NOTE TO USERS

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A Bifunctional Selectable Marker Gene for T-DNA Tagging of Plant Promoters

by

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ABSTRACT

Plant promoters are the principle cis-acting regulatory sequences responsible for the temporal and spatial expression of genes. These elements have become the focus of much research aimed at isolating regulatory elements necessary for the expression of a particular gene of interest in a target plant. One method for isolating plant promoters is based on the ability of a common soil bacterium, *Agrobacterium tumefaciens*, to transfer a specific segment of DNA (T-DNA) into plant cells. This specific T-DNA has been shown to integrate stably into the recipient plant genome, sometimes even inserting itself into, and thereby disrupting a resident gene. If the T-DNA is designed to contain a promoterless marker gene, then T-DNA integration events occurring adjacent and downstream to a promoter region can be detected by the activation of the marker gene. These T-DNA-mediated gene fusions, consisting of an unknown plant promoter sequence and the coding sequence of a marker gene, can be isolated using the marker gene as a promoter tag.

The key objective of this work was to develop a novel, bifunctional selectable marker gene and subsequently assess its use as a selectable marker gene in both bacterial and plant transformation systems, and as a promoter tag for T-DNA promoter tagging studies in dicots. A bifunctional fusion gene was produced between phosphinothricin acetyltransferase and neomycin phosphotransferase (*pat::nptII*), by fusing an *nptII* coding sequence to the 3' terminus of the *pat* gene. The *pat* gene product confers tolerance to the non-selective herbicide L-phosphinothricin (Ignite™, Hoechst AG). The expression of the neomycin phosphotransferase (*nptII*) gene allows for the direct selection of

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transformed cells with the antibiotic, kanamycin. Using an *in vivo Escherichia coli* selection system, a translational fusion gene between these two reporter genes was achieved. The resulting protein had the activities of both parent enzymes. This was demonstrated in transformed *Escherichia coli* as well as in transformed *Nicotiana tabacum* and *Brassica napus* plants.

Using this bifunctional selectable marker gene, a T-DNA promoter tagging vector, pBAU2, was constructed and its utility was demonstrated in *Nicotiana tabacum*. From leaf disk tissue transformed with pBAU2, eight tobacco plantlets were regenerated in the presence of kanamycin. Only three of these promoter tagged transformants were selected due to their ability to root on higher levels of L-PPT, and only one of these three regenerants was selected for subsequent promoter isolation studies. The promoter from this regenerant, pBAU2#15, was isolated by screening a Lambda subgenomic library and also by thermal asymmetric interlaced (TAIL-) PCR. The isolated upstream regulatory sequence was fused to a reporter gene, β-glucuronidase (*gus*), and its function was demonstrated in *Nicotiana tabacum* and in *Brassica napus*.

Future prospects of the *pat::nptII* bifunctional fusion gene could include selection of transformation events in a broad range of plant species (dicots and monocots), and its use in T-DNA and transposon vectors for finding useful promoters in a range of different plant species.

ACKNOWLEDGMENTS

This thesis is dedicated to my daughter,

Kyra Turkan,

who by being curious, loving, and inquisitive has been my motivator, my love and my reality check.

Dear Kyra,

"In 1515 Martin Luther translated the New Testament (all 30,000 words) in 2 weeks while sitting on a wooden stump using a quill. He was tempted many times by the devil to quit. Today visitors to the Wittenberg are shown the stains on the wall where Luther allegedly threw the ink pot at the devil."

Today your Mami sits here at the kitchen table and using her laptop computer she adds the final touches to a very long and arduous process of translating several years of research into a comprehensive and meaningful piece of text. As you know, there are more ink spots on our kitchen wall then in the tower at Wittenberg.

Whatever you start, little Maus, no matter how frustrating, insignificant or overwhelming, must be finished. Whatever you decide to tackle in life, please be assured that I am right behind you every step of the way.

With all of my love,

Mami

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Abbreviations	Descriptions
2,4-D	2,4-dichlorophenoxyacetic acid
als	acetolactate synthase gene
BA	6-benzylaminopurine
B5	Gamborg's salts and vitamins
bp	base pair
CaMV 35S	cauliflower mosaic virus 35S
CAT	chloramphenicol acetyl transferase
canola	*Brassica napus* cv. Westar
CTAB	cetyltrimethyl ammonium bromide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra acetic acid
g	gram
x G	centrifugal gravity
GA$_3$	gibberellic acid
GUS	β-glucuronidase
GUS::NPTII	bifunctional fusion protein of β-glucuronidase and neomycin phosphotransferase II
*gus*A (*gus.* syn. *uidA*)	β-glucuronidase gene
gus::nptII	β-glucuronidase and neomycin phosphotransferase II fused gene
Kb	kilo base pair
LB	T-DNA left border repeat
L-PPT	(L) form of phosphinothricin

IAA	indole-3-acetic acid
mg	microgram
μL	microlitre
mg	milligram
mL	millilitre
mM	millimolar
MS	Murashige and Skoog's salts and vitamins
NAA	naphtalenacetic acid
NPTII	neomycin phosphotransferase II
nptII	neomycin phosphotransferase II gene
PAT	phosphinothricin acetyl transferase
pat	phosphinothricin acetyl transferase gene
pat::nptII	phosphinothricin acetyl transferase and a neomycin phosphotransferase fusion gene
PCR	polymerase chain reaction
PVP	polyvinylpyrolidine
RB	T-DNA right border repeat
T-DNA	transferred DNA of Ti plasmid
Ti	tumor inducing plasmid of *Agrobacterium*
Tnos	transcriptional terminator of nopaline synthase gene
tobacco	*Nicotiana tabacum* cv. Xanthi
X-Gluc	5-bromo-4-chromo-3-indolyl-glucuronidase

Chapter 1:   Introduction

Gene expression is primarily regulated at the transcriptional level and is comprised of three stages: initiation, elongation and termination. These three stages are controlled by promoter and non-promoter elements.

Non-promoter mechanisms which can influence the level of gene expression include: enhancer elements which are orientation-independent and usually enhance transcriptional activity at distances as great as several kilobases (kb) 5' or 3' from the transcriptional start site (Khoury and Gruss, 1983), elements which act to regulate transcript termination (Hernandez et al., 1989; Mogen et al., 1990), sequences which regulate transcript stability (Newman et al., 1993), post-transcriptional modification (Callis et al., 1987) and translation efficiency (Murray et al., 1989).

Promoter elements used for genetic engineering can either originate from a species other than the host, or originate from the host species iteself. A promoter is usually defined as a nucleotide sequence which contains a TATA box and serves to determine the start site of transcription (Dynan and Tjian, 1985). Promoter function is dictated by the composition of the regulatory domain and by the transcription factors that interact with basal transcription factors acting as a multi-subunit complex. This multi-subunit complex, in turn, interacts with the "TATA" box by contacting the TATA Binding Protein (TAP) and the RNA polymerase II. Additional upstream factors, comprised of DNA-binding proteins, recognize short consensus elements (specific DNA sequences) upstream of the transcriptional startpoint. The activity of both basal and upstream DNA-binding factors may be regulated or constitutive and

influence the efficiency of transcription. They are necessary for a promoter to function. Inducible factors function like upstream factors except that they are subject to regulation. Inducible factors are synthesized or activated at specific times or in specific tissues. These latter factors are responsible for the control of transcription patterns in time and space.

A constitutive promoter, by its simplest definition, contains elements recognized by basal and upstream activators to initiate transcription in all tissues and at all times. An example of such a promoter may be that of a housekeeping gene (homologous) or that of a gene derived from a plant pathogen with the ability to infect a wide range of host cells (heterologous) (Lewin, 1994).

To date there a number of constitutive promoters available for the expression of reporter genes or other genes of interest in plants. Almost all of these promoters are protected by patents, therefore providing incentive to isolate novel constitutive promoters for future commercial use. Constitutive promoters are needed to express certain gene traits, such as herbicide tolerance (DeBlock *et al.*, 1987; Oelck *et al.*, 1991, 1993; Miki *et al.*, 1990; Freyssinet *et al.*, 1992), drought tolerance, heavy metal tolerance (Misra and Gedamu, 1989) some insect resistance (Cardineau *et al.*, 1993; Metz *et al.*, 1995; Puttick *et al.*, 1993; Mehra-Palta *et al.*, 1991) and fungal tolerance (Broglie *et al.*, 1991) traits, etc., throughout most plant organs and during most stages of a plant's development. Examples of plant promoters presently being used are listed in Table 1.1.

Two of the most common approaches for the isolation of promoter elements are cDNA based methods and T-DNA tagging methods. In cDNA methods the

desired mRNA is isolated and a cDNA clone produced. Adjacent regulatory regions can then be isolated by screening a genomic DNA population. The most commonly used methods for isolating constitutive promoters have been cDNA based. T-DNA tagging methods are based on the ability of *Agrobacterium tumefaciens* to insert it's T-DNA randomly into the plant genome (Zambryski *et al.*, 1988). If the T-DNA contains a promoterless reporter gene adjacent to the border region (right border is preferred), tagged plant promoters can be recovered through the selection and screening of transformed plants exhibiting reporter gene activity. The tagged promoter region can be isolated by screening the genome of the transformant for the presence of the reporter gene. Such tagging methods have been commonly used to isolate tissue and organ specific regulatory elements.

Most of the promoters used to express transgenes in plants are strong constitutive promoters, such as the cauliflower mosaic virus (CaMV) 35S promoter and its derivatives. However, evidence on targeted degradation of overproduced RNA and on other mechanisms of gene silencing (de Carvalho *et al.*, 1992; Flavell, 1994; Green, 1993; Mueller *et al.*, 1994; Van Hoof and Green, 1994) have stimulated the search for weaker native plant promoters and for promoters which can be controlled in a tissue-specific manner or switched on by an effector. The conventional cDNA based methods for promoter identification and isolation are not very ammenable for the isolation of such promoters. On the other hand, T-DNA tagging methods have been successfully used to isolate tissue specific promoters (Datla, R., personal communication). It is worthwhile to determine whether such a tagging method can also be used to isolate weaker constitutive-like plant regulatory sequences

or promoters. By modifying T-DNA tagging vectors and by applying a more stringent selection protocol, it may be possible to isolate such plant promoters from housekeeping genes. Once isolated and characterized, these 'constitutive' plant regulatory elements can be used to develop new promoter constructs for applications for expression of foreign genes in transgenic plants.

In this study, an efficient bifunctional fusion gene (*pat::nptII*) was constructed and used for tagging promoters in plants. This bifunctional fusion gene allowed the option to use two different *in vitro* tissue regeneration protocols (selection on kanamycin and selection on phosphinothricin, L-PPT). The kanamycin selection was preferred since it is a gentler selection, allowing for a higher shoot regeneration frequency. The L-PPT selection was used post-regeneration to eliminate nontransgenic regenerants (which had escaped the gentle kanamycin selection) and to screen *in vitro* plantlets for the strength of expression (relating to the strength of the tagged promoter sequence).

This fusion gene, prior to being used as a promoter tagging tool, was placed under the control of the tandem 35S Cauliflower Mosaic Virus (*CaMV*) promoter and expressed constitutively both in *Nicotiana tabacum* and in *Brassica napus* to ensure function. A promoterless *pat::nptII* was then introduced into *Nicotiana tabacum* for T-DNA tagging and subsequent promoter isolation.

Table 1.1:   Promoters Used for the Expression of Transgenes in Plants

Promoter	Tested In:	Reference
Cauliflower Mosaic Virus 35S RNA transcript (*35S*)	dicots and monocots	Guilley *et al.*, 1982. Odell *et al.*, 1985
Agrobacterium tumefaciens Nopaline Synthase Gene (*NosP*)	dicots	An *et al.*, 1986.
Agrobacterium tumefaciens Manopine Synthase Gene (*MasP*)	dicots	Langridge *et al.*, 1989.
Agrobacterium tumefaciens Octopine Synthase Gene (*OcsP*)	dicots	Fromm *et al.*, 1989.
Cauliflower Mosaic Virus 35S Promoter and 1st Intron of Maize Alcohol Dehydrogenase 1 Gene (*35S-Adh1intron1*)	monocots	Callis *et al.*, 1987.
Commelina Yellow Mottle Virus	dicots	Medberry & Olszewski, 1993.
Chimaeric Cauliflower Mosaic Virus 35S Promoter and Manopine Synthase Promoter (*35S-Mas*)	dicots	Comai *et al.*, 1990.
Modified Maize Alcohol Dehydrogenase I Promoter and First Intron (*Emu*)	monocots	Last *et al.*, 1991.
Rice Actin 1 Gene (*Act1-Act1 intron1*)	monocots	McElroy *et al.*, 1990.
Maize Ubiquitin 1 Gene (*Ubi1-Ubi1 intron 1*)	monocots	Christensen *et al.*, 1992.
A. thaliana Peroxidase Gene (*prxCa*)	dicots	Intapruk *et al.*, 1994.
A. thaliana atS1A ribulose-1,5-biphosphate carboxylase small subunit gene promoter (*pSsuAra*)	dicots	Krebbers *et al.*, 1988

Chapter 2: Literature Review

2.1 Fusion Genes

The process by which linear DNA is transcribed and translated into a precise amino acid sequence is well understood. The most consistent model used to describe the initiation of translation in the cytosol of eukaryotic cells is called the modified scanning model (Kozak 1981). In this model the 40S ribosomal subunits bind at or near the 5' cap structure of the mRNA and migrate downstream along the mRNA until the first AUG codon is reached. If this AUG codon exists in a favourable context, that is AACAAUGGC for plant mRNAs (Joshi 1987; Lutchke *et al.*, 1987), then the 40S ribosomal subunits are halted. The 60S subunits associate with the halted 40S subunits and translation is initiated. This AUG then becomes the translational start site and is translated into a methionine amino acid. If the context around the first AUG codon is less favourable, then some or most of the 40S subunits proceed to the next AUG. The ability for some of the 40S subunits to migrate to an AUG further downstream is the basis for a concept called reinitiation. Reinitiation occurs when an upstream AUG codon, even if it is in an optimal context, allows for the initiation of translation at a downstream AUG codon on the same mRNA. This only occurs provided that an in-frame termination codon exists between the two AUGs (Angenon *et al.*, 1989).

By manipulating the translation start and termination codons, messages which normally are separate can be joined together to produce a translational fusion protein. Cloning tools such as restriction endonucleases and T4 DNA ligase have been used to

join spatially separated genes. These tools are the basis for producing translational in-frame fusions between two proteins using modern molecular biology techniques.

Protein biochemists studying highly organized enzyme systems have often looked to the possibility of joining two or more enzymes to produce a single moiety carrying multiple enzymatic activities. Such a multifunctional enzyme would have the potential advantage of being able to catalyze several separate reactions. Numerous methods have been reported for obtaining these multifunctional enzymes. Typically, enzymes are either sequentially immobilized or chemically crosslinked, but these processes can be difficult to control and can result in a large decrease in enzyme activity. Another method involves the in-frame fusion of two genes which, when translated, produce an artificial bifunctional enzyme. This latter method has the advantage that large amounts of homogeneous bifunctional protein can be produced. In prokaryotic systems, these translational fusion enzymes often have the advantage of increased protein stability and retained activity (Bulow, 1993).

Translational in-frame fusions can be quite flexible. Two to five amino acid residues can frequently be removed from one or both termini of the enzymes without affecting their activity. Fusions can be made either to the amino or carboxy- terminal regions of the proteins depending largely on the availability of suitable restriction enzyme sites on the corresponding structural genes. If no such sites are present at the 5' or 3' ends of the genes, they can be generated by site-directed mutagenesis. Also, special linker regions can be used to bridge the two linked enzymes. These linker regions are chemically synthesized DNA fragments which are added during the cloning procedure. A functional linker region can be as small as only three nucleotides

or one amino acid residue (Hu *et al.*, 1992). When fusion enzymes are artificially constructed, the three-dimensional structure of the enzymes is often unknown. However, since the C and N termini are frequently located on the surface, an artificial gene fusion does not normally interfere with the folding of the protein. If interference is detected (i.e. loss in enzyme activity) and subunit interactions are disturbed, then the fusion can be made at the other end of the gene (Bulow, 1993).

An example of carboxy-terminal versus amino-terminal fusion proteins was demonstrated by Bernd Reiss and co-workers at the University of Heidelberg. They fused foreign DNA sequences to both the amino and carboxy termini of the neomycin phosphotransferase II (*nptII*) gene from transposon Tn5 (Beck *et al.*, 1982) and monitored the effect of the gene fusion on NPTII enzyme activity. Short additions to the amino terminus of the NPTII resulted in enzymatically active fusion proteins whereas long amino-terminal fusions often had to be proteolytically degraded to release active proteins. Fusions at the carboxy-terminal end, on the other hand, did not always produce an active NPTII enzyme. In the latter fusions, the nature of the junction sequence seemed to determine whether the product would be functional or not (Reiss *et al.*, 1984). These findings were in keeping with the discovery that the *nptII* gene tolerates extensive deletions at it's 5' end but not at its 3' end (Beck *et al.*, 1982).

The first bifunctional fusion enzyme was produced by mutating the intercistronic region (stop codon) between the second and third genes (*hisD* and *hisC*) of the histidine operon of *Salmonella typhimurium*. The single polypeptide produced by these fused genes demonstrated catalytic activity for both enzymes (Yourno *et al.*,

1970). This milestone predated the cloning era where spatially separated genes could be excised (using restriction endonucleases) and ligated to each other using T4 DNA ligase (Khorana *et al.*, 1976). With the implementation of modern cloning techniques, fusion proteins were routinely produced to study metabolic regulation in prokaryotic systems. By removing the naturally occurring stop signals at the 3' end of one gene and removing the promoter region at the 5' terminus of the other gene a DNA chimera encoding a hybrid protein with two active sites could be created.

The ability to produce fusion enzymes, such as ß-galactosidase::galactokinase (Bulow *et al.*, 1985; Bulow, 1987; Bulow, 1993), UDP-galactose 4-epimerase::galactose-1-P uridylyltransferase (Tamada *et al.*, 1994) and indoleglycerol phosphate synthase::phosphoribosyl anthranilate isomerase (Eberhard *et al.*, 1995), gave researchers the opportunity to study proximity effects, stabilizing effects and enzyme kinetics of bifunctional enzymes in comparison to their corresponding native enzymes. One of the reported advantages included the ability to recycle an expensive cofactor by covalently attaching it to the fusion protein through a flexible spacer. Another advantage was that the product of one enzyme, being a substrate for the next, might be captured by the second enzyme due to its proximity and therefore results in a kinetic advantage. Fusions consisting of two enzymes (di-enzymes) were often found to have the same activity as the native proteins and often showed much greater heat stability. This observation was reinforced by a study performed on di, tri and tetra-ß-galactosidase fusion enzymes expressed in a prokaryotic system (Kuchinke and Muller-Hill, 1985).

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Bifunctional fusion proteins have also been used to produce and study novel traits. A fusion gene between γ-glutamyl kinase:: γ-glutamyl phosphate reductase, when expressed in an *Escherichia coli* model system, coded for a bifunctional enzyme which increased the intracellular concentrations of proline. Proline is an amino acid found to accumulate in many microorganisms, algae and higher plants under physiologically extreme conditions with low water activity or high salinity. This amino acid, when expressed at enhanced levels, acts as a nontoxic osmolyte to protect the bacterial cell against desiccation (Meijer *et al.*, 1996).

One of the goals of biotechnology based research initiatives has been to manipulate ethylene production in agricultural and horticultural plants. Ethylene has profound effects on the growth and development of higher plants. It is involved in many physiological processes including fruit ripening, flower senescence, petal and leaf abscission, the inhibition of seedling elongation, root initiation, the promotion of flowering in pineapple and the increased flow of latex in rubber trees. Researchers at the Hong Kong University of Science and Technology constructed an artificial bifunctional enzyme by fusing the carboxy terminus truncated soybean 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene to the amino terminus truncated tomato ACC oxidase gene. The resulting fusion polypeptide was successfully expressed in a prokaryotic system where an increased ethylene production rate was observed (Li *et al.*, 1996).

The gene for metallothionein (MT) has been used to bind heavy metals such as cadmium (Cd) in plants. Cd is a frequent contaminant of agricultural soils and is absorbed and accumulated by the roots. Some plants, such as lettuce and tobacco,

translocate most of the Cd to the leaves where it may be eaten, or smoked, respectively, by humans. For this reason many researchers have attempted to overexpress MT in plant tissue. As a tool for improving sequestration of this heavy metal in plants, genes encoding fusion proteins between MT and an *E. coli* lipoprotein, for membrane localization (Jacobs *et al.*, 1989), and MT and streptavidin, for labelling of heavy metal ion-containing biologicals (Sano *et al.*, 1992), have been developed.

Besides novel traits, fusion proteins have also been used in the field of diagnostics. Several fusion systems have been designed to recover large quantities of fusion proteins using affinity chromatography purification. Most of the systems are based on fusing a gene of interest to the *lacA* gene of *E. coli*, which codes for β-galactosidase. By running the β-GAL fusion protein over an affinity column containing either APTG-Sepharose (Scholtissek and Grosse 1988; Germino *et al.*, 1983) or TPEG-Sepharose as a ligand, a large proportion of the hybrid protein can be purified to near homogeneity. Another reported gene fusion system uses vectors which allow fusion of any gene to the gene coding for Staphylococcal Protein A. The gene fusion product can then be purified by affinity chromatography on an IgG-Sepharose column which contains the Fc portion of the immunoglobulin cross-linked to Sepharose beads (Nilsson *et al.*, 1985). These purification systems, although they use translational gene fusion technology, do not require an in-frame fusion orientation nor do they require that the fused protein retain its native activity.

Translation fusion systems have also been used to target specific proteins to various organelles in both plant and animal cells. By fusing a passenger gene, such as

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nptII or the *gus* marker gene (β-glucuronidase) (Jefferson *et al.*, 1987), to the plant signal sequences for transport into the chloroplast (Van den Broeck *et al.*, 1985) or into the endoplasmic reticulum (ER) (Denecke *et al.*, 1990), scientists have been able to study and evaluate the mechanisms of protein transport in plants. Another study performed in mouse embryos examined the developmental control and targeting of a simian virus 40 large tumour nuclear location signal (NLS) by fusing it to a modified β-galactosidase gene (Bonnerot *et al.*, 1987).

Genes encoding bifunctional fusion proteins, where one of the proteins is a scorable or visual marker and the other is a non-selectable gene of interest, have been engineered as a tool to select for transformants which strongly express the gene of interest. For example, the cadmium (Cd)-sequestering gene metallothionein (MT) was fused to the visual marker gene coding for GUS (Jefferson *et al.*, 1987) and transformed into tobacco. Transformants which expressed the gene strongly could be easily selected. The targeting of this bifunctional gene to tissues most directly involved in Cd absorption could also be monitored (Elmayan and Tepfer 1994).

Marker genes have become an important instrument for the study of transformation processes in bacteria, fungi, plants and animals. A selectable marker gene can impart a selective advantage, such as antibiotic resistance, to a particular tissue or organism. A visual marker gene specifies an enzyme activity for which there is a strong chromogenic or luminescent product which can be visually or spectrophotometrically detected. To study a particular transformation event or transformation technology it is often desirable to combine two different marker genes into one. Using an in vivo selection approach, Datla and co-workers produced a

bifunctional fusion protein containing both NPTII and GUS activities (Datla *et al.*, 1991).

The NH₂-GUS::NPTII-COOH fusion gene was constructed by fusing the carboxy terminus of the *E. coli uidA* gene encoding GUS (EC 3.2.1.31) to the amino terminus of the *nptII* coding region (Beck *et al.*, 1982) whose first four codons including the start codon, were deleted. This configuration resulted in an out-of-frame nptII gene. When the *gus::nptII* fusion was expressed in a bacterial host only the gus gene was translated into an active protein. All of the bacteria which expressed the fusion gene were kanamycin sensitive. On the assumption that spontaneous genetic deletions were not uncommon, researchers maintained the *gus::nptII* containing bacteria on kanamycin (Km) selection medium until spontaneous Km resistant mutants appeared. GUS positive and Km resistant mutants were isolated and the intergenic region between the two marker genes was sequenced. A single base pair deletion near the 3' end of the *gus* gene was responsible for the in-frame orientation of the *nptII* gene. This sequence change was incorporated into future bifunctional gus::nptII fusion constructs (Datla *et al.*, 1991).

One of the constructs tested was a binary vector containing the *gus::nptII* bifunctional fusion gene under the control of the 35S promoter from Cauliflower Mosaic Virus (CaMV) (Odell *et al.*, 1985). This binary vector was used to transform tobacco. When this fusion gene was expressed in both plants and bacteria, it was shown to be bifunctional and its elution profile verified that it was a trimer. This observation corresponded well with gel filtration chromatography studies which suggested that the native NPTII protein is active as a monomer and the native GUS

protein is active as a dimer. This was the first reported study showing a successful C-terminus fusion to the *gus* gene (Datla *et al.*, 1991).

A fusion marker gene, such as the one described here, is an excellent candidate for a reporter system in plants. Its bifunctional fusion protein imparts both the ability to select directly for kanamycin resistance and the ability to assay histochemically for GUS activity. Due to the ability of both GUS and NPTII to function in a broad range of species, this construct can also be used to study the developmental biology of prokaryotes, fungi and higher eukaryotes (Datla *et al.*, 1991).

Other bifunctional marker enzymes were produced by fusing the carboxy terminus of the phosphinothricin acetyltransferase (*bar*) gene, isolated from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987), to the amino terminus of the *lacZ* gene and the *nptII* gene. *Bar* codes for an enzyme, PAT, which imparts resistance to the non-selective herbicide glufosinate ammonium. The *bar* gene has been commonly used as a selectable marker gene in monocots (Spencer *et al.*, 1990; Kramer *et al.*, 1993; Linscombe *et al.*, 1996; Nehra *et al.*, 1994; Vasil *et al.*, 1992) and in dicots (De Block *et al.*, 1987). The ability to detect the gene product using both a radiolabelled activity assay, a spectrophotometric assay (Thompson *et al.*, 1987) and a Western Blot immunoassay (Botterman *et al.*, 1991) have increased the versatility of this selectable marker gene.

To produce a C-terminus fusion, the *bar* gene was restricted at the *Sau3AI* site located next to the stop codon, and then fused to either the visual marker gene *lacZ* or to the selectable marker gene *nptII* to produce in-frame orientations. It was also found that fusions 16 amino acids upstream from the *bar* stop codon were inactive

(Botterman *et al.,* 1991). Both *bar::lacZ* and *bar::nptII* constructs were expressed in a bacterial host and the respective fusion proteins demonstrated bifunctional activity. Also, the binding constant of the native and fused BAR proteins for the herbicide, glufosinate ammonium, did not differ significantly. This indicated that geometry of the substrate-binding-site of the fusion protein did not fundamentally change as compared to the native BAR protein, when expressed in a prokaryote host (Botterman *et al.,* 1991).

The *nptII* gene codes for neomycin phosphotransferase (NPTII) which inactivates aminoglycoside antibiotics such as kanamycin, neomycin and G-418 by phosphorylation (Davies, 1980). This gene is extensively used a dominant selectable marker for the isolation of both prokaryotic and eukaryotic transformed cells resulting from gene transformation experiments (An *et al.,* 1993; DeBlock *et al.,* 1984; Herrera-Estrella *et al.,* 1983). The *nptII* gene is also used as a reporter since its functional gene product can be conveniently assayed using a NPTII activity assay (Platt and Yang, 1987; McDonnell *et al.,* 1987). The amount of NPTII protein can also be quantitated using a sensitive NPTII Enzyme Linked Immunosorbent Assay (ELISA) (Nagel et al., 1992), thereby enhancing the usefulness of such a gene for the study of transformed cell systems.

Besides providing important information on transformation systems and developmental stage analyses, bifunctional reporter genes have also been used to tag novel genes and promoters both in plants and in animals. In the past, bifunctional marker genes used for promoter tagging or trapping have often incorporated a selectable marker function along with a histochemical or luminescent detection

function so that tissue specific regulatory elements can be easily screened. For example, a *lacZ::nptII* fusion was used successfully in plants to tag shoot apex-specific promoters and genes (Suntio and Teeri, 1994). The same reporter gene fusion was used to tag promoters in embryonic stem cells of mice (Friedrich and Soriano, 1991). Other bifunctional fusion genes, which have been used for promoter tagging in plants, include the *gus::nptII* gene fusion (Datla *et al.*, 1991) and the visual marker::selectable marker combination of the luciferase firefly gene (*luc*) and the *nptII* gene (Barnes 1990).

The *nptII* and the *lacZ* genes are the most extensively studied candidates for gene fusions. The *lacZ* gene retains its activity when fused at either its carboxy or amino terminus, while the *nptII* gene retains its activity when fused at the amino terminus. Both are very powerful reporter genes and function well in bacteria, fungi, plants and animals.

2.2 Glufosinate Ammonium

L-phosphinothricin (L-PPT) [L-homoalanin-4-yl (methyl) phosphinic acid] is the active phytotoxic metabolite of the tripeptide bialophos [L-2-amino-4-((hydroxy)(methyl) phosphinoyl) butryl-L-alanyl-L-alanine] (Figure 2.1). This naturally occurring phytotoxic tripeptide was first isolated from cultures of *Streptomyces viridochromogenes* (Bayer *et al.*, 1972), and found to have activity against bacteria, fungi and higher plants. Before a herbicidal effect can be produced, the bialophos tripeptide is hydrolysed by the plant or bacterium. The active metabolite of this hydrolysis, L-phosphinothricin (L-PPT), is an analogue of glutamate and irreversibly inhibits glutamine synthetase (GS).

Chemically synthesized PPT, called glufosinate ammonium, is a racemic mixture of D- and L-isomers (Kocher, 1983). Only the L-isomer is active (Manderscheid and Wild, 1986). The chemically synthesized molecule is used as the active ingredient in a non-selective, postemergence, foliar applied herbicide called Basta^{TM} in Europe or Liberty® in North America. This herbicide, belongs to the amino acid derivative class of herbicides. It is formulated as a monoammonium salt with an anionic surfactant (AgrEvo AG, Frankfurt Germany). It is rapidly taken up by plants via the leaves and other green parts. No active ingredient is taken up from biologically active soil via the roots (Hoechst, Glufosinate Ammonium brochure).

Dissolution and redistribution of cuticular wax occurs upon contact of the glufosinate ammonium herbicide (Liberty®) with the leaf surface of a plant. Once the herbicide has penetrated the cuticle, there is a rapid (within 6 hours) distortion and rupturing of interveinal mesophyll cells. Other cellular alterations include a

disorganization of the bundle sheath cells and, in some species, a collapse of epidermal cells. Many of these cellular effects have been associated with a disruption in membrane function (Bellinder *et al.*, 1985)

Figure 2.1: Structural Formula of Bialophos, Glufosinate Ammonium (PPT) and Glutamate.

Bialophos:

$$
\begin{array}{cccc}
\text{O} & \text{NH}_2 & \text{CH}_3 & \text{CH}_3 \\
\| & | & | & | \\
\end{array}
$$

H$_3$C-P-CH$_2$-CH$_2$-CH-CONH-CH-CONH-CH-COOH
|
OH

Phosphinothricin (PPT):

$$
\begin{array}{cc}
\text{O} & \text{NH}_2 \\
\| & | \\
\end{array}
$$

H$_3$C-P-CH$_2$-CH$_2$-CH-COOH
|
OH

Glutamate:

$$
\begin{array}{cc}
\text{O} & \text{NH}_2 \\
\| & | \\
\end{array}
$$

C-CH$_2$-CH$_2$-CH-COOH
|
OH
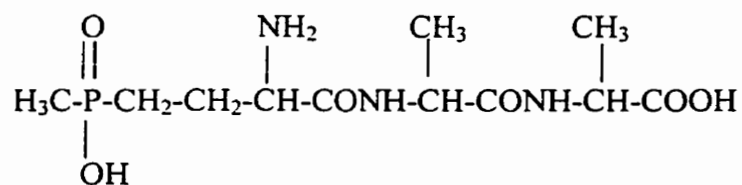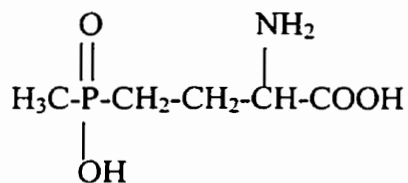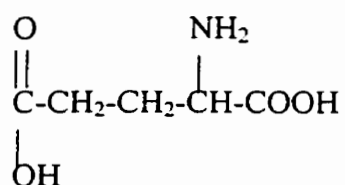
Once the glufosinate ammonium has penetrated the cell it has access to its target enzyme glutamine synthetase (GS). GS is the first enzyme involved in the capture of inorganic nitrogen in an organic form by catalyzing the condensation of glutamate and ammonia to form glutamine [Figure 2.2] (Miflin and Lea, 1976). The L-PPT molecule competes with the legitimate substrate, glutamate, for the same binding site on GS. Once bound, it is phosphorylated to produce an irreversible enzyme inhibitor complex. Though all isozymes of GS, cytosolic and chloroplast, are inhibited by L-PPT, the GS enzyme found in the root is more sensitive to the herbicide than the GS found in the shoots (Manderscheid and Wild, 1986). The resulting tissue damage is likely due to the combined effects of ammonia toxicity and an inhibition in photosynthesis.

Ammonia has been shown to accumulate to high levels in green tissue treated with glufosinate ammonium or Bialophos (Tachibana *et al.*, 1986; Ziegler and Wild, 1989; Wild and Ziegler, 1989). Plants obtain their nitrogen or ammonia from three major sources. They can obtain nitrogen in the form of nitrate which is taken up from the soil, or by nitrogen fixation which is carried out by *Rhizobium* bacteria in legume root nodules (Andrews, 1986). A third source of nitrogen originates from the metabolism of seed storage proteins during germination (Lea and Joy, 1983) and from the metabolism of transport compounds, such as asparagine and ureides, upon their arrival at the sink (Ta *et al.*, 1984). The ammonia obtained from all three sources is assimilated or reassimilated into amino acids via GS.

Figure 2.2:   Site of Action of Glufosinate Ammonium

[Glufosinate]

Nitrate reduction / Photorespiration / (light required) → NH₃⁺ --ATP-- GS --X-- Gln --2-oxoglutarate/GOGAT-- 2(Glu)

Glu

Many reactions

GS=Glutamine synthetase
Glu=Glutamate (glutamic acid)
Gln=Glutamine
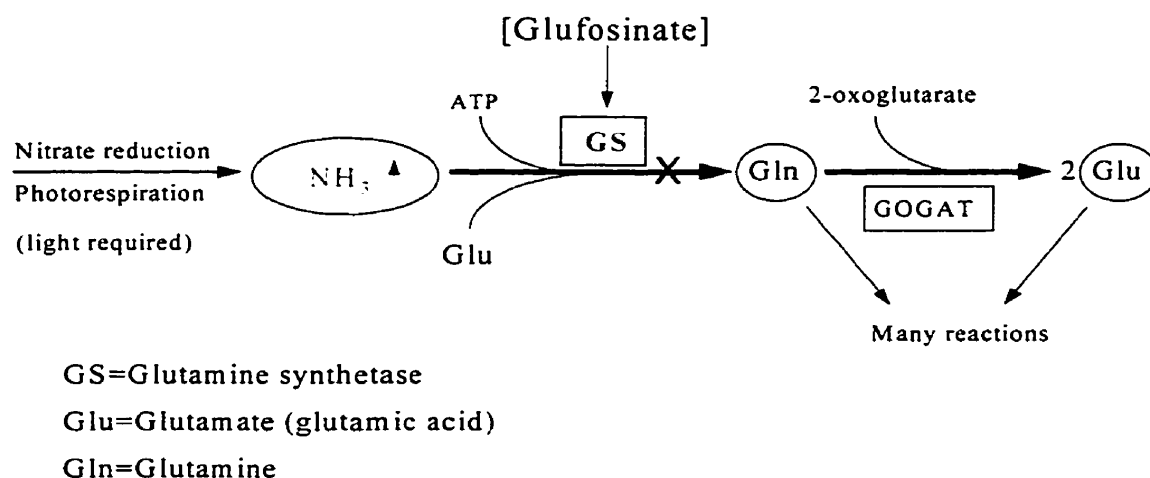
When GS is inhibited, ammonia accumulates in the cell.   Ammonia accumulation occurs at higher levels when the tissue is exposed to light. The effects of excess ammonia include leakage of potassium ion through membranes (Kocher, 1983), inhibition of ATP synthase, uncoupling of phosphorylation, inhibition of NADP+ reduction, inhibition of chlorophyll biogenesis, and inhibition of cell division (Sauer *et al.*, 1987).

Another major effect of glufosinate ammonium treatment is the inhibition of photosynthesis. Initially it was believed that ammonia accumulation was responsible for the inhibition. This was first demonstrated by a Meiji Seika Kaisha Ltd. study on barnyard millet, amaranth and crabgrass treated with bialophos (Tachibana *et al.*, 1986). A year later, Sauer and coworkers also studied the influence of L-PPT on photosynthesis. First, they investigated the effect of exogenously applied ammonia on the level of photosynthesis in chloroplasts, protoplasts and leaves. They discovered that

ammonia accumulation was not solely responsible for the inhibition of photosynthesis, but that a depletion in glutamine had a more detrimental effect (Sauer *et al.,* 1987). Other evidence supporting this observation was shown when no inhibition occurred under non-photorespiratory conditions despite ammonia accumulation. Also, supplying glutamine restored photosynthesis even in the presence of very high levels of ammonia (Wendler and Wild 1990; Krieg *et al.,* 1990).

There are several ways in which a reduction in glutamine can inhibit photosynthesis. One is by decreasing protein synthesis so that proteins with a high turn-over rate, such as the electron transport QB protein (Benett, 1984), fail to regenerate resulting in the collapse of electron transport. Glyoxylate, a reversible inhibitor of RubP carboxylase/oxygenase, may also accumulate to toxic levels within the cell and shut down photosynthesis (Sauer *et al.,* 1987). Furthermore, the inhibition of GS prevents the regeneration of carbon being channelled into the photorespiration cycle via the oxygenase reaction. This limits the amount of available Calvin cycle intermediates, such as RubP (Walker *et al.,* 1984). The effect of PPT on the various cellular processes is summarized in Figure 2.3.

Figure 2.3: Glufosinate Ammonium Mode of Action
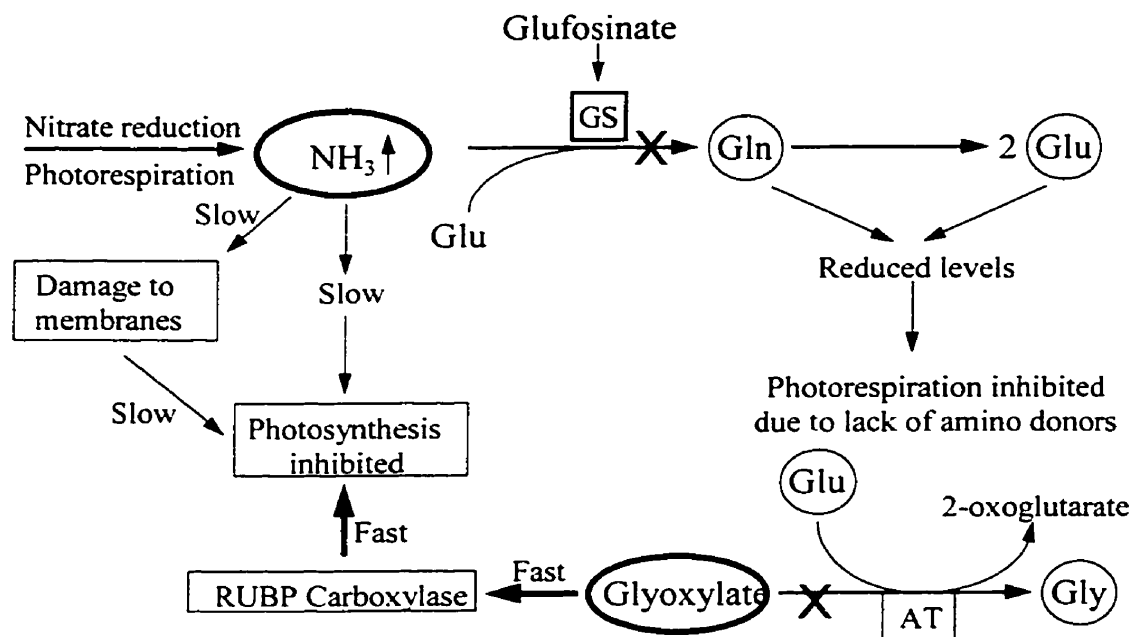
Kinetic studies of GS were done on seven species of plants representing five different families to test their susceptibility to glufosinate ammonium. Though a 70 fold variation in susceptibility to the herbicide was observed, only a five fold variation in susceptibility could be attributed to differences other than enzyme kinetics (Ridley and McNally, 1985). Other factors such as differences in herbicide uptake and translocation may also have contributed to the variation.

A surfactant is required for glufosinate ammonium uptake into the plant. The uptake process is stimulated by light and a pH of 5.0 or lower. There are no differences in the uptake between adaxial and abaxial leaf surfaces. Uptake rates have been shown to differ between plant species. For example, 85% of the herbicide is taken up by barley in a 24 hour period, as opposed to 64% by barnyard grass (Kocher, 1989).

In soybeans, glufosinate ammonium is mobile in both xylem and phloem (Shelp *et al.*, 1992). Translocation out of a treated leaf, though, is species dependent. Mersey *et al.* (1990) measured the amount of radiolabelled glufosinate ammonium absorbed and translocated from the third youngest leaf of barley and green foxtail plants. They found a strong correlation between species sensitivity and the amount of absorption and translocation of the herbicide to the rest of the plant. In barley, a more sensitive species, only 7% of the herbicide is translocated in a 24 hour period as opposed to 15% in green foxtail. There was no significant metabolic degradation of glufosinate ammonium in either species.

Glufosinate ammonium does not accumulate in plants as it is rapidly degraded to a non-phytotoxic metabolite called methyl-phosphinico-proprionic acid. In soil, the herbicide is rapidly degraded by microorganisms via deamination and oxidative decarboxylation into methyl-phosphinico-proprionic acid. Laboratory investigations of soils taken from freshly treated fields, ranging from well-aerated soils to soils with stagnant moisture, have yielded glufosinate ammonium half-lives of three to ten days. This first degradation step occurs very rapidly and is followed by a slower degradation to methyl-phosphinico-acetic acid down to carbon dioxide. In-house studies have shown that microorganisms are also able to exploit the amino group in glufosinate ammonium as a source of nitrogen. In the soil, both the active ingredient and its degradation products are adsorbed to clay particles and humus material. This restricts the mobility of these compounds and prevents leaching into deeper soil layers (*Glufosinate Ammonium: Information on the Active Ingredient*, brochure by Hoechst AG, Frankfurt).

Throughout the world, glufosinate ammonium is used as a non-selective herbicide to control a variety of annual and perennial weeds in fruit and vine plantations, forests, nurseries, in vegetables, field crops and in home gardens. It is used as a desiccant in a number of crops and is also used for the control of unwanted plants in non-crop areas and on industrial sites. Another application for this herbicide has arisen through the discovery of a unique glufosinate ammonium detoxification mechanism. Genes coding for glufosinate ammonium detoxification have been isolated from two strains of soil bacteria; the phosphinothricin acetyltransferase gene (*pat*) from *Streptomyces viridochromogenes* (Bayer *et al.,* 1972) and the Bialophos resistance gene (*bar*) from *Streptomyces hygroscopicus* (Kondo *et al.,* 1973; Murakami *et al.,* 1986). Both genes code for an almost identical enzyme called phosphinothricin acetyltransferase (PAT).

The gene(s) encoding PAT have been transferred into numerous commercially grown crops via genetic engineering. When expressed in a plant, PAT detoxifies the herbicide by transferring an acetyl group onto glufosinate ammonium. The acetylated glufosinate ammonium can no longer bind to the target enzyme, GS, and therefore has no herbicidal activity (Murakami *et al.,* 1986). Using this system, tobacco (DeBlock *et al.,* 1987), potato (DeBlock *et al.,* 1987), tomato (DeBlock *et al.,* 1987) canola (Oelck *et al.,* 1991), carrot (Droege *et al.,* 1992), maize (Kramer *et al.,* 1993; Spencer *et al.,* 1990), alfalfa (D'Halluin *et al.,* 1990), flax (McHughen and Holm 1994), rice (Linscombe *et al.,* 1996), soybean (D. Simmonds, personal communication) and wheat (Vasil *et al.,* 1992) have been rendered tolerant to the glufosinate ammonium herbicide. The *pat* gene, besides conferring herbicide resistance, has also been used as a selectable

marker whereby the herbicide is used as the sole selection agent in transformation experiments. This selection system, though, has some disadvantages when compared to other selection agents such as kanamycin. The glufosinate ammonium, due to its potency, causes immediate cell death. The accumulation of ammonia in untransformed cells could poison the whole explant or kill successfully transformed cells, thus making the glufosinate ammonium selection system not as desirable as gentler selection methods such as kanamycin.

Nevertheless, both *bar-* and *pat-*transformed plants have been shown to be highly resistant to both Basta™ and Liberty® herbicides at levels normally used to kill plants. Engineered glufosinate ammonium resistance displays Mendelian inheritance and behaves as a single dominant trait. The success of this system was clearly demonstrated in 1995 when Canada's first transgenic canola resistant to glufosinate ammonium (Liberty Link® Canola) was commercialized.

2.3 Plant Promoters

Promoters are cis-acting DNA sequences or protein coding regions which play an important role in the regulation of gene expression. These cis-acting DNA sequences, including enhancers, are commonly found upstream to the transcriptional start site of a gene, and in some cases in introns and in 3' regions.

There are three categories of eukaryotic genes, and they are classified according to the type of RNA polymerase which transcribes them. Ribosomal RNAs are transcribed by RNA polymerase I, messenger RNA (protein coding genes) by RNA polymerase II and tRNAs, as well as other small RNAs, by RNA polymerase III. For transcription to take place, the RNA polymerase interacts with basal transcription factors bound to the "TATA" box in the promoter region. This protein-DNA complex is called an initiation complex. It surrounds the transcriptional startpoint and determines the site for initiation of mRNA synthesis (Figure 2.4).

Promoters which utilize RNA polymerase II are modular in design and are made up of short sequence elements, upstream to the initiation site, that are recognized by transcription factors. These short sequence elements can either be positive regulatory elements, enhancers, tissue or developmental specific elements, or negative regulatory elements. In addition, through the interaction of numerous different cis-acting and trans acting regulatory factors, a promoter often directs tissue and development-specific expression of its associated genes' coding region. These transcription factors, or trans-acting factors, are basal and gene specific DNA-binding proteins which bind to DNA elements present in the promoter region and regulate transcription (Lewin 1994).

RNA polymerase II dependent promoters usually contain two core elements, namely a highly conserved TATA box and an Initiator box (Inr) which are located directly upstream of the protein coding sequence (Figure 2.4). The TATA box, located approximately 25 bp upstream of the transcriptional start-site, is a highly conserved 8bp sequence consisting of A-T base pairs and surrounded by GC rich sequences. This is the site where the RNA polymerase II, along with TATA-binding proteins and other associated factors, bind to the promoter region (Figure 2.4). The Inr box, located downstream of the TATA box, determines the transcriptional start-site where RNA polymerase II initiates mRNA synthesis. At this startpoint there is no extensive sequence homology, but there is a tendancy for the first base of the mRNA to be A, flanked on either side by pyrimidines. A generic promoter consisting only of the TATA box and the Inr box functions at a low efficiency (Lewin 1994). To function at its proper level, upstream regulatory elements are also required.

Gene transcription is complex and controlled by the interaction of specific proteins, transcription factors (TFs), with specific cis-acting DNA sequences. The nature of these interactions determines the functional state of the promoter. The induction or repression of gene expression by external and internal stimuli are suggested to be accomplished by alterations in these protein-DNA interactions.

In plants, there has been a concerted research effort to try to elucidate the transcriptional machinery needed to turn on or turn off specific genes. Much of this research, to date, parallels research already carried out in other model eukaryotic and prokaryotic systems. By attaching or adding a DNA element that binds a given factor into a different promoter region, it is possible to obtain novel transcription patterns by

combining properties of the donating and receiving promoters. Construction of such promoters is very attractive for the development of new regulatory elements for applications in engineering novel traits in biotechnologically important organisms. Reporter genes, such as β-glucuronidase (GUS) (Jefferson *et al.*, 1987) and firefly luciferase (luc) (Ow *et al.*, 1986), can be fused to such promoter constructs and introduced into plant cells via transformation vectors based on *Agrobacterium tumefaciens* (Hoekema *et al.*, 1983). The detection of the reporter gene product can demonstrate where in the plant, and how strongly a specific promoter sequence expresses.

In plants, the most commonly used heterologous gene promoter is the cauliflower mosaic virus 35S promoter (CaMV 35S) (Harpster *et al.*, 1988). Other promoters, such as those from *Agrobacterium tumefaciens* Tumour Inducing (Ti) plasmid genes (An *et al.*, 1990; Zhang and Singh 1994) and also those from plant viral genes (Odell *et al.*, 1985; Ruiz-Medrano *et al.*, 1994), have been routinely used for the expression of foreign genes in plants. These promoters all fall into the category of 'constitutive' promoters or promoters which are expressed in most tissues during different stages of development (Table 1.1).

Theoretically, a constitutive promoter functions in all cells and tissues throughout all stages of development. Practically, though, there have been no 'true' constitutive promoters isolated to date. Even the popular CaMV 35S promoter demonstrates some tissue specificity and differential expression levels (Williamson et al., 1989). Promoters which are not expressed at all times, notably regulated promoters, fall into one of four other categories: tissue-specific promoters, cell-

Figure 2.4:   **Formation of RNA Polymerase II Initiation Complex**
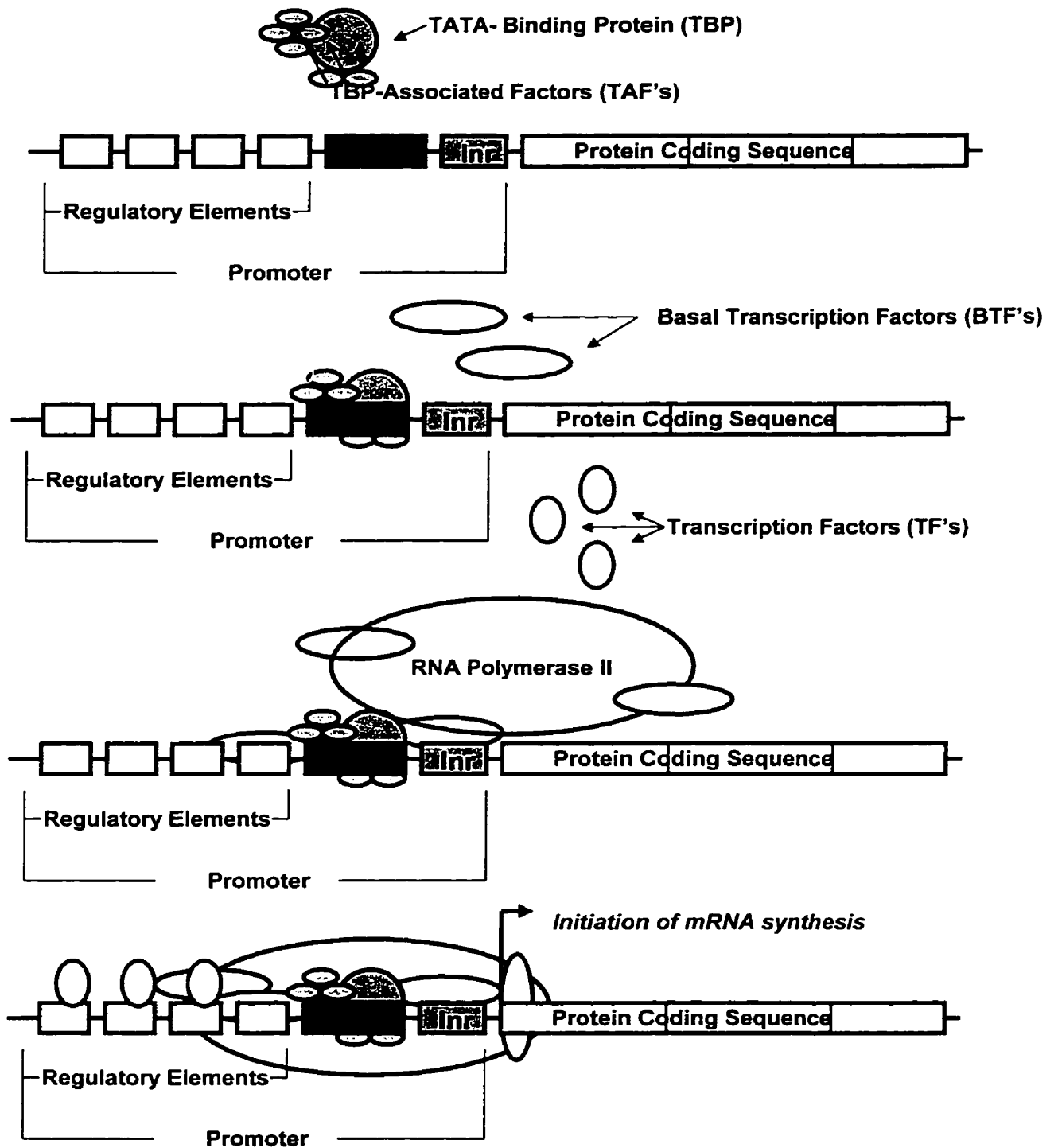
specific promoters, developmental stage-specific promoters and inducible promoters. A comprehensive list of these promoters is found in Table 2.1.

Constitutive Promoters

The most common and the most strongly expressing constitutive promoters used for heterologous gene expression in plants originate from either viral or bacterial DNA sources. Of these, the Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell *et al.*, 1985) and its derivatives are widely used to express foreign genes of interest in both dicots and monocots. Promoters isolated from the different T-DNA genes from *Agrobacterium tumefaciens* have also been used for foreign gene expression (An *et al.*, 1990; Zhang and Singh, 1994; Langridge *et al.*, 1989). In the last five years, viral promoters isolated from DNA-containing virions, such as the badnaviruses and the geminiviruses have been shown to be constitutive in nature, depending on the host plant (Mushegian and Shepherd, 1995). All of these heterologous promoters are functional and express strongly in most plant cells. Besides heterologous promoters, several homologous plant promoters have been studied and are presently being used to direct gene expression in transgenic plants. Two examples of such promoters are the Actin 1 gene promoter from rice (McElroy *et al.*, 1994) and the Ubiquitin 1 gene promoter from maize (Christensen *et al.*, 1992).

The cauliflower mosaic virus (CaMV), which belongs to the caulimovirus family, has a double stranded DNA genome which is approximately 8 kilobases (kb) in length. The complete nucleotide sequence of this genome has been determined (Hohn *et al.*,

1982). Within this genome, two distinct promoters, directing the expression of 19S and 35S transcripts, have been identified. During the viral life cycle, the 35S promoter is transcribed from the viral DNA minus strand to produce a major transcript in infected cells referred to as the 35S RNA. The 5' and 3' termini of this RNA have an overlapping sequence of about 200 nucleotides (Guilley *et al.*, 1982). This 35S RNA, besides serving as a template for translation, can also function as an intermediate molecule for viral DNA synthesis through a reverse transcription process (Pfeiffer and Hohn, 1983).

The presence of copious quantities of 35S transcripts in infected cells is not surprising, especially when considering the speed at which the CaMV can replicate in an appropriate host cell. The strength of the 35S promoter and its constitutive expression in most organs is also a reflection of this efficiency. For this reason, it has been used to express a number of foreign genes in transgenic plants (Odell *et al.*, 1985; Bevan *et al.*, 1983; Morelli *et al.*, 1985).

The increasing emphasis on the expression of desirable traits in transgenic plants has initiated numerous studies which have focused on isolating and characterizing CaMV 35S promoter regulatory complexes responsible for high and nominally constitutive activity. A general experimental design for studying such cis and trans elements is illustrated in Figure 2.5.

In 1985, Odell and coworkers identified specific proximal sequences required for the correct initiation of transcription from the 35S promoter. One of these proximal sequences included a TATA box, which is a necessary element for transcription initiation of other eukaryotic RNA polymerase II-transcribed genes (Nevins, 1983). Through 5' deletion analysis, Odell and coworkers (1985), identified that the rate of transcription

31

was determined by other sequences dispersed over 300 basepairs (bp) of upstream DNA. Within the -105 to -46 region, they identified a CAAT box, an inverted repeat and a sequence bearing homology to the SV40 core enhancer (Odell *et al.*, 1985). They further analyzed these sequences by placing isolated fragments in conjunction with the nopaline synthase (*nos*) promoter and tested for the presence of a plant enhancer element. A protoplast transient expression system was used to eliminate the problem of variation seen among independent stable transformants.

With such a system, they were able to demonstrate that a 338 bp DNA fragment, isolated from the region upstream of the TATA box, could increase expression of the *nos* promoter up to the level of the intact 35S promoter. This 338 bp DNA fragment was equally active in both orientations when placed 150 bp upstream of the transcription start site. When the distance from the transcription start site was increased, a decrease in promoter activity and a loss of orientation independent function was observed (Odell *et al.*, 1988).

Through 5' and 3' internal deletion studies, this upstream enhancer-like fragment was subdivided into three functional regions: -343 to -208, -208 to -90, and -90 to -46. The latter region, -90 to -46, demonstrated little activity by itself, but it played an important accessory role by increasing the transcriptional activity of the other two regions (Fang *et al.*, 1989). Domains which conferred tissue-specific expression were also identified within this enhancer region. Two domains, an upstream and a downstream domain, were found to confer different expression levels depending on the tissue type and developmental stage. The upstream domain could be further subdivided into five subdomains, each conferring a distinct expression pattern (Benfey *et al.*, 1990; Benfey

and Chua, 1989). These results provided important pieces of evidence for the suggestion that the CaMV 35S enhancer region is able to confer strong constitutive expression in plants due to the synergistic effects of the different cis elements within the enhancer region.

In the late 1980s and early 1990s, numerous studies began to appear identifying trans-acting factor binding sites and the respective DNA-binding proteins for the CaMV 35S promoter region. A binding site located at the -83 to -63 region of the 35S promoter was discovered in root and leaf nuclear extracts of tobacco and pea. This site, *as-1*, was found to contain two tandem TGACG-like motifs with close homologies to the cAMP responsive element found in mammalian systems. ASF-1 was later determined to be the factor which bound this *as-1* region (Lam *et al.*, 1989). In 1990, Yamazaki and coworkers also isolated a leucine zipper DNA-binding protein, TGA1a, having the same binding specificity as ASF-1. ASF-1, besides binding to the 35S promoter, also bound to functionally important regions of the *nos* and octopine synthase (*ocs*) promoters of the T-DNA from *Agrobacterium*. All of these binding sites demonstrated a common dimeric structure with two TGACG-like motifs, suggesting that this dimeric configuration for

Figure 2.5:   Experimental Design for Studying Cis and Trans Elements

```
┌─────────────────────────────────────────┐
│  Promoter deletion studies with reporter │
│  gene to define minimal region for function │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│  Identify factor binding sites by Dnase I │
│  Footprinting                            │
└─────────────────────────────────────────┘
            ↙               ↘
┌──────────────────────────┐   ┌──────────────────────────┐
│ Clone factor by labelled │   │ Characterize properties of│
│ binding site as probe    │   │ binding site in vivo      │
└──────────────────────────┘   └──────────────────────────┘
            │                               │
            ▼                               ▼
┌──────────────────────────┐   ┌──────────────────────────┐
│ Characterize properties  │   │ Selection schemes using  │
│ of DNA-binding protein   │   │ synthetic promoter        │
│ in vitro                 │   │ elements to isolate trans-│
└──────────────────────────┘   │ acting mutants defected in│
            │                   │ the regulation of the factor│
            ▼                   └──────────────────────────┘
┌──────────────────────────┐
│ Manipulation of factor   │
│ concentration in vivo by  │
│ overproduction of sense   │
│ and anti-sense mRNA       │
└──────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│  Model for the role and regulation of the │
│  factor in promoter function as well as the │
│  general developmental program           │
└─────────────────────────────────────────┘
```

(adapted from Lam, 1990).

ASF-1 binding sites may be conserved (Lam, 1990). In contrast to this, ASF-1 was also found to bind to a site within the promoter of a plant histone gene, *hex-1*, which contained only one TGACG-like motif (Katagiri *et al.*, 1989).

In 1990, Lam and coworkers identified a second binding region in the 35S promoter, *as-2*, located around the -100 position. The protein binding factor, ASF-2,

34

found only in leaf nuclear extracts, was identified as the factor which bound to two GATA motifs at the core of the *as-2* binding site. This same protein factor was also shown to bind the GATA repeat sequence of the petunia *Cab-22L* promoter (Lam, 1990). Interestingly, the GATA motif is also found in the binding site of an erythrocyte-specific factor (Tsai and Coruzzi, 1990), which may suggest that *as-2* shares some homology with a mammalian factor.

Recently, a tobacco gene, G13, believed to encode the *as-1* binding protein, TGA1a, was isolated and characterized. A TGA1a binding site (*as-1*) was found in its own upstream region, suggesting that G13 autoregulates its own transcription. The *as-1* binding site in G13, though, was found to be much weaker than the *as-1* binding site in the CaMV 35S promoter, *nos* promoter, or the *ocs* promoter (Fromm *et al.*, 1991). This difference in binding affinity may be due to the fact that enhancers of plant viral genes and of plant pathogen genes require strong binding sites for plant transcription factors to achieve maximal expression levels of the pathogen genes. On the other hand, plant genes such as those encoding regulatory proteins must be subjected to more subtle changes in their expression levels in response to a variety of cellular signals, or by post translational modifications such as phosphorylation.

Agrobacterium tumefaciens is a plant pathogen that causes crown gall tumours after infection of a wounded dicotyledonous plant. Large Ti plasmids are responsible for the oncogenicity of the bacterium. These plasmids transfer a segment of DNA, called T-DNA, into the plant cell whereupon it is stably integrated into the plant genome. Another region of the Ti plasmid is called the *vir* region, which is essential for tumour induction (Ooms *et al.*, 1982). There are a number of genes located within the T-DNA of the Ti

plasmid, and these genes are readily expressed in the plant tissues upon transfer to the plant chromosome (Gelvin *et al.*, 1982). Some of these genes are responsible for the production of low molecular weight compounds known as opines which serve as carbon and nitrogen sources for the infecting *Agrobacteria*. Other genes are involved in tumorigenesis (Thomashow *et al.*, 1986). The structure of these T-DNA genes is simple and well characterized. The flanking regions of T-DNA genes carry typical eukaryotic regulatory sequences, such as a TATA box and a poly[A] attachment signal (Barker *et al.*, 1983). Many also contain upstream elements, located further than 100 bp from the transcription start site. These upstream sequences are believed to modulate the levels of transcription. For example, the nopaline synthase (*nos*) gene, the octopine synthase (*ocs*) gene, and the 780 gene all contain upstream transcriptional activating sequences that can activate promoters in either orientation (Ellis *et al.*, 1987; Leisner and Gelvin, 1988; Bruce *et al.*, 1988; Mitra and An, 1989).

The nopaline synthase (*nos*) gene, which is located close to the right border of the T-DNA, is expressed in plants upon integration into the plant chromosome from the Ti plasmid of *Agrobacterium tumefaciens*. In plant cells, the NOS protein catalyses the synthesis of nopaline from arginine and α-ketoglutarate (Ellis and Murphy, 1981). The gene itself has been shown to be actively transcribed in all tissues examined in tobacco (DeBlock *et al.*, 1984; Horsch *et al.*, 1985). For this reason, the *nos* promoter was always thought to be constitutive in plants, and therefore, widely used to express foreign genes in transformed plant tissue (Bevan *et al.*, 1983; Herrera-Estrella *et al.*, 1983).

Further detailed studies reported that the *nos* promoter was found to be both organ specific and developmentally regulated in transgenic tobacco. High activity was seen in

lower parts of the plant, with a gradual decrease toward the upper parts. During flowering, the promoter strength decreased in all vegetative organs, while increasing differentially in the flowering organs (An *et al.*, 1988). The *nos* promoter was also shown to be inducible by mechanical wounding. This wound induction could be further enhanced by the phytohormone auxin (An *et al.*, 1990).

Three blocks of cis regulatory elements were initially identified within the *nos* promoter (Figure 2.6). The TATA and CAAT boxes were found to be important for the efficiency of *nos* promoter activity. Deletion of each element resulted in a ten fold reduction in promoter strength in cultured tobacco cells. Another upstream regulatory region, located approximately 20 bp up from the CAAT box, was essential for the *nos* promoter to function. Deletion of this latter region completely abolished promoter activity (An *et al.*,1986; Ebert *et al.*, 1987).

Closer analysis of this upstream regulatory region identified an eight bp sequence, CAGAAACC, at -106 to -113, along with its inverted repeat, GGTTTCTG, at -140 to -147. Between these repeat elements lay a ten bp potential Z-DNA-forming element (Z). When Z was deleted, the *nos* promoter was not active (Mitra and An, 1989). This Z-element, or an overlapping sequence (-114 to -123), was later found to be one of the regulatory elements involved in the promoter's inducibility by wounding and by auxin (An *et al.*, 1990). Furthermore, this DNA region (approximately -118) was also identified as a protein binding factor site, *nos-1*, for the tobacco nuclear factor ASF-1 (Lam *et al.*, 1990).

Figure 2.6. Sequence of *nos* Promoter Internal Deletions

```
1. GCGGGTTTCTGGAGTTTAATGAGCTAAGCACATACGTCAGAAACCATTAT
2. GC --------- AGTTTAATGAGCTAAGCACATACGTCAGAAACCATTAT
3. GCGGGTTTCTGGAGTTTAATGAGC ------- ATACGTCAGAAACCATTAT
4. GCGGGTTTCTGGAGTTTAATGAGC ------------ GTCAGAAACCATTAT
5. GCGGGTTTCTGGAGTTTAATGAGC -------------------------AT
6. GCGGGTTTCTGGAGTTTAATGAGCTAAGCAC ----------------- -TTAT
7. GCGGGTTTCTGGAGTTTAATGAGCTAAGCACATACGTC ------- -TTAT
```
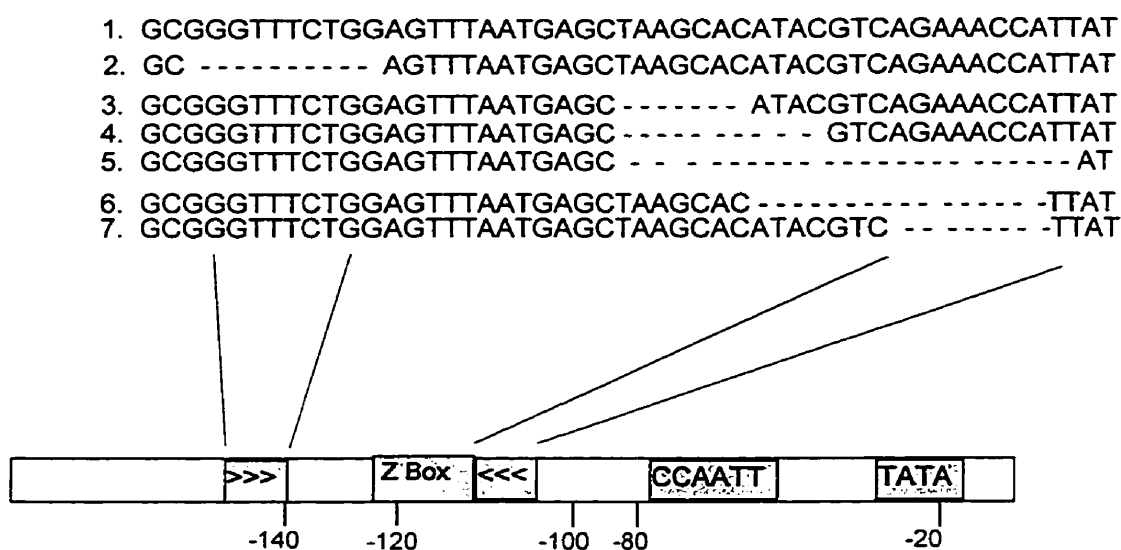

(Mitra and An, 1989).

The biosynthesis of mannopine involves two genes from the T_R-DNA, designated mannopine synthase (*mas*) 1' and 2' (Ellis *et al.*, 1984). The dual promoters from *mas* 1' and 2' were found to be functional in transformed plant tissue, and therefore could be used for developmental studies and for promoter deletion analyses.

The temporal and spatial distribution of *mas* promoter activity was determined throughout the development of transgenic tobacco plants using bacterial luciferase as a reporter gene. The activity of the *mas* dual promoter increased basipetally in developing plants and was wound-inducible in leaf and stem tissue. The activity of this dual promoter was also regulated by auxin and enhanced by cytokinin in both differentiated and nondifferentiated tissues (Langridge *et al.*, 1989).

By deletion analysis, sequences within 138 bp upstream of the initiation site for the *mas* gene were found to be sufficient for the accurate transcription initiation of a *mas-nptII* (neomycin phosphotransferase II) promoter-gene fusion in sunflower. Deletions

that left only 57 bp upstream of the transcription start site completely halted *mas* promoter activity, even though the CAAT and TATA sequences were intact. Optimum transcription levels, though, were obtained when 318 bp of upstream *mas* sequences were fused to the *nptII* gene (DiRita and Gelvin, 1987). In accordance with this, a 100 bp fragment from within the -138 to -318 region of the *mas* gene was able to fully restore expression of another truncated T-DNA promoter, namely that of the octopine synthase gene (Leisner and Gelvin, 1989). This indicates that sequences considerably upstream of the *mas* gene, CAAT box, and TATA box can enhance the expression not only of the *mas* gene, but also of other heterologous T-DNA gene promoters.

The agropine synthase (*ags*) promoter, like the *mas* promoter, requires the presence of other upstream elements in addition to the TATA box for optimal activity. The deletion of sequences in the TATA-proximal domain (-165 to -40) abolishes transcriptional activity of the *ags* promoter. Another T-DNA gene, the 780 gene, contains a promoter which can also be activated by an upstream element. Unlike the other opine gene promoters, the 780 gene promoter can be activated when separated by 613 bp. Other opine gene promoter elements cannot function when placed more than several hundred bp from a promoter (Bruce *et al.*, 1988).

It may be difficult to classify these T-DNA upstream elements as truly eukaryotic, even though they function in an orientation-independent manner. Their position relative to the promoter greatly influences promoter activity. Perhaps these elements could be more closely correlated with yeast upstream activation sites (Leisner and Gelvin, 1989), rather than with eukaryotic enhancer elements.

Further studies on the upstream regions of the *mas, ags* and 780 gene promoters are warranted. Perhaps the elucidation of DNA-protein and protein-protein interactions, at the molecular level, will lend some insights into the functional properties of these promoters.

The octopine synthase (*ocs*) gene encodes the enzyme involved in the biosynthesis of octopine from arginine and pyruvic acid in crown-gall tumours (Thomashow *et al.*, 1980). In transgenic plants, *ocs* was expressed constitutively in all tissues and it did not appear to be under any environmental or developmental control. In 1982, this gene was first isolated and characterized by DeGreve and coworkers. They determined that the *ocs* gene did not contain a consensus CAAT motif, but instead demonstrated two AGGA motifs at -59 and -78. A TATA box was also found at the -32 position (DeGreve *et al.*, 1982).

Other *ocs* gene sequences, located between -116 and -292, were found to be essential for *ocs* expression in tumours (Leisner and Gelvin, 1988). It was shown that this 176 bp upstream sequence had enhancer-like properties in transgenic tobacco plants. This sequence or element functioned independently of orientation and could activate the expression of heterologous promoters. One such study involved the expression of an alcohol dehydrogenase-1 (Adh-1) promoter in transgenic tobacco. When the *ocs* promoter, minus the 176 bp element, was assayed upstream of the promoter for the maize Adh-1 gene there was virtually no detectable Adh-1 promoter activity. When the 176 bp *ocs* segment was added to the promoter region in either orientation, there was a dramatic increase in the transcription rate of the Adh-1 gene. The 176 bp element was also shown to function as an enhancer when placed upstream of the Adh-1 promoter in protoplasts of

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both *Zea mays* (monocot) and *Nicotiana plumbaginifolia* (dicot). A 16 bp palindrome, ACGTAAGCGCTTACGT, contained within the 176 bp fragment was essential and sufficient for enhancing Adh-1 promoter activity in both monocot and dicot transient expression assays (Ellis *et al.*, 1987).

A plant protein, called OCSTF, which was shown to interact with the various ocs-elements, was identified in both monocots and dicots (Singh *et al.*, 1989). Another ocs-element binding factor was also isolated and characterized in maize. This latter factor, OCSBF-1, was a 21 kD protein which was encoded by a single copy, differentially expressed, intronless gene. It was found to contain a small basic amino acid region and a potential leucine zipper motif homologous to the DNA-binding domains of other eukaryotic basic leucine zipper factors, such as Jun (Hattori *et al.*, 1988) and GCN4 (Hinnebusch, 1984).

So far, proteins that recognize and bind *ocs*-elements (Katagiri *et al.*, 1989) or sequences within an ocs-element half site are basic-leucine zipper motif (bZIP) transcription factors (Tabata *et al.*, 1989). Due to the similarities in binding domains between many of these bZIP factors, it seems likely that there are different classes of bZIP proteins that recognize similar sequences, yet regulate different patterns of gene expression in plants (Singh *et al.*, 1990).

Badnaviruses belong to the plant pararetrovirus group and are similar to caulimoviruses in their replication strategy. Unlike caulimoviruses which only infect dicots, different members of the badnaviurs group can infect either monocots or dicots, and after infection the virus is usually phloem limited (Covey and Hull 1992). The first badnavirus promoter to be studied in detail was the commelina yellow mottle virus

(CoYMV) (Medberry *et al.*, 1992). This promoter was shown to be active in both dicots and monocots by transient expression assays. In stably transformed maize callus cells, the CoYMV promoter exhibited similar strength to the CaMV 35S promoter with a double enhancer (Medberry and Olzewski 1993). Using deletion analysis, researchers found that the region between -230 and +8 was responsible for most of the promoter's activity. More specifically, a transcriptional enhancer was detected between -230 and -200, and a vascular tissue-specific element was found at the -160 to -88 position.

Another badnavirus, the rice tungro bacilloform virus, has also been extensively studied. This virus is active mostly in the phloem tissue of rice and in transgenic tobacco plants (Bhattacharaya-Pakrasi *et al.*, 1993). The genomic promoter was found to have tissue specific determinants confined to sequences up to -169 from the transcriptional start site, whereas the upstream sequences enhanced transcription levels through interaction with two groups of transcription factors (Yin and Beachy 1994). A downstream enhancer sequence was also detected by deletion analysis at positions +8 to +83 (Chen *et al.*, 1994).

Geminiviruses are plant viruses with a single-stranded, circular DNA. Replication of these virus particles occurs in the nucleus of the host cell via a concerted action of virus-encoded and cellular activites using a rolling-circle mechanism (Ilyina and Koonin 1992; Koonin and Ilyina 1993; Stenger *et al.*, 1991). There are three groups of geminiviruses defined to date. Group I contains those monocot-infecting viruses which are transmitted by the whitefly, such as the maize streak virus (MSV) and the wheat dwarf virus (WDV). Group II geminiviruses, such as the beet curly top virus, infect dicots and are transmitted by leafhoppers. Group III members, like the tomato golden

mosaic virus (TGMV), are dicot infecting viruses which are also transmitted by the whitefly. The promoter regulating the transcription of the TGMV capsid protein gene, AR1, was fused to a reporter gene and transferred into tobacco. The resulting transgenic tobacco plants demonstrated reporter gene activity only in vascular bundle cells and in the meristem. When an AR1 transcriptional activator protein, AL2, was introduced into these transgenic tobacco plants by virus infection or by a cross with another transgenic plant, the AR1 promoter also demonstrated strong activity in mesophyll cells (Ruiz-Medrano *et al.*, 1994).

Many of the heterologous viral and bacterial promoters discussed so far have not been successful in instigating high-level foreign gene expression in transgenic monocots. For this reason, many research groups have focussed their efforts on the identification and isolation of constitutive plant monocot promoters for the application of gene transfer into target crops such as rice, wheat and maize. The maize alcohol dehydrogenase gene promoter, *Adh1* or *Emu*, was one of the first homologous monocot promoters to be used for transformation (Last *et al.*, 1991). This promoter demonstrated 10 to 20 times more activity than the CaMV 35S promoter in transformed rice protoplasts and calli, yet it was induced by anaerobic stress and not constitutively active in all transformed tissues (Kyozuka et al. 1991). The *Adh1* promoter also required a 5' intron for the efficient expression of foreign genes in monocots (Callis *et al.*, 1987).

Another monocot gene, the actin 1 gene, was found to be expressed at high levels in all rice tissues and at all stages of development. The abundancy of this transcript was to be expected since actin is one of the fundamental components of the plant cell cytoskeleton. Several fusions between the actin 1 (*Act1*) gene promoter and a reporter

gene were constructed and used in transient expression assays of transformed rice protoplasts. The regulatory elements necessary for maximum expression levels were located within a 1.3 kb region upstream of the translation initiation codon. These assays also verified the need for the 5' intron of the rice *Act1* gene as an essential unit for efficient reporter gene expression levels. Their results also suggested that the intron-mediated stimulation of gene expression was not a function of an enhancer-like activity within the intron sequence, but due to an *in vivo* requirement for efficient intron splicing (McElroy *et al.*, 1990).

A 5' intron, or untranslated, region immediately downstream of the promoter was also found to be necessary for efficient maize ubiquitin (Ubi-1) gene promoter activity. The maize Ubi-1 promoter was first used to express foreign genes in monocots via a transient protoplast expression system (Christensen *et al.*, 1991) and via stable cereal transformation studies. The Ubi-1 promoter was fused to a spectrum of selectable and scorable markers and transformed into a diverse number of monocot species such as rice (Toki *et al.*, 1992), wheat (Weeks *et al.*, 1993), *Lemna*, barley and other cereals (McElroy *et. al.*, 1994).

The isolation and utilization of monocot promoters will continue to be an important area of research, mainly due to differences in biochemistry, physiology and morphology between dicots and monocots, as well as due to the inefficient functionality of non-cereal promoter (regulatory) elements in transgenic cereals. Cereal promoters will become increasingly important for the expression of not just herbicide resistance, but also for disease resistance and for the optimization of grain quality (McElroy *et al.*, 1994).

Regulated Promoters

All of the constitutive promoters discussed so far contain numerous regulatory elements which together orchestrate expression levels in the majority of plant cells throughout all stages of development. Regulated promoters are those which, for all intents and purposes, require additional upstream or downstream regulatory elements to illicit an induced or a tissue-specific, cell-specific or developmental-stage-specific expression pattern. As an example of regulated promoters, inducible promoters will be discussed in some detail. Inducible promoters can be subdivided into two subclasses, those induced by intracellular signals, such as phytohormones, and those induced by external signals, such as light.

How internal plant messengers, such as phytohormones, act upon the cell's machinery has been the focus of many studies in the field of plant physiology and molecular biology (Ulmasov *et al.*, 1995; Van der Zaal *et al.*, 1991; Rogers *et al.*, 1994). It has been demonstrated that the expression of specific sets of RNA and protein molecules can be correlated with many of the plant's physiological responses to these hormone signals (Broglie *et al.*, 1989; Key, 1989). The expression of these hormone-regulated genes can be controlled at several levels, including transcription. The transcription machinery's interaction with the 5' promoter region will primarily determine the gene or genes' expression patterns. A fundamental understanding of this type of regulation, along with the participating factors and signalling molecules, would be an important step in elucidating the molecular basis of phytohormone action.

Auxins are plant phytohormones which play an important role in many plant developmental processes such as cell elongation and cell division. This class of growth regulators includes natural compounds such as indole-3-acetic acid (IAA) and synthetic

compounds such as 2,4-dichlorophenoxyacetic acid (2,4-D). IAA, which is the principal natural auxin produced by plant cells, is involved in the control of the rate of cell elongation, and therefore, is also involved in phototropic and geotropic responses (Evans, 1974).

One approach, aimed at determining how endogenously produced auxins mediate these plant responses, involves studying how gene expression is modified by auxin-induced growth and developmental responses. Such an approach has led to the identification of several auxin-responsive elements and their respective genes, namely those belonging to the soybean GH gene family (Li *et al.*, 1994), and the genes for the soybean small auxin up-regulated RNAs (SAURs) (Theologis *et al.*, 1985; McClure and Guilfoyle, 1987; van der Zaal *et al.*, 1987; Wong *et al.*, 1996; Gil *et al.*, 1994; Gil and Green 1996). Other auxin-inducible genes and regulatory elements which have been characterized are: the tobacco *par* gene (Takahashi *et al.*, 1990), the *Arabidopsis* AtAux2 genes (Conner *et al.*, 1990), the tobacco GST genes (Van der Zaal *et al.*, 1991; Van der Zaal *et al.*, 1996), and the soybean Aux genes (Ainley *et al.*, 1988). To date, only cis-acting regulatory elements have been characterized for many of these auxin-responsive genes. The identification of transcriptional binding factors will most likely occur in the very near future.

The GH3 gene, one of four members from the auxin-inducible GH multigene family, contains two introns and encodes a mRNA of 2.4 kb, which translates into a polypeptide of 593 amino acids. The GH3 promoter contains several auxin-inducible elements that are found in other auxin-responsive promoters (Li *et al.*, 1994). Three TGA1-like binding elements (TGACG) (Katagiri *et al.*, 1989) are found within 400 bp

from the start of transcription. One of these elements is identical to the *hex-1* sequence identified in a wheat histone gene, TGACGTGGCG, while the other two sites are TGACGTAA and TGACGC (Hagen *et al.*, 1991). The first two TGA1-like elements have been shown to bind nuclear proteins prepared from a wide variety of different plant and organ nuclear extracts, while the third site showed little if any binding (Guilfoyle *et al.*, 1992). Those elements which did show binding activity are potential auxin-regulatory elements which are found in a variety of other auxin-induced genes (An *et al.*, 1990). In addition to the TGA1-like elements, the GH3 promoter contains the sequence GTCGGCGGCGCCCATTAGT, which shares extensive homology with an auxin-inducible promoter in *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Slightom *et al.*, 1986). Another GH3 promoter element, CACCAT, is also found in the soybean Aux28 gene (Ainley *et al.*, 1988) and within the NDE element of two SAUR genes (McClure *et al.*, 1989).

The SAURs are a family of five highly homologous genes that are most strongly expressed in the zone of cell elongation in the soybean hypocotyl (McClure and Guilfoyle, 1987). When soybean organ sections were treated with 2,4-D the SAUR transcripts became more abundant in the epidermis, cortex, starch sheath, and pith. Without hormone induction, SAUR transcripts were detected in the same tissues, only to a lesser extent (Gee *et al.*, 1991).

Several regions of homology, which were speculated to be important for gene regulation, were identified among these SAUR genes. One prominent region of homology begins approximately 250 bp upstream of the ATG codon. This region was characterized by McClure and associates in 1989. For convenience, they divided this

homologous upstream region into two sections, which they referred to as DUE and NDE. The spacing between the NDE and the TATA box varied from 41 bp to 72 bp in the five SAUR genes. They also compared DUE and NDE to regulatory elements found in other auxin-inducible genes, but found no sequence homology, except for one TGA1-like element in the 5' intergenic region between SAUR genes 6B and 15A (McClure *et al.*, 1989). Even though this DUE/NDE region lacked homology with other auxin-regulated 5' upstream elements, these researchers were convinced that it contained all the cis-acting sequences necessary for auxin inducibility. They suggested that different factors could induce different auxin-regulated genes.

The differences in tissue-specific and organ-specific gene expression patterns, and the differences in promoter sequences, between GH3 and SAUR genes may suggest that the two are regulated by different transduction pathways in response to auxin. The GH3 promoter may even be representative of an auxin-inducible promoter that is most active in cells undergoing cell division. The SAUR promoter may be representative of an auxin-inducible promoter primarily active in cells undergoing cell elongation. In view of this and the variety of effects on plant growth and development, it seems likely that there are several mechanisms for auxin-regulated transcription (Abel *et al.*, 1996).

Abscisic acid (ABA) is a phytohormone which has been implicated in the control of many events such as embryogenesis, seed formation (Quatrano, 1987), storage protein synthesis (Finkelstein *et al.*, 1985), responses to osmotic stress (Zeevaart and Creelman, 1988), and in the onset and maintenance of dormancy (Koorneef, 1986). When tissue is subjected to osmotic stress, endogenous ABA levels have been shown to increase. Under these conditions, specific genes are expressed, yet these same genes can also be induced

in unstressed tissues by the application of exogenous ABA (Singh *et al.*, 1987; Mundy and Chua, 1988). Using this and similar approaches, a number of ABA-responsive genes have now been isolated. These are being studied in an attempt to understand the molecular mechanisms behind ABA action.

The amount of information available on the regulation of these particular genes is vast. As an example of research in this area, one gene system, namely the gene encoding a wheat embryo maturation protein (*Em*), will be discussed in detail.

During late seed development, a set of abundant mRNAs and their respective proteins are produced in conjunction with increases in endogenous ABA levels. These same RNAs can also be induced by applying exogenous levels of ABA to embryos cultured *in vitro* (Skriver and Mundy, 1990). In Arabidopsis, ABA-inducible RNAs have led to the discovery of two low temperature and ABA inducible genes and their respective promoters. The promoters from kin1 and cor6.6 have been shown to direct strong expression in guard cells, pollen, and in young developing seeds (Wang and Cutler 1995; Wang *et al.*, 1995). In wheat, one of these RNAs encodes the embryo maturation protein, *Em* (Williamson and Quatrano, 1988). In 1988, Marcotte and coworkers demonstrated that a mere 652 bp of the 5' flanking region of the *Em* gene was sufficient to express an *Em*:ß-glucuronidase (GUS) fusion gene in rice protoplasts in response to ABA. This same 652 bp promoter fragment was capable of directing the appropriate temporal and spatial expression pattern (i.e. in seed embryos) in transgenic *Em*-GUS tobacco plants. Using 5' deletion studies, they determined that deletions between -1800 and -163 bp from the transcriptional start site lowered the level of expression, but had no qualitative effect on ABA-mediated gene induction. However, deletion of an additional

63 bp of 5' flanking region drastically reduced the induction phenomenon (Marcotte *et al.*, 1989). It was suggested that an ABA response element (ABRE) resided within this 62 bp region.

Several sequence motifs were later identified within this *Em* promoter region. They all demonstrated homology to regions within the promoters of other seed protein genes. One of these motifs, the *Em1* box, was also found in all of the promoter regions of the ABA-regulated genes for which sequence data is available. In the *Em* gene, the *Em1* element was found to be an imperfect repeat (*a* and *b*) proximal to the 62 bp deletion which abolished ABA induction. When these *Em1* elements were placed upstream of a - 90 CaMV 35S-GUS construct, ABA inducible GUS expression was obtained in transient assays. A mutation in the *Em1a* box eliminated this induction phenomenon (Guiltinan *et al.*, 1990).

In the same study performed by Guiltinan and coworkers, a wheat nuclear binding protein was also identified and found to interact with the *Em1a* box, but not with the *Em1b* box. This nuclear DNA binding protein, EmBP-1, was found to contain features which have been found in a number of other transcription factors, in particular a conserved, highly basic region combined with a leucine heptad repeat (leucine zipper). Sequence analysis of this protein has shown it to be similar to a number of other basic-leucine zipper (bZIP) proteins in plants. Comparisons with the sequences for the G-box binding factor, histone DNA-binding protein (Tabata *et al.*, 1989), and the common plant regulatory factor show a high degree of conservation particularly in the basic region (Devos *et al.*, 1991). These similarities also correlated with similarities in the target

sequences for these proteins. All of the above mentioned binding factors were shown to bind a sequence which contained a CACGTGG core.

In a later study, this same group showed that the mRNA for EmBP-1 was present in most tissues of wheat, while the mRNA for *Em* was not present in roots and leaves. They found this result consistent with a model in which the EmBP-1 protein factor must first be activated, possibly through protein modification or interaction with accessory proteins, before being able to bind the ABA response element (*Em1a*) in the *Em* gene (Marcotte *et al.*, 1992).

By identifying a central point in the ABA signal transduction pathway (the *Ema1* ABA response element) it should now be possible to pursue the identification and characterization of an ABA receptor, and also to pursue further studies into the function of the proteins produced in response to ABA.

Light is one of the most important environmental cues in plant development. It is known to induce the activity of over 40 enzymes (Tobin and Silverthorne, 1985). Most of these enzymes have been characterized in greening etiolated plants whereby seeds are germinated and maintained in the dark for extended periods of time and then exposed to light. This experimental system was often chosen because the initial etiolated plant material contains very low amounts of photosynthetic components. Since then, gene cloning coupled with efficient systems for gene transfer have allowed for the direct characterization of a gene's photoinducibility in developmentally stable, mature leaves. Using this latter system, gene activities in normal diurnal light cycles or other regimes can be measured without the background complexity of a developmentally dynamic organ (Fluhr, 1988).

In higher plants, at least three photoreceptors exist: phytochrome (a far-red/red light receptor), cryptochrome (a blue/UV-A light receptor), and a UV-B light receptor. Phytochrome is the best characterized of these three pigments, consisting of a protein attached to a tetrapyrrole chromophore that can reversibly interconvert between two photochemically different forms. The P_r form (inactive) can absorb red light and be converted to the active P_{fr} form. P_{fr} can be converted back to P_r by absorbing far-red light. A gene family encodes a set of slightly different phytochrome apoproteins. The different patterns of expression of these genes may account for the diversity of phytochrome responses (Fluhr, 1988).

One of the most interesting effects of P_{fr} on plant growth and development involves the transcriptional regulation of specific nuclear genes, such as the chlorophyll a/b-binding proteins (*cab*) and the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcS*) (Gallagher and Ellis, 1982; Silverthorne and Tobin, 1984). In higher plants, the CAB proteins are encoded by multicopy nuclear gene families, ranging from ten to 20 members. The smallest of these families is found in *Arabidopsis thaliana*, and contains only three members. Each of these three members produces an identical mature polypeptide sequence. This is in contrast to other higher plant gene families which produce numerous variant polypeptides, raising the possibility that the amino acid variance is not essential for function (Leutwiler *et al.*, 1986).

The nuclear coded *rbcS* polypeptides, along with the chloroplast coded large subunit *rbc* polypeptides, account for approximately 50% of the soluble cellular protein in mesophyll cells. The *rbcS* polypeptide is initially synthesized on free cytoplasmic ribosomes and transported into the chloroplast, whereupon it undergoes post-translational

modifications (Ellis, 1981). The number of members in the *rbcS* gene family range from two in *Nicotiana plumbaginifolia* (Poulsen *et al.*, 1986) to eight in *Petunia* (Dean *et al.*, 1985). In pea, all five members of the *rbcS* family are linked and segregate as a single Mendelian unit (Polans *et al.*, 1985), but in tomato three different loci have been described (Vallejos *et al.*, 1986).

The mature *rbcS* polypeptide exhibits extensive interspecific divergence but marginal intraspecific change. This could suggest that these *rbcS* genes are evolving in concert within a genome. Several phytochrome mediated genes, including phytochrome itself, are transcriptionally repressed in response to light (Dehesh *et al.*, 1990; Tsai and Coruzzi, 1990). Early studies on several pea *rbcS* genes, though, show a phytochrome mediated gene induction (Fluhr and Chua, 1986). The ability of a photoreceptor to mediate opposite patterns of expression implies that there is a branch point in the signal transduction pathway leading to these different responses. The details of the signal transduction pathway are unknown. By analyzing the 5' upstream regulatory sequences and their respective trans-acting factors, research groups have tried to define the critical components of this pathway.

The study of *rbcS* promoter elements in dicots has been difficult due to the nature of the inducing agent, light. The effects of various wavelengths of light on particular tissue types and developmental stages have been studied only in a few species. The pea *rbcS* genes have been the most fully characterized of all the dicot *rbcs* genes, and researchers still do not fully understand the relationship between developmental stage and light induction. For example, two groups have presented evidence for a role of the TATA box and neighbouring sequences in mediating light regulation of pea *rbcS* genes.

Combining the results from both studies, it was suggested that a region from -35 to -2 contained a light-responsive element (LRE) (Morelli *et al.,* 1985; Timko *et al.,* 1985). Both sets of experiments were performed by assaying the gene constructs in transformed calli, clumps of cells which could not be induced to differentiate into whole plants because of the presence of the *Agrobacterium* tumour genes. Such an assay system could not be used as an accurate model for light-responsive gene transcription in mature, green leaves. A more serious complication is that the induction by light is extremely slow, in the order of one to two weeks. In that time the tissue either becomes green in the light (Morelli *et al.,* 1985) or is induced to become green by treatment with the plant hormone cytokinin (Timko *et al.,* 1985). From this, it is difficult to ascertain to what extent factors, other than light, contribute to the regulatory effect.

With this in mind, a number of studies on the cis and trans regulatory elements of pea *rbcS*-3A gene promoters will be reviewed. Three independent upstream regions (-410 to -330, -330 to -170, and -166 to -50) have been identified as light responsive (Gilmartin *et al.,* 1990). The region characterized by a -166 promoter deletion was shown to be phytochrome-responsive and also contained two physically separable LREs. One LRE was located between -166 and -50, and the other was located downstream of -50 (Kuhlemeier *et al.,* 1989). Other LREs have also been identified between -410 and -170 (Kuhlemeier *et al.,* 1988).

Six sequences, which bind the nuclear factor GT-1, have been isolated within the *rbcS*-3A promoter. Two of these sequences, within 170 bp from the transcriptional start site, are essential for transcriptional activity (Green *et al.,* 1987; 1988). Neither expansion nor contraction of the distance between these two GT-1 boxes affects binding

of GT-1 *in vitro*. By contrast, expansion of the distance between these sites by a mere two bp severely reduces promoter activity *in vivo*. These results suggest that GT-1, is necessary but not sufficient for light-responsive transcriptional activation in pea *rbcS*-3A (Gilmartin and Chua, 1990). When a tetramer of these GT-1 boxes (-153 to -140) is fused to the -90 CaMV 35S promoter, a light induced transcriptional activity is demonstrated. In the dark, the tetramer/35S fusion promoter acts to silence transcription, suggesting that a DNA-protein complex represses dark transcription at this site (Kuhlemeier *et al.*, 1987). Such a DNA-protein complex may also involve the activation sequence factor ASF-1, which interacts with the *as-1* element located between -83 and -65 of the CaMV 35S promoter (Lam *et al.*, 1989).

Two sequences with homology to the G box, a conserved sequence which binds the G box factor (GBF) in numerous *rbcS* genes of other species (Giuliano *et al.*, 1988), were identified in the pea *rbcS*-3A 5' region. G box regions are believed to be UV light-inducible, suggesting that GBF is either synthesized in response to UV, modified to facilitate its binding, or a second modified or labile factor facilitates its binding (Gilmartin *et al.*, 1990). Whether GBF can bind the G box elements of the pea *rbcS*-3A promoter is still under investigation.

Another region in the pea *rbcS*-3A promoter (between -51 and -31) has been shown to bind a protein factor, 3AF1, which is present in all cell types. The role of this regulatory element within the *rbcS*-3A promoter, though, is unclear. However, its contribution to *rbcS*-3A activity is probably derived from an interaction with other proteins bound to adjacent sequence elements (Gilmartin *et al.*, 1990).

Several GATA motifs are also present in this pea *rbcS* gene (-189 to -170). This motif is present in virtually all *rbcS* and *cab* genes (Castresana *et al.*, 1987; Gidoni *et al.*, 1989). In several other *rbcS* genes this element has been referred to as the I box (Giuliano *et al.*, 1988), and it has been shown to interact with a protein factor termed GAF-1. GAF-1 was found in greater abundance in extracts prepared from light-grown as opposed to dark-adapted plants. This protein was also distinct from ASF-2 and 3AF1, two factors which also bind to sequences containing GATA motifs. These observations point to a distinction between different GATA elements present within light-responsive promoters (Gilmartin *et al.*, 1990).

The mechanisms by which these trans-acting factors regulate the increases in transcription rates of *rbcS* and other light-responsive genes are presently not known. It can be speculated that these mechanisms involve *de novo* synthesis of the particular factor, constitutive expression of the factor and a modification of the DNA binding domain to facilitate binding, or a modification of the protein factor's binding domain so that it can interact with other components of the transcription complex (Gilmartin *et al.*, 1990).

The studies which have been discussed, in many cases, have identified the components involved in the transcriptional regulation of gene expression in response to a variety of signals. By understanding these fundamental cellular processes it will be possible to address more complex developmental problems. A general trend, which has been observed by many researchers in this field, is that general themes in regulation have been highly conserved during the evolution of divergent species of animals and plants. This conservation of structure and function, within the plant kingdom, is seen by the

similarity of factors and target sequences involved in responses to such diverse signals as light and plant hormones. The identification of these signal transduction pathways will eventually provide an understanding of the mechanisms by which an organism assimilates information into a coordinated response.

Table 2.1: Non-Constitutive Plant Promoters

Specificity	Promoter	Tested In:	Reference
Inducible	Heat inducible sunflower Polyubiquitin (*ubi1* & *ubi2*)	dicots	Binet *et al.*, 1991. Plant Science 79: 87-94
Inducible	Wound inducible promoter from potato (*wun1*)	dicots	Logemann *et al.*, 1989. Plant Cell 1: 151-158
Inducible	UV, fungal and wound inducible *pal* gene promoters from parsley	dicots and monocots	Logemann *et al.*, 1995. PNAS 92: 5905-5909
Inducible	Glucose inducible promoter from yeast *srp1* gene	dicots	Fantino *et al.*, 1992. Mol Gen Genet 236(1): 65-75
Inducible	Ethylene inducible prb-1b gene promoter (pathogenesis related protein)	dicots	Sessa *et al.*, 1995. Plant Mol Biol 28(1): 145-153
Inducible	Fungal and UV-light inducible gene promoter - bean NADP-malic enzyme	dicots	Walter *et al.*, 1994. Eur. J. Biochem. 224: 999-1009
Inducible	Pathogen induced promoter from parsley *pr2* gene	dicots and monocots	Korfhage *et al.*, 1994. Plant Cell 6(5): 695-708.
Inducible	Pathogen induced promoter isolated from flax infected with flax rust (*fis1*)	dicots	Roberts & Pryor 1995. The Plant J. 8(1): 1-8
Inducible	ABA inducible *hva22* gene promoter from barley	monocots	Shen and Ho, 1995. Plant Cell 7(3): 295-307
Inducible	Gibberellin inducible gene promoter from cereal high-PI alpha amylase	monocots	Rogers *et al.*, 1994. Plant Physiol. 105(1): 151-158
Inducible	Cold, drought and high salt responsive promoter from *Arabidopsis rd29* genes	dicots	Yamaguchi-Shinozaki & Shinozaki. 1994 Plant Cell 6(2): 251-264.
Inducible	Cold, drought and ABA responsive promoter from *Arabidopsis cor15a*	dicots	Baker *et al.*, 1994. Plant Mol Biol 24(5): 701-713.
Inducible	ABA and water stress inducible promoter from *rab28* maize gene	monocots	Pla *et al.*, 1993. Plant Mol Biol 21(2): 259-266
Inducible	Cold inducible promoter *bn115* from winter *Brassica napus*	dicots	Jiang *et al.*, 1996. Plant Mol. Biol. 30: 679-684

Inducible	Salt inducible promoter from a mutated *bnl15 B. napus* promoter	dicots	Jiang *et al.*, 1996. Plant Mol. Biol. 30: 679-684
Inducible	Osmotic stress responsive promoter from wheat *'Em'* gene	monocots	Onde *et al.*, 1994. J. Exp. Bot. 45(274): 561-566
Inducible	Stress inducible promoter from tobacco pathogenesis related osmotin gene	dicots	Raghothama 1993. Plant Mol Biol. 23(6): 1117-1128
Inducible	*gmhsp* 17.5-E heat shock promoter from soybean	dicots	Czarnecka *et al.*, 1989. Mol Cell Biol 9: 3457-3463
Inducible	Salicylic acid inducible cyclophilin gene promoter from maize	monocots	Marivet *et al.*, 1995. Mol Gen Genet 247: 222-228
Inducible	Low temperature & drought inducible *gblt4* gene promoter from barley	monocots	White *et al.*, 1994. J Exp Bot 45(281): 1885-1892
Inducible	Phytoalexin (disease response) induced promoter -chalcone synthase 15- bean	dicots and monocots	Arias *et al.*, 1993. Plant Cell 5(4): 485-496
Inducible	Salicylic acid inducible promoter(pathogenesis related)	dicots	Uknes *et al.*, 1993. Plant Cell 5: 159
Inducible	Abscisic acid inducible promoter from *Em* gene of wheat	monocots	Marcotte *et al.*, 1989. Plant Cell 1:969
Inducible	Auxin-inducible GH3 promoter from Soybean	dicots	Hagen *et al.*, 1991. Plant Mol Biol 17: 567-579
Inducible	Light inducible *elip* gene promoter from pea	dicots	Blecken *et al.*, 1994. Mol Gen Genet 245(3): 371-379
Inducible	Light inducible chalcone synthase (*chs*) promoter from parsley	dicots and monocots	Weisshaar *et al.*, 1991. EMBO J. 10(7): 1777-1786
Inducible	Light-inducible rubisco small subunit Pea Promoter (*rbcS-3A*)	dicots	Green *et al.*, 1987. EMBO J 6:2543-2549
Inducible	Light-inducible chloroplast *fbpase* gene promoter from wheat	monocots	Lloyd *et al.*, 1991. Mol Gen Genet 225: 209-216

Inducible	Light inducible promoters from spinach (*pc* and *psad* - strong)	dicots	Lubberstedt *et al.*, 1994. Plant Physiol. 104(3): 997-1006
Inducible	Light inducible promoters from spinach (*cab-1* gene - strong)	dicots	Lubberstedt *et al.*, 1994. Plant Physiol. 104(3): 997-1006
Inducible	Red-light inducible promoters from spinach (*fnr* gene)	dicots	Lubberstedt *et al.*, 1994. Plant Physiol. 104(3): 997-1006
Inducible and Tissue-Specific	Root and sepal specific (mature plant) and pathogen-induced *msr* gene prom.	dicots	Gough *et al.*, 1995. Mol Gen Genet 247: 323-337
Inducible and Tissue-Specific	Stress and developmentally regulated polyubiquitin promoter from tobacco	dicots	Rieping *et al.*, 1994. Plant Cell 6(8): 1087-1098
Inducible and Tissue-Specific	Seed specific and ABA inducible promoter - sunflower helianthinin genes	dicots	Nunberg *et al.*, 1994. Plant Cell 6(4): 473-486
Inducible and Tissue-Specific	Leaf specific and light inducible promoter from spinach rubisco activase	dicots	Orozco & Ogren 1993. Plant Mol. Biol 23(6): 1129-1138
Inducible and Tissue-Specific	Light inducible and tissue specific rice *cab* gene promoter	dicots	Luan & Bogorad 1992. Plant Cell 4(8): 971-981
Inducible and Tissue-Specific	Pathogen, UV and wounding inducible and developmental- 4-coumerate:CoA ligase (*4cl*) from parsley	dicots	Douglas *et al.*, 1991. EMBO J. 10(7): 1767-1775
Inducible and Tissue-Specific	Stress inducible and developmentally regulated *4cl* promoter from potato	dicots	Becker-Andre *et al.*, 1991. J Biol Chem 266(13):8551-8559
Tissue	Meristem, root and leaf specific promoter from *Arabidopsis a1 ef-1*	dicots	Curie *et al.*, 1993. Mol Gen Genet 238(3): 428-436
Tissue	Pea plastocyanin promoter (*pcp*)	dicots	Pwee & Gray 1993. Plant J. 3: 437-449
Tissue	Legume nodule specific promoter from soybean leghemoglobin (*lbc3*)	dicots	De-Bruijn *et al.*, 1989. Plant Mol Biol 13(3): 319-325
Tissue	Nodule specific Enhancer from soybean nodulin gene (*n23*)	dicots	Jorgensen *et al.*, 1991. Plant Cell 3(8): 819-827

Tissue	Napin seed-specific Promoter from *Brassica*	dicots	Baszcynski & Fallis, 1990. Plant Mol Biol 14: 633
Tissue	Seed specific promoter from *Vicia faba* non-storage seed protein gene (*usp*)	dicots	Fiedler *et al.*, 1993. Plant Mol Biol 22(4): 669-679
Tissue	Seed-specific promoter from a conifer2S albumin gene	dicots	McInnis *et al.*, 1996 IAPTC Canada poster presentation # 37
Tissue	Seed-specific promoter from beta- conglycinin gene (*7s*) from soybean	dicots	Fujiwara *et al.*, 1994. Plant Mol Biol. 24(2): 261-272 Chamberland *et al.*,1992. Plant Mol Biol. 19(6): 937-949
Tissue	Seed-specific (endosperm) of HMW glutenin subunit from wheat	monocots	Blechl & Anderson 1996. Nature Biotechnology14: 875-879
Tissue	Meristematic wheat *h3* gene promoter	monocots	Terada *et al.*, 1995. Plant Mol Biol 27(1): 17-26
Tissue	Wheat histone *h2b* gene promoter expressed in meristematic tissue	dicots	Yang *et al.*, 1995. Plant Mol. Biol. 28(1): 155-172
Tissue	Nodule and root specific lupin nodulin-45 gene promoter	dicots	Macknight *et al.*, 1995. Plant Mol. Biol. 27(3): 457-466
Tissue	Root specific promoter from *Arabidopsis* peroxidase *prxEa* gene	dicots (8 fold higher expression than 35S)	Intapruk *et al.*, 1994. Plant Cell Rep. 13(3/4): 123-129
Tissue	Root specific putative promoter from *Brassica oleracea*	dicots	Lee 1996 IAPTC Canada conference poster presentation #36
Tissue	Root specific promoter from soybean nodulin-26 gene (channel protein)	dicots	Miao & Verma 1993. Plant Cell 5(7): 781-794.
Tissue	Vascular tissue of root and stem specific promoter of bean (*grp 1.8*)	dicots	Keller & Baumgartner 1991. Plant Cell 3(10): 1051-1061
Tissue	Leaf and stem specific putative promoter from *Brassica oleracea*	dicots	Lee 1996 IAPTC Canada conference poster presentation #36

Tissue	Leaf mesophyll specific promoter phosphoenolpyruvate carboxylase from *Flaveria trinervia* (C4)	dicots (C3 and C4 plants)	Stockhaus *et al.*, 1994. Mol Gen Genet 245(3): 286-293
Tissue	Phloem specific sucrose synthase promoter from *Arabidopsis*	dicots	Martin *et al.*, 1993. Plant J. 4: 367
Tissue	Xylem-specific promoter from parsley 4CL gene (4-coumerate:CoA ligase)	dicots	Douglas, presentation at IAPTC Canada 1996
Tissue	Xylem-specific promoter from poplar *pal* (phenylalanine ammonia-lyase)	dicots	Gray-Mitsumune *et al.*, 1996 IAPTC Canada conference poster presentation #34
Tissue	Xylem specific promoter from *pal2* gene in bean	dicots	Leyva *et al.*, 1992. Plant Cell 4(3): 263-271.
Tissue	Xylem and phloem specific promoters from tobacco	dicots	Anderson & Datla 1996 IAPTC Canada conference poster presentation #27
Tissue	rol-A, rol-B & rol-C promoters from *Agrobacterium rhizogenes*	dicots	Schmuelling *et al.*, 1989. Plant Cell 1: 665
Tissue	Endosperm and pollen specific rice wx+ (amylose prod'n) gene promoter	dicots and monocots	Hirano *et al.*, Plant and Cell Physiol., 1995. 36(1): 37-44
Tissue	Leaf Guard cell specific promoter ADP-glucose pyrophosphorylase from potato	dicots	Mueller-Roeber *et al.*, 1994 Plant Cell 6: 60
Tissue	Arabin seed-specific promoter from *Arabidopsis thaliana*	dicots	Cornellisen *et al.*, 1989. Nucleic Acids Res 17: 19-29
Tissue	Flower specific promoter from petunia chalcone synthase (*chsA*) gene	dicots	Van der Meer *et al.*, 1990. Plant Mol Biol 15(1): 95-109
Tissue	Pollen (*sta44*) and tapetum (*sta41*) promoters from *B. napus*	dicots	Robert *et al.*, P.R.C., Ag. Canada, (personal communication)
Tissue	Tapetum specific promoter from tobacco (*ta29*)	dicots	Goldberg, 1988. Science 240: 1460-1467.
Tissue	Microspore specific promoter from *Brassica napus* (*bp4a*)	dicots	Albani *et al.*, 1990. Plant Mol Biol 15: 605-622

Tissue	Pollen specific promoters from *Brassica napus* (*bp10*)	dicots	Albani *et al.*, 1992. The Plant J 2: 1-12
Tissue	Pollen specific promoters from tomato (*lat52*, *lat56*, *lat59*)	dicots	Twell *et al.*, 1989, 1990, 1991 and Ursin *et al.*, 1989. Plant Cell 1: 727-736
Tissue	Pollen specific promoter from *Zea mays*	monocots	Hanson *et al.*, 1989. Plant Cell 1: 173-179
Tissue	Pollen and pistil specific promoter from *Brassica* S locus glycoprotein gene	dicots	Dzelzkalns, V. *et al.*, 1993. Plant Cell 5(8): 855-863
Tissue	Tobacco extensin gene promoter predominantly expressed in roots	dicots	Memelink *et al.*, 1993. Plant J 4: 1011-1022
Tissue	Peroxidase gene promoter from *A. thaliana*	dicots	Intapruk *et al.*, 1994. Plant Cell Rep 13: 123-129
Tissue	Sucrose synthase gene promoter	dicots	Maas & Werr, 1988 Maize Genet Coop Newsl 62: 48
Developmental Stage-specific	*Arabidopsis thaliana ef-1* alpha promoter	dicots	Curie *et al.*, 1993. Mol Gen Genet 238: 428-436
Developmental Stage-specific	*Arabidopsis* histone gene promoter[cell cycle dependant (*h3* and *h4*)]	dicots	Lepetit *et al.*, 1992. Mol Gen Genet 231:276-285
Plastid-specific	Mustard chloroplast *rrnB* operon promoter	dicots	Przybyl *et al.*, 1984 Plant Mol Biol 3: 147158
Plastid-specific	Mustard chloroplast *psbA* promoter	dicots	Link & Langridge, 1984 Nucleic Acids Res 12: 945-958
Plastid-specific	Mustard chloroplast tRNA(UUU) *trnK* promoter	dicots	Neuhaus & Link, 1987 Curr Genet 11:251-257
Plastid-specific	Mustard chloroplast trnQ and psbK promoters	dicots	Neuhaus & Link, 1990 Curr Genet 18: 377-383
Plastid-specific	Mustard chloroplast *rps16* and *trnS* promoters	dicots	Neuhaus *et al.*, 1989 Curr Genet 15: 63-70
Plastid-specific	Mustard chloroplast *trnH* promoter	dicots	Nickelsen & Link 1990 Nucleic Acids Res 18: 1051

Plastid-specific	Chlamydomonas *atpB* promoter	dicots	Klein *et al.*, 1992 Proc Natl Acad Sci 89: 3453-3457
Plastid-specific	Blue light responsive chloroplast promoter (*lrp*)	dicots and monocots	Christopher *et al.*, 1992 Plant Cell 4: 785-798.

Chapter 3: Construction and Analysis of a Bifunctional Selectable Marker Gene Vector for Plant Transformation

3.1 Introduction

Selectable marker genes are critical for plant transformation since they facilitate the recovery of plant tissues transformed with foreign genes. The neomycin phosphotransferase II (*nptII*) gene has been a commonly used selectable marker for dicot transformation (Horsch *et al.*, 1985; McCLean *et al.*, 1991; Fry *et al.*, 1987; Rotino and Gleddie 1990), while the phosphinothricin acetyltransferase (*pat* or *bar*) gene has often been used as a selectable marker for monocot transformation (Nehra *et al.*, 1994; Vasil *et al.*, 1992; Spencer *et al.*, 1990; Shimamoto *et al.*, 1989; D'Halluin *et al.*, 1992).

The *nptII* gene, from the bacterial transposable element Tn5 (Beck *et al.*, 1982), confers resistance to aminoglycoside antibiotics such as kanamycin, neomycin and G-418 by inactivation through phosphorylation (Davies, 1980). When the *nptII* gene is introduced into dicot species such as *Brassica* and tobacco, the selectable marker gene allows resistant tissue to regenerate in the presence of kanamycin at levels ranging from 20 mg/L in *Brassica* to 100 mg/L in tobacco (personal observation). The frequency of escapes (nontransformed tissues which regenerate into a shoot) is often higher at the lower selection levels.

The *pat* (Wohleben *et al.*, 1988) and *bar* (Thompson *et al.*, 1987) genes are isolated from naturally occurring soil bacteria, *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus*, respectively. Both genes code for the glufosinate

ammonium detoxifying enzyme, phosphinothricin acetyltransferase and confer tolerance to the non-selective herbicide glufosinate ammonium by acetylation. When glufosinate ammonium is used in *Brassica* and tobacco tissue culture it acts as a very stringent selection agent, blocking regeneration of nontransformed shoots at levels as low as 2 mg/L (personal observation).

Kanamycin is less stringent than the non-selective herbicide glufosinate ammonium. Since kanamycin is more easily tolerated than glufosinate ammonium, the frequency of dicot tissue regeneration in the presence of kanamycin is much higher than in the presence of glufosinate ammonium. Kanamycin selection, though, does allow the regeneration of some nontransformed shoots, or 'escapes', at a low frequency. This problem of 'escape' regeneration is eliminated when using glufosinate ammonium as a selection agent. A translational fusion between the *pat* and *nptII* genes would potentially produce a bifunctional marker gene conferring resistance to both kanamycin and glufosinate ammonium. Such a bifunctional marker gene would also be an ideal tag for conducting T-DNA promoter tagging studies in plants, where a stringent selection protocol is desired and a high regeneration frequency is also mandatory.

In 1991, Botterman and coworkers published a report in which they documented the production of a *bar::nptII* fusion gene which was expressed under the control of a P_R promoter element (Botterman *et al.*, 1991) in *E. coli*. Enzymatically active PAT and NPTII protein was purified from *E. coli* cultures containing the recombinant plasmid which housed the P_R-*bar::nptII* cassette. No further data on the expression of this fusion gene were reported.

It is the intent of this study to produce and express a *pat::nptII* fusion gene which is completely bifunctional in both a prokaryotic and a eukaryotic host. The *pat* sequence used to make this fusion gene was obtained from a synthetic gene with optimized plant codon bias (Peter Eckes, Hoechst AG). It's applicability as a plant selectable marker gene was also assessed in tobacco and in *Brassica*.

This is the first reported study of a bifunctional *pat::nptII* fusion gene which functions both in a prokaryotic host, *Escherichia coli*, and in two eukaryotic plant systems, namely in *Nicotiana tabacum* and in *Brassica napus*.

3.2 Materials and Methods

3.2.1 Construction of the *pat::nptII* Fusion Gene

The in-frame, translational fusion between the *pat* gene (Wohleben *et al.*, 1988) and the *nptII* gene (Beck *et al.*, 1982) was produced by fusing the carboxy-terminus of the codon biased synthetic *pat* gene (Eckes, P., AgrEvo GmbH, Frankfurt, Germany; modified by Datla, R., Plant Biotechnology Institute, National Research Council, 110 Gymnasium Pl., Saskatoon, SK, Canada) to the amino-terminus of the *nptII* gene.

The 5' end of the codon biased *pat* gene was modified by Raju Datla by introducing a *SalI* site inframe to the *NcoI* based ATG. During this modification process a few extra amino acids were added to the PAT protein. This new *pat* gene (Figure 3.1) was verified functionally in transgenic plants demonstrating high expression levels and conferring resistance to phosphinothricin (Datla, R., personal communication).

The cloning procedure to produce the final fusion gene was performed in an *Escherichia coli* host (DH5αMCR™ cells; Gibco BRL, cat. no. 18289-017) and the fusion gene product was ligated into the *E. coli* cloning vector pSE280 (InVitrogen; Brosius, 1989).

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Figure 3.1   Sequence of the Synthetic *pat* Gene

<u>*Sal*I</u>

GTC GAC ATG TCT CCG GAG AGG AGA CCA GTT GAG ATT AGG CCA GCT ACA GCA

        Met Ser Pro Glu Arg Arg Pro Val Glu Ile Arg Pro Ala Thr Ala

GCT GAT ATG GCC GCG GTT TGT GAT ATC GTT AAC CAT TAC ATT GAG ACG TCT

Ala Asp Met Ala Ala Val Cys Asp Ile Val Asn His Tyr Ile Glu Thr Ser

ACA GTG AAC TTT AGG ACA GAG CCA CAA ACA CCA CAA GAG TGG ATT GAT GAT

Thr Val Asn Phe Arg Thr Glu Pro Gln Thr Pro Gln Glu Trp Ile Asp Asp

CTA GAG AGG TTG CAA GAT AGA TAC CCT TGG TTG GTT GCT GAG GTT GAG GGT

Leu Glu Arg Leu Gln Asp Arg Tyr Pro Trp Leu Val Ala Glu Val Glu Gly

GTT GTG GCT GGT ATT GCT TAC GCT GGG CCC TGG AAG GCT AGG AAC GCT TAC

Val Val Ala Gly Ile Ala Tyr Ala Gly Pro Trp Lys Ala Arg Asn Ala Tyr

GAT TGG ACA GTT GAG AGT ACT GTT TAC GTG TCA CAT AGG CAT CAA AGG TTG

Asp Trp Thr Val Glu Ser Thr Val Tyr Val Ser His Arg His Gln Arg Leu

GGC CTA GGA TCC ACA TTG TAC ACA CAT TTG CTT AAG TCT ATG GAG GCG CAA

Gly Leu Gly Ser Thr Leu Tyr Thr His Leu Leu Lys Ser Met Glu Ala Gln

GGT TTT AAG TCT GTG GTT GCT GTT ATA GGC CTT CCA AAC GAT CCA TCT GTT

Gly Phe Lys Ser Val Val Ala Val Ile Gly Leu Pro Asn Asp Pro Ser Val

AGG TTG CAT GAG GCT TTG GGA TAC ACA GCC CGG GGT ACA TTG CGC GCA GCT

Arg Leu His Glu Ala Leu Gly Tyr Thr Ala Arg Gly Thr Leu Arg Ala Ala

GGA TAC AAG CAT GGT GGA TGG CAT GAT GTT GGT TTT TGG CAA AGG GAT TTT

Gly Tyr Lys His Gly Gly Trp His Asp Val Gly Phe Trp Glu Arg Asp Phe

                                                               <u>*Bgl*II</u>
GAG TTG CCA GCT CCT CCA AGG CCA GTT AGG CCA GTT ACC CAG ATC TGA
Glu Leu Pro Ala Pro Pro Arg Pro Val Arg Pro Val Thr Glu Ile STOP

<u>*Sal*I</u>
GTC GAC

(Eckes, P., AgrEvo GmBH. modified
by Datla, R., P.B.I., N.R.C., Canada)

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3.2.2 Expression and Verification of the *pat::nptII* Fusion Gene in *Escherichia coli*

In pSE280-*pat::nptII* (5.4 kb), the *pat::nptII* fusion gene was situated downstream from the prokaryote P_{TRC} promoter and upstream from the T1T2 termination signal (Brosius 1989). This prokaryote expression cassette made it possible to test the expression of the bifunctional fusion gene in an *E. coli* host.

Escherichia coli DH5α cells, originally transformed with pSE280-*pat::nptII*, were spread onto 2YT plates (Appendix III) containing 50 mg/L kanamycin and 50 mg/L ampicillin. Successful transformants containing a functional pSE280-*pat::nptII* plasmid were isolated on this selection medium and further analysed for the expression of the fusion gene by replica plating colonies onto different selection media ((M9 Minimal Media + 300 mg/L L-PPT, or + 300 mg/L L-PPT and 50 mg/L kanamycin) Appendix III) and by Western Blot Analysis (Appendix II) .

3.2.3 Construction of the *pat::nptII* Bifunctional Binary Vector

The *pat::nptII* fusion gene was isolated from the pSE280-*pat::nptII* plasmid vector and ligated into pRD300 (3.5 kb; based on pBI524, Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada; which is a pUC9 [2.8 kb] derivative) cloning vector containing the plant expression cassette: *35S35S P-AMV-* MCS − *nosT* where *35S35S P* is the duplicated *CaMV 35S* promoter sequence (Kay *et al.*, 1987), and *AMV* is the alfalfa mosaic virus enhancer sequence (Brederode *et al.*, 1980). The *35S35S* P-*AMV* enhanced promoter was constructed through site directed mutagenesis of the tandem or duplicated *35S* promoter element followed by

ligation to the synthetic sequence carrying the non-translated enhancer, *AMV*. The enhanced promoter was selected *in vivo* for optimized translation context (Datla, R., Plant Biotechnology Insitute, National Research Council, Saskatoon, SK, Canada; personal communication). MCS is the multiple cloning site, and *nosT* is the nopaline synthase (*nos*) polyadenylation site from the *nos* gene resident on a nopaline producing strain of *Agrobacterium tumefaciens* Ti plasmid (Bevan *et al.*, 1983).

The *35S35S-AMV-pat::nptII-nosT-EcoRI* cassette from pRD300-*pat::nptII* was ligated into pBI434 (BinSyn binary vector, 7.5 kb, Datla, unpublished) to derive pBAU1 (Figure 3.2).

Figure 3.2 Plasmid Map of the binary vector pBAU1

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3.2.4 Transformation of pBAU1 into canola and tobacco plants

The plant expression cassette *35S35SP-AMV-pat::nptII-nosT*, contained within pBAU1, was transferred into both *Brassica napus* cv. Westar and *Nicotiana tabacum* cv. Xanthi using *Agrobacterium tumefaciens* transformation. Tissue explants were inoculated with the *Agrobacterium tumefaciens* strain pMP90-pBAU1 or the positive control pMP90-pRD430 *(35S35SP-AMV-pat-nosT + nosP-nptII- nosT*; P.B.I., N.R.C., Saskatoon, SK) using the transformation and regeneration method of Moloney *et al.*, (1989) for cotyledonary petioles of *Brassica*, and the transformation and regeneration method of Horsch *et al.*, 1985, for tobacco leaf disks (refer to Appendix III for media and transformation protocols). Both *Brassica* and tobacco explants were regenerated in the presence of kanamycin (20 mg/L and 100 mg/L respectively), and regenerated shoots were induced to root in the presence of 20 mg/L and 60 mg/L L-PPT, respectively (Basta™, 25% L-PPT). Selection, regeneration and rooting cultures were all incubated in a growth room at 25°C, with a 16 hour photoperiod and a light intensity of 50 $\mu Em^{-2} s^{-1}$. Once rooted, transformants were transplanted to soil and grown to maturity in the greenhouse.

3.2.5 Analysis of pBAU1 transgenic plants

PCR Analysis

Primary putative transformants were evaluated for the presence of the fusion gene using PCR analysis. Leaves removed from primary transformants (T_0) which had rooted on L-PPT were subjected to a rapid plant DNA extraction protocol (Edwards *et al.*, 1991) and used in a polymerase chain reaction (PCR) assay. The two

fusion gene marker components, i.e. *pat* and *nptII*, if present in the leaf tissue, were amplified using primers specific for each marker gene. A 400 bp *pat* amplicon was amplified using a 22 mer and a 20 mer primer pair: 5'-AGACCAGTTGAGATTAGGCCAG-3' and 5'-GCCTCATGCAACCTAACAGA- 3' respectively. A 747 bp amplicon specific for the nptII gene was amplified using two 18 mer primers: 5'-GATGGATTGCACGCAGGT- 3' and 5'-TCAGAAGAACTCGTCAAG-3'. For each PCR reaction, 2.5 µl of plant DNA was used in a standard 50 µl reaction (0.25mM dNTPs (Pharmacia Biotech 27-2035-01), 1X Taq Polymerase Buffer (Pharmacia 27-0799A), 5 µM primer #1, 5 µM primer #2, 1 U Taq DNA Polymerase (Pharmacia 27-0799)). Amplifications were achieved after denaturation of the template DNA at 96°C for 2 minutes and 35 repeated cycles of 30 second denaturation at 94°C, 1 minute annealing at 55°C and 2 minute extension at 72°C, followed by a final 7 minute extension at 72°C and cooling to 15°C using the MJ Research Inc. PTC-100 thermocycler. The reaction products were directly analysed on a 0.8% agarose gel.

<u>Southern Hybridization</u>

Southern hybridization assays (Southern, 1975) were used to screen putative primary transformants for the presence of the *pat* and *nptII* marker genes and to estimate the copy number of the inserted fusion gene. Genomic DNA was isolated from leaves of *in vitro* plantlets using the method of Dellaporta (Dellaporta *et al.*, 1983, 1985; Appendix III for Dellaporta Buffer). DNA recovered from approximately

one gram of leaf tissue was dissolved in 100 µl of TE buffer (50 mM Tris, 10 mM EDTA, pH 8.0) and stored at -20°C for up to one year.

Approximately 5 µg of genomic *Brassica* DNA and 10 µg of genomic *Nicotiana* DNA was subjected to restriction endonuclease digestion using a high concentration (50 U per 30 µl reaction) of restriction enzyme (*HindIII*, Gibco BRL; *EcoRI*, Gibco, BRL) along with the appropriate 1X restriction buffer (React 2, Gibco BRL; React 3, Gibco BRL). The restriction enzymes *EcoRI* and *HindIII* were chosen since they only cut within the T-DNA at one site, therefore allowing for a copy number estimation. A *PstI* digest was also performed to verify the presence of the T-DNA insert. Genomic DNAs were incubated with the appropriate restriction enzymes at 37°C overnight. The resulting DNA fragments were then electrophoresed on a 0.8% agarose gel and blotted onto a nylon membrane (GeneScreen Plus, DuPont) according to the method of Southern (1975). Following transfer, the membrane was baked at 80°C for 1 hour and stored for subsequent hybridization.

Rooting Assays

Single copy T_0 transformants were subjected to *in vitro* rooting assays on increasing levels of L-PPT (0, 60, 120 and 240 mg/L of L-PPT; Basta™). Shoots containing at least two leaves and a shoot meristem were excised from rooted T_0 transformants (rooted on 20 mg/L L-PPT for *B.napus* and 60 mg/L for *N. tabacum*) and transferred to Rooting Medium (Medium V for *B. napus*, NTIII Medium for *N. tabacum*, Appendix III) containing the appropriate herbicide concentration. Each T_0 transformant was tested in triplicate on each herbicide level. Explants were incubated

in a growth room at 25°C, with a 16 hour photoperiod and a light intensity of 50 μEm^{-2} s^{-1}. Evaluations for rooting ability and leaf formation were scored at day 8, 12 and 20 post-transplant.

Enzyme Activity Assays

Two different enzyme activity assays were used to detect the presence of an active marker gene product. To detect the activities of the *pat* and *nptII* gene products in the fusion protein, leaf tissue samples from T$_0$ transformants were subjected to a crude extraction procedure designed to maintain the function of the enzyme. Extracts were incubated with a radiolabelled substrate, followed by a procedure which separated the incorporated substrate from the unincorporated substrate. The intensity of the radiolabel's signal was used as a qualitative indicator for the amount of active PAT or NPTII enzyme present in the tissue.

A modified version of the PAT activity assay (De Block *et al.*, 1987) is depicted in Figure 3.3. Approximately 20 μg of total leaf protein, relative to bovine serum albumin (BSA) (Bradford, 1976), was reacted with radiolabelled ^{14}C-acetyl CoA and L-PPT. Following a one hour reaction period at 37°C, the reaction was spotted onto a silicagel thin layer chromatography (t.l.c.) plate and subjected to ascendant chromatography. ^{14}C-substrates and reaction products were visualised by autoradiography. A detailed protocol is outlined in Appendix II.

The NPTII Activity or Dot Blot assay which was used to detect active NPTII protein in leaf extracts is a modification of the original assay by Reiss *et al.*, (1984). This modification uses the GUS Lysis Buffer for the protein extractions (Staebell *et*

al.,, 1990; Jefferson, 1987), followed by a proteinase K digestion as described by

Radke *et al.,* (1988) and the dot-blot method of Platt and Yang (1987). A schematic

representation of this assay is depicted in Figure 3.4 and a more detailed procedure

can be found in Appendix II.

Western Assay

To verify the size of the PAT::NPTII fusion protein, a western blot assay was

performed on leaf extracts from several *in vitro* T_0 transgenic canola and tobacco

plants (as well as nontransformed controls), and on bacterial protein extracts from *E.*

coli DH5α-pSE280-*pat::nptII* and *E. coli* DH5α (negative control). Leaf extracts were

ground and bacterial cells were sonicated in a chilled denaturing extraction buffer

containing sodium dodecyl sulphate (SDS). Duplicate samples were separated on two

12% SDS-polyacrylamide gels (PAG). One gel was stained with Coomassie Blue for

complete protein staining, while the other gel was transblotted onto nitrocellulose and

subjected to an immunoblotting procedure (Figure 3.5) with an antibody specific for

the NPTII protein (Rabbit anti-NPTII primary polyclonal antibody; 5'-3' Inc.) or for

the PAT protein (Rabbit anti-PAT primary polyclonal antibody; A. Schulz, Hoechst

AG, Frankfurt). The NPTII or PAT specific protein bands were visualized via a

secondary antibody linked to the alkaline phosphatase enzyme (Goat anti-Rabbit

IgG:alkaline phosphatase conjugate; BRL). A detailed protocol is outlined in

Appendix II.

Immunoassay

The amount of NPTII protein present in the leaves of T_0 tobacco pBAU1 transformants was quantitated using a two antibody sandwich immunoassay called an ELISA (enzyme linked immunosorbant assay). NPTII protein was quantified using the commercial 5 Prime -3 Prime Inc. NPTII ELISA Kit (cat. 5307-610101).

The NPTII ELISA is a two-antibody sandwich assay where the primary antibody is a polyclonal IgG antibody specific for the NPTII protein. The secondary antibody is a biotinylated polyclonal IgG also specific for NPTII. The antigen (NPTII protein) in a test solution, such as a cell extract, is allowed to bind to the first immobilized antibody. Unbound proteins are removed by washing, and a labelled secondary antibody (biotinylated anti-NPTII) is allowed to bind to the antigen. After washing, the assay is quantitated by flooding the well with streptavidin (SA) conjugated to an alkaline phosphatase (AP) enzyme. The streptavidin binds to the biotin molecules present on the labelled secondary antibody, and a colour reaction occurs by reacting the SA-AP complex with a soluble chromogenic substrate (Van Weemen and Schuurs, 1971). The amount of coloured reaction product produced is measured spectrophotometrically and is directly proportional to the amount of NPTII protein present in the original test sample.

The amount of NPTII protein in a given sample is calculated from the linear regression of the NPTII standard curve:

$$\boxed{y = mx + b}$$

m = slope   b = y intercept when x=0

y = spectrophotometric reading of the sample

x = the amount of NPTII protein in a given volume of your sample

The amount of NPTII protein in one milligram of total sample protein is calculated by dividing the amount of NPTII protein, in a given volume, by the number of mg of total protein present in the same volume (determined by a Bradford's assay; Bradford, 1976).

The NPTII ELISA works on the same principle as the PAT ELISA. A schematic diagram depicting the PAT ELISA is illustrated in Figure 3.6.

<u>Segregation Analyses</u>

In vitro segregation analyses were performed on the T_1 seed of the selfed T_0 room (25°C, 16 hr. photoperiod, 50 $\mu Em^{-2} s^{-1}$) and evaluated at day 21. The ratio of 'selective agent resistant' to 'selective agent sensitive' progeny was determined for each transformant and these values were subjected to a chi-squared analysis (Appendix II). These ratios were then checked for agreement with the results of Southern Blot hybridization.

Figure 3.3: PAT Activity Assay
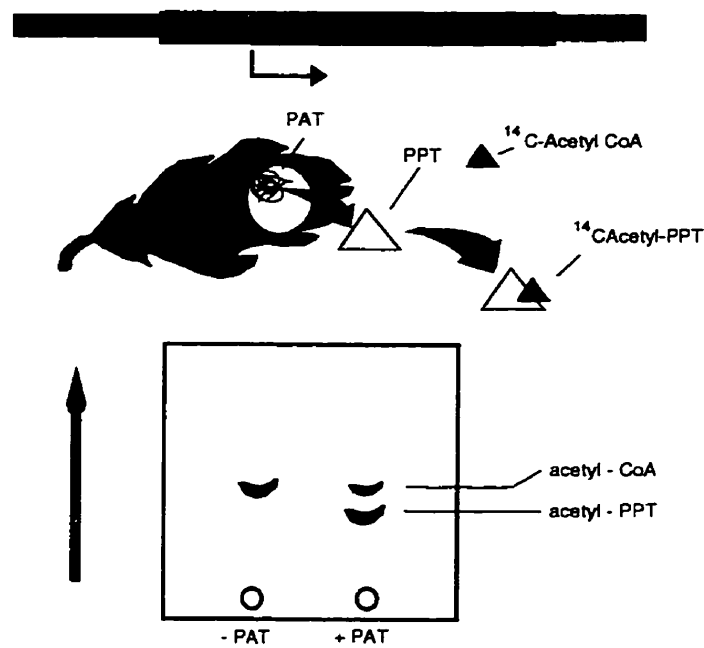
Figure 3.4: NPTII Dot Blot Assay
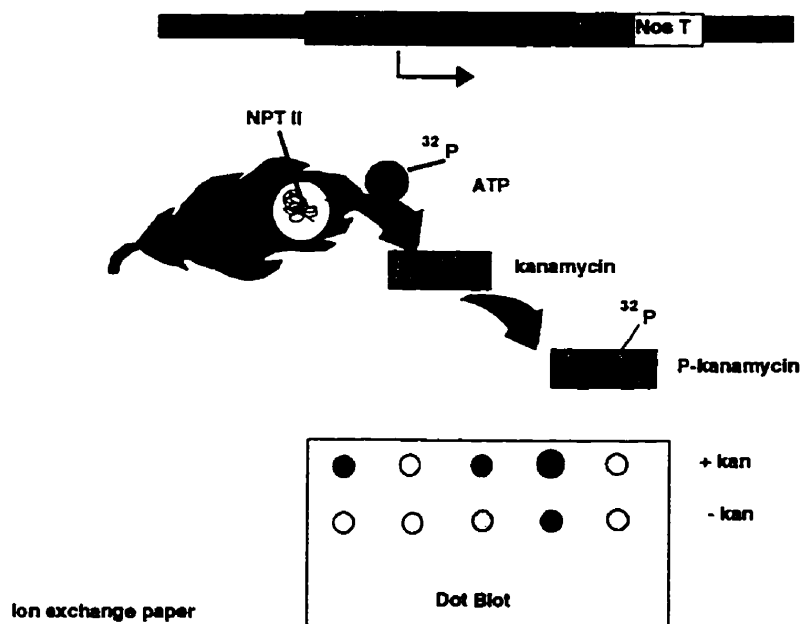
Figure 3.5: Western Blot Assay

leaf expressing PAT

non-transformed leaf

extract protein

+ bacterial PAT

Protein Standards

polyacrlamide gel electrophoresis   transblot onto membrane
& probe with labelled antibodies
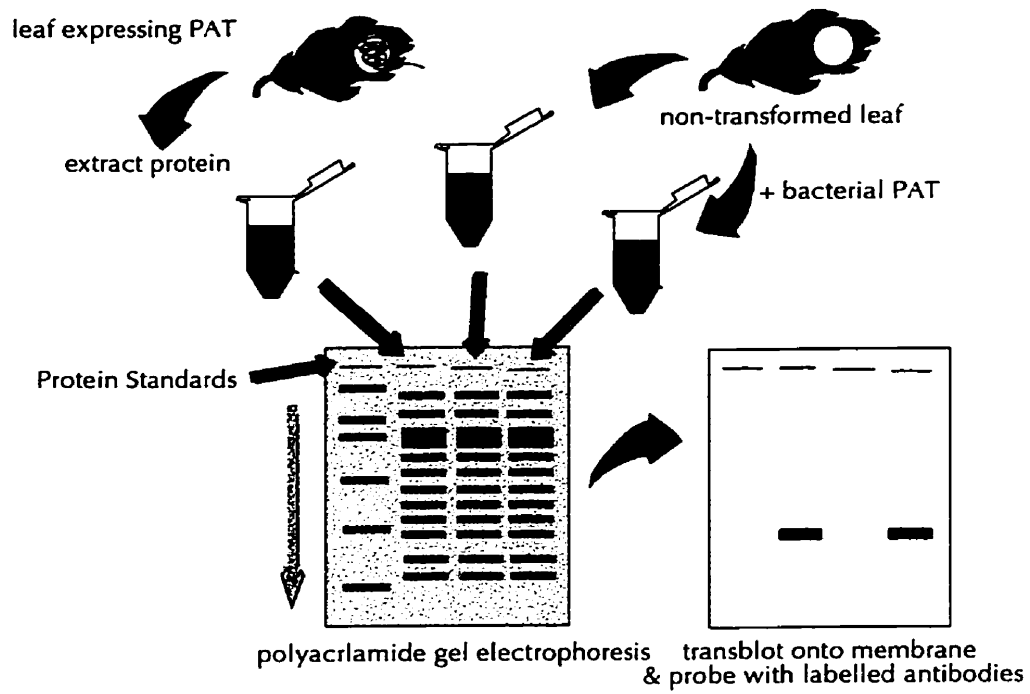
Figure 3.6: PAT Enzyme Linked Immunosorbant Assay

pnpp

IgG

PAT

biot-IgG

strep-AP
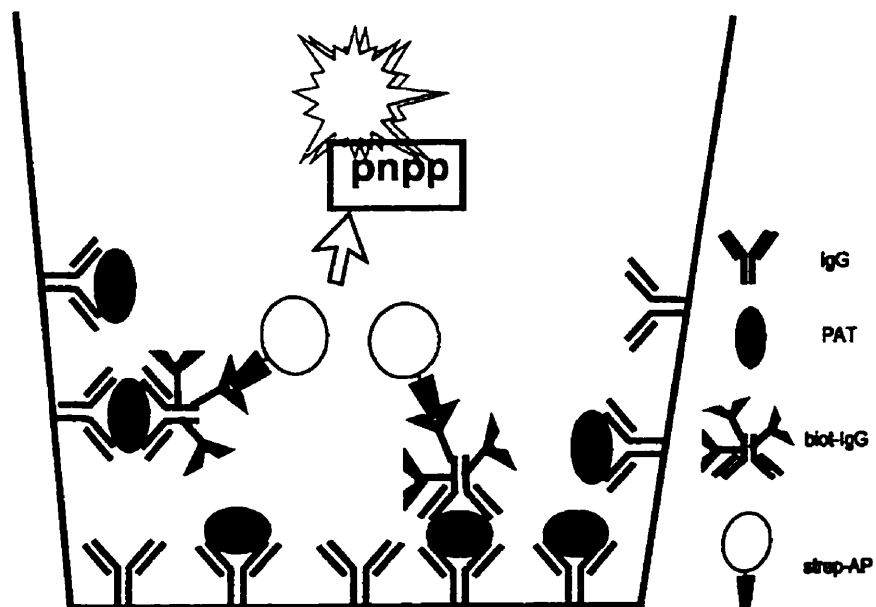
3.3 Results

3.3.1 Construction of the *pat::nptII* Fusion Gene

The construct containing the *pat* gene (Datla, R.; Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada) was digested with *NcoI* and *EcoRI* restriction endonucleases and ligated into the DNA polylinker of the *E. coli* cloning vector pSE280 (Brosius, 1989)(InVitrogen). The ligation product was transformed into competent *E. coli* DH5αMCR™ cells (Gibco BRL, cat. no. 18289-017) and successful transformants were isolated on 2YT plates containing 50 mg/L ampicillin.

The *pSE280-pat* plasmid was digested with *BglII* restriction endonuclease, followed by the removal of the 5'-phosphates using calf intestinal alkaline phosphatase (CIP) (Maniatis *et al.*, 1989) to prevent self-ligation. The linearized plasmid was then ligated to the *BglII* digested *nptII* gene from pTZ18:RD53-*nptII*-RD57 (Datla, R.; Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada) to produce an in-frame *pat::nptII* fusion. This ligation product was transformed into *E. coli* DH5αMCR™ cells and transformants were selected as described above.

The fusion junction between the *pat* and *nptII* genes was sequenced using the synthetic oligonucleotide primer OL-029 (5'-CGGCGGCATCAGAGCAG - 3'; Selvaraj, G., Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada). OL-029 binds near the 5'-end of the *nptII* gene and primes towards the 5'-end of the gene and upstream sequences (Figure 3.7). The sequencing data confirmed that the *pat* and *nptII* genes had undergone a translational in-frame fusion.

Figure 3.7   Sequence of the *pat::nptII* Gene Junction

ACA GCC CGG GGT ACA TTG CGC GCA **GCT** GGA TAC AAG CAT GGT GGA TGG CAT
Thr Ala Arg Gly Thr Leu Arg Ala Ala Gly Tyr Lys His Gly Gly Trp His

GAT GTT GGT TTT TGN CAA AGG GAT TTT GAG TTG CCA GCT CCT CCA AGG CCA
Asp Val Gly Phe ?   Gln Arg Asp Phe Glu Leu Pro Ala Pro Pro Arg Pro

GTT AGG CCA GTT ACC CAG ATC TCG AGC TTG GAT GGA TTG CAC GCA GCT TAC
Val Arg Pro Val Thr Gln Ile Ser Ser Leu Asp Gly Leu His Ala Gly Tyr

TCC ...
Ser

83

3.3.2   Expression and verification of the *pat::nptII* fusion gene in *Escherichia coli*

Escherichia coli DH5α cells, originally transformed with pSE280-*pat::nptII*, were spread onto 2YT plates containing 50 mg/L kanamycin and 50 mg/L ampicillin. Only colonies with pSE280 plasmid expressing a functional NPTII enzyme could grow on this selection medium. Seventeen colonies which grew on the kanamycin + ampicillin plates were selected and analyzed at the plasmid DNA level (analysed with *SalI*; *PstI*; *BglII*; and *SalI* + *EcoRI*) to verify the insertion of the *pat::nptII* into the pSE280 resident plasmid.

To determine whether the *pat::nptII* fusion construct produced a functional PAT and a functional NPTII protein, the seventeen colonies were subjected to a selection assay on plates containing kanamycin, L-PPT and kanamycin + L-PPT. An L-PPT killing curve was first produced to determine the level of L-PPT which would kill non-transformed DH5α cells. It was quickly determined that for the L-PPT to be toxic, the *E. coli* DH5α cells had to be grown on a minimal medium (M9 Minimal Medium, Appendix III) with a relatively high amount of L-PPT. The level of L-PPT needed to kill all non-transformed cells was determined by spreading *E. coli* DH5α cells on minimal medium containing increasing levels of L-PPT (Table 3.1).

From this killing curve, a level of 300 mg/L of L-PPT in minimal medium was found to be effective in preventing the growth of non-transformed *E. coli* DH5α cells.

Table 3.1: **Toxic Dose (TD_{100}) Determination for Phosphinothricin Against Non-transformed *E. coli* DH5α Cells**

Level of L-PPT (mg/L)	Amount of Growth
0	+++
20	+++
40	+++
60	+++
100	++
150	+
200	+ -
300	-

50 μl of 5.0 x 10^{8} cfu/mL (determined using a Petroff-Hausser bacteria counting chamber) were spread onto each M9 Minimal Media Selection plate; plates were incubated inverted at 37° C for 48 hours.

Legend: +++   thick lawn of bacteria   + faint growth visible (tiny colonies)

            ++   lawn of bacteria   - no visible growth

The TD_{100} of L-PPT on *E. coli* DH5α grown on M9 Minimal Media is 300 mg/L.

To determine the activity of the PAT and NPTII moieties in the *E. coli* pSE280-*pat::nptII* transformants, seventeen colonies (B1-B17, Table 3.2) were replica plated onto selection media consisting of:

- 50 mg/L ampicillin in 2YT

- 50 mg/L ampicillin + 50 mg/L kanamycin in 2YT

- 300 mg/L L-PPT in M9min

- 50 mg/L kanamycin + 300 mg/L L-PPT in M9min

Two controls, non-transformed *E. coli* DH5α, and *E. coli* transformed with phosphatased, self-ligated pSE280-*pat* (colonies A1-A6, Table 3.2.; only 6 colonies

grew when the entire ligation mix was plated out on 2YT + 50 mg/L ampicillin) were

also replica plated onto the selection plates.

Table 3.2   PAT::NPTII Activity Assay : Replica Plating onto Kanamycin and Phosphinothricin -Containing Selection Plates

Colony	2YT + 50 AMP	2YT + 50 amp + 50 KAN	M9 Min + 300 PPT	M9 Min + 300 PPT + 50 KAN
A1	+	-	-	-
A2	+	-	+	-
A3	+	-	+	-
A4	+	-	+	-
A5	+	-	-	-
A6	+	-	+	-
pSE280-pat	+	-	+	-
DH5α	-	-	-	-
B1	+	+	+	+
B2	+	+	+	+
B3	+	+	+	+
B4	+	+	+	+
B5	+	+	+	+
B6	+	+	+	+
B7	+	+	+	+
B8	+	+	+	+
B9	+	+	+	+
B10	+	+	+	+
B11	+	+	+	+
B12	+	+	+	+
B13	+	+	+	+
B14	+	+	+	+
B15	+	+	+	+
B16	+	+	+	+
B17	+	+	+	+

- growth on replica plates was assessed after 48 hours at 37 ° C

- A1 to A6 are Colonies containing the phosphatased, self-ligated pSE280-*pat* plasmid.

All seventeen transformed colonies (B1 to B17) grew in the presence of both kanamycin and L-PPT verifying that both products (PAT and NPTII) of the fusion gene construct were functional in the bacterial host. None of the non-transformed and self-ligated controls were able to grow in the presence of both selection agents (Table 3.2).

To verify the size of the fusion protein, a Western Blot analysis was performed on total protein isolated from non-transformed *E. coli* DH5α and *E. coli* DH5α-pSE280-*pat::nptII*. A sample of non-transformed *E. coli* DH5α was spiked with 20 ng of NPTII protein (5 Prime - 3 Prime Inc., cat.# 6-639134) and electrophoresed along with the other two samples. The electrophoresed protein banding patterns were visualised by staining one of the gels with Coomassie Brilliant Blue (Figure 3.8). The amount of protein loaded for each of the bacterial samples was 25 μg total protein. The other gel was subjected to an immunoblotting procedure and probed with the NPTII antibody. The non-transformed sample, spiked with the NPTII protein, demonstrated one NPTII specific band at 23 kilodaltons (kD). The *E. coli* DH5α-pSE280-*pat::nptII* protein sample demonstrated approximately four NPTII specific bands ranging in size from 30 kD up to 50 kD (Figure 3.9). These smaller antigenic peptides may have resulted from non-specific hybridisation or may have been caused by degradation of the fusion protein. Internal translation of the PAT and NPTII proteins in *E. coli* may have been another possibility for the presence of these smaller protein products.

Figure 3.8   Coomassie Brilliant Blue Stained SDS-Polyacrylamide

Gel of Bacteria and *Brassica napus* Protein Extracts
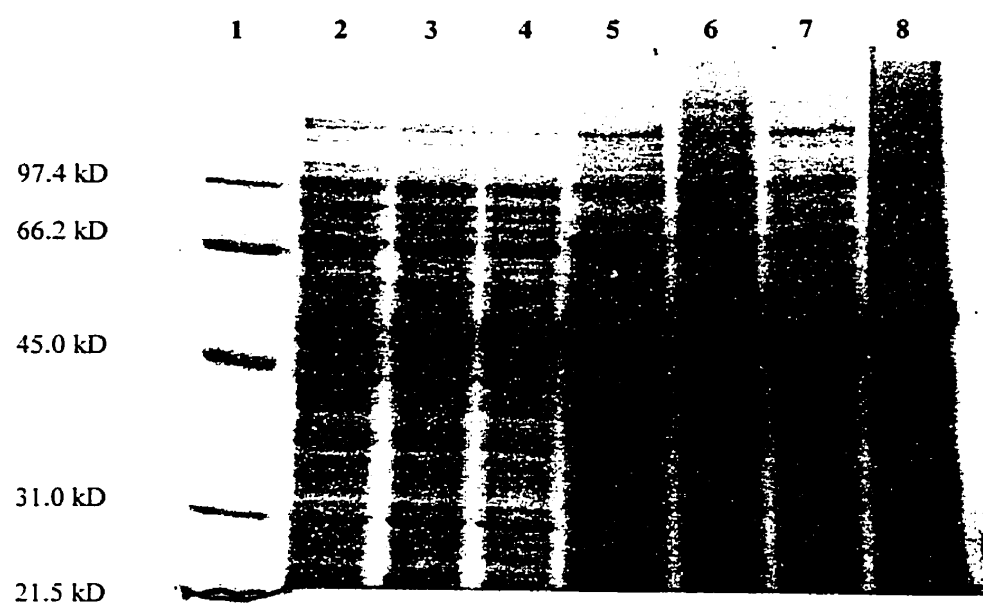
1. BioRad Low MW
2. *E. coli* DH5α pSE280-*pat::nptII*
3. *E. coli* DH5α wt
4. *E. coli* DH5α + 20 ng NPTII
5. HCN92
6. *B. napus* pBAU1 #4
7. *B. napus* pRD430 #2
8. *B. napus* wt

Figure 3.9   **Western Blot Assay of Bacteria and *Brassica napus***

Protein Extracts Containing the *pat::nptII* Fusion

Construct Probed with the NPTII Antibody
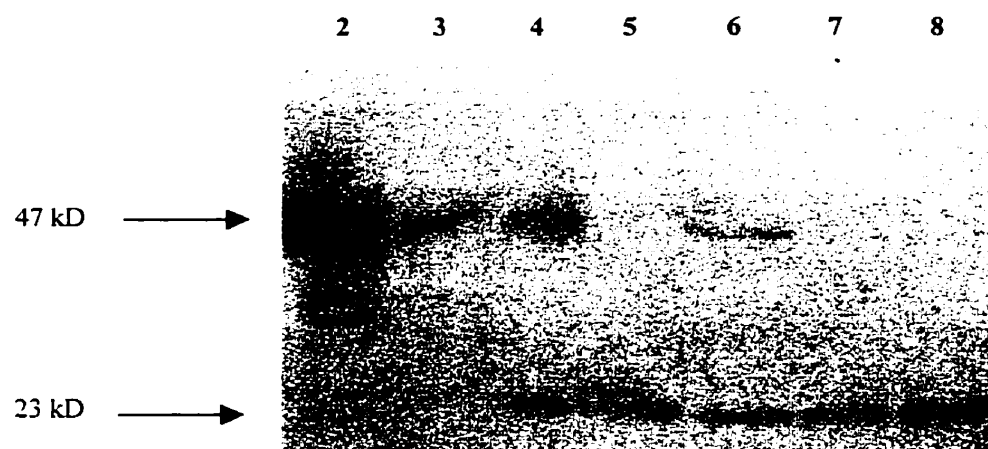
2   3   4   5   6   7   8

47 kD ──────►

23 kD ──────►

2. *E. coli* DH5α pSE280-*pat::nptII*
3. *E. coli* DH5α wt
4. *E. coli* DH5α + 20 ng NPTII
5. HCN92
6. *B. napus* pBAU1 #4
7. *B. napus* pRD430 #2
8. *B. napus* wt

Similarly, SDS-PAGEs were transblotted onto nitro-cellulose and subjected to immunoblot assays using a PAT specific primary antibody. The sample containing non-transformed *E. coli* DH5α + 50 ng of purified PAT protein (Schulz, A., Hoechst AG, Frankfurt, Germany) demonstrated only one PAT specific band at approximately 22 kD. The *E. coli* DH5α-pSE280-*pat::nptII* sample demonstrated two PAT specific bands at 50 kD and at 47 kD (Figure 3.10). The higher (approx. 50 kD) protein band which is reactive to both PAT and NPTII antibodies represents the fusion protein. The smaller bands (47 kD reactive to the PAT antibody and 30 kD reactive to the NPTII antibody) are most likely processed products or internally translated independent products.

Figure 3.10   Western Blot Assay of Bacterial Lysates

Containing the *pat::nptII* Fusion Construct

Probed with the PAT Antibody
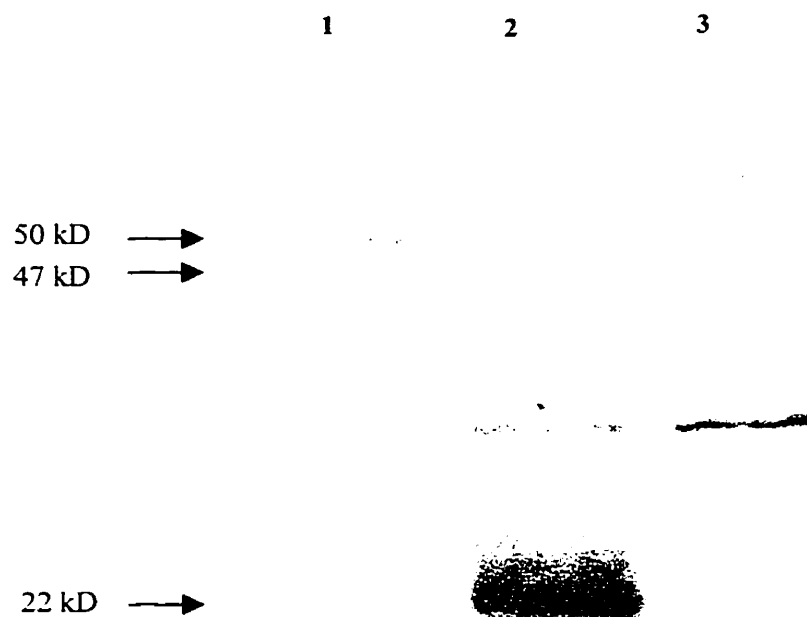
1. *E. coli* DH5α pSE280-*pat::nptII*
2. *E. coli* DH5α + 50 ng PAT
3. *E. coli* DH5α wt

3.3.3 Construction of the *pat::nptII* Bifunctional Binary Vector

The *pat::nptII* fusion gene was isolated from the pSE280-*pat::nptII* plasmid vector using a partial *BglII* digest to ensure that only the 3'-end of the *nptII* gene was restricted. This was followed by a *SalI* digest which cut at the 5'-end of the *pat* gene. The entire *SalI-pat::nptII-BglII* (1.3 kb) was ligated into a *SalI/BamHI* digested pRD300 (3.5 kb; based on pBI524, Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada; which is a pUC9 [2.8 kb] derivative) (Figure 3.2) cloning vector containing the plant expression cassette: *35S35S P-AMV-* MCS *– nosT*. Competent *E. coli* DH5αMCR™ cells were transformed with the ligation products and colonies containing the *pat::nptII* fusion gene cassette in pRD300 (4.8 kb) were selected.

pRD300-*pat::nptII*, as well as the binary vector pBI434 (BinSyn binary vector, 7.5 kb, Datla, unpublished) were digested with *HindIII* and *EcoRI* (Figure 3.11). The *HindIII-35S35S-AMV-pat::nptII-nosT-EcoRI* cassette was then ligated to the *HindIII/EcoRI* digested pBI434 binary vector to derive pBAU1, and subsequently transformed into competent *E. coli* DH5αMCR™ cells. Transformants were plated onto kanamycin 2YT plates (since pBI434 contains the kan[r] gene from *Streptococcus*) and colonies containing pBAU1 were selected. Selected recombinant colonies were then subjected to a triparental mating (Rogers *et al.*, 1986) to transfer the pBAU1 binary vector from *E. coli* into *A. tumefaciens* pMP90 (Koncz and Schell 1986).

Figure 3.11 The Construction of the Plant Binary Vector, pBAU1
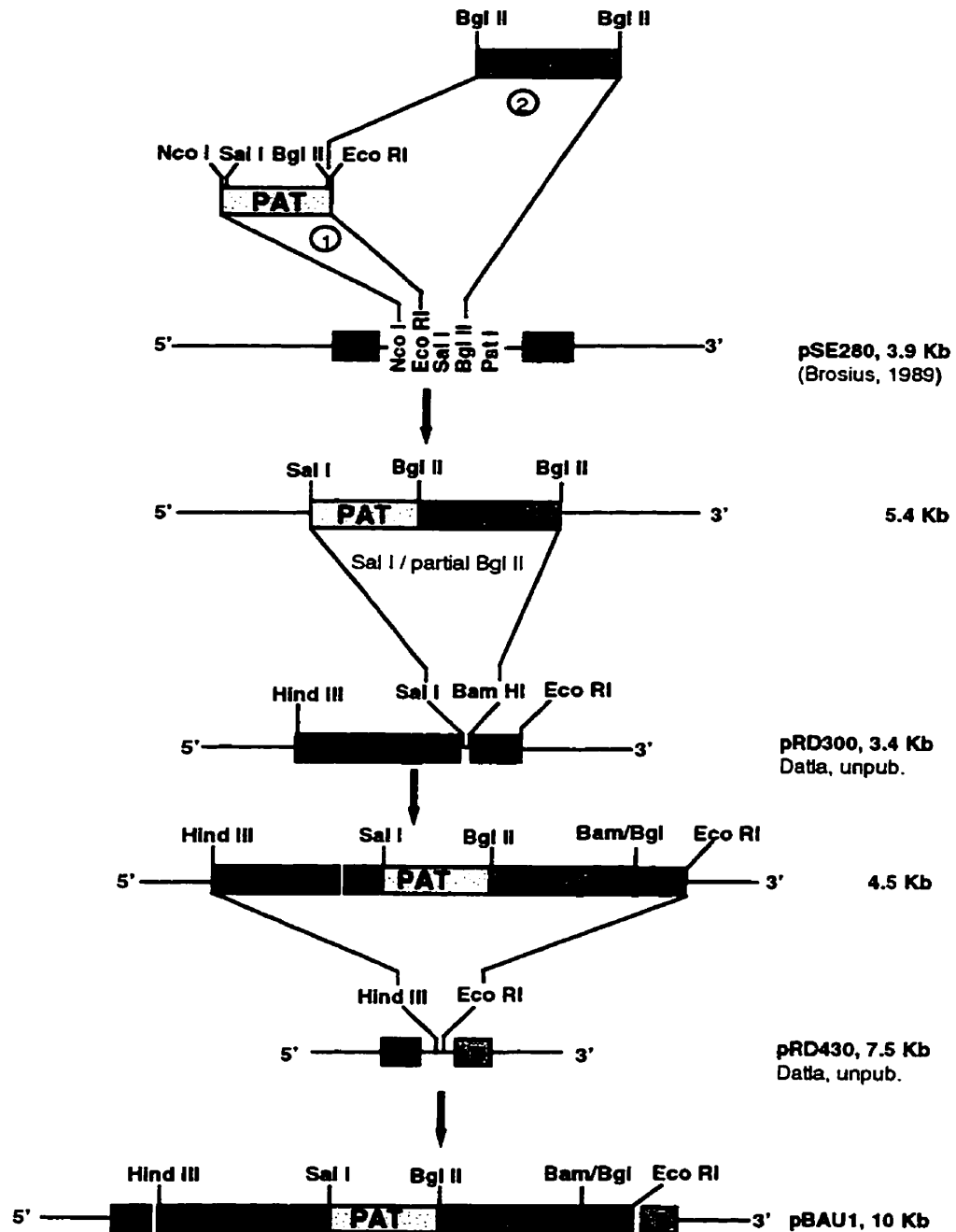
3.3.4 Transfer of the *pat::nptII* Fusion Gene into Plants

The *pat::nptII* fusion gene was transferred into the pBAU1 binary vector (Figure 3.2) and transformed into *Brassica napus* cv. Westar and into *Nicotiana tabacum* cv. Xanthi.

Brassica napus cv. Westar Cotyledonary Petiole Transformation

The cotyledonary petioles from 4 day old seedlings were inoculated with a diluted *Agrobacterium tumefaciens* pMP90-pBAU1 suspension (1:8 of *Agrobacterium* @ $OD_{590nm} = 1.0$: AB Minimal Medium), placed onto cocultivation medium for 3 days, followed by *Agrobacterium* Clean-Up medium for 7 days, and then transferred to selection medium (20 mg/L of kanamycin) or non-selection medium (0 mg/L kanamycin; control). After 3 weeks on selection medium, cotyledonary petiole explants were scored for their ability to regenerate shoots. The regeneration data from 3 separate experiments is summarised in Table 3.3. In the absence of a selection agent (0 mg/L kanamycin) the regeneration frequency of explants was the same for explants treated with *Agrobacterium* as for those not exposed to *Agrobacterium*. In the presence of kanamycin, explants treated with *Agrobacterium* containing pBAU1 demonstrated a slightly lower transformation frequency (1.0 ± 0.1%) than explants treated with the control *Agrobacterium* containing pRD430 (1.9 ± 1.4%). The percentage regeneration of nontransformed shoots in the presence of 20 mg/L of kanamycin (from explants not exposed to Agrobacterium) was 1.3 ± 2.2%. These shoots, often referred to as 'escapes', can only be distinguished from true transformants by PCR diagnostics or by placing these explants on a more stringent selection agent, such as L-PPT.

Table 3.3: **Regeneration and Transformation Frequencies of**
Brassica napus **cv Westar Cotyledonary Petioles Transformed with**
Agrobacterium tumefaciens **pMP90-pBAU1, along with Controls**

	Total # of Explants	# of Explants With Regenerating Shoots (green + purple)	# of Explants with Purple Shoots	# of Explants with Green Shoots	# of Green Shoots Rooting on 20 mg/L L-PPT	% Transformation Frequency ± Standard Deviation	% Regeneration Frequency ± Standard Deviation
Regeneration on 0 mg/L kanamycin							
no Agro.	129	113	0	113	0		88.5 ± 4.7 %
pRD430	92	82	0	82	0		89.6 ± 9.5 %
pBAU1	384	322	0	322	0		83.8 ± 3.1 %
Regeneration on 20 mg/L kanamycin							
no Agro.	181	116	113	3	0	0.0 ± 0.0 %	1.3 ± 2.2 %
pRD430	389	185	174	11	7	1.9 ± 1.4 %	3.1 ± 2.8 %
pBAU1	1595	654	608	46	16	1.0 ± 0.1%	2.8 ± 0.6 %

Standard Deviation =   the deviation between frequencies obtained from three different
                            experiments

Transformation Frequency = the # of green shoots rooting on 20 mg/L L-PPT divided by the total
                            number of explants

Regeneration Frequency =   the number of explants with green shoots divided by the total
                            number of explants

Nicotiana tabacum cv. Xanthi Leaf Disk Transformation

Leaf disks which had been inoculated with *Agrobacterium tumefaciens*
pMP90-pBAU1 and pMP90-pRD430 (control) were transferred to Cocultivation
Medium for 2 days and then directly onto Regeneration Medium containing 20 mg/L
kanamycin or 0 mg/L kanamycin (control). After 4 weeks, regenerating leaf disks
were scored and transferred to fresh Regeneration Medium. The regeneration data
from three separate transformation experiments are summarised in Table 3.4. As with
the *Brassica* petiole transformation experiment, the *Nicotiana* leaf disks also
demonstrated a slightly lower transformation frequency when transformed with
pBAU1 as with the control pRD430 (where both *pat* and *nptII* genes are regulated by
separate promoters).

Table 3.4: **Regeneration and Transformation Frequencies of**
Nicotiana tabacum cv. Xanthi Leaf Disks Transformed with
Agrobacterium tumefaciens pMP90-pBAU1, along with Controls

	Total # of Explants	# of Explants With Regenerating Shoots	# of Explants with Green Calli	# of Explants with Green Shoots	# of Green Shoots Rooting on 60 mg/L L-PPT	% Transformation Frequency ± Standard Deviation	% Regeneration Frequency ± Standard Deviation
Regeneration on 0 mg/L kanamycin							
no Agro.	144	139	141	139	0		96.5 ± 2.6 %
pRD430	52	48	51	48	0		92.4 ± 0.4 %
pBAU1	42	38	41	38	0		90.5 ± 4.1 %
Regeneration on 20 mg/L kanamycin							
no Agro.	142	1	1	1	0	0.0 ± 0.0 %	0.9 ± 1.6 %
pRD430	198	140	153	140	118	58.3 ± 42.2 %	69.0 ± 51.4 %
pBAU1	905	374	401	374	345	44.7 ± 29.4%	48.6 ± 32.6 %

Standard Deviation = the deviation between frequencies obtained from three different experiments

Transformation Frequency = the # of green shoots rooting on 60 mg/L L-PPT divided by the total number of explants

Regeneration Frequency = the number of explants with green shoots divided by the total number of explants

3.3.5 The Expression of the _pat::nptII_ Fusion Gene in Transgenic Plants

PCR Analysis

The presence of the _pat_ and _nptII_ sequences was evaluated in the primary _in vitro_ transformants that had rooted on L-PPT. Four _B. napus_ cv. Westar plantlets and six _N. tabacum_ cv. Xanthi plantlets were randomly selected and subjected to two PCR reactions; each reaction containing primers specific for either the _pat_ or the _nptII_ sequence. A _N. tabacum_ cv. Xanthi plantlet transformed with pRD430, a non-transformed _B. napus_ cv. Westar and a non-transformed _N. tabacum_ cv. Xanthi were used as controls. All of the tested plantlets which had rooted on the L-PPT (pBAU1

and pRD430) demonstrated a *pat* specific band (400 bp; as illustrated in Figure 3.12) and a *nptII* specific band (750 bp; not shown). The non-transformed controls did not show the presence of a PCR-product.

Southern Hybridization

Southern Blot assays (Southern 1975) were performed on DNA extracted from *B. napus* and *N. tabacum in vitro* plantlets. Copy number estimates were derived from banding patterns obtained from DNA digested with *EcoRI* (not shown) and *HindIII* (Figure 3.13) and probed with the random primed *pat* probe (550 bp). Most of the *N. tabacum* pBAU1 transformants were single locus insertions, whereas approximately 50% of the *B. napus* pBAU1 transformants tested demonstrated two or more insertion loci. A similar trend was also observed in tobacco and *B. napus* transformants produced using the control binary vector pRD430 (personal observation; results not shown).

A control *PstI* digest probed with *pat* was also performed which demonstrated the presence of the tag (Figure 3.14). Since *PstI* cuts twice within the T-DNA, no copy number estimate could be made. The *PstI* digest could be used to distinguish between transformants derived from the pRD430 construct versus those derived from pBAU1.

**Figure 3.12   PCR Products Following Amplification of the *pat* Gene
In Transformed Plant Tissue**

2036 bp
1636 bp
1018 bp

506 bp → 400 bp

1   2   3   4   5   6   7   8   9   10   11   12   13   14   15   16   17   18   19
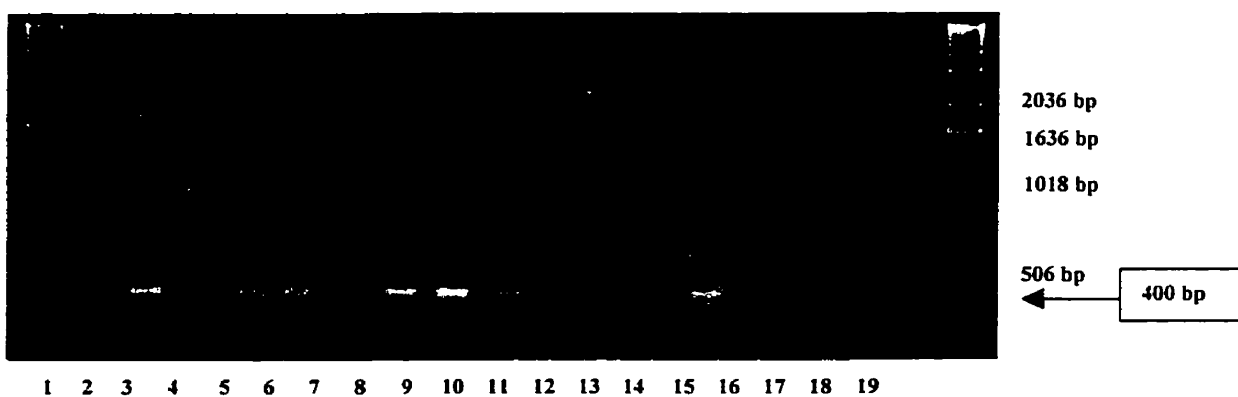
Lanes 1 and 19 contain the BRL 1kb molecular weight markers.

The remaining lanes are as follows:

2 = *B. napus* non-transformed

3 = *B. napus* pBAU1 #1

4 = *B. napus* pBAU1 #2

5 = *B. napus* pBAU1 #3

6 = *B. napus* pBAU1 #4

7 = *N. tabacum* non-transformed

8 = *N. tabacum* pBAU1 #1

9 = *N. tabacum* pBAU1 #2

10 = *N. tabacum* pBAU1 #3

11 = *N. tabacum* pBAU1 #4

12 = *N. tabacum* pBAU1#5

13 = *N. tabacum* pBAU1 #6

14 = *N. tabacum* pRD430 #1

15 = water (negative control)

16 = extraction buffer

17 = TE buffer

18 = blank

98

Figure 3.13 **Copy Number Estimate of pBAU1 Transformed Canola and Tobacco Plants Using Southern Blot Analysis**

1. *N. tabacum* non-transformed
2. *N. tabacum* pBAU1 #3; one locus
3. *N. tabacum* pBAU1 #18; one locus
4. *N. tabacum* pBAU1 #11; one locus
5. *N. tabacum* pBAU1 #2; one locus
6. *N. tabacum* pBAU1 #1; one locus
7. *N. tabacum* pRD430 #1; two loci
8. *B. napus* non-transformed
9. *B. napus* pBAU1 #4; two loci
10. *B. napus* pBAU1 #1; one locus
11. *B. napus* pRD430 #2; two loci
12. *B. napus* pRD430 #1; one locus
13. BRL 1 kb MW

A. **Ethidium Bromide Stained Agarose Gel of Genomic DNA digested with *HindIII***

1   2   3   4   5   6   7   8   9   10   1 1   12   13

B.   *HindIII* digest probed with the *pat* gene

1   2   3   4   5   6   7   8   9   10   11   12
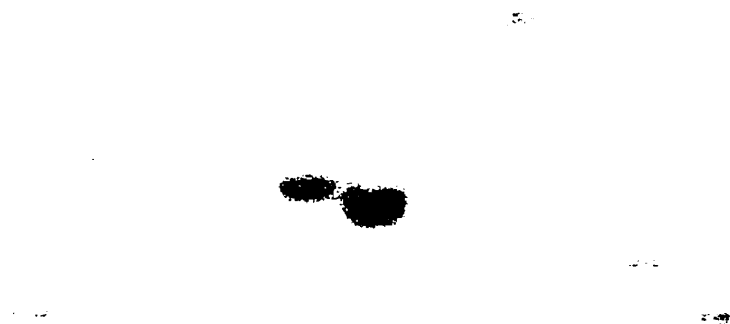
Figure 3.14 **Southern Blot Analysis of the T-DNA in Canola and Tobacco Plants Transformed with pBAU1:** *PstI* **digest probed with the** *pat* **gene**

1   2   3   4   5   6   7   8   9   10   11   12
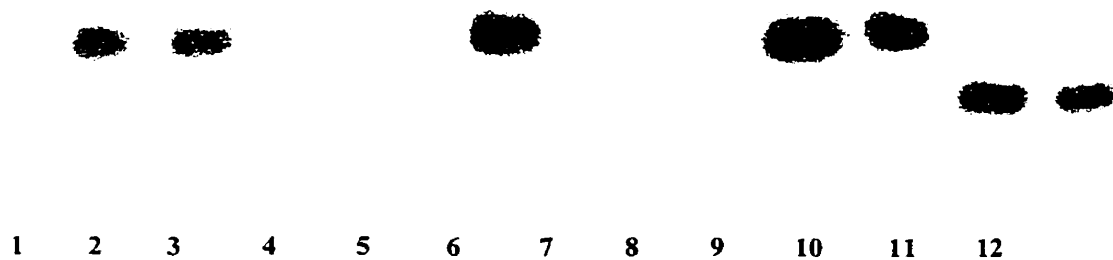
1 = *N. tabacum* non-transformed
2 = *N. tabacum* pBAU1 #3
3 = *N. tabacum* pBAU1#18
4 = *N. tabacum* pBAU1 #11
5 = *N. tabacum* pBAU1 #2
6 = *N. tabacum* pBAU1 #1
7 = *N. tabacum* pRD430 #1
8 = *B. napus* non-transformed
9 = *B. napus* pBAU1#4
10 = *B. napus* pBAU1 #1
11 = *B. napus* pRD430 #2
12 = *B. napus* pRD430 #1

Rooting Assays

The results from three rooting assays, each performed in triplicate is illustrated in Figure 3.15 for pBAU1 transformants in *Brassica napus*, and in Figure 3.16 for pBAU1 transformants in *Nicotiana tabacum* (raw data in Appendix IV). The most commonly used indicator for strength of *pat* expression is the ability for a transformant (containing the *pat* gene) to root in the presence of L-PPT. Two other parameters, average number of leaves produced and average amount of leaf necrosis, were also evaluated alongside the rooting ability to see whether these parameters could also be used as determinants for assessing the relative strength of transgene expression. Clones from transformed lines were tested on four levels of L-PPT (0, 60, 120 and 240 mg/L) and evaluated at 4 time points (day 0, 6, 12 and 20).

At day 12 and at day 20, all three determinants (rooting ability, number of leaves, amount of leaf necrosis) produced the same top four ranking for the *B. napus* transformed lines when tested at the highest L-PPT concentration (Figure 3.15, Appendix IV). The four *B. napus* lines which showed the highest average rooting ability, the largest average number of leaves and the lowest amount of leaf necrosis at 240 mg/L L-PPT were: HCN92 (highest rank), pBAU1#11 , pRD430#3 and pBAU#3. HCN92 was a sixth generation (T_6) transformed line (control) containing the pat gene regulated by the P35S-CaMV promoter (AgrEvo Canada Inc., Saskatoon, SK, Canada). pBAU1#11, pBAU1#3 and pRD430#3 were T_0 lines which had been clonally propagated to produce enough shoot material for the rooting assays.

The most relevant internal control, albeit only one, for this rooting assay was the pRD430#3 transformed line since it was the same generation level as the pBAU1 material and it was derived from a similar *Agrobacterium* transformation vector. In pRD430 the *pat* gene is regulated by the tandem 35S-CaMV promoter-AMV enhancer. The *pat::nptII* fusion gene in pBAU1 is regulated by the same promoter-enhancer combination.

The same three determinants were also used to evaluate *N. tabacum* pBAU1 transformed lines relative to a positive control (pRD430 line) and a negative control (non-transformed wild type). In the *N. tabacum* rooting assay, though, no correlation could be made either at day 12 or at day 20 for the three determinants. Based on the average rooting ability at 240 mg/L L-PPT at day 20 the two best lines were pBAU1#8 and pBAU1#11. At day 12, six different lines demonstrated the same high rooting ability (3.0) (Figure 3.16, Appendix IV), indicating that this evaluation date was too early to determine any variability between the lines. The highest herbicide concentration, 240 mg/L L-PPT, may have been too low to show variation for the other two determinants (average amount of leaf necrosis and average number of leaves).

Figure 3.15 *Brassica napus* pBAU1 Rooting Assay at Day 0, 6, 12 and 20, on Increasing Levels of Glufosinate Ammonium

<u>Legend:</u>

Green Bar = Average number of leaves
Orange Bar = Average Level of Necrosis
Blue Bar = Average Rooting Ability

<u>Level of Necrosis:</u>

1 = 0 to 5 % leaf surface area
2 = 6 to 25 % leaf surface area
3 = 26 to 50 % leaf surface area
4 = 51 to 75% leaf surface area
5 = 76 to 100% leaf surface area

<u>Rooting Ability:</u>

0 = no roots
1 = root bud formation or less than 3 roots per plant
2 = roots were 1 to 10 mm in length and more than 3 roots per plant
3 = roots were greater than 10 mm in length and more than 3 roots per plant

Standard deviations for the graphed data can be found in Appendix IV.

B. napus pBAU1: Day 6

0 mg/L : HCN92
0 mg/L: pBAU1#3
0 mg/L: pBAU1#2
0 mg/L: pBAU1#1
0 mg/L: pBAU1#4
0 mg/L: pBAU1#7
0 mg/L: pBAU1#11
0 mg/L: pBAU1#8
0 mg/L: pBAU1#10
0 mg/L: pRD430#3
0 mg/L: Bn wt

60 mg/L : HCN92
60 mg/L: pBAU1#3
60 mg/L: pBAU1#2
60 mg/L: pBAU1#1
60 mg/L: pBAU1#4
60 mg/L: pBAU1#7
60 mg/L: pBAU1#11
60 mg/L: pBAU1#8
60 mg/L: pBAU1#10
60 mg/L: pRD430#3
60 mg/L: Bn wt

120 mg/L : HCN92
120 mg/L: pBAU1#3
120 mg/L: pBAU1#2
120 mg/L: pBAU1#1
120 mg/L: pBAU1#4
120 mg/L: pBAU1#7
120 mg/L: pBAU1#11
120 mg/L: pBAU1#8
120 mg/L: pBAU1#10
120 mg/L: pRD430#3
120 mg/L: Bn wt

240 mg/L : HCN92
240 mg/L: pBAU1#3
240 mg/L: pBAU1#2
240 mg/L: pBAU1#1
240 mg/L: pBAU1#4
240 mg/L: pBAU1#7
240 mg/L: pBAU1#11
240 mg/L: pBAU1#8
240 mg/L: pBAU1#10
240 mg/L: pRD430#3
240 mg/L: Bn wt

Avg. Rooting Ability
Avg. Level of Necrosis
Avg. # of Leaves

B. napus pBAU1: Day 0

0 mg/L : HCN92
0 mg/L: pBAU1#3
0 mg/L: pBAU1#2
0 mg/L: pBAU1#1
0 mg/L: pBAU1#4
0 mg/L: pBAU1#7
0 mg/L: pBAU1#11
0 mg/L: pBAU1#8
0 mg/L: pBAU1#10
0 mg/L: pRD430#3
0 mg/L: Bn wt

60 mg/L : HCN92
60 mg/L: pBAU1#3
60 mg/L: pBAU1#2
60 mg/L: pBAU1#1
60 mg/L: pBAU1#4
60 mg/L: pBAU1#7
60 mg/L: pBAU1#11
60 mg/L: pBAU1#8
60 mg/L: pBAU1#10
60 mg/L: pRD430#3
60 mg/L: Bn wt

120 mg/L : HCN92
120 mg/L: pBAU1#3
120 mg/L: pBAU1#2
120 mg/L: pBAU1#1
120 mg/L: pBAU1#4
120 mg/L: pBAU1#7
120 mg/L: pBAU1#11
120 mg/L: pBAU1#8
120 mg/L: pBAU1#10
120 mg/L: pRD430#3
120 mg/L: Bn wt

240 mg/L : HCN92
240 mg/L: pBAU1#3
240 mg/L: pBAU1#2
240 mg/L: pBAU1#1
240 mg/L: pBAU1#4
240 mg/L: pBAU1#7
240 mg/L: pBAU1#11
240 mg/L: pBAU1#8
240 mg/L: pBAU1#10
240 mg/L: pRD430#3
240 mg/L: Bn wt

Avg. Level of Necrosis
Avg. # of Leaves

B. napus pBAU1: Day 20

Scale: 0 2 4 6 8 10 12 14 16 18

0 mg/L : HCN92
0 mg/L: pBAU1#3
0 mg/L: pBAU1#2
0 mg/L: pBAU1#1
0 mg/L: pBAU1#4
0 mg/L: pBAU1#7
0 mg/L: pBAU1#11
0 mg/L: pBAU1#8
0 mg/L: pBAU1#10
0 mg/L: pRD430#3
0 mg/L: Bn wt

60 mg/L : HCN92
60 mg/L: pBAU1#3
60 mg/L: pBAU1#2
60 mg/L: pBAU1#1
60 mg/L: pBAU1#4
60 mg/L: pBAU1#7
60 mg/L: pBAU1#11
60 mg/L: pBAU1#8
60 mg/L: pBAU1#10
60 mg/L: pRD430#3
60 mg/L: Bn wt

120 mg/L : HCN92
120 mg/L: pBAU1#3
120 mg/L: pBAU1#2
120 mg/L: pBAU1#1
120 mg/L: pBAU1#4
120 mg/L: pBAU1#7
120 mg/L: pBAU1#11
120 mg/L: pBAU1#8
120 mg/L: pBAU1#10
120 mg/L: pRD430#3
120 mg/L: Bn wt

240 mg/L : HCN92
240 mg/L: pBAU1#3
240 mg/L: pBAU1#2
240 mg/L: pBAU1#1
240 mg/L: pBAU1#4
240 mg/L: pBAU1#7
240 mg/L: pBAU1#11
240 mg/L: pBAU1#8
240 mg/L: pBAU1#10
240 mg/L: pRD430#3
240 mg/L: Bn wt

Avg. Rooting Ability
Avg. Level of Necrosis
Avg. # of Leaves

B. napus-pBAU1: Day-12

Scale: 0 2 4 6 8 10 12 14 16 18

0 mg/L : HCN92
0 mg/L: pBAU1#3
0 mg/L: pBAU1#2
0 mg/L: pBAU1#1
0 mg/L: pBAU1#4
0 mg/L: pBAU1#7
0 mg/L: pBAU1#11
0 mg/L: pBAU1#8
0 mg/L: pBAU1#10
0 mg/L: pRD430#3
0 mg/L: Bn wt

60 mg/L : HCN92
60 mg/L: pBAU1#3
60 mg/L: pBAU1#2
60 mg/L: pBAU1#1
60 mg/L: pBAU1#4
60 mg/L: pBAU1#7
60 mg/L: pBAU1#11
60 mg/L: pBAU1#8
60 mg/L: pBAU1#10
60 mg/L: pRD430#3
60 mg/L: Bn wt

120 mg/L : HCN92
120 mg/L: pBAU1#3
120 mg/L: pBAU1#2
120 mg/L: pBAU1#1
120 mg/L: pBAU1#4
120 mg/L: pBAU1#7
120 mg/L: pBAU1#11
120 mg/L: pBAU1#8
120 mg/L: pBAU1#10
120 mg/L: pRD430#3
120 mg/L: Bn wt

240 mg/L : HCN92
240 mg/L: pBAU1#3
240 mg/L: pBAU1#2
240 mg/L: pBAU1#1
240 mg/L: pBAU1#4
240 mg/L: pBAU1#7
240 mg/L: pBAU1#11
240 mg/L: pBAU1#8
240 mg/L: pBAU1#10
240 mg/L: pRD430#3
240 mg/L: Bn wt

Avg. Rooting Ability
Avg. Level of Necrosis
Avg. # of Leaves

Figure 3.16 *Nicotiana tabacum* pBAU1 Rooting Assay at Day 0, 6, 12 and 20

Legend:

Green Bar = Average number of leaves
Orange Bar = Average Level of Necrosis
Blue Bar = Average Rooting Ability

Level of Necrosis:

1 = 0 to 5 % leaf surface area
2 = 6 to 25 % leaf surface area
3 = 26 to 50 % leaf surface area
4 = 51 to 75% leaf surface area
5 = 76 to 100% leaf surface area

Rooting Ability:

0 = no roots
1 = root bud formation or less than 3 roots per plant
2 = roots were 1 to 10 mm in length and more than 3 roots per plant
3 = roots were greater than 10 mm in length and more than 3 roots per plant

Standard deviations for the graphed data can be found in Appendix IV.

N. tabacum pBAU1: Day-0

Avg. # of Leaves
Avg. Level of Necrosis

0 mg/L : NtWt
0 mg/L: pBAU1#5
0 mg/L: pBAU1#7
0 mg/L: pBAU1#8
0 mg/L: pBAU1#9
0 mg/L: pBAU1#10
0 mg/L: pBAU1#6
0 mg/L: pBAU1#11
0 mg/L: pBAU1#12
0 mg/L: pRD430#2

60 mg/L : NtWt
60 mg/L: pBAU1#5
60 mg/L: pBAU1#7
60 mg/L: pBAU1#8
60 mg/L: pBAU1#9
60 mg/L: pBAU1#10
60 mg/L: pBAU1#6
60 mg/L: pBAU1#11
60 mg/L: pBAU1#12
60 mg/L: pRD430#2

120 mg/L : NtWt
120 mg/L: pBAU1#5
120 mg/L: pBAU1#7
120 mg/L: pBAU1#8
120 mg/L: pBAU1#9
120 mg/L: pBAU1#10
120 mg/L: pBAU1#6
120 mg/L: pBAU1#11
120 mg/L: pBAU1#12
120 mg/L: pRD430#2

240 mg/L : NtWt
240 mg/L: pBAU1#5
240 mg/L: pBAU1#7
240 mg/L: pBAU1#8
240 mg/L: pBAU1#9
240 mg/L: pBAU1#10
240 mg/L: pBAU1#6
240 mg/L: pBAU1#11
240 mg/L: pBAU1#12
240 mg/L: pRD430#2

14 12 10 8 6 4 2 0

N. tabacum pBAU1: Day-6

Avg. # of Leaves
Avg. Level of Necrosis
Avg. Rooting Ability

0 mg/L : NtWt
0 mg/L: pBAU1#5
0 mg/L: pBAU1#7
0 mg/L: pBAU1#8
0 mg/L: pBAU1#9
0 mg/L: pBAU1#10
0 mg/L: pBAU1#6
0 mg/L: pBAU1#11
0 mg/L: pBAU1#12
0 mg/L: pRD430#2

60 mg/L : NtWt
60 mg/L: pBAU1#5
60 mg/L: pBAU1#7
60 mg/L: pBAU1#8
60 mg/L: pBAU1#9
60 mg/L: pBAU1#10
60 mg/L: pBAU1#6
60 mg/L: pBAU1#11
60 mg/L: pBAU1#12
60 mg/L: pRD430#2

120 mg/L : NtWt
120 mg/L: pBAU1#5
120 mg/L: pBAU1#7
120 mg/L: pBAU1#8
120 mg/L: pBAU1#9
120 mg/L: pBAU1#10
120 mg/L: pBAU1#6
120 mg/L: pBAU1#11
120 mg/L: pBAU1#12
120 mg/L: pRD430#2

240 mg/L : NtWt
240 mg/L: pBAU1#5
240 mg/L: pBAU1#7
240 mg/L: pBAU1#8
240 mg/L: pBAU1#9
240 mg/L: pBAU1#10
240 mg/L: pBAU1#6
240 mg/L: pBAU1#11
240 mg/L: pBAU1#12
240 mg/L: pRD430#2

14 12 10 8 6 4 2 0

N. tabacum pBAU1: Day 12

Avg. # of Leaves
Avg. Level of Necrosis
Avg. Rooting Ability

0 mg/L : N/wt
0 mg/L: pBAU1#5
0 mg/L: pBAU1#7
0 mg/L: pBAU1#8
0 mg/L: pBAU1#9
0 mg/L: pBAU1#10
0 mg/L: pBAU1#6
0 mg/L: pBAU1#11
0 mg/L: pBAU1#12
0 mg/L: pRD430#2

60 mg/L : N/wt
60 mg/L: pBAU1#5
60 mg/L: pBAU1#7
60 mg/L: pBAU1#8
60 mg/L: pBAU1#9
60 mg/L: pBAU1#10
60 mg/L: pBAU1#6
60 mg/L: pBAU1#11
60 mg/L: pBAU1#12
60 mg/L: pRD430#2

120 mg/L : N/wt
120 mg/L: pBAU1#5
120 mg/L: pBAU1#7
120 mg/L: pBAU1#8
120 mg/L: pBAU1#9
120 mg/L: pBAU1#10
120 mg/L: pBAU1#6
120 mg/L: pBAU1#11
120 mg/L: pBAU1#12
120 mg/L: pRD430#2

240 mg/L: N/wt
240 mg/L: pBAU1#5
240 mg/L: pBAU1#7
240 mg/L: pBAU1#8
240 mg/L: pBAU1#9
240 mg/L: pBAU1#10
240 mg/L: pBAU1#6
240 mg/L: pBAU1#11
240 mg/L: pBAU1#12
240 mg/L: pRD430#2

14 12 10 8 6 4 2 0

N. tabacum pBAU1: Day 20

Avg. # of Leaves
Avg. Level of Necrosis
Avg. Rooting Ability

0 mg/L : N/wt
0 mg/L: pBAU1#5
0 mg/L: pBAU1#7
0 mg/L: pBAU1#8
0 mg/L: pBAU1#9
0 mg/L: pBAU1#10
0 mg/L: pBAU1#6
0 mg/L: pBAU1#11
0 mg/L: pBAU1#12
0 mg/L: pRD430#2

60 mg/L : N/wt
60 mg/L: pBAU1#5
60 mg/L: pBAU1#7
60 mg/L: pBAU1#8
60 mg/L: pBAU1#9
60 mg/L: pBAU1#10
60 mg/L: pBAU1#6
60 mg/L: pBAU1#11
60 mg/L: pBAU1#12
60 mg/L: pRD430#2

120 mg/L : N/wt
120 mg/L: pBAU1#5
120 mg/L: pBAU1#7
120 mg/L: pBAU1#8
120 mg/L: pBAU1#9
120 mg/L: pBAU1#10
120 mg/L: pBAU1#6
120 mg/L: pBAU1#11
120 mg/L: pBAU1#12
120 mg/L: pRD430#2

240 mg/L: N/wt
240 mg/L: pBAU1#5
240 mg/L: pBAU1#7
240 mg/L: pBAU1#8
240 mg/L: pBAU1#9
240 mg/L: pBAU1#10
240 mg/L: pBAU1#6
240 mg/L: pBAU1#11
240 mg/L: pBAU1#12
240 mg/L: pRD430#2

14 12 10 8 6 4 2 0

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<u>Enzyme Activity Assays</u>

Two enzyme activity assays, one to determine PAT activity and the other to determine NPTII activity, were performed on leaf tissue from T_0 *in vitro B. napus* and *N. tabacum* transformants. Both PAT and NPTII enzyme activities were detected in all pBAU1 transformants tested. All tobacco pBAU1 transformants qualitatively demonstrated a higher activity level (a more intense radionuclide acetyl-PPT signal for the PAT assay and a more intense radionuclide ^{32}P-kanamycin signal for the NPTII assay) than the pRD430 control (Figure 3.17 and 3.18). The pRD430 plant transformants contained both marker genes regulated by their own respective promoters (35S-35SP-AMV-*pat-nos*T + *nos*P-*nptII-nos*T).

The level of PAT activity also does not appear to be compromised in the fusion protein (CaMV 35S-35S- AMV- *pat::nptII-nos*T in pBAU1) when compared to the PAT activity in the non-fusion control (CaMV 35S-35S-AMV-*pat-nos*T in pRD430). In Figure 3.17, the radionuclide acetyl-PPT signal from the pRD430 transformant qualitatively appears to be of the same intensity as those signals from the pBAU1 transformants. These comparisons, though, were made to only one pRD430 (control) transformant. More single-copy pRD430 transformants should be analysed for PAT activity in order to determine whether the observed PAT catalytic activity in the single-copy pBAU1 transformants has remained uncompromised by the translational fusion of the *pat* gene to the *nptII* gene.

Figure 3.17: *Nicotiana tabacum* **pBAU1 and pBAU2 Leaf Extracts**
Assayed for PAT Activity using the ^{14}C-Acetyl-CoA
Thin Layer Chromatography PAT Activity Assay
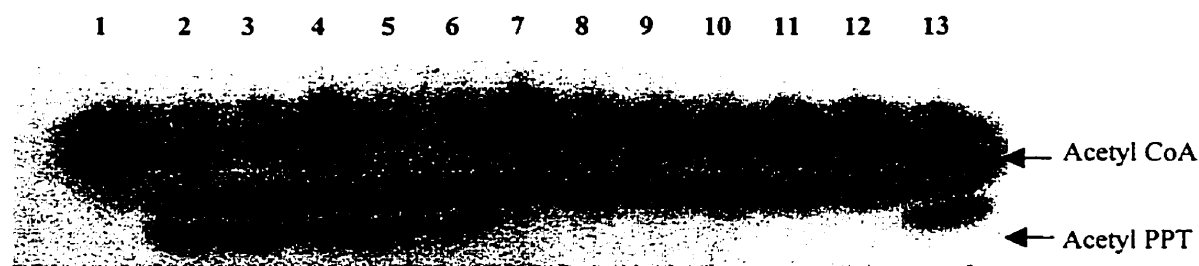
1. Extraction buffer
2. *N.tabacum* pBAU1 #17
3. *N. tabacum* pBAU1 #16
4. *N. tabacum* pBAU1 #13
5. *N. tabacum* pBAU1 #4
6. *N. tabacum* pBAU2 #1
7. *N. tabacum* pBAU2 #2
8. *N. tabacum* pBAU2 #3
9. *N. tabacum* pBAU2 #4
10. *N. tabacum* pBAU2 #5
11. *N. tabacum* non-transformed
12. *N. tabacum* pRD430 #1

Figure 3.18 *Nicotiana tabacum* and *Brassica napus* pBAU1 Leaf
Extracts Assayed for NPTII Activity using the
^{32}P-ATP NPTII Dot Blot Activity Assay
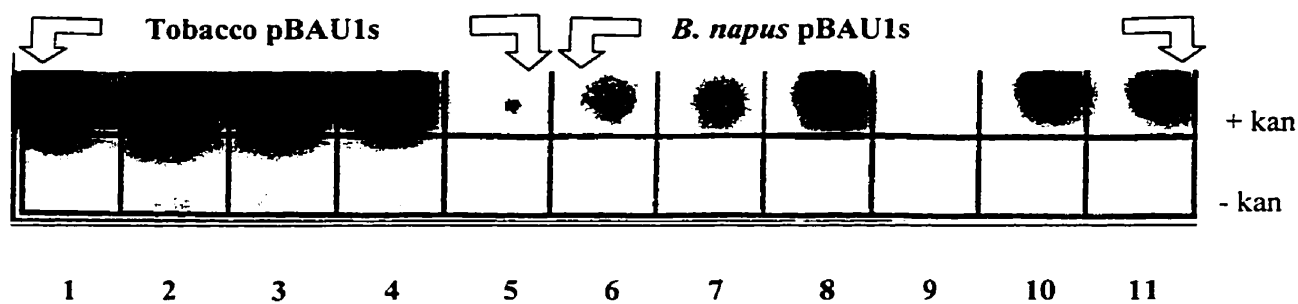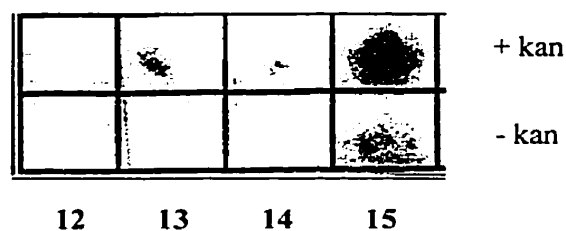
1. *N. tabacum* pBAU1 #1
2. *N. tabacum* pBAU1 #3
3. *N. tabacum* pBAU1 #2
4. *N. tabacum* pBAU1 #4
5. *N. tabacum* pRD400
6. *B. napus* pBAU1 #2
7. *B. napus* pBAU1 #7
8. *B. napus* pBAU1 #4
9. *B. napus* pBAU1#5
10. *B. napus* pBAU1 #11
11. *B. napus* pBAU1#1

Controls:

12. *B. napus* non-transformed
13. *B. napus* HCN92
14. *N. tabacum* non-transformed
15. *N. tabacum* pRD430 #1

<u>Western Assay</u>

To verify the size of the PAT::NPTII fusion protein, a Western Blot was performed on leaf extracts prepared from tobacco pBAU1 (Figure 3.19) and *Brassica* pBAU1 (Figure 3.9) T_0 transformants, and on leaf extracts from pRD430 and non-transformed control plants. A sample of non-transformed plant extract was also spiked with 20 ng of NPTII protein (5 Prime - 3 Prime Inc., cat.# 6-639134). The electrophoretically separated protein banding patterns were visualised by staining one of the gels with Coomassie Brilliant Blue (Figure 3.8). The amount of protein loaded for each of the plant samples was 75 µg total protein. The other gel was subjected to an immunoblotting procedure and probed with the NPTII antibody. The non-transformed sample which was spiked with the NPTII protein demonstrated one NPTII specific band at 23 kilodaltons (kD).

The assayed *Brassica* pBAU1 transgenics (one sample illustrated in Figure 3.9) demonstrated two NPTII specific bands, one at 47 kD and the other at 23 kD. The tobacco pBAU1 transgenics (Figure 3.19) demonstrated only one NPTII specific band at 47 kD. The 47 kD protein band was the fusion protein. The significance of the smaller 23 kD band in *B. napus* is not known.

<u>Immunoassay</u>

Tobacco pBAU1 transformants were also subjected to a two antibody sandwich immunoassay to quantitate the level of NPTII protein expressed in the leaves of T_0 *in vitro* transformants. The level of NPTII protein was calculated in

**Figure 3.19   Western Blot Assay of *Nicotiana tabacum* pBAU1
Plant Extracts Probed with the NPTII Antibody**

1.   *N. tabacum* pBAU1 #1
2.   *N. tabacum* pBAU1 #3
3.   *N. tabacum* pBAU1 #2
4.   *N. tabacum* pBAU1 #4
5.   *N. tabacum* pBAU1 #5
6.   *N. tabacum* wt + 20 ng NPTII Protein
7.   *N. tabacum* wt (non-transformed)
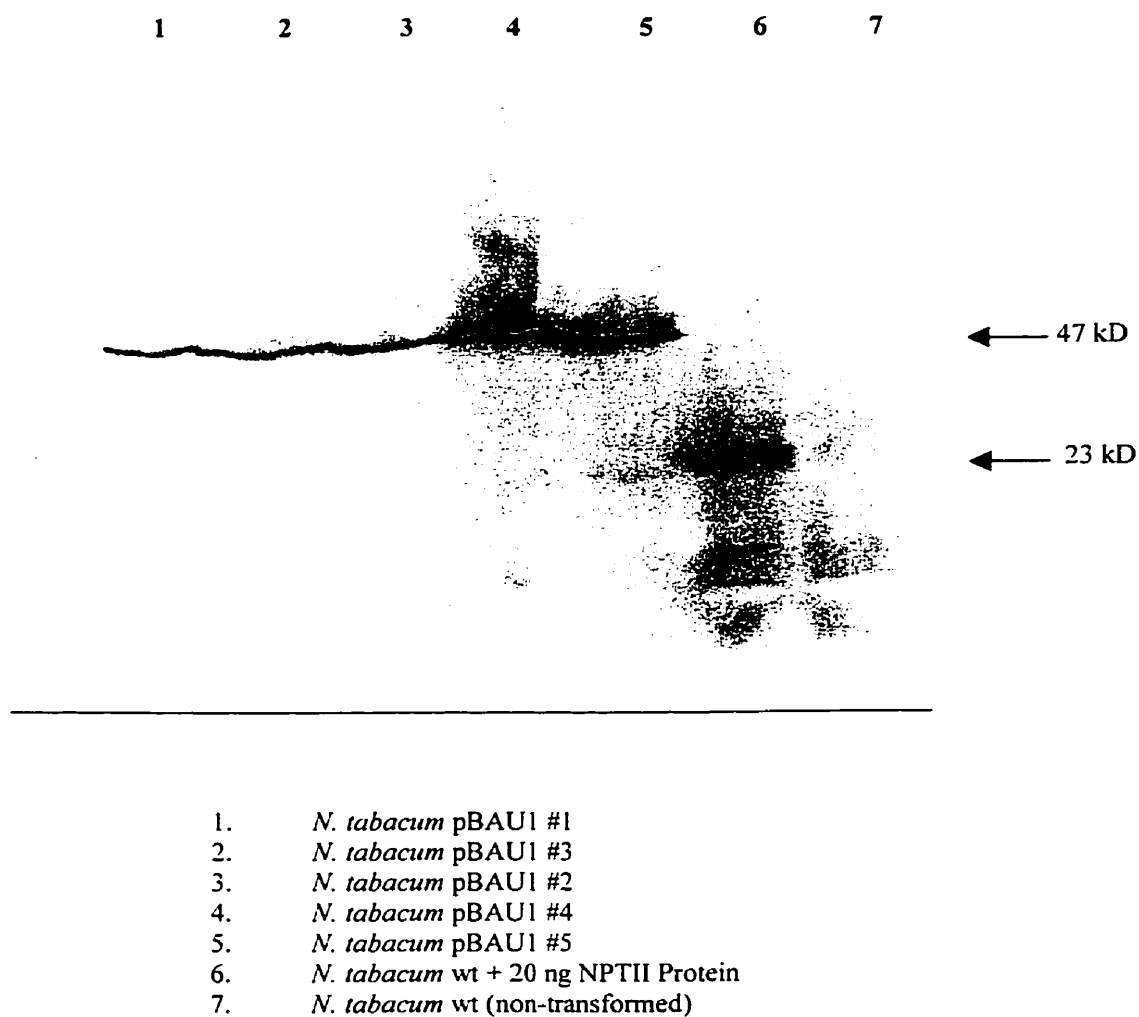
nanograms (ng) of NPTII per milligram (mg) total protein, according to the formula

outlined in Section 3.2.5 (Immunoassay). The level of NPTII protein detected in the

various T_0 pBAU1 transgenics demonstrated a twenty-fold variation ranging from 5.4

ng/mg to 105 ng/mg of NPTII protein per milligram total protein.

Table 3.5: The Quantity of NPTII Protein Present in the Leaves of *In Vitro* T_0
Nicotiana tabacum pBAU1 Transgenics as Determined by ELISA

Sample	ng NPTII/mg total protein
T_0:	
pBAU1 #13	5.4
pBAU1 #14	62.5
pBAU1#15	63.8
pBAU1 #16	105.0
pBAU1 #17	10.0
Nt wt	0.0

3.4 Discussion

The fusion of the carboxy terminus of the *pat* gene to the amino terminus of the *nptII* gene resulted in a translational in-frame fusion producing a bifunctional enzyme with activities for both PAT and NPTII. This was the first reported study of the introduction and successful expression of a PAT::NPTII fusion protein in plants.

In both *Brassica* and *Nicotiana*, the presence of the fusion gene and its respective product was verified using PCR (Figure 3.12), Southern Blot hybridisation (Figures 3.13 and 3.14) and Western Blot hybridisation (Figures 3.9 and 3.19), respectively. The PCR reaction only showed the presence or absence of the marker genes. On the other hand, the Southern Blot assay (i.e. the *PstI* digest probed with *pat*) produced an expected band size for all pBAU1 transformants which was distinct from the *PstI* band visualised in the pRD430 transformants (controls where each marker gene is independently regulated). The translational fusion was shown to be in-frame by sequencing the junction between the two selectable marker genes (Figure 3.7).

Western Blot assays were used to demonstrate whether the fusion gene produced an intact fusion protein in both bacterial and plant hosts. Bacterial lysates, extracted from *E. coli* colonies containing the pSE280-*pat::nptII* fusion gene construct, demonstrated numerous PAT and NPTII specific bands ranging in size from 30 to 50 kD (Figures 3.9 and 3.10). The smaller antigenic peptides may have resulted from non-specific hybridisation or may have been caused by the degradation of the

fusion protein. Internal translation of the PAT and NPTII proteins in *E. coli* may have been another reason for the presence of smaller protein products.

In tobacco and *Brassica napus* pBAU1 transformants, the translation product was a 47 kD fusion protein (Figures 3.9 and 3.19). This estimated size correlated closely with the sum of the NPTII (25 kD; Horsch *et al.*,1985) and the PAT (21 kD; Botterman *et al.*, 1991) proteins. The *Brassica* transformants also demonstrated a smaller translational product of 23 kD when hybridised to the NPTII specific antibody. The reason why this smaller translation product is present in *Brassica napus* pBAU1 transformants and not in tobacco pBAU1 transformants is not known.

These results suggest that the PAT::NPTII fusion protein is more stable in plants than in *E. coli*. Smaller than expected protein products are not uncommon in extracts prepared from *E. coli* expressing a translational fusion protein. Such products were also observed in *E. coli* GUS::NPTII (Datla *et al.*, 1991) and *E. coli* BAR::NPTII (Botterman *et al.*, 1991) protein extracts.

PAT and NPTII activities were verified in *E. coli*-pSE280-*pat::nptII* (Table 3.2), tobacco pBAU1 and in *Brassica* pBAU1 (Figures 3.17 and 3.18) transformants. It was expected that pBAU1 transformants demonstrate a higher NPTII activity than pRD430 transformants since the *nptII* gene in pRD430 is regulated by the *nos* promoter. The *nos* promoter has been shown to have a lower activity than the CaMV 35S-35S promoter (pBAU1), especially in leaf tissue (Sanders *et al.*, 1987). Rooting assays on the selective agent L-PPT were also used to determine the relative activity of the *pat* portion of the fusion gene construct in the pBAU1 transformants. These

three methods for determining the activity level of the fusion construct are compared

in Tables 3.6.

Table 3.6   **Comparison of the Relative PAT and NPTII Activities in *B. napus*
pBAU1 and *N. tabacum* pBAU1 Primary Transformants**

B. napus

Figure where Data is Shown:	Figure 3.13	* not shown	Figure 3.18	Figure 3.15 (240 mg/L; day 20)
Line	Copy Number Estimate	PAT Activity Assay	NPTII Activity Assay	L-PPT Rooting Assay
pBAU1#1	1	++*	+++	+
pBAU1#2	2	+*	+	++
pBAU1#3	1*	n.a.	n.a.	+++
pBAU1#4	2	n.a.	++	-
pBAU1#5	2*	n.a.	-	n.a.
pBAU1#7	>1*	n.a.	+	+
pBAU1#8	2*	n.a.	n.a.	++
pBAU1#10	1*	n.a.	n.a.	+++
pBAU1#11	1*	+++*	+++	+++
pRD430 #1	1	+*	n.a.	n.a.
pRD430 #2	2	n.a.	n.a.	n.a.
pRD430 #3	1	++*	n.a.	+++
HCN92	1*	+++*	+	+++
Wild type	0	-*	-	-

*   = data (Southern Blot or PAT Activity Assay) from these lines was not shown.
n.a. = assay not performed on these lines

Legend:
+++   strong expression relative to other entries
++   average expression relative to other entries
+   weak expression relative to other entries
-       no detectable expression

N. tabacum

Figure where Data is Shown:	Figure 3.13	Figure 3.17	Figure 3.18	Figure 3.15 (240 mg/L; day 20)
Line	Copy Number Estimate	PAT Activity Assay	NPTII Activity Assay	L-PPT Rooting Assay
pBAU1#1	1	n.a.	+++	n.a.
pBAU1#2	1	n.a.	+++	n.a.
pBAU1#3	1	n.a.	+++	n.a.
pBAU1#4	1*	+++	+++	n.a.
pBAU1#5	2*	n.a.	++*	++

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pBAU1#6	1*	n.a.	n.a.	++
pBAU1#7	1*	n.a.	n.a.	++
pBAU1#8	1*	+++	+++*	+++
pBAU1#9	1*	n.a.	n.a.	++
PBAU1#10	1*	n.a.	n.a.	++
pBAU1#11	1	n.a.	+++*	+++
pBAU1#12	1*	n.a.	n.a.	++
PBAU1#13	1*	++	n.a.	n.a.
pBAU1#16	1*	+++	n.a.	n.a.
pBAU1#17	1*	+++	n.a.	n.a.
pBAU1#18	1	n.a.	n.a.	n.a.
pRD430 #1	2	+	+	n.a.
pRD430 #2	1*	n.a.	n.a.	++
Wild type	0	-	-	-

*   = data (Southern Blot or NPTII Activity Assay) from these lines was not shown.
n.a. = assay not performed on these lines

Legend:
+++   strong expression relative to other entries
++   average expression relative to other entries
+   weak expression relative to other entries
-          no detectable expression

Previous studies have shown that C-terminal fusion proteins with PAT, such as BAR::NPTII, BAR::LACZ and BAR::BT, retain acetyl transferase activity when expressed in a prokaryote host (Botterman *et al.*, 1991). From the activity assays depicted in Figures 3.18 and 3.19, it is evident that the PAT::NPTII fusion protein retains both acetyl transferase and phosphotransferase enzyme functions in a eukaryotic host; namely in two unrelated dicot plant species, *B. napus* and *N. tabacum*.

The amount of fusion protein expressed in the leaves of various pBAU1 tobacco transformants was quantitated using an NPTII specific immunoassay. This assay demonstrated a twenty fold variability in the expression levels between different independant transformation events transformed with the same transgene expression construct. One of the main factors affecting the expression of a particular protein is

the promoter regulating the introduced gene. In the pBAU1 transgenics, the promoter was the tandem CaMV 35S promoter fused to the AMV enhancer. Since all of the transgenics possessed the same promoter, the variation in expression levels may have been due to other factors such as copy number (Hobbs *et al.*, 1993), DNA methylation (Matzke *et al.*, 1989; Bochart *et al.*, 1992), the effect of the surrounding genetic milieu on the transgene (positional effect) along with other possible epigenetic variations (Meyer 1995). This range in variation, from 5 to 105 ng NPTII/ mg total protein, is an important piece of information needed to establish a comparative baseline for future transgenics expressing the PAT::NPTII fusion protein under the control of novel promoters.

The *pat::nptII* fusion gene will be an ideal selectable marker gene especially for *Brassica napus* where the regeneration of non-transformed shoots on kanamycin selection (escapes) is a common problem. Following regeneration on the gentler selection agent, kanamycin, shoots can be transferred to medium containing the more stringent L-PPT, thereby eliminating any nontransformed escapes. Initial regeneration on kanamycin followed by more stringent selection on L-PPT results in higher transformation frequencies when compared to directly regenerating shoots in the presence of L-PPT (personal observation). Another advantage of the *pat::nptII* fusion as a selectable marker gene is that the initial transformants and their respective progeny can be selected in the greenhouse by directly spraying the L-PPT formulation (Liberty^{TM}) onto the plants. The zygosity of the progeny from selfed transgenic lines (S$_1$) can also be verified by subjecting S$_1$ seedlings to a Liberty^{TM} application in the greenhouse.

Chapter 4:   Construction and Implementation of a Bifunctional Promoter Tagging Vector

4.1 Introduction

Gene fusion techniques have been successfully used to study both prokaryotic and eukaryotic gene regulation. These techniques have involved the genetic fusion of an assayable, selectable marker gene to the promoter sequence of interest, thereby allowing the study of the promoter's regulatory information. In plants, promoters have been isolated using one of the following three methods: cDNA clone-based methods, genomic clone-based methods, and T-DNA tagging-based methods (An and Kim, 1993). T-DNA promoter tagging methods have the advantage of offering the researcher the ability to test numerous promoter fusions in an *in vivo* (plant) system prior to investing much time and effort in the isolation and cloning of a particular regulatory sequence. This method has also been an effective means for isolating spatially and temporally regulated promoters (Lindsey *et al.*, 1993; Fobert *et al.*, 1991; Teeri *et al.*, 1986; Koncz *et al.*, 1989; Kertbundit *et al.*, 1991). Weaker expressing or highly regulated promoters cannot be easily isolated using cDNA based promoter isolation techniques, since cDNA methods require large amounts of mRNA.

Recently, bifunctional fusion genes have been used to tag and subsequently isolate plant promoters. The *gus::nptII* fusion gene (Datla *et al.*, 1991) and the *lacZ::nptII* fusion gene (Suntio and Teeri, 1994) are two translational fusions which code for both a selectable phenotype and a histochemically detectable product. Using these visual marker:selectable marker gene combinations, tissue specific and

developmentally regulated promoter elements were tagged and isolated (Datla, R., personal communication; Suntio and Teeri, 1994).

It would be of significant importance to determine whether more constitutive and strongly expressing regulatory elements could be isolated using T-DNA promoter tagging methods. It is hypothesised that the stringency of the post-transformation *in vitro* selection regime may play a role in the types of promoters that can be isolated. If this assumption is true, then a more stringent selection protocol (a higher concentration or a more potent selection agent) would lead to the selection of stronger expressing T-DNA promoter tagged plants. To test such a hypothesis, a stringent selectable marker gene, such as the *pat* gene, would be critical for the selection of strongly expressing plant regulatory elements.

The *pat::nptII* fusion gene, under the regulation of the tandem CaMV 35S promoter-AMV enhancer sequence, was shown to be completely bifunctional in both *Brassica napus* cv. Westar and in *Nicotiana tabacum* cv. Xanthi. This fusion construct allowed for a relatively large number of *in vitro* shoots to regenerate in the presence of the gentler selection agent, kanamycin. Following regeneration, a more stringent *in vitro* selection protocol (L-PPT), which ensured that non-transformed tissues would not regenerate, was applied. This stringent selection system coupled with a promoterless *pat::nptII* T-DNA tagging vector was used to determine whether stronger and more constitutive plant promoters can be isolated. The target species for this promoter tagging study was tobacco. Tobacco explants transformed with the pBAU1 binary vector demonstrated a higher regeneration and transformation frequency, 48.6 ± 32.6% and 44.7± 29.4% respectively, than the *Brassica napus* explants, 2.8 ± 0.6% and

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1.0 ± 0.1% (Chapter 3.3.4). It is necessary to use a tissue source with a high regeneration and transformation capability, since the frequency of successfully regenerating a promoter tagged transformant is approximately ten fold lower than the regeneration frequency when using a transcriptionally active selectable marker gene (such as that found in pBAU1 or pRD430).

The production of the promoterless *pat::nptII* T-DNA tagging vector, pBAU2, its transformation into *Nicotiana tabacum* cv. Xanthi leaf disks, and the subsequent *in vitro* selection protocol used to select promoter tagged regenerants will be described and discussed. To better characterise the selected promoter tagged transformants, several molecular analyses and herbicide tolerance studies were also performed.

4.2 Materials and Methods

4.2.1 Construction of the *pat::nptII* bifunctional promoter tagging vector (pBAU2)

All DNA manipulations were as described by Maniatis *et al.*, (1989). The modified pRD300 plasmid, containing the tandem CaMV 35S promoter- AMV enhancer - *pat::nptII* gene - *nos* terminator, was linearized using the restriction enzyme *SalI* (Gibco BRL). The linearized plasmid was blunt-ended (filled in the protruding ends) using the Klenow Fragment of DNA Polymerase I (Pharmacia) and then digested with *EcoRI* restriction endonuclease (Gibco BRL) to isolate the blunt-ended *SalI- pat::nptII- nos* terminator - *EcoRI* fragment (Figure 4.1). This fragment was ligated into a blunt-ended *BamHI/EcoRI* digested plant binary vector, pBI430 (7.5 kb, P.B.I., N.R.C., Saskatoon, SK, Canada) which ensured that the *pat::nptII* fusion gene was placed adjacent to the right border sequence (RB) of the T-DNA (Figure 4.1). A T-DNA map of this novel plant binary vector, pBAU2, is depicted in Figure 4.2.

The pBAU2 binary vector was transferred into *A. tumefaciens* (MP90 strain) using triparental mating (Rogers *et al.*, 1986). Transformed colonies were selected based on their ability to grow on selection medium and whether they demonstrated the expected restriction endonuclease pattern. These selected colonies were used for plant transformations.

Figure 4.1 The construction of pBAU2 T-DNA Promoter Tagging Vector

Sal I   Bgl II   Bgl II

PAT   NPT II

Hind III

5'   AMV   Eco RI   3'

pRD300 (3.5 kb) (Datla, unpublished)

Hind III

5'   AMV   Sal I   Bgl II   Bgl II   Eco RI   3'

PAT   NPT II

Sal I/filled in/ Eco RI digest   (4.7 kb)

pBI430 (7.5 kb) Binary Vector
BamHI/filled in/ Eco RI digest

Kpn I   Kpn I

BamHI   HindIII   PstI   EcoRI   BglII   NotI

Kpn I   Eco RI   Kpn I

PAT   NPT II

pBAU2 Binary Vector (9.3 kb)
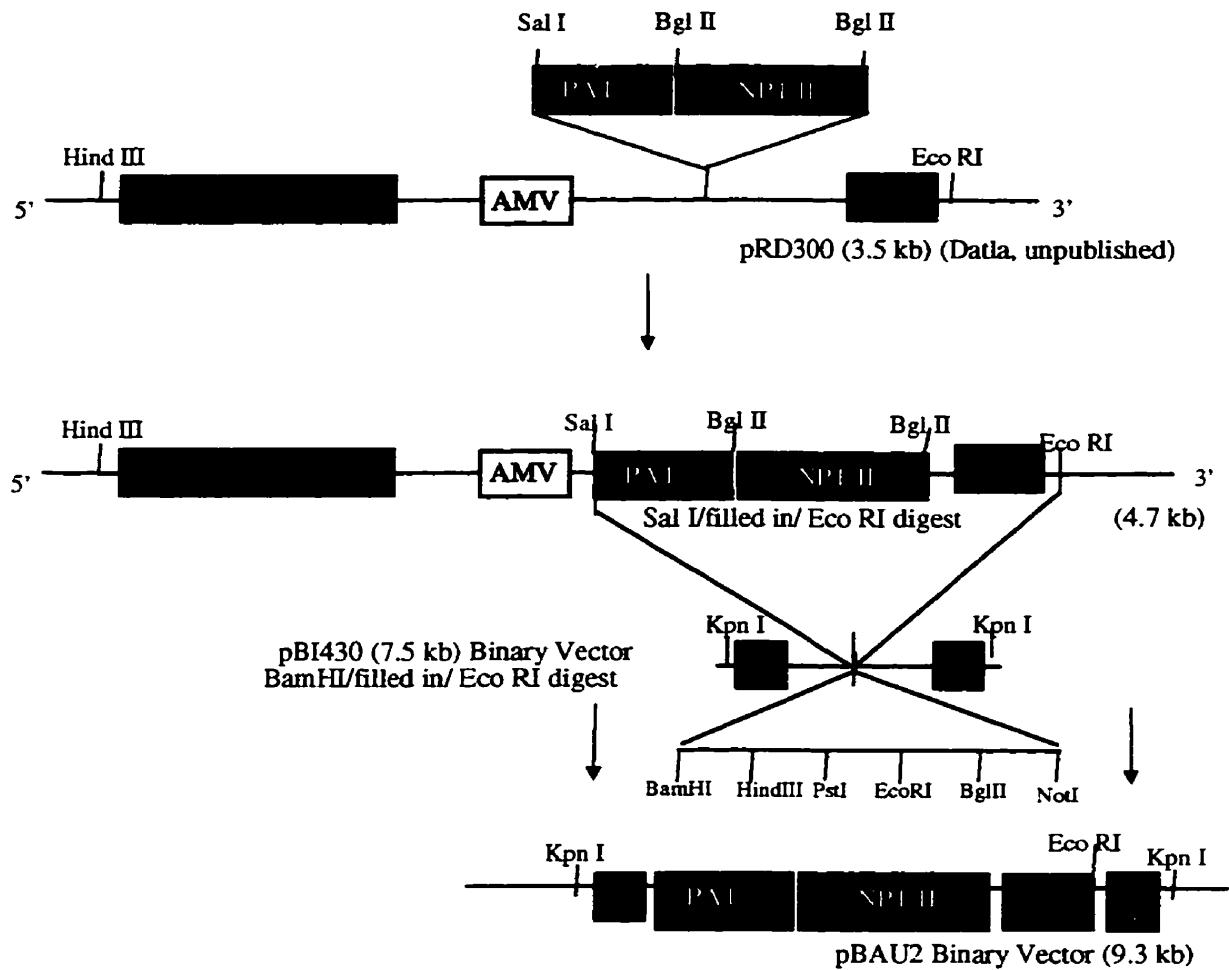
Figure 4.2 The T-DNA Map of the Plant Promoter Tagging Vector, pBAU2

(145)
XbaI

(57)   (91)   (308)   (546)   (725)   (1106)   (1334)   EcoRI
SstI   AatII   BamHI   BglI   PstI   NcoI   BglII/BamHI   BglI
Not I

PAT   NPT II   NosT

CATG
(1)

24   550   786   250   24
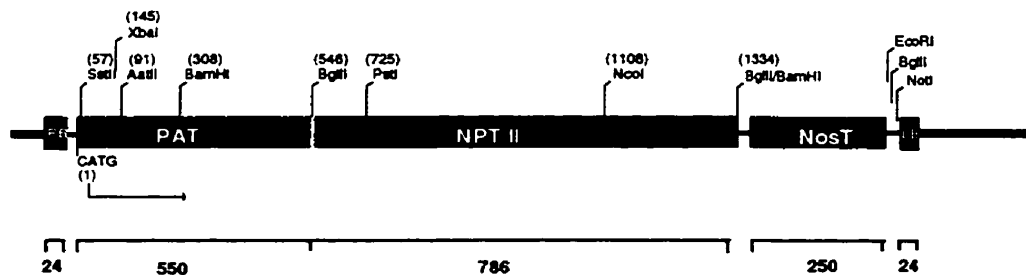
Length of T-DNA = approx. 1 650 base pairs

The left border may not be conserved following integration into genome

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4.2.2 Transformation of pBAU2 into tobacco plants

Nicotiana tabacum cv. Xanthi leaf disks were transformed with pBAU2 using the method of Horsch *et al.*, (1985). Transformation and regeneration protocols, as outlined in Appendix III, were followed with only slight modification to the selection regime. Regenerated shoots were placed onto NTIII Medium (Appendix III) containing 5 mg/L L-PPT, instead of 60 mg/L of L-PPT, for an initial screening. A secondary screening was carried out on NTIII Medium (Appendix III) containing 100 mg/L kanamycin + 5 mg/L L-PPT or 100 mg/L kanamycin + 10 mg/L L-PPT. Selection, regeneration and rooting cultures were maintained in a growth room at 25 °C, with a 16 hour photoperiod and a light intensity of 50 $\mu Em^{-2} s^{-1}$.

Those transformants which survived the 100 mg/L kanamycin + 10 mg/L L-PPT selection were subjected to *in vitro* L-PPT rooting assays, molecular analyses and *in vivo* greenhouse Liberty® (AgrEvo) spray assays.

4.2.3 Analysis of pBAU2 transgenic plants:

<u>PCR Analysis</u>

Primary pBAU2 putative transformants which rooted on 5 mg/L of L-PPT were evaluated for the presence of the *pat::nptII* fusion gene using PCR analysis. The protocol and PCR primers used were the same as those outlined in Chapter 3.2.3.

<u>Southern Hybridization</u>

To facilitate the application of promoter isolation techniques, it was important to identify those transformants that contained only one T-DNA insertion site. A copy number estimate, using the same protocol outlined in Chapter 3.2.3, was performed on T_0 pBAU2 transgenics. Genomic DNA was digested with *EcoRI*, which cuts only once at the 3' end of the *nos* terminator sequence in pBAU2, and with *HindIII*, which does not cut within the T-DNA. Single insertion events were also verified by segregation analyses and subjected to further molecular analyses.

<u>Restriction Mapping</u>

Three single copy T_0 pBAU2 transformants which had rooted on 100 mg/L kanamycin +10 mg/L of L-PPT were selected for restriction mapping studies. Genomic DNA from *N. tabacum* pBAU2 #13, *N. tabacum* pBAU2 #14, and *N. tabacum* pBAU2 #15 was subjected to restriction endonuclease digestion using a high concentration of restriction enzyme (50 U per 10 µg of genomic DNA). The restriction endonucleases used for this study were *HindIII, PstI, XbaI, BamHI, EcoRI, SstII* and *NotI*. Each digest was repeated three times. Southern hybridizations were carried out as outlined in

Chapter 3.2.3. Southern blot membranes were probed with a *pat* specific radioactively labelled DNA probe followed by exposure to Kodak X-OMAT film. Membranes were stripped in boiling deionized distilled water containing 0.1 % sodium dodecyl sulphate (SDS) for 15 minutes. They were reprobed with a *nptII*-specific radioactively labelled DNA probe (Gibco BRL Random Primers DNA Labelling System, cat. # 18187-013). The various restriction patterns were analysed and molecular maps were constructed. The band sizes were calculated from the molecular weight marker lane (BRL 1 kb Ladder, cat. # 15615-024, Appendix II) and using the computer software program 'Origin' (MicroCal). These maps were based on the assumption that the T-DNA region had remained intact during integration into the plant genome.

Rooting Assays

The three selected pBAU2 transformants, along with a non-transformed *N. tabacum* cv. Xanthi wt and a *N. tabacum* cv. Xanthi pHOE6 (pHOE6; AgrEvo AG; CaMV35S Promoter-*pat*-CaMV35S Terminator) control, were subjected to an *in vitro* rooting assay on 0, 5, 10 and 20 mg/L of L-PPT (Basta™, Hoechst AG, Frankfurt, Germany). The *in vitro* plantlets which had established roots on 100 mg/L kanamycin + 10 mg/L L-PPT (with the exception of the non-transformed control, which had rooted on 0 mg/L L-PPT) were the parental material for this assay. Shoots with 1 to 2 leaves were excised and transferred to NTIII Medium (Appendix III) containing the appropriate herbicide concentration. All rooting assays were performed in a growth chamber at 25 °C, with a 16 hour photoperiod and a light intensity of 50 $\mu Em^{-2} s^{-1}$. The rooting assays were replicated three times. The average rooting ability, level of leaf necrosis and number of leaves was calculated for the three rooting assays at 0, 4, 10 and 15 days.

Immunoassay

PAT and NPTII ELISAs were used to analyse the various tissues of three pBAU2 tobacco transgenics, pBAU2 #13, pBAU2 #14 and pBAU2 #15, along with a non-transformed *N. tabacum* cv. Xanthi control, a *N. tabacum* cv. Xanthi pHOE6 (pHOE6; AgrEvo AG; CaMV35S Promoter-*pat*-CaMV35S Terminator) control, and a *N. tabacum* pBAU1 #1 control. S_1 seeds were planted out into flats and germinated in soil in the greenhouse under high humidity (98%). Three sets of S_1 seed were planted out at two week intervals. Following germination, seedlings were assayed commencing

at 16 days post seeding. Seedlings were assayed before and after herbicide spray treatments. Spray treatments were performed at day 14 (no true leaves) for the first planting and at days 26 and 27 (at the 2 to 4 leaf stage) post-seeding for the second and third plantings with Liberty® herbicide (AgrEvo, 3 ml/L). Seedlings were transplanted into individual potting containers at approximately day 40 post-seeding (at the 5 to 6 leaf stage). Tissues were analysed from different parts of S_1 plants during the various stages of development. The protocols for the immunoassays used are outlined in Chapter 3.2.3 and in Appendix I. The PAT ELISA (Figure 3.8), based on the same principle as the NPTII ELISA, was developed as an adjunct to this project (Bauer-Weston *et al.*, 1996). The assay protocol and method validation can be found in Appendix I.

Greenhouse Spray Assay

Approximately thirty S_1 progeny from selfed *N. tabacum* pBAU2 #13, pBAU2 #14, pBAU2 #15, pBAU1 #1, pBAU1 #3, and non-transformed *N. tabacum* cv. Xanthi were subjected to different spray rates of glufosinate ammonium (Liberty®, AgrEvo). Greenhouse grown plants (25 °C, 30% relative humidity) were sprayed at the 4 to 6 leaf stage with 0 ml Liberty®/L, 3 ml Liberty®/L, 6 ml Liberty®/L, and 12 ml Liberty®/L. Plants were sprayed in three passes, until leaf drip off, to ensure even coverage. Genopol®, a wetting agent, was used in the spray mixture at a rate of 1 mL per 3 ml of herbicide. Plants were scored two weeks after spraying. The ratio of undamaged plants to damaged plants for the 3 ml Liberty®/L spray application was asessed for each pBAU2 and pBAU1 transgenic line.

<u>Seed Germination Assay</u>

Homozygous (S_2) seed from *N. tabacum* pBAU2 #13, pBAU2 #14, pBAU2 #15, pBAU1 #2 and non-transformed *N. tabacum* cv. Xanthi were sterilized and plated out onto Seed Germination Medium (Appendix III) containing varying levels of either kanamycin or L-PPT. Approximately 30 to 40 sterilized seeds were plated out onto each 60 x 15 mm petri dish containing either 0, 100, 200 mg/L of kanamycin or 0, 5, 50, 100, 250, or 500 mg/L of L-PPT (Basta ^{TM}, Hoechst AG, Frankfurt, Germany). Plates were incubated in a growth cabinet (25 °C, 16 hour photoperiod, 50 μEm^{-2} s^{-1}) and the percent germination was scored two weeks after seeding.

4.3 Results

4.3.1 Transformation of pBAU2 into tobacco

Leaf disks inoculated with *Agrobacterium tumefaciens* pMP90-pBAU2, or with

pMP90-pBAU1 as a control transformation, were plated out onto NTI Medium

(Appendix III) for two days and then onto NTII Medium (Appendix III) containing 100

mg/L of kanamycin. Regenerating explants were incubated in a growth chamber for a

period of four weeks, after which they were scored and transferred to fresh

Regeneration Medium for another four weeks. After four and eight weeks, regenerated

green shoots were excised and transferred to NTIII Medium (Appendix III) containing 5

mg/L of L-PPT (Basta®, AgrEvo AG, Frankfurt, Germany). Any escapes or weakly

expressing promoter tagged plantlets did not survive selection at 5 mg/L of L-PPT. The

regeneration and selection data are summarised in Table 4.1.

Table 4.1 **Transformation, Regeneration and Selection of *Nicotiana tabacum* cv. Xanthi Leaf Disks Transformed with *Agrobacterium tumefaciens* pMP90-pBAU2**

	Total # of Explants	# of Explants With Green Calli @ Week 4	# of Explants with Green Shoots @ Week 4	# of "Week 4" Shoots Which Rooted @ 5 mg/L L-PPT	# of Explants with Green Shoots @ Week 8	# of "Week 8" Shoots Which Rooted @ 5 mg/L L-PPT	% Regeneration Frequency (on 100 mg/L kanamycin)	% Transformation Frequency (on 5 mg/L L-PPT selection)
no Agro	63	2	0	0	0	0	0	0
pBAU1	58	45	32	28	11	10	72	66
pBAU2	592	201	30	1	36	7	12.8	1.2

Regeneration Frequency   = the number of explants with green shoots divided by the total number of explants

Transformation Frequency = the number of green shoots rooting on 5 mg/L of L-PPT divided by the total number of explants

A total of eight regenerated pBAU2 shoots were able to root on 5 mg/L of L-PPT. Most of these regenerated shoots developed after the second transfer to fresh NTII medium (week 8 shoots). Rooted plantlets were subjected to a secondary screening on Rooting Medium (NTIII, Appendix III) containing 100 mg/L kanamycin + 5 mg/L L-PPT and 100 mg/L kanamycin + 10 mg/L L-PPT. Only three pBAU2 transformed plantlets, *N. tabacum* pBAU2 #13, *N. tabacum* pBAU2 #14 and *N. tabacum* pBAU2 #15, were able to develop roots on 10 mg/L of L-PPT. These three plantlets were subjected to further characterisation.

4.3.2 The expression of the *pat::nptII* fusion gene in tagged transgenic plants

<u>PCR Analysis</u>

To demonstrate that the kanamycin and L-PPT resistant regenerants contained the T-DNA tag, a PCR analysis was conducted on the DNA extracted from the leaves of *in vitro* rooted plantlets (Chapter 3.2.3). The presence of the *pat* and *nptII* gene sequences was verified in the three pBAU2 transformants, pBAU2 #13, pBAU2 #14 and pBAU2 #15, using *pat* specific and *nptII* specific PCR primers (Section 3.2.5). All three transformants demonstrated a 400 bp PCR product when amplified with the *pat* specific primers and a 750 bp PCR product when amplified with the *nptII* specific primers. Also, the *pat::nptII* fusion sequence was amplified using the *pat*-specific primer 5'-AGACCAGTTGAGATTAGGCCAG-3' along with the *nptII*-specific primer 5'-TCAGAAGAACTCGTCAAG-3'. A 1150 bp PCR product was observed when this latter primer combination was used. There was no PCR product observed in the non-transformed control nor in the PCR reaction containing water instead of DNA (not shown).

<u>Southern Hybridization</u>

DNA was prepared from pBAU2 transformants and subjected to Southern Blot hybridization analysis. Figure 4.3 demonstrates a Southern blot from the plant DNAs digested with *EcoRI* and *HindIII*, and probed with the random primed *nptII* probe (750 bp). The same Southern blot membranes were stripped and reprobed with the random primed *pat* probe (550 bp) (not shown) and demonstrated the same banding pattern.

Since the T-DNA region does not contain a *HindIII* site and only one *EcoRI* site at the 3' end of the *nos* terminator sequence, the Southern blots can be used to estimate the number of T-DNA copies inserted into each of the transgenics. From the Southerns in Figure 4.3, only one individual, *N. tabacum* pBAU2 #1, showed the presence of two bands indicating the presence of two T-DNA insertion loci. Other individuals demonstrated one band when digested with either *HindIII* or *EcoRI*. The size of the *EcoRI nptII* hybridizing band was approximately 18 kb for *N.t.* pBAU2 #14 and 11 kb for *N.t.* pBAU2 #15. The size of the *HindIII* band was approximately 13 kb for *N.t.* pBAU2 #13, 20 kb for *N.t.* pBAU2 #14, and 15 kb for *N.t.* pBAU2 #15.

Figure 4.3 **Southern Blot Hybridization Analysis of T₀**
N. tabacum **pBAU2 Transformants.** DNA was
Digested with *EcoRI* and *HindIII* Restriction Enzymes
and Probed with an *nptII* Specific DNA Probe.
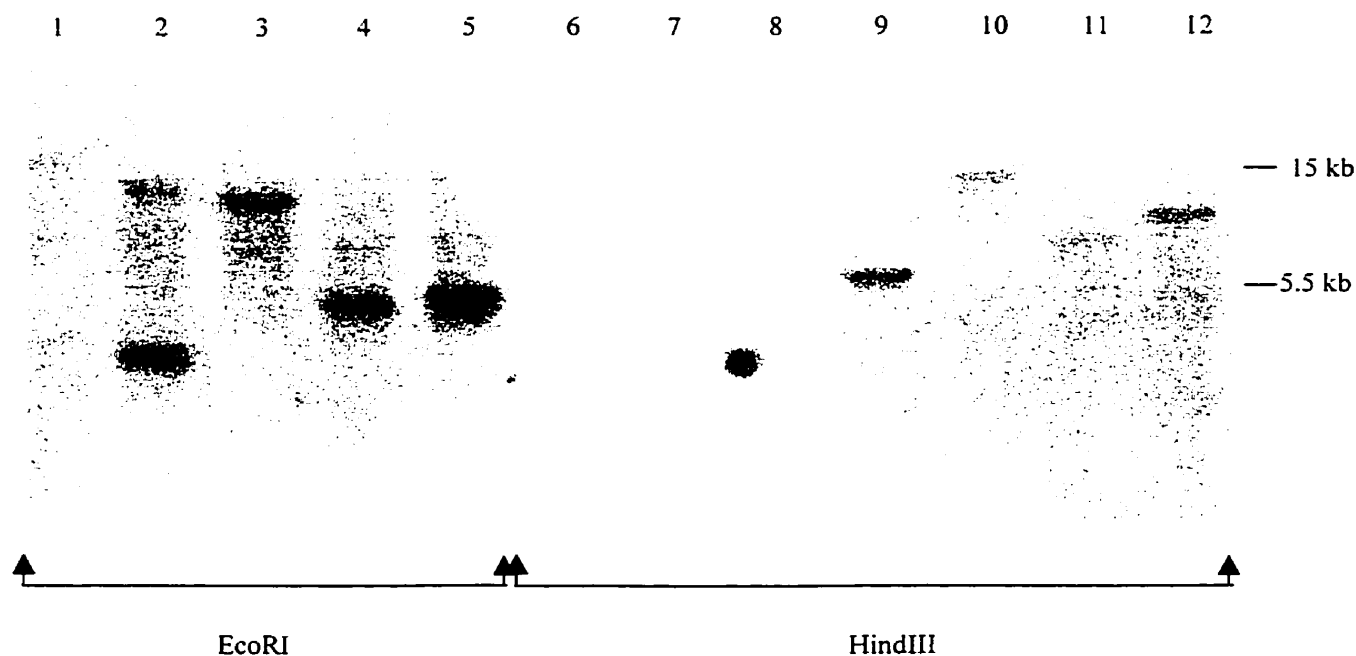
Legend:

1. *N. tabacum* pBAU2 #14 – *EcoRI* digest
2. *N. tabacum* pBAU2 #1   – *EcoRI* digest
3. *N. tabacum* pBAU2 #15 – *EcoRI* digest
4. *N. tabacum* pBAU2 #5   – *EcoRI* digest
5. *N. tabacum* pBAU2 #2   – *EcoRI* digest
6. *N. tabacum* wt   – *HindIII* digest
7. *N. tabacum* pBAU2 #13 – *HindIII* digest
8. *N. tabacum* pBAU2 #14 – *HindIII* digest
9. *N. tabacum* pBAU2 #1   – *HindIII* digest
10. *N. tabacum* pBAU2 #15 – *HindIII* digest
11. *N. tabacum* pBAU2 #5 – *HindIII* digest
12. *N. tabacum* pBAU2 #2 – *HindIII* digest

Restriction Mapping

Restriction mapping studies were performed on the genomic DNA isolated from

T_0 leaf tissue from the three pBAU2 promoter tagged plants: *N. tabacum* pBAU2 #13,

N. tabacum pBAU2 #14, and *N. tabacum* pBAU2 #15. The observed band sizes for

each restriction enzyme and each DNA probe are listed in Tables 4.2, 4.3 and 4.4.

Restriction maps for the three tagged plants are illustrated in Figure 4.4. These maps

were based on the assumption that the T-DNA region had remained intact during

integration into the plant genome.

Table 4.2 **Analysis of the T-DNA Insertion in *N. tabacum* pBAU2 #13 using Restriction Endonuclease Digestion of Genomic DNA**

Restriction Enzyme	PAT Probe		NPTII Probe	
	No. of Bands	Approx. Size of Band (kb)	No. of Bands	Approx. Size of Band (kb)
BamHI	1	2.2	2	17.3 >30
HindIII	1	11.2-12.2	1	12.2-13.0
XbaI	2	5.1-5.2 6.5	3	5.2 5.3-5.4 6.2-6.4
SstII	n.a.	n.a.	n.a.	n.a.
BamHI + HindIII	2	2.2 5.5-5.8	1	6.5-6.7
BamHI + XbaI	2	2.4 5.1-5.2	3	5.0-5.2 5.2-5.4 6.1
HindIII + NotI	2	6.1-6.2 11.6	2	6.9 14.0
HindIII + SstII	2	6.5-7.1 13.8-14.8	3	2.6 6.1-6.3 14.0
XbaI + SstII	2	12.5-13.8 17.0-18.5	3	12.2 13.8 19.2
EcoRI	1	10.0	1	10.0
PstI	1	20		
BglII	1	14		

Samples which were incompletely digested or which demonstrated variable restriction patterns between the three repeated digests are marked as 'n.a.'.

Table 4.3 **Analysis of the T-DNA Insertion in *N. tabacum* pBAU2 #14 using Restriction Endonuclease Digestion of Genomic DNA**

Restriction Enzyme	PAT Probe		NPTII Probe	
	No. of Bands	Approx. Size of Band (kb)	No. of Bands	Approx. Size of Band (kb)
Bam HI	2	10.8 -11.2 14.0-17.3	1	15.4
Hind III	1	> 30	1	>30
Xba I	2	5.5-5.6 3.02	2	5.6 6.1
Sst II	1	12.2	n.a.	n.a.
Bam HI + Hind III	2	1.4 > 30	1	>30
Bam HI + Xba I	1	5.5-5.6	2	6.9 8.4
Hind III + Not I	3	8.1 14.0 23.1	3	8.6 14.0 23.1
Hind III + Sst II	2	9.1-10.7 17.8	2	8.5 >30
Xba I + Sst II	2	9.7-11.0 15.3	2	10.2-10.7 16.0-16.5
Eco RI	1	11.0	1	11.0

Table 4.4 **Analysis of the T-DNA Insertion in *N. tabacum* pBAU2 #15 using Restriction Endonuclease Digestion of Genomic DNA**

Restriction Enzyme	PAT Probe		NPTII Probe	
	No. of Bands	Approx. Size of Band (kb)	No. of Bands	Approx. Size of Band (kb)
Bam HI	3	8.6 11.2 >30	1	>30
Hind III	1	14.0-17.3	1	15.4-19.7
Xba I	1	5.4-5.5	1	5.6-5.7
Sst II	1	12.0-12.2	1	12.2-13.2
Bam HI + Hind III	3	4.9 6.1 11.0	1	12.2
Bam HI + Xba I	1	5.3	1	5.6
Hind III + Not I	2	12.2 23.2	2	15.4 >30
Hind III + Sst II	2	8.2-9.7 11.7-12.7	2	7.1-7.6 10.2-11.2
Xba I + Sst II	4	8.7-9.2 11.7 13.8-14.5 17.2	2	9.2-10.2 14.8-16.0
Eco RI	1	8.5	1	8.5
Pst I	1	1.8		
Bgl II	1	1.1		

Figure 4.4   Restriction Maps for the T-DNA Insertion Loci
of *N. tabacum* pBAU2 #13, #14 and #15

N.t. pBAU2 #13
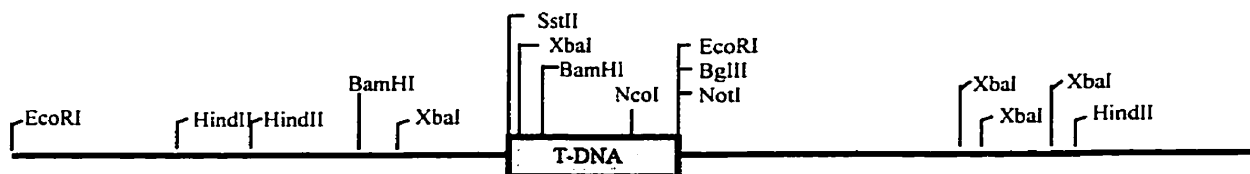
N.t. pBAU2 #14
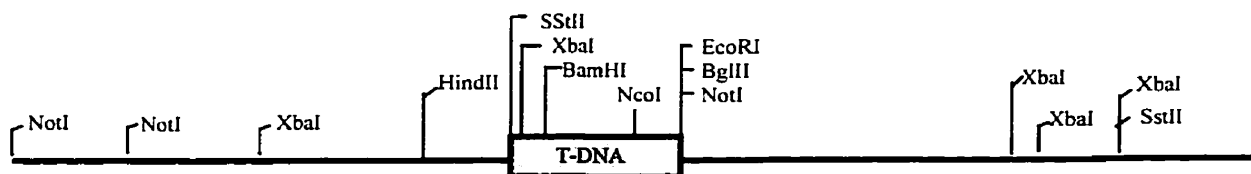
N.t. pBAU2 #15
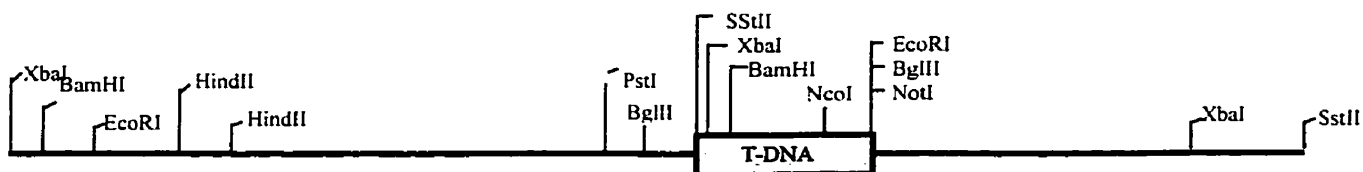
Scale: 1 cm~ 1 kb

Rooting Assays

The results from three rooting assays, as a function of rooting ability, degree of necrosis, and number of leaves, are presented in Appendix III (Raw Data). The parameter that best correlated to gene activity (rooting ability) in *N. tabacum* transformants (as determined in Section 3.3.5), is graphed in Figure 4.5. The three T_0 *N. tabacum* pBAU2 transformants were assayed as well as a non-transformed *in vitro* regenerant, *N. tabacum* cv. Xanthi, and a T_0 *N. tabacum* cv. Xanthi pHOE6 (CaMV35Spromoter – *pat* – CaMV35S terminator) transformant. A visual representation of the rooting assay for *N.t.*pBAU2#14 and *N.t.*pBAU2#15 is presented in Figure 4.6.

All three promoter tagged lines demonstrated a greater ability to root on 20 mg/L of L-PPT than the positive control line, T_0 *N. tabacum* cv. Xanthi pHOE6. The line that consistently produced the longest and largest number of roots per shoot explant, in all three rooting assays, was *N.t.*pBAU2#13.

Immunoassay

The results from three developmental analyses are recorded in Tables 4.5 and 4.6. For the first developmental analysis, seedlings were sprayed with 3 ml Liberty®/L, 14 days after seeding. Seedlings from the second and third developmental analyses were sprayed 26 days and 35 days after seeding, respectively. A comparison of the NPTII ELISA data and the PAT ELISA data (using the same tissue extracts for both ELISAs) was also performed and these results are presented in Table 4.7.

Figure 4.5 T$_0$ *Nicotiana tabacum* pBAU2 *In Vitro* Rooting
Assay
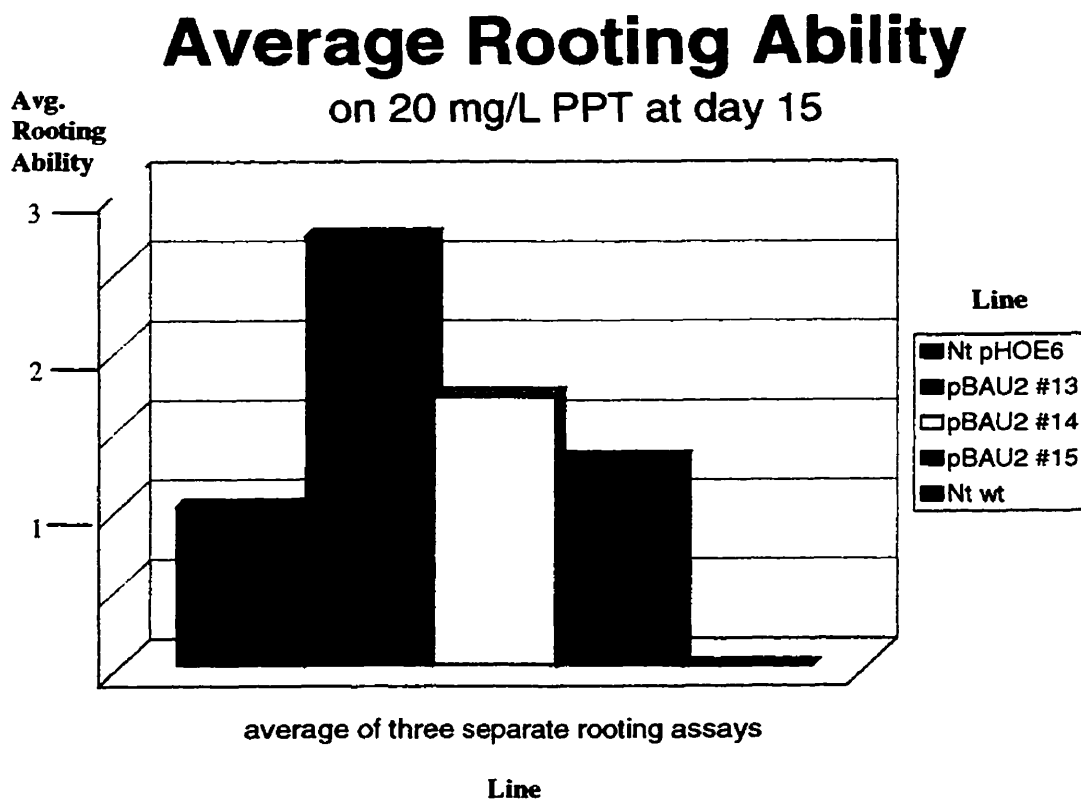
Average Rooting Ability

on 20 mg/L PPT at day 15

Avg.
Rooting
Ability

average of three separate rooting assays

Line

Legend:
- Nt pHOE6
- pBAU2 #13
- pBAU2 #14
- pBAU2 #15
- Nt wt

Rooting Ability:

0 = no roots
1 = root bud formation or less than 3 roots per plant
2 = roots were 1 to 10 mm in length and more than 3 roots per plant
3 = roots were greater than 10 mm in length and more than 3 roots per plant

Nt 110/2-PAT = *Nicotiana tabacum* cv. Xanthi transformed with pHOE6

(CaMV 35S Promoter-*pat*-CaMV 35S Terminator)

Nt wt = non-transformed *Nicotiana tabacum* cv. Xanthi

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Figure 4.6 T₀ *Nicotiana tabacum* pBAU2 *In Vitro* Rooting
Assay: 0, 5, 10 and 20 mg/L L-PPT at Day 15
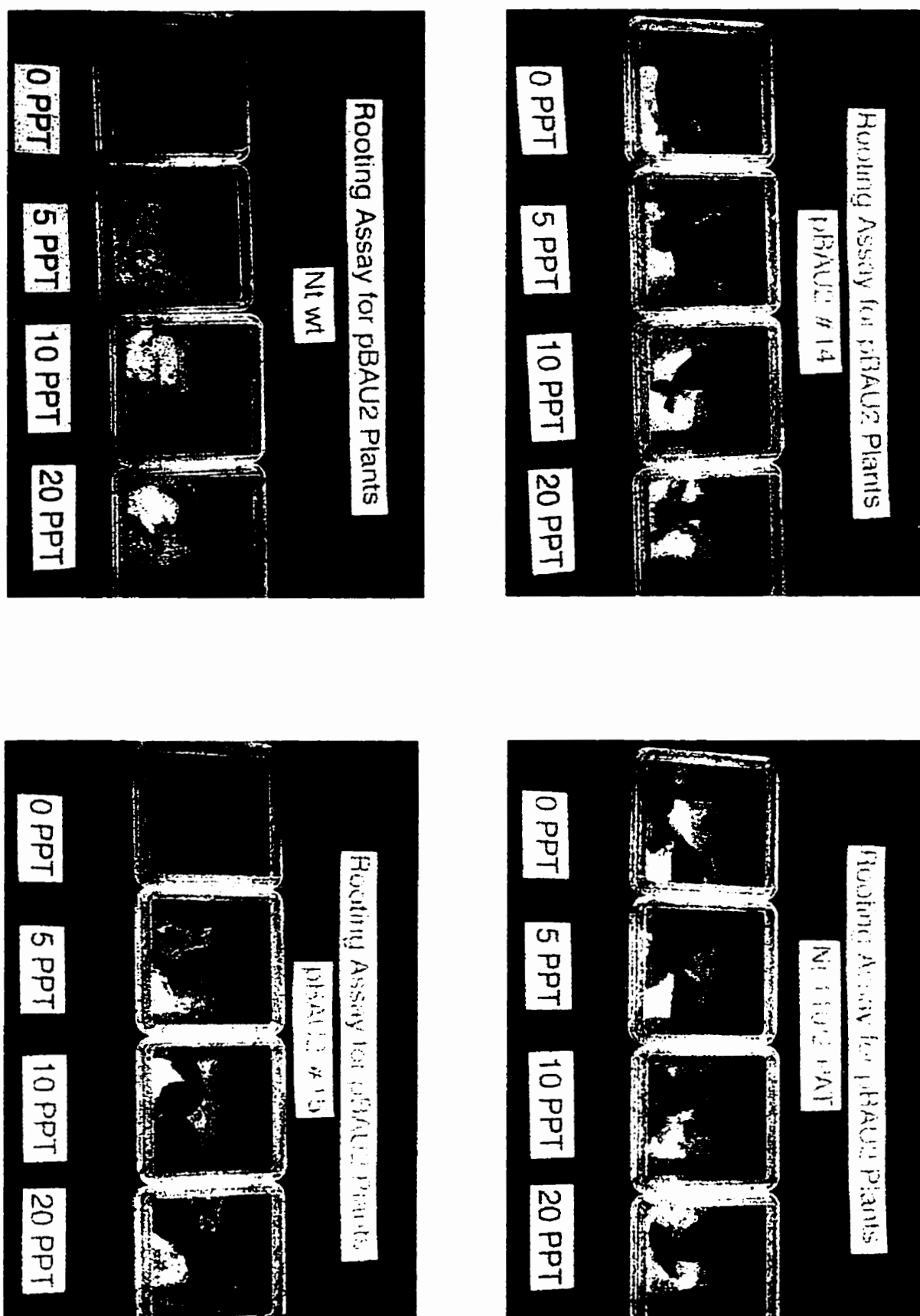
Table 4.5 Presence of NPTII in Various Tissues of S_1 Greenhouse Grown *N. tabacum* pBAU2 Promoter Tagged Plants Using an Enzyme Linked Immunosorbant Assay

Developmental Stage	ng NPTII/ mg total protein (relative to BSA)					
Tissue Type	Nt wt	pBAU2 #15	pBAU2 #13	pBAU2 #14	pBAU1 #1	pHOE6 110/2
Seedlings (0–4 true leaf stage)						
4 leaf stage individual seedlings (entire seedling; 25 days ps; pre- spray)	0		2.63	2.34		
	0	3.56	1.5		12.83	
	0	2.13		2.32	12.31	
	0	1.99	1.35		12.39	
	0	2.48	1.48	1.45	14.33	
*Average ± standard dev.	0 ± 0	2.54 ± 0.71	1.74 ±0.6	2.04 ±0.51	12.97 ±0.94	
4 leaf stage individual seedlings (entire seedling; 27 days ps; 2 days pL)	0	2.43	1.7	1.80	19.19	
	0	2.08	1.69	4.14	17.01	
	0	2.65	2.68	2.3	16.46	
	0	2.65	3.19	2.09	17.39	
	0	2.99	3.53	1.34	15.98	
Average ± standard dev.	0 ± 0	2.55 ± 0.32	2.56 ±0.85	2.34 ±1.07	17.21 ±1.23	
Young Plants Pre-Bolting (4-7 true leaf stage)						
L6 (50 days ps; 15 days pL)	0	0.29	0.01	0.33	2.20	0
L7 (53 days ps; 18 days pL)	0	3.21	3.79	0.46	29.02	0
L1 (53 days ps; 18 days pL)	0	2.58	2.31	2.12		0
stem (53 days ps; 18 days pL)	0	7.67	5.73	1.26	30.71	0
root (53 days ps; 18 days pL)	0	7.80	5.15	0.26		0
Mature Plants Bolting (7-8 true leaf stage)						
L4 (57 days ps; 22 days pL)	0	0.29	0.01	0.32	2.20	0
L4 (57 days ps; 22 days pL)	0	0.3	0.00	0.26	2.31	0
L4 tip(59 days ps;24 days pL)	0	1.79	0.82	2.63	9.41	0.04
L3 tip(58 days ps;30 days pL)	0	0.95	0.92	0.92	2.24	
Mature Plants Bolting (7-8 true leaf stage) cont'd						
L8 (60 days ps; 32 days pL)	0	0.33	0.38	0.61	6.75	0
L1 (60 days ps; 32 days pL)	0	0.4	0.28	0.52	6.73	
stem (60 days ps; 32 days pL)	0	0.86	0.52	0.68	6.55	
root (60 days ps; 32 days pL)	0	0.24	0	0	5.42	
Mature Plants Bolting (8-12 true leaf stage)						
L9 (60 days ps; 25 days pL)	0	0.7	0.54	1.48	0.96	0
L2 (60 days ps; 25 days pL)	0	0.73	0.16	0.58	2.31	0
stem (60 days ps; 25 days pL)	0	0.78	0.48	0.25	2.55	0
root (60 days ps; 25 days pL)	0	0.93	0.34	0.03	1.62	0

Mature Plants Flowering **(9-14 true leaf stage)**						
L12 (78 days ps; 64 days pL)	0	0.4	0.82	0.36	7.42	
L7 (78 days ps; 64 days pL)	0	0.84	0.44	1.07	8.97	
L4 (78 days ps; 64 days pL)	0	1.02	0.77	1.21	3.03	
L2 (78 days ps; 64 days pL)	0	0.78	0.33	0.85	7.77	
Mature Plants Flowering **(11-16 true leaf stage)**						
L12 (89 days ps; 63 days pL)	0	2.79	3.32	2.72	22.68	
L7 (89 days ps; 63 days pL)	0	2.31	1.9	4.71	22.98	
L4 (89 days ps; 63 days pL)	0	2.12	0.82	5.01	23.36	
L2 (89 days ps; 63 days pL)	0	1.53	0.95	4.30	24.51	
Mature Plants Flowering **(13-17 true leaf stage)**						
L2 (92 days ps; 78 days pL)	0	8.35	0.82	1.63	9.1	
L11 (92 days ps; 78 days pL)	0	1.57	1.84	0.34	7.71	
stem (92 days ps; 78 days pL)	0	4.24	3.31	1.32	10.53	
root (92 days ps; 78 days pL)	0	1.77	2.02	0	6.53	
Mature Plants Flowering and Setting Seed **(15-18 true leaf stage)**						
L16(155 days ps;128 days pL)	0	3.39	0	0.35	42.41	0
entire flower (155 days ps;128 days pL)	0			0	45.0	0

Legend: ps = post seeding pL = post Liberty® herbicide spray application
*Average = average of heterozygous and homozygous *pat::nptII* positive offspring
L1 to L18 = true leaf classification ranked according to internode # ; from oldest true leaf (L1) to youngest leaf (up to L18, depending on the plant's stage of development)

Nt wt = *Nicotiana tabacum* wild type (not transformed with *Agrobacterium*)

The amount of NPTII protein, presented in Table 4.5, was calculated from the linear regression of the NPTII standard curve as described in section 3.2.5 (Immunoassay).

Five seedlings that were not treated with Liberty (Table 4.5, pre-spray) were evaluated one month after seeding. For the transgenic lines (pBAU2 lines) only those unsprayed seedlings containing the *pat::nptII* fusion gene (fewer than five), as verified

by PCR, were evaluated using the NPTII ELISA. Five seedlings which survived Liberty spray in the greenhouse were also evaluated at the same developmental stage to determine whether the Liberty spray induced expression of the *pat::nptII* fusion gene. The *pat::nptII* containing seedlings (S_1) from both Liberty treated and untreated groups were a mixture of homozygotes (33%) and hemizygotes (66%). From the averages (and standard deviations) of the Liberty treated (pL) and Liberty untreated (pre-spray) seedlings, shown in Table 4.5, no significant induction effect could be detected. All of the pBAU2 seedlings demonstrated a level of NPTII ranging from 1.7 to 2.4 ng per mg total protein.

Only Liberty treated individuals (S_1) were used for subsequent analyses of the post-seedling stages of development. The non-transformed *N. tabacum* control plants (Nt wt) and the *pat* containing transgenic line pHOE6 110/2 did not show detectable NPTII protein in any of the tissues assayed nor during any of the developmental stages assayed. In all tissues and at all tested stages, the pBAU2 lines demonstrated lower NPTII protein levels than the pBAU1 positive control (tandem 35S promoter- AMV enhancer – *pat::nptII* – *nosT*). The pBAU2 line which demonstrated a slightly higher NPTII protein level, especially in the stem and root, was pBAU2#15. The presence of NPTII protein was very low in young leaves of pBAU2#13, except after bolting where the level of detectable NPTII was higher. Nine different individuals were assayed from each pBAU2 line. These individuals were either hemizygote or homozygote for the gene of interest (*pat::nptII*).

Table 4.6 Presence of PAT in S_1 Greenhouse Grown *N. tabacum* pBAU2 Promoter Tagged Plants Using an Enzyme Linked Immunosorbant Assay

Developmental Stage / Tissue Type	ng PAT/ mg total protein (relative to BSA)					
	Nt wt	pBAU2 #15	pBAU2 #13	pBAU2 #14	pBAU1 #1	pHOE6 110/2
Bulked Seedlings -Pre-Spray (cotyledon stage)						
entire seedlings (16 days ps)	0	0.21	0	0.09	1.31	9.75
cotyledons (21 days ps)	0	0.21	0.05	0	1.76	10.13
hypocotyls (21 days ps)	0	0.34	0.62	0.22	4.18	8.52
Bulked Seedlings -Pre-Spray (2-4 true leaf stage)						
entire seedlings :	0	0.01	0.05	0.03	0.62	1.44
(31 days ps)	0	0.01	0.04	0.04	1.02	1.52
	0	0.07	0.09	0.08	0.96	
Average ± Standard Dev.	0 ± 0	0.03 ±0.03	0.06 ±0.03	0.05 ±0.02	0.86 ±0.21	1.48 ± 0.06
Bulked Seedlings Post-Spray (2-4 true leaf stage)						
entire seedlings (31 days ps; 17 days pL)	0	0.17	0.14	0.08	1.66	2.55
	0	0.09	0.15	0.09	1.64	2.46
	0	0.05	0.04	0.03	1.91	2.50
	0	0.04	0.11	0	1.81	2.4
	0	0.11	0.02	0.03	0.7	0.93
Average ± Standard Dev.	0 ± 0	0.09 ±0.05	0.09 ±0.06	0.05 ±0.04	1.54 ±0.49	2.17 ±0.7
entire seedlings (44 days ps; 11 days pL)	0	0	0.03	0.02	1.39	1.41
hypocotyls:						
(24 days ps; 10 days pL)	0		0	0	4.09	9.14
(31 days ps; 17 days pL)	0		0.34		1.98	
cotyledons:	0	0	0.08	0	2.73	
(31 days ps; 17 days pL)						
roots:	0	0.12	0	0	1.54	
(31 days ps;17 days pL)	0	0.04	0	0	1.43	

Young Plants Pre-Bolting (5-7 true leaf stage)						
L5 (50 days ps; 36 days pL)	0	0.1	1.24	0.07		
	0	0.34	0.86	0.39		
	0	0.32	1.05	0.47		
	0	0.45	0.2	0.44		
Average ± Standard Dev.	0 ± 0	0.30 ±0.15	0.84 ±0.45	0.34 ±0.18		
Mature Plants Bolting (10-14 true leaf stage)						
L10 (45 days ps; 31 days pL)	0	0.03	0.06	0.08	1.01	0.93
L10 (45 days ps; 31 days pL)	0	0.02	0.05	0.12	1.07	1.09
L12 (53 days ps; 39 days pL)	0	0.19	0.1	0	1.7	2.53
L12 (53 days ps; 39 days pL)	0	0.05	0.06	0	1.72	2.48
L4 (57 days ps; 22 days pL)	0	0.07	0.03	0.01	3.44	1.78
L4 (57 days ps; 22 days pL)	0	0.1	0.06	0	1.91	1.22
L4 (59 days ps; 24 days pL)	0	0.37	0.11	0.22	3.84	17.12
Mature Plants Flowering and Setting Seed (15-18 true leaf stage)						
L16(155 days ps;128 days pL)	0	0.22	0.41	0.35	1.56	8.16
entire flower (155 days ps;128 days pL)	0			0.12	5.96	23.37

Legend: ps = post seeding   pL = post Liberty® herbicide spray application
^Bulked = 8 to 10 individuals were bulked together for analysis
L1 to L18 = true leaf classification ranked according to internode # ; from oldest true leaf (L1) to youngest leaf (up to L18, depending on the plant's stage of development)

Nt wt = *Nicotiana tabacum* wild type (not transformed with *Agrobacterium*)

The level of PAT protein in the various tissues was calculated from the linear regression of the PAT standard curve, in the same manner as for NPTII (section 3.2.5; Immunoassay). Again Liberty treated and untreated pBAU2 seedlings were compared to see if there was an induction of PAT expression after Liberty treatment (Table 4.6; Bulked Seedlings Pre-Spray versus Bulked Seedlings Post-Spray). From the detected PAT protein levels, no induction was observed (Table 4.6). The level of PAT in the pBAU1 positive control was often 10 fold higher in most tissues, as

compared to the pBAU2 lines. This was the same factor difference observed between

pBAU1 and pBAU2 lines assayed for the NPTII protein (Table 4.5).

In general, the PAT protein levels detected in all transgenic lines containing

the *pat::nptII* fusion gene (pBAU1 and pBAU2) are much lower than those detected in

the same tissue using the NPTII ELISA (Table 4.5). To better compare tissues

assayed by both ELISA methods (NPTII and PAT), the values were presented as a

function of the positive control used in each ELISA method (Table 4.7).

Table 4.7   Comparison of S₁ *N. tabacum* pBAU2 Plant Tissues Quantified Simultaneously for NPTII (NPTII ELISA kit) and for PAT (In-House PAT ELISA kit)

	Nt wt	pBAU2 #15	pBAU2 #13	pBAU2 #14	pBAU1 #1	pHOE6 110/2
		% of pBAU1 #1 (35S-35SP-AMV)				
Young Plants Pre-Bolting (5-7 true leaf stage) L5 (50 days ps; 36 days pL)						
PAT	0%	8%	22%	9%	100%	
NPTII	0%	13%	1%	14%	100%	0%
Mature Plants Bolting (10-14 true leaf stage) L4 (57 days ps; 22 days pL)						
PAT	0%	2%	1%	0%	100%	52%
PAT	0%	5%	3%	0%	100%	64%
NPTII	0%	13%	1%	14%	100%	0%
NPTII	0%	13%	0%	11%	100%	0%
Mature Plants Bolting (10-14 true leaf stage) L4 (59 days ps; 24 days pL)						
PAT	0%	9%	3%	6%	100%	446%
NPTII	0%	19%	9 %	28%	100%	1%
Mature Plants Flowering and Setting Seed (15-18 true leaf stage) entire flower (155 days ps;128 days pL)						
PAT	0%			2%	100%	392%
NPTII	0%			0%	100%	0%

Greenhouse Spray Assay

Greenhouse grown S_1 *N. tabacum* pBAU2 #13, pBAU2 #14, pBAU2 #15, pBAU1 #1, pBAU1 #3, and nun-transformed *N. tabacum* cv. Xanthi plants were sprayed with 0 ml Liberty®/L, 3 ml Liberty®/L, 6 ml Liberty®/L, and 12 ml Liberty®/L. The results were recorded two weeks post spray and are illustrated in Figure 4.7.

From the Liberty spray results, both pBAU2#13 and pBAU2#15 demonstrated the same level of post-spray symptoms as the pBAU1 controls for all levels of application. The pBAU2#14 line demonstrated a lower tolerance to Liberty at doses exceeding 3 ml Liberty®/L (Figure 4.7).

Figure 4.7: The Percentage Survival of S_1 *Nicotiana tabacum* pBAU2 Transgenics as a Function of Herbicide Spray Level
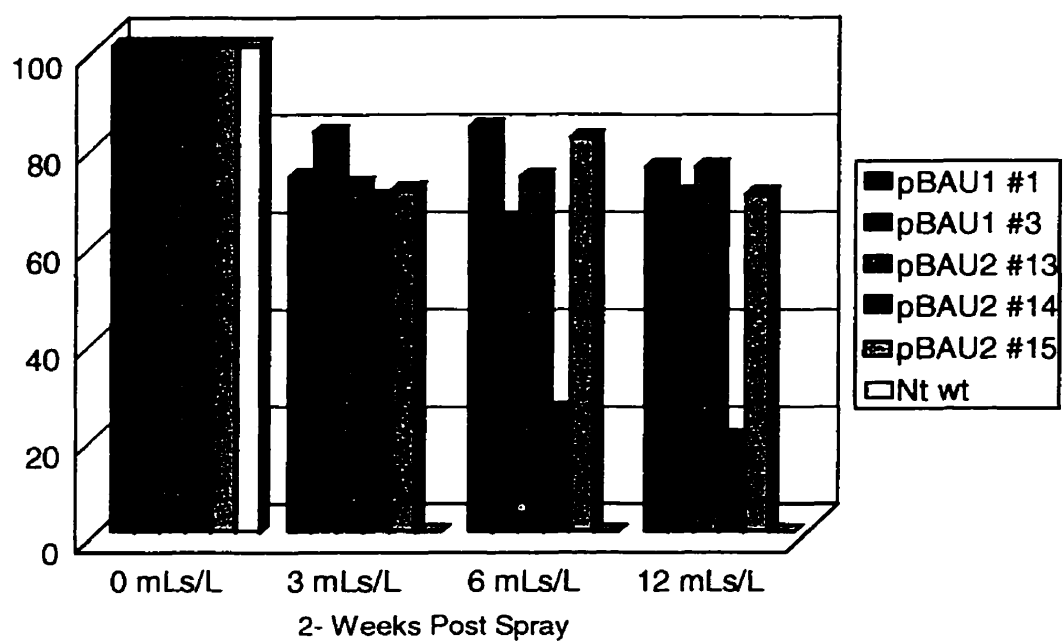
Seed Germination Assay

The percent germination of homozygous (S_2) (Table 4.8) *N. tabacum* pBAU2 seed plated onto varying levels of kanamycin or L-PPT were scored two weeks after seeding. The results from three separate seed germination assays are summarized in Table 4.8 and illustrated in Figure 4.8. Non-transformed *N. tabacum* seed which germinated in the presence of L-PPT often demonstrated an emerged root radicle but did not expand its cotyledons. At higher concentrations the hypocotyl and shrivelled cotyledons would turn white. A non-transformed *N. tabacum* seed which germinated in the presence of kanamycin (200 mg/L) demonstrated the same phenotype as one which germinated in the absence of kanamycin. Only after a period of two weeks would the seedling on kanamycin demonstrate smaller, discoloured (yellow) cotyledons while the control would have fully expanded green cotyledons.

Table 4.8   Seed Germination Assay of Homozygous S$_2$ *N. tabacum* pBAU2 Promoter Tagged Lines: Seedling Susceptibility to Increasing Levels of Kanamycin and Glufosinate Ammonium

Line	Selection Pressure (mg/L)	Average % Germination	Average % Seedlings Sensitive to Selection ± Standard Deviation
N.t. non-transformed	0 L-PPT	100	0 ± 0
	5 L-PPT	98.4	100 ± 0
	50 L-PPT	95.1	100 ± 0
	100 L-PPT	98.1	100 ± 0
	250 L-PPT	100	100 ± 0
	500 L-PPT	98.4	100 ± 0
	100 kanamycin	100	0 ± 0
	200 kanamycin	100	100 ± 0
N.t. pBAU2 #13	0 L-PPT	95.9	0 ± 0
	5 L-PPT	98.8	0 ± 0
	50 L-PPT	96	25.3 ± 32.6
	100 L-PPT	98.9	44.8 ± 38
	250 L-PPT	98.6	60.3 ± 49.7
	500 L-PPT	96.3	100 ± 0
	100 kanamycin	92.0	0 ± 0
	200 kanamycin	95.3	0 ± 0
N.t. pBAU2 #14	0 L-PPT	93.2	0 ± 0
	5 L-PPT	90.4	2.1 ± 3.6
	50 L-PPT	88.8	66.0 ± 36.5
	100 L-PPT	95.1	83.7 ± 17.1
	250 L-PPT	87.1	97.4 ± 3.6
	500 L-PPT	97.7	100 ± 0
	100 kanamycin	96.9	0 ± 0
	200 kanamycin	95.6	0 ± 0
N.t. pBAU2 #15	0 L-PPT	99.5	0 ± 0
	5 L-PPT	97.7	1.2 ± 3.4
	50 L-PPT	98.8	4.2 ± 9.2
	100 L-PPT	99.5	16.9 ± 19.9
	250 L-PPT	99.4	97 ± 5.3
	500 L-PPT	99.4	100 ± 0
	100 kanamycin	99.3	0 ± 0
	200 kanamycin	97.6	0 ± 0
N.t. pBAU1 #2	0 L-PPT	100	0 ± 0
	5 L-PPT	98.8	0 ± 0
	50 L-PPT	100	16.1 ± 14.5
	100 L-PPT	98.4	5.3 ± 3.2
	250 L-PPT	100	8.6 ± 2.9
	500 L-PPT	95.5	18.7 ± 5.3
	100 kanamycin	100	0 ± 0
	200 kanamycin	100	0 ± 0

Figure 4.8 *In Vitro* Seed Germination Assay of *N. tabacum*
pBAU2 S_2 Homozygous Material Two Weeks After Seeding
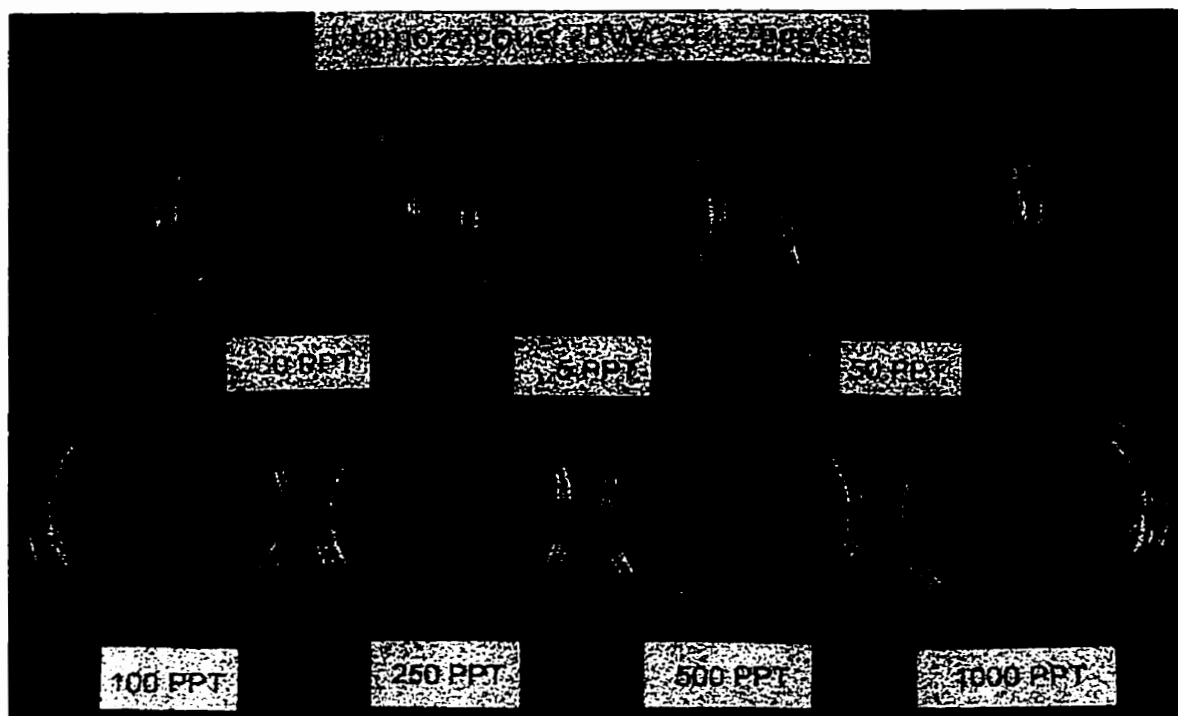
Legend:
N. t. pBAU2 #14 = BW 234 1Ø
N. t. pBAU2 #15 = BW 234 2ggg
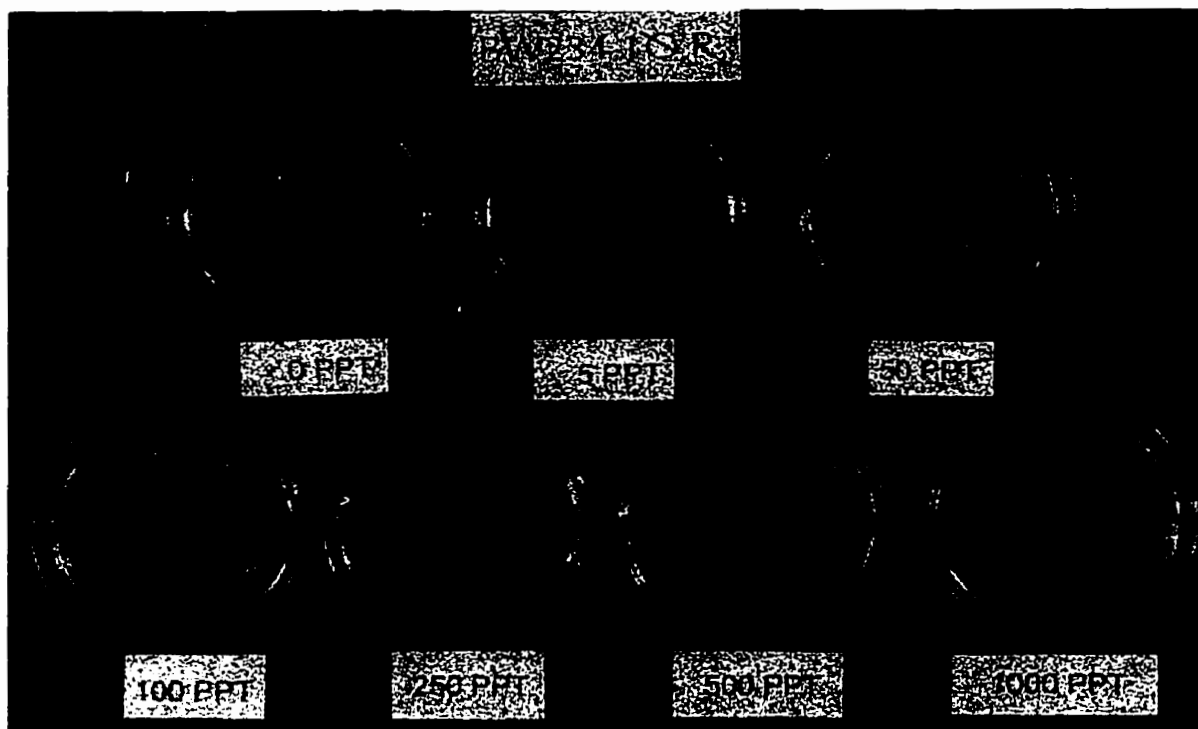
4.4 Discussion

Tobacco was chosen as the transformation system of choice since it has a high regeneration frequency when using the leaf disc method of Horsch *et al.*, (1985). From previous experiments it was shown that in the presence of kanamycin freshly transformed *Nicotiana tabacum* cv. Xanthi leaf disks (Table 3.4) had an almost ten fold higher regeneration frequency than *Brassica napus* cv. Westar cotyledonary petioles (Table 3.3). When using a promoterless selectable marker gene for transformation it can be expected that the regeneration frequency on the selection agent is much lower than when using a strong constitutive promoter fused to the selectable marker gene. Cells transformed with a promoterless selectable marker construct and transferred on to the respective selection agent can only result in a regenerant if the selectable marker gene has inserted itself downstream from an active promoter sequence. Transformations performed with the promoterless *pat::nptII* fusion construct (pBAU2) resulted in approximately a 6-fold lower regeneration frequency on kanamycin than the transformations which were performed with the CaMV35S-CaMV35S-AMV-*pat::nptII*-nos T construct (pBAU1) (Table 4.1).

From these promoter tagging transformations, a total of eight regenerants were able to root on 5 mg/L of L-PPT. Due to limited time and resources, one of the objectives of the subsequent screening process was to test the various promoter tagged transformants in such a manner as to be able to select one or more candidates for further promoter studies (Chapter 5). The minimum selection criteria included the following: a single locus insertion of the T-DNA into the plant genome (preferable for future promoter isolation studies), the presence of an intact *pat::nptII* selectable

marker gene, rooting on 20 mg/L of L-PPT (the standard rooting level used by AgrEvo Canada Inc. for selecting their CaMV-35S-*pat* transformants; Wang, HM, personal communication), and tolerance to a minimum of 1X (3ml/L) Liberty® (AgrEvo Canada Inc.; 3 ml/L is approximately equivalent to 1X field application of Liberty® herbicide; Gerwing, T., personal communication) foliar spray application level in the greenhouse. Only three of the eight regenerants were able to root on 10 mg/L and subsequently on 20 mg/L of L-PPT (Figure 4.5). These three transformants, pBAU2 #13, pBAU2 #14 and pBAU2 #15, were verified to be single copy transformants by Southern Blot hybridization (Figure 4.3). From restriction mapping studies (Figure 4.4) and from PCR analyses, the T-DNA region appeared to be intact. All three T_0 promoter-tagged plants showed no adverse effects when sprayed with 1X Liberty® herbicide in the greenhouse (Figure 4.7).

More stringent glufosinate ammonium (L-PPT) tolerance assays were implemented in an effort to select the best (strongest expressing) candidate from the three pBAU2 promoter tagged plants (#13, #14 and #15). A seed germination assay was performed on homozygous selfed lines (S_2) originating from each pBAU2 transformant (Table 4.8). A summary of both the molecular characterizations and the glufosinate ammonium tolerance assays is illustrated in Table 4.9. Single copy *Nicotiana tabacum* cv. Xanthi pBAU1 transformants (CaMV35S-CaMV35S-AMV-*pat::nptII*) and a single copy *Nicotiana tabacum* cv. Xanthi pHOE6 transformant (CaMV35S-*pat*) were used as positive controls for these assays. A non-transformed *Nicotiana tabacum* cv. Xanthi originating from tissue culture was used as a negative control.

Table 4.9   **Summary of Molecular Characterizations and Glufosinate Ammonium Tolerance Assays Performed on *Nicotiana tabacum* cv. Xanthi pBAU2 #13, #14 and #15**

Assay	N.t. pBAU2 #13	N.t. pBAU2 #14	N.t. pBAU2 #15
Copy Number on T_0 (Fig. 4.3)			
- Southern Analysis	1 copy	1 copy	1 copy
PCR Analysis on T_0			
(not shown) *- pat*	+	+	+
- nptII	+	+	+
-pat:nptII	+	+	+
Rooting Assay on T_0 (most resistant = 3; least resistant = 1) (Fig. 4.5)			
- @ 10 mg/L	2	1	3
- @ 20 mg/L	3	1	2
Greenhouse Spray Assay on S_1 (most resistant = 3; least resistant =1) (from Fig. 4.7)			
- @ 3 ml/L	3	3	3
- @ 6 ml/L	2	1	3
- @ 12 ml/L	3	1	2
Seed Germination Assay on S2 (% survival) (Fig. 4.8)			
- @ 100 mg/L	55.2 %	16.3 %	83.1 %
- @ 250 mg/L	39.7 %	2.6 %	3 %
- @ 500 mg/L	0 %	0 %	0 %

For these glufosinate ammonium tolerance assays (Table 4.9), pBAU2 #15 was clearly the best candidate (most vigorous and healthy) at the middle concentrations (10 mg/L L-PPT for rooting assay, 6 mL/L Liberty® for spray assay, and 100 mg/L L-PPT for seed germination assay). At the higher concentrations (20 mg/L L-PPT for rooting assay, 12 mL/L for Liberty® spray assay, and 250 mg/L L-PPT for seed germination assay) pBAU2 #13 was slightly more vigorous than pBAU2 #15 for the rooting and herbicide spray assays, and much more vigorous than pBAU2 #15 for the seed germination assay.

A more detailed analysis of the expression patterns in these three pBAU2 transgenics was carried out on S1 progeny (resulting from a T_0 selfing) in the greenhouse using an immunoassay technique. Two enzyme linked immunosorbant assays (ELISAs) were used to detect both PAT and NPTII proteins in various plant tissues and at various stages of development. The PAT ELISA was developed (Appendix I; Bauer-Weston et al., 1996), while the NPTII ELISA was a commercially available kit (5 Prime→3 Prime Inc.). PAT and NPTII Activity assays (Appendix II) could not be used to monitor the expression levels of the pBAU2 transformants since they were not sensitive enough (Figure 3.18).

The results from three separate developmental analyses are tabulated in Tables 4.5 and 4.6. A summary of the NPTII protein studies was prepared in order to simplify this data so that a comparison between the three transformants could be made (Table 4.10). Only the NPTII data were used for this summary since the sensitivity of the commercial NPTII ELISA kit was greater than the PAT ELISA, as demonstrated by the comparison of identical tissue samples using both assays (Table 4.7). The pBAU1 transformant, Nicotiana tabacum cv. Xanthi pBAU1 #1, was used as a reference point in Table 4.10 since it contained the identical gene construct as the pBAU2 transformants, except for the promoter region. pBAU1 #1, as well as the pBAU2 transformants, all contained a functional PAT::NPTII fusion protein (Figures 3.18 and 3.19 for pBAU1s and Table 4.9 for pBAU2s) . It was important to use a transformant expressing the larger PAT::NPTII fusion protein (47 kD) as a control, since this fusion protein may be less stable than the smaller monumeric PAT (22 kD) which is found in the pHOE6 110/2 control. If the fusion protein is less stable, then an

ELISA may detect lower quantities of the protein; these lower levels would not be related to the level of expression or promoter activity, but instead would be related to the protein's stability.

Seedlings were assayed before and after spraying to determine whether the promoter tagged transformants could be induced (expression increased) by a 1X Liberty® spray application. From the summary in Table 4.10 (and in Tables 4.5 and 4.6) there was no evidence of increased expression levels in any of the transgenics two days after spraying. The S1 seedling population was a segregating population (3:1 for herbicide tolerance:susceptibility). To accurately compare the expression levels before and after spraying, only *pat::nptII* positive segregants (homozygotes and heterozygotes) were used to calculate the average expression level before and after spray treatment (Table 4.5). After the spray treatment, all susceptible plants were discarded. Only those segregants which were heterozygous or homozygous for the *pat::nptII* selectable marker gene were maintained and used for subsequent developmental analyses.

Table 4.10   **The Relative Amount of NPTII Protein, as Determined by NPTII ELISA, Detected in the Various Tissues and Developmental Stages of *Nicotiana tabacum* cv. Xanthi pBAU2 #13, #14 and #15.**

Tissue	N.t. pBAU2 #13	N.t. pBAU2 #14	N.t. pBAU2 #15
	% of pBAU1 #1 NPTII Expression Level		
Entire Seedlings (from Table 4.5)			
Pre-Spray	13	16	20
Post-Spray	15	14	15
Plant Pre-Bolting (from Table 4.5)			
L1	0.5	15	13
L5	8	7	9
L7	13	2	11
stem	19	4	25
root	17	1	25
Plant Bolting (from Table 4.5)			
L1	4	8	6
L2	7	25	32
L4	5	20	16
L8	6	9	5
L9	23	64	30
stem	19	10	31
root	21	2	57
Plant Flowering and Setting Seed (from Table 4.5)			
L2	4	15	8
L4	4	21	9
L7	8	20	10
L11	24	4	20
L12	15	12	12
L16	0	1	8
stem	31	13	40
root	31	0	27

L1 to L18 = true leaf classification ranked according to internode # ; from oldest true leaf (L1) to youngest leaf (up to L18, depending on the plant's stage of development)

From the comparative analysis in Table 4.10 it is possible to draw some generalizations regarding the expression patterns of each promoter tagged line. In pBAU2 #13, expression appeared to be higher in younger leaves and approximately 5 fold lower in older leaves. After flowering, the leaves along the flowering racime demonstrated no expression, while the middle leaves, which had developed before flowering, demonstrated the highest expression levels. Both the stem and root tissue,

regardless of developmental stage, demonstrated relatively high expression levels equalling or exceeding those detected in the youngest leaf tissue prior to flowering. The ELISA results and the seed germination assay (Table 4.8) both indicated that the seedlings also possessed relatively high expression levels; high enough to permit germination on 100 mg/L and 250 mg/L of L-PPT.

The promoter tagged transformant line, pBAU2 #14, demonstrated lower expression levels in the younger leaves and higher expression levels in the older leaves throughout most stages of development. Using the PAT ELISA, expression was mostly detected in the hypocotyl tissue with relatively little expression in the cotyledons of the seedlings. This may explain why this transformant fared so poorly in the seed germination assay (Table 4.8). At the bolting stage, though, this line demonstrated a much higher expression level (greater than 2 fold) in its youngest leaf, relative to the older leaves. Expression in the roots was extremely low throughout all stages of development.

In pBAU2 #15, expression was relatively high in the seedling. Prior to bolting, expression levels were approximately the same in all leaves regardless of age. These levels were comparable to the highest leaf expression levels detected in the other transgenic line, pBAU2 #13. After bolting the highest leaf expression levels were detected in the middle leaves to youngest leaves with the lowest expression in the oldest leaves. During flowering the new leaves along the racime of the flower inflorescence demonstrated a lower expression level than that detected in the oldest leaves. The stem and root tissue consistently demonstrated the highest level of expression regardless of the developmental stage, often expressing two to three fold

higher than in the leaf tissue. Such variation in expression levels is not uncommon for constitutive promoters, such as the CaMV35S promoter, where expression is almost undetectable in pollen (Benfey et al., 1990).

From this general comparative analysis (Table 4.10), pBAU2 #15 was chosen as the candidate with the most constitutive-like expression patterns. In general, expression levels were higher than in the other two candidates, and they were more consistent throughout the various tissues, and stages of development. Conclusions regarding the nature of this tagged regulatory sequence cannot be drawn since this developmental analysis was only a preliminary screen performed on S_1 segregating heterozygous and homozygous material. In general, it was shown that all three promoter tagged plants demonstrated lower expression levels than the pBAU1 transformants which contained the CaMV35S-CaMV35S promoter linked to the AMV enhancer sequence. It was difficult to determine whether these pBAU2 transformants were weaker or stronger than a construct regulated by the CaMV35S promoter alone, since the pHOE6 control did not contain the same selectable fusion marker gene.

Taking all of the comparative studies (Tables 4.9 and 4.10) into consideration, *Nicotiana tabacum* cv. Xanthi pBAU2 #15 was chosen as the candidate for further promoter isolation studies (Chapter 5). This transgenic line demonstrated a glufosinate ammonium tolerance level which exceeded the 3 ml/L Liberty® greenhouse spray level (approximately equivalent to a 1X field application rate), as well as demonstrating a more consistent expression pattern throughout all of its tissues throughout the various developmental stages tested.

Chapter 5: Isolation and Characterization of a Tagged Regulatory Sequence from Tobacco

5.1 Introduction

There are different methods for isolating plant promoters or regulatory sequences, many being cDNA based. Tagging approaches are often more efficient since function can often be determined prior to isolation. Among the promoter tagged lines recorded in this study, pBAU2#15 exhibited higher expression levels in more tissues than the other two tagged lines (Chapter 4). The method chosen for this study was to clone a subgenomic DNA population isolated from *N. tabacum* pBAU2#15 into a bacteriophage lambda DNA vector. Bacteriophage lambda has been used as a recombinant DNA cloning vector since the early 1980s (Young and Davis 1983). Several advantages of lambda vectors include their relatively large cloning capacity and their highly efficient DNA packaging and infection process. The large cloning capacity of lambda vectors (up to 25 kb depending on the vector) is especially important when considering the total number of recombinant phage needed to represent an entire eukaryotic genome. To produce a representative library for a mammalian genome, one would require approximately 8×10^5 recombinants to provide a 99% probability of isolating a particular sequence (Kaiser and Murray 1985).

In this study, a subgenomic library was used to increase the probability of detecting the clone containing the *pat::nptII* tagged promoter sequence. Restriction mapping studies had indicated that the pBAU2#15 promoter tagged sequence plus the

tag was present on one 8.5 kb *EcoRI* fragment (Table 4.4). To produce a subgenomic library, all *EcoRI* fragments ranging in size from 8.0 kb to 10.5 kb were ligated into the lambda (λ)-ZAP cloning vector (Stratagene). This reduced the number of recombinants that had to be screened. Other necessary criteria were: that a maximum number of recombinants had to be obtained with a minimum number of background nonrecombinants, and that the genomic clone was surrounded by a multiple cloning site to facilitate subsequent restriction mapping and cloning strategies. These latter two criteria were met using Stratagene's high efficiency Lambda ZAP® genomic cloning kit. This kit provided high recombinant cloning efficiencies and low nonrecombinant backgrounds, as well as providing an *in vivo* excision method for the cloned fragments into a phagemid vector. Other cloning kits, such as LambdaGEM® by Promega, also offer similar features.

An alternate strategy, called thermal asymmetric interlaced (TAIL-) PCR (Liu *et al.*, 1995), was also used to recover the 5' genomic sequence flanking the *pat::nptII* insertion in the promoter tagged plant pBAU2#15. This strategy uses three nested specific primers in successive reactions together with a shorter arbitrary degenerate (AD) primer, as depicted in Figure 5.1. This method yields specific products of high purity that can be used directly as hybridization probes or as sequencing templates. TAIL-PCR does not require special DNA manipulations before PCR, nor laborious screening afterwards. The entire procedure was performed in three days, as opposed to the lengthy lambda library screening method which required sample preparation, packaging, titering, screening and finally excision of the identified fragment into a phagemid vector.

Figure 5.1   TAIL-PCR Procedure for Amplifying a Genomic Sequence Flanking a Known T-DNA Insert

Nested primers

AD primer   NP3   NP2   NP1

PAT   NPT II

genomic flanking sequence

Primary PCR with NP1 and an AD primer

5 high stringency cycles

1 low stringency cycle

TAIL-cycling
(14 super cycles)

1 reduced stringency cycle   2 high stringency cycles

specific product   nonspecific product I   nonspecific product II

product yield:

moderate   high   low
(generally undetectable)   (detectable)   (undetectable)

1000-fold dilution

Secondary PCR with NP2 and the same AD primer

specific product   nonspecific product

product yield:

high   low
(detectable)   (undetectable)

1000-fold dilution

Tertiary PCR with NP3 and the same AD primer

agarose gel analysis

(adapted from Liu *et al.*, 1995)

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The TAIL-PCR method was initiated as an alternative method for tag isolation, following months of unsuccessful screening with the Lambda library system. Finally, though, a 5' genomic flanking sequence was identified from the Lambda library, and it was cloned into a bacterial vector to facilitate restriction mapping and sequencing analyses. Due to the lack of a good *in vitro* transcription system for plants (An and Kim 1993), the tagged promoter sequence had to be introduced into intact plant cells in order to evaluate its activity. This was accomplished by cloning the 5' genomic flanking sequence along with its *pat::nptII-nosT* tag into a plant binary vector and observing its expression in transgenic *N. tabacum* cv. Xanthi and in transgenic *B. napus* cv. Westar. The 5' genomic sequence was also fused to the histochemical marker gene *gus* (Jefferson *et al.*, 1987) and again transformed into both *N. tabacum* cv. Xanthi and *B. napus* cv. Westar using *Agrobacterium*-mediated transformation.

5.2 Materials and Methods

5.2.1 Production of a Subgenomic Library in Lambda, Subsequent Screening and Tag Isolation

Three promoter tagged tobacco lines were identified as having strong expression of the tagged *pat::nptII* gene (Chapter 4.4). The strongest expressing line, pBAU2#15, as demonstrated by ELISA analysis, was chosen for further promoter isolation studies. A subgenomic library was produced from genomic DNA isolated from the line pBAU2#15. Genomic DNA was isolated from very young leaves of homozygous greenhouse grown plants (Dellaporta *et al.*, 1983, 1985; Appendix II). DNA recovered from approximately one gram of leaf tissue was dissolved in 100 µl of TE buffer (50 mM Tris, 10 mM EDTA, pH 8.0) and stored at -20°C.

EcoRI digested genomic DNA was electrophoresed on a 0.8% agarose gel and a subgenomic fraction between 8.5 and 10.5 kb was excised from the gel. This size range was chosen based on the *EcoRI* restriction mapping results determined in Chapter 4 (Table 4.4). The subgenomic DNA fraction was electroeluted from the 0.8% agarose using an Unidirectional Electroeluter (International Biotechnologies Inc. [IBI] Model UEA, Catalogue # B7300), then precipitated and washed.

The subgenomic library was produced using Stratagene's Lambda ZAP II - CIAP Vector Kit (Stratagene, category # 239212). The *EcoRI* subgenomic fraction (8.5 and 10.5 kb) from pBAU2#15 was ligated into the Lambda vector arms. The overnight ligation reaction (1 µl λ ZAP II *EcoRI*/CIAP predigested arms; 2.5 µl of pBAU2#15-*EcoRI* digested subgenomic DNA (200 ng/µl); 0.5 µl 10x ligase buffer; 0.5 µl of 10 mM ATP (pH 7.5); 0.5 µl T4 DNA ligase (40 000 U)) occurred at 12.5°C.

The ligase enzyme was heat killed and the ligation product was packaged into λ capsid heads using Stratagene's GigaPack II Gold phage packaging extract (Stratagene, Catalogue # 200214).

Each step of the cloning procedure (the insertion of the subgenomic fraction into the Lambda arms, and the subsequent packaging into the phage heads) was verified using PCR and Southern Hybridization to insure that the promoter tag was present in these fractions. The resulting library was titered, plated on NZY agar plates (0.5% NaCl (w/v), 0.2% $MgSO_4.7H_2O$ (w/v), 0.5% yeast extract, 1% casein hybrolysate (w/v), 1.5% Bactoagar (w/v), pH 7.5) and then screened by performing plaque lifts onto Hybond-N nylon membranes (Amersham Life Science, category # RPN 132N), followed by hybridization to the *nptII* probe. The random primed ^{32}P-CTP *nptII* probe was prepared according to BRL's Random Priming Kit (Catalogue # 18187-013). In total, over 1.5×10^6 plaque forming units (pfu) were screened.

A putative promoter tagged clone (a plaque which hybridized to the *nptII* probe) in Lambda was identified and purified, as specified in the Lambda ZAP II CIAP vector kit (Stratagene Catalogue # 239212). Phage from this plaque were subjected to secondary and tertiary screening procedures by isolating them and reinfecting them into XL1-Blue mrf- *E. coli* (Stratagene) cells. These cells were then plated onto NZY plates, and lifted onto nitrocellulose membranes, which were subsequently hybridized to a random primed *nptII* DNA probe (as previously described for the primary screening procedure).

These plaques were also subjected to a PCR reaction to verify that they contained the *pat::nptII* promoter tag. For PCR verification, 1 µl of a boiled plaque

lysate was used in a standard 30 µl reaction (0.25mM dNTPs (Pharmacia Biotech 27-2035-01), 1X Taq Polymerase Buffer (Pharmacia 27-0799A), 5 µM of each primer (refer to Section 3.2.5 for primer specifications), 1 U Taq DNA Polymerase (Pharmacia 27-0799)). Amplifications were achieved after denaturation of the template DNA at 96°C for 2 minutes followed by 35 repeated cycles of 30 second denaturation at 94°C, 1 minute annealing at 55°C and a 2 minute extension at 72°C. This cycle was ensued by a final 7 minute extension at 72°C and cooling to 15°C using the MJ Research Inc. PTC-100 thermocycler. The reaction products were directly analysed on a 0.8% agarose gel.

Using the excision kit from Stratagene's Lambda ZAP II vector, the *EcoRI* fragment (approx. 10 kb, containing the tagged promoter) was excised into the *E. coli* high copy number phagemid pBlueScript II SK + (Stratagene, Catalogue # 212205), according to the manufacturer's protocol (Stratagene Lambda Zap II, catalogue # 239212).

Numerous rearrangements were noted in some of the pBlueScriptII+ #15 *E. coli* clones, most likely due to the instability of such a large insert (10 kb) within a high copy number plasmid. As a result, the *EcoRI* piece from the pBlueScript-BAU2#15 vector was excised and cloned into pRD400 (a low copy number pBin19 binary vector derivative possessing a wild type *nptII* gene (Datla *et al.*, 1992)).

5.2.2 TAIL-PCR for the Isolation of Tagged Regulatory Sequences

Thermal asymmetric interlaced (TAIL-) PCR (Liu *et al.,* 1995), an efficient and relatively quick technique for amplifying DNA segments adjacent to known sequences, was used to isolate the flanking sequence 5' to the *pat::nptII* fusion gene. The resulting flanking sequence, a tertiary PCR product, was directly used as a sequencing template.

This TAIL-PCR protocol utilized three nested specific primers in successive reactions together with a shorter arbitrary degenerate (AD) primer, so that the relative amplification efficiency of the specific and non-specific products could be thermally controlled. The three nested primers that were used, along with the AD primer, are listed in Figure 5.2. The best three nested primers along with the best degenerate primer had to be determined. Five different nested primers and four different degenerate primers were tested. The entire procedure is illustrated in Figure 5.1.

The three subsequent PCR reactions were set up in the following manner:

Primary 20 μl rxn:	Secondary 20 μl rxn:	Tertiary 100 μl:
• 1x PCR buffer	• 1 μl of 1/50 diluted primary rxn product	• 1 μl of 1/10 diluted secondary rxn product
• 200 μM dNTPs	• 1x PCR buffer	• 1 x PCR buffer
• 20 ng genomic DNA	• 200 μM dNTPs	• 200 μM dNTPs
• 0.8 unit Taq polymerase	• 0.8 unit Taq polymerase	0.6 units Taq polymerase
• 0.2 μM Inv5'-nptII Primer	• 0.2 μM Pat4 Primer	• 0.2 μM revPAT3b Primer
• 2 μM degenerate primer	• 2 μM degenerate primer	• 2.0 μM degenerate primer

Figure 5.2   Nested and Arbitrary PCR Primers for the Amplification of the Flanking Sequence Upstream of the *pat::nptII* fusion gene
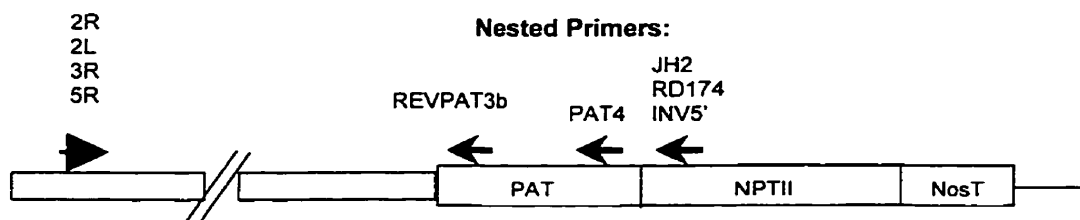
Nested Primers:

INV5':	5' - CGA TTG TCT GTT GTG CC - 3' (17 mer)
JH2:	5' – AAG AAG GCG ATA GAA GGC – 3' (18 mer)
RD174:	5' – TCA GAA GAA CTC GTC AAG – 3' (18 mer)
PAT4:	5' - GCC TCA TGC AAC CTA ACA GA - 3' (20 mer)
REVPAT3b:	5' - CTG GCC TAA TCT CAA CTG GTC T - 3' (22 mer)

Degenerate Primer:

2R: 5' - G^{A}AA A^{G}TT G^{A}AA G^{A}TT IGA C^{T}TC C^{T}TG - 3'
       (21 mer ; 64 fold degeneracy)

2L: 5' – CAG^A GAA^G TCI AAC^T TTC^T AAT^C TTC^T – 3'
       (21 mer; 64 fold degeneracy)

3R: 5' – C^{T}TT IGC A^{G}AA T^{C}TG IAC CAT IGG G^{A}TT - 3'
       (24 mer; 16 fold degeneracy)

5R: 5' – C^{A}AA IGT IGC T^{C}TC IAC A^{G}AA ICC C^{T}TC - 3'
       (24 mer; 16 fold degeneracy)

(courtesy of Dr. Jim MacPherson, Plant Biotechnology Institute,
N.R.C.,110 Gymnasium Pl., Saskatoon, SK, S7N 0W6, Canada)

Genomic DNA was isolated from young leaves of an S_1 pBAU2#15 plant using the method of Dellaporta *et al.*, (1983). The TAIL-PCR procedure, as well as the PCR thermal cycle settings (Amplitron II, Thermolyne) were followed as outlined in the publication by Liu *et al.*, (1995).

5.2.3 Sequence Analysis of Flanking Genomic DNA

Plant genomic DNA sequences, both 3' and 5' of the inserted T-DNA promoter tag, along with the tertiary TAIL-PCR product, were sequenced by the DNA Technologies Unit at the NRC-PBI (110 Gymnasium Place, Saskatoon, SK, S7N 0W6) using a Perkin Elmer Applied Biosystems 373 DNA Sequencer STRETCH™ in conjunction with the ABI PRISM™ Dye Terminator Cycle Sequencing Kit. λ-ZAP #15 DNA, pRD400 #15 (pBAU3) DNA, and the TAIL-PCR amplicon were used as a templates for sequencing. Each sequencing run produced a sequence of approximately 400 to 500 bp in length. A new 20 to 22 mer PCR primer (5' to 3') was designed near the 3' end of each new sequence and used for the subsequent sequencing run. The region 5' (upstream) of the *pat::nptII* tag was sequenced in both directions for both λ-ZAP#15 and for pRD400#15 (pBAU3). The TAIL-PCR amplification product was also sequenced in both directions. The sequence 3' (downstream) of the *pat::nptII* tag was sequenced in one direction only.

Sequence homology to already existing non-redundant GenBank, EMBL, DBJ and PDB sequences was investigated using the Blast Local Alignment Search Tool (Altschul *et al.*, 1990).

5.2.4 Southern Analysis of *N. tabacum* cv. Xanthi and *B. napus* cv.

Westar for the presence of Homologous Regions to the Putative BW1 Promoter Region

Genomic DNA was purified from young leaves of nontransformed *N. tabacum* cv. Xanthi, *N. tabacum* pBAU2#15 (positive control), and nontransformed *B. napus* cv. Westar according to the method of Dellaporta (Dellaporta *et al.*, 1983, 1985). DNA recovered from approximately 1 gram of leaf tissue was dissolved in 100 µl of TE buffer (50 mM Tris, 10 mM EDTA, pH 8.0) and stored at -20° C for up to one year. Genomic DNA was restricted with either *EcoRI*, *HindIII* or *BamHI*, electrophoresed through a 0.8% agarose gel, and transferred to a nylon membrane as described in Section 3.2.3. A template consisting of the 330 bp PCR amplicon (BW1-330) of the 5' *pat::nptII* flanking sequence (putative BW1 promoter region) amplified from λ-BAU2#15 was labelled with [α-^{32}P]dCTP using a Random Primer Labelling Kit (Life Technologies, USA) and used as a hybridization probe. Conditions for hybridization and washing were as described by Maniatis (1982).

5.2.5 Cloning of the Putative Promoter and T-DNA Tag into a Binary Vector (pBAU3): A Construct for a Functional Plant Transformation Assay

The 10 kb *EcoRI* fragment containing the *pat::nptII* fusion gene, along with the tagged 3' and 5' flanking sequences, was ligated into the *EcoRI*-digested *Agrobacterium tumefaciens* binary transformation vector, pRD400 (Datla *et al.*, 1992). The ligation product was transformed into competent DH5αMCR™ cells (Gibco BRL, catalogue no. 18289-017) and transformants were isolated on 2YT plates (Appendix III) containing 50 mg/L kanamycin and 40 mg/L 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-GAL). White colonies were checked for the presence of

the 10 kb insert by restriction enzyme digest and gel electrophoresis,. The new vector,

pBAU3, was mobilized by triparental mating (Rogers *et al.*, 1986) from *E. Coli* to

Agrobacterium tumefaciens pMP90 (Koncz and Schell 1986).

To determine whether the tagged flanking sequences (flanking the *pat::nptII*

gene) exhibited promoter activity, *B. napus* cv. Westar and *N. tabacum* cv. Xanthi

pBAU3 transformants were produced and tested for the expression of the *pat::nptII*

fusion gene by subjecting them to an *in vitro* rooting assay on varying levels of L-

PPT. Both species were regenerated *in vitro* in the presence of kanamycin (100 mg/L

for *N. tabacum* and 20 mg/L for *B. napus*). Half of the *N. tabacum* cv. Xanthi

explants were also regenerated in the presence of L-PPT (2 mg/L). Following

regeneration, these T_0 regenerants were subjected to a rooting assay on increasing

levels of glufosinate ammonium to determine the relative strength of the tagged

regulatory sequence at this stage of regenerant development.

To produce *B. napus* cv. Westar and *N. tabacum* cv. Xanthi pBAU3

transformants, both species were transformed with pBAU3 using *Agrobacterium*

transformation. As a positive control, both species were also transformed with

pBAU1 (CaMV35S-CaMV35S-AMV-*pat::nptII*-nosT) and subjected to the same *in*

vitro manipulations and assays. Explants not inoculated with *Agrobacterium* were also

regenerated in the absence of selective pressure to produce nontransformed negative

controls which had undergone in vitro regeneration. Tissue explants were transformed

and regenerated as described in Section 3.2.2. The ability of the tagged flanking

sequences to direct transcription of the *pat::nptII* fusion gene was tested by subjecting

T_0 pBAU3 transformants to an *in vitro* rooting assay on increasing levels of

glufosinate ammonium (from 0 mg/L to 2 000 mg/L for *N. tabacum*, and from 0 mg/L to 80 mg/L for *B. napus*). The protocol for the rooting assay was carried out as outlined in Section 3.2.3.

5.2.6 Histochemical Analysis of *In Vitro* Regenerants Containing the Tagged Putative Promoter Fused to GUS

Two in-frame fragments containing the putative 330 bp promoter, BW1, (373 bp and 403 bp) were fused to the β-glucuronidase gene *(gus)* in the GUS expression vector, pRD420 (Dr. R. Datla, Plant Biotechnology Institute, National Research Council, 110 Gymnasium Place, Saskatoon, SK, Canada). The 373 bp BW1-containing fragment was a *HindIII* digested PCR product resulting from the amplification of λ-ZAP#15 DNA with the following primer set:

REVPAT3B: 5' - CTG GCC TAA TCT CAA CTG GTC T - 3' (22 mer)

M13-20: 5'-   5' – GTA AAA CGA CGG CCA GT – 3' (17 mer)

The RevPAT3b binds at the 5' end of the *pat::nptII* fusion gene insert producing a blunt-ended amplification product whose ATG start codon is in-frame with the ATG start codon of the *gus* gene in pRD420. The 403 bp BW1-containing fragment was isolated by restricting pBSII-#15 with *HindIII* and *PvuII*. *PvuII* cuts in the 5' end of the *pat::nptII* gene producing a blunt end. Both 373 and 403 bp fragments were ligated into the pRD420 vector which had been restricted with *BamHI* and subsequently blunt-ended using the cloned Klenow Fragment of DNA Polymerase I (Pharmacia) and then digested with *HindIII*. The resulting pRD420-BW1 plasmids are described in Figure 5.15 (a and b).

Both binary plasmids, pRD420-BW1 a and b, were introduced into *Agrobacterium tumefaciens* via triparental mating and subsequently transformed into *B. napus* cv. Westar and *N. tabacum* cv. Xanthi as described in Section 5.2.5. As a control, both *B. napus* and *N. tabacum* were also transformed with a vector, pRD410 (Dr. R. Datla, Plant Biotechnology Centre, National Research Council, 110 Gymnasium Place, Saskatoon, SK, Canada), containing the constitutively regulated *gus* gene (CaMV35SP-*gus*-nosT). All shoots were regenerated in the presence of kanamycin (20 mg/L for *B. napus* and 100 mg/L for *N. tabacum*).

Histochemical β-glucuronidase (GUS) analysis was performed following the method described by Jefferson (1987) with minor modifications. Pieces of tissue from putative transgenic *N. tabacum* cv. Xanthi and *B. napus* cv. Westar plants were placed in a solution of 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt (X-Gluc; Biosynth AG, Catalogue # B7300) and incubated under vacuum overnight at 37°C. The samples were bleached with a 10-fold diluted commercial Javex™ solution for several hours to remove the pigments. The presence of blue colour was regarded as an indicator of β-glucuronidase *(gus)* gene expression. Details are described in Appendix II.

5.3 Results

5.3.1 Screening of the Lambda Subgenomic Library and Isolation of the Tagged Putative Promoter

In total, over 1.5×10^6 plaque forming units (pfu) were subjected to a primary screen before the one pfu, λZAP-#15, which tested positive for *nptII* in subsequent screenings, was isolated. The λZAP-#15 pfu was picked from a primary screening plate and subjected to a secondary and a tertiary screen. In the tertiary screen, all of the λZAP-#15 derived pfu's tested positive for *nptII*, as demonstrated by the hybridization of the plaques to a random primed ^{32}P-CTP-*nptII* probe (Figure 5.3).

The λZAP-#15 clone was subjected to a PCR analysis where it tested positive for the presence of a: 400 bp of a *pat* specific amplicon, 747 bp of a *nptII* specific amplicon, and 1150 bp of a *pat::nptII* specific amplicon. Following the procedures outlined by the Lambda ZAP II Kit manufacturer, the 10 kb *EcoRI* fragment was excised from the λZAP-#15 clone and recircularized to form a pBlueScript II SK + (pBSII) phagemid containing the cloned insert (pBSII-#15) (Figure 5.4). The resulting pBSII-#15 plasmid DNA also tested positive for the presence of intact *pat*, *nptII* and *pat::nptII* fusion gene sequences, using PCR analysis. It was observed that the 10 kb cloned insert was not stable in the high copy number pBlueScript plasmid background. Often rearranged restriction patterns were observed with different *E. coli* cultures originating from the same pBSII-#15 *E. coli* frozen stock. In an effort to introduce the cloned insert into a more stable background, the 10 kb *EcoRI* fragment was cloned into the low copy number *Agrobacterium* binary vector, pRD400 (Datla *et al.*, 1992).

Figure 5.3: Plaque Lift Hybridization of Tertiary Screened
Plaque Forming Units with the random primed
^{32}P-CTP-*nptII* DNA probe

Every plaque hybridized to the *nptII* probe; evidence for
a pure λZAP-#15 culture.
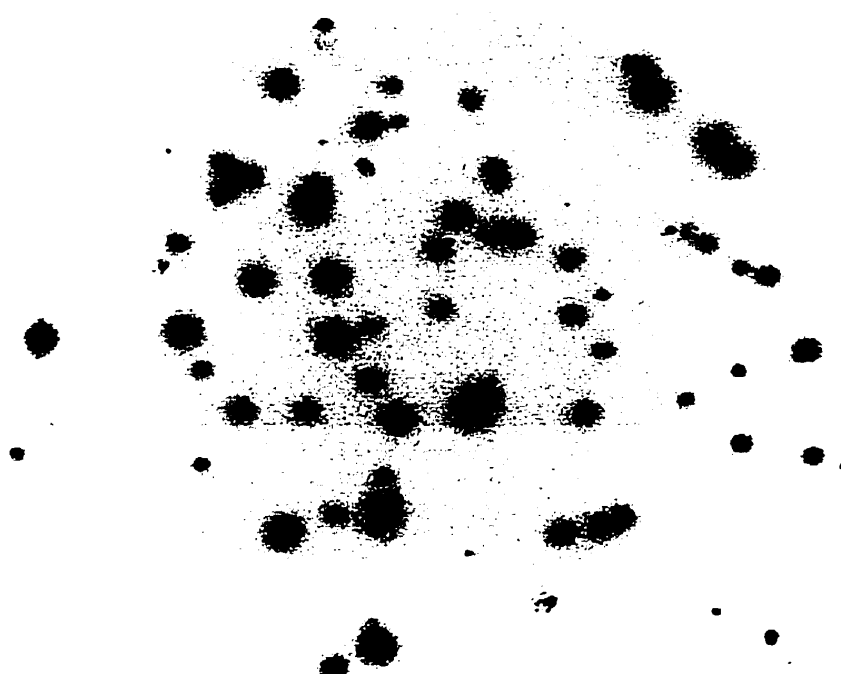
Figure 5.4: Plasmid Map of the pBlueScript II (SK+) Phagemid Containing the 10 kb *EcoRI* T-DNA Tag Insert: pBSII-#15
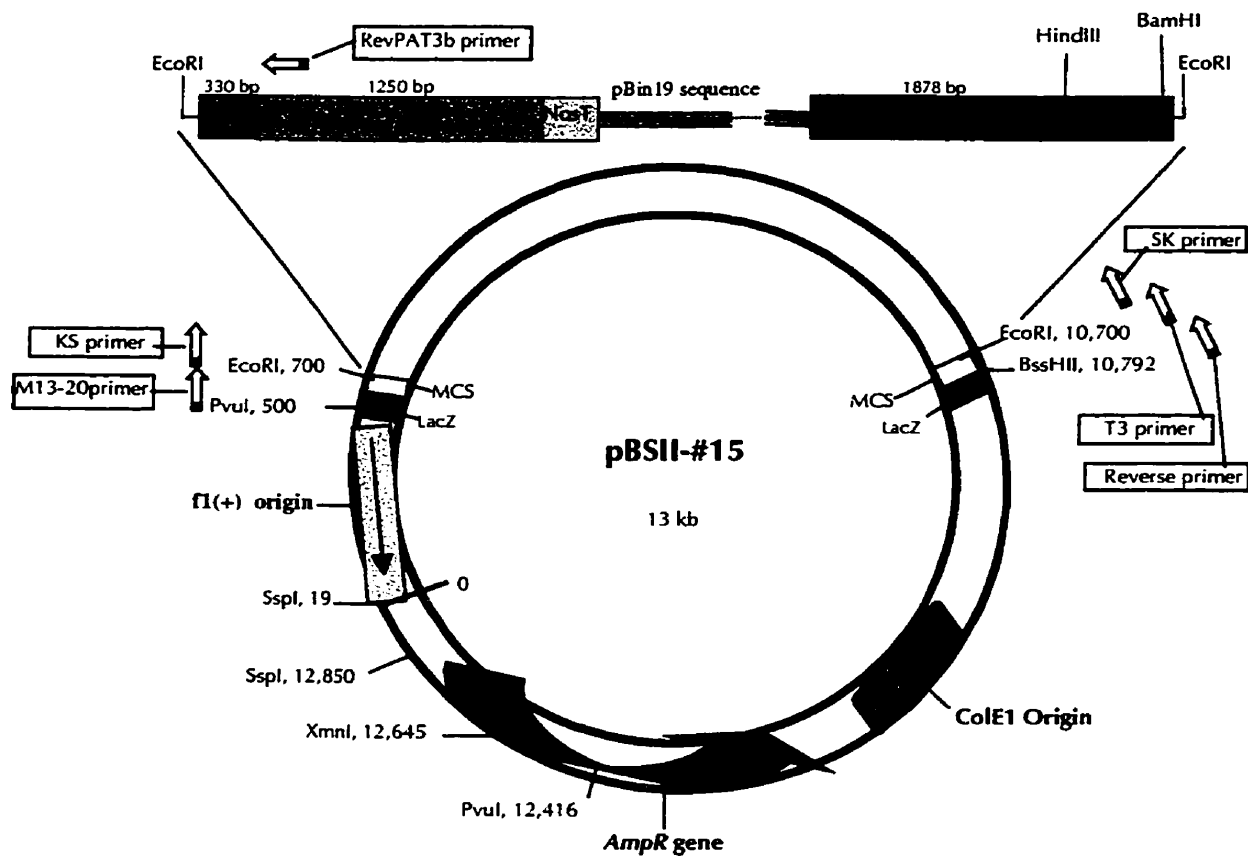
The 5' flanking sequence of the *pat::nptII* tag in the λZAP-#15 vector was

amplified and sequenced in both directions using the following PCR primers:

M13 – 20 Primer: 5' – GTA AAA CGA CGG CCA GT –3' (17 mer)

RevPAT3b :   5' - CTG GCC TAA TCT CAA CTG GTC T - 3' (22 mer)

The resulting 440 bp amplicon, of which 330 bp were tagged genomic sequence,

(330*BW1) was characterized (refer to section 5.3.3) and used as a Southern Blot

probe for the verification of the TAIL-PCR product(s) (Section 5.3.2).

5.3.2   TAIL-PCR for the Isolation of Tagged Regulatory Sequences

Four different degenerate primers (2R, 2L, 3R, 5R) in combination with three

different *nptII* gene specific primers (JH2, RD174, INV5') were tested on 20 ng of

genomic pBAU2#15 DNA. When the primary reaction products were run out on a

0.8% agarose gel, only the JH2 + four degenerate primers (lanes 3 to 7; Figure 5.5a)

and the INV5' + four degenerate primers (lanes 8 to 12; Figure 5.5a) combinations

produced distinct banding patterns.   The RD174 in combination with the four

degenerate primers (lanes 13 to 17; Figure 5.5a) did not result in a distinct banding

pattern, nor in any detectable radioactive signal on the Southern Blot. These latter

combinations were not continued in the secondary TAIL-PCR procedure. The primary

TAIL-PCR products arising from the degenerate primer combination 3R + INV5'

demonstrated a faint hybridizing band(s) to the 330*BW1 probe in a subsequent

Southern Blot (not shown).

The primary reaction products from the JH2 + degenerate primers (lanes 3 to 7; Figure 5.5a) and from the INV5' + degenerate primers (lanes 8 to 12; Figure 5.5a) were used for the secondary TAIL-PCR amplification procedure. The secondary reaction primer combinations which produced a relatively strong Southern Blot hybridization signal to the 330*BW1 probe were: PAT4 + (JH2 + 3R), PAT 4 + (INV5' + 3R) and PAT4 + (INV5' + 5R). The strongest of these signals was the PAT4 + 3R amplification of the (INV5' + 3R) primary reaction product (Figure 5.6b). This sample was used for the tertiary TAIL-PCR amplification procedure.

Figure 5.5: **Primary TAIL-PCR Amplification Products Electrophoresed on a 0.8% Agarose Gel**
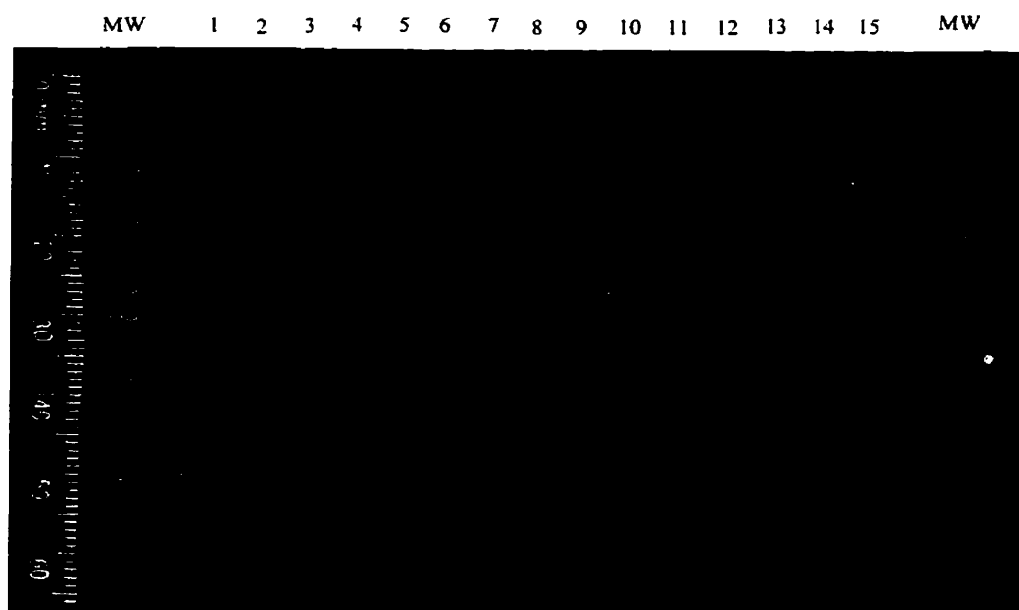
Lanes:
1. 2R + A1
2. 2L + A2
3. 3R + A3
4. 5R + A4
5. PAT3 + A5
6. 2R + B1
7. 2L + B2
8. 3R + B3
9. 5R + B4
10. PAT3 + B5
11. 2R + C1
12. 2L + C2
13. 3R + C3
14. 5R + C4
15. PAT3 + C5

Figure 5.6a: **Secondary TAIL-PCR Amplification Products Electrophoresed on a 0.8% Agarose Gel**
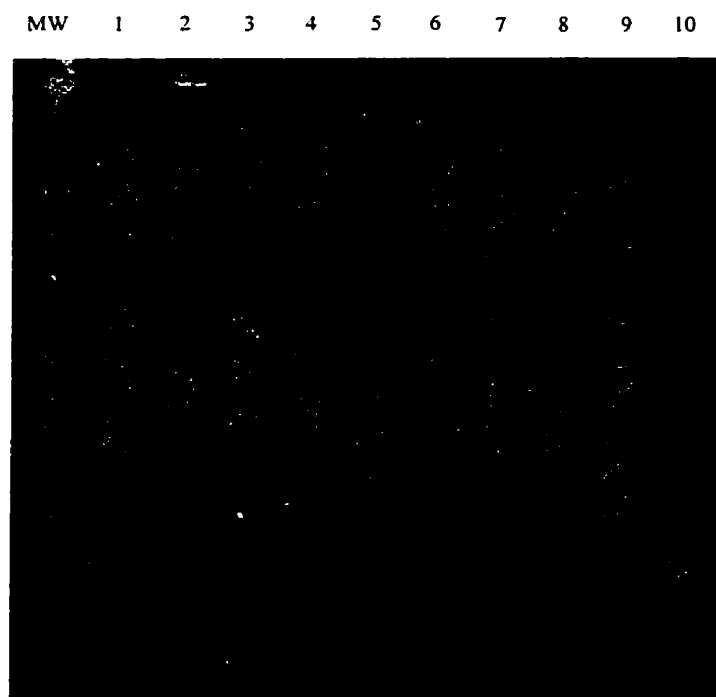
MW   1   2   3   4   5   6   7   8   9   10

MW.   BRL 1 kb Ladder
1. 2R + A1
2. 2L + A2
3. 3R + A3
4. 5R + A4
5. PAT3 + A5
6. 2R + B1
7. 2L + B2
8. 3R + B3
9. 5R + B4
10. PAT3 + B5

Figure 5.6b: Southern Blot Hybridization of the Electrophoresed Secondary TAIL-PCR Products using the Random Primed ^{32}P-CTP-*330*BW1* probe
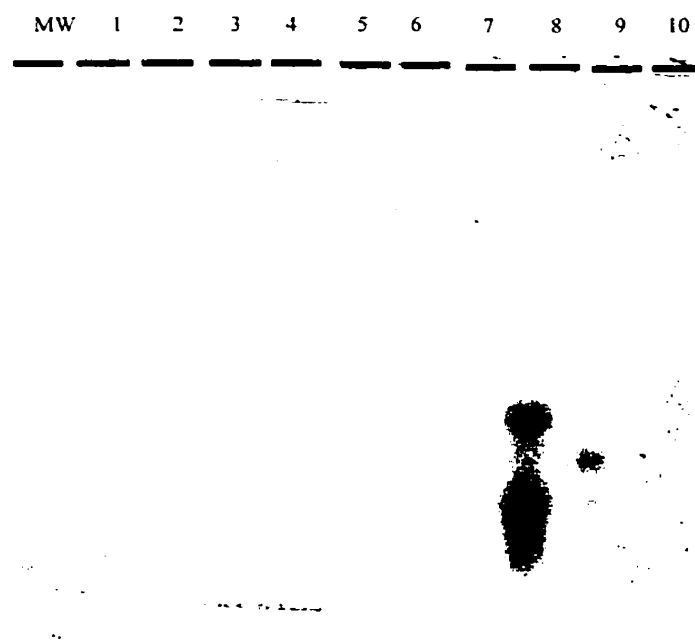
MW.	BRL 1 kb Ladder
1.	2R + A1
2.	2L + A2
3.	3R + A3
4.	5R + A4
5.	PAT3 + A5
6.	2R + B1
7.	2L + B2
8.	3R + B3
9.	5R + B4
10.	PAT3 + B5

Figure 5.7a: **Tertiary TAIL-PCR Amplification Products Electrophoresed on a 0.8% Agarose Gel**
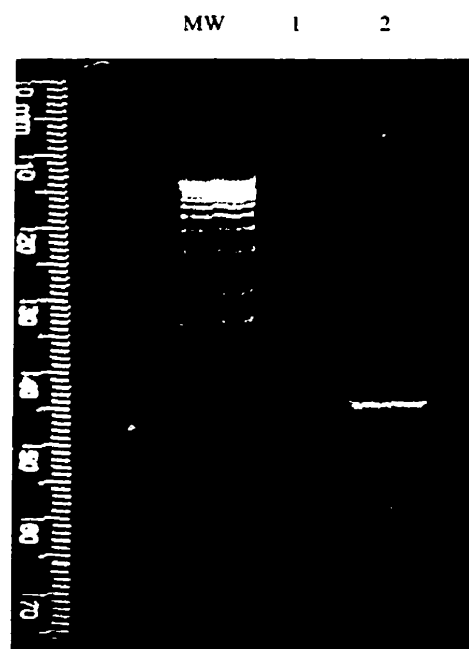
MW
1. 3R + REVPAT3b (+ PAT4 (+ JH2))
2. 3R + REVPAT3b (+ PAT4 (+ INV5'))

Figure 5.7b: Southern Blot Hybridization of the Electrophoresed Tertiary TAIL-PCR Products using the Random Primed ^{32}P-CTP-_330*BW1_ probe
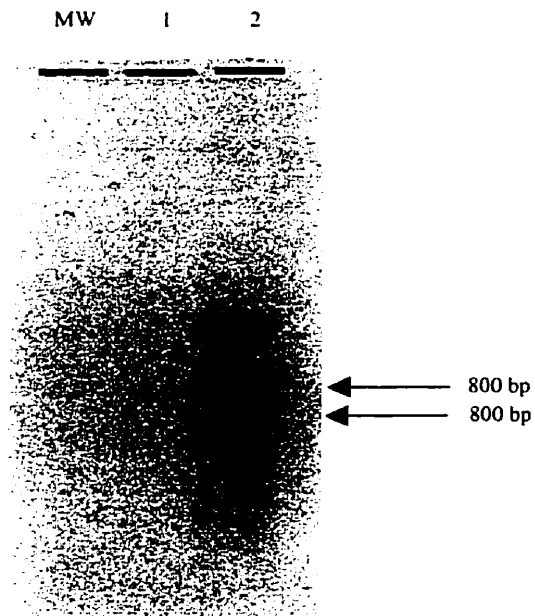
MW
1.   3R + REVPAT3b (+ PAT4 (+ JH2))
2.   3R + REVPAT3b (+ PAT4 (+ INV5'))

The tertiary REVPAT3b + {PAT4 + (INV5' + 3R)} reaction product, as visualized on the 0.8% agarose gel, was approximately 850 bp (lane 2; Figure 5.7a) (later determined to be 866 bp from sequencing analysis) and corresponded to a strong signal on the corresponding Southern Blot (lane 2; Figure 5.7b). This 850 bp band was purified from the agarose gel matrix and directly used as a sequencing template (Section 5.3.3). The entire TAIL-PCR procedure took three days, from start to finish.

5.3.3 Sequence Analysis of Flanking Genomic DNA

The sequence of the region upstream of the *pat::nptII* tag (330 bp), which was isolated from Lambda-ZAP (λZAP-#15), was sequenced in both directions. The sequence of the 866 bp region upstream of the *pat::nptII* tag, which was isolated using TAIL-PCR, was sequenced in one direction. Both sequences were identical for the overlapping regions at the 3' end (Figure 5.8). The larger 866 bp sequence displayed several N nucleotides (artifacts of sequencing) since the sequencing was only carried out in one direction.

From the sequencing data collected from the regions downstream of the tag in λZAP-#15 it was apparent that a large proportion of the pBAU#2 binary vector, past the left border, had been incorporated immediately after the nopaline synthase (*nos*) terminator sequence. In this process, the *EcoRI* site following the *nos* terminator was lost and some rearrangements at the 3' end of the *nos* terminator sequence had also occurred.

Expected 3' *nos* Terminator sequence:

RD144 Primer Site
…TCGCGCGC**GGTGTCATCTATGTTACTAGA**TCGGGGATCCGTCGACCTGCAGCC… 3'

Observed 3' nos Terminator sequence in λZAP-#15 and pBAU3:

RD144 Primer Site   pBAU2 binary vector
…TCGCGCGC**GGTGTCATCTATGTTACTAGA**TCGGGAATTGATCCTTATGACAGGATATA…3'

Approximately 1878 bp of tagged genomic sequence following the pBAU2

binary vector sequence was also included in the 10 kb *EcoRI* fragment cloned into

λZAP-#15. The sequence of this genomic downstream region (1878 bp) was

determined (Figure 5.9).

Some other rearrangements were also observed at the 5' end of the *pat::nptII*

fusion gene.

Expected 5' *pat* Gene Sequence:

5' **ATG**TCTCCGGAGAGGAGACCAGTTGAGATTAGGCCAG 3'

Observed 5' *pat* Gene Sequence in λZAP-#15 and pBAU3:

5' **ATG**AATTTCCGTGAGACATTGCGAAAATATGCAGTTGAGATTAGGCCAG 3'

The modified restriction map of the 10 kb *EcoRI* fragment isolated from pBAU2#15,

based on sequencing data, is illustrated in Figure 5.10.

Figure 5.8 Comparison of the 330 bp Tagged Sequence Isolated by Lambda Screening to the 866 bp Tagged Sequence Isolated by TAIL-PCR

5`GCnCCnGGnGATTnTTTTTGGTTTTTCTGGAGGTATTTTGGGGAGCGGAAATTTTA
866

ATGCTTTATTGGTGGCTGGGATGTGTGCAATTGGCGCTTAATCTTATATTTGTTTCGT

GGAATGTGCTTTAGGTCATGGCTGGGAAGCGAGTTATAGCCATATGTCAGTCAGGTGG

TGGTGAATTTGAGACTGATAAAGACGGTTTCCTTTCGTATAAAGGTGGAGATGCTCAT

GCTATGGAAATGGATGACAAAATGAACTATAATGATTTCAAGATGGAGGTAGCTGAGA

TGTTCAACTTCAnCCTTGCTACCATGTCAGTTAAATATTTTCTTCCTGGAAACAGGAA

GACACTTATAACAATCTCCAATGACAAAGACCTTAAGCGCATGATCAAATTCCATGGT

GACTCTGATTCTGCTGAGATCTATGTGATGATGACTGAAGAAGCTGTTGATCCTGATT

                                                                    5`

TCTCAAACATGCCTGGCAGTAGGTAGATTCCTTGCTGCTGCCTAAATATGTAGAATTC

TCCCTTTTTAAAGGATGAAATATATGGGGATATACTGTGTTGCAGGTCAAGCCGAACG
TCCCTTTTTAAAGGATGAAATATATGGGGATATACTGTGTTGCAGGTCAAGCCGAACG
330
330

ACTTTATCAGAAATGGCGATCCCTGTTGACGCTCCTCTTAGTGTCGTGGAGGATATCG
ACTTTATCAGAAATGGCGATCCCTGTTGACGCTCCTCTTAGTGTCGTGGAGGATATCG

TGGATGACCCCAACGAGCCTGGCCTCTTGCTTGATGCCAATTTTGATGTTGTAAGTGA
TGGATGACCCCAACGAGCCTGGCCTCTTGCTTGATGCCAATTTTGATGTTGTAAGTGA

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TACAAACAACATTGACGACACAATTGGGATAGAATCTGAAATGCCTGTTCCTGTTTCA

TACAAACAACATTGACGACACAATTGGGATAGAATCTGAAATGCCTGTTCCTGTTTCA

TTTGTTGCCGCTAATTATGACGAAAAGAATGCTAAAGCTGCTCAGCAGTGGCAGAATG

TTTGTTGCCGCTAATTATGACGAAAAGAATGCTAAAGCTGCTCAGCAGTGGCAGAATG

ATATAACTGGTGTTGGGTCCAAAGGTTTAATAGCGTACATGAATTTCCGTGAGACATT

ATATAACTGGTGTTGGGTCCAAAGGTTTAATAGCGTACATGAATTTCCGTGAGACATT
38
38

GCGAAAA←***TATG//CAGTTGAGATTAGGCCAG←3′**

GCG   **3′**

330 bp Sequence in BLACK (from Lambda library)

866 bp Sequence in RED (from TAIL-PCR)

// = plant DNA::T-DNA junction

* = revPAT3b primer (complementary strand)

TATA Box

.

Figure 5.9   **Sequence of the 1878 bpTagged Region Downstream of the**
***pat::nptII* and Binary Vector Tag**

3'TCATAACTGTAGAACATTCAAATCCTCAAGTAGTGATCTAAACCACNTTTT

CCCCTCCGGGATCCACCTACACATAACAGGACATAATACCTGAATACTTTC

AAATTAAATCAATTACGGAGACCGTCAAGCCTCGCAAAAACCATCACAAATC

AAGAGTAACTCAACACACTGAAAGAGCTGATCATTGCCAGCATCATGCCGA

TTCAACCCTTGCTAACCGATCCGACTTCTTCTTATTTACCCTTAACTTGACTT

AGAAATACTAATAGCTCCATTCAAAACATAGCGAATCAATCCGCACACGCC

CTGAGTGACCACATTAACAAGACCACATTGTCTCAAAACCCACGAACCGAT

TTATACCCTCCTTGTGCGCATAATCATATCTTGAACTAGTATACCCATTCTG

ACTTAGTCCTTAATAACAATCAACTCCAATGCTCTTCTATCACATGAGCACT

CTCTCGATGGAGTACATAACTGAATTACTTCCTTTAATAGTAATAATCCACC

AATACGTAGATAATCGGAAACTTCACTAATAGATAACCACTTAGCTTGGAGC

AACCCATTAAATAACTCTAGAACCACCAAGATTCTTCCTTCATCGTCGACCT

GATCTTACACAACCAACTTGCCAAACTCTTCGAAATCCTTCTGTTTCCTTAA

TTAACTATTTGGATCACCAACCATATCCATACATTTGACCTATTACCTGATAA

GCAGTAAAATCCTTCGCAAAAGCTTCATCAACCACGCGACTGCTAACCTGC

TCACAGGAGATAACCCACCTGTGGAAGTTATTCGCCGACATCTTTTAACGT

CGCCACANGGGGTACAATCATTACAATACCAATAACCCATCAGAAGCTCAT

GCTCATCCACCAACCATACTAGTTCGCCCACTCCTNGGGGACATCAACTAA

ATATGTGACAATATTTCCCAATTCAAAGCCATGTTGTATCCTTTTTCATATCA

AGCAACTCCTTTCTGTCGTATGATAGCCAATATTTCAAGTTAGGCCATGGAC

CAATCACCATCCATAAAACTCCCAAATCACTCTAAACTTTTCATCNNGGTGT

GTAGTTATCCTACCACTAAACCCATATGCTACTCTGCCACTCTCCCTCTTTT

GCCAAACTCGTCTTCTTTAAGTAACTACCTGACTTCCGCTTCTTACACATGG

CCTTCTAGAAGATTAACCATAGCATGACACTTCTCACNGGTTCTTCATCATC

CTTTGCTGCTCAGTTGATGCCTTAGATCAAAATCTATCTCAGTAGCACTGGA

ACCAATAGACTGCTAATAACTTTAAACCTTTTCGAAGATCATCTTTTCTCGA

GCCGTCGTCATTAGAAACATCGATTCAATCCTGAACCAACGCACCGCACGA

TCTCTGACAGCTGCTTCTTCAATACTATCTCGTAATATTCTATCATACNGGT

GATTTTTATAATATCATAACACCGGCAAACTTAACTCATTCAATCGAGGACG

189

ATGACACCACGCCGCAGATGAAAATTCCACCACGCTTCAAAACACCGAGTC

TCATTACTCTATCAACCCTAAACCTGATCATTTATAGTCCGATTGGCTTTCTT

TCGTCGGAAATATAATACGGAATCTCTAAATCACGCACTAAGAAAATCTCCT

TACAAGTCATACACTACTTCCACGCATGGACAAGCATCCCACCGTCACATC

GATACCGTGCGATAACCATTCATGTGCATCATAGAAACCTATGCATAGCNT

CAAGGNTCTCTGAAGTATAACTACGGAGCTGAAATGACAGAATATCCTTCN

GCAAGGCGACGACGATAGCCCAAACAACCACGCGAGGAAAAATATCTCGC

ACCACATCTGTTGTTACA **5'**

The tagged upstream and downstream sequences were compared to sequences in the GenBank, EMBL, DDBJ and PDB (November 10, 1998). The downstream 1878 bp tagged region had no homology to any known sequences. The following sequences found in the upstream 330 bp and 866 bp regions demonstrated homology to the following eukaryotic promoter sequences:

BW1:   TTATATCATTCTGCCACTGCT
Sg protamine C_II: TTAc ATCATc CTGCCACTGCT
(gn1/EPD/17043)
BW1:   GTGGAGGATATCGTGGATGACCCCAACGAGCCTGGCC
Ad5 Eib: GTGGA a t g TATCGa GGA c t t g C t t AACGAGCCTGGCC
(gn1/EPD/11198)
BW1:   AAACCTTTGGACCCAACACCAGTTAT
H-1 [+.40]:   AAAC aga T t GA a CCAACACCAGTTAT
(gn1/EPD/11212)

5.3.4 Southern Analysis of *N. tabacum* cv. Xanthi and *B. napus* cv. Westar for the Presence of Homologous Regions to the Putative BW1 Promoter Region

EcoRI digested genomic DNA isolated from nontransformed *N. tabacum* cv. Xanthi produced a single hybridizing band (approximately 500 bp) in an autoradiogram of a Southern Blot, when probed with *BW1*330* [^{32}P]-dCTP (Figure 5.11). *EcoRI* digested genomic DNA from the *N. tabacum* cv. Xanthi T-DNA tagged line (pBAU2#15) demonstrated two hybridizing bands; the expected 10 kb *EcoRI* band containing the T-DNA tag and a smaller 500 bp band (Figure 5.11). *B. napus* cv. Westar genomic DNA, digested with *EcoRI*, *HindIII* and *BamHI*, demonstrated no hybridizing band when probed with *BW1*330*.

The genomic DNAs were restricted with three restriction enzymes: *EcoRI*, *HindIII* and *BamHI*. The corresponding bands, visualized on a Southern Blot autoradiogram, are listed in Table 5.1.

Figure 5.10: Restriction map of the *EcoRI* Fragment Isolated from the T-DNA
Tagged Line: pBAU2#15
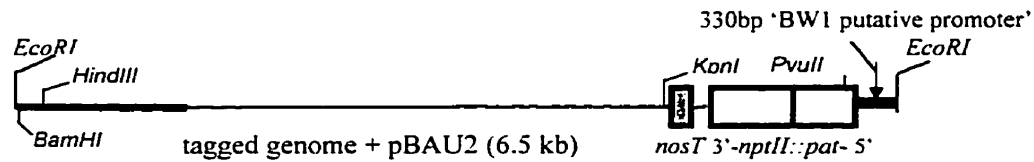
Table 5.1: Autoradiogram Signals from Southern Blot Hybridizations of
Genomic DNA from *N. tabacum*, *B. napus* and the T-DNA
Tagged Line pBAU2#15 Probed with the BW1*330 Putative
Promoter Sequence

Sample	Size of Hybridizing Restricted DNA (kb)		
	EcoRI	HindIII	BamHI
N. tabacum	0.5	5.0	5.0 – 6.0
		9.5	> 15
N. tabacum-pBAU2#15	0.5	10.5	5.5-6.0
	9.5		>15
B. napus	None	None	none

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Figure 5.11: **Southern Blot Hybridization of *EcoRI* digested Genomic DNA Probed with the Random Primed ^{32}P-CTP-*330*BW1* probe**

Lane 1 = *N. tabacum* cv. Xanthi DNA (nontransformed)
Lane 2 = *N. tabacum* cv. Xanthi- pBAU2#15
Lane 3 = *N. tabacum* cv. Xanthi DNA (nontransformed)
Lane 4 = *B. napus* cv. Westar (nontransformed)
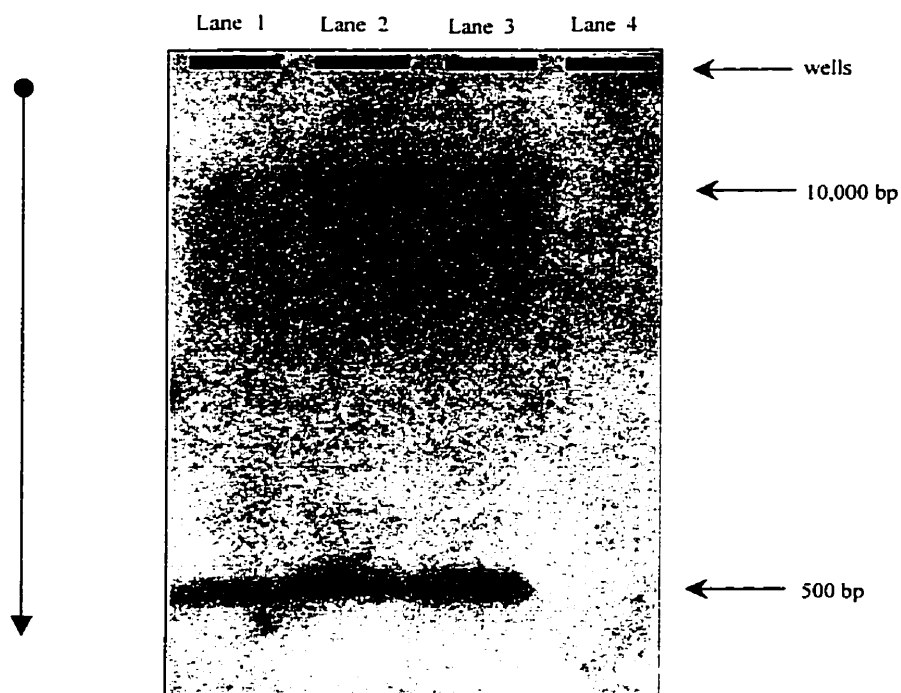
5.3.5 Cloning of the Putative Promoter and T-DNA Tag into a Plant Binary Vector (pBAU3): a Construct for a Functional Plant Transformation Assay

From the hybrization study in Table 5.1, it was observed that the 330 bp tagged upstream sequence (BW1) was present in wild type *N. tabacum* but not in wild type *B. napus*. To verify whether this tagged region functions both in a *N. tabacum* and a *B. napus* host, the entire tag was cloned into a plant binary vector and transformed into both these species. Function was determined using an L-PPT *in vitro* rooting assay.

The *Agrobacterium* binary vector, pBAU3 (10 kb *EcoRI* fragment + pRD400), as depicted in Figure 5.12, was used to transform both *B. napus* cv. Westar cotyledonary petioles and *N. tabacum* cv. Xanthi leaf disks. The average shoot regeneration frequency (from three separate transformation experiments) for *N. tabacum* cv. Xanthi leaf disks regenerating on 2 mg/L L-PPT was 38.5%, and for regeneration on 100 mg/L kanamycin it was 60.4%. The average shoot regeneration frequency for *B. napus* cv. Westar cotyledonary petioles (from two separate transformation experiments) was 8.3% on 20 mg/L kanamycin.

Seven *N. tabacum* pBAU3 transformants were subjected to a rooting assay on levels of L-PPT ranging from 0 to 80 mg/L. As a control, nine pBAU1 transformants, a clone of the original transformant pBAU2#15, and a non-transformed *in vitro* *N. tabacum* regenerant were also subjected to the same rooting assay conditions. Since all seven pBAU3 transformants rooted at 80 mg/L of L-PPT, three were selected for a subsequent rooting assay on levels of 0 to 400 mg/L (Figure 5.13). All three pBAU3 and all three pBAU1 transformants rooted at 400 mg/L L-PPT, while the clone of the original pBAU2#15 did not root at levels exceeding 50 mg/L (Figure 5.13).

Figure 5.12:   Plasmid Map of the *Agrobacterium* Binary Vector pBAU3
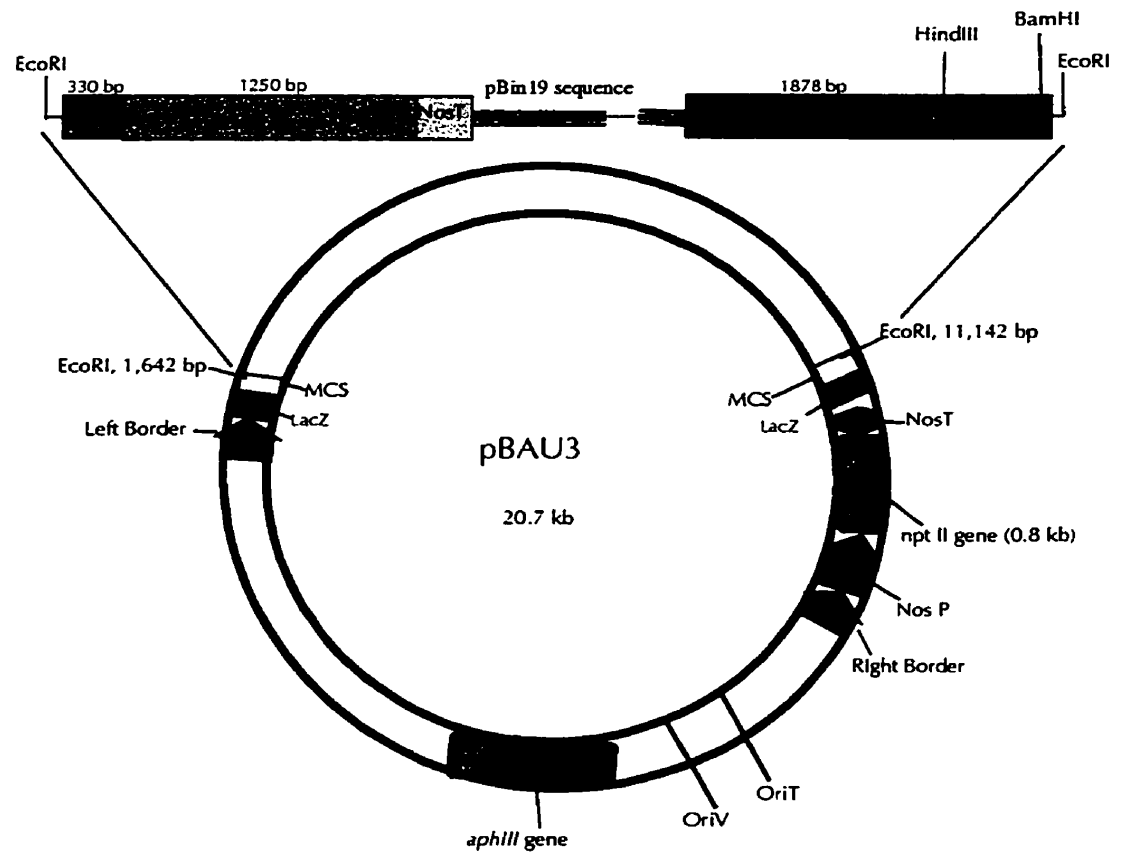
(based on pRD400; Datla *et al.*, 1992)

Nine individual *B. napus* pBAU3 transformants were also subjected to a rooting assay on 0 to 80 mg/L of L-PPT. None of the nine events were able to root at the lowest concentration of L-PPT (5 mg/L). Many of them, though, remained green or partially green at 5 mg/L L-PPT, but failed to develop any roots (Figure 5.14).

This was an interesting observation due to the fact that the BW1 region (330 bp upstream tagged genomic *N. tabacum* sequence) was not present in wild type *B. napus* (Table 5.1). This region is present in wild type *N. tabacum* and therefore it is expected that this tagged region function when reintroduced into a wild type *N. tabacum* host (Figure 5.13).

Figure 5.13: Average Rooting Ability of *N. tabacum*
cv. Xanthi pBAU3 Primary Transformants on
Different Concentrations of L-PPT at Day 15

Graph A
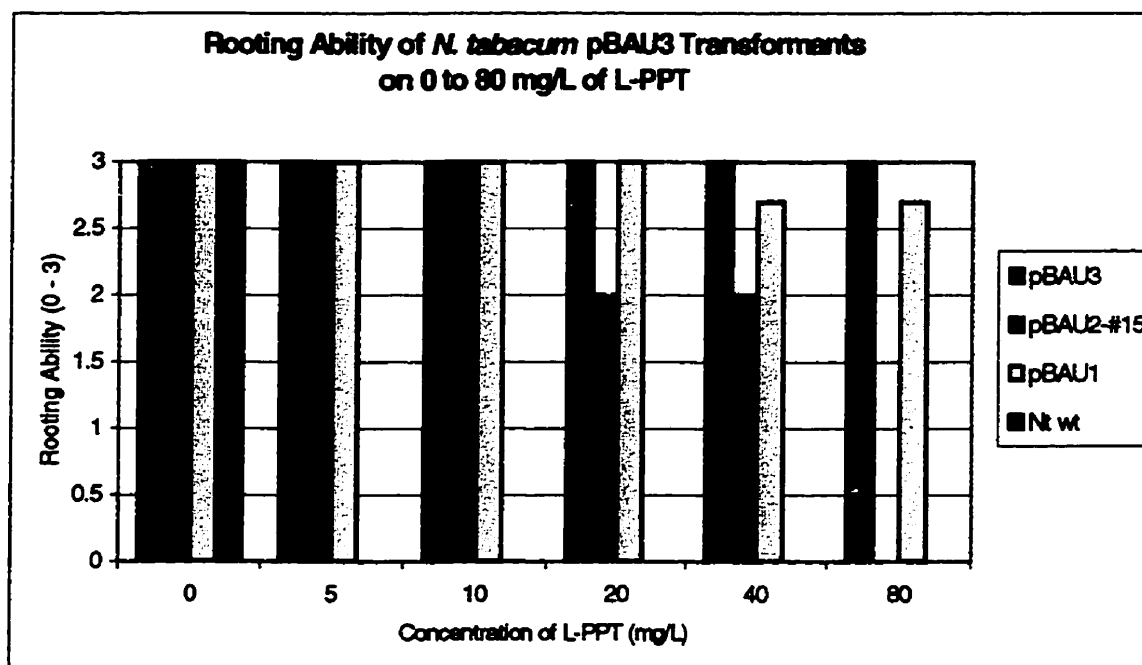
Rooting Ability is a score from 0 to 3 where:

0 = no roots
1 = visible root buds, yet no root formation
2 = < 10 roots (where each root is > 1 mm in length)
3 = > or = to 10 roots (where each root is > 1 mm in length)

Graph B
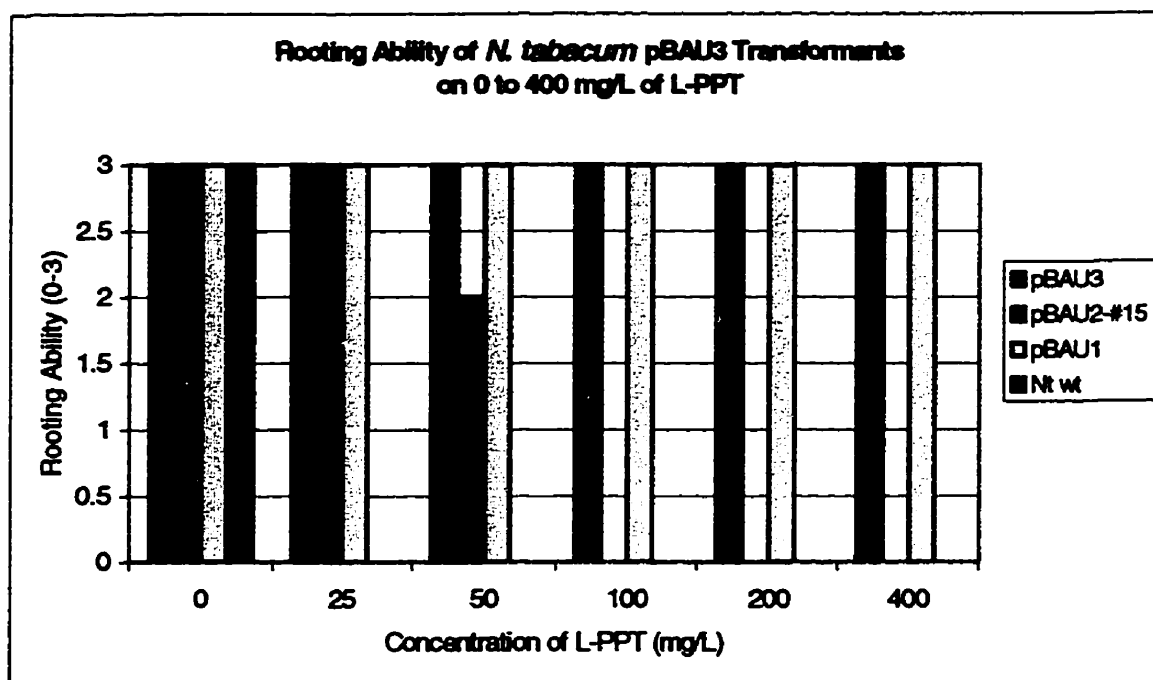
Rooting Ability of *N. tabacum* pBAU3 Transformants on 0 to 400 mg/L of L-PPT

Legend: pBAU3, pBAU2-#15, pBAU1, Nt wt

Y-axis: Rooting Ability (0-3)

X-axis: Concentration of L-PPT (mg/L): 0, 25, 50, 100, 200, 400

Rooting Ability is a score from 0 to 3 where:

0 = no roots
1 = visible root buds, yet no root formation
2 = < 10 roots (where each root is > 1 mm in length)
3 = > or = to 10 roots (where each root is > 1 mm in length)

The total number of T_0 transformants assessed for each given construct are :

Construct	No. of Transformants Assessed	
	Graph A.	Graph B.
pBAU3	7	3
pBAU2 - #15	1	1
pBAU1	9	3
Nt wt (nontransformed)	3	3

- 3 replicates of each T_0 transgenic line

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Figure 5.14: Rooting Ability of a *B. napus* cv. Westar pBAU3 Primary Transformant and a pBAU1 Primary Transformant on 0 to 80 mg/L of L-PPT at Day 15

Brassica napus cv. Westar pBAU3 T$_0$ Transformant (330BW1-*pat::nptII*-nosT)
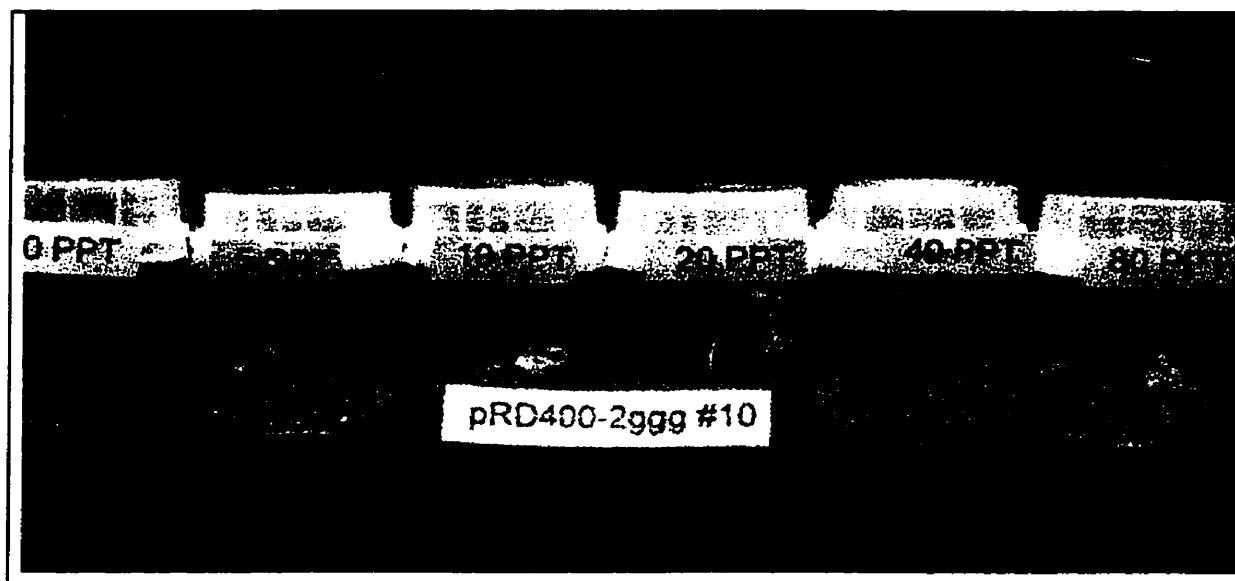
Brassica napus cv. Westar pBAU1 T$_0$ Transformant (Positive Control)
(CaMV35S-35S-AMV-*pat::nptII*-nosT)
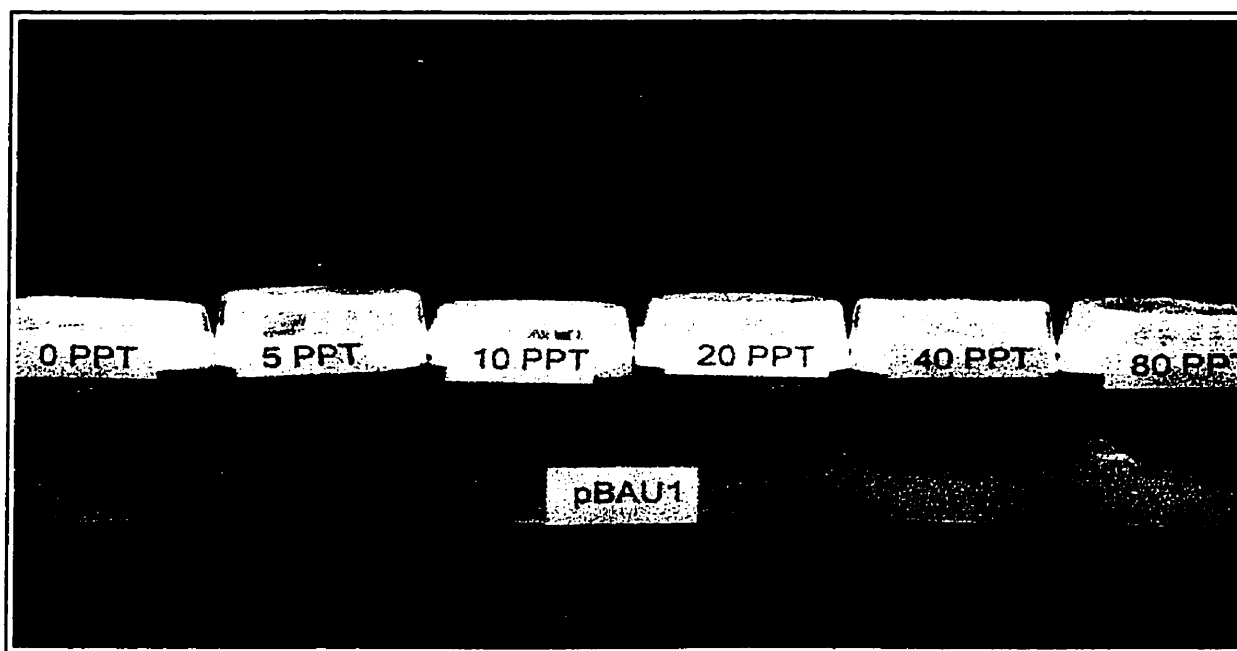
5.3.6 Histochemical Analysis of *in vitro* Regenerants Containing the Tagged Putative Promoter fused to GUS

Eight *B. napus* cv. Westar and eight *N. tabacum* cv. Xanthi T_0 *in vitro* regenerants, from each construct: pRD420a-BW1 and pRD420b-BW1 (Figure 5.15), were subjected to a GUS histochemical analysis. Three *B. napus* cv. Westar and three *N. tabacum* cv. Xanthi T_0 pRD410 (CaMV35Spromoter-*gus*) regenerants were also included as positive controls, along with several non-transgenic regenerants as negative controls. The pRD420a-BW1, pRD420b-BW1 and pRD410 transformants had regenerated and rooted in the presence of kanamycin, and had tested positive for the presence of the *nptII* gene using PCR.

Both pRD420a-BW1 and pRD420b-BW1 *in vitro* transformants in *B. napus* demonstrated faint GUS staining in the roots as well as the root crown (Figure 5.16), but no GUS staining in the shoots or stem (Figure 5.17). *B. napus* and *N. tabacum* pRD410 (CaMV35Spromoter-*gus*) transformants demonstrated GUS staining in all tissues of the rooted *in vitro* plantlets (Figure 5.18). The *N. tabacum* pRD420a-BW1 and the pRD420b-BW1 transformants did not demonstrate GUS staining in any of their *in vitro* tissues.

This observation was not expected since *N. tabacum* transformed with pBAU3 (promoter tag + tag, *nosP-nptII-nosT*) demonstrated a very strong expression of the *pat::nptII* tag in *in vitro* rooting assays on high levels of L-PPT (Figure 5.13). *The B. napus* pBAU3 transformants, on the other hand, were not able to root on low levels of

Figure 5.15: Plasmid Map of the *Agrobacterium* Binary Vector pRD420-BW1

(a. & b.), Containing the Putative 330 bp BW1 Promoter Fused to the *gus* Gene
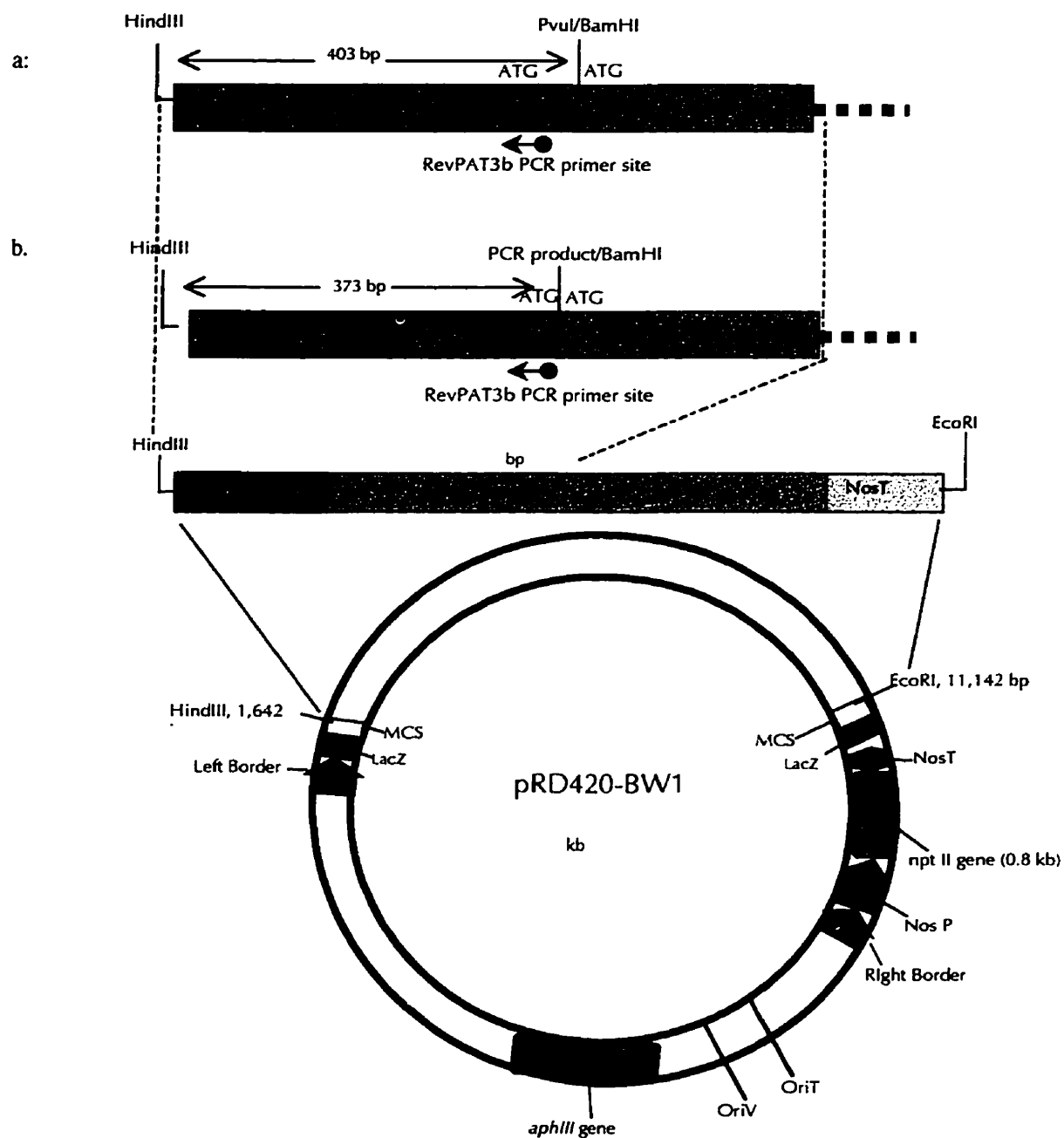
Figure 5.16   GUS Histochemical Analysis of the Roots (a., b.) and
the Root Crown (c.) of a *B. napus* cv. Westar
pRD420a-BW1 *in vitro* Transformant

a.
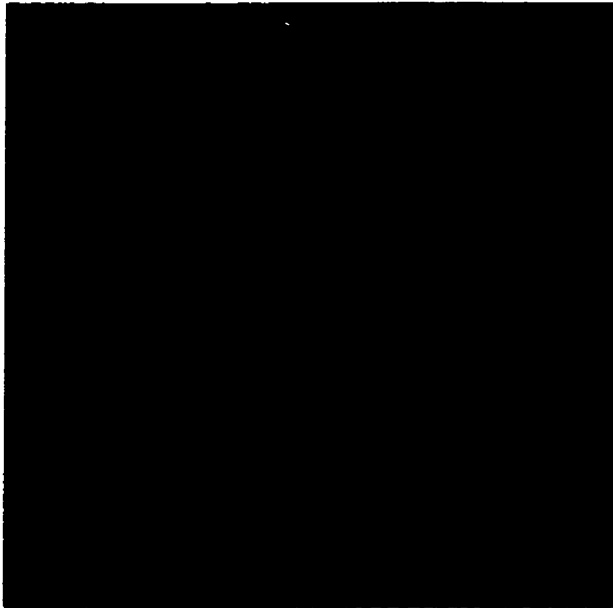
b.

c.

Figure 5.17   GUS Histochemical Analysis of an *In Vitro* Shoot of a
**   *B. napus* cv. Westar pRD420a-BW1 Transformant**

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**Figure 5.18   GUS Histochemical Analysis of an *In Vitro B. napus*
cv. Westar pRD410 Regenerant and of an *In Vitro Nicotiana
tabacum* cv. Xanthi pRD410 Regenerant**

Brassica napus cv. Westar pRD410

In Vitro Leaf

In Vitro Roots

Nicotiana tabacum cv. Xanthi pRD410

5.4 Discussion

The BW1 putative promoter (330 bp) was isolated from *N. tabacum* cv. Xanthi and subjected to analyses to determine whether it functions as a promoter element. The analyses performed so far have not conclusively shown that BW1 is a plant promoter.

The T-DNA tagged region upstream of the *pat::nptII* fusion gene was isolated using two methods: screening of a Lambda subgenomic library and TAIL PCR. The TAIL-PCR method was only used as a back-up isolation procedure in case the Lambda screening method did not function. Both methods were successful, but the Lambda screening took significantly longer. The TAIL PCR method was more efficient in terms of the amount of resources/materials it required and in terms of the amount of time needed to isolate the desired fragment. The advantage of the Lambda system was that the final product was a phagemid that could be used for subsequent cloning steps. For this reason, the Lambda product was used for all subsequent cloning steps (Section 5.3.5).

Both Lambda screening and TAIL PCR produced homologous upstream tagged sequence products (Figure 5.8). Both the 330 bp (Lambda isolation) and the 840 bp (TAIL PCR isolation) upstream plant genomic sequences showed no homology to any known sequence stored in the EMBL and the NIH databases. The upstream sequences, even though they contained certain promoter-like regulatory elements such as TATA Box elements, CAAT Box elements, *as-1* elements (TGACG) and AGGA elements (Figure 5.8), may not have been able to function as a promoter since the spatial arrangement of these elements is often more crucial than the mere

presence of these elements. In most RNA polymerase II specific promoters, the TATA box (an 8 bp consensus sequence consisting entirely of A*T base pairs, except in a minority of cases where a G*C pair is present) is usually located approximately 25 bp upstream of the transcriptional startpoint (*Inr*) (Lewin, 1994). In BW1, there is a putative TATA box present approximately 25 bps upstream from the ATG start site. There are other upstream regulatory elements (transcription factor binding sites), such as the CAAT box, which are also very important for an RNA polymerase II specific promoter to function. Often the CAAT box, which can function in either orientation, is located approximately 75 to 80 bp upstream of the *Inr* site. It can also function at distances that vary considerably from the startpoint (Lewin, 1994). In BW1, the first CAAT box-like motif is located approximately 115 bp upstream of the ATG. A detailed deletion analysis of the BW1 sequence is necessary to determine whether these regulatory elements actually contribute to the initiation and efficacy of transcription. It would also be interesting to combine these deletion studies of the upstream tagged region together with a deletion analysis of the downstream tagged region to see if there are any downstream enhancer-like elements which may influence transcription.

From the sequencing data it was apparent that numerous rearrangements had occurred both in the T-DNA insert and in the flanking region downstream of the insert (3' of the *nos* terminator). Originally it was thought that the entire *EcoRI* fragment (10 kb promoter tag region) consisted of the following (restriction mapping studies, Chapter 4):

EcoRI   *EcoRI*

tagged putative promoter (8.5 kb)

pat::nptII   *nosT*
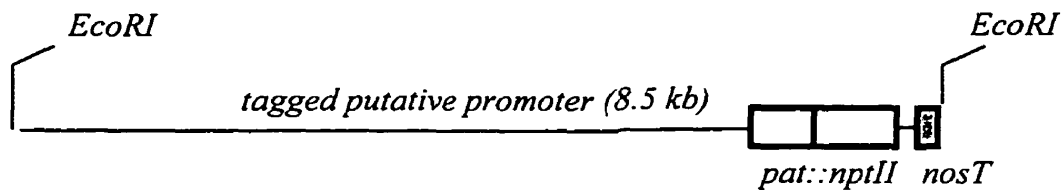
The sequencing results (Chapter 5) indicated the following orientation:

EcoRI   330bp 'BW1'   *EcoRI*

tagged genome (1.8 kb) + pBAU2 (6.2 kb)   *nosT   nptII::pat*
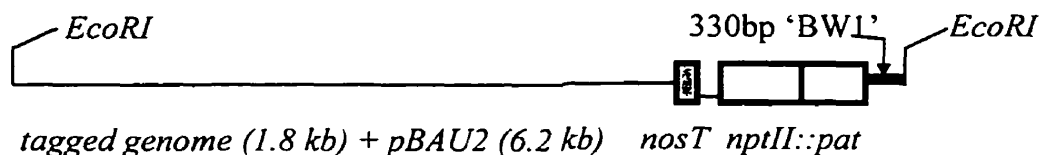
The *EcoRI* site downstream of the *nos* terminator was missing, as well as several basepairs from the 3' end of the terminator sequence. Approximately 6 kb of original pBAU2 vector sequence had inserted itself after the *nos* terminator region, as well as 1.8 kb of plant genomic DNA sequence. Approximately 20 bp at the 5' end of the *pat::nptII* fusion gene, including the ATG start codon, were missing. Instead there was a new ATG start codon, possibly from the plant genomic DNA, located approximately 60 bp upstream of the *PvuII* site which is situated in the 5' end of the *pat::nptII* coding region.

These type of complex rearrangements are not uncommon for T-DNA mediated transformations into plant cells. Duplications of short stretches of nucleotide sequences around the target site and deletions have been reported to be more common (Ohba et al., 1995) than previously expected. Other types of T-DNA insertion locus rearrangements, such as inverted repeat structures, have been described

in the literature. These inverted repeats were reported to be characteristic for transformation events caused by C58/pGV3850 strains of *Agrobacterium tumefaciens* (Jorgensen *et al.*, 1987), the same strain used for the transformation experiments outlined in this thesis. Other studies have shown that similarities in nucleotide sequence exist between target sites for the integration of T-DNA and the ends of the T-DNA. No consensus sequence for T-DNA integration has yet been recognized (Mayerhofer et al., 1991). An illegitimate recombination model for integration of T-DNA has been proposed (Mayerhofer et al., 1991; Gheysen et al., 1991).

In this study, these rearrangements were detected relatively late in the project. Instead of selecting another pBAU2 promoter tagged line for promoter isolation and analysis, it was decided to proceed with the rearranged pBAU2#15 candidate. If there had been no unexpected rearrangements in the 10 kb *EcoRI* tagged fragment isolated from pBAU2#15, then a larger upstream genomic region could have been isolated using the Lambda screening method. Also, if there had been no unexpected rearrangement, then the downstream tagged sequence would also have been absent. The 1.8 kb downstream tagged genomic sequence demonstrated no significant homology to any sequences stored in the EMBL and the NIH databases.

Functional assay studies (Section 5.3.5) did demonstrate that the large 10 kb *EcoRI* fragment, when reintroduced into tobacco, directed a strong expression of the promoterless *pat::nptII*, as shown by rooting assays on levels up to 400 mg/L of L-phosphinothricin (L-PPT). The same 10 kb *EcoRI* fragment, when transformed into *B. napus* cv. Westar did not effect the same level of *pat::nptII* expression, since primary *B. napus* transformants were not able to root on 5 mg/L of L-PPT. These *B. napus*

transformants were able to survive on 5 mg/L of L-PPT, yet they did not develop any roots, demonstrating a marginal expression of the fusion gene (Section 5.3.5). This was expected since the BW1 region was not detected in wild type *B. napus* (Table 5.1), indicating that *B. napus* plants possibly lack other components (i.e. transcription factors) necessary for the expression of open reading frames fused to the BW1 sequence.

Sequence analyses demonstrated that the 10kb *EcoRI* fragment contained only 330 bp of sequence upstream of the *pat::nptII* tag (Figure 5.10). This 330 bp upstream flanking sequence was subsequently fused to the *gus* visual marker gene and also transformed both into *N. tabacum* and into *B. napus*. In this experiment, the *in vitro N. tabacum* regenerants demonstrated no GUS activity in any of their tissues, while the *B. napus* regenerants demonstrated very weak GUS activity in the roots. There are several possible explanations for this observation. One explanation is that the 330 bp upstream flanking sequence is very small and may not be functional when introduced on its own into plants. Also, *N. tabacum* may require the downstream tagged sequence (1.8 kb) to effect transcription of the *pat::nptII* gene. A second explanation is that the 330 bp BW1-*gus* fusion was not functional due to the presence of two in-frame ATG start codons, one at the 5' end of the *gus* gene and the second one at the 3' end of the BW1 upstream sequence.

The first explanation may be the most plausible, since the BW1 region may require an enhancer region present in the downstream tagged genomic sequence (1.8 kb) to effect transcription. This downstream tagged region was present in the pBAU3 binary vector which was used for the functional assay (Section 5.3.5). In this

functional assay the *N. tabacum* pBAU3 transformants demonstrated a very strong expression of the tag (ability to root on 400 mg/L of L-PPT, Figure 5.13). Once this downstream region was removed (as in the GUS histochemical analysis for pRD420-BW1a and pRD420-BW1b) no activity was recorded.

The BW1 tagged region demonstrated the ability to drive the expression of the downstream fusion marker genes, but there is inconclusive evidence for promoter function. In light of the fact that there were rearrangements in the T-DNA of the promoter tagged line, pBAU2#13, it would have been more precautionary to analyze two or perhaps all three of the selected tagged lines instead of only one.

CONCLUSIONS

Construction and Analysis of a Bifunctional Selectable Marker Gene Vector for Plant Transformation

1. A novel bifunctional selectable marker gene, *pat::nptII*, was developed by fusing the carboxy terminus of the herbicide tolerance selectable marker gene *pat* (phosphinothricin acetyltransferase) to the amino terminus of the antibiotic resistance selectable marker gene *nptII* (neomycin phosphotransferase II).

2. The *pat::nptII* fusion gene was successfully expressed in a prokaryote host, *Escherichia coli*; protein extracts demonstrated both PAT enzyme and NPTII enzyme activities.

3. The *pat::nptII* fusion gene was successfully translated as a 47 kD fusion protein in the eukaryotic host systems, *Brassica napus* and *Nicotiana tabacum*.

4. *Brassica napus* and *Nicotiana tabacum* regenerants, transformed with the CaMV35S-CaMV35S (Cauliflower Mosaic Virus 35S) promoter-AMV (alfalfa mosaic virus) enhancer regulated *pat::nptII* fusion gene (pBAU1), as well as their progeny, demonstrated both PAT and NPTII enzyme activities.

5. A 20 fold variation in the transgene expression level was detected between individual transformants expressing the CaMV35S-CaMV35S promoter-AMV enhancer regulated *pat::nptII* fusion gene.

6. The novel *pat::nptII* fusion gene was successfully expressed in two unrelated dicot species demonstrating transformation frequencies and enzyme expression

levels comparable to those obtained from transformation experiments generated with non-fusion gene constructs.

Construction and Implementation of a Bifunctional Promoter Tagging Vector

1. The *pat::nptII* fusion gene was successfully used as a bifunctional selectable marker gene in a novel *Agrobacterium* T-DNA promoter-tagging vector, pBAU2, in *Nicotiana tabacum*.

2. Eight *Nicotiana tabacum* promoter tagged lines were generated from a stringent *in vitro* selection protocol involving shoot regeneration in the presence of 100 mg/L of kanamycin and root regeneration in the presence of 5 mg/L of L-phosphinothricin (L-PPT).

3. Three of the eight promoter tagged lines were subjected to detailed molecular characterizations, and based on these characterizations, one promoter tagged line (N.t. pBAU2#15) was selected for further promoter isolation studies.

4. An accurate and reproducible Enzyme Linked Immunosorbant Assay (ELISA), based on a two-antibody sandwich assay, was developed to quantitate the PAT enzyme in plant tissues.

5. To help characterize promoter tagged lines, both the PAT ELISA and the NPTII ELISA (commercially available) were used to quantitate the level of PAT::NPTII fusion protein expressed in different plant tissues and at different stages of development; expression levels of the PAT:NPTII fusion protein in these lines

were lower than those expressed by CaMV35S-CaMV35S promoter-AMV enhancer-*pat::nptII* transformants.

Isolation and Characterization of a Tagged Regulatory Sequence from Tobacco

1. The upstream region flanking the *pat::nptII* T-DNA tag in the line N.t.pBAU2#15 was isolated using two methods: Lambda screening of a subgenomic library and TAIL (Thermal asymmetric interlaced) PCR. Both methods produced identical upstream sequences, with the exception that the TAIL PCR product was larger (840 bp) and the TAIL PCR method was more efficient.

2. A 330 bp upstream tagged sequence was isolated via Lambda screening and reintroduced into *Nicotiana tabacum* and *Brassica napus*. This sequence contained promoter-like regulatory elements yet there was inconclusive evidence for promoter function.

3. Future prospects for the *pat::nptII* fusion gene could include:

   ▪ A model to study fusion gene function in eukaryotic systems.

   ▪ Use as a bifunctional selectable marker gene for transformation and selection studies in a broad range of dicot and monocot plant species.

   ▪ Use as a bifunctional selectable marker gene for integrating into T-DNA and transposon vectors for finding useful promoters and identifying knock-out gene mutants.

APPENDIX I

Development of a Two Antibody Sandwich Immunoassay Specific for the Phosphinothricin Acetyltransferase Protein

Introduction

The *pat* gene encoding the enzyme phosphinothricin *N*-acetyltransferase (PAT) was first isolated from a soil bacterium *Streptomyces viridochromogenes* (8). The *pat* gene is significantly homologous to the *bar* gene isolated from *Streptomyces hygroscopicus* (7). PAT deactivates the herbicide glufosinate ammonium by acetylation (7). The *pat* gene has been successfully introduced into various commercial crops including canola (*Brassica napus*)(6). These transgenic crops are tolerant of the non-selective herbicide glufosinate ammonium allowing for postemergent weed control without crop injury. In addition, the *pat* gene can function effectively as a selectable marker to assist in the introduction of other agronomically useful traits.

The detection and quantification of introduced gene products are among the regulatory requirements associated with commercializing genetically transformed crops. This capability is also useful as a research tool for quantifying marker proteins in various tissues and at various stages of plant development. Activity assays (1, 4) and immunoassays (6) have been developed for the detection of selectable marker gene proteins in tissues of transformed plants. An immunoassay has the advantage of being more quantitative than an activity assay.

A two-antibody sandwich immunoassay specific for the protein product of the *pat* gene was developed using a partially purified polyclonal goat antiserum. The following summary describes this immunoassay and demonstrates its utility for quantifying the PAT enzyme in transgenic canola seed.

Materials and Methods

Antiserum Production and Purification

PAT enzyme, purified to homogeniety from a transgenic *E. coli* cell line expressing the *pat* gene (J-118 Ac43; AgrEvo AG, Frankfurt, Germany), was used as the immunogen to obtain anti-PAT serum from a goat. Raw antiserum was saturated to remove antibacterial antibodies using the method of Gruber and Zingales (2). Following the saturation protocol, the IgG fraction of the antiserum was isolated by affinity chromatography using recombinant Protein G immobilized on agarose gel (Gibco BRL, Gaithersburg, MD, USA).

A portion of the isolated IgG was kept aside for biotinylation using a commercailly available kit (Gibco BRL Protein Biotinylation System, Gaithersburg, MD, USA). The remainder was mixed 1:1 with glycerol and stored at -20° C, or sodium azide was added to 0.05% and stored at 4° C.

Extraction of PAT Enzyme from Canola Seed

Canola seed (1 g) from transgenic canola (*Brassica napus* HCN92, AgrEvo Canada Inc, SK, Canada)(6) and non-transgenic canola (*Brassica napus* cv. Excel) was pulverized in a chilled mortar with a pestle. The pulverized seed was added to 10 mL of chilled extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA and 0.1 mg/mL leupeptin) and the mixture was homogenized with a polytron. The homogenate was centrifuged (10,000 x g) for 10 minutes at 4°C. The aqueous supernatant was used for analysis by immunoassay.

Immunoassay

Polystyrene 96-well microtiter plates (Corning Easy-Wash 96 Well Microtiter Plate, Corning, NY, USA) were prepared by adding 200 µL/well of anti-PAT IgG diluted 1:125 in carbonate buffer, pH 9.6. IgG was allowed to coat the surface of the wells for 4 hours at 37°C. The wells were emptied and washed with a solution of PBS three times. Any residual solution was removed by firm tamping on a stack of paper towels. Unoccupied sites on the polystyrene well surface were blocked by adding 300 µL/well of blocking dilution buffer (3% bovine serum albumin, BSA, in PBS) and allowing the plates to stand at room temperature for 30 minutes. The plates were then washed as described above using PBS-Tween wash solution (0.1% v/v Tween 20,

SIGMA, St. Louis, MO, USA, in PBS) for a total of three washes. After the third wash cycle, the inverted plate was tamped dry as described above.

A 10 ng/mL PAT standard was made by diluting a 25 μg/mL stock solution with blocking dilution buffer. Serial 1:1 dilutions of the 10 ng/mL standard solutions were made to obtain 5, 2.5, 1.25 and 0.625 ng/mL PAT standards. Blocking dilution buffer alone served as a 0 ng/mL standard. Aliquots of 200 μL of each standard were transferred to the microtiter plate in triplicate. Each seed protein extract was serially diluted 1:1 a total of 9 times. A 200 μL aliquot of each dilution was added to duplicate wells. Binding of PAT enzyme to immobilized IgG in the wells of the microtiter plate was allowed to occur overnight at 4° C.

After emptying and washing the wells of the plate six times using PBS-Tween, 200 μL/well of biotinylated IgG diluted in blocking dilution buffer was added. The plate was allowed to stand for 2 h at room temperature. The plate was then emptied and washed again (3 X PBS-Tween). Streptavidin-alkaline phosphatase conjugate (Gibco BRL, Gaithersburg, MD, USA) diluted in blocking dilution buffer was added (200 μL/well) and the plate was allowed to stand for a further 2 h at room temperature. After a final wash cycle (3 X PBS-Tween), p-nitrophenylphosphate (SIGMA, St. Louis, MO, USA) at a concentration of 1 mg/mL in a 10% diethanolamine buffer (BDH, Poole, England) with 10 mM magnesium chloride (pH 9.8) was added to the wells (200 μL/well) and color was allowed to develop at 37°C in the dark for 10 to 30 minutes. The color reaction was stopped with the addition of 50 μL of 2 N NaOH. The optical density at 405 nm of each well was determined on a microplate reader.

Data Handling

A first order regression was performed on the PAT standard data from which concentrations of samples were estimated. For samples, O.D. values were plotted against the log of the dilution factor. The O.D. value corresponding to the extract dilution at the midpoint of the resulting titration curve, as described by Harlow and Lane (3), was used for quantitation. The sample O.D. value was corrected for non-specific binding by applying a correction factor (CF):

$CF = O.D._{negative\ control} - O.D._{0\ ng/mL\ PAT\ std.}$

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The O.D. negative control value is obtained from the corresponding dilution of a non-transgenic seed extract. The corrected O.D. (O.D.corr.) is then:

O.D.corr. = O.D.midpoint - CF.

The concentration of PAT enzyme in the extract is calculated using the corrected O.D. value and the regression equation from the standard curve. Finally, the PAT concentration in the seed is calculated using the following equation:

$$PAT \ (ng \ / \ g) \ = \ \frac{PAT \ (ng \ / \ mL) \ x \ DF \ x \ Vol. \ extract \ (mL)}{Sample \ wt. \ (g)}$$

where DF is the midpoint dilution factor.

Results

Standard Curve

A typical standard curve is illustrated in Figure I. The relationship between O.D. and PAT enzyme concentration is first order over the 0 to 5 ng/mL concentration range shown.

The within-assay variability among replicate wells contributing to the standard curve was low (Table I). Coefficients of variability ranged from 0.4% to 5.9% with typical values falling below 5%. The assay to assay variability in the standard curve was also shown to be low (Table II). Over 3 separate assays, the coefficient of variability in the slope and y-intercept was 3.8% and 3.7%, respectively. These data indicate that the standard curve resulting from the two-antibody sandwich immunoassay for PAT enzyme determinations shows a high level of precision and reproducibility.

The limit of quantification (LOQ) for a particular assay run is described as the minimum O.D. value on the standard curve from which an estimate of PAT concentration can be made. The LOQ is calculated as the mean O.D. of the 0 ng/mL PAT standard plus 3 standard deviations of that mean. Using this formula, the LOQ would be determined as 0.652, 0.565, and 0.561 for Runs 1, 2, and 3, respectively.

Determination of PAT Enzyme in Transgenic Canola Seed

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The immunoassay signal given by a transgenic seed extract and a non-transgenic seed extract spiked with PAT enzyme compared to that given by a negative control (non-transgenic seed extract) is shown in Figure II. The canola seed extract matrix does not interfere with the immunoassay as the non-transgenic seed extract gives no greater signal than the 0 ng/mL PAT standard in blocking dilution buffer.

Determinations of PAT enzyme spiked into a non-transgenic seed extract in 3 separate assays fell within 80 to 120% recovery and were, therefore, considered acceptable (Table III). Transgenic canola seed was shown to contain approximately 400 ng PAT enzyme per g seed. The 8.1% coefficient of variability over 3 separate assays illustrates the high level of reproducibility of the immunoassay method.

Discussion

Detection of the selectable marker protein phosphinothricin N-acetyltransferase was previously possible using a Western blot method (unpublished results) or an enzyme activity assay (1). The Western blot method, employing the same antiserum used for immunoassay development, provides only qualitative detection of the PAT protein. The enzyme activity assay is based on the chromatographic separation and quantitation of ^{14}C-labelled acetyl-glufosinate following incubation of ^{14}C-labelled glufosinate herbicide with PAT enzyme. This method can give a semi-quantitative estimate of PAT enzyme in genetically transformed materials.

We have described the first truly quantitative method for determination of the selectable marker protein PAT based on a two-antibody sandwich enzyme immunoassay. Using transgenic canola seed, we have shown the method to be accurate, precise, and reproducible.

Although not reported here, the method has been successfully applied to several other transgenic canola, corn, and tobacco tissues including leaves, roots, and stems. This immunoassay method has also been applied to processed products from transgenic crops such as seed meal and oil from both canola and corn. As a research tool, the method is currently being employed to determine the expression of the *pat* gene in various tissues and at various stages of transgenic plant development.

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Table I. **Within-assay variability of PAT standards.**

PAT std., (ng/mL)	Run 1		Run 2		Run 3	
	Mean O.D.[1]	c.v.[2], %	Mean O.D.[1]	c.v.[2], %	Mean O.D.[1]	c.v.[2], %
0	0.554	5.9	0.513	3.4	0.517	2.8
0.625	0.703	0.4	0.661	2.2	0.679	3.3
1.25	0.819	2.5	0.803	4.1	0.808	1.2
2.50	1.022	0.8	1.010	3.2	1.029	1.8
5.00	1.321	0.7	1.338	2.6	1.321	1.3

[1] n = 3
[2] coefficient of variability

Table II. **Assay to assay variability in the standard curve.**

Assay Run	Slope	Y-intercept
1	0.150	0.604
2	0.162	0.562
3	0.157	0.577
Mean	0.156	0.581
Std. error	0.003	0.012
c.v.[1], %	3.8	3.7

[1] Coefficient of variability

Table III. **Determination of PAT enzyme in spiked non-transgenic and transgenic canola seed.**

Assay Run	Non-transgenic Spike (% recovered)[1]	Transgenic Seed (ng PAT/g seed)
1	112	392
2	85	378
3	98	440
Mean	98	403
Std. error	7.8	19
c.v.[2], %	14	8.1

[1] Non-transgenic canola seed extract was spiked with PAT enzyme to a concentration of 40 ng PAT / mL extract.
[2] Coefficient of variability.

Figure I. Two-antibody sandwich enzyme immunoassay standard curve for the determination of the PAT enzyme.
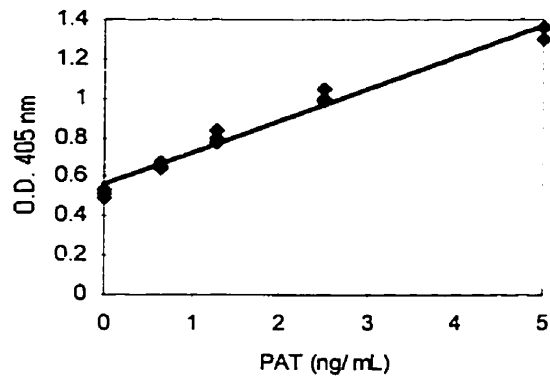
Figure II. Immunoassay signal of transgenic vs non-transgenic canola seed extracts. Spike refers to a non-transgenic canola seed extract spiked with PAT enzyme to a concentration of 40 ng PAT per mL extract. The 0 ng/mL PAT standard is included for reference.
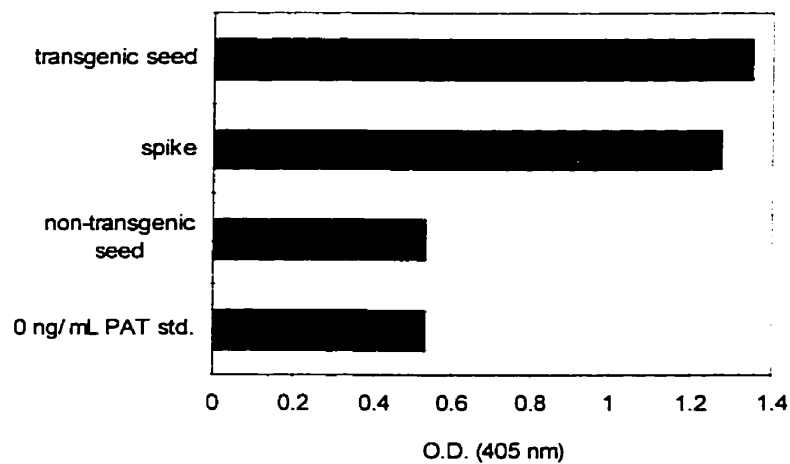
METHODS

BRL 1 Kb DNA Ladder

bp

—12,216
11,198 10, 180 9,162
8,144
7,126
— 6,108
— 5,090

— 4,072

— 3,054

— 2,036

— 1,636

— 1,018

— 506, 517

— 319
— 344
— 298

220
201
154
134
75

Hint I fragments
of the vector
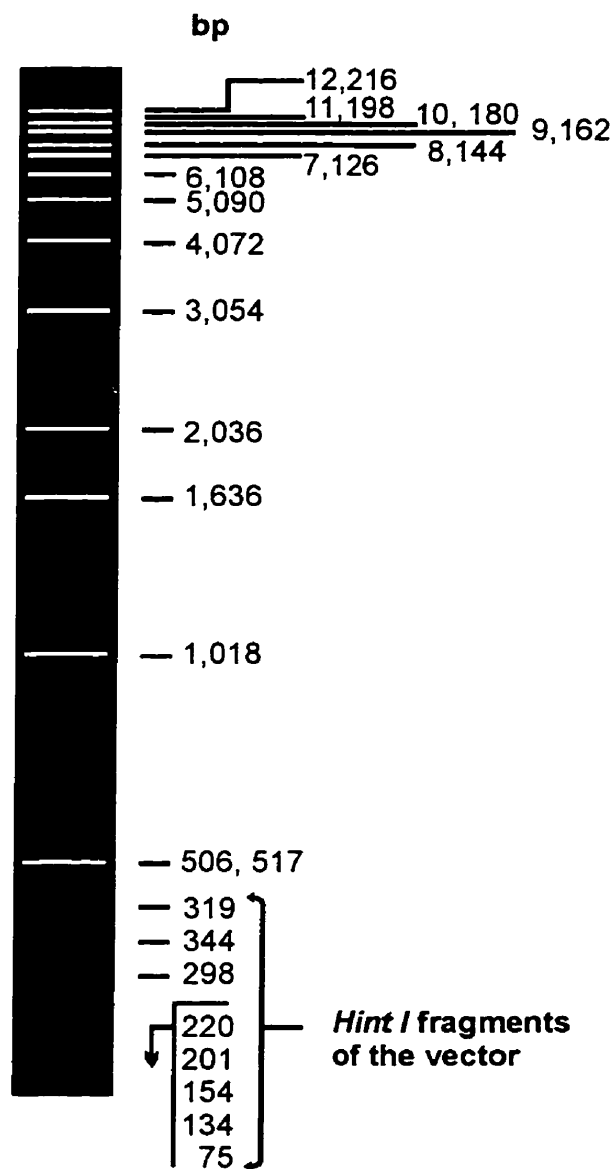
PAT Activity Assay

Reagents

1. Extraction Buffer:

    for 5 mls:

        -250 µL of 1M Tris-HCl, pH 7.5

        -20 µL of 500 mM EDTA, pH 8.0

        -50 µL of 1 mg/ml leupeptin (frozen stock)

        -4.68 mls ddH$_2$O

    Prepare fresh for each assay and store on ice.

2. Reaction Buffer:

    0.5M Tris-HCl, pH 7.5

3. ^{14}C-Acetyl CoA + L-PPT Reaction Cocktail:

    for 10 samples:

        -12.5 µL of 58.1 mCi/mmol ^{14}C-Acetyl CoA (NEN)

        -7.5 µL of 1.0 mM L-PPT

        -20 µL ddH$_2$O

    Prepare fresh just before adding to samples.

4. Developing Solvent:

    -3 parts 1-propanol to 2 parts 25% NH$_4$OH

Method

Extract Preparation:

    Approximately 2 cm^2 of the tissue sample was placed in an Eppendorf tube and kept on ice. 200 µL of the chilled extraction buffer was added and the tissue ground with a pellet pestle until homogenized. The samples were spun at 13,000 r.p.m. for 5 minutes at 4°C and the supernatant was transferred to a fresh tube on ice, and the pellet discarded.

Protein Quantification:

The total protein content in each sample was determined relative to a standard curve constructed using bovine serum albumin (BSA) according to the Coomassie Dye Binding Protein Assay (Bradford, 1976).

Reactions:

All plant samples were standardized to 20 µg total plant protein per reaction (1 µg/µl) by dilution with the reaction buffer.

For each reaction:

- 4µl reaction cocktail was added to 20 µl plant protein (20 µg total protein) in an Eppendorf tube, mixed gently and incubated at 37°C. After one hour, 26 µL of 12% (w/v) TCA (trichloroacetic acid) was added, the tubes were mixed well by vortexing, and centrifuged at 13,000 r.p.m. for three minutes to precipitate the proteins.

- 10 µL of the supernatant (5 x 2 µL aliquots) was spotted onto flexible Whatmann Silica Gel (PE SIL G, Cat # 4410 221) plates, dried, then placed upright in the developing solvent for approximately four hours.

- After the chromatography was complete, the plates were air dryed, and exposed to Kodak-X-Omat AR film with an intensifying screen for two days at room temperature.

NPT II Activity Assay

Reagents

1).Extraction Buffer: 5X GUS Lysis Buffer (Jefferson, 1987)

        for 100 mls:

                -3.32 g $NaH_2PO_4.H_2O$

                -0.23 g $Na_2HPO_4.H_2O$

                -0.5 ml Triton X-100

                -0.5 g Sarkosyl (n-lauroyl sarcosine)

                -0.35 ml ß-mercaptoethanol

                -1.46 g EDTA (free acid)

Adjust pH to 7.0 with NaOH and bring up volume to 100 mls with deionized, distilled water (ddH_2O) and store at 4°C.

2). Reaction Buffers: NPT II Reaction Buffers (Reiss et al., 1984)

        for 10 mls:

                -0.67 ml 1 M Tris-maleate buffer, pH 7.1[12.1 g Tris base, 11.6 g

                  maleic acid, 19.8 mls 5 N NaOH, ddH_2O to 100 mls]

                -0.42 ml 1 M $MgCl_2$

                -1.0 ml 4 M NH_4Cl

                -17 µl 1 M dithiothreitol (DTT)

                -7.49 mls ddH_2O

Split into 2 x 5 mls aliquots. To one, add 40 µl 100 mg/ml kanamycin sulfate (KAN^+ BUFFER). To the other, add 40 µl ddH_2O (KAN^- BUFFER). Buffers were made up fresh and stored on ice or at 4°C.

<u>3). ATP Solution</u>:

      for 500 µl:

                -3.71 µl "cold" ATP (100 mM)

                -5 µl [t ^{32}P-ATP] (3 000 Ci/mmol; 10 mCi/ml)

                -492 µl ddH$_2$O

                  made up immediately before use

Method

<u>Extract Preparation</u>:

Approximately 1 cm^2 of leaf tissue or other tissue was placed into an Eppendorf tube and kept on ice. 50 µl of 5X GUS Lysis Buffer and a pinch of clear, fine sand was added and the sample was ground with a pellet pestle. 200 µl ddH$_2$O was added and the tissue was thoroughly homogenized. The sample was centrifuged for 15 minutes at 4°C and the supernatant was transferred to a fresh tube on ice.

<u>Protein Quantification</u>:

The total protein content in each sample was determined (relative to BSA) according to Bradford's Protein Assay (Bradford, 1976).

<u>Reactions</u>:

For each plant sample, two reactions were prepared in Eppendorf tubes and stored on ice: one with KAN$^+$ BUFFER and one with KAN$^-$ BUFFER.

For each reaction 20 µl plant extract (40 µg of protein/ 20 µl sample), 10 µl reaction buffer (KAN$^+$ BUFFER and KAN$^-$ BUFFER), and 10 µl ATP solution were combined.

Tubes were gently mixed and incubated at 30°C for 30 minutes. Reactions were halted by returning them to ice. During the incubation, the Dot Blot Apparatus (BioRad) was assembled with 3 layers of Whatman P81 ion exchange paper on top of one layer of Whatman 3 MM paper (cut to fit the apparatus).

Reactions were pipetted into the wells of a Dot Blot apparatus (BioRad) and allowed to be completely absorbed by the paper layers. The 3 MM paper was discarded to the radioactive waste and the three P81 papers were washed as follows:

-2 x 5 minutes in ddH$_2$O at room temperature

-1 x 45 minutes in 0.5 mg/ml Proteinase K solution [25 mg Proteinase K, 2.5 mls of 20% SDS, 47.5 mls ddH$_2$O] (Radke et al., 1988)

The papers were dried, covered with Saran Wrap and autoradiographed with Kodak X-Omat AR film. The papers were exposed to the film at -70°C for 12 to 48 hours, and then developed.

Western Assay

Protein Extraction

Crude protein samples were extracted from seed of HCN 92 canola and from seed of Legend canola (negative control). Frozen leaf samples from mature HCN 92 and Excel canola (negative control) were also used as a source of crude protein extract.

Approximately 0.2 to 0.3 grams of tissue were placed into a crucible and ground with liquid nitrogen. Once the tissue was homogenized into a fine powder, 25 µl of ß-mercaptoethanol (note: for 1 gram of tissue add 100 µl of ß-mercaptoethanol) and 125 µl of Grinding Buffer (note: for 1 gram of tissue, add 500 µl of Grinding Buffer) were added . The homogenate was mixed with the buffer to produce a thick slurry. The slurry was transferred into an eppendorf tube (on ice) and centrifuged for 15 minutes (13 000 rpm) at 4°C.

The supernatant was removed to a fresh tube (on ice) and the amount of total protein, relative to bovine serum albumin (BSA), was quantitated using a Bradford's Protein Assay (Bradford, 1976).

SDS-Polyacrylamide Gel Electrophoresis

Two 12% polyacrylamide (PA) running gels, which separate polypeptides of 10 000 to 70 000 Daltons, were prepared using the BioRad Mini-Protean II Apparatus. The running gels were overlayed with a 4% PA stacking gel. The protein samples were diluted in cold Grinding Buffer to a final concentration of 3 µg total protein per µl of buffer. Twenty-five µl of diluted extract (75 µg total protein loaded in each well) was mixed with 5 µl of 6X SDS Loading Buffer. Some of the negative control samples were spiked with 50 ng of purified phosphinothricin acetyltransferase (PAT) protein (Dr. Arno Schulz, AgrEvo AG, Frankfurt, Germany) or 25 ng of purified neomycin phosphotransferase II (NPT II) protein (5'-3' Inc.). The molecular weight markers (BioRad SDS Protein-Low Molecular Weight Markers) were also mixed with 6x SDS Loading Buffer. All samples were boiled for 5 minutes, and then loaded into the wells of the stacking gel.

The SDS-PA gels were electrophoresed in Running Buffer at 200 volts for 40 minutes (at 4°C). After electrophoresis, the polyacrylamide gels were carefully pryed away from the glass plates. One gel was processed for total protein staining (Coomassie staining), while the other gel was processed for immunoblotting.

Coomassie Staining of Polyacrylamide Gels

One of the PA gels, and the molecular weight lanes from the second gel, were briefly rinsed in ddH$_2$O, and then immersed into a 1x solution of Coomassie Blue (0.05% Coomassie brilliant blue R-250 in ddH2O). The gel was allowed to stain overnight at room temperature with gentle agitation. The stained gel was destained for 6 hours in acetic acid:methanol:ddH$_2$O and then soaked for 2 hours in 10% glycerol. The gel was then dried under vacuum and photographed.

Immunoblotting

The second gel (minus the molecular weight marker outer lanes) was soaked (equilibriated) in Transfer Buffer for 15 minutes. The electrophoresed proteins on the PA gel were transferred onto a piece of nitrocellulose paper according to the protocol for wet electrophoretic transfer (Antibodies, a Laboratory Manual, 1992). The electrophoretic transfer occurred at 63 volts overnight at 4°C.

Following electrophoresis, the nitrocellulose membrane was rinsed in phosphate buffered saline (PBS) and placed into a blocking solution of 3% BSA (in PBS) for 2 hours (room temperature) with gentle agitation. The membrane was then rinsed in PBS and placed into a primary antibody solution (for NPT II: rabbit anti-NPT II (5'-3' Inc.) diluted 1 in 1000 in Ab Dilution Buffer (3% BSA, 0.02% NaN$_3$, in 1X PBS) for PAT: goat anti-PAT IgG (Dr. Arno Schulz, AgrEvo AG, Frankfurt, Germany) diluted 1 in 500 in Ab Dilution Buffer) for 3 to 4 hours at room temperature (with agitation). The membrane was washed 3 x 15 minute using PBS . Following the washing steps, the immunoblot was transferred to a secondary antibody solution (for NPT II: 1:3000 dilution of goat anti-rabbit-alkaline phosphatase (AP) conjugate (BRL) in Ab Dilution Buffer; for PAT: 1:3000 dilution of rabbit anti goat -AP conjugate (BRL) in Ab Dilution Buffer) for 1.5 hours. Again the membrane was

washed 3 x 15 minutes in PBS. Alkaline phosphatase activity was detected by immersing the membrane in a colour reagent buffer (20 mL of AP buffer plus 66 μl of Bromochloroindolyl Phosphate (BCIP) reagent (0.5 g in 10 mls of 100% dimethylformamide) and 132 μl of Nitro-blue Tetrazolium (NBT) reagent (0.5 g in 10 mls of 70% dimethylformamide) and incubating it in the dark at 27 °C for 20 minutes. To stop the colour development, the membrane was rinsed several times in ddH$_2$O and then stored in PBS.

Reagents:

Transfer Buffer
>for 1 L:
>-5.82 g of Tris (48 mM)
>-2.93 g glycine (39 mM)
>-3.75 mL of 10% SDS
>>dissolve these in 600 mL of water
>-200 mL methanol
>-adjust volume to 1 L with water
>(the buffer will already range in pH from 9.0 to 9.4- no adjustment neccessary)

Grinding Buffer
>for 10 mLs:
>-50 μl of 1 M DTT
>-50 μl of 20% SDS
>-10 mLs of 100 mM Tris, pH 7.5
>-100 μl of 1 mg/ml leupeptin
>(keep on ice)

12% PAG Running Gel
>for 15 mLs:
>-6 mLs 30% acrylamide stock (30% w/v acrylamide/bis in dH$_2$O-dark bottle)
>-3.75 mLs of 1.5 M Tris-HCl (pH 8.8)
>-5.025 mLs of dH$_2$O
>-150 μl 10% SDS
>degas the above mixture (under vacuum) for 15 minutes, then add:
>-7.5 μl of TEMED
>-75 μl of 10% APS (ammonium persulfate- prepared fresh)

4% PAG Stacking Gel

for 5 mLs:
-3.05 mls of ddH$_2$O

-1.25 mls of 0.5 M Tris-HCl, pH 6.8
-50 µl of 10% SDS
-650 µl of 30% acrylamide/bis
degas for 15 minutes under vacuum, then add
-37.5 µl 10% ammonium persulfate
-5 µl TEMED

PAGE Running Buffer 1X

for 1 L:
-60.6 g Tris base, anhydrous (100 mM)
-144 g glycine, anhydrous (380 mM)
-0.5 g SDS (0.1%) [or 100 mLs of 10% SDS]
dH$_2$O to 1 L (final pH should be 8.4)

Alkaline Phosphatase (AP) Colour Development Buffer

-100mM NaCl
-5 mM MgCl$_2$
-100 mM Tris pH 9.5

The BioRad Protein MW markers:

Phosphorylase b	97 400 Daltons
Bovine Serum albumin	66 200 Daltons
Ovalbumin	45 000 Daltons
Carbonic Anhydrase	31 000 Daltons
Soybean Trypsin Inhibitor	21 500 Daltons
Lysozyme	14 400 Daltons

Histochemical β-glucuronidase (GUS) assay

(modified from Jefferson 1987)

Reagents

X-Gluc assay buffer:

> 8.8 g Na_2HPO_4
> 5.24 g $NaH_2PO_4.H_2O$
> 10 mL 0.1 M $K_3[Fe(CN)_6]$
> 10 mL 0.1 M $K_4[Fe(CN)_6].3H_2O$
> 40 mL 0.5 M EDTA..Na_2
> bring up to 1000 mL with ddH_2O and adjust to pH 7.0

To prepare a working solution: weigh out 50 mg X-Gluc into an Eppendorf tube, dissolve in 1 mL NN-dimethylformide, then add to 100 mL X-Gluc buffer and mix well.

Method

Dispense 100 μL X-Gluc solution into wells of a 96 well microtiter plate. Place a piece of small tissue from putative transgenic plants into each well then add 1 μL of diluted Tween-20 into each well. Place plate into a vacuum chamber and apply vacuum for five minutes. Seal the plate with Saran Wrap® and incubate overnight at 37°C. To clear plant pigments from tissue, remove X-Gluc staining solution from the well and add 10-fold diluted commercial bleach (Javex®) solution and leave at room temperature for several hours then observe.

APPENDIX III

MEDIA

Brassica napus cv. Westar Cotyledonary Petiole Transformation and Regeneration Protocol

(based on method by Moloney et al., 1989; adapted by Joe Hammerlindl, PBI, NRC, 110 Gymnasium Place, Saskatoon, SK, Canada)

Seed Sterilization and Germination

To sterilize seeds, place approximately 15 mL of seed into a 50 mL screwcap tube. Add sufficient 95% ethanol to wet the seeds and leave for 15 seconds. Fill the tube with 70% Javex (or other commercial bleach) with a drop of wetting agent such as Tween 20. Gently agitate the tube for 15 minutes. Aspirate away the Javex solution and add 0.025% mercuric chloride along with a drop of Tween 20. Gently agitate for 10 minutes. Rinse well with sterile distilled water.

Plate 35 to 40 seeds onto ½ x strength hormone-free solid Ms medium (Linsmaier and Skoog 1965) with 1% sucrose (on a 15 x 60 mm petri dish). Place the MS petri dishes, with lids removed, into a larger sterile vessel (Majenta GA7 jars). This allows the germinating seeds to grow taller and straighter facilitating the harvest of the cotyledonary explants.

Store the plates at 25°C, with a 16 hour light/8 hour dark photoperiod (50 to 70 $\mu Em^{-1} s^{-2}$) for 4 days.

Explant Preparation and Cocultivation

The seedlings are used when they are 4 days old. Using forceps, gently excise both petioles of the two cotyledons and dip the cut end of each petiole into a suspension of *Agrobacterium tumefaciens* (*Agrobacterium tumefaciens*, OD 0.9, in AB Minimal Medium + appropriate antibiotics). Place 16 cotyledons (cut and inoculated petiole tip down into the agar) onto each 15 x 100 mm petri dish containing Medium I.

Incubate petri dishes with the cotyledons for 3 days at 25°C, 16 hour light/ 8 hour dark photoperiod, 50 – 70 $\mu Em^{-1} s^{-2}$ light intensity. After 3 days, transfer explants to plates containing Medium II (10 explants per 15 x 100 mm petri dish). Incubate for 7 days as before. After the 7 day incubation period, transfer the explants to petri dishes containing Medium III (10 explants per 25 x 100 mm petri dish). Incubate plates as specified before for a period of 14 days. Transfer explants to fresh Medium III every 14 to 21 days until shoots develop.

Regenerated shoots are transferred to Medium IV to grow out into shoots with normal morphology. Shoots may have to be transferred to fresh Medium IV several times until normal shoots are obtained. Once good shoots are obtained, remove any callus growth at the base and transfer to Medium V in tall sterile jars for rooting.

Medium I:

- Murashige Minimal Organics (Gibco Laboratories, cat. No. 510-3118)
- 3% sucrose
- 4.5 mg/L benzyl adenine (BA)
- 0.7% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.8

Medium II:

- Murashige Minimal Organics (Gibco Laboratories, cat. No. 510-3118)
- 3% sucrose
- 4.5 mg/L benzyl adenine (BA)
- 500 mg/L Carbenicillin (Pyopen, Ayerst Pharmaceutical)
- 0.7% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.8

Medium III:

- Murashige Minimal Organics (Gibco Laboratories, cat. No. 510-3118)
- 3% sucrose
- 4.5 mg/L benzyl adenine (BA)
- 500 mg/L Carbenicillin (Pyopen, Ayerst Pharmaceutical)
- 20 mg/L kanamycin
- 0.7% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.8

Medium IV:

- Murashige Minimal Organics (Gibco Laboratories, cat. No. 510-3118)
- 3% sucrose
- 1 mg/L benzyl adenine (BA)
- 500 mg/L Carbenicillin (Pyopen, Ayerst Pharmaceutical)
- 20 mg/L kanamycin
- 0.7% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.8

Medium V:

- Murashige Minimal Organics (Gibco Laboratories, cat. No. 510-3118)
- 3% sucrose
- 0.1 mg/L napthalene acetic acid (NAA)
- 20 mg/L of L-phosphinothricin (L-PPT) (Basta^{TM}, AgrEvo AG, Frankfurt, Germany)
- 0.7% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.8

Nicotiana tabacum cv. Xanthi Leaf Disk Transformation and Regeneration Protocol

(based on method by Horsch et al., 1985)

Leaf material

The youngest leaves (6-10 cm) from N. tabacum cv. Xanthi plants grown in a growth chamber with a 16 hour photoperiod at 20°C are excised prior to bolting.

Sterilization

Young leaves are rinsed in water and dipped in 70% ethanol for 15 to 20 seconds. Whole leaves are then placed into a solution of 8% Javex bleach with a drop of Tween 20 and gently agitated for 15 minutes. Leaves are then rinsed 4 times in chilled sterile distilled water.

Explant Preparation:

The distil 1/3 of the leaf is not used for leaf disk preparation. Leaf disks are punched out of the upper 2/3 of the young, sterile leaf using a sterile 7 mm paper punch device or a sterile scalpel. Leaf disks are collected in sterile chilled distilled water and then transferred to a solution of Agrobacterium tumefaciens (A. tumefaciens in $\frac{1}{2}$ x strength Ms medium (Linsmaier and Skoog, 1965), OD 0.9 diluted to an OD of 0.4, 0% sucrose, + appropriate antibiotic) for 5 minutes. Following inoculation with A. tumefaciens the leaf disks are blotted onto sterile filter paper to remove excess bacteria. The disks are then transferred to NTI medium for 2 days (approximately 15 explants per 15 x 100 mm petri dish) at 25°C with a 16 hour photoperiod.

Following 2 days of incubation, the leaf disks are transferred to NTII medium on 25 x 100 mm petri dishes for 14 to 21 days under the same conditions. If small shoots have not already regenerated after 21 days, then leaf disks are transferred to fresh NTII medium. Regenerated shoots are excised away from any callus material and transferred to rooting medium (NTIII medium) and incubated under the same growth chamber conditions.

NTI Medium:

- 4.44 g/L MSB5 salts/vitamins (SIGMA)
- 3% sucrose
- 2 mg/L 2,4-D
- 0.8% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.6

NTII Medium:

- 4.44 g/L MSB5 salts/vitamins (SIGMA)
- 3% sucrose
- 2.5 mg/L benzyl adenine (BA)
- 0.1 mg/L napthalene acetic acid (NAA)
- 100 mg/L kanamycin
- 500 mg/L Carbenicillin (Pyopen, Ayerst Pharmaceutical)
- 0.8% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.6

NTIII Medium:

- 4.44 g/L MSB5 salts/vitamins (SIGMA)
- 1.5% sucrose
- 0.1 mg/L napthalene acetic acid (NAA)
- 500 mg/L Carbenicillin (Pyopen, Ayerst Pharmaceutical)
- 60 mg/L L-phosphinothricin (L-PPT) (Basta^{TM}, AgrEvo AG, Frankfurt, Germany)
- 0.8% Phytagar (Gibco Laboratories, cat. No. 670-0675)
- pH 5.6

Dellaporta DNA Extraction Buffer

(based on the method by Dellaporta et al., 1983, 1985).

- 100 mM Tris.Cl
- 50 mM EDTA
- 500 mM NaCl
- 70 μl of β-mercaptoethanol (1.12 g/mL of MW 78.13) in 100 mLs

2YT Medium

Per liter:
- 16g bacto-tryptone
- 10 g bacto-yeast extract
- 5 g NaCl

pH to 7.0 with 5N NaOH, and adjust volume of the solution to 1 liter with deionized H2O. Sterilize by autoclaving 20 minutes at 15 lb/sq. in. on liquid cycle.

For solid Agar Plates:
- to 1 L of 2YT medium (prior to autoclaving) add 15 g of Bacto-Agar

M9 Minimal Medium

Per liter:

To 750 mL of sterile deionized H_2O (cooled to 50 °C or less) add:
- 200 ml of 5X M9 salts (sterile)
- 20 ml of 20% glucose (sterile)
- 500 μl of 1 mg/ml thiamine.HCl stock (sterile) [only for E.coli DH5α and other auxotrophs which requires a minimal supplement]
- 30 ml of sterile ddH_2O

5 X M9 Salts

Dissolve the following salts in ddH_2O to a final volume of 1 Liter:

- 64 g $Na_2HPO_4.7H_2O$
- 15 g KH_2PO_4
- 2.5 g NaCl
- 5.0 g NH_4Cl

DMSO Frozen Bacterial Stock Cultures

- grow bacteria overnight in 2YT plus antibiotic at 37 degrees C (for *Agrobacterium* grow 2 days in AB Min medium at 28 degrees C.)

- next day, remove 930 µl of culture (from an exponential growth phase culture) and place in a sterile screw-cap cryo-vial

- add 70 µl of DMSO and gently invert

- let sit at room temp. for 15 minutes then place at - 70 degrees C for storage

APPENDIX IV

Figure 3.15

B. napus pBAU1 Rooting Data at Day 0:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	0.00	0.00	0.00	0.00	HCN92	0.00	0.00	0.00	0.00
pBAU1#3	0.00	0.00	0.00	0.00	pBAU1#3	0.00	0.00	0.00	0.00
pBAU1#2	0.00	0.00	0.00	0.00	pBAU1#2	0.00	0.00	0.00	0.00
pBAU1#1	0.00	0.00	0.00	0.00	pBAU1#1	0.00	0.00	0.00	0.00
pBAU1#4	0.00	0.00	0.00	0.00	pBAU1#4	0.00	0.00	0.00	0.00
pBAU1#7	0.00	0.00	0.00	0.00	pBAU1#7	0.00	0.00	0.00	0.00
pBAU1#11	0.00	0.00	0.00	0.00	pBAU1#11	0.00	0.00	0.00	0.00
pBAU1#8	0.00	0.00	0.00	0.00	pBAU1#8	0.00	0.00	0.00	0.00
pBAU1#10	0.00	0.00	0.00	0.00	pBAU1#10	0.00	0.00	0.00	0.00
pRD430#3	0.00	0.00	0.00	0.00	pRD430#3	0.00	0.00	0.00	0.00
Bn wt	0.00	0.00	0.00	0.00	Bn wt	0.00	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	2.00	2.00	3.00	3.33	HCN92	0.00	0.00	0.00	2.31
pBAU1#3	2.67	2.33	2.33	2.33	pBAU1#3	0.57	0.57	0.57	0.57
pBAU1#2	2.33	2.00	2.33	2.66	pBAU1#2	0.57	0.00	0.57	0.57
pBAU1#1	2.00	3.33	2.33	2.33	pBAU1#1	1.00	1.52	0.57	0.57
pBAU1#4	2.66	2.00	2.66	2.00	pBAU1#4	0.57	0.00	1.52	1.00
pBAU1#7	2.33	2.00	2.33	2.33	pBAU1#7	1.15	1.00	0.57	0.57
pBAU1#11	3.00	2.66	3.00	2.33	pBAU1#11	0.00	0.57	1.00	0.57
pBAU1#8	2.66	3.00	3.33	2.33	pBAU1#8	1.52	0.00	1.52	0.57
pBAU1#10	3.00	2.33	3.33	2.00	pBAU1#10	1.00	0.57	1.15	0.00
pRD430#3	2.33	2.66	2.66	2.33	pRD430#3	1.15	0.57	0.57	0.57
Bn wt	2.66	2.33	2.66	3.00	Bn wt	0.57	0.57	0.57	0.00

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	1.00	1.00	1.00	1.00	HCN92	0.00	0.00	0.00	0.00
pBAU1#3	1.00	1.00	1.00	1.00	pBAU1#3	0.00	0.00	0.00	0.00
pBAU1#2	1.00	1.00	1.00	1.00	pBAU1#2	0.00	0.00	0.00	0.00
pBAU1#1	1.00	1.00	1.33	1.00	pBAU1#1	0.00	0.00	0.57	0.00
pBAU1#4	1.00	1.00	1.00	1.00	pBAU1#4	0.00	0.00	0.00	0.00
pBAU1#7	1.33	1.66	1.33	1.00	pBAU1#7	0.57	1.15	0.57	0.00
pBAU1#11	1.00	1.00	1.00	1.00	pBAU1#11	0.00	0.00	0.00	0.00
pBAU1#8	1.00	1.00	1.00	1.33	pBAU1#8	0.00	0.00	0.00	0.57
pBAU1#10	1.00	1.00	1.33	1.00	pBAU1#10	0.00	0.00	0.57	0.00
pRD430#3	1.00	1.00	1.00	1.00	pRD430#3	0.00	0.00	0.00	0.00
Bn wt	1.00	1.00	1.00	1.00	Bn wt	0.00	0.00	0.00	0.00

B. *napus* pBAU1 Rooting Data at Day 6:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	2.33	2.33	2.00	2.00	HCN92	0.58	0.58	1.00	0.00
pBAU1#3	2.33	2.33	2.67	2.00	pBAU1#3	1.15	0.58	0.58	0.00
pBAU1#2	1.67	1.00	1.67	0.00	pBAU1#2	0.58	1.00	0.58	0.00
pBAU1#1	1.00	1.00	0.00	0.00	pBAU1#1	1.00	1.00	0.00	0.00
pBAU1#4	1.33	1.33	0.00	0.00	pBAU1#4	1.53	1.15	0.00	0.00
pBAU1#7	2.00	2.00	0.67	0.00	pBAU1#7	0.00	1.00	1.15	0.00
pBAU1#11	2.33	2.33	2.67	2.00	pBAU1#11	0.58	0.58	0.58	1.00
pBAU1#8	1.33	2.33	1.00	0.00	pBAU1#8	1.15	0.58	1.00	0.00
pBAU1#10	2.33	1.67	1.00	0.67	pBAU1#10	0.58	0.58	1.00	1.15
pRD430#3	1.00	2.33	2.00	1.00	pRD430#3	1.00	1.15	1.00	1.00
Bn wt	1.67	0.00	0.00	0.00	Bn wt	0.58	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	4.67	4.00	5.33	4.33	HCN92	0.58	1.00	0.58	1.53
pBAU1#3	7.00	4.33	4.00	4.00	pBAU1#3	2.65	2.31	1.00	0.00
pBAU1#2	3.67	4.33	4.00	3.33	pBAU1#2	0.58	0.58	1.00	1.15
pBAU1#1	4.33	4.67	3.00	4.00	pBAU1#1	1.15	1.15	1.00	2.00
pBAU1#4	3.33	2.67	3.00	2.67	pBAU1#4	1.15	1.15	1.00	0.58
pBAU1#7	5.33	4.00	4.33	4.00	pBAU1#7	2.52	1.00	1.53	1.00
pBAU1#11	4.00	3.67	4.33	4.33	pBAU1#11	0.00	0.58	0.58	1.53
pBAU1#8	4.00	4.33	4.67	4.00	pBAU1#8	1.73	0.58	1.53	2.00
pBAU1#10	5.33	4.00	5.00	4.00	pBAU1#10	2.31	1.00	1.73	1.00
pRD430#3	5.67	7.00	5.67	5.33	pRD430#3	1.15	4.58	1.15	2.52
Bn wt	4.33	2.33	2.67	3.00	Bn wt	0.58	0.58	0.58	0.00

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	1.00	1.00	1.00	1.33	HCN92	0.00	0.00	0.00	0.58
pBAU1#3	1.00	1.00	1.00	1.67	pBAU1#3	0.00	0.00	0.00	0.58
pBAU1#2	1.33	2.00	1.00	2.33	pBAU1#2	0.58	0.00	0.00	1.15
pBAU1#1	1.33	1.00	3.00	3.33	pBAU1#1	0.58	0.00	1.73	2.08
pBAU1#4	1.33	1.33	2.33	2.33	pBAU1#4	0.58	0.58	0.58	1.53
pBAU1#7	1.00	1.67	1.33	2.00	pBAU1#7	0.00	1.15	0.58	1.00
pBAU1#11	1.00	1.00	1.67	1.67	pBAU1#11	0.00	0.00	0.58	0.58
pBAU1#8	1.33	1.67	2.00	2.33	pBAU1#8	0.58	1.15	0.00	0.58
pBAU1#10	1.67	1.33	1.67	2.33	pBAU1#10	0.58	0.58	0.58	0.58
pRD430#3	1.00	1.33	1.00	1.00	pRD430#3	0.00	0.58	0.00	0.00
Bn wt	1.00	2.67	3.67	4.00	Bn wt	0.00	0.58	1.15	1.00

B. napus pBAU1 Rooting Data at Day 12:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	3.00	2.50	2.50	2.50	HCN92	0.00	0.71	0.71	0.71
pBAU1#3	3.00	3.00	2.50	2.50	pBAU1#3	0.00	0.00	0.71	0.71
pBAU1#2	2.50	2.50	3.00	0.00	pBAU1#2	0.71	0.71	0.00	0.00
pBAU1#1	2.50	2.50	0.00	0.00	pBAU1#1	0.71	0.71	0.00	0.00
pBAU1#4	2.50	2.50	0.50	0.00	pBAU1#4	0.71	0.71	0.71	0.00
pBAU1#7	3.00	3.00	2.50	0.50	pBAU1#7	0.00	0.00	0.71	0.71
pBAU1#11	3.00	3.00	2.50	3.00	pBAU1#11	0.00	0.00	0.71	0.00
pBAU1#8	2.50	3.00	3.00	1.00	pBAU1#8	0.71	0.00	0.00	1.41
pBAU1#10	2.50	3.00	2.00	2.00	pBAU1#10	0.71	0.00	0.00	0.00
pRD430#3	2.50	1.50	2.50	1.00	pRD430#3	0.71	0.71	0.71	1.41
Bn wt	2.50	0.00	0.00	0.00	Bn wt	0.71	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	8.00	6.50	7.50	6.50	HCN92	2.83	0.71	0.71	0.71
pBAU1#3	7.50	7.00	5.00	4.50	pBAU1#3	3.54	4.24	1.41	0.71
pBAU1#2	4.50	5.00	6.00	4.00	pBAU1#2	0.71	1.41	1.41	0.00
pBAU1#1	5.50	4.00	3.50	3.00	pBAU1#1	0.71	0.00	0.71	1.41
pBAU1#4	4.00	3.00	5.00	3.00	pBAU1#4	0.00	1.41	2.83	1.41
pBAU1#7	7.00	4.50	5.50	4.00	pBAU1#7	1.41	0.71	2.12	1.41
pBAU1#11	5.50	4.00	8.00	6.50	pBAU1#11	0.71	0.00	4.24	0.71
pBAU1#8	4.50	6.00	5.00	5.00	pBAU1#8	0.71	0.00	2.83	1.41
pBAU1#10	6.00	5.00	5.00	4.50	pBAU1#10	1.41	0.00	0.00	0.71
pRD430#3	6.50	4.50	10.00	6.50	pRD430#3	0.71	2.12	2.83	3.54
Bn wt	7.00	2.00	2.50	3.00	Bn wt	1.41	0.00	0.71	0.00

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	1.50	1.00	1.00	1.50	HCN92	0.71	0.00	0.00	0.71
pBAU1#3	1.00	1.00	1.00	2.00	pBAU1#3	0.00	0.00	0.00	1.41
pBAU1#2	1.50	2.00	1.50	2.50	pBAU1#2	0.71	0.00	0.71	0.71
pBAU1#1	1.00	1.00	2.00	4.00	pBAU1#1	0.00	0.00	0.00	1.41
pBAU1#4	1.00	1.50	2.50	2.00	pBAU1#4	0.00	0.71	0.71	1.41
pBAU1#7	1.00	1.00	1.50	2.00	pBAU1#7	0.00	0.00	0.71	0.00
pBAU1#11	1.00	1.00	2.00	2.00	pBAU1#11	0.00	0.00	0.00	0.00
pBAU1#8	1.50	1.50	2.50	3.00	pBAU1#8	0.71	0.71	0.71	0.00
pBAU1#10	2.00	1.50	2.50	2.50	pBAU1#10	0.00	0.71	0.71	0.71
pRD430#3	1.00	1.00	1.00	1.50	pRD430#3	0.00	0.00	0.00	0.71
Bn wt	1.00	3.50	4.50	5.00	Bn wt	0.00	0.71	0.71	0.00

B. napus pBAU1 Rooting Data at Day 20:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	3.00	3.00	3.00	3.00	HCN92	0.00	0.00	0.00	0.00
pBAU1#3	3.00	3.00	2.67	3.00	pBAU1#3	0.00	0.00	0.58	0.00
pBAU1#2	3.00	2.00	3.00	1.67	pBAU1#2	0.00	1.73	0.00	1.53
pBAU1#1	2.67	2.67	0.67	0.33	pBAU1#1	0.58	0.58	0.58	0.58
pBAU1#4	2.67	3.00	1.33	0.00	pBAU1#4	0.58	0.00	1.15	0.00
pBAU1#7	3.00	3.00	2.33	1.00	pBAU1#7	0.00	0.00	1.15	1.73
pBAU1#11	3.00	3.00	3.00	3.00	pBAU1#11	0.00	0.00	0.00	0.00
pBAU1#8	3.00	3.00	2.67	2.00	pBAU1#8	0.00	0.00	0.58	1.00
pBAU1#10	3.00	3.00	2.00	2.67	pBAU1#10	0.00	0.00	1.00	0.58
pRD430#3	2.67	3.00	3.00	2.67	pRD430#3	0.58	0.00	0.00	0.58
Bn wt	3.00	0.00	0.00	0.00	Bn wt	0.00	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	9.00	8.33	8.67	7.33	HCN92	1.73	1.53	0.58	0.58
pBAU1#3	10.00	8.00	6.33	6.33	pBAU1#3	5.00	2.65	1.15	2.08
pBAU1#2	6.00	6.00	6.00	5.00	pBAU1#2	1.00	0.00	1.00	1.73
pBAU1#1	6.33	8.00	3.67	3.33	pBAU1#1	0.58	4.36	1.53	1.15
pBAU1#4	5.67	5.33	5.33	2.67	pBAU1#4	0.58	1.53	1.53	1.15
pBAU1#7	9.00	5.67	7.00	5.00	pBAU1#7	1.00	0.58	2.65	2.00
pBAU1#11	7.33	5.67	8.33	6.33	pBAU1#11	0.58	1.15	2.52	2.08
pBAU1#8	7.00	6.67	7.67	6.00	pBAU1#8	1.73	1.15	4.51	2.00
pBAU1#10	7.33	5.67	6.00	6.00	pBAU1#10	0.58	0.58	4.00	0.00
pRD430#3	10.00	10.33	11.67	7.00	pRD430#3	1.73	6.11	3.79	2.65
Bn wt	8.33	2.33	2.67	3.00	Bn wt	0.58	0.58	0.58	0.00

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
HCN92	1.33	1.00	1.00	1.67	HCN92	0.58	0.00	0.00	0.58
pBAU1#3	1.00	1.00	1.00	1.67	pBAU1#3	0.00	0.00	0.00	0.58
pBAU1#2	1.67	3.00	1.33	2.00	pBAU1#2	0.58	1.73	0.58	1.00
pBAU1#1	1.33	1.00	3.33	4.33	pBAU1#1	0.58	0.00	1.53	1.15
pBAU1#4	1.33	1.33	2.00	3.00	pBAU1#4	0.58	0.58	0.00	1.00
pBAU1#7	1.00	1.33	2.00	2.67	pBAU1#7	0.00	0.58	1.00	0.58
pBAU1#11	1.00	1.00	1.33	1.67	pBAU1#11	0.00	0.00	0.58	0.58
pBAU1#8	2.00	1.67	2.33	2.33	pBAU1#8	1.00	0.58	0.58	0.58
pBAU1#10	1.67	1.33	2.67	2.67	pBAU1#10	0.58	0.58	0.58	0.58
pRD430#3	1.00	1.00	1.00	1.67	pRD430#3	0.00	0.00	0.00	0.58
Bn wt	1.33	5.00	5.00	5.00	Bn wt	0.58	0.00	0.00	0.00

Figure 3.16

N. tabacum pBAU1 Rooting Data at Day 0:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	0.00	0.00	0.00	0.00	Nt wt	0.00	0.00	0.00	0.00
pBAU1 #5	0.00	0.00	0.00	0.00	pBAU1 #5	0.00	0.00	0.00	0.00
pBAU1 #7	0.00	0.00	0.00	0.00	pBAU1 #7	0.00	0.00	0.00	0.00
pBAU1 #8	0.00	0.00	0.00	0.00	pBAU1 #8	0.00	0.00	0.00	0.00
pBAU1 #9	0.00	0.00	0.00	0.00	pBAU1 #9	0.00	0.00	0.00	0.00
pBAU1 #10	0.00	0.00	0.00	0.00	pBAU1 #10	0.00	0.00	0.00	0.00
pBAU1 #6	0.00	0.00	0.00	0.00	pBAU1 #6	0.00	0.00	0.00	0.00
pBAU1 #11	0.00	0.00	0.00	0.00	pBAU1 #11	0.00	0.00	0.00	0.00
pBAU1 #12	0.00	0.00	0.00	0.00	pBAU1 #12	0.00	0.00	0.00	0.00
pRD430 #2	0.00	0.00	0.00	0.00	pRD430 #2	0.00	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	2.67	3.33	2.67	2.67	Nt wt	1.15	1.53	1.15	1.15
pBAU1 #5	2.67	3.00	2.00	2.67	pBAU1 #5	0.58	1.00	0.00	1.15
pBAU1 #7	2.67	1.67	2.33	2.33	pBAU1 #7	1.15	0.58	1.15	0.58
pBAU1 #8	2.33	2.00	2.33	3.00	pBAU1 #8	0.58	0.00	0.58	0.00
pBAU1 #9	2.00	3.00	2.33	2.67	pBAU1 #9	0.00	1.00	0.58	0.58
pBAU1 #10	2.67	3.33	2.33	2.33	pBAU1 #10	1.15	1.15	0.58	0.58
pBAU1 #6	2.67	2.67	2.00	3.00	pBAU1 #6	0.58	1.15	0.00	1.00
pBAU1 #11	2.33	2.67	2.00	3.00	pBAU1 #11	1.53	0.58	1.00	1.00
pBAU1 #12	2.33	1.67	2.00	1.67	pBAU1 #12	0.58	0.58	1.00	0.58
pRD430 #2	2.67	2.67	3.00	2.67	pRD430 #2	0.58	0.58	1.00	0.58

	Average Level of Necrosis					St. Dev. on Level Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	1.00	1.33	1.00	1.00	Nt wt	0.00	0.58	0.00	0.00
pBAU1 #5	1.00	1.00	1.00	1.00	pBAU1 #5	0.00	0.00	0.00	0.00
pBAU1 #7	1.00	1.00	1.00	1.00	pBAU1 #7	0.00	0.00	0.00	0.00
pBAU1 #8	1.00	1.00	1.00	1.00	pBAU1 #8	0.00	0.00	0.00	0.00
pBAU1 #9	1.00	1.00	1.00	1.00	pBAU1 #9	0.00	0.00	0.00	0.00
pBAU1 #10	1.00	1.00	1.00	1.33	pBAU1 #10	0.00	0.00	0.00	0.58
pBAU1 #6	1.00	1.00	1.33	1.00	pBAU1 #6	0.00	0.00	0.58	0.00
pBAU1 #11	1.00	1.00	1.00	1.00	pBAU1 #11	0.00	0.00	0.00	0.00
pBAU1 #12	1.00	1.33	1.00	1.00	pBAU1 #12	0.00	0.58	0.00	0.00
pRD430 #2	1.00	1.00	1.00	1.00	pRD430 #2	0.00	0.00	0.00	0.00

N. tabacum pBAU1 Rooting Data at Day 6:

Average	Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	1.33	0.00	0.00	0.00	Nt wt	0.58	0.00	0.00	0.00
pBAU1 #5	1.33	2.00	2.00	1.67	pBAU1 #5	1.53	0.00	1.00	1.53
pBAU1 #7	0.67	1.33	1.00	1.67	pBAU1 #7	1.15	1.53	1.00	1.15
pBAU1 #8	1.33	1.33	1.00	1.33	pBAU1 #8	1.15	1.53	1.00	1.15
pBAU1 #9	1.67	1.33	1.67	1.67	pBAU1 #9	0.58	1.15	1.15	1.15
pBAU1 #10	1.33	2.33	2.00	2.00	pBAU1 #10	1.15	1.15	1.00	0.00
pBAU1 #6	2.33	1.33	1.67	2.33	pBAU1 #6	0.58	1.15	0.58	0.58
pBAU1 #11	0.67	1.67	1.33	1.00	pBAU1 #11	1.15	1.53	1.15	1.00
pBAU1 #12	0.50	1.33	1.50	1.33	pBAU1 #12	0.71	1.15	0.71	1.15
pRD430 #2	1.33	2.67	1.67	1.00	pRD430 #2	0.58	0.58	1.15	1.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	3.67	3.00	3.33	3.33	Nt wt	1.15	1.73	1.53	2.31
pBAU1 #5	3.33	3.33	2.67	3.33	pBAU1 #5	0.58	0.58	0.58	1.53
pBAU1 #7	4.00	2.67	4.00	3.33	pBAU1 #7	1.73	1.15	1.00	0.58
pBAU1 #8	3.00	3.00	3.67	4.00	pBAU1 #8	1.00	1.00	0.58	1.00
pBAU1 #9	3.67	3.33	3.00	3.00	pBAU1 #9	1.53	0.58	1.00	1.00
pBAU1 #10	3.33	4.33	3.33	4.00	pBAU1 #10	0.58	1.53	0.58	2.65
pBAU1 #6	4.33	3.33	3.00	6.00	pBAU1 #6	0.58	0.58	1.00	3.46
pBAU1 #11	2.67	4.33	3.00	4.00	pBAU1 #11	2.08	1.53	1.73	1.00
pBAU1 #12	4.00	2.33	2.00	2.33	pBAU1 #12	1.41	1.15	1.41	1.53
pRD430 #2	3.00	3.67	4.33	4.33	pRD430 #2	1.00	1.15	1.53	0.58

Average	Level of Necrosis					St Dev on Level Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	1.00	3.33	2.33	3.00	Nt wt	0.00	1.53	1.15	1.00
pBAU1 #5	1.00	1.00	1.00	1.00	pBAU1 #5	0.00	0.00	0.00	0.00
pBAU1 #7	1.00	1.00	1.00	1.00	pBAU1 #7	0.00	0.00	0.00	0.00
pBAU1 #8	1.00	1.00	1.00	2.00	pBAU1 #8	0.00	0.00	0.00	1.00
pBAU1 #9	1.00	1.00	1.00	1.00	pBAU1 #9	0.00	0.00	0.00	0.0
pBAU1 #10	1.00	1.00	1.00	1.67	pBAU1 #10	0.00	0.00	0.00	0.58
pBAU1 #6	1.00	1.00	1.33	1.00	pBAU1 #6	0.00	0.00	0.58	0.00
pBAU1 #11	1.00	1.33	1.00	1.00	pBAU1 #11	0.00	0.58	0.00	0.00
pBAU1 #12	1.00	1.33	1.00	1.33	pBAU1 #12	0.00	0.58	0.00	0.58
pRD430 #2	1.00	1.00	1.33	1.33	pRD430 #2	0.00	0.00	0.58	0.58

N. tabacum pBAU1 Rooting Data at Day 12:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	3.00	0.00	0.00	0.00	Nt wt	0.00	0.00	0.00	0.00
pBAU1 #5	2.50	3.00	2.50	3.00	pBAU1 #5	0.71	0.00	0.71	0.00
pBAU1 #7	2.50	3.00	3.00	2.50	pBAU1 #7	0.71	0.00	0.00	0.71
pBAU1 #8	3.00	2.50	2.50	3.00	pBAU1 #8	0.00	0.71	0.71	0.00
pBAU1 #9	3.00	3.00	2.00	2.50	pBAU1 #9	0.00	0.00	1.41	0.71
pBAU1 #10	2.50	3.00	3.00	3.00	pBAU1 #10	0.71	0.00	0.00	0.00
pBAU1 #6	3.00	2.50	3.00	3.00	pBAU1 #6	0.00	0.71	0.00	0.00
pBAU1 #11	2.00	2.00	1.00	2.00	pBAU1 #11	1.41	1.41	1.41	1.41
pBAU1 #12	3.00	3.00	2.00	3.00	pBAU1 #12	0.71	0.00	1.41	0.00
pRD430 #2	1.50	3.00	2.00	3.00	pRD430 #2	2.12	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	5.00	4.00	3.50	4.00	Nt wt	2.83	1.41	2.12	2.83
pBAU1 #5	5.00	5.00	3.00	3.50	pBAU1 #5	0.00	2.83	0.00	2.12
pBAU1 #7	3.50	5.50	6.50	3.50	pBAU1 #7	0.71	0.71	2.12	0.71
pBAU1 #8	3.50	4.00	4.00	5.50	pBAU1 #8	0.71	1.41	1.41	0.71
pBAU1 #9	5.50	4.00	3.50	5.50	pBAU1 #9	0.71	1.41	2.12	3.54
pBAU1 #10	3.50	5.50	5.00	3.00	pBAU1 #10	0.71	2.12	0.00	1.41
pBAU1 #6	6.50	4.00	4.50	6.50	pBAU1 #6	0.71	1.41	2.12	3.54
pBAU1 #11	2.50	5.50	2.50	4.50	pBAU1 #11	2.12	2.12	2.12	0.71
pBAU1 #12	4.00	3.50	3.00	2.50	pBAU1 #12	2.83	0.71	0.00	0.71
pRD430 #2	5.00	6.00	4.00	6.50	pRD430 #2	4.24	0.00	1.41	0.71

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	1.00	4.50	4.50	5.00	Nt wt	0.00	0.71	0.71	0.00
pBAU1 #5	1.00	1.00	1.00	1.00	pBAU1 #5	0.00	0.00	0.00	0.00
pBAU1 #7	1.00	1.00	1.00	1.00	pBAU1 #7	0.00	0.00	0.00	0.00
pBAU1 #8	1.00	1.00	1.00	1.00	pBAU1 #8	0.00	0.00	0.00	0.00
pBAU1 #9	1.00	1.00	1.00	1.00	pBAU1 #9	0.00	0.00	0.00	0.00
pBAU1 #10	1.00	1.00	1.00	2.00	pBAU1 #10	0.00	0.00	0.00	1.41
pBAU1 #6	1.00	1.00	1.50	1.00	pBAU1 #6	0.00	0.00	0.71	0.00
pBAU1 #11	1.00	1.00	1.50	1.50	pBAU1 #11	0.00	0.00	0.71	0.71
pBAU1 #12	1.00	1.00	1.00	1.00	pBAU1 #12	0.00	0.00	0.00	0.00
pRD430 #2	1.00	1.00	1.00	1.50	pRD430 #2	0.00	0.00	0.00	0.71

N. tabacum pBAU1 Rooting Data at Day 20:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	2.00	0.00	0.00	0.00	Nt wt	1.41	0.00	0.00	0.00
pBAU1 #5	2.00	2.00	2.00	2.00	pBAU1 #5	1.41	1.41	1.41	1.41
pBAU1 #7	2.00	2.00	2.00	2.00	pBAU1 #7	1.41	1.41	1.41	1.41
pBAU1 #8	2.00	2.00	2.00	2.50	pBAU1 #8	1.41	1.41	1.41	0.71
pBAU1 #9	2.00	2.00	2.00	2.00	pBAU1 #9	1.41	1.41	1.41	1.41
pBAU1 #10	2.00	2.00	2.00	2.00	pBAU1 #10	1.41	1.41	1.41	1.41
pBAU1 #6	2.00	2.00	2.00	2.00	pBAU1 #6	1.41	1.41	1.41	1.41
pBAU1 #11	2.50	2.50	3.00	3.00	pBAU1 #11	0.71	0.71	0.00	0.00
pBAU1 #12	3.00	2.00	1.00	2.00	pBAU1 #12	1.41	1.41	1.41	1.41
pRD430 #2	1.50	2.00	2.00	2.00	pRD430 #2	0.71	1.41	1.41	1.41

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	6.00	2.00	2.00	2.00	Nt wt	1.41	0.00	0.00	0.00
pBAU1 #5	5.00	5.00	5.00	6.00	pBAU1 #5	1.41	0.00	1.41	0.00
pBAU1 #7	5.00	6.00	7.50	4.50	pBAU1 #7	1.41	0.00	0.71	2.12
pBAU1 #8	6.50	5.00	6.50	6.50	pBAU1 #8	2.12	1.41	2.12	2.12
pBAU1 #9	8.00	5.50	6.50	5.50	pBAU1 #9	0.00	2.12	0.71	2.12
pBAU1 #10	5.00	8.00	5.50	8.00	pBAU1 #10	0.00	1.41	0.71	5.66
pBAU1 #6	7.50	5.50	5.50	7.00	pBAU1 #6	0.71	0.71	2.12	0.00
pBAU1 #11	4.50	8.00	4.00	5.50	pBAU1 #11	4.95	1.41	0.00	2.12
pBAU1 #12	9.00	6.00	4.00	6.00	pBAU1 #12	2.12	0.00	0.71	1.41
pRD430 #2	3.00	7.50	7.00	7.50	pRD430 #2	1.41	0.71	4.24	0.71

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	60 mg/L	120 mg/L	240 mg/L		0 mg/L	60 mg/L	120 mg/L	240 mg/L
Nt wt	1.00	5.00	5.00	5.00	Nt wt	0.00	0.00	0.00	0.00
pBAU1 #5	1.00	1.00	1.00	1.00	pBAU1 #5	0.00	0.00	0.00	0.00
pBAU1 #7	1.00	1.00	1.00	1.00	pBAU1 #7	0.00	0.00	0.00	0.00
pBAU1 #8	1.00	1.00	1.00	2.00	pBAU1 #8	0.00	0.00	0.00	0.00
pBAU1 #9	1.00	1.00	1.00	1.00	pBAU1 #9	0.00	0.00	0.00	0.00
pBAU1 #10	1.00	1.00	1.00	1.00	pBAU1 #10	0.00	0.00	0.00	0.00
pBAU1 #6	1.00	1.00	1.00	1.00	pBAU1 #6	0.00	0.00	0.00	0.00
pBAU1 #11	1.50	2.00	2.00	2.50	pBAU1 #11	0.71	0.00	1.41	0.71
pBAU1 #12	1.00	1.00	1.00	1.00	pBAU1 #12	0.00	0.00	1.41	0.00
pRD430 #2	1.00	1.00	1.50	1.00	pRD430 #2	0.00	0.00	0.71	0.00

Figure 4.5

N. tabacum pBAU2 Rooting Data at Day 0:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	0.00	0.00	0.00	0.00	Nt wt	0.00	0.00	0.00	0.00
pBAU2 #13	0.00	0.00	0.00	0.00	pBAU2 #13	0.00	0.00	0.00	0.00
pBAU2 #14	0.00	0.00	0.00	0.00	pBAU2 #14	0.00	0.00	0.00	0.00
pBAU2 #15	0.00	0.00	0.00	0.00	pBAU2 #15	0.00	0.00	0.00	0.00
pHOE6 #110/2	0.00	0.00	0.00	0.00	pHOE6 #110/2	0.00	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	2.00	1.67	2.00	2.33	Nt wt	1.00	0.58	0.00	0.58
pBAU2 #13	2.33	2.00	2.00	2.00	pBAU2 #13	0.58	1.00	0.00	0.00
pBAU2 #14	2.00	2.67	2.33	2.67	pBAU2 #14	1.73	1.15	0.58	0.58
pBAU2 #15	2.00	2.67	3.00	2.33	pBAU2 #15	1.00	1.15	0.00	0.58
pHOE6 #110/2	2.00	1.67	1.67	2.00	pHOE6 #110/2	1.73	0.58	0.58	0.00

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	1.00	1.00	1.00	1.00	Nt wt	0.00	0.00	0.00	0.00
pBAU2 #13	1.00	1.00	1.00	1.00	pBAU2 #13	0.00	0.00	0.00	0.00
pBAU2 #14	1.00	1.00	1.00	1.00	pBAU2 #14	0.00	0.00	0.00	0.00
pBAU2 #15	1.00	1.00	1.00	1.00	pBAU2 #15	0.00	0.00	0.00	0.00
pHOE6 #110/2	1.00	1.00	1.00	1.00	pHOE6 #110/2	0.00	0.00	0.00	0.00

N. tabacum pBAU2 Rooting Data at Day 4:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	0.00	0.00	0.00	0.00	Nt wt	0.00	0.00	0.00	0.00
pBAU2 #13	0.33	0.00	0.00	0.00	pBAU2 #13	0.58	0.00	0.00	0.00
pBAU2 #14	0.00	0.00	0.00	0.00	pBAU2 #14	0.00	0.00	0.00	0.00
pBAU2 #15	0.67	0.00	0.00	0.33	pBAU2 #15	1.15	0.00	0.00	0.58
pHOE6 #110/2	0.00	0.00	0.00	0.00	pHOE6 #110/2	0.00	0.00	0.00	0.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	2.33	1.67	2.00	2.33	Nt wt	1.15	0.58	0.00	0.58
pBAU2 #13	2.67	2.33	2.00	2.00	pBAU2 #13	1.15	1.15	0.00	0.00
pBAU2 #14	2.67	2.67	2.67	3.00	pBAU2 #14	2.08	1.15	0.58	1.00
pBAU2 #15	2.33	2.67	3.33	2.67	pBAU2 #15	1.15	1.15	0.58	0.58
pHOE6 #110/2	2.00	1.67	1.67	2.33	pHOE6 #110/2	1.73	0.58	0.58	0.58

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	1.00	1.67	2.33	2.67	Nt wt	0.00	0.58	0.58	0.58
pBAU2 #13	1.00	1.33	1.00	1.00	pBAU2 #13	0.00	0.58	0.00	0.00
pBAU2 #14	1.00	1.00	1.00	1.33	pBAU2 #14	0.00	0.00	0.00	0.58
pBAU2 #15	1.00	1.00	1.00	1.33	pBAU2 #15	0.00	0.00	0.00	0.58
pHOE6 #110/2	1.00	1.00	1.00	1.00	pHOE6 #110/2	0.00	0.00	0.00	0.00

N. tabacum pBAU2 Rooting Data at Day 10:

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	3.00	0.00	0.00	0.00	Nt wt	0.00	0.00	0.00	0.00
pBAU2 #13	2.00	0.67	1.00	2.00	pBAU2 #13	1.73	1.15	1.73	0.00
pBAU2 #14	0.67	1.33	0.00	0.67	pBAU2 #14	1.15	1.15	0.00	1.15
pBAU2 #15	3.00	2.33	1.67	1.00	pBAU2 #15	0.00	0.58	1.53	1.73
pHOE6 #110/2	0.67	2.00	2.00	0.33	pHOE6 #110/2	1.15	1.00	1.73	0.58

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	2.67	1.67	2.00	2.33	Nt wt	1.53	0.58	0.00	0.58
pBAU2 #13	4.67	3.33	3.00	3.67	pBAU2 #13	2.08	0.58	1.00	0.58
pBAU2 #14	4.33	4.33	3.00	3.67	pBAU2 #14	2.52	2.08	0.00	1.15
pBAU2 #15	5.33	2.67	4.67	3.67	pBAU2 #15	1.15	1.15	0.58	1.15
pHOE6 #110/2	3.33	3.33	3.67	2.33	pHOE6 #110/2	2.08	1.15	2.52	0.58

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	1.00	4.00	5.00	5.00	Nt wt	0.00	1.00	0.00	0.00
pBAU2 #13	1.00	1.33	1.00	1.00	pBAU2 #13	0.00	0.58	0.00	0.00
pBAU2 #14	1.00	1.33	1.00	1.33	pBAU2 #14	0.00	0.58	0.00	0.58
pBAU2 #15	1.33	1.00	1.00	1.33	pBAU2 #15	0.58	0.00	0.00	0.58
pHOE6 #110/2	1.00	1.00	1.00	1.00	pHOE6 #110/2	0.00	0.00	0.00	0.00

	Average Rooting Ability					St. Dev. on Rooting Ability			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	3.00	0.00	0.00	0.00	Nt wt	0.00	0.00	0.00	0.00
pBAU2 #13	2.00	1.00	1.00	2.67	pBAU2 #13	1.73	1.73	1.73	0.58
pBAU2 #14	1.33	2.00	0.33	1.67	pBAU2 #14	1.53	1.73	0.58	1.53
pBAU2 #15	3.00	3.00	3.00	1.33	pBAU2 #15	0.00	0.00	0.00	0.58
pHOE6 #110/2	2.00	3.00	3.00	1.00	pHOE6 #110/2	1.73	0.00	0.00	1.00

	Average # Leaves					St. Dev. on # of Leaves			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	5.00	1.67	2.00	2.33	Nt wt	2.65	0.58	0.00	0.58
pBAU2 #13	6.67	3.33	3.33	5.67	pBAU2 #13	1.53	0.58	1.53	2.08
pBAU2 #14	6.67	6.33	3.33	6.33	pBAU2 #14	3.06	4.04	0.58	4.16
pBAU2 #15	7.33	4.67	5.33	3.33	pBAU2 #15	3.21	3.79	3.21	0.58
pHOE6 #110/2	4.00	4.67	5.67	3.67	pHOE6 #110/2	3.00	2.31	2.08	1.15

	Average Level of Necrosis					St. Dev. on Level of Necrosis			
	0 mg/L	5 mg/L	10 mg/L	20 mg/L		0 mg/L	5 mg/L	10 mg/L	20 mg/L
Nt wt	1.00	5.00	5.00	5.00	Nt wt	0.00	0.00	0.00	0.00
pBAU2 #13	1.00	1.33	2.00	1.33	pBAU2 #13	0.00	0.58	1.73	0.58
pBAU2 #14	1.00	1.33	1.00	1.33	pBAU2 #14	0.00	0.58	0.00	0.58
pBAU2 #15	1.33	1.33	1.33	1.67	pBAU2 #15	0.58	0.58	0.58	0.58
pHOE6 #110/2	1.00	1.33	1.00	1.33	pHOE6 #110/2	0.00	0.58	0.00	0.58

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