

Investigating the Introduction of a Broad-spectrum Antiviral Mechanism into Grapevine

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Abstract

Ribosome inactivating proteins (RIPs) are potent toxins produced by a wide range of evolutionarily diverse plants. These toxins cause cell death by physically dismantling ribosomal RNA and shutting down protein synthesis. They also have a strong antiviral activity. Some believe that the antiviral property of RIPs is a function of ribosomal inactivation, others believe that the two properties are unrelated. RIPs are non-specific in their antiviral activity. Transgenic RIP-expressing plants are resistant to a wide spectrum of viruses. Many different viruses threaten grapevine. It is not practical to design individual remedies for each of these viruses. In this study, we screen the grapevine genome for the presence of a RIP gene using degenerate PCR primers. If a RIP gene does exist in grapevine, it is not being expressed in a useful way. We also clone several well-documented RIP genes from various plants into pGEM-T Easy: dianthin from *Dianthus caryophyllus*; β -luffin from *Luffa octandra* and mirabilis antiviral protein (MAP) from *Mirabilis jalapa*. These isolated genes are then subcloned into a selection of expression vectors: dianthin into pKK223-3, a bacterial expression vector; β -luffin into pCambia3301, a plant expression vector; and MAP into pFLAG, a yeast expression vector. The constructs prepared in this project may be used for the synthesis of RIP molecules. The exogenous application of RIPs has been shown to protect plants from viruses. Transformation of grapevine with the RIP-containing plant expression vector may result in a variety of vine that is resistant to a wide range viruses. This thesis describes preliminary work in an attempt to impart broad-spectrum antiviral resistance to grapevine.

Opsomming

Ribosomale-inaktiverende proteïne (RIPs) is kragtige toksienes wat deur 'n wye verskeidenheid evolusionêr diverse plante verskaf word. Hierdie toksienes veroorsaak die dood van die selle deur fisies die ribosomale RNA af te breek en proteïensintese stop te sit. Hulle toon ook 'n sterk antivirale aktiwiteit. Sommige voel dat die antivirale eienskap van RIPs 'n funksie van ribosomale inaktivering is, terwyl ander glo dat die twee eienskappe onafhanklik optree. RIPs is in hul antivirale aktiwiteit onspesifiek. Transgeniese RIP-weergewende plante toon weerstand teen 'n wye spektrum virusse. Wingerd word deur baie verskillende virusse aangeval. Dit is onprakties om spesifieke teenmiddels vir elk van die virusse te ontwerp. In hierdie studie word die wingerdgenoom vir die voorkoms van 'n RIP-geen ondersoek, deur die gebruik van degeneratiewe PKR primers. As daar wel 'n RIP-geen in wingerd voorkom, word dit nie in 'n nuttige manier uitgedruk nie. Ons het ook 'n groep goedgeokumentêre RIP-gene vanuit verskeie plante in pGEM-T Easy gekloneer: dianthin vanuit *Dianthus caryophyllus*; β -luffin vanuit *Luffa octandra*; en mirabilis antivirale proteïen (MAP) vanuit *Mirabilis jalapa*. Hierdie geïsoleerde gene is toe in verskeie uitdruktingsvektore gesubkloneer: dianthin in pKK223-3, 'n bakteriële uitdruktingsvektor; β -luffin in pCambia3301, 'n plant uitdruktingsvektor; en MAP in pFLAG, 'n gis uitdruktingsvektor. Die constructs wat in hierdie projek voorberei is, kan gebruik word vir die sintese van RIP molekules. Dit is gevind dat die eksogeniese toepassing van RIPs plante teen virus-infeksie beskerm. Die transformasie van wingerd met die RIP-bevattende plant ekspressievektor kan 'n wingerd wat teen 'n wye verskeidenheid virusse bestand is tot stand bring. Hierdie tesis beskryf die voorlopige werk in 'n poging om breë-spektrum antivirale weerstand in wingerd deelagtig te maak.

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Abbreviations

A	adenine / adenosine
AmpR	ampicillin-resistance gene
ATP	adenosine 5'-triphosphate
BAP	bougainvillea antiviral protein
bp	base pair
BSA	bovine serum albumin
C	cytidine / cytosine
cDNA	complementary DNA
Da	dalton
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
G	guanine / guanosine
gDNA	genomic DNA
IPTG	Isopropylthio- β -D-galactoside
KanR	kanamycin-resistance gene
Kb	kilobase
MAP	mirabilis antiviral protein
ORF	open reading frame
PAP	pokeweed antiviral protein
PCR	polymerase chain reaction
PKR	polimerasekettingreaksie
RAPD	random amplified polymorphic DNA

RIP	ribosome-inactivating protein
RNA	ribonucleic acid
Rnase	ribonuclease
rRNA	ribosomal RNA
S	sedimentation coefficient
T	thymine / thymidine
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume per volume
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

Keywords

antiviral agent

disease resistance

expression vectors

grapevine

ribosome-inactivating proteins

pro meo immortale caro

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Chapter 1: Introduction

"When we asked Pooh what the opposite of an Introduction was, he said 'The what of a what?' which didn't help us as much as we had hoped, but luckily Owl kept his head and told us that the Opposite of an Introduction, my dear Pooh, was a Contradiction; and, as he is very good at long words, I am sure that that's what it is." - Milne, 1974.

Ribosome-inactivating proteins (RIPs) are potent toxins produced by a wide range of evolutionarily diverse plants (see **Table 1.1**). They bring about cell death by physically dismantling rRNA, which results in the inhibition of protein synthesis. RIPs also have an antiviral function. Since interest was first generated in this molecule, there have been numerous conflicting schools of thought as to the exact mechanism of action of RIPs and their biological role. These contrasting beliefs will be discussed in the following sections in an attempt to arrive at an accurate definition of RIPs.

Table 1.1 Examples of RIPs.

Plant of origin	RIP	Reference
<i>Bougainvillea spectabilis</i>	BAP	Balasaraswathi <i>et al.</i> , 1998
<i>Cucurbita pepo</i> (pumpkin)	pepocin	Yoshinari <i>et al.</i> , 1996
<i>Dianthus caryophyllus</i> (carnation)	dianthin	Stirpe <i>et al.</i> , 1981
<i>Phytolacca americana</i> (pokeweed)	PAP	Irvin, 1975
<i>Pisum sativum</i> (garden pea)	pisavin	Lam <i>et al.</i> , 1998
<i>Ricinus communis</i> (castor bean)	ricin	Olsnes <i>et al.</i> , 1972
<i>Viscum album</i> (mistletoe)	viscumin	Olsnes <i>et al.</i> , 1982

1.1 Historical background

Although the isolation and characterization of RIPs is a relatively new science, the toxic properties of these molecules have been exploited for many centuries. Trichosanthin, a RIP produced by *Trichosanthes kirilowii*, was the active ingredient in *Tian Hua Fen*, a potent medicine formulated by the Ancient Chinese to induce abortions and to treat hydatidiform moles, ectopic pregnancies and carcinomas (Wang *et al.*, 1986).

In the early 1900s, it was discovered that the mechanical transmission of viruses could take place by means of sap inoculation between infected and uninfected plants of the same type. However, transmission of the virus from one species to another was not always possible (Allard, 1918).

Gendron *et al.* (1954) proposed that some plants contain substances that inhibit virus infection in other plants, but are ineffective on the plants that produce them:

"Sap extracted from the leaves of *Phytolacca decandra*, sugar beet or carnation, contains substances that can act as virus inhibitors, that is, when mixed with viruses *in vitro*, the mixtures fail to infect when they are rubbed over the leaves of normally susceptible plants...The manner in which such inhibitors act is still obscure, but evidence is increasing that their action is on the inoculated plants rather than directly on the virus particles..."

- Gendron *et al.*, 1954.

In 1948, Kassanis *et al.* were the first to isolate a RIP (referred to as "an inhibitor of plant viruses") by means of a combination of biochemical techniques. The RIP was isolated from *Phytolacca esculenta*, and it was found to be a basic glycoprotein that could withstand numerous harsh conditions, but was denatured by heating at 100°C for 10 minutes. The molecule was combined with various

plant viruses. Its effect on infectivity was gauged by determining the number of local necrotic lesions (characteristic of viral infection) formed on tobacco plants inoculated with the mixtures. It was established that the isolated inhibitor had an immediate effect on the infectivity of a wide range of viruses and that the effect could be reversed by dilution. No satisfactory explanation could be given for these observations:

"...the simplest explanation of the phenomenon would be that in combining with the virus the inhibitors block some groups on it that are essential for the establishment of infection. However, there are various other facts that do not fit in with this view of a simple quantitative neutralization of the virus."

- Kassanis *et al.*, 1948.

Tomlinson *et al.* (1974), made a major breakthrough when they discovered that one such plant virus inhibitor is capable of preventing the infection of mammalian cells with animal viruses. This phenomenon generated a great deal of interest in the field of RIP research, as the potential medical benefits of these molecules were realized. The ability of several RIPs to inhibit HIV-1 replication and infection is being used to design anti-AIDS drugs (McGrath *et al.*, 1989; Lee-huang *et al.*, 1990, 1991(a) and (b); Zarlino *et al.*, 1990).

Virus-inhibiting extracts from the pokeweed plant were found to be capable of disrupting protein synthesis in cell-free protein synthesis systems (Owens *et al.*, 1973). The discovery of the dual nature of these molecules resulted in a better understanding of the actual mechanisms involved.

"The fact that PAP inhibits protein synthesis on ribosomes from plants other than pokeweed and inhibits mechanical transmission of virus to plants other than pokeweed suggests that its *in vivo* inhibition of virus replication results from interference with protein synthesis on host cell ribosomes."

- Owens *et al.*, 1973.

That same year Montanaro *et al.* (1973) realized that:

"...ricin brings about an irreversible modification of ribosomes which impairs their ability to interact with EF 2."

-Montanaro *et al.*, 1973.

More than a decade later, research performed by Endo *et al.* (1987(a) and (b)) in Japan elucidated the precise molecular mechanism involved in the inactivation of ribosomes. Their observations of the effect of ricin on rat liver ribosomes in *in vitro* protein synthesis systems brought them to the conclusion that ricin catalytically removes the adenine residue 4324bp from the 3' end of the 28S rRNA, which occurs in the 60S (large) ribosomal subunit of eukaryotic ribosomes. Ricin was also found to be active on naked rRNA, but at a reduced rate of activity. The removal of the adenine residue renders the surrounding phosphodiester bonds particularly susceptible to hydrolysis, which results in the disruption of the peptide chain elongation step of protein synthesis. The site of action is a sequence that is very well conserved in both eukaryotes and prokaryotes:

Adenine 4324
↓
AGUACGAGAGGAAC

It is thought that the central GAGA sequence forms a tetraloop structure that is recognized by ricin. Studies were extended to include five more RIPs: gelonin, saporin, PAP, PAP-II and PAP-S. Cleaving of the *N*-glycosidic bond at A4324 of 28S rRNA seemed to be the general mechanism of ribosomal inactivation employed by RIPs (Endo *et al.*, 1987(a) and (b)).

1.2 The categorization of RIPs

"...[RIPs] can be divided into two groups: single-chain proteins, type 1 RIPs, and two-chain proteins, type 2 RIPs."

- Barbieri *et al.*, 1993.

There is a third class of RIP, RIP type III, that is not very well known and hardly ever referred to. JIP60 (jasmonate-induced protein 60) from barley is expressed in response to jasmonates, which are volatile signal molecules secreted by plants under stress in order to communicate with neighbouring plants. JIP60 affects protein synthesis in microbes, mammals and other species of plant, but is only active on barley when stress prevails. JIP60 shares significant sequence homology with both types 1 and 2 RIPs and it seems to regulate protein synthesis in stressed plants - upregulating expression of certain proteins and shutting down synthesis of others. However, due to its vastly different mode of action - instead of dismantling rRNA, it breaks polysomes down into their subunits - this RIP has been grouped in a class of its own (Reinbothe *et al.*, 1994(a) and (b)).

Type 1 RIPs are basic single-chain molecules with a molecular weight of about 30 000 Da. They are by far the most abundant type of RIP and are characterized by their RNA *N*-glycosidase activity on 28S rRNA of the large subunit of rat liver ribosomes. These molecules are often glycoproteins. However, the carbohydrate portion is not considered to be involved in the enzymatic activity, as some RIPs have no sugar moiety and are totally functional (Barbieri *et al.*, 1982(b)).

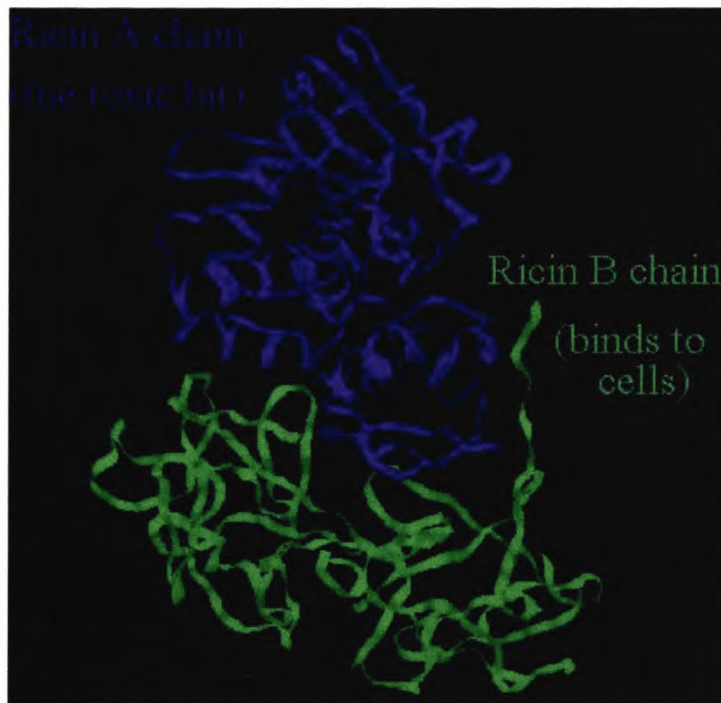
Type 2 RIPs consist of either two or four chains and have a molecular weight in the region of either 60 000 or 120 000 Da respectively. At least one of these chains is an A (Active) -chain, with the same enzymatic activity as type 1 RIPs and a pI value of between 4.6 and 8.6, and at least one chain is a B (Binding) -chain, with lectin properties. The chains are linked together by disulphide bonds (reviewed by Barbieri *et al.*, 1993). Although type 1 and type 2 RIPs are equally destructive in cell-free systems, type 2 RIPs are approximately 10 000 times more toxic than type 1 RIPs (Monzingo *et al.*, 1993) due to the presence of the B-chain, which facilitates entry into cells. There are $\sim 8 \times 10^7$ ricin binding sites on the surface of a murine neuroblastoma cell (Gonatas *et al.*, 1980) and $\sim 3 \times 10^7$ per HeLa cell (Sandvig *et al.*, 1976). The internalization of just one ricin molecule is sufficient to kill a HeLa cell (Eiklid *et al.*, 1980).

The A-and B-chains must separate before the RIP becomes catalytically active (Olsnes *et al.*, 1976). Ricin seems to be taken up into the cell by means of receptor-mediated endocytosis and is then reduced in the cytosol (Frénoy *et al.*, 1992).

Type 1 and type 2 RIPs have been reported to occur concurrently in the leaves of the same plant (*Sambucus ebulus*). However, this is to be regarded as the exception, rather than the rule (De Benito, *et al.*, 1995).

Comparative studies of amino-terminal sequences suggest that type 1 RIPs and the A-chain of type 2 RIPs have evolved from a common ancestor. It seems that the B-chain evolved independently and that its encoding gene fused with the enzymatic gene during the course of evolution, to form a heterodimer encoding a cytotoxin. In some cases the two genes may not have fused yet, resulting in the simultaneous expression of type 1 RIPs and galactose-specific lectins (Ready *et al.*, 1986).

PAP and ricin may be considered to be the prototype type 1 and 2 RIP molecules respectively. Although there is just 31% sequence homology between PAP and ricin A-chain (Lin *et al.*, 1991), X-ray crystallography reveals that their three-dimensional structures and folding patterns are remarkably similar (Monzingo *et al.*, 1993). The proteins are globular with extensive secondary structures and a prominent cleft - thought to be the active site. The B-chain of ricin houses two shallow lactose-binding clefts (Montfort *et al.*, 1987). **Figure 1.2.1** is a picture of ricin.



<http://www.oikos.warwick.ac.uk/~biojo/work/ricin.html>

Figure 1.2.1 Three dimensional protein structure of ricin.

RIPs do not generally contain introns (Barbieri *et al.*, 1993). However, PAP-II has an intron of 734 bp which divides the gene into roughly equal-sized exons. It has been suggested that PAP-II is the ancestral member of the PAP family (Poyet *et al.*, 1997), as introns tend to get lost, and not created through the centuries (Gilbert, 1978). MAP (mirabilis antiviral protein) also contains an intron (Kataoka *et al.*, 1993).

1.3 Distribution in nature and within the plant

"Many, and possibly all, plants contain ribosome-inactivating proteins..."

- Kataoka *et al.*, 1992(c).

It is not only plants that produce toxins that catalytically depurinate the adenine residue in the well-conserved GAGA-tetraloop: the prokaryote, *Shigella shigae*, produces such a toxin that has significant homology to ricin (Jackson *et al.*, 1990). *Shigella* toxin depurinates ribosomes at a rate of 40 ribosomes per minute (Reisbig *et al.*, 1981) - a figure not all that impressive if one considers that an average *E. coli* cell has 15 000 ribosomes (Mathews *et al.*, 1990) and that ricin A-chain depurinates ribosomes at a rate of 1 500 per minute (Piatak *et al.*, 1988)!

RIPs have been isolated from more than 30 different plants and are expressed at vastly different concentrations: some plants produce a few micrograms of RIP per 100 g of plant tissue, whereas others may contain hundreds of milligrams of RIP (Barbieri *et al.*, 1993). Some RIP genes have evolved over the years to give rise to different RIP isoforms (Ready *et al.*, 1984). And thus, a single plant may have a multigene family of RIPs (Leah *et al.*, 1991; Lin *et al.*, 1991), expressing various forms of RIP in different tissues, or at different times.

Three distinct RIP isomers together represent the most abundant protein in iris bulbs (Van Damme *et al.*, 1997). *Phytolacca americana* (pokeweed) produces PAP-S in the seeds (Barbieri *et al.*, 1982(a)), α -PAP in the leaves, PAP-II in late Spring leaves (Irvin *et al.*, 1980) and PAP-R in the roots (Bolognesi *et al.*, 1990). Furthermore, pokeweed cells in culture produce a distinct form of pokeweed antiviral protein, known as PAP-C (Barbieri *et al.*, 1989). Dianthin 30 occurs throughout the carnation plant, whereas dianthin 32 is found exclusively in the leaves and growing shoots. Both proteins are more abundant in old plants, where they may constitute up to 3% of the extractable proteins (Reisbig *et al.*, 1983).

RIPs are most often found in the leaves, roots, sap, seeds and bulbs of plants. There is also a report of a RIP having been isolated from the flesh of a pumpkin (Yoshinari *et al.*, 1996). Although RIPs have been isolated from both monocotyledonous and dicotyledonous plants, no RIP has been found in Gymnospermae (Barbieri *et al.*, 1993).

Many RIPs have an amino-terminal signal peptide which targets the protein to a specific location (Kataoka *et al.*, 1991; Legname *et al.*, 1991; Lin *et al.*, 1991). Electron microscopy studies reveal that PAP, which has a 22 amino acid signal peptide (Lin *et al.*, 1991), is found in the cell wall matrix of the leaf mesophyll (Ready *et al.*, 1986).

Saporin, a type 1 RIP from soapwort, occurs in the perisperm (storage tissue) of the seed and in the intercellular spaces of leaf tissue (Carzaniga *et al.*, 1994). Ricin is located in the soluble matrix of vacuolar protein bodies of castor beans (Tully *et al.*, 1976).

The degree to which RIP expression is influenced by external factors is a matter of conjecture.

"To investigate if RIP activity changed during virus infection, we examined infected and non-infected leaves of *Chenopodium amaranticolor*; RIP activity was almost identical in the two specimens, indicating therefore that RIPs have probably to be considered to be different from interferon-like substances."

- Barbieri *et al.*, 1982(b).

However, there is evidence that RIP expression can indeed be upregulated under certain environmental conditions. Girbés *et al.* (1996) report that the expression of RIPs in sugar beet is induced by viral infection.

Furthermore, Rippmann *et al.* (1997) used reverse transcription differential display (RT-DD) to investigate the variable expression of genes in stressed and unstressed ice-plants. They discovered that a RIP gene transcript was 10-fold more abundant in salt stressed plants than in unchallenged plants. Perhaps the regulation of RIP expression differs from plant to plant.

1.4 Suitable substrates

"...the wider the evolutionary gap between the source of RIP and of its target ribosomes, the greater the inactivation is likely to be..."

- Barbieri *et al.*, 1982(b).

This statement is supported by the observation that ricin is approximately 23 000 times more active on mammalian expression systems than on plant expression systems (Harley *et al.*, 1982). For many years it was the sincere belief that RIPs are inactive on the plants that produce them:

"Not surprisingly, RIPs are inactive on ribosomes from their own plant species..."

- Stirpe *et al.*, 1989.

Today we know that most RIP-producing plants are indeed sensitive to their endogenous RIP. PAP depurinates pokeweed ribosomes (Taylor *et al.*, 1990; Prestle *et al.*, 1992), dianthin 29 depurinates *Dianthus barbatus* ribosomes

(Prestle *et al.*, 1992) and MAP depurinates *Mirabilis jalapa* ribosomes (Kataoka *et al.*, 1992(b)) - to name but a few examples.

Since plants are sensitive to their endogenous RIPs, several mechanisms exist for the safe synthesis and storage of these potent toxins...

The spatial separation of RIPs from ribosomes is a mechanism commonly employed in nature. As mentioned earlier, many RIPs have terminal targeting peptides, which are responsible for transporting the pre-protein out of the cytosol and away from contact with the ribosomes (Kataoka *et al.*, 1991; Lin *et al.*, 1991; Legname *et al.*, 1991). These peptides are not present in mature RIPs and do not affect the protein's enzymatic activity.

It is possible that RIPs are temporarily sheathed in enzymatically inactive complexes. Desvoyes *et al.* (1997), detected the relatively inactive PAPI complexes in *P. americana* leaf extracts. These are either PAP dimers or compound molecules consisting of PAP-and-an-unidentified-component. Under certain conditions, PAPI dissociates to yield fully active PAP.

"In all cases examined RIPs acted on ribosomes in the absence of any cofactor..."

- Barbieri *et al.*, 1982(b).

It has recently been demonstrated that the activity of certain RIPs increases by more than 100-fold in the presence of certain cofactors. Wheat germ ribosomes are relatively resistant to depurination by their endogenous RIP, tritin-S (Taylor *et al.*, 1990), although slight levels of depurination have been detected during the isolation process of wheat germ ribosomes (Kataoka *et al.*, 1992(b)). There is no amino-terminal signal peptide encoded in the cDNA of tritin-S, and it is therefore probably stored in the cytosol - in direct contact with the germ ribosomes. The

activity of tritin increases 300-fold in the presence of certain cofactors (including ATP). Perhaps wheat germ ribosomes are resistant to their endogenous RIP under normal physiological conditions, but become susceptible as soon as trauma alters the prevailing concentrations of chemicals within the cell.

Cofactor requirements vary greatly. RIP isoforms within the same plant may have different dependencies on cofactors: PAP-S, PAP-R and PAP-C are all stimulated by cofactors, whereas the activities of PAP and PAPII are not (Carnicelli *et al.*, 1997).

Another popular misconception regarding RIPs is that:

"...RIPs do not act on bacterial ribosomes..."

- Stirpe *et al.*, 1989.

This assumption was probably made on the grounds that *E. coli* ribosomes are insensitive to ricin. However, it was known that ricin depurinates naked *E. coli* ribosomal RNA: 23S rRNA at position A2660, corresponding to A4324 of rat liver rRNA; and 16S rRNA at A1014 (Endo *et al.*, 1988). Many type 1 RIPs - with the notable exception of saporin (Ippoliti *et al.*, 1992) - are indeed active on *E. coli* ribosomes (Habuka *et al.*, 1990, 1991; Hartley *et al.*, 1991). Much higher concentrations of RIP are required to have the same affect on bacterial protein synthesis systems as on rabbit reticulocyte lysates (Habuka *et al.*, 1991). Girbés *et al.* (1993) commented that all RIPs have a degree of toxicity on bacterial systems above a concentration of 5000 nM (as would most any substance!).

Mutation analysis of the GAGA-tetraloop region reveals that the tetraloop structure - in either the wild type or reverse orientation - is a basic requirement for ricin depurinaton. On the other hand, PAP activity is not dependent on a tetraloop structure (Marchant *et al.*, 1995).

It has been suggested that there are elements on the ribosomes that act as recognition sites for RIPs. These elements could account for the different preferences in ribosomal substrates. Ippoliti *et al.* (1992), discovered that saporin forms a covalent complex with a protein in the 60S subunit of yeast ribosomes. This complex was not formed with *E. coli* ribosomes, which are resistant to inactivation by saporin. However, this finding offers no explanation for the general activity of RIPs on naked ribosomes.

Time and again, RIPs have been defined as:

"...*N*-glycosidases which depurinate ribosomal RNA by cleaving the *N*-glycosidic bond of a single adenine in a specific position of rRNA (A₄₃₂₄ of rat liver 28S rRNA)..."

- Barbieri *et al.*, 1994.

Although the universally-conserved A4324 is always preferentially depurinated, it was found that a few RIPs release more than one mol of adenine per mol of depurinated rat liver ribosomes. The fact that some RIPs liberate as much as 33 mol adenine per mol of depurinated ribosomes strongly suggests that other adenine residues are being released, too (Barbieri *et al.*, 1992).

Recently, several new RIP substrates have emerged: RIPs from Bougainvillea hydrolyse adenine residues from herring sperm DNA and artichoke mottled crinkle virus (Bolognesi *et al.*, 1997); saporin-L1 cleaves adenine from several RNA, DNA and poly-(A) origins (Barbieri *et al.*, 1994, 1996); a few RIPs cleave supercoiled double-stranded DNA (Ling *et al.*, 1994); and gelonin has deoxyribonuclease activity on single-stranded DNA (Nicolas *et al.*, 1997).

However, it appears that it is always only adenine residues that are depurinated by RIPs (Barbieri *et al.*, 1992)- without exception.

To sum up: there is great diversity in the substrates recognized and depurinated by RIPs. All RIPs inactivate eukaryotic ribosomes, but only some are active on the ribosomes of prokaryotes. The precise factors determining substrate preference remain obscure.

1.5 Antiviral mechanism

For many years, RIPs were referred to as "antiviral agents". The realization that these "agents" catalytically inactivate ribosomes has resulted in much dispute as to the exact mode of antiviral activity of RIPs. Two main theories have emerged...

The first theory claims that the antiviral activity is the result of ribosomal inactivation. According to what one might call "The Defensive Suicide Model", compartmentalized RIP leaks into cells infected with virus and shuts down protein synthesis. The virus, which relies on the cellular machinery of the host for its own survival, is unable to replicate and the viral infection is contained. In the process, the infected plant cell dies - a small price to pay for the welfare of the plant as a whole. Ready *et al.* (1986) proposed that PAP (which is stored in the extracellular spaces of leaf mesophyll tissue) leaks into the cytoplasm with the virus when an aphid, or other such viral vector, bites into the plant cell. RIP internalization may be even simpler than this. Viruses require higher concentrations of monovalent ions than cells do. To meet this need, viruses increase the permeability of cell membranes, facilitating the redistribution of ions (Carrasco *et al.*, 1978). RIPs may pour into these leaky, virus-infected cells. There is evidence that the antiviral and ribosomal inactivation activities are tightly intertwined: the *in vitro* depurination activity of several RIPs correlates with their ability to inhibit tobacco mosaic virus infection (Taylor *et al.*, 1994).

The second theory of antiviral activity states that RIPs attack the virus directly:

"...our results show that saporins, at least, acting on substrates other than rRNA, could directly inhibit the replication of viruses by damaging their genomic or messenger RNA. Because these saporins depurinate TMV genomic RNA at concentrations similar to those present in plants, it is possible that this is a mechanism whereby these RIPs exert their antiviral activity in nature."

- Barbieri *et al.*, 1994.

In support of this theory is the phenomenon that ricin A-chain loses its RNA *N*-glycosidase activity when it is denatured by boiling, but retains its ability to cleave supercoiled DNA. The ability of RIPs to disrupt protein synthesis (i.e. RIP toxicity) and to dismantle nucleic acid (i.e. resistance to disease) therefore seem to be regulated by separate mechanisms acting independently of each other (Ling *et al.*, 1994).

Furthermore, an intact RIP active site was found to be an absolute requirement for both antiviral activity and *in vitro* depurination of tobacco ribosomes. In contrast, a normal RIP C-terminal is not essential for the antiviral (or antifungal) function, but is necessary for depurination (Tumer *et al.*, 1997; Zoubenko *et al.*, 1997).

Simple experiments conducted in the 1970s suggest that viruses are not permanently damaged or altered by contact with RIPs.

"Both viruses become non-infective when mixed with preparations of the extracts but infectivity was regained when the viruses were separated from them by centrifuging."

- Tomlinson *et al.*, 1974.

Subsequent research contradicts this finding:

"To determine if antiviral activity of PAP could be due to direct effects on viral RNA or DNA, we inoculated PVX RNA with purified PAP, and showed that the infectivity of PVX RNA decreased significantly upon treatment with PAP. Gel analysis indicated that the PVX RNA is hydrolyzed after incubation with PAP."

-Wang *et al.*, 1997.

Perhaps the action of RIPs depends on the particular virus involved. However, it seems unlikely that different antiviral mechanisms are encapsulated in the same RIP molecule.

The exact mode of RIP antiviral activity remains a topic of hot debate.

1.6 Biological roles and extended applications

"The wide distribution and the abundance of RIPs suggest a very important role in plant life, since a protein would have been so widely conserved throughout the diversity of species only if the evolutionary advantage was very large."

- Barbieri *et al.*, 1982(b).

Many have pondered over the precise function of RIPs in nature and there is much speculation as to what their actual purpose is. RIPs have been called by many names, including antiviral agents, antifungal agents (Zoubenko *et al.*, 1997) and repellants of seed-eating predators. Due to the extraordinarily high concentration of RIPs in seeds, some have suggested a role in food storage. Others are of the opinion that RIPs shut down protein synthesis, and consequently stop other vital functions, in cells about to die - due to either

senescence or trauma (Stirpe *et al.*, 1986). Perhaps RIPs play a role in the regulation of protein synthesis, or are responsible for eliminating foreign or diseased ribosomes. RIPs have been known to have larvicidal activity: a RIP from *Eranthis hyemalis* is lethal to southern corn rootworms (Kumar *et al.*, 1993). Barbieri *et al.* (1982(b)) formulated a most creative suggestion:

"...RIPs may play an important role in reducing the frequency of spontaneous, successful grafts in nature, thus strengthening integrity and therefore diversity of plant species."

- Barbieri *et al.*, 1982(b).

However, it should be noted that no rejection occurs during the grafting of wild type scions onto transgenic PAP-expressing tobacco plants. Granted, the levels of PAP present in the transgenic scions are lower than those occurring naturally in pokeweed plants (Smirnov *et al.*, 1997).

Perhaps RIPs have a range of functions, each type of RIP being expressed to fulfil a specific purpose.

Fortunately, a good understanding of the natural function of RIPs is not a requirement for exploiting their useful properties.

Ricin eliminates tumours in mice (Lin *et al.*, 1970). Much work has been done to target RIPs to specific cells and to reduce their general toxicity. Type 1 RIPs may be made more cytotoxic by insertion into liposomes, or by conjugation to molecules that recognize cell surface receptors: such as lectins, sugars, antibodies and hormones (Barbieri *et al.*, 1982(b)). RIPs conjugated to tumour-associated surface antigens make extremely toxic and highly selective immunotoxins capable of eliminating lymphomas in mice (Olsnes, 1981).

Unfortunately, such 'magic bullets' are highly antigenic and elicit an immune response: the body recognizes the RIP as alien and forms antibodies against it (Olsnes *et al.*, 1982; Strocchi *et al.*, 1992).

The fact that RIPs seem to be more toxic to virus-infected cells than to healthy cells (Watanabe *et al.* (1997), gauged the activity of PAP on healthy and virus-infected protoplasts), creates potential for the development of therapies for viral infections as diverse as herpes simplex virus, influenza and HIV.

PAP inhibits HIV-1 replication at concentrations too low to affect the proliferation of healthy CD4+ cells. Combining PAP with a monoclonal antibody that recognizes antigens on CD4+ cells both increases the toxicity and the half-life of this medicine. Clinical trials show that HIV-1 replication is inhibited for several weeks after administration of the drug (Zarling *et al.*, 1990).

Furthermore, there are many useful applications of RIPs in agriculture, which will be discussed in due course.

1.7 Defining a RIP

The fact that there is such variation amongst RIPs makes the formulation of a definition a formidable task. There is a danger in making generalizations: each apparent characteristic is matched by a host of exceptions. On a molecular level, there may be as little as 17% amino acid homology between two given RIPs (Barbieri *et al.*, 1994). RIPs may have different structures: some contain sugar moieties, some have lectin-binding subunits and others have both or neither of these. There is huge diversity in the distribution, substrate preference and cofactor requirements of RIPs.

Pioneers in the field modify their definition of RIPs from year to year:

"The ribosome-inactivating proteins (RIPs) from plants are RNA N-glycosidases that depurinate the major rRNA, thus damaging ribosomes, and arresting protein synthesis..."

- Barbieri *et al.*, 1993.

"These proteins would be more appropriately called polynucleotide: adenosine nucleosidases and the question arising from this is what is their natural substrate?"

- Barbieri *et al.*, 1994.

The precise role of RIPs in nature remains a mystery. RIPs tend to inhibit viral infection in other plants more efficiently than in their own. The evolutionary advantage of synthesizing these molecules in such abundance and variety of form is difficult to comprehend. The gene, after all, is "the basic unit of selfishness" (Dawkins, 1989).

We define RIPs thus:

RIPs are potent toxins that catalytically and preferentially depurinate eukaryotic 28S rRNA at A4324, or at the equivalent site in the conserved sequence of prokaryotic rRNA (A2660). This renders the rRNA particularly susceptible to hydrolysis and makes it an unsuitable substrate for binding of elongation factor 2 (EF2). The result of this is disruption of protein synthesis and cell death.

However, if one were to include type III RIPs in this definition, it would have to read as follows:

RIPs are potent toxins that catalytically disrupt protein synthesis and result in cell death.

Current research just seems to add to the list of paradoxes, making a succinct definition of RIPs all the more complicated.

1.8 Aims of this study

"...The dark red vine leaves seemed like the perfect harbinger of succulent red wines stuffed with the goodness of the southern sun. Far from it. This memorable sight was nothing more than that of an entire vineyard region suffering from virus infection...."

- O. Clarke, 1995.

Viruses pose a major threat to the grapevine industry. At present, attempts to control viruses are archaic and largely unsuccessful: insecticides are used to destroy aphids and other such viral vectors; herbicides eradicate the weeds that host the vectors during the lean winter months and traditional plant breeding techniques are employed to select varieties of vine with a natural resistance to viruses.

"Transgenic plants carrying nucleotide sequences derived from plant viruses can exhibit increased resistance to viral disease."

- Fitchen et al., 1993.

Molecular approaches - such as coat or movement protein induced resistance and antisense therapies - are only just beginning to emerge in the vineyard (Martinelli *et al.*, 1994; Ling *et al.*, 1997). These therapies offer varying degrees of protection against a narrow range of closely related strains of virus. There is, however, some concern about the far-reaching effects that such programs could have on agroecosystems (De Zoeten, 1991). **Figure 1.8.1** lists some of the viruses known to infect grapevine.

Grapevine fanleaf nepovirus
Sowbane mosaic sobemovirus
Tomato black ring nepovirus
Grapevine corky bark-associated closterovirus
Grapevine Bulgarian latent nepovirus
Grapevine leafroll-associated viruses I-VII
Grapevine stem pitting associated closterovirus
Peach mosaic nepovirus
Grapevine fleck virus
Grapevine stunt virus
Arabidopsis mosaic nepovirus
Shiraz disease viruses
Grapevine trichovirus A+B
Grapevine ajinashika disease luteovirus
Grapevine Algerian latent tombusvirus
Grapevine chrome mosaic nepovirus
Grapevine line pattern ilarvirus
Petunia asteroid mosaic tombusvirus
Strawberry latent ringspot nepovirus

Figure 1.8.1 A few grapevine viruses.

To design a remedy for each and every one of these would be very time-consuming and labour-intensive, not to mention the effects that spontaneous virus mutations would have on these therapies.

In this study, we investigate three alternative strategies by which RIPs may be used to impart resistance to the entire spectrum of grapevine viruses:

1. We search the grapevine genome for a naturally occurring RIP. If this gene does exist in grapevine, it is not being expressed in a useful way.
2. We clone two RIPs into bacterial and yeast expression vectors respectively. Topical application or intercellular injection of biologically-manufactured RIP protects plants from viral infection (Chen *et al.*, 1991; Lodge *et al.*, 1993) in an environment-friendly fashion.
3. We clone a RIP into a plant expression vector. Transgenic lines of PAP expressing potato are resistant to a wide range of viruses (Lodge *et al.*, 1993). The cloned RIP gene may ultimately be used to transform grapevine, with the hope of giving rise to RIP-expressing, virus-resistant varieties of vine.

The following chapter addresses our attempt to establish the presence of a RIP in grapevine. Chapter 3 deals with the cloning of several well-documented RIPs into various expression vectors. A description of the methods used forms the last section of both of these chapters. Chapter 4 concludes this study.

Chapter 2: Screening the grapevine genome for a RIP



*"...he bent down, put his head into the hole, and called out:
'Is anybody at home?'
There was a sudden scuffling noise from inside the hole, and then silence.
'What I said was, "Is anybody at home?" called out Pooh very loudly.
'No!' said a voice; and then added, 'You needn't shout so loud. I heard you quite well
the first time.'
'Bother!' said Pooh. 'Isn't there anybody here at all?'
'Nobody.' " - Milne, 1973.*

Traditionally, new RIPs are isolated by means of a combination of biochemical and molecular techniques. Once RIP activity is detected in a plant, the protein is isolated and sequenced. Degenerate primers are designed from the amino acid sequence and these are used to amplify portions of the gene, synthesize radioactive probes and screen cDNA libraries for RIP-containing clones.

"These proteins [RIPs] seem to be widely distributed and possibly ubiquitous in the plant kingdom."

- Stirpe *et al.*, 1986.

A general PCR-based method for screening plant genomes for RIPs would be a great contribution to the quest for novel RIPs. The availability of such a diagnostic tool would both facilitate and accelerate the process of isolating these proteins.

"It is useful to look for further RIP (*i*) to increase our knowledge of these enzymes, (*ii*) to identify those with the highest antiviral or antifungal activity, (*iii*) to select the most suitable for human therapy and (*iv*) to overcome the immune response following clinically oriented administration of RIP-conjugates."

- Hornung *et al.*, 1996.

"..the search for new RIPs is of interest as new structural and biological data may contribute to the explanation of their role *in vivo*."

- Di Maro *et al.*, 1999.

Perhaps our motivation for searching for a RIP in grapevine is less noble than these: we would ultimately like to produce a variety of grapevine that is resistant to a wide range of viruses. Before considering the introduction of a foreign RIP into grapevine, we would like to establish whether grapevine contains a RIP of its own.

2.1 Degenerate primer design

The amino acid sequences of several RIPs, originating from a range of plants, were downloaded from GenBank and aligned by ClustalX (Jeannmougin *et al.*, 1998). **Figure 2.1.1** is a section of this alignment, showing two regions of reasonable homology. These regions are approximately 300 bp apart. There is tremendous diversity in the amino acid sequences of RIPs produced by different plants. These differences are amplified on the nucleotide level. In order to eliminate some of the variations, we decided to base the design of degenerate PCR primers on the RIPs from *Phytolacca americana* (pokeweed) and *Sambucus nigra* (Japanese elderberry). Since these plants both bear berries, we believe that they are more closely related to grapevine than many other RIP-producing plants. In fact, the local name for pokeweed is "*bobbejaan druiwe*" (monkey grapes). Conventional wisdom dictates that the closer the evolutionary link between two species, the greater the homologies in their genetic makeup. Furthermore, we calculated the codon preference of grapevine (**Appendix I**). This could be used to decrease the degeneracy of the primers when alternative codons were used in PAP and the RIP from sambucus to encode the same amino acid. The regions highlighted in **Figure 2.1.1** were used to design the pair of degenerate primers. A tetrapeptide within the second highlighted sequence - EAAR - is thought to form the active site of the enzyme (Legname *et al.*, 1991). **Table 2.1.1** gives nucleotide equivalents of parts of the highlighted regions in PAP and the sambucus RIP, as reported by several researchers (the Genbank accession numbers are included for each sequence).

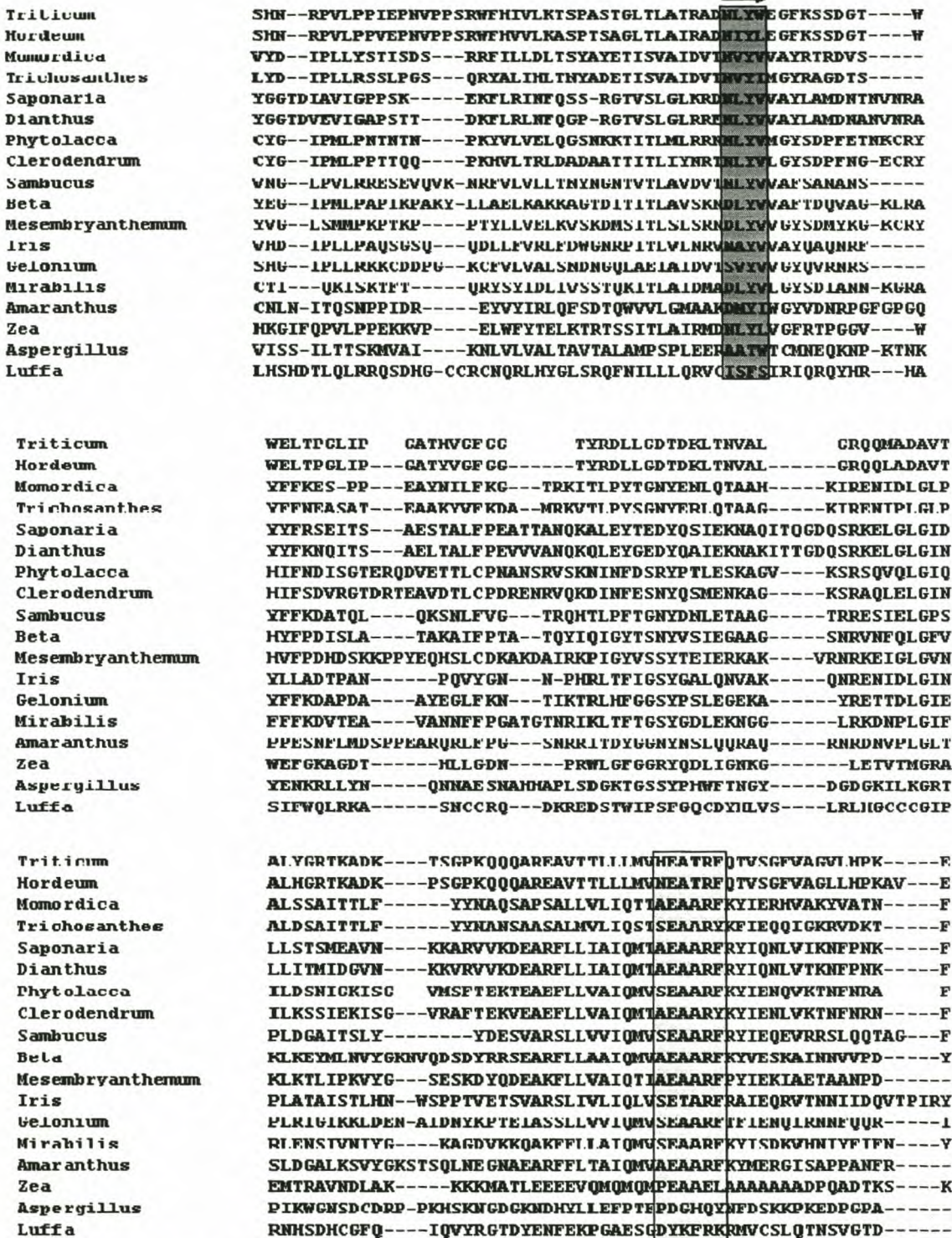


Figure 2.1.1 Amino acid alignment of RIPs from several plants. The highlighted boxes were deemed the most suitable regions from which to design PCR primers.

Table 2.1.1 Nucleotide sequences on which degenerate PCR primers are based.

RIP	GenBank Accession number	Nucleotide sequence
FOR THE DESIGN OF FORWARD PRIMER:		
Sambucus RIP	U76524	AAC CTT TAC GTG
Sambucus RIP	D25317	AAC CTT TAT GTG
Sambucus RIP	U66191	ACC TTT TAT GTG
PAP	X55383	AAT TTG TAT GTG
PAP	X98079	AAC TTA TAC GTG
FOR THE DESIGN OF REVERSE PRIMER:		
Sambucus RIP	D25317; U76524	GCG GCA AGG TTC
Sambucus RIP	U66191	GCG GCC AGG TTC
PAP	X55383	GCA GCA AGA TTC
PAP	X98079	GCA GCG CGA TTC

In order to facilitate the cloning of amplification products, *EcoRI* and *XbaI* sites were engineered into the 5' ends of the forward and reverse primers respectively. It is thought that these flaps may contribute to the stability of the PCR complexes:

"It may be that the common practice of adding an arbitrary 5' extension to a degenerate primer in order to introduce a restriction site is inadvertently responsible for many successful amplifications of unknown sequences in the past."

- Rose *et al.*, 1998.

The pair of degenerate PCR primers that we designed from the relatively homologous regions is given in **Table 2.1.2**. The design of such degenerate primers is largely a subjective process.

Table 2.1.2 Pair of degenerate PCR primers designed for RIP detection in grapevine. The restriction enzyme recognition sites are in *italics*.

Forward Primer 5' *CCGAATTC*AMYYTDTAYGTG 3'

Reverse Primer 5' AGTCTAGAGAAYCKBGMTGC 3'

2.2 Isolation of 300 bp fragment from *Vitis* gDNA

The pair of primers was used in a PCR reaction using the low-stringency conditions typical of RAPD amplification cycles. PCR amplification of *Vitis* DNA produced a series of bands characteristic of RAPD reactions. Amongst these bands was a fragment of the expected size - in the order of 300 bp. The desired band was isolated and used as template in successive rounds of PCR amplification. This process eliminated unwanted background bands and provided us with a homogenous product. **Figure 2.2.1(a)** and **(b)** are photographs of the PCR amplification and re-amplification products respectively, fractionated on an agarose gel.

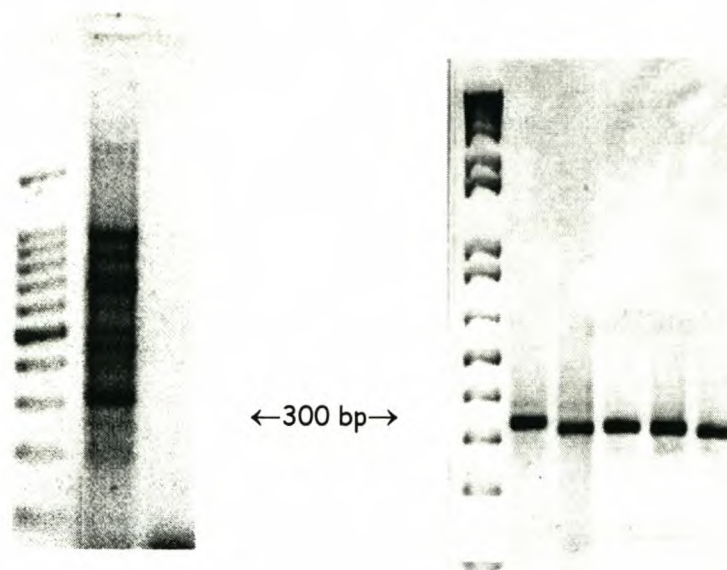


Figure 2.2.1 Fragments amplified from *Vitis* gDNA using degenerate primers. PCR products were fractionated on a 0.8% 1X TAE agarose gel. (a) Amplification products of expected size (~300 bp) (b) Fragment was re-amplified to homogeneity.

The fragment was cloned into pGEM-T Easy (see **Figure 2.2.2** and **Appendix V11**) and the nucleotide sequence was determined by means of automatic DNA sequencing. The sequence is recorded in **Appendix IV**.

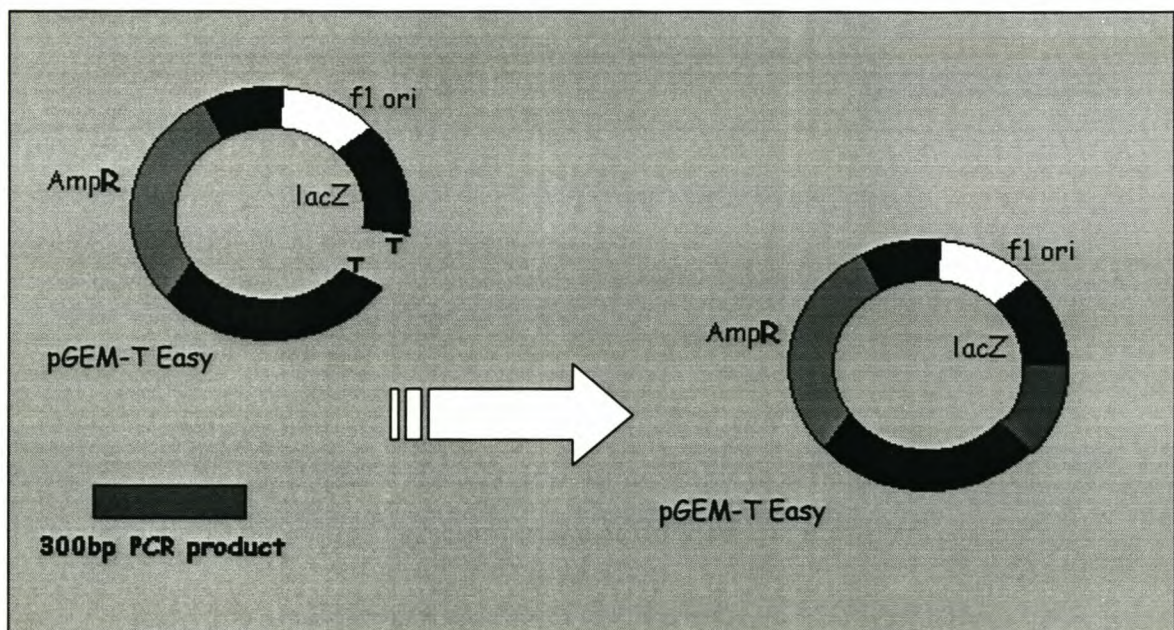


Figure 2.2.2 Blunt-end cloning of PCR product into pGEM-T Easy.

The sequence was analyzed by the BLAST (Altschul et al., 1990; <http://www.ncbi.nlm.nih.gov/blast>) server and showed no homology with any reported RIP. A sequence alignment with that of pokeweed antiviral protein revealed absolutely no homology (see **Figure 2.2.3**).

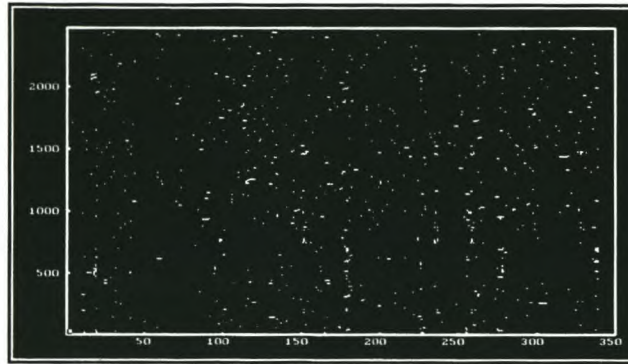


Figure 2.2.3 Dot plot nucleotide sequence alignment of the amplified fragment and the published PAP-gene .

A different set of amplification conditions was tested: several rounds of low stringency annealing (to facilitate the incorporation of the 5' flap into the PCR product) initiated PCR amplification, followed by the regular number of cycles at higher stringency (to reduce the overall levels of non-specific primer binding). The amplification products are shown in **Figure 2.2.4**. Once again, a band of expected size was amplified from the *Vitis* DNA. This time the band was only just visible. However, the same band was not amplified from the gDNA of any of the positive controls; namely *Phytolacca*, *Dianthus*, *Luffa* and *Mirabilis* - plants all known to produce RIPs. Furthermore, several "background" bands were amplified from the *Vitis* gDNA that were of much greater clarity than the desired 300 bp fragment. These facts cast serious doubt on the notion that the 300 bp fragment may indeed be part of a RIP gene. It was decided that this set of results did not merit any further investigation.

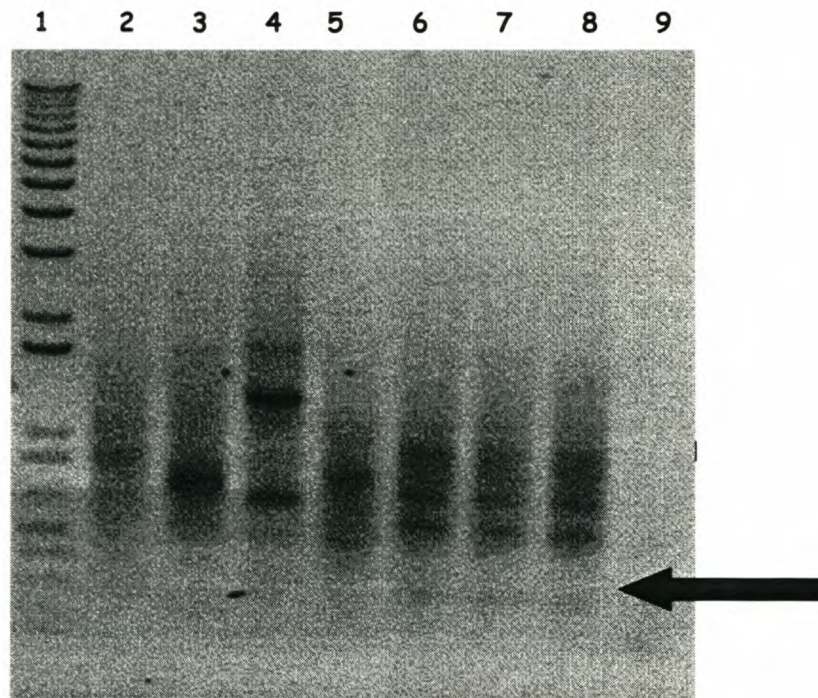


Figure 2.2.4 Fragments generated from various plants using degenerate PCR primers. Lane 2: PCR of phytolacca DNA; Lane 3: PCR of dianthus DNA; Lane 4: PCR of luffa DNA; Lane 5: PCR of mirabilis DNA; Lanes 6-8: PCR of Vitis DNA. The arrow indicates faint bands of size 300bp, amplified from Vitis, but not from the other plants.

2.3 Discussion

It is still not known whether a RIP gene lurks in the 475 Mbp (Lodhi *et al.*, 1995) that constitute the grapevine genome. No member of the Vitaceae family is known to produce a RIP and there is no biological evidence of RIP expression in grapevine:

"The extracts of *V. vinifera*..., either green or senescent, did not inhibit protein synthesis or brought about release of adenine from DNA..."

- Stirpe *et al.*, 1996.

The tremendous amount of diversity between RIPs produced by different species casts doubt on the theory that a pair of degenerate PCR primers may be designed for the detection of new RIPs. The challenge is to detect regions of high sequence homology encoded by codons of low degeneracy (Rose *et al.*, 1998). This transpires to be a formidable task in the case of the RIP family.

A major problem in our efforts was the ease with which the primers bound to the template DNA - at multiple sites. Increasing the annealing temperature did little to improve the situation. The primers that we designed have a combined degeneracy of 576-fold. Rose *et al.*, 1998, state that the maximum level of acceptable degeneracy is 128. Furthermore, the primers are based on regions of just 12 nucleotides. These factors contribute to the high degree of non-specific primer binding and the concurrent background effect.

The inadequacy of our design was highlighted by the fact that the primers were unable to amplify the expected portion of RIP from a series of plants known to contain the gene. Hardly an appropriate tool for the detection of novel RIPs, then!

However, all hope should not be cast aside. Useful algorithms for the design of degenerate primers are constantly being developed and made available to the public in the form of computer programs. A recent example of this is CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer), which can be accessed on the World Wide Web (<http://www.blocks.fhcrc.org/codehop.html>). According to the authors:

"The practical utility of the hybrid method is demonstrated by successful amplification of unknown sequences that are too diverged from known sequences to be readily isolated by standard methods."

- Rose *et al.*, 1998.

Even CODEHOP had no suggestions for the design of primers from an alignment block of RIPs originating from different sources. Until such time that degenerate primer design becomes a well-defined science, detection of novel RIPs will most probably do best by following conventional routes.

2.4 Methods

2.4.1 Isolation of gDNA

DNA was extracted from grapevine, pokeweed, luffa, dianthin and mirabilis as stipulated in **Appendix II**.

2.4.2 PCR amplification

Reagents in PCR reaction mixes were combined at the concentrations given in **Appendix III**.

Two sets of amplification programs were used. The first (see **Table 2.4.1**) is much the same as a typical RAPD reaction. The second (see **Table 2.4.2**) begins with six cycles of low-stringency annealing for the incorporation of the additional 5' flaps into the PCR products. The T_m of the whole forward primer is 49.2°C and that of the reverse, 52.3°C (as stated on the primer supplier's dispatch note). However, in order to reduce the high level of non-specific primer binding, the annealing temperature in the main amplification section was raised to 65°C.

Temperature	Time	Number of cycles
94°C	2 minutes	1
94°C	30 s	
37°C	30 s	40
72°C	1 minute	
72°C	1 minute	1
4°C	Indefinitely	HOLD

Table 2.4.2.1 First PCR program tested on degenerate primers.

Temperature	Time	Number of cycles
94°C	5 minutes	1
94°C	30 s	
39°C	45 s	6
72°C	1 minute	
94°C	30 s	
65°C	45 s	36
72°C	1 minute	
72°C	5 minute	1
4°C	Indefinitely	HOLD

Table 2.4.2.2 Modified program for amplification with degenerate PCR primers.

2.4.3 Re-amplification of desired fragment

Amplification products were separated by means of agarose gel electrophoresis, at 80 V on a 0.8% 1X TAE gel. The 300 bp fragment was excised from the gel and frozen solid. The block of DNA-containing agarose was thawed and

centrifuged (10,000 g for 5 minutes) through a sintered glass column in order to remove all traces of agarose. A microlitre of the purified flow-through was re-amplified using the initial amplification program (**Table 2.4.1**).

2.4.4 Cloning fragment into pGEM-T Easy and automatic DNA sequencing

The 300 bp fragment was purified from agarose (as described in the previous section) and cloned into pGEM-T Easy according to the instructions prescribed by the manufacturers. This vector system has both an ampicillin resistance gene (for the selection of clones containing the vector) and blue/white colour screening, in the form of a *lacZ* gene surrounding the site of cloning (for the selection of recombinant clones). Positive (white) clones were used to inoculate ~5 ml of LB medium (100 µg/ml ampicillin selection). The cultures were shaken overnight (~225 rpm) at 37°C and plasmid DNA was isolated according to the method described in **Appendix V**. There is an *EcoRI* recognition site on either side of the vector's cloning site. Isolated plasmid DNA (~5 µg) was digested with 1 U *EcoRI* in the presence of 1X buffer H for one hour, at 37°C. The excision of the 300 bp fragment from plasmid DNA served as confirmation of successful cloning (results not shown).

About 350 ng of isolated recombinant plasmid was submitted to the University's Core Sequencing Facility for automatic DNA sequencing, using standard M13 primers.

Chapter 3: The isolation and cloning of RIPs from several plants

"Pooh Bear...went round to Piglet's house to see what Piglet was doing. It was still snowing as he stumped over the white forest track, and he expected to find Piglet warming his toes in front of his fire, but to his surprise he saw that the door was open, and the more he looked inside the more Piglet wasn't there. 'He's out,' said Pooh sadly. 'That's what it is. He's not in. I shall have to go a fast Thinking Walk by myself. Bother!' But first he thought he would have to knock very loudly just to make quite sure . . . and while he waited for Piglet not to answer, he jumped up and down to keep warm, and a hum came suddenly into his head..."

- Milne, 1974.

This chapter deals with the isolation of several well-documented RIPs from various species of plant. Unique PCR primer pairs were designed for the cloning of RIPs from pokeweed, carnation, luffa and mirabilis. Pokeweed antiviral protein (PAP), dianthin, β -luffin and mirabilis antiviral protein (MAP) are all examples of type 1 RIPs. **Figure 3.1** outlines our general strategy. The following four sections contain the details of each cloning. The last section is a combined account of the methods used.

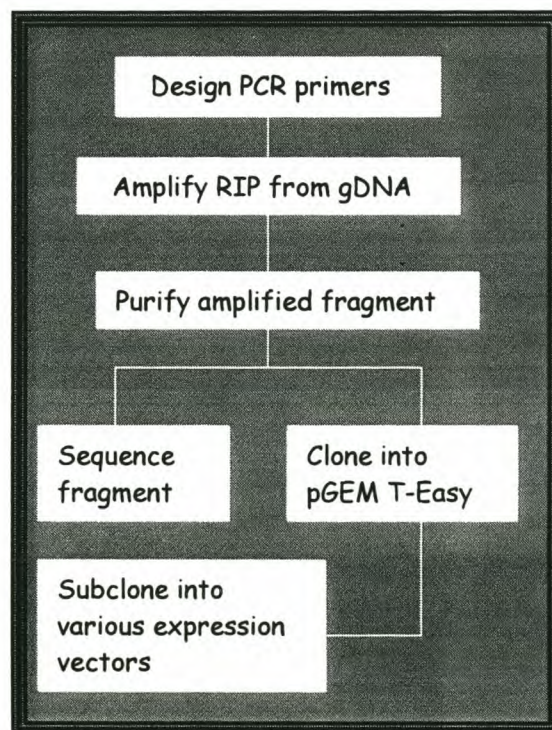


Figure 3.1 Sequence of procedures followed.

3.1 Attempted isolation of PAP from *Phytolacca octandra*



Pokeweed antiviral protein is the "...best characterized RIP..." (Chen *et al.*, 1991). Numerous isoforms of PAP have been isolated, including α -PAP, PAP-S, PAPII, PAP-C (Barbieri *et al.*, 1982(a); Houston *et al.*, 1983; Barbieri *et al.*, 1989; Poyet *et al.*, 1994). The exogenous application of PAP protects heterologous plant species from a range of viral infections (Lodge *et al.*, 1993).

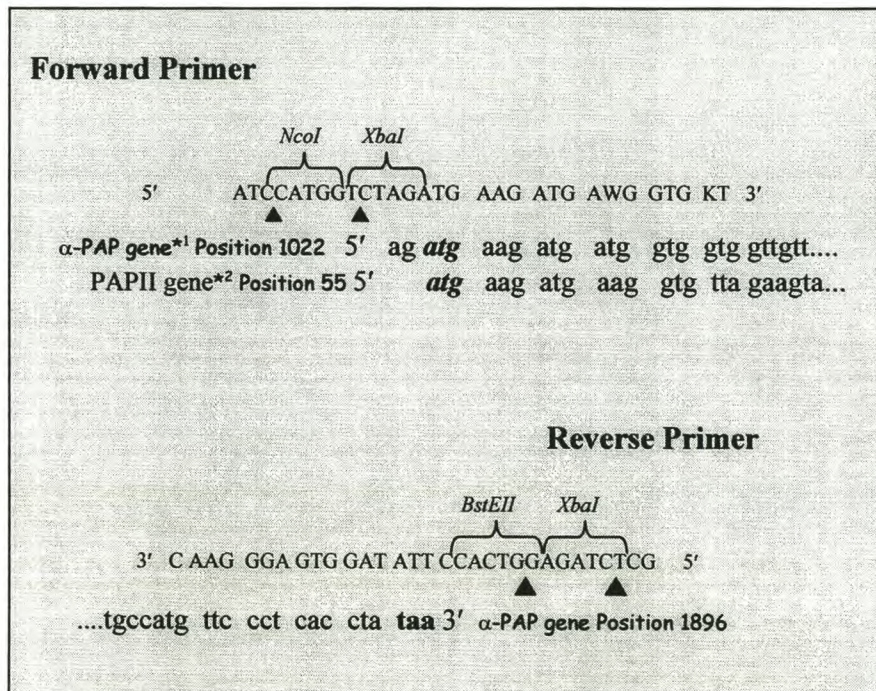
Furthermore, potato and tobacco plants transformed with PAP are resistant to both the mechanical and aphid transmission of viruses (Lodge *et al.*, 1993).

"Previous methods for creating virus-resistant plants have been specific for a particular virus or closely related viruses. To protect plants against more than one virus, multiple genes must be introduced and expressed in a single [transgenic] line. Expression of PAP in transgenic plants offers the possibility of developing resistance to a broad spectrum of plant viruses by expression of a single gene."

- Lodge *et al.*, 1993.

Antiviral proteins have been isolated from several species of pokeweed: *Phytolacca americana* (Wyatt *et al.*, 1969), *Phytolacca esculenta* (Kassanis *et al.*, 1948) and *Phytolacca dioica* (Parente *et al.*, 1993). We attempt to clone a RIP gene from *Phytolacca octandra*, the variety of pokeweed growing wild in South Africa, using PCR primers based on α -PAP from the American weed.

The nucleotide sequence of both PAP and PAP-II were downloaded from GenBank. There is much homology between the 5' ends of these two genes. **Figure 3.1.1** illustrates the primers designed from the selected segments of nucleotide sequence. A forward primer, with a four-fold degeneracy, was designed for the amplification of both PAP and PAP-II. A reverse primer was designed solely for the amplification of PAP.



*¹ α -PAP sequence as submitted to GenBank (Kataoka *et al.*, 1992(a)). Available under accession number X55383.

*² PAPII sequence as submitted to GenBank (Poyet *et al.*, 1994). Available under accession number X78628.

Figure 3.1.1 PCR primer design for amplification of PAP. The forward primer may also be used to isolate PAPII at a later stage- a reverse primer was designed specifically for this purpose (not shown). The **start codon** and **stop codon** are in bold.

DNA was isolated from the South African species of pokeweed and used in a standard PCR reaction. Various bands were amplified - typical of a RAPD reaction. No bands of the expected size (874 bp) were generated. There was, however, a very prominent band in the 500 bp region. This band was amplified consistently, over a range of annealing temperatures. It was thought that the reduced size might be the result of a truncation event during the evolutionary history of the plant. The 500 bp fragment was purified from an agarose gel and re-amplified to homogeneity (see **Figure 3.1.2**).

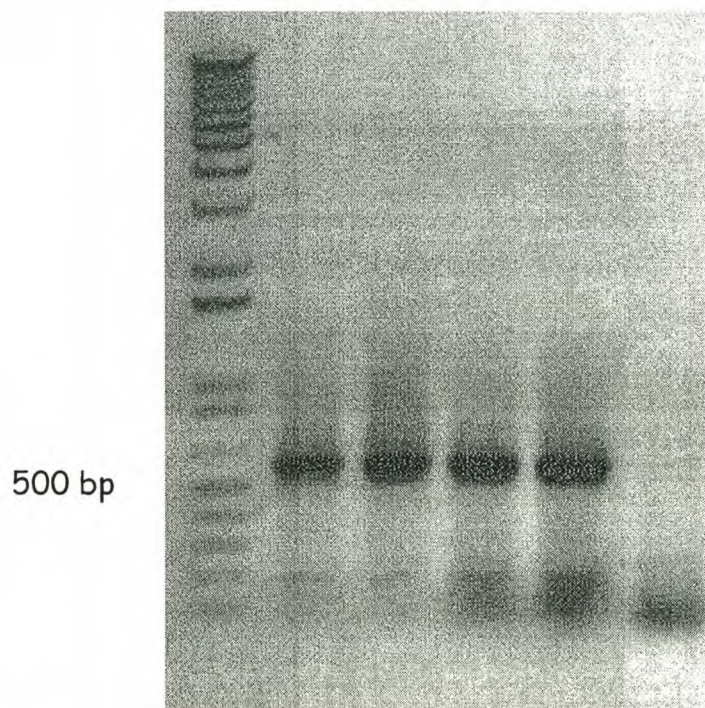


Figure 3.1.2 Amplification products of pokeweed DNA. The bands produced were about 400 bp smaller than expected.

Automatic DNA sequencing (see **Appendix IV**) revealed that this fragment does not encode a RIP: the results of a Genepro sequence alignment of our fragment with that of the published PAP sequence are given in **Figure 3.1.3**.

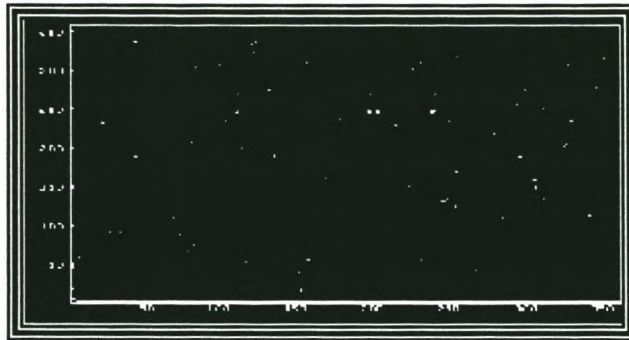


Figure 3.1.3 Dot-plot sequence alignment of our sequence with that of the published PAP sequence. There is clearly no relationship between the two sequences.

Furthermore, a BLAST analysis showed no homology between our fragment and any published RIP. Our attempt to clone a RIP from the South African variety of pokeweed was unsuccessful. Perhaps this should come as no great surprise: PAPII shares just 33% amino acid homology with PAP and PAP-S (Poyet *et al.*, 1994). And these three RIP isoforms are all to be found in the very same plant.



3.2 Cloning dianthin 30 into pKK223-3, a bacterial expression vector

The carnation family has long been known to produce "a highly potent inhibitor of virus infection" (Ragetli *et al.*, 1962). Evidence of carnation's RIP activity had already been published by the early 1950s :

"...an inhibitory substance, present in the leaves of *D. barbatus*, might be responsible for suppression or delay of virus multiplication...the inhibitor was generally present in the leaves of members of the genus *Dianthus*."

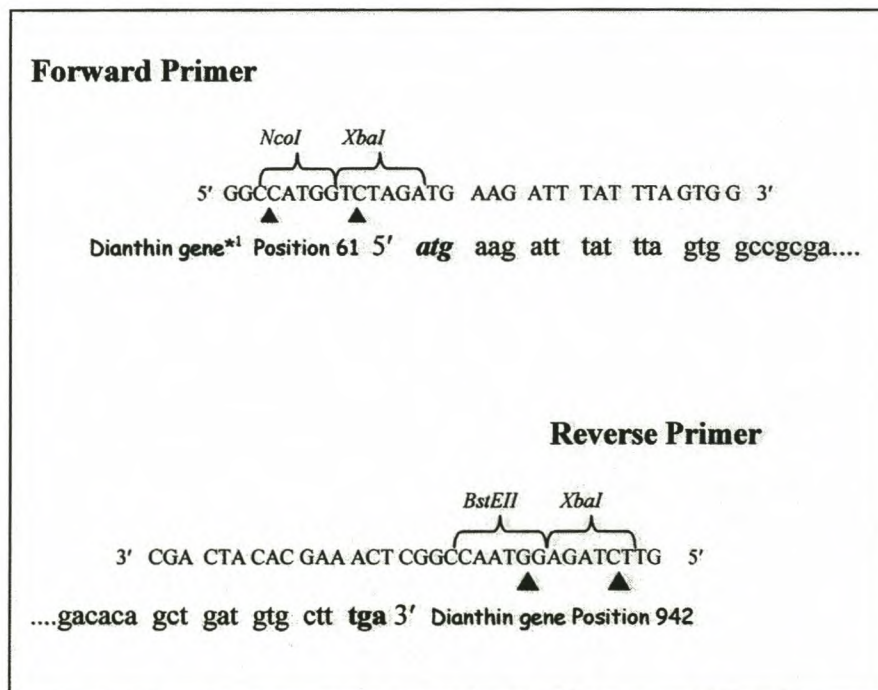
- Weintraub *et al.*, 1952.

The potency of dianthins has been exploited in the production of genetically engineered tobacco plants that are resistant to African cassava mosaic virus (ACMV). The plants were transformed with a dianthin-construct that is driven by an ACMV virion-sense promoter, which is transactivated by a viral gene product. Although the range of viruses to which these plants are resistant is extremely narrow, the regulated expression of dianthin ensures that the toxin is produced only in those cells infected with virus. Constitutive RIP expression may be hazardous for the host plant and result in atypical phenotypes (Hong *et al.*, 1996).

In this section we discuss the cloning of dianthin 30 into pKK223-3, a bacterial expression vector.

The pKK223-3 vector is 4584 bp in size and has a very powerful, IPTG-inducible *tac* promoter. The inducible promoter ensures that bacterial cultures can grow to saturation before production of the toxic dianthin gene is initiated. An ampicillin resistance gene facilitates the isolation of positive transformants. This vector requires a *lac* I^q strain of bacterium (such as JM109 or JM105) - it is not compatible with DH5 α cells.

Figure 3.2.1 gives the pair of PCR primers we designed for the isolation of dianthin 30 from the standard carnation plant, *Dianthus caryophyllus*. The published dianthin 30 sequence is 882 bp from the start to the stop codon.



*¹ Dianthin sequence as submitted to GenBank (Legname *et al.* 1991). Available under accession number X59260.

Figure 3.2.1 Primers for the amplification of dianthin 30.

A fragment of the expected size was amplified from gDNA using standard PCR techniques (see **Figure 3.2.2**). The PCR reaction mix was fractionated by means of agarose gel electrophoresis. A series of less prominent bands was also generated. It is possible that some of these may encode other members of the dianthin family. Perhaps the slightly larger band (of ~1000 bp) encodes dianthin 32. The target fragment was fractionated by agarose gel electrophoresis, purified from the gel and re-amplified to homogeneity using the same set of primers. This was submitted for nucleotide sequencing.

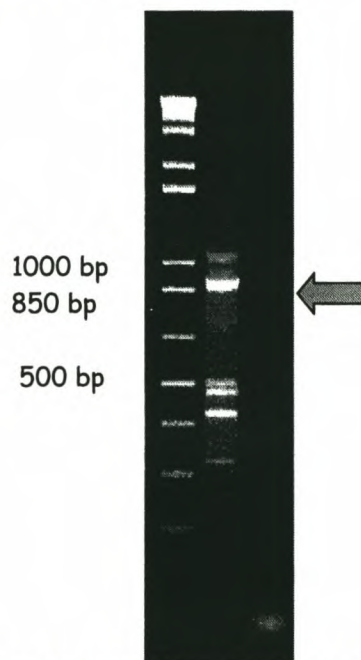


Figure 3.2.2 Fragment of expected size amplified from carnation gDNA.

The nucleotide sequence (see **Appendix IV**) shares 94% homology with dianthin 30, with an E value of 0.0 (BLAST analysis). It also has significant homology with saporin, the RIP from *Saponaria officinalis*. **Figure 3.2.3** is an alignment of our sequence with the published dianthin 30 sequence.

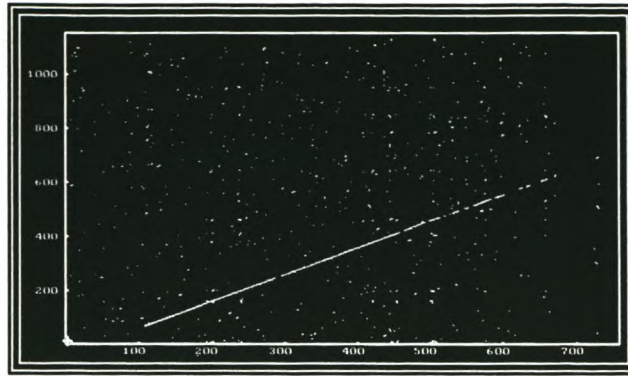


Figure 3.2.3 Dot plot sequence alignment to confirm that the amplified fragment is dianthin.

The fragment was cloned into pGEM-T Easy; recombinant clones were selected by PCR analysis; and freezer cultures were made of pGEM-T Easy-dianthin.

The general strategy for subcloning dianthin 30 from pGEM-T Easy into pKK223-3 is illustrated in **Figure 3.2.4**.

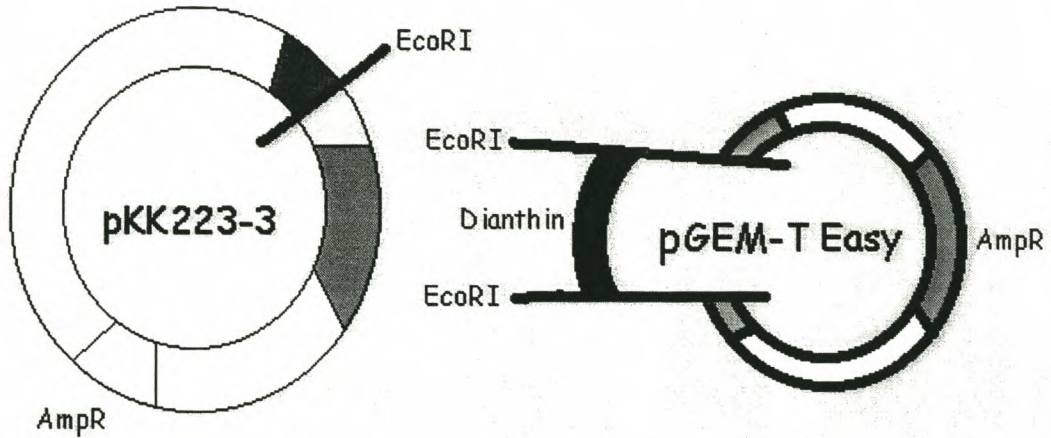


Figure 3.2.4 Subcloning dianthin into pKK223-3.

pGEM-T Easy contains two *EcoRI* recognition sites: one on either side of the cloned dianthin gene. Expression depends on the presence of a ribosome binding site in the construct. Since the dianthin gene does not have one of its own, it has to be situated within 13 bp of the vector's ribosome binding site. This condition is met by cloning the gene at the *EcoRI* site. The orientation of the cloned insert cannot be controlled, and must be assessed for each clone by means of restriction enzyme analysis. Dianthin 30 has an internal *EcoRI* recognition site about 11 amino acids upstream of the stop signal, which results in a truncated form of dianthin being cloned into the bacterial vector. It will be interesting to establish whether the carboxy-terminus is required for the enzymatic activity of the protein. The active site of the gene is intact.

Isolated pKK223-3 was linearized by digestion with *EcoRI* (see **Figure 3.2.5**) and dephosphorylated to prevent it from re-ligating upon itself. Purified pGEM-T Easy-dianthin was digested with *EcoRI* to liberate the *dianthin* gene.(see **Figure 3.2.6**).

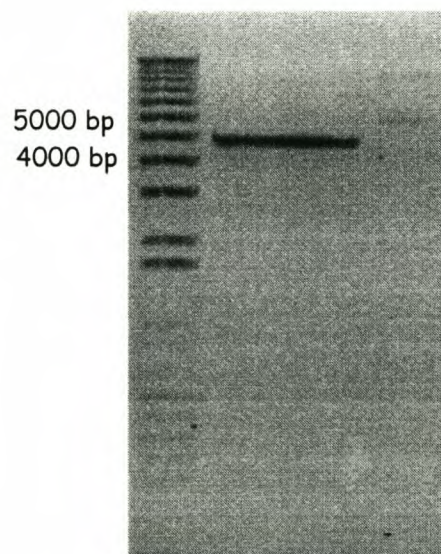


Figure 3.2.5 pKK223-3 digested with *EcoRI*. Lane 3 contains undigested pKK223-3.

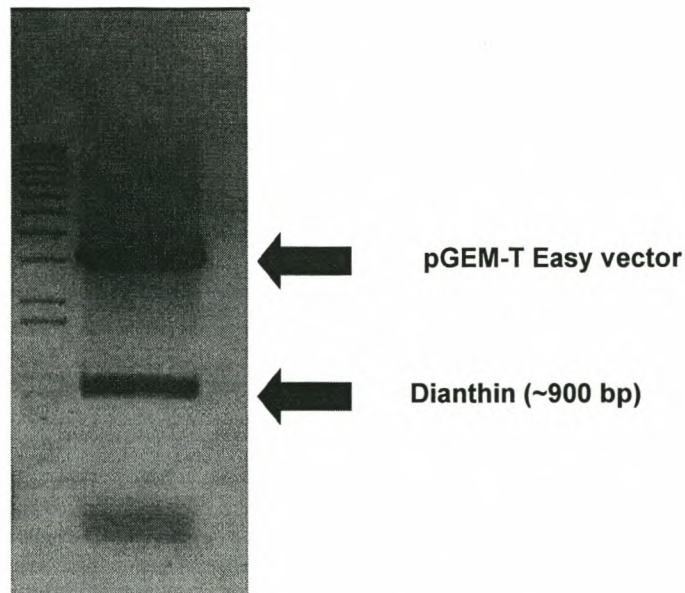


Figure 3.2.6 pGEM-T Easy-dianthin digested with *EcoRI*.

Insert and vector were ligated at a ratio of 5:1 (the insert is approximately one-fifth the size of the vector). The efficiency of the dephosphorylation reaction was gauged by two control ligation reactions: one ligation contained undephosphorylated vector and the other contained dephosphorylated vector (neither control had any insert). JM109 was transformed and positive clones were selected by means of digestion analysis of isolated plasmid DNA (see **Figure 3.2.7**).

The control reactions verified that dephosphorylation had been successful: many colonies resulted from transformation with the undephosphorylated vector control, whereas hardly any colonies occurred on the other control plate.

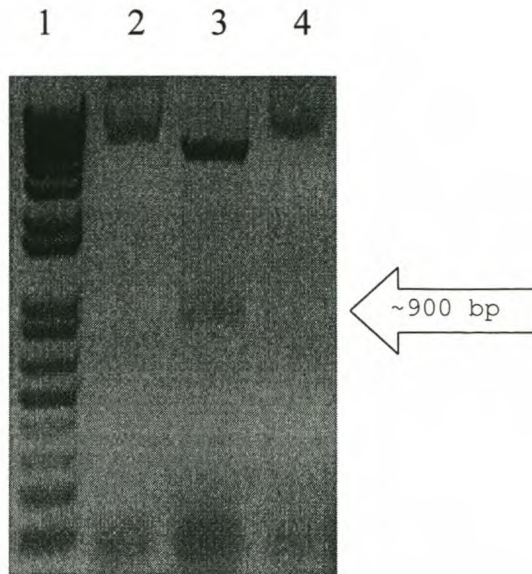


Figure 3.2.7 Confirmation of cloning dianthin into pKK223-3. Digestion of plasmid DNA from clones with *EcoRI* released the ~900 bp dianthin fragment from successful transformants. Lane 2: uncut pKK223-3; Lane 3: recombinant pKK223-3-dianthin, digested with *EcoRI*; Lane 4: unsuccessful transformant (no dianthin present in clone), digested with *EcoRI*.

The orientation of the *dianthin* gene insert in the clones was determined by further restriction enzyme analysis of isolated plasmid DNA (see **Figure 3.2.8**). Dianthin contains a *StyI* recognition site 245 bp from the start codon. The pKK223-3 vector has a *StyI* site at position 1252 and a *HindIII* recognition site at position 4554.

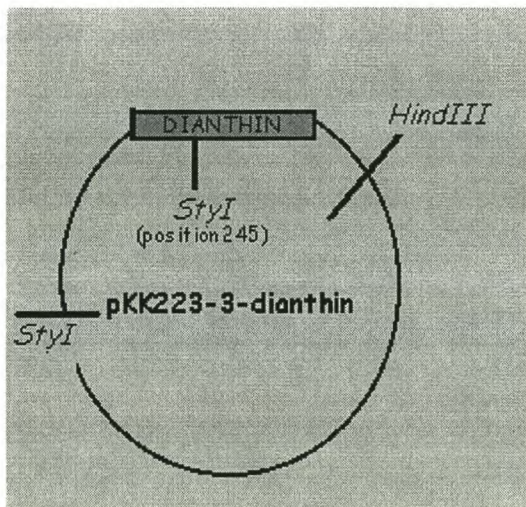


Figure 3.2.8. A schematic representation of pKK223-3-dianthin, with dianthin cloned in the correct (5'→3') orientation). The distance between dianthin's *StyI* and *HindIII* is ~650 bp.

If the *dianthin* gene is cloned in the correct 5'→3' orientation, a double digest of pKK223-3-*dianthin* with *HindIII* and *StyI* will liberate three fragments: one of ~650 bp (the region between the insert's *StyI* site and the vector's *HindIII*); another of ~1000 bp (the region between the two *StyI* sites) and the third a fragment of ~3000 bp (the region between the vector's two restriction enzyme recognition sites). Those clones in which the *dianthin* gene is cloned in the reverse orientation would yield the following fragments: ~250 bp; ~1400 bp, and ~3000 bp. **Figure 3.2.9** is a photograph of the double digests fractionated on an agarose gel.

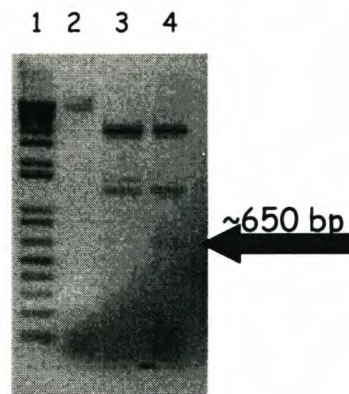


Figure 3.2.9 Determining the orientation of the cloned *dianthin* gene. Lane 2: uncut pKK223-3-*dianthin*; Lane 3: pKK223-3, with *dianthin* in the incorrect (3'→5') orientation digested with *StyI* + *HindIII* (ambiguous result: the ~250 bp fragment is not visible and the middle band is smaller than expected); Lane 4: pKK223-3 with *dianthin* cloned in the correct (5'→3') orientation digested with *StyI* + *HindIII* (the liberated band ~650 bp is only just visible).

Recombinant clones containing the *dianthin* gene in the correct orientation may be cultured and induced to express the RIP. The activity of the purified *dianthin* can then be assessed by monitoring its affect on virus infection in plants.

3.3 Cloning β -luffin into pCambia3301, a plant expression vector



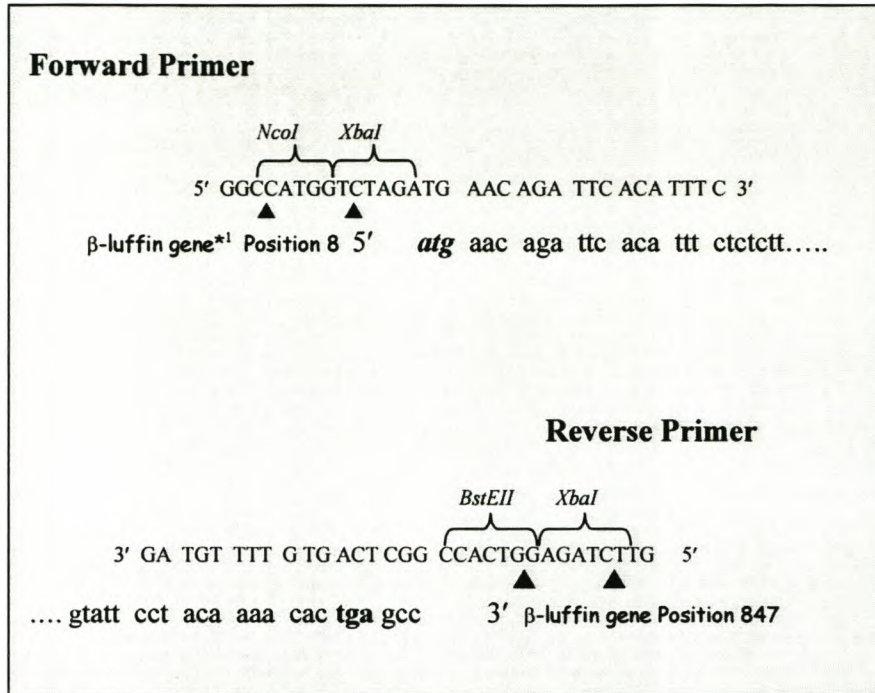
In 1983, Kishida *et al.* discovered and purified a RIP from *Luffa cylindrica*, a cucurbit of Asian origin, the fruit of which is frequently used as bath sponges. They called the protein luffin. In the years that followed, numerous RIP isoforms have been isolated from this plant, having varying degrees of similarity to each other (Kataoka *et al.*, 1992(c); 1992(d); Kishida *et al.*, 1983).

In this section, we isolate a gene encoding luffin from *Luffa octandra*, a different species of luffa, using PCR primers designed for the isolation of β -luffin from *Luffa cylindrica*. Our luffin gene shares 97% homology (E value: 0.0) with the published sequence of β -luffin (BLAST analysis). There is also significant sequence homology with three other RIPs: alpha-trichosanthin (87% homology); α -luffin (86%); and alpha-momorcharin (84%). We clone this gene into pCambia3301 (Jefferson *et al.*, *personal correspondence*), a binary plant expression vector that is 11307 bp in size.

"Because grapevine is a vegetatively-propagated perennial crop with production and marketing rooted in traditional cultivars, genetic engineering is a very promising approach for the development of pest resistant cultivars."

- Kikkert *et al.*, 1996

A pair of PCR primers was designed for the amplification of the β -luffin gene (see **Figure 3.3.1**). The size of the β -luffin gene is ~844 bp.



*¹ β -luffin sequence as submitted to GenBank (Katakaoa *et al.*, 1991). Available under accession number X62372.

Figure 3. 3.1 PCR primers designed for the amplification of β -luffin.

A fragment of the expected size was amplified from the gDNA of *Luffa octandra* using standard PCR techniques- see **Figure 3.3.2**.

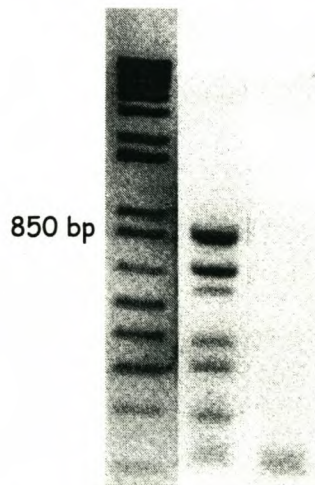


Figure 3.3.2 Fragment amplified from *Luffa octandra*.

PCR products were resolved by means of agarose gel electrophoresis and the desired fragment was excised from the gel, purified and re-amplified to homogeneity. Gel-purified amplification product was submitted for automatic DNA sequencing. The sequence of this fragment appears in **Appendix IV**. The identity of the molecule was confirmed: **Figure 3.3.3** shows an alignment of our sequence with the published one.

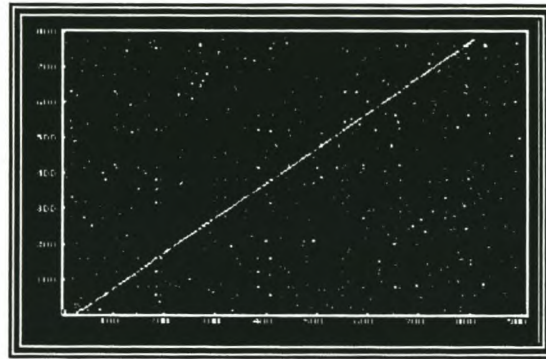


Figure 3.3.3 DNA sequence alignment of amplified fragment with that of published sequence of β -luffin.

The gene was cloned into pGEM-T Easy and stored in glycerol stocks. **Figure 3.3.4** outlines our strategy for subcloning β -luffin into pCambia3301.

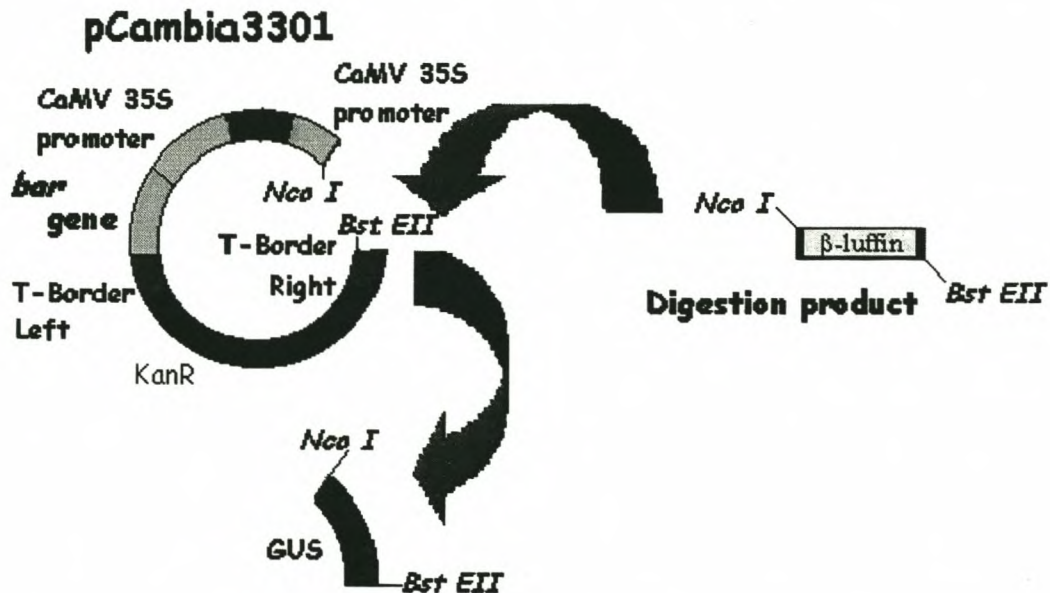


Figure 3.3.4 Cloning the β -luffin gene into pCambia3301. The GUS gene is excised and replaced with the β -luffin gene.

Liquid cultures of pGEM-T Easy-luffin and pCambia3301 were grown on selection (100 µg/ml ampicillin and 50 µg/ml kanamycin, respectively) overnight. Plasmid DNA was isolated and digested with *BstEII* and *NcoI*. Digestion products were resolved by agarose gel electrophoresis (see **Figure 3.3.5**). Both the β -luffin gene and the linearized pCambia3301 (minus GUS) were purified from the gel.

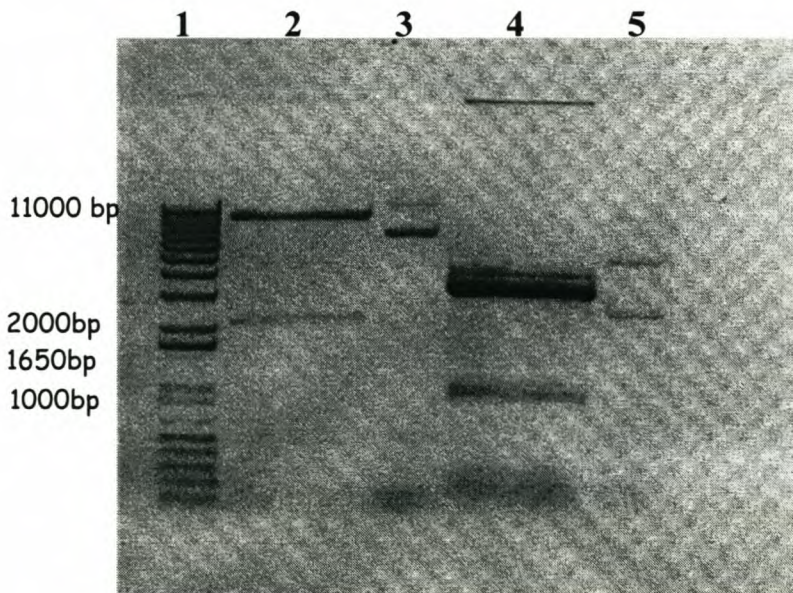


Figure 3.3.5 Preparation of reagents for ligation reaction. Lane 2: pCambia3301, digested with *BstEII* + *NcoI* (the 2049 bp GUS fragment is excised); Lane 3: uncut pCambia3301; Lane 4: pGEM-T Easy- β -luffin digested with *BstEII* + *NcoI* (the β -luffin gene is liberated); Lane 5: uncut pGEM-T Easy- β -luffin.

DNA concentrations were determined on a spectrophotometer and insert and vector were ligated at a ratio of 10: 1. Competent DH5 α cells were transformed. PCR amplification of the β -luffin gene from the resulting colony provided preliminary evidence that the cloning had been successful (**Figure 3.3.6**).

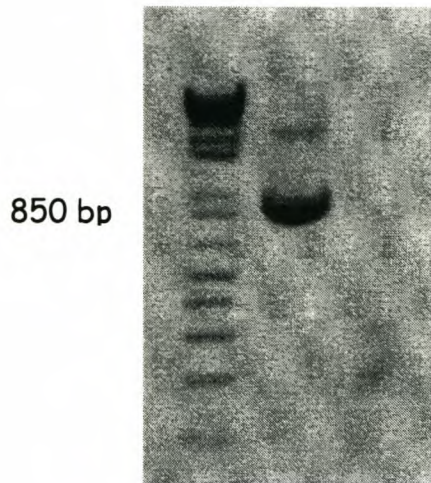


Figure 3.3.6 PCR amplification of pCambia3301-luffin. A fragment of ~850 bp was amplified from the clone.

Plasmid DNA was isolated from cultured clones and digested with *NcoI* and *BstEII*. Restriction analysis confirmed that the clone was indeed recombinant.

Figure 3.3.7 shows the various digestion products of plasmid DNA from this colony.

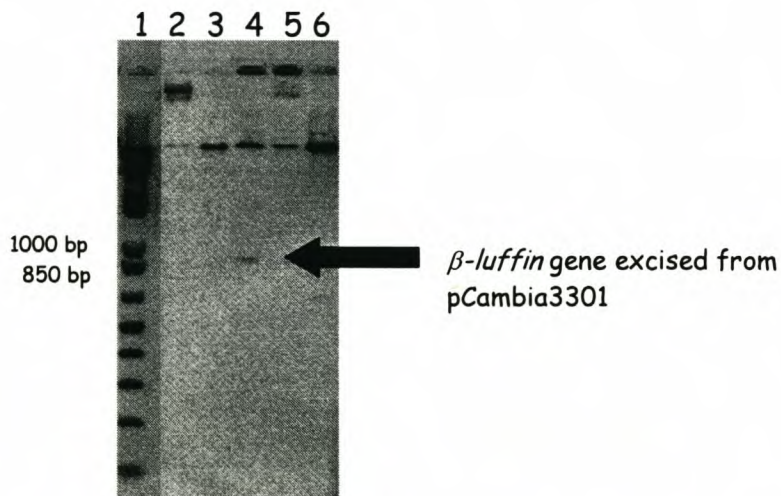


Figure 3.3.7 Confirmation of cloning into pCambia3301. Lane 2: undigested pCambia3301; Lane 3: linearized pCambia3301- β -luffin (digested with *NcoI* + *BstEII*); Lane 4: pCambia3301- β -luffin digested with *NcoI* and *BstEII* (β -luffin gene is liberated); Lane 5: pCambia3301- β -luffin linearized with *BstEII*; Lane 6: pCambia3301- β -luffin linearized with *NcoI*.

We have cloned the β -luffin gene into a plant expression vector, pCambia3301. The fact that this gene is derived from *Luffa octandra*, and not from the documented *Luffa cylindrica*, is noteworthy. These two species of *Luffa* must be close relatives for the sequence of one of their ribosome-inactivating protein genes to be so well conserved.

"Genetic transformation of grapevines has been difficult, particularly for *V. vinifera*. Most attempts have utilized *Agrobacterium*-mediated transformation..."

- Kikkert *et al.*, 1996.

This construct may now be used to transfect either *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, with the ultimate aim of expressing the RIP in grapevine. The expression of an agronomically important gene in hairy root cultures of grapevine was first reported in 1997, when cultures were genetically engineered to produce a viral coat protein (Torregrosa *et al.*, 1997). The biolistic transformation of grapevine (Kikkert *et al.*, 1996) offers an alternative approach. The pCambia3301-luffin construct is also a suitable candidate for bombardment by gene gun.

3.4 Cloning MAP into pFLAG, a yeast expression vector

Mirabilis jalapa is an ornamental plant indigenous to South America, and is commonly known as the four o'clock plant. MAP has very potent RIP activity and is thought to be an ancestral member of the RIP family (Kataoka *et al.*, 1993).



"Root extracts of *M. jalapa* sprayed over potato plants conferred stable inhibition of viral infection...The viral inhibition was attributable to MAP and was based on its RIP activity and its biochemical stability."

-Vivanco *et al.*, 1999.

Despite the fact that crude plant extracts are sufficient to impart the viral resistance characteristic of potent MAP, researchers have developed strategies for the molecular production of this toxin. This proved to be a rather complex task::

"When MAP genes were expressed in *Escherichia coli*, the growth of the transformants was completely inhibited."

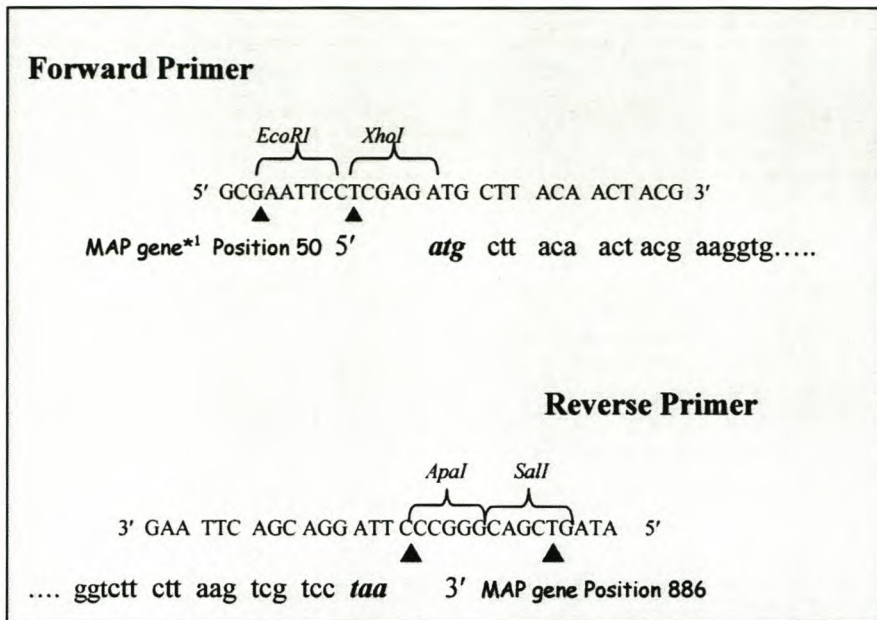
-Habuka *et al.*, 1991.

This prompted the researchers to make use of a vector system with a temperature sensitive repressor - enabling bacteria to grow to saturation before being subjected to the toxic gene. And thus, relatively good yields of active MAP have been generated.

However, since MAP is one of the few RIPs known to contain an intron (Kataoka *et al.*, 1993), it would be interesting to gauge the effects of the post-translational cleavage of MAP. Such modifications are best done in eukaryotic expression systems. We decided to clone MAP into a yeast expression vector. Yeast is the simplest of these systems. YEpFLAG-1 (henceforth referred to as pFLAG), 7205 bp in size, was our choice of expression vector.

This system has many appealing features. An octapeptide fused to the amino terminal of the expressed protein facilitates detection and purification of the protein. Although the tiny tag is unlikely to interfere with the quality of the protein, it may be proteolytically removed from the expressed protein. Furthermore, the vector is equipped with a glucose repressor. As yeast colonies grow to saturation, the glucose in the growth medium is metabolized, and expression of the foreign protein is initiated. An α -factor leader peptide transports the protein out of the cell. The cloning construct is generated in *E. coli*. This may then be transformed into yeast (not done in this study). Ampicillin resistance and TRP1 genes permit the selection of transformed bacteria and yeast cells respectively. In short, this vector is well-suited for the production and isolation of toxic molecules, like MAP.

PCR primers were designed to amplify the entire gene (see **Figure 3.4.1**).



*1 MAP sequence as submitted to GenBank (Katakoa *et al.*, 1991). Available under accession number D90347.

Figure 3.4.1 PCR primer design for the amplification of MAP.

Standard PCR techniques were used to amplify the MAP gene from total genomic DNA. The ~1000 bp fragment generated was fractionated by agarose gel electrophoresis and re-amplified to homogeneity. **Figure 3.4.2** is a photograph of the isolated gene.

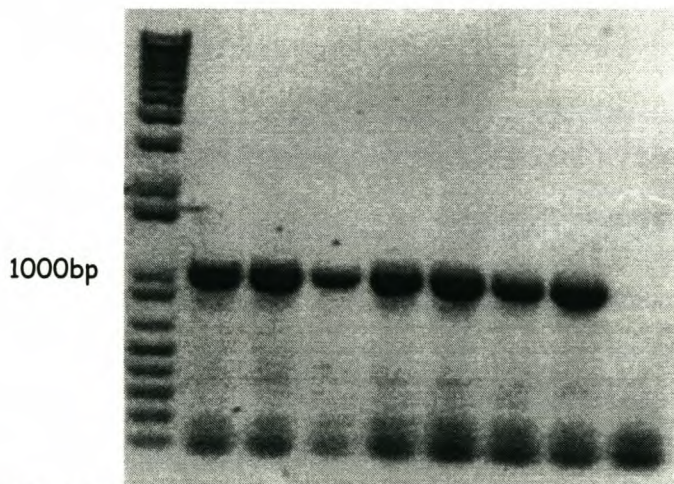


Figure 3.4.2 Isolation of MAP from gDNA. A prominent fragment in the region of the expected size was consistently amplified from mirabilis DNA.

Automatic DNA sequencing confirmed the identity of the fragment (sequence appears in **Appendix IV**). A BLAST analysis of the fragment registered 96% homology (and an E value of 0.0) with the sequence of MAP. Sequence alignments of the isolated fragment and published sequence were generated by Genepro (Version 6.10, Riverside Scientific Enterprises, CT, USA).

Figure 3.4.3 illustrates the linear relationship between these two sequences.

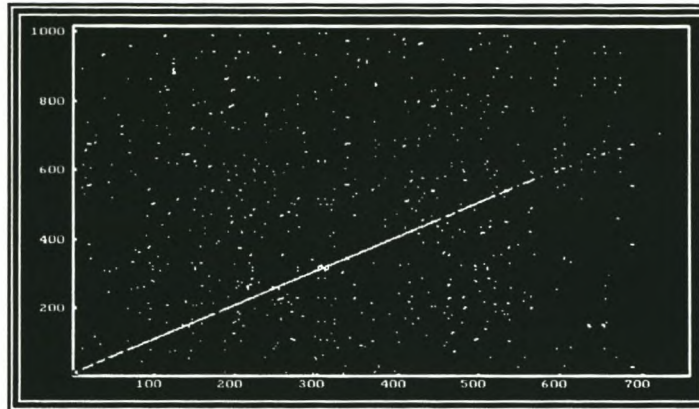


Figure 3.4.3 A dot plot alignment of the nucleotide sequence of our fragment with that of the published MAP sequence. The linear relationship confirms the identity of our fragment as MAP.

The amplified MAP gene was purified from agarose gel and cloned into pGEM-T Easy using blunt-end ligation. Recombinant colonies were identified by PCR amplification of the MAP gene from bacterial cells (results not shown). These transformed clones, pGEM-T Easy-MAP, are being stored in the form of glycerol freezer cultures for future use.

A simplified overview of our scheme for cloning MAP into pFLAG is given in **Figure 3.4.4**. The vector provides an ADH2 promoter, and a start codon occurs within the MAP ORF.

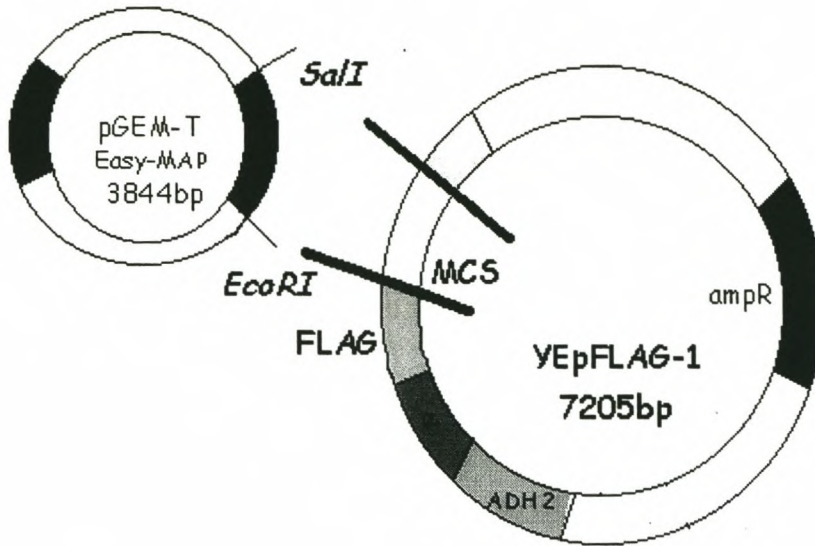


Figure 3.4.4 Construction of pFLAG-MAP. MAP is excised from pGEM-T Easy by means of restriction enzyme digestion (*EcoRI* + *SalI*) and ligated into the pFLAG vector.

Plasmid DNA was extracted from overnight liquid LB cultures (100 $\mu\text{g/ml}$ ampicillin selection) of pGEM-T Easy-MAP and pFLAG. DNA was digested with *EcoRI* and *SalI* simultaneously. This liberated MAP from pGEM-T Easy-MAP and linearized the pFLAG vector. Digestion products were fractionated by means of agarose gel electrophoresis (see **Figure 3.4.5**).

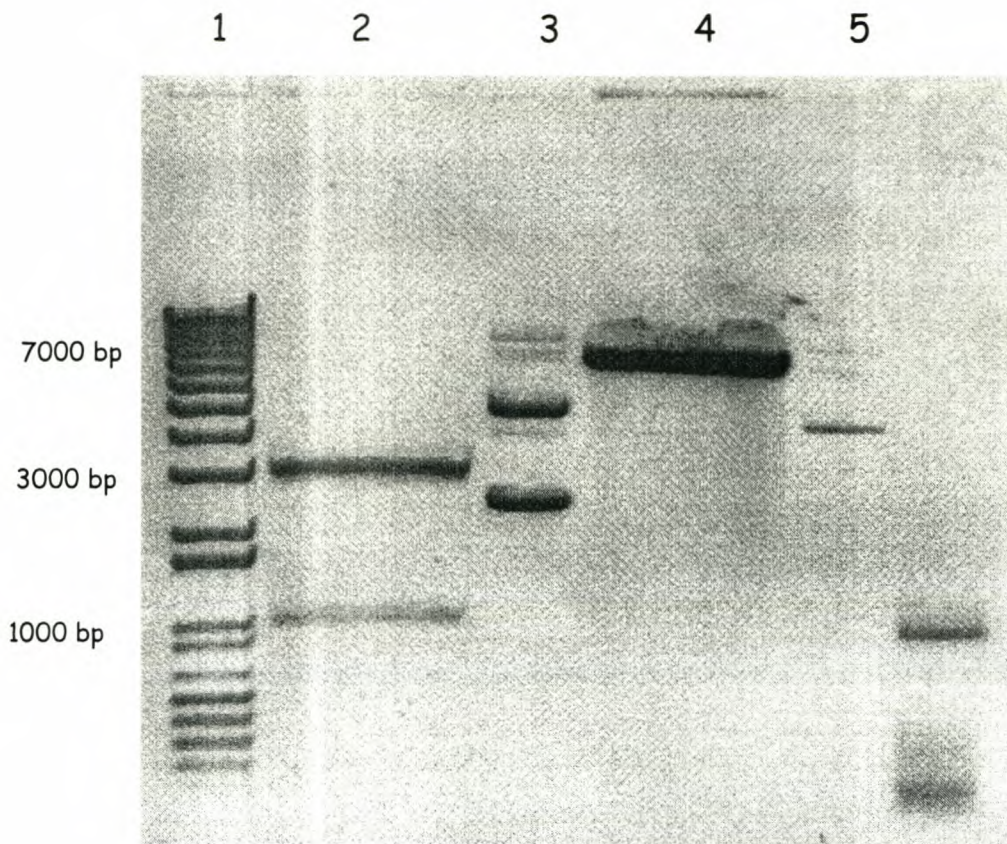
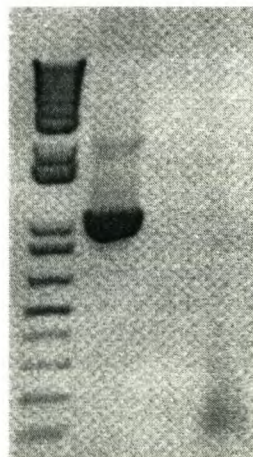


Figure 3.4.5 Preparation of MAP insert and pFLAG vector. Lane 2: pGEM-T Easy-MAP digest (*EcoRI* + *Sall*); Lane 3: pGEM-T Easy-MAP uncut; Lane 4: pFLAG digest (*EcoRI* + *Sall*); Lane 5: pFLAG, uncut; Lane 6: MAP.

MAP and pFLAG were purified from the gel and quantified on a spectrophotometer. Insert and vector were ligated at a ratio of 10:1 and competent DH5 α cells were transformed. Recombinant clones were identified by PCR analysis (see **Figure 3.4.6**).

Figure 3.4.6 PCR amplification of MAP from isolated pFLAG-MAP. MAP was amplified from *E. coli* transformed with pFLAG-MAP.



Digestion analysis provided further proof that the cloning had been successful. Plasmid DNA was extracted from overnight cultures inoculated with cells from the transformed colonies. Digestion with *EcoRI* and *Sall* produced two fragments: a 7205 bp pFLAG fragment and the MAP fragment. **Figure 3.4.7** shows the digestion products of a positive transformant.

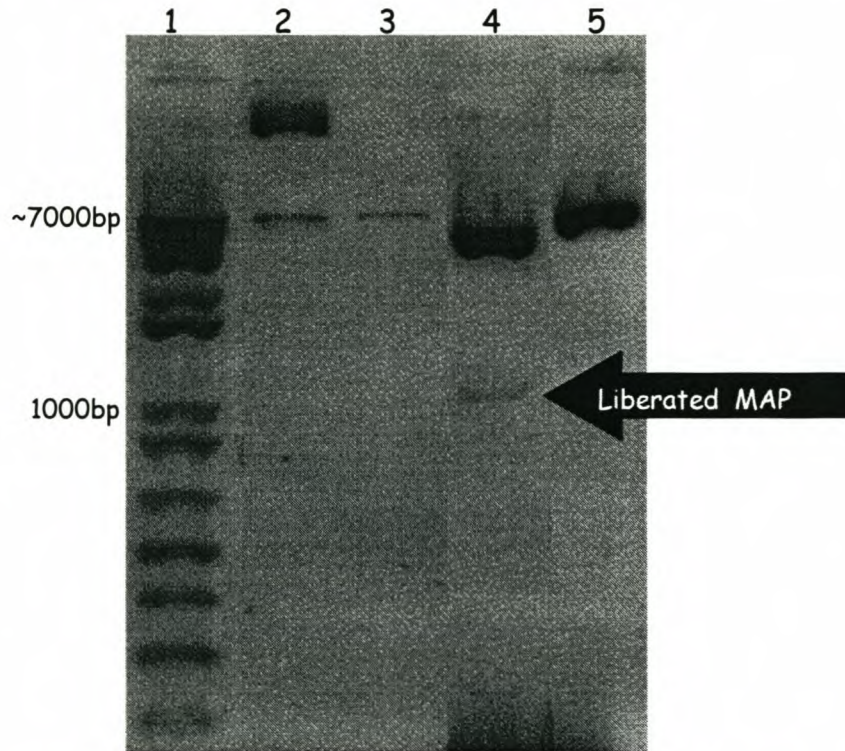


Figure 3.4.7 Proof of cloning MAP into pFLAG. MAP is liberated from pFLAG-MAP during digestion analysis with *EcoRI* and *Sall*. Lane 2: undigested pFLAG; Lane 3 pFLAG digested with *EcoRI* and *Sall*; Lane 4 isolated pFLAG-MAP digested with *EcoRI* and *Sall*; Lane 5: isolated pFLAG-MAP linearized with *Sall*.

Freezer cultures of pFLAG-MAP await further manipulation. Transformants may be used for the transfection of yeast (strain BJ3505) by means of electroporation. The yeast may then be cultured to produce active MAP.

3.5 Methods

3.5.1 Isolation of gDNA

The details of gDNA extraction are given in **Appendix II**.

3.5.2 PCR primer design

Nucleotide sequences encoding the RIPs were downloaded from GenBank and PCR primers were designed to amplify the entire coding regions. Restriction enzyme recognition sites were included in the 5' region of the primers to facilitate cloning into the various expression vectors. The composition of the primers is given in **Table 3.5.2**.

Table 3.5.2 PCR primer composition. Each primer consists of a portion that is specific to the gene and a flap engineered to incorporate restriction enzyme recognition sites.

RIP	Size ^{*1} of 5' engineered flap	Size ^{*1} of gene-specific region	Total size ^{*1} of primer
PAP	F: 11	F: 19	F: 30
	R: 15	R: 16	R: 31
Dianthin	F: 13	F: 19	F: 32
	R: 18	R: 15	R: 33
Luffin	F: 13	F: 14	F: 32
	R: 15	R: 15	R: 32
MAP	F: 14	F: 15	F: 29
	R: 15	R: 15	R: 30

^{*1} given in bp; F = forward primer; R = reverse primer

3.5.3 PCR amplification

The composition of PCR cocktails is specified in **Appendix III**. The amplification conditions were designed to include an initial phase of low-stringency annealing, to incorporate the 5' add-ons in the PCR product. The annealing

temperatures during the second phase of amplification varied for each set of primers. A gradient of annealing temperatures was programmed. Initially, each annealing reaction took place at a temperature approximately 5°C below the average T_m value of the primer pair (PrimerCalculator: nearest neighbour method). In some cases, this temperature was raised slightly in order to eliminate some of the background caused by unspecific primer binding. The amplification program is given in **Table 3.5.3(a)**. The T_m values and annealing temperatures used are in **Table 3.5.3(b)**.

Table 3.5.3(a) PCR amplification program

<i>Temperature</i>	<i>Time</i>	<i>Number of cycles</i>
94°C	5 minutes	1
94°C	30 s	6
39°C	45 s	
72°C	1 minute	
94°C	30 s	36
59.9-69.7°C	45 s	
72°C	1 minute	
72°C	5 minute	1
4°C	Indefinitely	HOLD

Table 3.5.3(b) T_m values of primers and annealing temperatures for PCR amplification.

RIP	T_m of Forward Primer	T_m of Reverse Primer	Annealing temperature used
PAP	69.13°C	67.84°C	65.2°C
Dianthin	67.77°C	67.12°C	62.4°C
β -luffin	71.12°C	70.69°C	65.2°C
MAP	62.80°C	64.03°C	60.4°C

3.5.4 Reamplification of desired fragment

PCR products were separated on a 0.8% 1X TAE agarose gel, by electrophoresis at 80 V. The fragment of expected size was excised from the gel and extracted from the agarose by means of the freeze-thaw-spin method: the gel slice was placed in a glass-wool spin column and frozen solid; it was then thawed and centrifuged at 12,500 g for 25 minutes. One microlitre of the flow-through was amplified using the PCR cycle specified in **Table 3.5.4**. This program is a condensed form of the one described in **Table 3.5.3(a)** - the low-stringency phase is omitted as the entire primer is now specific to the DNA fragment that is to be amplified.

Table 3.5.4 Program for the re-amplification of a PCR fragment.

<i>Temperature</i>	<i>Time</i>	<i>Number of cycles</i>
94°C	5 minutes	1
94°C	30 s	
59.9-69.7°C	45 s	36
72°C	1 minute	
72°C	7 minutes	1
4°C	Indefinitely	HOLD

3.5.5 Sequencing of amplified fragment

Once the fragment had been amplified to homogeneity, it was extracted from the gel using the freeze-thaw-spin method described in the previous paragraph. Purified PCR product (30 ng) was submitted for automatic DNA cycle sequencing. Samples were sequenced in both the forward and reverse orientation, using 3.2 pmol of specific primer, and these

sequences were aligned with the aid of computer software (Sequence Navigator).

3.5.6 Cloning of RIP into pGEM-T Easy

When the identity of the RIP fragment had been confirmed by sequencing, purified PCR products were cloned into pGEM-T Easy according to the instructions issued by the manufacturer. Clones were cultured on LB agar plates (Sambrook *et al.*, 1989) under 100 µg/ml ampicillin selection and in the presence of 40 µg/ml X-gal and 0.5 mM IPTG. Positive (white) clones (based on the blue-white colour screening described in the manual) were confirmed by re-amplification of the gene by means of PCR (using primers specific for the cloned gene). A few cells were picked from white colonies with sterile toothpicks and added to PCR cocktails (**Appendix III** - an extra microlitre of water was added per reaction to compensate for the omission of DNA in solution). The amplification program in **Table 3.5.4** was used to screen for the insert. Freezer cultures of positive clones were produced by combining overnight liquid cultures (grown on 100 µg/ml ampicillin selection) with 15% (v/v) sterile glycerol in Eppendorf tubes. These stocks are being stored at -80°C. pGEM-T Easy provides many unique restriction sites, which may be useful for cloning the genes into other vectors at a later stage.

3.5.7 Preparation of components for subcloning

Transformed pGEM-T Easy colonies (either 5 µl of freezer culture or cells picked from a single colony) were used to inoculate ~5 ml liquid LB (with 100 µg/ml ampicillin selection) and cultured overnight (37°C, ~225 rpm). Plasmid

DNA was isolated from transformed pGEM-T Easy colonies, according to the method described in **Appendix V**.

Simultaneously, expression vectors were harvested from liquid cultures (the various selection agents are specified in **Table 3.5.7(a)**) using the same technique.

Table 3.5.7(a) Selection media for the expression vectors.

Expression vector	Selection
pKK223-3	100 µg/ml ampicillin
pCambia3301	50 µg/ml kanamycin
pFLAG	100 µg/ml ampicillin

Both the RIP-containing pGEM-T Easy vector and the expression vector were digested with the appropriate set of restriction enzymes (sites were originally included in the 5' ends of the PCR primers). The specific enzymes used in each digest are given in **Table 3.5.7(b)**. Approximately 5 µg of DNA was digested with 1 U of enzyme for 2 hours, in the presence of the appropriate reaction buffer (1X concentration). Reactions took place at 37°C, with the exception of the *BstEII* digest, which occurs at 60°C. Furthermore, *BstEII* and *NcoI* require different reaction buffers. The pH of the reagents was adjusted for *NcoI* after the *BstEII* digest reaction, by the addition of 1 µl of 1 M Tris, pH 7.5.

Table 3.5.7(b) Restriction digests for cloning. Enzymes used to digest both the vector and the appropriate insert in preparation for ligation reactions.

Vector	Insert	Restriction enzyme(s)
pKK223-3	dianthin	<i>EcoRI</i>
PCambia3301	luffin-b	<i>BstEII; NcoI</i>
pFLAG	MAP	<i>EcoRI; SalI</i>

Since pKK223-3 was digested with a single restriction enzyme, the vector was dephosphorylated in order to prevent it from re-ligating upon itself. One pmol of digested pKK223-3 was incubated (37°C) with 1 U calf thymus phosphatase in the presence of 1X buffer for an hour. The enzyme was denatured by a 30 minute heat-shock period at 65°C. Digesting this plasmid with a single enzyme yielded a uniform product (no fragment was excised from the vector) and it was therefore not necessary to gel-purify the linearized vector.

Digestion products (with the exception of pKK223-3) were fractionated on a 0.8% 1X TAE gel, by electrophoresis at 80 V. In each case the liberated RIP and the linearized expression vector were excised from the gel and isolated according to the freeze-thaw-spin method previously described. DNA concentrations of both the insert (RIP) and vector were determined. Initially, the concentration of dianthin was exceptionally low. Yields were increased by precipitating the excised fragment with 0.15 M NaOAc, 20 µg linear polyacrylamide (GenElute™ LPA) and 2.5 volumes ethanol. The sample was centrifuged at 12000 g for 5

minutes and the pellet was washed with 70% ethanol and resuspended in 20 μl ddH₂O. Quantification was done either using standard size markers and agarose gel electrophoresis (as in the case of *dianthin* and pKK223-3) or with the use of a spectrophotometer. The concentrations recorded are given in **Table 3.5.7(c)**.

Table 3.5.7(c) DNA concentrations of insert and vector.

Construct	Insert	Vector
Dianthin + pKK223-3	10 ng/ μl	25 ng/ μl
Luffin + pCambia3301	13.912 ng/ μl	12.243 ng/ μl
MAP + pFLAG	19.413 ng/ μl	19.411 ng/ μl

3.5.8 Ligation Reactions

To begin with, insert and vector were combined in a series of ratios ranging from 1:1 to 30:1. The *dianthin* gene was successfully ligated into pKK223-3 at a ratio of 5:1. Undephosphorylated pKK223-3 vector and dephosphorylated pKK223-3 vector without insert were used in control reactions, to ensure that the dephosphorylation had been successful. A ratio of 10:1 was found to work best for ligations involving the two larger vectors. Ligations were set up in either 10 μl or 12 μl volumes and contained 1X reaction buffer. The ligation reaction of *dianthin* and pKK223-3 utilized 5 U of Pharmacia ligase, with 10 mM ATP. The other two ligations used 12 U of New England BioLabs ligase. Ligation took place overnight at 4°C.

3.5.9 Transformation of competent *E. coli*

Two microlitres of the dianthin-pKK223-3 ligation mix was transferred to a 2 ml Eppendorf tube in ice slurry. Competent JM109 cells were removed from storage at -80°C and thawed in an ice-bath. Fifty microlitres of cells were carefully added to the aliquot of ligation mix and the tube was gently flicked. The tube was left in the ice slurry for 20 minutes. The cells were heat-shocked at exactly 42°C for 50 s and returned to the ice for another 2 minutes. Nine hundred microlitres of room-temperature SOC medium (no selection) was added and the reaction mix was incubated at 37°C, while shaking at ~225 rpm. Cultures were concentrated by centrifugation (5000 g for 10 minutes) and resuspended in approximately 100 µl of the LB medium. This was plated onto an agar plate, containing the appropriate selection antibiotic (see **Table 3.5.7(a)**) and incubated overnight at 37°C.

The other two transformations (involving pCambia3301 and pFLAG) followed the same procedure, with the following exceptions: 5 µl of ligation mix was used to transform 100 µl of competent **DH5α** cells, which were incubated in **LB** medium.

3.5.10 Confirmation of positive clones

Clones were screened for insert by means of PCR. Cells were picked from the single colonies and added to PCR cocktails (see **Appendix III**), containing the primers specific to the gene being cloned. The thermocycle conditions used were the same as those given in **Table 3.5.4**. Reaction products were electrophoresed at 125 V on a 1.4% 0.5X TBE gel, stained

with ethidium bromide, and visualized under UV light. Positive clones were confirmed by restriction analysis: plasmid DNA was isolated from overnight cultures (grown on selection), as described in **Appendix V**, and digested for 2 hours at 37°C with the appropriate enzyme(s) - see **Table 3.5.7(b)**. The liberation of RIP from the isolated vector confirmed that the cloning had been successful.

The orientation of *dianthin* in recombinant pKK223-3 was determined by digesting ~5 µg of plasmid DNA for two hours at 37°C, with 1 U of *HindIII* and 1 U of *StyI*, in the presence of 1X buffer B. Digestion products were fractionated by agarose gel electrophoresis at 125 V on a 1.4% 0.5X TBE gel. The liberation of a 650 bp fragment was proof that the *dianthin* gene was cloned in the correct orientation.

Chapter 4: Conclusion

*" 'Now then, Piglet, let's go home.'
'But, Pooh,' cried Piglet, all excited, 'do you know the way?'
'No,' said Pooh. 'But there are twelve pots of honey in my cupboard,
and they've been calling to me for hours.
I couldn't hear them properly before because Rabbit would talk,
but if nobody says anything except those twelve pots,
I think, Piglet, I shall know where they're coming from. Come on.' "*
- Milne, 1974.

Our attempt to isolate a RIP from grapevine was unsuccessful. Future attempts may either revert to more conventional methods of isolating new genes with low levels of homology to existing genes in the same class (such as Southern blot), or involve much perseverance in optimizing degenerate primers and PCR conditions. If grapevine does contain a RIP, or some remnant of a RIP gene, it is clearly not being expressed in a useful way. Inducing the useful expression of a truncated or mutated RIP gene may be a huge problem in itself, making the search for a RIP in grapevine little more than an academic exercise. However, the benefits of a general set of degenerate PCR primers for the detection of novel RIPs would be numerous and well worth the effort. South Africa has a host of plants renowned for their medicinal value. Perhaps RIPs are the active ingredients in some of these.

"Now just in time for the millenium scientists have become involved with the genes of the vine. However, it will take many years before wine is commercially produced in Germany from grapes grown on genetically modified vines - if at all....The promise of gene technology is to produce cultivars with improved resistance to infestation and so ensuring more consistent crops."

*Translated from:
Was werden wir trinken?
Spekulationen über die Weine der Zukunft
Von Mario Scheuermann
23 September 1999 Die Zeit Nr. 39*

The clones prepared in this project will be used in future research, with special emphasis on protein studies. RIPs may be produced by the bacterial and yeast expression systems. The expressed proteins may either be used in an investigation of these fascinating molecules, or for commercial applications. Perhaps one day, the β -luffin construct will emerge in the vineyard. An understanding of RIP expression and action is required for this purpose, as the constitutive expression of RIPs in transgenic plants may have serious effects on the general well-being of the plant. Furthermore, grapes engineered to express RIPs may not be suitable for human consumption: the only separation between type I RIPs and their highly toxic and lethal type II relatives, is a lectin molecule.

The intricate mechanisms of these diverse molecules must be unraveled before we can truly exploit their magical qualities.

Appendix I

Codon preference of Vitis

A comprehensive list of all the Vitis gene sequences available on GenBank was downloaded in January 1998. The frequency that each codon was used to encode a particular amino acid was scored manually and the use of each codon was presented as a percentage of all possible codons encoding a particular amino acid.

Codon	Score	% Codon usage per amino acid
Phe TTT	265	45.77
TTC	314	54.23
Leu TTA	138	10.53
TTG	290	22.12
CTT	319	24.33
CTC	248	18.92
CTA	126	9.61
CTG	190	14.49
Ile ATT	360	44.89
ATC	274	34.16
ATA	168	20.95
Met ATG	309	100.00
Val GTT	312	31.61
GTC	214	21.68
GTA	148	14.99
GTG	313	31.71

Ser	TCT	172	17.29
	TCC	205	20.60
	TCA	210	21.11
	TCG	91	9.15
	AGT	156	15.68
	AGC	161	16.18
Pro	CCT	248	33.93
	CCC	156	21.34
	CCA	244	33.38
	CCG	83	11.35
Thr	ACT	264	31.24
	ACC	295	34.91
	ACA	197	23.31
	ACG	89	10.53
Ala	GCT	462	38.73
	GCC	309	25.90
	GCA	339	28.42
	GCG	83	6.96
Tyr	TAT	223	47.65
	TAC	245	52.35
Stop	TAA	18	40.91
	TAG	11	25.00
	TGA	15	34.09
His	CAT	166	54.97
	CAC	136	45.03
Gln	CAA	217	52.29
	CAG	198	47.71

Asn	AAT	336	52.26
	AAC	307	47.74
Lys	AAA	358	40.59
	AAG	524	59.41
Asp	GAT	461	22.05
	GAC	269	36.85
Glu	GAA	407	47.49
	GAG	450	52.51
Cys	TGT	147	48.20
	TGC	158	51.80
Trp	TGG	197	100
Arg	CGT	100	16.10
	CGC	75	12.08
	CGA	66	10.63
	CGG	58	9.34
	AGA	166	26.73
	AGG	156	25.12
Gly	GGT	346	29.03
	GGC	282	23.66
	GGA	325	27.27
	GGG	239	20.05

Appendix II

Genomic DNA Extraction

The procedure described by Steenkamp *et al.* (1994) was modified for small-scale extractions of DNA. This protocol was selected as it was specifically formulated for the extraction of DNA from grapevine, which is a notoriously difficult process due to the high levels of polyphenols and polysaccharides present in this leaf tissue (Lodhi *et al.*, 1994). The method worked well for the extraction of DNA from all the plants used in this project. In each case, the starting material was between 0.02 and 0.05 g of fresh leaf tissue. Cape Riesling leaves were gathered from the University vineyard; *Luffa octandra* was grown from seed; *Mirabilis jalapa* and *Phytolacca octandra* shoots were gathered from the wild and rooted in pots; and *Dianthus caryophyllus* was the serendipitous gift of various suitors. All extractions were performed in 1.5 ml Eppendorf tubes and leaf-tissue was pulverized with tissue-grinders. One major adaptation was made to the extraction protocol: due to excessive oxidation during the grinding of *Mirabilis* leaves, leaf-tissue was ground in 10 μ l of lemon juice.

The extraction buffer consists of the following:

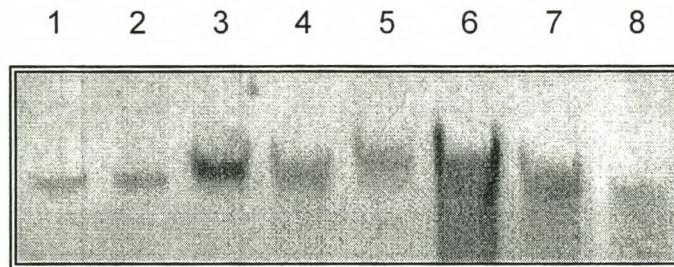
Stone's Extraction Buffer
1 M Tris, pH8
3% CTAB
1.4 M NaCl
20 mM EDTA
0.02% β- mercaptoethanol^{*1}
50 μg/ml Rnase^{*1}

^{*1} Added fresh

A synopsis of the extraction process follows:

- ♣ A few leaf disks were punched out using the lid of an Eppendorf tube and finely ground in lemon juice.
- ♣ 400 μ l of extraction buffer was added and the samples were mixed thoroughly, but gently.
- ♣ The ground tissue was digested at 60°C for half an hour, with gentle, intermittent mixing.
- ♣ One volume of chloroform-isoamyl-alcohol (24:1) was added and samples were centrifuged at 10 000 g for 10 minutes (room-temperature).
- ♣ The supernatant was carefully transferred to fresh tubes and a second chloroform-isoamyl-alcohol extraction was performed.
- ♣ A two-thirds volume of icy cold isopropanol was added to the aspirated supernatant and the samples were mixed gently. (In some cases, DNA strands were observed at this point.)
- ♣ The samples were left in the freezer (-20°C) for about half-an-hour, to facilitate precipitation of DNA.
- ♣ The DNA was pelleted by centrifugation at 8000 g for 10 minutes (room-temperature).
- ♣ Pellets were washed with 70% ethanol and spun down at 8000 g for 10 minutes.
- ♣ DNA was left on the lab-bench to dry and resuspended in 30-50 μ l ddH₂O.

Isolated DNA was analyzed on a 0.8% 0.5X TBE agarose gel by electrophoresis at 80 V:



Products of gDNA extractions. Lane 1: 15 ng standard DNA; Lane 2: 63 ng standard DNA; Lane 3: 125 ng standard DNA; Lane 4: *Phytolacca octandra* DNA; Lane 5: *Dianthus caryophyllus* DNA; Lane 6: *Luffa octandra* DNA; Lane 7: *Mirabilis jalapa* DNA. Lane 8: *Vitis* DNA. 10 μ l of DNA sample was loaded.

Appendix III

PCR specifications

PCR cocktail mixes consisted of the following reagents:

Reagent	Final concentration
ddH₂O	*1
NH₄⁺ buffer	1X
MgCl₂	2.5 mM
Forward Primer	0.75 μM
Reverse Primer	0.75 μM
Template DNA	~50 ng
dNTPs	200 μM
Taq polymerase	0.05 U

*1 ddH₂O topped reaction volumes up to 15μl.

Reaction mixes were aliquoted in 0.2 ml Eppendorf tubes and the lid temperature was set at 105°C.

Appendix IV

Nucleotide sequences of cloned fragments

The 300 bp fragment amplified from grapevine using degenerate primers. The fragment was cloned into pGEM-T Easy and sequenced with standard M13 primers, in the forward and reverse orientation. The sequences were assembled.

```

TAGTCTAGAGAACCTTGCTGCCAAGTTTAAGCCAGTTGACTATGTAGTTGTC
AAGGGAAAGAAGGTGCAATATGACTCCCTGCTATAAATGTCGTTTTGGGAAG
CACCAAGGCTCCTGAGGACAACAGTTAGATTATGATCAGAAAGACGTCACT
GGAGGATATGAAAGAATGGTTGTCCCCATTAATATCAGATGGAACCTCTTAAG
TGGCTTGAGGCTGGAGCAGTTATCGAGAAGAAGGACCTAAATATGGCAACA
AGGTATTGGTTTGGCTTCATCAGCAGCCCGATTCTCTAGACT

```

Just the forward sequence of the 500 bp fragment amplified from pokeweed was determined:

```

TTTGAANCCCNCGGAGATCMGGACTIONWATAACWAAAGGAAA GACGATTWT
ATCGCCAAGTCTCTAATCAGGGGATCTATCGCGGTAGCGAATATAAGGAGT
GCCTTAACTGTGATATACTAGATTCACAGAAAAAACCACTCCTCGGCGTTTG
AGTTCCTTTGCTAGAGTCACACTCGAGAGCCTCACATTCTTCTTTCTATACG
TTACATCTCTTCCTTGTACTTATATACGTGACCATACCGGAGCAAATAAACCT
TATTTGACCGGAAATAACTGATCTCGCTACCGGGTGAGGGAAGAAGAGCT
CTACCAAGAGGTACACTCTTTTCGCATGACTTTCTTTNCNGCAATAACGMA
TAAAGTAGAACGMAGGACYATAAGCCYTMGCTTAGAACAAAGAGYCANYN
MNGYSCTCNSSTAASAANCYNYCANRYTCKYC

```

PCR products were sequenced with both the forward and reverse primer and the two sequences were aligned. The primer sequences are included **in bold**.

Dianthin 30 Sequence

The sequence of dianthin was totally illegible at the 3' end. The recognition sequence of *Styl*, used to determine the orientation of the cloned dianthin gene in pKK223-3-dianthin, is highlighted. The internal *EcoRI* site occurs in the illegible 3' region.

```

GGCCAATGGTCTAGATGAAGATTTATTTAGTGGTCTAGAGGAAGCTGTATT
AGGCTTCATCTTGGACAACACTGATGCGGTCACAGCATAACATTAATCTGGC
AAATCCATCCGCGAGTCAATACTCATCTTTTCTGGATCAAATCCGAAACAAT
GTGAGGGATACCAGCCTCATATACGGTGGGACAGACGTAGCCGTGATTGGT
GCGCCTTCTACTACTGATAAATTCCTTAGACTTAATTTCCAAGGTCCTCGAG
GAACGGTCTCTCTTGGCCTTAGGCGCGAGAACTTATACGTGGTTCGCGTATC
TTGCAATGGATAACGCAAATGTTAACCGTGCATATTACTTCAAAAACCAAATC
ACTTCTGCTGAGTTAACCGCCCTTTTCCCCGAGGTTGTGGTTGCAAATCAAA
AACACTTAGAGTACGGGGAAGATTTCCACGCCGATAGAACGCCCTGTTAAC
AATCAGCCGTCCAAAGTAGAACGGACCTCGCGTTTGAGGATCAATCTACTTA
GTAACGAGTGAATTGATGAGGTGAATAAGAAGGTACGGTGTAGTCTAGACA
CGAGGCAGGCAAGGTTTATGTTAATCGCAAGTTCAAATGACGGCTGAGGCC
GCGCGATTTAGGTACATACAGAACTTGGTTACCAAGAACTTCCCAAACAAGT
TCGGACTCAGAAAATAAGGTTATTCAATTTCAAGTTAGTTGGAGTAAGATTTT
TACGGCAATATTTGGGGATTGCAAAAACGGCGTGTTTAATAAAGATTACTGA
TTTCGGGTTTGGGAAAGTGAGGCAGGCAAAGACCTTCAAATGGGGCTCCT
TAA ... GCTGATGAGCTTTGAGCCGGTTACCTCTAGAAC

```

β-luffin Sequence

GGCCATGGTCTAGATGAACAGATTACATTTCTCTCTTTGCTAATTCTCATT
GCTTTCTTTACCGCTGAAGGTGCTAATGTGAGCTTCAGTTTGTGAGGGGCTG
ATTCCAACCTCTACAGCAAGTTCATCACAGCTCTGAGGAAGGCTCTTCCATC
TAAGGAGAAAGTGTCCAATATACCTCTATTGCTTCCCTCCGCTTCCGGCGCA
AGTCGCTACATACTGATGCAACTCTCCAATTACGACGCCAAAGCCATCACAA
TGGCTATAGATGTAACAAACGTTTACATTATGGGCTATCTTGTGAATTCAACA
TCCTACTTTTTCAACGAGTCTGATGCTAACTAGCTTCTCAATACGTATTCAA
AGGTAGTACGATCGTTACACTTCCATATTCTGGCAATTACGAAAGGCTTCAA
AATGCTGCAGGCAAAGTGAGAGAAAAAATTCCACTTGGATTCCGAGCTTTTCG
ACAGTGCAATTACCTCCTTGTTTCACTACGACTCCGCGGCTGCTGCTGGGG
CATTTCTCGTAATCATTGAGGACTGCTGAGGCTTCCAGATTCAAGTACAT
TGAGGGACAGATTATTGAGAGAATTTCCAAAACGAGGTGCCGAGTCTAGC
GGCTCTGAGTTTAGAAAACGAATGGTCTGCTCTCTCCAAACAGATTCAGCTA
GCACAACTAACAATGGAGCGTTTGAAGTCTGTTGTGATTATAGACAATA
AAGGCCAACGAGTTGAAATAAAGGACGTTAATTCAAAGTTGTAACCAACAA
CATCAAGTTGCTTCTAAACAAACAAAATATTGCAGCTTTTGACGGATGGTATT
CCTACAAAACACTGAGCCGGTGACCTCTAGAAC

MAP Sequence

ATGCTTACAAC TACGAGGGTGT TTTTTCTCCTATTGACGACATGGATCACTTG
GTACGCGATTGTGAATCCACAATCAAGGGCAGCACCGACACTCGAAACAAT
AGCATCTTTGGACCTAAATAATCCCACCACATATCTATCATTATCACAAATA
TAAGAACAAAAGTTGCTGATAAAACCCTGCAATGTACAATTCAGAAAATAAG
CAAACATTTACTCAAAGATATTCATACATCGACTTGATAGTCTCAAGCACTC
AAAAGATTACATTAGCAATAGATATGGCTGATCTGTATGTGCTTGGATATAG
CGATATAGCGAATAACAAAGGGCGTGCTTTCTTCTTTAAAGATGTCACCGAG
GCAGTCGCAAACAAC TTTTTCCCTGGTGCAACCGGAACTAATCGAATTAAGT
TAACATTCACAGGAAGTTATGGTGACCTTGAAAAAATGGTGGTTCACCGAA
AAGATAATCCACTAGGAATATTCAGACTGGAGAATAGCATAGTTAACATATAT
GGTAAAGCAAGTGATGTAAAAAACAAGCCAAATTTTTnTTACTTGCCATTCA
GATGGTGTCCGAAGCAGCCAGATTCAAGTATATTTCTTGATAAGGTACATAA
TATATACTTATCATCTTCAACTATTTATCAATCTGTGTTGTTTCAACTTAAGTT
TTTTCTTTGATCAACTAGTTACGAGTTTGAATCCTATCTCATGACAAAATATGA
ATCATGAGTTTTACAACACTTAGTTCTTATTGATTGATAATGTAACAGATTCC
ATCAGAAAAATATGACGAAGTTACGGTGGATGACTATATGACTGCGTTGGAA
AATAATTGGGCTAAACTTTCAACTGCGGTATACAATTCAAAGCCTTCCACCA
CCACGGCCACCAAGTGTCAATTGGCCACCAGTCCAGTCACCATAAGTCCAT
GGATCTTCAAAC TGTGAGGAGATCAAGCTAGTTATGGGTCTT**CTTAAGTCG**
TCCTAA

Appendix V

Plasmid DNA extraction: Johan's WIZ-Prep Protocol

- ♣ Each 5 ml overnight *E. coli* culture (grown on selection) is pelleted in an Eppendorf tube by successive centrifugation steps at maximum speed for 20 s periods.
- ♣ The pellet is resuspended in 200 μ l of WIZ-1 solution (50 mM Tris, pH 8.0; 10 mM EDTA; and 50 μ g/ml RNase, added freshly) by vortex mixing.
- ♣ 200 μ l of WIZ-2 solution (200 mM NaOH; 1% SDS) is added; the sample is inverted several times and left on the bench-top for 4 minutes.
- ♣ 200 μ l of WIZ-3 solution (3.0 M potassium acetate, pH set at 5.5 with acetic acid) is added, the sample is mixed by inversion and left on ice for 4 minutes.
- ♣ Sample is centrifuged at maximum speed for 5 minutes.
- ♣ Supernatant is transferred to a second Eppendorf tube containing 200 μ l of homogenized WIZ-Matrix (silica granules) and inverted several times.
- ♣ Sample is centrifuged at maximum speed for a minute and the supernatant is discarded.
- ♣ The pellet is resuspended in 500 μ l WIZ-Wash solution (50 mM NaCl; 10 mM Tris, pH 7.5; 2.5 mM EDTA; 50% ethanol) by vortex mixing.
- ♣ Sample is centrifuged at maximum speed for a minute and supernatant is aspirated.
- ♣ Sample is briefly centrifuged to remove all traces of WIZ-Wash solution.
- ♣ Sample is left to dry on bench-top for 10 minutes.
- ♣ Pellet is resuspended in 35 μ l ddH₂O by vortex mixing.
- ♣ Sample is warmed in heating-block (55°C) for 2 minutes.
- ♣ Sample is centrifuged at maximum speed for 30 s and 30 μ l of supernatant is carefully removed. This is the purified plasmid DNA.

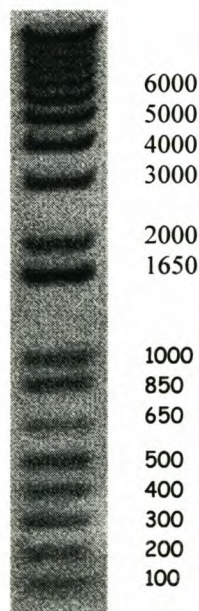
Appendix VI

Technical notes

Agarose gel electrophoresis

Genomic DNA was analyzed on a 0.8% agarose gel (at ~80 V) and PCR products, plasmid DNA and restriction digests were fractionated on 1.4% agarose gels (at 115 V~125 V). Ethidium bromide was added to the gels and the DNA was visualized under UV light. 0.5X TBE buffer was used for analytical gels and 1X TAE buffer was used when fragments were to be purified from the gel. (Although the clarity of the bands was greater with TBE buffer, lower yields of DNA were purified from gels made with this buffer than with TAE.)

A 1 Kb⁺ ladder was used (see figure below) to determine the size of the fragments and the negative (water) control of PCR reactions was loaded in the last lane of the gel. Total PCR reaction volumes were loaded onto the gels. Samples were mixed with 2 µl of blue juice as loading buffer.



DNA Sequencing

All DNA sequencing reactions were done at the University of Stellenbosch's Core Sequencing Facility. Cycle sequencing was performed on an ABI PRISM™377 DNA sequencer. 350 ng of DNA cloned into pGEM-T Easy, or 30 ng of gel-purified PCR product were sequenced using 3.2 pmol of each primer (M13 universal pUC primers in the case of the pGEM clones). Samples were sequenced in both the forward and reverse orientation (with the exception of the fragment amplified from pokeweed, which was only sequenced in the forward orientation). Sequences were aligned using Sequence Navigator (ABI Prism software).

Culture media

Sterile LB (liquid and solid media) and SOC were prepared according to the specifications of Sambrook *et al.*, 1989.

Antibiotics (ampicillin or kanamycin) were added to the cooled media (before setting, in the case of solid medium).

X-Gal and IPTG were rubbed onto agar plates after they had set, to final concentrations of 40 µg/ml and 0.5 mM, respectively.

Appendix VII

List of materials

Item	Supplier
Agarose (molecular grade)	Whitehead Scientific
Acetic acid	Merck
ATP	Boehringer Mannheim
Biotaq™ DNA polymerase	Bioline
<i>BstEII</i> (+buffer D + BSA)	Promega
Chloroform	SARCHEM
DH5 α cells	Gibco BRL
dNTPs	Bioline
DNA size standard	Gibco BRL
<i>EcoRI</i> (+buffer H)	Promega
EDTA	Merck
Ethanol	Merck
Glycerol	BDH
<i>HindIII</i> (+buffer B)	Promega
Isoamyl alcohol	Merck
JM109	Promega
Kanamycin	Sigma
Magnesium chloride	Merck
MicropH 2001	CRISON
μ Quant universal microplate spectrophotometer	BIO-TEK Instruments, Inc.
10X NH ₄ Buffer (no Mg ²⁺)	Bioline
<i>NotI</i> (+buffer H)	Boehringer Mannheim
1Kb Plus DNA ladder™	Gibco BRL
pCambia3301	CAMBIA
pFLAG	IBI/KODAK
pKK223-3	Pharmacia
PCR Mastercycler® gradient	Eppendorf
pGEM-T Easy Vector	Promega Corporation
Phosphatase, alkaline	Boehringer Mannheim
Potassium acetate (extra pure)	Merck
Primers: Degenerate	Perkin Elmer
PAP	Gibco BRL
Dianthin	Whitehead Scientific; Gibco BRL
β -luffin	Gibco BRL
MAP	Whitehead Scientific; Gibco BRL

Sal I (+buffer H)	Boehringer Mannheim
Sty I (+buffer B)	Promega
SDS	BDH
Sodium chloride (standard analysis)	Merck
Sodium hydroxide pellets	SARCHEM
T4 DNA ligase	New England BioLabs
10X ligase buffer with 10mM ATP	New England BioLabs
T4 DNA ligase FPLC <i>pure</i> ®	Pharmacia Biotech
One-Phor-All PLUS ligase buffer	Pharmacia Biotech
Tris	Boehringer Mannheim
TruLem	Brookes
Tryptone powder	Biolab
Yeast extract (select)	Gibco BRL

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