The expression of Dianthin 30, a Ribosome Inactivating Protein

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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.

Summary

Ribosome inactivating proteins (RIPs) are currently classified as rRNA *N*-glycosidases, but also have polynucleotide: adenosine glycosidase activity. RIPs are believed to have anti-viral and anti-fungal properties, but the exact mechanism of these proteins still need to be elucidated. The mechanism of resistance however, appears to be independent of the pathogen. For resistance the RIP terminates virus infected plant cells and stops the reproduction and spread of the virus. Transgenic plants containing RIPs should thus be resistant to a wide range of viruses. The ultimate goal of the larger project of which this forms part is the development of virus resistant plants. To monitor the expression of a RIP in a transgenic plant a detection method had to be developed. Antibody detection of the RIP was decided upon as the most cost effective method. The RIP, Dianthin 30 from *Dianthus caryophyllus* (carnation), was used and expressed in bacterial and insect expression systems. The bacterial expression experiments were done using the pET expression system in BL21(DE3)pLysS cells. The expression in this system yielded recombinant protein at a very low concentration. Expression experiments were also performed in insect tissue culture with the baculovirus vector BAC-TO-BAC™. With this system the expression was also too low to be used for the production of antibodies. A Dianthin 30 specific peptide was then designed and then produced by Bio-Synthesis. This peptide was then used to raise antibodies to detect Dianthin 30. These antibodies were tested on *Dianthus caryophyllus* proteins. To establish if this detection method was effective to monitor the expression in plants, tobacco plants were transformed with *Agrobacterium tumefaciens* containing *Dianthin 30* in the pART27 plant expression vector. The putative transformed plants were analysed with PCR and Southern blots.

Opsomming

Tans word Ribosomale-inaktiverende proteïene (RIPs) geklassifiseer as rRNA *N*-glikosidase wat ook polinukleotied: adenosien glikosidase aktiwiteit bevat. Daar word geglo dat RIPs anti-virale en anti-fungus eienskappe bevat, maar die meganisme van beskerming word nog nie ten volle verstaan nie. Dit is wel bewys dat die meganisme van weerstand onafhanklik is van die patogeen. Virus geinfekteerde plantselle word deur die RIP gedood om die voortplanting en verspreiding te bekamp en sodoende word weerstand bewerkstellig. Transgeniese plante wat dan 'n RIP bevat sal dus weerstandbiedend wees teen 'n wye spektrum virusse. Die hoofdoel van die breër projek, waarvan die projek deel uitmaak is die ontwikkeling van virusbestande plante. Om die uitdrukking van die RIP in die transgeniese plante te kontroleer, moes 'n deteksie metode ontwikkel word. Die mees koste effektiewe deteksie metode is met teenliggame. Die RIP, Dianthin 30 from *Dianthus caryophyllus* (angelier) was gebruik vir uitdrukking in bakteriele- en insekweefselkultuur. Die bakteriele uitdrukkingseksperimente was gedoen met die pET uitdrukkings sisteem in BL21(DE3)pLysS selle. Die uitdrukking in die sisteem het slegs rekombinante proteïene gelewer in uiters lae konsentrasies. Uitdrukkingseksperimente was ook gedoen in insekweefselkultuur met die baculovirus vektor BAC-TO-BAC™. Met die sisteem was die uitdrukking ook veels te laag om bruikbaar te wees vir die produksie van teenliggame. Daar is toe 'n peptied ontwerp wat Dianthin 30 kan verteenwoordig vir die produksie van teenliggame. Die teenliggame is getoets teen *Dianthus caryophyllus* proteïene. Om vas te stel of die deteksiemetode wel die uitdrukking van Dianthin 30 sal kan monitor, is tabak ook getransformeer met *Dianthin 30*. Die transformasies is gedoen met die hulp van *Agrobacterium tumefaciens* en die pART27 plant uitdrukkings vektor. Die plante is getoets met die polimerase ketting reaksie en Southern klad tegnieke.

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ABBREVIATIONS

A	Adenine
a a	amino acid
Amp	Ampicillin
BAP	6-Benzylaminopurine
bp	base pair
BSA	Bovine Serum Albumen
CaMV	Cauliflower Mosaic Virus
cDNA	complimentary DNA
CTAB	N-Cetyl-N,N,N,-Trimethyl Ammonium Bromide
Cx	Cefotaxime
DIG	Digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylene-Diamine-Tetra-Acetic acid
EtOH	Ethanol
GLRaV-3	Grapevine Leafroll Associated Virus 3
HRP	Horse Radish Peroxidase
IPTG	Isopropylthio-β-D-galactoside
KLH	Keyhole Limpet Hemocyanin
kb	kilobase
Km	Kanamycin
lac Z	β-galactosidase
LB	Luria-Bertani
MCS	Multiple Cloning Site
MS	Murashige and Skoog
NAA	1-Napthalene-Acetic Acid
nptII	neomycin phosphotransferase II
OD	Optical Density
ORF	Open Reading Frame

PAP	Pokeweed Antiviral Protein
PAG	Polyacrylamide Gel
PCR	Polymerase Chain Reaction
pDNA	plasmid DNA
PDR	Pathogen Derived Resistance
RBS	Ribosomal Binding Site
RE	Restriction Enzyme
Rif	Rifampicin
RIP/s	Ribosome Inactivating Protein/s
SAP	Shrimp Alkaline Phosphatase
SAR	Systemic Acquired Resistance
SDS	Sodium Dodecyl Sulphate
SDS-PAG	SDS- Polyacrylamide Gel
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
Sp	Spectinomycin
St	Streptomycin
TAE	Tris, Acetic Acid and EDTA
T-DNA	transfer DNA
Ti	Tumór inducing
Tris	Tris(hydroxymethyl)aminomethane

"As being is to becoming, so is pure intellect to opinion. And as intellect is to opinion, so is science to belief, and understanding to the perception of shadows."

Plato, Republic BookVII

Contents

Chapter 1: Introduction

1.1. Background to the study

1.1.1. Why this study?

Vitis vinifera belongs to the family Vitaceae and is economically the most important grapevine. Viticulture in South Africa started with the arrival of Simon van der Stel as governor to the Cape in 1679 and since then South Africa has grown to the eighth largest producer of wine in the world [1]. According to a study commissioned by the SA Wine Industry Information and Systems (SAWIS) the South African wine industry employs more than 348 500 people with wine tourism adding an additional 48 350 people. In the Western Cape alone the industry generates over R18 billion per annum [1].

Grapevine is host to many pathogens of which viruses are currently the most devastating in South Africa. Unlike other pathogens like bacteria or fungi there is no "cure" for infected plants. All efforts are presently being put into preventing the disease in vineyards by spraying for vectors and by planting uninfected vines once the infected vines are removed. Infected vines will usually not die from the disease but the yield and quality of the fruit will be affected with delayed and uneven ripening and low sugar content [2].

The virus problem in South Africa is taking on epidemic proportions affecting harvests in all the mayor regions. In 2001, Winetech identified viruses and more specific GLRaV-3 as the most devastating pathogen on South African vines.

1.1.2. This thesis

At the current rate, conventional agricultural methods of introducing resistance to viral pathogens in grapevine will soon not be able to keep up with the growing problem. This is the main reason why research into transgenic crops and in our case grapevine is a very relevant and active field. Currently, there is a limited acceptance of transgenic crops due to the perceived hazards, but that perception should change in the future. It is thus necessary to

research the fundamental principals now, so that crops and products can be ready or in an advanced state of testing by the time transgenic crops do get accepted [3].

To combat the onslaught of the viral pathogens on our grapevine, Winetech launched a large project in collaboration with the University of Stellenbosch Genetics Department and Institute for Wine Biotechnology. In this project, the aim was to introduce virus resistance into grapevine using various approaches. This thesis is the third on this project, the first two are briefly described in 1.1.3 and 1.1.4.

1.1.3. The construction of plant expression vectors for the introduction of leafroll resistance in grapevine. *C.D. van Straten*

This project was performed by C.D. van Straten as part of an MSc study. The aim was to engineer virus resistance, specifically to GLRaV-3, into grapevine. To achieve this resistance, a pathogen-derived method of coat protein-mediated resistance was used. Plant expression vectors were constructed to express the sense and antisense of the coat protein gene of Grapevine leafroll associated virus III and tested it in tobacco. She was unable to produce transgenic tobacco or grapevine plants [4].

1.1.4. Investigating the introduction of a broad-spectrum antiviral mechanism into grapevine. *K.L. Wilsen*

This project was done by K.L. Wilsen as part of an MSc study. The aim of this project was to investigate the possibility of using a heterologous resistance gene to engineer resistance into tobacco and grapevine. The first steps were to isolate different ribosome inactivating protein (RIP) genes and clone them into expression vectors. She was unable to isolate a RIP gene from the grapevine genome but was able to do so from other plants that were known to contain these proteins. She was unable to express these genes in bacteria [5].

1.2. Current study

1.2.1. Current project outline

In this study, the use a heterologous resistance gene to engineer virus resistance into tobacco as a model for grapevine was investigated. The project, involved the introduction of a ribosome inactivating protein (RIP) into tobacco as well as the development of a detection method to monitor the expression of the RIP in tobacco. For this project the RIP, Dianthin 30 from *Dianthus caryophyllus* was used.

1.2.2. Aims

The main focus of this thesis was the expression of Dianthin 30 for the development of a detection method that would monitor the expression of the protein in transgenic plants. An immunological detection method was chosen, and for this purpose rDianthin 30 had to be expressed. Expression was initially targeted only in bacteria, but insect cells were later also included. The recombinant protein then had to be purified for the production of antibodies in rabbits. The antibodies then had to be evaluated on carnation and transgenic plants. The transgenic plants were generated by *Agrobacterium tumefaciens* mediated transformation and a suitable plant expression vector.

1.3. Chapter layout

1.3.1. Chapter 2

In this chapter, ribosome inactivating proteins (RIPs) are reviewed, focussing on their properties and their current applications in agriculture.

1.3.2. Chapter 3

Chapter 3 describes the expression of Dianthin 30 in bacterial expression systems. The system used was the pET expression system, developed by Novagen.

1.3.3. Chapter 4

This chapter describes the expression of Dianthin 30 in insect tissue culture using a baculovirus vector for the expression. For this the BAC-TO-BAC™ system from Monsanto was used.

1.3.4. Chapter 5

In Chapter 5, the design of a unique peptide to represent Dianthin 30 as an antigen is described as well as the analyses of the serum from the inoculated rabbits.

1.3.5. Chapter 6

Agrobacterium mediated transformation of tobacco leaf material with the plant expression vector pART27 containing the Dianthin 30 gene is described in Chapter 6.

1.3.6. Chapter 7

Chapter 7, contains the concluding remarks on the thesis and the research done.

Chapter 2: Review of Ribosome inactivating proteins (RIPs)

2.1. Introduction

During the past 15 years, scientists have been able to increase our understanding into the molecular mechanisms of protein toxins that inhibit protein synthesis. These toxins inhibit eukaryotic cellular protein synthesis by either NAD^+-dependent adenosine diphosphate ribosylation of elongation factor 2 (EF-2) or by functioning as an *N*-glycosidase to remove adenine 4324 (A^{4324}) from 28S rRNA. In both cases the target is permanently altered and unable to continue with protein synthesis, and if enough EF-2s or ribosomes are damaged the cell will die [6].

2.1.1. History

The toxic properties of RIPs are not a new discovery. In ancient Chinese medicine, the root tuber of *Trichosanthes kirilowii* Maxim. was an ingredient in *Tianhuafen* that was used to induce abortions [7, 8, 9]. Modern science later confirmed the activity of this potent medicine and discovered that the active ingredient is the RIP, Trichosanthin [9, 10].

Western scientists have known about toxins such as ricin (from the seeds of *Ricinus communis,* the castor bean) and abrin (from the seeds of *Abrus precatotius,* the jequirity bean) for a long time, before it was discovered that these toxins were RIPs. In fact, in 1887, Dixon suspected that the toxicity of the castor beans could be attributed to a protein, and in 1888, Stillmark purified the protein and called it ricin. Three years later Hellin purified another RIP, abrin [reviewed by 11 and 12]. The toxicity of these proteins was then attributed to their ability to agglutinate erythrocytes.

2.1.2. RIPs as virus transmission inhibitors

Gendron *et al.* have observed the effect of RIPs on mechanical transmission of plant viruses in 1954 [13]. They concluded that the sap of *Phytolacca decandra*, sugar beet and carnation contains substances that can act as virus inhibitors. From their studies, they concluded that there was some evidence that the action of inhibition was on the plant rather than the virus [13]. Thomlinson *et al.* made some significant discoveries when they observed that the "plant virus inhibitor" from *Phytolacca americana* (American

pokeweed) was not only able to inhibit virus infection in plants but was also able to inhibit the transmission of animal viruses (influenza) in mammalian tissue culture [14].

Owens *et al.* (1973) found that the virus inhibiting extracts of pokeweed was able to disrupt protein synthesis in cell free protein synthesis systems and concluded that the mechanism of inhibition must be on the ribosomes [15]. Montanaro *et al.* came to the same conclusion in 1973 but narrowed the mechanism of inhibition down to the interaction between the ribosome and EF-2 [16].

2.1.3. Renewed interest in RIPs and subsequent classification

Studies on these toxins (RIPs) came under the spotlight in 1970 when Lin *et al.* reported that these toxins were more toxic to tumor cells (Ehrlich ascites) inoculated intraperitoneally to mice than normal cells [17].

This discovery was followed by a revival in the research of these toxins and led to the definition of the two-chain structure for ricin and abrin (later referred to as type-2 RIPs). Obrig *et al.* described the first type-1 RIP in 1973 [18]. The RIP they found was isolated from leaf extracts of *Phytolacca americana* and called it pokeweed antiviral protein (PAP). Since then many other type-1 RIPs have been found (see Table 2.1) from a wide range of plants of different plant families. Another RIP, Modeccin (from *Adenia (Modecca) digitata*), was found by Stirpe *et al.* in 1978 and classified as a type-2 RIP. Since then very few type-2 RIPs have been discovered [19](see Table 2.2).

It was proposed in 1983 by Barbieri and Stirpe that this group of proteins be referred to as Ribosome-Inactivating Proteins (RIPs) [20]. The general consensus at that time was that RIPs could be defined as proteins "...that inactivate eukaryotic ribosomes in a catalytic (enzymatic) manner, rendering the 60S ribosomal subunit incapable of binding the elongation factor 2, and thus arresting protein synthesis." [21].

They also proposed that RIPs be divided into two major categories: type-1 RIPs which have a single chain of protein (similar to the A-chain of type-2), and the type-2 RIPs which have two chains of protein, the **A**ctive- and **B**inding-chain (see Figure 2.1). Studies now suggest that type-2 RIPs might have evolved from type-1 RIPs [20, 22].

TABLE 2.1

Purified type-1 ribosome inactivating proteins

Family, genus and species		Plant tissue	Name	Yield (mg/100g)
Asparagaceae	*Asparagus officinalis*	Seeds	asprin 1	5.4
			asprin 2	4.9
Caryophyllaceae	*Agrostemma githago*	Seeds	Agrostin 2	8.4
			Agrostin 5	34.2
			Agrostin 6	18.4
	Saponaria officinalis	Leaves	Saporin - L1	35
		Leaves	Saporin - L2	13
		Roots	Saporin - R1	20
		Roots	Saporin - R2	5
		Roots	Saporin - R3	9
		Seeds	Saporin - S5	63
		Seeds	Saporin - S6	268-414
		Seeds	Saporin - S8	15
		Seeds	Saporin - S9	24
Cucurbitaceae	*Cucumis melo*	Seeds	Melonin	
	Momordica charantia	Seeds	Momordin I	150-180
			Momordin II	
	Trichosanthes kirilowii	Roots	Trichosanthin	140
		Roots	α - trichosanthin	
		Roots	TAP 29	2
		Seeds	Trichokirin	5.7
Eyphorbiaceae	*Gelonium multiflorum*	Seeds	Gelonin	250-300
	Hura crepitans	Latex	*Hura crepitans* RIP	146
Nyctaginaceae	*Mirabilis jalapa*	tissue culture	MAP	3
		Roots	MAP	90
Phytolaccaceae	*Phytolacca americana*	Leaves	PAP	9.2
			PAP II	3.6
		Seeds	PAP – S	100-180
		tissue culture	PAP – C	17.5
		Roots	PAP – R	7
	Phytolacca dodecandra	Leaves	Dodecandrin	3.4
		tissue culture	Dodecandrin – C	1
Poaceae	*Hordeum vulgare*	Seeds	Barley RIP	139
	Triticum aestivum	Germ	Tritin	3
		Seeds	Tritin	32
	Zea mays	Seeds	Maize RIP	12.1-20

Table adapted from Barbieri *et al.* (1993) [11].

TABLE 2.2

Purified type-2 ribosome inactivating proteins

Family, genus and species		Plant tissue	Name	Yield (mg/100g)
Euphorbiaceae	*Ricinus communis*	Seeds	ricin D	120
			ricin E	
			Ricinus agglutinin	
Fabaceae	*Abrus precatorius*	Seeds	abrin a	
			abrin c	75
Passifloraceae	*Adenia digitata*	Roots	Modeccin	20-180
			Modeccin 6B	7
	Adenia volkensii	Roots	Volkensin	0.2
Sambucaseae	*Sambucus ebulus*	Leaves	ebulin 1	3.2
Viscaceae	*Phoradendron californicum*	Leaves	*P. californ.* Lectin	
	Viscum album	Leaves	Viscumin	7

Table adapted from Barbieri *et al.* (1993) [11].

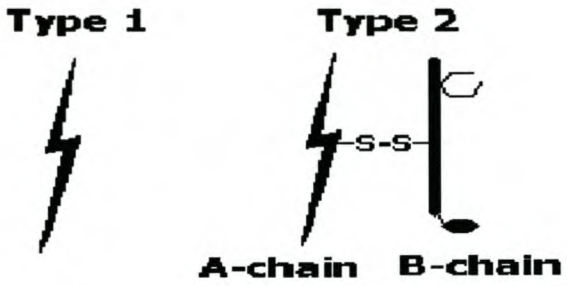


Figure 2.1. Schematic representation of the structure of type-1 and type-2 ribosome-inactivating proteins. The A-chain is a polypeptide with *N*-glycosidase activity. The B-chain is a polypeptide with lectin activity [11].

From Figure 2.1. the differences between the two types of RIPs, a type-1 and a type-2 can clearly be seen. Ricin and abrin are both type-2 RIPs. The discovery of new RIPs that did not fit this mold lead to the classification of a type-3 RIP [23]. At first the "type-1" RIPs that underwent proteolytic modification were also part of this group but re-evaluation of this classification suggested that type-1 RIPs be re-grouped so that type-1 RIPs now consist of one-chain and two-chain RIPs [12].

This restructuring now allows for all the type-1 RIPs that undergoes proteolytic modification to be named two-chain type-1 RIPs. The term type-3 RIP is reserved for the truly different RIPs like, JIP60 from barley, (*Hordeum vulgare*) and other not yet discovered proteins with properties similar to JIP60. JIP60 is a 60 kDa protein that consists of two linked domains: an N-terminal domain with the properties of a type-1 RIP and a C-terminal of unknown function [23]. For this suggested re-classification see Figure 2.2.

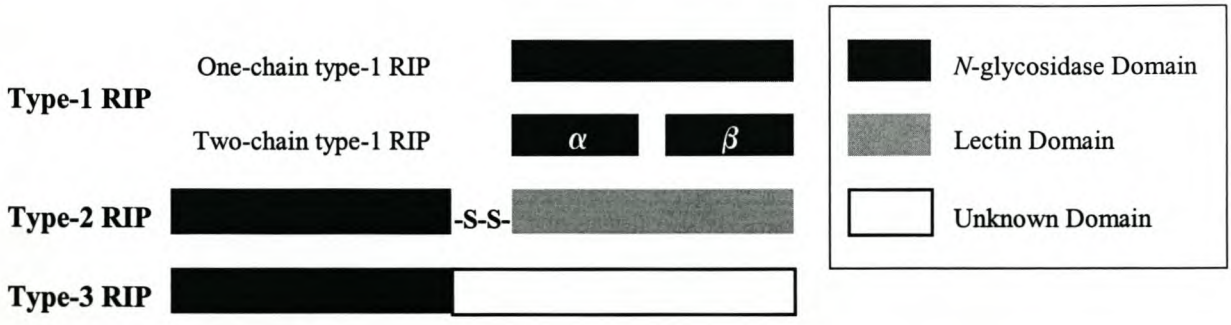


Figure 2.2. Schematic representation of the molecular structure of different types of RIPs as suggested by Peumans *et al.* [12].

After the structure of RIPs was elucidated, the enzymatic activity had to be determined. Endo *et al.* described the RNA *N*-glycosidase activity of ricin A-chain on ribosomes and rRNA [24, 25, 26]. Ribosome inactivating proteins are presently classified as rRNA *N*-glycosidases [EC 3.2.2.22].

2.2. General

2.2.1. Distribution

The distribution of RIPs in the plant kingdom has not been studied intensively, mainly because scientists were searching for new RIPs in related plants, rather than in new classes. Most RIPs discovered up to 1993, were from the Angiospermae class and from both monocotyledonae and dicotyledonae. No RIP has yet been isolated from the Gymnospermae [11].

The similarities in activity between shiga toxin (Stx), produced by some *E. coli* strains, and RIPs in general lead some scientists to group these toxins together [27, 28]. Similarly, N-terminal sequence homology of the proteins, Flammulin (from the winter mushroom, *Flammulina velutipes*) and Lyophyllin (from *Lyophyllum shimeji*) to RIPs make it possible to categorize these proteins with the plant RIPs [29, 30]. Other fungus toxins such as α-sarcin (from *Aspergillus giganteus*) are sometimes also grouped with RIPs [31]. The latest twist to this tale is the discovery of RIP-like proteins in mammalian tissue culture cells that suggests that RIPs might even be more widely spread than originally thought [32].

2.2.2. Structure

The type-1 RIPs are very similar to each other in structure. All consist of only a single polypeptide that has the same properties as the A-chain of the type-2 RIPs. The structural similarities between type-1 RIPs and the A-chain of the type-2 RIPs are illustrated in Figure 2.3.

Figure 2.3. Superimposition of the 3-D models of irisRIP.A3 (thick line) with the A-chains of ricin and abrin as well as the type-1 RIPs α-momorcharin and trichosanthin (thin lines) [33].

The type-2 RIPs are composed of an A-chain that is bound to a B-chain via a disulphide bond and other non-covalent bonds (see Figure 2.1). The type-2 RIPs can consist of two or four polypeptide chains linked together, and have an estimated molecular mass of 60 000 or 120 000 Da. Type-2 RIPs such as ricin, abrin and modeccin are heterodimers whilst viscumin is a tetramer consisting of two heterodimers held together by non-covalent bonds [34, 35].

The three-dimensional structure of ricin was examined after crystallization at 2.8 Å and 2.5 Å [36]. This revealed that the A-chain was globular with a well-defined cleft (see Figure 2.4). This cleft was investigated and it was found that it held the enzymatic site.

The B-chain was examined and was shown to be a gene duplication product. It was also shown that the B-chain consisted of two domains, and that it has a binding site for lactose in a shallow cleft. With X-ray diffraction it was confirmed that the A-and B-chain are held together by a disulphide bridge and other non-covalent bonds (hydrophobic interactions). This can be seen in the ribbon structure in Figure 2.5.

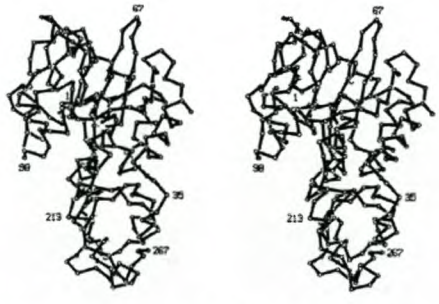


Figure 2.4. The putative active site cleft of the ricin A-chain [37].

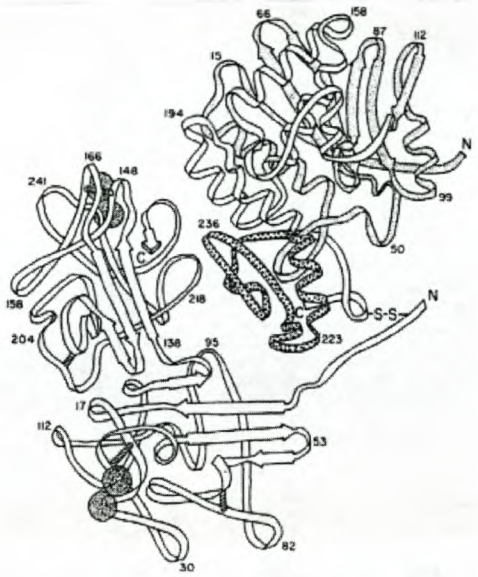


Figure 2.5. Ribbon representation of the ricin backbone. The A-chain is in the upper right and the B-chain is the lower left [37].

2.2.3. Physicochemical properties

The sizes of RIPs have been determined using SDS-PAGE and were found to be approximately 30 000 Da for the A-chain and the B-chain within a 10% error (see Table 2.3). The pI of the A-chain cannot be measured but is basic (>9.5) [11]. The pI of the type-2 RIPs has been determined and varies from 4.6-8.8. As can be seen in Table 2.3, most of the RIPs are glycoproteins that do not seem to play a role in their enzymatic activity but rather with their way of internalization. The genes of many RIPs have been sequenced (see Table 2.4), and very little nucleic sequence homology between the different RIPs was found. However, with amino acid sequence alignment it was observed that the N-terminal and core sequences were more conserved than the C-terminal, which might then explain why some activities are conserved throughout the range of RIPs and other activities are confined to certain groups [38].

TABLE 2.3

Physico-chemical characteristics

RIP	Molecular Mass (Da)	Neutral sugar Content (%)	Extinction Coefficient at 280 nm (M)
TYPE-1 RIPs			
Agrostin 2	30600	6.68	
Agrostin 5	29500	6.87	
Agrostin 6	29600	7.17	
Asprin 1	30500	Traces	30500
Asprin 2	29800	Traces	29800
Barley RIP	29976		27600
Dodecandrin	29000	0	
Dodecandrin – C	31-32000	4.46	
Gelonin	30000		21000
Hura crepitans RIP	27500	0.94	25200
Maize RIP	25000	0	26500
MAP	27833	0	24000
Momordin I	29092	1.74	
Momordin II	30000	1.3	
PAP	29000	0	24000
PAP – C	29000	Traces	
PAP – R	29800	0	23800
PAP – S	29167	0	22500
PAP II	30000	0	26700
Saporin – L1	31600		29400
Saporin – L2	31600		26400
Saporin – R1	30200		23200
Saporin – R3	30900		24700
Saporin – S5	29500		23800
Saporin – S6	29500	0	
Saporin – S8	29500		
Saporin – S9	29500	0	
Trichokirin	27000	1.27	23330
Trichosanthin	24-29000	0	
α - trichosanthin	27215		
Tritin	30000		
TYPE-2 RIPs			
Abrin a	60100	5.5	70700
A chain	31000	Present	
B chain	35500	Present	
Abrin c	63800	7.4	82500
A chain	30000	0	23600
B chain	36000	7.4	63700
Ebulin 1	56000		
A chain	26000		
B chain	30000		
Modeccin	60000	2.7	
A chain	27800		
B chain	31000		
P. californ. Lectin	69000	14.1	
A chain	31000		
B chain	38000		
Ricin D	63-66000	5.5-6.2	77900
A chain	30000	4.5	23000
B chain	33000	6	50700
Ricin E	63000	Present	
Ricinus agglutinin	120000	4.5	140400
A chain	31000		
B chain	34000		

Table adapted from Barbieri *et al.* (1993) [11].

TABLE 2.4

RIPs of which the sequence is known

RIP	Genome	CDNA	Protein
Abrin	*		
Abrin-a			*
Abrin c	*		
b 32/ maize RIP	*	*	
Barley RIP		*	*
Dianthin 30		*	
Luffin a		*	*
Luffin b		*	*
MAP	*	*	*
Momordica balsamina RIP		*	
Momordin I		*	
PAP-S			*
PAP	*	*	
Ricin D	*	*	*
Rinus agglutinin		*	
Saporin-S6	*	*	*
Trichosanthin	*	*	*
-Trichosanthin			*

Table adapted from Barbieri *et al.* (1993) [11].

2.2.4. Biosynthesis

To determine where and how RIPs are synthesized is quite difficult, because RIPs have been found in all plant tissues, and at levels of expression that vary quite considerably. Because of their enzymatic activities, RIPs must be synthesized in such a way that they are not harmful to the cell. There are three possible ways in which the plants handle this problem: i) the ribosomes of the cell are resistant to the RIP that is produced by the same cell, ii) the RIP is first synthesized in a inactive pro-form, iii) the synthesis of a preproform with an N-terminal signal sequence that directs the nascent peptide chain to the lumen of the reticulum before it can be folded into its active form (see Figure 2.6).

Plants producing ricin seem to make use of the third pathway. The active ricin is then stored in single membrane organelles in the endosperm of seeds [38]. In the case of pokeweed, Ready *et al.* used electron microscopy and RIP specific antibodies to show that the RIP PAP was produced and secreted over the cell membrane into the cell wall matrix and was thus trapped in the intercellular matrix [38, 39].

It was observed with Beetins 27 and 29, from *Beta vulgaris* (sugar beet), that expression is only induced when this plant has a virus infection, and in this case it is proposed that the RIP has a cell death role and does not have to be compartmentalized [40].

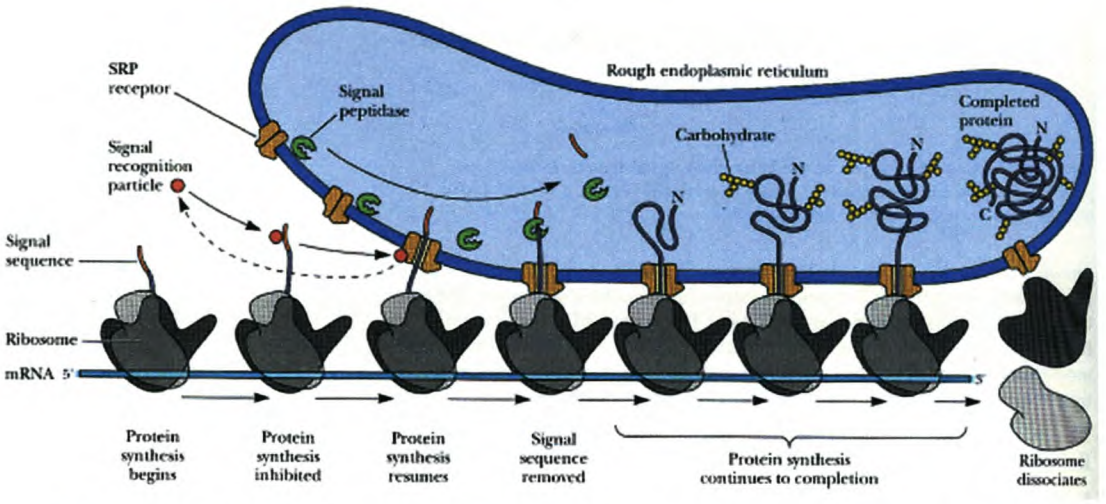


Figure 2.6. Synthesis of a protein and its translocation via the endoplasmic reticulum [41].

2.3. Biological Activity

2.3.1. Enzymatic activity

2.3.1.1. rRNA-N glycosidase activity

Endo *et al.* (1987 (a),(b) and 1988) demonstrated that the functional damage by RIPs on ribosomes is due to a modification of the rRNA of the ribosome. *N*-glycosidase activity of both type-1 RIPs and the A-chain of type-2 RIPs were observed to cleave the *N*-glycosidic bond of A^{4324} (see Figure 2.7) of 28S mammalian rRNA. It was also discovered that the adenine residue that is cleaved is in a loop and stem sequence in rRNA that contain a GAGA sequence. This sequence is the recognition site for EF-2, indicating that the RIPs target a specific structure [11, 24, 34]. Some of these loop structures are shown in Figure 2.8.

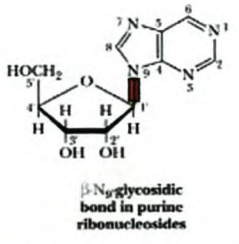


Figure 2.7. Glycosidic links (marked in red) are between the nitrogen-base and sugar to form nucleoside. This is where the RIP depurinates the rRNA [41].

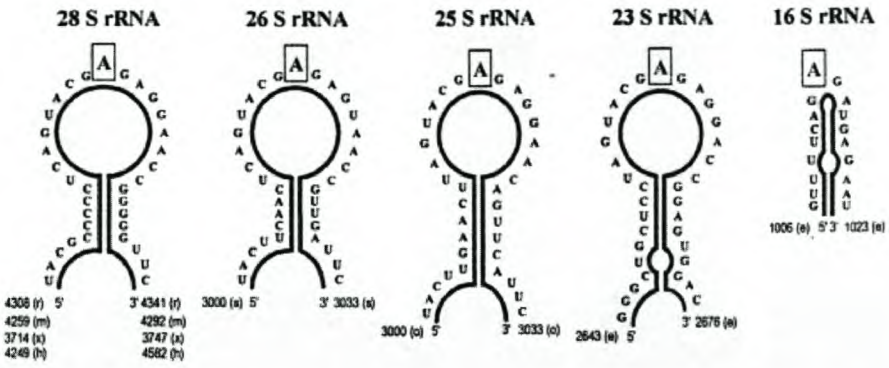


Figure 2.8. Structure of rRNA substrates for *N*-glycosidase activity of RIPs. (e) *E. coli.*, (h) *Homo sapiens,* (m) mouse, (o) *Oryza sativa,* (r) rat, (s) *S. cerevisiae,* (x) *X. laevis* [11, 37].

2.3.1.2. Site of action

The RNA *N*-glycosidase activity of RIPs that depurinate rRNA, is thus damaging the ribosome and ultimately stopping protein synthesis [20, 24, 33, 34, 42-44]. Protein synthesis cannot take place on ribosomes that have been damaged by RIPs. During translation the function of the EF-2 is to translocate the uncharged tRNA when hydrolysing the bound GTP [41]. The inability of EF-2 to bind to the ribosome will halt protein synthesis and because the damage is irreversible, the ribosome will be inactive and unable to continue with protein synthesis.

The site of cleavage is highly specific and is adjacent to the site of cleavage by a group of specific RNAases from fungi [34, 45]. α-Sarcin from the mould *Aspergillus giganteus* is one of these RNAases that cleave the phosphodiester bond in rat liver rRNA between G^{4325} and A^{4326} [34, 45, 46]. There are also some toxins from bacteria that show RIP type-2 like activities. *Corynebacterium diphtheriae* and *Pseudomonas aeruginosa* are two bacteria that associate a lectin and an EF-2 ADP ribosylase. This type of toxin will then inactivate EF-2 by binding ADP-ribose covalently, a reaction which is irreversible under normal cell conditions (see reaction below)[45, 47, 48].

$$\text{EF-2} + \text{NAD}^+ \rightarrow \text{ADP-ribosyl-EF-2} + \text{nicotinamide} + \text{H}^+$$

2.3.1.3. Other factors influencing RIP activity

The variation in RIP's activity on ribosomes in general can be due to a number of factors not yet considered, such as resistance to the RIP and/or that there are some cofactors needed for the RIPs' enzymatic activity [49].

Many studies have been done on the site of recognition and conflicting results have been obtained which were due to some unexpected activities of RIPs. It seems that some RIPs do not always inactivate ribosomes of their host plant [43]. In the cases where RIPs were observed to inhibit protein synthesis of their own cells (e.g. ricin [50] and PAP [51, 52]) it was shown that the concentrations of RIPs needed to inhibit protein synthesis were much higher than was needed for cells of non-host species. This also leaves room for speculation on the involvement of a possible cofactor.

As with most other activity studies, the activity on bacterial ribosomes had conflicting results. However, the main consensus now is that RIPs do indeed inactivate ribosomes of *E. coli* [38, 53-57]. In the case of yeast ribosomes, RIPs always displayed good activity (it has been proven that *Saccharomyces cerevisiae* is sensitive to both type-1 and type-2 RIPs [11]) and newly found RIPs are now generally tested for reactivity on *E. coli* and yeast ribosomes [58]. Activity towards other bacterial ribosomes (*Streptomyces lividans*) has also been identified [59].

Some studies reported the weak anti-fungal activity of type-1 RIPs but these are so low that activity may originate from other contaminating proteins [12].

Although all RIPs share the same activity of RNA *N*-glycosidase activity there seems to be a difference in substrate specificity as is clearly demonstrated by the above-mentioned studies. The type-2 RIP, Ricin, is very active on mammalian cells and yeast ribosomes with no activity on plant and bacterial ribosomes. In contrast, the type-1 RIP, PAP, inhibits protein synthesis in bacteria, plants, yeast as well as in lower and higher animals [12]. This difference in target ribosomes, leads us to believe that these two types of RIPs might have different functions in the native plant.

2.3.1.4. Alternative substrates of RIPs

Until recently the above-mentioned activities were believed to be the only mechanisms of RIPs. Ribosomal-RNA was believed to be the only substrate for this protein but it was since proven that ricin, cinnamonin, luffin and camphorin (also α-sarcin and shiga toxin) removed the adenosine residues in supercoiled DNA, with no effect on linear DNA [11, 28, 60]. Dianthin 30, PAP and Gelonin were also proved to cut supercoiled DNA specifically [61, 62]. Later reports suggest that certain RIPs (gelonin) do cut linear DNA [63].

Barbieri *et al.* (1996, 1997), tested most of the known RIPs for alternative substrate affinity. The substrates tested for were DNA, RNA from different sources and poly (A) RNA. It was found that RIPs do interact with these substrates and release an adenosine by cutting the glycosidic bond. The activity of the RIPs tested, varied but it was proposed (by Barbieri *et al.*) that the term ribosome-inactivating protein (RIP) should be changed to polynucleotide: adenosine glycosidase [64, 65]. In 2000 Barbieri *et al.* confirmed that polynucleotide: adenosine glycosidase activity is the sole activity of RIPs on DNA [66].

2.3.1.5. Summary

In short, the activity of RIPs can be summarised as follows: the RIP enters the cell (type-2 RIPs need to be reduced for the A-chain to be active), and they then recognize the GAGA site on rRNA of 28S ribosome subunit (binds to rRNA with protein-protein binding or protein-RNA binding [44]). The RIP then cuts the glycosidic bond of the adenine with the active site in the prominent cleft in the A-chain (*N*-glycosidase activity). The ribosome is thereby inactivated and no protein synthesis can continue, resulting in cell death.

2.3.2. The entrance of RIPs into the cell

Type-2 RIPs are more potent than their single chain relatives, because of the B-chain, with the lectin properties that bind to galactosyl-terminated receptors and allows RIPs to enter the cell and deliver the A-chain, with enzymatic activities to the cytosol where it inactivates the ribosomes [11, 20, 67-69]. Thus type-2 RIPs do not require cell damage before they can enter.

The RNA *N*-glycosidase activity for the type-1 RIP and the A-chain of the type-2 RIP is identical. It is very specific and in rat liver, it recognizes the A^{4324} base residue on the 28S rRNA [11, 24, 34, 44].

2.3.2.1. Internalization of type–2 RIPs

The first step for the RIP in entering the cell is to bind to the receptor site [70]. Type-2 RIPs have a B-chain, which has lectin activity (galactose-specific) which lets the RIP bind to the galactose containing glycoproteins and glycolipids that are present on the membrane surface [71]. Most RIPs are glycoproteins and some receptors might interact with these carbohydrates and mediate entrance into the cell in this way.

Evidence indicates that ricin, and most likely all other type 2 RIPs enters the cell through endocytosis, and more specifically receptor-mediated endocytosis by way of clathrin-coated pits, mannose receptors or galacosyl residues. After being internalized by endocytosis, the vesicle will loose its protein and become an endosome [11].

After endocytosis, the RIP enters the cell via the endosomes, which are then turned into lysosomes. In the lysosomes the contents can be degraded and the receptors can be recycled to cell membrane. On this route the toxin must find a way to enter the cytosol. It seems that some types of receptors might be more effective in delivering the toxin into the cell than others. The toxin-ligand complex needs an acidic environment (lysosome) to dissociate and it has been suggested that acidity is needed when ricin enters the cytosol via the mannose receptor and ricin bound to galactose enters the cytosol via a neutral compartment [69]. This could be attributed to the specific receptor and its pathway in the cell. It was also noted that a flux of Ca^{2+} over the membrane is needed for successful transport to the cytosol [70].

The internalization of type-2 RIPs can be summarized as follows: The RIP binds to a receptor on the cell membrane and enters the cell through endocytosis. It is then routed to intracellular compartments where the active chain is transferred to the cytosol after dissociation from the binding chain.

2.3.2.2. Internalization of type–1 RIPs

The transport of type-1 RIPs to the cytosol is not clearly understood. It may be that some of the type-1 RIPs enter the cell like type-2 RIPs via their carbohydrate receptors. This seems to be the exception and might not be true because not all type-1 RIPs are glycoproteins. Saporin S6 is one the most toxic type-1 RIPs and is void of all sugars. The uptake of type-1 RIPs seems to be fluid-phase endocytosis. No specific binding of receptors is required for this uptake [11]. The difference in cytotoxicity of the type-1 RIPs might be ascribed to their different intracellular routes that they take to enter the cytosol. Some of these routes are discussed later under the antiviral activity in the host plant.

2.3.3. Effects of ribosome-inactivating proteins

The cytotoxicity of RIPs has been studied extensively, and it is clear that type-1 RIPs are far less cytotoxic than type-2 RIPs. The concentration at which 50% inhibition of protein synthesis is induced (IC_{50}), for type-1 RIPs are in the *micro*-molar range, whilst in the *pico*-molar range for type-2 RIPs. This can be ascribed to the different ways by which the two RIPs enter the cytosol. From this it could be said that for the RIP to enter the cell properly it needs a molecule to bind with the cell membrane. The entrance of type-1 RIPs thus needs to be facilitated by either another molecule, physical break in the cell wall or heightened permeability [72].

Data on the effect of RIPs on different organisms are conflicting and it seems that there is still a lot to be unraveled about the mechanism of inactivation. For example, in a study on the effect of barley-RIPs on fungus it was discovered that the RIP needs the help of two other enzymes (α-glucanase and chitinase) to be able to inhibit protein synthesis [73]. It was also observed that some RIP's activity are up-regulated or dependant on ATP as a co-factor [49, 74] and others are up-regulated by specific tRNAs [75]. Osmotic and heat stress also up-regulates the expression of some RIPs [76, 77].

It can be seen from all these effects that RIPs have a wide range of possible applications, of which broad-spectrum virus resistance and specific toxicity, might be the most important. It can also be seen that the exact mechanism of operation and effects of RIPs have not been elucidated. The study on RIPs is an active field and many of the mechanisms of inactivation need to be clarified.

2.3.4. Antiviral activities

The antiviral activities of certain plants have been known since 1925 and in experiments done by Duggar, cell extracts were mixed with viruses and applied to the leaves of another species [11, 12, 20]. From the observed results it was concluded that plant extracts (RIPs) acted on the other plants and not on the virus. PAP was the first RIP to be purified and shown to inactivate the ribosomes of other plants, which was consistent with the other experiments [78]. Subsequent studies have shown that all type-1 RIPs as well as most type-2 RIPs have antiviral activity [19].

The antiviral activities of RIPs on DNA and RNA viruses that infect animal cells were proven the same as in plant viruses where the infected cells are selectively killed [14, 79, 80]. The virus seems to increase the permeability of the cell [72, 81, 82], allowing the RIP to enter, protein synthesis is halted and the virus is kept from spreading [83]. This describes the possible mechanism of activity of RIPs on non-host plant or animal cells, but what happens in plants where these RIPs are present?

2.3.5. Physiological role

All evidence supports the thought that RIPs play a vital role in plant defense. The type-2 RIPs have a different mechanism of defense in that they have the ability to facilitate movement over membranes with the help of their Binding-chain (lectin) [11]. This activity the type-2 RIPs makes it highly toxic to lower and higher animals but ineffective towards bacteria and fungi with their protective cell walls. It is thus believed that type-2 RIPs (mainly found in seeds) protect the seeds of the host plant from plant eating organisms [12].

Type-1 and 3 RIPs do not have the ability to move over membranes and is not cytotoxic to higher animals and invertebrates and it is thus believed that type-1 and 3 RIPs play a role in plant defense against pathogenic fungus and viruses and not predation [12,39]. It is questionable whether RIPs have a role in nature as a broad-spectrum pathogen resistance protein because of its tissue specific expression [84].

2.3.5.1. Antiviral activity in host plants

The mechanism of virus inhibition in RIP containing plants has not been fully elucidated, but three proposals have been put forward on how this might occur [12].

1. RIPs act directly on the viral nucleic acid by means of its polynucleotide: adenosine glycosidase activity [85]. As direct activity on intact viruses are unlikely because DNA/RNA is physically protected by the virus coat proteins, deadenylation can only take place as soon as the virus disassembles for reproduction [12].
2. The local suicide model where RIPs gain access to the cytosol through a break in the cell wall through damage caused by a virus vector such as an aphid [39, 68]. The spread of the virus to the neighboring cells are limited, as the virus cannot replicate.
3. The activation of the plants defense mechanism by a RIP [40].

Conflicting opinions exist on the first two proposals because of their time of supposed activity. After the cell integrity has been breached the endogenous lyases liberated from the lysosomes will result in almost total nucleic and protein degradation. The observed contradiction that plants containing RIPs are also susceptible to plant viruses indicates that there is no direct antiviral activity but rather an indirect activity [12]. It is important to note that the challenges put to these proposals only have bearing on the mechanism of action in plants where RIPs are naturally occurring and do not contradict the findings on the activity of RIPs in cell free experiments.

An alternative to the second proposed mechanism is a local suicide model that is only activated after the infection has take place. It has been observed that the cell membranes of mammalian cells become more permeable after virus infection [83, 85]. The plasmodesmata of plant cells become over-dilated with virus infection and this could also be possible way by which the RIPs in the intercellular space leak into the cytoplasm of the cell and by killing the cell prevents further spreading of the virus [11, 39, 72].

With the contradictory results and findings of the first two proposals, the third proposed mechanism needs more investigation. No such mechanism has yet been observed in RIP-containing plants but studies on transgenic tobacco expressing recombinant RIPs might pave the way to a better understanding of the mechanisms involved in antiviral activity

[12]. Such mechanisms are currently being proposed after the observations in PAP transgenic tobacco. The proposed mechanism of resistance is independent of the salicylic acid and pathogenesis-related protein synthesis pathways [69, 87-89]. However, the exact mechanism for antiviral activity still needs to be elucidated and it is more than likely a combination of the above-mentioned mechanisms.

2.3.5.2. Anti-fungal activity in host plants

Anti-fungal activity has been observed by some RIPs but unreliable purification techniques might have given misleading results and it has been suggested that all RIPs accredited with anti-fungal properties be re-evaluated [12]. Cereal seed RIPs and RIPs that are inactive on conspecific ribosomes cannot bring about local cell death for virus resistance [38]. It has been proposed that these RIPs might hold an anti-fungal role as they are more active on fungal ribosomes than mammalian ribosomes [90].

Logemann *et al.* transformed tobacco plants to study the anti-fungal activity of the barley RIP. The transformed plants were resistant to the infection to *Rhizoctonia solani* a phytopathogenic fungus [91]. In general, plants respond to fungus infection by producing chitinases and glucanases as part of their systemic acquired resistance (SAR) [92]. These enzymes inhibit fungal growth by degrading the major structural polysaccharides in the fungal cell wall [73, 92]. The observation that barley RIPs increased the inhibitory effect of plant chitinases and glucanases on fungi leads scientists to believe that this is the mechanism of action for RIPs in plants towards fungus [73]. This at least seems to hold true for the barley RIPs.

2.4. Applications

The most studied applications of RIPs are their direct use as immunotoxins or as conjugates with other suitable molecules. The possibilities of this technology are numerous and the only limitation is that the molecule that binds to the cell must be highly selective to malignant (cancer or parasitic) cells. In theory then, RIPs can be used as a 'magic bullet' that kills only unwanted cells.

In agriculture the application lies mainly with transgenic crops where the RIP is used to give a crop resistance to a range of invading viruses or fungi. The RIPs do not act directly on the pathogen but on the plant itself and the resistance attained would be broad spectrum. Discussed below are some specific applications that have been studied thus far.

2.4.1. Immunotoxins

RIPs can be coupled to many molecules that will give it target-specific toxicity, one of these types of molecules are immunoglobulins (usually monoclonal antibodies). The coupling of a RIP will then target the RIP to specific cells that will act as the antigen of that specific antibody. Such a conjugate is called an immunotoxin. The RIP is linked to the antibody via a disulphide bond or sometimes via a thioether bond. Some interesting techniques have been developed that exploit the properties of the immunotoxins and many medical applications have been developed. The field of immunotoxins although interesting is not relevant to this study [review 93].

2.4.2. Applications in agriculture

The application of RIPs in the agriculture is in principle very simple. The aim is to create transgenic plants that produce the RIP and can function normally. The ideal situation would be where transgenic crops are produced that will express the RIP protein in such a way that they would be resistant to a broad spectrum of viruses [68]. In most of the cases tobacco was used as a model plant to observe the effects on the plant as well as to ensure that resistance is generated in the plant.

By using the RIP, PAP resistance towards PVX (Potato Virus X, Potexvirus) and PVY (Potato Virus Y, Potyvirus) was engineered into tobacco and potato plants [68]. Other groups followed up on these experiments in an attempt to elucidate the mechanism of resistance. It was shown the active site of PAP was needed to give resistance to PVX, but that the resistance is independent of the salicylic acid and pathogenesis-related protein synthesis pathways [88]. A less toxic RIP, PAPII was also used in transgenic experiments and resistance was obtained to PVX, TMV and *Rhizoctonia solani* [89]. The introduction of a non-toxic mutant of PAP (PAPn) indicated that the activity on ribosomes is a requirement for resistance of the plant against PVX, PVY and *Rhizoctonia solani* reiterating the independence of resistance to the salicylic acid pathway [89, 94].

Lam *et al.* obtained complete resistance to turnip mosaic virus in transgenic tobacco by introducing the RIP trichosanthin [95]. Recently, the effects of trichosanthin in transgenic tobacco were studied in an attempt to unravel the mechanism of resistance. Transgenic plants generated in this study were resistant to Cucumber mosaic virus (CMV) and TMV. The mode of action could not be demonstrated [96].

African cassava mosaic virus (Geminivirus) resistant Dianthin 30 transgenic tobacco plants were produced by the Hong *et al.* from the John Innes institute [97, 98].

Recently, a type-2 RIP, SNA-I' (*S. nigra* agglutinin I' from *Sambucus nigra*) was transformed into tobacco and expression of functional type-2 RIP observed. Resistance to TMV was attained in transgenic plants but a different mode of action is suspected [99].

To date, there is only one case where transgenic work was continued beyond tobacco or any other model plant. Kim *et al.* produced transgenic rice containing a maize RIP (maize-RIP b-32) [100, 101]. The R2 plants were tested for resistance to *Rhizoctonia solani* and *Magnaporthae grisea* and found more resistant than the non-transformed control plants [100, 101].

2.5. Dianthin 30

2.5.1. History

The *Dianthus* genus has been known to contain inhibitors of virus infection since the early 1950's. Scientists from Holland (van der Want, 1951) and Canada (Weintraub and Gilpatrick, 1952) mixed the sap of *Dianthus caryophyllus* L. and *Dianthus barbatus* L. with plant viruses such as tobacco mosaic and tobacco ring spot and found reduced symptoms. Ragetli showed in 1957 that the inhibition effect was most likely via the host plant [102]. More recently the same type of studies were conducted and found that Dianthin 32 was highly effective in the prevention of TMV infection [103].

In 1962, Ragetli and Weintraub were the first to partially isolate and characterise the "Virus inhibitor protein" now known as Dianthin 30 from *Dianthus caryophyllus* L. [102, 104].

Stirpe and his team were the first to isolate Dianthin to homogeneity by chromatography and proposed the name Dianthins for the two RIPs (Dianthin 30 and 32) isolated from *Dianthus caryophyllus* L.[105]. The molecular weight of Dianthin 30 was calculated at 29.5 kDa using SDS-PAGE, and an IC_{50} on rabbit reticulocytes of 9.15 ng/ml [105]. From multiple protein sequence alignments the putative enzymatic site of Dianthin 30 was shown to be between amino acids 152 and 165 [54].

2.5.2. Properties and activity

Reisbig *et al.* investigated the tissue distribution of Dianthin 30 and 32 in young and old *Dianthus caryophyllus* plants, and found that Dianthin 32 was only present in the leaves and the young shoots while Dianthin 30 was found throughout the plant. It is also interesting to note that they found that Dianthins constituted as much as 3% of the total extracted protein in older plants (Table 2.5)[106].

The Dianthin 30 ORF is 879 bp long and translates to 293 amino acids. From sequence homologies, the first 23 amino acids might contain a secretion signal. If the polypeptide undergoes post-translational modification and it is possible that at least the 23 N-terminal amino acids that are within this signal sequence might be cleaved of and will not be present in the mature protein. Sequence homology to the C-terminal (last 18 a a) also indicated a possible vacuole-targeting signal but this remains to be confirmed [11, 107]. In Figure 2.9. the different predicted forms of Dianthin 30 are depicted. It is important to note that all referrals to Dianthin 30 in this thesis are to the unmodified pre-Dianthin 30 form.

Initially it was believed that RIPs have no activity on endogenous plant ribosomes [13]. After also investigating the effects of RIPs on their own ribosomes, Stirpe at first came to the same conclusion [21]. This has later been proven incorrect and some RIPs were indeed found to be active on their own ribosomes, as Taylor *et al.* demonstrated for PAP [51] and Kataoka *et al.* for MAP [108].

TABLE 2.5
Tissue distribution of Dianthins

Tissue	µg of RIP/mg of protein	
	Dianthin 30	Dianthin 32
Old Leaves	12.5	3.9
Young Leaves	2.9	1.7
Shoots	3.0	2.1
Stems	17.9	
Roots	30.2	
Branch Roots	33.3	
Seeds	10.1	

Table adapted from Reisbig *et al.* (1983) [106].

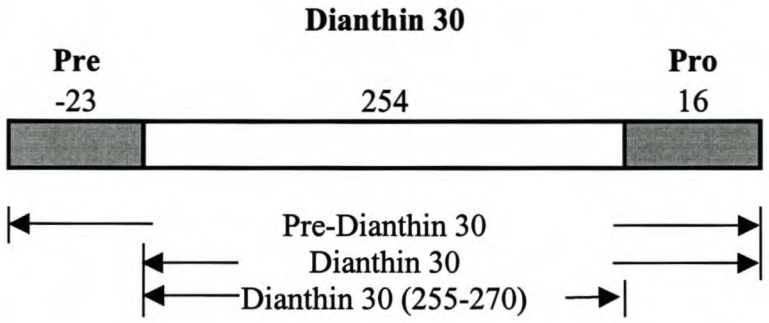


Figure 2.9. Schematic representation of the Dianthin 30 gene [11, 107]

Dianthin 30 was believed to have no activity on cell-free ribosomes of *E. coli* [109], but with continued study Legname *et al.* demonstrated that the expression of Dianthin 30 in a bacterial system was extremely difficult, but achievable with vectors that have tighter initiation control [107].

The first "type-1" RIP to be expressed was ricin A (Ricin without the B chain) and all conclusions drawn from these expression studies indicated that type one RIPs might have no activity on *E. coli* ribosomes. Expression of type 1 RIPs were not so successful and lead Hartley *et al.* to investigate further [54]. They found that PAP, PAP-S, Dianthin 30 and 32 inhibit the activity of *E. coli* 23S ribosomal RNA [38, 54, 59]. The same was also observed for Dianthin 29 from *Dianthus barbatus* [55] and for MAP from *Mirabilis jalapa* [56]. Dianthin 32 was also observed to have a strong action on yeast ribosomes [103].

2.5.3. Expression

The first attempts to express Dianthin 30, done by Legname *et al.* in a bacterial expression system (pKK233.2), yielded very low concentrations of recombinant protein (20 μg/L) [110, 111]. Higher concentrations of the rDianthin 30 were however achieved with the use of the pET/T7 expression system that has tighter transcription initiation control [107]. Expression of a related RIP cDsRIP2 (79% homology to Dianthin 30) from *Dianthus sinesis* proved unsuccessful and the possible toxicity of this protein to the *E. coli* ribosomes seems the most likely explanation [112].

2.6. Conclusion

It is indisputable that ribosome-inactivating proteins (RIPs) of plants are an interesting family of proteins. They were first discovered as toxins and this function was exploited extensively. The reason for their toxicity was only later discovered to be the RNA *N*-glycosidase activity that depurinates the 28S eukaryotic mammalian rRNA. It was also shown that the type-1 RIPs and type-2 RIP's A-chains both have *N*-glycosidase activity that cleaves the *N*-glycosidic bond of adenine$_{4324}$ of rat 28S rRNA. The site where the adenine is removed is highly conserved and named the GAGA-site (next to α-sarcin's cut site on the α-sarcin loop). The removal of this adenine then inhibits the binding of EF-2 thus stopping the translocation of the tRNA (the hydrolyzing of a GTP resulting in the translocation is catalyzed by EF-2).

More recently it was discovered that RIPs release the adenine of different rRNA as well as DNA, implicating that the name should be changed to polynucleotide: adenosine glycosidase. The fact that these new substrates have been found does not change the current applications of these proteins, it might actually broaden the application.

In the field of agriculture, the possibility of using RIPs in transgenic plants is very alluring with the reward of possible broad-spectrum virus resistant plants.

Chapter 3: Expression of Dianthin 30 in bacteria

3.1. Introduction

An intricate part of studying the expression of any protein in transgenic plants is a detection method for the protein of interest. For this study, it was decided that detection via polyclonal rabbit antibodies would be the most effective.

Either purified or recombinant protein can be used as antigen but to avoid any cross-reaction between the polyclonal antibodies and other plant proteins, the route of recombinant expression was chosen. In a bacterial expression system, a reduced possibility of epitope similarities to the plant proteins exists as all possible contaminating proteins are bacterial and the only plant protein is the one that is being investigated.

Two systems for the large-scale expression of rDianthin 30 were considered: a yeast expression system and a bacterial expression system. The reactivity of Dianthin 32 on yeast ribosomes was found by Taylor *et al.* to be >8 times higher than Ricin and >14 times higher than PAP [103]. Considering these data and the sequence similarities between Dianthin 30 and 32, yeast expression was not attempted for Dianthin 30 [103]. The expression of Dianthin 30 has largely been unsuccessful and various reasons are given for this. The toxicity of the protein to ribosomes is the most likely explanation. However, some researchers were able to claim limited success with the expression of Dianthin 30 in bacterial expression systems [107, 110, 111].

Legname *et al.* (1991) was first to attempt the expression of rDianthin 30 [110]. They expressed 20 µg/L rDianthin 30 in JM109 *E. coli* cells using the expression vector pKK 233.2. In 1995, Legname *et al.* described the expression of Dianthin 30 and a deletion mutated Dianthin (Δ255-270) using the pET/T7 expression system with BL21(DE3) and BL21(DE3)pLysS *E. coli* cells. They were able to express Dianthin 30 (first 23 N-terminal a a removed) to a level of 10 mg/L [107].

For recombinant expression, *in vitro* expression was also considered. Commercial expression systems are available that promises high-level expression in a cell-free

environment (e.g. TNT from Promega). The drawback to these systems are that they are very expensive and with the observed activity of RIPs on ribosomes of various organisms combined with the limited success already achieved in an *in vivo* expression system [110], it was decided not to attempt expression this way. Wheat germ expression was also discarded as a possible option because of the inhibition of the native RIP, tritin, that inhibits expression [113].

The expression vector pET14b was chosen, mainly for its T7 promoter driven expression as well as its Histidine-tag [114]. The vector has the advantage that with the help of an in frame *Nde*I restriction site, a gene of interest can be cloned behind a 6x His-Tag that could be helpful during detection and purification of the expressed protein. One more advantage of using the His-tag is that no ATG start codon is needed as the start codon is already situated 8 bp from the ribosomal binding site. One drawback to pET14b is that it does not contain many restriction sites to clone into and cloning strategies need to be planned carefully to ensure success.

The mechanisms utilised by the pET expression systems are illustrated in Figure 3.1. The DE3 *E. coli* cell line that was used contains a *lac* promoter driven *T7RNA polymerase* as well as a repressor gene in the genome that allows for stricter expression (i.e. less basal expression) than *lac* promoter driven systems. The cell line BL21(DE3) was used. For even more stringent expression the BL21(DE3)pLysS cell line was used. BL21(DE3)pLysS cells contain an additional plasmid, pLysS that down regulates the T7 RNA polymerase that helps with the expression of toxic genes. It is thus clear from Figure 3.1. that induction with IPTG will lead to the production of T7 RNA polymerase that can then in turn transcribe the gene of interest on the introduced plasmid that contains a T7 promoter for translation. In our case it was pET14b [114].

The strategy was to utilize the His-tag for detection and purification purposes, therefore a cloning strategy was planned around it. *Dianthin 30* has an internal *Xho*I site and that made directional cloning into pET14b difficult. However, with the 5' overhang compatibility of *Xho*I and *Sal*I it was decided to design a 5' primer with the *Nde*I restriction site and a 3' primer with the *Sal*I restriction site. With *Nde*I and *Xho*I being so close to one another, this made the restriction enzyme digestions extremely difficult but its was nevertheless attempted and achieved.

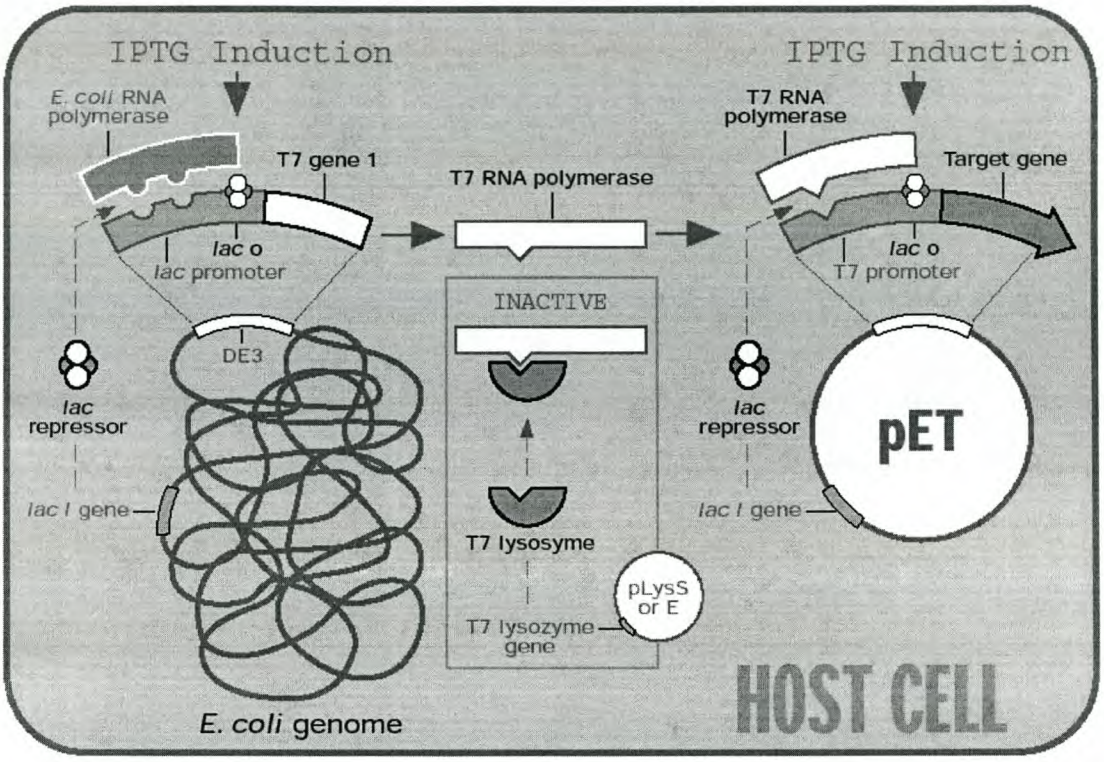


Figure 3.1. This diagram illustrates the working of the pET system. The diagram illustrates the mechanism in an *E.coli* strain containing the DE3 construct. The insert shows the mechanism of down regulation attained when using *E.coli* strains containing the pLysS plasmid [115].

To visualize the product of expression the His-tag was used in Western blots of SDS-PAGE gels. There are a number of products available to detect or purify 6xHistidine-containing proteins - they use either antibodies or nickel. The nickel based systems are more cost effective and are just as sensitive as antibody based products. For these expression experiments the KPL ExpressDetector™ Nickel-HRP Chemiluminescent Blotting Kit was used in the detection of His-tag containing proteins.

3.2. Experimental procedures

3.2.1. Cloning and sequencing of *Dianthin 30* in pGEM T Easy.

As starting material a Dianthin 30 pGEM T Easy clone was used [5]. To validate the clone it was first sequenced with the SP6 primer (ABI prism® 3100 genetic

analizer). For comparison the sequence for Dianthin 30 was obtained from GenBank under accession number X59260 [110].

Primers were designed to amplify the entire sequence of *Dianthin 30* as well as incorporating restriction enzyme sites on the 5' and 3' ends that would facilitate the cloning into pET14b. The primers were designated pET-For: 5'-GG GAG CAT ATG AAG ATT TAT TTA GTG G-3' with the recognition site for *Nde*I underlined, and pET-Rev: 5'-AA GTC GAC TCA AAG CAC ATC AGC-3' with the recognition site for *Sal*I underlined. The T_m for this primer set was calculated at 63°C using a Nearest Neighbor algorithm [116].

Using PCR [117], (16 mM NH_4SO_4, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 1.5 mM $MgCl_2$, 10 mM dNTP, 5 U DNA polymerase and 10 mM Primer) and the pET primers (T_A 58°C) *Dianthin 30* was amplified from the pGEM-T Easy Dianthin 30 clone [5]. Buffers and DNA polymerase used was from Bioline and dNTPs from Promega. The resulting PCR product was then re-cloned into pGEM T Easy. Ligations were performed as recommended by manufacturer [118]. The ligation product was then transformed into competent *E. coli* DH5α cells, which were rendered competent with the $CaCl_2$ method [119]. Transformations were done as described by Promega technical manual [118]. The new plasmid was named pGEM-Dian.

3.2.2. Cloning *Dianthin 30* into pET14b

pET14b, derived from the original vector developed by Studier and Moffatt in 1986, was obtained from Novagen [120, 121, 122]. pGEM-Dian was digested with *Nde*I and *Sal*I and pET14b with *Nde*I and *Xho*I restriction enzymes. With the *Nde*I and *Xho*I restriction sites so close to one another partial digestion of pET14b can be expected. The fragments resulting from the digest of pGEM-Dian were then separated on a 1.4% agarose-TAE gel (Figure 3.3) and the insert (*Dianthin30*) purified by centrifugation through synthetic glass wool (5', 12 000 g).

The *Nde*I and *Sal*I flanked *Dianthin 30* was ligated into the *Nde*I and *Xho*I digested pET14b. All resulting colonies were screened with PCR and RE digests. To

confirm the integrity of *Dianthin 30* in pET14b, it was sequenced with a T7 promoter primer and a T7 terminator primer. The T7 terminator primer was designed from the sequence of pET14b (5'-GCTAGTTATTGCTCAGCGG-3') [115]. The new *Dianthin 30* containing plasmid was referred to as pET-Dian.

3.2.3. Expression in *E. coli.*

pET-Dian was then transformed onto BL21(DE3) and BL21(DE3)pLysS competent cells. These cells were rendered competent using the RbCl method [123].

One milliliter saturated overnight BL21(DE3) and BL21(DE3)pLysS, pET-Dian cells were inoculated into 50 mL LB media (100μg/mL ampicillin). The following expression studies were performed:

1) Culture was induced at time 0 with 0.4 mM IPTG and samples taken at 0, 2, 4 hour after induction (Figure 3.6.).
2) The same procedure as in 1 but with 0.8 mM IPTG.
3) The culture was allowed to grow to an OD_{600} of 0.8 before induction with 0.4 mM IPTG. To establish if the protocol is indeed capable of giving positive results a positive control was included. The positive control was the Hsp70 gene from Grapevine Leafroll associated virus 3 in pET14b obtained from M-J Freeborough from the Institute for Wine Biotechnology (Figure 3.7.).
4) Identical to 3 but add 100 μg/mL rifampicin 30' after induction – only for BL21(DE3)pLysS cells.

A growth curve comparison was drawn between BL21(DE3) pET14b and BL21(DE3) pET-Dian (see Graph 3.1.).

3.2.4 SDS-PAGE and Western blots

Crude protein extracts were prepared and denatured at 95°C for 2min in 2x loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% Glycerol, 10% β-mercaptoethanol, 0.25% Cresol red). Denatured protein was then separated on a discontinuous PAG (6% stacking and 12% resolving) in electrode buffer (250 mM

Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) and Coomassie staining protocol as described by Promega [124].

Soluble and insoluble protein was purified from other cell debris as follows:
Cells were harvested and resuspended in TNE buffer (20 mM TRIS; 50 mM NaCl; 1 mM EDTA, pH 8, 0.5 mM DTT) at a ratio of 1 g : 7 mL. When cells were fully resuspended, 10 mg/mL lysozyme and 50 mM PMSF were added and incubated for 30 min on ice and vortexed every 10 min. Three freeze/thaw cycles for 30 min at –80°C and 37°C were performed. The sample was then frozen overnight at –80°C. Samples were sonicated 90" (50% duty micro tip 5) on ice in 1.5 mL volumes in 2mL microcentrifuge tubes and centrifuged for 10 minutes maximum rpms. The supernatant containing the soluble proteins was decanted from the pellet. The pellet was washed in TNE buffer and 2% Na-DOC, and incubated for 10 minutes and centrifuged. The supernatant containing soluble protein was kept. The pellets were resuspended in TNE buffer with 0.5 mM DTT and 0,4% Sarcosyl, and vortexed 30 min. Sample was spun down maximum rpms for 10min, supernatant contained insoluble proteins.

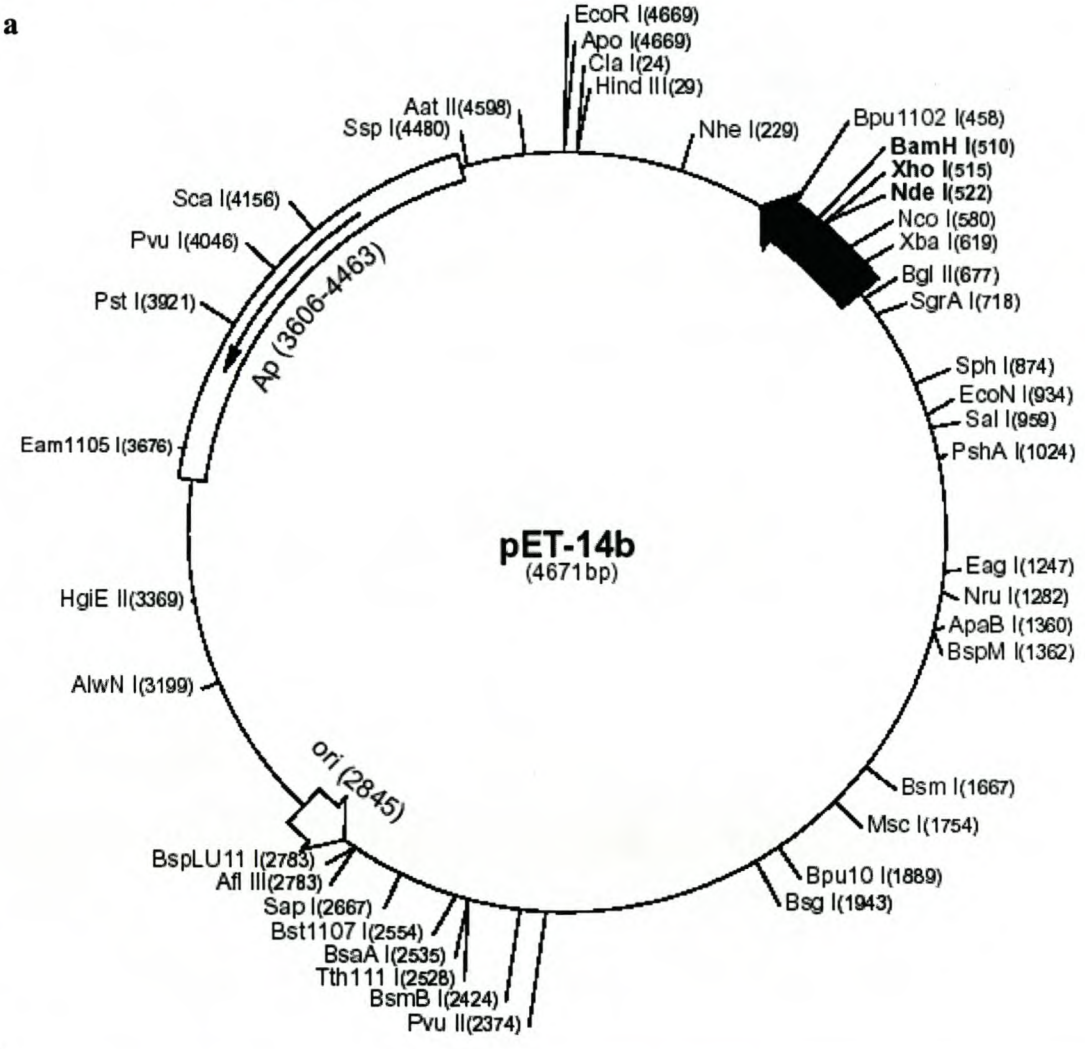
For Western blots, SDS-PAGE gels were electroblotted to PVDF-membrane (1 amp, 6 V, 30'). The KPL ExpressDetector™ Nickel-HRP Chemiluminescent Blotting Kit was used to detect His-tag proteins on the membrane. No alterations were made to the protocols and the carbonic anhydrase molecular weight marker (30 kDa) was used as the positive control [125].

3.3. Results and discussion

3.3.1. Cloning and sequencing of *Dianthin 30* in pGEM T Easy.

To validate the clone from K.L Wilsen it was first sequenced with the SP6 and T7 primers. The results indicated a four base pair mutation affecting the stop codon of *Dianthin 30.* From these results, it was clear that the 3' primer was mutated and new primers were then designed to aid cloning into pET14b.

The primers that were designed were called pET-For and pET-Rev and they incorporated *Nde*I and *Sal*I restriction sites respectively. With these restriction sites *Dianthin 30* could be directionally cloned into pET14b taking into account that the *Sal*I and *Xho*I restriction sites have compatible overhangs. See Figure 3.2 for restriction map of pET14b.



b

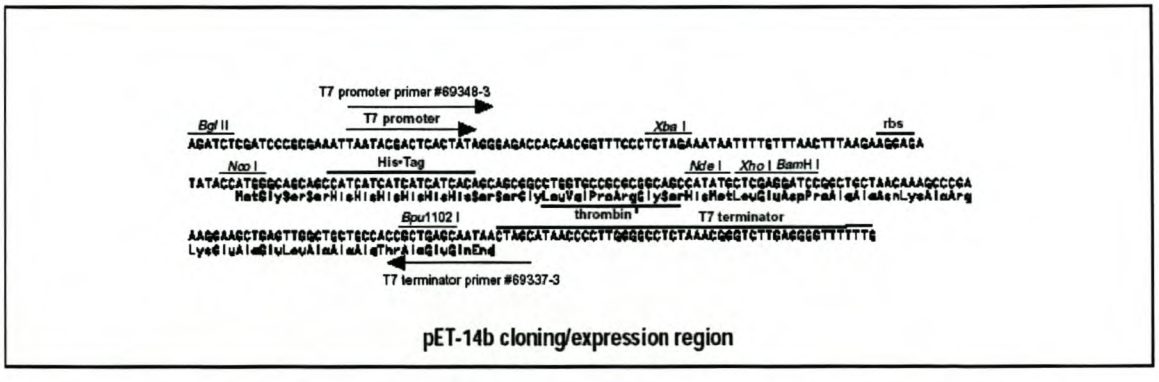


Figure 3.2. a) Restriction map of pET14b. b) Sequence and RE site details of expression region indicated in a) with a black arrow [115].

Using PCR and the pET primers, *Dianthin 30* was amplified and re-cloned into pGEM T Easy. This was done to ensure that the restriction enzyme digests of the flanking regions of *Dianthin 30* could be monitored. pGEM-Dian, the resulting plasmid was confirmed with restriction digests (see Figure 3.3.).

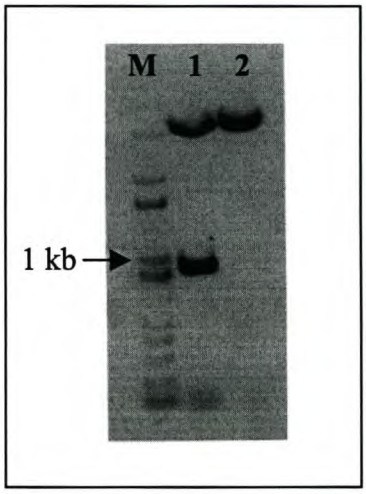


Figure 3.3. pGEM-Dian digested with *Nde*I and *Sal*I and visualised on an ethidium bromide stained, 1.4% agarose-TAE gel. Lane 1 1 kb+ molecular marker (GibcoBRL), Lane 2 Digest product.

3.3.2. Cloning *Dianthin 30* into pET14b

Two important advantages of the pET14b expression vector are the T7 promoter driven expression and the His-tag. The start codon is preceded by sequence coding for a 6xHis-tag and thrombin site with the ribosome-binding site (RBS) 8 bp upstream. The 5' overhangs of *Xho*I and *Sal*I are compatible and because *Dianthin 30* has an internal *Xho*I site this strategy was chosen as this was the only way of directionally cloning *Dianthin 30* into pET14b.

With the vector quantity kept constant a positive correlation was found with the amount of insert and number of colonies formed. After numerous isolations only one colony was found that tested positive with PCR and RE digests (see Figure 3.4.). This was due to the fact that the two restriction sites used were too close to each other which was to be expected (see Figure 3.5.).

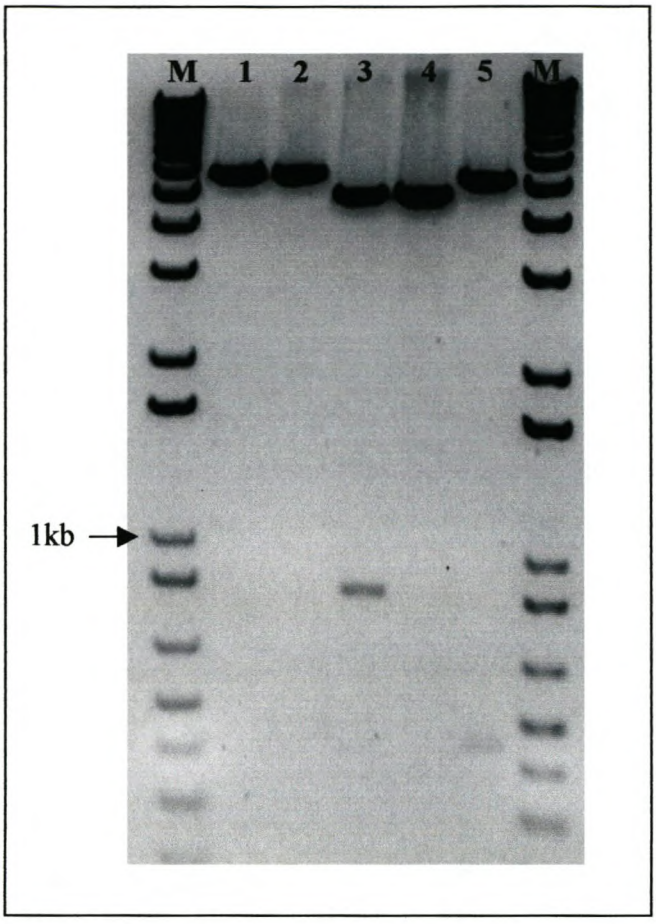


Figure 3.4. Figure shows RE digests of pET-Dian. In lane 1 and 7 1 kb+ marker, Lane 2 pET-Dian digested with *Nde1*, Lane 3 pET-Dian digested with *Sal*I, Lane 4 pET-Dian digested with *Bam*HI, Lane 5 pET14b digested with *Bam*HI, Lane 6 pET-Dian *Nde*I and *Sal*I.

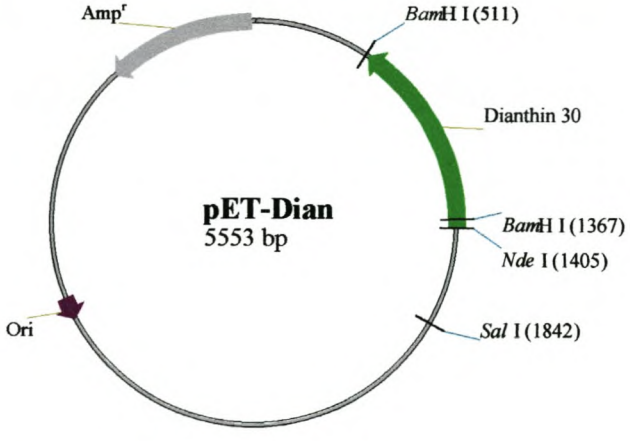


Figure 3.5. Restriction map of pET-Dian.

Restriction enzyme digests yielded fragments of the expected sizes and indicated that the insert might be *Dianthin 30*. The sequencing results of *Dianthin 30* in the pET14b vector with the T7 promoter and the T7 terminator primers confirmed the results of the RE digests. The T7 terminator primer was designed on the sequence of the pET14b, vector to have a T_A of 55°C. The sequencing results showed no mutations in the sequence of *Dianthin 30*. With the sequence of *Dianthin 30* confirmed in pET14b the new plasmid pET-Dian could be used for expression in various *E. coli* strains.

3.3.3. Expression in *E.coli*, SDS-PAGE and Western blots

The confirmed vector pET-Dian was transformed into BL21(DE3) cells made competent with the RbCl method. As explained earlier, only cell lines containing the DE3 construct can be used for expression of vectors driven by a T7 promoter. For these expression experiments, a protein at 32 kDa is expected and not 30 kDa as *Dianthin 30* is now preceded by the His-tag and the thrombin site in the expression vector.

For the first expression experiment, 1 mL overnight culture was inoculated in fresh media containing selection antibiotics and 0.4 mM IPTG. Samples were taken at regular intervals (0, 2, 4 hours) and processed for SDS-PAGE separation and Coomassie staining (see Figure 3.6.).

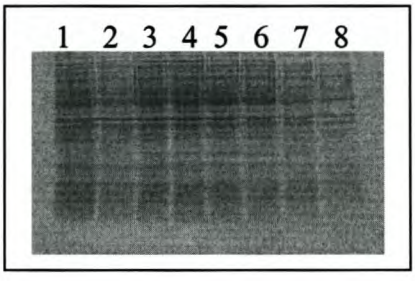
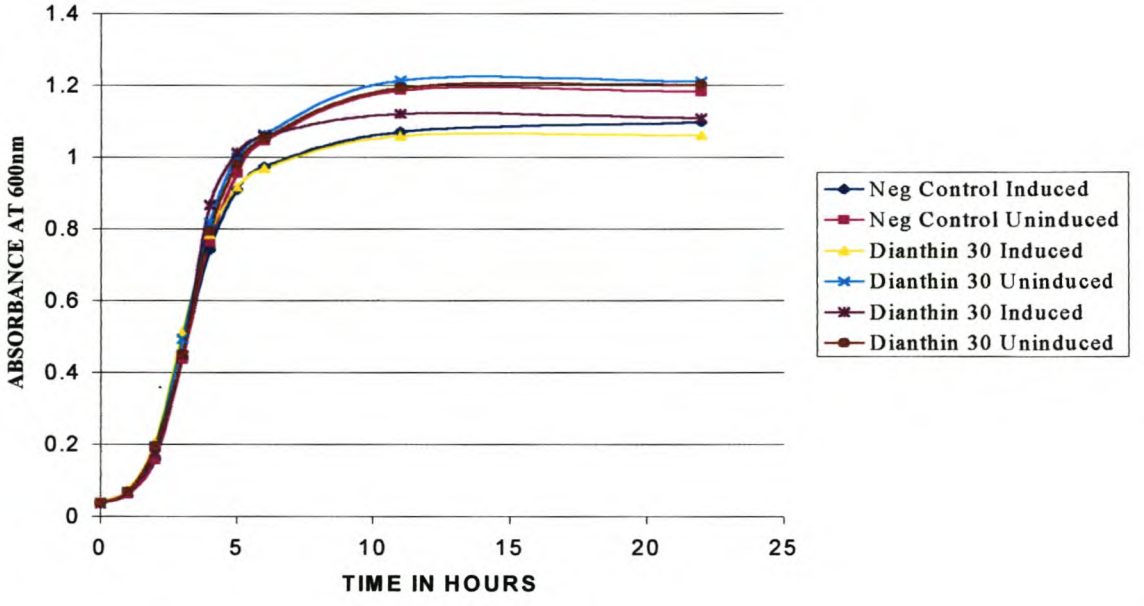


Figure 3.6. Coomassie stained SDS-PAGE of crude protein extracts of *E. coli* (BL21(DE3)) containing pET14b and pET14b-Dian. Lanes 1, 3, 5 and 7 are pET14b-Dian, uninduced, 4 h, 2 h, and 0 h. Lanes 2, 4, 6 and 8 are pET14b uninduced, 4 h, 2 h, and 0 h.

Cells from the expression studies were boiled in loading buffer to denature proteins and separated in a discontinuous PAG. The proteins in the PAG were then stained with Coomassie brilliant blue for visualization. From the first expression experiment (see Figure 3.6.) it was clear that no expression of induced protein is visible in the protein band patterns. It might be that the expression was too weak to be detected by the insensitive Coomassie staining technique or that the expression was unsuccessful.

A growth curve comparing the growth of BL21(DE3) cells containing the two different plasmids pET14b and pET-Dian was drawn (see Graph 3.1). From this graph it is clear that there is a slight difference in final OD_{600} reading between induced and uninduced samples, with the induced samples not reaching the same optical density.

GRAPH 3.1 Absorbance at 600 nm of Dianthin 30 clones



To test the expression efficiency, the experiments were repeated with a higher concentration of IPTG (0.8 mM), but from the Coomasie stained SDS-PAGs no induction was detectable.

The protocol for expression was then changed as the culture was allowed to grow to an OD_{600} of 0.8 before it was induced with 0.4 mM IPTG. To confirm that the

protocol was able to produce results a positive control was added. The positive control (Hsp70 gene from Grapevine Leafroll associated Virus 3) from M-J Freeborough yielded a protein at 59 kDa, which is at the expected size. Again no detectable induction was observed except for the positive control (see Figure 3.7.). Successful expression from the positive control suggested that the levels of Dianthin 30 expression might be too low to be detected with the Coomassie staining technique or that the abundance of other proteins in the crude cell extracts masked the visualisation of any expression product. The possibility that the expression product is toxic to the bacterial cells was also considered and expression experiments were redone with BL21(DE3)pLysS cells that has the ability to deal with toxic gene products better as these cells have more control over the induction of expression. The results however were the same.

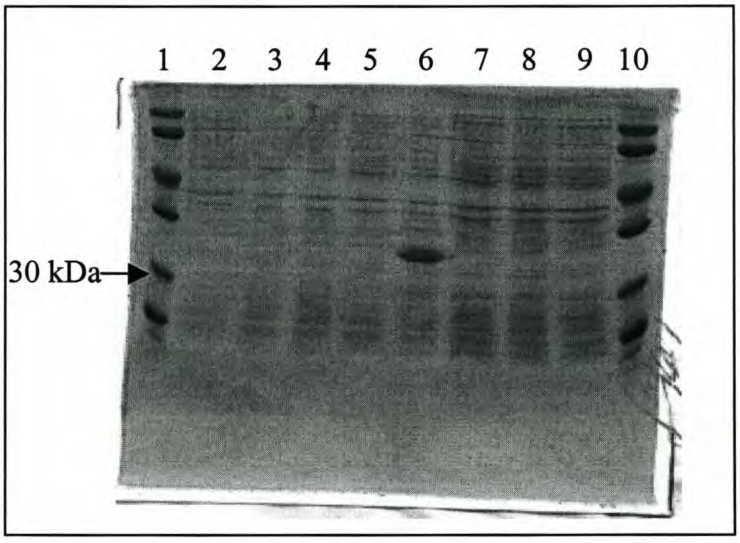


Figure 3.7. Coomassie stained SDS-PAGE crude protein extracts of *E. coli* (BL21(DE3)) containing pET14b (Negative control), pET14b-Hsp70 (Positive control) and two samples of pET14b-Dian. All before and after induction. Lanes 1 and 10 is the Molecular weight marker from Amersham, Lanes 2 and 6 pET14b-Hsp70 (Pos) uninduced and induced. Lanes 3 and 3 pET14b (Neg) uninduced and induced. Lanes 4 and 5 pET14b-Dian samples uninduced and Lanes 8 and 9 pET14b-Dian samples induced.

Another induction strategy was used where rifampicin was added to the culture, 30 minutes after induction. Rifampicin inhibits transcription and thus ensures that all

cell function focuses on expression of existing mRNA in the bacterial cell and no additional mRNA is produced [126].

Soluble and insoluble proteins was purified from the harvested cells in the expression studies and separated with SDS-PAGE. The results again did not indicate a clear induction (see Figure 3.8.).

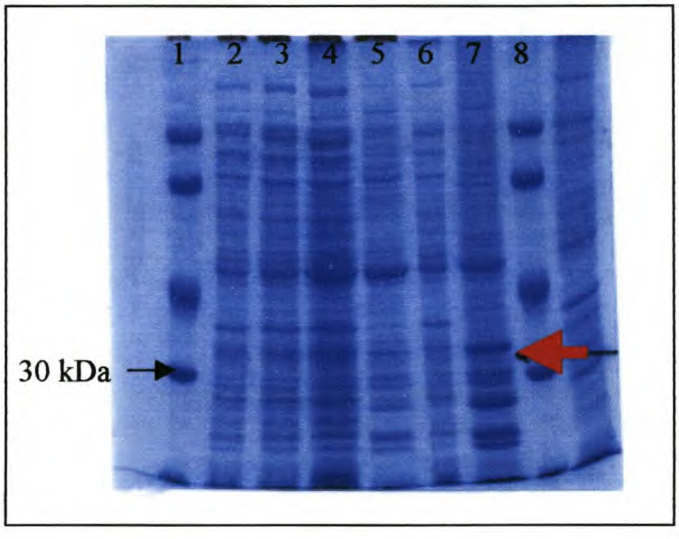


Figure 3.8. Coomasie stained SDS-PAGE gel; red arrow indicates position where Dianthin 30 is expected. Lanes 1 and 8 Low Molecular weight marker, lane 2 pET14b uninduced, lane3 pET14d-Dian uninduced, lane4 pET14b induced soluble fraction, lane 5 pET14b induced insoluble fraction, lane 6 pET14d-Dian induced soluble fraction, lane 7 pET14d-Dian induced insoluble fraction.

With the advantages of the His-tag, it was possible to do Western blots to detect smaller quantities of the expressed protein. The KPL ExpressDetector™ Nickel-HRP Chemiluminescent Blotting Kit was used to detect the His-tag on the N-terminal of rDianthin. Carbonic anhydrase (30 kDa) was used as molecular weight marker and positive control for detection with nickel HRP. The nickel binds to the zinc binding motifs of carbonic anhydrase that contains high concentrations of histidines. Very weak expression of Dianthin 30 was detected by the HRP (see Figure 3.9.). From the figure, a faint band can be seen at the expected size of 32 kDa (30 kDa and the addition of the His-tag with the thrombin site), as well as

the marker at of 30 kDa. The faint band seen here was reproducible but attempts to increase the concentration failed.

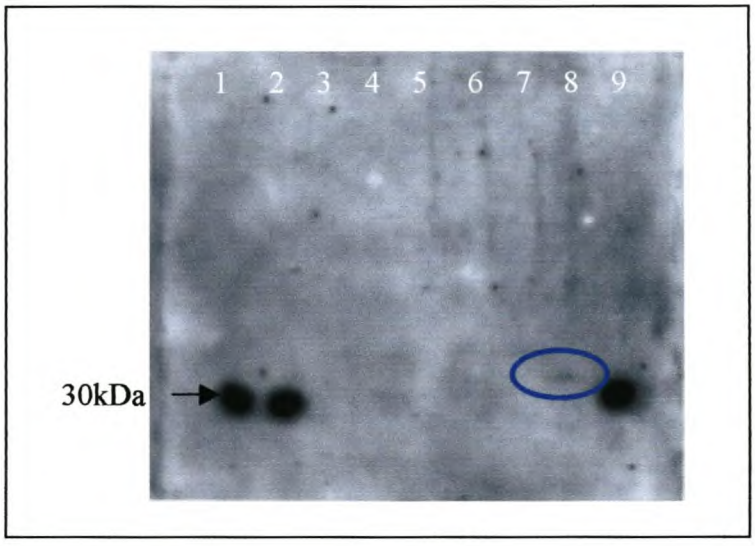


Figure 3.9. Chemiluminescent Western blot using Nickel-HRP to detect His-tag. The blue ellipse shows faint band that consistently detected. Lanes 1, 2 and 9 Low Molecular weight marker, lane 3 pET14b uninduced, lane 4 pET14d-Dian uninduced, lane 5 pET14b induced soluble fraction, lane 6 pET14b induced insoluble fraction, lane 7 pET14d-Dian induced soluble fraction, lane 8 pET14d-Dian induced insoluble fraction.

With all the permutations in the expression parameters and cell lines the best results were obtained with the BL21(DE3)pLysS cell line in the induction protocol were rifampicin was added 30 minutes after induction. However, the results obtained was far from being adequate to be used as antigen for antibody production - this was in line with the weak expression observed by Legname *et al.* [107]. The most logical conclusion from these results would be that the bacterial ribosomes are extremely sensitive to Dianthin 30 as also found by other researchers [38, 54, 59].

The low level of expression of Dianthin 30 in a bacterial expression system leads us to conclude that expression in prokaryotes will not lead to Dianthin 30 expression levels that could be used for antibody production without extensive

purification. However, the possibility does exist that an eukaryotic expression system might be better equipped to deal with this toxicity, and it needed to be investigated. In chapter 4, the expression of Dianthin in insect tissue culture is described.

Chapter 4: Expression of Dianthin 30 in insect tissue culture

4.1 Introduction

Previous attempts at expressing Dianthin 30 in a bacterial expression system were described in Chapter 3. Very low levels of expression were observed that were inadequate to be used as antigen for the production of antibodies. From these results, it seemed that the prokaryotic system was unable to deal with the toxicity of the gene product and alternative methods of expression were investigated.

A eukaryotic system was considered that would be better equipped to deal with the toxicity of Dianthin 30 in a foreign cell environment. There were three considerations for these expression studies: plant, mammalian and insect tissue culture.

The expression of foreign proteins in plant tissue culture has only recently come to the forefront with the advances in biotechnology and plant tissue culture techniques. It is an option to consider for future applications [127]. A major drawback of this system would be the difficulty to purify the recombinant protein to homogeneity, i.e. to eliminate contaminating plant proteins for antibody production.

Mammalian tissue culture was not considered since it requires considerable technical skill and equipment that we did not have to our disposal.

The expression of Dianthin 30 in a eukaryotic system was attempted in insect tissue culture for a number of reasons. Although RIPs have never been expressed in insect tissue culture the ability of insect cells to deal with toxic genes generally is better than that of bacteria. With the use of a virus vector these expression experiments are less time consuming and simpler to perform than mammalian tissue culture. It also has the added advantage that it has less chance of contributing contaminating proteins than a plant tissue culture expression system.

For the expression in insect tissue culture, the BAC-TO-BAC™, baculovirus expression system developed by Monsanto (see Figure 4.1.), now distributed by Invitrogen was chosen [128]. This rapid and efficient system makes use of the site-specific transposition of an expression cassette into the baculovirus shuttle vector (bacmid) propagated in DH10BAC

E. coli cells. The bacmid propagates as a large plasmid that contains all the essential elements for the virus to be functional as well as a kanamycin resistance gene. The plasmid also contains the *lacZα* gene with an attachment site for the Tn7 bacterial transposon in the N-terminal region. This can thus indicate if the transposition event did indeed occur. The bacterial plasmid pFASTBAC1 contains the transposition elements and the expression cassette and is used to clone the gene of interest.

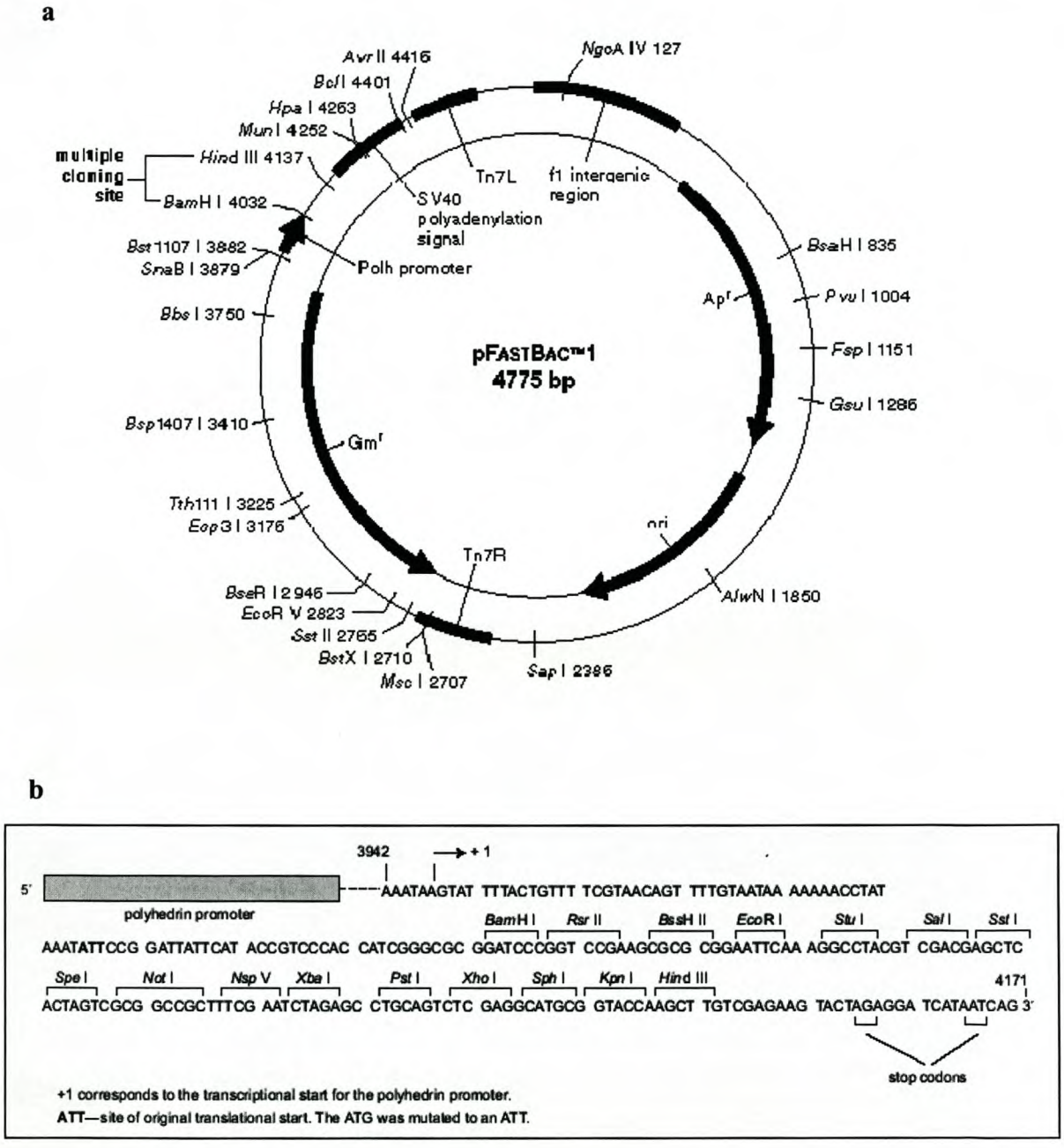


Figure 4.1. a) Restriction map of pFASTBAC1. b) RE and primer binding details of multiple cloning site [129].

The expression cassette between the left and right arms of Tn7 consists of a Gm^r gene, the polyhedrin promoter from *Autographa californica* nuclear polyhedrosis virus (AcNPV), a multiple cloning site and a SV40 poly(A) region. Recombinant bacmid DNA can be used to transfect Sf21 insect cells from the fall armyworm, *Spodoptera frugiperda* to produce recombinant baculoviruses. Once the cells are infected they produce mature recombinant virus particles that infect neighboring cells and the gene of interest is thus expressed during this process. The gene of interest is under the control of the polyhedrin promoter (AcNPV) that is abundantly expressed in the late stages of infection [129].

4.2. Experimental procedures

4.2.1. Cloning of *Dianthin 30* into pFastBac1

As starting material the pET-Dian clone was used. With the T7 promoter primers and pET Rev primers, *Dianthin 30* with the His-tag was sub cloned into pGEM-T Easy. *Dianthin 30* with the His-tag was then liberated from pGEM-T Easy Dianthin by digesting with *Not*I and cloned into pFASTBAC1 (see Figure 4.3.). To confirm the insert and orientation, pFASTBAC1-Dian was digested with *Xba*I and *BamH*I (see Figure 4.4.). pFASTBAC1-Dian was sequenced with a *Dianthin 30* internal reverse primer (391Rev)(5'-CTATCGCCTGGTAATCTTCC-3').

4.2.2. Expression in insect tissue culture

The pFASTBAC1-Dian plasmid was used for the transformation of competent DH10BAC *E. coli* cells as described by the manufacturer [129]. White colonies contain the expression cassette as the transposition disrupted the *lacZ* gene. Recombinant bacmid DNA was isolated as recommended and used for the transfection of Sf21 insect cells. The transfection of the bacmid DNA was aided with the addition of CELLFECTIN, a lypolysing agent that aids the movement of the bacmid DNA over the cell membrane. Recombinant virus particles were isolated and used to infect Sf21 insect cells. The growth medium used for the cells was TC100 with 10% FCS. Infected insect cells were harvested at 4 days [129]. For a summarised protocol see Figure 4.2.

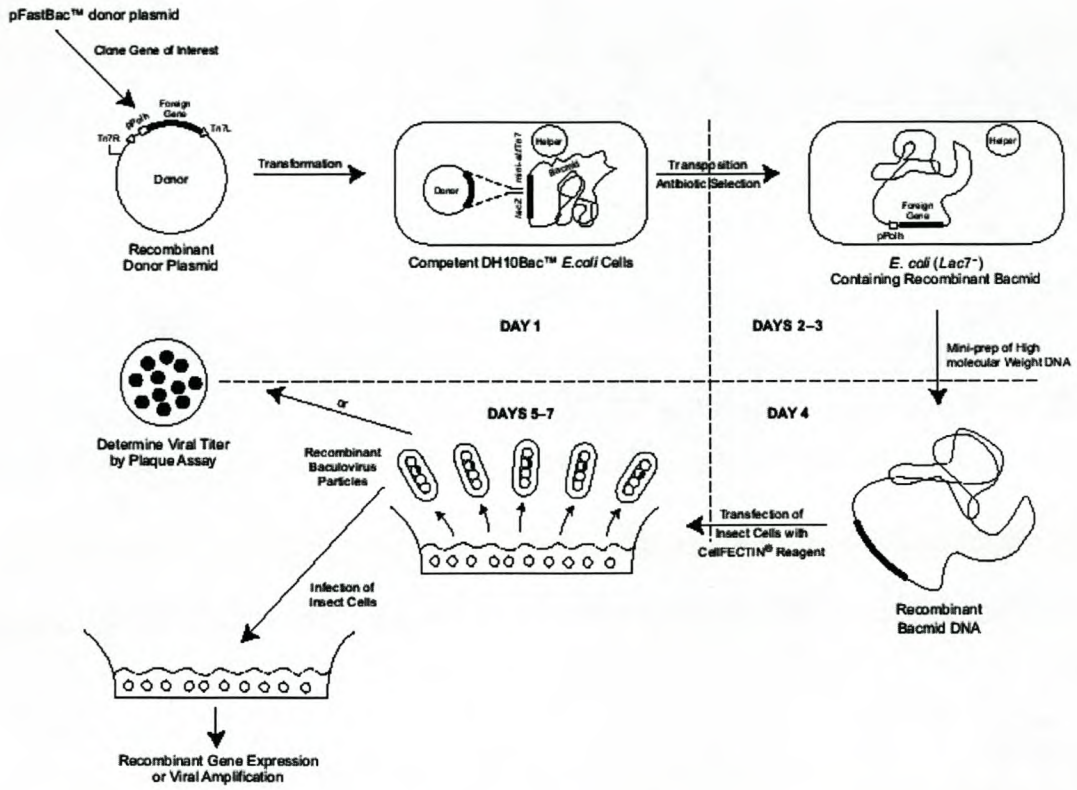


Figure 4.2. Summary of protocol for the generation of recombinant baculoviruses and gene expression with the BAC-TO-BAC ® Baculovirus expression system [129]

4.2.3. SDS-PAGE and Western blot analyses

From the harvested insect cells, crude protein extracts were prepared and denatured at 95°C for 2min in 2x loading buffer (0.125 M Tris-HCl, pH 6.8, 6 M Urea, 1 mM DTT, 0,25% Bromophenol Blue). Denatured proteins were then separated on a discontinuous SDS-PAGE (6% stacking 12% resolving) in electrode buffer (250 mM Tris, 192 mM Glycine, 0.1% SDS pH 8.3).

Western blots were performed with the ECL Plus Western blotting detection system from Amersham LIFE SCIENCE and Anti-His_6 Peroxidase (mouse monoclonal antibody) from Roche. All procedures were performed as recommended by Amersham [130].

4.3. Results and discussion

4.3.1. Cloning of *Dianthin 30* into pFastBac1

For the expression of Dianthin 30 in insect cells, it was important to have a tag to trace the protein as it is being expressed. The His-tag from the pET-Dian plasmid was utilized by amplifying the entire sequence of *Dianthin 30* with the pET-Rev primer on the 3' end, and the T7 promoter primer on the 5'end. For restriction map see Chapter 3 Figure 3.5. The amplified sequence was then sub cloned in to pGEM-T Easy to acquire flanking *Not*I sites that were then used for cloning into pFASTBAC1. See Figure 4.3. for restriction map of pFASTBAC1-Dian. The insert and orientation was confirmed with RE digests (See Figure 4.4.). pFASTBAC1-Dian was sequenced with an 20 bp internal primer (391 Rev) that bound at bases 531 to 550 of *Dianthin 30.* From the sequencing results it was clear that *Dianthin 30* was intact and in the right orientation in pFASTBAC1.

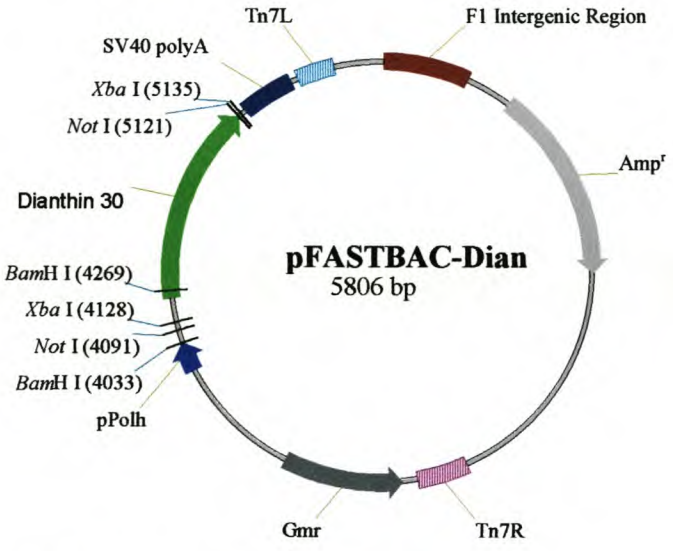


Figure 4.3. Restriction map of pFASTBAC1-Dian.

4.3.2. Expression in insect tissue culture

pFASTBAC1-Dian was transformed into competent DH10BAC *E. coli* cells for the transposition to take place. White colonies, indicating that transposition took place since it disrupted the *lacZ* gene, were detected after 48 hours. Bacmid DNA was isolated from these colonies and used with the lypolising agent, CELLFECTIN to transfect Sf21 cells. After 4 days recombinant virus stock was collected from the

transfection experiments and used to infect Sf21 cells for the expression. After 4 days, the cells were infected as judged visually and harvested.

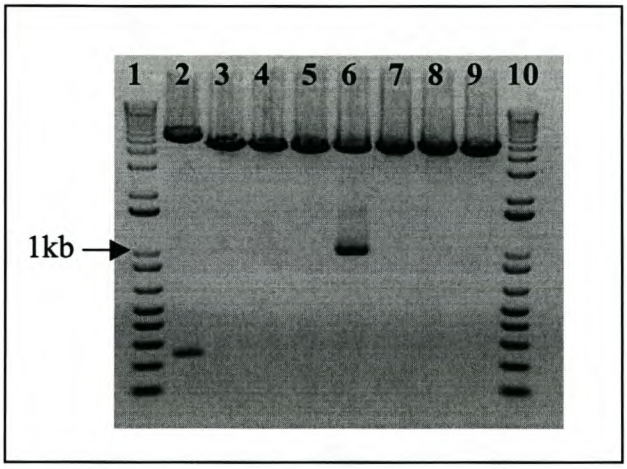


Figure 4.4. RE-Digest of four putative pFASTBAC1-Dian plasmids. Lanes 1 and 10 1 kb+ molecular marker, lanes 2, 3, 4 and 5 are *Bam*HI digests of samples 1-4 and lanes 6, 7, 8 and 9 the *Xba*I digests. From this digest it is clear that only sample 1 (lanes 2 and 6) contains the insert and that it is in the right orientation (See Figure 4.3. for RE map).

4.3.3. SDS-PAGE and Western blot analyses

An anti-His antibody detected very low levels of expression in the insect cells tissue culture. In Figure 4.5., it is clear that the antibody detected a protein at the expected size but that the concentration of this protein is very low. Again the toxicity of the protein might be blamed, but with no reference study except for the positive controls of unrelated proteins, it was difficult to say exactly why such low concentrations were expressed.

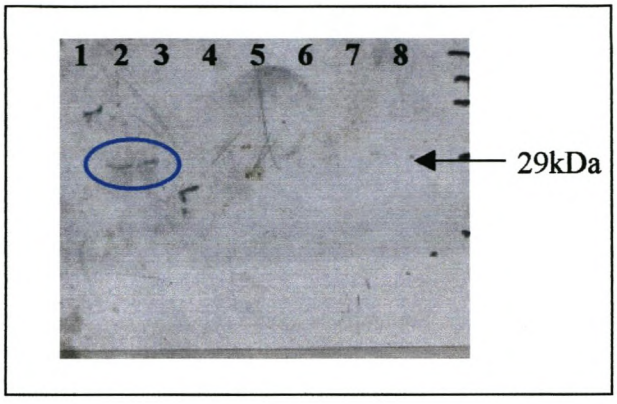


Figure 4.5. Chemiluminescent Western blot using Anti-His Peroxidase to detect His-tag. The blue ellipse shows faint bands that were detected. Lanes 1 and 8 Rainbow Marker (Amersham), Lanes 2 and 3 supernatant and cell protein respectively. Lanes 4 to 9 supernatant and cells of two other proteins that do not contain His-tags (Negative controls).

Chapter 5: Peptide design and antibody analysis

5.1. Introduction

The low concentration recombinant protein obtained from the bacterial and insect tissue culture expression experiments was insufficient to be used as antigen for the production of antibodies. As an alternative to expressing the whole protein and using it as the antigen, a peptide may be synthesized that would mimic an epitope on the native protein. With the advent of the computer age came the opportunity to predict the properties of proteins based on their amino acid sequence and this is particularly useful in the prediction of the possible antigenic sites on the mature protein of interest.

There are various algorithms available on the Internet and in Software packages that help with these predictions. The first step in predicting which sequence areas are the most antigenic, would be to determine which parts of the protein are on the outside and subsequently available for interaction with the antibody. Using an algorithm that predicts the hydrophilic areas in the protein, these areas can be identified. This would not be critical for our purpose, as we will analyse only denatured protein. Other algorithms predicting the flexibility and antigenicity can also be useful to predict peptide sequences that would be antigenic [131, 132].

The longer the synthesised peptide the greater the conformational similarity is to the native protein. This increases the chance that antibodies raised against the peptide as antigen would result in antibodies that would recognise the natural protein. The smallest antigenic peptide is between 5 and 8 amino acids - thus a peptide of 15-20 amino acids would at least contain 1 epitope and would adopt a limited amount of conformation [133].

The peptide selected must be soluble in an aqueous buffer for conjugation and use in biological buffers. The selection of a sequence in the hydrophilic regions should produce peptides that are soluble. Hydrophobic residues like W, V, L, I, and F, must still be avoided and the selected peptide must contain as little as possible of these amino acids. Multiple glutamine residues must also be avoided as they could form hydrogen bonds between peptide chains and thus cause insolubility of the peptide [133].

The inclusion of proline (P) and tyrosine (Y) in the peptide sequence would enhance the immunogenic properties of the peptide. Proline can adopt a cis-amide bond structure that gives the peptide a structure that could represent the natural conformation of the epitope more accurately. Tyrosine is a large amino acid that with its ring structure can also induce structural motifs that would contribute to the success of the peptide to simulate the epitope of the natural protein. Transmembrane and glycosylation sites should be avoided, as these sites may be modified or unavailable in the natural protein [133]. The sequence chosen must also not have sequence similarities to other proteins as this will lead to cross reaction of the antibodies to these proteins.

A peptide (10-20 a a) by itself is too small to raise an immune response and needs to be coupled to a carrier protein such as BSA or KLH for the immune system of the rabbit to respond to the antigen [131].

The serum collected from the rabbits at different intervals after inoculation with the antigen can be used in Western blots to detect the protein of interest.

5.2. Experimental procedures

5.2.1. Selection of a suitable peptide sequence in Dianthin 30 and antibody production.

The amino acid sequence of Dianthin 30 was obtained from GenBank (Accession number X59260). The sequence was submitted to three independent peptide synthesis companies for predictions on which areas would be most suitable for peptide synthesis for the purpose of raising antibodies. Their proposals for the most likely antigenic peptides are set out in Figure 5.1.

From these results it appears that there are three possible areas from which peptides can be synthesized as potentially useful antigen. Although the algorithms are useful they still are just predictions and do not take into account any post translation modification. With the use of the web based algorithms TargetP V1.0 Prediction Results and PSORT, a signal sequence was predicted on the N-terminal of Dianthin 30 [133-135]. For the purposes of a potential antigen, the a a sequence

of residues, 174-189, which was also the first prediction by Bio-Synthesis and Princeton Biomolecules was chosen.

Bio-Synthesis Incorporated was contracted to synthesise the Dianthin 30 specific peptide and to conjugate it to a carrier protein for the immunisation of 2 rabbits. The antibody production of the two animals was then verified with ELISA testing by Bio-Synthesis. The purity of the peptide was determined by Laser Desorption Mass Spectrometry.

Dianthin 30 protein sequence

MKIYLVAAIA WILFQSSSWT TDAATAYTLN LANPSASQYS SFLDQIRNNV 50
RDTSLIYGGT DVEVIGAPST TDKFLRLNFQ GPRGTVSLGL RRENLYVVAY 100
LAMDNANVNR AYYFKNQITS AELTALFPEV VVANQKQLEY GEDYQAIEKN 150
AKITTGDQSR KELGLGINLL ITMIDGVNKK VRVVKDEARF LLIAIQMTAE 200
AARFRYIQNL VTKNFPNKFD SENKVIQFQV SWSKISTAIF GDCKNGVFNK 250
DYDFGFGKVR QAKDLQMGLL KYLGRPKSSS IEANSTDDTA DVL 293

α-Diagnostics Princeton Biomolecules Bio-Synthesis

Figure 5.1. Protein sequence of Dianthin 30 showing the different peptide sequence predictions of three companies. The sequence used in highlighted in red.

5.2.2. Western blot analyses

Antibody binding in the serum received from Bio-Synthesis was verified using the peptide as the positive control in a suitable detection system (ELISA). The next step was to determine if the antibodies would recognise native Dianthin 30. *Dianthus caryophyllus* plants from different cultivars were used as positive controls and tobacco and *Dianthus barbatus* (a related species) as negative controls in the Western blots that were used to optimise the conditions for the blots.

Crude protein extracts of the plants were made by grinding the material up in an extraction buffer (0.5 M Tris-HCl, pH 7.4, 0.5% β-Mercapto-ethanol and 1% Triton X100) and centrifuged at 16 000 g for 10 minutes. The supernatant was then

loaded onto a discontinuous (6% - 12% comp) SDS-PAG for electrophoresis with 6x Loading buffer (0.750 M Tris-HCl, pH 6.8, 3% SDS, 40 mM DTT, 30% Glycerol, 0.25% Cresol Red).

The separated proteins were then transferred to a PVDF membrane as suggested by the manufacturer, MSI [136]. The procedure that was followed for the western blots was similar to the one described in Chapter 4 of this thesis [130]. The only difference was that the KPL substrate LumiGlo was used as the substrate for HRP.

To optimise the western blots, a dilution series of the primary antibody was used to detect Dianthin 30 that ranged from 1:500 to $1:10^6$. The sera of the two rabbits were also compared.

5.3. Results and discussion

5.3.1. Selection of a suitable peptide sequence in Dianthin 30 and antibody production.

The peptide predictions from the different companies highlighted three areas that would possibly be antigenic. Using the TargetP V1.0 Prediction Results and PSORT web based algorithms, a signal peptide was predicted in the first 23 N-terminal amino acids of Dianthin 30, which correlates with the findings by Legname *et al.* [107, 133-136]. Dianthin 30 also contains a putative vacuole target sequence on the 16 C-terminal amino acids [107]. The existence of these signal peptide sequences and the possibility that they might be modified in the mature protein makes these areas undesirable as antigenic sites. The sequence chosen is clear of any signal sequences and only has sequence similarity to Saporin another type-1 RIP that is only found in *Saponaria officinalis* plants.

The peptide that was synthesised (NH_2-GCDGVNKKVRVVKDEAR-OH) also contained the least amount of hydrophobic amino acids.

The HPLC results received from Bio-Synthesis, showed a clear peak at 14.83 min at the following conditions: Column C18, 120Å, 5 µ, Column size 4.6 X 150 mm, Solvent A H_20/0.05% TFA, Solvent B Acetonitrile/0.05% TFA, Flow rate 0.5 mL/Min and %Gradient 10-60% B.

The Laser Desorption Mass Spectrometry results showed that the purified peptide was more than 70% pure and has a Mass Spectrum of 1875.6 and a Molecular Weight of 1872.18 (Bio-Synthesis).

The ELISA results indicated that both rabbits had a titer of 1:25600 towards the peptide. Bio-Synthesis described these results as excellent.

5.3.2. Western blot analyses

At a dilution of 1:500 of the primary antibody an array of bands was detected with a main band at 30 kDa, which is the expected size of Dianthin 30 in crude protein extracts of different cultivars of *Dianthus caryophyllus*. The pre-bleed also gave bands at various sizes including at 30 kDa, but this could all be attributed to protein-protein interactions between the proteins on the membrane and the antibodies (See Figure 5.2.). The main reason for this interaction probably is the relative high concentration of the primary antibody used.

A dilution had to be determined where the pre-bleed detected no bands. The dilution series was taken to the extreme for the pre-bleed and the 8-week bleed, both was diluted from 1:5000 to $1:10^6$. With the increase in dilution all the other background bands disappeared. Both sera still detected a band at 30 kDa (See Figure 5.3.).

Both rabbit samples were tested at a dilution of $1:10^5$ of the primary antibody. Both samples detected a protein at 30 kDa in the pre-bleed as well as in the 8-week bleed (See Figure 5.4.).

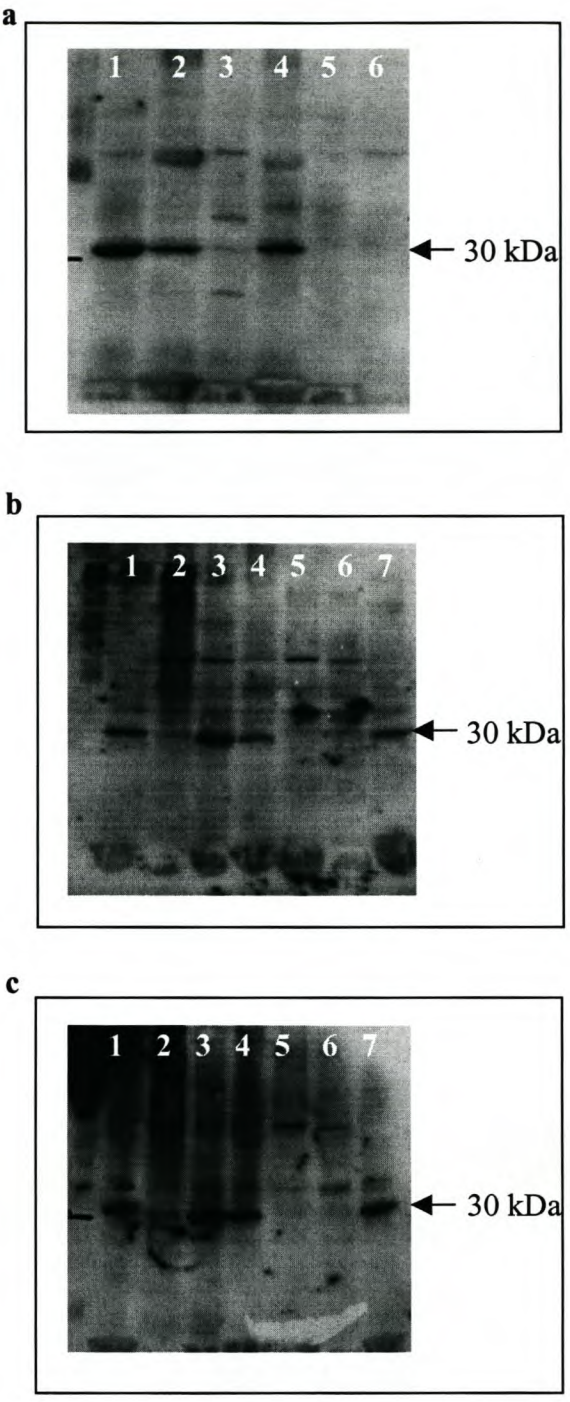


Figure 5.2. Chemiluminescent Western blots of different *Dianthus caryophyllus* cultivars. The primary antibody used in these blots was rabbit serum at different stages of the inoculation (Pre-bleed, 8-week and 10-week) and at a concentration of 1:500. **a)** Lane 1 *Dianthus caryophyllus* cv Fenbow Nutmeg (FN), lane 2 *Dianthus caryophyllus* cv Early Mother's Day (EM), lane 3 *Dianthus barbatus* (Barb) lane 4 *Dianthus caryophyllus* cv Giant (G), lane 5 Tobacco and lane 6 Rainbow Marker. **b** and **c)** Lane 1 G, lane 2 Barb, lane 3 FN, lane 4 EM, lane 5 and 6 tobacco and lane 7 G.

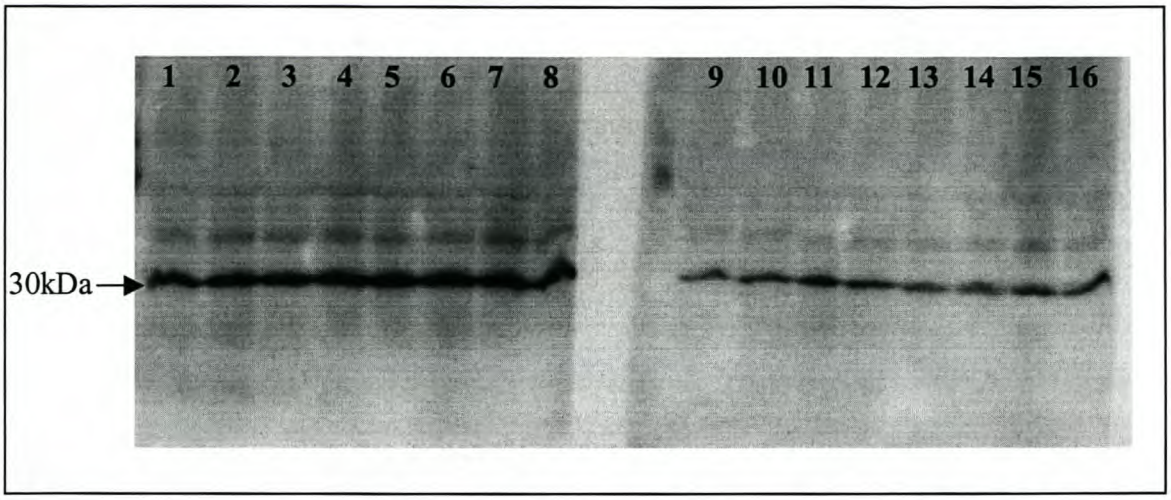


Figure 5.3. Chemiluminescent Western blots with Pre-Bleed and 10-week bleed sera as primary antibody. All lanes were loaded with *Dianthus caryophyllus* cv Fenbow Nutmeg and after blot membranes were cut and treated separately. Lanes 1-8 were treated with the pre-bleed at concentrations 1:100 000 (lane 1 and 2), 1:200 000 (lane 3 and 4), 1:600 000 (lane 5 and 6), 1:1000 000 (lane 7 and 8). Lanes 9 – 16 were treated with the 10-week bleed at concentrations 1:100 000 (lane 9 and 10), 1:200 000 (lane 11 and 12), 1:600 000 (lane 13 and 14), 1:1000 000 (lane 15 and 16).

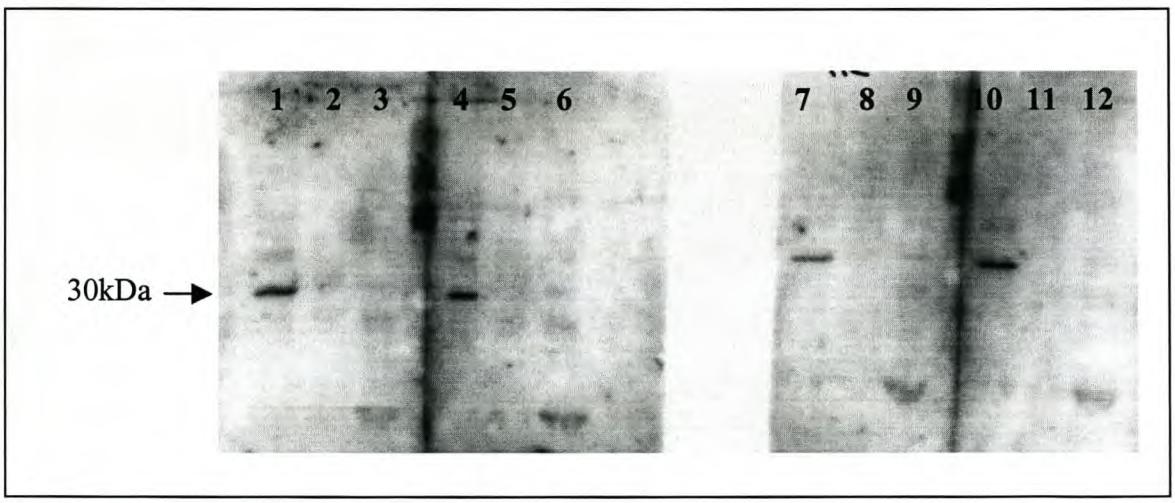


Figure 5.4. Chemiluminescent Western blot to compare the different sera of the two rabbits. Lanes 1, 4, 7 and 10 were loaded with *Dianthus caryophyllus* cv Fenbow Nutmeg and the remaining lanes with tobacco negative controls. Lanes 1-6 are the results with the antisera of Rabbit 2850 and Lanes 7-12 of Rabbit 2851. Lanes 1, 2, 3 and 7, 8, 9 are the results of the different rabbits with the pre-bleed sera and Lanes 4, 5, 6 and 10, 11, 12 from the 10-week sera.

A 30 kDa protein band was detected throughout all the experiments in only *Dianthus caryophyllus* and not in tobacco or in other *Dianthus* species. Unfortunately the band was also detected with the pre-bleed sera from both rabbits used in the inoculations. From the results obtained it seems unlikely that the band detected was not Dianthin 30. However, the question then still remains why the band was also detected with the pre-bleed sera. There is a slight possibility that the rabbits have inherent antibodies to one of the epitopes presented by the peptide that would generate cross reactivity. It might also be a simple case of sera being labeled wrong. Whatever the case might be, the fact now remains that it is impossible to tell if the sera were labeled incorrectly and no conclusive results can be obtained using these antibodies.

Chapter 6: Expression of Dianthin 30 in Tobacco

6.1. Introduction

Genetically transformed plants have revolutionized plant molecular biology in fundamental and applied science [137]. The transformation of important crop plants has not been perfected and with the use of model plants, constructs can be tested for their effects and efficiency. Model plants like *Nicotiana tabacum* are ideal, as they are easy to transform with foreign DNA and regenerate to form mature seed producing fertile plants. The most commonly used method to transform tobacco is by means of the pathogenic bacterium *Agrobacterium tumefaciens,* other methods like particle bombardment can also be used but require more skill and resources.

Agrobacterium tumefaciens is a soil-borne, plant pathogen that induces gall formation on infected plants [138]. *A. tumefaciens* contains a large tumor inducing (Ti) plasmid that is responsible for the delivery of the transfer-DNA (T-DNA) that contains the phytohormone genes that cause disease symptoms [139]. The T-DNA is randomly incorporated into the nucleic DNA of the plant cell.

The mechanism of gene transfer of this naturally occurring plant pathogen has been utilized extensively by scientists to transform plants in its host range with genes of scientific and economic importance. To achieve this, the Ti plasmid had to be modified to prevent disease symptoms from occurring on the transformed plants [138]. The T-DNA that carried these genes was largely removed and new gene constructs with all the control and selection elements were introduced by recombination. The alternative to the recombination approach came with the observation that the left and right borders are the only *cis* acting elements necessary for the transfer of the T-DNA, and that all the other elements can function in *trans* [140]. This led to the development of the intermediate vector or the binary vector system. In the binary vector system, the T-DNA region of the Ti-plasmid is removed to create a non-oncogenic *A. tumefaciens* strain. The binary vector can carry the T-DNA and contains all the elements to be replicated in *A. tumefaciens* and *E. coli,* as well as all the elements for selection in the bacterial and plant stages of the experiments [141]. The binary vector can then be introduced into the *A. tumefaciens* by

electroporation, transformation or triparental mating that requires a third plasmid (helper). For the expression in plants in this study, the binary expression system, pART, developed by Gleave in 1992 was used [142].

The pART system consists of a primary cloning vector pART7 and the binary vector pART27. Genes of interest are cloned into pART7 which contains a small MCS flanked by a CaMV35S promoter and the ocs terminator. The whole construct can be liberated with the *Not*I restriction enzyme. The pART27 binary vector contains all the elements for replication in *A. tumefaciens* and *E. coli* with resistance to spectinomycin, streptomycin and kanamycin. Flanked by the RB and LB, the vector also has the *lac*Z gene and a nptII gene driven by the pnos promoter [142]. The construct can then be cut from pART7 with *Not*I and cloned into pART27 at the internal *Not*I site that will disrupt the *lac*Z gene.

The expression of RIPs in tobacco has been largely unsuccessful with very few laboratories reporting positive results. The main problems occurring are very low transformation ratios, stunted growth and the production of sterile plants. Most of these reports come from studies into PAP expression and although very little homology exists with *Dianthin 30,* the possibility exists that similar results might be obtained [68, 89, 98].

The aim of expressing Dianthin 30 in tobacco was to engineer broad-spectrum virus resistance into tobacco as a model for grapevine. Previous studies by Hong *et al.* indicated that this approach had the potential to produce virus resistant plants [97, 98]. Our study differed from that of Hong *et al.* in that we were expressing Dianthin 30 constitutively and they used the virion-sense promoter of the African cassava mosaic virus (ACMV) that was transactivated by the gene product of the viral gene AC2 [97, 98]. Hong *et al.* maintained that the advantage of using an inducible promoter was that the expression of the RIP gene was restricted to the infected cells. They were however unable to show any Dianthin 30 specific transcript in resistant plants [98]. One of the disadvantages of having an inducible promoter is that any resistance gained from the introduced construct will be limited to the infection of one virus or virus family. This is one of the limitations of pathogen-derived resistance [137]. With the shortcomings of PDR and similar approaches and in our attempts to engineer broad-spectrum virus resistance, it was decided that the constitutive expression of Dianthin 30 would be attempted in this study.

6.2. Experimental procedures

6.2.1. Cloning of *Dianthin 30* into pCambia3301

Before using the pART system, an attempted was made to clone into a 11307 bp binary vector with the *bar* gene for resistance *in planta* developed by Cambia pCambia330. The plasmid was isolated and confirmed with RE digests. *Dianthin 30* was amplified from pGEM Dian (Chapter 3) with primers that amplified the whole Dianthin 30 gene and incorporated the restriction sites *Nco*I and *Pml*I on the 5' and 3' ends respectively. The GUS gene was liberated from pCambia3301 using *Nco*I and *Pml*I, and the amplified *Dianthin 30* was also digested with the same enzymes, for ligation. The vector to insert ratios used for the ligation ranged from 1:225-1:0.5 with two different ligation systems, T4 ligase from New England Biolabs (NEB) and All Phor One T4 ligase (Pharmacia). Both enzymes were used as suggested by the manufacturers and transformed into competent DH5α cells.

6.2.2. Cloning of *Dianthin 30* into pART27

Cloning into pART27 is made easier with the help of the cloning vector pART7. To facilitate the cloning into pART7 the restriction sites *Kpn*I and *Xba*I had to be added to the 5' and 3' ends of *Dianthin 30. Dianthin 30* was subcloned into pBluescriptSK, a phagemid from Stratagene, to obtain these restriction sites. This was done by amplifying *Dianthin 30* with the DNA polymerase pfx from GibcoBRL and ligated into pBluescriptSK that was digested with the *Eco*RV. Positive (white) colonies from the blue/white selection were selected and the pDNA isolated for RE digests to confirm insert and check orientation. A positive plasmid, designated pSK-Dian and pART7 was then digested with *Kpn*I and *Xba*I and purified after separation by gel electrophoresis (1.4% Agarose-TAE). The purified digestion products were then ligated with T4-ligase (NEB). Colonies from the transformation were screened with PCR (*Dianthin 30* specific primers) and pDNA from positive colonies were isolated and confirmed with a *Bam*HI digest.

pART7-Dian was then digested with *Not*I to excise the construct containing the CaMV35S promoter, *Dianthin 30* and the ocs terminator. The construct and the backbone DNA were the separated on a 1.4% Agarose-TAE gel. The construct was

carefully purified from the gel and used for ligation into pART27. pART27-Dian binary vector was isolated from colonies generated from the transformation and screened with PCR and confirmed with RE digests.

6.2.3. Transformation of *A. tumefaciens*

Two methods were used to introduce pART27-Dian into *A. tumefaciens;* triparental mating and direct transformation [141, 143].

The triparental mating procedure was used for the transformation of the *A. tumefaciens* strain LBA4404. pART27-Dian was introduced into LBA4404 with the help of the helper plasmid pRK2013 [141]. The *A. tumefaciens* colonies produced from the triparental mating were screened with PCR and the positive colonies were used in the transformation of tobacco leaf discs.

The least time consuming and simpler method of direct transformation was described by Tzfira *et al.* and this method was also used for the transformation of *A. tumefaciens* with pART27-Dian [143]. In this method the *A. tumefaciens* strain GV3101 was made competent with a 20 mM $CaCl_2$ solution and the plasmid DNA (pART27-Dian) transformed into the cells with freeze/thaw steps. The colonies obtained from this transformation were tested with PCR.

6.2.4. Transformation of *Nicotiana tabacum*

Several batches of tobacco leaf discs were transformed with the two strains of *A. tumefaciens.* With the LBA4404 strain about 200 *Nicotiana tabacum* cv Xanthi leaf discs were transformed and with the GVA3101 about *Nicotiana tabacum* cv Petit Havana (SR1) 600 discs were transformed. One set of 100 *Nicotiana tabacum* cv. Petit Havana (SR1) leaf discs from *in vitro* material were also transformed by ARC-Infruitech/Nietvoorbij with the GV3101 strain.

All transformations were performed as set out in Section 4 of Methods in plant molecular Biology written by Svab *et al.* [141]. Sterile *in vitro* plant material was used for the transformation of *Nicotiana tabacum* cv Xanthi. For the transformation of *Nicotiana tabacum* cv Petit Havana (SR1) leaves were surface

sterilized by submerging leaves in 70% EtOH for one minute and 20 minutes in 0.5% sodium hypochlorite and thoroughly rinsed with autoclaved water.

A. tumefaciens strains containing pART27-Dian were grown to a density of OD_{600}=1 at 28°C in LB with antibiotic selection (Rif 100 µg/mL, Sp 100 µg/mL, St 50 µg/mL). The cells were harvested and suspended in medium without antibiotics. Leaf discs were incubated at room temperature in the cell suspension for 20 minutes. The leaf discs were then blotted dry and placed on co-cultivation MS medium (See Table 6.1 for all medium compositions). After 4 days of co-cultivation *A. tumefaciens* growth was visible and leaves were dipped in water containing 400 µg/mL cefotaxime and placed on regeneration MS medium. Leaf discs were replanted every 2 weeks and when shoots formed, leaves were plated onto root MS medium.

Table 6.1.
Media used in the transformation of *Nicotiana tabacum* leaves

Co-cultivation MS medium	MS Salts and Vitamins 8 g/L Phyto Agar 1.0 mg/L BAP 0.1 mg/L NAA pH 5.8 KOH 30 g/L Sucrose
Regeneration MS medium	MS Salts and Vitamins 8 g/L Phyto Agar 1.0 mg/L BAP 0.1 mg/L NAA pH 5.8 KOH 30 g/L Sucrose 50 ug/mL Km and 400 ug/mL Cx
Rooting MS medium	MS Salts and Vitamins 8 g/L Phyto Agar 0.1 mg/L BAP 1.0 mg/L NAA pH 5.8 KOH 30 g/L Sucrose 50 ug/mL Km and 400 ug/mL Cx

6.2.5. Molecular analysis of putative transformed plants

Genomic DNA extractions were performed according to the CTAB method as described by C van Straten [4]. Very little material was available and DNA pellets were dissolved in minimum volumes of sterile water containing 20 μg/mL RNAase A (Roche).

PCR was performed on plants that rooted on antibiotic containing media with *Dianthin 30* specific primers and CaMV35S primers. The PCR product was then separated on a 1.4% Agarose-TAE Gel.

For the Southern blot analysis the maximum volume of isolated gDNA was RE digested with *Hind*III overnight at 37°C. The digested gDNA was then separated on a 0.8% Agarose-TAE gel for 10 hours. The DNA was then transferred to a positively charged nylon membrane, Hybond + (Amersham) using downward capillary transfer in an alkaline buffer as described in the FMC Guide to DNA transfer and analysis [144].

The DNA was detected using DIG Filter Hybridization (Roche). The 730 bp probe was PCR labeled with *Dianthin 30* specific primers as described in the DIG applications Manual [145]. All techniques followed for the detections of DNA on the membrane were done as described in the DIG-Manual [145].

6.3. Results and discussion

6.3.1. Cloning of *Dianthin 30* into pCambia3301

After initial experiments that yielded no transformed colonies, a range of ratios and two ligation systems were used. None of these yielded any colonies except for the control experiments that indicated that the transformation of DH5α cells with pCambia3301 was possible and that the ligation of pCambia3301 was possible after it was digested. All attempts to clone *Dianthin 30* into pCambia3301 failed, probably because of the large size difference between the vector and the insert.

6.3.2. Cloning of Dianthin 30 into pART27

The pART system was used for the transformations as it had the advantage of a cloning vector that aided transformation into the plant expression vector. *Dianthin 30* was first cloned into pBluescriptSK to obtain the restriction sites *Kpn*I and *Xba*I that could be used to clone into the cloning vector pART7 (see Figure 6.1a). This was done by amplifying *Dianthin 30* with a DNA polymerase, *pfx* that did not leave an A overhang on the PCR product and cloned into *Eco*RV (blunt cutter) digested pBluescriptSK. The MCS of pBluescriptSK is in a *lacZ* gene and thus gives the added advantage of blue/white selection. The positive (white) colonies were tested for orientation with *Bam*HI digests. *Dianthin 30* in the correct orientation (to be used for cloning into pART7) would yield an 850 bp fragment if digested with *Bam*HI (see Figure 6.1.a) and from Figure 6.1.b it is clear that of all the plasmids tested, only one did not give the correct band pattern.

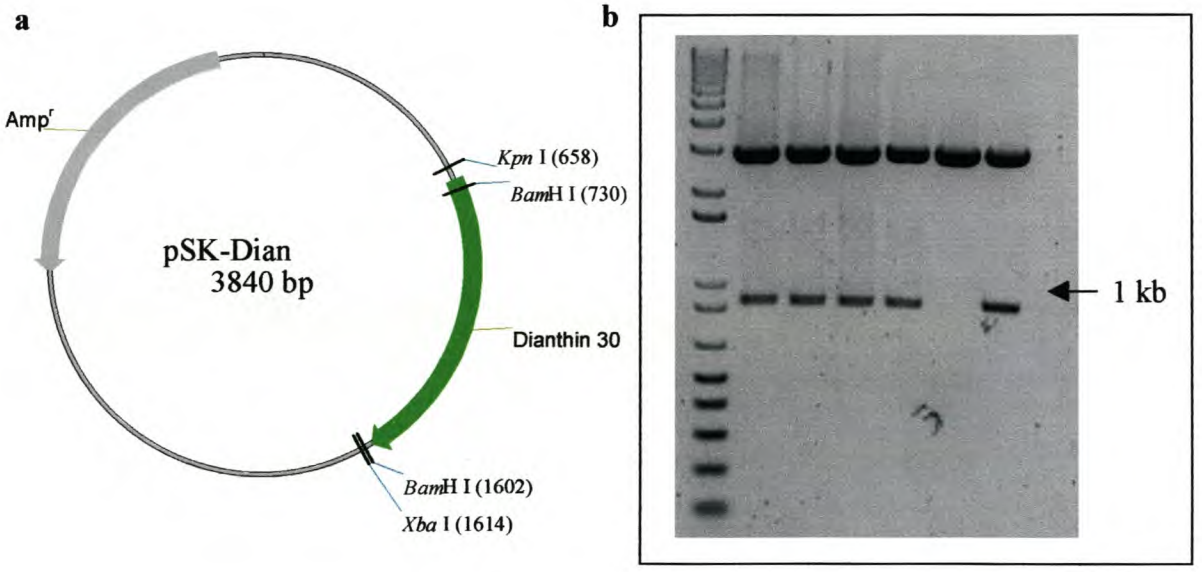


Figure 6.1. a) Restriction map of pSK-Dian indicating RE sites and orientation needed for *Dianthin 30* to gain *Kpn*I and *Xba*I on the 5' and 3' ends. b) RE digest of different pSK-Dian isolates from white colonies digested with *Bam*HI (Note one escape).

Both pSK-Dian and pART7 were digested with *Kpn*I and *Xba*I and the band with *Dianthin 30* was purified from an agarose gel. The transformation yielded many

colonies, as the digested pART7 plasmid was not SAP treated thus allowing the plasmid that was only cut with one enzyme to re-ligate without the insert. Treatment with Shrimp Alkaline Phosphatase (SAP) will catalyse the dephosphorylation the 5' phosphates of the DNA strand and thus prevent any re-circulization of liniarized plasmid. All the colonies were screened with PCR and it was found that about 20% did contain the insert. The positive colonies were confirmed with RE digests and one was used for cloning into pART27. See Figure pART7-Dian for digest of pART7-Dian.

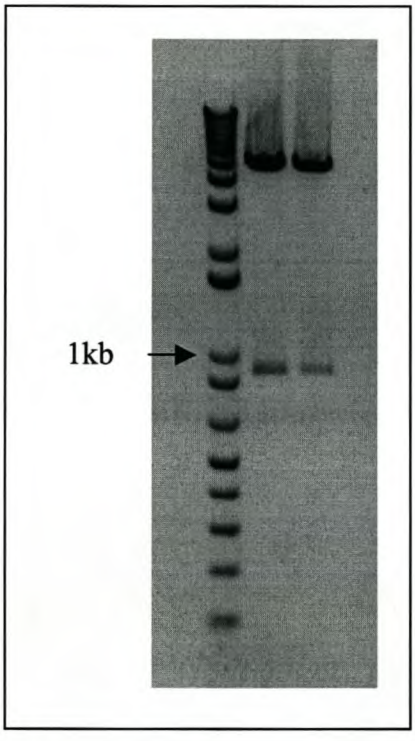


Figure 6.2. pART7-Dian digested with *Bam*HI to confirm insert of *Dianthin 30*.

pART7-Dian and pART27 were digested with *Not*I and the fragment containing *Dianthin 30* was ligated into the digested pART27 (See Figure 6.3). The transformation was confirmed with PCR (See Figure 6.4.). Only 50% of tested colonies contained the insert and this could be attributed to the fact that the vector was not SAP treated. The colonies tested positive by PCR were also verified to be positive with RE digests (See Figure 6.5.)

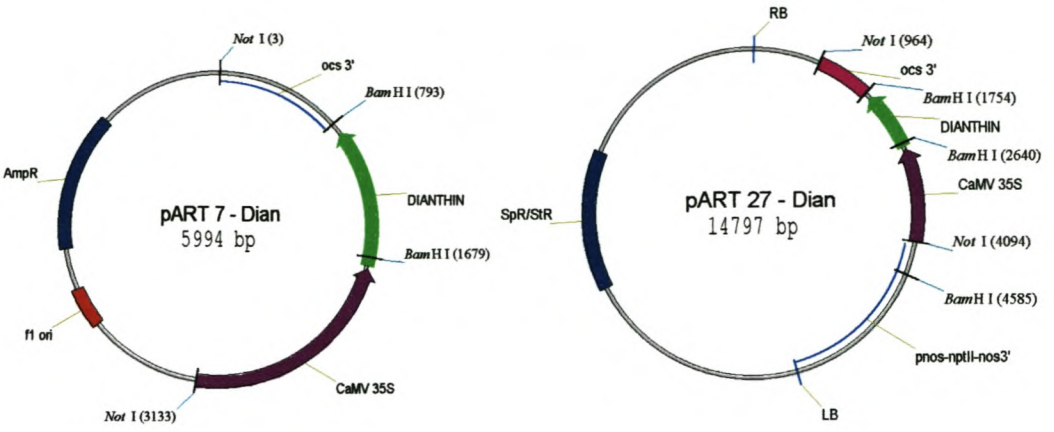


Figure 6.3. Restriction maps of pART7-Dian and pART27-Dian

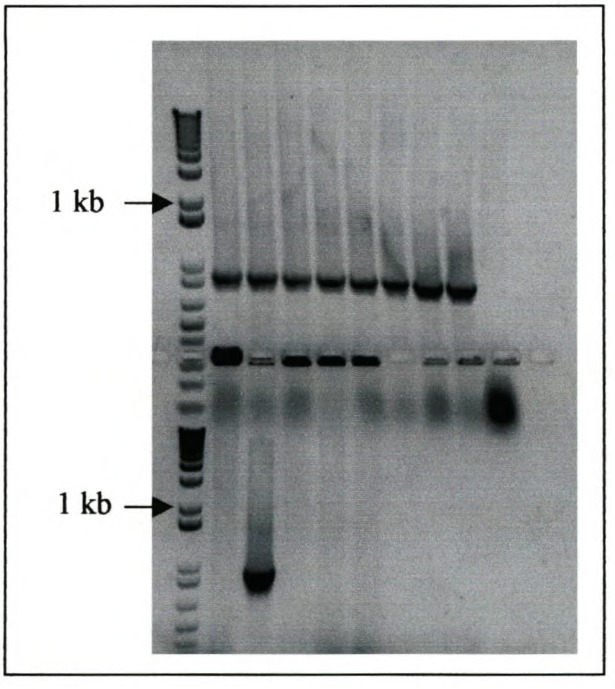


Figure 6.4. PCR screening of pART27-Dian colonies. From the photo it is clear that not all the colonies did contain the construct.

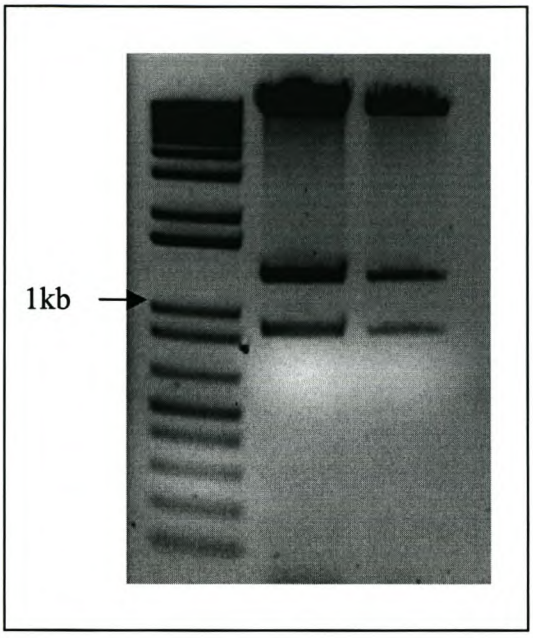


Figure 6.5. pART27-Dian digested with *BamH*I.

6.3.3. Transformation of *A. tumefaciens*

The transformation of *A. tumefaciens* was successful with both methods. The triparental mating yielded transformed LBA4404 cells (see Figure 6.6.) and the direct transformation, transformed GV3101 cells. Both transformations were confirmed with stringent antibiotic selection and PCR (see Figure 6.7.).

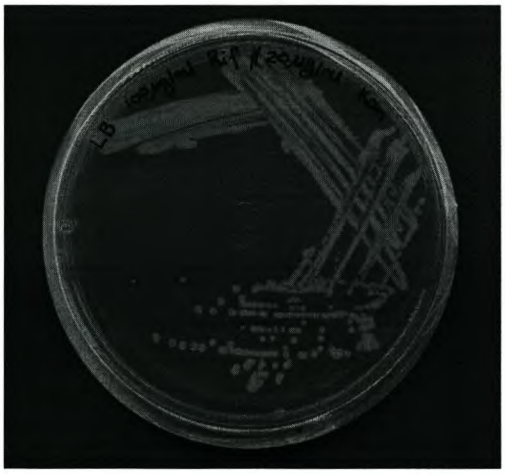


Figure 6.6. LB plate with transformed *A. tumefaciens* cells after tri-parental matings.

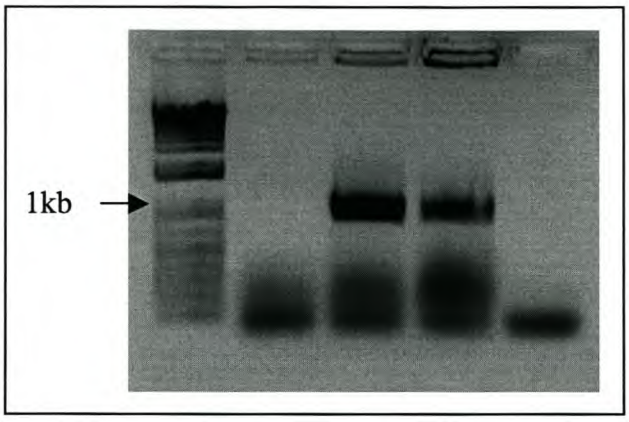


Figure 6.7. PCR screen *A. tumefaciens* strains for pART27-Dian with primers that would amplify the whole sequence. Lane 1 1 kb+ Marker (GibcoBRL), lane 2 and 5 negative controls, 3 LBA4404, lane 4 GV3101.

6.3.4. Transformation of *Nicotiana tabacum*

Transformation of pART27-Dian was performed on *Nicotiana tabacum* cv Xanthi and Petit Havana (SR1) leaf discs. From the approximately 200 Xanthi leaf discs (*in vitro* material) transformed with LBA4404, 56 plants were generated. With the Petit Havana (SR1) leaves, no plants were generated after almost 600 leaf discs that were transformed with LBA4404 and GV3101. The stringent sterilization might have been too robust for the young leaves, but attempts with less stringent sterilization yielded too much fungal contamination. Petit Havana (SR1) leaf discs (*in vitro* material) transformed with GV3101 yielded 20 plants from 100 discs.

6.3.5. Molecular analysis of putative transformed plants

The PCR screening of the putative positive plants yielded positive results with the *Dianthin 30* specific primers and the CaMV35S primers (See Figure 6.8.). The PCR also included the necessary negative controls.

A 730 bp probe was PCR labeled using *Dianthin 30* specific primers (see Figure 6.9.) From the Figure it can be deduced that the labeling was successful and very efficient. The gDNA isolated was digested with *Hind*III overnight and separated (see Figure 6.10). The smears indicated that the concentration of gDNA isolated might be too low to detect any bands.

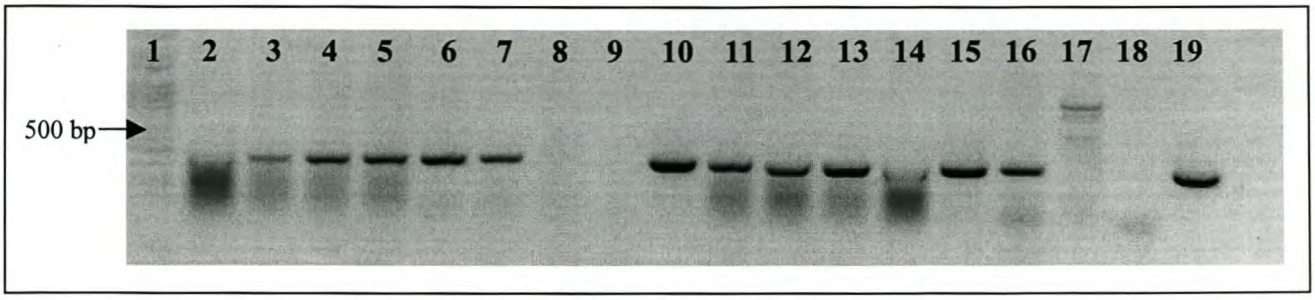


Figure 6.8. Results of PCR screen of putative transformed tobacco plants. Lanes 2-10 were tested with the Dianthin internal primers and lanes 11-19 with the CaMV35S primers. Lanes 8 and 17 were DNA negatives and 9 and 18 were water negatives. The samples were loaded in lanes 2 –7 and same samples in 10-16 but with different primers.

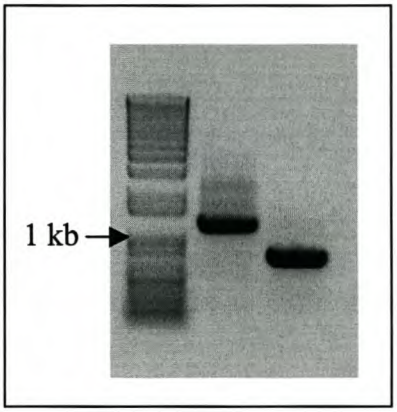


Figure 6.9. PCR products of Dianthin 30 specific primers for the production of a DNA probe. The size difference between labeled and unlabeled PCR product is clearly visible.

The Southern blot was only able to detect a band in the positive control. The main reason why all plants tested negative, apart for the possibility that the plants might not be transformed was that relative concentration of gDNA used in the blots was too low (See Figure 6.10. and 6.11.).

From all the plants tested with the Southern blots, no transformed plants were detected. There is a possibility that the DNA concentration on the blots was too low but it is more likely that the plants were not transgenic even though the plants were tested positive with PCR. An explanation might be that the plant cells that were transformed were not able to grow into embryos. Another reason that supports the conclusion that no transformed plants were generated, is the

observation that some plants that rooted on antibiotic containing media died when replanted. This was a common occurrence and could only be explained as residual *Agrobacterium* growth that helps the plant to cope with the antibiotics in the media. The positive PCR results could also be explained in view of this conclusion.

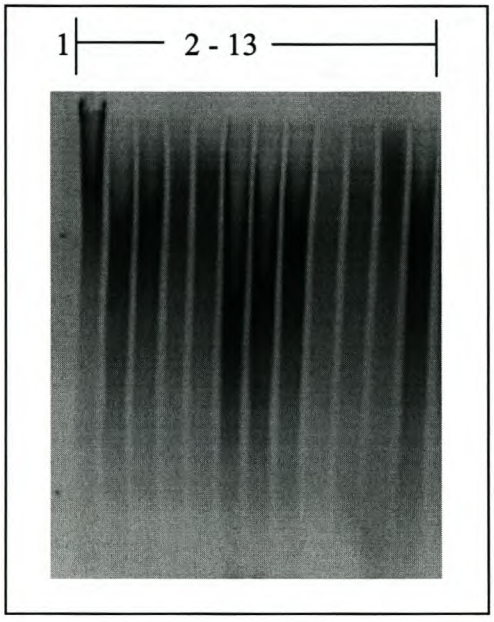


Figure 6.10. *Hind*III Digest of gDNA of putatively transformed tobacco plants. Lane 1 is the positive control and lanes 2-13 are the putative transformed plants.

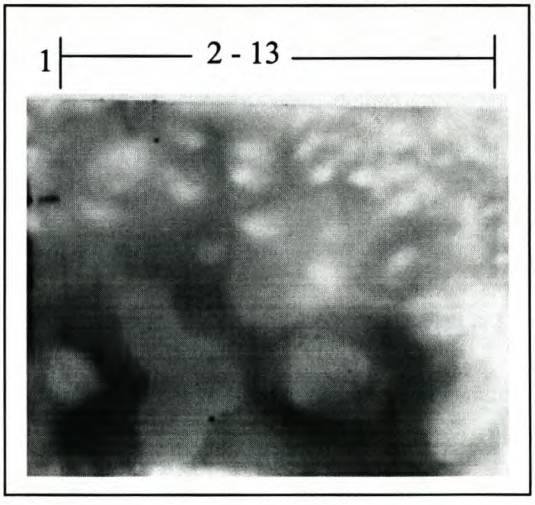


Figure 6.11. Southern blot of the above shown agarose gel indicating only the positive control with less than 0.4 ng DNA.

Chapter 7: Conclusion

RIPs hold great promise to be one of the major role players in broad-spectrum virus resistance in transgenic agricultural crops. This initial study highlights some of the hurdles that first need to be cleared before such crops can be produced.

In an attempt to express rDianthin 30, a bacterial expression system was initially used. The bacterial expression with the pET14b vector was successful but at such a low concentration that it was not useful for the main aim of the project, which was to produce Dianthin 30 antibodies. It appears that the toxicity of the protein might be too great for the bacteria to deal with sufficiently. Other researchers also observed similar low expression levels.

To rDianthin 30 at higher concentrations, expression in insect tissue culture was attempted. It was hoped that the eukaryotic system would be better equipped to deal with the toxicity of the protein, but this system also yielded recombinant proteins at such low levels that they were insufficient for use as an antigen. To my knowledge this is the first attempt at expressing Dianthin 30 in insect cells.

Despite the above failures in expressing sufficient protein for antibody production to detect Dianthin 30 at least in carnations, a peptide was designed that would represent Dianthin 30 and could be used as antigen for the production of Dianthin 30 specific antibodies. The synthesis and rabbit immunisation were sub-contracted to Bio-Synthesis Incorporated. The antibodies received were tested and did detect a protein at 30 kDa (size of Dianthin 30) only in *Dianthus caryophyllus* cultivars and not in tobacco or even *Dianthus barbatus*. Unfortunately, the pre-bleed serum also detected the same protein. The only reasonable explanations for these results would be that the rabbits have inherent antibodies to one of the epitopes presented by the peptide that would generate cross reactivity or that the "pre-bleed" received was one of the later bleeds and was labeled incorrectly.

The plant transformations yielded no transformed plants. Repeated efforts to produce these plants were unsuccessful. Other studies also indicated that the expression of a RIP in transgenic plants could have an adverse effect on the growth of the host plant. The only other

study that used Dianthin 30 was unable to show Dianthin 30 specific transcripts in the transgenic plants.

In an attempt to reach the goal of producing transgenic crops that would have broad-spectrum virus resistance this study has shown that the choice of gene is extremely important. The toxicity of the exogenous protein to the plant physiology needs to be taken into account. A more toxic RIP does not necessarily mean more resistance would be gained from it as it might not result in transgenic plants. An alternative approach would be to express the RIP under the control of an inducible promoter that might lessen the impact of the RIP on the plant's system.

It is possible to use RIPs in transgenic plants to gain resistance to a range of pathogens. To achieve this might be more difficult than other methods but the possible rewards do warrant the risk and difficulty in this approach.

It might not have been the answer you were hoping for but here it is.....
".....'Forty-two' said Deep Thought, with infinite majesty and calm."
The Hitch Hiker's Guide to the Galaxy, Douglas Adams

References

1. Wines of South Africa. http://www.wosa.co.za.
2. Krake, L.R. (1993) Aust. N.Z. Wine Ind. J. 8. 40-44.
3. Shah, D.M. (1997) Current opinion in Biotechnology. 8. 208-214.
4. Van Straten, C.D., (2000) *The construction of plant expression vectors for the introduction of leafroll resistance in grapevine.* MSc thesis, University of Stellenbosch.
5. Wilsen KL, (1999) *Investigating the introduction of a broad-spectrum antiviral mechanism into grapevine.* MSc thesis, University of Stellenbosch.
6. Murphy, J. (1997) In: *Guidebook to protein toxins and their use in cell biology.* Rappuoli, R. and Montecucco, C. ed. Sambrook and Tooze publication at Oxford University press. p47.
7. Wang, Y., Qian, R., Gu, Z., Jin, S., Zhang, L., Xia, Z., Tian, G. and Ni, C. (1986) Pure & Appl. Chem. 58(5), 789-798.
8. Shaw, P.C., Chan, W.L., Yeung, H.W. and Ng, T.B. (1994) Life Sci 55. 253-62.
9. Wang, H.X. and Ng, T.B. (2000) Comparative Biochemistry and Physiology Part B 127, 519-524.
10. Maraganore, J.M., Joseph, M. and Bailey, M.C. (1987) J. Biol. Chem. 262, 11628-11633.
11. Barbieri, L., Batelli, M.G. and Stirpe, F. (1993) Biochim. Biophys. Acta 1154, 237-282.
12. Peumans, W.J., Hao, Q. and Van Damme, E.J.M. (2001) FASEB 15(9), 1493-1506.
13. Gendron, Y. and Kassanis, B. (1954). Ann. Appl. Biol. 41(1), 183-188.
14. Thomlinson, J.A., Walker, V.M., Flewett, T.H. and Barclay, G.R. (1974) J. Gen. Virol. 22, 225-232.
15. Owens, R.A., Bruening, G. and Shepherd, R.J. (1973) Virology 56, 390-393.
16. Montanaro, L., Sperti, S. and Stirpe, F. (1973) Biochem. J. 136, 677-683.
17. Lin, J.-Y., Tserng, K.-Y., Chen, C.-C., Lin, L.-,T and Tung, T.-C. (1970) Nature 227, 292-293.
18. Obrig, T.G., Irvin, J.D. and Hardesty, B. (1973) Arch. Biochem. Biophys. 155, 278-289.

19. Stirpe, F., Gasperi-Campani, A., Barbieri, L., Lorenzoni, E., Montanaro, L., Sperti, S. and Bonetti, E. (1978) FEBS Letters 85, 65-67.
20. Barbieri, L. and Stirpe, F. (1982) Cancer Surveys 1(3), 490-520.
21. Stirpe, F. (1982). Biochem. J. 202, 279.
22. Ready, M., Wilson, K., Piatak, M. and Robertus, J.D. (1984) J. Biol. Chem. 259, 15252-15256.
23. Reinbothe, S., Reinbothe, C., Lehmann, J., Becker, W., Apel, K. and Parthier, B. (1994) Proc. Natl. Acad. Sci. USA 91, 7012-7016.
24. Endo, Y. and Tsurugi, K. (1988) J. Biol. Chem. 263, 8735-8739.
25. Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) J. Biol. Chem. 262(12), 5908-5912.
26. Endo, Y. and Tsurugi, K. (1987) J. Biol. Chem. 262(17), 8128-8130.
27. Ferens, W.A. and Hovde, C.J. (2002) Infect. Immun. 68(8), 4462-4469.
28. Brigotti, M., Accorsi, P., Carnicelli, D., Rizzi, S., Gonzalez-Vara, A., Montanaro, L. and Sperti, S. (2001) Toxicon. 39(2-3), 341-348.
29. Wang, H.X. and Ng, T.B. (2000) Biochem. Cell. Biol. 78(6), 699-702.
30. Lam, S.K. and Ng, T.B. (2001) Arch. Biochem. Biophys. 393(2), 271-280.
31. Hwu, L., Huang, K., Chen, D. and Lin, A. (2000) J. Biomed. Sci. 7(5), 420-428.
32. Barbieri, L., Valbonesi, P., Bondioli, M., Alvarez, M.L., Dal-Monte, P., Landini, M.P. and Stirpe, F. (2001) FEBS Letters 505(1), 196-197.
33. Van Damme, E.J.M., Barre, A., Barbieri, L., Valbonesi, P., Rouge, P., Van Leuvens, F., Stirpe, F. and Peumans, W.J. (1997) Biochem. J. 324, 963-970.
34. Pallanca, A., Mazzaracchio, R., Brigotti, M., Carnicelli, D., Alvergna, P., Sperti, S. and Montanaro, L. (1998) Biochim. Biophys. Acta 1384, 277-284.
35. Obrig, T.G., Irvin, J.D. and Hardestry, B. (1973) Arch. Biochem. Biophys. 155, 278-289.
36. Montfort, W., Villafranca, J.E., Monzingo, A.F., Ernst, S.R., Katzin, B., Rutenber, E., Xuong, N.H., Hamlin, R. and Robertus, J.D. (1987) J. Biol. Chem. 262, 5398-5403.
37. Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. and Igarashi, K. (1988) Eur. J. Biochem. 171, 45-50.
38. Hartley, M.R., Chaddock, J.A. and Bonness, M.S. (1996) TIPS 1(8), 254-260.
39. Ready, M.P., Brown, D.T. and Robertus, J.D. (1986) Proc. Natl. Acad. Sci. USA 83, 5053-5056.

40. Girbés, T., De Torre, C., Iglesias, R., Ferreras, J.M. and Méndez, E. (1996) Nature 379, 777-778.
41. Garret, R.H. and Grisham, C.M. (1995) *Biochemistry,* Saunders College Publishing, Harcourt Brace College Publishers.
42. Marchant, A. and Hartley, M.R. (1995) J. Mol. Biol. 254, 848-855.
43. Taylor, B.E. and Irvin, J.D. (1990) FEBS Letters 273, 144-146.
44. Ippoliti, R., Lendaro, E., Bellelli, A. and Brunori, M. (1992) FEBS Letters 298, 145-148.
45. Zubay, G. (1993) *Biochemistry 3RD edition,* Columbia University.
46. Endo, Y., Huber, P.W. and Wool, I.G. (1983) J. Biol. Chem. 258, 2662-2667.
47. Murphy, J. (1997) In: *Guidebook to protein toxins and their use in cell biology.* Rappuoli, R. and Montecucco, C. Ed. Sambrook and Tooze publication at Oxford University press. p48-50.
48. Olsnes, S. (1997) In: *Guidebook to protein toxins and their use in cell biology.* Rappuoli, R. and Montecucco, C. Ed. Sambrook and Tooze publication at Oxford University press. p50-52.
49. Carnicelli, D., Brigotti, M., Alvergna, P., Pallance, A., Sperti, S. and Montanaro, L. (1997) Journal of Experimental Botany 48, 1519-1523.
50. Harley, S.M. and Beevers, H. (1984) Proc. Natl. Acad. Sci. USA 79, 5935-5938.
51. Taylor, B.E. and Irvin, J.D. (1990) FEBS Letters 273, 144-146.
52. Marchant, A. and Hartley, M.R. (1995) J. Mol. Biol. 254, 848-855.
53. Irvin, J.D. (1975) Arch. Biochem. Biophys. 169, 522-528.
54. Hartley, M.R., Legname, G., Osborn, R., Chen, Z. and Lord, J.M. (1991) FEBS Letters 290(1,2), 65-68.
55. Prestle, J., Hornung, E., Schönfelder, M. and Mundry, K. (1992) FEBS Letters 297(3), 250-252.
56. Habuka, N., Akiyama, K., Tsuge, H., Miyano, M., Matsumoto, T. and Noma, M. (1990) J. Biol. Chem. 265(19), 10988-10992.
57. Gribés, T., Ferreras, J.M., Iglesias, R., Citores, L., De Torre, C., Carbajales, M.L., Jiménez, P., De Benito, F.M. and Muñoz, R. (1996) Cell. Mol. Biol. 42(4), 461-471.
58. Di-Maro, A., Valbonesi, P., Bolognesi, A., Stirpe, F., De-Luca, P., Siniscalco-Gigliano, G., Gaudio, L., Delli-Bovi, P., Ferranti, P., Malorni, A. and Parente, A. (1999) Planta 208(1), 125-131.

59. Ferreras, J.M., Iglesias, R., Barbieri, L., Alegre, C., Bolognesi, A., Rojo, M.A., Carbajales, M.L., Escarmis, C. and Girbes, T. (1995) Biochim. Biophys. Acta 1243(1), 85-93.

60. Ling, J., Liu, W. and Wang, T.P. (1994) FEBS Letters 345, 143-146.

61. Roncuzzi, L. and Gasperi-Campani, A. (1996) FEBS Letters 392(1), 16-20.

62. Wang, P. and Tumer, N.E. (1999) Nucleic Acids Res. 27(8), 1900-1905.

63. Nicolas, E., Beggs, J.M., Haltiwanger, B.M. and Taraschi, T.F. (1997) FEBS letters 406, 162-164.

64. Barbieri, L., Valbonesi, P., Gornini, P., Pession, A. and Stirpe, F. (1996) Biochem. J. 319, 507-513.

65. Barbieri, L., Valbonesi, P., Bonora, E., Gornini, P., Bolgnesi, A. and Stirpe, F. (1997) Nucleic Acids Res., 25(3), 518-522.

66. Barbieri, L., Valbonesi, P., Righi, F., Zuccheri, G., Monti, F., Gorini, P., Samorí, B. and Stirpe, F. (2000) J. Biochem. 128, 883-889.

67. Magnusson, S., Berg, T., Turpin, E. and Frenoy, J.-P. (1991) Biochem. J. 277, 855-861.

68. Lodge, J.K., Kaniewski, W.K. and Tumer, N.E. (1993) Proc. Natl. Acad. Sci. USA 90, 7089-7093.

69. Simmons, B.M., Stahl, P.D. and Russel, J.H. (1986) J. Biol. Chem. 261, 7912-7920.

70. Stirpe, F., Sandvig, K., Olsnes, S. and Phil, A. (1982) J. Biol. Chem. 257, 13271-13277.

71. Olsnes, S., Saltvedt, E. and Phil, A. (1974) J. Biol. Chem. 249, 803-810.

72. Wolf, S., Deom, C.M., Beachy, R.N. and Lucas, W.J. (1989) Science 246, 377-379.

73. Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991) J. Biol. Chem. 266, 1564-1573.

74. Massiah, A.J. and Hartley, M.R. (1995) Planta 197, 633-640.

75. Brigotti, M., Keith, G., Pallanca, A., Carnicelli, D., Alvergna, P., Dirheimer, G., Montanaro, L. and Sperti, S. (1998) FEBS Letters 431, 259-262.

76. Rippmann, J.F., Michalowski, C.B., Nelson, D.E. and Bohnert, H.J. (1997) Plant Mol. Biol. 35, 701-709.

77. Stirpe, F., Barbieri, L., Gorini, P., Volbonesi, P., Bolognesi, A. and Polito, L. (1996) FEBS Letters 382, 309-312.

78. Dallal, J.A. and Irvin, J.D. (1978) FEBS Letters 89, 257-259.

79. Foa-Tomasi, L., Campadelli-Fiume, G., Barbieri, L. and Stirpe, F. (1982) Arch. Virol. 71(4), 323-332.

80. Barbieri, L., Aron, G.M., Irvin, J.D. and Stirpe, F. (1982) Biochem. J. 203, 55-59.

81. Ussery, M.A. and Irvin, J.D. (1974) Fed. Proc. 33, 1544.

82. Yamaizumi, M., Uchida, T. and Okada, Y. (1979) Virology 95, 218-221.

83. Fernández-Puentes, C. and Carrasco, L. (1980) Cell 20, 766-775.

84. Maleck, K. and Lawton, K. (1998) Current Opinion in Biotechnology 9, 208-213.

85. Barbieri, L., Valbonesi, P., Bonora, E., Gorini, P., Bolognesi, A. and Stirpe, F. (1997) Nucleic Acids Res. 25(3), 518-522.

86. Zarling, J.M., Moran, P.A., Haffar, O., Sias, J., Richman, D.D., Spina, C.A., Myers, D.E., Keubelbeck, V., Ledbetter, J.A. and Uckun, F.M. (1990) Nature 347, 92-95.

87. Zoubenko, O., Hudak, K. and Tumer, N.E. (2000) Plant Mol. Biol. 44, 219-229.

88. Smirnov, S., Shulaev, V. and Tumer, N.E. (1997) Plant Physiol. 114, 1113-1121.

89. Wang, P., Zoubenko, O. and Tumer, N.E. (1998) Plant Mol. Biol. 38, 957-964.

90. Roberts et al 1986 Biochim. Biophys. Acta 880 161-170.

91. Logemann, J., Jach, G., Tommerup, H., Mundy, J. and Schell, J. (1992) Bio/technol. 10, 305-308.

92. Enyedi, A.J., Yalpani, N., Silverman, P. and Raskin, I. (1992) Cell 70, 879-886.

93. Ramakrishnan, S., Fryxell, D., Mohanraj, D., Olson, M. and Li, B.Y. (1992) Ann. Rev. Pharmacol. Toxicol. 32, 579-621.

94. Tumer, N.E., Hwang, D. and Bonness, M. (1997) Proc. Natl. Acad. Sci. USA 94, 3866-3871.

95. Lam, Y., Wong, Y., Wang, B., Wong, R.N., Yeung, H. and Shaw, P. (1996) Plant Sci. 114, 111-117.

96. Krishnan, R., McDonald, K.A., Dandekar, A.M., Jackman, A.P. and Falk, B. (2002) J. Biotech. 97, 69-88.

97. Hong, Y., Saunders, K. and Stanley, J. (1997) Virology 228, 383-387.

98. Hong, Y., Saunders, K., Hartley, M.R. and Stanley, J. (1996) Virology 220, 119-127.

99. Chen, Y., Pneumans, W.J. and Van Damme, E.J.M. (2002) FEBS Letters 516, 27-30.

100. Giri, C.C. and Laxmi, G.V. (2000) Biotechnology Advances 18, 653-683.

101. Kim, J.K., Duan, X., Wu, R., Seok, S.J., Boston, R.S., Jang, I.C., Eun, M.Y. and Nahm, B.M. (1999) Mol. Breed. 5, 85-94.

102. Ragetli, H.W.J. and Weintraub, M. (1962a) Virology 18, 232-240.

103. Taylor, S., Massiah, A., Lomonossoff, G., Roberts, L.M., Lord, J.M. and Hartley, M. (1994) The Plant Journal 5(6), 827-835.

104. Ragetli, H.W.J. and Weintraub, M. (1962b) Virology 18, 241-248.

105. Stirpe, F., Williams, D.G., Onyon, L.J. and Legg, R.F. (1981) Biochem. J. 195, 399-405.

106. Reisbig, R.R. and Bruland, Ø. (1983) Arch. Biochem. Biophys. 224(2), 700-706.

107. Legname, G., Fossati, G., Monzini, N., Gromo, G., Marcucci, F., Mascagni, P. and Modena, D. (1995) Biomedical Peptides, Proteins & Nucleic Acids 1, 61-68.

108. Kataoka, J., Habuka, N., Furuno, M., Miyano, M., Takanami, Y. and Koiwai, A. (1991) J. Biol. Chem. 266(13), 8426-8430.

109. Girbés, T., Barbieri, L., Ferreras, M., Arias, F. J., Rojo, M.A., Iglesias, R., Alegre, C., Escarmis, C. and Stirpe, F. (1993) J. Bacteriol. 175(20), 6721-6724.

110. Legname, G., Bellosta, P., Gromo, G., Modena, D., Keen, J.N., Roberts, L.M. and Lord, M. (1991) Biochim. Biophys. Acta 1090, 119-122.

111. Legname, G., Gromo, G., Lord, J.M., Monzini, N. and Modena, D. (1993) Biochemical and Biophysical Research Communications 192(3), 1230-1237.

112. Cho, H.J., Lee, S.J., Kim, S. and Kim, B.D. (2000) Mol. Cells 10(2), 135-141.

113. Madin, K., Sawasaki, T., Ogasawara, T. and Endo, Y. (2000) Proc. Natl. Acad. Sci. USA 97(2) 559-64.

114. PET system manuel 9th Edition May 2000, Novagen

115. i*Nova*tions Vol 1 Number 1 May 1994, Novagen

116. Breslauer, K.J., Frank, R., Blocker, H. and Marky, L.A. (1986) Proc. Nat. Acad. Sci. 83. 3746-50.

117. Mullis, K. and Faloona, F. (1987) Meth. Enzymol. 55, 335-350.

118. *Promega Technical Manual* (1998) Promega USA

119. Sambrook, J., Fritsch, E.F. and Maniatis,T. (1989) *Molecular cloning 2nd Edition,* Cold Spring Harbour Laboratory Press, NY.

120. Studier, F.W. and Moffatt, B.A. (1986) J. Mol. Biol. 189, 113.

121. Rosenberg, A.H., Lade, B.N., Chui, d., Lin, S., Dunn, J.J. and Studier, F.W. (1987) Gene 56, 125.

122. Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Meth. Enzymol. 185, 60-89.

123. Hanahan, D. (1985) In: *DNA Cloning Vol1*, Glover, D. ed. IRL Press Ltd, UK.

124. *Protocols and applications guide, 3rd Edition* (1996), Promega

125. *ExpressDetector manual*, KPL Virginia Commonwealth University, USA.

126. Burgess, R.R. (1996) Methods in Enzymology 273, 145-149.

127. Doran, P.M. (2000) Current Opinion in Biotechnology 11 199-204.

128. Luckow, V.A., Lee, S.C., Barry, G.F. and Olins, P.O. (1993) J. Virol. 67, 4566.

129. BAC-TO BAC ® Baculovirus expression systems. http://www.lifetech.com/content/sfs/manuals/bac.pdf.

130. Amersham International (1997) Little Chalfont Buckinghamshire England.

131. Princeton Biomolecules corporation. http://www.pbcpeptide.com/Antibody.htm

132. ePitope informatics. http://www.epitope-informatics.com/whetwedo.htm

133. Emanuelsson, O., Nielson, H., Brunak, S. and Von Heijne, G. (2000) J. Mol. Biol. 300. 1005-1016.\

134. Nielson, H., Engelbrecht, J., Brunak, S. and Von Heijne, G. (1997) Protein. Eng. 10. 1-6.

135. Von Heijne, G. (1986) Nucl. Acids Res. 14. 4683.

136. PVDF plus Protocols, Micron Separations Inc.

137. Sanford, J.C. and Johnson, S.A. (1985) J. Theor. Biol. 113, 395-405.

138. McClean, P. (1998) http://www.ndsu.nodak.edu/instruct/mcclean/plsc731/transgenic.htm

139. Cado, I.C. (1991) Crit. Rev. Plant. Sci. 10, 1-32.

140. Hoekema, A., Hirsch. P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) Nature. 303. 179-180.

141. Svab, Z., Hajdukiewicz, P. and Maliga, P. (1995) In: *Methods in plant molecular Biology*. Maliga, P., Klessig, D.F., Cashmore, A.R., Gruissem, W. and Varner, J.E. ed. Cold Spring Harbour Laboratory Press, NY.

142. Gleave, A.P. (1992) Plant Molecular Biology. 20. 1203-1207.

143. Tzfira, T., Jensen, S.J., Wang, W., Zuker, A., Vinocur, B., Altman, A. and Vainstein, A. (1997) Plant Molecular Reporter 15, 129-235.

144. FMC BioProducts, *Your complete guide for DNA separation and analysis*, Rocklands ME.

145. Eisel, D., Grunewald-Janho, S., Hloch, P., Hofner, P., Keesey, J., Krunchen, B. and Ruger, B. (Ed) (2000) *Roche Molecular Biochemicals, DIG Applications Manual for Filter Hybridization.*