of the hygromycin resistance gene. This was accomplished in a similar way to the construction of pJC7S and pJC7AS; the AL1 fragment was ligated into the Sal I site of pJC3. DNA from recombinants containing the insert were digested with a variety of enzymes to check the integrity of the constructs (Figure 3.14, Table 3.14).

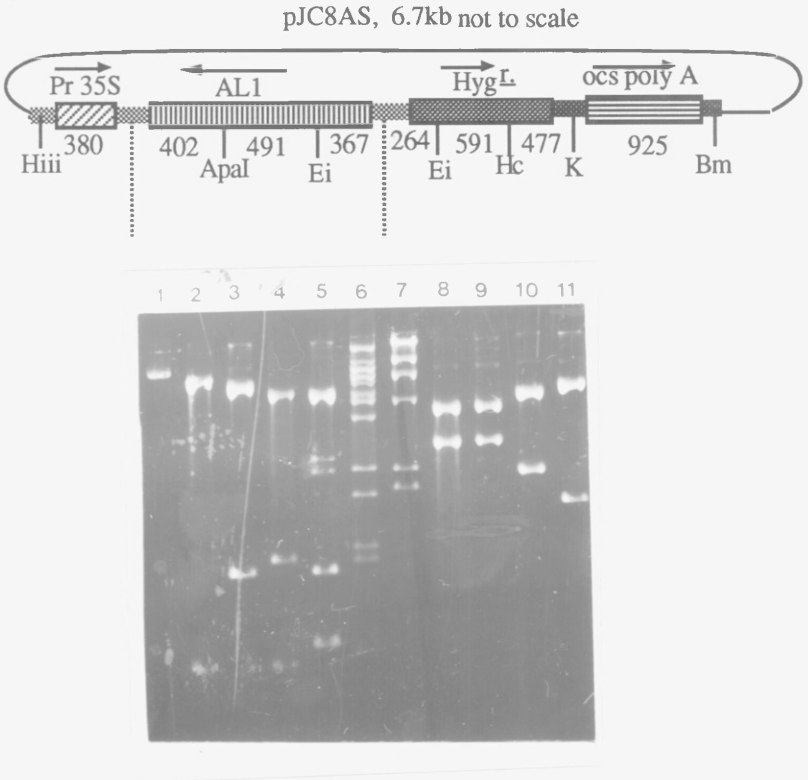


Figure 3.14. Map of pJC8AS and restriction digests. Lane 1, AS-Hind III; lane 2, AS-Eco RI; lane 3, S-Eco RI; lane 4, AS-Eco RI/Hind III; lane 5, S-Eco RI/Hind III; lane 6, lambda Bst EII markers; lane 7, lambda Hind III markers; lane 8, AS-Bam HI/Hind III; lane 9, S-Bam HI/Hind III; lane 10, AS-Apa I/Kpn I; lane 11, S-Apa I/Kpn I.

Digest	Predicted sizes (kb) (Sense)	Predicted sizes (kb) (antisense)
Hind III	6.7	6.7
Eco RI	1.15, 5.6	0.63, 6.1
Hind III + Eco RI	0.75, 1.15, 4.9	1.27, 0.63, 4.9
Bam HI + Hind III	3.88, 2.86	3.88, 2.86
Apa I + Kpn I	1.73, 5.0	2.19, 4.55

Table 3.14. pJC8 digests.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis. Recombinants carrying the insert in the sense orientation were called pJC8S and those carrying it in the antisense orientation were called pJC8AS.

3.2.11 pJC9S and pJC9AS.

Following the cloning of BL1 downstream of the *npt II* gene (pJCVI, 3.2.6), the BL1 gene was cloned upstream of the *npt II* gene.

This was accomplished in an analogous manner; the BL1 fragment was ligated into the Sal I site of pJC5. DNA from recombinants containing the insert were digested with a variety of enzymes to check the integrity of the constructs (Figure 3.15, Table 3.15).

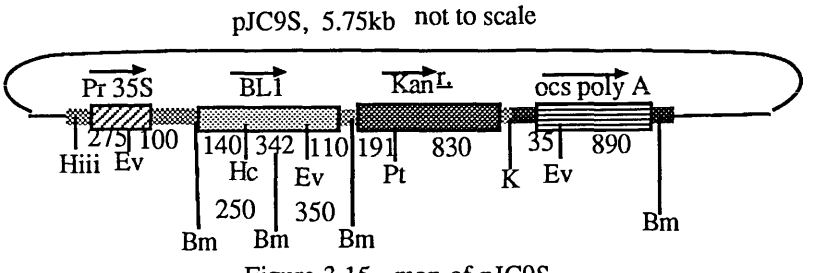


Figure 3.15. map of pJC9S.

Digest	Predicted sizes (kb) (Sense)	Predicted sizes (kb) (antisense)
Hind III	5.75	5.75
Eco RV	0.58, 1.16, 4.0	0.21, 1.54, 4.0
Bam HI	0.25, 0.35, 1.94, 3.2	0.25, 1.94, 3.6
Pst I + Eco RV	0.58, 0.30, 0.86, 4.0	0.21, 0.67, 0.85, 4.0

Table 3.15. pJC9 digests.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis. Recombinants carrying the insert in the sense orientation were called pJC9S and those carrying it in the antisense orientation were called pJC9AS.

3.2.12 pJC10S and pJC10AS.

Following the cloning of AL1 upstream of the hygromycin resistance gene (pJC8, 3.2.10), the AL1 gene was cloned downstream of the hygromycin resistance gene.

This was accomplished in an analogous manner; the AL1 fragment was ligated into the Sma I site of pJC3. DNA from recombinants containing the insert were digested with a variety of enzymes to check the integrity of the constructs (Figure 3.16, Table 3.16).

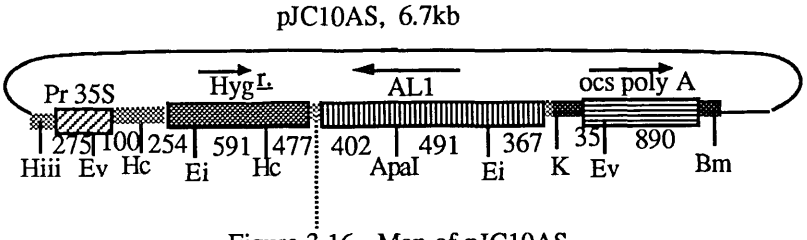


Figure 3.16. Map of pJC10AS.

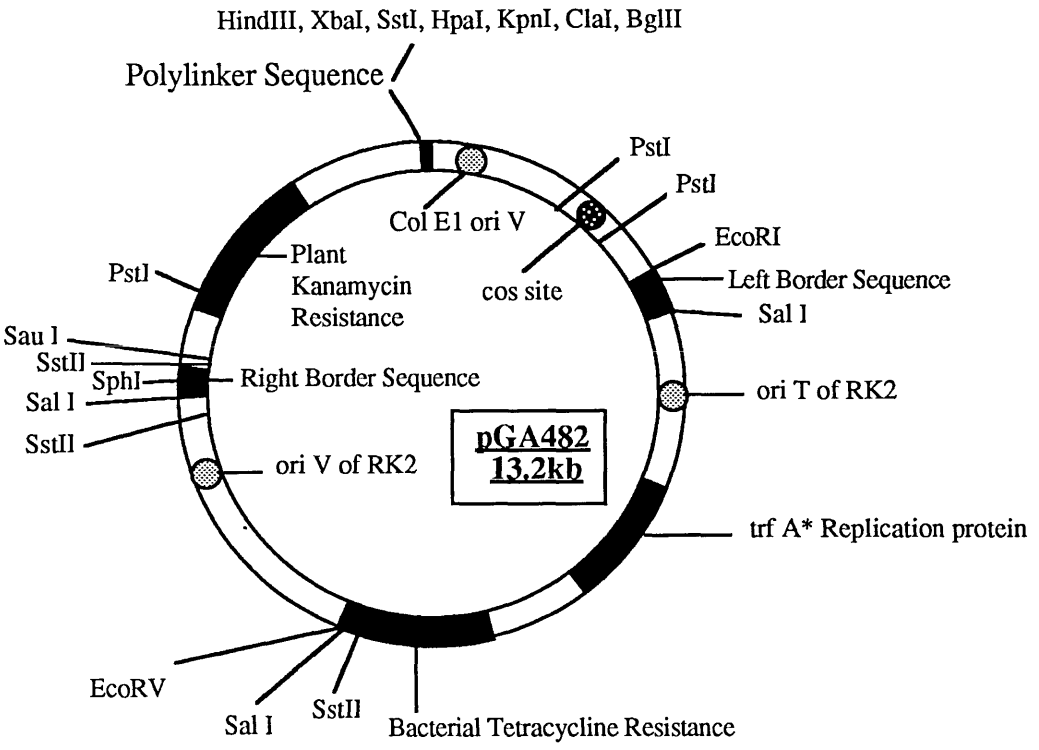
Digest	Predicted sizes (kb) (sense)	Predicted sizes (kb) (antisense)
Eco RI	1.44, 5.3	1.96, 4.8
Eco RI + Eco RV	0.35, 1.44, 0.93, 4.0	0.35, 1.96, 0.40, 4.0
Bam HI + Hind III	3.88, 2.86	3.88, 2.86

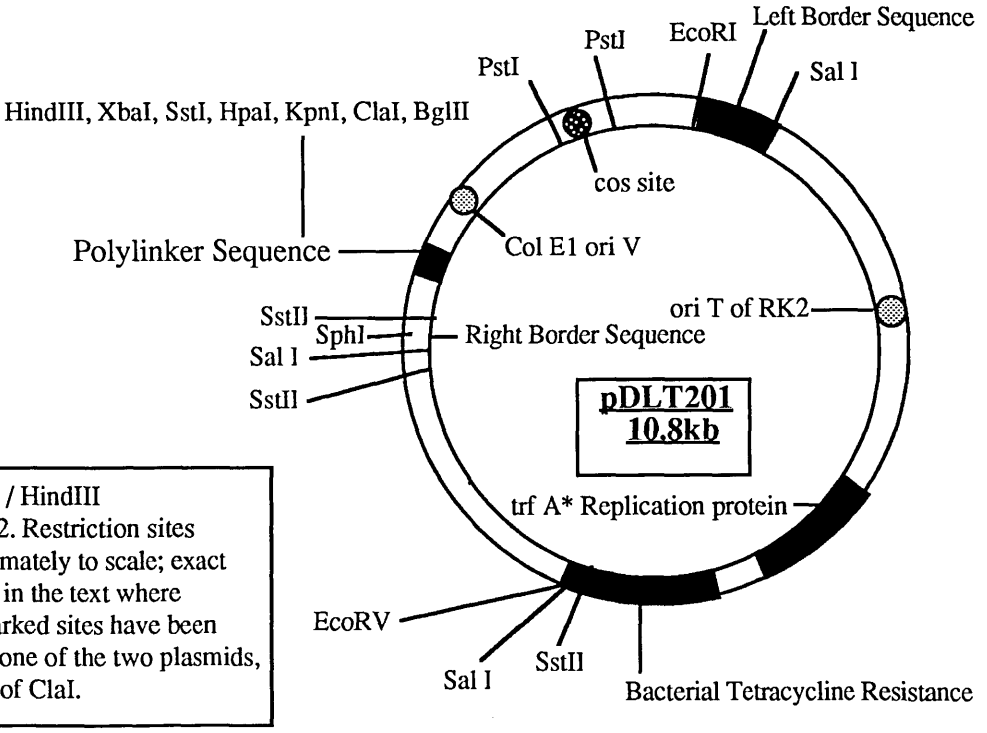
Table 3.16 pJC10 digests.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis. Recombinants carrying the insert in the sense orientation were called pJC10S and those carrying it in the antisense orientation were called pJC10AS.

3.3 The pPlant Series (and related plasmids).

Having constructed the bifunctional chimaeric plant genes in pBS+, it was necessary to transfer the genetic units to a plant binary vector able to replicate in *A.tumefaciens* and containing T-DNA borders which allow transfer of the T-DNA from *A.tumefaciens* to the plant genome. The vector chosen was based on pGA482[125] (see Appendix I, section 7.2.3.2 for a description). This vector has the *npt II* gene under the control of plant regulatory signals cloned between the T-DNA borders. The chimaeric plant genes encode a bifunctional transcript which expresses drug resistance and it was therefore essential to delete the *npt II* gene from pGA482. This was done by removing a Sau I/Hind III fragment containing the whole *npt II* gene (this regenerated the Hind III site)[123]. The resultant vector was called pDLT201. Both pGA482 and pDLT201 were extensively restriction endonuclease mapped (data not shown) and the maps in Figure 3.17 generated.





pDLT201 is a SauI / HindIII deletion of pGA482. Restriction sites are marked approximately to scale; exact distances are given in the text where appropriate. All marked sites have been checked in at least one of the two plasmids, with the exception of ClaI.

Figure 3.17. pGA482 and pDLT201.

3.3.1 pPlant1A, pPlant1B, pPlant2A and pPlant2B.

pDLT201 was cut with Xba I, rendered blunt ended with Klenow polymerase, and treated with CIP.

pJC8S and pJC8AS, which contain AL1 (sense and antisense respectively) upstream of the hygromycin resistance gene, were cut with Hind III and partially cut with Xba I. The 3.89kb fragments released (containing the chimaeric plant constructs) were gel purified, rendered blunt ended with Klenow polymerase, and ligated into the now blunt, former Xba I site of pDLT201. The mixture was transformed into *E.coli* strain, TG2. DNA from 24 transformants from each of the two ligation mixtures was cut with Hind III + Bgl II to identify recombinants containing the insert. These were then digested with a variety of enzymes to check the integrity of the constructs (Figures 3.18-3.19, Tables 3.17-3.18).

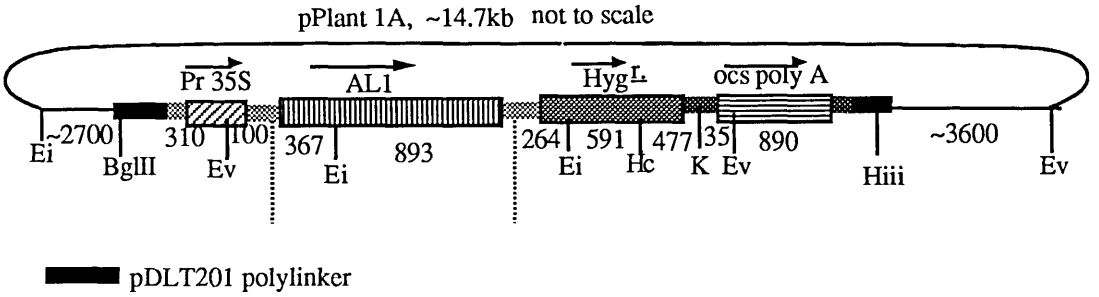


Figure 3.18. Map of pPlant 1A.

Digest	Predicted sizes (kb) (clockwise)	Predicted sizes (kb) (anticlockwise)
Eco RV	2.72, 3.9, 8.1	2.72, 4.5, 7.5
Eco RI	1.15, 4.7, 8.8	1.15, 3.5, 10.0
Hind III + Eco RV	3.6, 0.27, 2.72, 8.1	3.6, 0.89, 2.72, 7.5
Bgl II + Hind III	10.8, 3.9	10.8, 3.9

Table 3.17. pPlant1 digests.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis. Recombinants carrying the insert in the clockwise orientation were called pPlant1B or pPlant2B and those carrying it in the anticlockwise orientation were called

pPlant1A or pPlant2A. Clockwise and anticlockwise are arbitrarily defined by the direction of transcription of the drug resistance markers as they would be viewed cloned into pDLT201 (Figure 3.17).

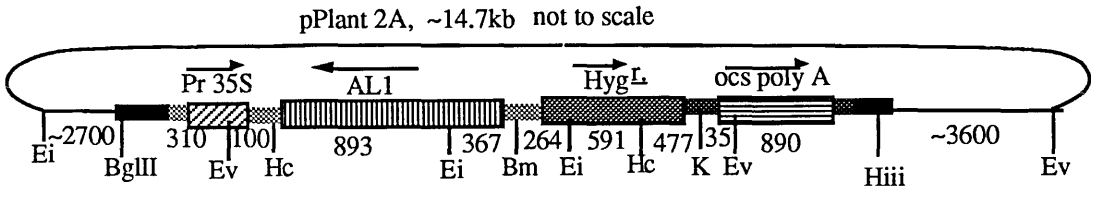


Figure 3.19. Map of pPlant2A.

Digest	Predicted sizes (kb) (clockwise)	Predicted sizes (kb) (anticlockwise)
Eco RV	2.72, 3.9, 8.1	2.72, 4.5, 7.5
Eco RI	0.63, 4.7, 9.3	0.63, 3.0, 10.0
Hind III + Eco RV	3.6, 0.27, 2.72, 8.1	3.6, 0.89, 2.72, 7.5
Bgl II + Hind III	10.8, 3.9	10.8, 3.9

Table 3.18. pPlant2 digests.

3.3.2 pPlant3A, pPlant4A and pPlant4B.

These plasmids were prepared in a similar way to pPlant1A-2B. Blunt Hind III/Xba I 2890bp fragments from pJC9S and pJC9AS, which contain BL1 (sense and antisense respectively) upstream of the *npt II* gene, were cloned into the blunt Xba site of pDLT201. A construct equivalent to pPlant3B was not obtained. DNA from recombinants which appeared to contain the insert were digested with a variety of enzymes to check the integrity of the constructs (Figures 3.20-3.21, Tables 3.19-3.20).

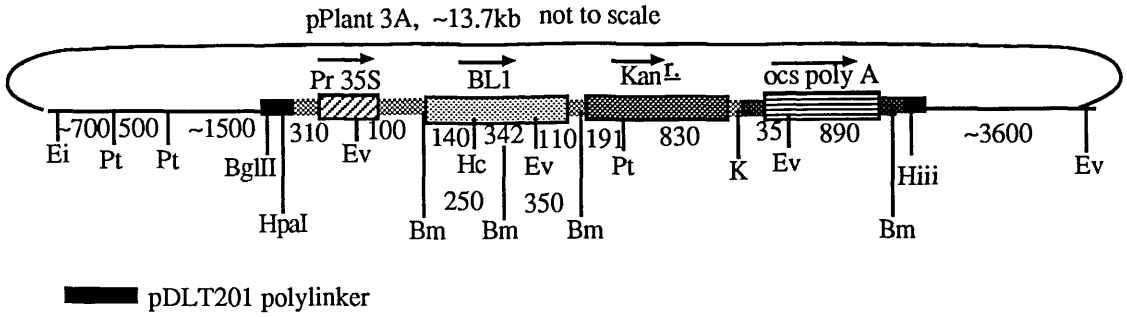


Figure 3.20. Map of pPlant3A.

Digest	Predicted sizes (kb) (anticlockwise)
Eco RV	4.5, 1.16, 0.58, 7.5
Bam HI	0.25, 0.35, 1.94, 11
Hind III + Hpa I	2.9, 10.8
Pst I + Hind III	0.5, 2.6, 1.75, 8.9

Table 3.19. pPlant3A digests.

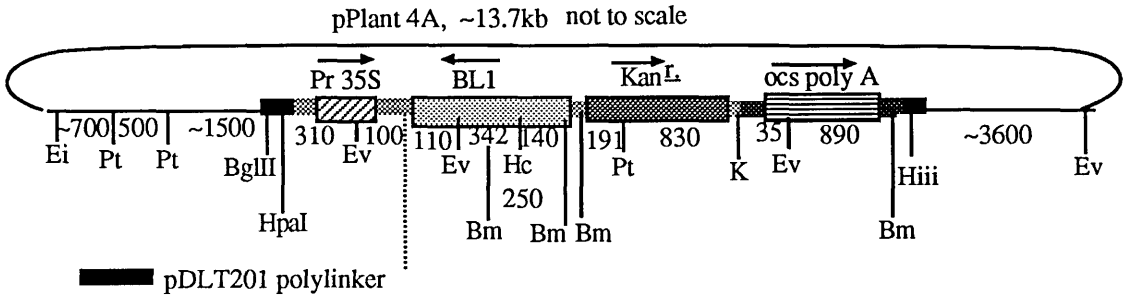


Figure 3.21. Map of pPlant4A.

Digest	Predicted sizes (kb) (clockwise)	Predicted sizes (kb) (anticlockwise)
Eco RV	3.9, 0.21, 1.54, 8.2	4.5, 0.21, 1.54, 7.5
Bam HI	0.25, 1.94, 11.4	0.25, 1.94, 11.4
Hind III + Hpa I	2.89, 10.8	2.89, 10.8
Pst I + Hind III	0.5, 3.24, 1.14, 8.9	0.5, 2.6, 1.75, 8.9

Table 3.20. pPlant4 digests.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis. Recombinants carrying the insert in the clockwise orientation were called pPlant4B and those carrying it in the anticlockwise orientation were called pPlant3A or

pPlant4A.

3.3.3 pPlant5A, pPlant5B, pPlant6A and pPlant6B.

These plasmids were constructed in a similar way to pPlant1A-4B. Blunt Hind III/Bam HI 3880bp fragments from pJC10S and pJC10AS, which contain AL1 (sense and antisense respectively) downstream of the hygromycin resistance gene, were cloned into the now blunt, former Xba I site of pDLT201. DNA from recombinants containing the correct insert were digested with a variety of enzymes to check the integrity of the constructs (Figure 3.24, Table 3.21-22).

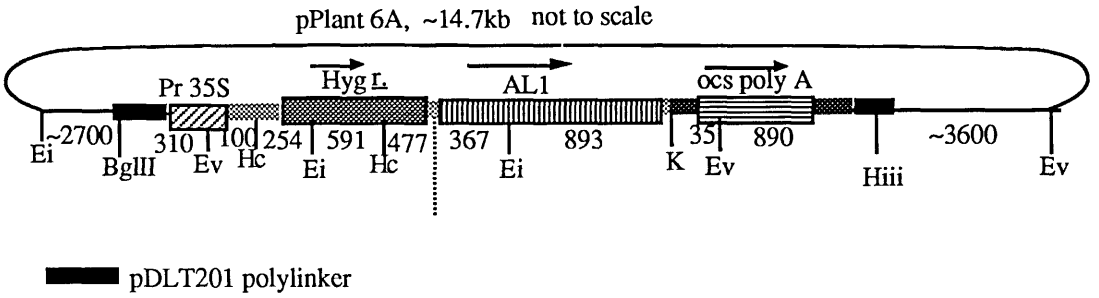


Figure 3.22. Map of pPlant6A.

Digest	Predicted sizes (kb) (clockwise)	Predicted sizes (kb) (anticlockwise)
Eco RV	3.9, 2.75, 8.2	4.5, 2.75, 7.5
Eco RI	1.44, 4.5, 8.7	1.44, 3.3, 9.9
Hinc II	0.85, 13.9	0.85, 13.9

Table 3.21. pPlant6 digests.

Except in the case of Hinc II, bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis; between 6 and 8 bands were seen indicating at least 5 Hinc II sites in pDLT201. There were bands in the expected region for the internal fragment from pJC10S and pJC10AS. Recombinants carrying the insert in the clockwise orientation were called pPlant5B or pPlant6B and those carrying it in the anticlockwise orientation were called pPlant5A or pPlant6A (Figure 3.22-3.23).

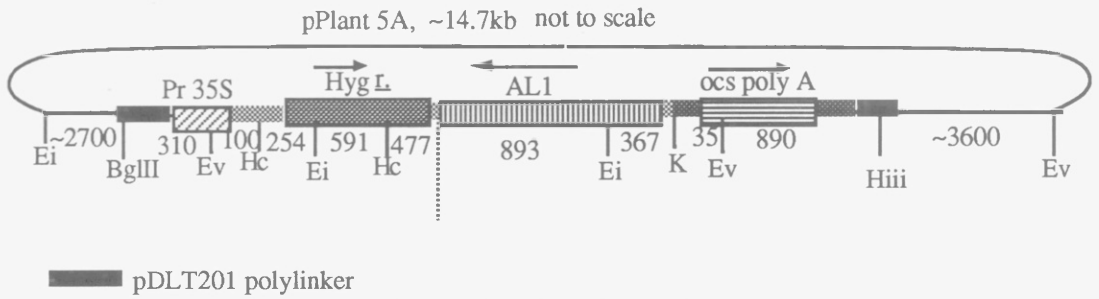


Figure 3.23. Map of pPlant5A.

Digest	Predicted sizes (kb) (clockwise)	Predicted sizes (kb) (anticlockwise)
Eco RV	3.9, 2.75, 8.2	4.5, 2.75, 7.5
Eco RI	1.97, 4.0, 9.2	1.97, 3.3, 9.4
Hinc II	0.85, 13.9	0.85, 13.9

Table 3.22. pPlant5 digests.

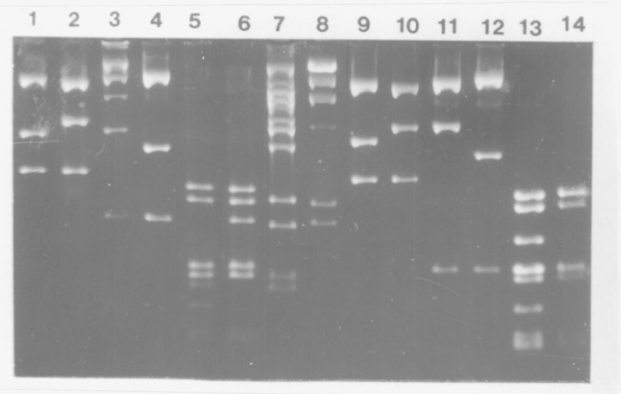


Figure 3.24 pPlant5 and pPlant6 digests. Lane 1, pPlant5B-Eco RV; lane 2, pPlant5A-Eco RV; lane 3, pPlant5B-Eco RI; lane 4, pPlant5A-Eco RI; lane 5, pPlant5B-Hinc II; lane 6, pPlant5A-Hinc II; lane 7, lambda Bst EII markers; lane 8, lambda Hind III markers; lane 9, pPlant6B-Eco RV; lane 10, pPlant6A-Eco RV; lane 11, pPlant6B-Eco RI; lane 12, pPlant6A-Eco RI; lane 13, pPlant6B-Hinc II; lane 14, pPlant6A-Hinc II.

3.3.4 pPlant7A, pPlant7B and pPlant8A.

These plasmids were constructed in a similar way to pPlant1A-6B. Blunt Hind III/Xba I (partial Xba I digests) 2890bp fragments from pJCVIS and pJCVIAS, which contain BL1 (sense and antisense respectively) downstream of the hygromycin resistance gene, were cloned into the now blunt, former Xba I site of pDLT201. A construct equivalent to pPlant8B was not obtained. DNA from recombinants which appeared to carry the insert were digested with a variety of enzymes to check the integrity

of the constructs (Figure 3.27, Tables 3.23-24).

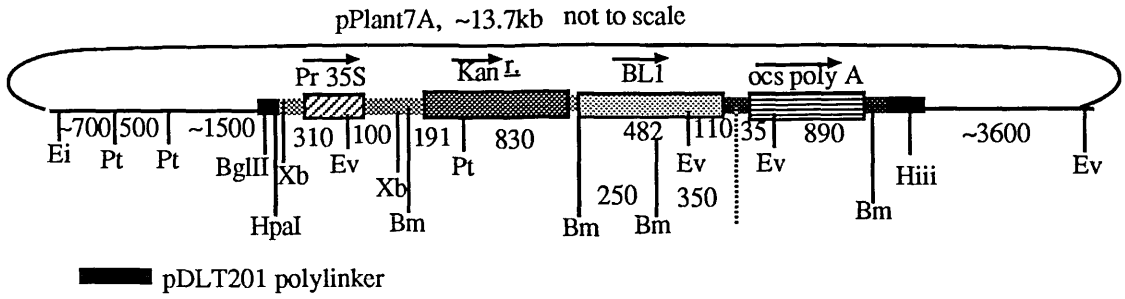


Figure 3.25. Map of pPlant7A.

Digest	Predicted sizes (kb) (clockwise)	Predicted sizes (kb) (anticlockwise)
Pst I	0.50, 3.84, 9.4	0.50, 2.08, 11
Eco RV	3.9, 1.60, 0.14, 8.1	4.5, 1.60, 0.14, 7.5
Bam HI	1.02, 0.25, 1.27, 11.2	1.02, 0.25, 1.27, 11.2
Xba I + Hind III	0.4, 2.54, 10.8	0.4, 2.54, 10.8

Table 3.23. pPlant7 digests.

Bands of the expected sizes were seen after separation of the DNA by agarose gel electrophoresis; except for the 140bp band expected in the Eco RV digest which was probably not visible because not enough DNA was loaded onto the gel. Recombinants carrying the insert in the clockwise orientation were called pPlant7B and those carrying it in the anticlockwise orientation were called pPlant7A (Figure 3.25).

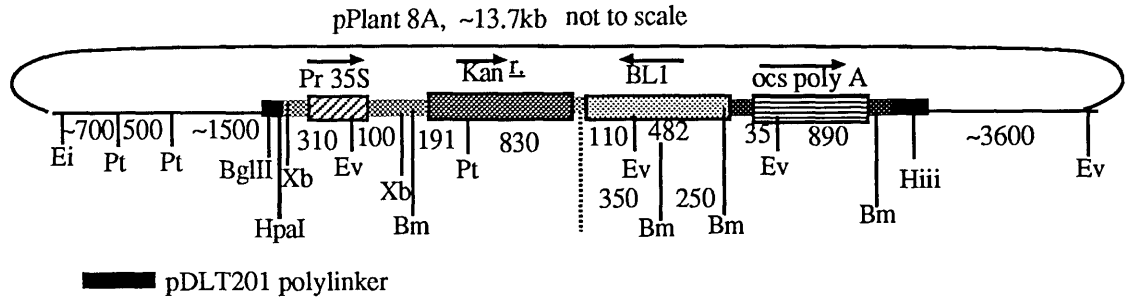


Figure 3.26. Map of pPlant8A.

Digest	Predicted sizes (kb) (anticlockwise)
Pst I	0.50, 2.08, 11
Eco RV	4.5, 1.23, 0.52, 7.5
Bam HI	1.37, 0.25, 0.92, 11.2
Xba I + Hind III	0.4, 2.54, 10.8

Table 3.24. pPlant8A digests.

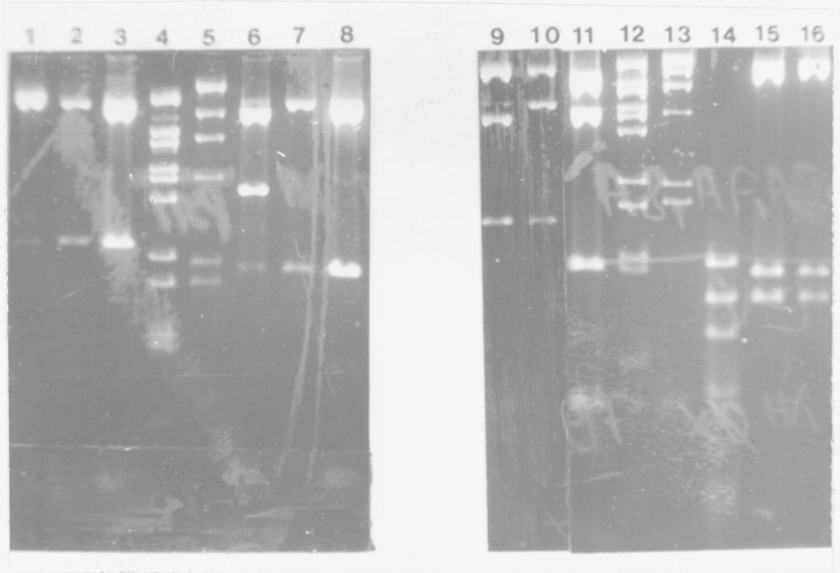


Figure 3.27. pPlant7 and pPlant8 digests. Lane1, pPlant7B-Xba I/Hind III; lane 2, pPlant7A-Xba I/Hind III; lane 3, pPlant8A-Xba I/Hind III; lane 4, lambda Bst EII markers; lane 5, lambda Hind III markers; lane 6, pPlant7B-Pst I; lane 7, pPlant7A-Pst I; lane 8, pPlant8A-Pst I; lane 9, pPlant7B-Eco RV; lane 10, pPlant7A-Eco RV; lane 11, pPlant8A-Eco RV; lane 12, lambda Bst EII markers; lane 13, lambda Hind III markers; lane 16 , pPlant7B-Bam HI; lane 15, pPlant7A-Bam HI; lane 14, pPlant8A-Bam HI.

Bands of the expected sizes were seen. Recombinants carrying the insert in the the anticlockwise orientation were called pPlant8A (Figure 3.26).

3.4 The Enhanced (EN) Series of Plasmids.

It has been reported that the upstream 260bp of the CaMV 35S promoter fragment contains an enhancer sequence, and that, if this sequence is duplicated, expression levels increase by 3-10 fold[126,127,128]. I decided to clone this enhancer fragment upstream of the CaMV 35S promoter in order to increase the antisense transcript levels.

3.4.1 pENH SE and pENH HE.

Before carrying on with the construction of the enhanced constructs, I wanted to have a readily accessible source of the CaMV 35S enhancer, flanked by convenient

restriction sites, for use in future constructs.

The plasmid, pJCD2, containing the CaMV 35S promoter and the incorrect fragment from pN19, was digested with Hind III and Eco RV to release the 260bp enhancer fragment from the CaMV 35S promoter. This fragment was gel purified, electroeluted and rendered blunt ended by the action of Klenow polymerase. It was then ligated into the Sma I site of pBS+ and transformed into *E.coli* strain, TG2. DNA from transformants appearing to carry the insert were digested with a variety of enzymes to determine the integrity of the construct and the orientation of the insert (Table 3.25).

Digest	Predicted sizes (kb) (SE)	Predicted sizes (kb) (HE)
Xba I + EcorI	0.31, 3.2	0.31, 3.2
SphI	0, 3.45	0.31, 3.2
SphI +Eco RI	0.30, 3.2	0.31, 3.2

Table 3.25. pENH digests.

The digests were run in 1.7% agarose gels against Hae III cut ØX 174 DNA . Bands of the expected sizes were seen. Recombinants carrying the insert in the the 'Hind III- Eco RI' orientation were called pENH SE and those carrying it in the reverse orientation were called pENH HE.

3.4.2 pBSCamBEN.

In order to have a readily accessible source of the enhanced CaMV 35S promoter, flanked by convenient restriction sites, for use in future constructs, the enhancer fragment (described above) was ligated into the Sac I site of pCamBS (which carries the unenhanced CaMV 35S promoter cloned into pBS+, 3.1.3) and transformed into *E.coli* strain, TG2. DNA from transformants appearing to carry the insert were digested with a variety of enzymes to determine the integrity of the construct and the orientation of the insert (Table 3.26).

Digest	Predicted sizes (kb)
SphI	0.66, 3.2
SphI + Eco RV	0.53, 0.11, 3.2
Eco RI + Hind III	0.68, 3.2

Table 3.26. pBSCamBEN digests.

The digests were run in 1.7% agarose gels against Hae III cut ØX 174 DNA . Bands of the expected sizes were seen. Recombinants carrying the insert as a direct repeat were called pBSCamBEN.

3.4.3 pJC2EN.

I also thought it desirable to have an enhanced CaMV 35S promoter upstream of polylinker sites and the octopine synthase polyadenylation signal.

In order to accomplish this, the enhancer fragment described in 3.4.1 was ligated into a now blunt, former SphI site of pJC2 and transformed into *E.coli* strain, TG2. DNA from transformants appearing to carry the insert were digested with a variety of enzymes to determine the integrity of the construct and the orientation of the insert (Figure 3.28, Table 3.27).

pJC2EN, 4.4kb not to scale

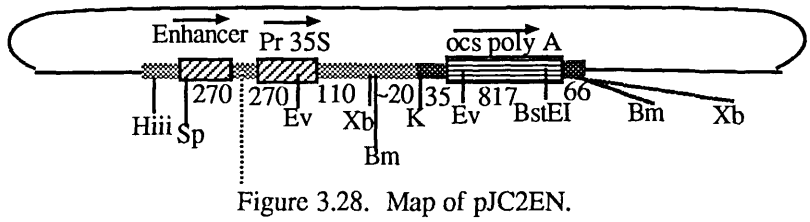


Figure 3.28. Map of pJC2EN.

Digest	Predicted sizes (kb)
Bam HI + Hind III	0.93, 0.64, 2.85
SphI + Eco RV	0.54, 0.14, 3.7
Bam HI	0.92, 3.5

Table 3.27. pJC2EN digests.

Bands of the expected sizes were seen following agarose gel electrophoresis. Recombinants carrying the insert as a direct repeat were called pJC2EN (Figure 3.28).

3.4.4 pPlant1AEN, pPlant2AEN, pPlant5AEN and pPlant6AEN.

The most convenient way to generate an enhanced CaMV 35S promoter in the binary vector pPlant series of constructs was thought to be to clone the enhancer fragment upstream of the bifunctional constructs in a polylinker site originating with the binary vector.

This was accomplished as follows. The blunt ended enhancer sequence was cloned directly into the former Bgl II sites (rendered blunt by the action of Klenow polymerase and treated with CIP) of pPlant1A, pPlant2A, pPlant5A and pPlant6A. DNA from transformants carrying the insert were digested with a variety of enzymes to determine the integrity of the construct and the orientation of the insert (Figure 3.31, Tables 3.28-29).

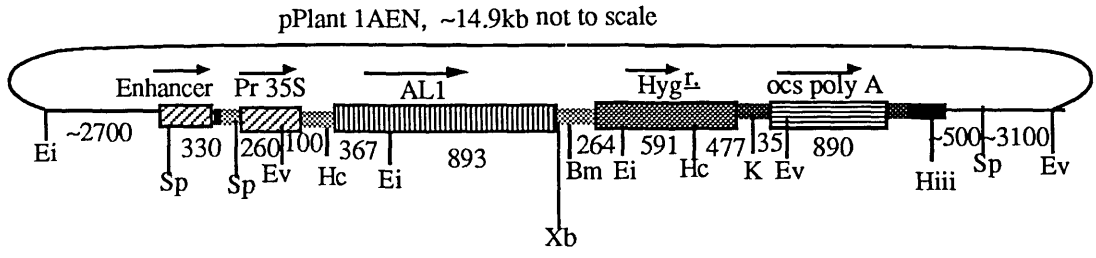


Figure 3.29. Map of pPlant1AEN.

Digest	Predicted sizes (kb) (pPlant1AEN)	Predicted sizes (kb) (pPlant2AEN)
Eco RV + Eco RI	3.3, 0.46, 1.2, 1.1, 4.5, 4.7	3.3, 0.99, 0.63, 1.1, 4.5, 4.7
SphI	4.4, 0.33, 10.3	4.4, 0.33, 10.3
Xba I +Eco RV	0.28, 1.36, 1.36, 4.5, 7.5	0.28, 1.36, 1.36, 4.5, 7.5

Table 3.28. pPlant1AEN and pPlant2AEN digests.

Bands of the expected sizes were seen following agarose gel electrophoresis (except for the 460bp band expected for the Eco RI/Eco RV digest of pPlant2AEN, which was probably not visible because not enough DNA was loaded onto the gel). Recombinants carrying the insert as a direct repeat were called pPlant1AEN and pPlant2AEN (Figure 3.31).

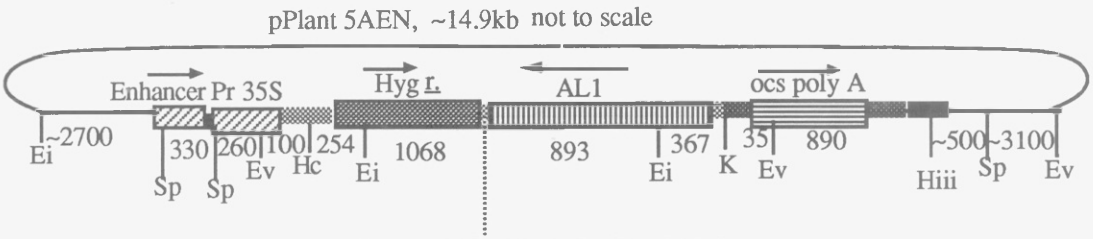


Figure 3.30. Map of pPlant5AEN.

Digest	Predicted sizes (kb) (pPlant5AEN)	Predicted sizes (kb) (pPlant6AEN)
Eco RV + Eco RI	3.3, 0.36, 1.97, 0.40, 4.5, 4.7	3.3, 0.36, 1.44, 0.93, 4.5, 4.7
SphI	4.4, 0.33, 10.3	4.4, 0.33, 10.3
Xba I +Eco RV	not determined	0.28, 0.10, 2.62, 4.5, 7.5

Table 3.29. pPlant5AEN and pPlant6AEN digests.

Bands of the expected sizes were seen following agarose gel electrophoresis (except for the 100bp band expected for the Xba I/Eco RV digest of pPlant6AEN, which was probably not visible because not enough DNA was loaded onto the gel). Recombinants carrying the insert as a direct repeat were called pPlant5AEN and pPlant6AEN (Figure 3.30).

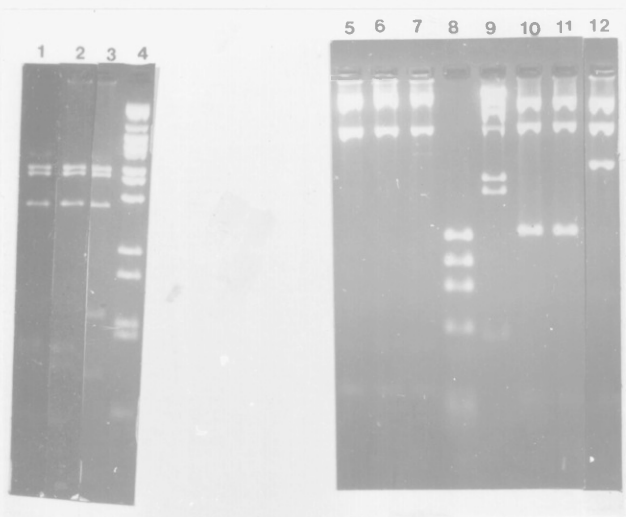


Figure 3.31. pPlant1AEN, Plant2AEN and pPlant6AEN digests. Lane1, pPlant1AEN-Eco RI/Eco RV; lane 2, pPlant2AEN-Eco RI/Eco RV; lane 3, pPlant6AEN-Eco RI/Eco RV; lane 4, lambda Bst EII markers; lane 5, pPlant1AEN-Sph I; lane 6, pPlant2AEN-Sph I; lane 7, pPlant6AEN-Sph I; lane 8, ØX 174 markers; lane 9, lambda Hind III markers; lane 10, pPlant1AEN-Xba I/Eco RV; lane 11, pPlant2AEN-Xba I/Eco RV; lane 12, pPlant6AEN-Xba I/Eco RV.

3.5 Junction Sequences and Coding Regions.

The exact sequences across the various cloning junctions were calculated and the chimaeric plant genes were re-constructed in the IBM program 'MicroGenie'; the sequences were then analysed for translation initiation and termination as related to the expression of the drug resistance markers (Figure 3.32). Stop codons were only considered 'in frame' to the ORF that they terminated. Spurious upstream initiation codons can diminish translational efficiency (discussed in 1.3) and this analysis was carried out in order to assess the degree to which the translational efficiency of the drug resistance genes might be decreased by the presence upstream TGMV genes.

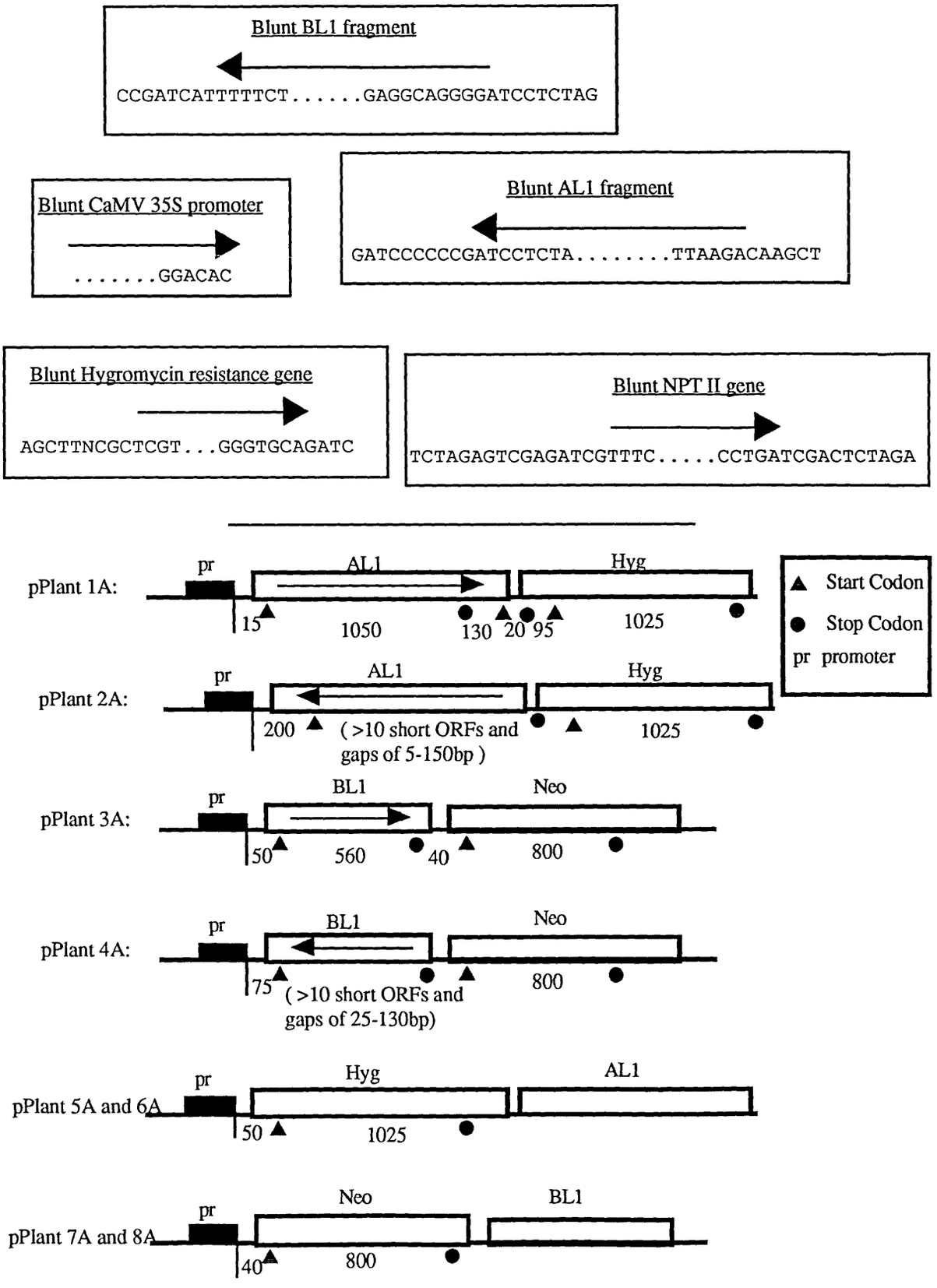


Figure 3.32. Junction sequences and coding regions. Note that where the antisense genes are upstream of the drug resistance genes there are many start and stop codons and these are not marked in detail.

3.6 Construct functionality.

The transformation of plants by *A.tumefaciens* to give whole transgenic plants or seeds can take many months (see Appendix I, Section 7.2.4). Furthermore, at this time there were no reports on the use of bifunctional constructs in plants. For these reasons, I decided, as far as was reasonably possible, to check the functionality of the constructs before transforming plants. The *npt II* coding region was tested as described in 3.2.5.

It has been observed[123] that the CaMV 35S promoter is slightly 'leaky' in *A.tumefaciens*; once the constructs pPlant 1A-8A had been transferred to the *A.tumefaciens* strain, LBA4404, they were streaked out on nutrient agar plates containing antibiotics as follows, Table 3.30.

Strain	no antibiotics	kanamycin, 5µg/ml	hygromycin, 80µg/ml
LBA4404	+	-	-
Plant1A	nd	nd	+
pPlant2A	nd	-	+
pPlant3A	nd	+	nd
pPlant4A	nd	+	nd
pPlant5A	nd	nd	+
Plant6A	nd	nd	+
pPlant7A	nd	+	nd
pPlant8A	nd	+	nd
pGA482	+	+	-

nd = not determined.

Table 3.30. Antibiotic resistance of *A.tumefaciens* strains.

Clearly both of the drug resitance coding regions are functional in these constructs.

It was also necessary to check that the plant regulatory signals to be used were functional. This was done using pCAT35[90] which contained the *cat* coding region cloned into the Sma I site of pJC2. This plasmid was electroporated into petunia protoplasts as

described (2.2.2vi); after culturing for 48 hours, protein was extracted and assayed for CAT activity (2.2.2v; Figure 3.33). This suggested that the promoter, at least, was functional in plants. A *caveat* to this experiment is that a negative control in which a promoterless *cat* region was electroporated into the protoplasts should have been carried out and was not. (Such a construct did not exist at the time of practical course where this experiment was carried out, see 2.2.2(vi)).

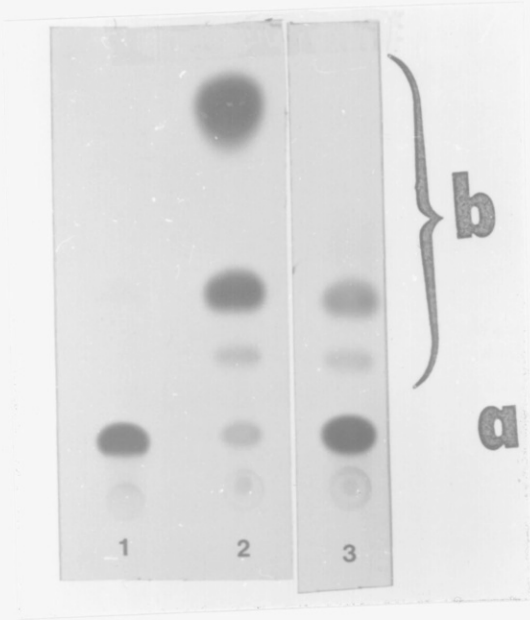


Figure 3.33. CAT assays. a: chloramphenicol; b: chloramphenicol acetates. Lane 1, plant negative control (no plasmid); lane 2, bacterial positive control; lane 3, pCAT35.

4. PLANT TRANSFORMATION

N.tabacum was transformed by the leaf disc method as described in 2.2.2.i.

The pPlant series of plasmids were transformed into *Agrobacterium* strain LBA4404 by triparental mating (described in 2.2.1.iii) to yield the 'AgroPlant' series of *Agrobacterium* strains. Two independent *Agrobacterium* transformants were, in general, used for each plant transformation. DNA was prepared from the cultures used for each transformation and the integrity of the strain confirmed by Southern blotting.

I have described the first transformation in some detail in order to give an idea of the chronology of events in a plant transformation.

4.1 Transformation 1.

The first series of transformations was carried using the *Agrobacterium* series, Plant 1A to Plant 4A, derived from pJC8S, pJC8AS, pJC9S and pJC9AS. These constructs contain the AL1 and BL1 coding regions upstream of the drug resistance marker in the bifunctional constructs. Total DNA from *Agrobacterium* strains generated by triparental mating was analysed by Southern blotting. Strain LBA4404 harbouring the plasmid pGA482 (conferring kanamycin resistance in plants) was used as a positive control of the plant transformation, and was designated Agro E. LBA4404 alone was used as a negative control and, in the absence of selection, to regenerate wild type plants which had undergone the rigours of tissue culture. The plant line used for this transformation was *N.tabacum*, cultivar Petit Havana SR1.

Between ten and fifteen 0.25-0.5cm sterile leaf pieces from 1 month old, healthy, greenhouse grown, plants were used per petri dish. The culture medium used was MSD 4x2 supplemented with 250μg/ml of Cefotaxime and drug selection as described in Table 4.1.

Strain	Number. of Petri Dishes		
	Hyg., 25μg/ml	Kn.,.100μg/ml	No Selection
AgroPlant1A	10	1	1
AgroPlant 2A	12	1	1
AgroPlant 3A	1	10	1
AgroPlant 4A	1	10	1
Agro E (pGA482)	1	10	1
LBA 4404	1	1	10

Table 4.1. Conditions for plant transformation.

Total DNA was prepared from the *Agrobacterium* cultures used in the plant transformation: AgroPlant 1A and AgroPlant 2A were cut with Eco RI and Eco RV, AgroPlant 3A and AgroPlant 4A were cut with Eco RV and Bam HI, Agro E was cut with Sal I and Pst I, and LBA4404 was cut with Eco RV. Southern blots were carried out and then probed with a labelled mixture of pGA482, pJC9S and pJC8S. Bands of the expected size were seen (data not shown).

Ten days after inoculation of leaf discs, callus started to appear on the edges of the leaves growing in the absence of selection and a few bright green spots appeared on the edges of some leaves grown on selective media. After twenty–one days, some shoots and larger amounts of callus had begun to appear. Some shoots were transferred to petri dishes containing MSO media (to root) and the appropriate selective antibiotics. The results at this point are shown in Table 4.2.

After twenty–eight days, no more growth was seen on leaf discs growing on the incorrect selective media and they were discarded. During this time eighteen further shoots from AgroPlant1A, twenty from AgroPlant2A, none from AgroPlant3A, fourteen from AgroPlant4A and nineteen from Agro E were transferred to MSO media on petri dishes to root.

Strain	Number of plates	Total number of shoots	Number transferred to fresh plates.
	9 Hyg	108 shoots, 0-2mm	15 onto Hyg 25µg.
	1 Kn	1 odd looking shoot	-
AgroPlant1A	1 No Selection	Many shoot >5mm and much callus.	Plate discarded
	12	103 shoots, 0-2mm,	13 onto Hyg 25µg
	1 Kn	No Shoots	-
AgroPlant2A	1 No Selection	Many shoot >5mm and much callus.	Plate discarded
	10 Kn	4 shoots ~5mm, 4 shoots ~10mm	8 onto Kn 100µg
	1 Hyg	8 shoots ~2mm	-
AgroPlant3A	1 No Selection	Many shoot >5mm and much callus.	Plate discarded
	10 Kn	15 shoots ~5mm, 5 shoots ~2mm	15 onto Kn 100µg
	1 Hyg	No shoots	-
AgroPlant4A	1 No Selection	Many shoot >5mm and much callus.	Plate discarded
	10 Kn	~ 20 shoots/plate each 5-10mm	30 onto Kn 100µg
	1 Hyg	2 tiny shoots	-
Agro E (pGA482)	1 No Selection	Many shoot >5mm and much callus.	Plate discarded
	10 No Selection	Many shoot >5mm and much callus.	26 transferred to no selection
	1 Hyg	3 tiny shoots	
LBA 4404	1 Kn	No Shoots	

Table 4.2. Plant transformation at 21 days.

Thirty–seven days after the initial inoculation, no more growth was observed on the leaf discs, which had all turned brown, and they were discarded. Following transfer from the leaf discs, all shoots from AgroPlant1A, AgroPlant2A and AgroPlant3A had bleached (on selective media) and were discarded. Nine shoots from AgroPlant4A had rooted and looked healthy. The nine healthy shoots were subcultured into fresh selective media in 500ml screw cap jars. Of the thirty Agro E shoots, seventeen rooted. Eighteen of the twenty–six LBA4404 control plants rooted and were subcultured.

The rooted plantlets were subcultured once more over the next three weeks, and the following kept for further study: seven lines from AgroPlant4A (called PD1-7), three from Agro E (called PE1-3), and ten from LBA4404 (called PF1-10). These plants were propagated in tissue culture and were hardened off in the greenhouse prior to TGMV infection and generation of F1 seeds.

The concentration of hygromycin used in this transformation (25μg/ml), while sufficient to select against rooting of untransformed plants, was insufficient to select against the initial formation of shoots. In subsequent transformation experiments 35-50μg/ml of hygromycin were used.

4.2 Transformation 2.

This transformation was carried out in essentially the same manner as transformation 1, with the same number of plates. The differences were as follows. Hygromycin was used at a concentration of 40μg/ml instead of 25μg/ml. *Agrobacterium* strains AgroPlant 5A to AgroPlant 8A, derived from pJC10AS, pJC10S, pJCVIS and pJCVIAS, were used. These strains contain the AL1 and BL1 coding regions downstream of the drug resistance marker in the bifunctional constructs. Total DNA was prepared from the *Agrobacterium* cultures used in the plant transformation and cut with a variety of enzymes to check the integrity of the constructs (Table 4.3, Figure 4.1). Southern blots were carried out and probed with a labelled mixture of pGA482, pJC9S and pJC8S.

Bands of the expected size were seen with the following exceptions. No fragments smaller than 0.6kb were seen as they were run of the end of the gel. There was a faint, unexpected band at about 3.3kb in all of the Bam HI digests; the reason for the appearance of this band is unclear, but it may be due to DNA contamination in the probe or in the enzyme stock solution.

Agrobacterium strain	Digest	Predicted sizes (kb)
AgroPlant5A	Bam HI	14.7
	Eco RI	1.97, 3.3. 9.4
	Eco RV	2.75, 4.5, 7.5
AgroPlant6A	Eco RI	1.44, 3.3, 9.9
AgroPlant7A	Bam HI	0.25, 1.02, 1.27, 11.2
	Eco RV	0.14, 1.6, 4.5, 7.5
AgroPlant8A	Bam HI	0.25, 0.92, 1.37, 11.2
	Eco RI	13.7
	Eco RV	0.52, 1.23, 4.5, 7.5
Agro E	Sal I	2.8, 4.1, 6.4
LBA4404	Eco RI	-

Table 4.3. *Agrobacterium* DNA digests.

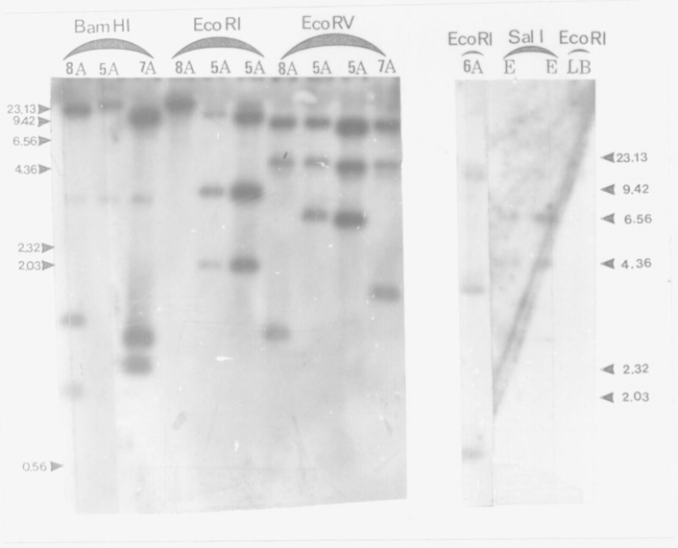


Figure 4.1. Agrobacterium Southern blots. 5% of a total DNA miniprep (2.2.3Biii) per lane. 24 hour exposure at -70°C with intensifying screens.

This transformation experiment yielded the following transgenic lines, Table 4.4.

Agrobacterium strain	# shoots	# rooted	Name of plant line
AgroPlant5A	2*	2	P5.1, P5.2
AgroPlant6A	22	6	P6.1, P6.2, P6.3, P6.6, P6.10, P6.S1
AgroPlant7A	0	0	-
AgroPlant8A	38	5	P8.1 - P8.5
AgroE	~100	~50	discarded

Table 4.4. Plant transformations.

*AgroPlant5A yielded two pieces of callus on one leaf disc. These calli were transferred to shoot inducing media and one transformed plant was regenerated from each piece of callus. No other transformants were obtained from any of the other hundred or so leaf discs inoculated with this strain.

N.B. Other nomenclature is also used for these transformants[129].

These transgenic plants were micro-propagated, hardened off and F1 seeds were generated. The following lines were taken for further study; F4 (SR1, LBA 4404 mock transformation); P5.1 and P5.2; P6.10, P6.6 and P6.S1.

In order to analyse the structure of foreign DNA in transgenic plants, genomic DNA was isolated from leaves of F1 plants (2 plants per transformant in most cases). These DNAs were cut with Eco RI and Eco RV and Southern blotted. (Figure 4.2, Table 4.5).

Plant line	Digest	Predicted sizes (kb)
P5A	Eco RI	1.97, **3.36**, Border fragments*
	Eco RV	2.75, **Border fragments***
P6A	Eco RI	1.44, **3.36**, Border fragments*
	Eco RV	2.75, **Border fragments***

Table 4.5. Plant DNA digests. Predicted fragment sizes; **bold** indicates that these fragments do not have homology to the AL1 probe.

*Border fragments would clearly be variable as one site is in the plant, genomic DNA.

The filters were probed with pJC3, containing the CaMV 35S promoter, the hygromycin resistance coding region and the octopine synthase polyadenylation signal. The expected internal fragments were observed in all cases except for P6.10, Eco RI digest (lane 6, Figure 4.2A) where the band which was expected at 3.36kb appears to be shifted to 3.6kb. However, the expected 2.7kb band is seen in the Eco RV digest (lane 6, Figure

4.2A), implying that both coding regions and part of the regulatory signals are intact. One explanation is that the Eco RI site adjacent to the left border has been deleted during T-DNA transfer.

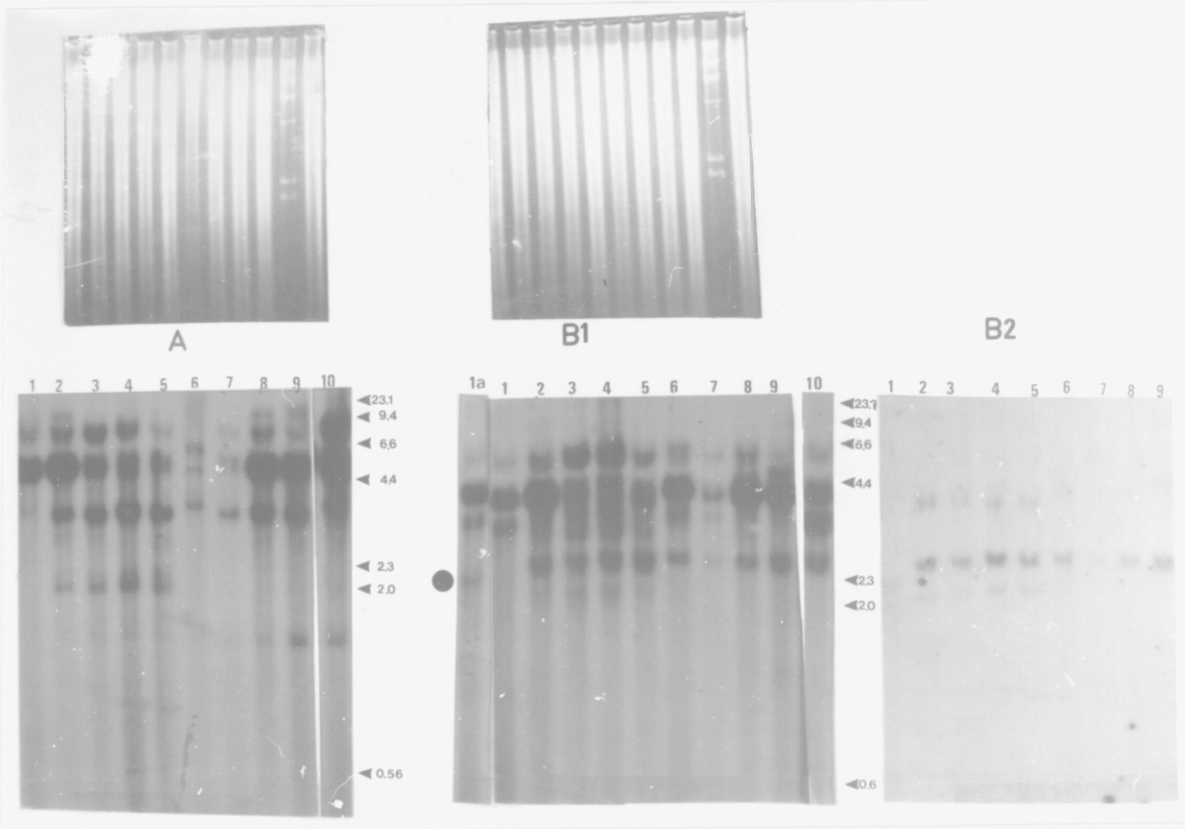


Figure 4.2. Plant Southern blots. 10µg of plant DNA per lane. A, Eco RI digest probed with pJC3: Lane 1, F4 (SR1); lane 2, P5.1i; lane 3, P5.1ii; lane 4, P5.2i; lane 5, P5.1ii; lane 6, P6.10; lane 7, P6.6i; lane 8, P6.6ii; lane 9, P6.S1i; lane 10, 15ng PJC10S spiked into 10µg F4 DNA. B1, Eco RV digest probed with pJC3: As A with the folowing exceptions; lane 1a, F4 probed with pBSALS; lane 8, P6.S1i, lane 9, P6.S1ii. Both A and B1 exposed for 4 days with intensifying screens at -70°C. B2: as B1 but probe with gel purified AL1 fragment; exposed for 2days with intensifying screens at -70°C.

There are intense bands between 4 and 9kb, in both transformed and wild type DNA found with all probes and digests; this suggests either contamination or spurious homology with probe sequences. It was not possible to distinguish border sequences from these signals.

The filters were stripped and re-probed with pBSALS, containing the AL1 fragment. The signals obtained were identical to those obtained with pJC3 with two exceptions (data not shown except for lane 1a, Figure 4.2B1). The signals at 3.36kb in the transformant Eco RI digests decreased markedly; the presence of this signal with a pBSALS probe (also seen with a purified AL1 probe) may be due to homology with

polylinker sequences in the probe. There was an additional band in the F4 Eco RV digest at 2.3kb (marked with a filled circle, lane 1a, Figure 4.2B1); since there were no corresponding bands in the transformants, this was assumed to be an artifact.

In order to check for spurious homologies, the filters were probed with gel purified AL1 fragment (Figure 4.2B2, Eco RV digests only shown). The relative intensity of the 4-9kb bands decreased almost to nothing, implying that vector sequences were responsible for the bulk of these signals; it was still not possible to identify border sequences. However, there were faint signals at 2.3kb and 4.0kb in the Eco RV digests of P5.1 and P5.2 (lanes 2-5, Figure 4.2B2). The AL1 probe should light up only the internal 2.7kb fragment. One explanation for the presence of these extra bands, which can also be seen with both of the other probes, is the integration of deleted and/or rearranged copies of pPlant5A T-DNA in addition to unrearranged copies. If this is true, it follows that P5.1 and P5.2 are clones of each other. This is not completely unexpected, as they arose from two neighbouring pieces of callus on a single leaf disc and no other transformants were obtained on ~200 more leaf pieces. However, the possibility that these bands are artifacts cannot be ruled out.

A reconstruction experiment was carried out by spiking 75pg of pJC10S into 10μg of SR1 (F4) DNA (lane 10, Figure 4.2 A and B1); this is a 5x reconstruction based on a genome size of 3.9pg[130]. P5.1, P5.2 and P6.S1 appeared to have ~4-8 copies/genome. P6.10, P6.6 appeared to have ~1-4 copies/genome.

From the Southern blot data it is possible to conclude that all of the transformants analysed contain unrearranged copies of the T-DNA insert, with the possible exception of P6.10. In the latter case, it is very likely that the bifunctional construct encoding the AL1 and hygromycin resistance genes is intact.

In order to check that the chimæric genes were being transcribed, northern blots were run of polyA$^+$ RNA prepared from greenhouse grown seedlings (Figure 4.3). 4μg per lane were run, except for SR1 and P5.1, where only 1μg was run due to limited supplies of RNA. The blots were probed with the AL1 fragment and the hygromycin resistance coding region. Identical bands were seen in both cases. No signal was seen in

SR1 RNA even after over exposure (Figure 4.3A, second panel). All transformants had bands at ~3.45kb and ~1.9kb (cf. single stranded lamba Hind III markers). P6.6 had an additional band at ~2.2kb. The transcript expected from these lines is 3.07kb plus the polyA tail. The upper band on the northern blot is in reasonable agreement with this. There are a number of possible explanations for the smaller transcripts. These are chimaeric constructs and it is possible that the larger transcript is being processed in some way to give the smaller transcript. In the case of the 2.2kb band, which is unique to P6.6, this processing may be directed by flanking plant chromosomal sequences, or by transcription from a deleted insert; although a deleted insert was not detected in the Southern blots.

The bands observed are similar in size to the ribosomal RNA bands. The following is evidence that cross hybridisation between the probes and the ribosomal RNA is not taking place. There was no signal for the SR1 RNA and there is no correlation between the intensity of the signals and the intensity of the ribosomal bands in the corresponding ethidium bromide stained gel (Figure 4.3A). A northern blot was run on a number of RNA samples and probed with AL1 (Figure 4.2B). This gel was run in the presence of ethidium bromide and consequently has run abnormally; discrete bands may only be discerned in lane 3, and possibly lane 2. However, the intensities of the signals may be compared with the intensity of the smear in the corresponding ethidium bromide stained gel. Lanes 1-3 are polyA$^+$ RNA from transgenic plants and show intense signals. Lanes 4 and 6-9 are poly A$^-$ RNA and lane 5 is total RNA; clearly the signals are polyA$^+$ specific and not related to amount of ribosomal RNA.

In order to be completely certain that the corrrect sense and antisense RNAs are being transcribed, the northern blots should have been probed with strand specific probes. However, the strength of the signals obtained decreased each time the filters were stripped. There was not enough RNA to run further northern blots, nor enough time to prepare more. The combination of the Southern blots and the double stranded probed northern blot data is strong circumstantial evidence that the correct transcripts are being synthesised. Preparation of fresh RNA and probing of northern blots with strand specific probes needs to be carried out.

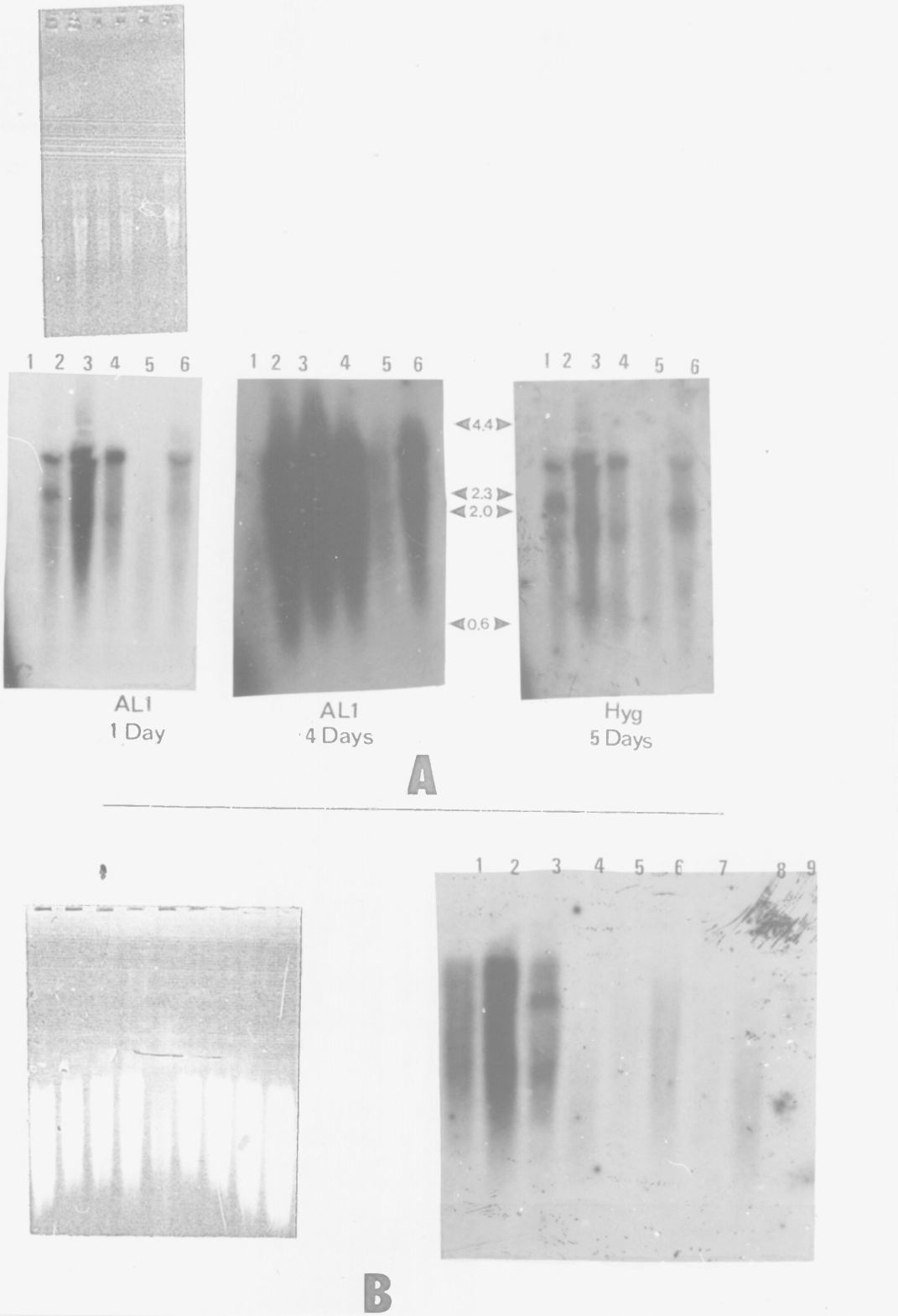


Figure 4.3. Plant northern blots. Poly A^+ RNA used. A: probed with AL1 fragment or hygromycin coding region (exposure times at -70°C with intensifying screens marked). Lane 1, 1µg SR1 RNA; lane 2, 4µg P6.6 RNA; lane 3, 4µg P6.10 RNA; lane 4, 4µg P6.S1 RNA; lane 5, 1µg P5.1 RNA, 4µg P5.2 RNA. B: Gel prestained with ethidium bromide. Probed with AL1 fragment (exposed for 3 days at -70°C with intensifying screens marked), 5µg per lane unless stated. Lane 1, P6.S1 polyA$^+$; lane 2, P6.10 polyA$^+$; lane 3, P6.6 polyA$^+$; lane 4, P5.1 polyA$^-$; lane 5, 1µg P6.S1 total; lane 6, P6.10 polyA$^-$; lane 2, P6.6 polyA$^-$; lane 8, P5.1 polyA$^-$; lane 9, P6.S1 polyA$^-$.

4.3 Transformation 3.

The following strains of *Agrobacterium* yielded no transformed plants; AgroPlant1A, AgroPlant2A, AgroPlant3A and AgroPlant7A. AgroPlant5A yielded only 2 (possibly 1) line of transformed plants. In order to ask whether this failure to produce transformed plants was due to the nature of the DNA construct and not to the experimental conditions pertaining to a particular inoculation, plant transformations were repeated with AgroPlant1A, AgroPlant 2A, AgroPlant5A, AgroPlant6A, AgroPlant7A, AgroPlant8A and AgroE. The transformations were carried out as described above (4.1 and 4.2) except that only 6 petri dishes of leaf discs were used for each strain instead of 10-12. The DNA from the *Agrobacterium* strains used for the transformations was Southern blotted to confirm the integrity of the constructs (data not shown). As before, no shoots were obtained from AgroPlant1A, AgroPlant 2A, AgroPlant5A or AgroPlant7A. AgroPlant6A, AgroPlant8A and AgroE produced shoots in the expected quantities; they were all discarded.

4.4 Transformation 4.

This transformation was carried out in the same manner as transformation 1, with the same number of plates and the same controls. The differences were as follows. Hygromycin was used at a concentration of 35μg/ml instead of 25μg/ml. *Agrobacterium* strains AgroPlant1AEN, AgroPlant2AEN, AgroPlant5AEN and AgroPlant6AEN were used in this transformation. They contain the the AL1 region, in the sense and antisense orientation, upstream and downstream of the hygromycin resistance coding region, with the CaMV 35S promoter, enhancer region cloned upstream of the promoter (see 3.4). Total DNA was prepared from the *Agrobacterium* cultures used in the plant transformation and cut with the a variety of enzymes to check the integrity of the constructs (Table 4.6, Figure 4.4). Southern blots were carried out and probed with labelled pPlant2A.

Agrobacterium strain	Digest	Predicted sizes (kb)
Plant1AEN	Eco RI + Eco RV	0.46, 1.1, 1.2, 3.3, 4.5, 4.7
	Xba I + Eco RV	0.28, 1.36, 1.36, 4.5, 7.5
Plant2AEN	Eco RI + Eco RV	0.63, 0.99, 1.1, 3.3, 4.5, 4.7
	Xba I + Eco RV	0.28, 1.36, 1.36, 4.5, 7.5
Plant5AEN	Eco RI + Eco RV	0.36, 0.40, 1.97, 3.3, 4.5, 4.7
	Xba I + Eco RV	0.10, 0.28, 2.62, 4.5, 7.5
Plant6AEN	Eco RI + Eco RV	0.36, 0.93, 1.44, 3.3, 4.5, 4.7
	Xba I + Eco RV	0.10, 0.28, 2.62, 4.5, 7.5

Table 4.6. *Agrobacterium* DNA digests.

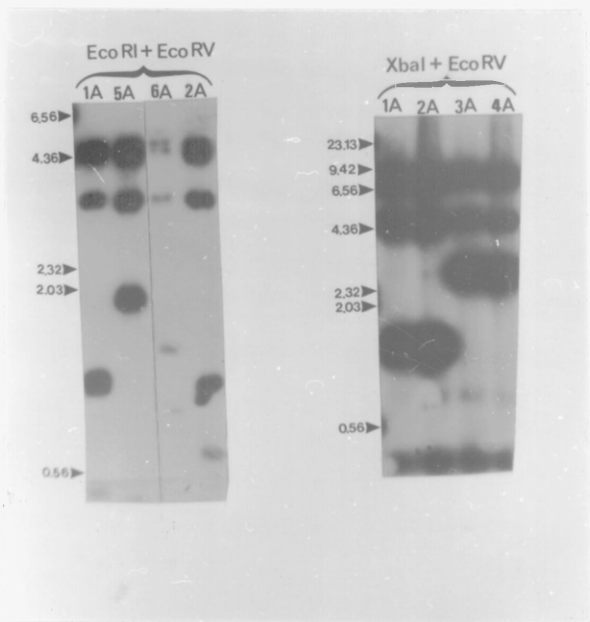


Figure 4.4. Agrobacterium Southern blots. 5% of total DNA miniprep (2.2.3Biii) per lane. 48 hour exposure at -70°C with intensifying screens.

Bands of the expected size were seen with the following exceptions. The 0.36kb band was not seen in the Eco RI/Eco RV digest of Plant6A. The 0.1kb bands were not seen in the Xba I/Eco RV digests of Plant5A and Plant6A.

This transformation experiment yielded a number of transformed plants (Table 4.7).

Agrobacterium strain	# shoots	# rooted	Name of plant lines
AgroPlant1AEN*	0	0	-
AgroPlant2AEN*	†	2	P5AEN.1, P5AEN.6
AgroPlant5AEN*	35	5	P2AEN.1-P2AEN.5
AgroPlant6AEN	26	6	P6AEN.3, P6AEN.5, P6AEN.6, P6AEN.9, P6AEN.10, P6AEN.12
AgroE	~100	~50	discarded

Table 4.7. Plant Transformations.

*AgroPlant2AEN and AgroPlant5AEN transformations were all contaminated with a coliform bacteria which was recalcitrant to antibiotics and bleach treatments. These transformations were repeated, as well as AgroPlant1AEN, using sterile axenically grown *N.tabacum* cultivar Samson N.N.; consequently lines P2AEN and P5AEN are in the genetic backround of cultivar Samson N.N., while P6AEN are in the genetic background of cultivar Petit Havana SR1.

†Plant2AEN yielded 46 calli and 4 shoots. Calli were transferred to shooting media. 6 shoots were finally obtained and were micropropagated for 5 or 6 months. However, only two rooted. These were called P5AEN.1 and P5AEN.6. The other transformants were almost certainly hygromycin resistant or they would have died on 35µg/ml hygromycin; it is possible that hygromycin phosphotransferase was being expressed at a level sufficient to render the shoots resistant, but not the more sensitive roots.

N.B. To avoid confusion, it should be noted that the *Agrobacterium* strains, AgroPlant2AEN and AgroPlant5AEN were accidently swopped, and this was only discovered when the plant lines were Southern blotted; for this reason ***Agrobacterium* strain Plant5aAEN gives rise to plant line P2AEN** and ***Agrobacterium* strain Plant2aAEN gives rise to plant line P5AEN.**

Genomic DNA was isolated from the leaves of greenhouse grown plants which had been transferred to soil from tissue culture. These DNAs were cut with a variety of enzymes and Southern blotted (Figure 4.5, Table 4.8).

Plant line	Digest	Predicted fragment sizes (kb) (**AL1 probe** *Hyg probe*)
P2AEN (from Plant5AEN)	Eco RI	***1.97***, *3.64*, ***Border fragments***
	Eco RV	***2.75***
	Hind III	***Border fragments***
P5AEN (from Plant2AEN)	Eco RI	***0.63***, **4.28**, *Border fragments**
	Eco RV	***2.75***
	Hind III	***Border fragments****
P6AEN (from Plant6AEN)	Eco RI	***1.44***, *3.64*, ***Border fragments****
	Eco RV	***2.75***
	Hind III	***Border fragments****

Table 4.8. Plant DNA digests. Predicted fragment sizes; **bold** indicates homology to the AL1 probe and *italic* indicates homology to the hygromycin resistance probe.
*Border fragments would clearly be variable as one site is in the plant, genomic DNA.

It was found that the AL1 probe has homology to wild type tobacco; bands common to wild type and transgenic lines are discussed elsewhere (see Chapter 5).

The intensity of the signals obtained in these Southern blots made it possible to attempt a more detailed interpretation than in the previous experiments. In some cases the pattern obtained defied analysis.

In all of the transformants examined, the expected internal fragments (Table 4.8) were seen, with the following exceptions. The 1.44kb bands expected in the Eco RI digests of P6AEN were occluded by a band arising from the tobacco genome when AL1 was used as probe (Figure 4.5b). The 4.28kb fragment which is expected when P5AEN is probed with AL1 was also observed (albeit at a lower intensity) when the hygromycin fragment is used as probe; this may be due to homology with polylinker sequences in the probe.

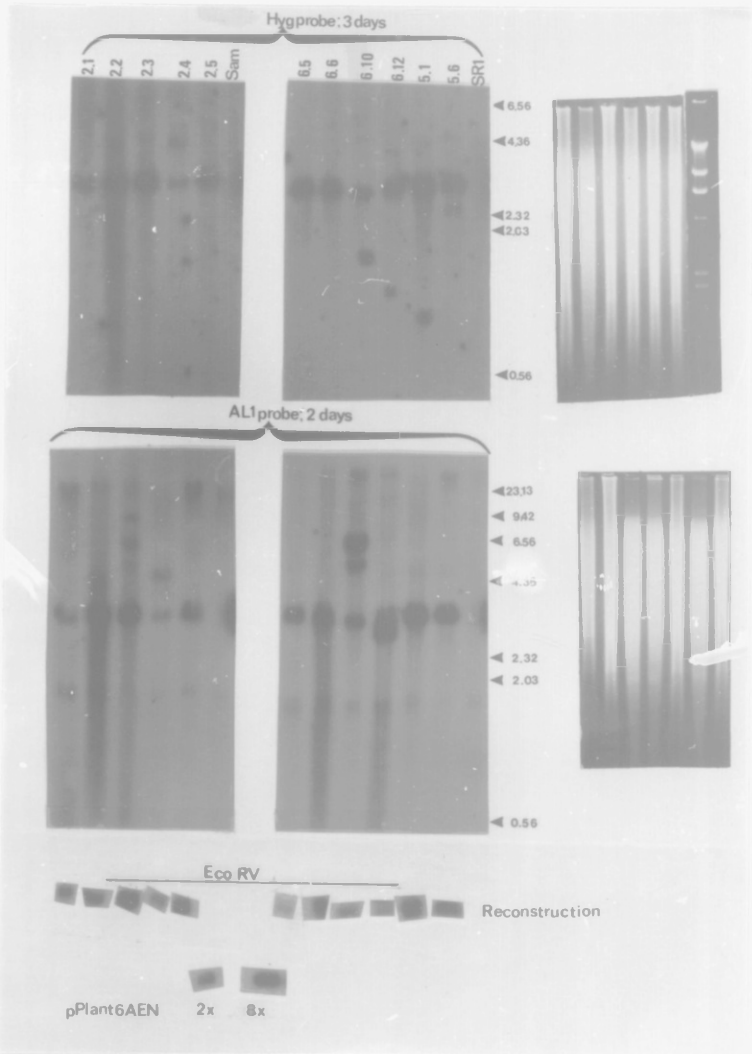


Figure 4.5a. Southern blots of plant ENhanced lines, Eco RV digests; 10µg DNA/lane.

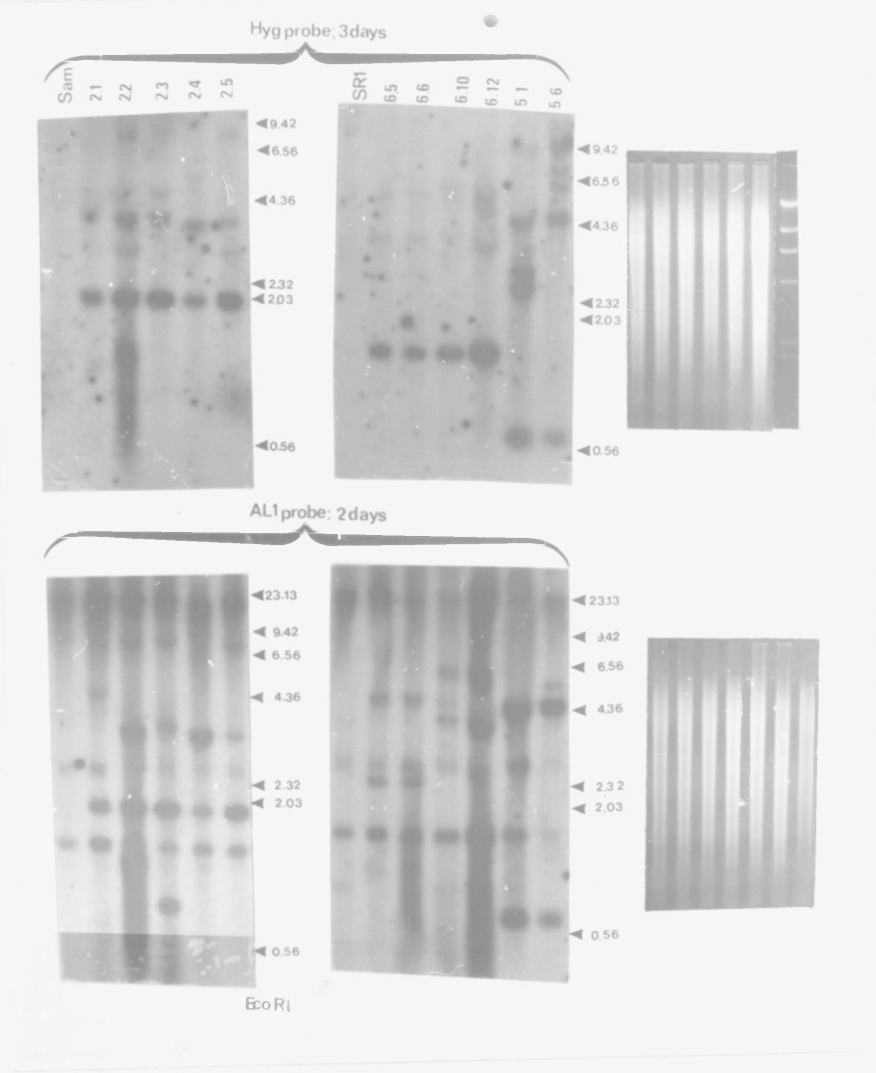


Figure 4.5b. Southern blots of plant ENhanced lines, Eco RI digests; 10µg DNA/lane.

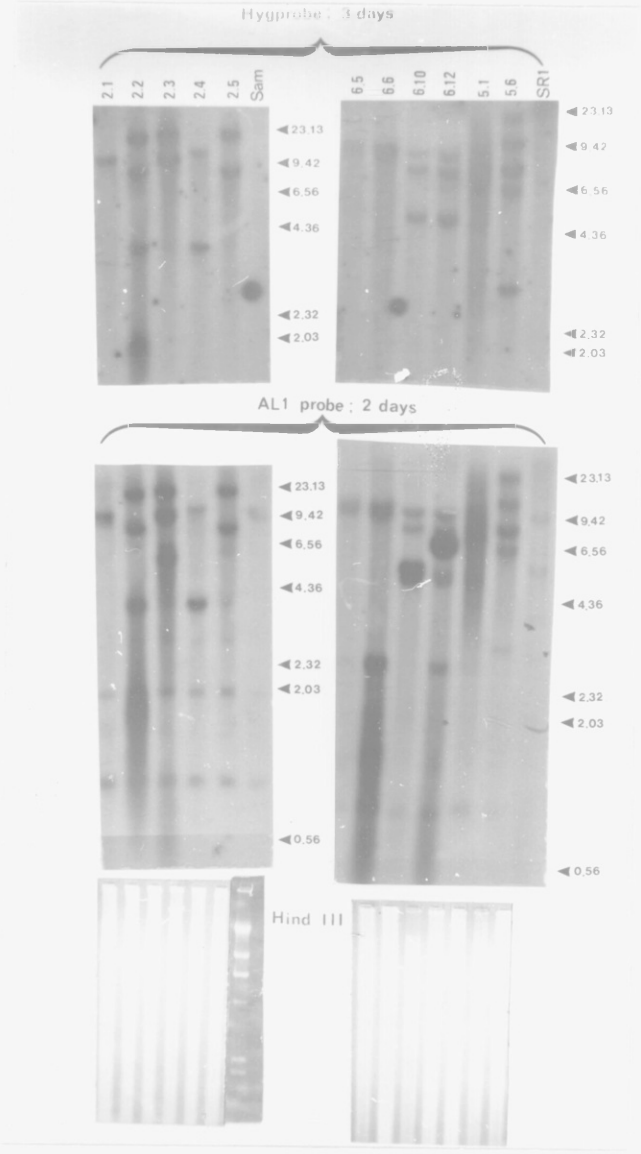


Figure 4.5c. Southern blots of plant ENhanced lines, Hind III digests; 10µg DNA/lane.

Let us consider each transgenic line in turn.

P2AEN.1

When digested with Eco RI, an additional (border?) fragment at ~4.6kb is observed on probing with AL1 (Figure 4.5b). When digested with Hind III, a fragment is observed at ~12kb with both probes (Figure 4.5c). A reconstruction experiment with 2 and 8 copies of pPlant6AEN (Figure 4.5a) suggests 1-2 copies of T-DNA. This data is consistent with an insert of 1 copy of unrearranged T-DNA in P2AEN.1.

P2AEN.2

When digested with Eco RI, the 3.6kb fragment is seen with both probes, and is considerably more intense than in P2AEN.1 (Figure 4.5b); this band is also present in P2AEN3-5 and is consistent with T-DNA insertions as tail to tail dimers (Hind III sites adjacent). In addition, bands are observed at ~7kb when probed with AL1 and ~2.7kb when probed with hygromycin, presumably these are border fragments. Less easy to explain is the strong band at ~1.2kb, visible on probing with both probes; the facile explanation of rearranged T-DNA is not consistent with the Eco RV digests. When digested with Hind III, 4 fragments are observed at ~20kb, ~8kb, ~4.0kb and ~1.9kb on probing with hygromycin or AL1 (Figure 4.5c). The reconstruction experiment suggests 2-4 copies of T-DNA. The interpretation of this data is not obvious; clearly, there are unrearranged T-DNA inserts, perhaps as head to tail dimers. The possibility of additional rearranged insertions cannot be ruled out.

P2AEN.3

When digested with Eco RV, and on probing with AL1, two additional bands are observed at ~6.5kb and 9kb, suggesting the integration of rearranged copies of T-DNA (Figure 4.5a). When digested with Eco RI, the 3.6kb fragment is seen with both probes and, as above, is considerably more intense than in P2AEN.1 (Figure 4.5b); this band is consistent with T-DNA insertions as tail to tail dimers (Hind III sites adjacent). In addition, bands are observed at ~0.7kb and ~7kb when probed with AL1, and ~4.5kb

when probed with hygromycin, presumably these are border and rearrangement fragments. When digested with Hind III, and on probing with hygromycin or AL1, 2 fragments are observed at ~20kb and ~14kb; in addition, fragments are observed at ~6.6kb and ~6.0kb only when AL1 is used as a probe (Figure 4.5c). The reconstruction experiment suggests 6-10 copies of T-DNA. An explanation consistent with this data is a series of 2 or 3 head to head (14kb Hind III band) and tail to tail (3.6kb Eco RI/ AL1 probe fragment) dimers in addition to one or more rearranged insertions in P2AEN.3. Further diagnostic digests would be necessary to confirm this interpretation.

P2AEN.4

When P2AEN.4 is digested with Eco RV, an additional band is observed with both probes at ~5.5kb, suggesting the integration of rearranged copies of T-DNA (Figure 4.5a). When digested with Eco RI, the 3.6kb fragment is also seen with both probes, and is considerably more intense than in P2AEN.1 (Figure 4.5b); this band is consistent with T-DNA insertions as tail to tail dimers (Hind III sites adjacent). No border fragments can be seen, but they may be of very high molecular weight and not seen due to poor transfer. When digested with Hind III, 2 fragments may be observed at ~16kb and ~4.0kb on probing with hygromycin or AL1 (Figure 4.5c). The reconstruction experiment suggests 1-3 copies of T-DNA. This is consistent with 1 or 2 copies of unrearranged T-DNA inserted (possibly as a tail to tail dimer), and one copy of deleted and/or rearranged DNA in P2AEN.4.

P2AEN.5

When digested with Eco RI, a 7kb fragment is seen in addition to the predicted 1.9kb fragment with both probes (Figure 4.5b). The 3.6kb fragment is also seen with both probes, and is more intense than in the P2AEN.1 line (Figure 4.5b); this band is consistent with T-DNA insertions as tail to tail dimers (Hind III sites adjacent). When digested with Hind III, 2 fragments are observed at ~20kb and ~8kb on probing with hygromycin or AL1 (Figure 4.5c). The reconstruction experiment suggests 6-10 copies of T-DNA. This high

apparent copy number is not easy to reconcile with the 2 border fragments observed in the Hind III digests. The digests are not consistent with multiple head to head/ tail to tail inserts or multiple head to tail inserts. All that it is possible to conclude in this case is that there are no obvious rearrangements and more than 1 copy of T-DNA has been integrated (possibly including one tail to tail dimer).

P6AEN.5/6

Examination of the Southern blots reveal almost identical patterns for these two lines and they are probably clonally identical. The only difference is the presence of a strong additional band and smear at ~2.4kb when P6AEN.6 is probed with AL1 (Figure 4.5c). As there are no differences observed in the other digests, this is presumed to be an artifact

When digested with Eco RI, additional (border?) fragments are seen at ~2.5kb and ~4.6kb with AL1 as probe (Figure 4.5b). Digestion with Hind III yields a fragment at ~14kb with both probes;(Figure 4.5c). The reconstruction experiment suggests 1-2 T-DNA inserts in P6AEN.5 and 2-4 in P6AEN.6. This data is not unequivocal, but is reasonably consistent with the integration of one unrearranged T-DNA insert.

P6AEN.10

P6AEN.10 has extra bands at ~5kb and 6.5kb when digested with Eco RV with AL1 as probe; this suggests the integration of rearranged and deleted T-DNA (Figure 4.5a). When digested with Eco RI and probed with AL1, additional fragments are seen at ~4.1kb, ~4.6kb and 6.5kb (Figure 4.5b). Digestion with Hind III yields fragments of ~14kb, ~8.5kb and ~5.0kb with both probes. An additional band is seen at ~5.5kb on probing with AL1. The copy number reconstruction suggests 2-3 T-DNA inserts. This data is difficult to interpret; there are probably 1-2 unrearranged copies of T-DNA integrated and at least 1 rearranged copy.

P6AEN.12

The Southern blot patterns of P6AEN.12 are very similar to p6AEN.10.

The Eco RV digest reveals an extra band at ~2.5kb and no bands at ~5kb and 6.5kb when AL1 is used as probe. The Eco RI digests are essentially identical. The Hind III digests are identical, except for the absence of the 5.5kb band and the appearance of a ~6.6kb and ~3.3kb band on probing with AL1. The copy number reconstruction is about the same. Th interpretation of this data is not clear, particularly the similarity with P6AEN.10. Possible explanations are: i) P6AEN.10 and P6AEN.12 are identical and the observed differences are artifacts; ii) both lines arose from the same transformation event, but rearrangements occurred later during differentiation; iii) they are independent transformants, but some copies have integrated in identical sites in the chromosome; iv) the transformants are independent and the similarities in pattern are due to an underlying similarity in structure (dimers or multimers) which is difficult to interpret from the available Southern blot data.

P5AEN.1

The Eco RI digest shows 2 (border?) fragments at ~2.5kb and ~2.7kb with the hygromycin probe. Hind III digests show a smear between 6.5kb and 9.5kb with both probes. The copy number reconstruction suggests 6-10 copies of T-DNA are integrated. This data is consistent with at least 2 unrearranged copies of independently integrated T-DNA (the 2.5kb and 2.7 kb bands in the Eco RV digest probed with hygromycin). Further interpretation would require additional digests.

P5AEN.6

The Eco RI digest shows 3 additional (border?) fragments at ~6kb, ~7kb and ~9.5kb with the hygromycin probe. Hind III digests show 4 fragments between 6.5kb and 23kb with both probes, and an additional band at ~3kb which is much more intense when probed with hygromycin than with AL1. The copy number reconstruction suggests 4-6 copies of T-DNA integrated. This data is consistent with at least 4 unrearranged copies of

independently integrated T-DNA (the 4 Hind III bands above 6.5kb) and the possibility of at least one rearranged insert (the 3kb Hind III band).

Plant line	relevant genotype	Possible DNA organisation
PD	SR1, anti-BL1 upstream of *npt II*	not known
PE	SR1, p*nos-npt II* control	not known
PF	SR1	-
P5.1	SR1, anti-AL1 downstream of hyg^r gene	4-8 unrearranged copies in addition to possible rearranged copies. Possibly identical to P5.2.
P5.2	SR1, anti-AL1 downstream of hyg^r gene	4-8 unrearranged copies in addition to possible rearranged copies. Possibly identical to P5.1.
P6.6	SR1, AL1 downstream of hyg^r gene	1-4 unrearranged copies; rearranged copies not seen
P6.10	SR1, AL1 downstream of hyg^r gene	1-4 copies with possible rearrangement or deletion at non-functional end of the transferred T-DNA
P6.S1	SR1, AL1 downstream of hyg^r gene	4-8 unrearranged copies; rearranged copies not seen
P5AEN.1	Samson, anti-AL1 upstream of hyg^r gene, enhanced promoter	6-10 unrearranged copies in 2 independant loci, no evidence of rearranged copies
P5AEN.6	Samson, anti-AL1 upstream of hyg^r gene, enhanced promoter	4 unrearranged copies in 4 independent loci, possibly 1 rearranged copy
P2AEN.1	Samson, anti-AL1 downstream of hyg^r gene, enhanced promoter	1 unrearranged copy, no evidence of rearranged copies
P2AEN.2	Samson, anti-AL1 downstream of hyg^r gene, enhanced promoter	2-4 unrearranged copies, possible tail-to-tail dimers
P2AEN.3	Samson, anti-AL1 downstream of hyg^r gene, enhanced promoter	6-10 unrearranged copies, possibly in 2 or 3 tail-to-tail and head-to-head dimers, evidence of rearranged copies
P2AEN.4	Samson, anti-AL1 downstream of hyg^r gene, enhanced promoter	1-2 unrearranged copies, 1 deleted or rearranged copy
P2AEN.5	Samson, anti-AL1 downstream of hyg^r gene, enhanced promoter	>1 unrearranged copy, no evidence of rearranged copies
P6AEN.5/6	SR1, AL1 downstream of hyg^r gene, enhanced promoter	1 unrearranged copy, no evidence of rearranged copies
P6AEN.10	SR1, AL1 downstream of hyg^r gene, enhanced promoter	1-2 unrearranged copy, 1 rearranged copy
P6AEN.5/6	SR1, AL1 downstream of hyg^r gene, enhanced promoter	1 unrearranged copy, data difficult to interpret

Table 4.9. Summary of plant lines.

Northern blots were not carried out on RNA from these transformants due to lack of time. This needs to be done. However, all lines have at least one copy of T-DNA which is unrearranged (at the resolution of Southern blotting) and all are resistant to hygromycin. The antisense lines (P5AEN and P2AEN) show additional phenotypes: virus resistance in some cases, and abnormal morphology, which are discussed in more detail in Chapters 5 and 6. These observations are strong circumstantial evidence for synthesis of the expected transcript.

4.5 Conclusion.

Southern blotting of all of the strains used for the *Agrobacterium* transformations indicated no gross DNA rearrangments and the functionality assays described in 3.5 suggest that the coding regions of the drug resistance genes and the regulatory sequences are functional. There were a relatively large number of transformants obtained from AgroE (pGA482), where the drug resistance gene is monocistronic and driven by the relatively weak *nos* promoter, compared with the other lines, where the drug resistance genes are driven by the strong constitutive CaMV 35S promoter, but are bicistronic. It is tempting to speculate that the bicicistronic nature of the transcript decreases expression of the drug resistance gene, either by decreased transcript stability and/or decreased translational efficiency as has been discussed in the introduction.

No transformants were obtained for AgroPlant1A, AgroPlant2A, AgroPlant3A. AgroPlant7A AgroPlant1AEN. AgroPlant5A yielded only 1 or 2 transformants. How might this be explained (other than the trivial explanation of mutation in the construct)? AgroPlant1A and AgroPlant1AEN both have the AL1 coding region upstream of the hygromycin resistance gene. One explanation is that translation of the downstream drug resistance gene is so inefficient that it is never expressed at a high enough level to confer resistance on transformants. This is unlikely as AgroPlant4A (with the anti-BL1 gene upstream of the kanamycin resitance gene) and AgroPlant2AEN (with the anti-AL1 gene upstream of the hygromycin resitance gene) both yielded transformants; it would be expected that translation would be more seriously inhibited by an antisense gene, with

many start and stop codons, than an upstream sense gene (see 1.3). Other possible explanations are a much decreased stability for this transcript, due to its sequence, or that over-expression of AL1 is lethal. It should be noted that suppression of an endogenous plant gene with high homology to AL1 appears to cause altered morphology (see Chapter 6), so the latter explanation has some slight circumstantial support. It is also possible that in the case of AgroPlant6A and AgroPlant6AEN, where the AL1 gene is downstream of the drug resistance gene, inhibition of translation of the second gene mitigates any toxic effect. It is not possible to distinguish easily between the two possibilities using the available data.

As both AgroPlant2A and AgroPlant2AEN should give rise to the same transcript, the most likely explanation for the lack of transformants with AgroPlant2A is translational inefficiency of the drug resistance gene; in AgroPlant2AEN, it is expected that the presumed increase in transcription rate due to the enhancer sequence would ameliorate inefficient translation. Fewer transformants were obtained from AgroPlant2AEN than from AgroPlant5AEN or AgroPlant6AEN which have hygromycin resistance as the first gene in the bifunctional constructs.

AgroPlant3A and AgroPlant7A have the BL1 gene upstream and downstream, respectively, of the kanamycin resistance gene. It is not possible with the present data to distinguish between the possibilities of transcript instability and the BL1 gene product being toxic. However, it should be noted that one of the symptoms of a TGMV infection is cell death ('necrotic lesions') and that, while replication can take place in the absence of BL1 and BR1, both genes are needed for cell to cell transmission and symptom development, it is, therefore, not beyond the realms of possibility that the product of one or both of these genes is toxic.

5: TGMV RESISTANCE.

One of the limiting factors in this work has been the assay for TGMV resistance. *N.tabacum* is a poor host for TGMV and it was sometimes difficult to obtain reproducible symptoms; plants often displayed mild symptoms and often grew out of the symptoms so that only the lower leaves remained symptomatic. In order to overcome this problem dot/slot blots were carried out, where necessary, on total DNA prepared from leaves to determine if the virus was replicating in symptomless plants.

Infection with TGMV was carried out by mechanical inoculation of plants with a viral pellet, by agroinfection, or by a combination of both. For agroinfection, a mixture of four strains were used. These contained cassettes with dimers of TGMV A and TGMV B cloned in both orientations into the polylinker site of pGA482. These plasmids were constructed by E.Bejarano (Imperial College) from precursors supplied by R.Hayes (Imperial College).

They were then transformed into *Agrobacterium* and checked for infectivity in *N.benthamiana* (a more permissive host than *N.tabacum).*

5.1 Statistical analysis.

The statistical technique of 'empirical logistic transforms'[131] was used to analyse the data from the infected plants. This technique was recommended as appropriate for the data by L.White (Mathematics Department, Imperial College). The calculations were carried out using a short program written in MSBASIC on a Macintosh Plus computer.

For n plants, of which R show symptoms, the empirical logistic transform, Z is given by:

$$Z = \mathrm{Ln}\left(\frac{R+\frac{1}{2}}{n-R+\frac{1}{2}}\right) \qquad (5.1)$$

The variance in Z is V, where:

$$V = \frac{(n+1)(n+2)}{n(R+1)(n-R+1)} \qquad (5.2)$$

To compare two lines, the value T, which has a standard normal distribution, is :

$$T = \frac{Z_1 - Z_2}{\sqrt{V_1 + V_2}} \qquad (5.3)$$

The confidence limits for a given value of T may be found from tables of the standard normal distribution[132]. For example if T>1.96 or T<-1.96 the symptom rates for the two lines are different with a confidence limit >95%. If T>2.33 or T<-2.33 the symptom frequencies for the plant lines are different with a confidence limit >99%. If the confidence limit found was less than 95% the lines were considered to have the same symptom frequency (ie. the diference in symptom frequency is statistically insignificant).

Where applicable, the wild type and sense control lines were compared and found to be statistically identical. They were then summed together and treated as one block for comparison with the antisense lines.

In this statistical analysis, plants were considered to be symptom bearing or symptomless; no consideration was taken of the severity of the symptoms.

5.2 Resistance of plant lines PD, PE and PF.

PD were transformed with AgroPlant4A (containing anti-BL1 upstream of the *npt II* gene), PE with AgroE (containing pGA482, encoding *npt II*)) and PF were untransformed but had undergone the rigours of tissue culture.

After transfer to soil, PD1-7, PE1-3 and PF1-10 were hardened off over a two week period in a greenhouse with a 16 hour day length. At ~6 inches high (rather large for infection) the lower leaves were removed and they were inoculated with a TGMV viral pellet. Symptoms appeared in some plants after 13 days, and no more symptoms developed after a further 14 days. Symptom severity was scored as nil (-), medium (*), or strong(**). DNA was prepared from the leaves of all plants (leaf material was taken from a number of leaves from each plant). DNA was also prepared, in the same way, from an uninoculated plant as a negative control; this DNA was also spiked with 10ng of pBH604

(containing TGMV B) as a positive control. The DNA was quantitated by visual comparison of ethidium bromide stained gels and 2.5µg of each sample was dot blotted and probed with labelled pBH604 (data not shown). The results are summarized in Table 5.1.

Where the symptoms are defined as 'medium', it is possible that inoculation damage was the cause of the apparent symptoms. In these cases plants were only considered to be infected if infection was confirmed by dot blot. The results are: PD 5/7 (71%) infected; PE 2/3 (67%) infected; and PF 6/10 (60%) infected (statistical analysis not carried out).

This experiment was intended as a preliminary experiment. Only one plant from each line was tested; in addition, PD and PE were grown in the presence of antibiotics and PF was not. However, it could still be ascertained that at least 5 of the 7 antisense lines developed TGMV symptoms; not all of the negative control plants developed symptoms. If the transformants are considered as a block there is little difference between the control plants and the PD plants.

Plant	Symptom severity	Dot blot intensity	Plant	Symptom severity	Dot blot intensity
D1	**	**	F1	*	?
D2	-	-	F2	*	*
D3	**	**	F3	**	**
D4	**	**	F4	*	-
D5	**	**	F5	*	*
D6	*	-	F6	**	*
D7	-	*	F7	-	*
E1	**	**	F8	-	-
E2	**	*	F9	-	-
E3	-	-	F10	-	-

Table 5.1. Infectivity assay for PD, PE and PF.

As only one plant from each line was examined, this data does not preclude the possibility that the two uninfected PD lines are resistant, or that the other PD lines are

partially resistant, which would not be seen unless more plants from each line were assayed. Neither can we be sure that any of these lines contain unrearranged T-DNA inserts nor, if they do, that they are synthesizing the predicted antisense transcript. However, the results of the above assay, together with other data that became available at this time (see 7.1) persuaded me to give up targeting BL1 as an antisense target and concentrate on AL1. No further work was carried out on BL1.

5.3 Resistance of plant lines P5 and P6.

A preliminary experiment was carried out as follows. F1 seeds from P6.6, P6.10, P6.S1 (from AgroPlant6A, containing AL1 downstream of the hygromycin resistance gene), P5.1, P5.2 (from AgroPlant5A, containing anti-AL1 downstream of the hygromycin resistance gene) and F10 (SR1) were germinated in MSO medium (supplemented with hygromycin at 35μg/ml where appropriate). One week after germination, it was possible to determine which seedlings were resistant to hygromycin and a number of plants were transferred to soil in seed trays to harden off in a greenhouse with a 16 hour day length over a four day period. Following this (at ~2.5 inches) the plants were transferred to pots (13 each of P6.6, P6.10 and P6.S1; 7 of P5.1; 11 of P5.2; 10 of F10), the lower leaves were removed and the plants were inoculated with a TGMV viral pellet. One plant from each line was inoculated with water instead of TGMV as a negative control. After 11 days there were no symptoms and, after removal of lower leaves, the plants were re-inoculated. Ten days later a few of the plants had developed symptoms. However, these symptoms were mild, and in most cases were difficult to distinguish from inoculation damage. In order to determine whether or not the plants had been infected, DNA was prepared from leaves from each plant (leaf material was taken from a number of leaves from each plant). DNA was also prepared, in the same way, from an uninoculated plant, as a negative control; this DNA was spiked with 1ng and 10ng of pBH604 (containing TGMV B) as a positive control. The DNA was quantitated by visual comparison of ethidium bromide stained gels and 2.5μg of each sample was slot blotted and probed with labelled pBH604 (Figure 5.1, Table 5.2).

Line	Number infected (by slot blot)	Percentage
P5.1	3/6	50%
P5.2	0-1/10	0-10%
P6.6	5-6/12	42-50%
P6.10	5/12	42%
P6.S1	5/12	42%
PF10 (SR1)	1-5/9	11-55%

Table 5.2. Infectivity assay for P5, P6 and PF.

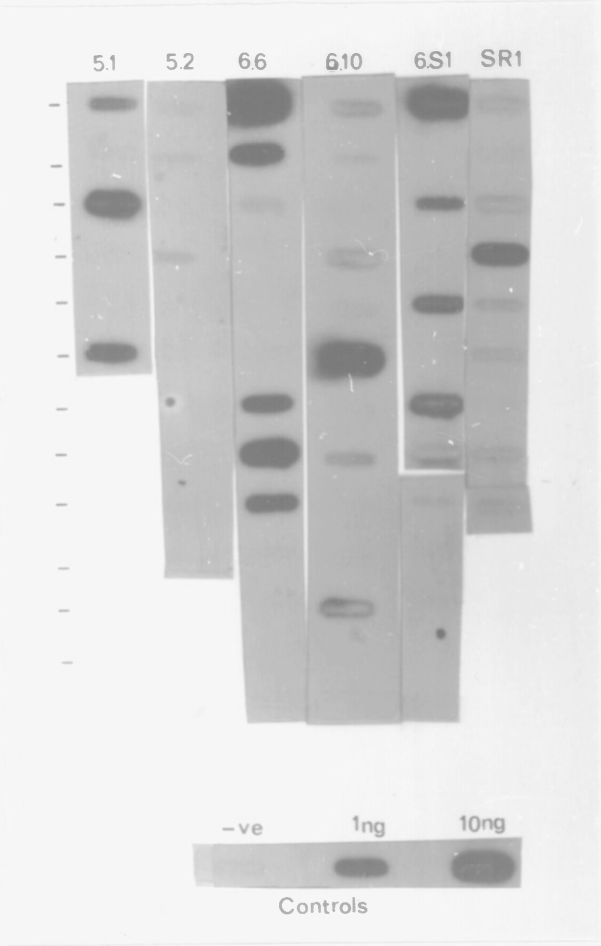


Figure 5.1. Slot blots of DNA from TGMV infected plants. Four hour exposure at -70°C with intensifying screens. Probed with pBH604 (containing TGMV B).

In the cases of P5.2 and PF.10, the signals obtained were generally only slightly more intense than the negative control (see Figure 5.1) and, for this reason, a range of percentages has been given for TGMV infection (statistical analyses not carried out). The

control line, PF10, had fewer infected plants than the other control lines; perhaps because they were more healthy; they were germinated and grown in the absence of selective antibiotics. P5.2 had a low level of infection, and whether or not P5.2 is genetically different from P5.1 or the two have to be considered one line, they still appear (intuitively) to be partially resistant to TGMV compared with the control lines.

Another experiment was carried out with more plants. F1 seeds from P6.6, P5.2 and F10 were germinated in MSO medium (supplemented with hygromycin at 35µg/ml where appropriate). As soon as resistant seedlings could be distinguished, they were transferred to fresh media which contained no antibiotics. After a further 2 weeks they were transferred to soil and hardened off in the containment growth room of Advanced Technologies Cambridge (ATC) at 24°C with a 16 hour day length and illuminated at ~10,000 lux with high pressure Sodium lamps. Eighteen days after transfer to soil, the plants were kept in the dark for 24 hours prior to removal of the lower leaves and inoculation with TGMV viral pellet; after inoculation the plants were kept in the dark for a further 24 hours. Because no symptoms were seen two weeks after inoculation, the process was repeated. Three weeks after the second inoculation, clear symptoms had developed in a number of the plants (see Figure 5.2 for an example of TGMV infected tobacco plants). These results are summarized in Table 5.3.

Line	Severe symptoms	Medium symptoms	No symptoms	Percentage with symptoms	Resistance and confidence limit
PF10	25	3	12	70%	-
P6.6	20	5	15	63%	-
controls	45	8	27	66%	-
P5.2	13	6	20	49%	resistant, >95%

Table 5.3. Symptom development of P5.2, P6.6 and PF10.

Slot blots were not carried out in this case. With symptom development as the assay, P5.2 appears to be resistant to TGMV relative to the control lines with a confidence limit >95%. Repeating this experiment with a larger number of plants would increase the confidence limits of the result obtained.

5.4 Resistance of enhanced plant lines P2AEN, P5AEN and P6AEN.

Seedlings from P5AEN.1, P5AEN.6 (from AgroPlant2AEN, containing anti-AL1 upstream of the hygromycin resistance gene), P2AEN.1-P2AEN.5 (from AgroPlant5AEN, containing anti-AL1 downstream of the hygromycin resistance gene), P6AEN.5, P6AEN.6 (clonally identical; from AgroPlant6AEN, containing AL1 downstream of the hygromycin resistance gene), and wild type, SR1 and Samson N.N., were micropropagated in tissue culture through two generations without antibiotic selection . *N.tabacum* cultivars Petit Havana SR1 and Samson N.N. were used as controls because the antisense lines are in Samson N.N. and the sense lines are in Petit Havana SR1. The seedlings were transferred to soil and hardened off in the containment growth room of ATC at 24°C with a sixteen hour day length and illuminated at ~10,000 lux with high pressure Sodium lamps. One week after transfer to soil, the plants were kept in the dark for 24 hours prior to removal of the lower leaves and mechanical inoculation with a TGMV viral pellet; following inoculation the plants were kept in the dark for a further 24 hours (2 plants from each line were 'mock' inoculated with water). Seventeen days after inoculation no symptoms were seen and the process was repeated. Thirteen days following the second infection there were still no symptoms and the plants were kept in the dark for 24 hours prior to removal of the lower leaves and infection by agroinoculation (the mock infected plants were injected with water); following agroinoculation the plants were kept in the dark for a further 24 hours. Ten days later clear symptoms had developed in a number of plants (Table 5.4, Figure 5.2). In some plants the symptoms were ambiguous; in these cases DNA was prepared and slot blots used for clarification (data not shown). The results of this experiment are summarized in Table 5.4.

Lines P2.1AEN and P2.2AEN appear to have a degree of resistance to TGMV (confidence limit >95%). The level of symptom development in the P2.3AEN line is less than the control plants; this difference is statistically insignificant. However, there is not a large difference in the percentage of symptom bearing plants in the latter three lines and the experiment should be repeated to confirm these results. Line P2.5AEN appears to be more resistant than the P2.1AEN-P2.3AEN lines. P2.4AEN has no symptom bearing plants at

all; the odds against this occurring by chance in an unresistant line are less than 1 in 10,000. P5.6AEN is also resistant (confidence limit >99%), while P5.1AEN appears unresistant.

A statistical comparison of the two cultivars, Petit Havana SR1 and Samson NN, and the sense control plants suggests that the differences in the percentage of symptom bearing plants in these lines were statistically insignificant.

To further confirm the results given by the statistical analysis above, the assays should be repeated with controls which are in the same cultivar as the antisense lines.

In order to see how symptom development correlated with TGMV replication, slot blots were carried out on 2-4 DNA samples isolated from symptom bearing and symptomless plants of each line, and from 18 plants of the symptomless line P2.4AEN. DNA was quantitified using the Hoechst fluorimeter as described (2.2.3B(iv)) and the quanti fication was checked for 14 samples by visual comparison of ethidium bromide stained gels. The results are shown in Figure 5.3. All of the plants which had symptoms had intense signals in the slot blot. Slightly surprising was the observation that between one-third and two-thirds of the inoculated plants which did not have symptoms had intense signals in the slot blots. A comparison of the number of signals obtained in the slot blots of DNA from all symptomless plants except P2.4AEN (counted as one block), with the number of signals obtained in the slot blots of DNA from eighteen P2.4AEN plants suggest that there is no significant difference.

It was noticed that tobacco plants often only show symptoms on one or two leaves, and appear to grow out of the infection. In order to see if there was a correlation between symptom severity and viral titre in individual leaves on a single plant, slot blots were carried out on DNA prepared from individual leaves (Figure 5.4 shows a typical series of leaves). This experiment was carried out on four plants and, in all cases, DNA from all leaves had an intense signal in the slot blots (data not shown). There is no correlation between symptom severity and viral titre within individual leaves of an infected plant.

The observation of resistance in some lines is most encouraging, although it will be neccessary to repeat some infectivity assays and plant transformations to confirm these results.

Figure 5.2. Upper panel: TGMV infected tobacco plants, showing that symptoms are similar in all lines. lower panel: Inoculated and uninoculated P2.4AEN, showing that no symptoms are discernible in the inoculated plant.

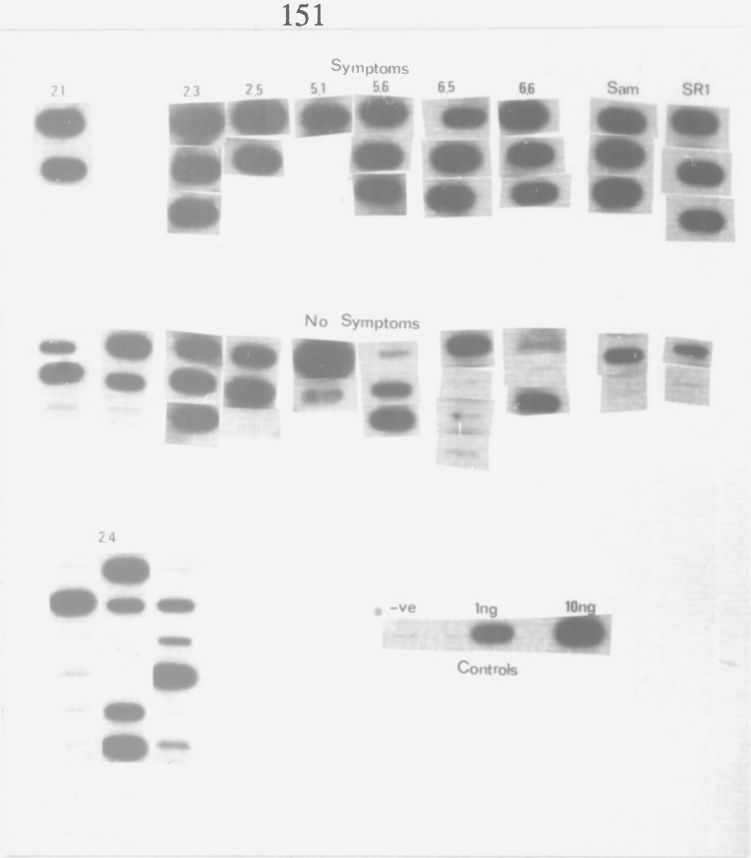


Figure 5.3. Slot blots of DNA from TGMV infected plants symptom-showing and symptomless plants. Overnight exposure at -70°C with intensifying screens. Probed with pBH604 (containing TGMV B).

Line	Severe symptoms	Medium symptoms	No symptoms	Percentage with symptoms	Resistance and confidence limit
SR1	7	12	8	70%	-
Sam.	4	3	4	64%	-
P6.5AEN	6	10	12	57%	-
P6.6AEN	7	9	9	64%	-
controls	24	34	33	64%	-
P2.1AEN	5	8	16	45%	resistant, >95%
P2.2AEN	4	4	11	42%	resistant, >95%
P2.3AEN	5	6	12	48%	not resistant
P2.4AEN	0	0	21	0%	resistant, >99.99%
P2.5AEN	0	3	15	17%	resistant, >99.95%
P5.1AEN	6	5	6	65%	not resistant
P5.6AEN	0	7	14	33%	resistant, >99%

Table 5.4. Symptom development of P2AEN, P5AEN, P6AEN and wild type tobacco.

Figure 5.4. Variation in TGMV symptom severity on leaves within a single plant.

6: SUPPRESSION OF AN ENDOGENOUS DEVELOPMENTAL GENE?

When the P2AEN and P5AEN plant lines were transferred to soil, several morphological abnormalities were noticed *viz.*

i) Shortening of the internode distance and reduced apical dominance (increased 'bushiness', compare Figure 6.1a with Figure 6.1b).

ii) All leaves have a cleft at the tip (Figure 6.1c).

iii) A pronounced 'frill' continuing down the stem from the base of the leaves (compare Figure 6.1d with Figure 6.1e).

iv) Mottling and browning of lower leaves when plant is starved of nutrients (Figure 6.1f). The plant seems to actively remove nutrients from the lower leaves, which dry and change colour in a manner similar to deciduous trees in Autumn. The upper leaves on these plants continue to grow and appear healthy. In morphologically normal tobacco plants the leaves of plants which are starved of nutrients just go yellow and rot. This observation may or may not be directly related to the transgenic nature of the plants; it may not have been observed before because most tobacco plants (grown in our greenhouse conditions) flower before reaching this stage.

v) The P2AEN lines are all vegetative. They do not flower under the conditions prevailing in the Biotechnology or Pure and Applied Biology greenhouses, and have not yet been shown to be capable of flowering under any conditions. This failure to produce flowers has been observed in at least three separate transfers of micropropagated lines to soil in greenhouses. The oldest plants have been in soil for 11 months and have reached a size unprecedented for the conditions in our greenhouse (Figure 6.1g); normal tobacco plants under similar conditions flower after between two and three months. The two P5AEN lines both flower, however, while P5.1AEN plants produce viable seeds, P5.6AEN plants produce empty seed pods. This observation was made initially after transfer of one P5.6AEN plant to

soil (all flowers were covered with one bag to ensure self pollination), and subsequently after transfer of three more P5.6AEN plants to soil (each flower was covered with a separate bag to ensure self pollination).

There are three obvious explanations for the presence of abnormal morphologies in these transgenic plants. i) Somaclonal variation; the rigours of tissue culture give rise to altered, heritable genetic changes by a poorly understood mechanism (see[133]). This is a rare event and is unlikely to have occurred in seven independent transformants. ii) T-DNA insertion into a developmental (but non-essential) gene causing gene disruption; this is also unlikely to have occurred in seven independent transformants. iii) Antisense RNA being expressed by the transgenic plants is suppressing expression of an endogenous developmental gene; this is also an unlikely event. However, it only has to be true once in order to explain the abnormal morphology observed in all seven transformants. This is the explanation that I favour.

Homology has been observed between the AL1 probe and wild type tobacco DNA (see 4.4). A Southern blot was carried out on genomic DNA prepared from *N.tabacum* cultivars Samson N.N. and Petit Havana SR1, cut with Eco R1, Hind III and Eco RI + Hind III. The filter was probed with the AL1 fragment and washed at high stringency; 0.2x SSC, 0.2%SDS (Figure 6.2).

Both cultivars gave identical patterns. The Eco RI digest yielded a strong band at high molecular weight, and some weaker bands at lower molecular weights. The Hind III digest yielded a strong band at ~1kb in addition to fainter bands at higher molecular weights. The double digest was almost identical to the Hind III digest suggesting that the Hind III sites in the area of homology are bounded by the Eco RI sites. A faint band did appear at ~0.6kb in the double digest which was not present in the single digest. Copy number reconstructions using the AL1 fragment (data not shown) suggest a single copy gene by comparison with the strongest band in each lane. This does not rule out the possibility of more copies if the signal observed is reduced in intensity due to incomplete homology between the probe and the plant sequences.

No bands were visible in the northern blot carried out on RNA from wild type tobacco seedlings (Figure 4.3). This is not surprising; any transcript produced by the putative endogenous gene may be present at a much lower level than the transcript produced by the chimaeric gene in the transgenic plants and would therefore not be seen without a longer exposure. Furthermore, the gene may be expressed in a tissue specific or temporally regulated manner (not unlikely if it is a developmental gene), and the transcript may not be present in RNA prepared from seedlings.

The Southern blot has been repeated with different DNAs and different enzymes, in addition to DNA from *N.benthamiana*[134]. Homology was observed in all cases and appears to be real and not an artifact arising from contamination. In the case of *N.benthamiana*, the signals were weaker than in *N.tabacum*. The AL1 probe was cut into three parts and these were used to probe the filter separately[134]. There is homology in *N.tabacum* to both ends of the AL1 probe but not the centre region.

It is interesting to note that homology has been observed at the amino acid level between the predicted AL1 gene product and a number of mammalian proto-oncogenes[135].

Figure 6.1. a: (upper) Wild type tobacco plant, cultivar Samson N.N.; b: (lower) P2.3AEN tobacco plant, showing 'bushiness'.

Figure 6.1. c: (upper) Comparison between wild type (right) and abnormal (left) 'cleft' leaf morphology; d: (lower) close up of wild type tobacco plant, cultivar Samson N.N.

Figure 6.1. e: (upper) P2AEN tobacco plant, showing 'frilled leaf' morphology; f: (lower) Abnormal plants showing mottling and withering of lower leaves.

Figure 6.1. g: 11 month old vegetative P2.1AEN plant (left) with the author (right).

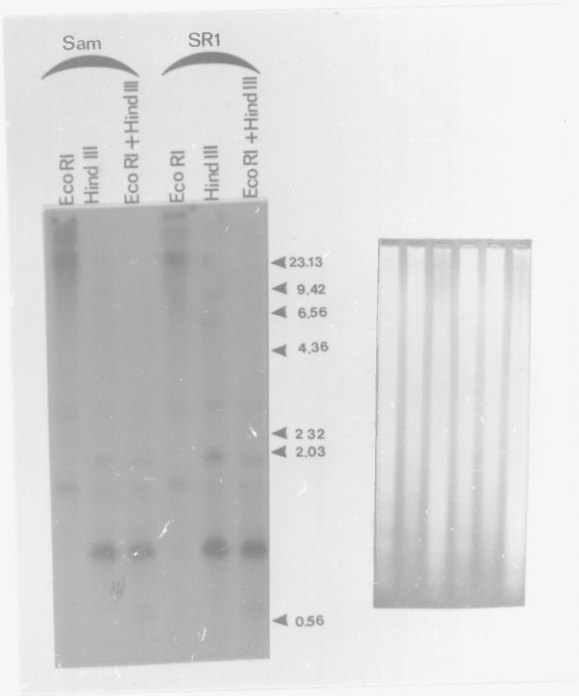


Figure 6.2. Southern blot of DNA from wild type tobacco probed with the AL1 fragment. 10µg DNA/lane, 4 days exposure at -70°C with intensifying screens.

7: DISCUSSION.

7.1 Experimental design.

The technique of antisense RNA to suppress gene expression is, in some respects, one of brute force in that the higher the level of antisense RNA, the greater the effective suppression one gets[eg.39, 59]. Our approach, therefore, has been to attempt to maximize the expression of the antisense transcript. As has already been discussed (1.4), we used bifunctional constructs, in which the antisense region is encoded on the same transcript as the antibiotic resistance gene. In this way expression of the antibiotic resistance gene ensures concomitant expression of the non-selected antisense gene.

The use of a bifunctional construct in one mammalian system studied enabled an antisense effect where no effect was observed with monofunctional constructs[50]; selection on high levels of antibiotic led to an increase in the level of antisense RNA by virtue of gene duplication. This observation was, in part, our inspiration for the use of bifunctional antisense constructs in plants (although we did not select for gene duplication).

The use of a bifunctional construct in plants has subsequently enabled an antisense effect where none had been observed with monofunctional constructs[59]; in this case gene duplication was not explicitly observed; selection for high rates of transcription of the bifunctional constructs was at the level of primary transformation.

We decided to use bifunctional constructs exclusively; it is well known that position effects can cause non-selected genes to be under-expressed in transgenic plants (see Appendix I) and in this research group we had already experienced these problems[90]. If the antisense effect were dependent on steady state RNA levels as opposed to transcription rate, the advantage of a bifunctional construct may be outweighed by the possibility of a decrease in stability of the bifunctional transcript relative to the monofunctional transcript. However, there is no reason, *a priori*, to expect that a bifunctional construct will be less stable and an any case the evidence suggests (see 1.2.3) that transcription rate plays a greater role than steady state asRNA level in the antisense effect. We felt that these factors, coupled with the consideration that preparation and testing of monofunctional constructs in

transgenic plants would have added considerable time to the project, justified our decision to use bifunctional constructs alone.

We attempted to increase the selection for high levels of transcription by putting the antibiotic resistance gene downstream of the antisense gene in order to decrease translational efficiency and thus to increase the transcript level required for an equivalent degree of antibiotic resistance. This approach was not completely successful. It is not possible to determine from the present data whether the P2AEN (AL1 downstream) or P5AEN (AL1 upstream) transgenic lines are more resistant to the virus. However, P5.6AEN plants are resistant to TGMV and produce empty seed pods, while P5.1AEN plants are not resistant to TGMV and produce viable seeds. In this case it appears that the degree of abnormal morphology is correlated with the degree of TGMV resistance, and presumably with gene suppression by asRNA. If this argument is valid it may be extended. The P2AEN lines are more morphologically abnormal than the P5AEN lines, as the former produce no flowers at all. If this is related to a stronger asRNA effect, it follows that the transcript in which AL1 is upstream of the drug resistance gene is less efficient. It is possible that the transcript in which the anti-AL1 gene is upstream is less stable than the transcript in which it is downstream, or that the conformation of this transcript is such that the anti-AL1 region is sterically hindered from approaching its target.

We could also have attempted to select for the most transcriptionally active transgenic plants by increasing the concentration of selective antibiotic in the media during transformation. However, the transformation efficiency obtained with the bifunctional antisense constructs was relatively low (see 4) and it was felt that, if we increased the concentration of antibiotic, we might reduce the number of transformants to zero.

Other factors affecting transcript levels are the regulatory sequences, the promoter and polyadenylation signal. Little is known of the effect of polyadenylation signalson plant transcript levels and, therefore, the choice was decided by availability. When this project was started very little was known about the biology of TGMV and, in particular, in which cell types it replicates; to be sure of suppressing the virus, the promoter had to be active in all cell types at all times. This dictated the use of a constitutive promoter. The strongest

constitutive promoter available at the time was the CaMV 35S promoter. Dissection of this promoter subsequently revealed an upstream enhancer-like sequence, the duplication of which can lead to a four to ten fold increase in transcript level[126,127,128]. This 'enhanced' promoter was used in the constructs that gave rise to the trangenic plants which showed resistance to TGMV.

The choice of target genes for the asRNA is also an important consideration. When the project was started, little was known about the function of the TGMV genes other than that all were essential, with the exception of the coat protein gene, AR1. The choice of AL1 was based on its size; it is the only ORF big enough to encode a polymerase. BL1 was chosen because it was felt to be desirable to target genes on both viral components. After the project had been underway for some time, evidence became available that AL1 is transcribed at low levels and is the only gene absolutely required for replication; BL1 is transcribed at high levels (comparable to the coat protein gene, AR1) and is involved in cell-to-cell spread and/or symptom development (see 1.1.2). This makes it a poor target for two reasons. i) If the transcript level of BL1 is high, the level of asRNA also has to be high to have an effect; thus a smaller percentage of transformants might be expected to express asRNA at a level capable of suppressing BL1 (compared with the percentage expected to express asRNA at the level required to suppress AL1 or any other gene with a low transcript level). ii) If the BL1 gene product is not required in the TGMV life cycle until it is ready to spread to another cell, the virus can be expected to have replicated to an average copy number (over all cells) of >1000 copies per cell[22]; if, at this point, BL1 was being transcribed from 1000 copies per genome, it would almost certainly swamp out any asRNA being transcribed from only a few copies integrated in the chromosome unless there were a very large favourable disparity in promoter strengths. This argument applies whether or not the expression of the TGMV genome is temporally regulated, since suppression of BL1 at an early stage would have no effect on viral replication. AL1, on the other hand, is needed at the start of the life cycle (mutagenesis of AL1 abolishes the presence of detectable levels of virus) and suppression of AL1 at an early stage should stop high levels of virus accumulating in the cell and, thus, suppress infection.

When this information became available, BL1 was abandoned as a target gene for asRNA suppression; it was thought that the time could be better spent on AL1. The BL1 transgenic plants were not checked by Southern or northern blotting for integrity of T-DNA inserts or transcription of the asRNA, nor were infectivity assays carried out on large numbers of each transgenic line. Clearly however, it could be seen from the preliminary infectivity assay that 4 of 7 BL1 antisense transgenic lines were less than completely resistant to TGMV (Table 5.1), and, by comparison with the untransformed lines, it is likely that the other 3 were not resistant either. In the absence of nucleic acid data, this can not be conclusively correlated with a BL1 being a poor target for suppression as there is no evidence that the transformants were transcribing anti-BL1 transcript. I hoped that there would be time for the nucleic acid analyses to be carried out towards the end of the project; there was not.

7.2 TGMV resistance.

Transgenic plants containing four different antisense constructs were assayed for susceptibility to infection by TGMV.

The first of these (PD) contains anti-BL1 downstream to a kanamycin resistance gene; the construct is driven by a CAMV 35S promoter. In preliminary experiments (Table 5.1) these lines did not appear to be resistant and, as discussed above (7.1), were not pursued.

The next antisense lines to be analysed were P5 which have anti-AL1 downstream to a hygromycin resistance gene, the construct is driven by a CaMV 35S promoter. The initial results were promising(Table 5.2) and a more careful experiment (Table 5.3) confirmed that there was some degree of resistance in one of the lines (with >95% confidence). Statistically there is ~1 in 20 chance that this apparent resistance has arisen by accident: the infectivity assay would have to be repeated with a larger number of plants to be more certain that the observed resistance is genuine.

Following these were the P2AEN lines, which should produce the same transcript as the P5 lines but are driven by the stronger, enhanced, CaMV 35S promoter. The results

here are very promising (Table 5.4); 4 of 5 lines appear to have reduced symptom rates compared to the control plants at a statistically significant level. In one case, the odds against this occurring by chance are less than 1 in 10,000. The increased level of resistance seen with these 'enhanced' lines is presumably due to the stronger promoter used. It is not unlikely that the predicted four to ten fold increase in transcriptional efficiency could make the difference between resistant and non-resistant plants. However there are some *caveats* to this result.

i) Only one line was examined in the P5 series, five lines were examined in the P2AEN series.

ii) P5 were transformed into cultivar SR1, P2AEN were transformed into cultivar Samson N.N.

iii) P5 was inoculated with a viral pellet, P2AEN was inoculated by agroinfection.

Taking these points in turn:

i) It might be argued that because only one line was examined for P5, the limited degree of resistance observed was atypical for lines transformed with this construct. In fact, as only two transformants were obtained from hundreds of leaf discs (and these may be clonally identical) it is more likely that this transformant is particularly transcriptionally active for the construct.

ii) Similarly it might be said that one cultivar of tobacco is more susceptible to TGMV than the other; examination of the data would suggest that the two lines are both equally susceptible.

iii) The question of different methods of viral inoculation is more difficult to address. It is not clear what the exact mechanism of infection is in agroinfection. It is conceivable that the asRNA blocks infection in agroinfected plants at a stage of infection which does not occur if a viral pellet is used. However, the existence of varying levels of virus in the symptomless line, P2.4AEN would suggest that symptom development was being inhibited during the later stages of infection, which are presumably identical whichever method of inoculation is used.

I would expect the P2AEN transformants to be more resistant than the P5 transformants, for not only are they driven by a stronger promoter, but they also have an abnormal morphology which may be related to high levels of the anti-AL1 transcript (see 6 and 7.3).

The final set of transformants is P5AEN, which carries the anti-AL1 gene upstream of the hygromycin resistance gene. The two members of this set were analysed in tandem with P2AEN and are in the same genetic background (cultivar Samson N.N.). We can see (Table 5.4) that one line, P5.1AEN, is not resistant, while the other, P5.6AEN, is resistant at an intermediate level, between the best and worst of the P2AEN lines. It is not possible to say conclusively that the P5AEN lines are more or less resistant as a group than the P2AEN lines.

It might also be suggested that the absence of symptoms in some lines is correlated biochemically with the abnormal morphology and not directly correlated with the presence of asRNA. This is unlikely, the lines having abnormal morphology show between 0% and 65% (wild type) symptom development; if abnormal morphology gave rise to the resistance, all lines with abnormal morphology might be expected to be resistant.

Slot blots were carried out on DNA from 2-4 of the inoculated symptom-bearing and symptomless plants from each enhanced line and each control line, and on DNA from 18 of the symptomless P2.4AEN plants (Figure 5.3). All of the symptom bearing plants had a strong signal and ~50% of the symptomless plants had a strong signal.

Before we can address the question of the mechanism by which asRNA effects TGMV resistance, we must address the question of the mechanism of viral infectivity. In the case of inoculation with a viral pellet, the virus must enter the wounded cell and become (passively or actively) uncoated. The ssDNA uncovered is the minus-strand and so second strand synthesis must be carried out by host encoded proteins as the AL genes are encoded on the plus-strand. It may be assumed that at this stage AL1, 2 and 3 are expressed and viral replication begins in earnest.

In this experiment, agroinfection was used. It is thought that a functional virus is

produced either by homologous recombination between the duplicate genomes, or by replication from the first to the second common region (which may be indistinguishable from moving around a circular molecule back to the same common region for the putative replicase). There is no evidence to distinguish between these possibilities or to tell whether they occur as the (single stranded) T-strand is transferred from the *Agrobacterium* to the plant cell, or after integration has taken place. After this stage there should be nothing to distinguish between an infection arising from either type of inoculation. If AL1 is suppressed at this stage, infection should be prevented in either case. If enough AL1 is expressed to allow the virus to replicate, I would suppose that the asRNA would soon be swamped as transcription of the viral gene takes place from thousands of viral genomes. The fact that we see replication in a line which shows no symptoms (P2.4AEN) implies that the 'decision' of whether viral replicatation is suppressed or the asRNA is swamped is taken each time the virus spreads to a new cell; thus the virus may replicate to some extent, but be contained by the asRNA below some threshold level at which macroscopic symptoms become visible.

I should reiterate at this point that anti-AL1 includes the 5' region of AL2 and AL3, and it is possible that these regions contribute to, or even are alone responsible for, the observed resistance. My bias is that the AL1 gene is the most important, as it is the only one essential for viral replication (see 1.1.2).

7.3 Abnormal morphology.

The abnormal morphology observed in the 'enhanced' antisense lines P2AEN and P5AEN has already been discussed in some detail in Chapter 6. Homology was observed between both ends of the AL1 probe and the *N.tabacum* genome. It is not unreasonable to assume that this homology is related to the abnormal morphology, and that the 3' homology is due to AL1 sequences and not the AL2 or AL3 sequences in this probe (as this would require fortuitous homology of plant DNA sequences to two viral genes!). If this is so, it suggests a common or related function for the gene products of AL1 and the putative endogenous plant gene as homology between the opposite ends of two functionally

unrelated genes is unlikely to have arisen purely by chance. It is possible that the central region of the gene is not functionally important, or that homology at the nucleic acid level is not found because of a combination of limited homology at the amino acid level and third base degeneracy. It is not immediately obvious what common functions there might be between a plant viral gene essential for viral replication, and a gene involved in flowering and leaf and stem development. If the relationship between the anti-AL1 gene and the abnormal morphology is real, then there is a large amount of work to be carried out in the future.

It should be noted that there are a variety of potential commercial applications for a gene which can suppress flowering in crop plants, particularly if this gene can be induced. For example: Tobacco plants will grow to a much larger size if they are vegetative, so increasing yield. Plant varieties with commercially desirable characteristics may be rendered sterile; seed would then have to be purchased from the owner of the variety, thus protecting her/his investment in the development of that variety.

7.4 Future work related to TGMV resistance.

There are a number of experiments that need to be carried out in order to confirm that the resistance to TGMV observed in some lines is both genuine and also related to the presence of asRNA. Northern blots should be carried out on the enhanced lines and P5.1 with strand specific probes in addition to double stranded hygromycin and AL1 probes. This will show whether the correct sequence and asRNA strand is being transcribed, and if, as appears to be the case in the P5 and P6 lines, this transcript is processed or truncated in any way. It will also be interesting to see if there is any correlation between the amount of asRNA each line is producing and the degree of resistance seen. The steady state asRNA levels should be directly proportional to the transcriptional rate as both constructs should give rise to the same transcript. However, it may be necessary to carry out nuclear run-on experiments in order to determine if there is a correlation at the level of transcription rate since Cornellisen[61] has observed an increased antisense gene dosage giving rise to increased gene suppression without a corresponding increase in the steady state asRNA

level. One interpretation is that, if the level of asRNA increases beyond a certain level, it is degraded much more rapidly. If the asRNA were at this (hypothetical) level in all lines, no correlation would be seen between steady state asRNA level and degree of TGMV resistance.

The assays for infectivity should be repeated with more plants so as to obtain a result with a higher confidence limit. The assay should also be repeated for inoculation with viral pellet and agroinoculation to see if there is any difference in the degree of resistance obtained with these different methods of infection. Slot blots should be carried out on DNA prepared from all inoculated, symptomless plants in order to determine whether the resistance observed at the level of symptoms is paralleled at the molecular level. This might be a more precise way of scoring TGMV resistance as it is difficult and somewhat subjective attempting to take symptom severity into account (symptom severity was lower in symptom bearing plants from resistant lines than in symptom bearing plants from control lines).

Assays of viral replication could also be carried out by agroinoculation of leaf discs[21]. This is a direct assay of viral replication in a process which only requires the viral gene product from AL1[21]. If this technique showed promise in lines known to be resistant at the symptom level, it would be an easier way of assaying for resistance in large numbers of independent transformants.

More plant transformations should be carried out with the enhanced lines in order to have the antisense lines and the sense control lines in the same genetic background. In addition, this would enable a comparison to be made to determine whether transformation with AgroPlant5AEN or AgroPlant2AEN, yield more resistant plant lines. *N.benthamiana*, which is a more permissive host, should also be transformed to see if symptoms (which are much more severe in this host) could be suppressed in this species.

It might also be possible to increase the antisense effect by varying parameters such as duplication of the antisense region and flanking of it by secondary structure. I suspect that this sort of optimisation would have to be carried out empirically for each sequence.

There is a significant homology between AL1 in different gemini viruses; 70%-80%

homology at the nucleic acid level with CLV and BGMV[24]. In view of this, lines resistant to TGMV should be assayed for resistance to other gemini viruses.

Now that the inhibition of geminivirus in the model system of TGMV and *N.tabacum* has been demonstrated, it is possible that this work may be extended to commercially more important geminivirus pathogens of crop plants. The *caveats* on such an extension are as follows: i) Many important crop plants are refractory to transformation, particularly monocots (see Appendix I, Section 7.2.6). ii) It remains to be seen if expression of the asRNA would continue in the field through many generations; the utility of this technique would be substantially decreased if it was necessary to maintain antibiotic selection to ensure viral resistance.

7.5 Future work related to the abnormal morphology.

It is most important to repeat the plant transformations with the *Agrobacterium* strains which gave rise to the abnormal transgenic plants in order to be sure that the observed morphology is really due to transformation with those lines, and not to some unusual conditions prevailing at the time of the first transformation.

Southern blots of DNA from wild type tobacco should be probed with oligonucleotides homologous to different regions of the AL1 probe in order to define the regions of homology between AL1 and the endogenous plant gene more accurately.

A genomic library should be constructed and the endogenous gene cloned using the AL1 region as a probe. Once this clone has been obtained, it can be sequenced and also used to probe northern blots of RNA isolated from different tissues at different times; in this way, the tissue specificity, the temporal regulation and the level of expression of the transcript can be determined. The gene can be put under the control of a strong constitutive promoter in the sense and antisense orientations and transformed back into tobacco plants to see the effects of over expression and of more efficient inhibition of this gene.

Probes based on the sequence can be made and used to look for an equivalent gene in other plant species. The gene could be over expressed in a heterologous system and a biochemical functional analysis could be carried out on the gene product.

The P2AEN lines, which do not flower, can be exposed to a variety of enviromental conditions (day length, temperature, nutrients, etc) in order to determine if, and under what conditions, flowering can be induced; exogenous application of plant hormones may also be tried.

7.6 Conclusion.

When this project was started in December, 1985 there was little known about TGMV, other than its sequence, and there were no reports of asRNA applied to plants. It was with trepidation and excitement that I embarked on this project. As time progressed, the trepidation increased. It was nearly three years later that the abnormal morphology was first observed and the TGMV resistance was not observed until after I had started to write this thesis. The project has been (to at least some degree) a success. At this stage the project is blossoming and could continue in a number of exciting directions. I am looking forward to following the progress of this work in future years.

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APPENDIX I

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It is presented here as it was submitted, only the formatting has been changed. Figures and Figure legends are at the end.

(Chapter 7)

Plant Genetic Transformation

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Chapter 7
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7.1 INTRODUCTION

Plant genetic engineering is a very young science. It was only in 1983 that chimaeric genes were first expressed in plant tissue (Herrera–Estrella *et al.*, 1983; Bevan *et al.*, 1983). Only after this development and the concimitant availability of selectable, and non–selectable marker genes which express in plant tissue, did work on plant genetic transformation systems begin in earnest. Since that time there has been an explosion in the literature of plant genetic transformation. We propose to review some of that literature and, hopefully give a feeling for the areas where most of the work and the most promising work is going on.

The *Agrobacterium* system was the first used to transform plant tissue; there have been many developments since then and we will try to cover them. We will also discuss direct gene transfer (DGT) of naked DNA to plant cells; a technique that has also been developed to a high degree of sophistication. Finally, we will review some of the techniques and approaches which have not yet (eg. organelle transformation) or have only just begun (eg. transposon and geminivirus vectors) to yield the fruits of their early promise.

7.2 *AGROBACTERIUM TUMEFACIENS*

7.2.1 A Natural Genetic Engineer

It could be said that the birth of modern plant genetic engineering stems from the recognition of the natural genetic engineering ability of the bacteria, *Agrobacterium tumefaciens* (Chilton et al., 1977; Chilton et al., 1980; Willmitzer et al., 1980; Yadav et al., 1982; Zambryski et al., 1980). This opportunistic soil phytopathogen causes crown gall tumours in wounded gymnosperms and dicotyledonous angiosperms (dicots). Oncogenic strains contain a single copy of a large (150-250kb) tumour-inducing (Ti) plasmid (Fig. 1). Part of this plasmid DNA (the 'transfer' or T-DNA) is transferred to the wounded plant cell and stably integrated into the genome.

The genes encoded on the T-DNA, whilst bacterial in origin, contain eukaryotic plant regulatory signals that are expressed in infected plant cells. The expression of these genes has the following two consequences: (i) the synthesis of phytohormones and (ii) the synthesis of opines. The former are necessary for the neoplastic transformation of the infected tissue, which proliferates to produce the characteristic tumorous gall of crown gall disease. The latter are amino acid derivatives which diffuse from the tumour into the surrounding soil where they may serve as a sole carbon source for *Agrobacteria* harbouring a Ti plasmid; they also induce the *tra* operon which allows conjugal transfer of the Ti plasmid to other *Agrobacteria.*

Thus, the Ti plasmid of *A.tumefaciens* has evolved an elegant genetic parasitism, in which infection results in the hijacking of plant metabolic resources to produce food (opine) which is metabolisable only by the plasmid and the plasmid itself can spread only in the presence of infected tissue. Furthermore infected tissue proliferates thereby increasing the amount of opine available indefinitely.

Ti plasmids can be adapted to make useful vectors for the genetic transformation of plant cells with recombinant DNA; in many cases these transgenic plant cells can be regenerated into whole plants (see Chapter 12). The construction of Ti based vectors has been greatly facilitated by the observation that all of the material on the T-DNA, with the exception of two 25bp border regions, is inessential for T-DNA transfer (Garfinkel et al.,1981; Zambryski et al., 1983) and that it can be deleted (phytohormone synthesis interferes with plant regeneration) and replaced with recombinant DNA.

7.2.2 *Agrobacterium* Biology

Before discussing in any detail the development of Ti based vectors for plant transformation we will first give an overview of the biology of T-DNA transfer and crown gall disease as it is understood at present. Comprehensive reviews on the subject have recently been published (Memelink *et. al.*, 1987; Melchers and Hooykaas, 1987; Hooykaas, 1988;Lichtenstein and Fuller, 1987; Stachel and Zambryski,1986) and extensive literature references will be found therein.

For successful infection of a plant cell by oncogenic *Agrobacteria* the following four steps must take place (Fig. 2).

(i) The *Agrobacterium* recognises some signal molecule exuded by the susceptible (wounded) plant cells and, in a chemotactic response, moves up the concentration gradient towards these plant

cells. The *Agrobacterium* must then become attached to the cell.
(ii) In response to the same, or different, signal molecules the *vir* regulon on the Ti plasmid is induced to express the genes necessary for T-DNA transfer to the plant cell.
(iii) The T-DNA is integrated into the plant genome and the T-DNA genes are expressed in the plant cell. The expression of the *onc* genes give rise to cell proliferation and expression of the opine genes whose products are responsible for the synthesis of opine amino acid derivatives. Ti plasmids are classified according to the opine for which they encode. At least six *Agrobacterium* strains have been distinguished on this basis(Fig. 3).
(iv) Finally, the opine(s) diffuses from the tumour tissue and into the surrounding *Agrobacteria* containing soil, where it induces the two Ti plasmid encoded operons, *tra* and *oc*. The former encodes the genes necessary for conjugal transfer of the Ti plasmid to other *Agrobacteria*, while the latter encodes the genes required for opine catabolism.

7.2.2.1. Attachment

Initially the *Agrobacterium* has a chemotactic response towards phenolic compounds released by wounded plant tissue and moves up the concentration gradient towards the wounded cell. There is some contradiction in the literature at present regarding the molecular basis for this chemotaxis.
A phenolic compound, acetosyringone (Fig. 4), has been shown to induce the *vir* genes at a concentration of $1.5\text{-}10 \times 10^{-6}$ M (Stachel et al.,1985). At a lower concentration (10^{-7} M) this same compound has been shown to induce a chemotactic response in *Agrobacterium* (Ashby et al; 1987) and this response has been seen to be dependent on the constitutivly expressed, Ti plasmid encoded vir genes, *vir A* and *G* (Shaw et al. 1988). However, Parke et al. (1988) obtained contradictory results. They found that acetosyringone produced no chemotaxis in agrobacteria; furthermore, other *vir* inducing phenolics which did produce a chemotactic response, did so in strains which harboured no Ti plasmid. Different strains of *Agrobacteria* were used for the two sets of experiments and it is possible that this is the reason for the apparently contradictory results. Perhaps *in vivo*, some, as yet unelucidated, compound is the major chemotactic attractant signalling the presence of wounded plant cells to *Agrobacteria* and the observed strain dependent responses to the compounds examined are, therefore, fortuitous.
Following the chemotactic response the *Agrobacterium* attaches itself to the plant cell (the bacterial-plant cell recognition process has been reviewed by Halverson and Stacey,1986). It has been shown (Douglas *et al.*1986), by deletion and complementation analysis, that two neighbouring, constitutivly expressed, chromosomally encoded genes, *chvA* and *chvB*, are required for attachment. Dylan et al. (1986) have shown that *chvA* and *chvB* have functional equivalents in the *Rhizobium ndvA* and *ndvB* loci and can be complemented by them (these *Rhizobium* loci are required for normal nodulation and nitrogen fixation in the root nodules of legumes). It has similarly been shown by Thomashow *et al.* (1987) that mutations in the chromasomally encoded *pscA* locus abolish *Agrobacterium* virulence by virtue of preventing attachment. Mutants in this locus produce none of the four species of exopolysaccharide normally synthesised by the wild type strain.
Very little is known about the plant components necessary for attachment. It is difficult to distinguish between productive and non-productive binding in the absence of a marker for productive binding during *in vitro* studies on plant cells and components. It may be that the immunity of monocotolydenous plants (monocots) to *Agrobacterium* infection is due, in part, to the absence of suitable receptors for binding (an alternate explanation is that monocots, once infected, fail to respond to the phytohormones synthesised). Although Douglas *et al.*(1986) have some evidence that *Agrobacterium* can bind specifically to suspension cells of the monocot, bamboo.

7.2.2.2. T-DNA transfer and integration

This work has recently been reviewed in detail (Melchers and Hooykaas, 1987; Hooykaas, 1988; Koululíková–Nicola *et al.*, 1987; Lichtenstein and Fuller, 1987) and the experimental basis for the conclusions presented here are not discussed in any detail.
In response to plant phenolics the constitutivly expressed **VIR** protein, **VIR A**, is thought to be a membrane protein which acts as a signal messenger and transmits the extracellular signal to the (also constitutivly expressed) intracellular *virG* gene product, thus leading to its expression at an increased level. The **VIR G** protein acts as an inducer for the rest of the vir genes. Once activated the *vir* genes act on the T-DNA. T–DNA is defined by the presence of two 25bp imperfect repeats (the left and right borders), and possibly by the T-region transfer enhancer, the 'overdrive' sequence

(Perealta *et al.*1986;Van Haaren *et al.*, 1987a;1987b) which increases the efficiency of transfer; transfer of T-DNA is completely independent of the sequence between the borders. The transfer is polar, going from right border to left border. If the right border is deleted, transfer is abolished; however, if the left border is deleted, transfer takes place and the T-DNA transfer continues to some pseudo-border, or in the case of smaller plasmids (<50kb), perhaps all the way around back to the right-border (Wang *et al.* 1984).

Upon induction the T-DNA is nicked precisely in the lower strand within the border repeats by **virD** protein(s) (Yanofsky *et al.* 1986;Melchers and Hooykaas, 1987 and references therein). Following this single stranded T-strand is produced, corresponding to the bottom strand of the T-DNA. Production of this strand is also thought to be associated with DNA replication or repair as the T-region of the Ti plasmids have partial single and/or triple stranded structures at this time (replication intermediates?).

How is the bacterial/plant conjugation carried out? It is likely to be similar to bacterial conjugation and Buchanan-Wollaston *et al.*(1987) have shown that bacterial *mob* and *oriT* functions, together, allow conjugal transfer to plants in the absence of T-DNA borders. During bacterial conjugation *oriT* is the origin of transfer and *mob* supplies the mobilisation functions, including nicking at *oriT* and single stranded transfer of the DNA into recipient bacterium (reviewed by Willets and Wilkins, 1984). In *Agrobacterium* the following model has been proposed for transfer of T-DNA to plants (Lichtenstein & Fuller, 1987 and references therein).

(i) A strand specific nick is made in each of the borders by **virD** proteins.

(ii) Rolling circle DNA replication is initiated from the right border nick and (perhaps with the aid of a helicase activity) the T-strand is extruded in a 5' to 3' direction. The left border nick signals the end of transfer.

(iii) In some manner, as yet unclear, the single stranded DNA is transferred to the wounded plant cell, transported to the nucleus (do *vir* encoded proteins allow passage through the nuclear membrane?) and integrated into the plant nuclear genome. There is some evidence for the role of a single stranded DNA binding protrin in T–strand transfer (Gietl *et al.*, 1987). It is probable that the integration of the T-DNA is carried out, at least in part, by plant encoded functions which are expressed during replication and repair of the wounded plant cell. The sites of integration in the plant DNA appear to be random.

(iv) Once integrated into the plant nuclear genome, the T-DNA encoded functions are expressed as described above (**7.2.1**).

7.2.3 Ti-Plasmid Based Vectors (reviewed by An, 1987; Fraley *et al.*,1986; Klee *et al.*, 1987; Lichtenstein and Fuller, 1987)

The use of Ti-plasmid based vectors was facilitated by the observation that tissue infected by *Agrobacterium* was able to grow *in vitro* in the absence of phytohormones. This was used as a dominant selectable marker for transformation. Furthermore, recombinant DNA inserted into the T-region is expressed in plants transformed with that T-DNA if the recombinant DNA is attached to the correct plant regulatory signals. These signals were initially taken from T-DNA genes, notably the octopine (*ocs*) and nopaline (*nos*) genes. After unsuccessful attempts to express foreign genes in plants (with non-plant regulatory signals) (eg. Barton *et al.*, 1983; Shaw *et al.*, 1983), the first successes were reported by Herrera-Estrella *et al* (1983) and Bevan *et al.*(1983). Herrera-Estrella *et al* (1983) expressed the octopine synthase and chloramphenicol acetyl transferase (CAT) genes regulated by the nopaline synthase promoter in transgenic tumour tissue; they also reported the expression of the neomycin phosphotransferase II gene and dihydrofolate reductase gene; thus providing dominant selectable markers in the absence of oncogenicity. Bevan *et al.*(1983) reported the expression of neomycin phosphotransferase in transgenic tumours at about the same time.

The Ti plasmids themselves are too large (150-250kb) for direct genetic manipulation and it is fortuitous that the *vir* region of Ti plasmids will act *in trans* on T-DNA carried on a another plasmid. This allows the use of small plasmids more amenable to the manipulation of recombinant DNA in *E.coli.*

All vectors require a selectable marker for introduction into *Agrobacterium*, a selectable marker functional in plants and borders for T-DNA transfer to plants.

There are two types of Ti-based vectors currently in use, *viz.* co–integrate vectors and Binary vectors.

7.2.3.1. co–integrate or shuttle vectors

The co–integrating vectors have a region of homology between themselves and the accepting Ti-plasmid. One of the main systems in use today is the pGV3850 system (Zambryski *et al.*, 1983). In this system the acceptor Ti-plasmid, pGV3850 has the *onc* genes replaced by pBR322; the rest of the plasmid, including the borders and the *nos* gene (used as a screen for transformation), are intact. Any plasmid containing pBR322 DNA (into which foreign genes for expression in plants can be cloned) can be used as the shuttle vector. This plasmid, having a ColE1 origin of replication, cannot be maintained in *Agrobacterium*. It can be mobilised to *Agrobacterium* by a 'triparental mating' with a plasmid which supplies the *mob* and *tra* genes in *trans* (eg. pRK2013, Ditta *et al.*, 1980), but only the Ti plasmid will be maintained in *Agrobacterium;* selection for an antibiotic resistance gene from the shuttle vector will select for a single crossover event in which the shuttle vector has become integrated between the border sequences of pGV3850. A similar system has been developed by Fraley *et al.* (1985), in which all except a small region of T-DNA 'the limited internal homology' (LIH) and the left border have been deleted from the accepting Ti-plasmid. The shuttle vector contains the LIH region and the right border; upon integration, a functional T-DNA with intact left and right borders is created (Fig. 5).

7.2.3.2. Binary vectors

Binary vectors rely on the observation that the *vir* region is *trans* acting. They contain a broad host range origin of replication and a bacterial antibiotic resistance gene, for selection and maintenance in *Agrobacterium;* they also have T-DNA borders between which they typically carry a selectable marker which expresses in plants and a polylinker region, containing multiple, unique restriction endonuclease sites for cloning genes of interest. Many binary vectors have been designed (eg. An, 1986; Bevan, 1984; Hoekema *et al.*,1985; Klee *et al.*,1985; Matzke and Matzke, 1986; Simoens *et al.*,1986; Töpfer *et al.*, 1987). As an example, we will describe the binary vector most often used in our laboratory, pGA482 (An, 1986) (Fig. 6). This plasmid is 13.2 kb in size and contains the following features:

a. A broad host range origin of transfer (*oriT*) and replication (*oriV*) allowing conjugation to, and maintenance in, *Agrobacterium.* The mobilisation (*mob*) and transfer (*tra*) functions must be supplied in *trans,* by a helper plasmid as described above (i) for the shuttle vectors. As the frequency of introduction into *Agrobacterium* is so much higher for binary vectors, direct transformation by chemical means, which is less efficient, can also be used (reviewed by Lichtenstein and Draper, 1985).

b. The high copy number origin of replication from plasmid ColE1 (*oriV*) which, whilst being non-functional in *Agrobacterium*, facilitates recovery of high yields from *E.coli.*

c. A bacterial tetracycline resistance gene, for selection in *E. coli* and *Agrobacterium.*

d. A kanamycin resistance gene which expresses in plants, to allow for selection of transformed plant tissue. This gene has the coding region of NPTII from transposon Tn5 flanked by the nopaline (*nos*) promoter and polyadenylation signal.

e. A polylinker sequence for the cloning of foreign DNA.

f. A bacteriophage l-*cos* site. This, together with *oriV*, will allow the integrated DNA, and flanking plant DNA to be cloned as a cosmid by l-*in vitro* packaging of plant nuclear DNA fragments. As far as we are aware, no reports of this have yet been published.

g. The left and right T-DNA borders flanking d, e and f.

7.2.3.3. Relative merits of the shuttle and binary vector systems

The shuttle vector systems have the disadvantage that the frequency of introduction into *Agrobacterium* is only about 10^{-5}-10^{-7}, compared with 10^{0}-10^{-1} for the binary vector systems (Fraley *et al.*, 1986). Shuttle vectors are also dependent on a specific Ti plasmid, or set of Ti plasmids with which they have homology; there is evidence that the host range varies with *Agrobacterium* strain (Byrne *et al.*, 1987; Owens and Cress, 1985). On the other hand shuttle vectors are, in contrast to binary vectors, completely stable in *Agrobacterium* in the absence of drug selection and there is evidence that shuttle vectors can transform (tomato at least) at higher frequency than binary vectors (McCormick *et al.*, 1986). Perhaps the latter observation is due to the so called 'proximity effect', in which the *trans* acting *vir* factors are at a higher local concentration when the T-DNA is on the same plasmid as the *vir* region.

7.2.4 Transformation with *Agrobacterium* (reviewed Rogers *et al.*, 1986a; Rogers and Klee, 1987)

There are four major, established techniques for transformation of plants with *Agrobacterium, viz.* i) inoculation of whole or decapitated plants, ii) co–cultivation of protoplasts with *Agrobacterium* and iii) 'leaf disc transformation'. The first is useful only in the study of oncogenic strains and will not be discussed further here (the laboratory manual written by Draper *et al.* (1988) covers this technique in some detail).

7.2.4.1. co–cultivation

In this procedure two or three day old protoplasts (plant cells that have had their cell wall removed enzymatically)(10^4-10^5 per ml) that are undergoing their first round of cell division are inoculated with *Agrobacterium* at 10^7-10^8 per ml and cultured for between 36 and 48 hours. At this time the cells are washed and treated with antibiotics to kill the *Agrobacterium* and microcolonies are allowed to develop before transferral to media containing the appropriate regime to select for transformation (eg. antibiotic resistance or phytohormone independent growth). Very large numbers of transformants can be obtained in this way; transformation efficiency can approach 50% of viable calli (An *et al.*.1985). Protein or RNA can be extracted from a large number of transformed calli in order get results that are an independent of position effects by virtue of being an average of many independent transformation events (Fraley *et al.*,1986). The main drawbacks of this technique are the relative technical difficulty of preparing protoplasts, the long time required to regenerate reasonable amounts of callus or whole plants (3-4 months), and susceptibility of the procedure to cell culture induced chromasomal abnormalities (Evans and Sharpe, 1983). Perhaps more important is the difficulty of regenerating whole plants from the protoplasts of most crop plants (see chapter 12).

Co–cultivation systems in which suspension culture cells (not protoplasted) are inoculated with *Agrobacterium* have been developed and in tobacco give similar efficiencies to systems that use protoplasts (An, 1985) . This may be a route for efficient transformation and regeneration of whole plants from species not amenable to regeneration from protoplasts (eg. embryogenic carrot suspension cells have been efficiently transformed and plants regenerated by somatic embryogenesis (Scott and Draper, 1987)).

7.2.4.2. Leaf disc transformation

This term was originally applied to the use of surface sterilised tobacco, petunia or tomato leaf pieces (Horsch *et al.*, 1985), but the term has since been applied to other axenic explants, the choice of which is dependent on the ability to regenerate well in tissue culture, eg. stem sections of *Brassica napus* (Fry *et al.*, 1987) and developing hypocotyl sections of flax (Basiran *et al.*, 1988). A typical protocol for tobacco leaf discs would be as follows. Surface sterilised leaves from greenhouse grown plants or axenically grown leaves are cut into 1 cm pieces and transferred to plates containing an appropriate, agar solidified plant media; these plates may also contain feeder protoplast cells although in our hands reasonable transformation can be obtained without them. The plates are incubated for two days to condition the media (presumably with *vir* inducing signal substances) and then dipped in a 50 fold dilution of an overnight *Agrobacterium* culture and returned to the plates for a further 48 hours to allow bacteria to bind to the wounded plant cells and T-DNA transfer to occur. At this time the leaf pieces are transferred to media containing a) carbanecillin (to kill the *agrobacterium*); b) the appropriate antibiotic to select for transformation and phytormones to allow either shoot or callus regeneration. Callus or callus+shoots (depending on the media used) will start to appear at about one week. After a further two weeks callus can be excised and transferred to separate plates for further culture and shoots can be excised and transferred to rooting media. Once the transformed shoots have rooted they may be propagated in tissue culture or, when they reach a reasonable size, transferred to soil in the greenhouse or growth room. Most plants obtained in this way will be morphologically normal and will go to seed.

It should be noted at this point that not all of the shoots generated by this method will be transformed; this is perhaps due to cross feeding from adjacent transformed tissue. The ability to root on media containing the selective drug, however, is almost always indicative of transformation.

The advantages of leaf disc transformation over co–cultivation are its relative simplicity, the enhanced ability to regenerate whole plants from species which are not amenable to regeneration from protoplasts and the rapidity with which transgenic plants can be obtained, typically 4-6 weeks. Horsch and Klee (1986) have developed a method by which leaf discs can be assayed for

transformation within two days of inoculation!

7.2.5 The Structure and Stability of T-DNA Introduced into Plants

7.2.5.1. Mendelian segregation

It has been shown in a number of studies that once T-DNA is integrated into the plant genome it segregates stably in a Mendelian fashion (reviewed by Fraley *et al.* (1986)).

Budar *et al.* (1986) carried out a series of genetic crosses with 44 independently transformed plants and analysed the segregation of the phenotypic marker, kanamycin resistance. It was found that 40 of these plants were amenable to analysis by Mendelian genetics; 35 segregated as having a single kanamycin resistance locus and 5 as having two independent resistance loci. Interestingly two of the single locus plants appeared to have the T-DNA inserted such that it caused a recessive lethal mutation; perhaps this will be a way into the use of T-DNA for mutational analysis. Müller *et al.*, (1987) analysed two transgenic tobacco plants carrying single copies of T-DNA. The marker segregated according to Mendelian genetics. Extremely low levels of instability were observed; less than 1 seed in 1800 appeared to have lost kanamycin resistance by a mechanism of meiotic instability (seed were produced by back crossing homozygotes to untransformed plants). They predict from their observations that plants carrying more than one copy of a foreign DNA sequence will, by virtue of homologous recombination, be less stable. It will be interesting to see if this prediction is born out by unambiguous experiments.

Peerbolte and co-workers (1987a) studied a large number of transformed F1 seedlings at the DNA level and also observed fidelity of T-DNA transfer; furthermore, this was true in three states of differentiation (shoot, callus and graft).

Although T-DNA has been shown to to be faithfully inherited in F1 and F2 progeny, the same is not completely true of gene expression. Czernilofsky *et al.* (1986a) examined the level of NPTII activity in eight independent transformants (NPTII activity segregated as a single genetic locus in the F1 generation in all cases) and their progeny (which was shown to have T-DNA identical to that of the parent). There was an approximately 20 fold variation in levels of expression between the eight primary transformants. While there was some correlation between parental and progeny levels of expression, the variation in expression between six F1 progeny (from the same parent) was upto ten fold. This may be due to methylation (**7.2.5.4**). It was also shown that plants that were homozygouse for NPTII expressed it at higher levels than heterozygous plants.

7.2.5.2. The fate of simultaneously transferred unlinked T-DNAs

Another factor that may be important in the design of experiments is the fate of two unlinked T-DNAs transferred to the same plant during a single inoculation with *Agrobacterium*.

It had been found that co–cultivation using *Agrobacterium* strains containing oncogenic helper plasmids (with binary vectors) sometimes gives rise to higher efficiencies of transformation than those containing non-oncogenic helpers (An *et al.*, 1985). In these experiments it was also observed that, by phenotype at least, 20-60% of transformants (depending on whether phytohormone independence or kanamycin resistance was the initial selection) had integrated and were expressing both the wild type and recombinant T-DNAs. In a similar system de Framond et al. (1986) demonstrated that, in the single plant which they analysed, two unlinked T-DNAs transferred to the same cell segregated in the F1 generation. This suggests the possibility of co–transforming a plant cell with a selectable and non-selectable gene, taking segregants which have lost the selectable gene in the F1 generation and re-co–transforming with a second gene and the same selectable marker gene.

It was not possible in either of the above cases to distinguish between transfer of two T-DNAs from one bacteria and transfer of one T-DNA from two bacteria. To address this problem Depicker *et al.* (1985) compared the frequencies of co–transformation of protoplasts by T-DNAs carried in separate *Agrobacteria* with T-DNAs on the the same Ti plasmid in a single *Agrobacterium*. The results suggest that the frequency of double transformation by two *Agrobacteria* is equal to the product of their independent transformation frequencies. The number of double transformants arising from two T-DNAs resident on one Ti-plasmid in a single *Agrobacterium* is about 60% of the total transferred, independent of the overall transformation efficiency. This suggests that the rate limiting step in *Agrobacterium* infection is the formation of a successful plant cell/bacteria interaction and not DNA integration and transfer or plant cell competency (Depicker *et al.*, 1985).

From the point of view of experimental design, however, the important question is 'Can we get reasonable levels of double transformation events from separate *Agrobacterium*?'. If we can, it

greatly reduces the difficulty of carrying out experiments which require more than one independent transformations of a single plant cell. Recently it was shown (McKnight *et al.* ,1987) that, when two *Agrobacterium* strains containing different T-DNAs (in the same genetic background) were used in a leaf disc inoculation, about 20% of the transformants contained both T-DNAs and the markers were shown to segregate independently in the F1 generation. When they used the related *Agrobacterium Rhizogenes* (see **7.2.8**) they obtained roots (a phenotype equivalent to crown gall in *A.tumefaciens*) containing one marker (in addition to rootiness itself) in 50% of the transformants and both markers in 11%. This indicates that in 11% of the transformants three(!) independent T-DNAs had been transferred to the plant cell. Segregation in the *A.Rhizogenes* case was not examined. Work carried out by Petit *et al.*(1986) gave similar results.
The above work indicates that it is not necessary to put two unlinked T-DNAs in the same *Agrobacterium* in order to get reasonable rates of co–transformation.

7.2.5.3. Structure, stability and rearrangements in transferred T-DNAs

A number of studies have been carried out on the structure of inserted T-DNA in plant genomes and the occurrence of truncated or rearranged integrations has been noted. Some of these studies are discussed below.
Van Lijsbettens *et al.*(1986) demonstrated that of nearly 1000 subclones from a *Nicotiana tabacum* callus produced by the wild type *Agrobacterium* C-58 only 6 were morphologically different from the parental callus, and none of these differences could be correlated with gross rearrangements of T-DNA structure. The implication of this is that once the T-DNA had been integrated into the plant genome it was stable through mitosis; this, together with the meiotic stability discussed in section **7.2.5.1** , confirms the potential utility of transgenic crop plants to commercial breeders. Inactivation of T-DNA in transformed tissue at the DNA level has, however, been shown to occur, albeit at very low frequency, in at least one instance. Peerbolte *et al.*(1987d) found a shooty crown gall line which reverted to wild type after three years in tissue culture due to T-DNA rearrangements and deletions (so called 'somaclonal variation'). In the light of Van Lijsbettens' *et al.*(1986) study this is probably not important so far as application to commercial strains are concerned.
An attempt has been made (Czernilofsky *et al.*, 1986b) to amplify an NPTII gene in transformed tobacco callus by exposure to increasing levels of kanamycin, both in small steps (0-700µg/ml in 100µg steps) and in single jumps to high concentration. No amplification was found. In animal cells this technique works well (eg. Schimke, 1984).
It has been found (Van Lijsbettens *et al.*, 1986) that in calli produced by co–cultivation with protoplasts about 12% had morphological abnormalities associated with truncated T-DNA and it was proposed that this is related to the presence of 'pseudoborders' within the T-DNA. Furthermore, if calli are produced by the inoculation of whole plants then the incidence of truncated T-DNA is considerably less (although expression of the T-DNA genes in these tumours is less than perfect, discussed below, **7.2.5.4**). If this observation can be extended to recombinant T-DNA, it serves as another reason to choose leaf disc transformation over co–cultivation wherever possible.
The arrangement of T-DNA integrated into the plant chromosome seems to be partly dependent on the *Agrobacterium* strain and vector system (binary or co–integrate) used. Early work (Zambryski *et al.*, 1980) on a tumour line derived from the nopaline strain, T37, showed that T-DNA was integrated as an inverted repeat and several direct repeats. More recently Jorgensen *et al.*(1987) have shown, for the nopaline strain C58, that arrays of inverted repeats are the predominant structure in transformed plants; both head–to–head and tail–to–tail repeats being equally well represented. T-DNA junctions were also analysed and right ends were considerably more homogenous than left ends. This is consistent with the right to left end polarity of T-strand transfer if initiation of T-strand transfer is more precise than termination.
These results are in contrast to those found for octopine type strains where inverted repeat structures are relatively uncommon (Jorgensen *et al.*, 1987 and references therein). An analysis of T-DNA structure in plants transformed by the disarmed octopine strain LBA4404 harbouring a binary vector has been carried out (Spielmann and Simpson, 1986). Between one and six copies of the T-DNA had been transferred to the nine plants analysed and approximately 30% of the transferred T-DNAs were incomplete or rearranged. Seven of the nine plants had T-DNA in more than one genetic locus. A study of the literature shows that insertion at multiple loci is quite unusual (Spielmann and Simpson, 1986 and references therein); it was suggested that it occurred because the binary vector, containing the T-DNA, is maintained at 4-7 copies per *Agrobacterium* cell (most of the literature

examples cited used wild type or co–integrate Ti-plasmids). This hypothesis is entirely consistent with the high degree of co–transformation found when two different T-DNAs are present in the same *Agrobacterium* (Depicker *et al.*, 1985) (**7.2.5.2**). If true, the difference in the number of integration loci obtained by use of either co–integrate or binary vectors is another factor to be considered in experimental design.

An extremely detailed analysis of T-DNA transferred from the octopine strain LBA1501 has been carried out by Peerbolte *et al.* (1987b,c) and quite a high degree of infidelity of transfer was observed. It is recommended that the reader refer to the original papers as there is not room in this review to go into the details.

Only recently has a thorough investigation of the plant sequences involved in T-DNA integration been carried out (Gheysen *et al.*,1987) with the cloning of a virgin target site. This allowed duplications and rearrangements in both T-DNA and plant DNA to be elucidated. The tissue used was from a C58 nopaline strain derived tumour. Plant/T-DNA junction sequences were cloned and used as probes to screen a genomic library constructed from untransformed tissue. The results may be summarized as follows (Fig. 7). At the right junction there is a direct transition from T-DNA to plant DNA; however there is a deletion of 27bp at a position 165bp to the right of the junction followed by a single C to T transition. At the left end the transition is not direct, there is a 33bp 'filler' sequence between the T-DNA and the plant DNA and this sequence is an imperfect, inverted repeat of two regions from the right end junction. Following this filler sequence is a duplication of 158bp of target sequence (ie. identical to the first 158bp of plant DNA from the right junction). It is not possible to propose a mechanism for T-DNA integration from analysis of a single event, although from the degree of rearrangement it can be presumed that it is a multi step event. It was also noted that the target site was particularly AT rich around the point of integration, whether this is coincidental will not be known until further analyses have been carried out. Clearly, in the light of this work, experiments based on the assumption of precise insertion of T-DNA into the plant genome (cf. bacterial transposons) will have to be approached cautiously.

7.2.5.4. The role of methylation in transgenic gene expression

Although gene expression is discussed elsewhere (Ch. 2), it is pertinent at this point to discuss briefly the stability of levels of gene expression in transgenic plants.

It has been observed (An, 1986) that the activity of the nopaline synthase (*nos*) promoter can vary 200 fold in transgenic plants and that the activity of closely linked genes under the control of identical *nos* promoters have no apparent correlation with each other. This has been attributed by various workers to some sort of little understood 'position effect'. Although chromatin structure and position effects probably play a role in the level of transgenic gene expression, an equally, if not more, important factor is cytosine methylation. Methylation of T-DNA was first reported by Gelvin *et al.* (1983). Subsequently (Amasino *et al.*, 1984; ; Hepburn *et al.*,1983; Peerbolte *et al.*, 1986b and Van Lijsbettens *et al.* 1986), T-DNA methylation has been correlated with aberant tumour phenotype (suggesting under expression of certain T-DNA genes) and directly with low levels of transcription; in both instances suppression of gene expression was found to be reversible by the action of the demethylating drug 5–azacytidine. We have regenerated plants (by leaf disc transformation in the presence of the selective drug but in the absence of *Agrobacterium*) from a transformant which was expressing CAT from a *nos* promoter at reasonable levels (Day and Lichtenstein, unpublished) and approximately 50% of the retransformants failed to express the non-selected CAT gene at a detectable level. When the process was repeated, using one of the plants not expressing CAT, about 50% of the regenerated plants **did** express CAT. We assume in this case that the plants are all clonal copies of one another and that the variation in CAT expression is due to methylation, although this has yet to be confirmed. It appears that the *nos* promoter is particularly prone to these high levels of variation and the stronger promoters, such as the CAMV 35S and 19S promoters, have a much lesser degree of variation of expression (Negrutiu *et al.*, 1987a). It is possible that the *nos* promoter directs its own methylation.

7.2.6 Transformation of Monocots

One of the major shortcomings of *Agrobacterium* mediated plant transformation has been its limited host range; for a long time monocots, which includ the majority of important crop plants (maize, wheat and rice), had, been considered refractive to *Agrobacterium* mediated transformation (De Cleene and De Ley, 1976).

However, it was reported (Hooykaas-Van Slogteren *et al.*, 1984) that inoculation of both

Chlorophytum capense and *Narcissus*, (Liliaceae and Amaralyllidaceae respectively) with oncogenic *Agrobacterium* produced small tumours with which opines were associated. Similar results have been reported by Graves and Goldman for *Gladiolus* sp. (Iridaceae)(1987) and *Zea mays* (Graminaceae) (1986).

As yet, proof at the nucleic acid level of T-DNA integration into the monocot host genome has not been forthcoming in the above species; although it has been shown for the Liliaceae *Asparagus officinalis* (Bytebier *et al.*,1987) and for *Dioscorea bulbifera* (yam)(Scäfer *et al.*, 1987). In the latter case tumour formation only occurred if the *Agrobacteria* were preinduced by incubation with *Solanum tuberosum* (the common dicot, potato) wound exudate. It is possible that this preinduction is a general requirement for *Agrobacterium* mediated transformation of the majority of monocots. If this turns out to be the case we can look forward to a deluge of reports on monocot transformation.

Opine producing tumours have been produced by inoculation of stem segments of *Asparagus officinalis* (Liliaceae) (Hernalsteens *et al.*, 1984) and the T–DNA integration has been shown to occur normally (Bytebier *et al.*,1987). In the same paper the regeneration of transformed asparugus plants, after transformation with disarmed Ti plasmid vectors, was reported; *Asparagus officinalis* is one of the few monocots amenable to regeneration from single cells.

The practical difficulties of detecting *Agrobacterium* mediated monocot transformation have recently been reviewed by Hooykaas and Schilperoort (1987).

It is also germane at this point to mention the 'Agroinfection' of maize by the single stranded DNA virus, maize streak virus (MSV) (discussed more fully in the next section) (Grimsley *et al.*, 1987). Normally MSV can only be transmitted by its insect vector; naked DNA and even whole virus isolate are uninfectious by mechanical inoculation. However, if the virus is cloned between T-DNA borders and the resulting *Agrobacterium* strain used to inoculate maize, a systemic infection results. From these results it is clear that the *Agrobacterium* must be binding to the plant cells and T-DNA transfer taking place; however the fate of the T-strand after transfer is not clear.

It may be seen from the results discussed above that the problem of transformation of monocots by *Agrobacterium* is gradually being solved; the Liliaceae, Amaralyllidaceae, Graminaceae and Dioscorea family all show some degree of susceptibility to Ti plasmid transformation and it is our opinion that it will not be too long before these transformations become routine. Perhaps a more intractable problem in the transformation of the important monocot crop plants will be that of whole plant regeneration from single cells or protoplasts (see Ch.12). The first reports of transformed, regenerated maize plants from protoplasts have recently been published (Rhodes *et al.*, 1988).

7.2.7 Viral Infection of Plants by *Agrobacterium:* "Agroinfection"

The use of *Agrobacterium* as a vector to facilitate infection of plants by virus has been termed Agroinfection (Grimsley *et al.*, 1986) and has been reviewed elsewhere (Grimsley and Bisaro, 1987).

The first experiments were carried out using the double stranded DNA virus, Cauliflower mosaic virus (CAMV) (Grimsley *et al.*, 1986)(see **7.4.1**).It had previously been observed that cloned CAMV was not infectious by mechanical inoculation unless the viral DNA was first excised from the bacterial vector or a cloned tandem duplication of CAMV was used. This implied that duplication of the viral genome allowed it to escape from the vector and replicate. *Agrobacterium* containing Ti-plasmids were constructed containing 1.4 and 2 tandem genomes of CAMV and these were both found to give rise to systemic infections when inoculated onto leaves or cut crown of *Brassica rapa* (turnip). Furthermore, the role of the *Agrobacterium* was confirmed by the observation that, while inoculation with less than 1μg of plasmid DNA rarely led to infection, agroinfection with bacteria containing the equivalent of 10ng of CAMV DNA consistently caused systemic infection.

As mentioned above (**7.2.6**) agroinfection has also been used to infect the graminaceous monocot, maize, with the single stranded DNA gemini virus, maize streak virus (MSV)(Grimsley *et al.*, 1987); again a tandem repeat was used. This virus is very recalcitrant to infection by mechanical inoculation and previously the only reports of successful infection used an insect vector. The same technique has also been applied to the related (and similarly recalcitrant) digitaria streak virus (DSV) (Donson *et al.*, 1988), to wheat dwarf virus (WDV) (Hayes *et al.*, 1988a) and also to the more distantly related virus, tomato golden mosaic virus (TGMV) (Hayes *et al.*, 1988b; Elmer *et al.*, 1988).

TGMV has a bipartite genome of two circular single stranded DNA molecules approximately 2.5kb in length, both of which are required for systemic infection. The only homology between the two molecules is the so called 'common region' of about 200bp. It has previously been shown that when dimers of TGMV DNA A are cloned into the chromosome of petunia plants (*via Agrobacterium* mediated transformation) a small number of unit length DNA A molecules are produced (Rogers *et al.*, 1986b), suggesting that replication takes place. In the same work, trimers of TGMV B were cloned into the chromosome of petunia, but no unit length DNA molecules were found. However, when the A2 and B3 plants were crossed, a systemic infection resulted. This suggests that the A particle encodes the functions necessary for replication and the B particle those necessary for cell to cell spread and symptom development. Hayes *et al.* (1988b) found that when they agroinfected wild type plants with *Agrobacteria* containing (within the same T-DNA) 1. dimers of DNA A and DNA B ; 2. a partial dimer (1.6mer) of A and a dimer of B or 3. a dimer of B and a partial dimer of A with part of the coat protein gene missing, they obtained systemic infection. They also obtained systemic infection when they agroinfected plants with a mixture of a strain containing a dimer of A and a strain containing a dimer of B. The latter strain was also found to complement A2 transgenic plants (containing dimers of A in the genome) and the former B3 plants (containing trimers of B in the genome). This gave rise to systemic infection in both cases. Similar work has been carried out by Elmer *et al.* (1988). The results were almost the same, with one exception, they found that A2 plants were not complemented by agroinfection with the B component.

The mechanism of agroinfection is not clear from these experiments and hopefully further work will help to elucidate whether infections arise by a replicative mechanism or by homologous recombination between repeated sequences and ii. whether integration of the T-DNA into the plant chromasomal DNA occurs and/or is necessary during infection. Mechanism not withstanding, agroinfection provides an efficient and reproducible means of infecting plants with virus, already it has shown it's utility with regards to viral vectors (**7.4.2**) and may one day help to answer questions about interactions between *Agrobacteria* and monocots.

7.2.8 *Agrobacterium rhizogenes*

Agrobacterium rhizogenes is a species very closely related to *A.tumefaciens*. The only major difference between the two is that while *A.tumefaciens* harbours a Ti plasmid (which causes a crown gall on infection), *A.rhizogenes* harbours a Ri plasmid which causes a rooty phenotype on infection (Fig. 8) (reviewed in Hooykaas, 1988; Melchers and Hooykaas, 1987). Avirulent *Agrobacteria* are called *A.radiobacter*.

Ri plasmids (in common with octopine type Ti plasmids) have two T–regions, the T_L and T_R regions, either or both of which may be transferrerd to plants on infection. Integration of T-DNA from Ri plasmids gives rise to a characteristic 'hairy-root' phenotype; adventitious roots grow from the infected wound site whether it is on stem or leaf. Several genes responsible for this neoplastic transformation have been identified, they have no homology with the three *onc* genes present in the T-DNA of Ti plasmids, and their functions are, as yet, unknown (Hooykaas, 1988).

Ri plasmids have been used to transform plants. Whole plants may be regenerated from the adventitious roots arising from integration of Ri plasmid T-DNA under the appopriate conditions. Tepfer (1984) showed that plants with altered morphology were produced and that the altered phenotype and genotype is heritable. In all species studied, the altered phenotype consisted of i. high root growth rate in culture, ii. reduced apical dominance in roots and stems, iii. wrinkled leaves and iv. increased width to length ratio in leaves; other, species dependent, alterations in phenotype were also observed. This altered morphology could be used as a marker for transformation.

Simpson *et al.* (1986) have shown that a binary vector resident in an Ri strain of *Agrobacterium* succesfully co–transfers T-DNA to hairy roots. The rate of co-transformation was dependent on both the plant species and the marker/selection. When nopaline synthase was the marker, co-transfer took place in 33-55% of cases in alfalfa, 33% in tomato and 2% in soybean; when kanamycin resistance was the selection, the rates of co-transfer were 4% in alfalfa, 19% in tomato, 18% in tobacco and 0% in soybean. The lower rate of co-transformation observed with selection on kanamycin may have been due to the use of too high levels of the drug. However, in three of the four plant species examined, the rate of co–transfer of a non-selected marker was reasonable. Similar results have been obtained by eg. Hoekma *et al.* (1984) and Shahin *et al.* (1986).

A number of different workers have constructed shuttle vectors (see **7.2.3.1**) based on Ri plasmids

(eg Comaiet al.,1985; Jensen *et al.*,1986; Morgan *et al.*,1987; Stougard *et al.*,1987;Tepfer and Casse-Delbert,1987). We will take the work of Stougard *et al.*(1987) as an example. They used replacement mutagenesis to insert pBR322 DNA into the T_L region of an Ri plasmid at such a position that the plasmid was still virulent. co–integrates could then be formed by homologous recombination with pBR322 vectors containing DNA to be cloned into plants. Transformed roots (and regenerated plants) were shown to contain the cloned DNA in addition to the Ri DNA. When they used a binary vector in conjunction with their co–integrate vector, the rate of co–transformation (as assayed by selection on kanamycin) was 60% of the root lines tested.
The work discussed above shows that border sequences from Ti plasmids are functional in *A.rhizogenes* Ri plasmids and in *A.tumefaciens*. The combination of the two systems holds great potential for work involving plant genetic transformation.

7.2.9 Conclusion

The *Agrobacterium* system for plant transformation has been, and will continue to be, of great utility in the analysis of plant gene function and in the engineering of crop plants, particularly as the problems of its use in monocots are being solved. It has already been shown to be possible to rescue a single gene from a plant by shotgun cloning using leaf disc transformation (DNA from a plant having a single copy of a NPTII gene was shotgun cloned into a Ti plasmid which was then used to transform leaf discs; one disc in twenty gave rise to kanamycin resistant tissue)(Klee *et al.*, 1987), and plants have been engineered which are resistant to herbicide (Comaiet al.,1985; De Block *et al.*,1987), virus (eg.Abel *et al.*, 1986; Hemenway *et al.*, 1988) and insect (Vaeck *et al.*, 1987).

7.3 DIRECT GENE TRANSFER

7.3.1 A Physico-Chemical Method of Genetic Transformation

Direct gene transfer (DGT) involves the application of DNA containing the gene(s) of interest directly to the tissue to be transformed. The uptake and/or integration and/or expression of the foreign genes is mediated by the physical and chemical conditions of application. These techniques have been applied to yeast and mammalian systems for some time (eg. Hinnen *et al.*, 1978; Chu and Sharp, 1981). However, it is only recently that DGT has been applied to plants (for recent reviews see Lichtenstein and Fuller, 1987; Potrykus *et al.*, 1987a; Potrykus *et al.*, 1987b; Potrykus *et al.*, 1985a; Negrutiu *et al.*, 1987a; Saul *et al.*, 1987; for a review of the techniques see Fromm *et al.*, 1987; Langbridge *et al.*, 1987; Shillito and Potrykus, 1987a).
The are a number of advantages of DGT over *Agrobacterium* mediated gene transfer. One is the general applicability of DGT to plant species; whereas *Agrobacteria* have a limited host range, particularly with respect to monocots, DGT is applicable to any species from which it is possible to make protoplasts. A further advantage is the utility of DGT in transient expression systems where it is possible to check the expression of DNA or RNA transferred to plant protoplasts within two days of initiating the experiment. The main disadvantage of DGT is the necessity (in most cases) of using protoplast; this is technically more demanding and many species of cereal cannot yet be regenerated from protoplasts. However, the regeneration of genetically transformed, whole maize plants from protoplasts has recently been reported (Rhodes *et al.*, 1988). All of the transgenic plants (38) were sterile; it is not clear at present if this is intrinsic to regeneration, or a factor of some other experimental parameter (eg. culture line or age). Another disadvantage of DGT is the complex patterns of integration often observed (**7.3.3**).
The two techniques most often associated with DGT in plants are electroporation and polyethylene glycol (PEG) mediated DNA transfer; both of these techniques have been applied to transient and stable transformation of plant cells. In both, the cell membrane is temporarily made permeable and DNA uptake by the cell is possible. In the latter, it is chemical conditions that allow the uptake, whilst in the former it is an electrical impulse of high field strength that reversibly permeabilises the cell membrane (Zimmermann *et al.*, 1984) and allows DNA uptake by the plant protoplasts.

7.3.2 Transformation of Plant Protoplasts by Eletroporation and Chemically Stimulated DNA Uptake

Early experiments, in which naked DNA was incubated with plant protoplasts in the presence of various transforming agents, gave rise to transformants at a low frequency of $\sim 10^{-6}$ (Lurqin and Kado, 1977; Davey *et al.*, 1980; Draper *et al.*,1982; Krens *et al.*, 1982). When naked Ti-

plasmids were used as the transforming DNA, the function of the T-DNA borders were not retained and the vir region was not required for the transformation integration event (Krens *et al.*, 1985). Since this time the procedure has been optimized and transformation efficiencies of 10^{-2}–10^{-4} are routinely obtained (Potrykus *et al.*, 1985a; Fromm *et al.*, 1986; Negrutiu *et al.*, 1987b).

A number of factors appear to effect the efficiency of DGT by naked DNA. Negrutiu *et al.* (1987a,b) systematically studied seventeen parameters affecting direct gene transfer in over one hundred combinations for the two species, *Nicotiana tabacum* and *N.plumbaginifolia*. Factors investigated included i.plant species; ii. growth conditions; iii. protoplast source and method of isolation; iv. bathing media for transformation; v. state, size and concentration of plasmid and carrier DNA; vi. PEG molecular weight, concentration and pH; vii. timing and order of steps; viii. heat shock; ix. electroporation; x. cell cycle stage; xi. irradiation, and more besides (Negrutiu *et al.*, 1987a,b). The major parameters found to be important for efficient transformation are: 1) the plant species, 2) the concentration and state of the plasmid DNA, 3) the presence and concentration of carrier DNA, 4) Cations and PEG concentration, and 5) electroporation (Negrutiu *et al.*, 1987a,b). Synchronization of the cell cycle by aphidicolin, which inhibits alpha–like DNA polymerase, has been claimed to increase efficiency in both stable (Meyer *et al.*, 1985) and transient (Okada *et al.*, 1986a) transformation systems. However, Negrutiu *et al.*, (1987b) found that the use of this compound gave a tenfold drop in efficiency. It is probable that other differences in protocol led to these apparently contradictory results.

7.3.2.1 Stable transformation

A typical experiment to obtain stable transformation of plants by PEG mediated DNA uptake might be as follows (Shillito and Potrykus, 1987): 1ml of *N.tabacum* protoplasts at $2x10^6$/ml in an appropriate media are placed in 10ml sterile plastic tubes and heat shocked for 5 minutes at 45°C followed by cooling on ice. Linearized plasmid DNA is added (10µg) together with calf thymus carrier DNA (50µg) in 50µl distilled water; after mixing, 0.5ml of 40% PEG 6000 is added. The mixture is incubated for 30 minutes at room temperature and following dilution by 5 successive additions of media (2ml) the protoplasts are centrifuged. The protoplasts are then resuspended and plated in media containing agarose. After a number of days the recovering protoplasts may be transformed to media containing selective agents where appropriate. Transformation efficiencies are reported to be of the order 10^{-4}-10^{-3} of surviving protoplasts.

A protocol for the transformation of protoplasts by elecroporation is given in the same reference (Shillito and Potrykus, 1987). Protoplasts are resuspended in buffered 0.4M mannitol (to maintain the correct osmoticum) containing 6mM magnesium chloride at a concentration of $1.6X10^6$/ml. 0.37ml is transferred to the electroporator chamber and magnesium chloride is added until the resistance is 1-1.1KΩ. Heat shock is carried out as before for 5 minutes at 45oC . 0.25ml aliqots of the suspension are removed to polycarbonate tubes, 4µg of linearized plasmid DNA and 20µg of calf thymus DNA are added in 20µl of water and a half volume of 24% PEG 6000 (previously adjusted to 1.2kΩ with magnesium chloride) is added. After ten minutes the samples are transferred back to the electroporation chamber and pulsed three times at ten minute intervals with a field strength of 1.4–15kV/cm. Following this they are plated as described above. This protocol is reported to routinely yield transformants at an efficiency of 1–3%.

Negrutiu *et al.*(1987b) have reported a method of transforming Nicotiana protoplasts by the synergistic interaction of Mg^{2+}, PEG and possibly Ca^{2+}. The optimal concentration of Mg^{2+} was different for *N.tabacum* and *N.plumbaginifolia*. This protocol was reported to give transformation efficiencies as high, or higher, than those obtained under optimum electroporation conditions (upto 4.8% for *N.tabacum* protoplasts); this is 100–fold higher than anything previously reported for a chemical salt+PEG transformation method.

A number of points should be made with regard to the above described experimental protocols. First of all, it should be stressed that conditions described for one species will not necessarily give as high efficiencies in other species, and when identical conditions were used, efficiencies varied by as much as an order of magnitude (Negrutiu *et al.*, 1987a,b). At present, however, there have been no reports of protoplasts from any species which are completely refractory to transformation; and species from which protoplasts have been transformed include: *Nicotiana tabacum, Nicotiana plumbaginifolia, Lolium multiflorum, Triticum monococcum, Oryza sativa, Petunia hybrida, Hyoscyamus muticus, Brassica campestris* and *Zea mays* (Negrutiu *et al.*, 1987a; Potrykus *et*

al., 1987a; and references therein). Electroporation is in many respects more of an art than a science, it is difficult to compare results from different laboratories or to use published protocols without further optimization. Electroporators may differ in either giving an exponentially decaying pulse discharged through a capacitor (usually home made) or a step wave pulse (commercial electroporators); Voltages, discharge time, number of pulses and pre/post treatments can all vary. Construction of cells varies both in terms of shape and material. The intensity of electrical pulse required for efficient transformation is inversely proportional to the diameter of the protoplasts (Zimmermann *et al.*, 1984), which varies from species to species. Finally, even such things as trace amounts of ions in water used to make up solutions can effect results (purer not neccessarily being better). Clearlly, protocols have to be optimized in one's own laboratory and, once good results have been obtained, it is a good idea to use materials from the same batch for subsequent experiments (particularly for crude substances such as enzymes used in the preparation of protoplasts).

7.3.2.2 Transient transformation (reviewed by Frommand Walbot, 1987)

Different conditions are required for transient expression of foreign DNA and RNA (cf. stable transformation). Circular plasmids and metabolically active, non-dividing cells seem to represent optimal conditions for transient expression (Negrutiu *et al.*, 1987; Okada *et al.*, 1986a); conditions harsh enough to reduce a cell's ability to divide may allow ingress of larger amounts of nucleic acid and leave transcriptional and translational ability unimpaired.

Conditions for the transient expression of DNA in both dicot and monocot protoplasts, mediated by electroporation, have been investigated using a number of reporter genes (Neomycin phosphotransferase, NPTII; nopaline synthase, nos; and chloramphenicol acetyl transferase, CAT) and optimal activity could be monitored 36 hours after electroporation (after which activity decayed exponentially) (Fromm *et al.*,1985; Hauptmann *et al.*,1987; Ou-Lee *et al.*,1986).

The expression of mRNA electroporated into both plant and animal cells has been studied by Callis *et al.* (1986). It was found in monkey fibroplasts, and in maize and carrot protoplasts, that CAT mRNA is only efficiently expressed if it is both 5' capped and 3' polyadenylated; expression *in vitro* (rabbit reticulocyte lysate) was much less affected by these parameters.

Electroporation has also been successfully used to mediate infection of protoplasts with viral RNA (Okada *et al.*,1986b; Nishiguchi *et al.*,1985; Watts *et al.*,1987). Attempts to infect protoplasts with whole virus particles by this method have been less successful (Watts *et al.*,1987) unless large amounts of virus are used (Okada *et al.*,1986b) (500µg/ml cf. 10µg/ml for RNA).

Detailed methods for the electroporation and transient expression of DNA and RNA into plant protoplasts, including instructions for the building of simple electroporators, are given by Fromm *et al.* (1987) and Langbridge *et al.* (1987).

7.3.3 The Structure and Stability of DNA Stably Introduced into Plants by DGT

A number of workers have analysed the fate of DNA transferred to plant tissue by DGT. Hain *et al.* (1985) obtained kanamycin resistant tobacco callus by means of calcium phosphate mediated protoplast transformation. Southern [1] blot analysis of two of these clones demonstrated that the DNA had integrated in a complex pattern (cf. *Agrobacterium* mediated transfer of the same gene), possibly including concatenation of the input DNA. One regenerated plant was analysed and kanamycin resistance was shown to be inherited in Mendelian fashion as a single dominant locus. Similar work was carried out by Potrykus *et al.*, (1985b); two transformed tobacco cell lines were subjected to a rigorous genetic analysis, in addition to clonal (ie propagation of plants in tissue culture) and Southern analyses. In both cases kanamycin resistance was inherited as a single dominant Mendelian trait, also the gene was stably maintained during clonal propagation. Southern analysis demonstrated that all kanamycin resistant tissue had the expected band as well as extra bands corresponding to rearranged DNA. The additional bands were shown to be genetically linked to the expected band. Instability was very occasionally observed in clonaly propagated plants; in one case, all of the rearranged bands had disappeared (but not the expected band) and in another, the expected band had disappeared and the other bands remained. In the latter case, the kanamycin resistant phenotype was also lost. This instability has not been observed in *Agrobacterium* transformed plants (**7.2.5**) and may be due to homologous recombination between rearranged

[1] Southern blot analysis is a technique in which DNA is cut by restriction endonucleases, the fragments separated electrophoretically, and bands (fragments) with homology to a specific probe visualised. Northern blot analysis is the analogous technique applied to RNA. A fuller explanation will be found in any introductory text on molecular biology.

(inactive) and non-rearranged foreign DNA sequences. Detailed analyses of tobacco transformed by direct gene transfer have also been carried out by Riggs and Bates (1986) and Czernilofsky *et al.* (1986a,b). The former analysed 26 plants. Most plants transmitted kanamycin resistance as a single dominant marker, although three exhibited patterns consistent with the inheritence of homozygous lethal mutations. Southern blot analysis of nine plants suggested that one of these plants had integrated the transforming DNA at more than one site. Evidence for plasmid recircularisation or concatemer formation was found in most of the plants and in every case the EcoRI used to linearise the plasmid prior to transformation was lost; head-to-head and head-to-tail (incomplete) concatemers were seen. In six of the nine plants, an unexpected 6.5kb fragment was observed suggesting that, in addition to ligation, the plasmid had undergone some sort of regular rearrangement. It was suggested that the observed plasmid ligations were more likely to have occurred prior to integration. Similar results were obtained by Czernilofsky *et al.* (1986a,b); the integrated DNA occurred in multiple arrays of 10–25, mostly identically altered units of donor DNA.

Potrykus *et al.*, (1985c) also obtained G-418 resistant colonies after transformation of the cereal monocot, *Lolium multiflorum* by PEG treatment; the two colonies analysed by Southern blot both gave bands of the expected size (ie undeleted) when probed with an internal fragment with homology to the NPTII gene at copy number greater than five per haploid genome. This again suggested concatenation (although heredity could not be investigated as only callus was produced). Additional bands were not seen. Similarly, Fromm *et al.*(1986) transformed maize by electroporation and analysed four of the kanamycin resistant calli by Southern blot. All four contained between one and five copies of the intact gene; two of the calli also had rearranged copies of the gene present.

In 220 independent transformants genetically analyzed by Potrykus *et al.* (1987a) (see also Saul *et al.*, 1987) 77% passed on the kanamycin resistant phenotype as a single dominant locus, 8% as two independent loci, 2% as two linked loci, 2% as three or more linked or unlinked loci, 3% passed on the marker only when crossed as the female parent and 8% had patterns difficult to assign.

In the experiments described above, the foreign DNA segregated mostly as a single dominant genetic locus. The observation of primarily single loci inheritance has been confirmed at the molecular level in an elegant experiment in which metaphase plates from two independent transformants were examined by *in situ* hybridization using a ^{3}H labelled probe (Mouras *et al.*, 1987). After 12 weeks exposure it was possible to see in both cases a signal localised on (different) homologous chromosome pairs. This confirms that the transforming DNA had integrated into the plant chromosomes in single blocks.

It had previously been observed that calf thymus carrier DNA was integrated along with the DNA containing the selectable marker (Krens *et al.*, 1985) and this DNA was shown to be genetically linked to the selected DNA (Peerbolte *et al.*, 1985). This suggested that it should be possible to co-transform protoplasts with non-linked, selected and non-selected DNA's. This is indeed the case. Czernilofsky *et al.* (1986a) have successfully co-transformed tobacco (using calcium phosphate/PEG) with the selectable NPTII gene and the non-selected maize transposable element *Ac*. The rates of co-transformation are not given. Schocher *et al.* (1986) carried out similar experiments with tobacco protoplasts (transformed by electroporation) in which they co-transformed a zein genomic clone with an NPTII gene and a nopaline synthase (nos) gene with an NPTII gene. They found sequences from the zein clone in 88% of kanamycin resistant calli; the zein clone was full length in 33% of the cases. 47% of the kanamycin resistant calli co-transformed with the nos gene were found to have a complete and active nos gene present. These results further extend the potential utility of chemically and electroporation mediated DGT for the investigation of plant gene expression and crop improvement, particularly in species which are not amenable to *Agrobacterium* mediated transformation.

A cautionary note about DGT has been sounded by Negrutiu *et al.* (1987a) who pointed out a possible mutagenic effect of exogenous DNA; reversion rates in nitrate reductase-less plant cells were an order of magnitude higher in cells treated with DNA than the spontaneous reversion rates, and indeed even higher than the reversion rates induced by UV or gamma radiation. It is suggested that this may pose a problem when attempts are made to transfer native genes (particularly those for which there is no tight selection or specific assay) by means of transformation with total genomic DNA.

7.3.4 DGT by Other Means

7.3.4.1 Liposome and spheroblast mediated DGT

Artificial lipid vesicles (liposomes) and bacterial spheroblasts (bacteria from which the cell wall has been enzymatically removed) harbouring plasmids have both been used to transform plant protoplasts.

Expression of RNA introduced into tobacco protoplasts by liposome mediated transformation has been observed (Fraley *et al.*, 1982; Rouze *et al.*, 1983) at claimed efficiencies of between 20 and 70%. Tonaka *et al.*(1984) used *E.coli* spheroblasts to successfully infect *Brassica chinensis* mesophyll protoplasts with cloned cauliflower mosaic virus at an efficiency of between 1 and 5%.

Perhaps more interesting are the lipsome mediated stable transformations obtained by Deshayes *et al.* (1985) and the *E.coli* spheroblasts mediated gene transfer observed by Hain *et al.* (1984). In the former case a plasmid carrying a kanamycin resistance gene was successfully transferred to tobacco protoplasts and stably integrated at a frequency of ~10^{-5}. Three transformants analysed further all passed on kanamycin resistance as a single dominant trait; Southern analysis suggested that the transferred DNA was present in 4–5 tandemly repeated, essentially unrerarranged copies.

The potential advantages of liposome (and spheroblast) mediated DGT are the protection of sequestered nucleic acids from nuclease digestion during introduction into the cellular enviroment, no carrier DNA is required and it is possible that less reaarangement takes place. In a transient expression system, Rosenberg *et al.* (1988) found that if liposome mediated transformation was used to transfer CAT DNA to tobacco protoplasts, then CAT activity could be detected upto nine days post transformation (cf. 48 hours for naked DNA transformation).

On the other hand simpler protocols (electroporation and PEG mediated DNA uptake) give higher transformation efficiencies, preparation of liposomes requires sonication which may well shear DNA larger than 25kb (Deshayes *et al.*, 1985) and there is always the possibility that the components of the liposome system are toxic and/or mutagenic to plant cells.

7.3.4.2 Microinjection (reviewed by Miki *et al.*, 1987)

The physical microinjection of DNA directly into the cytoplasm or nucleus of plant protoplasts has been successfully attempted a number of times (Crossway *et al.*, 1986; Reich *et al.*, 1985; Steinbiss *et al.*, 1985).

The technique has progressed from the production of one transformant per scientist per year of intensive work (Steinbiss *et al.*, 1985) to the point where upto 10% of injected protoplasts give rise to transformants (Crossway *et al.*, 1986). The protoplasts were held on a holding pippette by slight suction while the DNA could be introduced selectively into either the cytplasmic or nuclear space using a microcapilliary pippette (Fig. 9). Following injection, individually injected protoplasts could be cultered in a hanging drop of media. A 30kb T-DNA containing plasmid was used and 1-2 copies of forcign DNA was integrated. In all cases the integrated DNA was less than full length and in only four of the eleven transformants obtained were sequences homologous to the NPTII gene found; it was suggested that micro injection through a small capillary shears the DNA.

Considering the extreme technical difficulty, high cost of micromanipulators and other equipment needed to carry out the experiments and relatively poor transfomation efficiencies (per man hour at least) compared to DGT mediated by PEG and electroporation, microinjection is unlikely to be of general use. This situation may change if technical difficulty decreases, or if it becomes possible to microinject plastids, nuclei or whole chromosomes, or into whole cells rather than protoplasts.

7.3.4.3 Electroinjection into whole cells

Recently Morikawa *et al.* (1986) introduced TMV RNA into intact tobacco mesophyll cells by electroporation in the absence of PEG. The RNA was shown to be expressed and a homogenate of the electroporated cells was shown to be infectious when inoculated onto tobacco leaves. The applicability of this technique to transient expression or stable transformation has been questioned by Negrutiu *et al.* (1987a); they asked whether the technique was applicable to cells that had been isolated by non–enzymatic treatment and observed that the electroporation conditions used would give extremely low transformation rates with tobacco protoplasts. However, Lindsay and Jones (1987) obtained a low level of CAT activity after transient transformation of intact sugar beet suspension cells by electroporation.

Further work will no doubt tell if this method is generally applicable; if it is it will be a step towards the transformation of plants which at present cannot be regenerated from protoplasts.

7.3.4.4 DGT into whole plants

Ohta (1986) reported efficient gene transfer to maize by the application of a viscous mixture of (foreign) total genomic DNA and ungerminated pollen onto silks. However, the process led to mutations at a high level (~9%) and there was no proof of transformation at the DNA or RNA level. Also transmission of the marker gene was unstable and crosses to determine the Mendelian (or otherwise) segregation of the marker gene in progeny were not reported. We await further reports on this technique with interest.

de la Peña *et al.* (1987) have reported the transformation of rye plants by injection of young floral tillers with DNA. Their previous work had demonstrated that 14 days prior to the first meiotic metaphase, archesporail cells are highly sensitive to exogenously applied chemicals injected into the developing floral tillers. They injected the developing floral tillers with an aqueous solution containing ~30μg of plasmid DNA carrying the NPTII gene. Seeds from the injected tillers were obtained by cross pollination with other injected tillers (rye is self incompatible). 3,023 seeds from the cross pollination of 98 plants were plated out on media containing kanamycin and of these 7 seedlings appeared to be resistant to kanamycin. From these 7 seeds 2 were shown to express NPTII enzyme activity and the presence of the gene was confirmed by Southern blot analysis where it was found to be integrated in multiple copies, some rearranged. This transformation at about 0.1% was the first confirmed report of a transgenic, graminaceous, monocotyledonous plant. It may turn out to be the method of choice for transforming monocots, avoiding, as it does, the need for tissue culture.

7.3.5 Conclusion

From the experiments described above it is clear that DGT complements *Agrobacterium* mediated transformation. It is unlimited by host range and allows the transient expression of introduced DNA and RNA; this is a great advantage in the investigation of plant gene function when stable transformation experiments take a minimum of months. It is possible that due to the different mechanism of DNA uptake and integration (cf. *Agrobacterium* mediated transformation) some experiments, such as gene targetting by homologous recombination, will be more facile by the DGT approach.

7.4 VIRUS VECTORS

Plant viruses have not yet fulfilled their promise as vectors for the genetic engineering of plants. They have the potential to complement the existing technologies of DGT and *Agrobacterium* effectively. They systematically infect whole plants and thus gene expression can be investigated in differentiated tissue as opposed to protoplasts or callus (particularly in plants recalcitrant to regeneration). Systemic infection typically takes place over days or weeks, not months, and viral particles accumulate to high levels leading to gene amplification and thus, possibly expression at high levels (see **7.4.2**). However, no plant virus so far discovered integrates into the host genome and transmission through the germline is not possible; transmission by vegetative propagation (cf. abutilon mosaic virus which is passed on in vegetatively propagated *Abutilon sellovianum*, and gives rise to the decorative mosaic of the leaves (Abouzid, 1986)) is possible and may even be an advantage to commercial plant breeding and genetic engineering companies. There are also problems with regard to limited host range and packaging constraints on the size of the cloned insert in viral vectors.

There are two broad groups of plant viruses, the DNA and the RNA viruses. The DNA viruses make up only 1-2% of known plant viruses (Lebeurier, 1986). They may be subdivided into two groups, the double stranded caulimoviruses and the single stranded gemini viruses. Because it is easier to work with DNA viruses most of the work (although not all) on vector development has been carried out on these viruses and we shall consider this group first.

7.4.1 Caulimoviruses

There are at least twelve members of the caulimovirus family (Hull and Davies, 1983) and of these the cauliflower mosaic virus (CaMV) is the best characterized. The biology of this virus has been recently well reviewed (Lichtenstein and Fuller, 1987; Gronenborn, 1987) and extensive literature references will be found therein. Reviews have also been published on the potential of this virus for vector development (Fraley *et al.*, 1986; Hull and Davies, 1983; Lebeurier, 1986; Lichtenstein and Fuller, 1987). We shall only describe the biology as it pertains to vector development and the evidence for the described biology will be found in the reviews cited above.

CaMV has a double stranded DNA genome of approximately 8kb in length with a host range

limited, in the main, to cruciferous plants such as cauliflower and turnip. Aphids are the natural transmission vector; however it is also infectious by mechanical inoculation and agroinfection (**7.2.7**).

The viral DNA is transcribed in the nucleus of infected cells by host encoded RNA polymerase II. There are two major transcripts produced (Fig. 10), a 35S RNA corresponding to the entire viral DNA plus a terminal repeat of 180 bp and a subgenomic messenger 19S RNA terminating at the same point as the 35S RNA. There are eight potential open reading frames (ORFs)(Fig. 10). The 19S transcript probably encodes ORF VI, respnsible for symptom development (Baughman *et al.*, 1988). It has been proposed that the other ORFs are translated from the polycistronic 35S transcript by a 'relay race' mechanism in which the ribosomes pause after passing the termination codon of one transcript and reinitiate at the next initiation codon on the transcript. It has also been suggested that ORFs IV and V could be expressed as a long precursor protein which is subsequently cleaved to produce two functional proteins. In support of the relay race mechanism, and of impotrtance to vector design, is the observation that a nonsense mutation in ORFII affects the translation of the downstream ORFIII in a polar fashion (there is other evidence for the relay race mechanism, see Lichtenstein and Fuller, 1987); clearly, any foreign DNA cloned CaMV must take account of the polycistronic nature of the viral 35S transcript.

The virus replicates by priming of the 35S transcript with a host encoded t–RNA^{met} followed by minus strand DNA synthesis catalysed by a virus encoded reverse transcriptase.

ORFII has been shown to be an aphid transmission factor (Daubert *et al.* (1983) and is not essential for viral infectivity as a naturally occurring, severely deleted mutant has been isolated; ORFVII has also been shown to be dispensible (Howarth *et al.*, 1981; Howell *et al.*, 1981). Gronenborn *et al.*, (1981) successfully cloned foreign DNA into ORFII and found that the upper limit for foreign DNA propagated by CaMV in this way was ~250bp. Brisson *et al.* (1984) replaced ORFII with a small (234bp) bacterial dihydrofolate reductase (dhfr) gene and it was expressed and stably propagated in turnip plants. This gene confers resistance to the compound methotrexate, and turnip plants infected with CaMV containing the dhfr gene were resistant to methotrexate. It was noted that when one of the constructs used had a slightly longer non coding region (47bp) between the end of ORFI and the start of the dhfr gene than the other (9bp); the former construct was unstable to passage through the plant and expressed the dhfr gene at a reduced level compared to the latter. This sensitivity to intergenic sequences found in CaMV shows the care that has to be taken when designing vectors of this kind.

In the simple CaMV vector approach described above the limit for cloned inserts is probably about 800bp (by deletion and addition). A different approach is to construct complementing defective mutants, such that the 'vector' mutant carries the foreign DNA and only a few CaMV functions, and the other mutant carries all of the complementing CaMV ORFs (but is deficient in the ORFs carried on the 'vector'). This approach has been tried (Choe *et al.*, 1985); pairs of complementing, non-infective CaMV mutants were constructed and used in a mixed infection. Successful infection occurred and **wild type virus was rescued in all cases**; recombination events had deleted the foreign DNA. This high frequency of recombination in CaMV has been observed before (Walden and Howell, 1982). Perhaps if complementing defective mutants can be constructed with little or no homology this approach to vector design will bear fruit. Pazkowski *et al.*, (1986) have also attempted to use complementation as a route to using CaMV as a vector; they replaced the essential ORFVI (translated from the 19S transcript) with the coding region from NPTII. Unfortunately, this mutant was non–infective both on its own and when complemented by the wild type virus. The NPTII gene was expressed from the viral promoter when the mutant virus was transferred to turnip protoplasts by direct gene transfer but there was no escape of the virus and viral sequences were only associated with high molecular weight genomic DNA.

Another approach to complementation in CaMV vectors would be to put a complementing defective virus genome into the plant genome by means of *Agrobacterium* mediated transformation. This would give a system equivalent to the COS cell system for the propagation of defective mammalian SV40 virus based vectors (Gluzman, 1981) (see **7.4.2** for a successful use of this approach in plants). The feasibility of this approach in CaMV based vectors is beginning to be demonstrated. The work of Pazkowski *et al.* (1986) described above demonstrates that gene products of mutant CaMV genomes cloned into plants can be expressed; Shewmaker *et al.* (1985) have cloned a full length copy of CaMV into a variety of plant species and shown that both the 19S and 35S transcripts are expressed (at varying levels in different species). Furthermore, Walden (pers.

comm.) has made transgenic tobacco plants (outside the normal hostrange of CaMV) containing dimers of CaMV and shown that leaf homogenate from these plants is infectious when inoculated onto turnip; it is not yet clear if the virus replicates in tobacco, or if the infection in turnip is mediated by the 19S and 35S transcripts. Perhaps, tobacco, which is more amenable to genetic transformation, can be used as a model system for this work. Clearly, much still needs to be done before CaMV becomes the basis of a useful vector system; in the light of the exciting work on gemini virus based vectors recently reported (see below) we should seriously consider whether it is worth expending such effort.

7.4.2 Geminiviruses

Gemini viruses (reviewed by Stanley, 1985; Harrison, 1985; Davies *et al.*, 1987) are single stranded DNA viruses that replicate in the nuclei of cells via a double stranded DNA intermediate. The name derives from the bisegmented nature of the virions as they appear in electron micrographs. Cloned copies of several members are, between them, transmissible to a wide host range including both monocots and dicots (Harrison, 1985). Gemini viruses may be subdivided into whitefly and leafhopper transmitted virus; leaf hopper transmitted viruses in general have monopartite genomes, whilst whitefly transmitted viruses have bipartite genomes as described in **7.2.7** for TGMV (genome lengths for both groups are ~2.5kb). It is members of this latter class that have recently been reported as plant vectors.

It has previously been shown that the bipartite cassava latent virus (CLV) DNA 1 encodes functions essential for replication but not systemic infection or cell–to–cell transmission (Townsend *et al.*, 1986); it has also been shown that DNA 1 encodes the coat protein which is not essential for infectivity (Stanley and Townsend, 1986). Ward *et al.* (1988) have shown that although deletion of the coat protein gene abolishes infectivity by manual inoculation with linear DNA 1 and DNA 2 molecules, mutants in which it is replaced by sequences of similar size are fully infectious. When the coat protein gene was replaced by the bacterial CAT gene and the engineered DNA 1 inoculated onto *Nicotiana benthamiana* together with wild type DNA 2, the plants developed normal symptoms of infection and CAT was expressed systemically at high levels (80units/mg soluble protein) 10 days post inoculation. The level of CAT activity was monitored over a period of four weeks and found to be maintained. Complementing defective mutants of CLV undergo frequent recombination to give wild type virus (Ward *et al.*, 1988). However, no instability was observed in the virus expressing the CAT gene, presumably because there is no selective pressure for deletion of this gene.

Similar work has also been carried out on the closely related tomato golden mosaic virus (TGMV) by Hayes *et al.* (1988c). It is useful to recap some of the work on this virus described in **7.2.7** before describing the results.

When dimers of TGMV DNA A were cloned into the chromosome of petunia plants (*via Agrobacterium* mediated transformation) freely replicating DNA A components were found (Rogers *et al.*,1986b). Trimers of TGMV B were cloned into the chromosome of petunia, but no freely replicating DNA B molecules were found. However, if these A2 and B3 plants were crossed, a systemic infection resulted. This suggested that perhaps the A particle encodes the functions necessary for replication and the B particle those neccesary for cell to cell spread and symptom development. *Agrobacterium* strains containing B dimers complemented A2 plants (containing dimers of A in the genome), and *Agrobacterium* strains containing A dimers complemented the B3 plants (containing trimers of B in the genome); systemic infection was seen in both cases. The virus coat protein is not required for systemic infection of plants (Brough *et al.*, 1988; Hayes *et al.*, 1988b).

Hayes *et al.* (1988c) constructed vectors in which the NPTII coding region replaced the coat protein coding region. These vectors contained either 1.6 copies of tandemly repeated NPTII encoding TGMV A DNA (pBIN19A1.6*neo*) or 1.6 copies of tandemly repeated NPTII encoding TGMV A DNA plus 2 copies of TGMV B DNA (pBIN19A1.6*neo*B2) between T–DNA borders, analogous to those described in the agroinfection experiments (**7.2.7**) (Hayes *et al.*, 1988b). Wild type tobacco plants were agroinfected with pBIN19A1.6*neo*B2, and B2 transgenic plants (containing TGMV B DNA integrated into the genome) were agroinfected with pBIN19A1.6*neo*; four out of twenty of the former and eighteen out of twenty of the latter were systemically infected as determined by DNA dot blot analysis using probes specific for TGMV DNA A and DNA B. None of the *neo* infected plants showed any symptoms. The infections were confirmed by

Southern blot; single stranded, supercoiled and open circular forms of viral DNA of the expected size were found. When the 'wildtype' *neo*less pBIN19A1.6B2 construct was used to infect tobacco plants, infection was more efficient and nineteen out of twenty developed symptoms.
Plants containing A1.6*neo* DNA integrated into the chromosome were also constructed. On Southern analysis the expected monomeric forms of TGMV A *neo* DNA (with the NPTII coding region replacing the coat protein gene) were found in the plant DNA.
NPTII activity was assayed and the relative activities were as follows: transgenic plant containing 7 copies of NPTII regulated by the nos–promoter, 1; transgenic plant containing A1.6*neo* DNA, 40; wildtype plant agroinfected with pBIN19A1.6*neo*B2, 80; and transgenic B2 plant agroinfected with pBIN19A1.6*neo*, 240!
The size of the NPTII expressing TGMV A DNA is 2708bp compared to the wild type DNA A which is 2588bp. The size limit of this vector has since been extended by inclusion of the coding region from the glucuronidase (GUS) gene in place of the NPTII coding region (Hayes, pers. comm.); this is an increase of 600bp.
There are some interesting differences between the work on CLV and that on TGMV. The loss of infectivity observed in CLV coat protein deletion mutants was not observed in TGMV coat protein deletion mutants, suggesting that perhaps the size constraints in CLV are tighter than those in TGMV. Also, the TGMV construct produced symptomless plants while the CLV construct did not. As the marker gene, host, and method of infection differed in the two experiments, it is not possible at this stage to say whether the differences observed are due to the viruses or the way in which the experiments were carried out.
Gemini virus based vectors appear to hold great promise. They seem to provide useful means of gene amplification in plants; either by systemic infection of plants with the vector to provide a rapid assay of gene expression, or by producing plants transgenic for a partial dimer of the vector to provide inheritable gene amplification. The upper limit of foreign DNA that can be replicated by a gemini virus vector has not yet been determined but is not limited by packaging constraints as it is in the caulimovirus vectors. Furthermore, geminiviruses infect a wide range of plant species.

7.4.3 RNA Viruses

The potential general utility of RNA virus based vectors is uncertain. To our knowledge, there have been no demonstrations of directly infectious cDNA forms of RNA viruses; thus infection by *in vitro* synthesised RNA transcripts is the only obvious route to engineered RNA viruses (Fraley *et al.*, 1986 and references therein). It has also been suggested that high error frequency during viral RNA synthesis will cause instability in foreign genes (van Vloten–Doting *et al.*, 1985). There is no selective pressure to correct errors induced in foreign genes, mutations in endogenous viral genes will be lethal. This suggestion has been disputed (Siegel,1985).
There have been two recent reports of foreign genes expressed in RNA viral vectors. French *et al*, (1986) have reported the expression of CAT mediated by RNA transcripts of a coat protein fusion of Bromo mosaic virus in barley protoplasts. Since protoplasts were used, it is difficult to compare the results to those obtained with other viral vectors. Takamatsu *et al.* (1987) made similar constructs in tobacco mosaic virus which produced local lesions when the RNA transcripts were inoculated on tobacco leaves. CAT was detected (0.1unit/mg of soluble protein); this is 0.125% of the activity obtained with the gemini virus CLV based vector. Also, the coat protein is necessary in TMV for systemic infection and no CAT or virus was detected on leaves distal to the site of inoculation.
These results suggest that that a great deal more work must be done if RNA virus based vectors are to have any realistic future.

7.5 TRANSPOSON VECTORS (reviewed by Wienand and Saedler, 1987)

Transposons are pieces of DNA (often encoding drug resistance in bacteria) that can jump from one piece of DNA to another *in vivo*. Transposons were first observed in maize by Barbara McClintock (McClintock, 1951), andfor this work she won the Nobel prize. The transposon she discovered, the *Ac* element, creates an 8bp duplication at the site of integration, and it can excise itself precisely from one part of the genome and integrate in another, distal position.
This pioneering work by McClintock has recently led to the possibility of transposon vectors. The *Ac* element from maize has been transferred to tobacco by *Agrobacterium* mediated transformation where it was shown to excise and reintegrate elsewhere as in its natural 'host', maize (Baker *et al.*, 1986). The same group has developed a phenotypic assay for excision of the *Ac*

element in tobacco (Baker *et al.*, 1987). The *Ac* element has been cloned between the promoter and coding region of the NPTII gene so as inactivate it; upon excision of the *Ac* element, a functional NPTII gene is recreated. After *Agrobacterium* mediated transfer to tobacco, excision events could be monitored by the appearance of kanamycin resistant colonies. If selection was applied 10–12 days after co–cultivation kanamycin resistant calli were detected in the *Ac* transformed clones at between 13 and 40% the number found with a wild type NPTII gene. If, however, selection was not applied until 3–4 months following co–cultivation, the number of kanamycin resistant calli went up to 70% suggesting that *Ac* excision continues for >2weeks after its introduction into tobacco cells. The results were confirmed by Southern blot analysis.

Several plant genes have been cloned by gene tagging using transposons. A mutated gene carrying a transposon insert is first isolated using a previously cloned transposon as a probe to find the element and flanking DNA; the flanking DNA is then used as a probe to isolate the gene from the wild type line (references in Baker *et al.*, 1987). Gene tagging has previously been limited to the few plant species which possess well characterized transposable elements. In principle, this work could extend the usefulness of gene tagging to any plant species which is amenable to genetic transformation (by *Agrobacterium* or DGT) and whole plant regeneration, so long as the *Ac* element functions in that species.

7.6 ORGANELLE TRANSFORMATION

Organelle transformation is of interest, both because such commercially important things as cytoplasmic male sterility and resistance to the triazine herbicides are encoded there, and also because much of a plant's fundamental biochemistry (eg. photosynthesis) occurs in organelles.

Transfer and expression of foreign genes in plant organelles is difficult; much of the unpublished (and unsuccessful) work on organelle transformation carried out in the last few years has been reviewed by Cornelissen *et al.* (1987). There are upto a thousand organelle genome copies in a single plant cell (Cornelissen *et al.*, 1987); by comparison with bacteria, we can see that there are strong possibilities that selective replicative disadvantage and recombination events will quickly remove a recombinant plastid or mitochondrial genome. Furthermore, differential codon usage in mitochondria poses a potential problem.

De Block *et al*, (1985) have reported a successful chloroplast transformation mediated by *Agrobacterium* co–cultivation. A chimaeric gene consisting of the promoter from the nopaline synthase gene (*Pnos*) fused to the coding region of the CAT gene. Chloramphenicol resistant plants obtained from chloramphenicol resistant calli were unable to transmit the phenotype through pollen and a typically maternal inheritance pattern was observed. The CAT activity was shown to be localised exclusively in the chloroplast fraction by Southern blot analysis and CAT assay on isolated nuclei, mitochondria and chlorolplasts. The CAT activity and associated DNA was lost from plants not grown under chloramphenicol selection.

Numerous attempts to extend this work using chloroplast specific promoters (Cornelissen *et al.*, 1987) have been unsuccessful; it has been suggested that the promoters used were too strong and the success enjoyed by De Block *et al*, (1985) was due to the use of the Ti plasmid derived *nos* promoter (Cornelissen *et al.*, 1987); this eukaryotic promoter is weakly functional in bacteria (and thus presumably, plant organelles). Progress in organelle transformation is probably going to be slow and difficult.

An alternative approach is to target nuclear gene products into chloroplasts (reviewed by Szabo and Cashmore, 1987). A particularly clever example is given by the work of Cheung *et al.* (1988). The coding region from a chloroplast gene *psbA* (encoding resistance to the herbicide, atrazine) was fused to the transcriptional and transit peptide sequences of the nuclear, rubisco small subunit gene. This chimaera was transferred to the nuclear genome of tobacco by *Agrobacterium* mediated transformation and the foreign protein was made and transported to the chloroplasts. Atrazine tolerant tobacco plants resulted.

Another type of organelle transformation, which is discussed in chapter 9, is the transfer of organelles from one species to another by protoplast fusion. An example is the transfer of terbutryn (herbicide) resistant plastids from *Nicotiana plumbaginofolia* to *N.tabacum* without transer of the accompanying mitochondrial encoded cytoplasmic sterility (Menczel *et al.*, 1986). This could not have been accomplished by standard plant breeding techniques.

7.7 CONCLUDING REMARKS.

As stated in the introduction, plant genetic engineering is yet a young science. Although it has progressed by leaps and bounds, is still in its infancy. The development of techniques for plant genetic transformation have been rapid, and progress continues to be rapid. The complementary technique of plant tissue culture (Ch. 12) has to catch up before the full commercial and scientific potential of plant genetic engineering can be realised.

Our knowledge of plant biochemistry and development is, as yet, not as advanced as our knowledge of bacterial,fungal, *Drosophila* and mammalian systems. As the technology of plant genetic manipulation becomes as versatile and sophisticated as that available in other systems, so our understanding of plants at the molecular level will increase accordingly. As the function of new genes is elucidated, so our ability to engineer plants for specific traits will increase. In the study of developmental biology plants have a number of advantages over other systems; they are totipotent, differentiated cells can be de– and re–differentiated, sometimes into whole plants or isolated tissue types. Furthermore, fully differentiated transgenic plants can be produced from a single cell in a matter of only weeks.

We feel that the future of plant genetic engineering will be both exciting and intellectualy challenging.

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FIGURE HEADINGS

Figure 1: Octopine Type Ti–plasmid.

Figure 2: Mechanism of *Agrobacterium* infection: i. *Agrobacterium* move up a plant wound exudate concentration gradient towards wounded plant tissue. Once they reach the wounded plant cells, they attach themselves.; ii. (A) Wound exudate stimulates the vir–region of the Ti–plasmid to (B) act upon the T–region and initiate (C) T–strand transfer and integration.; iii. Undifferentiated callus tissue proliferates from the wound site and synthesises opines. The opines are catabolised by the *Agrobacterium* and induce conjugal transfer of the Ti–plasmid from one *Agrobacterium* to another.

Figure 3: Opines

Figure 4: Acetosyringone

Figure 5: Transfer of shuttle vectors to *Agrobacterium* by triparental mating: i. The *mob* and *tra* genes of pRK2013 act on it's own origin of transfer (*ori T*) and it is transferred to the vector strain.; ii. Once in the vector strain the *mob* and *tra* genes of pRK2013 can act *in trans* on *ori T* of the shuttle vector and transfer it to *Agrobacterium*.; iii. Recombination (1) between homologous regions on the shuttle and Ti–plasmids gives rise to transfer of DNA from the shuttle vector to the Ti plasmid.; iv. The shuttle vector does not have an origin of replication which functions in *Agrobacterium* and is lost (2), leaving a recombinant Ti–plasmid behind.

Figure 6: An Example of a Binary Vector, pGA482.

Figure 7: Rearrangements Made by T–DNA Insertion (after Gheysen *et al.*, 1987): (*upper*) The unnocupied target site. (*Lower*) Diagram of the left and right border junctions of the T–DNA insertion. T–DNA is shown as an interrupted black box, and the 158bp target duplication is shown as an empty box. On the right there is the 158 bp duplication (open box), an unchanged 7bp sequence (black square), the deletion of target sequences (dotted line) and the CoT transition (filled circle). On the left, rearrangements include the 158bp duplication and the 'filler' DNA (wavy line). Arrows indicate homology of 'filler' DNA to other sequences.

Figure 8: A. Crown Gall Tumour.on a sugar beet stem; B. Rooty (Ri) Tumour.on carrot discs **(Not shown in Appendix).**

Figure 9: Microinjection: The protoplast is held with gentle suction by a holding pipette and DNA is injected directly into the nucleus with a micro–capilliary pipette.

Figure 10: The CaMV Genome.

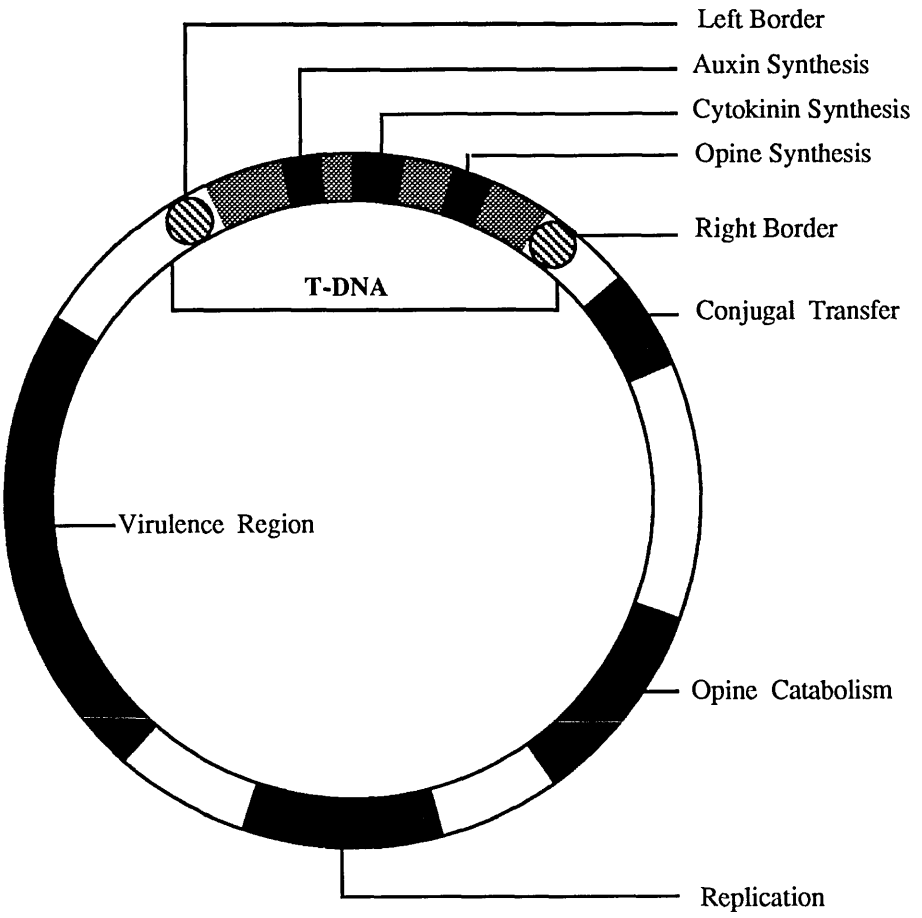
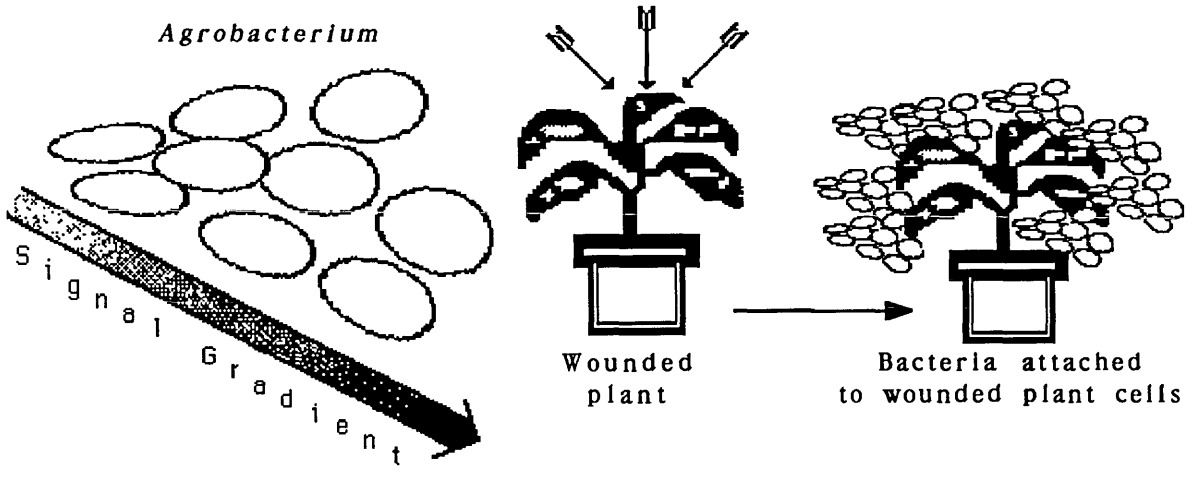
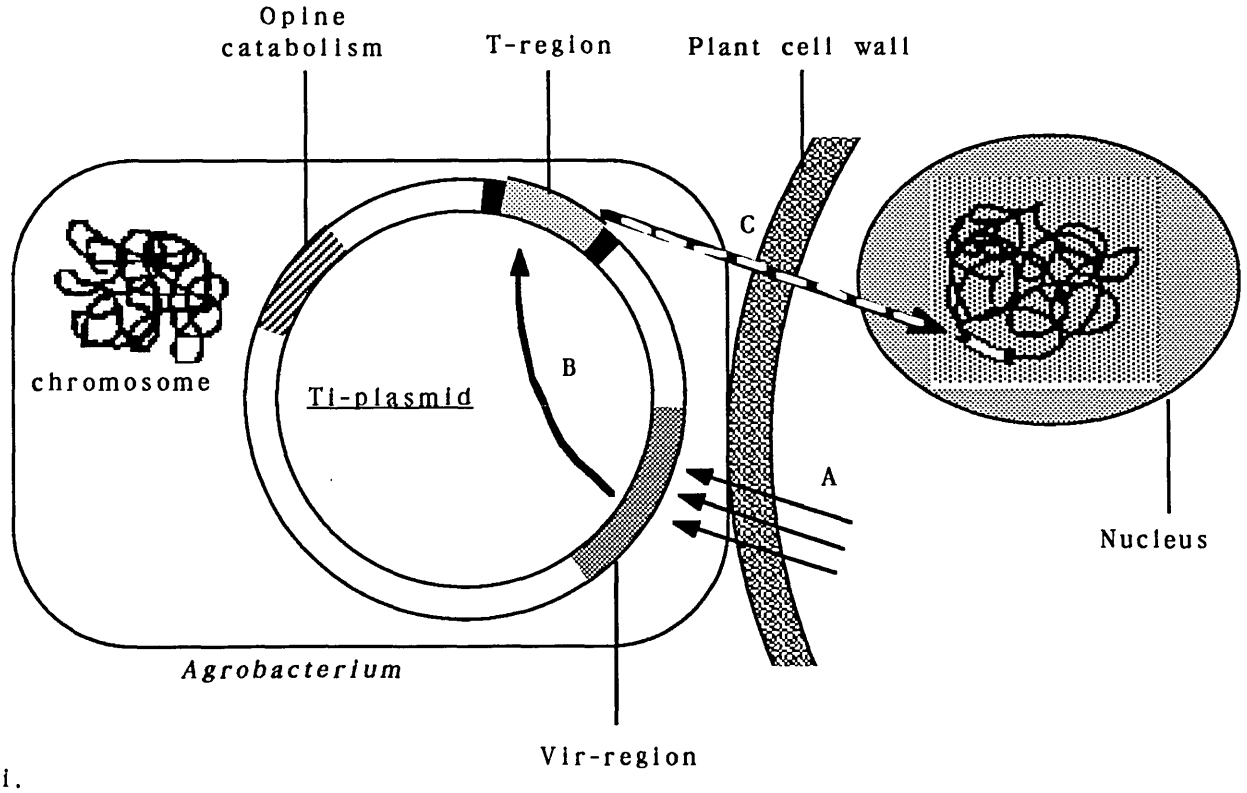

Left Border
Auxin Synthesis
Cytokinin Synthesis
Opine Synthesis
Right Border
T-DNA
Conjugal Transfer
Virulence Region
Opine Catabolism
Replication

Figure 1.

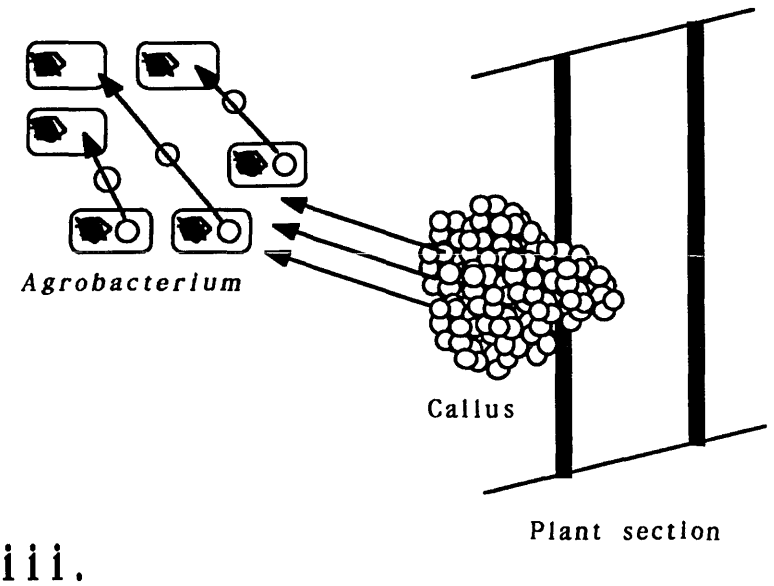


i.



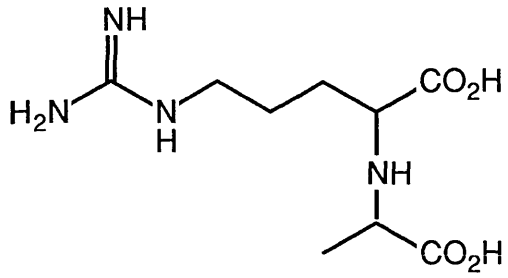
ii.

Figure 2.i and Figure 2.ii.

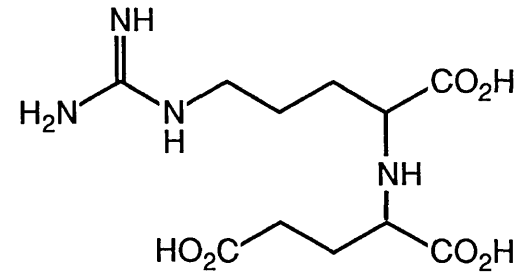


iii.

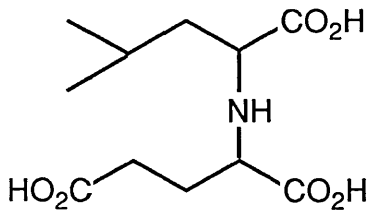
Figure 2.iii.



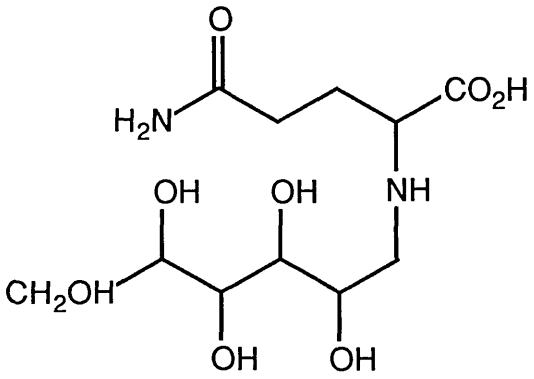
Octopine



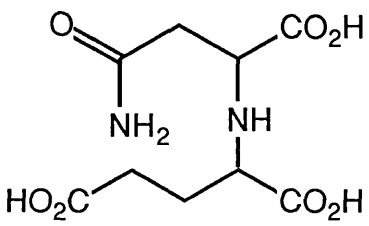
Nopaline



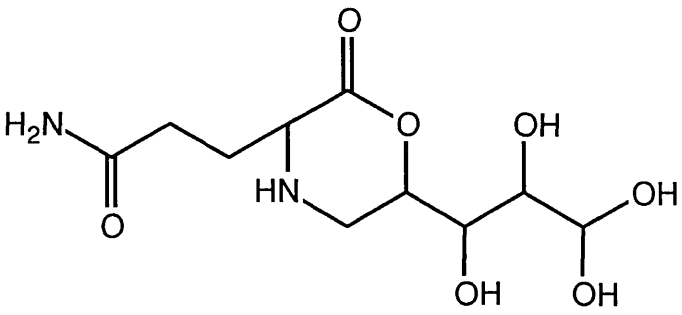
Leucinopine



Mannopine



Succinamopine
(Asparaginopine)



Agropine

Figure 3.

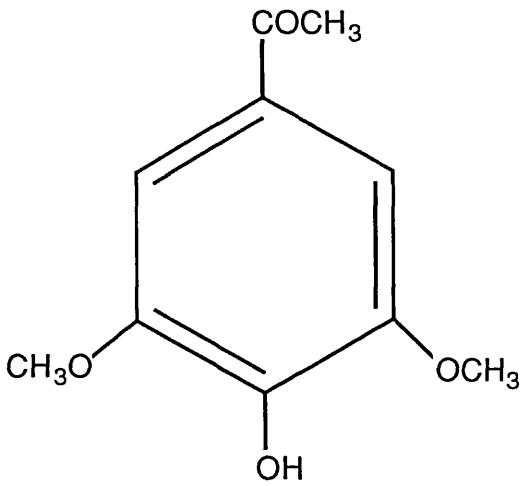


Figure 4.

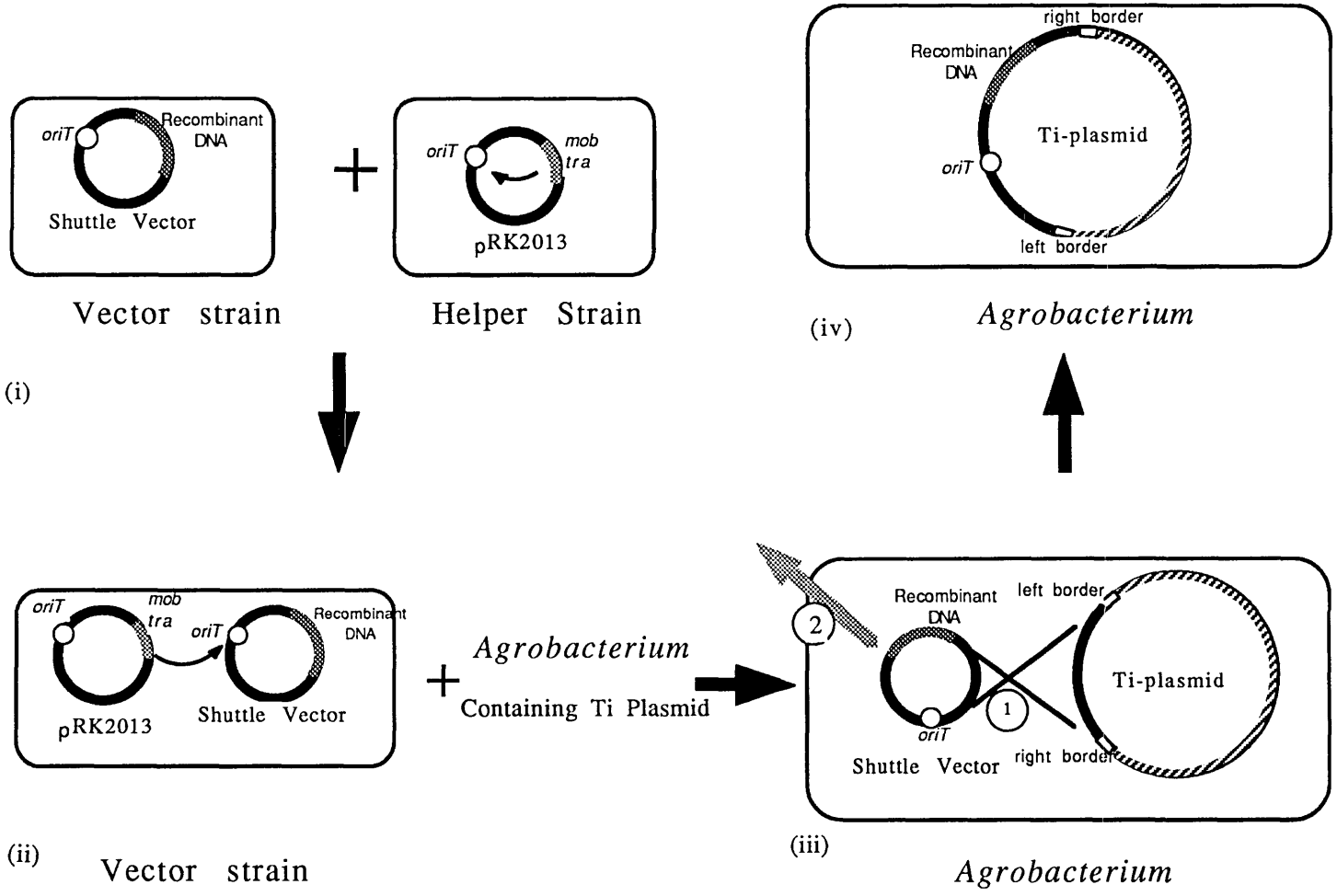

oriT
Recombinant DNA
Shuttle Vector
oriT
mob
tra
pRK2013
Vector strain
Helper Strain
(i)
right border
Recombinant DNA
Ti-plasmid
oriT
left border
(iv)
Agrobacterium
oriT
mob
tra
oriT
Recombinant DNA
pRK2013
Shuttle Vector
Agrobacterium
Containing Ti Plasmid
2
Recombinant DNA
left border
Ti-plasmid
1
oriT
Shuttle Vector
right border
(ii)
Vector strain
(iii)
Agrobacterium

Figure 5.

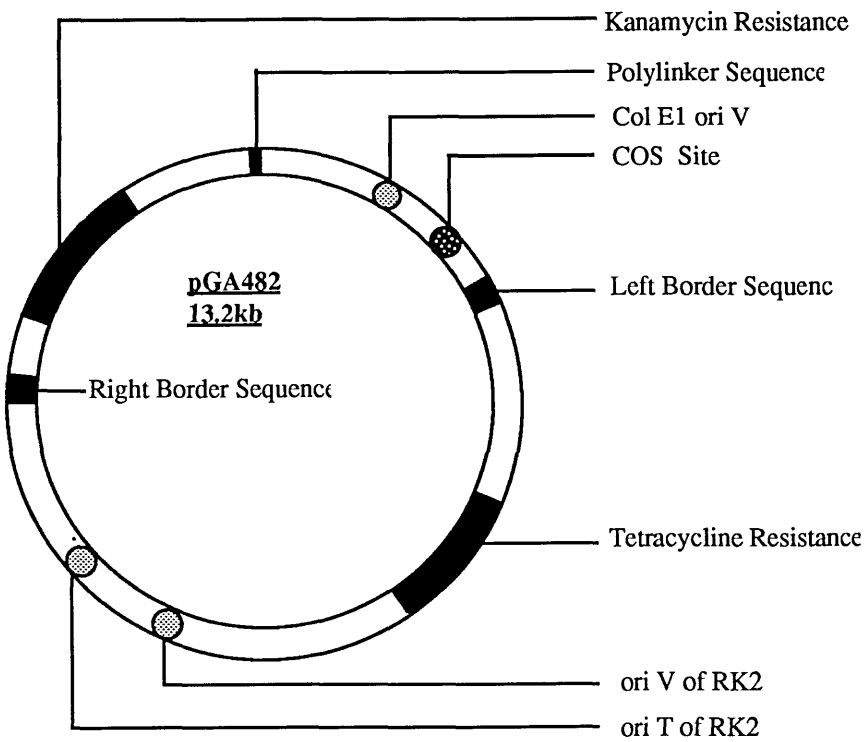

Kanamycin Resistance
Polylinker Sequence
Col E1 ori V
COS Site
pGA482
13.2kb
Left Border Sequenc
Right Border Sequenc
Tetracycline Resistance
ori V of RK2
ori T of RK2

Figure 6.

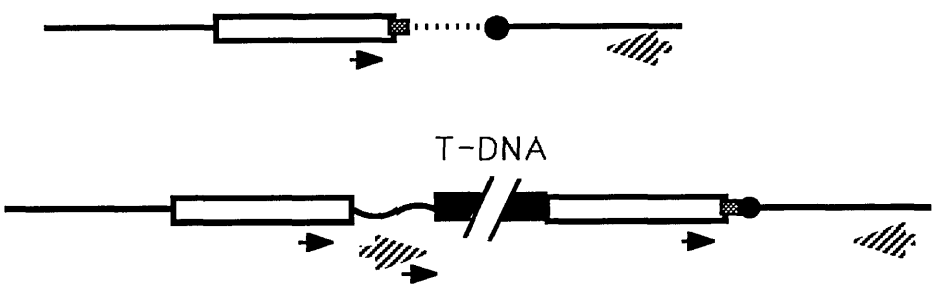


Figure 7.

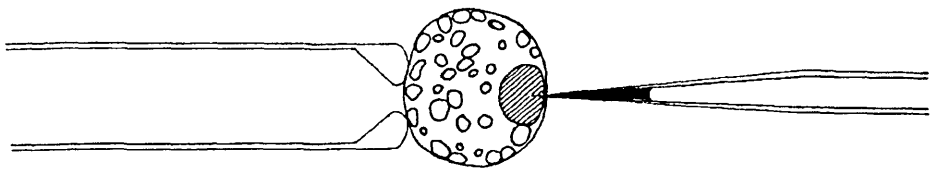


Figure 9.

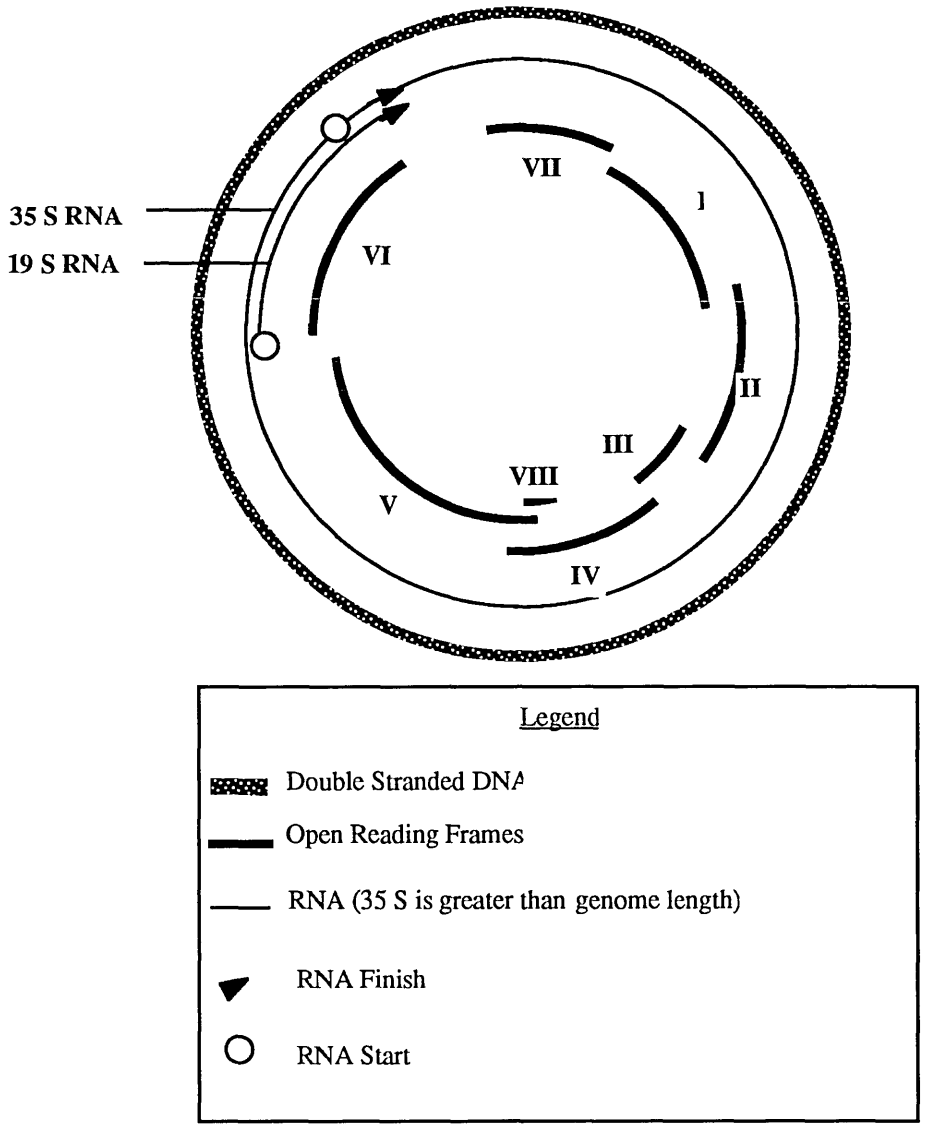

35 S RNA
19 S RNA
VII
I
VI
II
III
VIII
V
IV
Legend
Double Stranded DNA
Open Reading Frames
RNA (35 S is greater than genome length)
RNA Finish
RNA Start

Figure 10.

APPENDIX II

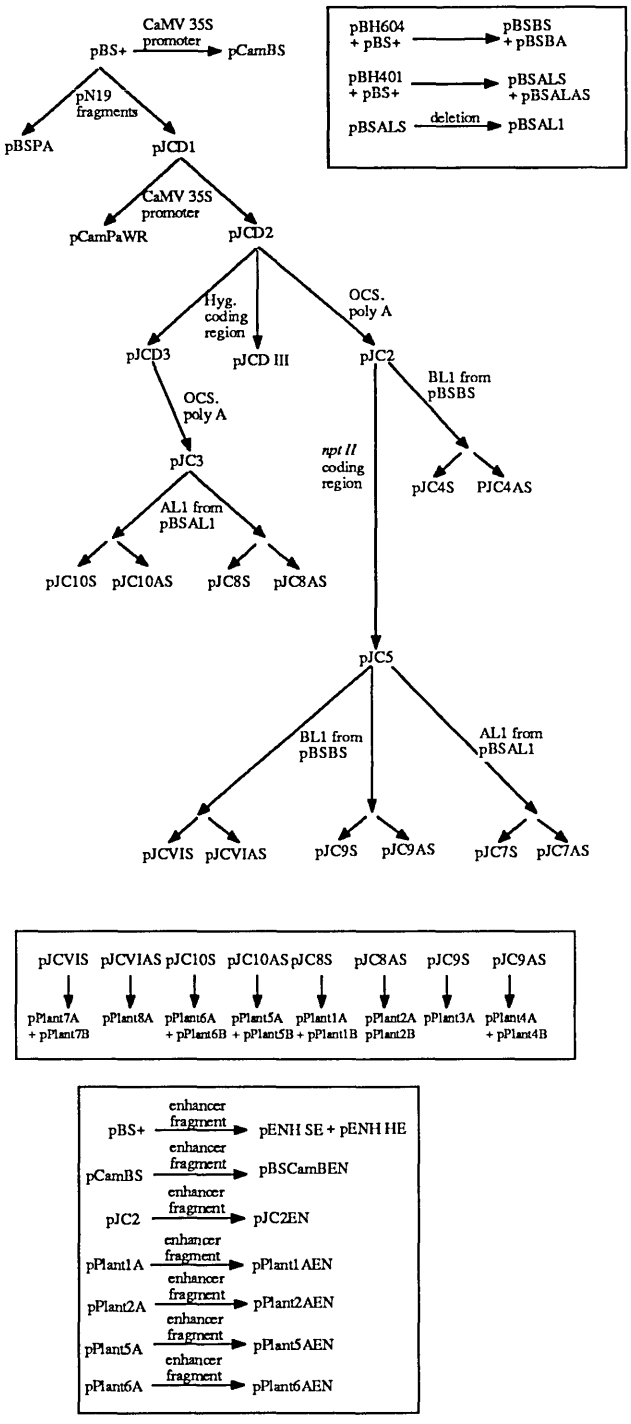


Figure II.1. Lineage of plasmids constructed.

Plasmid	Brief description
pJCD1	incorrect fragment cloned into pBS+
pJCD2	CaMV 35S promoter & incorrect fragment cloned into pBS+
pJCD3	hygromycin resitance gene cloned between the CaMV35S promoter and the incorrect fragment in pBS+
pCamBS	CaMV 35S promoter cloned into the Sma I site of pBS+
pBSPA	*nos* polyadenylation signal cloned into the Sma I site of pBS+
pJC2	CaMV 35S promoter & *ocs* polyadenylation signal cloned into part of pBS+
pJC3	hygromycin resitance gene cloned between the CaMV35S promoter and the *ocs* polyadenylation signal in part of pBS+
pBSBS & pBSBA	BL1 coding region cloned in both orientations in pBS+
pJC4S & pJC4AS	BL1 coding region cloned in both orientations between the CaMV 35 S promoter and *ocs* polyadenylation signal
pJC5	*npt II* coding region cloned between he CaMV 35 S promoter and *ocs* polyadenylation signal
pJCVIS & pJCVIAS	BL1 coding region cloned downstream of the *npt II* coding region in both orientations in pJC5
pBSALS & pBSALAS	undeleted AL1 gene cloned into the Eco RI site of pBS+
pBSAL1	deleted AL1 gene cloned into the Sma I site of pBS+
pJC7S & pJC7AS	deleted AL1 gene cloned upstream of the *npt II* coding region in both orientations in pJC5
pJC8S & pJC8AS	deleted AL1 gene cloned upstream of the hygromycin resistance coding region in both orientations in pJC3
pJC9S & pJC9AS	BL1 coding region cloned upstream of the *npt II* coding region in both orientations in pJC5
pJC10S & pJC10AS	deleted AL1 gene cloned downstream of the hygromycin resistance coding region in both orientations in pJC3
pPlant and pPlantAEN series	refer to main text
pENH SE & pENH HE	CaMV 35S enhancer sequence cloned into the Sma I site of pBS+
pBSCamBEN	enhanced CaMV 35 S promoter cloned into pBS+
pJC2EN pJC2 with enhancer sequence cloned upstream of the CaMV 35S promoter	

Table II.1. Summary of plasmids constructed in this thesis.

APPENDIX III

During the course of this project, I supervised a M.Sc. student, Esther Cabanes. The following paper arose from work carried out during her M.Sc. course and the early part of her Ph.D. course.

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Short Communications

A sensitive and simple assay for neomycin phosphotransferase II activity in transgenic tissue

(Thin-layer chromatography; kanamycin phosphate; NPTII; autoradiography; aminoglycoside antibiotics)

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SUMMARY

A new assay for the detection of the enzyme neomycin phosphotransferase II (NPTII) in crude cell extracts is described. The method is based on the chromatographic separation of the compounds resulting from the reaction of NPTII with kanamycin (Km) and [γ-^{32}P]ATP; the labelled Km · phosphate is subsequently detected by autoradiography. Chromatography is carried out on polyethyleneimine cellulose plates. This assay has been tested with bacterial, plant and animal crude extracts and comparisons with the assays in current use have been made. Our assay has several advantages: it is simple, rapid, sensitive and safe.

INTRODUCTION

A widely used selectable marker in many different transformation systems is the enzyme NPTII. NPTII is encoded by the *nptII* gene, present in the bacterial transposon Tn*5*, and confers resistance on its host cells to several aminoglycoside antibiotics, such as neomycin, Km and G418. Due to its utility as a selectable marker in prokaryotes and eukaryotes, including both plant and animal cells, considerable efforts have been made in trying to develop an easy assay for this enzyme.

Currently, two different assays are commonly used; both of them are based on the use of ATP labelled at the γ-phosphate. During the reaction, the γ-phosphate is transferred from ATP to Km. However, the [γ-^{32}P]ATP is also used as a substrate for the phosphorylation of a variety of other compounds (e.g., proteins) by other enzymes. In one assay, the so-called 'dot' assay (Haas and Dowding, 1975), the phosphorylated antibiotic is separated from ATP and the other contaminating compounds by virtue of

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Abbreviations: DTT, dithiothreitol; Km, kanamycin; K_m, Michaelis constant; nd-PAGE, non-denaturing polyacrylamide gel electrophoresis; NPTII, neomycin phosphotransferase II; *nptII*, gene coding for NPTII; PEI, polyethyleneimine; Rf, relative front, TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; Tn, transposon; wt, wild type.

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its affinity for phosphocellulose ion-exchange paper, which preferentially binds Km-phosphate. Free ATP is then simply washed from the paper and the amount of immobilised radioactivity is measured by scintillation counting. This method has been successfully used to measure NPTII activity in bacteria.

However, we and others (Draper et al., 1988), have found that this assay is not sensitive enough to detect NPTII in plants. The enzyme activity found in transgenic plants is usually considerably lower than that found in bacteria. 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 is below the limits of detection. There is also a strong background due to the binding to the phosphocellulose paper of other compounds that have been phosphorylated during the reaction.

To overcome the background problem, a modified assay was developed by Reiss et al. (1984) and tested on bacterial and animal cell extracts. Here a crude extract of tissue is fractionated by nd-PAGE to separate NPTII from other proteins, e.g., phosphorylases. Then NPTII activity is detected in situ, by phosphorylation of Km with [γ-^{32}P]ATP in an agarose gel overlay. After the reaction, the phosphorylated Km is transferred to phosphocellulose paper by capillary blotting and, after washing, visualised by autoradiography. This is the commonly used procedure for the detection of NPTII in plant tissue, but it is complicated, time-consuming and involves the spreading of large amounts of radiolabelled ATP on the gel; hence the need for a cleaner, more rapid and convenient assay.

Recently, some improvements were introduced to the dot assay, described above, to prevent non-specific adsorption to the phosphocellulose paper. Platt and Yang (1987) suggested that the use of a nitrocellulose membrane combined with the phosphocellulose paper, eliminates the background, since phosphorylated proteins bind to the nitrocellulose. McDonnell et al. (1987) also introduced some modifications to the original dot assay. First of all, the phosphocellulose paper was pre-treated with unlabelled ATP, in order to reduce non-specific binding; secondly, the [γ-^{32}P]ATP was diluted with non-labelled nucleotides; finally, sodium fluoride (an inhibitor of phosphatases) was added to inhibit the hydrolysis of Km · phosphate and ATP.

We have developed a new, rapid and simpler assay for the detection of NPTII, based on the chromatographic separation of Km · phosphate from the rest of components of the reaction mixture on PEI-cellulose plates. Radiolabelled Km · phosphate is visualised by autoradiography. This assay is more sensitive than those reported previously.

EXPERIMENTAL AND RESULTS

(a) Materials, bacterial strains, plant tissue and animal cells

PEI-cellulose plates were purchased from Camlab and [γ-^{32}P]ATP (10 mCi/ml, > 6000 Ci/mmol) from Amersham. All other chemicals were obtained from Sigma.

The bacterial strain (*Escherichia coli*) used as a negative control was DH5α (Jessee, 1986). As positive controls, either of two different *E. coli* strains were used, harbouring the plasmids pRK2013 (Figurski and Helinski, 1979) or pGS9 (Selvaraj and Iyer, 1983); both plasmids encode NPTII.

The plant material used were leaves and calli from *Nicotiana tabacum*, cultivar petit havana SR1. The test material, transgenic SR1 plants expressing NPTII, were generated by transformation with a chimæric gene comprising the coding region of the bacterial *nptII* gene flanked by a promoter and polyadenylation signal sequences allowing expression in plants (obtained from the nopaline synthase gene; An et al., 1985). This test material was harvested from plant tissue grown on media containing 100 μg/ml Km. Both transformed and non-transformed animal cells were mouse L-cells (gift of A. Smith).

(b) Preparation of cell extracts and assay conditions

All extractions were carried out at 4 °C. Bacterial and animal crude extracts were prepared by centrifugation of a 1-ml culture; the cell pellet was resuspended in 200 μl of extraction buffer (0.5 M sucrose/0.1 M Tris · HCl/0.1% ascorbic acid/0.1% cysteine · HCl pH 7.5) (An et al., 1985) and sonicated for 30 s. After centrifugation at 14000 rev/min for 10 min in a microcentrifuge, the supernatant was used without further purification.

In the case of plant leaves and calli, the tissue (either fresh or frozen) was ground with a pestle and mortar in the extraction buffer described above (1 ml buffer/1 g tissue), in the presence of aluminium oxide. They were then centrifuged at 14000 rev/min for 10 min and the supernatant was used as above.

Bradford (1976) protein assays were carried out with all the extracts to determine total protein concentration.

The reaction mixture contained 10 μl reaction buffer (67 mM Tris · malate pH 7.1; 42 mM $MgCl_2$; 400 mM NH_4Cl; 1.67 mM DTT); 10 μl crude extract (1–10 μg protein); 2 μl Km sulfate (1 mg/ml); 3 μl ATP (200 μM); 7.5 μl H_2O and 0.6 μl [γ-^{32}P]ATP (10 mCi/ml; >6000 Ci/mmol).

The components of the reaction were mixed and incubated for 30 min at 35°C before application to the PEI-plates.

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

One-μl aliquots from the reaction mixture were applied to the plate, which was then developed in a 50 mM Na · formate/formic acid buffer pH 5.4.

Once the solvent front had reached the top ($\approx$ 14 cm), the PEI-plate was air-dried prior to autoradiography.

(c) Development of a suitable TLC system

Initial attempts using silica plates and various organic mobile phases did not give adequate resolution. We therefore turned our attention to an ion-exchange system. This used PEI-cellulose plates as stationary phase and a 0.75 M K · phosphate buffer,

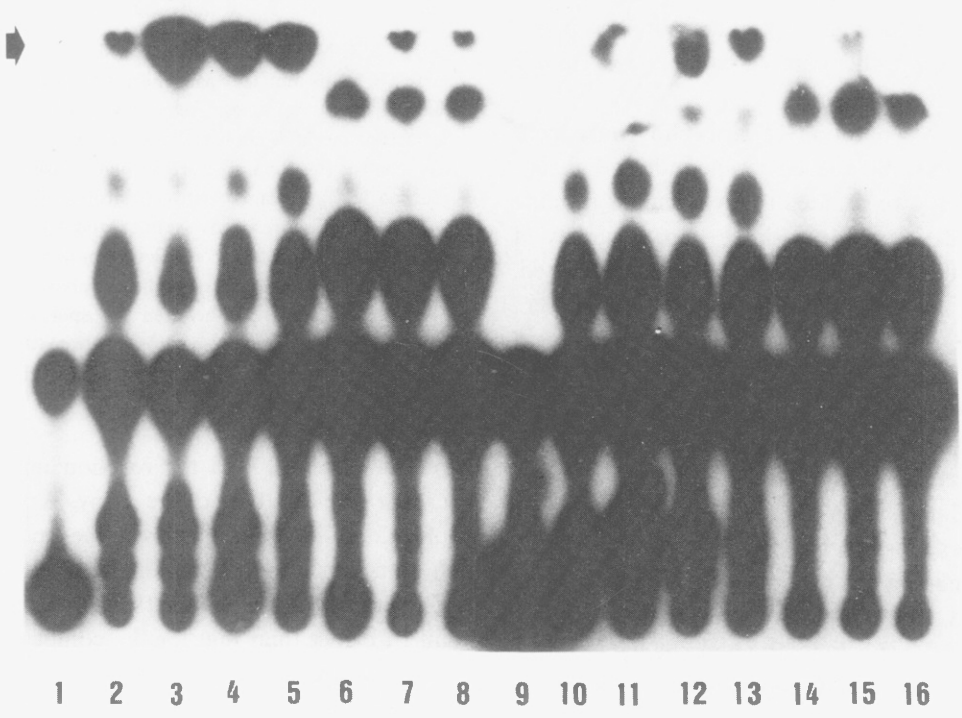

Fig. 1. TLC-NPTII assay following a stepwise development. The buffer used was formic acid/sodium formate pH 3.4, in concentrations of 0.5 M, 1.5 M and 4 M. The plate was developed in the 0.5 M buffer to a height of 3 cm; then, without intermediate drying, it was developed in the 1.5 M buffer to a height of 9 cm and finally, in the 4 M buffer to a final height of 12 cm. The autoradiograph was exposed for 24 h, at −70°C in the presence of intensifying screens. Lanes: 1, control (no enzyme extract added); 2, bacterial negative control (wt *E. coli*); 3–5, bacterial positive (transformed *E. coli*); 6, plant negative control (wt tobacco leaf); 7 and 8, plant positive (transformed tobacco leaf); 9–16, as lane 1–8, respectively, but without Km in the reaction mixture. The spot corresponding to Km · phosphate appears at the solvent front. See EXPERIMENTAL AND RESULTS, sections **a–c** for details.

pH 3.5 as mobile phase (Efstratiadis et al., 1977). The results obtained were somewhat encouraging but not entirely satisfactory so we decided to use the same stationary phase and vary the mobile phase. We tested: 0.5 M LiCl, 1 M formic acid; solutions of LiCl in increasing concentrations (0.1 M, 0.3 M, 0.7 M, 1.5 M); 2% boric acid : 2 M LiCl (2 : 1); 4% boric acid : 4 M LiCl (4 : 3); 1 M LiCl for a few centimetres followed by 1.5 M LiCl + 0.2 M Na · acetate + 4 M urea; solutions of formic acid/sodium formate, pH 3.4 in increasing concentrations (0.5 M, 1.5 M, 4 M, successively). The best overall resolution was given using Na · formate buffer in different concentrations in a stepwise development (Fig. 1). The phosphorylated Km appears at the solvent front (marked by the arrow). As expected, the signal from the bacterial sample is more intense than that for the plant samples. When Km is omitted from the reaction mixture, some spots are present, albeit at lower intensity, at the same position as those corresponding to Km · phosphate in the bacterial positive controls (although not in the bacterial negative controls). This suggests that the NPTII is phosphorylating some other compound that has a similar Rf to Km · phosphate.

To improve separation of Km · phosphate from contaminating radiolabelled compounds and to simplify the assay, we decided to try a similar formate buffer, but developed in a single step. A variety of different buffer concentrations and pHs were tested, viz. 4 M, 0.5 M and 50 mM and the pHs 2.9, 3.4, 3.6, 3.8, 4.2, 4.8, 5.4. The optimal condition for the assay was found to be pH 5.4 and 50 mM. Under these conditions, most of the phosphorylated contaminants remain at the origin (at higher pHs, most compounds tend to bind tightly to the PEI-cellulose and remain at the origin). However, Km-phosphate still migrates close to the solvent front (see signal above the marked line in Fig. 2), thus the separation is increased. The phosphorylated compound associated with NPTII activity (compare lanes 1 and 2), in the absence of Km (compare lanes 2 and 7) has a lower Rf than Km · phosphate.

Sodium fluoride, a phosphatase inhibitor, was added to the reaction mixture and SDS to the extraction buffer, as these conditions have been reported to increase sensitivity (McDonnell et al., 1987). In our hands no improvement was observed and we omitted them in subsequent assays. However, the addition of

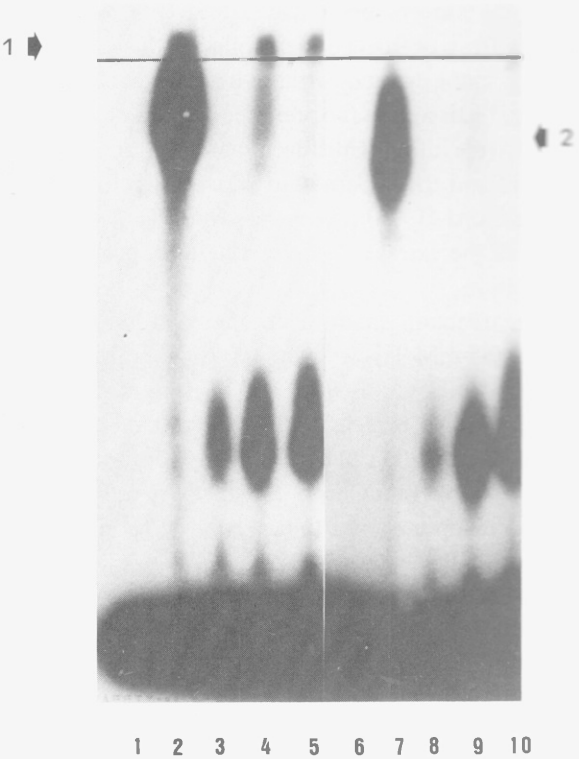

Fig. 2. TLC-NPTII assay using 50 mM formic acid/sodium formate pH 5.4, as mobile phase. The autoradiograph was exposed for 16 h, at −70 °C in the presence of intensifying screens. Lanes: 1, bacterial negative control; 2, bacterial positive; 3, plant negative control (wt tobacco leaf); 4, plant positive (transformed tobacco leaf); 5, plant positive (transformed tobacco callus); 6–10, as lane 1–5, respectively, but without Km in the reaction mixture. The spot corresponding to Km-phosphate appears at the top of the chromatograph (indicated above the line by arrow 1). The difference in Rf between this spot, corresponding to Km · phosphate, and the competing substrate (the spot indicated by arrow 2) is most easily seen by comparing lanes 2, 5 and 7. See sections **a–c** for details.

non-labelled ATP to the reaction mixture did increase the sensitivity of the assay. We tried a range of cold ATP concentrations; the optimal concentration was found to be 20 μM (data not shown). The reason might be that the K_m for the NPTII is much higher than that for the competing enzymes; since the concentration of radiolabelled ATP is low, as we increase the total ATP concentration, so the velocity of the reaction of NPTII with its substrates increases.

(d) NPTII assay with animal cells

The thin-layer chromatography-NPTII assay was tested on animal cells. Under identical conditions to

those used with plant and bacterial cells no NPTII activity was detected in transgenic, Km-resistant, animal cells. This suggests either that the levels of expression of the *nptII* gene in the animal cells tested are very low, under the limits of detection of this assay, or that some other compound(s) in these cells is preventing the formation of Km · phosphate.

Bacterial extracts expressing NPTII activity and animal cell extracts were mixed and then assayed. This time NPTII activity was clearly detected (see arrow, Fig. 3a), indicating either that addition of exogenous NPTII is titrating out the putative inhibitor(s), or more likely, that there is nothing in the animal cell extracts that inhibits NPTII activity, but that levels of expression of the *nptII* gene in these animal cells tested is very low. Increased time of reaction and longer exposure allowed us to detect NPTII activity in animal cells (see arrow, Fig. 3b).

(e) Comparison between TLC assay and gel assay

As discussed above, the gel assay (Reiss et al., 1984) is the commonly used assay for the detection of NPTII activity in plants. For this reason we decided to compare our method with that of Reiss et al. To make this comparison, both types of assay were carried out using leaves from transformed plants as well as different dilutions of a bacterial extract expressing NPTII activity. For the gel assay, dilutions of bacterial extract were made in such a

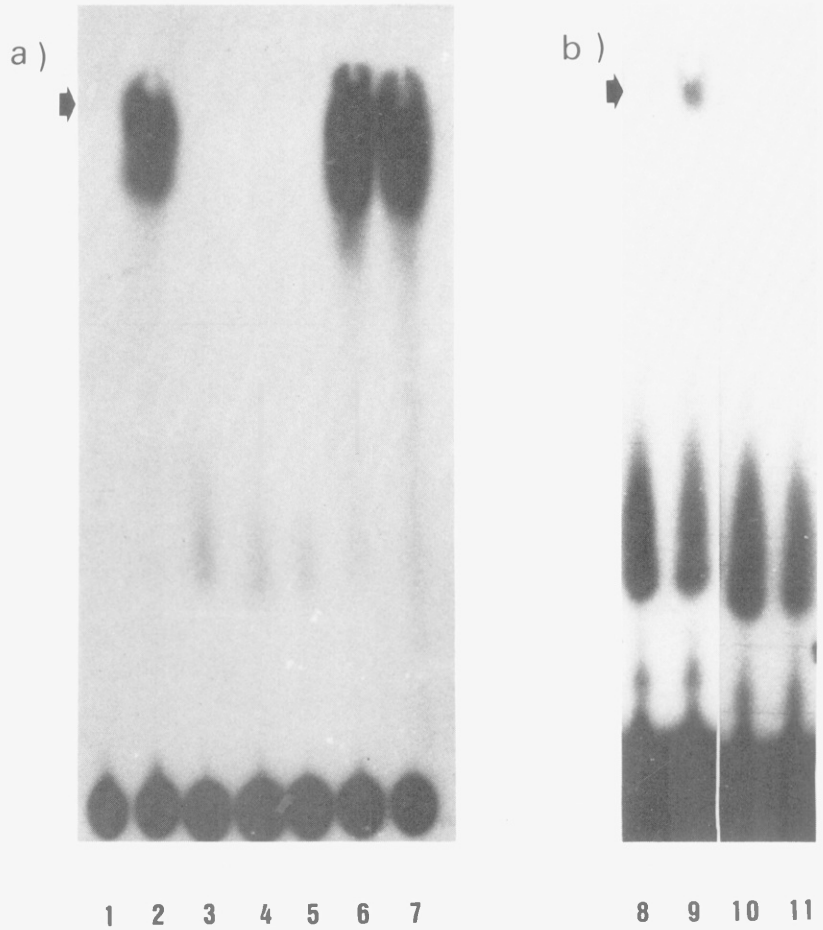

Fig. 3. TLC-NPTII assay for animal cells. (**a**) The autoradiograph was exposed for 16 h, at −70 °C in the presence of intensifying screens. Lanes: 1, bacterial negative control; 2, bacterial positive; 3, animal negative control; 4 and 5, animal positive (transformed L-mouse cells); 6, animal negative + bacterial positive; 7, animal positive + bacterial positive. (**b**) The reaction time in this case was 3 h and the autoradiograph was exposed for three days, at −70 °C in the presence of intensifying screens. Under these conditions it was possible to detect NPTII activity in animal cells. Lanes: 8, animal negative control; 9, animal positive; 10 and 11, as 8 and 9, respectively, but without Km in the reaction mixture. See section **d** for details.

way that the amounts of total protein/well were in the range 0.05 ng to 50 μg. It should be noted that due to the nature of the TLC-NPTII assay the amount of protein per reaction used in this assay was one-third of the amount of protein per well in the gel assay; moreover, the actual amount of protein per spot applied to the TLC-plate is just $^1/_{33}$ of that used in the reaction. Therefore, in the case of the TLC assay the range of total protein per spot is 0.5 pg to 500 ng, i.e., 100 times less protein than in the gel assay. The results can be seen in Fig. 4.

It can be seen that, whereas in the gel method it is only possible to detect NPTII when the total amount of protein loaded is equal to or higher than

Fig. 4. Comparison between gel assay and TLC assay. (**a**) Gel NPTII assay (10% nd–PAGE, following the method of Draper et al., 1988). The autoradiograph was exposed for 16 h at –70°C in the presence of intensifying screens. Lanes: 1–7, bacterial positive with: 50000 ng, 5000 ng, 500 ng, 50 ng, 5 ng, 0.5 ng and 0.05 ng protein/well, respectively; 8, bacterial negative; 9 and 10, plant positive (leaf); 11, plant positive (callus); 12, plant negative (leaf). (**b**) TLC-NPTII assay. The autoradiograph was exposed for 16 h at –70°C in the presence of intensifying screens. Lanes: 1, plant negative; 2 and 3, plant positive (leaf); 4, plant positive (callus); 5, bacterial negative; 6–10, bacterial positive with: 500 ng, 50 ng, 5 ng, 0.5 ng and 0.05 ng protein per lane, respectively. See section **e** for details.

500 ng (see arrow, lane 3, Fig. 4a), in the TLC assay there is a detectable signal when the amount of protein loaded is just 0.5 ng per spot (see arrow, lane 9, Fig. 4b). Thus, the TLC-NPTII assay is more sensitive than the method in current use. The concentrated bacterial extracts, lanes 6–8, give a strong signal below the Km · phosphate, which is NPTII-dependent but Km-independent (seen also in Fig. 2; compare lanes 1, 2 and 7). This signal drops sharply with an additional 10 × reduction in crude extract (see Fig. 4b, lane 9) where only the Km · phosphate signal is visible. One explanation for this is that, in this dilution, the concentration of the competing substrate in the extract is reduced to below its K_m. Furthermore, since the contaminating compound is presumably competing for the same binding site as Km; as this compound is diluted, the sensitivity of the assay should increase.

Apart from its sensitivity the TLC-NPTII assay has other important advantages over other assays: first, it requires lower amounts of radioactivity. In the gel assay 8.8 μCi ^{32}P per sample are needed, whereas the TLC assay uses 6 μCi per reaction and only 0.18 μCi per sample for the chromatography. Thus the same reaction can be used many times (up to 33 times) as only 1 μl is spotted onto the plate from a total of 33 μl of reaction mixture. There is also the possibility of reducing the total volume of the reaction mixture; thus the amounts of protein and of radioactivity involved could be further minimised. It would also be possible to apply the 1-μl sample several times on the same spot on the plate, letting it dry between the consecutive applications; in this way the sensitivity of the assay would be increased as long as overloading of the plate is avoided.

(f) Conclusions

In conclusion, we have developed a new, sensitive method to assay NPTII in crude cell extracts. The method has greater sensitivity than assays currently in use. The reproducibility of our assay is also better; for example, we have found that NPTII activity is not detectable in the gel assay of Reiss et al. (1984) unless a fresh solution of acrylamide/bisacrylamide is used to cast the gel.

Interestingly, in *E. coli* extracts, the assay indicates the presence of another substrate (other than Km) which is specifically phosphorylated by NPTII; however, we cannot rule out the possibility that some other plasmid encoded gene product (other than NPTII) is phosphorylating this other substrate.

In addition to increased sensitivity, the assay is faster and easier and with less radioactive hazard than the others. This is due, both to the use of lower amounts of radioactivity per sample and to the nature of the procedure itself; it does not involve tedious, repeated washes of a filter, possibly involving loss of signal. The method can also be quantitative by cutting out the spots from the chromatographic plate and measuring the amount of radioactivity by scintillation counting.

Perhaps the only disadvantage of the TLC-NPTII assay is that it does not allow the determination of the M_r of the protein; this information is not necessary for most routine assays.

Finally, it should be pointed out that we have observed a variation in NPTII activity in relation to the age of the transformed plant, being the highest in the early stages of the development of the plant and becoming very low in the late stages, even if the plant is growing in a selective medium containing Km.

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