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Development of the polygalacturonase inhibiting protein (PGIP) for delivery of foreign proteins to the surfaces of plant cells

by

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To my darling husband, Fergus
and adorable daughter, Emma-Jo

List of Contents

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Summary

Polygalacturonase-inhibiting proteins (PGIPs) are cell wall-associated plant proteins that inhibit endopolygalacturonases (endo-PGs) from phytopathogenic fungi. For proteins to confer resistance to invading plant pathogens, it is preferred that they are either associated with the plant cell wall or secreted into the intercellular spaces where they can act almost immediately upon pathogen attack. The bactericidal efficacy of the Hen Egg White Lysozyme (HEWL) has previously been unequivocally demonstrated in transgenic plants; however, most of the protein remains intracellular. It was hypothesized that bean PGIP1, that has previously been expressed correctly in transgenic tomato plants and was found to inhibit the endopolygalacturonase activity of *Stenocarpella maydis* in a reducing sugar assay, would deliver the HEWL protein to the intercellular spaces due to its inherent translocation to the plant cell wall by means of a translational fusion between bean *pgip1* and *hewl* genes.

In this study, the efficacy of such a translational fusion was determined. The bean *pgip1-hewl* fusion was inserted into the binary vector pCAMBIA2300 and transformed into *Nicotiana tabacum* cv. LA Burley 21 plants by *Agrobacterium*-mediated transformation. Phenotypically normal transgenic plants were produced. Stable transgene insertion into the transgenic *N. tabacum* genomes was verified by PCR and Southern blot analyses.

To demonstrate the efficacy of the bean PGIP1-HEWL fusion, independent homogenate and intercellular fluid protein extracts were prepared from transgenic *N. tabacum* leaf material. Protein extracts prepared so as to enrich for PGIP activity were tested *in vitro* for inhibition of *S. maydis* endo-PGs whereas protein extracts for HEWL activity were tested for lysis of *Micrococcus luteus* cells. Biochemical assays showed that bean PGIP1-HEWL inhibited *S. maydis* endo-PGs and cleaved *M. luteus* cell walls sufficiently to suggest that the PGIP1-HEWL fusion was structurally and functionally stable. Total protein extracts from the PGIP-HEWL and HEWL transgenic plants showed similar levels of HEWL specific activity, whereas intercellular fluid samples from PGIP-HEWL transgenic plants showed high activity in contrast to HEWL plants. With the success of showing protein activity *in vitro* of HEWL in intercellular spaces, bean PGIP1 can be recommended as a vehicle for delivery of other proteins to cell surfaces.

List of Abbreviations

ADA	Agarose Diffusion Assay
ARC	Agricultural Research Council
ARC-Infruitec	ARC-Fruit, Vine and Wine Research Institute
ARC-PPRI	ARC-Plant Protection Institute
ARC-Roodeplaat	ARC-Roodeplaat Vegetable and Ornamental Plant Institute
AS	Ammonium sulphate
avr	avirulence
bp	basepair
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
CIAA	Chloroform: isoamylalcohol
CSPD	Disodium 3-(4-methyoxyspiro {1,2-dioxethane-3,2'-(5'-chloro) tricycle [3,3,1,$1^{3,7}$]decan}-4-yl) phenyl phosphate
CTAB	Hexadecyl trimethyl ammonium bromide
cv	cultivar
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyquanidine triphosphate
dH_20	distilled water
DIG	Digoxygenin
DIG-11-dUTP	Digoxygenin-II-deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
endoPG	Endopolygalacturonase
IF	Intercellular fluid
kb	kilobasepair
kDa	kilodalton
LB	Luria Broth
LRR	Leucine-rich repeat
MS	Murashige and Skoog
MWM	Molecular Weight Marker

nptII	Neomycin phosphotransferase II
onc	oncogenic
ORF	Open reading frame
PAHBAH	ρ-4-amino-2-hydroxybenzoic acid hydrazide
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PG	Polygalacturonase
PGA	Polygalacturonic acid
PGIP	Polygalacturonase-inhibiting protein
R gene	Resistance gene
RNase	Ribonuclease
rpm	revolutions per minute
TAE	Tris-acetate ethylenediamine tetraacetic acid
T-DNA	Tranfered deoxiribonucleic acid
TE	Tris ethylenediamine tetraacetic acid
TEV	Tobacco etch virus
Tm	Temperature
TNE	Tris-sodium chloride ethylenediamine tetraacetic acid
Tris	Tris hydroxy methyl aminoethane

List of Figures

List of Tables

Chapter 1

Aim of the study

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Microbial diseases lead to significant crop losses in the agricultural industry. This is usually characterised by corresponding financial losses. A possible approach to this problem is to develop crops with resistance to the diseases caused by these microorganisms by introducing genes that confer resistance to pathogens, a prospect that could be invaluable in preventing such occurrences. With the advancement in genetic modification of several important crops, this approach has become more feasible.

In bacterial infection, the pathogen invades the intercellular spaces of the host plant and accumulates to high numbers before initiating disease (Düring, 1996). Lysozymes (muramidases or peptidoglycan N-acetylmuramoyl-hydrolase) are the enzymes that are capable of breaking down bacterial cell walls by cleaving the peptidoglycan backbone of the cell wall, thereby making lysozyme a potentially invaluable bactericidal protein. Since most of the plant lysozymes are bi-functional enzymes displaying a much stronger chitinase than lysozyme function (Jollès and Jollès, 1984), nearly all known characterized plant enzymes with lysozyme function are strongly basic proteins and are classified as vacuolar basic chitinases (Majeau *et al*., 1990; Stintzi *et al*., 1993; Düring, 1996). These endogenous plant lysozymes located in the vacuoles would not come into contact with the bacteria until plant cells became disrupted and the vacuoles burst. By then, the number of bacterial cells would probably be too large for enzymes to be effective in limiting disease development. Integrating foreign lysozyme genes into transgenic plants and directing these lysozymes to the intercellular spaces through fusion to a signal peptide may be a strategy to meet the requirements of spatial and temporal coincidence of pathogen and resistance protein (Düring, 1993).

The anti-microbial activity of Hen egg white lysozyme (HEWL) has been reported in transgenic tobacco (Trudel *et al*., 1992) and potato (Destfano-Beltran *et al*., 1992). Further studies by Trudel *et al*., (1995) on the fate of active HEWL in transgenic tobacco showed that secreted HEWL is tightly bound to some tobacco component(s). They reported recovery of less than 10% of secreted HEWL in the intercellular fluid of transgenic tobacco and also observed that the HEWL activity level varied with the vector construct used in transforming tobacco. There is thus a need to target the HEWL to the intercellular spaces.

With this in mind, the aim of this study was to examine the difference in the expression of *hewl* when introduced into constructs having its full sequence (including its native signal peptide) and another where the signal peptide has been replaced with a gene whose products are destined for the intercellular spaces in transgenic tobacco plants. The gene encoding the bean polygalacturonase inhibitor protein (PGIP1) is one such gene that is important in plant resistance against fungal pathogens. The bean *pgip1* gene, previously cloned at ARC-Roodeplaat, inhibits plant cell wall-degrading polygalacturonases produced by invading fungi. It was already correctly expressed in transgenic tomato plants yielding a PGIP that appeared to inhibit the polygalacturonase activity of the maize pathogen *Stenocarpella maydis* in a sugar-reducing assay (Berger *et al*., 2000). In designing a construct where the bean *pgip1* gene occurs in place of the *hewl* signal peptide, the hypothesis that the *hewl* product will be deposited at the desired location on the surfaces of plant cells could be achieved since the *pgip* gene possesses its own signal peptide that is targeted to the plant cell wall. Additionally, the PGIP-HEWL protein may be more stable than that of HEWL existing on its own and is hoped that the end result would be that resistance against both fungal and bacterial diseases would be achieved.

Chapter 2

Literature Review

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Literature Review

2.1 Introduction

Plants have the potential to respond to pathogen attack by employing one of two broad strategies. Firstly, structural and pre-formed chemical barriers such as saponins prevent the pathogen from gaining sustenance from the host (Hammerschmidt, 1999). Secondly, the plant can engage a number of defence mechanisms aimed at limiting pathogen spread. These defence mechanisms include the hypersensitive response (HR), a rapid increase of reactive oxygen species (Bell, 1981), structural barriers (Hammond-Kosack and Jones, 1996) such as the reinforcement of cell walls by cross-linking hydroxyproline-rich glycoproteins (Bradley *et al*., 1992), forming cell wall papillae (Kovats *et al*., 1991) and lignification (Ride, 1980), the expression of a number of genes including those encoding pathogenesis-related (PR) proteins, thionins, defensins, glutathione-S-transferases, proteinase inhibitors such as chitinases and peroxidases and enzymes involved in phytoalexin biosynthesis (Hammond-Kosack and Jones, 1996). Figure 2.1 below depicts the model proposed by Albersheim and co-workers on the role of oligosaccharide and PGIPs in the induction of plant defence responses. The type of responses generated by the plant differs with the specific pathogen that could initiate disease.

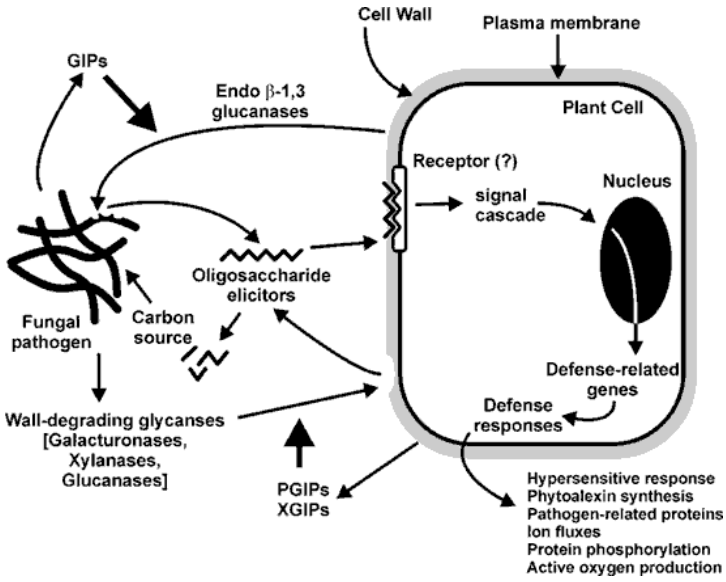

Figure 2.1 Model depicting the role of oligosaccharides and PGIPs in the induction of plant defence response. (Proposed by Albersheim and co-workers, Complex Carbohydrate Research Centre, University of Georgia, USA; www.ccrc.uga.edu/-ao/plapath/Pptext.htm). **GIPs:** endo-β-1,3-gluconase inhibitor proteins; **PGIPs:** polygalacturonase inhibiting proteins. **XGIPs:** other glycanses inhibiting proteins yet to be discovered.

The ability of a plant to respond defensively to attempted invasion by pathogenic microorganisms involves the perception of the presence of the pathogen (recognition), the transmission of this information into infected and to the adjacent plant cells, and the elicitation of a number of biochemical changes that act cooperatively to limit invasion of the pathogen. The threat of pathogens on plants also makes it important that they react in a timely manner with the appropriate defence response upon pathogen recognition in order that an infection does not proceed (Somssich and Hahlbrock, 1988). If the defense reactions occur too late or are suppressed, the infection process will proceed successfully, resulting in disease and often plant death.

Not all plant-pathogen interactions lead to disease. Those that do not lead to disease mostly occur on plants that are not hosts to the pathogen, perhaps because of it lacking the pathogenicity factors to cause disease or because of the plant possessing preformed structural barriers or toxic compounds that confine successful infection to specialised pathogen species, or that upon recognition of the attacking pathogen, defence mechanisms are elaborated and the invasion remains localized. Hence, plant-pathogen interactions are either known as compatible or incompatible (Laugé and De Wit, 1998). Compatible interactions are those that lead to disease due to susceptibility of the plant when being attacked by a virulent pathogen and incompatible interactions are those where the host is resistant to the pathogen.

The survival of the plant depends on its ability to resist marauding pathogens while at the same time, a pathogen's success hinges on its ability to either avoid recognition or to overcome the resistance mechanisms of the plant.

The specificity of plant responses to pathogens can be classified into two broad categories. Non-specific resistance (general, non-host or basic resistance) is a response to all races of a particular pathogen and occurs in all cultivars of a host plant species. In contrast, specific resistance is dependent upon the presence of a particular pathogen race, a particular host plant cultivar, or both. If both pathogen and host specificity are involved, plant disease resistance is termed race-cultivar-specific resistance, since it results only from the interaction of a particular pathogen race with a particular cultivar of the host plant. This type of resistance is usually referred to as gene-for-gene resistance, because in most cases it requires the presence of both a race-specific avirulence (*avr*) gene in the pathogen and one or more corresponding cultivar-specific resistance (*R*) genes in the host plant (Flor, 1971) The underlying genetic

basis of each type of plant disease resistance differs according to the genetic makeup of both plant and pathogen.

Effective and sustained control of bacterial and fungal pathogens is an important issue for all agricultural systems. Bacterial diseases are of high economic importance in many crop plant species including different vegetable species, fruit trees, rice, cotton and grapevine. Furthermore, fungi are continually becoming resistant to existing resistance genes and fungicides and in addition to reducing crop yield; fungal diseases often lower crop quality by producing toxins that affect humans and human health (Rommens and Kishore, 2000).

Plant *R* genes have been used for a long time in breeding for disease control (Rommens and Kishore, 2000). Although originally believed to provide durable resistance, only a few exceptional *R*-genes proved able to control pathogens for an extended period of time. Therefore, the limited durability of single *R*-genes for many of the agronomically important diseases make it necessary to continue the discovery and introgression of new *R*-genes, a process that is extremely time consuming and laborious.

Because of the ongoing losses of the world's crop harvests due to pests and diseases, despite breeding programs that enhance resistance by exploiting endogenous resistance genes, additional methods of disease control are highly desirable. Genetic modification allows a new approach to this unending problem. Besides *R*-gene mediated resistance where genes can be transferred within and across plant species, other foreign proteins or peptides can be expressed in transgenic plants and might introduce new resistance factors or complement the natural mechanisms.

The last decade has witnessed a remarkable increase in our understanding of the molecular events that take place during the response of plants to microbial attack. These exciting developments in molecular plant pathology include the isolation of the first major race-specific resistance genes from plants (Dangl, 1995), progress in understanding the molecular events associate with the hypersensitive response (HR) i.e., the programmed death of challenged host cells producing a visible area of cell death around the site of attempted pathogen invasion, and the establishment of systemic acquired resistance (SAR) i.e., the establishment of immunity to secondary infections in systemic tissues that is long lasting and extends to tissues distant from the initial infection site providing protection against a broad

spectrum of microbial pathogens (Delaney *et al*., 1994, Levine *et al*., 1994 and Ryals *et al*., 1995).

Genetic modification can complement and accelerate breeding programs by introducing resistance genes from diverse sources, such as other plants, bacteria and even animals. Many organisms other than plants produce proteins or small peptides with antimicrobial activity. These compounds either slow down growth of microbes or kill them. Peptides or proteins that have been expressed in different transgenic plants include defensins from plants, cecropins from insects, attacins from insects, lysozyme from chicken or other sources and polygalacturonase inhibiting proteins from plants (Düring, 1996).

To take a step further, the stable introduction of more than one useful gene into a plant genome by transformation, a concept known as "transgene pyramiding", could be the key to the future need of providing transgenic solutions to many complex traits (Bano-Maqbool *et al*., 2001). Strides in that direction would entail the introduction of several genes into a plant that could confer improvements in different traits such as having both antibacterial and antifungal resistance. The introduction of multiple genes could involve either of the following approaches: 1.) the construction of multi-gene transcripts that encode several polypeptides and that are linked as a translational fusions under the control of a single promoter contained on a single T-DNA together with a single selectable marker or 2.) the construction of multi-gene transcripts under the control of its own promoters contained on a single T-DNA with a single selectable marker.

This chapter describes the roles of two unrelated foreign genes i.e., the hen egg white lysozyme (*hewl*) and the bean polygalacturonase inhibitory protein1 (bean *pgip1*) in their participation of offering both antimicrobial and antifungal resistance when being introduced as a translational fusion on a single T-DNA into *N. tabacum* cv. LA Burley 21. More specifically this chapter will firstly give a general overview of each of the said gene products individually before portraying their potential usefulness as a combination when integrated within the genome of crops.

2.2 Lysozymes

Alexander Fleming discovered lysozymes in 1922 while he was suffering from a cold and let some of his nasal secretions fall on a plate of bacteria and later noticed that the bacteria in the vicinity of the mucous from his nose had dissolved. He was able to establish that the

anti-bacterial activity in the mucous was due to an enzyme, the enzyme he called lysozyme because it breaks down the cell walls of bacteria and causes them to lyse. Lysozyme belongs to a large class of enzymes known as glycosidases. These enzymes are extremely efficient catalysts, capable of hydrolysing the glycosidic bond, which is the most stable of the linkages occurring in natural biopolymers (Davies and Henrissat, 1995; Zechel and Withers 2000). Lysozymes (muramidases or peptidoglycan N-acetylmuramoyl-hydrolase) have a specific hydrolytic activity directed against the bacterial cell wall component peptidoglycan that consists of the alternating polysaccharide copolymers of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) or of the homopolymer of GlcNAc (chitin). Most bacteria, with only a few exceptions, are enclosed in this unique crosslinked polymer peptidoglycan that endows the cell wall with enough mechanical strength to withstand the high intracellular pressure (Rogers *et al*., 1980). However, the actions of lysozymes are to cleave at the β(1-4) glycosidic linkages, connecting the C_1 carbon of NAM to the C_4 carbon of NAG. The optimal substrate is a $(NAG\text{-}NAM)_3$ hexasaccharide, with lysozyme cleaving at the NAM_4-O-NAG_5 glycosidic bond. Lysozyme cleavage of the peptidoglycan is shown in Figure 2.2.

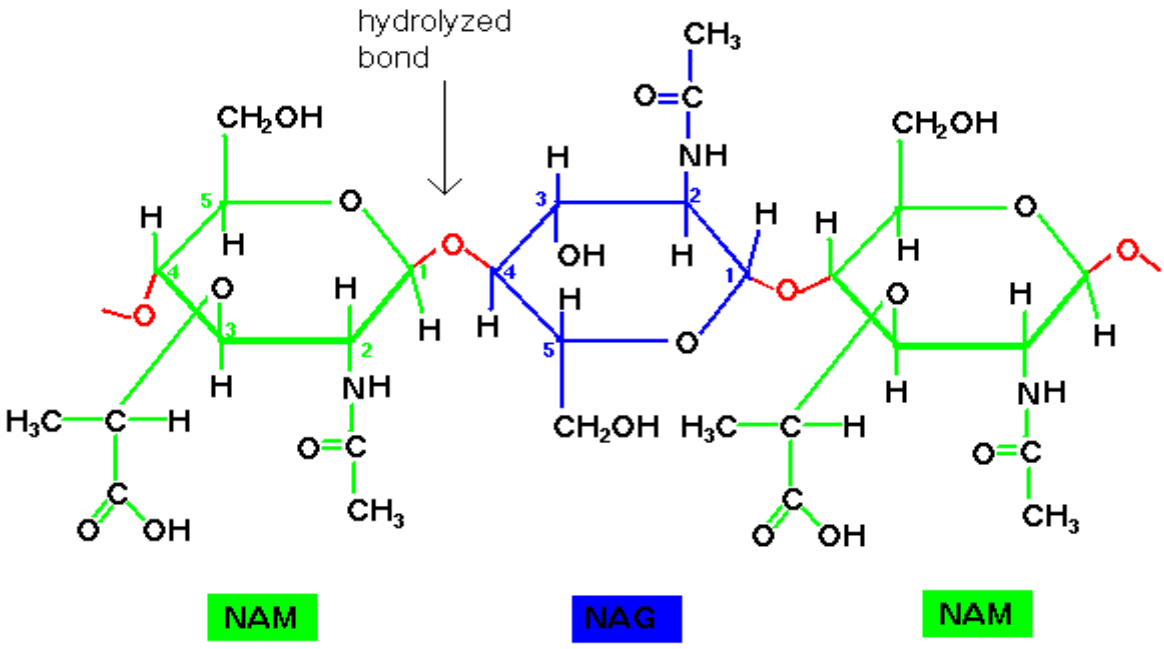

Figure 2.2 Cleavage of peptidoglycan. Lysozyme splits the molecule at the places indicated by the arrow**. (NAM)** N-acetylmuramic acid (**NAG)** N-acetylglucosamine

Lysozymes are widely distributed in nature and their sources include plants (Audy *et al*., 1988; Audy *et al*., 1990; Beintema and van Scheltinga, 1996), birds, mammals, microorganisms and bacteriophages (Fastrez, 1996; Jollès and Jollès, 1984; Prager and Jollès, 1996). The bacteriolytic *in vitro* activity of these different lysozymes is well known for a long time. In birds, lysozyme is exceptionally abundant in the egg whites, while in vertebrates this glycosidase is found mainly in biological secretions (such as tears) where it serves as an anti-bacterial agent by digesting and weakening the rigid bacterial cell wall thereby rendering the bacteria susceptible to osmotic lysis. Bacteriophage genomes encode lysozymes whose role is to favour the release of virions by lysis of the host cells or to facilitate infection (Young, 1992). As for its presence in bacteria where it has the potential to act as a suicidal enzyme, the physiological function is still a matter of debate, although it is clear that they are involved in the metabolism of the bacterial cell wall (Shockman and Holtjie, 1994).

Lysozyme belongs to a supergene family that also includes the genes for lactalbumins and calcium-binding lysozymes (Dautigny *et al*., 1991; Grobler *et al*., 1994; Nitta and Sugai, 1989; Prager and Wilson, 1988). The chicken lysozyme *c* gene from hen egg white (HEWL) was among the first genes to be isolated and characterised in structural and mechanistic studies (Blake *et al*., 1965; Blake *et al.,* 1967; Canfield, 1963); and of all the members of the gene family, it is the gene that has been the most extensively studied at the molecular level (Baldacci *et al*., 1979; Sippel *et al*., 1978).

2.2.1 Bacterial lysozymes

Lysozymes are not only the most powerful antibacterial agents found in various tissues and secretions of higher organisms, it is also present in a number of bacteria where it is referred to as an autolytic enzyme (autolysin). How the bacterial cell controls this dangerous enzyme from premature or unbalanced action remains unknown. The basic structure of the murein of bacterial cells has shown a number of variations in the peptide moieties and the type of crosslinkage that exists for different bacterial species yet the polysaccharide structure is always a poly-(Glc-NAc-β-1.4-MurNAc)$_n$. The only modifications that occur are the presence or absence of O- and /or N-acetylation (Schleifer and Kandler, 1972).

The murein net is tailored into a covalently closed bag-shaped structure, called a sacculus, which completely encloses the cell (Rogers *et al*., 1980). Hence enlargement and division of the murein sacculus is a prerequisite for the propagation of the cell. Since it is difficult to imagine how this could be done without bond breakage in the murein network, it is generally

assumed that both growth of the sacculus and division into two intact daughter sacculi is accomplished by a tightly controlled co-operation of murein polymerising and hydrolysing enzymes. Although not yet experimentally proven, enzymes hydrolysing bonds in the bacterial exoskeleton murein such as lysozyme are believed to be crucial for the growth and division of bacteria (Weidel and Pelzer, 1964).

2.2.2 Phage lysozymes

As previously mentioned for bacteriophage lysozymes, the release of lytic phages from host cells at the end of an infection cycle has been identified as being facilitated by these enzymes. This strategy holds true for larger phages such as λ, T4 and T7 that in contrast to the smaller phages, devote more than one gene to the lysis system. The smaller phages have lysis systems that are lysozyme-independent where a single gene encoding a holin is involved with the destabilization of the cytoplasmic membrane or to form trans-membrane tunnels through which virions are released. Numerous phages have been shown to encode lytic enzymes but the level of understanding of the lytic machinery varies greatly (Fastrez, 1996).

2.2.3 Plant lysozymes

In plants, the involvement of endogenous plant lysozymes in plant-bacteria interactions has not been demonstrated however, because of its enzymatic activity, a participation in plant defence responses seems conceivable.

The first studies on pure plant lysozymes were those on the enzymes of lattices of dicotyledons such as fig (Glazer *et al.*, 1969; Meyer *et al.*, 1946) and papaya (Dahlquist *et al.*, 1969; Howard and Glazer, 1969; Smith *et al.*, 1955), which contained, besides large amounts of proteolytic enzymes, other hydrolases. Lysozyme activity since has then been detected in various plant species such as wheat germ, tobacco, bean, cucumber and potato. However, like most other lysozymes, these plant enzymes also have chitinase activity. Hence, most of the plant lysozymes were found to be bi-functional enzymes displaying a much stronger chitinase than lysozyme function. Nearly all known characterized plant enzymes with lysozyme function are strongly basic proteins and are classified as vacuolar basic chitinases (Düring, 1996; Majeau *et al.*, 1990; Stintzi *et al.*, 1993). However an acidic and a basic lysozyme with strong bacteriolytic activity, which seems to be localized in the intercellular spaces was detected in tobacco (Stintzi *et al.*, 1993). Also shown and found to be quite interesting is that tobacco displays a significant extracellular bacteriolytic potential since

halos of lysed bacteria rapidly appeared upon the growth of tobacco calli on lawns of *Erwinia carotovora* (Düring, 1993).

Even though conceivable, findings of endogenous lysozyme activities are mostly correlated to vacuolar chitinases. This suggests that the involvement of lysozymes in defence reactions against phytopathogenic bacteria growing in the intercellular spaces cannot be expected. Lytic action against these phytopathogenic bacteria can therefore only be exerted after cell disruption and breakage of the vacuole, which occurs when these bacterial cells reach high densities. For this high levels of lysozyme activity are necessary for overcoming the bacterial infection. The criteria of co-incidence in time course and location of pathogen and resistance factor during early stages of the interaction is not met by endogenous lysozymes. The result of this is that its involvement in defence reactions occur rather late thereby allowing the bacteria to invade the plant cell and cause disease.

2.2.4 Animal lysozymes

Two types of higher animal lysozymes have been described. The chicken (*c*)-type represented by the hen egg white lysozyme (HEWL) which was among the first genes to be isolated and characterized which lead to it becoming one of the first model genes for studies of regulation of gene expression (Sippel and Renkawitz, 1989). This lysozyme c from other species such as human, mouse, cow, as well as from two orders of insects have also been extensively studied enzymatically, structurally and functionally with the growth of molecular genetics. The other lysozyme type, known as the goose (*g*)-type, was named after the Embden goose, which is the species in whose egg white it was first discovered by Canfield and Mc Murry, 1967. The g-type lysozyme will not be further discussed here.

2.2.4.1 The *hewl* gene

HEWL, representing one of the best-characterized avian c-type lysozymes, (Trudel *et al*., 1992) contains all twenty of the common amino acids. It is a rather small enzyme comprising of only 129 amino acid residues with a molecular weight of 14 000 kDa. It is characterized by a deep crevice that contains the substrate-binding site and divides the molecule into two domains: one has almost entirely a β-sheet structure and the other is mostly helical in nature. Six sugar units (termed A through to F) of the polysaccharide substrate can be accommodated within the enzyme active site cleft. The cleavage of the glycosidic bond occurs between unit D and E (Bottoni *et al*., 2005). Two or more carboxyl groups of the type c lysozymes, which correspond to the Glu-35 and Asp-52 of HEWL, occur in similar

positions in all c-type lysozymes. These groups are essential for lysozyme activity. A third carboxyl group, which corresponds to Asp-101 is involved in a substrate binding interaction, and occurs in most c-type lysozymes.

The enzyme is encoded by an mRNA of approximately 700 bases (Jung *et al*., 1980) and comprise of an open reading frame of 586 bases and a poly (A) tail. The translation of chicken lysozyme mRNA results in a pre-lysozyme, with an 18-residue amino terminal signal peptide that is removed *in vivo* by a signal peptidase to yield the mature lysozyme c protein (Prager and Jollès, 1996). The final mature secreted HEWL protein consists of 129 amino acid residues (Irwin *et al*., 1996).

The structure of the chicken lysozyme c gene, Figure 2.3, was found to be composed of four exons separated by three introns and to be spread over approximately 3745 bases of genomic DNA (Jung *et al*., 1980). Mature coding sequences were found within all four exons, and the signal peptide was within exon 1. Non-coding (untranslated) sequences were found in both exon 1 (the 5' untranslated sequence of 29 bases) and exon 4 (the 3' untranslated sequence of 113 bases).

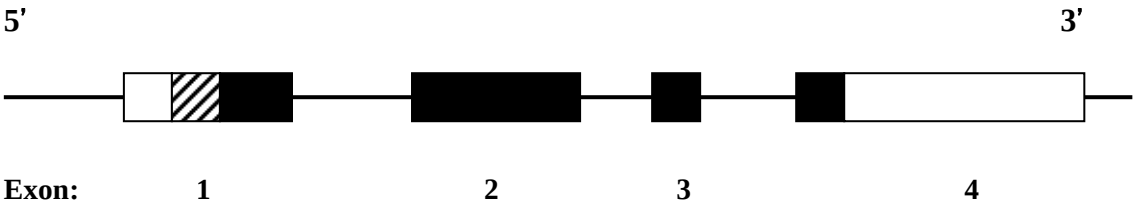

Figure 2.3 Structure of the vertebrate lysozyme c (Jung *et al*., 1980).

Lysozyme genes are made up of four exons separated by three introns. Exons are shown as boxes with the mature coding region shown as solid boxes, signal peptide as the hatched box, and untranslated regions as open boxes. Introns are shown with a thin line, and are in homologous positions in all genes. Exons and introns are not to scale.

2.2.4.2 Uses of HEWL

In addition to its lysozyme and chitinase activity, HEWL shows promising use as a pharmaceutical (Nakumara *et al*., 1990) and preservative for food (Nakumara *et al*., 1990; Proctor and Cunningham, 1988). It has also been evaluated for protection against bacterial contamination of *in vitro* embryo cultures (Eudes *et al*., 1995).

2.2.4.3 HEWL expression in other systems

HEWL expression in systems such as *E. c*oli (Miki *et al*., 1987), *Saccharomyces cerevisiae* (Oberto and Davidson, 1985) and *Aspergillus niger* (Archer *et al.*, 1990) has been reported. Successful expression of HEWL was observed in both *S. cerevisiae* and *A. niger.* Expression in *E. coli* resulted in enzymatically inactive aggregates due to its lack of the proper eukaryotic secretory pathway thus resulting in improper disulphide bond formation that is normally catalysed by the enzyme, protein disulphide isomerase, present in the endoplasmic reticulum.

2.3 Development of resistance strategies against phytopathogenic bacteria in transgenic plants using lysozymes

Because of the moderate knowledge about lysozyme action in plants, exploitation of the endogenous mechanisms for transgenic plants has not yet been performed. However, applications involving the use of foreign lysozymes in transgenic plants have been investigated.

Studies involving the use of foreign lysozymes include applications on the anti-microbial activity of HEWL in transgenic tobacco (Trudel *et al*., 1992 and 1995) and potato (Destfano-Beltran *et al*., 1992), of bacteriophage T4 lysozyme in potato (Düring 1996), and human lysozyme in tobacco (Nakajima *et al*., 1997).

The investigations done by Trudel and co-workers in 1992 involved the use of the full-length *hewl* gene (including its native signal peptide) for enhanced resistance in transgenic tobacco plants. Expression and partial secretion of HEWL to the intercellular spaces has been reported although the native signal peptide was thought not to efficiently direct the foreign lysozyme to the apoplast. However, Trudel and co-workers went on to report recovery of less than 10% of secreted HEWL in the intercellular fluid of transgenic tobacco. They also reported no phenotypic changes due to the expression of HEWL and suggested that HEWL be used as a reporter gene since its quantification involved the use of a simple assay.

Further studies by Trudel and co-workers (1995) on the fate of active HEWL in transgenic tobacco showed that optimising extraction methods for the recovery of HEWL from intercellular fluid (IF) of foliar tissue yielded up to 38% for six successive IF extracts using histamine. The rationale for using histamine in IF extracts was that HEWL could possibly bind to substrate-like plant components that could be competed with known inhibitors (such as histamine) of HEWL activity. The inclusion of 1% histamine allowed the partial

recovery of HEWL (approximately 20% as quantified by the lysoplate assay) when compared to HEWL in a leaf homogenate made in the presence of hot SDS. Other HEWL inhibitors such as N-acetyl-D-glucosamine were tested and results showed that they were not as effective as histamine for recovering both forms of HEWL in IF extracts. Similar results were achieved when using 50 mM $CaCl_2$ where recovery of approximately 10% of HEWL was achieved. The same was true for phosphate buffer at neutral pH and when using 1% (w/v) lysine. Such results indicated that mature secreted HEWL is tightly bound to some tobacco component(s).

Other interesting outcomes of their investigations were the detection of HEWL released around growing tobacco seeds. This was the first report of a potential antimicrobial enzyme in transgenic plant organs that could be released in the extracellular environment. Important to note was the fact that HEWL could then influence the microbial population surrounding growing organs in the soil and since HEWL has the capacity to act as both a lysozyme as well as a chitinase so peptidoglycan containing bacteria and chitin-containing fungi or invertebrates could be inhibited by the release of active HEWL.

After examining the findings of his work with transgenic T4 lysozyme producing potato plants Düring (1996) opined that the native signal peptide might not efficiently direct the foreign protein to the apoplast. Integrating foreign lysozyme genes into transgenic plants and directing these lysozymes to the intercellular spaces through fusion to a signal peptide may be a strategy to meet the requirements of spatial and temporal coincidence of pathogen and resistance protein (Düring, 1996). He suggested a construction of a chimeric gene using a plant signal peptide that might enhance the efficiency of secretion. This was demonstrated in a fusion protein consisting of the signal peptide of barley α-amylase and the bacteriophage T4 lysozyme. Expression of this fusion protein in transgenic potato plants resulted in low-level synthesis of the foreign lysozyme localized in the intercellular spaces of the transgenic plants.

The spectrums of inhibition for the different lysozymes used in these applications differ with regards to the specific pathogen being targeted. There are many plant diseases derived from phytopathogenic bacteria and fungi, and harvest loss caused by these diseases is a serious problem. Transgenic plants expressing the T4 lysozyme were found to be more resistant to Gram-negative bacteria (such as *Erwinia carotovora*) whereas transgenic plants expressing HEWL were more resistant to Gram-positive bacteria (such as *Clavibacter michiganense*) and to some fungi (such as *Fusarium oxysporum*, *Verticillium albo-atrum* and *Rhizoctonia solani*)

(Trudel *et al*., 1995). Transgenic plants expressing both types of lysozymes (T4 against Gram negative bacteria and HEWL against Gram-positive ones) could be useful in enhancing the spectrum of bacteria sensitive to exogenous lysozymes in addition to providing putative resistance to some fungi.

However, besides having good inhibition potential against these Gram negative bacteria, subsequent studies shows evidence of T4 lysozymes in transgenic potatoes showing significantly higher killing activity on root absorbed *B. subtilis* cells, meaning that T4 lysozyme is released from the root epidermis cells and is active in the fluid film on the root surface (Ahrenholtz *et al*., 2000). This implied that the agricultural growth of these potatoes might have harmful effects on soil microbiota as a result of T4 lysozyme release into the rhizosphere.

The evidence from transgenic plants demonstrates that in general lysozymes possess the ability to effectively interact with invading bacteria. In order to exploit this defence capacity optimally, these factors and redirecting highly active lysozymes to the extracellular compartments may convert various susceptible plant-bacteria interactions, which are of agricultural importance, into resistant ones. The achieved quantitative resistances might be sufficiently strong for agricultural practices.

Even so, based on the foregoing, different *hewl* constructs may be designed with and without its native signal peptide to study their expression patterns in transgenic plants. In the search for a gene that could act as a signal peptide for lysozyme to ensure its localization in the intercellular spaces the polygalacturonase-inhibiting protein (PGIP), a cell wall-associated protein was identified.

2.4. Polygalacturonase-inhibiting proteins

Polygalacturonase-inhibiting proteins (PGIPs) are cell wall-associated plant proteins found mostly in dicotyledonous plants and in pectin-rich monocotyledonous plants such as onion and leek. Although structurally related to several resistance gene products already cloned in plants and belonging to the super-family of leucine-rich repeat (LRR) proteins (Mattei *et al*., 2001), PGIP is not an *R*-gene product as its levels are induced as a defence response against non-race specific elicitors (Hammond-Kosack and Jones, 1996). PGIP is a specific, reversible and saturable high affinity receptor for fungal endopolygalacturonases and is therefore capable of inhibiting the activity of these fungal endopolygalacturonases (PGs),

which are thought to play a role in the early stages of plant pathogenesis (De Lorenzo *et al*., 2001).

2.4.1. Fungal endopolygalacturonases

Fungal endopolygalacturonases (endo-PGs) are produced by a large variety of organisms such as bacteria, fungi and plants. They are pectin-degrading enzymes thought to be an important factor in fungal pathogenicity. Endo-PGs are amongst the first enzymes to be secreted by fungal pathogens (English *et al*., 1971) since during fungal pathogenesis the order of enzyme secretion and degradation is meticulously sequenced. The reason for this is that the endo-PGs are responsible for cleaving the α-1-4 glycosidic linkages between galacturonic acids in the homogalacturonan and providing useful substrates that assists other glycosides, cellulases, hemicellulases and pectinases in further degrading the cell wall (Karr and Albersheim, 1970).

2.4.2. The PGIP hypothesis (action)

Complete hydrolyses of homogalacturonan by fungal endo-PGs can be hampered by PGIPs by it modulating the activity of the fungal endo-PGs and favouring the release of elicitor-active oligogalacturonides (De Lorenzo *et al*., 1994). Essentially, oligogalacturonides with a degree of polymerization of four and higher are associated with eliciting active defence responses in plants. PGIP facilitates this occurrence by preventing further PG action (hydrolysis of these existing oligogalacturonides). As mentioned earlier PGIPs have high affinities for PGs and thus form complexes with them to prevent this hydrolysing action. These complexes are formed at an optimum pH of 5.0 and could be dissociated at pH values lower than 4.5 and higher than 6.0 showing that the complex PGIP-PG complex is reversible. Both proteins were shown to maintain their isolelectic points and their catalytic and inhibitory properties after dissociation of the complex, indicating that neither PG nor PGIP are irreversibly modified by formation of the PGIP-PG complex (Cervone *et al*., 1987, 1989). Therefore the hypothesis then is that the complexes formed between the endo-PG and the PGIP leads to more stable oligogalacturonides (Cervone *et al*., 1989; De Lorenzo *et al*., 1990) with elicitor activity accumulating at the site of infection and increasing the elicitation of the plant defence responses (Cervone *et al*., 1987).

2.4.3. Leucine-rich repeats

All PGIPs isolated to date comprise of approximately 10 leucine-rich repeat (LRR) domains. LRR's are versatile structural motifs and are found in many proteins of diverse origin being responsible for protein-protein interactions (De Lorenzo *et al*., 2001). These LRR domains

consist of a modification of 24 amino acids. Leucine residues are regularly spaced in the consensus sequence of GxIOxxLxxLKnLxxLdLSxNxLx, with residues conserved in at least four repeats indicated with capital letters. The x's represents the non-conserved residues and the lower case letters represents the residues identical in five or less repeats. The LRR domains match the extracytoplasmic consensus also found in R genes that participate in gene-for-gene resistance.

In plants, LRR proteins play an important role in both development and defence, where specificity of recognition is a fundamental prerequisite. Although PGIP is not directly involved in pathogen recognition, it does bind to specific fungal endo-PGs (Stotz *et al*., 2000). It is one of the few plant LRR proteins for which the ligand is known (Leckie *et al*., 1999). The proposal is that PGIP is the secreted receptor component of the cell-surface signalling system involved in the recognition event between the plant and fungi and it is this LRR domain of PGIP that may be required for interaction with and inhibition of fungal PGs (Stotz *et al*., 1994).

2.4.4. Isolation of bean *pgip1* gene

The first isolation of the gene encoding bean *pgip1* was cloned from a genomic library of *P. vulgaris* cv. Pinto (Toubart *et al*., 1992). A set of degenerate primers were designed from parts of the amino acid sequence and were used to amplify part of the coding sequence for PGIP from the total DNA isolated from *P. vulgaris* cv. Pinto. This partial PGIP sequence consisting of 0.758 kb was then used as a hybridisation probe to screen the genomic library of *P. vulgaris* cv. Pinto and *P. vulgaris* cv. Saxa where a full length PGIP gene was detected. The nucleotide and deduced amino acid sequences of the *pgip* genes from the two different cultivars shared 96.4% and 97.4% identify, respectively. The differences observed could be due to the difference in cultivar (Toubart *et al*., 1992).

In an independent study to determine whether bean PGIP could inhibit the maize pathogen *Stenocarpella maydis*, the isolation of the gene encoding for bean PGIP1 was subsequently achieved by PCR amplification of the genomic DNA from *P. vulgaris* L. cv. Wintergreen (Berger *et al*., 2000). A specific set of primers was designed that incorporated an *Xba*I site in the left primer ($PGIP_L$) and a *Sac*I site in the right primer ($PGIP_R$) and was used to amplify the bean *pgip1* gene from the genomic DNA isolated from *P. vulgaris* L. cv. Wintergreen. The restriction enzyme sites *Xba*I and *Sac*I were incorporated into the primers to accommodate the

subsequent cloning events of the bean *pgip1* gene in the specific pBI221 vector. The newly constructed vector was called pLD1.

2.4.5. The gene structure of bean *pgip*1

Bean PGIP1 is synthesized as a precursor of 345 amino acids of which the first 29 amino acids constitutes a highly hydrophobic signal peptide (De Lorenzo and Cervone, 1997). The potential cleavage site (Ala-Leu-Ser) for the signal peptidase is conserved in all PGIPs (Yao *et al*., 1999). The signal peptide targets the PGIP through the endomembrane system (Von Heintjie, 1985) for its translocation across endoplasmic reticular membranes (De Lorenzo *et al*., 2001). This is consistent with the proposed cell wall localisation of PGIP (Abu-Goukh *et al*., 1983), the observation that PGIP is in the extracellular matrix in bean hypocotyls and that it is secreted into the medium by suspension-cultured bean cells (Salvi *et al*., 1990).

10 M S S S L S I I L V I L V S L R T A H S

Figure 2.4 Amino acid sequence of bean PGIP1 signal peptide (Leckie *et al*., 1999).

The mature PGIP polypeptide consists of three domains, namely the central LRR region, and two cysteine-rich flanking regions (Mattei *et al*., 2001). The protein has a molecular mass of 34 kDa and contains several basic amino acids (lysines and arginines) that confer a high isoelectric point to the protein (Toubart *et al*., 1992) allowing interaction of its basic nature with the acidic pectins in the cell wall matrix (Johnston *et al*., 1993).

2.4.6. The specificity of PGIP inhibition

PGs are produced by almost all phytopathogenic fungi and exhibit a variety of isoenzymatic forms, differing in terms of stability, specific activity, pH optimum, substrate preference and types of oligosaccharides released. Plants have evolved different PGIPs with specific recognition capabilities against the many PGs produced by fungi. Bean PGIP is significantly more effective against PGs of *Colletotrichum lindemuthianum* than against the PGs of the related pathogen of cucurbits *C. lagenarium* (Lafitte *et al*., 1984). This observation suggests that a PGIP is more effective against a pathogen of the plant from which it is derived.

The different PGIPs from the same plant source also have been shown to have different specificities towards PGs from different fungi (De Lorenzo and Cervone, 1997). Although

two bean PGIPs showed nearly identical biochemical characteristics their inhibitory activity were shown to be quite distinct. Desiderio *et al*., (1997) demonstrated that PGIP1 from bean showed different inhibitory activities against PGs from *Fusarium moniliforme* and *Botrytis cinerea* than the bulk bean PGIP. The bulk bean PGIP could interact with and completely inhibit the PGs from both *F. moniliforme* and *B. cinerea*. The product of the *pgip1* gene exhibited different specificities to the purified bulk bean PGIP when expressed in both transgenic tomato and tobacco plants. PGIP1 was unable to interact with and inhibit a PG from *F. moniliforme* and had limited inhibition activity against a PG from *B. cinerea* (Desiderio *et al*., 1997).

These observations were further supported with the isolation of two *pgip* genes with different specificities from bean cDNA library (Leckie *et al*., 1998). They showed that when expressed in tobacco plant, PGIP2 is able to interact with and inhibit PGs from *A. niger* and *F. moniliforme* whereas PGIP1 could only interact with and inhibit PGs from *A. niger*. In another experiment by Berger *et al*., (2000), PGIP1 was shown to inhibit PGs from *Stenocarpella maydis*. The two proteins differ in eight amino acids, three of which are thought to be involved with binding of the ligand (PGs) (Leckie *et al*., 1998). Speculations of Frediani *et al*., (1993) that multiple PGIP forms with different functions exist in *P. vulgaris,* were thus supported. It is then concluded that the sum of different PGIPs with different specificities enables the bulk bean PGIP to inhibit a wide spectrum of fungal PGs (Desiderio *et al.,* 1997).

2.4.7. Uses of PGIP in developing disease resistant crops

Studies to determine whether inhibition against the pectic enzymes from microbial or plant origin were possible revealed that PGIP from *P. vulgaris* could only inhibit endopolygalacturonases from fungi (Brown and Adikaram, 1983; Hoffman and Turner, 1982, 1984; Johnston *et al*., 1993). More specifically it completely inhibited the endo-PGs purified from *A. niger, F. moniliforme,* three races of *C. lindemuthianum* and three isoforms of the endo-PGs purified from *A. japonicus*. Discoverers of PGIP in immature raspberry fruit proposed to use this gene in breeding programs to enhance the disease resistance of ripe fruit against *B. cinerea* (Williamson *et al*., 1993). To follow on from this proposal, studies on the development of genetically modified crops are ongoing. More specifically studies involving the production of plants transformed with a variety of *pgip* genes are in progress. From previous studies showing the spectrum of organisms that the bean PGIP1 is active against, the developing a bean PGIP1 transgenic plant is conceivable.

Another application for PGIP in developing disease resistance crops could be to use PGIP as a signal to target enzymes that play a vital role in plant defence to the site (cell wall) where it could function almost immediately after the recognition of a foreign body. In doing so, plants could confer dual resistance, meaning that transgenic plants could confer resistance to two unrelated groups of organisms. To elaborate further, one gene could confer resistance to specific fungi and another could confer resistance to specific bacteria that are common pathogens to a particular crop.

In this study a totally unrelated gene such as the *hewl* is fused to the bean *pgip1* gene and subsequently transformed into tobacco. The study aims to determine bean *pgip1* as a viable gene to transform into a crop to first confer resistance to fungi and secondly to determine whether PGIP, since it is localised in the cell wall, could support the movement of an independent enzyme (bound to PGIP through a translational fusion) from their original localisation within the plant to the site (cell wall) where defence against infection is required.

This chapter provided some background information to the roles of lysozyme and PGIP in the defence against fungal and bacterial pathogens. It also reviewed the different roles of endogenous lysozymes within a particular host, with most emphasis placed on the lysozyme isolated from hen egg white. A brief overview of PGIPs, with emphasis on the isolation and characterization of bean PGIP was presented.

CHAPTER 3

The subcloning of the bean *pgip1-hewl* fusion cassette into pCAMBIA2300

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3.1 Introduction

This chapter describes the construction of the plant transformation vectors that would harbour the target genes to be stably integrated into the genomic DNA (genomic DNA) of *Nicotiana tabacum* cv. LA Burley 21. It involves the subcloning of the bean *pgip1–hewl* fusion gene as well as the *hewl* gene containing its own signal peptide, into the plant transformation vector pCAMBIA2300. A third construct (plant transformation vector, pGA482-*pgip1*), previously cloned at ARC-Roodeplaat by P. Fourie, is incorporated along with the two novel constructs in the transformation of the *N. tabacum* cv. LA Burley 21 plant for completeness of the study. This construct will contribute the positive control when demonstrating expression of the bean *pgip1* gene and the negative control when demonstrating expression of the *hewl* gene (Chapter 5). The novel plant transformation vector possessing the full-length *hewl* gene (mentioned above) is to play a role as a positive control during expression studies of the *hewl* gene and the negative control during bean *pgip1* expression studies (Chapter 5). Also described here is the transformation of *Agrobacterium tumefaciens* with the newly constructed binary vectors for subsequent *N. tabacum* cv. LA Burley 21 transformation.

3.1.1 Construction of intermediate plasmids

The construction of the plasmids from which the expression cassettes are to be used for the subcloning of the plant transformation vector pCAMBIA2300 had previously been cloned at ARC-Roodeplaat. A brief description of these cloning events is given below and is supported by a cloning strategy provided by Dr. M. Cloete, ARC-Roodeplaat depicted in Figure 3.1.

3.1.1.1 pMOS-*hewl* containing the *hewl* gene without its signal peptide

The *hewl* gene (0.393 kb) lacking its signal peptide was amplified from pHEWL [pUC19 containing a cloned copy of the *hewl* gene (Langenhoven, 1999)] with specifically designed primers to aid in subsequent cloning events. This PCR fragment was subcloned into the blunt ended cloning vector pMOS*Blue* (Amersham International plc, Little Chalfont, UK) and re-named pMOS-*hewl* (Kaimoyo and Cloete, 1999, unpublished). This vector was designed to provide the *hewl* gene in the construction of the bean *pgip1–hewl* gene fusion.

3.1.1.2 pRTL2 containing the gene fusion (bean *pgip1–hewl*)

The gene fusion was constructed in two steps. In the first step, the bean *pgip1* gene (1.042 kb) lacking its stop codon was amplified from pLD1 [pBI221 (Clontech Laboratories, Inc., USA) containing the cloned copy of the bean *pgip1* gene (Berger *et al*., 2000)] with specifically designed primers that included the appropriate restriction sites for subsequent cloning. This PCR fragment was subcloned as an *Nco*I/*Kpn*I fragment into pRTL2 [pUC19 containing the dual enhanced 35S Cauliflower Mosaic Virus (CaMV) promoter, the *N. tabacum* cv. LA Burley 21 etch virus translational leader and the CaMV 35S terminator elements (Restrepo *et al*., 1990)] and named pRTL2-bean *pgip1* (Kaimoyo and Cloete, 1999, unpublished).

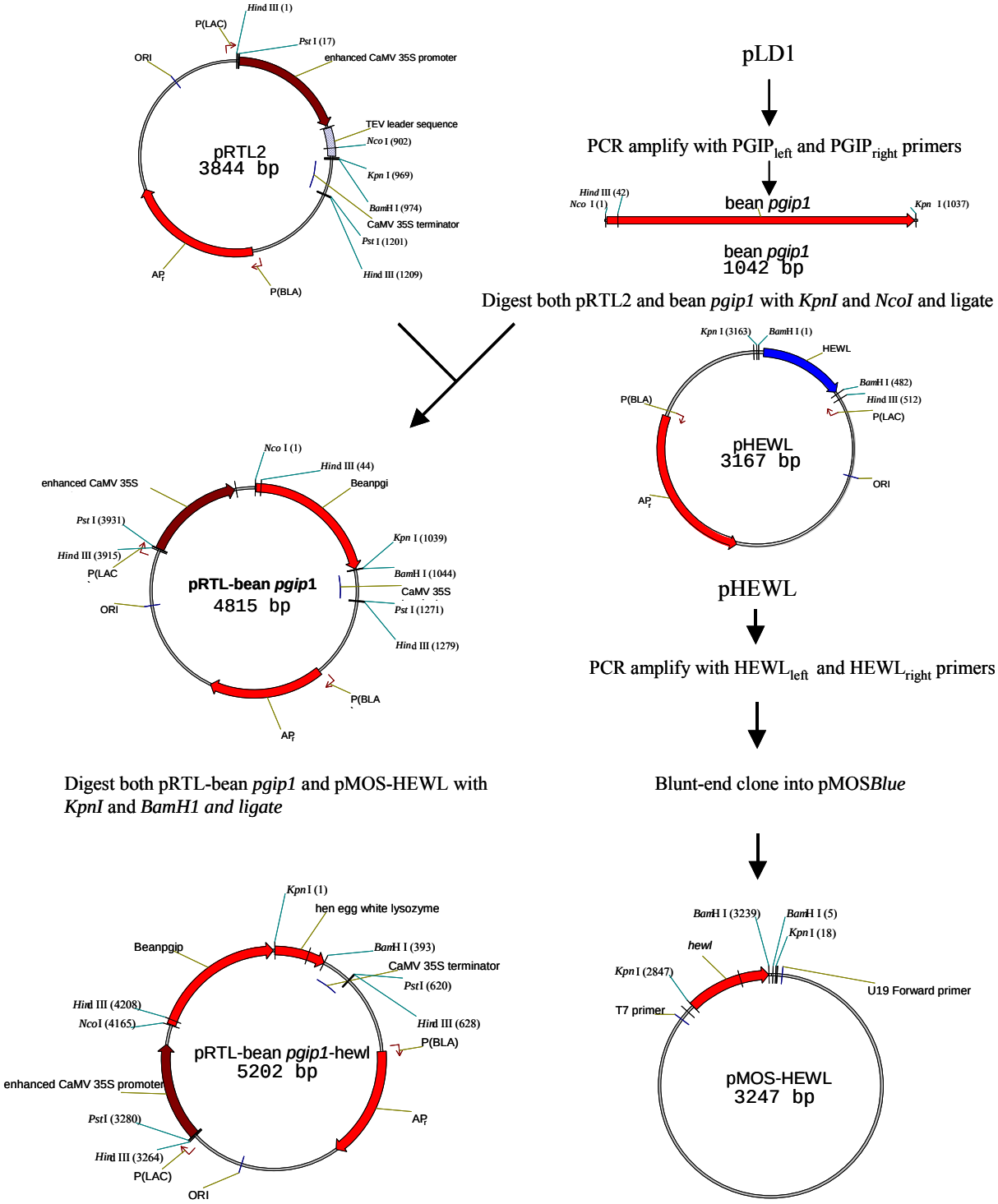


Figure 3.1. Presentation of the main cloning strategy used to clone the bean *pgip1* and *hewl* genes into pRTL2, generated with the aid of the Vector NTI program. The final step to produce pRTL2-bean *pgip1-hewl* was carried out with an intermediate step using a stuffer fragment as shown in Figure. 3.2. pRTL2-bean *pgip1-hewl* vector shows the position of the two genes in the cassette.

The second step, Figure 3.2, involved the cloning of a stuffer fragment from the Ornithogalum Mosaic Virus (OrMV) coat protein (OMV CP) gene (0.779 kb) cloned in pA7 [pBI221 (Clontech Laboratories Inc., USA) containing the O*rMV CP* gene as a *Bam*HI fragment (Burger et al., 1990) into the *Bam*HI restriction site of pRTL2-bean *pgip1*. The reason for this is that the *Kpn*I and the *Bam*HI restrictions sites were too closely located and that the use of two enzymes with restriction sites that are directly adjacent to one another reduces the efficiency of cleavage of the second site after the first site has been cleaved (Sambrook *et al*., 1989). The resulting construct was named pRTL2-bean *pgip1*$_{mod}$. This modification step does not appear in Figure 3.1, since at the time of the strategy design this information was unknown. This modified construct ensured effective restriction digestion with *Bam*HI of the previously *Kpn*I linearised pRTL2-bean *pgip1*$_{mod}$ to produce a linearised pRTL2-bean *pgip1* with *Bam*HI/*Kpn*I ends. This was the vector used into which the *hewl* gene lacking its signal peptide, excised from pMOS-*hewl* as a *Bam*HI/*Kpn*I fragment, was subcloned. The resulting construct was named pRTL2-bean *pgip1–hewl* (Ogundiwin and Cloete, 2000, unpublished).

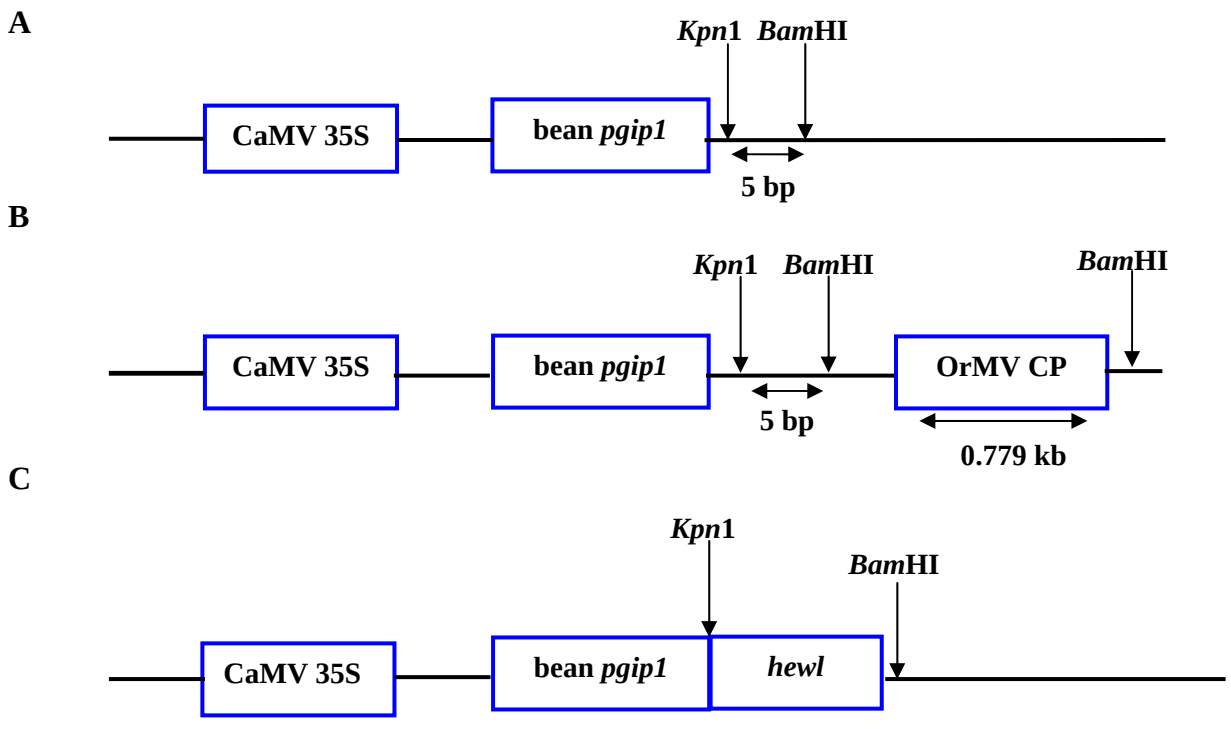


Figure 3.2 A schematic representation of expression cassettes to illustrate the cloning strategy employed for developing pRTL2-bean *pgip1-hewl*. A. pRTL2-bean *pgip1* with 5 base pairs between *Bam*HI and *Kpn*I sites. **B.** pRTL2-bean *pgip1*$_{mod}$ with *Bam*HI fragment from pA7. **C.** pRTL2-bean *pgip1-hewl* with one site each for *Bam*HI and *Kpn*I. OrMV CP: Ornithogalum Mosaic Virus Coat Protein.

3.1.1.3 pRTL2 containing the *hewl* gene with its signal peptide

The *hewl* gene containing its signal peptide was amplified from pHEWL (Langenhoven, 1999) with specifically designed primers containing the appropriate restriction sites for subcloning into pRTL2 as an *Nco*I/*Bam*HI fragment. The resulting construct was named pRTL2-*hewl*$_{signal}$. This vector was designed to act as the positive control for determining the presence and activity of the *hewl* gene during subsequent molecular analysis and expression studies of putative transgenic *N. tabacum* cv. LA Burley 21 plants (Kaimoyo and Cloete, 1999, unpublished).

3.1.2 Construction of plant transformation binary vectors

The two plant transformation systems currently used are the co-integrate vector system and the binary vector system. The binary vector system has been employed in this study because the *in vitro* or *in vivo* manipulations of large co-integrative Ti plasmids are much more difficult to achieve than that of the *in vitro* manipulations of small binary vectors. To facilitate immediate transfer into plant cells, the binary vector system consists of two autonomously replicating plasmids within *A. tumefaciens:* a binary vector containing the target gene between T-DNA borders that is equipped to be replicated in both *E. coli* and *Agrobacterium*, and a helper Ti plasmid that lacks the T-DNA and 25 bp border repeat sequences but rather provides the *vir* gene products that facilitates transfer of the T-DNA to the plant genome (Rogers *et al*., 1992). Only the T-DNA is eventually transferred into the plant genome.

3.1.2.1 pGA482 containing the bean *pgip1* gene

The cloning of the bean *pgip1* gene into pGA482 (An *et al*., 1992) involved two steps. The first step included the excision of the bean *pgip1* gene containing its stop codon from pLD1 (Berger *et al*., 2000) by restriction enzyme digestion using specific enzymes that enabled cloning of the gene into pRTL2 (Figure 3.2). Once purified the gene was placed under the control of the dual enhanced 35S Cauliflower Mosaic Virus (CaMV) promoter and ligated between the *N. tabacum* cv. LA Burley 21 etch virus translational leader and the CaMV 35S terminator elements and named pRTL2-8 (Figure 3.2). In the second step the expression cassette of pRTL2-8 was excised by restriction enzyme digestion, purified and cloned into pGA482 as shown in Figure 3.3 by P. Fourie, ARC-Roodeplaat. This vector was designed to act as the positive control for determining the presence and activity of bean *pgip1* during subsequent molecular analysis and expression studies of putative transgenic *N. tabacum* cv. LA Burley 21 plants.

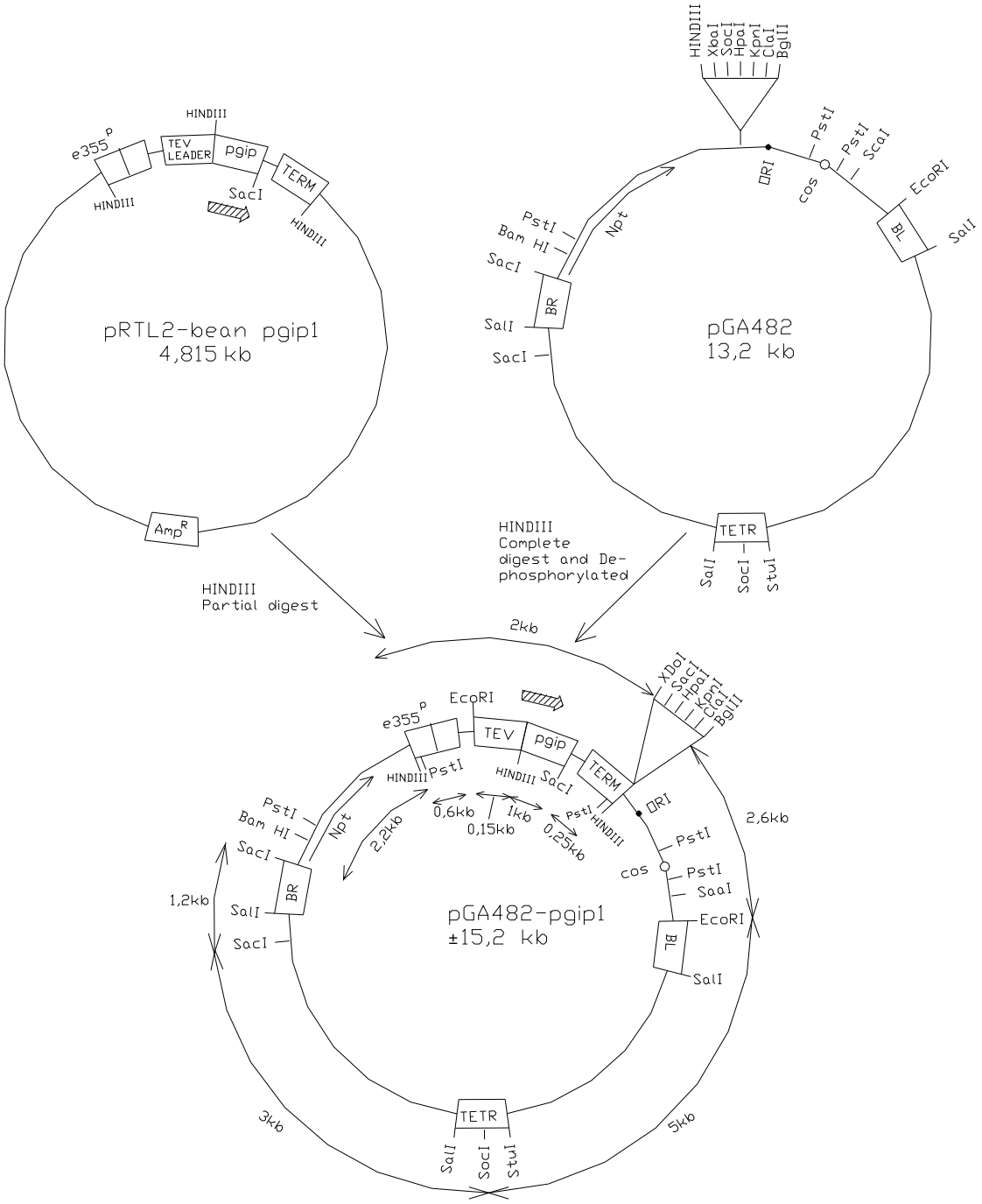


Figure 3.3 Cloning of bean *pgip1* into pGA482.

3.1.2.2 pCAMBIA2300 containing either the bean *pgip1–hewl* gene fusion or the $hewl_{signal}$ expression cassettes

pCAMBIA2300 (www.cambia.org) contains the neomycin phosphotransferaseII (*nptII*) gene encoding kanamycin resistance under the control of the plant expression signals situated between the T-DNA borders. This feature enables selection of transformed plants on kanamycin containing media. The binary vector also has a unique *Pst*I site within a multiple cloning site situated between the right and left border sequences that allows the cloning of the two expression cassettes i.e., bean *pgip1-hewl* and $hewl_{signal}$. The ability to construct recombinant molecules by inserting foreign DNA into a vector is simplified by making use of restriction enzymes. Restriction endonucleases are highly specific enzymes that cleave double-stranded DNA in a sequence dependant manner. These enzymes cut DNA by making two single stranded breaks, one in each strand. There are two distinct arrangements of these breaks namely sticky ends or blunt ends.

This chapter describes the cloning of the bean *pgip1–hewl* gene fusion and the $hewl_{signal}$ expression cassettes into pCAMBIA2300. The genes were excised from pRTL2 by restriction enzyme digestion with *Pst*I to accommodate ligation into the *Pst*I restriction site of pCAMBIA2300.

3.2 Materials and Methods

All chemicals and reagents used were either analytical or molecular biology grade. Buffers, solutions, and media were all prepared using distilled water and either autoclaved or filter-sterilized through 0.2µm sterile syringe filters. All buffers, solutions, and media used in this study are outlined in **Appendix A**.

Restriction endonucleases, RNase A, and antibiotics were obtained from Roche Diagnostics GmbH, Germany.

All Polymerase Chain Reactions were conducted in 0.2 ml thin-walled tubes in a MJ Research PTC-100 Peltier Thermal Cycler (MJ Research, USA).

All plasmid maps are shown in **Appendix B.**

3.2.1 Verification of the bean *pgip1–hewl* fusion cassette in pRTL2

3.2.1.1 Plasmid DNA isolation

Mini preparations of plasmid DNA from *E. coli* were done using the Qiagen Mini plasmid purification kit and larger preparations were done using the Qiagen Midi plasmid purification kit (Qiagen, Germany) according to the manufacturer's instructions. Qiagen plasmid purification is based on the optimised alkaline lysis method of Birnboin and Doly, 1979. The DNA is purified by selective absorption to a silica column in the presence of a chaotropic salt. For midi preparations the starter culture of 5 ml was diluted 1/500 into Luria Bertani (LB) liquid medium and the cells allowed reaching a cell density of 3-4x 10^9 cells per ml which typically corresponds to a pellet weight of approximately 3 g/litre medium.

For screening of possible recombinants, mini preparations of plasmid DNA were done by a modified alkaline lysis method (Sambrook *et al.,* 1989). A single bacterial colony was grown overnight at 37°C with shaking in 5 ml LB liquid medium containing the appropriate antibiotic (100 µg/ml ampicilin for the pRTL2 constructs and 50µg/ml kanamycin for pCAMBIA2300 constructs). The cells were harvested by centrifugation at 6500xg for 15 min at 4°C and resuspended in 200 µl Solution I (**Appendix A**), containing 10 mg/ml RNase A (**Appendix A**). The cells were lysed by the addition of 400 µl Solution II (**Appendix A**) and

incubated on ice for 5 min. The sample was neutralised by the addition of 300 µl chilled Solution III (**Appendix A**) and incubated on ice for 15 min. The debris was pelleted by centrifugation at 6500xg for 20 min at 4°C and the supernatant containing plasmid DNA promptly removed. The DNA was precipitated with 750 µl isopropanol and pelleted at 6500xg for 30 min at 4°C. The DNA pellet was washed with 70% ethanol and re-pelleted. After air-drying, the DNA was resuspended in 30 µl 1x TE buffer (pH 8.0) or 10 mM Tris (pH 8.5).

Plasmid DNA concentration was determined fluorometrically on a Hoefer™ DyNA Quant™ 200 (Hoefer, San Fransisco, USA) according to the manufacturer's instructions. The fluorometer was calibrated with 1x TNE buffer, pH 7.4, containing 0.2 µg/ml Hoechst H33258 DNA binding dye (Hoefer, San Fransisco, USA). Calf thymus DNA was used as a DNA standard.

3.2.1.2 Restriction enzyme digestion of plasmid DNA

All restriction enzyme digestions were carried out as described by Sambrook *et al.*, (1989). For verification of plasmid DNA integrity, 500 ng of plasmid DNA was digested with approximately 3U of the appropriate enzyme and 1x of the restriction buffer in a total volume of 15 µl. Where two different enzymes requiring different buffering conditions were used, buffer changes were done using 0.025 µM membrane filters (Millipore, USA). The reactions were incubated at 37°C for 1 h 30 min and an aliquot of the digests were analysed on a 1% (w/v) agarose gel. Recognition sites for the restriction enzymes used in this study are provided in Table 3.1.

Table 3.1. The recognition sequences of restriction endonucleases

Enzyme	Organism	Recognition sequence
*Nco*I	*Nocardia corralina*	C↓TGCAG GACGT↑C
*Bam*HI	*Bacillus amyloliquefaciens*	G↓G ATCC CCTAG↑G
*Kpn*I	*Klebsiela pneumonia*	GGTAC↓C C↑CATGG
*Pst*I	*Providencia stuartii*	CTGCA↓G G↑ACGTC

3.2.1.3 Nucleotide sequence analyses

To ascertain that the correct sequences have been cloned into the pRTL2 vector, the clones, pRTL2-bean *pgip1-hewl* and pRTL2-$hewl_{signal}$, were subjected to automated sequencing at the University of Stellenbosch, Department of Genetics. Sequencing of pRTL2-bean *pgip1-hewl* was done using pBI121, $IPGIP_L$, $IPGIP_R$, $PGIP_R$ $HEWL_L$, $HEWL_S$, $HEWL_R$ and $M13_F$ sequencing primers and pRTL2-$hewl_{signal}$ using pBI121, $HEWL_S$ and $M13_F$ sequencing primers. All primers used for sequencing are given in Table 3.2. Sequences of the expression cassettes for pRTL2-bean *pgip1-hewl* and pRTL2-$hewl_{signal}$ were analysed and aligned with its computer generated ORF sequences using the computer software *Genepro Version 6.1* (Riverside Scientific Enterprises, WA, USA).

Table 3.2. Characteristics of primers used for sequencing and PCR amplifications

Primer	Sequence 5'-3'	Length	Tm[a](°C)
$HEWL_L$	CATAGGTACCAAAGTCTTTGGACGATGTC	29	84
$HEWL_R$	TAATGGATCCTCACAGCCGGCAGCC	25	80
$HEWL_S$	TCTGTAGGATTCCGTAGTCG	20	60
$PGIP_R$	GCATGGTACCAGTGCAGGAAGGAAGAGG	28	88
$IPGIP_L$	CGAATTCC(C/A)GC(C/T)AT(C/T)GC(T/C)AA(A/G)CT	24	ND[b]
$IPGIP_R$	CAGGATCC(C/T)TC(C/G)AGC(A/T)TGTT(C/A)CGA	24	ND
PBI121	GACGCACAATCCCACTATCC	20	40
$M13_F$	GTTTTCCCAGTCACGAC	17	52
$Hewl_{signal}$	GCATCCATGGTCAGGTCTTTGCTAATC	27	80
$Hewl_R$	CATAGGTACCAAAGTCTTTGGACGATGTC	29	84
$IPGIP_L$	CGAATTCC(C/A)GC(C/T)AT(C/T)GC(T/C)AA(A/G)CT	24	ND[b]
$NPTII_{left}$	GAGGCTATTCGGCTATGACTG	21	64
$NPTII_{right}$	ATCGGGAGCGGCGATACCGTA	21	68
BPG_{left}	CACCGACACCCAAACATATC	20	60
BPG_{right}	GCAGGAAGGAAGAGGAGAAC	20	60
$HEWL_{sig}$	TGGTCAGGTCTTTGCTAATC	20	60
$HEWL_{right}$	CATCGCTGACGATCTTCTTC	20	60
MIA_{Fwd}	CCGGCCCGACGGCAAGCGGC	20	72
MIA_{Rev}	CGGCTGGATGCGCGTCCAG	20	72

a-Tm calculated using formula: Tm=4(GC)+2(AT)

b-ND, not determined

3.2.2 Cloning of bean *pgip1-hewl* and *hewl$_{signal}$* expression cassettes into the plant transformation vector

3.2.2.1 Excision of the bean *pgip 1-hewl* and *hewl$_{signal}$* expression cassettes

To clone the expression cassettes into the *Pst*I site of pCAMBIA2300, pRTL2-bean *pgip1-hewl* and pRTL2-*hewl$_{signal}$* had to be digested with *Pst*I. For each restriction digestion, 10 µg of DNA was used with approximately 40U of *Pst*I and the appropriate 1x restriction buffer in a final volume of 100 µl. Digestions were done at 37°C for 1 h 30 min. An aliquot of each digest was analysed on a 1% (w/v) agarose gel.

3.2.2.2 Purification of DNA from agarose gels

Restriction enzyme digested DNA was run on a 1% (w/v) agarose gel. The fragment of interest was excised from the gel under Ultraviolet light (350 nm) using a sterile scalpel blade. The weight of the gel slice was determined and the DNA recovered from the gel using the QIAquick Gel Extraction Kit (Qiagen, Germany) as described by the manufacturers. DNA concentrations were estimated by co-electrophoresis with Lambda DNA (Life Technologies Inc., Gaithersburg, MD, USA) on an agarose gel. This was done by the comparison of the DNA band intensity of the sample to the intensity of the DNA bands of λ DNA of increasing concentrations ranging from 5-80 ng/µl.

3.2.2.3 Restriction enzyme digestion of pCAMBIA2300

The pCAMBIA2300 vector was linearised by restriction enzyme digestion with *Pst*I. For each restriction digestion, 10 µg of DNA was used with approximately 40U of *Pst*I and the appropriate 1x restriction buffer in a final volume of 100 µl. The digestion was done at 37°C for 1 h 30 min. An aliquot of the digest was analysed on a 1% (w/v) agarose gel.

3.2.2.4 Dephosphorylation of linearised pCAMBIA2300

Prior to dephosphorylation of pCAMBIA2300, the restriction enzyme used in the digestion reaction was inactivated by incubating the reaction tube at 65°C for 15 min and the restriction buffer extracted using a 0.025µM membrane filter (Millipore, USA). The linearised vector was dephosphorylated using calf thymus alkaline phosphatase (Roche Diagnostics GmbH, Germany) to prevent recircularisation in subsequent ligation reactions. The dephosphorylation reaction contained 10 µg linearised vector, 5 µl phosphatase reaction buffer (supplied with the enzyme), 3 µl dH_2O and 4 µl phosphatase (1U/µl). The reaction was incubated at 37°C for 30

min after which the enzyme was inactivated at 65°C for 15 min. Linearised dephosphorylated vector was then purified as described in 3.2.2.2.

3.2.2.5 Cloning of the expression cassettes into pCAMBIA2300

The 2.542 kb expression cassette containing the bean *pgip1-hewl* gene and the 1.561 kb expression cassette containing the $hewl_{signal}$ gene were ligated to the linearised pCAMBIA2300 vector in a ligation reaction set at 5:1 insert-to-vector ratio. Two 10 µl ligation reactions were prepared containing 80 ng pCAMBIA2300 with the different inserts of 116,5 ng for the *pgip1-hewl* cassette and 148 ng for the $hewl_{signal}$ cassette, 1x ligase buffer, 2.5U T4 DNA ligase and was made up to a final volume of 10 µl with sterile dH_2O. The amount of insert DNA was calculated as follows:

$$\frac{insert\,(bp) \times vector\,(ng)}{size\,vector} \times \frac{insert}{vector}$$

Two ligation controls were included. First, a control ligation reaction was included in which the insert and the T4 DNA ligase were omitted to determine whether uncut vector DNA was present in the digested pCAMBIA2300 preparation. The presence of uncut vector would result in background colonies in subsequent transformation events. Second, a control ligation was included in which insert was omitted to determine whether the vector re-ligated and formed background colonies. The ligation reactions were incubated at 16°C overnight after which it was stored at 4°C.

3.2.2.6 Preparation of competent *Escherichia coli* DH5α cells

Competent *E. coli* DH5α cells were prepared according to the Ca^{2+}/Mn^{2+} method (Sambrook *et al*., 1989). A single colony of *E. coli* DH5α cells was inoculated into 25 ml 2x LB medium containing 0.2% sterile glucose and allowed to grow overnight at 37°C with shaking. One millilitre of the overnight culture was inoculated into 100 ml preheated 2x LB medium and grown for 1h at 37°C with shaking. Incubation of the culture for another 35 min continued with the addition of 1 ml sterile 2 M $MgCl_2$ followed by quick cooling and incubation on ice for 1 h 30 min. The cells were pelleted by centrifugation at 2000xg for 5 min at 4°C in 40 ml sterile centrifuge tubes. The supernatant was discarded and the pellets incubated on ice for 1 h 30 min once resuspended in 50 ml ice-cold Ca^{2+}/Mn^{2+} solution (40 mM NaOAc, 100 mM $CaCl_2$, 70 mM $MnCl_2.4H_2O$; pH 5.5 and filter sterilized). Cells

were pelleted by centrifugation at 700xg for 5 min at 4°C and resuspended in 3 ml ice-cold Ca^{2+}/Mn^{2+} solution containing 15% (v/v) sterile glycerol and kept on ice. These resuspended cells were pooled, aliquotted in 100 µl quantities into pre-chilled sterile 1.5 ml Eppendorf tubes and frozen in liquid nitrogen for storage at -70°C.

3.2.2.7 Transformation of ligation reaction into *Escherichia coli* DH5α

Five microlitres of each ligation reaction was added to 100 µl of competent cells and incubated on ice for 10 min. The cells were then heat-shocked at 37°C for 1 min followed by the addition of 1 ml LB liquid media and incubated at 37°C for 1 h. A transformation control in which 100 µl competent cells were transformed with 1ng of $pBSSK^+$ DNA was included to determine the transformation efficiency of the competent cells. 150 µl aliquots of the transformed cells were spread onto LB agar plates containing 50 µg/ml kanamycin. The plates were incubated inverted at 37°C overnight. Each sample was duplicated (Sambrook *et al*., 1989).

3.2.2.8 Screening of positive *Escherichia coli* DH5α transformants by PCR

Colonies were screened for the presence of the insert by the direct colony PCR method as set out in the pMOSB*lue* blunt ended cloning kit instruction manual (Amersham International plc, Little Chalfont, UK). 10 colonies were picked with sterile toothpicks and transferred to 1.5 ml Eppendorf tubes containing 100 µl sterile water. The mixtures were vortexed to disperse the pellets and boiled for 5 min to lyse the cells and denature DNAses. The samples were centrifuged at 6500xg for 1 min at 4°C to pellet the cell debris. Two microlitres of the supernatant containing the DNA was used as the template in PCR amplifications. The transgene specific primer combinations used for amplification were as follows:

a) $IPGIP_L$ and $Hewl_R$ for amplification of the bean *pgip1-hewl* gene and
b) $Hewl_{signal}$ and $Hewl_R$ for amplification of the $hewl_{signal}$ gene

Characteristics of these primers are given in Table 3.2. *Taq* DNA polymerase (from *Thermus aquaticus* BM, expressed in *E. coli*) was used in all PCR amplifications (Takara Shuzo Co., LTD, Japan). Each reaction mixture contained 1x reaction buffer, 2.5 mM $MgCl_2$, 250 µM dNTP, 0.5 µM of each primer and 1U *Taq* DNA polymerase. Reaction volumes were made up to 10µl using sterile dH_2O. PCR cycling conditions included an initial denaturation step of 94°C for 1 min. This was followed by 35 cycles with denaturation at 94°C for 30 sec,

annealing at 65°C for 30 sec and elongation at 72°C for 1 min. A final extension step at 72°C for 1 min was included. The PCR products were electrophoresed on a 1% (w/v) agarose gel.

3.2.2.9 Restriction enzyme digestion to determine the insert orientation

DNA was isolated from 10 PCR positive *E. coli* DH5α clones and restriction enzyme digestions were performed as described in section 3.2.1.2 on the isolated plasmid DNA, first with *Pst*I and then with the enzymes that specifically excised the genes, i.e., *Nco*I and *Bam*HI, to verify the presence of the correct insert. The digestion mix was resolved on a 1% (w/v) agarose gel. Colonies containing the recombinant plasmids were selected and stored as bead cultures at -70°C.

3.2.3 Transformation of *Agrobacterium tumefaciens*

In the natural environment *Agrobacterium* introduces its T-DNA, located on a large extrachromosomal element, into compatible host plant cells and via highly evolved molecular mechanisms stably integrates the new DNA into the plant genome. With the great discovery of the molecular mechanisms of this large extrachromosomal element in *A. tumefaciens,* harbouring genes involved in crown gall induction, a natural transfer system for genetically engineering plants was born. In this study *A. tumefaciens* LBA4404, transformed with suitable binary vectors harbouring the genes of interest, is the strain employed to transform *N. tabacum.*

3.2.3.1 Preparation of competent *Agrobacterium tumefaciens* LBA4404 cells

A. tumefaciens LBA4404 was streaked from a bead culture stored at -70°C onto LB agar plates containing 50 µg/ml rifampicin. The plated cells were grown inverted at 27°C for 48 hrs. A single colony was inoculated into 5 ml LB liquid media containing 50 µg/ml rifampicin. These starter cultures were grown overnight at 27°C on a shaker and used to inoculate 100 ml LB liquid media containing 50 µg/ml rifampicin. The culture was again grown at 27°C until an $O.D_{680}$ of 0.5 to 1.0 was reached. The cultures were then chilled on ice before brief centrifugation to remove unwanted clumps of cells. The cell suspensions were poured into sterile 1.5 ml Eppendorfs and henceforth maintained on ice between activities. The cell suspensions were then centrifuged at 1600xg for 10 minutes at 4°C after which the supernatant was discarded and the remaining cells resuspended in a total volume of 2.5 ml ice cold 20 mM $CaCl_2$. For long-term storage the cells were dispensed in 0.3 ml aliquots into pre-chilled sterile 1.5 ml Eppendorf tubes.

3.2.3.2 Transformation of *Agrobacterium tumefaciens* LBA4404

A. tumefaciens LBA4404 competent cells, were transformed with the binary vectors pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA*, pCAMBIA2300-$hewl_{signal}A$, and pGA482-bean *pgip1,* the latter containing the bean *pgip1* gene. Five micrograms of each binary vector was added to 300 µl *A. tumefaciens* competent cells, mixed and quick-frozen in liquid nitrogen. The cells were thawed by incubation at 37°C for 5 min followed by the addition of 1 ml of LB liquid media and incubated at 27°C for 4 hours on a shaker. A negative control was included where no DNA was added to the competent cells. 100 µl aliquots were spread onto LB agar plates containing 50 µg/ml rifampicin and 50 µg/ml kanamycin. Tetracycline (5 µg/ml) was used instead of kanamycin for *A. tumefaciens* colonies transformed with pGA482-bean *pgip1*. The plates were incubated for 48 to 72 hrs at 27°C. Each sample was duplicated.

3.2.3.3 Verification of the transgenes in *Agrobacterium tumefaciens*

To test for the presence of the transgenes in *A. tumefaciens*, the colonies were screened using the direct PCR screening method as described in section 3.2.2.8 except that single colonies were picked and each was resuspended in 30 µl dH_2O. Two microlitres of the supernatant prepared from each colony was used as the template in PCR amplifications. The transgene specific primer combinations used for amplification were as follows:

a) $IPGIP_L$ and $Hewl_R$ for amplification of the bean *pgip1-hewl* gene,
b) $Hewl_{signal}$ and $Hewl_R$ for amplification of the $hewl_{signal}$ gene,
c) $IPGIP_L$ and $IPGIP_R$ for amplification of the bean *pgip1* gene and
d) $NPTII_L$ and $NPTII_R$ for the amplification of the *nptII* gene.

The characteristics of the PCR primers are summarized in Table 3.2 The reaction mixture in a total volume of 10 µl contained 1 x reaction buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 1U DNA polymerase (Promega, Madison, WI, USA) and template DNA. The reaction volume was made up to 10 µl using sterile dH_2O. PCR cycling conditions consisted of 1 cycle of 94°C for 30 sec, annealing at x°C for 30 sec and elongation at 72°C for 45 sec, and a final extension step at 72°C for 3 min. Annealing temperatures (x) used was 65°C for the HEWL and NPTII primers. PCR amplifications using the $IPGIP_L$ and $IPGIP_R$ were carried out using special conditions to amplify primers with degeneracy. An initial

denaturation at 94°C for 90 sec was followed by 2 cycles of 94°C for 30 sec, annealing at 37°C and an extension step at 72°C for 45 sec. The ramping rate between the annealing-extension steps was 0.33°C per second. This was followed with 34 cycles consisting of 94°C for 20 sec, 56°C for 30 sec and 72°C for 45 sec (Compton, 1990). A final extension step of 3 min at 72°C was included. Positive controls (1 ng of either pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA,* pCAMBIA2300-*hewl*$_{signal}$*A* or pGA482-bean *pgip1*) and negative controls (sterile water and non-transformed *A. tumefaciens* LBA4404) were always included. Positive colonies were selected and bead cultures made for storage at -70°C. PCR products were analysed on a 1% (w/v) agarose gel.

3.3 Results

3.3.1 DNA integrity determination of previously cloned constructs

This section verifies the proper insertion of the significant genes in the appropriate positions within pRLT2 and confirms them to have the correct nucleotide sequences.

3.3.1.1 Restriction enzyme digestion

The integrity of the starting materials used for cloning was tested by restriction enzyme digestion with the relevant enzymes. Agarose gel analyses of the digestions are shown in Figure 3.4. Digestions of pRTL2-*hewl*$_{signal}$ with *Nco*I and *Bam*HI (lanes 2 and 3) yielded the expected pRTL2 vector (3.772 kb) and the *hewl*$_{signal}$ gene (0.448 kb) fragments. Digestions of pRTL2-bean *pgip1-hewl* with *Nco*I, *Bam*HI and *Kpn*I (lanes 4 and 5) yielded the bean *pgip1-hewl* fusion gene (1.430 kb), the bean *pgip1* gene (1.037 kb) and the *hewl* gene (0.393 kb) fragments. This ascertained that the starting materials contained the relevant target genes and that they were amenable to manipulation with the appropriate restriction enzymes.

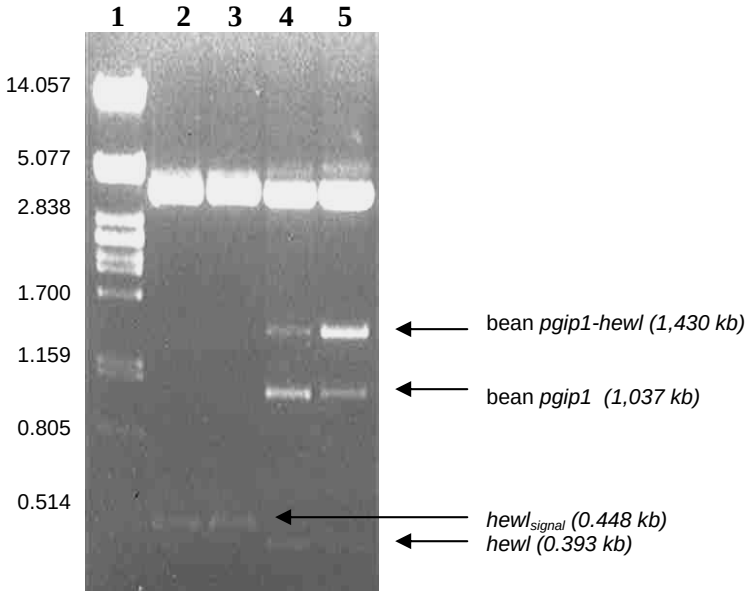
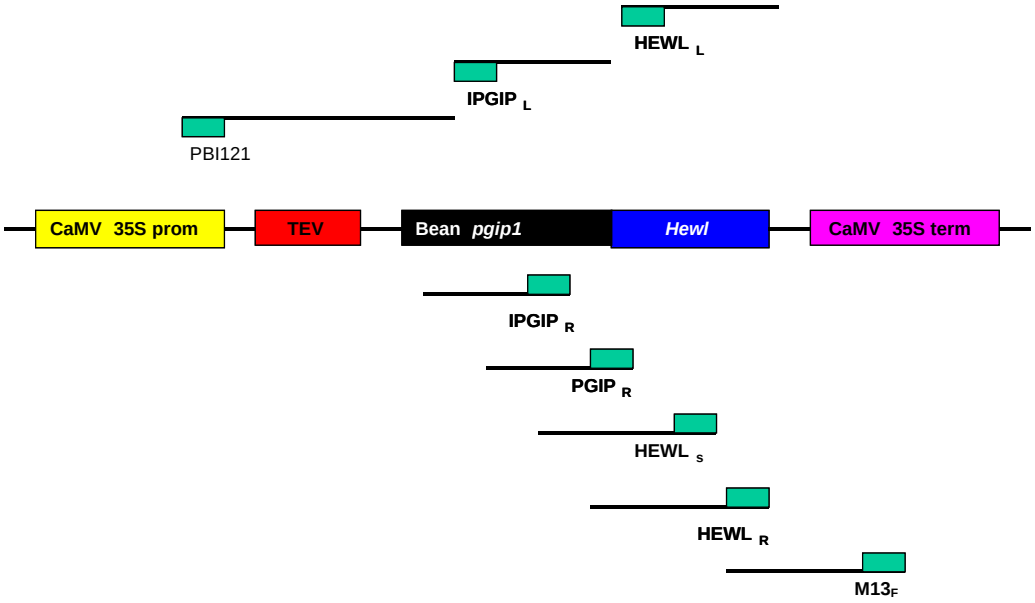


Figure 3.4 Restriction enzyme digestions of pRTL2-bean *pgip1-hewl* and pRTL2-*hewl*$_{signal}$. Lane 1, λ*Pst*I; Lanes 2 and 3, pRTL2-*hewl*$_{signal}$ digested with *Nco*I and *Bam*HI, Lanes 4 and 5, pRTL2-bean *pgip1-hewl* digested with *Nco*I, *Bam*HI and *Kpn*I. The positions of the 1.430 kb bean *pgip1-hewl*, 1.037 kb bean *pgip1*, 0.448 kb *hewl*$_{signal}$ and 0.393 kb *hewl* genes are indicated with arrows.

3.3.1.2 Sequence analyses of expression cassettes

The recombinant plasmids, pRTL2-bean *pgip1-hewl* and pRTL2-*hewl*$_{signal}$, were sequenced to verify the nucleotide sequences of the bean *pgip1-hewl* and *hewl*$_{signal}$ genes. pRTL2-bean *pgip1-hewl* was sequenced using nine different primers and pRTL2-*hewl*$_{signal}$ with three different primers. All primers were previously designed except for the HEWL$_s$ primer that was designed specifically to read the DNA junction between the bean *pgip1* and *hewl* genes. Alignment of the newly sequenced data with that of a previously cloned and sequenced gene revealed that no errors occurred during PCR amplification and subsequent cloning of the genes. The sequencing strategy is depicted in Figure 3.5.

A



B

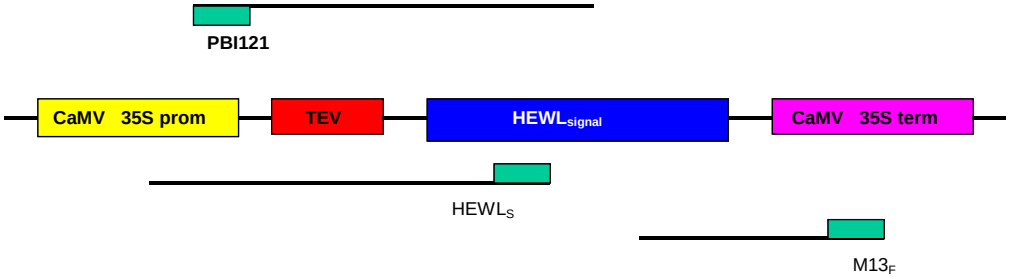


Figure 3.5 Schematic representations of the regions and the primers used in automated sequencing of the bean *pgip1-hewl* and *hewl*$_{signal}$ expression cassettes in pRTL2-bean *pgip1-hewl* and pRTL2-*hewl*$_{signal}$ respectively.

Comparison of the sequence data obtained for the bean *pgip1-hewl* and *hewl*$_{signal}$ genes within pRTL2 with the sequences of plasmid vectors constructed *in silico* using the computer program *Vector NTI*™. The sequences were confirmed to be identical through alignments done using the computer program *Genepro Version 6.1* (Riverside Scientific Enterprises, WA, USA). The sequence alignments mentioned above are presented in Figure 3.6.

A

```
Seq 1   Sequencing of pRTL2-bean pgip1-hewl
Seq 2   Vector NTI generated bean pgip1-hewl gene sequence

Seq 1   ATGACTCAATTCAATATCCCAGTAACCATGTCTTCAAGCTTAAGCATAATTTTGGTCATT    60
        ************************************************************
Seq 2   ATGACTCAATTCAATATCCCAGTAACCATGTCTTCAAGCTTAAGCATAATTTTGGTCATT    60

Seq 1   CTTGTATCTTTGAGAACTGCACTCTCAGAGCTATGCAACCCACAAGATAAGCAAGCCCTT   120
        ************************************************************
Seq 2   CTTGTATCTTTGAGAACTGCACTCTCAGAGCTATGCAACCCACAAGATAAGCAAGCCCTT   120

Seq 1   CTCCAAATCAAGAAAGACCTTGGCAACCCAACCACTCTCTCTTCATGGCTTCCAACCACC   180
        ************************************************************
Seq 2   CTCCAAATCAAGAAAGACCTTGGCAACCCAACCACTCTCTCTTCATGGCTTCCAACCACC   180

Seq 1   GACTGTTGTAACAGAACCTGGCTAGGTGTTTTATGCGACACCGACACCCAAACATATCGC   240
        ************************************************************
Seq 2   GACTGTTGTAACAGAACCTGGCTAGGTGTTTTATGCGACACCGACACCCAAACATATCGC   240

Seq 1   GTCAACAACCTCGACCTCTCCGGCCATAACCTCCCAAAACCCTACCCTATCCCTTCCTCC   300
        ************************************************************
Seq 2   GTCAACAACCTCGACCTCTCCGGCCATAACCTCCCAAAACCCTACCCTATCCCTTCCTCC   300

Seq 1   CTCGCCAACCTCCCCTACCTCAATTTTCTATACATTGGCGGCATCAATAACCTCGTCGGT   360
        ************************************************************
Seq 2   CTCGCCAACCTCCCCTACCTCAATTTTCTATACATTGGCGGCATCAATAACCTCGTCGGT   360

Seq 1   CCAATCCCCCCCGCCATCGCTAAACTCACCCAACTCCACTATCTCTATATCACTCACACC   420
        ************************************************************
Seq 2   CCAATCCCCCCCGCCATCGCTAAACTCACCCAACTCCACTATCTCTATATCACTCACACC   420

Seq 1   AATGTCTCCGGCGCAATACCCGATTTCTTGTCACAGATCAAAACCCTCGTCACCCTCGAC   480
        ************************************************************
Seq 2   AATGTCTCCGGCGCAATACCCGATTTCTTGTCACAGATCAAAACCCTCGTCACCCTCGAC   480

Seq 1   TTCTCCTACAACGCCCTCTCCGGCACCCTCCCTCCCTCCATCTCTTCTCTCCCCAACCTC   540
        ************************************************************
Seq 2   TTCTCCTACAACGCCCTCTCCGGCACCCTCCCTCCCTCCATCTCTTCTCTCCCCAACCTC   540

Seq 1   GGAGGAATCACATTCGACGGCAACCGAATCTCCGGCGCCATCCCCGACTCCTACGGCTCG   600
        ************************************************************
Seq 2   GGAGGAATCACATTCGACGGCAACCGAATCTCCGGCGCCATCCCCGACTCCTACGGCTCG   600

Seq 1   TTTTCGAAGCTGTTTACGGCGATGACCATCTCCCGCAACCGCCTCACCGGGAAGATTCCA   660
        ************************************************************
Seq 2   TTTTCGAAGCTGTTTACGGCGATGACCATCTCCCGCAACCGCCTCACCGGGAAGATTCCA   660

Seq 1   CCGACGTTTGCGAATCTGAACCTGGCGTTCGTTGACTTGTCTCGGAACATGCTGGAGGGT   720
        ************************************************************
Seq 2   CCGACGTTTGCGAATCTGAACCTGGCGTTCGTTGACTTGTCTCGGAACATGCTGGAGGGT   720
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Seq 1   GACGCGTCGGTGTTGTTCGGGTCAGATAAGAACACGAAGAAGATACATCTGGCGAAGAAC    780
        ************************************************************
Seq 2   GACGCGTCGGTGTTGTTCGGGTCAGATAAGAACACGAAGAAGATACATCTGGCGAAGAAC    780

Seq 1   TCTCTTGCTTTTGATTTGGGGAAAGTGGGGTTGTCAAAGAACTTGAACGGGTTGGATCTG    840
        ************************************************************
Seq 2   TCTCTTGCTTTTGATTTGGGGAAAGTGGGGTTGTCAAAGAACTTGAACGGGTTGGATCTG    840

Seq 1   AGGAACAACCGTATCTATGGGACGCTACCTCAGGGACTAACGCAGCTAAAGTTTCTGCAA    900
        ************************************************************
Seq 2   AGGAACAACCGTATCTATGGGACGCTACCTCAGGGACTAACGCAGCTAAAGTTTCTGCAA    900

Seq 1   AGTTTAAATGTGAGCTTCAACAATCTGTGCGGTGAGATTCCTCAAGGTGGGAACTTGAAA    960
        ************************************************************
Seq 2   AGTTTAAATGTGAGCTTCAACAATCTGTGCGGTGAGATTCCTCAAGGTGGGAACTTGAAA    960

Seq 1   AGGTTTGACGTTTCTTCTTATGCCAACAACAAGTGCTTGTGTGGTTCTCCTCTTCCTTCC   1020
        ************************************************************
Seq 2   AGGTTTGACGTTTCTTCTTATGCCAACAACAAGTGCTTGTGTGGTTCTCCTCTTCCTTCC   1020

Seq 1   TGCACTGGTACCAAAGTCTTTGGACGATGTGAGCTGGCAGCAGCTATGAAGCGTCACGGA   1080
        ************************************************************
Seq 2   TGCACTGGTACCAAAGTCTTTGGACGATGTGAGCTGGCAGCAGCTATGAAGCGTCACGGA   1080

Seq 1   CTTGATAACTATCGGGGATACAGCCTGGGAAACTGGGTGTGTGCCGCAAAATTCGAGAGT   1140
        ************************************************************
Seq 2   CTTGATAACTATCGGGGATACAGCCTGGGAAACTGGGTGTGTGCCGCAAAATTCGAGAGT   1140

Seq 1   AACTTCAACACCCAGGCTACAAACCGTAACACCGATGGGAGTACCGACTACGGAATCCTA   1200
        ************************************************************
Seq 2   AACTTCAACACCCAGGCTACAAACCGTAACACCGATGGGAGTACCGACTACGGAATCCTA   1200

Seq 1   CAGATCAACAGCCGCTGGTGGTGCAACGATGGCAGGACCCCAGGCTCCAGGAACCTGTGC   1260
        ************************************************************
Seq 2   CAGATCAACAGCCGCTGGTGGTGCAACGATGGCAGGACCCCAGGCTCCAGGAACCTGTGC   1260

Seq 1   AACATCCCGTGCTCAGCCCTGCTGAGCTCAGACATAACAGCGAGCGTGAACTGCGCGAAG   1320
        ************************************************************
Seq 2   AACATCCCGTGCTCAGCCCTGCTGAGCTCAGACATAACAGCGAGCGTGAACTGCGCGAAG   1320

Seq 1   AAGATCGTCAGCGATGGAAACGGCATGAACGCGTGGGTCGCCTGGCGCAACCGCTGCAAG   1380
        ************************************************************
Seq 2   AAGATCGTCAGCGATGGAAACGGCATGAACGCGTGGGTCGCCTGGCGCAACCGCTGCAAG   1380

Seq 1   GGCACCGACGTCCAGGCGTGGATCAGAGGCTGCCGGCTGTGAGGATCCT              1429
        ***********************************************
Seq 2   GGCACCGACGTCCAGGCGTGGATCAGAGGCTGCCGGCTGTGAGGATCCT              1429
```

B

Seq 1 Sequencing of pRTL2-*hewl*$_{signal}$
Seq 2 Vector NTI generated *hewl*$_{signal}$ gene sequence

```
Seq 1   ATGGTCAGGTCTTTGCTAATCTTGGTGCTTTGCTTCCTGCCCCTGGCTGCTCTGGGGAAA     60
        ************************************************************
Seq 2   ATGGTCAGGTCTTTGCTAATCTTGGTGCTTTGCTTCCTGCCCCTGGCTGCTCTGGGGAAA     60

Seq 1   GTCTTTGGACGATGTGAGCTGGCAGCAGCTATGAAGCGTCACGGACTTGATAACTATCGG    120
        ************************************************************
Seq 2   GTCTTTGGACGATGTGAGCTGGCAGCAGCTATGAAGCGTCACGGACTTGATAACTATCGG    120

Seq 1   GGATACAGCCTGGGAAACTGGGTGTGTGCCGCAAAATTCGAGAGTAACTTCAACACCCAG    180
        ************************************************************
Seq 2   GGATACAGCCTGGGAAACTGGGTGTGTGCCGCAAAATTCGAGAGTAACTTCAACACCCAG    180
```

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Seq 1   GCTACAAACCGTAACACCGATGGGAGTACCGACTACGGAATCCTACAGATCAACAGCCGC   240
        ************************************************************
Seq 2   GCTACAAACCGTAACACCGATGGGAGTACCGACTACGGAATCCTACAGATCAACAGCCGC   240

Seq 1   TGGTGGTGCAACGATGGCAGGACCCCAGGCTCCAGGAACCTGTGCAACATCCCGTGCTCA   300
        ************************************************************
Seq 2   TGGTGGTGCAACGATGGCAGGACCCCAGGCTCCAGGAACCTGTGCAACATCCCGTGCTCA   300

Seq 1   GCCCTGCTGAGCTCAGACATAACAGCGAGCGTGAACTGCGCGAAGAAGATCGTCAGCGAT   360
        ************************************************************
Seq 2   GCCCTGCTGAGCTCAGACATAACAGCGAGCGTGAACTGCGCGAAGAAGATCGTCAGCGAT   360

Seq 1   GGAAACGGCATGAACGCGTGGGTCGCCTGGCGCAACCGCTGCAAGGGCACCGACGTCCAG   420
        ************************************************************
Seq 2   GGAAACGGCATGAACGCGTGGGTCGCCTGGCGCAACCGCTGCAAGGGCACCGACGTCCAG   420

Seq 1   GCGTGGATCAGAGGCTGCCGGCTGTGAGGATCCTCTAG                         458
        ***********************************
Seq 2   GCGTGGATCAGAGGCTGCCGGCTGTGAGGATCCTCTAG                         458
```

Figure 3.6 A. Nucleotide sequence alignments of bean *pgip1-hewl* from pRTL2-bean *pgip1-hewl* and the computer generated bean *pgip1-hewl* sequence. *B.* Nucleotide sequence alignments of *hewl*$_{signal}$ from pRTL2-*hewl*$_{signal}$ and the computer generated *hewl*$_{signal}$ sequence.

3.3.1.3 Cloning bean *pgip1-hewl* and *hewl$_{signal}$* expression cassettes into pCAMBIA2300

The 2.542 kb bean *pgip1-hewl* and the 1.561 kb *hewl$_{signal}$* expression cassettes were transformed into the plant transformation vector, pCAMBIA2300. The expression cassettes were cloned into the vector in a unique *Pst*I site situated between the T-DNA borders. Prior to cloning, *Pst*I restriction digestions of pCAMBIA2300, pRTL2-bean *pgip1-hewl* and pRTL2-*hewl$_{signal}$* were analysed on a 1% agarose gel (Figure 3.7). *Pst*I digestions of pRTL2-bean *pgip1-hewl* (lane 6) show the overlapping of the pRTL2 vector (2.660 kb) and the expression cassette (2.542 kb) fragments. True separation of these two fragments was not achieved photographically (Figure 3.7, lane 6) due to the extreme closeness in their sizes. However, separation of the fragments was visualized on an agarose gel in preparation for subsequent excision of the relevant band containing the gene to be used in the ligation reaction (Results not shown) from which the bottom fragment was excised. *Pst*I digestion of pRTL2-*hewl$_{signal}$* (lane 8) shows the pRTL2-vector (2.660 kb) and the *hewl$_{signal}$* expression cassette (1.561 kb).

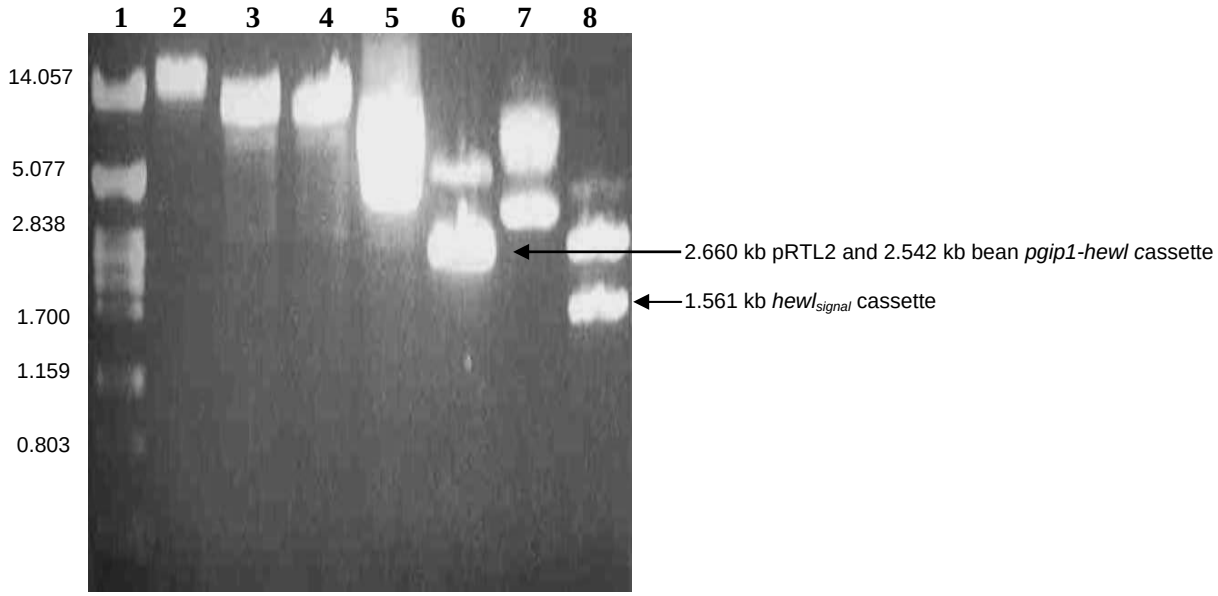


Figure 3.7 Restriction enzyme digestions of pRTL2-bean *pgip1-hewl* and pRTL2-*hewl$_{signal}$* with *Pst*I. Lane 1, *Pst*I λ; Lane 2, uncut pCAMBIA2300; Lanes 3 and 4, *Pst*I digested pCAMBIA2300; Lane 5, uncut pRTL2-bean *pgip1-hewl*; Lane 6, *Pst*I digested pRTL2-bean *pgip1-hewl*; Lane 7, uncut pRTL2-*hewl$_{signal}$*; Lane 8, *Pst*I digested pRTL2-*hewl$_{signal}$*. The positions of the 2.660 kb pRTL2 vector, the 2.542 kb bean *pgip1-hewl* expression cassette and the 1.561 kb *hewl$_{signal}$* expression cassette are indicated with arrows.

The expression cassettes with the relevant genes under the control of the enhanced cauliflower mosaic virus (CaMV) 35S promoter and terminator fragments were successfully cloned in the *Pst*I site of pCAMBIA2300. The T-DNA also contained the *nptII* plant selection gene under the control of the CAMV 35S promoter.

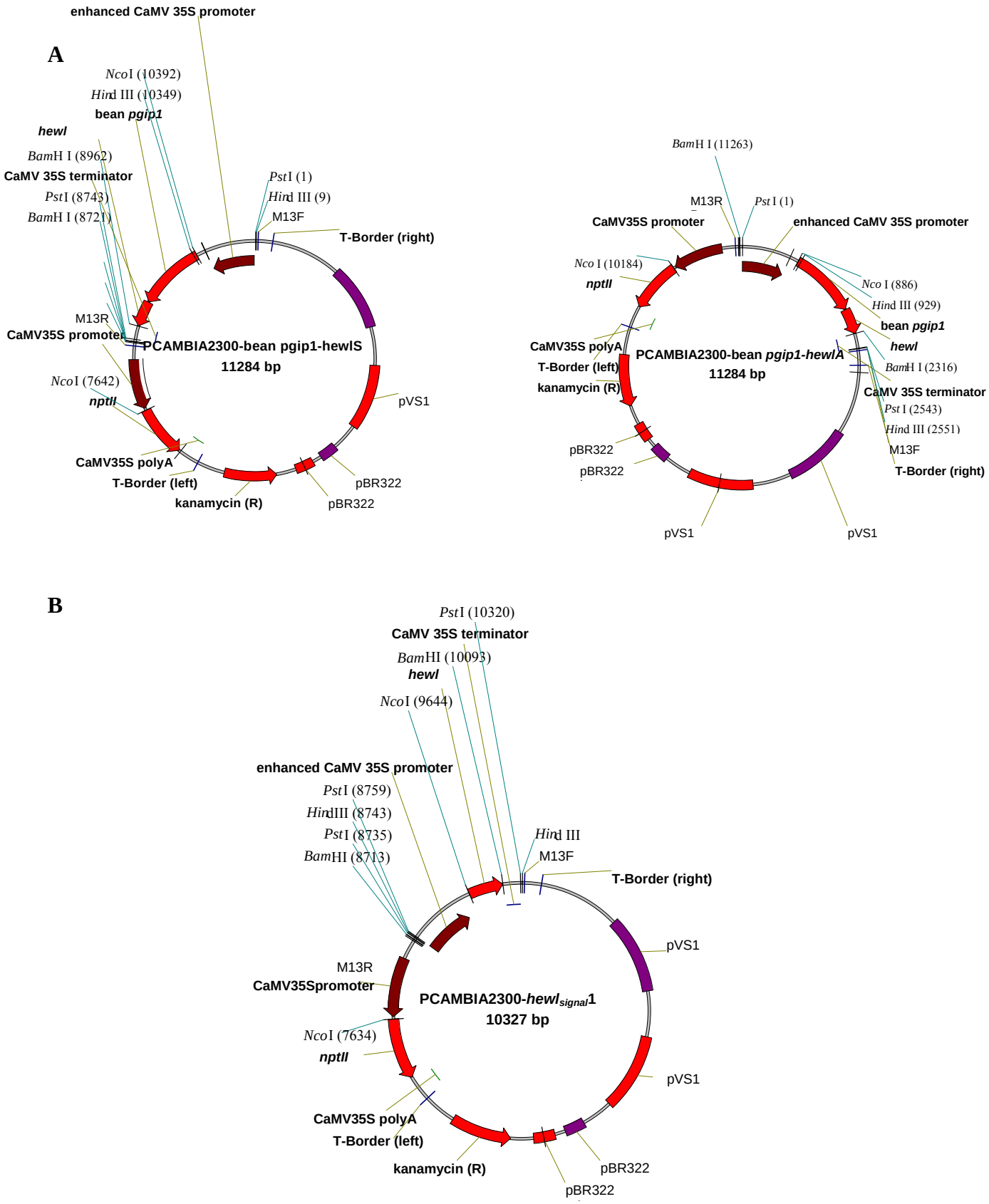


Figure 3.8 Plasmid maps of pCAMBIA2300 containing relevant expression cassettes. A. pCAMBIA2300-bean *pgip1-hewlS* and pCAMBIA2300-bean *pgip1-hewlA.* B. pCAMBIA2300-*hewl*$_{signal.}$

A selection of 10 colonies from each transformation event was screened by PCR for the presence of the transgenes bean *pgip1-hewl* and *hewl*$_{signal}$. Amplification products were obtained with PCR performed on three colonies per clone are presented in Figure 3.9. The control reactions with dH_2O showed no amplification products, indicating that there were no contaminants present in the PCR reagents (Figure 3.9, lanes 6 and 11). PCR performed with $IPGIP_L$ and $HEWL_R$ primers on transformed *E. coli* DH5α colonies resulted in the amplification of the 1.037 kb bean *pgip1-hewl* gene fragment (Figure 3.9, lanes 2, 3 and 4). Additional PCR products appearing in lanes 2-5 are possibly non-specific amplification since $IPGIP_L$ is a degenerate primer. PCR performed with $HEWL_{signal}$ and $HEWL_R$ primers on transformed *E. coli* DH5α colonies resulted in the amplification of the 0.448 kb *hewl*$_{signal}$ gene fragment (Figure 3.9, lanes 7, 8 and 9). Two control PCR amplifications were performed on pCAMBIA2300-bean *pgip1-hewl* and pCAMBIA2300-*hewl*$_{signal}$ plasmid DNA (Figure 3.9, lanes 5 and 10) and show the respective 1.037 kb and 0.448 kb PCR products. Thus all the colonies chosen for colony PCR were positive for the relevant genes.

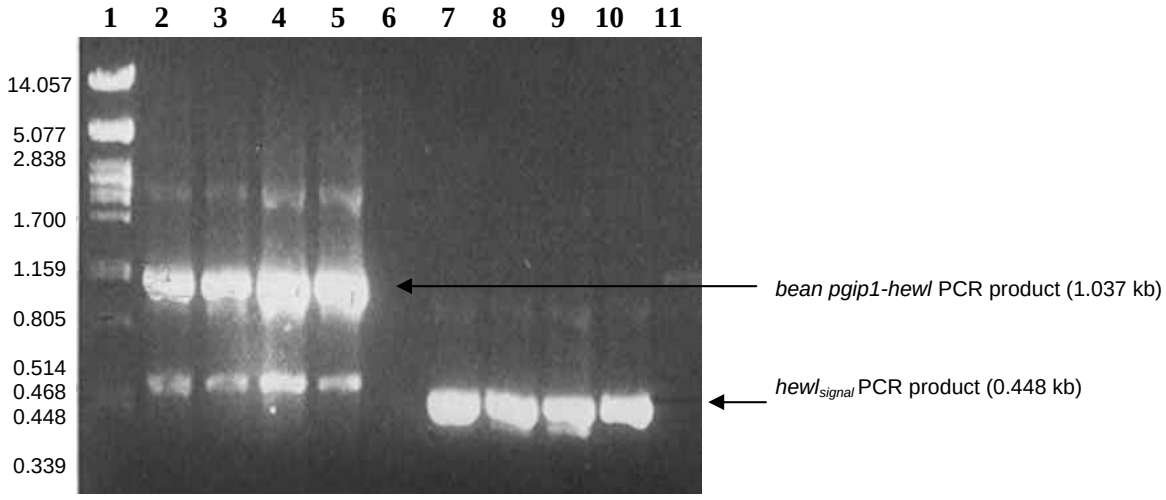
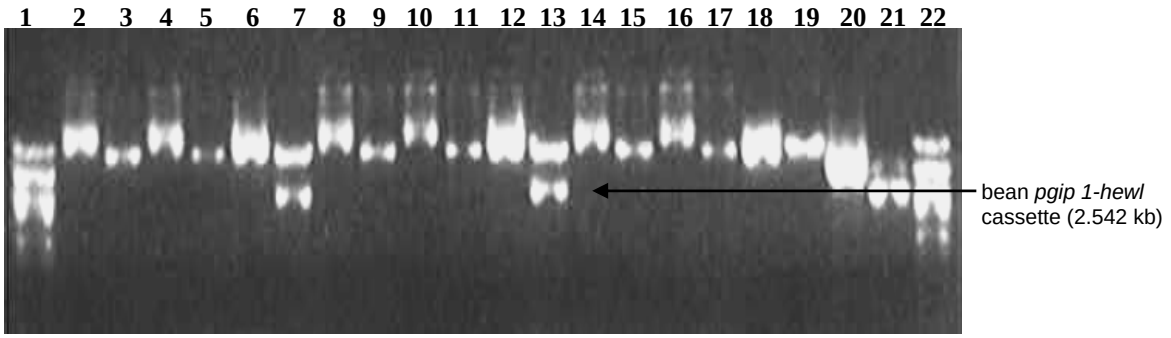


Figure 3.9 PCR analyses of *Escherichia coli* DH5α recombinants using $IPGIP_L$ and $HEWL_R$ primers for bean *pgip1-hewl* amplification and $HEWL_{signal}$ and $HEWL_R$ primers for *hewl*$_{signal}$ amplification. Lane 1, *Pst*I λ; Lane 2 to 4 shows the PCR products of *E. coli* DH5α recombinant colonies transformed with the pCAMBIA2300-bean *pgip1-hewl* ligation reaction ratios 1:1, 1:5 and 1:10 respectively; Lane 5, is a control PCR reaction with pRTL2-bean *pgip1-hewl* DNA; Lane 6, is a water control PCR reaction; Lane 7 to 9 shows the PCR products of *E. coli* DH5α recombinant colonies transformed with the pCAMBIA2300-*hewl*$_{signal}$ ligation reaction ratios 1:1, 1:5 and 1:10 respectively; Lane 10, is a control PCR with pRTL2-*hewl*$_{signal}$ DNA; Lane 11, is a water control PCR reaction.

10 PCR positive clones from both the bean *pgip1-hewl* and $hewl_{signal}$ subcloning were then further analysed by restriction enzyme digestion with *Pst*I (Figure 3.10 A and B). DNA isolated from two of the ten bean *pgip1-hewl* transformed colonies showed the expected fragments of 8.742 kb and 2.542 kb (Figure 3.10A, lanes 7 and 13). DNA isolated from three of the ten $hewl_{signal}$ transformed colonies showed the expected fragments of 8.742 kb and 1.561 kb when digested with *Pst*I (Figure 3.10B, lanes 7, 9, 11. Two of the recombinant pCAMBIA2300-bean *pgip1-hewl* and three recombinant pCAMBIA2300-$hewl_{signal}$ plasmids were selected for further analysis. Figure 3.11, shows *Pst*I restriction digestions of the recombinant plasmids confirming the presence of correct inserts. **Appendix C** shows an *in silico* electrophoresis gel of the expected products produced after *Pst*I digestion.

A



B

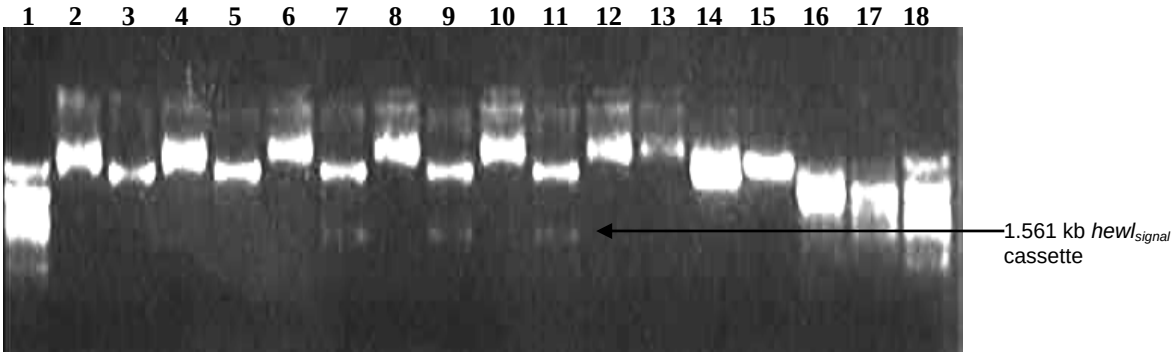


Figure 3.10 Restriction enzyme digestion analysis of A: Putative *Escherichia coli* DH5α recombinants pCAMBIA2300-bean *pgip1-hewl* with *Pst*I. Lane 1, *Pst*I λ. Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 represents undigested pCAMBIA2300-bean *pgip1-hewl*. Lanes 3, 5, 7, 9, 11, 13, 15, 17 and 19 represents *Pst*I digested pCAMBIA2300-bean *pgip1-hewl*. Lanes 7 and 13 shows the 8.742kb pCAMBIA2300 vector and 2.542kb bean *pgip1-hewl* expression cassette. Lane 20, is undigested pCAMBIA2300; Lane 21, *Pst*I digested pCAMBIA2300 and Lane 22, *Pst*I λ. **B. Putative *Escherichia coli* DH5α recombinants pCAMBIA2300-$hewl_{signal}$ with *Pst*I**. Lane 1, *Pst*I λ. Lanes 2, 4, 6, 8, 10, 12 and 14 represents undigested pCAMBIA2300-$hewl_{signal}$. Lanes 3, 5, 7, 9, 11, 13 and 15 represents *Pst*I digested pCAMBIA2300-$hewl_{signal}$. Lanes 7, 9 and 11 shows the 8.742kb pCAMBIA2300 vector and 1.561kb $hewl_{signal}$ expression cassette. Lane 16, undigested pCAMBIA2300; Lane 17, *Pst*I digested pCAMBIA2300 and Lane 18, *Pst*I λ.

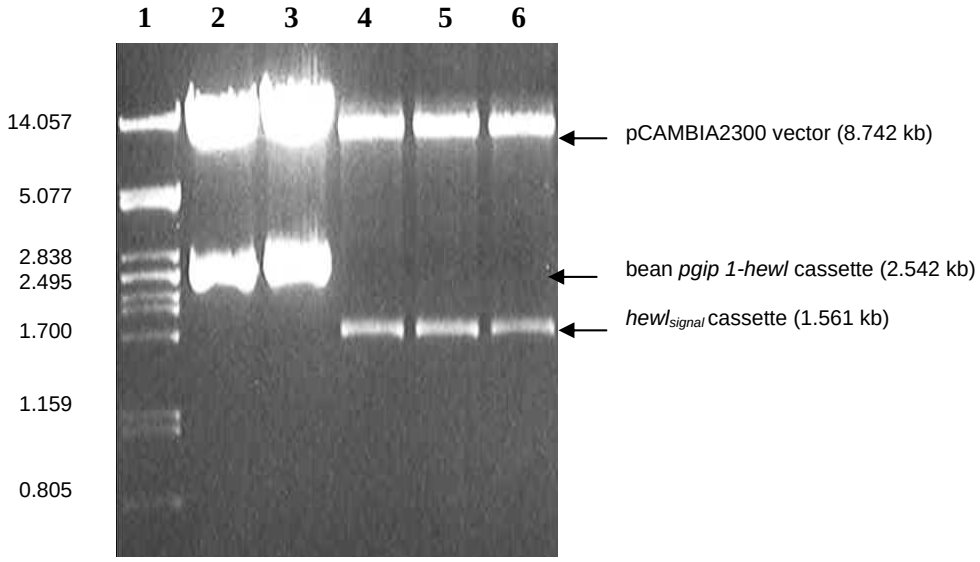


Figure 3.11 Restriction enzyme digestions analysis of positive *Escherichia coli* DH5α recombinants, pCAMBIA2300-bean *pgip1-hewl* and pCAMBIA2300-*hewl*$_{signal}$, with *Pst*I. Lane 1, *Pst*I λ; Lane 2 and 3, *Pst*I digested pCAMBIA2300-bean *pgip1-hewl* (pCAMBIA2300-bean *pgip1-hewl*-3 and pCAMBIA2300-bean *pgip1-hewl*–7) respectively; Lane 4 to 6, *Pst*I digested pCAMBIA2300-*hewl*$_{signal}$ (pCAMBIA2300-*hewl*$_{signal}$-4, pCAMBIA2300-*hewl*$_{signal}$-5 and pCAMBIA2300-*hewl*$_{signal}$-6) respectively.

The recombinant plasmids were subsequently digested with *Nco*I and *Bam*HI. pCAMBIA2300-bean *pgip1-hewl* digested with *Nco*I and *Bam*HI were shown to have the expression cassettes containing the bean *pgip1-hewl* gene ligated to pCAMBIA2300 in different orientations. The results are shown on three different agarose gels in Figures 3.11, 3.12 and 3.13 as well as *in silico* in **Appendix C**. The expression cassette containing the bean *pgip1-hewl* gene fusion is shown in the sense orientation or the antisense orientation (Figure 3.8A) in respect to the *nptII* gene within the pCAMBIA2300 binary vector and the expression cassette containing the $hewl_{signal}$ gene is shown in either the antisense orientation (Figure 3.8B). The 0.241 kb band in Figure 3.12, lane 7 does not appear very clearly. The clones were subsequently renamed pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA* and pCAMBIA2300-$hewl_{signal}A$ and were selected for *A. tumefaciens* transformation.

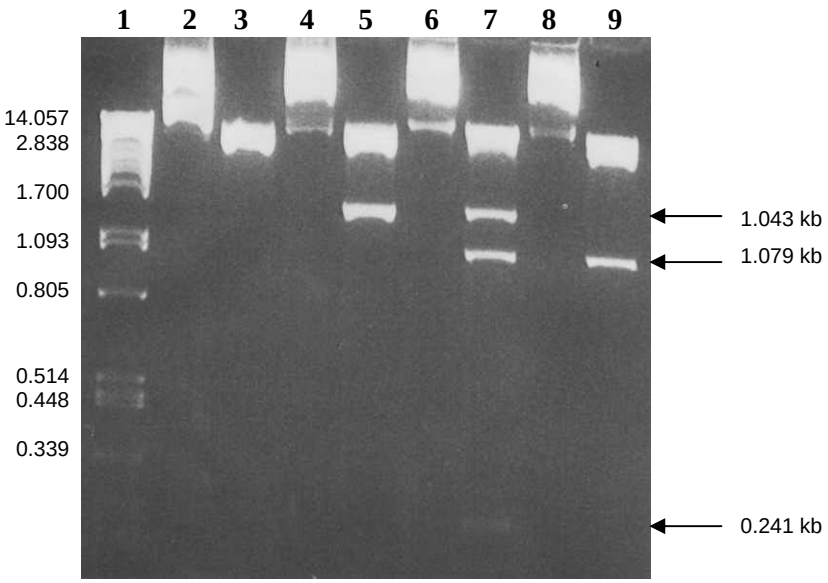


Figure 3.12 Restriction enzyme digestion analyses of pCAMBIA2300-bean *pgip1-hewlS* with *Nco*I and *Bam*HI. Lane1, *Pst*I λ; Lane 2, pRTL2 uncut; Lane 3, pRTL2 digested with *Nco*I/*Bam*HI; Lane 4, pRTL2-bean *pgip1-hewl* uncut; Lane 5, pRTL2-bean *pgip1-hewl* digested with *Nco*I/*Bam*HI; Lane 6, pCAMBIA2300-bean *pgip1-hewlS* uncut; Lane 7, pCAMBIA2300-bean *pgip1-hewlS* digested with *Nco*I/*Bam*HI; Lane 8, pCAMBIA2300 uncut; Lane 9, pCAMBIA2300 digested with *Nco*I/*Bam*HI.

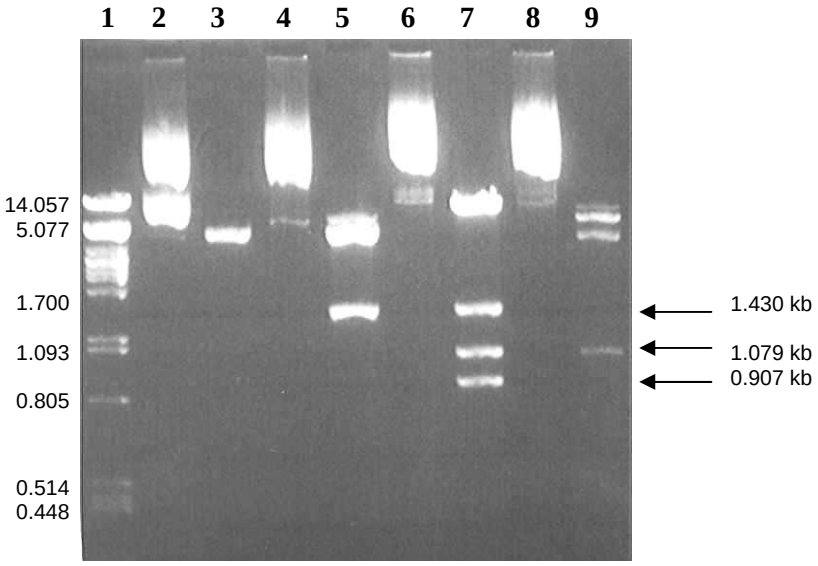


Figure 3.13 Restriction enzyme digestion analyses of pCAMBIA2300-bean *pgip1-hewlA* with *Nco*I and *Bam*HI. Lane1, *Pst*I λ; Lane 2, pRTL2 uncut; Lane 3, pRTL2 digested with *Nco*I/*Bam*HI; Lane 4, pRTL2-bean *pgip1-hewl* uncut; Lane 5, pRTL2-bean *pgip1-hewl* digested with *Nco*I/*Bam*HI; Lane 6, pCAMBIA2300-bean *pgip1-hewlA* uncut; Lane 7, pCAMBIA2300-bean *pgip1-hewlA* digested with *Nco*I/*Bam*HI; Lane 8, pCAMBIA2300 uncut; Lane 9, pCAMBIA2300 digested with *Nco*I/*Bam*HI

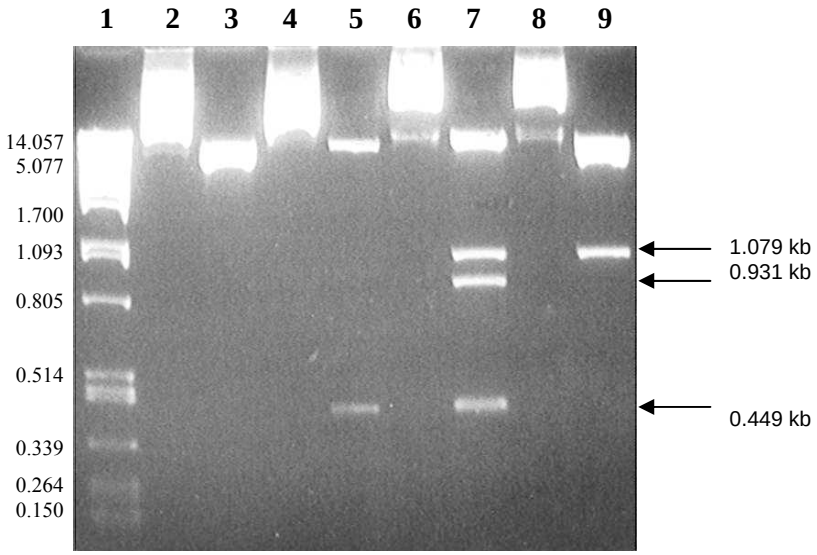


Figure 3.14 Restriction enzyme digestion analyses of pCAMBIA2300-*hewl*$_{signa}$*A* with *Nco*I and *Bam*HI. Lane1, *Pst*I λ; Lane 2, pRTL2 uncut; Lane 3, pRTL2 digested with *Nco*I/*Bam*HI; Lane 4, pRTL2-bean *pgip1-hewl* uncut; Lane 5, pRTL2-bean *pgip1-hewl* digested with *Nco*I/*Bam*HI; Lane 6, pCAMBIA2300-*hewl*$_{signal}$*A* uncut; Lane 7, pCAMBIA2300-*hewl*$_{signal}$*A* digested with *Nco*I/*Bam*HI; Lane 8, pCAMBIA2300 uncut; Lane 9, pCAMBIA2300 digested with *Nco*I/*Bam*HI.

3.3.2 Transformation of *Agrobacterium tumefaciens* LBA4404 with binary vectors pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA*, pCAMBIA2300-*hewl*$_{signal}$*A* and pGA482-bean *pgip1*

The binary vectors (pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA*, pCAMBIA2300-*hewl*$_{signal}$*A* and pGA482-bean *pgip1*) were directly introduced into competent *A .tumefaciens* LBA4404 and grown on plates containing the antibiotics rifampicin and kanamycin for pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA* and pCAMBIA2300-*hewl*$_{signal}$*A* and rifampicin and tetracycline for pGA482-*pgip1* as described in the Materials and Methods 3.2.3.2.

The presence of the binary vectors carrying the T-DNA with the specific transgenes in *A. tumefaciens* was verified by PCR analyses as described in the Materials and Methods 3.2.3.3. Amplification products obtained with PCR performed on colonies are presented in Figure 3.14. The negative control reaction with sterile dH_2O showed no amplification products, indicating that there were no contaminants present in the PCR reagents (Figure 3.15, lanes 5, 8, 13 and 18). PCR performed with the $HEWL_{signal}$ and $HEWL_R$ primers on *A. tumefaciens* colonies that had been transformed with pCAMBIA2300-*hewl*$_{signal}$*A* resulted in the amplification of the 0.448 kb *hewl*$_{signal}$ gene fragment (Figure 3.15, lane 4). PCR performed with $IPGIP_L$ and $HEWL_R$ primers on *A. tumefaciens* colonies transformed with either of the constructs pCAMBIA2300-bean *pgip1-hewlS* and pCAMBIA2300-bean *pgip1-hewlA* resulted in the amplification of the 1.037 kb bean *pgip1-hewl* gene fragment (Fig.3.14, lane 7). Only one of these PCR amplifications is shown on the agarose gel. PCR performed with $IPGIP_L$ and $IPGIP_R$ primers on *A. tumefaciens* colonies transformed with pGA482-*pgip1* resulted in the amplification of the 0.351 kb *ipgip* gene fragment (Fig.3.14, lane 7). Additional bands appearing in lanes 7 and 11 of Figure 3.15 could be due to the use of degenerate primers. No amplification products were obtained when PCR was conducted on non-transformed *A. tumefaciens* colonies (Fig. 3.14, lanes 2, 9, 14 and 19).

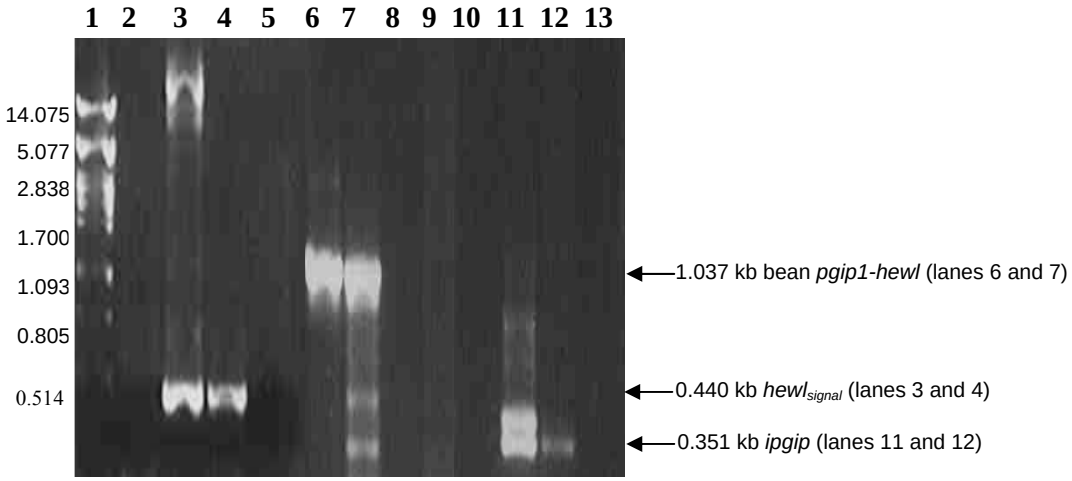


Figure 3.15 Colony PCR analyses of *Agrobacterium tumefaciens* LBA4404. Lane 1, *Pst*I λ. Lanes 2 to 5 shows the PCR analysis of *A. tumefaciens* LBA4404 using $HEWL_{signal}$ and $HEWL_R$ primers where lane 2 shows a PCR reaction with non-transformed *A. tumefaciens* LBA4404 colony as template, lane 3 is a control PCR reaction with pCAMBIA2300-$hewl_{signal}A$ plasmid DNA as template, lane 4 shows a PCR reaction with *A. tumefaciens* LBA4404-PCAMBIA2300-$hewl_{signal}A$ colony and lane 5 is a dH_2O control PCR reaction. Lanes 6 to 9 shows the PCR analysis of *A. tumefaciens* LBA4404 using $IPGIP_L$ and $HEWL_R$ primers where lane 6 is a control PCR reaction with pCAMBIA2300-bean *pgip1-hewl* plasmid DNA as template, lane 7 show a PCR reaction with *A. tumefaciens* LBA4404-PCAMBIA2300-bean *pgip1-hewl* colony, lane 8 is a dH_2O control PCR reaction and lane 9 show a PCR reaction with non-transformed *A. tumefaciens* LBA4404 colony as template. Lanes 10 to 13 shows the PCR analysis of *A. tumefaciens* LBA4404 using $IPGIP_L$ and $IPGIP_R$ primers where, lane 10 is a control PCR reaction with pGA482-*pgip1* plasmid DNA as template, lane 11 show a PCR reaction with *A. tumefaciens* LBA4404-pGA482-*pgip1* colonies, lane 12 is a dH_2O control PCR reaction and lane 13 show a PCR reaction with non-transformed *A. tumefaciens* LBA4404 colony as template.

3.4 Discussion

This chapter focused on the construction of the plant transformation vector pCAMBIA2300 containing the relevant genes of interest i.e., bean *pgip1*, bean *pgip1-hewl* and $hewl_{signal}$ in preparation for *Agrobacterium*-mediated transformation of *N. tabacum* cv. LA Burley 21 plants. Three different constructs were produced including pCAMBIA2300 with the expression cassettes containing the bean *pgip1-hewl* gene in either a sense or antisense orientation with respect to the *nptII* gene and pCAMBIA2300 with the expression cassettes containing the $hewl_{signal}$ gene in the anti-sense orientation with respect to the *nptII* gene. pGA482 containing the bean *pgip1* gene had been previously constructed. The pCAMBIA2300 constructs were all successfully transformed into *A. tumefaciens* LBA4404.

3.4.1 Integrity of specific genes for subcloning

The starting material provided for this study included pRTL2-bean *pgip1-hewl* (Kaimoyo and Cloete, 1999; Ogundiwin and Cloete, 2000) and pRTL2-$hewl_{signal}$ (Kaimoyo and Cloete, 1999). However, no evidence authenticating the proper positioning of the genes within the said vectors were done or that any PCR-related errors had occurred (Maruyama, 1990). Therefore, before proceeding with any subcloning the plasmid DNA extracted from *E. coli* DH5α cells provided as the starting materials were subjected to studies that would generate this sought after information. The relevance of this information finds its importance in determining whether these clones had been correctly constructed and to nullify any reason to repeat the cloning experiments.

Analysis of clones included restriction enzyme digestion and automated sequencing analysis. The restriction enzyme digestion reactions employed enzymes that allowed for the digestion of the plasmids at the specific sites of insertion that yielded information on the exact size of the particular fragment in question. For both the plasmids pRTL2-bean *pgip1-hewl* and pRTL2-$hewl_{signal}$ the specific genes were cloned as *Nco*I and *Bam*HI fragments thereby distinguishing the two genes apart only on the bases of the sizes of the fragments with the bean *pgip1-hewl* fusion being the larger of two (Figure 3.4). The bean *pgip1-hewl* gene cloned into pRTL2 could further then be analysed since the two genes bean *pgip1* and *hewl* without its signal peptide is fused at a unique *Kpn*I site. Upon subsequent digestion with all three enzymes (*Nco*I, *Bam*HI and *Kpn*I), four different fragments namely bean *pgip1*, *hewl* without its signal, the pRTL2 vector and a band that corresponds with the expected size of the bean *pgip1-hewl* fusion were produced (Figure 3.4, lanes 4 and 5). The migration of the restriction

digestions on an agarose gel corresponded to the expected sizes of the individual fragments obtained from predictions generated using *Vector NTI*™ software except for the bean *pgip1-hewl* fusion gene that occurred after digesting pRTL2-bean *pgip1-hewl* with the three enzymes. The explanation for the occurrence of this fourth band (the 1.430 kb bean *pgip1-hewl*; Figure 3.4, lanes 4 and 5) could be a result of incomplete restriction enzyme digestion. However it is still deduced that the plasmid starting material had not lost its integrity.

Since PCR was employed in cloning of some of the DNA fragments, a small chance of a mutation occurring is likely and therefore the actual sequence of the relevant genes had to be determined. On aligning these nucleotide sequences to previously published sequences, exactness was achieved and thus a conclusion that the proper sequences containing no deviations from the original sequence had indeed been cloned, Figure 3.6A and B.

3.4.2 Subcloning of the bean *pgip1-hewl* and $hewl_{signal}$ expression cassettes into pCAMBIA2300

Initial subcloning attempts of the bean *pgip1-hewl* fusion into pCAMBIA2300 were carried out with the restriction enzyme *Hind*III. A partial digestion was performed since the bean *pgip1* gene already contained an internal *Hind*III site. However, the partial digestion of pRTL2-bean *pgip1* using *Hind*III yielded bands that were too close to distinguish between after gel electrophoresis thereby causing problems for excision of the expression cassette from the gel (*Hind*III digestion results not shown). This route was thus discontinued.

In re-examining the pRTL2-bean *pgip1-hewl* plasmid, *Hind*III digestion was substituted with *Pst*I digestion and again the result was two fragments of very similar size (2.660 and 2.542 kb). However, after enough persistence, sufficient separation between the expression cassette and the intermediate vector was eventually achieved by running the pRTL2-*pgip1-hewl Pst*I digestion on a 0.8% agarose gel at a much lower voltage. Thus, upon isolation of the two expression cassettes containing the bean *pgip1-hewl* fusion and $hewl_{signal}$ genes from the gel it was used for subcloning into pCAMBIA2300 at the *Pst*I site. Successful subcloning of the expression cassettes into pCAMBIA2300 were thus achieved and confirmed by PCR (Figure 3.9) and restriction enzyme digestion with *PstI* (Figures 3.9 and 3.10). Further restriction enzyme digestion of pCAMBIA2300-bean *pgip1-hewl* and pCAMBIA2300-$hewl_{signal}$ constructs with *Nco*1 and *Bam*HI revealed that the only two pCAMBIA2300-bean *pgip1-hewl* clones produced were different with regards to the orientation of the expression

cassettes. The presence of the recombinant plasmids within *A. tumefaciens* LBA4404 was confirmed by colony PCR as seen in Figure 3.15.

One of the most useful tools in this cloning chapter was the availability of full sequences of cloning vectors pRTL2 and pCAMBIA2300 as well as that of the genes of interest. It considerably facilitated in the prediction of the cloning strategy to be used and the confirmation of the clones produced using *Vector NTI*™. To illustrate the usefulness of the above-mentioned information a description of an essential part of the cloning events regarding the success to producing the bean *pgip1-hewl* fusion gene within pRTL2 is specified below.

The closeness of the *Kpn*I and *Bam*HI restriction sites in the initial cloning strategy (Figure 3.1) was not considered at the time. Thus, because the digestion sites for the two enzymes were too close to each other, numerous cloning attempts yielded unsuccessful results. The reason for this could be that each of the enzymes made it difficult for the other to bind to their specific sites of activity due to steric hindrance. Although steric hindrance occurs when the sizes of groups within a single molecule prevent certain chemical reactions, the fact that restriction enzyme sites are located very close to each other within the DNA molecule could be considered as a reason for unsuccessful enzyme digestion. To overcome this problem the idea of inserting a stuffer fragment as a *Bam*HI insert was introduced as shown in Figure 3.2 (Ogundiwin and Cloete, 2000). A stuffer fragment is simply a piece of DNA inserted into a plasmid that does not have any adverse effects on the plasmid and that can be excised with the aid of one or more restriction enzymes, thus making allowance for subsequent substitution with a specific gene of interest. The fragment used as the stuffer in this study was from the Ornithogalum Mosaic Virus (OrMV) coat protein gene provided by J. Burger, ARC-Roodeplaat that made possible the insertion of the *hewl* gene lacking its signal peptide adjacent to the bean *pgip1* gene. This cloning was done previously, yet plays an essential role in the success of subsequent cloning experiments done in this study.

This chapter concludes successful subcloning of the expression cassette containing the bean *pgip1–hewl* fusion gene as well as the expression cassette containing the $hewl_{signal}$ gene and the transformation of *Agrobacterium tumefaciens* with the novel plant transformation vectors i.e., pCAMBIA2300-bean *pgip1-hewl* and pCAMBIA2300-$hewl_{signal}$. *Agrobacterium*-mediated transformation of *N. tabacum* and the molecular analysis of the transformants will be reported in Chapter 4.

CHAPTER 4

Transformation and Molecular Analysis of Transgenic *Nicotiana tabacum* cv. LA Burley 21 plants

CHAPTER 4

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Transformation and Molecular Analysis of Transgenic *Nicotiana tabacum* cv. LA Burley 21 plants

4.1 Introduction

Genetic modification of plants provides an opportunity to alter the properties or performance of plants in order to improve their usefulness. Genetic modification may also be used to modify the expression of genes already present in the plant. New genes may be introduced from species with which the plant cannot be bred conventionally, and totally novel or synthetic genes may be added (Henry, 1997).

A variety of techniques that can be defined as either direct or indirect can be implemented to introduce foreign DNA into plants. Direct transfer methods, including chemical treatment (calcium chloride and polyethylene glycol), electroporation, microinjection and particle gun bombardment (biolistics) (De Block, 1993), are techniques where the DNA is directly delivered into the plant cell. The indirect gene transfer method involves the transfer of the DNA into an *Agrobacterium* strain prior to its transfer into the plant (Schaff, 1991).

Agrobacterium tumefaciens may be viewed as a natural genetic engineer. As a soil pathogen this bacterium causes crown gall tumours and hairy root disease at wounded sites of plants. Gall-inducing strains contain a single copy of a plasmid (the transfer or Ti plasmid) that includes a segment (the transfer or T-DNA) that, during transfer, is stably integrated into the plant nuclear genome. The T-DNA region contains an oncogenic region (*onc*) with genes encoding phytohormones (auxins and cytokinin) and opines which are metabolised by the A*grobacteria* present in the crown gall (Bevan, 1984). Replacing the T-DNA with a particular gene sequence of interest to be introduced into the plant produces vectors for plant transformation, since it capitalises on several inherent characteristics of the *Agrobacterium*–mediated transformation process.

Of the three most commonly used methods for gene delivery i.e., electroporation, biolistics and *Agrobacterium*-mediated gene transfer, the latter is probably the most effective approach in those plants amenable to the technique. Although the range of species that can be transformed using *Agrobacterium* was initially restricted to dicotyledonous plants however,

species previously considered outside the range of this technique have shown progress of being successfully transformed. All the above-mentioned techniques of gene transfer require that the gene encoding the desired trait be under the control of a promoter that will be active in plants and a transcriptional terminal signal that is functional in a plant system (Songstad *et al*., 1995) and that the transgene conform to the plants preferred codon usage.

Plant selectable markers can be used for identifying transformed tissue (Chee *et al*., 1991; Schaff, 1991). The selectable functions on most general transformation vectors are prokaryotic antibiotic resistance enzymes which have been engineered to be expressed constitutively in plant cells such as the neomycin phosphotransferaseII II (*nptII*) gene, one of the most commonly used to detect transformed plant tissues. The *nptII* gene confers resistance to the antibiotics kanamycin, neomycin and geneticin (Angenon *et al*., 1992), and like other selectable markers, alters the conformation of the smaller subunit of prokaryotic ribosomes thereby preventing normal interaction between the mRNA and the anticodon of tRNA. This causes the production of a nonsense protein. Selectable markers allow the elimination of maintenance and analysis of plants that do not carry the gene of interest since the selections of plants containing the desired gene(s) are done during regeneration. Putatively transformed tissue will only be resistant to kanamycin if the *nptII* gene is functional and expressed.

Additional screening of putative transgenic plants grown on selection medium can be conducted to gain information about the presence and structural integrity of the transgenes. A useful technique for screening large numbers of putative recombinants is the Polymerase Chain Reaction (PCR). About 21 years ago researchers developed this *in vitro* method to amplify DNA fragments and scientists in many different disciplines quickly recognized this major advancement where they then made the necessary modifications to apply this technique to resolve many problems that were unapproachable by other techniques (Slightom *et al*., 1995). PCR is used for the rapid determination of the presence and structural integrity of any transferred target gene and thus provides a solution for screening these large numbers of putatively transformed plants (Chee *et al*., 1991). Other plant molecular biology-related uses for PCR include inverse PCR of unknown plant DNA regions that flank an *Agrobacterium* T-DNA and the engineering of a plant expression cassette (Slightom *et al*., 1995).

Southern blot hybridisation analyses of the DNA integrated into the plant genome using the *Agrobacterium*-mediated transformation system can be used to verify the PCR result and will yield information not only on the presence or absence of the transferred gene but also the copy number, whether multiple inserts are linked in tandem or dispersed and the stability of this DNA in the F1 generation of the transformed plants. This technique requires the isolation and digestion of large amounts of plant DNA that is then separated by gel electrophoresis and then immobilised onto a membrane to allow hybridisation with a specific labelled DNA probe. Several methods including capillary, vacuum and electro transfer have been developed to efficiently transfer DNA from an agarose gel to a nylon membrane, however the capillary transfer, applied in this study, is still the most popular because of its simplicity and reliability (Southern, 1975).

Many variations of labelling DNA probes exist where in most cases hybridisation probes have been labelled with radioisotopes such as ^{32}P and ^{125}I yielding high sensitive probes. However, the health hazards involved and the limited shelf-life of radioactively labelled reagents have led to a great interest in the development of non-radioactive nucleic acid labelling methods. Among the non-radioactive markers developed, biotin has gained the widest use since it can be linked to DNA enzymatically or chemically. Other non-isotopic markers include the horseradish peroxidase system and the digoxigenin-anti-digoxygenin system. The non-radioactive digoxygenin (DIG) system from Roche Diagnostics GmbH, Germany, applied in this study, uses a cardenolide steroid hapten (a molecule that acts as an antigen when conjugated to a protein) isolated from *Digitalis* plants to label the DNA probe. The use of this system offers the advantage of safety and stability for long periods at -20°C.

DIG can be incorporated into DNA probes by nick translation, random primed labelling or during PCR. In this study, DIG coupled to dUTP, is incorporated into the nucleic acid probe by means of a PCR reaction. The DIG-labelled DNA probe is then hybridised to the membrane under conditions of appropriate stringency along with a suitable blocking reagent containing a range of complex long-chain macromolecules that stick to any sites on the membrane not occupied by DNA and thus block non-specific attachment during hybridisation. The probe only binds to DNA bands with a high degree of homology. Detection of the hybridised probe is carried out with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase of which either a chromogenic or chemiluminescent substrate for alkaline phosphatase can be used. In this study, a chemiluminescent signal is visualized upon enzymatic dephosphorylation of CSPD (Disodium 3-(4-methyoxyspiro {1,2-dioxethane-3,2'-

(5'-chloro) tricycle [3,3,1,$1^{3,7}$]decan}-4-yl) phenyl phosphate) by alkaline phosphatase that delivers a light emission at a maximum wavelength of 477 nm, which is then recorded on an X-ray film.

The *Agrobacterium*-mediated transformation method usually results in a low copy number of the T-DNA in transformed plants lines. They are most likely to vary from one to only a few copies (Hooykaas and Schilperoort, 1992), with a dozen copies being a rare occurrence. Multiple copies may be located at different loci in the plant genome, or occur at a single locus as direct or inverted repeats.

The introduction of the target genes (bean *pgip1-hewl*, $hewl_{signal}$ and bean *pgip1*) via *Agrobacterium*-mediated transformation and the confirmation of its integration into the genomic DNA of the *Nicotiana tabacum* cv. LA Burley 21 plant are described in this chapter.

4.2 Materials and Methods

All chemicals and reagents used were either analytical or molecular biology grade. Buffers, solutions, and media were all prepared using distilled water and either autoclaved or filter-sterilized through 0.2µm sterile syringe filters. All buffers, solutions, and media used in this study are outlined in **Appendix A**.

Restriction endonucleases, RNase A, and antibiotics were obtained from Roche Diagnostics GmbH, Germany.

All Polymerase Chain Reactions were conducted in 0.2 ml thin-walled tubes in a MJ Research PTC-100 Peltier Thermal Cycler (MJ Research, USA).

All plasmid maps are shown in **Appendix B**

4.2.1 *Agrobacterium*-mediated transformation of *Nicotiana tabacum* cv. LA Burley 21

It is easier to regenerate shoots from organ explants than from protoplast-derived tissues or established callus culture in many plant tissue culture systems. In this study, leaf discs from *N. tabacum* cv. LA Burley 21 (Legg and Collins, 1971) will be used for the transformation procedure.

4.2.1.1 Preparation of leaf explants

In order to obtain a source of leaf discs, *N. tabacum* cv. LA Burley 21 seeds of LA Burley 21 were germinated *in vitro*. The seeds were sterilised in a 3.5% hypochlorite solution for ten minutes followed by three washes with sterile distilled water. The sterile seeds were then germinated on MS media (**Appendix A**) and leaves produced *in vitro* were cut into 1cm^2 discs to produce wounded edges. A pre-culture phase where the discs are pre-incubated for 2 to 4 hrs on MS media was done in a growth room set at 26°C.

4.2.1.2 Preparation of *Agrobacterium tumefaciens*

A. tumefaciens LBA4404 harbouring the vectors pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA*, pCAMBIA2300-*hewl*$_{signal}$*A* or pGA482-bean *pgip1* were grown on a shaker in 20 ml LB liquid media supplemented with 50 µg/ml kanamycin and 50 µg/ml rifampicin at 28°C for 36 hours. Tetracycline (5 µg/ml) was used instead of

kanamycin for *A. tumefaciens* colonies transformed with pGA482-*pgip1*. The cells were collected by centrifugation at 700xg for 25 minutes at 4°C and the supernatant containing the antibiotics were discarded. The cells were then resuspended in 20 ml LB liquid media without antibiotics with an OD_{620} of approximately 0.6 for infection of *N. tabacum* cv. LA Burley 21 leaf discs. Non-transformed *A. tumefaciens* cultures were used as a negative control.

4.2.1.3 Leaf disc transformation

The leaf disc transformation procedure was performed using an adapted method from Horsch *et al*., (1992). The leaf explants were immersed in the prepared *A. tumefaciens* cell suspensions for 15 min and thereafter blotted on sterile filter paper to remove excess bacteria before being replaced onto MS agar plates.

4.2.1.4 Co-cultivation

Co-cultivation took place for 48 hrs at 26°C during which time the T-DNA regions were transferred from bacterial cells to the *N. tabacum* cv. LA Burley 21 cells.

4.2.1.5 Regeneration

Following co-cultivation, the leaf discs were transferred to Regeneration medium (**Appendix A**) to allow shoots to form. Regeneration medium contained 50 µg/ml kanamycin for selection of transgenic *N. tabacum* cv. LA Burley 21 plants and 250 µg/ml cefotaxime to eliminate any *Agrobacterial* growth. The explants were then subcultured to fresh medium once a week and after one month, when the regenerated shoots were approximately 0.5 to 1 cm in size; they were placed on rooting medium (MS media containing 50 µg/ml kanamycin) and allowed to root for approximately 2 to 3 weeks. The negative controls that were included during regeneration comprised of two groups namely, one group that was incubated on regeneration medium without any antibiotic to determine the regeneration efficiency and a second group that was incubated on regeneration medium supplemented with kanamycin and cefotaxime to test the efficiency of the antibiotics.

4.2.2 Screening of putative transgenic T_0 *Nicotiana tabacum* cv. LA Burley 21

PCR analysis was performed to verify the insertion of the transgene into the genome of putative transgenic plants. Plant genomic DNA was isolated from transformed and non-transformed *N. tabacum* cv. LA Burley 21 plants using the small-scale Cetyl Trimethylammonium Bromide (CTAB) method, a modification of the genomic isolation method described by Murray and Thompson (1980). This method relies on the fact that under high salt conditions, nucleic acids form stable but soluble complexes with the detergent CTAB. Reducing the salt concentration causes the CTAB-nucleic acid complex to precipitate, leaving the majority of polysaccharides in solution. CTAB in total nucleic acid preparation is removed by ethanol precipitation.

4.2.2.1 Small-scale genomic DNA isolation

Three leaf discs per plant were collected and ground thoroughly with an Ultra TURRAX (Janke & Kunkel, IKA®-Labortechnik, Stanfen, Germany) in a 1.5 ml Eppendorf tube containing a pinch of 400 grit carbarundum. 400 µl CTAB DNA extraction buffer (2% (w/v) CTAB, 5 M NaCl, 0.5 M EDTA and 1 M Tris-HCl) pre-heated at 60°C was added to the ground material together with 15 µl of 10µg/ml RNase A and was then incubated at 60°C for 1 h. After cooling to room temperature, the sample was extracted with an equal volume of phenol-chloroform:isoamylalcohol (25:24:1) and the phases separated by centrifugation at 10 000xg for 10 min at 4°C. The DNA-containing aqueous phase was recovered and extracted a second time with an equal volume of chloroform:isoamylalcohol (24:1). The recovered supernatant (350 µl) was precipitated by the addition of 0.6 volumes (210 µl) ice cold isopropanol, mixed gently and left at -20°C for 30 min. The precipitated DNA was then pelleted by centrifugation at 10 000xg for 10 min at 4°C, followed by a wash with 500 µl 70% ethanol and air-dried. Once the pellet was sufficiently dried it was resuspended in 50 µl TE buffer (pH 7.25) and the concentration was determined fluorometrically as described in section 3.2.1.1.

4.2.2.2 PCR analyses

A new set of primers was designed for PCR analysis of plant genomic DNA (genomic DNA) material. The characteristics of these new primers are shown in Table 3.2. NPTII primers remained unchanged as shown in Table 3.2 although an additional set of primers namely the MIA primers (Grayburn and Vick, 1995) were included in PCR analysis to confirm the

absence of *A. tumefaciens* contamination. Negative controls, i.e. water and DNA extracts from non-transformed plant material were also included.

PCR cycling conditions were as previously described consisting of 1 cycle of 94°C for 30 sec, annealing at x°C for 30 sec and elongation at 72°C for 45 sec, and a final extension step at 72°C for 3 min. The annealing temperatures (x) used were 60°C for the new BPG and HEWL primers and 65°C for NPTII primers. For MIA primers the cycling conditions were slightly different in that it first included an initial denaturation step of 94°C for 3 min. This was then followed by 34 cycles with denaturation at 94°C for 20 sec, annealing at 72°C for 30 sec and elongation at 72°C for 45 sec. The final extension step was the same as described above. Positive controls (1 ng of either pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA,* pCAMBIA2300-*hewl*$_{signal}$*A* or pGA482-bean *pgip1*) and negative controls (sterile water and non-transformed *N. tabacum* cv. LA Burley 21 DNA) were always included. PCR products were analysed on a 1% (w/v) agarose gel.

4.2.3 Production of T_1 transgenic *Nicotiana tabacum* cv. LA Burley 21

4.2.3.1 Hardening off

The transgenic *N. tabacum* cv. LA Burley 21 plants that were confirmed as positive by PCR were taken to the glasshouse set at 24°C. Traces of agar were washed from the roots and the plantlets were placed in a sterile vermiculture: potting soil mixture (1:1). The pots were then covered with plastic bags and the plantlets allowed to acclimatise gradually to ambient humidity by piercing holes in the plastic bags over a period of time until they were strong enough for the plastic bags to be removed completely. The plants were watered once a week together with a 205g/l Multifeed® (Plaaskem Ltd., SA) solution. After a two-month growth period in the glasshouse the plants began to produce flowers. To prevent cross-pollination, the flowers were sealed with brown paper bags and allowed to form seeds. After three months, dried seedpods were removed and stored in brown envelopes. Seeds were then harvested from the pods.

4.2.3.2 *In vitro* kanamycin selection for transgenic *Nicotiana tabacum* cv. LA Burley 21 seeds

The seeds were treated as previously described in section 4.2.2.1 and spread on MS agar selection plates containing 50 μg/ml kanamycin. The seeds were then allowed to germinate and after a suitable period a count of germinated vs. non-germinated seeds were taken. A time course for the *N. tabacum* cv. LA Burley 21 leaf disc transformation is presented in Table 4.1.

Table 4.1. *Nicotiana tabacum* cv. LA Burley 21 leaf disc transformation time course

	Period	Event
Day	**1**	Cut and pre-cultured leaf discs
	2	Started Agrobacterium culture
	3	Inoculated discs with Agrobacterium; cultured leaf discs
	5	Transferred leaf discs to selection medium
Week	**2-4**	Divided independent events and transferred first shoots
	4-15	Transferred shoots to rooting medium; subcultured every 28 days
	15-18	Transplanted to soil and hardened off
	18-21	Collected seeds

4.2.4 Screening of putative transgenic T_1 *Nicotiana tabacum* cv. LA Burley 21

The presence of the transgene in the T_1 generation was verified by PCR with the specific transgene primers described previously. This was followed by Southern blot analysis to confirm that the transgene had been integrated into the plant genome by providing information about the number of insertion events that have occurred and the amount of copies of the specific transgene.

4.2.4.1 Large-scale genomic DNA isolation

Total genomic DNA was isolated from *in vitro* plants of transformed and non-transformed *N. tabacum* cv. LA Burley 21 plants using a modification of the method described by Dellaporta *et al*., (1985). Plant extracts (approximately 10 g) were prepared by macerating fresh leaf material to a fine powder in liquid nitrogen using a mortar and pestle. The resulting powder extract was resuspended in 60 ml extraction buffer [100 mM Tris (pH 8.0), 0.5 M NaCl. 50 mM EDTA (pH 8.0), 0.07% β-mercaptoethanol] in a 250 ml centrifuge tube. After a fine suspension had been achieved, 8 ml of 20% SDS was added and the sample incubated for 30 min at 65°C with shaking. This was followed by the addition of 20 ml 5 M KOAc, incubation on ice for 20 min and centrifugation at 6500xg for 20 min at 4°C. The supernatant was then filtered through muslin cloth into 40 ml ice-cold isopropanol and incubated for 30 min at -20°C. The pellet recovered from centrifugation was then allowed to air-dry and was followed by resuspension in 3 ml TE buffer (pH8.0). Once the pellet was dissolved, 150 µl RNase A (100 mg/ml) was added to the sample which was then incubated for 30 min at 37°C in a water bath to remove any traces of RNA. The DNA was split into three 2.2 ml Eppendorf and extracted twice with an equal volume of phenol: chloroform (1:1). The phases were separated by centrifugation at 6500xg for 10 min at 4°C. Each tube was then extracted once with 1000 µl chloroform: isoamylalcohol (CIAA 24:1) and the phases separated by centrifugation. The DNA was precipitated by the addition of 1/10 of the volume (100 µl) of 3 M NaOAc (pH 5.2) and an equal volume (1000µl) of cold 100% ethanol followed by incubation at -70°C for 10 min. The DNA was then pelleted by centrifugation at 4500xg for 10 min at 4°C, washed with 1 ml 70% ethanol, and re-pelleted. The DNA was allowed to air-dry and was then resuspended in 150 µl TE (pH8.0) each and pooled. The DNA concentration was determined fluorometrically as previously described.

4.2.4.2 PCR analyses

PCR analysis of putative transgenic T_1 *N. tabacum* cv. LA Burley 21 was carried out as previously described in section 4.2.3.2.

4.2.5 Southern blot hybridisation

Additional screening of putative transgenic plants grown on selection medium was conducted to gain information about the presence and organization of the transgenes (Chee *et al*., 1991). This was achieved by Southern blot hybridisation.

4.2.5.1 Preparation of the bean *pgip1* fragment for non-transformed *Nicotiana tabacum* genomic DNA spiking

4.2.5.1.1 Restriction digestion of plasmid

Previously isolated pRTL2-bean *pgip1* was digested in order to isolate the expression cassette containing the gene that was to be used for spiking non-transformed *N. tabacum* cv. LA Burley 21 genomic DNA during Southern blot hybridisation. Fifteen micrograms of DNA was digested overnight at 37°C with 100U of *Pst*1 and the appropriate 1x restriction buffer in a final volume of 100 µl. The entire digest was analysed on a 1% (w/v) agarose gel from which the fragment of interest (bean *pgip1*) was excised under UV light (350 nm) using a sterile scalpel blade and purified as previously described in section 3.2.2.4.

4.2.5.1.2 Copy number determination for spiking

In order to provide an accurate control, the amount of the bean *pgip1* expression cassette corresponding to the number of genome copies in 20 µg *N. tabacum* cv. LA Burley 21 DNA had to be calculated. To determine the number of copies of bean *pgip1* that were integrated into the *N. tabacum* cv. LA Burley 21 genome, one, ten, and twenty copy number reconstruction samples were prepared as follows.

For *Nicotiana tabacum*:

Constant amount of DNA in a tetraploid *N. tabacum* cv. LA Burley 21 (*Nicotiana tabacum* (2n=4X) cell = 15.6pg (Arumuganathan *et al*., 1991)

Amount of genomic DNA loaded on the gel = 20µg
= 20×10^{-6}

Number of *N. tabacum* cv. LA Burley 21 genomes loaded = $20 \times 10^{-6} / 15.6 \times 10^{-12}$
= 1.2×10^{6}

Calculation for bean *pgip1-hewl* expression cassette

Since 1pg of DNA = 0.965×10^{6} kb
1kb = 1.03×10^{-6}
Size of the bean *pgip1-hewl* expression cassette = 2542 bp
= 2.542 kb

Therefore = $2.542 / 0.965 \times 10^{6}$
= 2.634×10^{-6}pg
The mass of 1.2×10^{6} copies = $2.634 \times 10^{-6} \times 1.2 \times 10^{6}$
= 3.2 pg

Therefore 1 copy of bean *pgip1-hewl* = 3.2 pg,
10 copies = 32 pg, and
20 copies = 64 pg of bean *pgip1-hewl*

Calculation for bean *pgip1* expression cassette

Since 1pg of DNA = 0.965×10^{6} kb
1kb = 1.03×10^{-6}
Size of the bean *pgip1* expression cassette = 2155 bp
= 2.155 kb

Therefore = $2.155 / 0.965 \times 10^{6}$
= 2.233×10^{-6}pg
The mass of 1.2×10^{6} copies = $2.233 \times 10^{-6} \times 1.2 \times 10^{6}$
= 2.7 pg

Therefore 1 copy of bean *pgip1* = 2.7 pg,
10 copies = 27 pg, and
20 copies = 54 pg of bean *pgip1*

4.2.5.3 Digoxygenin (DIG) labelling of the bean *pgip1-hewl* probe

The probe (bean *pgip1-hewl* expression cassette) was labelled using the PCR DIG Probe synthesis kit (Roche Diagnostics GmbH, Germany) that involved the incorporation of DIG-dUTP (alkali labile). Reaction mixes contained 30 ng bean *pgip1-hewl* purified expression cassette as template, 1 x reaction buffer with $MgCl_2$, 1 x DIG nucleotide mix (dATP, dGTP, dCTP, dTTP and DIG-110dUPT), 0.5µM of each of the primers BPG_{left} and $HEWL_{right}$ and Expand High fidelity enzyme mix. The PCR reaction resembled those previously described using the two mentioned primers. Two PCR controls were included; a negative control containing all the reagents except the template and a control containing all the PCR components except the DIG-11-dUTP. PCR products were analysed on a 1% agarose gel.

4.2.5.4 End labelling of the molecular weight marker with DIG-dUTP (λDNA/ *Hind*III)

The molecular weight marker (MWM) II from Roche Diagnostics GmbH, Germany was labelled with DIG by filling in the ends with Klenow enzyme in the presence of dATP, dCTP, dGTP and DIG-11-dUTP (alkali labile). A reaction mix containing 1 µg λDNA, 1 x buffer B (restriction buffer from Roche Diagnostics GmbH, Germany), 200 µM each of dATP, dCTP, dGTP, 40 µM DIG-11-dUTP and 3U Klenow enzyme (Roche Diagnostics GmbH, Germany) was set up and incubated for 3 hrs at 37°C. By inactivating the enzyme at 65°C for 15 min the reaction was stopped. A 100 ng of the labelled marker was electrophoresed together with samples to be used in Southern blot analysis.

4.2.5.5 Restriction digestion of *Nicotiana tabacum* cv. LA Burley 21 genomic DNA

The DNA of transgenic *N. tabacum* cv. LA Burley 21 was digested with *Pst*1 to cut out the insert *(*bean *pgip1-hewl* expression cassette). This is done to provide information of the copy number of the insert. Another digestion that allowed only one cut within the T-DNA that would tell the number of insertion events of the bean *pgip1-hewl* into the transgenic *N. tabacum* cv. LA Burley 21 genome was done with *BamH*I. Digestion reactions contained 20 µg genomic DNA with 5U of enzyme per µg genomic DNA and the appropriate 1x restriction buffer in a total volume of 200 µl. This was then incubated overnight at 37°C. An aliquot of each digestion reaction was analysed on a 1% agarose gel. The digested DNA samples were precipitated by the addition of 1/10 of the volume (20 µl) of 3 M NaOAc (pH 5.2) and an equal volume (200 µl) of cold 100% ethanol followed by incubation at -70°C for 10 min. The DNA was then pelleted by centrifugation at 4500xg for 10 min at 4°C,

washed with 1 ml 70% ethanol, and re-pelleted. The pellet was allowed to air dry and was subsequently resuspended in a 20 µl 1 x TE (pH8.0). The DNA samples (digested, reconstructed and MWM) were separated by electrophoresis at 5°C on a 1% agarose gel at a voltage of 40 V.

4.2.5.6 Southern blotting of DNA onto a nylon membrane

After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide (EtBr) for 20 min while shaking gently, viewed under UV light and photographed. The DNA fragments in the gel were depurinated in 0.25 M HCl with gentle shaking for 10 minutes, denatured in denaturation solution (0.4 N NaOH, 0.6 M NaCl) for 2 x 15 min and neutralised in neutralising solution (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl) for 2 x 15 min at room temperature. The gel was rinsed with sterile water in between each of the treatments. This was followed by capillary transfer of the DNA (Southern, 1975) to a nylon membrane (Osmonics Magnacharge nylon transfer membrane, Amersham-Pharmacia Biotec, Little Chalfont, UK) using 20 x SSC as transfer buffer (3 M NaCl, 0.3 M Sodium citrate, pH 7.4) Figure 4.16. DNA transfer was done overnight after which the membrane was washed with 2 x SSC for 5 minutes to remove any collected debris and the DNA fixed onto the membrane by baking the membrane between two sheets of Whatman 3MM filter paper (Whatman International, UK) at 80°C for 2h. The membrane was stored in aluminium foil at room temperature until hybridisation.

4.2.5.7 Hybridisation and detection of DIG-labelled probes

Hybridisations were performed at 42°C in a Techne Hybridizer (Techne, Barloworld Scientific Ltd., Staffordshire, UK) The membrane was pre-hybridised in 20 ml DIG Easy Hyb solution (Roche Diagnostics GmbH, Germany) containing denatured salmon testes DNA to a final concentration of 125 µg/ml (Sigma D-9156, GmbH, Germany) for 4h. Denaturation of the salmon testes DNA was done by boiling for 10 min and quick cooling on ice. For hybridisation, the pre-hybridisation solution was discarded and 20 ml fresh Easy Hyb solution containing the respective denatured probe (DIG-labelled bean *pgip1-hewl* expression cassette) and salmon testes DNA were added to the membrane. Following overnight hybridisation, the membrane was washed 2 x 5 min at room temperature in 2 x SSC, 0.1% SDS and 2 x 15 min at 65°C in 0.5 x SSC, 0.1% SDS.

Chemiluminescent detection on membranes with anti-DIG-AP and CSPD as a substrate was used according to the DIG Luminescent detection kit manual (Roche Diagnostics GmbH, Germany). The DIG Wash and Block Buffer Set was used together with the DIG Luminescent detection kit for nucleic acids (Roche Diagnostics GmbH, Germany) for the detection of DIG labelled nucleic acids. All the incubations were performed at room temperature with gentle agitation. The membrane was equilibrated in 1 x washing buffer for 5 min, followed by incubation in 100 ml 1 x blocking solution for 30 min. The membrane was then incubated for 30 min with the anti-DIG-AP (the Fab fragment of polyclonal sheep anti digoxigenin, conjugated to alkaline phosphatase) that was diluted in blocking buffer to 75 mU/ml (1:10 000). The antibody solution then was discarded and the membrane washed 2 x 15 min in washing buffer. The membrane was then equilibrated in detection buffer for 5 min. The CSPD was diluted in detection buffer and spread evenly over the membrane in a sealed plastic bag. After 5 min of incubation, the excess CSPD was removed, the plastic bag resealed and incubation allowed for a further 15 min at 37°C to enhance the luminescent reaction. For detection of the chemiluminescent signal, the membrane was exposed for 1 hr to Hyperfilm ECL High Performance chemiluminescence film (Amersham-Pharmacia Biotec, Little Chalfont, UK) and developed.

4.3 Results

4.3.1 Production of transgenic *Nicotiana tabacum* cv. LA Burley 21 plants

Transgenic plants were produced by the *Agrobacterium*-mediated transformation procedure that involved the dipping of *N. tabacum* cv. LA Burley 21 leaf discs in the *A. tumefaciens* liquid culture transformed with the relevant binary vectors harbouring the specific genes of interest i.e., pCAMBIA2300-bean *pgip-hewlS*, pCAMBIA2300-bean *pgip-hewlA*, pCAMBIA2300-$hewl_{signal}$ and pGA482-bean *pgip1* as described in section 4.2.1. Figure 4.1A is a representation of the regeneration of putative transgenic shoots from *N. tabacum* cv. LA Burley 21 leaf discs transformed with one of the *A. tumefaciens* LBA4404 strains on MS agar media containing kanamycin. Figure 4.1 B and C are two non-transformed *N. tabacum* cv. LA Burley 21 controls, where B shows the *N. tabacum* cv. LA Burley 21 leaf discs grown on MS agar media with kanamycin (negative control) and C those grown on MS agar media without kanamycin (positive control). The non-transformed *N. tabacum* cv. LA Burley 21 leaf discs (Figure 4.1B) did not show growth of shoots on MS media containing kanamycin since the leaf discs show a yellow colouration representative of tissue death. In contrast, non-transformed *N. tabacum* cv. LA Burley 21 leaf discs grown on MS media without kanamycin showed the expected formation of shoots (Figure 4.1C). Therefore, shoots produced on leaf discs shown in Figure 4.1A are representative of its survival on MS agar media containing kanamycin proven by the fact that it has been successfully transformed with the relevant *Agrobacterium* strains.

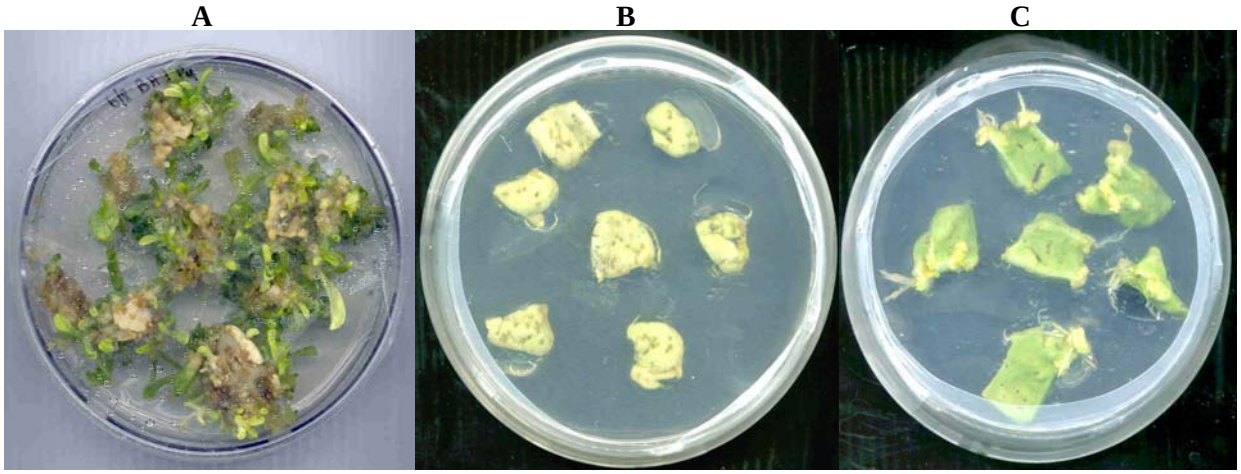

Figure 4.1 Regeneration of transgenic *Nicotiana tabacum* cv. LA Burley 21 on MS agar media. A. Regeneration of transgenic *N. tabacum* cv. LA Burley 21 shoots transformed with an *A. tumefaciens* transconjugant. **B.** Regeneration of non-transformed *N. tabacum* cv. LA Burley 21 leaf discs on MS agar media containing kanamycin. **C.** Regeneration of non-transformed *N. tabacum* cv. LA Burley 21 leaf discs on MS agar media without kanamycin.

The transformation results of *N. tabacum* LA Burley 21 with the different *Agrobacterium* strains harbouring the binary vectors pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA*, pCAMBIA2300-*hewl*$_{signal}$*A* and pGA482-bean *pgip1* are summarized in Table 4.2. Fifty shoots of each transformation experiment were transferred to jars containing MS media supplemented with 100 µg/ml kanamycin. One shoot per explant was transferred to ensure that no sibling shoots (from one transformation event) were propagated.

Table 4.2 Regeneration of putative transgenic plantlets from *Nicotiana tabacum* LA Burley 21 leaf discs

Construct in *Agrobacterium tumefaciens*	**#. leaf discs**	**# explants producing shoots**	**% explants producing shoots**	**# explants on rooting medium**	**# explants rooting**	**% Explants rooting**
pCAMBIA2300-bean *pgip1-hewlS*	56	50	89	50	35	70
pCAMBIA2300-bean *pgip1-hewlA*	56	55	98	50	37	74
pCAMBIA2300-*hewl*$_{signal}$*A*	56	54	96	50	25	50
pGA482-bean *pgip1*	56	52	92	50	35	70
Control[a]	24	23	96	20	19	95
Control[b]	24	0	0	0	0	0

a–non-transformed leaf discs cultured on MS media containing no antibiotics

b–non-transformed leaf discs cultured on MS media containing antibiotics

All of the shoots transferred to jars that produced roots on the medium containing kanamycin were shown to be phenotypically normal (Figure 4.2). All of the surviving plantlets were taken further for screening by PCR from which a selected few were chosen for further experimental analyses.

Figure 4.2 Rooting of *Nicotiana tabacum* cv. LA Burley 21 plantlets on MS media. A. Non-transformed *N. tabacum* cv. LA Burley 21 control plantlet. **B.** Transgenic *N. tabacum* cv. LA Burley 21 line on MS media containing 100 µg/ml kanamycin and 250 µg/ml cefotaxime.

4.3.2 Verification of transgene insertion

4.3.2.1 PCR screening analysis of putatively transformed T_0 *Nicotiana tabacum* cv. LA Burley 21 tissue

Rooting of transformed plantlets on MS media containing kanamycin indicated the expression of the *nptII* gene that is present on the T-DNA. Since the complete DNA fragment between the T-DNA borders is usually transferred to the plant cell during *A. tumefaciens*-mediated transformation, it is expected that the other transgenes (bean *pgip1-hewl*, $hewl_{signal}$ and bean *pgip1*) should also be present in the transgenic plantlet.

Agrobacterium specific primers were used to detect traces of *Agrobacterium* still present on the plantlets. PCR analysis of genomic DNA confirmed no *Agrobacterium* contamination since none of the specific *Agrobacterium* amplification products appeared on the agarose gel (Figure 4.3) except for primer dimers caused by the primers annealing with each other and not with any of the template DNA sequences.

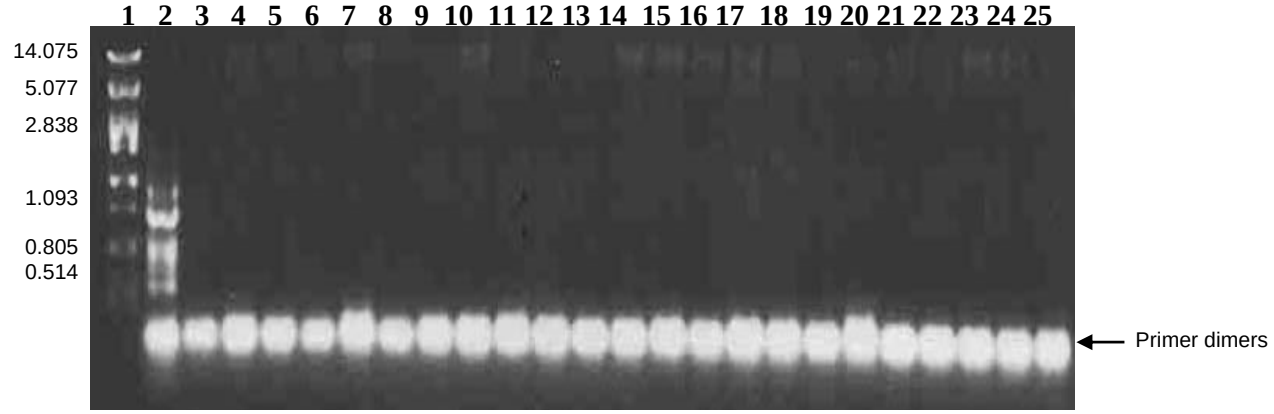

Figure 4.3 PCR analyses of transgenic lines using MIA primers specific for *Agrobacterium* detection. Lane 1, *Pst*I λ. Lane 2, *A. tumefaciens* LBA4404 positive control. PCR amplifications using genomic DNA from transgenic *N. tabacum* cv. LA Burley 21 lines produced with pGA482-bean *pgip1* (PG 2A, 6B, 7E, 8A and 9A; lanes 3-7); pCAMBIA2300-bean *pgip1-hewlS* (PGHS 2C, 3A, 5C, 7A, 7B, 9A and 10B; lanes 8-14); pCAMBIA2300-bean *pgip1-hewlA* (PGHA 1A, 2A, 3A, 6A and 8A; lanes 15-19) and pCAMBIA2300-$hewl_{signal}A$ (HSA 7, 10, 1A, 2A and 8A; lanes 20-24) constructs as template. Lane 25, water control.

Transgene specific primers were used to screen the genomic DNA for the presence of *nptII*, bean *pgip1-hewl*, $hewl_{signal}$ and the bean *pgip1* genes and a summary of the results shown in Figures 4.4-4.7 is given in Table 4.3.

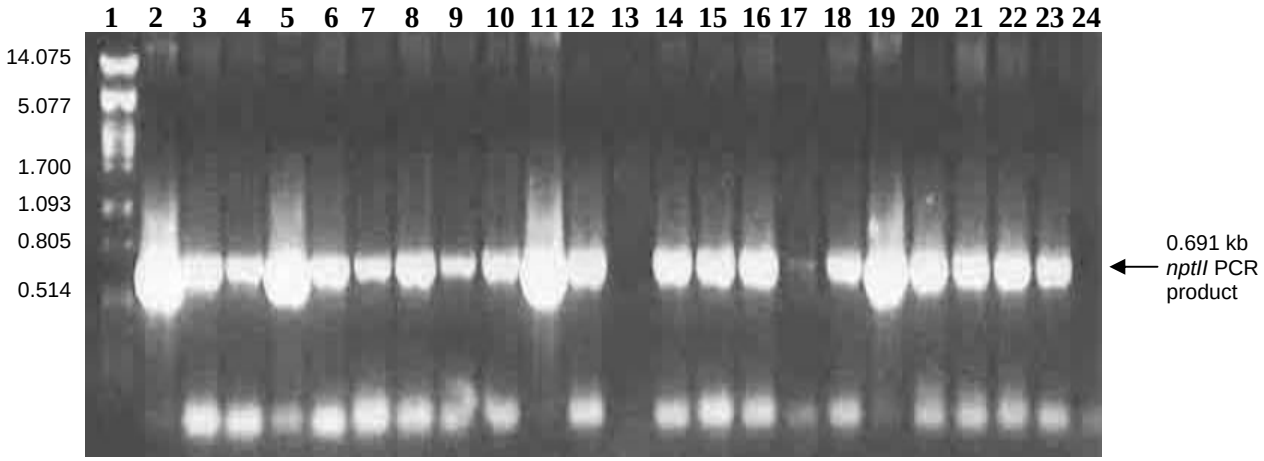

Figure 4.4 PCR analyses of transgenic lines using the *nptII* primers.

Lane 1, *Pst*I λ. Lanes 2, 5, 11 and 19 shows PCR amplifications using *nptII* gene specific primers of plasmid DNA pCAMBIA2300-$hewl_{signal}$*A*, pGA482-*pgip1*, pCAMBIA2300-*bean pgip1-hewlS* and pCAMBIA2300-bean *pgip1-hewlA* respectively as the template DNA which constitutes the positive controls showing the expected 0.691 kb PCR product. Genomic DNA isolated from lines transformed with pCAMBIA2300-$hewl_{signal}$*A* (HSA 7 and 10 respectively) was used as the template in PCR amplifications shown in Lanes 3 and 4. Genomic DNA isolated from lines transformed with pGA482-*pgip1* (PG 2A, 6B, 7E, 8A and 9A respectively) was used as the template in PCR amplifications shown in Lanes 6 to 10. Genomic DNA isolated from lines transformed with pCAMBIA2300-*bean pgip1-hewlS* (PGHS 2C, 3A, 5C, 7B, 9A and 10B respectively) was used as the template in PCR amplifications shown in Lanes 12 to 18. Genomic DNA isolated from lines transformed with pCAMBIA2300-bean *pgip1-hewlA* (PGHA 2A, 3A, 6A and 8A respectively) was used as the template in PCR amplifications shown in Lanes 20 to 23. A control PCR reaction with no template DNA is shown in Lane 24.

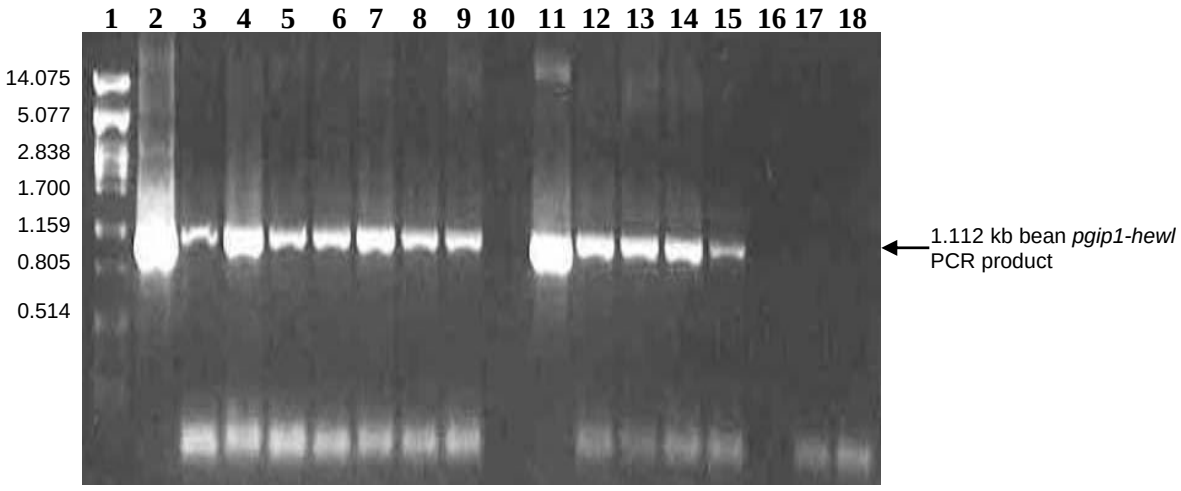

Figure 4.5 PCR analyses of transgenic lines produced from constructs pCAMBIA2300-bean *pgip1-hewlS* and pCAMBIA2300-bean *pgip1-hewlA*. Lane 1, *Pst*I λ. Lanes 2 and 10 shows PCR amplifications using bean *pgip1-hewl* gene specific primers of plasmid DNA pCAMBIA2300-bean *pgip1-hewlS* and pCAMBIA2300-bean *pgip1-hewlA* respectively as the template DNA that constitutes the positive controls. Genomic DNA isolated from transgenic lines (PGHS 2C, 3A, 5C, 7A, 7B, 9A and 10B) respectively, transformed with *A. tumefaciens* pCAMBIA2300-bean *pgip1-hewlS* was used as template in PCR amplifications shown in Lanes 2 to 8. Genomic DNA isolated from transgenic lines (PGHA 2A, 3A, 6A and 8A) respectively, transformed with *A. tumefaciens* pCAMBIA2300-bean *pgip1-hewlA*, was used as template in PCR amplifications shown in Lanes 11 to 14. Lanes 16 and 17 shows a PCR reaction containing non-transformed *N. tabacum* LA Burley 21 genomic DNA as the negative control and a water control, respectively.

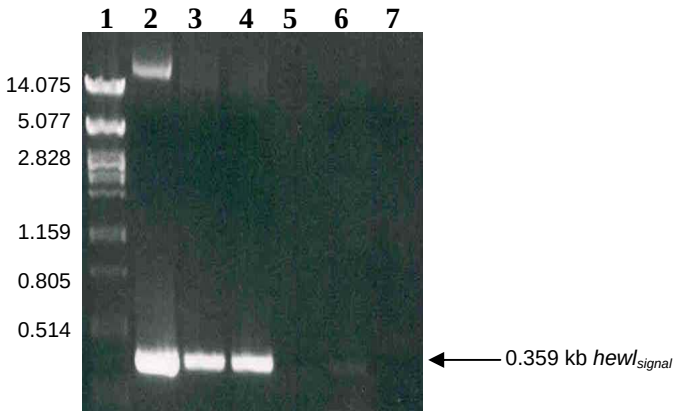

Figure 4.6 PCR analyses of transgenic lines produced from the construct pCAMBIA2300-*hewl*$_{signal}$*A*. Lane 1, *Pst*I λ. Lane 2 shows the PCR reaction using *hewl*$_{signal}$ gene specific primers of plasmid DNA pCAMBIA2300-*hewl*$_{signal}$*A* as the template DNA that constitutes the positive control. Lanes 3 and 4 shows PCR amplifications of transgenic lines HSA 7 and HSA 10 respectively that were produced by *A. tumefaciens* (pCAMBIA2300-*hewl*$_{signal}$*A*)–mediated transformation. Lanes 6 and 7 shows a PCR reaction containing non-transformed *N. tabacum* LA Burley 21 genomic DNA as the negative control and a water control, respectively.

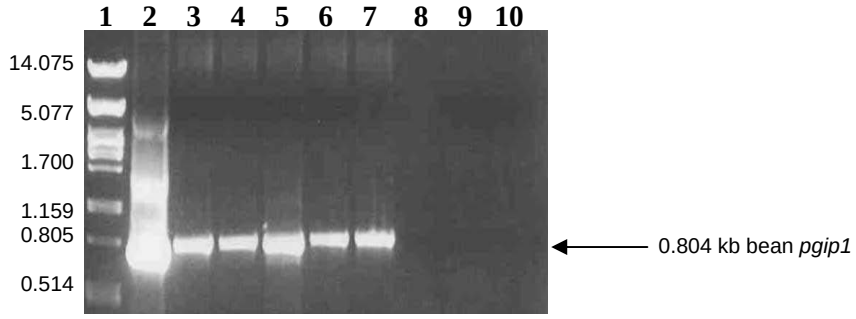

Figure 4.7 PCR analyses of transgenic lines produced from the construct pGA482-*pgip1*. Lane 1, *Pst*I λ. Lane 2 shows the PCR reaction using bean *pgip1* gene specific primers of plasmid DNA pGA482-*pgip1* as the template DNA that constitutes the positive control. Lanes 3 to 7 shows PCR amplifications of transgenic lines (PG 2A, 6B, 7E, 8A and 9A), respectively that were produced by *A. tumefaciens* (pGA482-*pgip1*)–mediated transformation. Lane 8 is empty. Lanes 9 and 10 shows a PCR reaction containing non-transformed *N. tabacum* LA Burley 21 genomic DNA as the negative control and a water control, respectively.

Table 4.3. Summary of results of PCR analyses of transgenic T_0 *Nicotiana tabacum* cv. LA Burley 21 tissue

Figure #.	Target DNA	PCR product (kb)	Transgenic lines	Number tested	Names of transgenic lines
4.4	*nptII*	0.691	PGHS	6	2C, 3A, 5C, 7B, 9A and 10b
		0.691	PGHA	4	2A, 3A, 6A and 8A
		0.691	HSA	2	7 and 10
		0.691	PG	5	2A, 6B, 7E, 8A and9A
4.5	bean *pgip1-hewl*	1.112	PGHS	7	2C, 3A, 5C, 7A, 7B, 9A and 10b
		1.112	PGHA	4	2A, 3A, 6A and 8A
4.6	$hewl_{signal}$	0.359	HSA	2	7 and 10
4.7	bean *pgip1*	0.804	PG	5	2A, 6B, 7E, 8A and9A

PGHS Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene.

PGHA Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene.

HSA Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene.

PG Transgenic lines transformed with pGA482-*pgip1*.

4.3.3 Production of T_1 transgenic *Nicotiana tabacum* cv. LA Burley 21 plants

4.3.3.1 Hardening off of *in vitro Nicotiana tabacum* cv. LA Burley 21 plants in the glasshouse

Positive transgenic plants were selected from the analyses of PCR results and were hardened off in the glasshouse to allow self-fertilisation to occur. Figure 4.9 shows that there was no phenotypic difference between the control plant and a representative transgenic plant. All the plants selected for self-fertilisation were phenotypically normal.

A **B**

Figure 4.8 Control and transgenic *Nicotiana tabacum* cv. LA Burley 21 plants in the glasshouse. A. *N. tabacum* cv. LA Burley 21 control. **B.** *N. tabacum* cv. LA Burley 21 transgenic plant (PGHS 5C).

4.3.3.2 Germination of T_1 transgenic *Nicotiana tabacum* cv. LA Burley 21 seeds

Seed produced from plants that had self-fertilized in the glasshouse were allowed to germinate on MS media containing kanamycin. Table 4.4 gives a summary of the percentages of T_1 seed germination. The surviving T_1 plants were all phenotypically normal. These plants were then selected for further analysed.

Table 4.4. T_1 seed germination

T_0 *N. tabacum* cv. LA Burley 21 transgenic plants	# sowed	# germinated	% germinated
PG 2A	224	29	12.9
PG 6B	269	77	28.6
PG 7A	263	104	39.5
PG 8A	253	72	28.4
PG 9A	244	32	13.1
PGHS 2C	284	0	0
PGHS 3A	280	10	3.5
PGHS 5C	214	1	0.4
PGHS 7A	333	77	23.1
PGHS 7B	412	4	0.9
PGHS 9A	289	29	10
PGHS 10B	329	7	2.1
PGHA 2A	233	39	16.7
PGHA 3A	298	4	1.3
PGHA 6A	311	143	45.9
PGHA 8A	289	5	1.7
HSA 7	229	1	0.4
HSA 10	258	56	21.7
NT	257	121	47.1

PGHS Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene.

PGHA Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene.

HSA Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene.

PG Transgenic lines transformed with pGA482-*pgip1*.

NT Non-transformed *Nicotiana tabacum* LA Burley 21

4.3.4 Verification of transgene insertion

4.3.4.1 PCR screening analysis of putatively transformed T_1 *Nicotiana tabacum* cv. LA Burley 21 tissue

Two transgenic plants each transformed with the pCAMBIA2300-bean *pgip1-hewlS* and pCAMBIA2300-bean *pgip1-hewlA* constructs, one transformed with pCAMBIA2300-*hewl*$_{signal}$*A* and one transformed with pGA482-*pgip1* were shown to be positive when analysed by PCR and run on an agarose gel depicted in Figure 4.9.

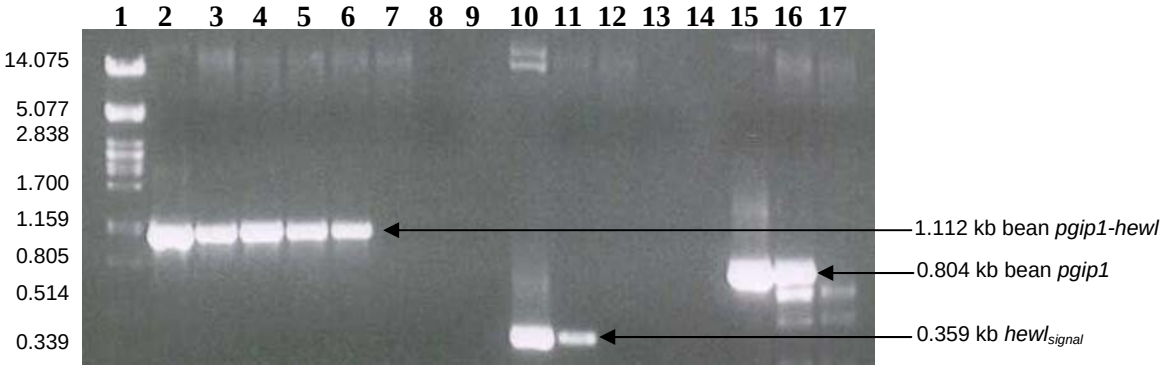

Figure 4.9 PCR analyses of T_1 transgenic lines produced from pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA*, pCAMBIA2300-*hewl*$_{signal}$*A* and pGA482-bean *pgip1* constructs. Lane 1, *Pst*I λ. Lanes 2, 10 and 15 shows PCR amplifications using gene specific primers of plasmid DNA pCAMBIA2300-*bean pgip1-hewlS,* pCAMBIA2300-*hewl*$_{signal}$*A*, pGA482-*pgip1*, respectively as the template DNA which constitutes the positive controls showing the expected 1.112 kb, 0.359kb and 0.804 kb PCR products. Genomic DNA isolated from transgenic lines PGHA 6A-1 and 8A-1 produced from *A. tumefaciens* (pCAMBIA2300-bean *pgip1-hewlA*)–mediated transformation and PGHS 5C-1 and 7A-1 produced from *A. tumefaciens* (pCAMBIA2300-bean *pgip1-hewlS*)–mediated transformation was used as template in PCR amplifications shown in lanes 2 to 6 respectively. Genomic DNA isolated from the transgenic line HSA 10 produced from *A. tumefaciens* (pCAMBIA2300-*hewl*$_{signal}$*A*)–mediated transformation was used as template in PCR amplifications shown in Lane 11. Genomic DNA isolated from transgenic line PG 7E produced from *A. tumefaciens* (pGA482-bean *pgip1*)–mediated transformation was used as template in PCR amplifications shown in Lane 16. Lanes 7 and 8, lanes 12 and 13 and lanes 17 and 18 shows PCR amplifications containing non-transformed *N. tabacum* LA Burley 21 genomic DNA as the negative control and a water control, respectively.

4.3.5 Southern blot hybridisation

Randomly selected lines were used to analyse transgene insertion into the plant genome. These lines comprised of four transgenic plants from independent transformation events transformed with the bean *pgip1-hewl* gene fusion (PGHA 6A and 8A and PGHS 5C and 7A), control transgenic plants transformed with the bean *pgip1* (PG 7E) and the $hewl_{signal}$ (HSA 10) genes as well as untransformed *N. tabacum* cv. LA Burley 21 as a negative control.

4.3.5.1 Digestion of pRTL2-bean *pgip1* in preparation for spiking non-transformed *Nicotiana tabacum* cv. LA Burley 21 genomic DNA during Southern blot hybridisation

pRTL2 *pgipl* was digested completely with *Pst*1 to release the expression cassette containing the bean *pgip1* gene fragment of 2.155 kb in length. Figure 4.10 shows in lane 3 the complete *Pst*I digestion of pRTL2-bean *pgip1* yielding 2 bands of 2.660 kb and 2.155 kb in length in comparison with undigested pRTL2-bean *pgip1* in lane 2 being 4.815 kb. Lane 4 and 5 has been loaded with a control undigested and *Pst*I digested pRTL2 respectively without any cloned cassette. This shows that linearised pRTL2 is indeed 2.660 kb in length depicted in lane 5.

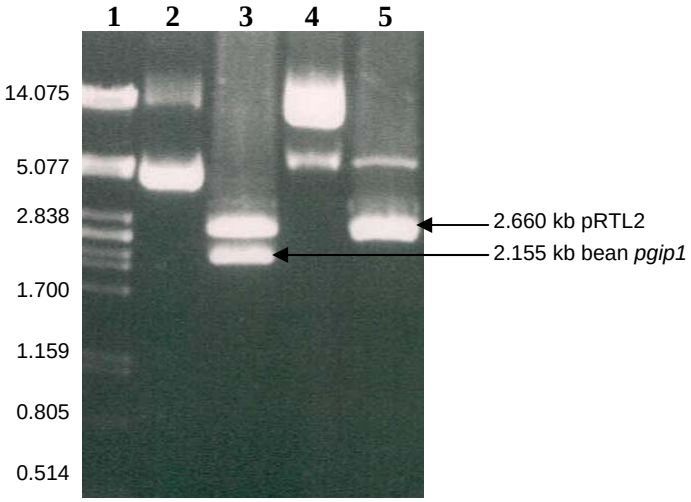

Figure 4.10 *Pst*I restriction digestion of pRTL2-bean *pgip1.* Lane 1, *Pst*I λ. Lane 2, undigested pRTL2-bean *pgip1;* Lane 3, *Pst*I digested pRTL2-bean *pgip1;* Lane 4, undigested pRTL2; Lane 5, *Pst*I digested pRTL2.

4.3.5.2 DIG-labelling of the bean *pgip1-hewl* probe

Labelling of the bean *pgip1-hewl* fusion gene with DIG in a PCR reaction yielded a non-radioactively labelled probe (Figure 4.11, Lane 3) of higher molecular weight than the unlabelled PCR product (Figure 4.11, Lane 2). This is due to the incorporation of DIG-dUTP during the PCR process. The unlabelled PCR product yielded a fragment of ± 0.6 kb using the BPG_{left} and $HEWL_{right}$ primers. Since the PCR product yielded is smaller than expected it could be that an incorrect primer was used for this PCR reaction and that another primer that specifically bound to the DNA at approximately 0.600 kb upstream or downstream with respect to the other primer was used. The primer used could have been one of the PGIP degenerate primers, which could explain the additional bands seen in lane 2 of Figure 4.11. Nonetheless, the probe, which constitutes a part of the bean *pgip1* gene, was definitely labelled with DIG, since (as seen in lane 3) a higher molecular weight PCR product was obtained.

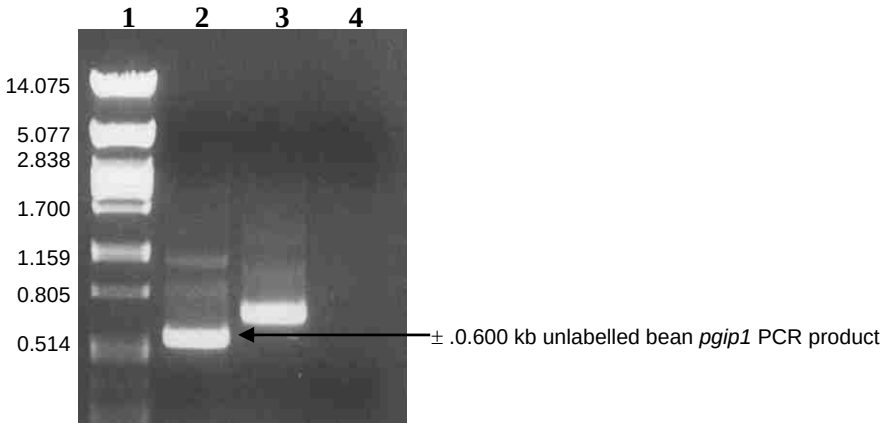

Figure 4.11 DIG-labelled bean *pgip1* PCR product. Lane 1, *Pst*I λ. Lane 2, unlabelled PCR product using the bean *pgip1* expression cassette as the template DNA along with the BPG_{left} and an unknown or degenerate PGIPright primer. Lane 3, DIG labelled bean *pgip1* PCR product.

4.3.5.3 Optimisation of restriction enzymes for the digestion of *Nicotiana tabacum* cv. LA Burley 21 genomic DNA

In choosing the appropriate restriction enzymes to obtain information regarding copy number and number of insertion events of the specific gene transformed into the *N. tabacum* cv. LA Burley 21 plant, it was important to test the digestion efficiency to ascertain complete digestion of *N. tabacum* cv. LA Burley 21 genomic DNA. For this, a small-scale restriction digestion experiment using the restriction enzymes *Pst*I and *Bam*HI separately was carried out using 500 ng of genomic DNA in order that the expected smear of fragments could be visualized after agarose gel electrophoresis. Selection of these two enzymes were based on the following: *Pst*I digestion excised the entire bean *pgip1* expression cassette and thus yielded information on the copy number of the gene in the *N. tabacum* cv. LA Burley 21 genome and *Bam*HI digestion allows one cut within the bean *pgip1* expression cassette and therefore yielded information that allowed the determination of the number of insertion events of the bean *pgip1* gene. Figure 4.12 shows a small degree of digestion of *N. tabacum* cv. LA Burley 21 genomic DNA thus failing to reveal complete digestion. However, it was proposed to continue using these enzymes in a large-scale restriction digestion experiment to seek out whether there would be a difference in digestion potential between the two experiments, which vary only in the length of the incubation of the reaction.

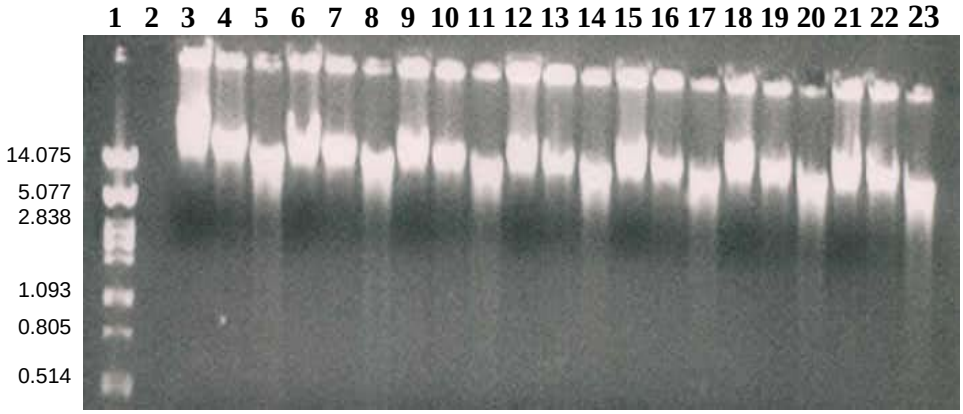

Figure 4.12 Small-scale restriction digestion of *Nicotiana tabacum* cv. LA Burley 21 genomic DNA. Lane 1, *Pst*I λ. Lanes 3, 6, 9, 12, 15, 18 and 21: undigested *N. tabacum* cv. LA Burley 21 genomic DNA of transgenic lines PG 7e, PGHA 6A, PGHA 8A, PGHS 5c, PGHS 7a, HSA 10 and non transformed *N. tabacum* cv. LA Burley 21 genomic DNA respectively. Lanes 4, 7, 10, 13, 16, 19 and 22: 500 ng *Pst*I digested *N. tabacum* cv. LA Burley 21 genomic DNA of above-mentioned transgenic lines. Lanes 5, 8, 11, 14, 17, 20 and 23: 500 ng *Bam*HI digested *N. tabacum* cv. LA Burley 21 genomic DNA of the same above-mentioned transgenic lines.

The enzyme digestion efficiency in the large-scale experiment described in detail in section 4.2.6.5 shows a slight alteration in the high molecular weight genomic DNA found at the top of specific wells in Figure 4.13. This was assumed to be an indication of possible digestion. However, complete digestion is still not evident yet the use of the samples digested with these two enzymes was continued in a large gel preparation for subsequent Southern blotting experiments.

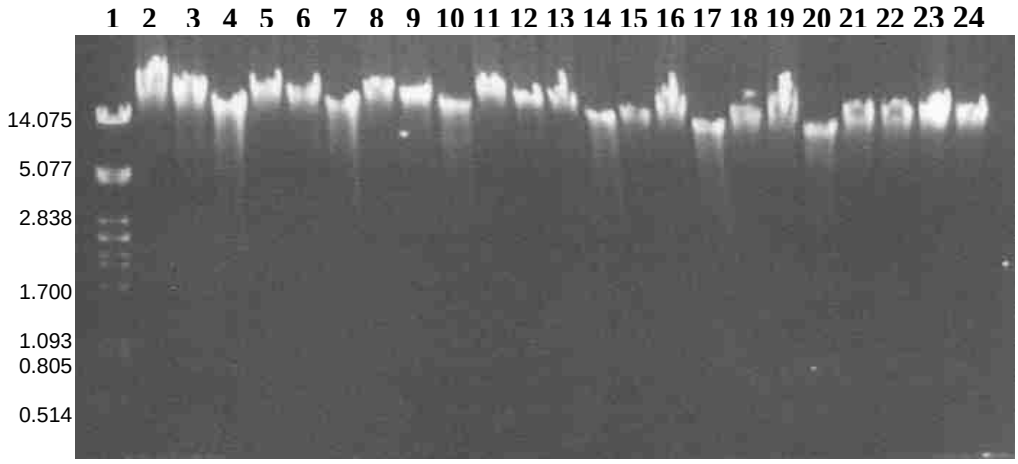

Figure 4.13 Large-scale restriction digestion of *Nicotiana tabacum* cv. LA Burley 21 genomic DNA. Lane 1, *Pst*I λ. Lanes 2, 5, 8, 11, 14, 17 and 20 and: undigested *N. tabacum* cv. LA Burley 21 genomic DNA of transgenic lines PG 7e, PGHA 6A, PGHA 8A, PGHS 5c, PGHS 7a, HSA 10 and non transformed *N. tabacum* cv. LA Burley 21 genomic DNA respectively. Lanes 3, 6, 9, 12, 15, 18, 21, 23 and 24: 500 ng *Pst*I digested *N. tabacum* cv. LA Burley 21 genomic DNA of above-mentioned transgenic lines. Lanes 4, 7, 10, 13, 16, 19 and 22: 500 ng *Bam*HI digested *N. tabacum* cv. LA Burley 21 genomic DNA of digested *N. tabacum* cv. LA Burley 21 genomic DNA of the same above-mentioned transgenic lines.

After electrophoresis at low voltage and ethidium bromide staining of the digested samples in a larger gel, a better representation of digestion efficiency was visualized, Figure 4.14. Digestion of *N. tabacum* cv. LA Burley 21 genomic DNA appeared as long smears for some of the samples indicative of complete digestion and for others digestion did not appear to be complete.

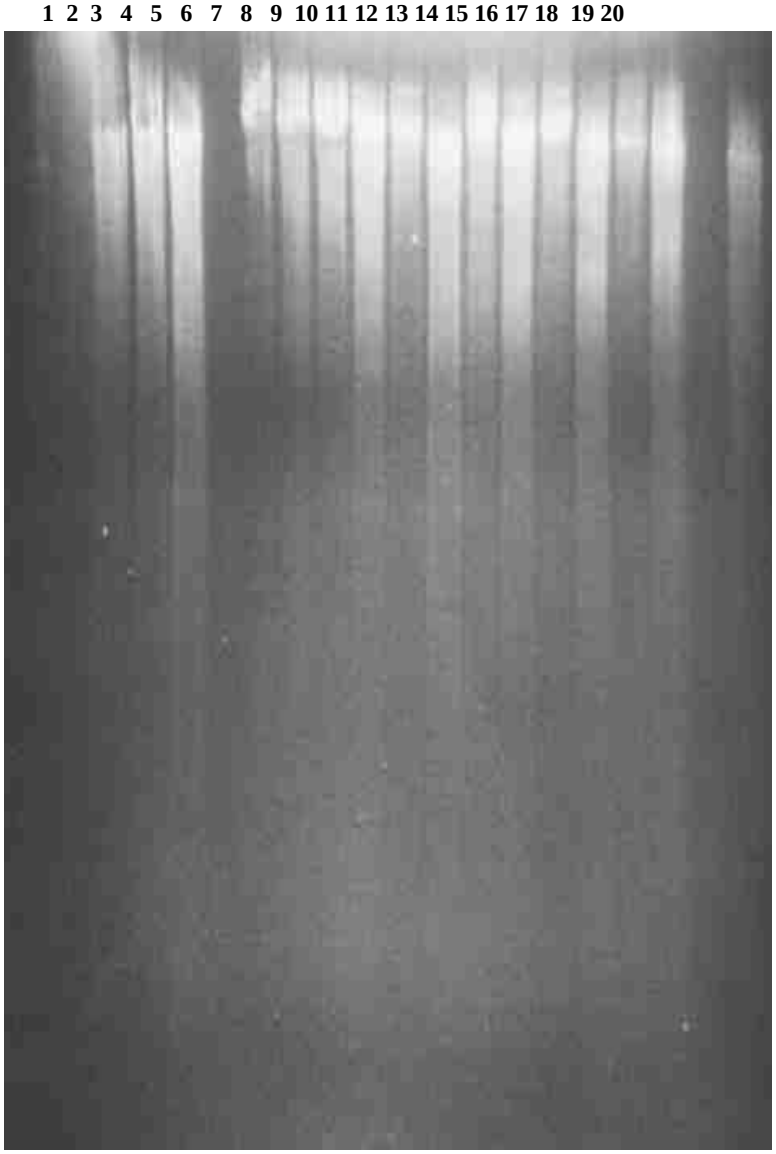

Figure 4.14 Agarose gel electrophoresis of large-scale digestion of transgenic *Nicotiana tabacum* cv. LA Burley 21 lines for Southern blot analysis. Contents of lanes are indicated in Table 4.5.

Table 4.5. Loading order of *Nicotiana tabacum* cv. LA Burley 21 genomic DNA T_1 samples for Southern hybridisation

Lane	DNA	Restriction enzyme[a]
1	DIG-λDNA (60ng)	*Hind*III
2		
3	Non-transformed *N. tabacum* cv. LA Burley 21	*Pst*I (1 x copy bean *pgip1*)[b]
4	"	*Pst*I (10 x copy bean *pgip1*)[b]
5	"	*Pst*I (20 x copy bean *pgip1*)[b]
6		
7	PG[f] 7E-1	*Pst*I
8	"	*Bam*HI
9	PGHA[d] 6A-1	*Pst*I
10	"	*Bam*HI
11	PGHA[d] 8A-1	*Pst*I
12	"	*Bam*HI
13	PGHS[c] 5C-1	*Pst*I
14	"	*Bam*HI
15	PGHS[c] 7A-1	*Pst*I
16	"	*Bam*HI
17	HSA[e] 10-1	*Pst*I
18	"	*Bam*HI
19		
20	Non-transformed *N. tabacum* cv. LA Burley 21	Undigested

a: specific restriction enzyme used for DNA digestion

b: bean *pgip1* fragment of 2.155 kb prepared as described in section 4.3.5.1

c: PGHS: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene.

d: PGHA: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene.

e: HSA: Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene.

f: PG: Transgenic lines transformed with pGA482-*pgip1*.

4.3.5.4 Southern blotting, hybridisation and detection of DIG-labelled probe

The gel containing separated fragments was blotted onto a nylon membrane as described in the Materials and Methods section 4.2.5.6.

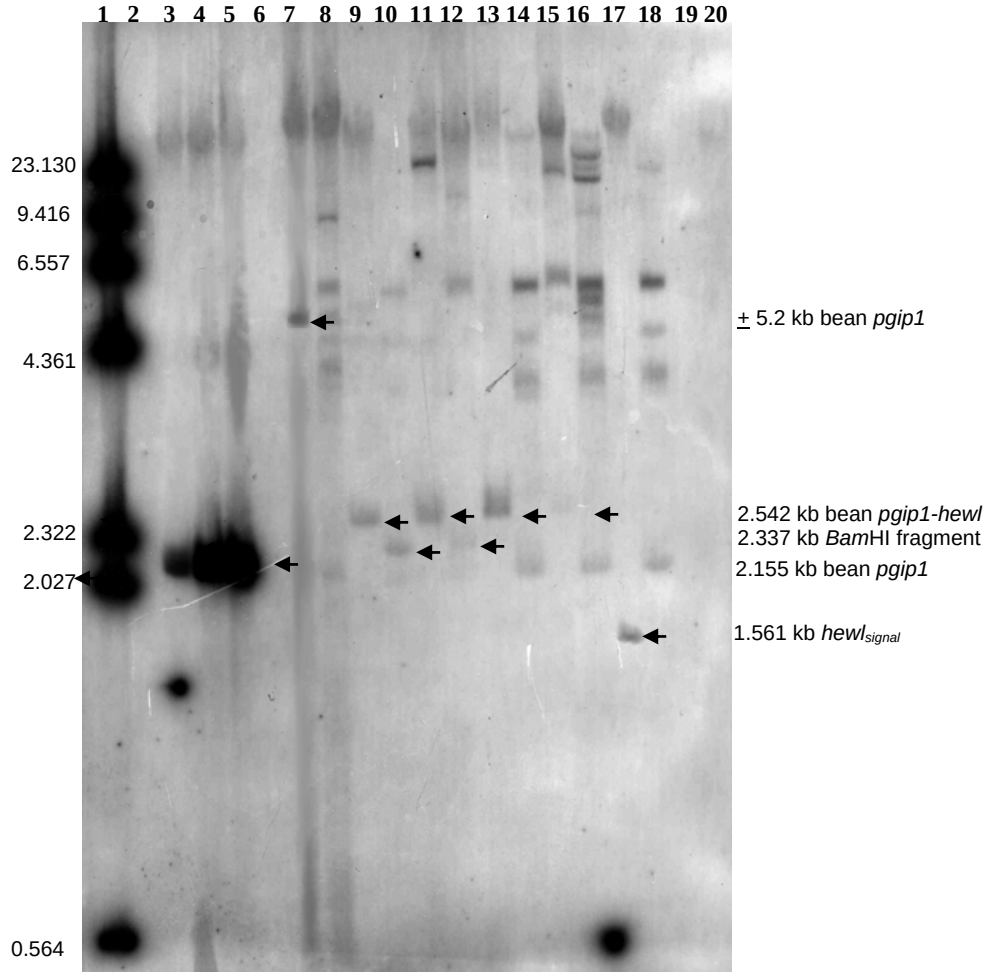

Figure 4.15 Autoradiograph showing the Southern blot analysis of DIG labelled bean *pgip1-hewl* gene probe hybridisation to digested genomic DNA from transgenic *Nicotiana tabacum* cv. LA Burley 21 lines. Lane1: DIG-λDNA (60ng) *Hind*III; lanes 3-5: 1, 10 and 20 copies of apple pgip1 spiked into 10 µg of *Pst*I digested untransformed *N. tabacum* cv. LA Burley 21 genomic DNA, respectively; lanes 7-18: 10 µg of the following transgenic *N. tabacum* cv. LA Burley 21 genomic DNA digested with the indicated restriction enzymes; lanes 7 and 8: PG 7E-1 digested with *Pst*I and *Bam*HI, respectively; lanes 9 and 10: PGHA 6A-1 digested with *Pst*I and *Bam*HI, respectively; lanes 11 and 12: PGHA 8A-1 digested with *Pst*I and *Bam*HI, respectively; lanes 13 and14: PGHS 5C-1 digested with *Pst*I and *Bam*HI, respectively; lanes 15 and 16: PGHS 7A-1 digested with *Pst*I and *Bam*HI, respectively; lanes 17 and 18: HSA 10-1 digested with *Pst*I and *Bam*HI, respectively; lane 20: 10 µg *Pst*I digested untransformed *N. tabacum* cv. LA Burley 21 genomic DNA. The contents of lanes are indicated in Table 4.5.

Klenow end labelling of λDNA/ *Hind*III with DIG was successful, since 60 ng of the labelled λDNA/ *Hind*III was sufficient for detection after electrophoresis and blotting to a membrane during Southern blot (Figure 4.15, lane 1). Sharp bands formed where the bean *pgip1-hewl* probe hybridised to the membrane and the anti-DIG antibody/ alkaline phosphatase conjugate subsequently bound. One copy of the bean *pgip1* gene spiked into 10 µg *Pst*I digested non-transformed *N. tabacum* cv. LA Burley 21 genomic DNA was successfully detected (Figure 5.15, lane 3). The fragment of DNA used for spiking was digested from pRTL2-bean *pgip1* and was 2.155 kb. The fragment that hybridises to the bean *pgip1-hewl* probe consists of the CaMV e35S promoter, TEV leader and the bean *pgip1* gene. Since the probe was a product of PCR comprising a fusion between the bean *pgip1* and the *hewl* gene it is expected that only the part consisting of the bean *pgip1* gene hybridised to the bean *pgip1* gene used to spike the transgenic *N. tabacum* cv. LA Burley 21 genomic DNA. Therefore, the expected size of the fragment detected on the Southern blot is 2.155 kb.

*Pst*I digestion of the transgenic *N. tabacum* cv. LA Burley 21 bean *pgip1* line yielded a single hybridising fragment of approximately 5.200 kb (Figure 4.15, lane 7). For bean *pgip1-hewlA and S* transgenic *N. tabacum* cv. LA Burley 21 lines digested with *Pst*I, a single hybridising fragment of approximately 2.542 kb was detected (Figure 4.15, lanes 9, 11, 13 respectively) with PGHS 7a only giving signals in the large molecular weight region (Figure 4.15, lane 15). The expected fragment size for *Pst*I digestion of the bean *pgip1-hewl* transgenic *N. tabacum* cv. LA Burley 21 lines is 2.542 kb. Transgenic *N. tabacum* cv. LA Burley 21 $hewl_{signal}$ lines digested with *Pst*I gave a faint signal corresponding to a single hybridisation fragment of approximately 1.500 kb. The fragment size expected was 1.561 kb.

Digestion of the transgenic *N. tabacum* cv. LA Burley 21 lines with *Bam*HI yielded hybridising fragments of varying sizes. *Bam*HI digestion was done to give an indication of the number of insertion events; however the Southern Blot revealed *Bam*HI not to be the best candidate restriction enzyme to have used to determine the number of insertion events for PGHA transgenic tobacco plants. The search for a single cutter within the T-DNA of the constructs below was done using *Vector™ NTI*. At the time of the search only one *Bam*HI site was recognized, however upon analysis of the Southern blot autoradiograph a second *Bam*HI restriction enzyme recognition site gives explanation for a 2.337 kb band (Figure 4.15; lanes 10 and 12) obtained for *Bam*HI digested PGHA 6A-1 and PGHA 8A-1 genomic DNA that hybridised to the bean *pgip1-hewl* hybridisation probe. These *Bam*HI recognition sites are shown in Figure 4.16. The bands occurring in Figure 4.15; lanes 14, 16 and 18 could give an

indication of the number of insertion events. A 1.380 kb band expected in lane 18 for *Bam*HI digested HSA genomic DNA was not detected.

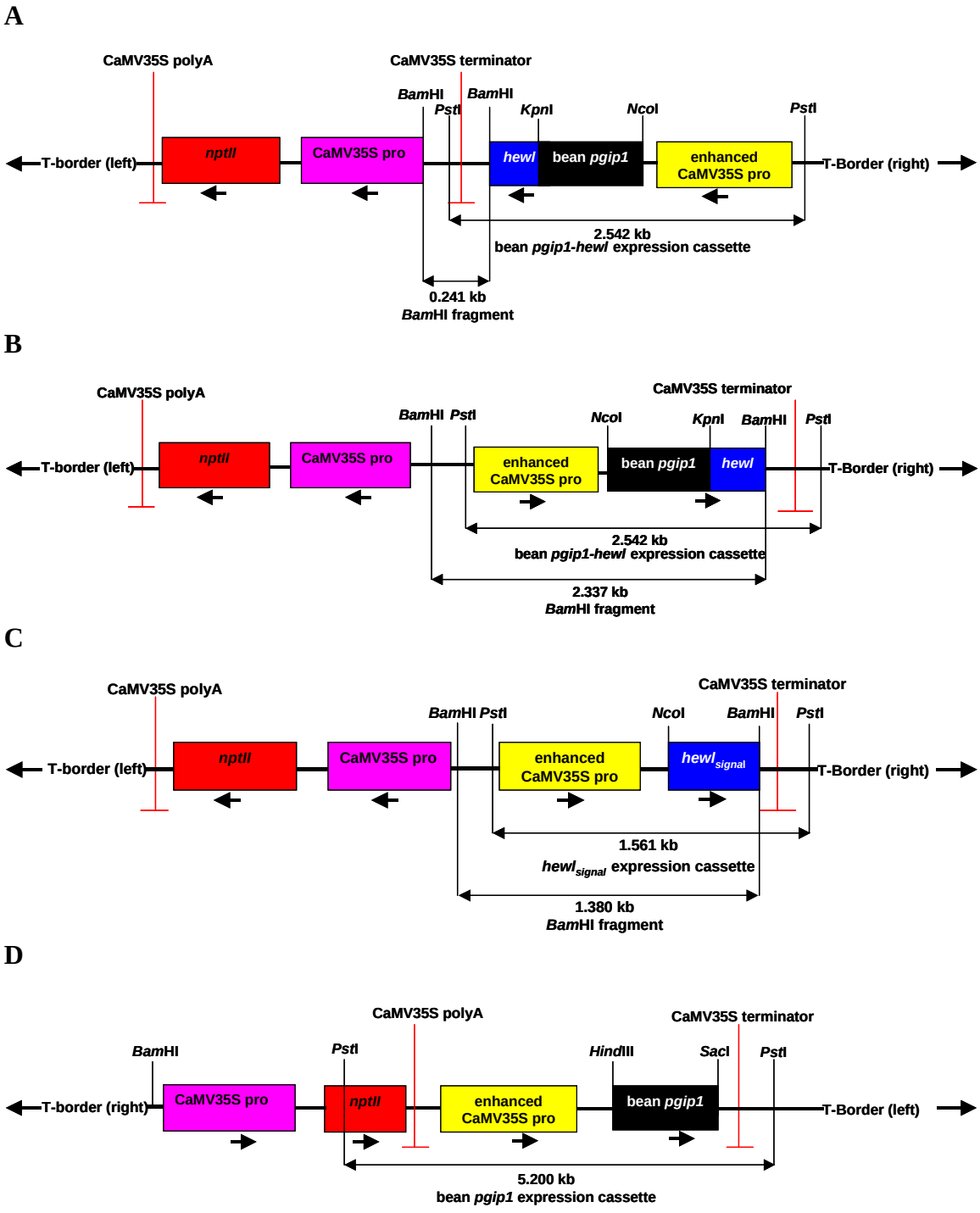

Figure 4.16 Schematic depiction of the T-DNAs integrated into *Nicotiana tabacum* cv. LA Burley 21 genome. A. pCAMBIA2300-bean *pgip1-hewlS;* **B**. pCAMBIA2300-bean *pgip1-hewlA;* **C** pCAMBIA2300-$hewl_{signal}A$**;** **D**. pGA482-bean *pgip1*.

4.4 Discussion

This chapter focused on the transformation of *N. tabacum* cv. LA Burley 21 plants. Transformations were done using the *Agrobacterium*-mediated method to transform *N. tabacum* cv. LA Burley 21 independently with each of the following genes, the bean *pgip1-hewl* gene fusion, the *bean pgip1* gene and the $hewl_{signal}$ gene. Verification of gene integration was done by molecular characterization of transgenic plants, which included PCR and Southern blot hybridisation respectively. The resultant visually normal putative T_0 transgenic *N. tabacum* cv. LA Burley 21 plants were scrutinized for the presence of the transgene by PCR, a selection procedure for the identification of positive transgenics to be further characterised. The lines chosen to be self-fertilised included PGHS 2C, 3A, 5C, 7B, 9A and 10b, PGHA 2A, 3A, 6A and 8A; HSA 7 and 10 and PG 2A, 6B, 7E, 8A and9A. After a process of T_0 self-fertilisation, the putative T_1 transgenics were analysed to confirm transgene integration in the *N. tabacum* cv. LA Burley 21 genome by both PCR and Southern blot hybridisation respectively.

4.4.1 Rooting of transgenic plantlets on kanamycin containing MS media

The successful rooting of transformed plantlets on MS media containing kanamycin suggested that the expression of the *nptII* gene was present on the T-DNA. Since the complete DNA fragment between T-DNA borders is transferred to the plants during *Agrobacterium*-mediated transformation, it is expected that the other transgenes will be present in the transgenic plantlets. To corroborate this theory, genomic DNA from transgenic plants was subjected to PCR using primers that were specific for the *nptII* gene. The presence of the *nptII* gene was shown in all transgenic lines (Figure 4.4) except in PGHA 3A. There are two possible reasons for this occurrence, one being that the plant was either unsuccessfully transformed and the other that the DNA template used in the PCR reaction was insufficient for the amplification reaction to produce a PCR positive product. The latter reason is a more accepted theory since the specific plant line that tested negative during PCR survived on kanamycin containing MS media, which gives an indication of kanamycin resistance.

4.4.2 PCR verification to verify the presence of the transgenes

After establishing and selecting transgenic plants that successfully expressed the *nptII* gene, subsequent screening for the presence of specific genes were carried out also by means of PCR using specifically designed primers unique for its corresponding gene. All lines tested showed present the specific transgenes namely the bean *pgip1-hewl* gene fusion, the *bean pgip1*

gene and the *hewl*$_{signal}$ gene (Figures 4.4 to 4.7). The outcome of this result facilitated in choosing lines that was to be taken through to the next generation. Plants were then allowed to self-fertilise in a glasshouse to initiate the generation of T_1 progeny. Again, subsequent analysis on these T_1 progeny by PCR was a means to determine whether or not the genes of interest could be passed on from one generation to the next. At this stage of experimentation though only a few lines were chosen to represent a particular set of transformed *N. tabacum* cv. LA Burley 21. This was done to reduce the amount of samples that would subsequently be examined for transgene integration in the genomic DNA of *N. tabacum* cv. LA Burley 21 by Southern blot. Again all the T_1 lines tested were positive for the specific transgenes by PCR.

4.4.3 Southern blot hybridisation

Accurate measurement of the copy number demands that all the DNA of the inserted gene is contained on a single restriction fragment so that their signal can be compared with that of an artificial reconstruction DNA, i.e., non-transformed *N. tabacum* cv. LA Burley 21 genomic DNA spiked with (bean *pgip1*), a fragment which includes sequences with homology to the probe DNA, so that the probe hybridises to only one band on the final blot. The copy number of the transgene that was inserted into the *N. tabacum* cv. LA Burley 21 genome was determined by *Pst*I restriction digestion that excised the entire transgene expression cassette located between the T-DNA border sequences. The bean *pgip1-hewl* probe hybridised to fragments of approximately 5.200 kb (Figure 4.15; lane 7), 2.155 kb (Figure 4.15; lanes 9, 11, 13 and 15) and 1.561 kb (Figure 4.15; lane 17) for *Pst*I digested pGA482-bean *pgip1*, pCAMBIA2300-bean *pgip1-hewlS*, pCAMBIA2300-bean *pgip1-hewlA* and pCAMBIA-*hewl*$_{signal}$ T-DNAs. The insertion of the transgenes, bean *pgip1*, bean *pgip1-hewl* and *hewl*$_{signal}$ into the genomic DNA of *N. tabacum* was thus successful. In determining the copy number of each of the specific transgenes, which is achieved by comparing the hybridising densities of the resulting fragments to non-transformed samples spiked with a known number of copies (Figure 4.16, lanes 3-5), it was discovered that a *Pst*I digested fragment from pRTL2-bean *pgip1* was used instead of a *Pst*I digested fragment from pRTL2-bean *pgip1-hewl* containing the bean *pgip1-hewl* fusion expression cassette to be used for spiking the non-transformed *N. tabacum* genomic DNA. Therefore because of this experimental error, the copy number calculation in section 4.2.5.2 was incorrect, meaning that instead of spiking with 3.2 pg of bean *pgip1-hewl*, 3.2 pg of bean *pgip1* was used. This did not constitute a 1x copy of the fragment containing the bean *pgip1* gene since upon calculating the amount of DNA to represent a 1x copy of the fragment containing bean *pgip1* the picograms of DNA was reduced to 2.7 as shown in section 4.2.5.1.2. Thus in Figure 4.16 it is seen that the intensity of

the bands of the transgenic *N. tabacum* plants were lighter than that of the non-transformed *N. tabacum* genomic DNA spiked with a bean *pgip1* containing fragment. Another reason for the appearance of the intense band in lane 3 of Figure 4.16 could be due to contamination from wells 4 and 5 containing a 10x and a 20x copy of the expression cassette. Therefore the lanes containing the spiked copy numbers does not yield promising results for the estimation of the number of copies of the transgene in the *N. tabacum* cv. LA Burley 21 transgenic lines.

To determine the number of insertion events the excised fragment should contain T-DNA border sequences, which could result in dispersion of the signal if multiple insertions have occurred at more than one site in the plant DNA. It was thought that insertion events could be determined by digesting the transgenic plant genomic DNA with the restriction enzyme *Bam*HI, since at the time of searching for this appropriate candidate an error occurred revealing *Bam*HI to have one recognition site between the T-borders of pCAMBIA2300-bean *pgip1-hewlS,* pCAMBIA2300-bean *pgip1-hewlA,* pCAMBIA2300-*hewl*$_{signal}$*A* and pGA482. This was not the case and instead a second *Bam*HI restriction enzyme site was found that resulted in the bean pgip1-hewl probe to hybridise to a specifically sized fragment of 2.337 kb.

Using the above mentioned enzymes, restriction digestions of the *N. tabacum* cv. LA Burley 21 genomic DNA for Southern blot is shown to be incomplete in Figure 4.14 since even though a long smear indeed has appeared on the gel after electrophoresis, the smears do not run all the way to the end of the gel. A possible reason for this incomplete digestion could be that the recognition sites for these enzymes might occur at low frequencies in the genome of *N. tabacum* cv. LA Burley 21. These enzymes then are known as rare cutters. Even though restriction digestions didn't seem complete, the Southern blot was continued since even extra units of enzyme and increased incubation time could not be improve digestions. Irrespective of this, the DIG-detection kit produced signals sufficient to detect 1 copy of the bean *pgip1-hewl* fusion gene spiked into 10 µg *Pst*I digested non-transformed *N. tabacum* cv. LA Burley 21 genomic DNA. The sensitivity of detection with the DIG-system was thus sufficient to detect single copy insertions of the transgene into the genome of the transgenic *N. tabacum* cv. LA Burley 21 lines.

Bean *pgip1-hewl* transgenics digested with *Pst*I gave the expected size fragment (2.542 kb) and the Southern blot results related well with this. Fragments of approximately 2.500 kb were obtained for lines PGHA 6A, PGHA 8A and PGHS 5c (Figure 5.14, lanes 9, 11 and 13

respectively). The transgenic line PGHS 7a showed only a faint indication of the fact that a hybridising fragment could be present, however since additional bands in the large molecular weight region of the gel is present, it can be assumed that because of incomplete digestion, migration of the specific sized bands were retarded.

The results of the Southern blot of genomic DNA digested with *Bam*HI shows multiple bands indicative of the number of insertion events of the transgene within the *N. tabacum* cv. LA Burley 21 genomic DNA. However, true bands and those that could be as a result of non-specific binding or because of incomplete digestion cannot be distinguished with certainty.

The bean *pgip1-hewl* probe did not hybridise to the non-transformed *N. tabacum* cv. LA Burley 21 genomic DNA (Figure 4.16, lane 20). The assumption then is that the bean *pgip1-hewl* gene sequence is sufficiently different from any similar endogenous gene sequences present in *N. tabacum* cv. LA Burley 21 genome. Therefore it is clear that the bands formed on the Southern blot for the different transgenic lines are due to the presence of the transgenes.

This chapter then concludes the confirmation of successful transformation of *N. tabacum* cv. LA Burley 21 with the relevant transgenes by showing its presence through PCR and Southern blot. The positive lines include PGHS (2C, 3A, 5C, 7B, 9A and 10b); PGHA (2A, 3A, 6A and 8A); HSA (7 and 10) and PG (2A, 6B, 7E, 8A and9A). Even though Southern blot results do not give precise indication of the number of insertion events, it does however prove transgene integration to be successful. Further analysis of transgene expression in transgenic *N. tabacum* cv. LA Burley 21 lines will be discussed in Chapter 5.

CHAPTER 5

Transgene expression analysis of bean PGIP1-HEWL in transgenic *Nicotiana tabacum* cv. LA Burley 21

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5.1 Introduction

The aim of this chapter is to assess the expression of transgenic *N. tabacum* cv. LA Burley 21 cv. LA Burley 21 lines produced by transformation with the bean *pgip1-hewl* fusion, the bean *pgip1* and the $hewl_{signal}$ genes. Assessment would involve evaluating the ability of plant protein extractions from bean *pgip1-hewl* fusion and bean *pgip1* transgenic to show PGIP activity by inhibiting endopolygalacturonases (endo-PGs) of the *Stenocarpella maydis* fungus and from bean *pgip1-hewl* fusion and $hewl_{signal}$ transgenics to show HEWL activity by hydrolysing the cell wall of the bacterium *M. luteus*. The preceding chapter focussed on the molecular characterisation of these transgenic plants where it was shown that the genes had been successfully integrated into the *N. tabacum* cv. LA Burley 21 genomic DNA under the control of the constitutive enhanced CaMV 35S (e35S) promoter. Then, to further characterise the transgenic plants, besides investigating whether both gene products are functional, completeness of the study relies on the localization of the lysozyme gene products hypothesised to be translocated to the intercellular spaces. This is essential in providing conclusive evidence on whether the *pgip* gene could act as a signal peptide for a variety of valuable proteins to be localized in the intercellular spaces, by using *hewl* as the model gene, in order that its functioning could be optimally executed.

Mature PGIP, a cell wall-associated protein forms reversible high-affinity complexes with fungal endo-PGs and it is because of this very nature of the enzyme that allows for *in vitro* activity studies to be performed. Several methods have been developed to determine the polygalacturonase activity of culture filtrates. One method, known as the agarose diffusion assay (Taylor and Secor, 1988), is a rapid, qualitative assay where the key to its performance lies in the ability of the endo-PGs present in the culture filtrates to degrade polygalacturonic acid (a substrate for endo-PGs). The end result of most agarose diffusion assays is either formation of clear zones or no zone that is representative of a particular reaction. In the case with assaying PG activity, upon applying the filtrate into a suitably sized well, the PG either diffuses out into the agarose or not. The latter is indicative of the absence of PG activity and a clear zone would represent PG activity. Activity of the endopolygalacturonases on the

substrate is detected by staining the agarose plates with ruthenium red dye. The ruthenium red dye interacts with unhydrolysed polygalacturonic acid. Alternatively, with the incorporation of a PGIP specific for the particular endo-PG, before application of the filtrate into the wells, the expected outcome would include the absence of a clear zone, meaning that the PGIP interacted with the endo-PG preventing it from degrading the polygalacturonic acid substrate within the agarose.

Another method, the reducing sugar assay, is a quantitative assay that measures the reducing sugars present in the test reaction. In this assay, the endopolygalacturonases degrade the substrate polygalacturonic acid to produce the reducing sugars. A colour reagent, added to the test reaction, reacts with the reducing sugars. The product of the reaction, which is proportional to the amount of reducing sugars present, can then be quantified spectrophotometrically.

Various colour reagents for the determination of reducing sugars have been described. In 1944, Nelson described a colorimetric method using arsenomolybdate reagent, which when used with a copper reagent (Somogyi, 1937; 1952), produced a colour precipitate. The principle of the reaction is based on the reduction of the copper reagent by the reducing sugars resulting in cuprous oxide as the product. The arsenomolybdate reagent is added and a reaction between the cuprous oxide and the chromogen results in the development of a colour product. The product, which absorbs at 500 nm, is proportional to the reducing sugars present in the reaction. The reagent that is being used to date for colorimetric determination of carbohydrates is ρ-4-amino-2-hydroxybenzoic acid hydrazide (PAHBAH; Lever, 1972). PAHBAH reacts with reducing sugars under alkaline conditions to produce yellow anions. The carbohydrate derivatives (yellow anions) absorb at a wavelength of 410 nm and the colour is proportional to the amount of reducing sugars present. The reaction between PAHBAH and reducing sugars is an easy assay to perform and is sensitive to detect less than 1 μg monosaccharide sugar.

Polygalacturonase-inhibiting proteins (PGIPs) play an important role in plant defences by forming specific, reversible, high-affinity complexes with the endopolygalacturonases (endo-PGs) of invading fungi that catalyse the fragmentation and solubilisation of the plant cell wall polysaccharides. Since, the activities of PGIPs are extremely specific, a study of bean PGIP1 has shown it to inhibit the endo-PGs of the fungus *Stenocarpella maydis* (Berger *et al*., 2000). *S. maydis* was formerly known as *Diplodia maydis* and is the cause of

ear rot on corn during warm, humid weather. The endo-PGs of *S. maydis* are used in this study for the relevant enzyme assays to test the activity of the bean PGIP1.

Bacterial cell walls are of two types: Gram negative and Gram positive. Gram-positive bacteria have a cell wall composed of a thick layer of peptidoglycan overlaid by a thinner layer of teichoic acid. In contrast, Gram-negative bacteria have a thinner layer of peptidoglycan, which is enclosed in a second lipid bilayer. The 'Gram' designation comes from whether or not the specific bacteria are stained by a reaction series developed by a Danish physician, Hans Christian Gram. Lysozyme is most effective against Gram-positive bacteria since the peptidoglycan layer is relatively accessible to the enzyme and affects Gram-negative bacteria only after the outer membrane has been compromised.

Lysozymes attack bacterial cell walls by degrading the sugar backbone of the high molecular weight, insoluble peptidoglycan polymer. It does this by specifically adding water to the glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) (hydrolysis). Accordingly, the most widely used substrate for measuring lysozyme activity is this gigantic polymer, employed in the form of dried cells of UV-inactivated *M. luteus,* which is a Gram-positive bacterium (Shugar, 1952; Smolelis and Hartsell, 1949). As a result of the insolubility of so complex a substrate as the cell wall, the reaction kinetics cannot be interpreted as true Michaelis-Menten parameters. Measurement of the degradation of the high molecular weight substrate is a rather simple and quite sensitive method of following the enzyme reaction.

Commonly, three different assay procedures are employed: (1) a turbimetric assay, (2) a viscosimetric assay and (3) the lysoplate assay. The turbimetric assay follows the decrease in optical density of a turbid cell suspension (about 0.2 mg dried *M. luteus* cells/ml) photometrically at about 650, 520 or 420 nm. The method has been substantially improved by using a microtiter plate reader to determine the changes in the turbidity of the bacterial suspension. The procedure allows the detection of enzyme activity in the range of 1 to 100 ng/ml (Moreira-Ludewig and Healy, 1992). A major problem with these turbimetric assays using *M. luteus* cells (as a substrate) is its poor reproducibility due to lack of homogeneity of the cell suspensions. Recently, an interesting modification of the classical method has been presented. By the use of *M. luteus* labelled with Remazol brilliant blue, the release of soluble blue products by the action of lysozyme can easily be measured spectrophotometrically at 600 nm (Ito *et al*., 1992).

In the second method, fragmentation of the insoluble peptidoglycan into soluble muropeptides is accompanied by a decrease in the viscosity of the substrate suspension. Thus, the changes in the viscosity during depolymerisation of the peptidoglycan have been exploited to measure lysozyme activity. Due to the endo-muramidase activity of lysozyme the viscosity decreases in a non-linear fashion with only a narrow range of linearity (Meyer and Hahnel, 1946).

The decrease in the turbidity of cell walls or dried cells is also utilized to measure lysozyme activity in the lysoplate method (Osserman and Lawlor, 1966). Heat-killed *M. luteus* cells in phosphate buffer are added to 1% agarose and poured into Petri dishes. Enzyme samples are applied to wells punched into the solidified agarose (about 3 mm in diameter) and the zones of clearance around the wells that develop upon incubation are measured. The diameters of the zones of clearance are proportional to the log of concentration of enzyme. The great advantage as compared to the turbimetric measurements with *M. luteus* suspensions of this spot test on plates is its linearity over a much wider range of enzyme concentrations. In addition, the method can be automated and is therefore easily applicable for analysing large numbers of samples.

In an attempt to confirm lysozyme activity in transgenic *N. tabacum* cv. LA Burley 21 leaf extracts a commercial lysozyme sensitive assay kit [The EnzCheck® Lysozyme Assay Kit, E–20013, Molecular Probes (Invitrogen), USA] that measures levels of lysozyme activity in solution was exploited. This is a fluorescence-based assay that measures the levels of lysozyme activity in solution down to 20 U/ml. The assay measures lysozyme activity on *M. luteus* cells walls, which are labelled to such a degree that the fluorescence is quenched. Lysozyme action can relieve this quenching; yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. Fluorescence is then measured in a fluorescence microplate reader using excitation/emission of ~485/538 nm that can detect fluorescein.

The various assays described above will form the bases of this chapter where it will be determined whether either bean PGIP1 or HEWL or both are successfully expressed within the transgenic *N. tabacum* cv. LA Burley 21.

5.2 Materials and Methods

All chemicals and reagents used were either analytical or molecular biology grade. Buffers, solutions, and media were all prepared using distilled water and either autoclaved or filter-sterilized through 0.2µm sterile syringe filters. All buffers, solutions, and media used in this study are outlined in **Appendix A.**

5.2.1 Crude protein extractions from *Nicotiana tabacum* cv LA Burley 21

5.2.1.1 Crude protein extractions from *Nicotiana tabacum* cv LA Burley 21 for bean PGIP1 expression studies

Protein extractions to test for PGIP activity using a method adapted from Desiderio *et al.*, (1997) were prepared from transgenic and non-transformed *N. tabacum* cv. LA Burley 21 leaf material.

Leaves were collected from plants that were grown *in vitro* and stored at -70°C. Extraction of the proteins used in the reducing sugar assay involved the grinding of leaf material to a fine powder in liquid nitrogen using a mortar and pestle followed by the addition of 2 volumes of 1 M NaCl, 20 mM NaOAc buffer (pH 4.7) and allowed to shake for 2 h at 4°C. Insoluble material were pelleted at 6500xg for 20 min at 4°C and the supernatants dialysed extensively against 20 mM NaOAc buffer (pH 4.7) at 4°C in a 12000 molecular weight cut-off dialysis membrane (Sigma D-9277, GmbH, Germany) to remove salts that could influence the level of accuracy during spectrophotometer readings. Dialysed supernatants were centrifuged at 6500xg for 20 min at 4°C and stored at -20°C.

For screening purposes, a more rapid extraction method was applied where the leaf material was ground directly in a 1.5 µl Eppendorf using carborundum powder (400grit) and an ultra Turrax. The same extraction buffer and volume was employed to extract the proteins of which the supernatant was subsequently used without dialyses in the agarose diffusion assay.

5.2.1.2 Crude protein extractions from *Nicotiana tabacum* cv LA Burley 21 for lysozyme expression studies

Proteins extracted for lysozyme activity assays were prepared from transgenic and non-transformed *N. tabacum* cv. LA Burley 21 leaf material similarly to the proteins extracted for PGIP activity assays with the only difference being the pH of the NaOAc buffer which had been altered to 6.5.

Intercellular fluid (IF) extractions prepared according to the method of Rathmell and Sequeira (1974) for the study of HEWL localization were also performed on transgenic and non-transformed *N. tabacum* cv. LA Burley 21 leaf material however only from the F1 generation. Freshly collected leaves were cut with a scissors into pieces of 4 to 5 cm^2 and was placed in 50 ml Erlenmeyer flasks and evenly covered with 20 ml filter sterilised buffer (60 mM MES, pH 6.5; 50 mM $CaCl_2$). The leaves were then infiltrated *in vacuo*, with gentle agitation for three periods of 30 s each. Leave pieces were then gently blotted dry rolled up and placed in 5 ml syringe barrel that had been blocked up with glass microfibre filters with the syringe aperture placed on top of a 1.5 ml Eppendorf tube of which the cap had been snipped off. After centrifugation at 1000xg for 10 min at 4 °C, the extract was recovered in the Eppendorf tube (Parent and Asselin, 1984).

5.2.1.3 Protein concentration determination

The protein concentration of PGIP extracts from *in-vitro* transgenic *N. tabacum* cv. LA Burley 21 leaves was determined according to the method of Bradford (1976) using the microassay of the Biorad protein assay kit (Biorad Laboratories, USA). Bovine serum albumin (BSA) was used as a standard. The method uses a dye, Coomassie brilliant blue G-250 which has a negative charge and binds with a positive charge on the protein. The dye exists in a red form (A_{max} 465nm) and a blue form (A_{max} 595nm). The red form is the predominant form in solution, and when its negative charge binds to the positive charge on the protein, it is converted into the blue form. Bovine serum albumin (BSA) is used at a range of known concentrations to construct a standard curve from which the sample concentration could be calculated by matching the correct concentration with the particular absorbance reading of a specific sample. More specifically, the standard curve is created by setting out a standard series of 1.5 ml Eppendorf tubes containing 800 µl BSA in triplicate arranged in increasing concentrations of 0, 1, 2, 3, 5, and 10 µg/ml. Keeping the volume of 800 µl, the same samples (also in triplicate) were made up of a specific amount of sample diluted with dH_2O. The dye reagent was then added in equal amounts to each of the tubes and mixed and

allowed to incubate at room temperature for 10 min. The absorbencies were measured at 595 nm that in turn allowed for a standard curve to be plotted and the concentration of the samples determined by comparison of its absorbance value to the standard curve. The samples used in subsequent assays were diluted to 300 µg/ml.

5.2.2 *Stenocarpella maydis* PG isolation

5.2.2.1 Fungal isolate

Stenocarpella maydis was isolated from an infected maize cob collected in the Potchefstroom region of South Africa. The culture was stored as isolate PPRI accession no. 03061 in the culture collection of ARC-Plant Protection Institute, Pretoria, South Africa. It was plated and maintained on potato dextrose agar (PDA) plates. Fresh cultures were initiated by transferring a plug of mycelia from one plate to fresh plates and incubating at 27ºC overnight.

5.2.2.2 *Stenocarpella maydis* growth and PG production

S. maydis fungal isolate was grown in Potato dextrose broth containing 100 µg/ml ampicillin to inhibit bacterial growth. Potato dextrose broth was prepared from 20 g potatoes that had been peeled, sliced and microwaved for 4 min in 50 ml sterile dH_2O. The liquid broth was strained through muslin cloth into a clean Scott bottle after which 2 g of dextrose was added and the volume made up to 100 ml with sterile dH_2O. The culture was incubated at 27ºC with shaking for three days, after which pieces of mycelium were used to inoculate a number of flasks containing pectin medium for PG production. The pectin medium was prepared by adding 0.25 g pectin (Sigma P-9135, GmbH, Germany), washed with 0.1M HCl in 70% ethanol and dried) to 25 ml of citrate/phosphate buffer (pH 6.0), and autoclaving before the addition of the sterile inorganic salt solutions. The buffer was prepared from 17.9 ml 0.1 M acetic acid and 32.1 ml 0.2 M Na_2HPO_4 per 100 ml. The following volumes of sterile salt solutions were added to each 24 ml pectin medium-containing flask: 50 µl of 1 M $MgSO_4$, 250 µl of 0.001% SO_4H_2O, 625 µl of 0.01% $ZnSO_4.7H_2O$, 250 µl of 0.0015% $CuSO_4.5H_2O$ and 250 µl of 0.01% $FeSO_4.7H_2O$. Ampicillin was added to a final concentration of a 100 µg/ml. The flasks containing the inoculated pectin medium were incubated at 27ºC with shaking at 100 rpm.

5.2.2.3 Isolation of *Stenocarpella maydis* PG

The cultures in the flasks were harvested each day in triplicate for 13 days by suction filtration through weighed Whatman #1 filter paper (Whatman International, UK) and stored at -20ºC. The mycelia collected on the filter paper were dried at 37ºC overnight and the dry weight determined. The culture filtrate was centrifuged at 3500xg for 10 min at 4ºC. The supernatant was filter-sterilised sequentially through 0.45µm and 0.22 µm disposable syringe filters (Millipore, USA) and stored at 4ºC until further use.

5.2.2.4 Determination of PG activity of culture filtrates

The agarose diffusion assay, adapted from Taylor and Secor (1988), was used to determine endopolygalacturonase activity of the culture filtrates. Sixty-five millimetre diameter Petri dishes containing 10 ml of the assay medium [1% Type II agarose, 0.01% Polygalacturonic acid (PGA) and 0.5% ammonium oxalate in a buffer containing 100 mM citrate, 200 mM Na_2HPO_4, pH 4.6] was prepared and allowed to solidify. Wells were punched in the solidified medium using a no. 1 cork borer. The wells were filled with 40 µl of the culture filtrates from the different days and the plates incubated at 27ºC overnight. The fungal endopolygalacturonase activity was visualised by staining the plates with 10 ml 0.05% ruthenium red (Sigma, GmbH, Germany) for 1 h at 37ºC after which the plates were rinsed with dH_2O to remove excess dye and left overnight at 4ºC before the zones were measured. Two perpendicular diameter measurements were used to calculate the area in mm of the zones.

5.2.2.5 Ammonium sulphate (AS) precipitation of culture filtrates

The filtrates with the highest endopolygalacturonase activity were pooled and subjected to AS precipitation. This was done to remove the medium-derived pectin and to concentrate endopolygalacturonases. The exact volume of sterilised supernatant was determined and the amount of AS to give a final AS concentration of 85% (55.9 g AS per 100ml supernatant) was added. The mixture was allowed to dissolve completely by gentle mixing at 4ºC overnight on a roller (Stuart Scientific, UK.). The samples were subsequently centrifuged at 15300xg for 40 min at 4ºC. The supernatant was decanted and the pellet drip-dried inverted on absorbent paper. The pellet was resuspended in 20 mM sodium acetate buffer (pH 4.7), a twentieth of the original sterilised supernatant volume, and stored aliquots at -20ºC.

5.2.2.6 Protein concentration of *Stenocarpella maydis* PG

The protein concentrations of PGIP was determined as for *S. maydis* PG using the Biorad protein assay kit (Biorad Laboratories, USA) according to the method of Bradford (1976) for the purpose of calculating the exact amount of protein to be used in subsequent protein assays.

5.2.3 PGIP activity assays

5.2.3.1 Inhibition of *Stenocarpella maydis* PGs by PGIP extracts from *Nicotiana tabacum* using the agarose diffusion assay

The agarose diffusion assay used to determine PG activity of culture filtrates was used to determine inhibition of *S. maydis* PG activity by transgenic *N. tabacum* cv. LA Burley 21 PGIP extracts. Petri dishes containing the assay medium were prepared as described in section 5.2.1.4. For this experiment 15 µl of the *S. maydis* PG was incubated with 15 µl of either 20 mM NaCl buffer (pH 4.7) or various PGIP extracts. A positive control, purified bean PGIP2, (provided by Dr. D. Oelofse, ARC-Roodeplaat) was used. The reactions were incubated and the plates stained as described previously.

5.2.3.2 Reducing Sugar Assays

This method was employed to determine the linear trend for the fungal PG activity as well as the inhibition of *S. maydis* by transgenic *N. tabacum* cv. LA Burley 21 PGIP extracts. Polygalacturonase activity was determined by measuring the increase in reducing end-groups with time by the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (Lever, 1972; York *et al*., 1985).

5.2.3.2.1 Linear range of *Stenocarpella maydis* PG activity

This experiment involved running a series of dilutions (1+1, 1+4, 1+9, 1+14 and 1+19) of the *S. maydis* PG extract (AS precipitated) with 20 mM NaOAc buffer (pH4.7) as well as an undiluted sample in triplicate over time and the collection of samples at six different time points (t=0', t=20', t=40', t=80' and t=100'). The PG (40 µl) was mixed with an equal volume of 20 mM NaOAc buffer (pH 4.7) (40 µl) and incubated for 20 min at 25°C before the addition of the substrate. A 72 µl aliquot of this sample was then added on ice to the substrate 108 µl of 0.42 % PGA in a citric acid/sodium phosphate buffer (pH 4.7) to give a final concentration of 0.25 %. At time zero, 25 µl of this sample was removed and immediately boiled for 10 min. After boiling the sample was kept on ice. The remainder of the sample mix was incubated at 30°C for the total time course of the experiment (up to 100 min). This

continued for each time point where a 25 µl sample was removed and the reaction stopped by boiling for 10 min and subsequently kept on ice. To sediment the condensate a pulse spin was performed and the volume increased by the addition of 225 µl sterile dH_2O and 750 µl 1 % PAHBAH (p-4-amino-2-hydroxybenzoic acid hydrazide, Sigma, GmbH, Germany) reagent. The PAHBAH reagent was made fresh each time by mixing 1 volume of 5 % PAHBAH in 0.5 M HCl with 4 volumes of 0.5 M NaOH to give a final PAHBAH concentration of 1 %. The samples were then boiled for 10 min and cooled on ice. The absorbance values of the samples were determined at 410 nm in a spectrophotometer. The spectrophotometer was blanked with dH_2O. The average and standard deviation of each triplicate sample was calculated and subtracted from the 0 min time point. A graph of polygalacturonase activity (A_{410nm} values) against time was plotted for each PG dilution. Linear regression was applied to all graphs by calculating the R^2 values using Microsoft Excel. The R^2 value is an indicator that ranges in value 0 to 1. It reveals how closely the estimated values for the trendline correspond to the actual data. The trendline is the most reliable when its R^2 value is at or near to 1.

5.2.3.2.2 Inhibition of *Stenocarpella maydis* PGs by PGIP extracts from *Nicotiana tabacum* using the reducing sugar assay

The method used to determine the linear trend for *S. maydis* was adapted to test for the inhibition of the *S. maydis* PGs by the PGIP extracts prepared from transgenic *N. tabacum* cv. LA Burley 21. In this experiment, the control reaction contained equal vols. of PG and 40 mM NaOAc buffer (pH 4.7) whereas the test reaction contained equal volumes of PG and the dialysed PGIP extracts from transgenic *N. tabacum* cv. LA Burley 21. Again all reactions were done in triplicate. Upon mixing the PGs with the PGIP extracts it was incubated at 25°C for 20 min followed by the addition of the substrate and incubation at 30°C for the appropriate time period. Another adaptation was that samples were only taken at two time points, 0 and 60 min. The average percentage activity relative to PG activity in the presence of NaOAc buffer as well as the standard deviation was calculated for each triplicate sample.

5.2.4 Lysozyme activity assays

5.2.4.1 Lysozyme lysoplate assay with extracts from transgenic *Nicotiana tabacum*

Leaf extracts prepared for the quantification of lysozyme activity were assayed in a 1% (w/v) agarose slab gel containing 0.05% (w/v) lyophilised *M. luteus* cells as substrate in a 60 mM 2-[*N*-morpholino]ethane-sulphonic acid (MES)-NaOH buffer (pH 6.5). Agarose gels were made from 100 ml agarose solution and poured into a 25.5 x 19.5 cm gel tray. Wells were punched out of the gels using a no.1 cork borer. 15 µl of sample were put into the wells and the plate incubated for 16 hrs at 37° sealed off in a plastic covering to prevent drying out.

A commercially purified HEWL (9001-63-2 MP Biomedicals, Aurora, USA) was used as the standard to assist in determining the relationship between the surface of the lysis zone and the amount of HEWL. An increasing series of HEWL concentration ranging from 0 to 500 ng was prepared from the commercial HEWL and concurrently tested with the extracted samples by the lysoplate assay described above. According to MP Biomedicals, the lyophilised lysozyme had a specific activity of ~60 000 units/mg protein. (One unit of lysozyme produces a decrease in A_{450} of 0.0001 per minute at pH 6.24 and 25°C using *M. luteus* as substrate. From this data an exponential standard curve was plotted with the lysis zone area (mm^2) of the commercial HEWL standard series that provided a means to quantify HEWL activity in the various protein extracts from transgenic *N. tabacum* cv. LA Burley 21 leaf material. Lysis zones were visualized and photographed against a black background. Two perpendicular diameter measurements were used to calculate the area of the lysis zones.

5.2.4.2 The EnzCheck® Lysozyme Assay Kit (E–20013)

The kit was used according to the manufacturer's instructions (Molecular Probes-Invitrogen, USA). Briefly, a series of lysozyme dilutions was prepared from a 1000 U/ml stock solution of lysozyme. According to the manufacturer one unit is defined as the amount of enzyme required to produce a change in the absorbance at 450 nm of 0.0001 units per min at pH 6.24 and 25°C, using a suspension of *M. luteus* as the substrate. The lysozyme concentration ranged from 500 U/ml to 0 U/ml in 50µl volumes, for a range of 250 U/ml to 0 U/ml in the final 100 µl volume Experimental samples were optimally diluted in 1x reaction buffer (0.1 M NaP, 0.1 M NaCl, pH 7.5 and 2mM sodiumazide as a preservative). Both the standard lysozyme and the diluted extracts from transgenic *N. tabacum* cv. LA Burley 21 plants were loaded into a multiwell microplate. To this the lysozyme substrate 50 µg/ml *M. luteus* labelled

with fluorescein was added. This initiated the reaction. The plates were then incubated for 1 hour at 37°C, protected from the light. After incubation, the fluorescence intensity was measured in a fluorescene microplate reader at absorption maximum of ~485 nm and fluorescence emission maximum of ~538 nm.

5.3 Results

5.3.1 Expression studies of bean PGIP1-HEWL in transgenic *Nicotiana tabacum* cv. LA Burley 21

Crude protein extractions were made from transgenic and non-transformed *N. tabacum* cv. LA Burley 21 leaf material for the subsequent studies relating to the functioning of the specific transformed genes. These took place in the form of assays pertaining either to PGIP activity or lysozyme activity. PGIP activity was tested by the agarose diffusion assay and the reducing sugar assay and lysozyme was assayed using the lysoplate lysozyme assay as well as the EnzCheck® Lysozyme Assay Kit.

5.3.2 Growth and PG activity of *Stenocarpella maydis*

A culture of *S. maydis* was prepared for the agar diffusion and reducing sugar assays as described in section 5.2.2.2. Growth of *S. maydis* in a liquid culture with pectin as its sole carbon source was determined by measuring the mycelial dry weight over a period of 13 days and the results are shown in Figure 5.1. A substantial increase in dry mycelial weight was observed from day 2 to day 3 and thereafter increased at a steady pace with no extreme peaks through to day 14. The polygalacturonase activity in the culture supernatants was assessed using the agarose diffusion assay (ADA) and the results are shown in Figure 5.1. PG activity reached maximum after 10 days of growth.

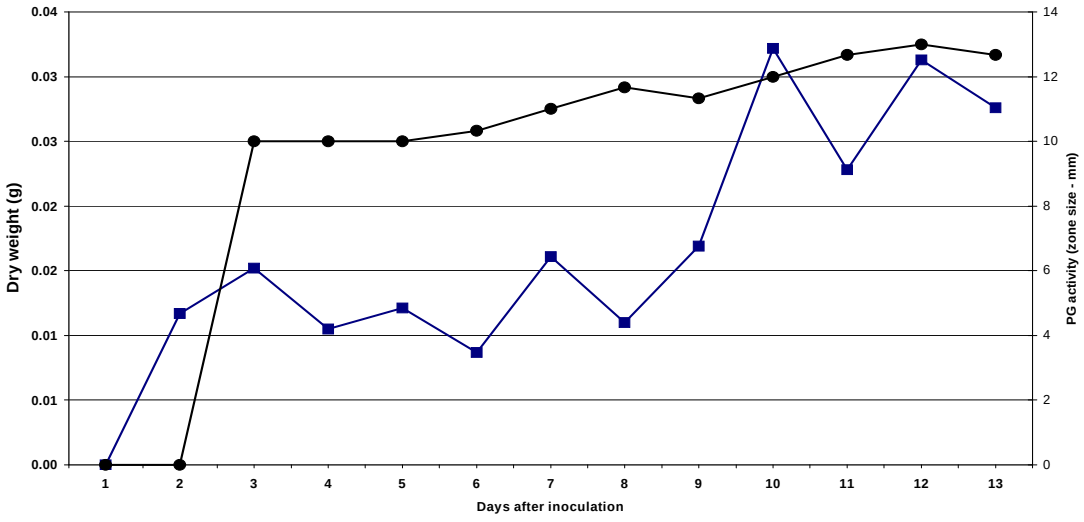

Figure 5.1 Mycelial dry weight and agarose diffusion assay of *Stenocarpella maydis*. Growth and polygalacturonase activity of *S. maydis* grown on pectin. The growth curve was determined by measuring mycelial dry weight (■). PG activity was determined by ADA using the culture supernatants (●).

5.3.3 Ammonium sulphate precipitation of culture filtrates

Filtrates of day 9 to 13 were pooled and subjected to AS precipitation for pectin removal as well as to a concentrate the enzyme. Polygalacturonase activity recovered was verified by ADA, as shown in Figure 5.2. Clear zone diameters were measured in millimetres. The interpretation of these zones is that the larger the appearance of the clear zone the more active is the PG. The zones of the concentrated PG are shown to have a larger diameter (higher activity) than when the culture filtrates containing PGs produced each day were tested individually. When measuring diameter of the zones for ADAs, the well diameter of 6 mm should be taken into consideration and the zone diameters corrected accordingly. The total protein concentration was determined to be 31 µg/ml.

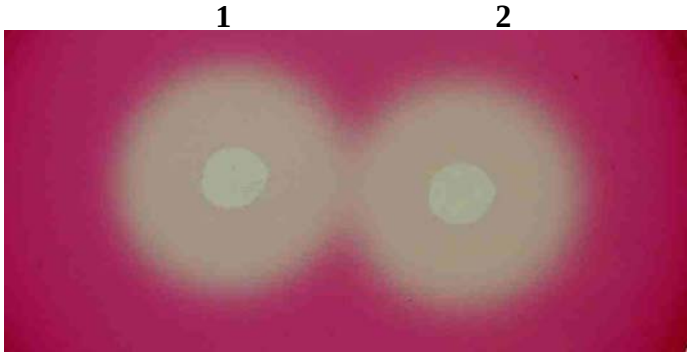

Figure 5.2 ADA of *Stenocarpella maydis* PG pools after ammonium sulphate precipitation. Well 1 and 2 both represent the AS-culture filtrates of the *S. maydis* PG pools that had been resuspended in 20 mM NaOAc buffer (pH 4.7).

5.3.4 Inhibition of *Stenocarpella maydis* PG by the bean *pgip1*, bean *pgip1-hewl* and $hewl_{signal}$ transgenic *Nicotiana tabacum* cv. LA Burley 21 extracts using the agarose diffusion assay

5.3.4.1 ADA of *Stenocarpella maydis* PG activity with extracts from *in vitro* T_0 leaf material

ADA was performed on non-dialysed PGIP extracts from *in-vitro* transgenic *N. tabacum* cv. LA Burley 21 T_0 leaf material. All the samples shown in Figure 5.3 were diluted to 300 µg/ml of which 15 µl per sample was loaded per well. A reduction in zone diameter, indicative of a decrease in PG activity, was observed for all transgenic lines transformed with either bean *pgip1* i.e., PG 6B, 7E, 8A and 9A or bean *pgip1-hewl* genes i.e. PGHS 3A, 6A, 8A except for PGHS 9A. However, the reduction in zone sizes observed was not of great significance with regards to inhibitory effects. The extracts from transgenic lines transformed with the $hewl_{signal}$ gene (a negative control) showed no inhibitory effect on PG activity. Also as expected, the extract from non-transformed *N. tabacum* cv. LA Burley 21 showed no inhibitory effect on the PG activity indicating that *N. tabacum* cv. LA Burley 21 does not contain any endogenous inhibitors specific for *S. maydis* endo-PGs.

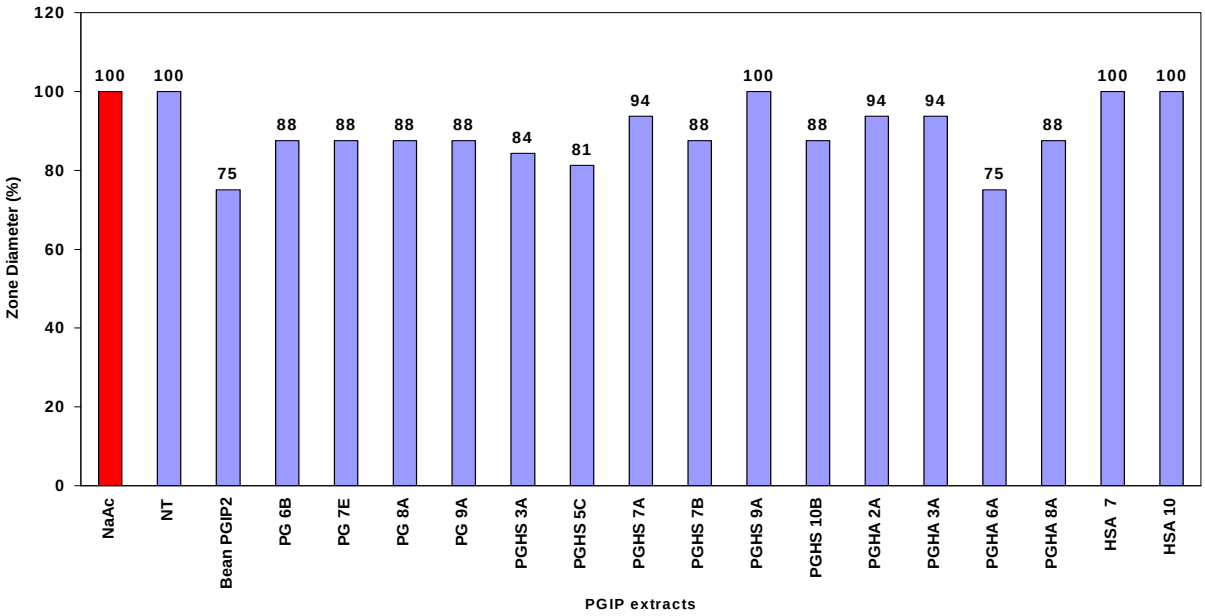

Figure 5.3 ADA of *Stenocarpella maydis* PG activity. Homogenate extracts from T_0 leaf material. Each bar represents the mean of duplicate samples. No standard deviations between samples were obtained for this assay. PGHS: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene. PGHA: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene. HSA: Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene. PG: Transgenic lines transformed with pGA482-*pgip1*.

5.3.4.2 ADA of *Stenocarpella maydis* PG activity with both homogenates and intercellular fluid PGIP extracts from *in vitro* T_1 leaf material

ADA was also performed on non-dialysed PGIP extracts from *in-vitro* homogenate and intercellular fluid (IF) extractions from transgenic *N. tabacum* cv. LA Burley 21 T_1 leaf material. Again all samples were diluted to 300 µg/ml of which 15 µl per sample was loaded per well. A reduction in zone diameter was observed for all extracts (both homogenate and IF) from transgenic lines transformed with either the bean *pgip1* or bean *pgip1-hewl* genes, including PGIP extracts from PGHS 9A-1. Interestingly, a greater reduction in zone diameter was observed for intercellular fluid extractions. Consistent with that of ADAs performed on the T_0 transgenic plants, homogenate extracts from transgenic lines transformed with the $hewl_{signal}$ gene as well as from non-transformed *N. tabacum* cv. LA Burley 21 showed no inhibitory effect on PG activity. Figure 5.4 summarises the results of the ADA of transgenic *N. tabacum* cv. LA Burley 21 lines of the T_1 generation.

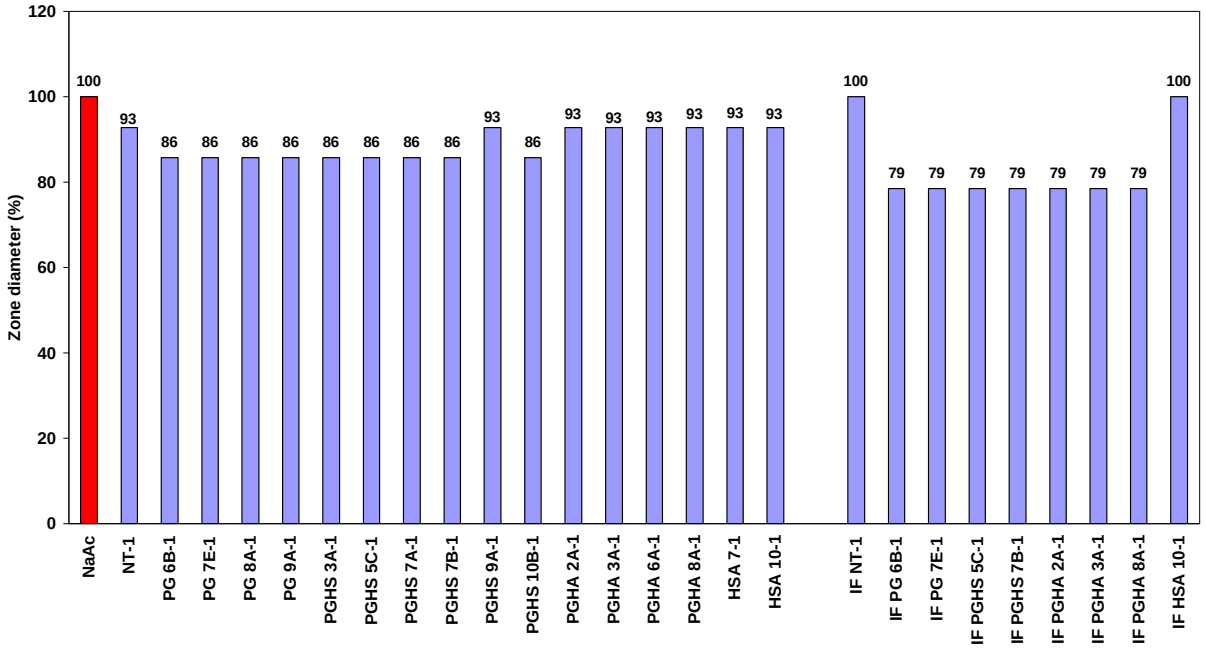

Figure 5.4 ADA of *Stenocarpella maydis* PG activity. Homogenate and intercellular fluid extracts from T_1 leaf material. Each bar represents the mean of duplicate samples. No standard deviations between samples were obtained for this assay. PGHS: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene. PGHA: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene. HSA: Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene. PG: Transgenic lines transformed with pGA482-*pgip1*.

5.3.5 Inhibition of *Stenocarpella maydis* PG by the bean *pgip1*, bean *pgip1-hewl* and $hewl_{signal}$ transgenic *Nicotiana tabacum* cv. LA Burley 21 extracts by the reducing sugar assay

5.3.5.1 Determination of *Stenocarpella maydis* polygalacturonase activity by the production of a linear range curve using the reducing sugar assay

The agarose diffusion assay was found to be inadequate for the quantitative determination of polygalacturonase activity in extractions from *S. maydis* and in determining the change in activity upon inhibition from extracts from transgenic *N. tabacum* cv. LA Burley 21. To overcome this, the reducing sugar assay was employed. In performing a sugar reducing assay consisting of different dilutions of *S. maydis* PG the specific PG dilution at which there was a linear increase in the release of reducing sugars was determined. This activity can be observed in Figure 5.5. The averages of the triplicate samples were determined and plotted together with the standard deviation. Regression analysis showed that there was a linear increase in the release of reducing sugars of *S. maydis* PG from 0 to 100 min, when the PG was diluted 1:10 or less (1:5; 1:2 and undiluted).

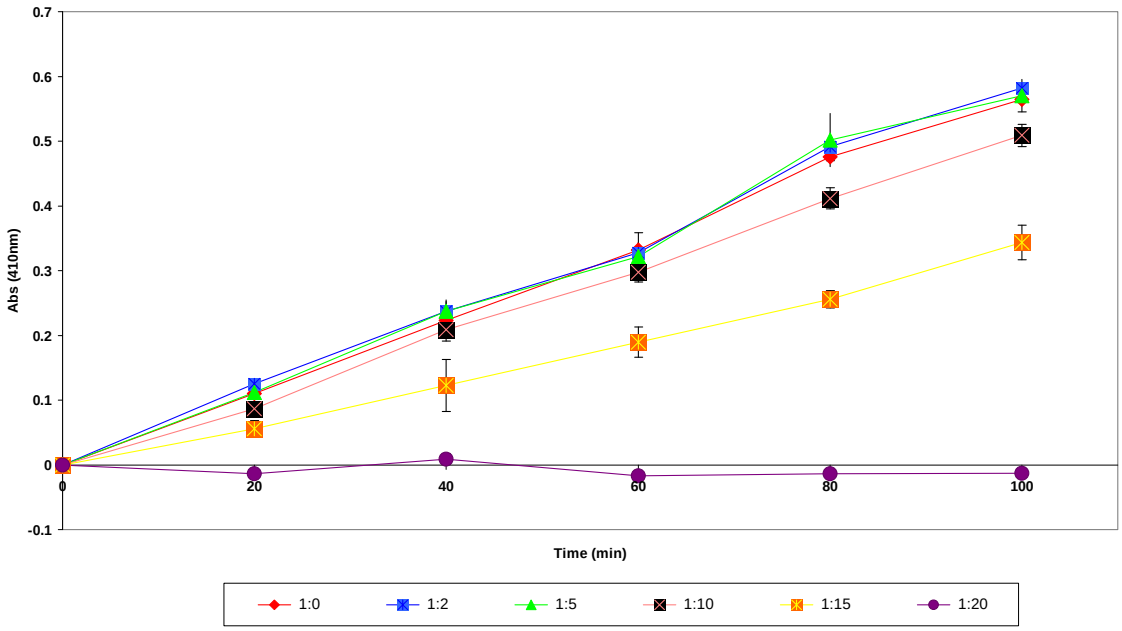

Figure 5.5 Determination of time points at which different dilutions of *Stenocarpella maydis* PG exhibit a clear increase in activity in the reducing sugar assay. *S. maydis* PG activity is respresented by the mean values of three replicate reactions. Standard deviations are plotted as vertical bars.

From the graph shown in Figure 5.5, a decision was made to run PG inhibition studies over a time period of 60 min using the PG at a dilution of 1:10. These parameters were chosen since it yielded an absorbance difference of 0.2–0.3 between the time points T_{60} and T_0.

The specific activity of *S. maydis* PG could then be calculated using the information provided in Figure 5.5. In order to do this calculation it was necessary to determine the concentration of PG used in a 1:10 dilution and since the concentration of *S. maydis* PG was determined as 31 mg/ml, the amount of PG used in the reaction (1:10 dilution) was 3.1 µg/µl. The PG concentration was then tracked throughout the experiment as follows:

40 µl of the PG stock (3.1 µg/µl) was added to 40 µl 20 mM NaOAc yielding a final volume of 80 µl and 124 µg PG. From this diluted sample 72 µl (111.6 µg) PG was added to 108 µl 0.42% PGA, yielding a final volume of 200 µl. A 25 µl aliquot removed from the reaction at 60 min was then added to 225 µl dH_2O and 750 µl 1% PAHBAH, yielding a final volume of 1 ml. The OD reading for this reaction was determined from the graph in Figure 5.5 to be 0.2975 OD units. The conversion factor from OD units to µmoles reducing ends is 0.1458 (determined from a standard curve of galacturonic acid dilution series, data not shown).

$$\text{Therefore Specific Activity} = \frac{\mu\text{moles reducing ends}}{\text{min } \mu\text{g protein}}$$

$$= \frac{0.1458 \times 0.2975}{60 \times 27.9}$$

$$= 0.000259 \ \mu\text{moles reducing ends min}^{-1} \ \mu\text{g protein}^{-1}$$

$$= 25.9 \ \text{pmoles reducing ends min}^{-1} \ \mu\text{g protein}^{-1}$$

$$= 26 \ \text{nmoles reducing ends min}^{-1} \ \text{mg protein}^{-1}$$

5.3.5.2 Inhibitory activity of the extracts from transgenic *Nicotiana tabacum* cv. LA Burley 21 lines

Transgenic *N. tabacum* cv. LA Burley 21 extracts were screened for inhibitory activity towards *S. maydis* PG using the reducing sugar assay. The *S. maydis* PG activity in NaOAc buffer at 60 min was set at 100% to compare inhibitory effects of the different PGIP extracts. The PG activities of the test reactions were set as a percentage of the control reaction (100%). The same concentration (300 µg/ml) of protein extract from transgenic *N. tabacum* cv. LA Burley 21 for all lines was used in the assay. The results are shown in Figure 5.6. The inhibitory activity of bean PGIP1 on *S. maydis* PG was observed for all the reactions containing extracts prepared from the transgenic lines transformed with the bean *pgip1* gene i.e., PG: 6B-1, 7E-1, 8A-1 and 9A-1 (Figure 5.6). From the test transgenic *N. tabacum* cv. LA Burley 21 plants transformed with the bean *pgip1-hewl* fusion gene inhibition of *S. maydis* PGs again was observed in samples PGHS: 3A-1, 5C-1, 7A-1, 7B-1, 9A-1 AND 10B-1 and PGHA: 2a-1, 6a-1 and 8A-1. The inhibitory activity for samples PGHS 7A-1 and PGHA 3A-1 were of little or no significance. Interestingly, a stronger inhibitory effect was observed by the sample PGHS 7B-1 (that was transformed with the fusion gene) than most of those tested that had been transformed solely with the bean *pgip1* gene. It shared inhibitory activities within more or less the same range as PG 6B-1, 7E-1 and 9A-1 but much higher than that of PG 8A-1. No PG inhibitory activity was observed in the reactions containing the extracts prepared from the transgenic lines (HSA 7-1 and HSA 10-1) and the non-transformed *N. tabacum* cv. LA Burley 21 (NT-1) extract (Figure 5.6).

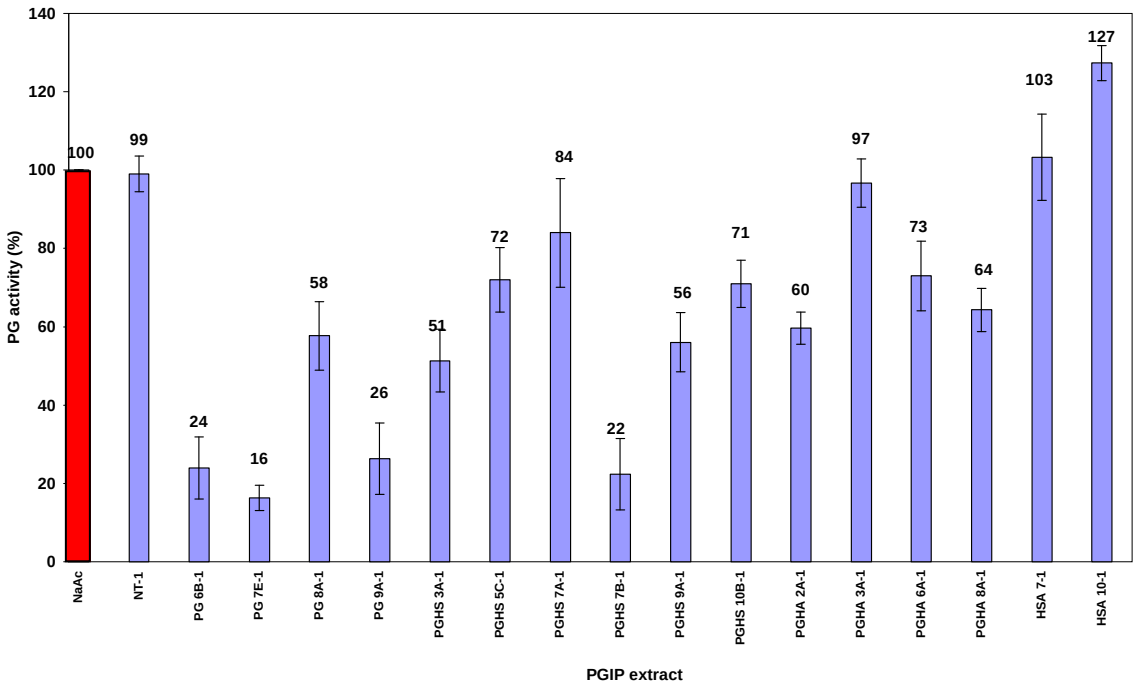

Figure 5.6 Inhibition of *Stenocarpella maydis* PGs by PGIP extracts from *in vitro* T_1 leaf material. SmP:NaOAc was set to 100%. Each bar and value above represents the mean of triplicate samples. Standard deviations are plotted as vertical bars. Specific activity of *S. maydis* = 26 nmoles min^{-1} (mg $protein^{-1}$). PGHS: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene. PGHA: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene. HSA: Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene. PG: Transgenic lines transformed with pGA482-*pgip1*.

5.3.6 Lysoplate assay of HEWL activity

The lysozyme lysoplate assay used was a well-known and routine (Lie *et al*., 1986) lysoplate assay for lysozyme activity in order to quantify the amounts of HEWL expressed in transgenic *N. tabacum* cv. LA Burley 21 extracts. Assaying increasing amounts of purified HEWL ranging from 0-500 ng (Figure 5.7) was used to generate a lysozyme standard curve (**Appendix D**). The amount of HEWL (ng) was converted to HEWL (U) and the diameter measurements for each of the lysis zones formed was calculated as the lysis zone areas (mm^2). The standard curves were then plotted as Lysis zone area (mm^2) vs. HEWL units (U). The lysozyme standard curves shown in **Appendix D** were generated simultaneously i.e., on the same lysoplate with the samples being tested, therefore each of the graphs shown are the standard curves for the specific experiment assaying for either T_0 (lysozyme standard curve 1) or T_1 (lysozyme standard curve 2) protein extracts. The standard curve trends for both lysoplate assays were similar and for this only the photograph of one standard curve lysoplate assay is shown (Figure 5.7) to provide a typical visual appearance of the lysis zones.

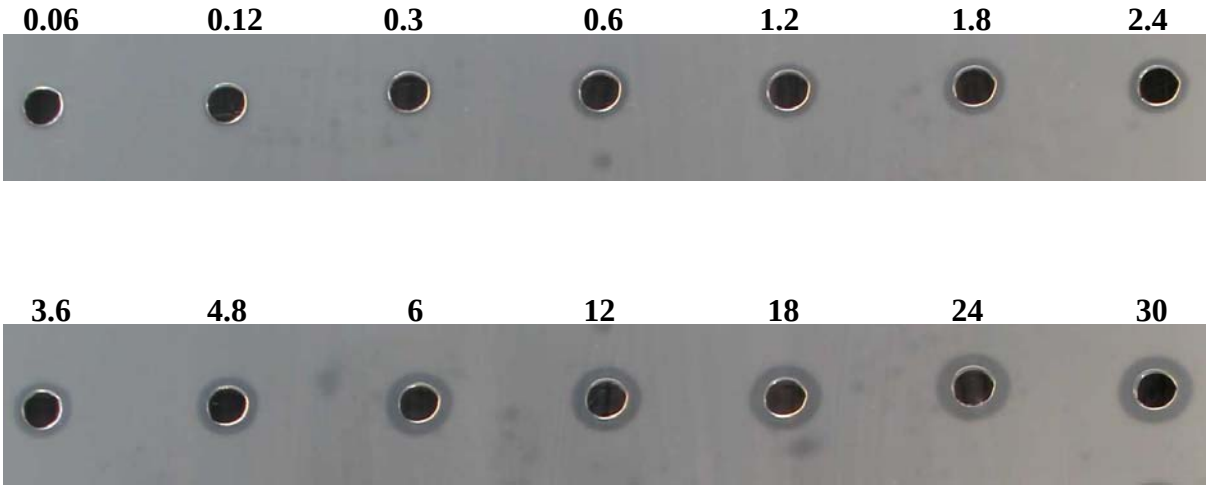

Figure 5.7 Lysozyme assays of known amounts of purified HEWL (9001-63-2 MP Biomedicals, Aurora, USA). Commercial purified HEWL ranging from 0.06 to 30 U was tested by the lysoplate assay for determining the relationship between the surface of the lysis zone and the amount of HEWL. Commercial purified HEWL (0 U) is not shown. The standard curves are generated from lysoplate assays for T_0 and T_1 are shown independently in **Appendix D**.

5.3.6.1 Lysoplate assay of *in vitro* transgenic *Nicotiana tabacum* cv. LA Burley 21 T_0 leaf material

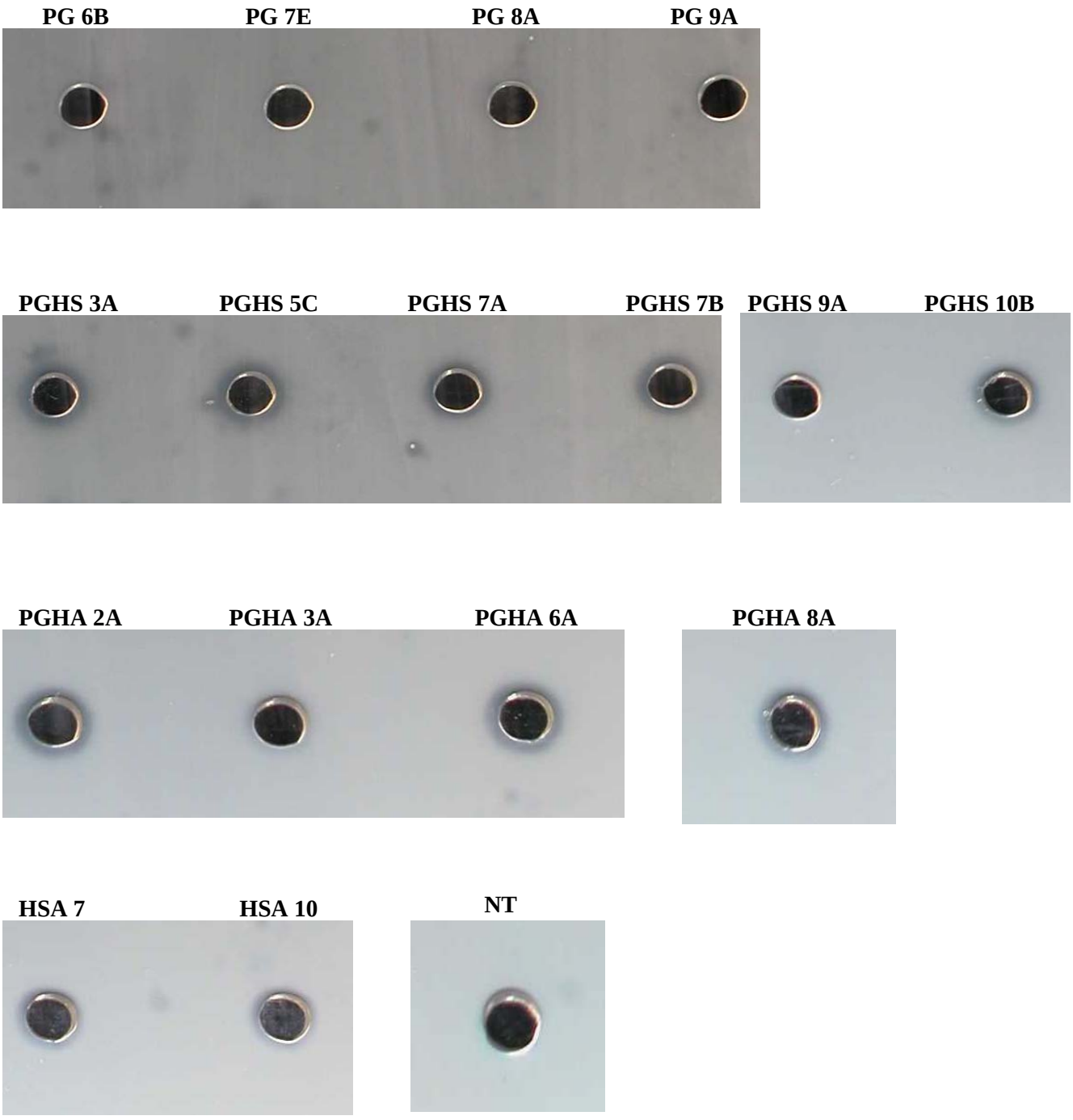

Figure 5.8 Lysozyme assays of independent transgenic *Nicotiana tabacum* cv. LA Burley 21 T_0 plants.

5.3.6.2 Lysoplate assay of *in vitro* transgenic *Nicotiana tabacum* cv. LA Burley 21 T_1 leaf material

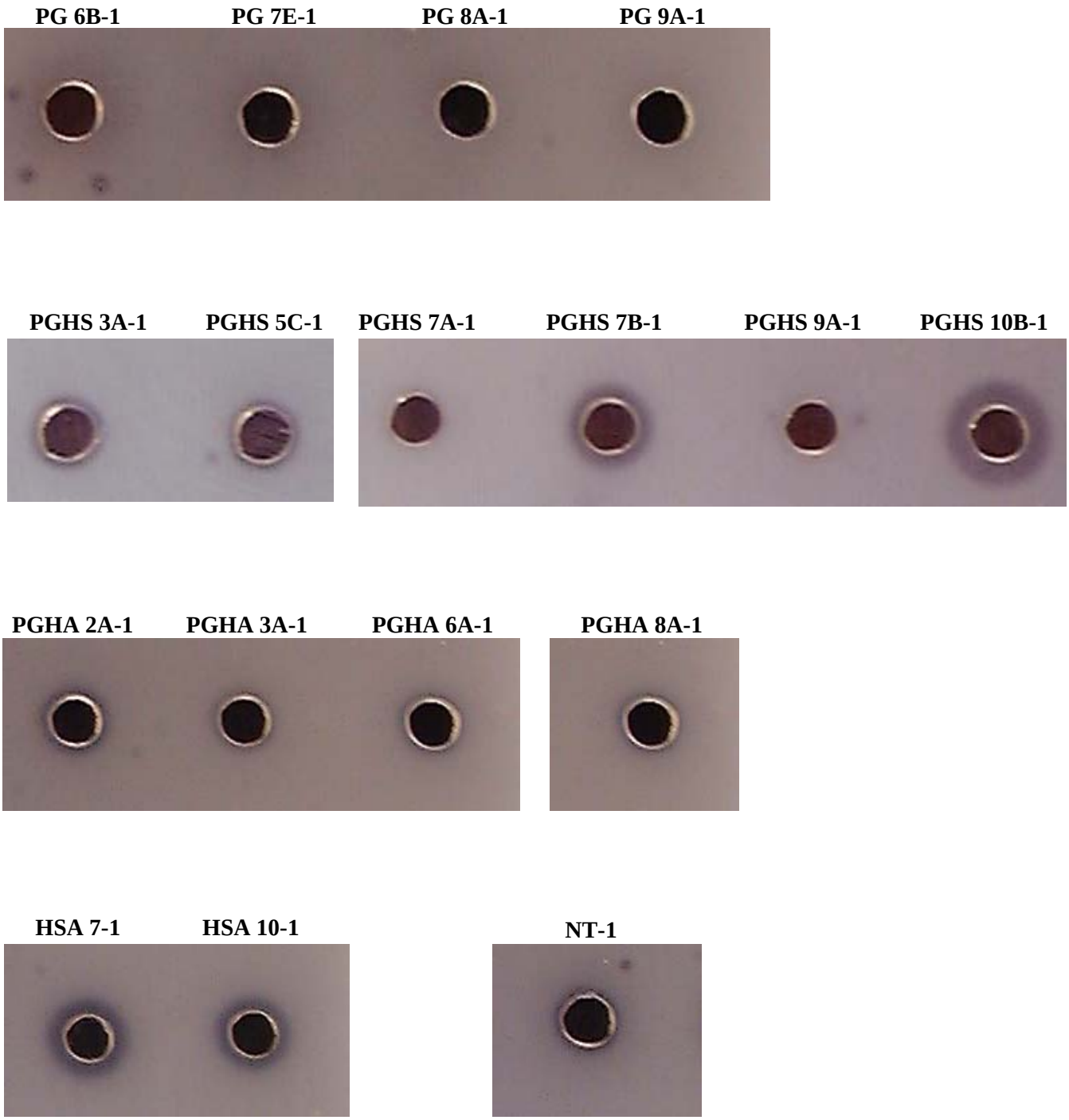

Figure 5.9 Lysoplate assays of transgenic *Nicotiana tabacum* cv. LA Burley 21 T_1 plants. The lysoplate assay was performed with homogenate extracts.

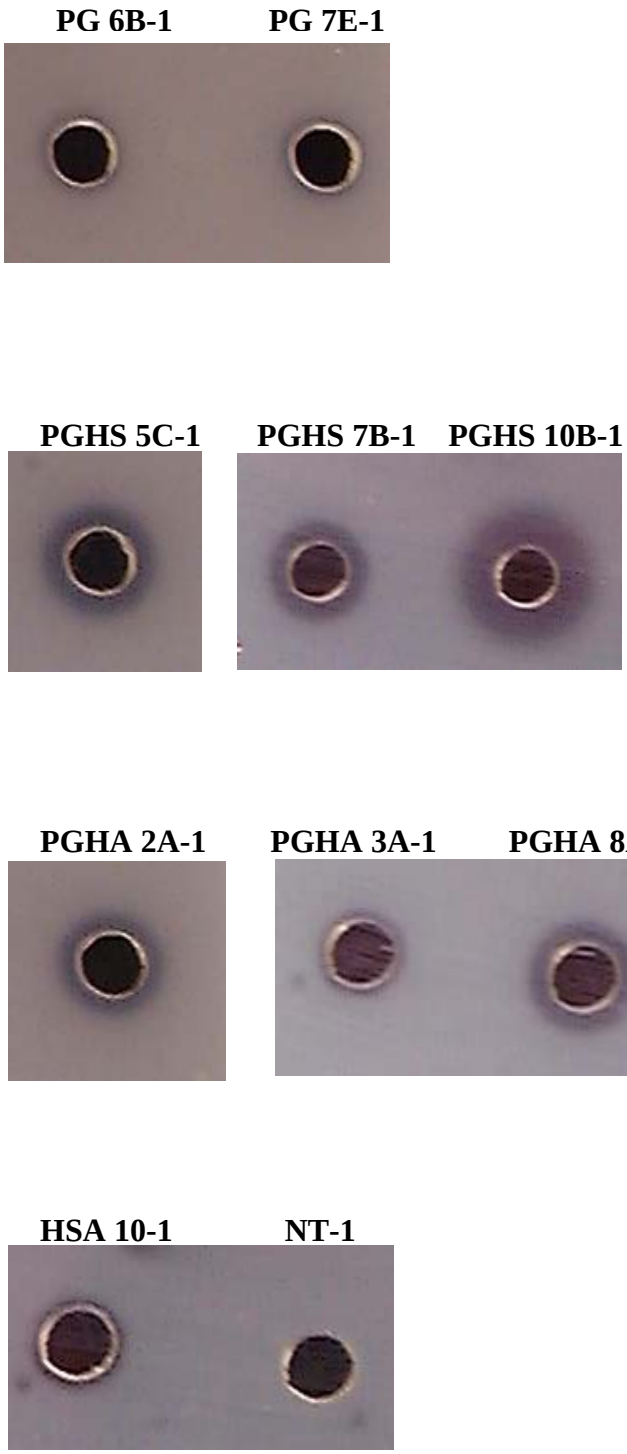

Figure 5.10 Lysoplate assays of transgenic *Nicotiana tabacum* cv. LA Burley 21 T_1 plants. The lysoplate assay was performed with intercellular fluid (IF) extracts.

5.3.6.3 Quantifying HEWL by the lysoplate assay

From the standard curve a correlation between the lysis zone area (LZA) in the lysoplate assay (mm^2) and the amount of HEWL was established. The amount of HEWL was found to be equal to $0.1333 \times 10^{(0.0883 \times LZA\ (mm2))}$ with an R^2 correlation coefficient value of 0.9576 for standard curve 1 (T_0 protein extracts) and $0.2783 \times 10^{(0.07 \times LZA\ (mm2))}$ with a correlation coefficient value of 0.8965 for standard curve 2 (T_1 protein extracts). From such calculations, histograms of the specific activity of HEWL for transgenic *N. tabacum* cv. LA Burley 21 plants were generated and are shown in Figures 5.8-5.10). The same total protein concentration (300 µg/ml) of protein the extracts from transgenic *N. tabacum* cv. LA Burley 21 lines were used in the lysoplate assays.

Of the 16 transgenic T_0 plants assayed for lysozyme activity, those transformed with only the bean *pgip1* gene did not exhibit lysozyme activity (as expected). All except two (PGHS 9A and PGHA 3A) of the plants transformed with either the bean *pgip1-hewl* fusion gene or the *hewl* gene with its own signal peptide exhibited lysozyme activity. As expected, no HEWL activity was observed in the non-transformed *N. tabacum* cv. LA Burley 21 extracts (NT).

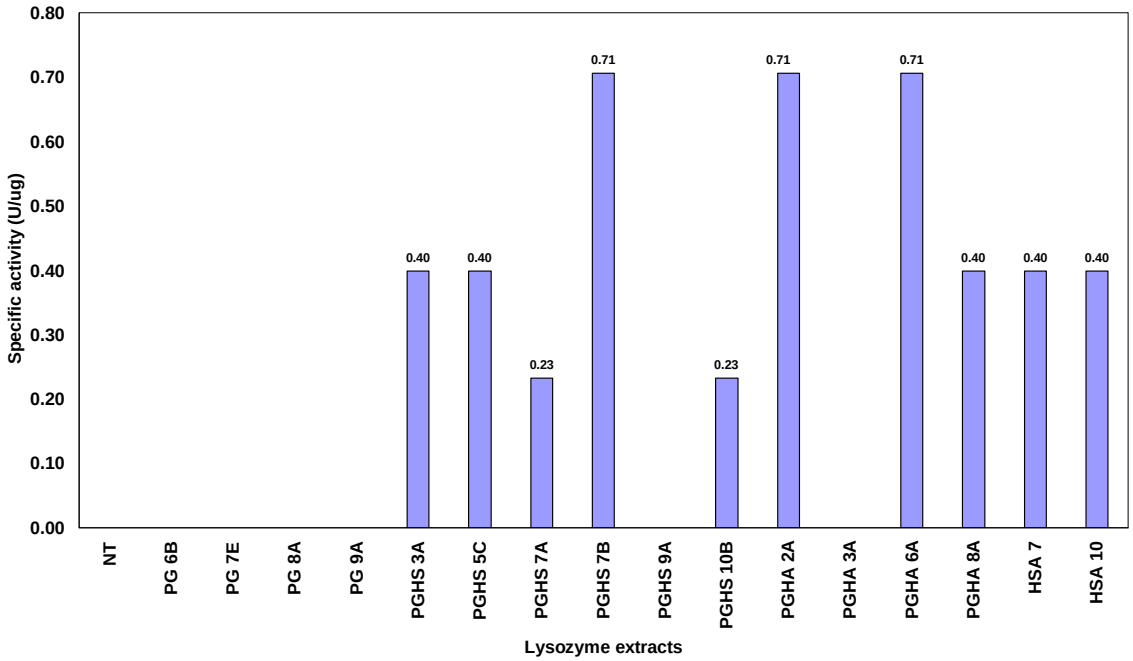

Figure 5.11 Histogram of HEWL activity in T_0 transgenic *Nicotiana tabacum* cv. LA Burley 21 leaf homogenate extracts using the lysolpate assay. All HEWL amounts were expressed as U/μg. A Unit of lysozyme enzyme activity is defined as the amount of enzyme which decreases the absorbance at 450 nm by 0.001 per minute at pH 6.24 and 25°C. Each bar represents the mean of duplicate samples. Specific activity values were derived from the lysozyme standard curve 1 (**Appendix D**). PGHS: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene. PGHA: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene. HSA: Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene. PG: Transgenic lines transformed with pGA482-*pgip1*.

In the assay performed with extracts from T_1 transgenic plants, lysozyme activity was not observed for homogenate or IF extracts from plants transformed with only the bean *pgip1* gene. Lysozyme activity was observed for PGHS 3A-1, 5C-1, 7A-1 (very low), 7B-1 and 10B-1 and PGHA 2A-1 homogenate extractions from plants transformed with the bean *pgip1-hewl* fusion gene samples. Activity was observed for HSA 10-1 of the two homogenate extractions from plants transformed with the *hewl* gene having its own signal peptide. No HEWL activity was observed in the non-transformed (NT-1) *N. tabacum* cv. LA Burley 21 extracts.

During the analysis of the IF extractions, no activity was observed for IF NT-1 plants (Figure 5.12). All extractions made from plants transformed with either the bean *pgip1-hewl* fusion gene or the *hewl* gene with its own signal, showed lysozyme activities. Interestingly, on comparing this with activities from homogenate samples two transgenic plants had lysozyme activity in the IF extracts that had not been detected in the homogenate samples (Figure 5.12, IF PGHA 3A-1 and 8A-1). Lysozyme activity of lysozyme extractions from the intercellular fluid of transgenic IF PGHS 5C-1, 7B-1 and 10B-1 and IF PGHA 2A-1 were higher that that of the homogenate extracts from the same transgenics whereas lysozyme activity for IF extract from HSA 10 transgenic was decreased.

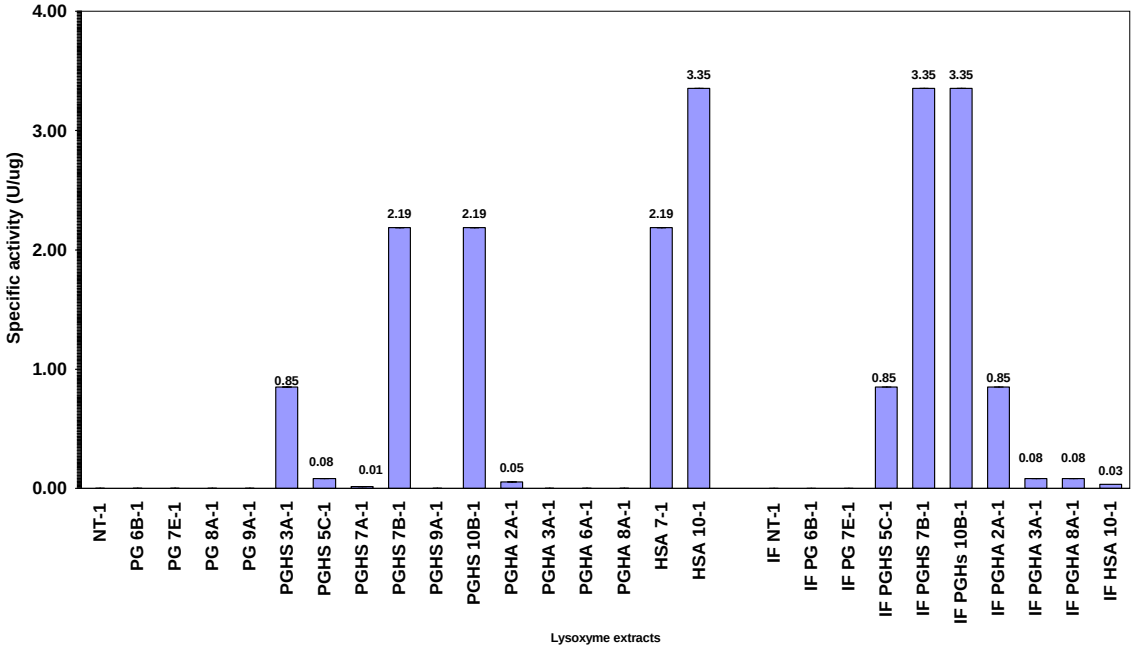

Figure 5.12 Histogram of HEWL activity in T_1 transgenic *Nicotiana tabacum* cv. LA Burley 21 leaf homogenate and IF extracts using the lysolpate assay. All HEWL amounts were expressed as U/µg. Each bar represents the mean of duplicate samples. Specific activity values were derived from the lysozyme standard curve 2 (**Appendix D**). PGHS: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene. PGHA: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene. HSA: Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene. PG: Transgenic lines transformed with pGA482-*pgip1*.

5.3.6.4 Verification of lysozyme activity in T_1 transgenic *Nicotiana tabacum* cv. LA Burley 21 using the EnzCheck® Lysozyme Assay Kit (E–20013)

To verify results obtained from the lysoplate assay, a lysozyme activity kit was used. The standard curve for lysozyme is shown in **Appendix D**. Lysozyme activity was observed in all samples except for those transformed with the bean *pgip1* gene only as well as the non-transformed *N. tabacum* cv. LA Burley 21. A relatively similar trend of lysozyme activity was observed for T_1 transgenic *N. tabacum* cv. LA Burley 21 using the EnzCheck® Lysozyme Assay Kit when compared to lysoplate results.

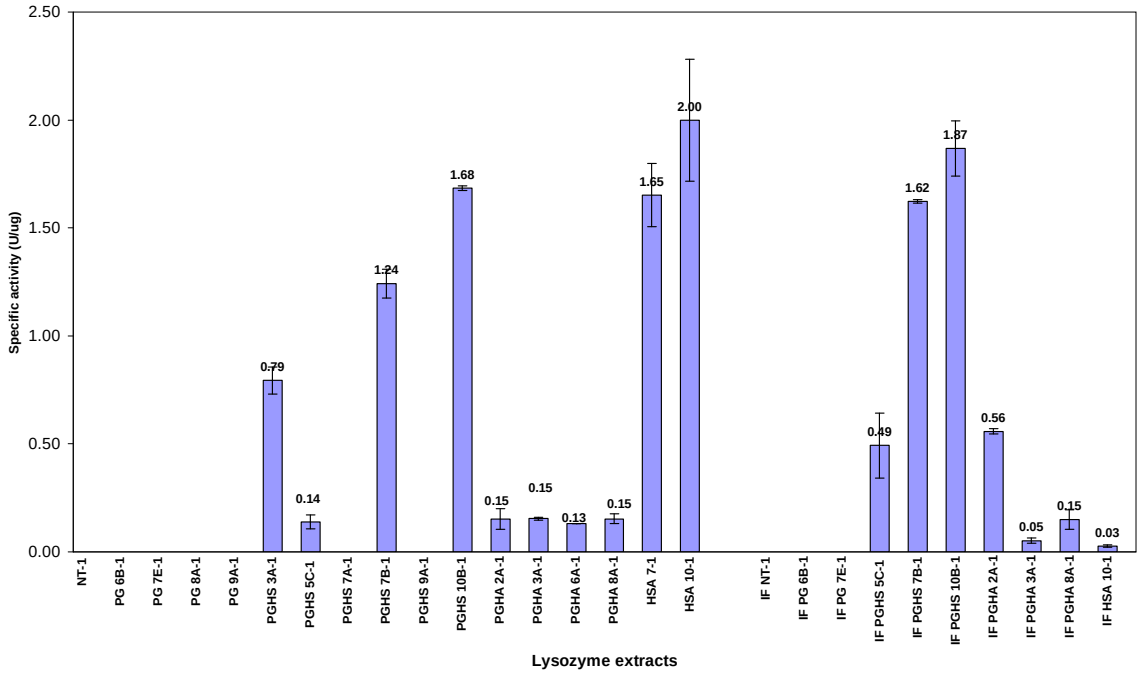

Figure 5.13. Histogram of HEWL activity in T_1 transgenic *Nicotiana tabacum* cv. LA Burley 21 leaf material using the EnzCheck® Lysozyme Assay Kit (E–20013). IF: Intercellular fluid. PGHS: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in a sense orientation with regards to the *nptII* gene. PGHA: Transgenic lines transformed with pCAMBIA2300-bean *pgip1-hewl* with the bean *pgip1-hewl* expression cassette in an antisense orientation with regards to the *nptII* gene. HSA: Transgenic lines transformed with pCAMBIA2300-$hewl_{signal}$ with the $hewl_{signal}$ expression cassette in an antisense orientation with regards to the *nptII* gene. PG: Transgenic lines transformed with pGA482-*pgip1*.

5.4 Discussion

The aim of this chapter was to analyse transgene expression in *N. tabacum* cv. LA Burley 21 plants transformed with the bean *pgip1-hewl* fusion gene by examining its ability *in vitro* to inhibit the endopolygalacturonase activity of *S. maydis* as well as express the *hewl* gene products in the intercellular spaces.

Crude protein extractions from transgenic plants were optimised to allow maximum yield of either protein that would be required for assaying its activity. For this, protein extraction buffers were set at the most favoured pH at which each of the specific proteins functioned best, i.e., pH 4.7 for bean PGIP1 and pH 6.5 for HEWL. This was also the pH at which the specific assays for each protein were carried out.

Proteins that inhibit the activities of PGs have been found in the cell walls of a variety of plants yet their role *in vivo* is not clear. The bean polygalacturonase inhibiting protein (PGIP1), isolated and characterized by Cervone *et al*., 1987 has been applied in various investigations to study the mechanistic phenomena of this protein. With all the available information on the bean PGIP1 protein, the idea of exploiting its localisation within the plant as a means to facilitate another important protein that plays a role in bacterial resistance to function where its impact would be considered significant proved viable.

Extractions from transgenic plants were first subjected to polygalacturonase inhibiting protein assays. Polygalacturonases were produced in *S. maydis* cultures grown on pectin as the sole carbon source. Culture filtrates of each of the growth days were tested for PG activity by the agarose diffusion assay to determine which of the fractions would yield the best activity and therefore contain the most PGs. Normally the filtrates with the highest PG activities are pooled and used in subsequent inhibition assays however, in the case of *S. maydis* PG, activity was found to be very low. The culture filtrate having the highest activity presented clear zone diameters of not more than 13 mm after staining with ruthenium red, a stain that reacts with unhydrolysed polygalacturonic acid. PGs produced for each of the different growth days are likely to be a mixture of both exo-and endo polygalacturonases; hence activities observed are therefore a reflection of the action of the bulk PGs present in the extracts. Even so, PG activity of *S. maydis* was still low and therefore all of the triplicate samples from day 9 to day 13 were pooled. Also observed over the growth period and represented in Figure 5.1 is a fluctuation of PG activity between days. This could be so due to

the fact that there was variation in the sizes of mycelia inoculated into the pectin medium thereby leading to either faster or slower fungal growth in some of the flasks resulting in a variation in the amount of PGs secreted. Yet another reason for this variation could be because fungi secrete different PGs at different stages of growth.

The agarose diffusion assay was performed on PGIP extracts prepared from leaves of *in vitro* transgenic *N. tabacum* cv. LA Burley 21 from T_0 and T_1 generations. As mentioned above the ADA was used as a screening tool to assist in fast elimination of inactive extracts. Since it does not yield quantitative results, assays were done only in duplicate, which upon measurement showed no difference between the two; hence a standard deviation is not shown in Figures 5.3 and 5.4. However, room has to be kept for human error in taking measurements with the ruler since depending on the angle at which your eye meets the edge of the zone a millimetre could easily be mistaken, leading either the addition to or subtraction of a millimetre from another person's readings.

The agarose diffusion assays performed did not include positive bean PGIP1 control samples. Purified bean PGIP1 samples were unavailable at the time of experimentation. A purified bean PGIP2 sample that was available at the time of the first ADA was omitted in subsequent assays due to quantity limitations. The negative controls included were reactions where the addition of PGIP was omitted and instead substituted with the NaOAc buffer, reactions with extracts from non-transformed *N. tabacum* cv. LA Burley 21 plants and reactions with extracts from transgenic *N. tabacum* cv. LA Burley 21 transformed with the *hewl* gene. The data is presented as a percentage of the negative control (NaOAc) to simplify the presentation of the inhibition effects of PGIPs contained in the transgenic *N. tabacum* cv. LA Burley 21 crude protein extractions.

Evident in the histograms of zone diameter vs. PGIP (a form of representing results of ADAs) is the fact that the extractions from transgenic plants transformed with the bean *pgip1* gene slightly inhibited the PGs of *S. maydis* and in contrast, those extracts from transgenic plants transformed with the *hewl* gene, showed no inhibitory effects. This result was expected and however negligible the inhibitory effects from bean PGIP1 transgenics, the difference in zone sizes is indicative of a specific reaction being present that could not be demonstrated for HEWL transgenics. Together with the other negative controls a conclusion can be drawn that *N. tabacum* cv. LA Burley 21 does not possess endogenous PGIPs specific for *S. maydis* PGs. On the other end of this is proof of successful expression of the bean PGIP1 transgene under

the control of the enhanced CaMV 35S (e35S) promoter in the leaves of transgenic *N. tabacum* cv. LA Burley 21.

Analysing the bars of the ADA histograms, PG inhibition by PGIP extracts of transgenics transformed with the bean *pgip1-hewl* fusion were detected except from one sample PGHS 9A-1. However, the PG inhibition range produced by these samples was shown to be between 12 and 25% activity only. A possible reason for this very low inhibition activity could be because of the low affinity of bean PGIP1 for *S. maydis* PGs.

The transgenic plant extract, PGHS 9A that showed no inhibitory activity against the *S. maydis* PG in ADAs could be due to several reasons. For one, it could be possible that for the specific line the lack of protein activity was an indication of the absence of a translatable mRNA, since no expression studies were performed to verify this. The occurrence of such a phenomenon is referred to as gene silencing since, when multiple copies of a transgene are closely linked, it could lead to the transgene being turned off (Gelvin, 1988). Other reasons could be mutations that could have an effect on the amino acid sequence and ultimately the structure causing that the protein either be active or not. A strange occurrence however was that when testing this same line after self-fertilisation and production of the T_1 generation, inhibition was detected against the *S. maydis* PG.

In comparing PGIP inhibition trends between the two generations examined by ADA i.e., the T_0 generation and the T_1 generation, an inhibition inconsistency was observed amongst the same lines of different generations meaning that for a line having a high inhibition activity in the T_0 generation altered to having a low inhibition activity in the T_1 generation. As far as possible the same samples were tested in different assays. Where samples were omitted it was mainly due either to a loss in extracted protein material due to degradation or contamination of the specific line. Also observed is that even the lines showing the best inhibition activity, none of the samples illustrated more than 50% inhibition with respect to the negative NaOAc control. Inhibition activity of IF extractions prepared from the T_1 generation gave no variation between lines. Again all lines tested had inhibitory effects on the PGs of *S. maydis*. However, inhibition activities were higher as compared to whole cell extractions. This result is expected since PGIP is a cell wall bound protein occurring in the intercellular spaces where it acts as the line of defence against one of the early infectious secretions from fungi.

As a more quantitative demonstration of the inhibition activity of PGIP extracts, the reducing sugar assay was employed. Besides inhibition studies of PGIPs it was also used to determine the activity of *S. maydis* PG over time to facilitated in the determination of the specific time points between which *S. maydis* PG activity had a linear trend. On obtaining this information, the reducing sugar assays with PGIP extracts could be run over a time period of 60 min with a 1:10 dilution of the PG. The inhibition activity of PGIP extracts in the reducing sugar assay once again yielded similar results as the ADAs with regards to the transgenics included as negative or positive controls in relevant assays. Test samples (bean *pgip1-hewl* fusion transgenic extracts) all were shown to contain an active PG inhibitor. This result corresponded well with that of the ADA done for the T_1 generation including the inhibition activity of the extracts prepared from PGHS 9A-1. The reason for it not showing inhibition in the T_0 generation is yet to be explained and understood since upon repetitive runs of the assays using a freshly prepared sample, the trend of inhibition remained constant even though the exact zone sizes varied (results not shown). The reducing sugar assay also reveals a much higher activity of bean PGIP1 in extracts from bean *pgip1* transgenic *N. tabacum* cv. LA Burley 21 lines. This corresponds well with the work done by Berger and co-workers (2000) where they showed that leaf extracts from tomato plants transformed with the bean *pgip1* gene did indeed express bean PGIP1 protein. Inhibition of *S. maydis* PGs by approximately 80% was achieved as compared to the control tomato extract that had no effect on the PG activity.

Samples were then subjected to assays to investigate the lysozyme activity. This was done using the lysoplate assay, where the degree of clear zone was indicative of the hydrolysis of the peptidoglycan in the cell walls of the substrate *M. luteus*. In the lysoplate assays it was observed that no lysozyme activity could be detected in neither non-transformed nor transgenic plant transformed with the bean *pgip1* gene since it was used as the negative controls to indicate that there were no endogenous *N. tabacum* cv. LA Burley 21 lysozymes capable of initiating enzyme activity on the cell walls of *M. luteus*. Specific activities of whole cell extracts from transgenic *N. tabacum* cv. LA Burley 21 transformed with the *hewl* gene possessing its own signal peptide far exceeded those from transgenic *N. tabacum* cv. LA Burley 21 transformed with the bean *pgip1-hewl* fusion gene and in comparison to the IF extracts. Very little activity was shown in $HEWL_{signal}$ transgenic IF extracts (line HSA 10). This could be due to the fact that since possessing its native signal peptide, lysozyme localisation was destined for the vacuole. As a result, no vacuolar bound lysozyme was extracted along with IF extractions and hence no activity could be detected. Consistent with this result, was that of the lysozyme assay done using an optimised lysozyme assay kit.

Comparison of active lysozyme yields with those of previously published results of Trudel and co-workers (1992) are difficult to make because of the lack of the specific activity of the commercial lysozyme used to produce a standard curve and so also then the lack of a reference to quantify the specific activity of the various lysozyme extracts. However it is clear that when bound to the bean *pgip1* gene HEWL expression is increased in the intercellular fluid of the *N. tabacum* cv. LA Burley 21 leaf material.

In combining all the results obtained in assays used to investigate the expression of transgenes the observation made was that transgenics transformed with the bean *pgip1-hewl* fusion could express both the bean *pgip1* and the *hewl* gene products, meaning that both proteins were functionally expressed in both the cell wall and intercellular spaces and that HEWL did not interfere with the bean PGIP1 functionality upon expression as a fusion and therefore could yield combined resistance against fungal and microbial plant pathogens.

CHAPTER 6

Concluding Discussions

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Polygalacturonase inhibiting proteins (PGIPs), the extracellular plant proteins responsible for inhibiting fungal endopolygalacturonases have promoted huge interest amongst many research groups. These interests include topics ranging from PGIP distribution in nature, its recognition abilities, specific genes encoding PGIPs to its role in defence, the structural basis of PG-PGIP interaction and the regulation of the *pgip* gene. This study however looks at something totally different from the above-mentioned topics where the focus is on employing the bean *pgip1* gene as a means of delivering useful proteins to the surfaces of cell walls.

Many proteins synthesized within the cytoplasm are destined for other locations. These proteins are 'ear'-marked for intracellular or intercellular translocation by N-terminal (5′) peptide sequence additions, which are excised, often during the translocation process, to form the mature protein. Hen egg white lysozyme (HEWL) with its distinguished history of being among the first enzymes to be sequenced, the first for which a three-dimensional crystal structure was determined by X-ray diffraction techniques and the first for which a mechanism of action was detailed has been selected to demonstrate bean *pgip1* gene as a means of delivering proteins to the cell wall.

However vast the knowledge base of HEWL and the ease with which the enzyme can be assayed, the rationale for using HEWL lies with previous experiments to clone and transform HEWL into tobacco that yielded a very low activity (10%) in the intercellular fluid (Trudel *et al*., 1995). It can therefore be confirmed that the native HEWL signal peptide does not sufficiently direct the secretion of mature HEWL to the intercellular spaces. This study aims to improve the secretion of HEWL to the intercellular spaces by first removing its native signal peptide and secondly fusing the part of the gene that codes for the mature protein downstream of the bean *pgip1* gene. In doing so, the *hewl* gene is then placed under the influence of the bean *pgip1* gene control elements, which targets the mature PGIP protein for the plant cell wall, and it is hypothesized that it would do the same for the mature HEWL protein. To demonstrate HEWL location, lysozyme activity in intercellular fluid extractions of *N. tabacum* transgenic plants transformed with the bean *pgip1-hewl* fusion gene was determined.

The first part of this study (Chapter 3) involved the construction of the plant transformation vectors pCAMBIA2300-bean *pgip1-hewlS* and *A* and pCAMBIA2300-*hewl*$_{signal}$*A*. Success of this cloning step was verified with PCR and restriction enzyme digestion of the relevant constructs (bean *pgip1*, bean *pgip1-hewl* and *hewl*$_{signal}$) with specific primers and restriction enzymes that were then compared to *in silico* predictions done using *Vector NTI*™ software. PCR was then also used to confirm the presence of the transgenes in transgenic *N. tabacum* cv. LA Burley 21 plants after *Agrobacterium*-mediated transformation as well as after self-fertilisation of the T_0 generation to produce the T_1 progeny (Chapter 4).

Once a new DNA sequence has been inserted into the chromosome, it becomes a stable part of the chromosome and will be replicated and passed on like a normal gene. During genetic modification, a single gene is normally inserted into one chromosome in a pair and the other chromosome does not have a copy of that gene. These plants are termed hemizygous for this particular gene since it only has one copy of one allele of that gene. Therefore T_0 plants that tested positive by PCR had a hemizygous genotype. Plants that tested negative were thus untransformed. After self-fertilisation, four possible combinations of chromosome pairs were produced in the T_1 progeny, i.e., one in four (25%) progeny had two copies of the transgene, two in four (50%) progeny had one copy of the transgene and one in four (25%) had no copies of the transgene and were therefore be considered non-transgenic. Therefore PCR analyses of the T_1 generation had a 25% chance of testing negative for the relevant transgenes. It could thus be concluded that the PCR negative T_1 lines were homozygous non-transgenic lines meaning that both T_0 pollen cell and T_0 ovule cell did not have a copy of the transgenic gene.

PCR positive lines of the T_1 generation were further analysed by Southern blot using a DIG-labelled bean *pgip1-hewl* probe and performed on six different lines including a bean *pgip1* transgenic line, 2x bean *pgip1-hewlS* and 2x bean *pgip1-hewlA* transgenic lines and a *hewl*$_{signal}$ transgenic line (Chapter 4). Due to difficulty in yielding complete digestion with the restriction enzymes chosen, hybridising fragment sizes were not entirely accurate with regards to size and could be as a result of retardation of the incompletely digested transgenic *N. tabacum* cv. LA Burley 21 genomic DNA. To improve digestion quality the DNA could be cleaned up more and Bovine Serum Albumen (BSA) can be added to the digestion reaction. It was concluded that the chosen lines contained a single copy of the gene and that the number of insertion events were unclear due to a number of non-specific fragments that hybridised to the bean *pgip1-hewl* probe.

Chapter 5 reported on the PG inhibition studies and lysozyme activity of the different extractions made from transgenic lines. In comparing results produced from the ADAs between the T_0 and T_1 generation inhibition was shown to be higher in the T_1 generation of approximately the same lines tested. Comparison of the whole cell extracts with the IF extracts from the T_1 generation it is clear that inhibition was higher for IF extracts in those lines tested. Quantitatively T_1 transgenic lines were shown to possess PG inhibitory activity in all relevant lines (PG 6B-1, 7E-1, 8A-1 and 9A-1). Especially interesting was that lines transformed with the bean *pgip1-hewl* fusion gene had lower inhibitory effects than those that had been transformed with only the bean *pgip1* gene. A possible reason for this could be that since the *hewl* gene was fused to the bean *pgip1* gene, it could induce some interference with the expression of the protein. However, confirmation of the inhibitory effects means that the fusion did not entirely disrupt the functioning of the bean PGIP1 protein since some activity was detected.

Lysozyme activity was also shown in both the T_0 and T_1 generations. The specific activities of the T_1 lines were much lower than that of the T_0 generation. Therefore it can be deduced that self-fertilisation of transgenics has an effect on the functioning of the transgenic protein. Also interesting is that the lines for which the IF extractions were tested showed that the transgenics that had been transformed with the intact $hewl_{signal}$ gene gave almost no activity. For this it can be concluded that bean *pgip1* successfully delivered the lysozyme gene into the intercellular spaces. In comparison to these $hewl_{signal}$ transgenic lines, lines transformed with the bean *pgip1-hewl* genes showed lysozyme activity and additionally the activity in the intercellular spaces were higher than those from whole cell extract assays. A summary of the T_1 events giving an indication of all the tests performed are listed in Table 6.1. The table shows that PCR and Southern blot hybridisation were not conducted on all the events for which the activity assays were performed. However, since all of the lines grew on media containing kanamycin proof of lines being transgenic had been achieved.

Table 6.1 Summary of results for *Nicotiana tabacum* cv. LA Burley 21 T_1 events

T_1 event	Kan[a]	PCR product[b] (kb)	SB[c] *Pst*I	SB[c] *BamH*I	PGIP activity WC[d]	Lysozyme activity WC	Lysozyme activity IF[e]
PG 2A-1	+	x	x	x	x	x	x
PG 6B-1	+	x	x	x	+++	-	-
PG 7E-1	+	0.804	5.2	-	+++	-	-
PG 8A-1	+	x	x	x	++	-	x
PG 9A-1	+	x	x	x	+++	-	x
PGHS 2C-1	+	x		x	x	+	x
PGHS 3A-1	+	x	x	x	++	x	x
PGHS 5C-1	+	1.112	2.542	-	+	-	+
PGHS 7A-1	+	1.112	2.542	-	+	-	x
PGHS 7B-1	+	x	x	x	+++	++	+++
PGHS 9A-1	+	x	x	x	++	-	x
PGHS 10B-1	+	x	x	x	+	++	+++
PGHA 2A-1	+	x	x	x	++	-	+
PGHA 3A-1	+	x	x	x	+	-	-
PGHA 6A-1	+	1.112	2.542	2.337	+	-	x
PGHA 8A-1	+	1.112	2.542	2.337	++	-	-
HSA 7-1	+	x	x	x	+	++	x
HSA 10-1	+	0.359	1.561	-	+	+++	-

a: Inheritance of the ability to grow on media containing kanamycin

b: PCR confirmation of presence of transgene

c: Southern Blot Hybridisation

d: Whole cell extractions

e: Intercellular fluid extractions

+: Poor activity

++ Average activity

+++ Good activity

x: Not tested

-: Negative result

Future studies would be to evaluate the efficacy of the two proteins independently against specific problem pathogens *in vitro*, which could lead to the challenging of these transgenic *N. tabacum* cv. LA Burley 21 plants with both bacterial and fungal tobacco pathogens. Other studies following on from this could involve the transformation of this particular construct into other crops and render some resistance against certain bacteria susceptible to lysozyme action. Transgenic plants expressing T4 lysozyme were more resistant to Gram-negative bacteria such as *Erwinia carotovora* (During *et al*., 1993). Transgenic plants expressing HEWL could possibly be more resistant to Gram-positive bacteria (such as *Claviabacter michiganense*) and to some fungi (such as *Fusarium oxysporum*, *Verticillium albo-atrum* and *Rhizoctonia solani*). Transgenic crops expressing both HEWL and PGIP could be useful in enhancing the spectrum of microorganisms sensitive to exogenous lysozymes in addition to

providing resistance to more fungi. Furthermore, experiments could involve the cloning of different essential genes required at the intercellular spaces downstream of perhaps a different *pgip* gene, for example the gene that codes for apple PGIP1, which has a broader fungal PG sensitivity range than bean PGIP1. This would therefore increase the spectrum of resistance a plant can offer.

Chapter 7

Appendices

CHAPTER 7

Appendices

Appendix A

Buffers, Solutions, Reagents and Culture media

Agarose diffusion Assay (ADA) medium

1.0% (w/v) Type II agarose (Sigma A-6877, GmbH, Germany)

0.01% (w/v) Polygalacturonic acid (PGA) (Sodium polypectate, Sigma P-1879, GmbH, Germany)

0.5% (w/v) Ammonium oxalate (Sigma A-8545, GmbH, Germany)

For 100 ml of buffer with pH 4.6:

26.7 ml 0.1 M Citric acid

23.2 ml 0.2 M Na_2HPO_4

Antibiotic stocks

Ampicillin	100 mg in 1ml dH_2O
Kanamycin	100 mg in 1ml dH_2O
Rifampicin	25 mg in 1ml 100% methanol
Cefotaxime	250 mg in 1 ml dH_2O
Tetracycline	10 mg in 50% ethanol

All antibiotics dissolved in dH_2O were filter sterilized through 0.2µm sterile filters. Rifampicin was made fresh just before use. Antibiotic stocks were stored at -20°C.

BSA (1mg/ml)

1mg in 1ml dH_2O

Ca^{2+}/Mn^{2+} solution for preparation of competent *Escherichia coli* cells

40 mM NaOAc

100 mM $CaCl_2$

70 mM $MnCl_2 4H_2O$

Adjust to pH 5.5 with HCl. Be careful not to over-acidify the solution, as precipitation will occur. Filter-sterilise and store at 4°C.

Citrate / Phosphate buffer

For 100 ml of buffer with the following pH:

pH 4.6	pH 6.0	
26.7 ml	17.9 ml	0.1 M Citric acid
23.3 ml	32.1 ml	0.2 M Na_2HPO_4
50 ml	50 ml	dH_2O

Coating buffer – 0.05M sodium bicarbonate, pH 9.6

1.59 g	Na_2CO_3
2.93 g	$NaHCO_3$
0.20 g	NaN_3

Dissolve in 1 l dH_2O

Dellaporta DNA extraction buffer

1 M	Tris-HCl (pH 8.0)
5 M	NaCl
0.5 M	EDTA (pH 8.0)

Denaturing solution

0.4 M	NaOH
0.6 M	NaCl

Depurinating solution

0.25 M	HCl

DIG Blocking buffer

1% (w/v) Blocking reagent in maleic acid buffer (Roche Diagnostics GmbH, Germany)

DIG Detection buffer

0.1M	Tris-HCl
0.1M	NaCl

pH 9.5

DIG Maleic Acid buffer

0.1 M	Maleic Acid
0.15M	NaCl
pH 7.5	

DIG Washing buffer

0.3% (v/v) Tween 20 in maleic acid buffer.

DNA reference standard

100 ng/µl calf thymus DNA

Ethidium Bromide (10 mg/ml)

Dissolve 10 mg ethidium bromide powder in 1ml dH_2O. Cover tube with aluminium foil and store at 4°C.

N HCl / 70% Ethanol

For 600 ml:

6 ml	10 N HCl
420 ml	Ethanol
174 ml	dH_2O

Luria-Bertani (LB) Medium

Composition per l:

10 g	Tryptone
5 g	Yeast extract
5 g	NaCl

For LB agar, add 15g Bacto-agar

2x LB Medium for competent cells

Composition per l:

20 g	Tryptone
10 g	Yeast extract
1 g	NaCl

Adjust the pH to 7.0 and autoclave. Before use, add 1/100th volume 20% sterile glucose

MS media (Murashige and Skoog, 1962)

Composition per l:

4.418 g	MS Basal Salt mixture (Highveld Biological Ltd)
30 g	Sucrose
8 g	Agar
pH 5.8	

Minimal salts medium

Composition for 100 ml:

0.2 ml	1 M $MgSO_4.7H_2O$
1 ml	0.001% $MnSO_4.H_2O$
2.5 ml	1 M KNO_3
1 ml	0.01% $ZnSO_4$
1 ml	0.0015% $CuSO_4$
1 ml	0.01% Fe $SO_4.H_2O$
91.5 ml	Citrate-PO_4 buffer (pH 6.0)

Add 1% (w/v) pectin (Sigma P-9135, GmbH, Germany) washed with 0.1 N HCl / 70% Ethanol and dried) to the citrate-phosphate buffer (pH 6.0) and autoclave. Add the filter-sterilised salts just before use.

Neutralising solution

0.5 M	Tris-HCl, pH 7.5
3.0 M	NaCl

1% PAHBAH reagent

5% p-hydroxybenzoic acid hydrazide (PAHBAH) in 0.5 M HCl, store at -20°C.

Just before use, mix 1 volume of 5% PAHBAH in 0.5 M HCl with 4 volumes of 0.5 M NaOH to give a final PAHBAH concentration of 1%.

0.42% PGA (in solution phosphate / citric acid buffer, pH 4.6)

For 10 ml:

42 mg	PGA (Sodium polypectate, Sigma P-1879, GmbH, Germany)
2.33 ml	0.2 M $NaHPO_4$
2.67 ml	0.1 M Citric acid
5 ml	dH_2O

Aliquot and store at -20°C

Pre-hybridisation and hybridisation solution

5 ml	Formamide
2.5 ml	20 x SSC
400 µl	50 x Denhardts solution
100 µl	10% SDS
2 ml	sterile H_2O

Just before use, 100 µg/ml Herring sperm DNA is boiled for 5 min, quick-chilled on ice and added to both the pre-hybridisation and hybridisation solutions.

Regeneration media

Composition per l:

4.418 g	MS Basal Salt mixture (Highveld Biological Ltd)
30 g	Sucrose
0.5 mg	3-Indolylacetic acid (IAA)
1 mg	6-Benzylaminopurine
7.5 g	Agar
pH 5.8	

RNase A (10mg/ml)

Dissolve 10 mg RNase A in 1ml TE buffer, pH 8.0

Heat to 100°C for 10 min. Allow cooling slowly to RT

Store at -20°C.

Ruthenium Red dye solution

0.05% (w/v) in dH_2O

Solution I

25 mM Tris-HCl, pH 8.0

10 mM EDTA

50 mM Glucose

Solution II

0.2 NaOH

1% SDS

Solution III

2.5 M Potassium acetate

2.5 M Acetic acid (MW=60.05 g/mol; 11=1.05 kg ∴ 3.574 ml per 25 ml solution III)

pH 5.4 with Acetic acid

20 x SSC

3.0 M NaCl

0.3 M Sodium citrate

pH 7.0

For 2 x SSC: 1 part 20 x SSC + 9 parts dH_2O

Stringency wash solution 1

2x SSC

0.1% SDS

Stringency wash solution II

0.1%	SSC
0.1%	SDS

50 X TAE

Composition per l:

242 g	Tris
57.1 ml	Glacial acetic acid
100 ml 0.5 M	EDTA

For 1 x TAE: 40 ml 50 X TAE + 1960 ml dH_2O

TE buffer (pH 8.0)

10 mM	Tris-HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

1x TNE buffer

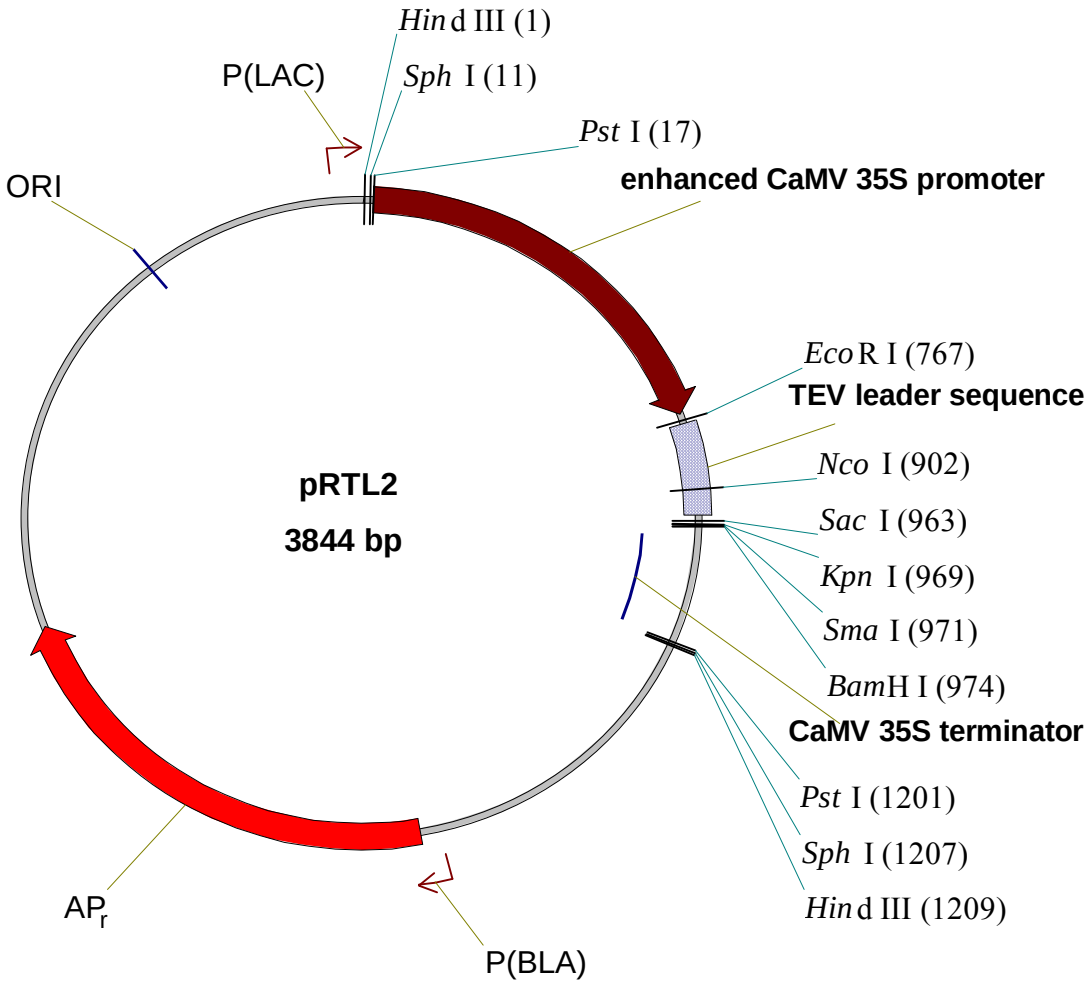
10 mM	Tris-HCl (pH 8.0)
1 mM	EDTA (pH 8.0)
0.2 M	NaCl
pH 7.4	

Appendix B

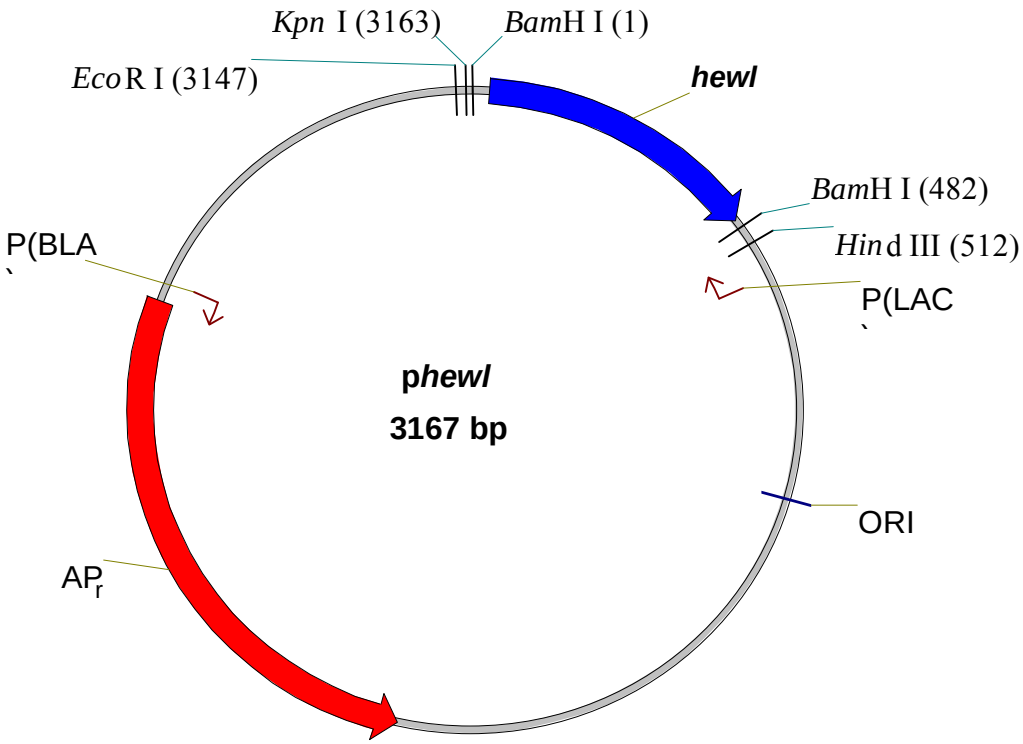
Plasmid Maps

1. pRTL2 (Restrepo *et al*., 1990)
2. pHEWL (Langenhoven, 1999)
3. pMOS-*hewl* (Kaimoyo and Cloete, 1999, unpublished)
4. pRTL2-bean *pgip1* (P. Fourie, ARC-Roodeplaat)
5. pRTL2-bean *pgip1-hewl* (Ogundiwin and Cloete, 2000, unpublished)
6. pRTL2-$hewl_{signal}$ (Ogundiwin and Cloete, 2000, unpublished)
7. pGA482 (An *et al*., 1992)
8. pGA482-bean *pgip1* (P. Fourie, ARC-Roodeplaat)
9. pCAMBIA2300 (www.cambia.org)

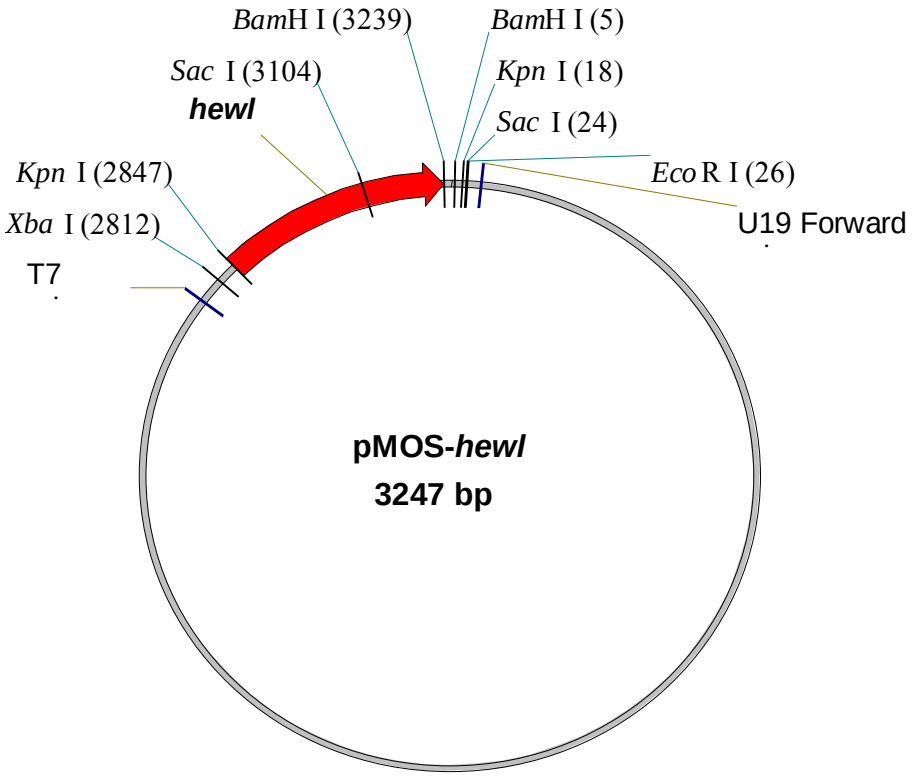
1


Hin d III (1)
P(LAC)
Sph I (11)
Pst I (17)
enhanced CaMV 35S promoter
ORI
Eco R I (767)
TEV leader sequence
Nco I (902)
pRTL2
3844 bp
Sac I (963)
Kpn I (969)
Sma I (971)
BamH I (974)
CaMV 35S terminator
Pst I (1201)
Sph I (1207)
Hin d III (1209)
AP$_r$
P(BLA)

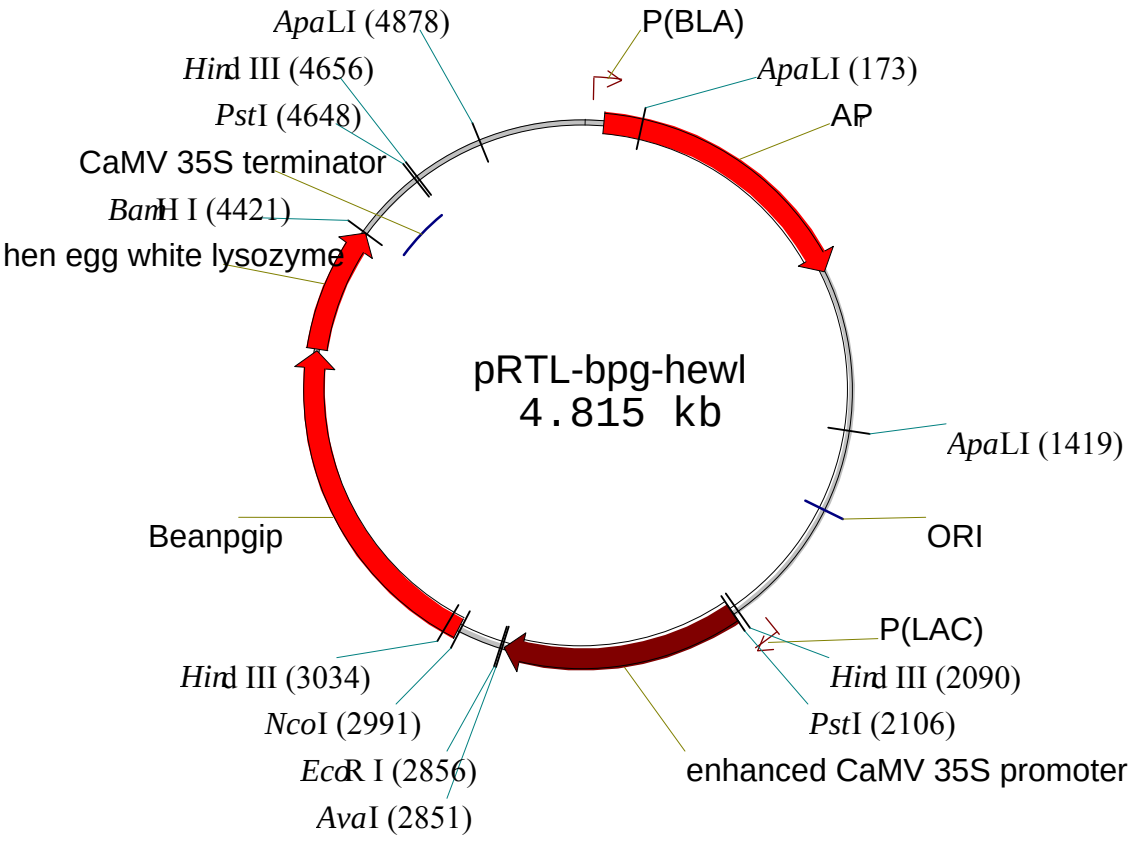
2


Kpn I (3163)
*Bam*H I (1)
Eco R I (3147)
hewl
*Bam*H I (482)
Hin d III (512)
P(BLA
`
P(LAC
`
p*hewl*
3167 bp
ORI
AP$_r$

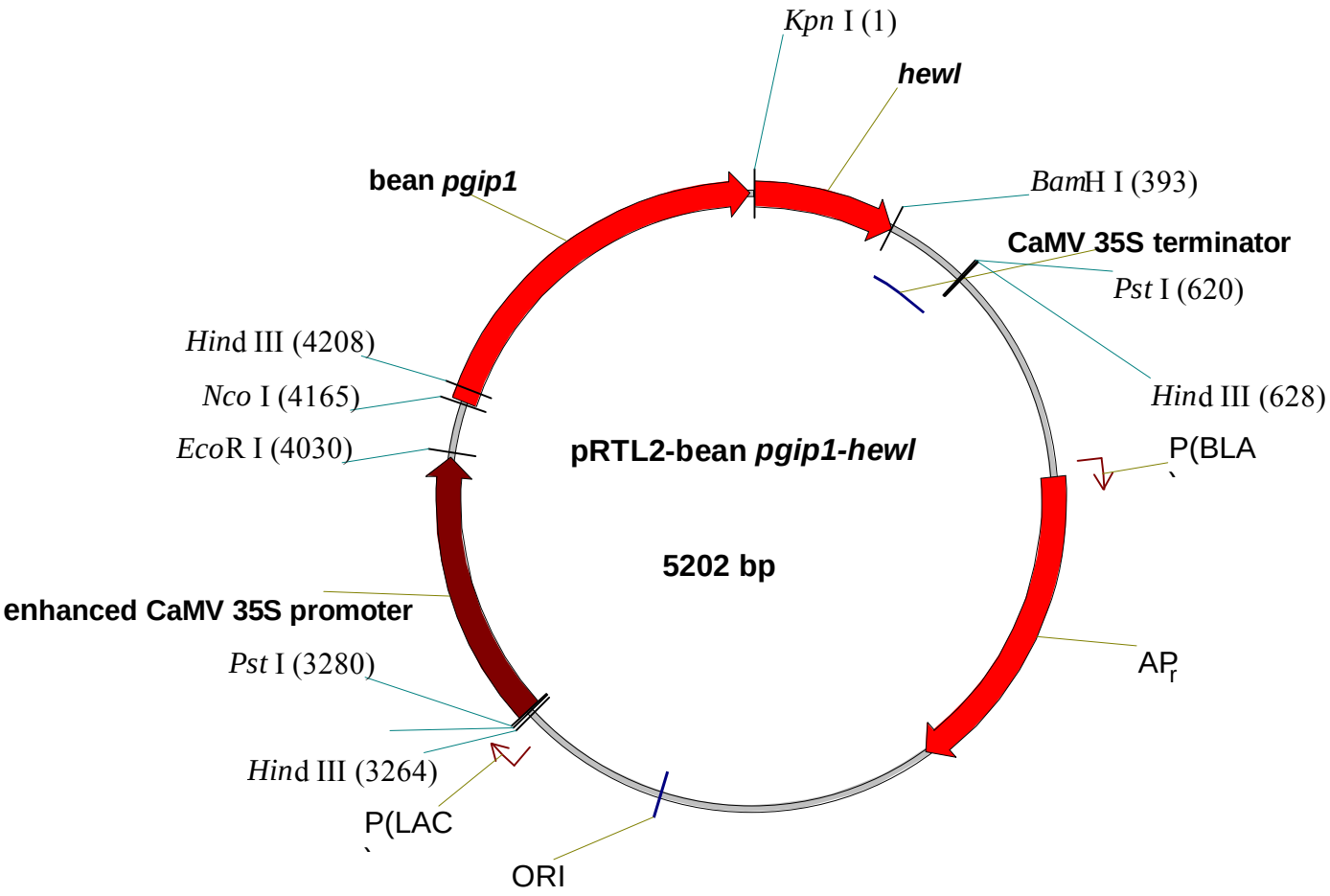
3


BamH I (3239)
BamH I (5)
Sac I (3104)
hewl
Kpn I (18)
Sac I (24)
Kpn I (2847)
Eco R I (26)
Xba I (2812)
U19 Forward
T7
pMOS-hewl
3247 bp

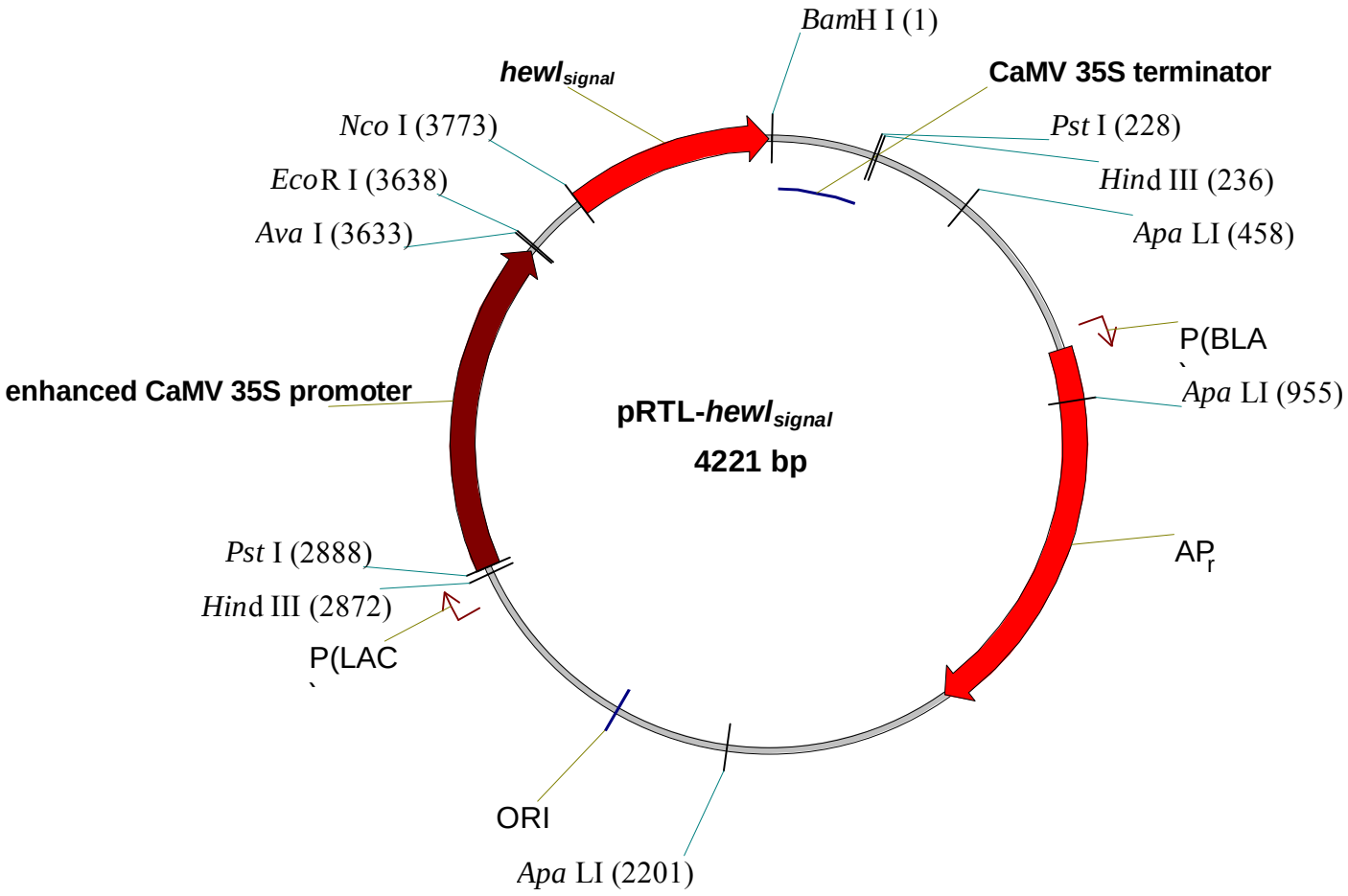
4


ApaLI (4878)
P(BLA)
HindIII (4656)
ApaLI (173)
PstI (4648)
AMP
CaMV 35S terminator
BamHI (4421)
hen egg white lysozyme
pRTL-bpg-hewl
4.815 kb
ApaLI (1419)
Beanpgip
ORI
P(LAC)
HindIII (3034)
HindIII (2090)
NcoI (2991)
PstI (2106)
EcoR I (2856)
enhanced CaMV 35S promoter
AvaI (2851)

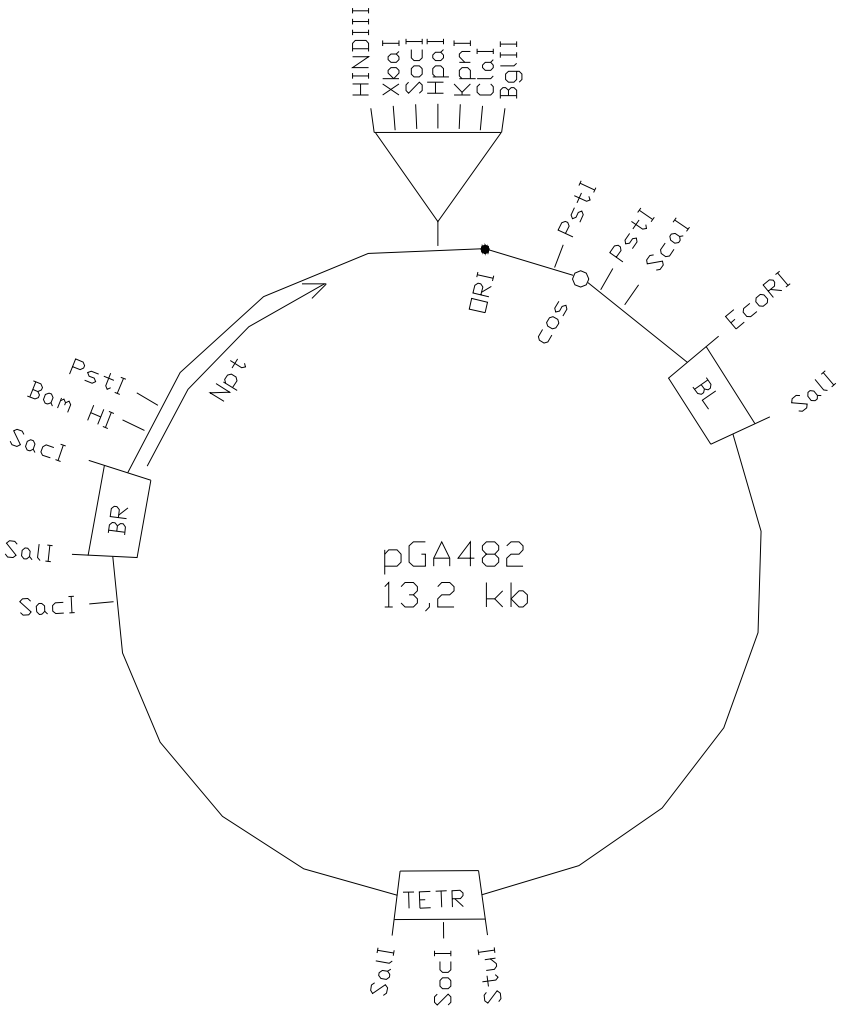
5.


Kpn I (1)
hewl
bean pgip1
BamH I (393)
CaMV 35S terminator
Pst I (620)
Hind III (4208)
Nco I (4165)
EcoR I (4030)
Hind III (628)
P(BLA
pRTL2-bean pgip1-hewl
5202 bp
enhanced CaMV 35S promoter
Pst I (3280)
AP
r
Hind III (3264)
P(LAC
ORI

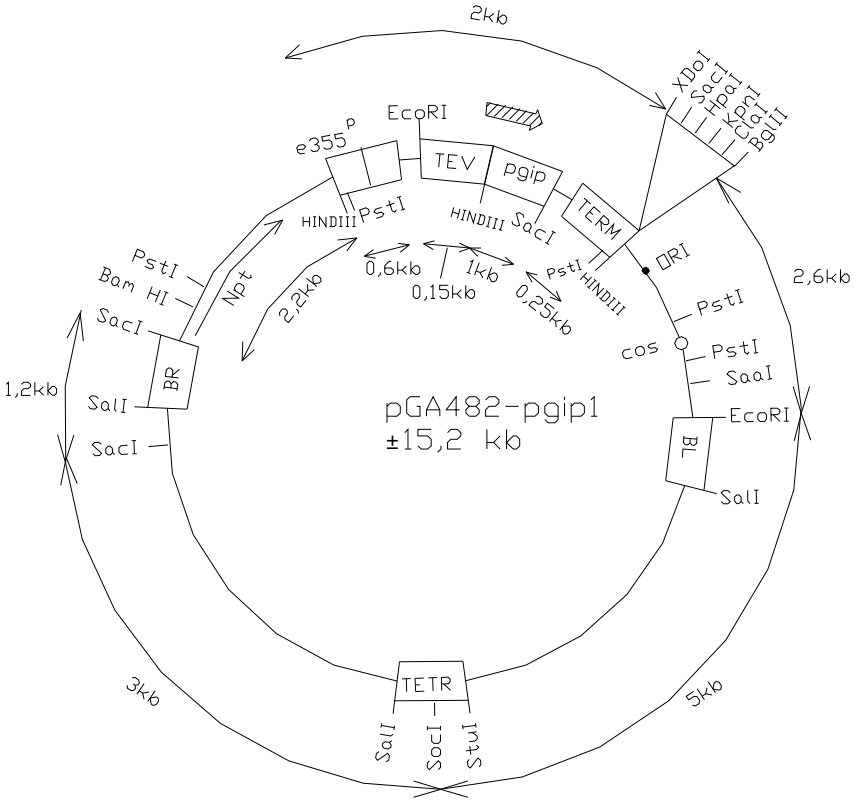
6


BamH I (1)
hewl signal
CaMV 35S terminator
Nco I (3773)
Pst I (228)
EcoR I (3638)
Hind III (236)
Ava I (3633)
Apa LI (458)
P(BLA
enhanced CaMV 35S promoter
pRTL-hewl signal
4221 bp
Apa LI (955)
AP r
Pst I (2888)
Hind III (2872)
P(LAC
ORI
Apa LI (2201)

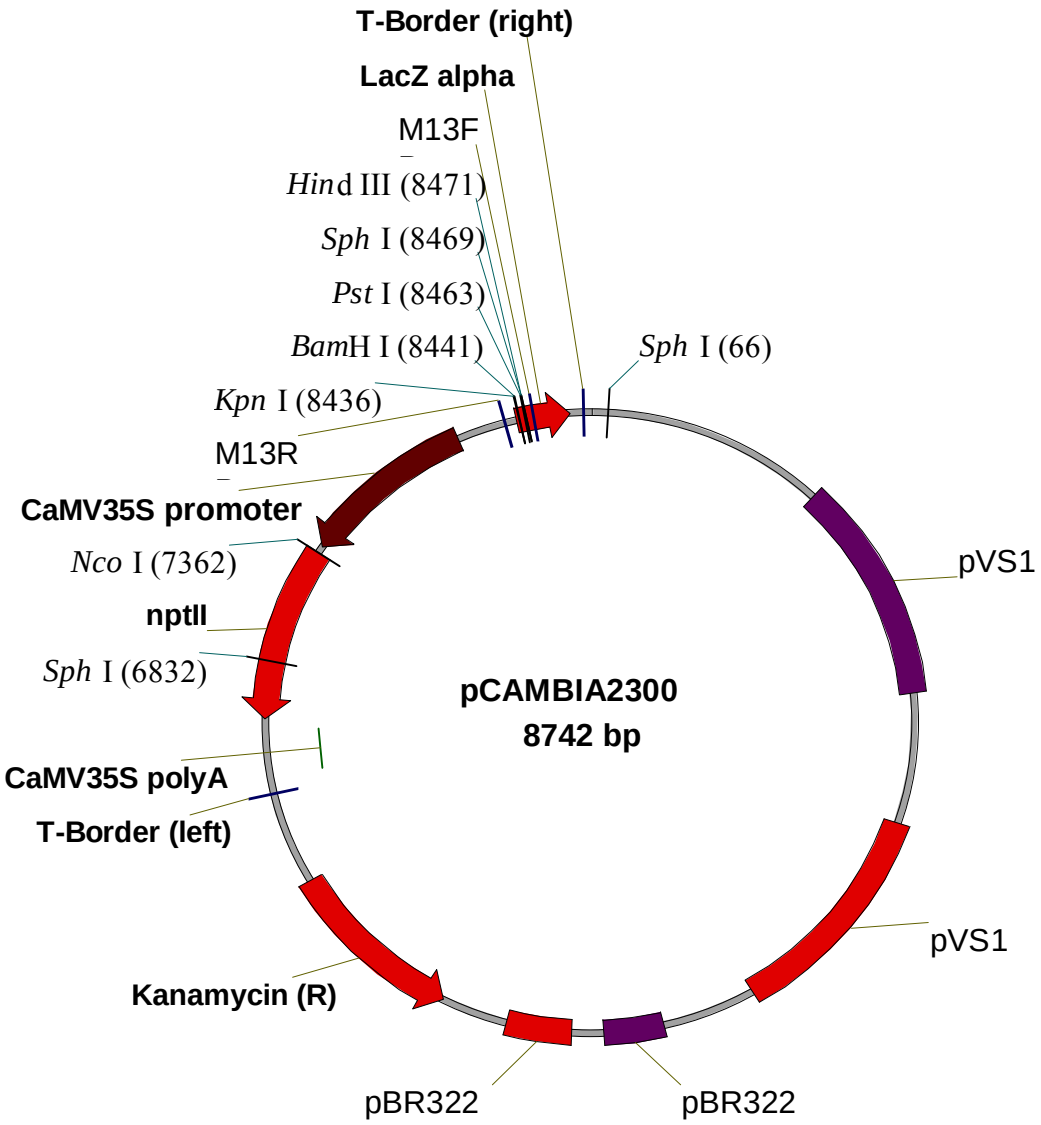
7


HINDIII
XbaI
SacI
HpaI
KpnI
ClaI
BglII
PstI
PstI
ScaI
EcoRI
SalI
BL
ORI
cos
Npt
PstI
Bam HI
SacI
BR
SalI
SacI
pGA482
13,2 kb
TETR
SalI
SacI
StuI

8


2kb
XbaI
SacI
HpaI
XhoI
BglII
EcoRI
e35S p
TEV
pgip
TERM
HINDIII
PstI
HINDIII
SacI
ORI
2,6kb
PstI
Bam HI
Npt
SacI
BR
1,2kb
SalI
SacI
2,2kb
0,6kb
0,15kb
1kb
PstI
HINDIII
0,25kb
cos
PstI
SaaI
EcoRI
BL
SalI
pGA482-pgip1
±15,2 kb
TETR
SalI
SocI
StuI
3kb
5kb

9


T-Border (right)
LacZ alpha
M13F
Hind III (8471)
Sph I (8469)
Pst I (8463)
BamH I (8441)
Kpn I (8436)
M13R
CaMV35S promoter
Nco I (7362)
nptII
Sph I (6832)
CaMV35S polyA
T-Border (left)
Kanamycin (R)
pBR322
pBR322
pVS1
pVS1
Sph I (66)
pCAMBIA2300
8742 bp

Appendix C
Restriction Enzyme Digestions of predicted plasmids constructed *in silico* using Vector NTI 6.0

Plasmid DNA digestions with *Pst*I

1. Lambda *Pst*I (28 fragments)

2. pCAMBIA2300-bean *pigp1-hewlS* (2 fragments)

8742

2542

3. pCAMBIA2300-bean *pgip1-hewlA* (2 fragments)

8742

2542

4. pCAMBIA2300-*hewl*$_{signal}$*A* (3 fragments)

8742

1561

24

5. pRTL2-bean *pgip1-hewl* (2 fragments)

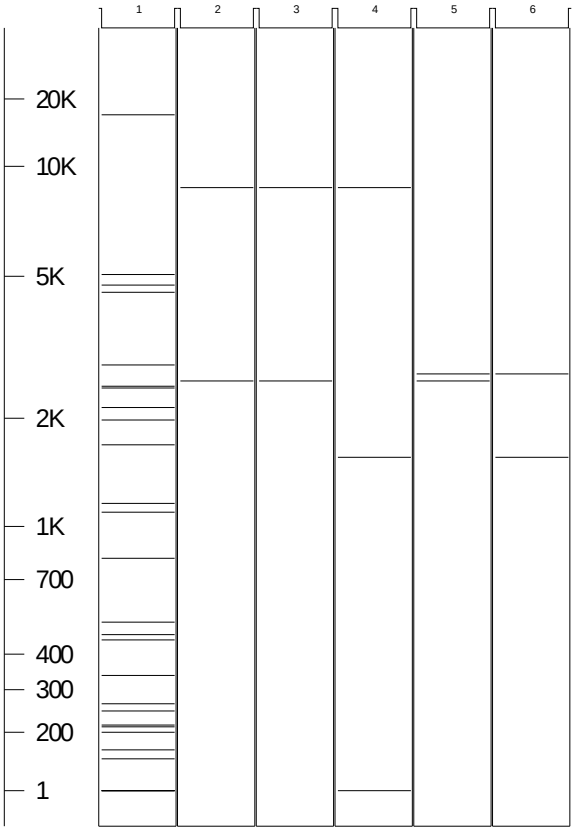
2660

2542

6. pRTL2-*hewl*$_{signal}$ (2 fragments)

2660

1561



Plasmid DNA digestions with *Nco*I and *Bam*HI

1. Lambda *Pst*I (28 fragments)

2. pCAMBIA2300-bean *pgip1-hewlA* (4 fragments)

7868
1430
1079
907

3. pCAMBIA2300-bean *pgip1-hewlS* (4 fragments)

8534
1430
1079
241

4. pCAMBIA2300-*hewl*$_{signal}$*A* (4 fragments)

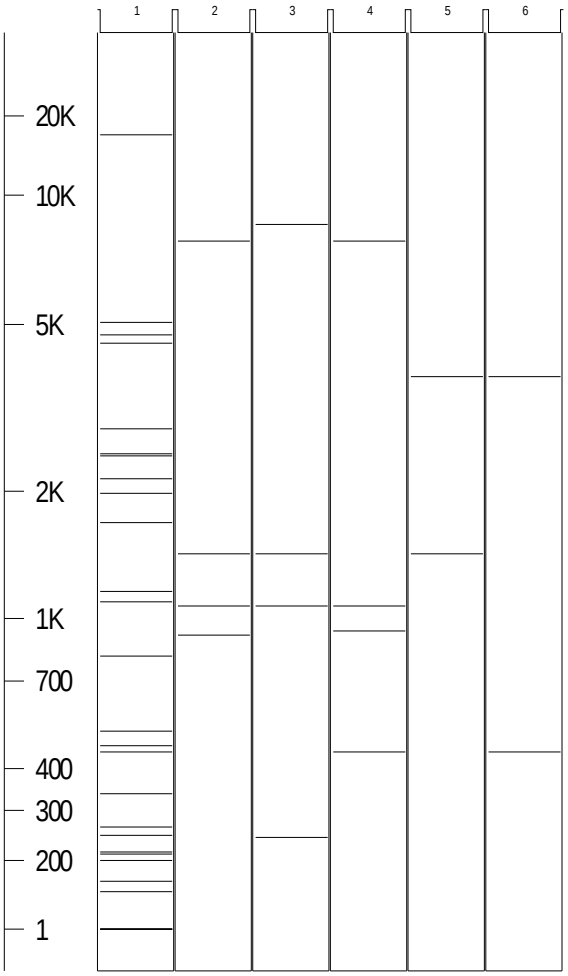
7868
1079
931
449

5. pRTL2-bean *pgip1-hewl* (2 fragments)

3772
1430

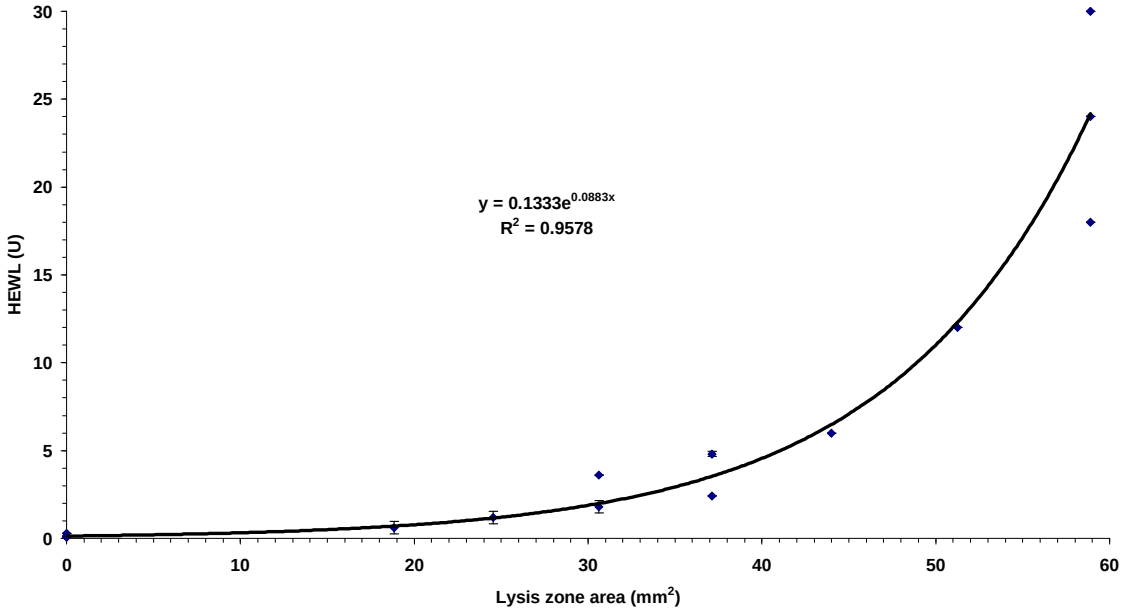
6. pRTL2-*hewl*$_{signal}$ (2 fragments)

3772
449

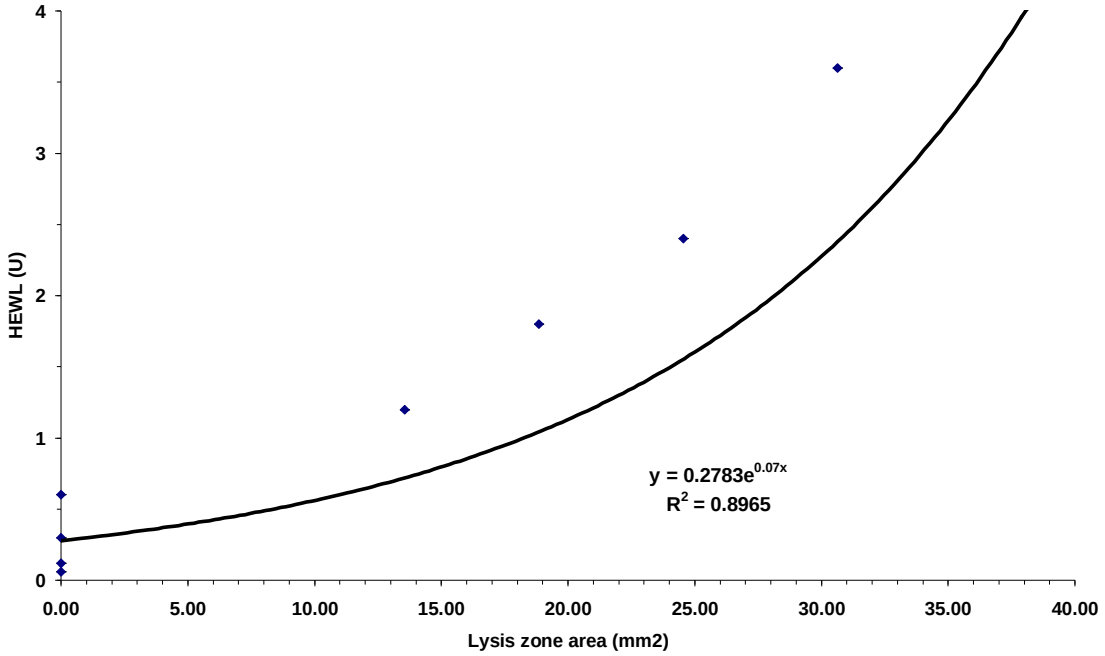


Appendix D
Standard curves

Lysozyme standard curve 1

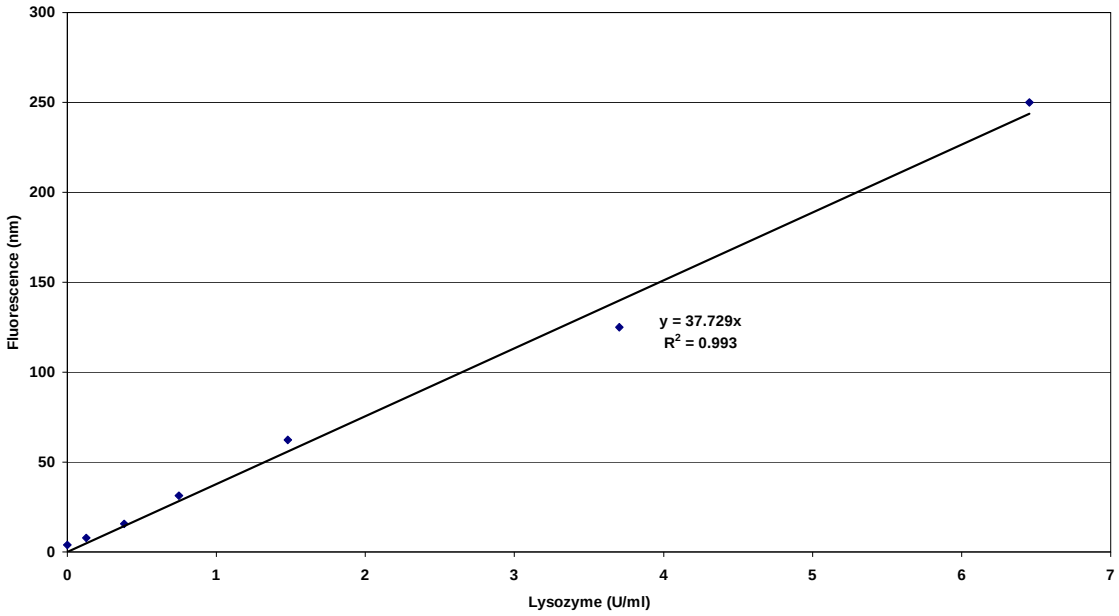


Lysozyme standard curve 2



Appendix D (continued)
Standard curves

EnzCheck® lysozyme standard curve



Chapter 8

References

CHAPTER 8

References

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