Establishment of a transformation procedure to study the role of trypsin inhibitors in soybean

By

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DECLARATION

I the undersigned hereby declare that the thesis submitted herewith, for the degree

Magister Scientia, to the University of Pretoria, contains my own independent work.

This has hitherto no been submitted for any degree anywhere else.

Tinyiko Mokoena

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June 2009

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Research Aim and Objectives

The main objective of this study was to reduce the trypsin inhibitor (serine proteinase inhibitor) activity in soybean seeds. This would allow enhancing the nutritional value of soybean. Serine protease inhibitors are anti-nutritional compounds found in soybean feed-cake as a by-product. The inhibitor alters the metabolism of animals when feeding on soy cake which leads to physiological disorders such as pancreatic hypertrophy. In this study, RNAi constructs have been created and transferred into soybean by plant transformation allowing silencing/reducing the expression of serine proteinase inhibitors (Kunitz- and Bowman-Birk-type) in soybean seeds. In particular for silencing/reducing gene expression, the conserved regions of these inhibitors have been targeted.

The specific objectives of this study were:

❖ Identification of known gene sequences for soybean serine proteinase inhibitors by using a database search and identification of conserved regions useful for RNAi silencing/suppressing serine proteinase inhibitor expression.

❖ Design and production of a vector allowing *gus* gene expression in soybean for monitoring soybean transformation.

❖ Design and production of RNAi vectors allowing silencing/suppressing serine proteinase inhibitor expression.

❖ Isolation of a seed specific promoter allowing expression of the *gus* gene and of RNAi constructs to silence/suppress serine proteinase inhibitor expression.

❖ Development of tissue culture protocol to facilitate transformation of soybean.

ABSTRACT

The major serine proteinase inhibitors Kunitz and Bowman-Birk-type trypsin are key anti-nutrients responsible for the low nutritional value of raw soy cake, the by product of oil expression from soybean. Traditionally, proteinase inhibitors are eliminated from soy cake through intensive heating, which is highly costly. The long term goal is to generate soybean seeds devoid of trypsin inhibitors through tissue culture and genetic modification of soybean. The RNAi technology has been selected in this study as a technique for down-regulation or silencing these two major serine trypsin inhibitors. Conserved regions, which have been identified by searching NCBI and EMBL database, were targeted for down regulation. Seed specific promoters were also isolated to drive the expression of hairpin constructs designed to down-regulate selected conserved regions of the inhibitors in soybean seeds. RNAi silencing constructs were designed for use in soybean transformation. Ultimately, a tissue culture and transformation protocol for a local soybean variety PAN 512 was established for transformation with two designed RNAi constructs. Suitability of selected promoters was tested by attaching promoters to the *gus* gene and evaluating specificity of seed expression after soybean transformation using the Agrobacterium tumefaciens strain EHA101. Future work will focus on further optimisation of the transformation protocol and generation of transformed plants carrying the designed silencing vectors.

THESIS COMPOSITION

Chapter 1 of this thesis explains briefly the role of proteinase inhibitors as antinutritional factors in plants. The significance of proteinase inhibitors in nutrition, health, is also discussed, and we explain the genetic relatedness of Kunitz and Bowman-Birk families of inhibitors. The last part of this chapter reviews literature on soybean transformation and RNAi technology for gene silencing. **Chapter 2** embodies the materials and methods used in this study. The use of various molecular biology techniques such as RNA isolation, genomic DNA extraction, PCR, Cloning, Genome walking, cDNA synthesis, designing of RNAi constructs, plant transformation mediated by Agrobacterium and Histochemical Gus Assay is outlined, **Chapter 3** presents the results obtained on identification of the major soybean serine proteinase inhibitors using database research, determination of which of the antinutritional factors could be silenced via a genetic approach. The isolation of seed specific promoters that will drive the expression of selected conserved regions of trypsin inhibitor genes in seeds and verifying its seed specific expression using the Gus marker gene, designing and construction of silensing vectors and the development of tissue culture protocol to facilitate genetic modification of soybean cultivars are outlined. **Chapter 4** presents a discussion of the results obtained related to the literature. In the annex recipes of media and solutions used during the study are given and finally, a list of literature used and cited in this thesis is provided under References.

ACKNOWLEGDEMENT

I would like to thank all people who have helped and inspired me during my Masters study.

I especially want to thank my supervisors, Dr. Rachel Chikwamba, and Prof. Karl Kunert for their guidance and support during my study. Their perpetual energy and enthusiasm in research had motivated me. In addition, they were always accessible and willing to help me in research. As a result, research life became smooth and rewarding for me.

Last but not least, thanks be to God for my life through all tests in the past two years. You have made my life more bountiful. May Your name be exalted, honoured, and glorified.

DEDICATION

This thesis is dedicated to my family especially, my grandmother who has supported me all the way since the beginning of my studies.

ABBREVIATION AND SYMBOLS

%	Percentage
µg	Microgram
µl	Microlitre
bp	Base pair
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
T-DNA	Transfere Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
E. coli	Escherichia coli
h	Hours
kb	Kilo base pair
L	Litre
LB	Luria broth
M	Molar
Min	Minute
mL	Millilitre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
ºC	Degree Celcius

PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Rotations per minute
sec	Seconds
HCl	Hydrochloric acid
GA_3	Gibberellic acid
pH	Potenz hydrogen
mg	Milligrams
MES	2-(N-morpholino) ethanesulfonic acid
NaEDTA	Sodium Ethylenediamine tetra-acetic acid
BAP	Benzylaminopurine
B5	Pantothenic acid
^oC	Degree Celsius
μF	Micro Farads
Ω	Ohm
UV	Ultraviolet
OD	Optical density
nm	Nano metre
$CaCl_2$	Calcium Chloride
$MgCl_2$	Magnesium Chloride
NaAc	Sodium Acetate
dH_2O	Distilled water

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DNA marker, 1-4 represent digested plasmid with EcoR1 restriction enzyme, plasmids isolated from different colonies. Plasmid from *E. coli* colony 4 has the correct insert with the expected fragment size of 312 bp for KTi3 and 212 bp for BBi is in a box since it faint.

expected size 809 bp. after digestion. (B) Lanes 1-4 represents pSK-C-II/β-Conglycin-Ntcp2-vsp-KTi3-BBi-KTi-1. this is an expected fragment of 809 bp (C) 1-4 represents IR KTi3-BBi-KTi1 amplification product with an expected size 809 bp. (D) 1-6 represents pSK- C-II/β-Conglycinin- KTi3-BBi-KTi -Ntcp2- IR KTi3-BBi-KTi-1-Tvsp. This is an expected fragment of 809 bp.

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CHAPTER 1

INTRODUCTION

1.1   Importance of soybean as livestock feed

Soybean [*Glycine max* (L.) Merrill, family Leguminose, subfamily papilionoidae] originated in Eastern Asia, probably in north and central China. (Baldwin and Fulmer, 1985) Soybean is an important source of high quality, inexpensive protein and oil for both humans and animals, especially in the formulation for non-ruminants, such as poultry, swine and cattle (Baldwin and Fulmer, 1985). Soybeans contain approximately 40% protein and 20% oil (Krober and Cartter, 1962; Nielsen, 1996; Krishnan, 2000). In spite of the relatively high concentration, the quality of soybean protein is not optimal for human and animal nutrition (Friedman and Brandon, 2001). Soybeans contain significant amounts of anti-nutrition factors, such as protease inhibitors, lectins, alkoloids and phytic acid (Friedman and Brandon, 2001).

1.2   Anti-nutrients in soybean

1.2.1   Lectins

Lectins in soybeans are believed to be an antinutritional factor (Schulze *et al*., 1995) that depresses growth rate in young animals (Pusztai *et al.,* 1990). Lectins are glycoproteins that have the ability to bind to cellular surfaces via specific oligosaccharides or glycopeptides (Oliveira *et al*., 1989) and have a relatively high binding affinity to small intestinal epithelium (Pusztai, 1991). As a result, lectins can produce structural changes in the intestinal epithelium and resist gut proteolysis (Pusztai *et al*., 1990). These changes can result in impairment of brush border continuity and ulceration of villi (Oliveira *et al*., 1989), which may result in increased endogenous nitrogen losses (Oliveira and Sgarbierri, 1986; Schulze *et al*., 1995) and depressed growth rate in young animals (Pusztai *et al*., 1990). Thus, the growth depressant effect of lectins is believed to be due primarily to their damaging impact

on intestinal enterocytes (Pusztai, *et al*., 1979; Lorenzsonn and Olsen, 1982) and through appetite depression (Liener, 1986). Research has shown that the detrimental effects of lectins may be lessened with proper heat processing of conventional soybeans (Higuchi *et al*., 1984).The lectins in soybeans are tetrameric glycoproteins that have a specific affinity to terminal N-acetyl-D-glucosamine and D-galactose (Schulze *et al*., 1995). These lectins were originally referred to as hemagglutinating factor or soyin, and it was estimated that they accounted for one-half of the growth inhibition produced by raw soybeans fed to rats (Liener, 1953).

1.2.2   Phytic acid

Phytic acid is a compound found in many cereals and legumes that allow the plants to store phosphorus. Phytic acid is known to bind with proteins in feeds making the proteins unavailable to the animal (Zhou *et al.,* 1981). In soybeans, phosphorus is stored in the form of phytic acid. Phytic acid can not be digested by animals. Therefore most of the phosphorus in soybeans cannot be used by animals for their nutrition and is excreted. This is a major source of phosphorus pollution in water systems, particularly in places with large concentrations of livestock that have soybean meal in their diets (Zhou *et al.,* 1981).

1.2.3   Proteinase inhibitors

Proteinases are classified according to their catalytic mechanisms. Four mechanistic classes have been recognized by the International Union of Biochemistry and Molecular Biology (IUBMB): the serine proteinases, the cysteine proteinases, the aspartic proteinases, and the metallo proteinases. This classification by catalytic types has been extended by a classification by families based on the evolutionary

relationships of proteases (Rawlings and Barret, 1993). The main focus of this study is the serine proteinase inhibitor as they are the major antinutritional factors in soybean. Soybeans contain two types of serine proteinase inhibitors, Kunitz-type trypsin inhibitor (KTi) and Bowman-Birk trypsin inhibitor (KTi) both of which inhibit chymotrypsin. These protease inhibitors account for approximately 6% of the total soybean seed protein (Rackis *et al.*, 1986).

1.2.3.1   *Serine proteinase inhibitors*

This study is targeting serine proteinase inhibitors because they can alter the body metabolism of animals and humans consuming raw soy, and exert a negative impact on the nutritional quality of seed protein (Friedman and Brandon, 2001). Serine proteinase inhibitors have a strong affinity for human digestive enzymes (Weder, 1986). Studies by Liener *et al*. (1953) revealed that in humans, BBI induced 2-3- fold stimulation in the production of the enzymes trypsin, chymotrypsin, elastase, and amylase. Evidently, BBi caused a negative feedback inhibition of pancreatic exocrine secretion in humans (Gallaher and. Schneeman, 1986; Holm *et al.,* 1992; Toskes, 1986). Feeding studies have demonstrated that proteinase inhibitors can cause pancreatic hypertrophy or hyperplasia in rats (Booth *et al*., 1960; Myers *et al.,* 1991; Roedbuck *et al.,* 1987). Similar effects on the pancreas have also been reported in chickens and growing guinea pigs (Friedman and Brandon, 2001; Hasdai *et al.,* 1989; Schneeman and Gallaher, 1986). It has also been suggested that dietary trypsin inhibitor induces the chronic release of cholecystokinin from the intestinal wall into the blood stream, and in doing so mediates pancreatic enlargement which results to death (Green *et al*., 1972).

1.2.4   Serine proteinase inhibitors in plants

The main function of plant proteinase inhibitors is thought to be in plant defense (Mosolov *et al.*, 2001) and regulation of endogenous proteinases, they may also function as storage proteins (Mosolov *et al.*, 2001; Birk, 2003; Shewry, 2003). The possible role of proteinase inhibitors in plant protection was investigated by Mickel and Standish (1947) who observed that the larvae of certain insects were unable to develop normally on soybean products (Haq *et al.*, 2004). They are of interest as potential sources of resistance against pests and pathogens in transgenic plants and as drugs with antiviral properties, as well as in providing markers for studies of plant diversity and evolution (Konarev *et al.*, 2002; Lawrence and Koundal, 2002; Korsinczky *et al.*, 2004). It is well established that proteinase inhibitors protects plants from insects and other pathogens.

In very early research work, trypsin inhibitors in soybean have been already shown to be toxic to the larvae of flour beetle, *Tribolium cofusum* (Lipke *et al.*, 1954). For use in pest control in plants, proteinase inhibitors encoded by single gene have practical advantages over genes encoding for complex pathways for example by transferring a single defensive gene from one plant species to another and expressing it under the regulation of their own wound inducible or constitutive promoters may imparting resistance against insect pests, such as coleopterans (*Diabrotica*), orthoptera (*Locust*) and lepidopterans (*Heliothis*) (Boulter, 1993). Transfer of an alfalfa Bowman-Birk inhibitors gene to tobacco plants also showed directly that this proteinase inhibitor can function as an endogenous insecticide (Hilder *et al.*, 1986). In addition to insect pests, proteinase inhibitors also have great potential in generating transgenic plants with enhanced resistance to other pathogens, e.g. nematodes, fungi, bacteria, and viruses,

the survival and /or invasion of which require proteolytic activities. Plant proteinase inhibitors are known to confer natural as well as engineered protection against nematode attack (Atkinson *et al.*, 2003; Cai *et al.*, 2003; McPhode and Harrison, 2001).

1.2.5 Serine proteinase inhibitor characteristics

1.2.5.1 *Kunitz-type inhibitor*

The Kunitz-type inhibitor gene family contains 12 gene members; many of them are closely linked on the chromosome in tandem pairs. The three Kunitz-type trypsin inhibitor genes are designated as KTi1, KTi2, and KTi3 are expressed during embryogenesis and in mature plants. The KTi 3 represents 8 gene members of trypsin inhibitors which share high sequence similarities of about 97-98%. The KTi1 and KTi 2 genes have nearly identical nucleotide sequences. For example, the coding regions of these genes differ by only 18 out of 608 nucleotide which indicates approximately a 97% sequence similarity (Figure 1.1). By contrast, the KTi3 gene has diverged by 20% from the KTi1 and KTi2 genes, and encodes the prominent Kunitz-type trypsin inhibitor found in soybean seeds. The KTi3 has the highest expression level during embryogenesis, and is also represented in the mRNA of the leaf (Walling *et al.*, 1986).
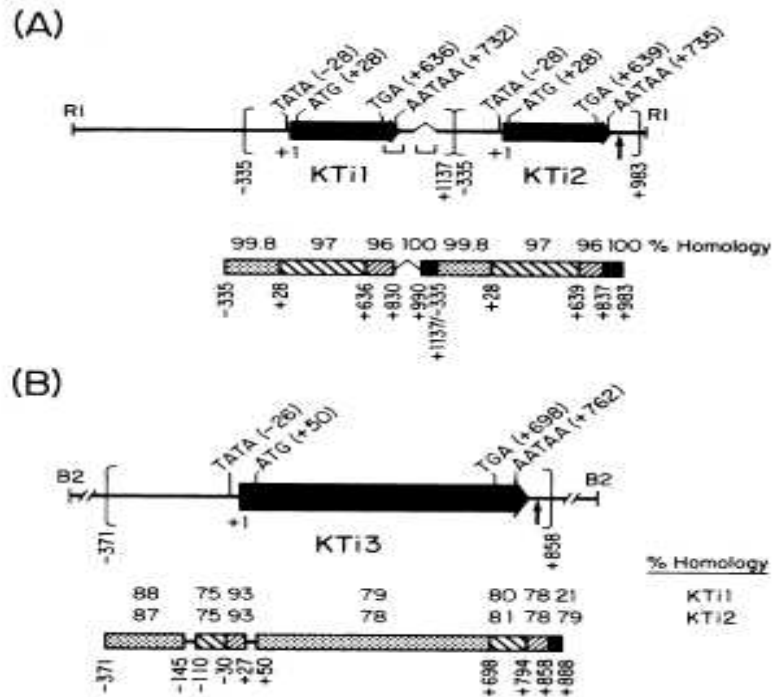
Figure 1.1: Kunitz-type trypsin inhibitor gene similarities. (A) KTi1 and KTi2 gene sequence comparison. Boxes filled with similar patterns indicate compared gene regions. Percentage homology and nucleotide reference points are shown above and below the designated gene region, respectively. (B) KTi1, KTi2, and KTi3 gene sequence comparisons. Percentage homology of KTi3 gene regions with corresponding KTi1 or KTi2 sequences is shown above the designated region. The 5' and 3' ends of compared gene regions are shown below the designated gene region. (Jofuku *et al.*, 1989)

KTi1 and KTi3 are expressed at different levels during embryogenesis are represented in leaf, root and stem mRNAs (Walling *et al.*, 1986). Analysis of the winged bean Kunitz-type chymotrypsin inhibitor (WCI) protein shows that it is encoded by a multi-gene family that includes four putative inhibitor coding genes and three pseudogenes. It is believed that a 5' proximal insertion occurred after duplication of the ancestral

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WCI gene (Habu *et al.,* 1997). According to Habu *et al.* (1997) several gene conversion events contributed to the evolution of this gene family. The 5' end of the pseudo-gene. The WCI proteinase inhibitor contains frame shift mutations. This indicates that the 5' region of the inhibitor gene may have spontaneously acquired new regulatory sequences (Habu *et al.,* 1997). Gene conversion is a relatively frequent event and the homology between the WCI-P1 and the other inhibitor genes WCI-3a/b is disrupted at a 5' proximal site by remnants of an inserted sequence. The WCI inhibitor gene thus appears to be a possible intermediate that could have been converted into a new functional gene with a distinct pattern of expression by a single gene-conversion event (Habu *et al.*, 1997)

1.2.5.2   *Bowman Birk-type inhibitor*

The Bowman Birk-type serine proteinase inhibitor (BBi) and proteinase inhibitor I to IV comprise a closely related group of proteinase inhibitors. They contain 20% sulphur amino acids which are the most limiting of the essential amino acids in soybean seeds. Both groups have molecular weight less than 10,000 (Hwang *et al.*, 1977) and comparison of their amino acid sequence reveals that the BBi and P1 I-IV are about 70% homologous. The amino acids sequences of PI I-IV are nearly identical and differ primarily in the length of their N terminal (Hwang *et al.,* 1977; Odani and Ikenaka, 1978).

BBIs are classified as double-headed inhibitors because each contains two reactive site domains. BBis has both a trypsin (lys-Ser) and a chymotrypsin (Leu-Ser) inhibitory site (Birk, 1961), whereas PI I-IV have two trypsin (Arg-Ser) inhibitory sites (Hwang *et al*., 1977). The sequences of the amino acids surrounding the reactive

sites both within the same polypeptide and between the BBi and PI I-IV are homologous. This homology suggests that the BBi and PI I-IV genes may have evolved from a common ancestral moo-valent inhibitor (Tan and Stevens, 1971). The occurrence of multiple genes, low molecular weight protease inhibitors among other members of the legume family (Chu and Chi, 1965; Jones *et al*., 1963; Wilson and Laskowski, 1973) as well as distantly related plants (Bryant *et al*., 1976; Kanamori *et al.*, 1976) suggests that the genes may have been conserved throughout the evolution of several plant species (Bryant *et al*., 1976; Kanamori *et al.*, 1976).

The BBi and PI I-IV do not appear to be expressed in any plant tissue other than in the developing seed (Hwang *et al.,* 1978). The proteins are rapidly released within the first 8 hours of imbibition. The kinetics of their release suggests that the inhibitors may be eluted from a barrier-free pool. Consistent with this suggestion is the observation that some BBis are found in intercellular spaces of cotyledons in un-germinated seeds, but are absent from this location in four day–old seedlings (Horisberger and Tacchini-Vonlanthen, 1983).

BBi double-headed inhibitors are assumed to have risen by duplication of an ancestral single headed protease inhibitor gene. These subsequently diverged into different classes of inhibitors, for example, trypsin/trypsin (T/T), trypsin/chymotrypsin (T/C) and trypsin/elastase (T/E) inhibitors (Odani *et al*., 1983). The mature proteins comprise a readily identifiable 'core' region, covering the invariant cysteine residues and active centre serines. These are bound by highly variable amino and carboxy-terminal regions. There is a core region of 62 amino acids, both between and within the different classes of inhibitor in soybean (Odani and Ikenaka, 1976). The average

number of amino acid replacements in the core region shows, using all pair-wise comparisons, that the differences between the various classes of inhibitor within a species (around 16.5/62 residues) are much greater within a class than between species (around 11/62 residues). Considering that 18 of the residues in this region are obligatorily invariant for proteins to be classified as BBis, these are very high rates of amino acid substitutions.

1.2.6   Expression of inhibitors in soybean

The Kunitz-type and Bowman-Birk-type gene family are highly regulated during the soybean life cycle (Goldberg *et al*., 1981; Walling and Goldberg, 1986; Jofuku *et al*., 1989). These genes are expressed at precise times during embryogenesis. They have different expression levels in the embryo cotyledon and axis, and are either inactive or expressed at low levels in mature plant organ systems (Goldberg *et al*., 1981; Walling *et al*., 1986). Both transcriptional and posttranscriptional processes regulate Kunitz trypsin inhibitor gene expression (Walling *et al*., 1986).

1.3   Strategies for reducing anti-nutrients

1.3.1   Heat strategy

Inhibitors of digestive enzymes adversely affect the nutritional quality of soy protein. To improve nutritional quality, inhibitors are generally inactivated by heat treatment during food processing or are partially removed by fraction. However, heat treatment has limitations. Heat treatment is expensive, the more protracted heating required to destroy all inhibitor activity the more damage the nutritive value of soy proteins, by destroying or changing structure of certain amino acids, such as cysteine, methionine,

and lysine. Most of the time the inhibitors retain their activity after heat treatment (Friedman and Brandon, 2001).

1.3.2 <u>Plant breeding</u>

Breeding strategies have also been used eliminate inhibitors from soybean. Breeding work using the wild soybean types as donors of the low serine proteinase inhibitor have been reported, this has resulted in the development of soybean cultivars with lower serine proteinase content. To date, there is no soybean variety devoid of these anti-nutrition factors that is commercially grown. This has been due to the presence of multiple gene families coding for both BBi and KTi, and possibly other negative attributes in these lines as a result of linkage drag on poor pest resistance.

1.4 Silencing of inhibitor expression

RNAi technology might be used to effectively silence multigene families of the trypsin inhibitors since it mechanism targets conserved regions within members of the families in plants (Waterhouse *et al.,* 1998; Chuang and Meyerowitz, 2000; Azevedo *et al.,* 2002; Hayama *et al.,* 2003; Morioh *et al.,* 2005). The term 'RNA world' was first coined to describe a hypothetical stage in the evolution of life some four billion years ago when RNA may have been the genetic material and catalyst for emerging life on earth (Timmons and Fire, 1996). This original RNA world, if it ever existed, is long gone. But this insight deals with a process that reflects an RNA world that is alive and thriving within our cells-RNA silencing or RNA interference (RNAi). Many organism when exposed to foreign genetic material (RNA or DNA), mount highly specific counter attacks to silence the invading nucleic acid sequences before these sequences can integrate into the host genome or subvert cellular processes. At the

heart of this sequence directed immunity mechanism is double stranded RNA (dsRNA) (Timmons and Fire, 1996).

dsRNA does more than help to defend cells against foreign nucleic acids. It also guides endogenous development gene regulation and can even control the modification of cellular DNA and associated chromatin (Timmons and Fire, 1996). dsRNA was thought to be a non-specific silencing agent that triggers a general destruction of messenger RNAs and the complete suppression of protein translation in mammalian cells (Proud, 1995; Williams, 1997). Secondly dsRNA is energetically stable and inherently incapable of further specific Watson and Crick base pairing. So a model in which dsRNA activates sequence-specific silencing implies the existence of cellular mechanisms for unwinding the dsRNA and promoting the search for complementary base pairing partners among the vast pool of cellular nucleic-acid sequences.

RNA silencing is a form of gene suppression that occurs at the level of RNA and includes post-transcriptional gene silencing in plants and fungi. Double-stranded (dsRNA) and short interfering RNA (siRNA) are two types of RNA that play key roles in this phenomenon (Fire *et al.,* 1998; Waterhouse *et al*., 1998; Hamilton and Baulcombe, 1999). Extensive genetic and biochemical studies in various species have yielded a model of RNA silencing in which the trigger is dsRNA (Waterhouse *et al*., 1998).

From the double stranded genomic DNA, only one strand is transcribed and translated to protein (Timmons and Fire, 1996). The other strand serves as a back up copy that a

cell can use to repair genetic damage. However, in many cases, such as when viruses infect cells or when transposons and transgenes integrate into the host genome, the antisense strand also transcribes giving rise to double stranded RNA. Various species have yielded a model of RNA silencing in which trigger dsRNA, either introduced into the cell or transcribed from transgenes, is cleaved into small siRNA of 21 to 26 nucleotides (nt) by an RNAse termed Dicer (Figure 1.3)
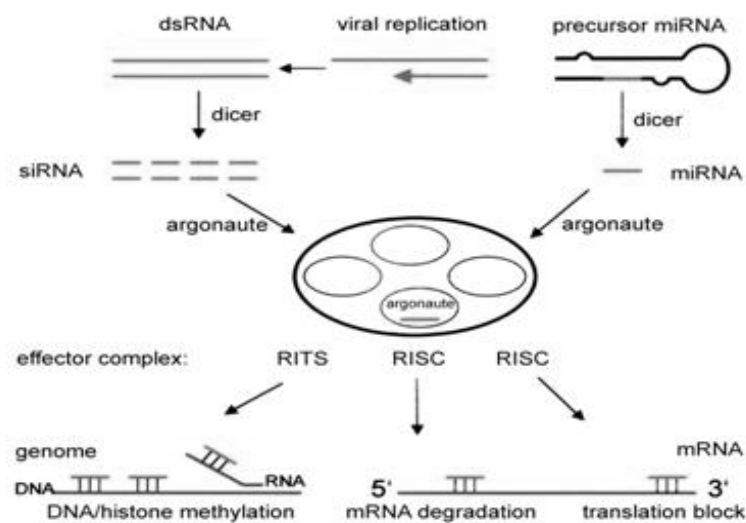
Figure1.3: The enzyme dicer trims double stranded RNA, to form small interfering RNA or microRNA. These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation (Waterhouse *et al*., 1998; Bernstein *et al.,* 2001).

The siRNA is incorporated into an RNA-induced silencing complex (RISC) to be associated with the target mRNAs (Hammond *et al.,* 2000), activating RISC functions to degrade target mRNAs and suppress gene expression at various levels (Matzke *et al*., 2001; Waterhouse *et al*., 2001; Hannon, 2002; Plasterk, 2002; Meister and Tuschl, 2004). Features of RNAi include:

- RNAi is a sequence-specific and thus can target, requiring only a few transformants per target gene.

- RNAi can be controlled in a tissue-specific or time-dependent manner (Alonso *et al.,* 2006).

- RNAi is dominant, so phenotype can observed in the T1 generation (Waterhouse *et al.,* 2001).

- RNAi often leads to partial knockdown and to a range of phenotypes of differing severity, this facilitate the study of essential genes whose inactivation would lead to lethality or extremely severe pleiotropic in a tissue-specific or time dependent manner (Smith *et al.*, 2000).

- RNAi can be quickly and easily used in a wide range of genotypes or even species, whereas insertion mutant collections are limited to just a few due to the effort involved (Matzke *et al.,*2001).

- RNAi can be used to reduce the expression of several related genes in parallel by targeting conserved regions of the genes, facilitating the study of redundant gene functions. (Waterhouse *et al.,* 1998; Chuang and Meyerowitz, 2000; Azevedo *et al.*, 2002; Hayama *et al.*, 2003; Morioh *et al.,* 2005).

1.5   Promoters for RNAi

The promoter sequence of β-Conglycinin might be useful tool to drive gene silencing of serine proteinase inhibitors in soybean seeds. β-Conglycinin is a major component of seed storage proteins in soybean. Expression of these genes is spatial regulated in a stringent manner and occurs during seed development. β-Conglycinin, a major component of seed storage proteins in soybean (Glycine max), is a trimetric protein

composed of various combinations of three subunits: α, ά and β. These proteins occupy up to 30% of the total seed protein in soybean (Thanh and Shibasaki, 1978; Higgins, 1984). The genes for these proteins appear to be transcribed in a coordinated but not identical manner during seed development. Regulatory elements involved in the transcription of the ά and β subunit genes have been studied extensively by means of a reporter gene assay in transgenic plants and a binding assay using nuclear extracts (Chen *et al*., 1989; Allen *et al*., 1989; Lessard *et al.,* 1991; Chamberland *et al*., 1992; Fujiwara and Beachy, 1994). Early studies on the ά and β subunit genes of β-conglycinin suggested an involvement of protein factors, designated SEF3 and/or SEF4, and their binding sites located within several hundred bp upstream of these genes in the control of seed specific gene expression (Allen *et al*., 1989; *Lessard et al*., 1991).

1.6 Soybean transformation

There are two modes of DNA delivery are currently utilized to transform soybean. One is particle bombardment of embryogenic tissue (Trick *et al.,* 1998; Maughan *et al.,* 1999; Droste *et al.,* 2002). This technique often requires a prolonged tissue culture period to prepare target tissues and yields complex insertion patterns of transgenes into the plant genome. The other method involves *Agrobacterium*-mediated transformation of plant tissue (Olhoft *et al*., 2003; Zhang *et al*., 1999; Paz *et al*., 2005). *Agrobacterium*-mediated transformation offers several advantages, such as defined integration of transgenes, preferential integration of transgenes, preferential integration into transcriptionally active chromosomal regions, and potentially a single or low copy number with relatively rare rearrangement (Birch *et al*., 1998).

Fast and efficient systems of transformation and regeneration of transgenic plants are necessary to successfully use RNAi constructs for plants functional genomics. However, soybeans are still difficult to transform compare with other plants and efficient methods are required for genetic transformation (Trick and Finer, 1998). The difficulties of soybean transformation include poor sensitivity to *Agrobacterium tumefanciens* and absence of a regeneration system adapted to *Agrobacterium*-mediated transformation. Therefore much effort has been made to enhance the efficiency of soybean transformation, including the utilization of different explants (Hinchee, 1988; Chee *et al*., 1989; Parrott *et al.,* 1989; McCabe and Christou, 1993; Stewart *et al.,* 1996) and improvement of transformation conditions such as changes of hormone concentration and supplement of silver nitrate and antioxidants into culture medium (Olhoft *et al*., 2001; Olhoft and Somers, 2001).

1.6.1 Cotyledonary node system

The most commonly used method for soybean transformation is the cotyledonary node system. This system is based on *A. tumefaciens*-mediated transferred DNA (T-DNA) delivery of genes into regenerated cells of the axillary meristem of the cotyledonary node (Hinchee, 1988; Zhang *et al*., 1999; Clemente *et al*., 2000; Liu *et al*., 2004). After inoculation with *Agrobacterim*, the meristem of the cotyledonary node regenerates adventious shoots. Recently, this method has been improved by adding a mixture of thiol compounds, such as L-cysteine, dithiothreitol, and sodium thiosulfate, into the co-cultivation medium. These compounds are efficient in decreasing the necrosis during the cotyledonary node transformation and dramatically increase the number of cells transiently transformed with T-DNA (Olhoft *et al.,* 2001; Olhoft and Somers, 2001). However transformation frequency under improved

conditions is still low compared to frequencies found in other plant species. Moreover, the cotyledonary node transformation requires making precise cuts on the explants for effective transformation and regeneration (Paz *et al*., 2006). The ideal transformation system should result in:

- High quality and fertility of transformed plants.
- Integration of low numbers of copies of genes into the plant genome.
- Transfer of pieces of DNA with defined ends with minimal rearrangements.

1.6.2   *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation has been selected as the method of choice for delivery of RNAi inverted repeats into the soybean genome. *Agrobacterium* is a soil-borne bacterium that, in the presence of a wounded plant, moves toward the plant, attaches itself to the wound site, and proceeds to transform the cell. The sugars and phenolic compounds exuded by the wounded plant not only signal the pathogenic opportunity to the bacterium but also induce transcription of the virulence genes. These virulence genes are located on a specific plasmid known as the tumour–inducing (Ti) plasmid, which also contains the transferred DNA (T-DNA). Virulence proteins have roles ranging from transcriptional activation to T-DNA processing and export, with certain proteins also having a function in the host. *Agrobacterium* has evolved to transfer the T-DNA, which codes for plant hormone producing enzymes that stimulate growth of a tumour and metabolic enzymes responsible for producing opines, metabolizable only by *Agrobacterium*. The resultant crown gall is a microcosm where the bacteria can thrive (Zhang *et al*., 1999).

Many features of T-DNA and the nature of its delivery and integration have made it an invaluable tool in plant biotechnology. The actual transfer and integration do not rely on genes encoded by the T-DNA itself. In fact, the only necessary sequence on the T-DNA are the border sequences (right and left) that delineate it. Between the border sequences, genes of scientific interest can be cloned, allowing for great flexibility in what is transferred to the host cells. At the chromosomal level, fewer T-DNA s were found at the centromeric region, which is consistent with the observation that there is a correlation between T-DNA insertion and gene density. Specific preferences within a gene were also analyzed, with promoter and coding exons making up nearly 50% of all insertion sites (Brunaud *et al.*, 2002). This result may be skewed, however, by the selection of kanamycin-resistant plantlets in the T1 generation, resulting in the analysis of insertion sites with transcriptionally active T-DNA. These observations are consistent with a study that looked specifically at the conservation of the T-DNA borders and the genomic sequences after integration (Brunaud *et al.*, 2002). The authors concluded that there are micro-similarities involved in the integration of both the right and left borders. These similarities, however, need only occur over a stretch of 3 to 5 bp and can be between any T-DNA and genomic sequence. This allows T-DNA to integrate at any locus in the genome.

CHAPTER 2

MATERIAL AND METHODS

2.1   **Construct preparation**

2.1.2   Materials

2.1.1.1   Genomic DNA preparation

Genomic DNA was isolated from plant material using the using the Qaigen DNA Easy mini kit according to the manufacture's instruction (Qaigen, Germany) and quantified with the nano-drop technique using a spectrophotometer (ND-1000 spectrophotometer, Inqaba biotech, SA).

2.1.1.2   cDNA synthesis

Total RNA was extracted from developing soybean seeds and pods of cultivar Williams using a RNA isolation kit (Roche, Switzerland). Double-stranded cDNA was then synthesised using a cDNA synthesised kit (Roche, Switzerland). All DNA primers for gene amplification were designed using the web-based PRIMER3 software (Rozen and Skaletsky 2000). Isolation of plasmids from bacterial cells was done using the Qaiprep kit (QAIGEN, Germany).

2.1.1.3   PCR product purification

All amplified PCR products were separated by eletrophoresis on a 1.2% agarose gel and visualized with ethidium bromide. PCR products were purified using a DNA band purification kit according to manufacture's instruction (GE Healthcare, US) and purified DNA was T/A-cloned into the vector pGEM-T Easy according to the manufacture's instruction (Promega, UK) and PCR amplification reaction was carried out using a GeneAmp 2720 Thermal cycler (Inqaba, SA).

2.1.2   Bacterial transformation

2.1.2.1 *E. coli transformation*

For isolation of the DNA fragment from the agarose gel, the DNA fragment with the correct size was cut from the agarose gel using a sterile blade. The fragment was purified using a High Pure PCR product Purification kit (Roche, Switzerland). The linearized pGem-T Easy vector was purified after digestion with the phenol/chloroform method according to Marniatis *et al.*, 1986. Recirculation of pGEM-T Easy vector by removing 5′ phosphates from both DNA ends by alkaline phosphatase treatment Maniatis *et al.*, 1986. Ligation of the cut pGEM-T Easy vector with the purified insert was carried in a reaction mixture (25 μl) containing insert, linearized pGem-T Easy vector, 1 μl of ligase (Fermantas, SA) and 2.5 μl of ligase buffer. The reaction was incubated at 16ºC for 16 hrs to facilitate ligation of insert into pGem-T Easy vector.

2.1.2.2 DNA fragment cloning

To insert the ligated plasmid into E *coli*, a bacterial transformation procedure was carried out to multiply the plasmid within a bacterial cell. For transformation, commercially available competent *E.coli* cells (Roche, Switzerland) were removed from -80ºC freezer and placed on ice for 10 min to defrost. Competent cells (50μl) were mixed with 5 μl of the ligation reaction mixture and incubated on ice for 30 min. the mixture was exposed for 30 sec to a 42 ºC heat shock treatment in the presence of 0.1M $CaCl_2$. This treatment results in wounding of the bacterial membrane allowing the uptake of ligated DNA into bacterial cells. After heat shock, the cells were place on ice for 2 min, after which 1 ml of SOC medium (20 g bactotryptone, 5 g bacto yeast, 0.5 NaCl and 20 ml of 1 M glucose solution) was added to the sample and

shaken at 200 rpm at 37ºC for 60 min in a bench-top shaker. A cell suspension (20 µl) was streaked out onto a LB plate containing 50µg/ml kanamycin; 2.5 Mm of IPTG (Isopropyl-β-D-thiogalactoside) and 1 Mm X-gal (5-bromo-4 chloro-3indolyl-β-D galactoside) and plates were incubated overnight at 37ºC. The plasmid pGem-T Easy contains the LacZ gene which codes for galactosidase which converts X-Gal into a colored blue product. In order for the gene to be actively transcribed to produce and for the enzyme, to be produced, the activator called IPGT has to be added. Within the LacZ gene there is a multiple cloning site and any insert cloned into the multiple cloning site will disrupt expression and activity of the LacZ gene, which prevents the formation of the blue colour product (Rubens *et al.,* 1998). Plasmid containing pGem-T Easy cells containing the cloned insert were identified by blue/white selection. White colonies were screened by growing each colony in 5 ml liquid LB medium with selection antibiotic carbenicillin (50mg/ml) added for plasmid carrying cells in an incubator at 37˚C overnight.

2.1.2.3 Dephosphorylation

The binary vector digested with Sma1 restriction enzyme was dephosphorylated in the 50 µl reaction mixture containing 30µl DNA solution, 5 µl 10X reaction buffer, 1 µl Calf Intestine Alkaline phosphatase. The reaction mixture was incubated at 37ºC for 30min. then ran about 50 ng in the agarose gel to check if the dephosphorylayed vector was still there.

2.1.2.4 *Agrobacterium transformation*

Electroporation was used to transfer plasmid DNA into *Agrobacterium* cells. For transformation, 2 µl plasmid DNA (130ng DNA) was added to 40 µl EHA101 competent cells, the mixture was gently shaken, transferred to a plastic cuvette and placed on ice. The electroporation machine was set to 140 Ω and a voltage of 1.44 kV. The plastic cuvette was placed into the electroporation chamber and a pulse of 5ms was applied to transfer DNA into the cells. Immediately after electroporation 1 ml of YEP medium was added to the cuvette. The mixture was subsequently transferred to a 2 ml Eppendorf tube and the tube was incubated at room temperature for 1 hr with agitation. An aliquote was then plated onto a LB plate containing the following antibiotics chloramphenicol 25 l^{-1}, kanamycin 20 l^{-1}, spectinomycin 100 l^{-1}, rifampicin 20mg l^{-1} for selection of transformed *Agrobacterium* cells. Plates were incubated at 28°C for 2 days, and colonies that appeared were placed for plasmids isolation and analyses.

2.1.3   Gene amplification and cloning

2.1.3.1 *Amplification of KTI3, BBI and KTI1 from cDNA*

The coding region of the KTi3 gene was amplified by PCR using the forward 5′-CCCAAAAATGAAGAGCACCA-3′ and the reverse 5′-CTTTGCATTCGGCCTTAGAA-3′ primer pair. The coding region of the BBi gene was amplified using the forward 5′-ATGGTGGTGCTAAAGGTGTG-3′ and reverse 5′-TTAGTTTTCCTTGTCATCCTC-3′ primer pair. The KTi1 conserved gene region (281bp) was amplified using the forward 5′-CGGCATGCCTACTGAGTG-3′ and reverse 5′-TCACGCTTCATGCAGTTGAT-3′ primer pair. The PCR amplification reaction contained 50 ng of template cDNA, 0.2 µM of each primer, 5 µl of dNTPs (2

mM), 5 μl of 10 X pfu polymerase reaction buffer and 0.5 μl of *pfu polymerase* (Fermentas, South Africa) in a 50 μl total reaction mixture. The PCR amplification reaction was carried out at an initial time 94˚C for 4 min, for DNA denaturation, 58˚C for 1 min (annealing), 72˚C for 1 min (extension) and a final DNA extension at 72˚C for 10 min in for 35 cycles. The amplified PCR products were separated by electrophoresis on a 1.2% agarose gel and visualized with ethidium bromide and purified DNA was then T/A- cloned into the vector pGEM-T Easy vector. Plasmids were finally isolated from resulting *E. coli* colonies and sequenced to identify correct DNA insertion into the plasmid.

2.1.3.2 *Amplification of KTi1*

The conserved gene region of KTi1 was amplified by introducing a BglII restriction site on the forward BglII-KTi1 5'-GAAGATCTTCCGGCATGCCTACTGAGTG-3' and a BamHI site on the reverse BamH1-KTi1 5'-CGGGATCCCGTCACGCTTCATGCAGTTGAT-3' primer. For amplification, 2 μl (160 ng) of cDNA was used as a template mixed with 5 μl of 10X reaction buffer, 5 μl of dNTPs, 2 μl of $MgCl_2$, 0.2 μM of forward primer, 0.2 μM of reverse primer, 2.5 units Taq polymerase in 50 μl total volume reaction mixture. The PCR amplification reaction was carried out under the following conditions: DNA denaturation at 94˚C for 2 min followed by 5 cycles consisting of 94˚C for 30 sec, 58˚C for 30 sec, 72˚C for 1 min followed by 30 cycles with 94˚C for 30 sec, 64˚C for 30 sec. and 72˚C for 1 min. The PCR product (BglII-KTi1-BamH1) was purified DNA using band purification kit according to manufacture's instruction (GE Healthcare, US) and the fragment was then digested with BamHI for 4 hr, purified and then cloned as a BamHI fragment into the p Bluescript pSK (Stratagene, USA) vector.

2.1.3.3 *Isolation of C-II-protease inhibitor promoter*

Genomic DNA was extracted from soybean leaves (cultivar Williams). Isolation of the C-II promoter was made carried out by genome walking according to the published method (Siebert *et al*., 1995). Genomic DNA (3 µg) from soybean was digested with either of the 6 bp blunt-cutting restriction enzymes EcoRV, ScaI or StuI overnight using 80 units of each enzyme to generate a genomic DNA fragment library for genome walking. The digested DNA was precipitated using 2.5X volume of ethanol and 1/10X volume of 3M NaAc pH 5.2. The precipitated DNA was then re-suspended in 25 µl of sterile and distilled water. To ligate adaptors to the 6 µl each adaptor 1 (5′CTAGTCATCATCTTCATCACTGGACTTGC3′) and adaptor 2 (5′GAGGTTCAGTTCCATGCGTGCATT3′) 6 µl each corresponding to 5 units were first mixed and heated for 3 min at 96ºC and cooled at room temperature for 5 min. Then 4 µl of the adaptor mixture was added to 25 µl of blunt-ended DNA digested either with EcoRV, ScaI or StuI, which was followed by ligation of adaptors to blunt-ended digested DNA. The ligation reaction mixture contained blunt-end DNA (45ng DNA) mixed with adaptors 1 and 2 (29 µl), 10X ligation buffer (3.3 µl) and T4 *DNA* polymerase (1 µl corresponding to 5 units of enzyme). The total reaction volume of 33 µl was then incubated at 16°C overnight. The ligation reaction was then denatured at 70°C for 5 min to stop the enzymatic reaction. The ligated product was 10-times diluted with dH_2O before used as a template in a PCR reaction. The C-II promoter sequence was then amplified from the ligated product using two nested gene specific primers designed to amplify part of the coding region of C-II. Nested primers for amplification used were 5′-CTAGTCATCATCTTCATCACTGGACTTGC-3′ and 5′-GAGGTTCAGTTCCATGCGTGCATT-3' after amplification these primers were

used together with adapter primers 1 and 2 mentioned above. 2 µl of the ligation product was used as the template, 1 µl of a gene specific primer 1 and 0.2 µM of adaptor primer 1, and 0.5 µl of *pfu polymerase* in a total PCR reaction mixture of 50 µl. The amplification reaction was carried out under the following conditions: Denaturation at 94°C for 2 min for DNA denaturation followed by 35 cycles of 94°C for 20 sec 68°C for 5 min and 68°C for 10 sec. Amplified DNA was run on an agarose gel and the DNA band with the correct size was purified, cloned into a pGEM-T Easy vector and *E.coli* cells were transformed with the plasmid and streaked onto a LB plate with carbenicillin ($50mgl^{-1}$) as a selective antibiotic. Plasmid DNA was isolated from resulting *E. coli* colonies, and subsequently sequenced using M13 forward and reverse primers (Inqaba, SA).

2.1.3.4   *Cloning of KTI3-BBi conserved regions*

The selected conserved regions from each of the three genes included a 313 bp sequence region from the 5′ region of KTi3 gene, a 212 bp sequence from 3′ region of BBi and a 285 bp sequence from the 3′ region of the KTi1. The fragments were joined together sequentially by introducing an extra restriction site at the end of primers used to amplify the conserved regions. In a first step the conserved region of KTi3 was cloned into the pGEM-T Easy vector with the introduction of a BamHI site at the 3′ end of KTi3. The orientation of the insert was determined by digesting the plasmid with BamHI and two further enzymes, NcoI or SacI, which are unique in the plasmid pGEM-T Easy. Then the BBi conserved region was introduced down-stream of KTi3. For that the pGEM-T Easy vector containing KTi3 was digested with PstI and blunt-ends were created by pfu polymerase treatment, followed by digestion with BamHI. The conserved region of BBi was amplified by introducing a BglII site on the forward

primer and BamHI on the reverse primer. The resulting PCR product was digested with BglII for 4 hr and ligated with the BamHI/PstI digested and blunt-ended (at PstI site) pGEM-T Easy-KTi3 vector and then cloned into *E.coli*. The two conserved regions were then amplified as one fragment using a forward primer containing a XhoI site at KTi3 and a ClaI restriction site on the reverse BBI side. The amplified fragment was finally T/A-cloned into the pGEM-T Easy vector.

The KTi3-BBi conserved region fragment was then cut from the vector pGem-T Easy as a ClaI/XhoI fragment and cloned into the ClaI/XhoI restriction sites of the vector pSK. The pSK-KTi3-BBi vector was then digested with BamHI and EcoRV to recover a pSK-KTi3-BBi fragment with BamH1 site. In addition, the KTi1 PCR fragment was digested with BamHI and cloned into the BamHI site of the vector pSK-KTi3-BBi to join the three conserved regions.

2.1.3.5   *Cloning of β-conglycinin promoter*

The extracted genomic DNA of 2 μl (160 ng) was used as a template and sequence information from published work of Yoshino *et al.* (2006) was used to design the forward 5′-TGCTTGGATTTGGACCAGAC-3′ and reverse 5′-TAGGATATTGAACTAGTTCTCG-3′ primer pairs. PCR reaction contained 5 μl of 10X reaction buffer, 5μl of dNTPs, 2 μl of $MgCl_2$, 0.2 μM for the forward primer, 1 μM for the reverse primer, and 2.5 units (0.5 μl) of *Taq polymerse* in a total 50 μl reaction mixture. The PCR product of 1.4 kb was then purified from an agarose gel and was T/A-cloned into the vector pGEM-T Easy.

2.1.3.6 _Cloning of KTi3-BBI-KTi1 into gene silencing vector_

The three contagious conserved regions KTi3-BBi-KTi sequence on the vector pSK were amplified using the forward (containing a restriction site for BamHI and SalI) IR (inverted repeat) primer 5′-CG*GGATCCGCTAGC*GTCACGCTTCATGCAGTTGAT-3′ (restriction sites are in italics) and the reverse (containing NheI and XhoI sites) IR-primer 5′-AA*CTGCAGGTCGAC*CTTTTGTGCCTTCACCACCT-3' to amplify KTi3-BBi-KTi and the amplified product was purified on an agarose gel and T/A-cloned into the vector pGEM-T Easy. This insert was then sequentially introduced into customized vectors (see section 2.1.4.3). For that, the three contagious conserved regions KTi3-BBi-KTi1 were cut out as a BamHI/XhoI fragment from the vector pGEM-T Easy and cloned into the BamHI/XhoI sites of the two customized vectors (C-II/β-conglycinin-NtcP2-Tvsp). After confirming the presence of the insert, the pGEM-T Easy vector was digested by SalI/NheI (the two reactions had been previously created, to cut out the three contagious conserved regions KTi3-BBi-KTi and cloned into SalI/NheI of the customized vectors to introduce the insert between the _NtCP2_ intron and VSP terminator. This completes the construction of trypsin inhibitor silencing vectors driven by two different promoters.

2.1.3.7 _Cloning of C-II and β-conglycinin-trypsin inhibitor silencing construct into pTF101_

E coli cells harboring the C-II/-β-conglycinin-silensing construct were cultured from glycerol stocks in the incubator at 37˚C overnight. Isolated plasmid DNA from cells was digested with NotI to release the β-conglycinin-trypsin inhibitor silencing construct from the pSK vector which was purified and blunt-ends were created by

filling DNA overhangs up in reaction mixture consisting of 30 μl of template plasmid, 2 μl of dNTPs, 2.5 μl of 10X reaction buffer and 0.3 μl of pfu polymerase. For DNA extension, the reaction mixture was placed into an Applied Biosystems 2720 Thermal cycler for 10 min at 72°C and the resulting product was purified again The C-II silencer band was also released from pSK by NotI digestion, then purified and blunt-ends were created by the polymerase reaction. Plasmid pTF101 which provided the vector backbone was digested with the restriction enzyme SmaI to create blunt-ends and the cut plasmid was dephosphorylated using calf intestine alkaline phosphatase. The dephosphorylated plasmid was then ligated in a 3:1 ratio with either the β-conglycinin-trypsin inhibitor silencing or C-II silencing insert in the following reaction mixture containing 10 μl of vector DNA, 5 μl of insert (10ng DNA), 2.5 μl 10X reaction buffer, 5 μl of T4 ligase enzyme. Ligation was carried out at 16°C overnight and *E. coli* cells were then transformed with the ligation product. Resulting colonies were screened by growing each colony in a 5 ml liquid LB with carbenicillin (50mgl^{-1}) in an incubator at 37°C overnight and resulting colonies were screened for transformation by digestion of plasmids with BamHI and visualizing the correct insert on a agarose gel containing ethidium bromide for DNA staining.

2.1.3.8 *Cloning of C-II-gus int-T35S and β-conglycinin-gus int-T35s into pTF101 for Agrobacterium transformation*

Binary plasmid pTF101 vector containing the *bar* gene driven by CaMV 35S promoter was used for Agrobacterium transformation. The pSK–C-II and pSK β-conglycinin–gus int-T35S plasmids were digested with NotI and blunt-ends were created with *pfu polymerase* at 72°C for 15 min. The plasmids were then digested with EcoRI and the band was purified after separation on an agarose gel. Plasmid

pTF101 was digested with the restriction enzymes SmaI and EcoRI and then dephosphorylated with calf intestine alkaline phosphatase. The pSK–C-II, β-conglycinin–gus int-T35S and pTF101 were ligated at 16˚C overnight. The ligation product (2.5 μl) was transformed into *E. coli* cells for confirmation of cloned C-II, β-conglycinin–gus int-T35S in pFT101. Isolated plasmids from resulting colonies were digested with BamHI.

2.1.4   Vector design for GUS expression

2.1.4.1   *C-II and β-conglycinin promoters*

The *gus-intron* gene with T35S terminator was cut out of vector pTF102 vector with the restriction enzymes BamHI and PstI and cloned into BamHI/PstI sites of the vector pSK. Digestion reaction for pTF102 consisted of 5 μl plasmid DNA (10ng of DNA), 10X BamHI reaction buffer (1 μl), 10 units BamHI (1µl) and 10 units PstI (1µl) in a total 20 μl digestion reaction. In parallel, the vector pSK was digested in a reaction mixture containing 3 μl of pSK vector, 10 units BamHI (1µl), 20units PstI (2µl) in a total 20 μl digestion reaction. The *gus-intron* gene derived from pTF102 together with the T35S terminator sequence was cloned into pSK vector. For ligation, the reaction mixture contained 2 μl pSK (25ng DNA), 6 μl (125ng) *gus intron* gene-T35S terminator sequence insert, T4 ligase buffer, 200 unit T4 ligase (1µl) in a total reaction volume of 20 μl. The ligation product was transferred to *E. coli* cells and resulting colonies were screened by growing each colony in a 5 ml liquid LB with carbenicillin in the incubator at 37˚C overnight. To confirm the insertion of the *gus-intron* gene with the T35S terminator sequence in pSK, isolated plasmids from transformed *E. coli* cells were digested with BamHI/PstI for insert confirmation.

In order to create the *gus-intron* sequence driven by a seed specific promoter, vector pGEM-T Easy containing the C-II promoter was digested with NotI/BamHI and cloned in front of the *gus* gene as a NotI/BamHI fragment in pSK. Also, the β-conglycinin promoter was cut out of pGEM-T Easy as a NheI/BamHI fragment and cloned in front of the *gus* gene as at XbaI/BamHI fragment.

The plasmid pSK–*gus intron*-T35s was digested with NotI or BamHI. The C-II promoter was released as a XbaI fragment and β-conglycinin was released as a BamHI fragment from pGEM-T Easy and both were then ligated into the vector pSK–*gus intron*-T35S. The ligation reaction containing: 4 μl pSK-Gus-T35S (vector 96ng DNA), 6 μl of insert (β-conglycinin 45ng promoter and C-II promoter 39ng DNA), 2 μl of 10X T4 ligase buffer, 1 μl of T4 ligase. *E. coli* cells were transformed with the ligation product. To confirm the insertion of the promoters respectively in front of the *gus-intron* with T35S (pSK), the plasmid was digested with NotI and BamHI.

2.1.5   Vector design for gene silencing

2.1.5.1   *Construction of soybean seed specific vector*

A *Nicotiana tabacum* NtCP2 intron1 (GenBank AY881010 for cDNA) was amplified from plasmid TOPO4 PCR. For amplification three restriction sites (BamHI, PstI and XhoI) were introduced into the forward 5'-CGC*GGATCCCTGCAGCTCGAG*GTGGTAAGAAAGAGTAGCA-3' and three (SalI, NheI and KpnI) into the reverse primer 5'-*GTCGACGCTAGCGGTACC*ACTTCCTATAACAGGCAAAAGG-3'.These enzymes were used to introduce inverted repeats flanking the intron. The PCR reaction contained 5 μl of 10X reaction buffer, 5 μl of dNTPs, 2 μl of the template plasmid, 0.2

µM of the forward primer, 0.2 µM of the reverse primer, and 0.5 µl of the pfu polymerse in the total reaction volume of 50 µl. The obtained amplified DNA band was purified and the amplified fragment was cloned into pGEM-T Easy vector and sequenced. The cloned NtCP2 intron was then cloned as a BamHI/SalI fragment into the vector pSK vector. The C-II and β-conglycinin promoters were amplified from the previous vectors (see section 2.1.2.3 and 2.1.3.3) and cloned into the BamHI site in front of the NtCP2 intron. For amplification, primers contained restriction sites and for the amplification of the C-II promoter the primer contained a NotI site at the 5' primer end was used as forward along with reverse primer BamHI-CII-Rev 5'-CG*GGATCC*CGAACCCCCACAAGGAAAAGAAC-3' that contained BamHI site. For the amplification of β-conglycinin promoter a forward NheICongFF 5'-CTAGCTAGCTAGGCTTGGATTTGGACCAGAC that contained NheI site and a reverse CongRev 5'-CG*GGATCC*CGTAGGATATTGAACTAGTTCTCG-3' primer that contained *BamHI* site were used. The pGEM-T Easy plasmids containing the C-II and β-conglycinin were used as template. The PCR amplification conditions were 1 cycle at 94°C for 2 min, followed by 5 cycles at 94°C 30 sec, 55°C for 30 sec and 72°C for 2 min, followed by 30 cycles at 94°C 30 sec, 64°C for 30 sec and 72°C for 2 min. The PCR product was gel purified and T/A cloned in to pGEM-T Easy vector. The C-II promoter was then cloned in front of the NtCP2 intron at NotI and BamHI site and β-conglycinin promoter was cloned in front of NtCP2 intron at XbaI (compatible with NheI) and BamHI sites.

A soybean vegetative storage protein (VSP) terminator was used as terminator for this customized vector. For that the plasmid pRCBar that contained VSP was digested with KpnI and the vector was refilled. Then the VSP terminator insert was cut out by

digesting with EcoRI and cloned in to PstI digested and blunted and EcoRI digested pGEM-T Easy vector. Finally the VSP terminator insert from pGEM-T Easy vector was digested as SalI and ApaI and cloned behind the NtCP2 intron on pSK at SalI and ApaI sites. This finishes the construction of customized soybean seed specific vector containing two seed specific promoters and a tobacco intron and soybean VSP terminator with intron flanked by three restriction sites for cloning of inverted repeats in front and behind the intron.

2.1.6   Gene analytical work

2.1.6.1   *Phylogenetic tree analysis*

To establish a phylogenetic tree relationship of the two serine proteinase inhibitor family- Bowman-Birk and Kunitz family, the Bowman-Birk family sequence (s) (X68704, CA 819 763, AB 081834, AB 081833, AAA 33952, CK 768695, CN 472434, X68707) and Kunitz family sequence(s) (S45035 Kti1, S45035 Kti-2, AF233296 Kti3, S45092 Kti3+, AF314823, AF314824, X64447(Ti-A), AB112032 (Ti-B), AB070269 (TiE), AB112033(TiC), X80039 (KTi-S) and AB029441 (P20-1) were retrieved from GenBank and aligned using the Multiple sequence alignment software (MAFFT) (Katoh *et al.*,2002). To obtain the correct reading frame the sequences were edited using BioEdit. Phylogenetic and molecular analyses were conducted using MEGA version 4 Neighbouring Joining (NJ) and Maximum Parsimony (MP) (Tamura et al., 2007). Phylogenetic maximum likelihood (ML) version 2.4.4 program was used to obtain the maximum likelihood (ML) (Guindon and Guascuel, 2003). The ML tree was displayed using TreeView (Win32) version 1.64b (Page, 1996). For each analysis, the bootstrap was set at 1000 replicates.

The KTI3, BBi and KTi1 were targeted by identifying highly similar sequence region from per group, which will represent each group. After the identification of similar conserved region from the EST data base search, the RNA was extracted from the leaf and pods to isolate cDNA.

2.2 <u>Plant Work</u>

The amplification reaction was carried out under the following conditions: One cycle at 94°C for 4 min, (5 cycles) 94°C for 1 min (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (extension); and a final extension at 72°C for 10 min (20 cycles).

2.2.2 <u>Plant characterization</u>

2.2.2.1 *Histochemical analysis of GUS expression*

The potential T_1 seeds were further analysed for GUS activity. The histochemical assay of GUS activity was carried out as described by Jefferson (1987) on cotyledons after three days of cocultivation with Agrobacterium and on matured potential transgenic seeds. Explants were incubated at 37°C for 24 h in buffer (pH 7.0) containing, 10 Na_2EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100, to visualize the blue stain.

2.2.1 <u>Plant transformation</u>

2.2.1.1 *Plant material, bacterial strains and plasmids*

The *Agrobacterium tumefaciens* strain EHA 101, harboring a binary vector pTF 101 containing a *bar* gene as a selectable marker, carrying C-II promoter/ β-conglycinin

and *gus intron* as a marker gene were used for transformation of soybean seeds of genotype PAN 512.
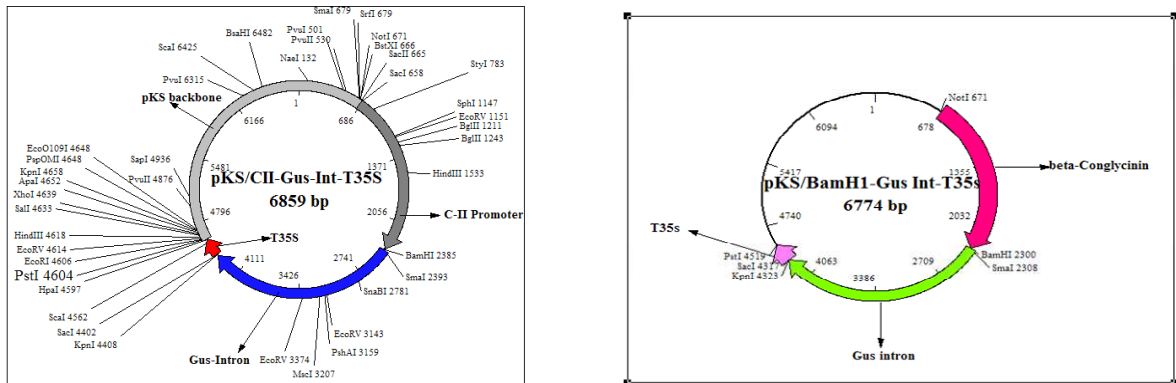
Figure 2.1: C-II/β-conglycinin-Gus construct plasmids schematic representation of restriction sites. The two plasmids were used for soybean transformation

2.2.1.1 Explant sterilization

Mature soybean seeds were sterilised by exposing them in a chlorine gas for overnight in the desiccator within the fume hood. Agrobacterium of the vector system, pTF101-C-ll silencer was collected by pelleting at 3.500 rpm for 10 min at 20˚C from the -80˚C glycerol stocks 3 days prior to an experiment. The pellet was resuspended in the infection medium, and the bacteria cell density was adjusted to a suitable $OD_{650} = 0.6$ to 1.0. The resulting infection medium was agitated at 60 rpm for 30 min. Approximately 20 hr prior to the experiment; the sterilized seeds were inhibited by adding deionised sterile water. Then the plates were completely covered with aluminium foil to block out light.

2.2.1.2 *Explant preparation and infection*

Explants were prepared by making a longitudinal cut along the helium to separate cotyledons and removed the seed coat. The embryonic axis found at the nodal end of the cotyledon was excised, and remaining axial shoots attached to the cotyledonary node was removed. Then cotyledons were wounded by either by sonication, heat shocking or vacuuming. Wounded cotyledons were placed on 50 ml tube added about 30ml of infection medium. Allowed the explants to incubate at room temperature for 30 min with occasional gentle agitation.

After *Agrobacterium* infection, the half seed explants were transferred to co-cultivation medium for 3-5 days. After the period of 5 days the half seed- explants were washed in the shoot induction washing medium. Explants were placed on shoot induction medium 1 for 14 days. After 14 days the large shoots were cut and discarded and then the fresh cut was made at the base of the shoot pad flush to the shoot induction medium ll.

2.2.1.3 *Shoot elongation and rooting*

After 4 weeks on shoot induction medium, the cotyledons were removed from the explants and a fresh cut was made at the shoot pad flush to the medium. Then the tissue was transferred to the shoot elongation medium every 2 weeks. Shoots were excised from the shoot pad when they survived glufosinate selection and reach at least 3cm long.

2.2.1.4 *Plant acclimatization*

When more than 2 roots develop after 2 weeks, it is then transplanted into soil. Potential transgenic plants were screened by the polymerase chain reaction (PCR) for the integration of T-DNA. Genomic DNA was isolated from the leaf tissues using a Sigma's DNA (Sigma, UK) extraction kit according to the protocol provided. The 786-bp coding region of the C-II promoter was amplified using 20-bp oligonucleotide primers for the forward reaction (5'- TCGCGAGAGGAATGAAGAGT-3', and 3′-CGGCTTCAAATGGCGTATAG-5′. PCR amplification reaction contained 50 ng of template DNA, 1 μl of each primer. 2 μl of dNTP (2 mM) mixture, 5 μl of 10X Taq DNA polymerase reaction buffer and 1 μl of Taq DNA polymerase in a 50 μl final volume.

CHAPTER 3

RESULTS

3.1   Serine protease inhibitor gene analysis

Nucleotide sequences of the two major type of serine protease inhibitor of soybean (Kunitz and Bowman-Birk-type) were retrieved from public databases, GenBank and EMBL. In order to determine the target sequence for silencing, relative abundance, diversity/similarity and organ specificity of expression of the individual member sequences were determined from the NCBI Unigene EST profile viewer. A database search revealed a number of individual database entries (accession numbers) and diversity in both KTi and BBi group of serine protease inhibitors in soybeans.

3.1.1   *Bowman-Birk type inhibitors*

Table 3.1 shows tissue specificity of 8 members of Bowman-Birk family retrieved from Gene Bank. The phylogenetic tree in Figure 3.1 further shows the evolutionary relationship among the members of the Bowman-Birk type gene family. Bowman-Birk type double-headed protease inhibitors are assumed to have risen by duplication of an ancestral (X68704) single-headed protease inhibitor gene (Odani *et al.*, 1983). These subsequently diverged into different classes of inhibitors (Odani *et al.,* 1983). The first four members (CA81976, AB081834, AB081833 and X68704) fall into one group and they share a sequence similarity of 97%. Since they are well researched in comparison to C4 (X68707 and CN472434 PI4) and C-II (AAA33952 PI CII and CK768695), they were targeted in this study.

Table 3.1: Gene Bank representatives of Bowman-Birk protease inhibitor gene members.

Gene Bank/EMBL Acc. No	Tissue specificity
1. X68704/ AY233800/AA 089509	Pods and seeds
2. CA 819763	Pods and seeds
3. AB 081834	Pods and seeds
4. AB 081833	Pods and seeds
5. AAA 33952	Pods and seeds
6. CK768695	Seeds
7. CN472434	Seeds
8. 68707	Pods and seeds
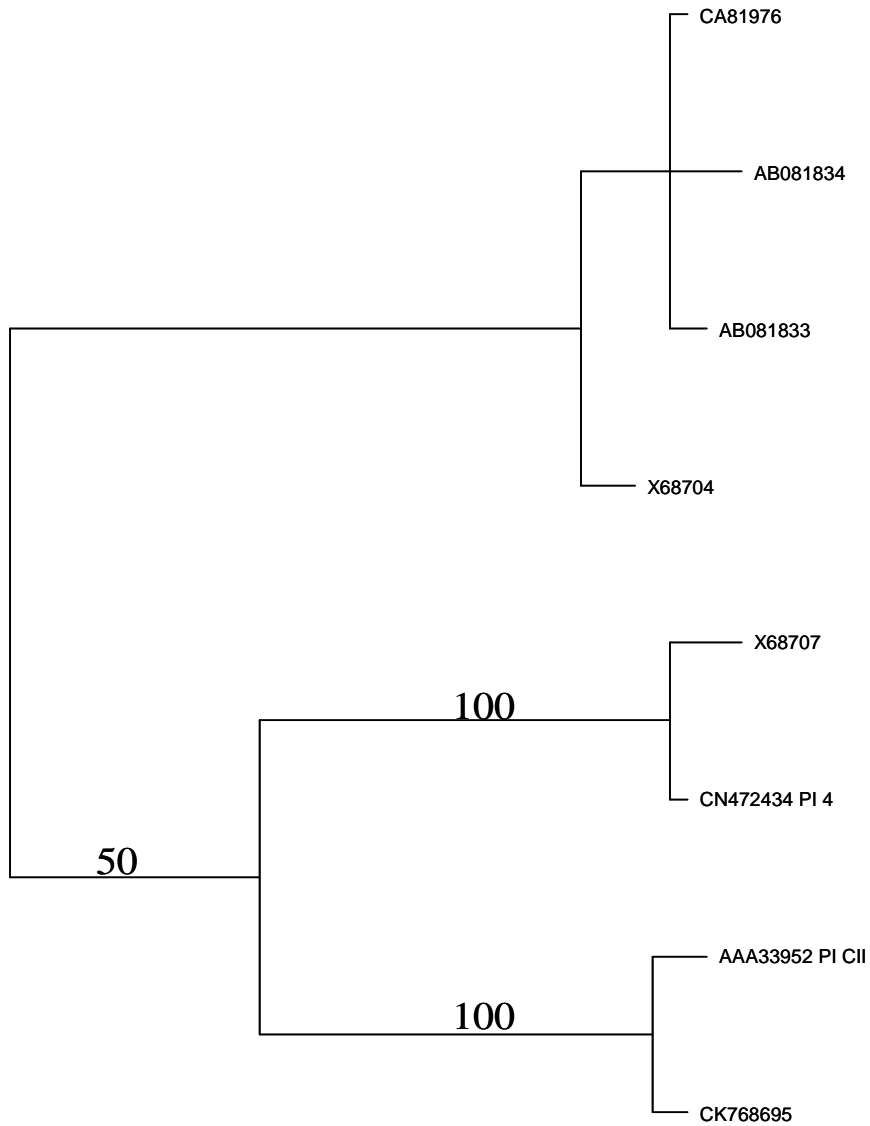
Figure 3.1: Phylogenetic tree of Bowman-Birk type family of serine protease inhibitors of soybean.

3.1.2   *Kunitz-type inhibitors*

Table 3.2 shows the tissue specificity of 12 members of Kunitz-type trypsin inhibitor family retrieved from Gene Bank. Figure 3.2 further shows the phylogenetic tree demonstrating the evolutionary relationship among the Kunitz-type inhibitors in soybean. According to Habu *et al.* (1997) several gene conversion events contributed to the evolution of this gene family. The Kunitz-type trypsin inhibitor gene class in soybean is designated as KTi1/KTi2 and KTi3 has about 712 expressed sequence tags. These genes differ by only 18 out of 608 nucleotides indicating an approximately 97% sequence similarity (Jofuku and Goldberg, 1989). The KTi3 group is represented by 8 genes (Figure 3.2). They diverge by 20% at the DNA level from the KTi1 and KTi2 group (Jofuku and Goldberg, 1989).

Table 3.2: Gene Bank representative of Kunitz trypsin protease inhibitors type gene members.

Gene Bank/EMBL Acc. No	Tissue specificity
S45035 (Kti-1)	Pods, seeds, leaf, roots
S45035 (Kti-2)	Pods, seeds, leaf, roots
AF233296 (Kti-3)	Pods, seeds and stem
S45092 (Kti3+)	N/D
AF314823	N/D
AF314824	N/D
X64447(Ti-A)	N/D
AB112032 (Ti-B)	N/D
AB070269 (Ti-B)	N/D
AB112033 (TiC)	N/D
X80039 (Kti-S)	N/D
AB029441 (P20-1)	N/D
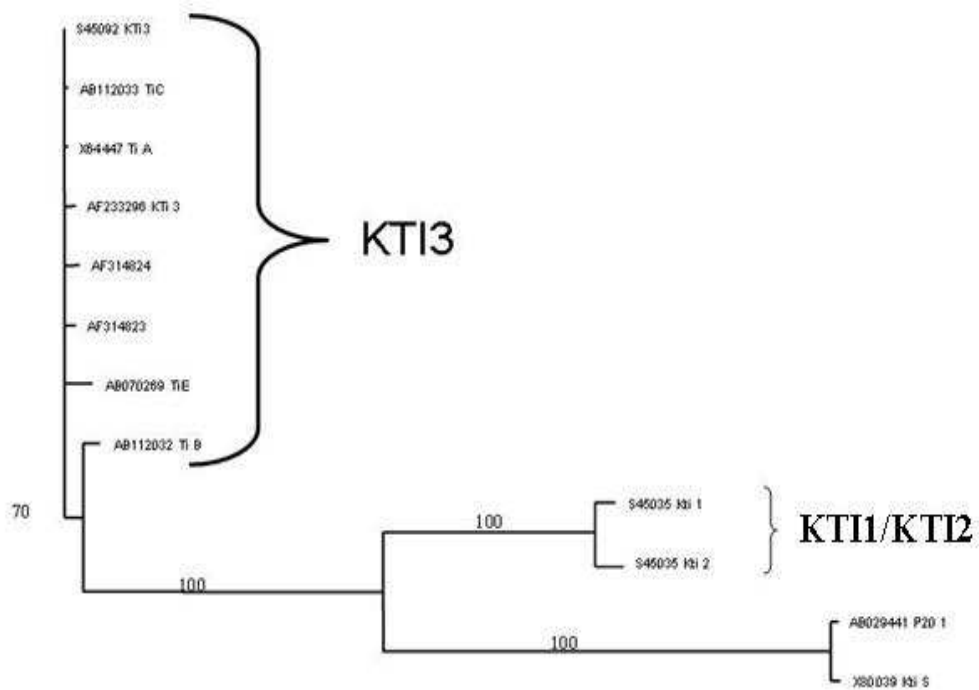
Figure 3.2: Phylogenetic tree of the Kunitz-type serine protease inhibitor family of soybean.

3.2   Serine protease inhibitor isolation

All sequences of BBi and KTi represented by GenBank accession numbers were downloaded. The sequences were aligned using the multiple alignment software ClustalW (Thompson *et al.*, 1994) to determine the similarity between the members and also to select a highly conserved sequence among the individual members. Based on the information acquired from database searches (NCBI and EMBL) digital Northern blot analysis and literature, three sequences were chosen to target as many inhibitor sequences as possible. The targeted sequences for creation of the RNAi silencing element included:

- Bowman-Birk protease inhibitors (GenBank: AY233296)

- Kunitz-type trypsin inhibitors, KTi-3 (GenBank: AF233296)

- Kunitz-type trypsin inhibitors, KTi-1 (GenBank: AF233296)

Figure 3.3 shows the multiple gene sequence alignment of the BBi conserved regions. Primers were designed from the region (underlined) to amplify a 212 bp fragment (Figure 3.4) from a cDNA isolated from soybean seeds and pods with the following BBi primers: forward primer 5'TCCGGCATGCCTACTGAGTG3' and 5' CGTCACGCTTCATGCAGTTGAT3' as a reverse primers designed with the PRIMER3 program. The 212 bp DNA fragment was purified and T/A cloned into the vector pGem-T Easy.
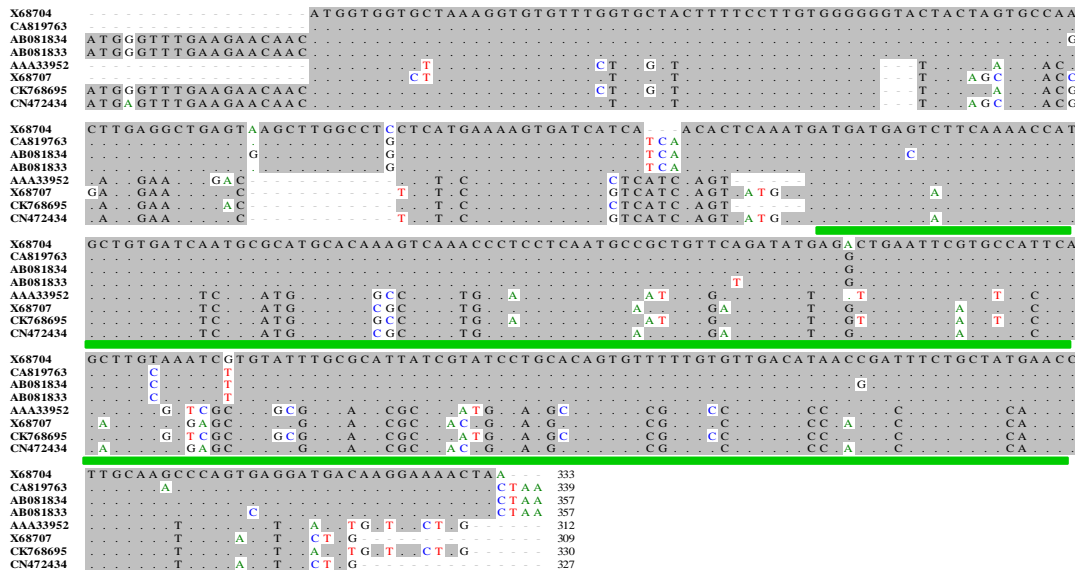
Figure 3.3: Multiple gene alignment showing similarity among the BBi serine protease inhibitors. Green line shows BBi conserved regions.
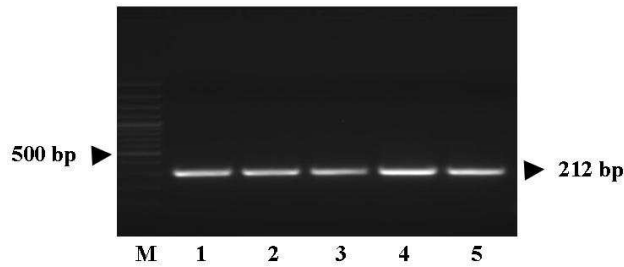
Figure 3.4: PCR amplification of the conserved BBi coding region from a cDNA isolated from soybean seeds and pods. M represents a DNA marker and 1-6 represents the 212 bp BBi fragment amplified by PCR.

3.3   Isolation and characterization of Kunitz-type trypsin inhibitors

Figure 3.5 shows the multiple gene sequence alignment of the KTi3 and KTi1 conserved regions. Primers were designed from the regions (underlined) to amplify a 312 bp and 285 bp fragment (Figure 3.6) from a cDNA isolated from soybean seeds and pods with the following primers: KTi3 forward primer 5'CCCAAAAATGAAGAGCACCA-3' and 5' CTTTGCATTCGGCCTTAGAA-3' as reverse primer, and KTi1 forward primer 5' CGGCATGCCTACTGAGTG-3' and 5'-TCACGCTTCATGCAGTTGAT-3' as reverse primer designed with the PRIMER3 program. The 312 bp and 285 bp DNA fragment was purified and T/A cloned into the vector pGem-T Easy.
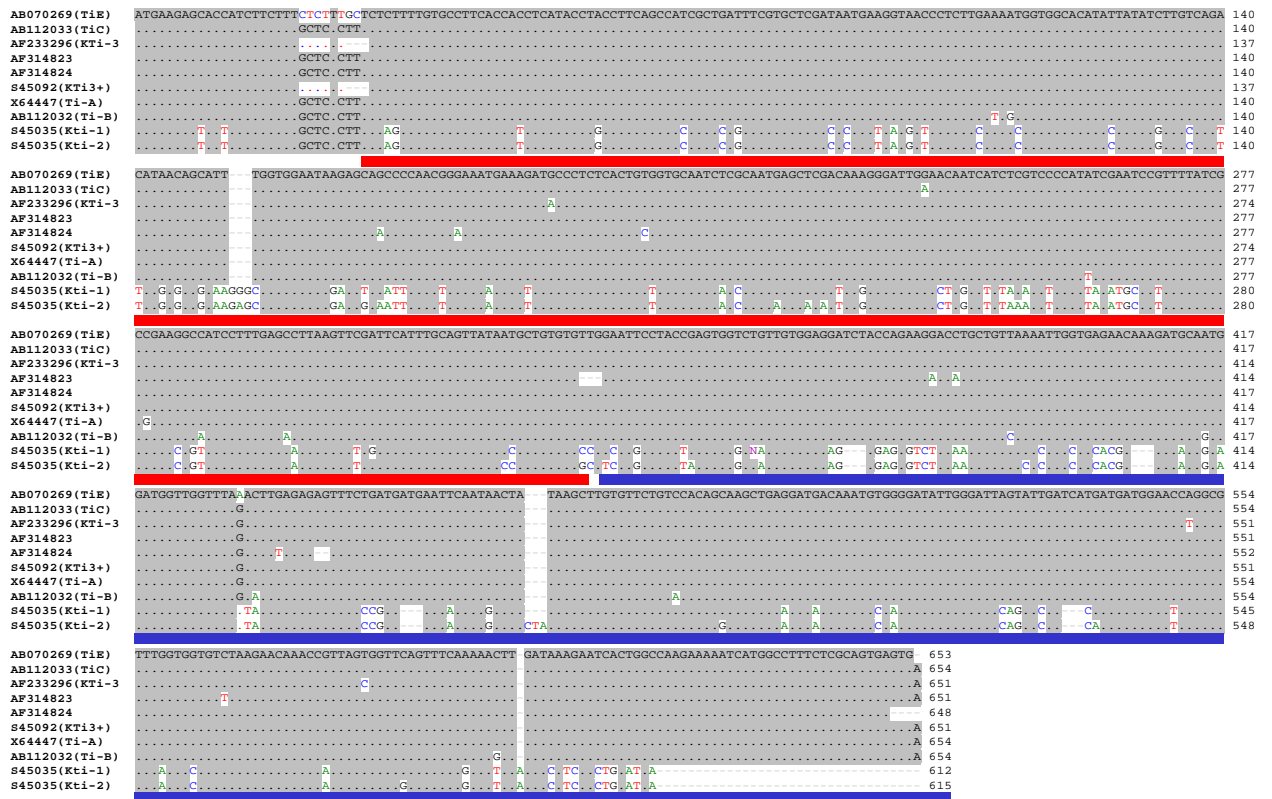
Figure 3.5: Multiple gene alignment similarities among the KTi3 and KTi1 and KTi2 sequences. Red line shows the KTi3 conserved region and the blue line shows the KTi1 and KTi2 conserved regions.
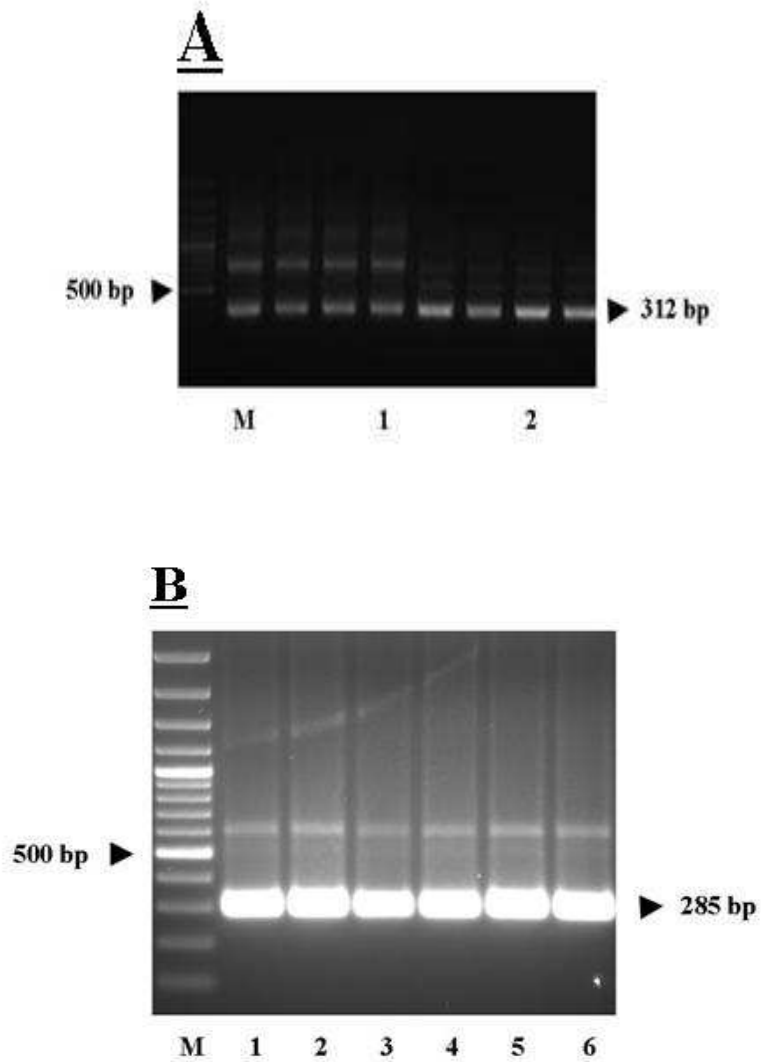
Figure 3.6: (A) KTi1 and (B) KTi3 coding regions amplified from cDNA isolated from soybean seeds and pods. M represents DNA marker and 1-6 represents PCR product of KTi3 and KTi1 with the predicted size of 285 bp.

3.5. β-Conglycinin isolation and characterization

Figure 3.7 shows the 1800 bp PCR amplification product of the β-Conglycinin promoter sequence derived from leaf tissue genomic DNA. For amplification, primers were designed using the reporter sequence information with the following primers: β-Conglycinin forward primer 5'-TGCTTGGATTTGGACCAGAC-5' and 5'-TAGGATATTGAACTAGTTCTCG-3' as reverse primer designed with the PRIMER3 program. The 1800 bp DNA fragment was purified and T/A cloned into the vector pGem-T Easy. Figure 3.8 shows the sequence analysis of β-Conglycinin in pGem-T Easy vector which confirmed the correct sequence of the alpha subunits according to Yoshino *et al.* (2006).
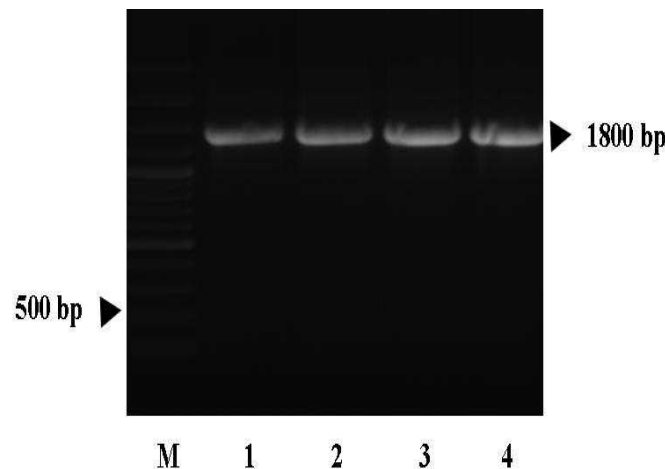
Figure 3.7: PCR amplification of β-Conglycinin promoter derived from leaf tissue genomic DNA. M represents DNA marker and 1-4 represent PCR amplification product of the β-conglycinin promoter sequence with the predicted size of 1800 bp.

TCTTTTCAATTTTTTTGGCCACATATTATTCGGGTTCTGTGACCTTTTCAAAATGAC
TGCTATTACCTCCTGACCTTGCTATTACATCTTGACCATCACTAGGCATTTAAAAG
TATTAGTCATAGTCACATATTACTACAAAGCGAGATTGATCTCTCAATCTAATG
GGTGGGAAAACACTTATAATATATGATTCAAGAAAGAAAGTAAATAAAACAAT
TTTATTATATAAAGACTATTAGGATAAAAAAAACCTTAAAAGTGCTTGGATTTGG
ACCAGACTTGAATTTTAATTTAATGATATTATAATATGTGAATATATTTTTGAGAC
AATTGTAAATTTCAGATAAAAAATAATGTAATTAAAATTGTAATAACTATATCG
TATACTTAATTAATTATTAAATGTGACAAAAA[GATATACATCAAAACTTAATGT
TTCATGACTTTTTTTTTAATGTGTGTCCTAAATAGAAATTAAAAATAAAAATTATT
ATATCCAAATGAAAAAAACATTTAATACGTATTATTTAAGAAATAACAATATATT
TATATTTTAATATGTATTCACATGTAAATTTAAAAACAAAAACAAAATTTCTCTTT
TATTGATTAATTAAAATAATTTTATAACTACATTTATTTTCTATTATTATCAATTTT
CTTCTGTTTTTTTATTTGGCATATATACCTAGACAAGTCAAAAAATGACTATTCTT
TAATAATCAATCATTATTCTTACATATTGTTCGAACTACGAGTTATGAAGTGTCAA
TTGCACCTTAGTGTTTTGATAGGCCTCCATTTGCCGCTCATTAATTAATTTGATAA
CAGCCGTACCGATCAATTACTTATGCTTCTTCCATCGTAATTATATGCATGTCGGT
TCTTTTAATCTTGGTACTCTCGAATGCCACCACAACACTGACTAGTCTCTTGGATC
ATGAGAAAAGCCAAAGAACAAAAAGACAACATAAAGAGTATCCTTTGCAAA
AAAATGTCTAAGTTCATAAAATACAAACAAAAACGCAATCACACACAGTGGACC
CAAAAGCCATGCACAACAACACGTACTCACCAAGGTGCAATCGTGCTGCCCAAA
AACATTCACCAACTCAATCCATGATGAGCCCACACATTTGTTGTTTGTAACCAAA
TCTCAAACGCGGTGTTCTCTTTGGAAAGCAACCATATCAGCATATCACACTATCT
AGTCTCTTGGATCATGCATGCGCAACCAAAAGACAACACATAAAGTATCCTTTCG
AAAGCAATGTCCAAGTCCATCAAATAAAATTGAGACAAAATGCAACCTCACCCC
ACTTCACTATCCATGGCTGATCAAGATCGCCGCGTCCATGTAGGTCTAAATGCCA
TGCACATCAACACGTACTCAACATGCAGCCCAAATTGCTCACCATCGCTCAACAC
ATTTCTTGTTAATTTCTAAGTACACTGCCTATGCGACTCTAACTCGATCACAACCA
TCTTCCGTCACATCAATTTTGTTCAATTCAACACCCGTCAAACTTGCATGCCACCC
CATGCATGCAAGTTAACAAGAGCTATATCTCTTCTATGACTATAAATACCCGCAA
TCTCGGTCCAGGTTTTC

Figure 3.8: Sequence analysis of the β-conglycinin alpha subunit cloned into the pGem-T Easy vector.

3.7   C-II promoter isolation and characterization

Figure 3.9 shows the 2 kb PCR amplification product of the C-II promoter derived from leaf tissue genomic DNA. The isolation of C-II promoter was carried out according to the published method by Siebart *et al.* (1995) using the genome walking technique. Genomic DNA (3 µg) from soybean digested with either of the 6 bp blunt-cutting restriction enzymes EcoRV, ScaI or StuI overnight using 80 units of each enzyme to generate a genome walk library. Nested primers were designed based on a C-II coding region sequence to amplify a 2 kb DNA fragment (Figure 3.9) using the following primers: C-II forward primer 5'-CTAGTCATCATCTTCATCACTGGACTTGC-3' and 5'-GAGGTTCAGTTCCATGCGTGCATT-3' as reverse primer. The 2kb ScaI digested fragment was purified and T/A cloned into the vector pGem-T Easy. Figure 3.10 shows the sequence analysis of the C-II promoter with restriction sites in the pGem-T Easy vector.
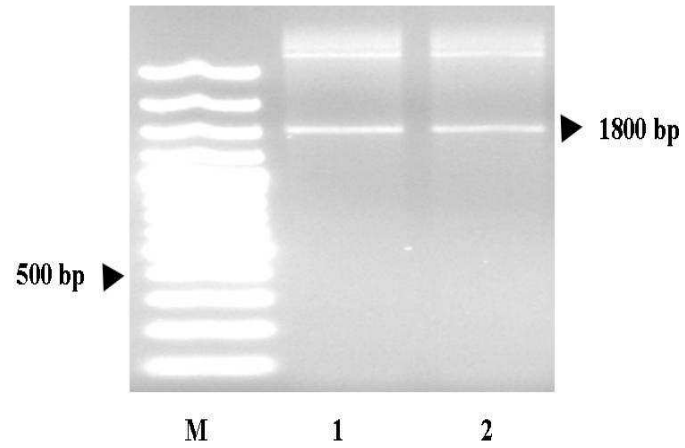
Figure 3.8: PCR amplification of C-II promoter sequence that was cut before with ScaI fragment. M represents DNA marker and 1-2 represents the PCR amplification product of C-II promoter with the predicted size of 1800 bp.

TACTAATAATACTAAAAATTAATAAAATCTAAGATGATGGTGGTGGTGGTGGTGA
TAGATCAAAGCATGTGCGGATGAAAGTGACATCTCCTTCAACCTTGGTGATCCTT
GAGTCCATCTCGTCGAATTGCATGTCCACTTGTTGTTCCAAAGCATCAAACCTTTC
ACCAACATAGGTTTGAAGTCCATCAAACCTGTCCAAAATACTCTGAAGGAGAGA
ATAATCCTCTCCTACTGGTAAGTGTCCTTCATCATCATTGGGTTGAGCACCCTTTT
TTATCCAAGAGCCATCACACTCTTTGTGGTAACCAAAAGATGCAATCACAGCAGC
ACCGATCAAGAAGGATCTCTTGATTGGAACATAAGGTTCAGAATCAAAAGGGAT
GTTAAAGTGTTGAAGGAAAAGAGTGACTAGATGTAGATATGGTAAAGGAGCATT
TAATCGTAATGCCTTATGCATGCGATATCGAACTAAGTGTGCCCAATCAATTTGT
CGGTCGGTATGAAAAGCCCACATGACAATAAGATCTTCTTCAGAAACTTGTGCAA
GGTTTGAAGATCTTGGAAGTAAAATACGCACTATAAGATAGTGAAGGATGCGGC
TTTCAAGAGCCAATGAACCGGCAAGAAGCCTTCCGGTCATATCCGCTTGGTTGGT
GCAAACCAACCGGCGGGCATCATGCACAGAGAAATCAAATTTCCAATCATCATT
AAGTGCACCTTCAAATGGTACACCTTCACTAGATAATTGGGTCAAGTCATAGAAT
AGGGATCGGTCAATGACCATTTTTATCCCAAAAACCTCATAAATCAAAGTTCTTT
CTTGAATTTCGAGGTGAGAATAAAAAGCTTTAACTAATTCAGAATAAACAAGCA
ATTTCAAAGACATAAAAGGAGTGAGATTTGAGTTCTGAAATGCCTGAATGCATTC
AAAACTCTCATTGGAAAAGAAATCCATGTCTATGAATTTAGGATCGATAATGGAA
CGAGAGGAGAAAAGGTTTGTGTACCGTATCCGTTGTTCTTCTGATGAGAACAGCA
AGGAAGAGGAAATGGAGGAGGGAATCTATGTTTCCTGAGCCTCGAAGTGCCGCT
GGCTTCGACTCGAAGAACCCTTGTGCTTTTTTTATGGTTCCTCCATTTGAGAGAGT
TATTTGAAATTTCAATCGG**T**TAAAGTGATAGAGAATGAAAAAAAGAATTTTGGG
ATTTGTGGGACTTGATTTGGACAAGAAATGAGCAAGATATGACAGAAATATGAA
TTGGGAACGAGGGTTCGCGAGAGGAATGAAGAGTTGCAGATTTCTAATTTTGAA
ATCTGAATATATAGGAGCAAGGACGTGTTGTAATCGATTACAGCATTTGGTAATC
GATTACACTCTTCCTGACTGTCTGTAATCGATTACACTATTGTAGCAATCGATTAC
CAGAGAGCATTTTAGCCAAACTAAATGCTGAATGACTCTTAAGTGTAAAATGGAT
TTGAATGAAAACCAAAATACTTTTAAAAAATAAATATATATAACATTAAATTTTT
TAAAACACTTTATGAAAGAACTTTAAGCAAGTAATTCACTTATCACATTCAAATC
ACGGGTTCTTTTCTCTTCACACTTGACAGGAAAAACAGTAAAGATGGGTTTGAAG
AACAACATGGTGGTGTTAAAGGTGTGTTTCTTGGTTCTTTTCCTTGTGGGGGTTAC
TAATGCACAC

Figure 3.10: Sequence analysis of C-II promoter in the pGem-T Easy vector.

3.7 Cloning of BBi and KTi3 hybrids

For confirmation of cloning of the BBi conserve region into the vector pGem-T Easy vector, isolated plasmids from different colonies were screened for the BBi insert with the following primers: forward primer 5'TCCGGCATGCCTACTGAGTG3' and 5' CGTCACGCTTCATGCAGTTGAT3' as reverse primer. The amplification product was separated on an agarose gel and a 212 bp BBi fragment with the expected size was identified (Figure 3.11) which was then purified and sequenced at Inqaba (SA). The cloned 212 bp BBi sequence contained some mis-matches when compared to the already reported conserved region of BBi (AY233800; Figure 3.12). This might indicate differences in the BBi conserved region between different soybean cultivars or might indicate the existence of a BBi gene family.
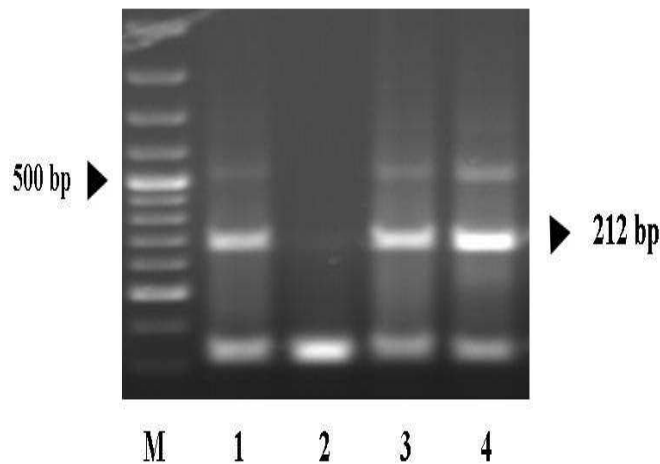
Figure 3.11: PCR screening of colonies for insertion of the conserved BBi region into the vector pGEM-T Easy. M represents DNA marker and 1-4 represents BBi amplification products from plasmids isolated from different colonies with the expected size of 212 bp.
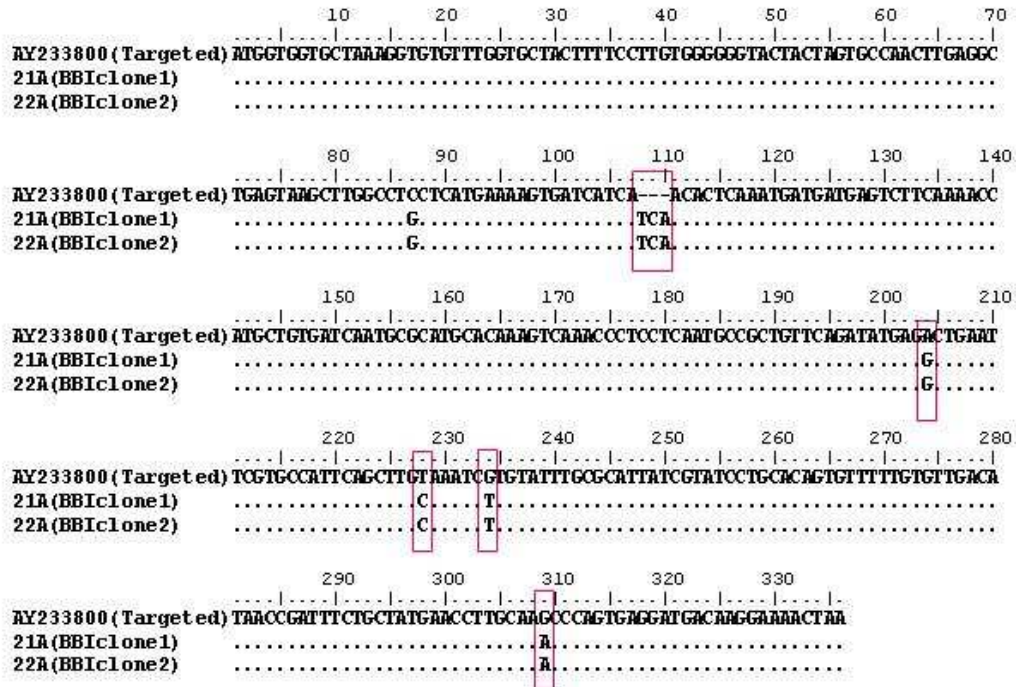
Figure 3.12: Multiple sequence alignment of already reported targeted sequence AY233800 and cloned sequences of the BBi conserved region. Red boxes indicate gene sequence differences.

For confirmation of cloning of KTi3 into the vector pGem-T Easy, isolated plasmids from different colonies were screened with the following primers: KTI3 forward 5'-CCCAAAAATGAAGAGCACCA-5' and 5'-CTTTGCATTCGGCCTTAGAA-3' as reverse primer. The amplification products were separated on an agarose gel and a KTi3 fragment with the expected size was identified (Figure 3.13) which was then purified and sequenced at Inqaba (SA). The cloned sequence KTi3 had also some mismatches when compared to the targeted already sequenced conserved region of KTi3 (Figure 3.14). This might also be due, as already found for the BBi sequence, to differences between cultivars or due to the existence of a gene family.
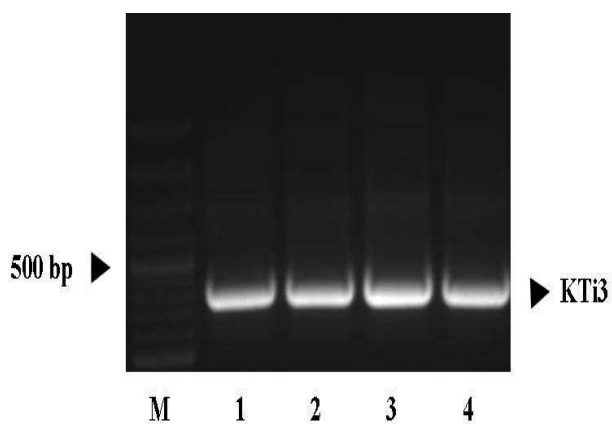
Figure 3.13: Amplification of KTi 3 region from isolated plasmids from different colonies. M represents DNA marker and 1-4 represent KTi3 amplification products from isolated plasmids from different colonies with the expected size of 312 bp.

```
KTI-3 targeted      CCCAAAAAATGAAGAGCACCATCTTCTTT---CTCTTTCTCTTTTGTGCCTTCACCACCTCATACCTACCTTCAGCCATCG
23A (KTI-3 clone 1) ............................GCT.................................................
24A (KTI-3 clone 2) ............................GCT.................................................

KTI-3 targeted      CTGATTTCGTGCTCGATAATGAAGGTAACCCTCTTGAAAATGGTGGCACATATTATATCTTGTCAGACATAACAGCATTT
23A (KTI-3 clone 1) .............G.................................................................
24A (KTI-3 clone 2) .............................................................................

KTI-3 targeted      GGTGGAATAAGAGCAGCCCCAACGGGAAATGAAAGATGCCCTCTCACTGTGGTGCAATCTCGCAATGAGCTCGACAAAGG
23A (KTI-3 clone 1) .............................................................................
24A (KTI-3 clone 2) .............................................................................

KTI-3 targeted      GATTGGAACAATCATCTCGTCCCCATATCGAATCCGTTTTATCGCCGAAGGCCATCCTTTGAGCCTTAAGTTCGATTCAT
23A (KTI-3 clone 1) .............................................................................
24A (KTI-3 clone 2) .............................................................................

KTI-3 targeted      TTGCAGTTATAATGCTGTGTGTTGGAATTCCTACCGAGTGGTCTGTTGTGGAGGATCTACCAGAAGGACCTGCTGTTAAA
23A (KTI-3 clone 1) .............................................................................
24A (KTI-3 clone 2) .............................................................................

KTI-3 targeted      ATTGGTGAGAACAAAGATGCAATGGATGGTTGGTTTAGACTTGAGAGAGTTTCTGATGATGAATTCAATAACTATAAGCT
23A (KTI-3 clone 1) .............................................................................
24A (KTI-3 clone 2) .............................................................................

KTI-3 targeted      TGTGTTCTGTCCACAGCAAGCTGAGGATGACAAATGTGGGGATATTGGGATTAGTATTGATCATGATGATGGAACCAGGC
23A (KTI-3 clone 1) .............................................................................
24A (KTI-3 clone 2) .............................................................................

KTI-3 targeted      GTTTGGTGGTGTCTAAGAACAAACCGTTAGTGGTTCAGTTTCAAAAACTTGATAAAGAATCACTGGCCAAGAAAAATCAT
23A (KTI-3 clone 1) ...........................................................................G......
24A (KTI-3 clone 2) .............................................................................

KTI-3 targeted      GGCCTTTCTCGCAGTGAGTGAGACACAAGTGTGAGAGTACTAAATAAATGCTTTGGTTGTACGAAATCATTACACTAAAT
23A (KTI-3 clone 1) .............................................................................
24A (KTI-3 clone 2) ..'..........................................................................

KTI-3 targeted      AAA TAATCAAAGCTTATATATGCCTT---CTAAGGCCGAATGCAAAG-
23A (KTI-3 clone 1) ......,....................CTT.................A
24A (KTI-3 clone 2) ..........................T--.................A
```

Figure 3.14: Multiple gene alignment of already known targeted KTi3 and cloned KTi3 gene sequences. Red boxes indicate differences in the gene sequences.

The 312 bp sequence of the conserved 5′ region of KTi3 gene, and the 212 bp conserved sequence region of BBi were joined together sequentially by introducing an extra restriction site at the end of primers used to amplify the conserved regions. In a first step, the conserved region of KTi3 was cloned into the vector pGEM-T Easy with the introduction of a BamHI site at the 3′ end of KTi3. The orientation of the insert was determined by digesting the plasmid with BamHI and two further enzymes, NcoI or SacI, which are unique in the vector pGEM-T Easy. Then the BBi conserved region was introduced down-stream of KTi3. For that, the pGEM-T Easy vector containing KTi3 was digested with PstI and blunt-ends were created by treatment with *pfu* polymerse, followed by digestion with BamHI. The conserved region of BBi was amplified by introducing a BglII site on the forward primer and BamHI on the reverse primer. The resulting PCR product was digested with BglII for 4 hr and ligated with the BamHI/PstI digested blunt-ended (at PstI site) pGEM-T Easy-KTi3 vector and then cloned into *E. coli*. The two conserved regions were then amplified as one fragment using a forward primer containing a XhoI site at KTi3 and a ClaI restriction site on the reverse BBi side. The amplified fragment was finally T/A-cloned into the vector pGEM-T Easy. Figure 3.15 shows the 312 bp (KTi3) and 212 bp (BBi) amplification product confirming insertion of the hybrid into the pGem-T Easy vector, which was done by digesting the pGem-T Easy plasmid with EcoRI (colony 4). The isolated plasmid containing the hybrid was then sequenced (Figure 3.16).
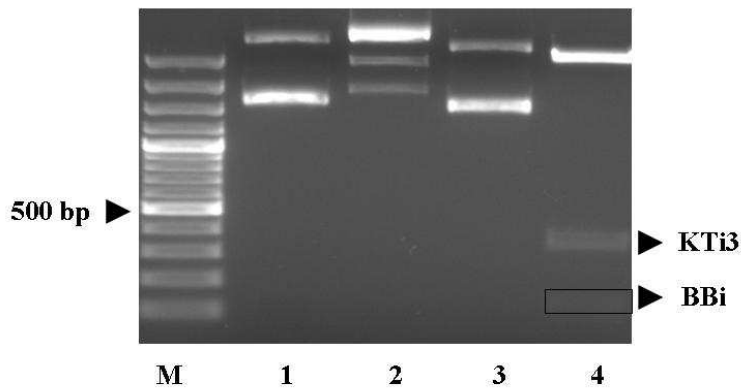
Figure 3.15: Conserved joined KTi3 and BBi regions in the vector pGem-T Easy. M represents DNA marker, 1-4 represents digested plasmid with EcoRI restriction enzyme, plasmids isolated from different colonies. Plasmid from *E. coli* colony 4 has the correct insert with the expected fragment size of 312 bp for KTi3 and 212 bp for BBi (boxed).

CTTTTGTGCCTTCACCACCTCATACCTACCTTCAGCCATCGCTGATTTCGTGCTCG
ATAATGAAGGTAACCCTCTTGAAAATGGTGGCACATATTATATCTTGTCAGACAT
AACAGCATTTGGTGGAATAAGAGCAGCCCCAACGGGAAATGAAAGATACCCTCT
CACTGTGGTGCAATCTCGCAATGAGCTCGACAAAGGGATTGGAACAATCATCTCG
TCCCCATATCGAATCCGTTTTATCGCCGAAGGCCATCCTTTGAGCCTTAAGTTCGA
TTCATTTGCAGTTATAATGCTGTGTGTTGGAATTCCTCAAATGATGATGAGTCTTC
AAAACCATGCTGTGATCAATGCGCATGCACAAAGTCAAACCCTCCTCAATGCCGC
TGTTCAGATATGAGGCTGAATTCGTGCCATTCAGCTTGCAAATCTTGTATTTGCGC
ATTATCGTATCCTGCACAGTGTTTTTGTGTTGACATAACCGATTTCTGCTATGAAC
CTTGCAAACCCAGTGAGGATGA

Figure 3.16: Gene sequence analysis of joined conserved regions for KTi3 and BBi. Gene sequence not underlined represents the KTi3 conserved region; gene sequence underlined represents the BBi conserved region.

For confirmation of KTi1 insertion as part of the hybrid into the pGem-T Easy vector, the plasmid (Figure 3.17) was screened with the following gene specific primers: KTi1 forward 5'- TCCGGCATGCCTACTGAGTG-3' and 5' TCACGCTTCATGCAGTTGAT-3' as reverse primer and viewed on an agarose gel. The PCR product (BglII-KTi1-BamHI) was purified and the fragment was then digested with BamHI for 4 hr, purified and then cloned as a BamHI fragment into the vector pBluescript pSK (Stratagene, USA).
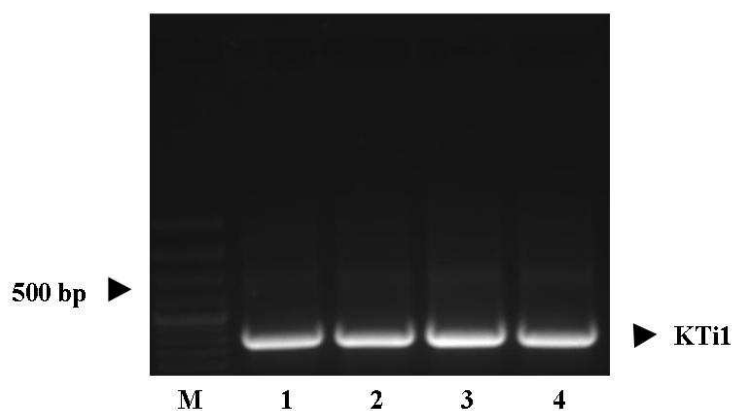
Figure 3.17: PCR amplification of conserved KTi1 coding region. M represents DNA marker and 1-4 represents KTi1 amplified fragments with the expected fragment size of 285 bp.

3.7.1 *NtcP2 intron*

A *Nicotiana tabacum* NtcP2 intron1 (GenBank AY881010 for cDNA) was amplified from plasmid TOPO4 PCR so that it could be used to separate the two one-arm sense and one-arm antisense of the inverted repeats from designed conserved regions of KTi3, BBi and KTi1. For amplification, three restriction sites (BamHI, PstI and XhoI; italics and underlined in the primer sequence) were introduced into the forward 5'-

CGC*GGATCCCTGCAGCTCGAG*GTGGTAAGAAAGAGTAGCA-3'   and   three

(SalI, NheI and KpnI; italics and underlined in the primer sequence) into the reverse

primer   5'-   *GTCGACGCTAGCGGTACC*ACTTCCTATAACAGGCAAAAGG-3'.

Figure 3.17 shows the amplified PCR product of the NtcP2 intron containing 3

restriction sites for both forward and reverse primers, which was then T/A cloned into

the vector pGem-T Easy.
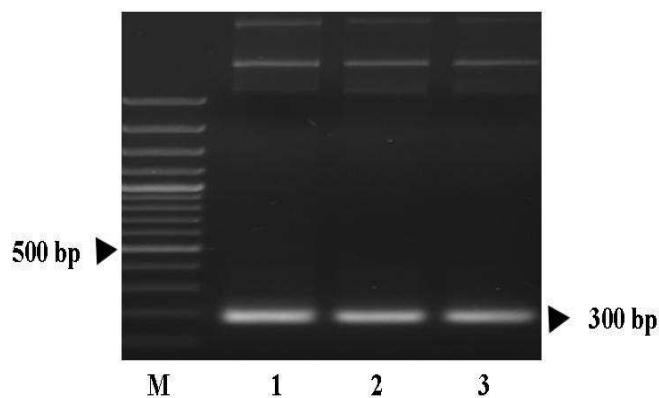
Figure 3.18: PCR amplification of the NtcP2 intron with specific primers containing 3

different restriction sites for both the forward and the reverse primer. M represents

DNA marker and 1-3 represents the NtcP2 intron amplified fragment with the

expected fragment size of 300 bp.

Figure 3.18 (lane 3) shows confirmation of the correct cloning of the NtcP2 intron

(300 bp) into the vector pGem–T Easy. For confirmation, the plasmid was digested

with the restriction enzymes BamHI and SalI (Figure 3.18).
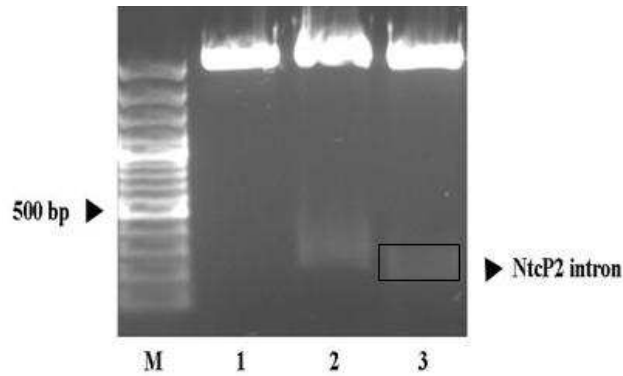
Figure 3.19: NtcP2 intron cloned into the vector pGem–T Easy. M represents a DNA marker. Lane 1 and 2 represents failed detection of NtcP2 into the vector pGem-T Easy and lane 3 represents NtcP2 fragment correctly cloned (boxed) into the vector pGem-T Easy with the expected fragment size of 300 p.

3.8   Cloning into pBlueScript (pSK) vector

3.8.1   *NtcP2 intron*

The C-II and β-conglycinin promoter insert were cloned into pSK-NtcP2 vector. For that, the C-II promoter was cloned as a NotI/BamHI fragment into the pSK-NtcP2 vector (Figure 3.20; lanes 3 and 4) and the β-Conglycinin promoter as an XbaI/BamHI fragment into the pSK-NtcP2 vector (Figure 3.19; lanes 1 and 2).
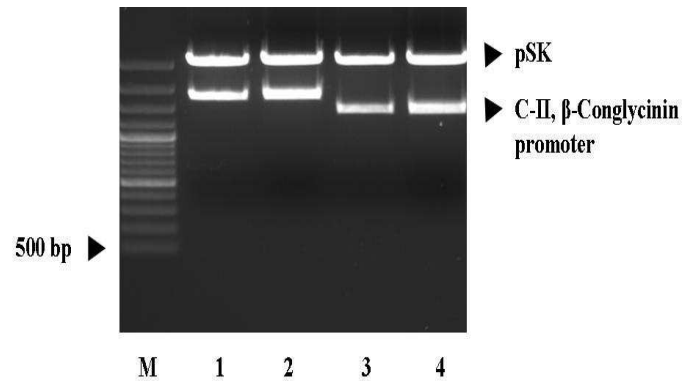
Figure 3.20: β-Conglycinin and C-II promoter insert into pSK–NtcP2 intron. M represents a DNA marker, lanes 1-2 represent C-II-NtcP2 and lanes 3 and 4 represent the β-conglycinin-NtcP2 fragment cloned into pSK vector with the expected fragment size of 2010 bp and 2100 bp, respectively, after cutting with restriction enzymes BamHI and NotI.

3.8.2 VSP terminator

A soybean vegetative storage protein (VSP) terminator sequence was used as a terminator for these customized vectors pSK- C-II/β-Conglycinin-NtcP2 (Figure 3.20). For that, the plasmid pRCBar that contained the vsp terminator sequence was digested with KpnI and the vector DNA overhang was refilled using *pfu polymerase*. The VSP terminator sequence was cut out of the plasmid pRCBar by digesting the plasmid with EcoRI and the sequence was cloned into PstI-digested pGEM-T Easy plasmid after filling in overhangs using *pfu polymerase*. Finally, the VSP terminator insert was cut from the vector pGEM-T Easy vector as a SalI/ApaI fragment and cloned behind the NtcP2 intron in the vector the pSK-C-II/ β-conglycinin-NtcP2 (Figure 3.21). This created a vector containing either C-II or a β-Conglycinin promoter sequence together with a tobacco intron and the soybean VSP terminator

sequence where the intron sequence is flanked by three restriction sites for cloning of KTi3-BBi-KTi1 inverted repeats. This produced a sense and antisense arm of a short hairpin RNA in front and behind the intron.
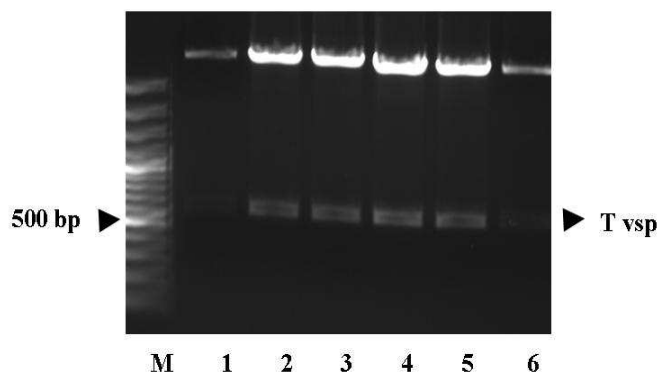
Figure 3.21: Detection of cloned Tvsp terminator sequence in C-II/β-Conglycinin-NtcP2 vector. M represents DNA marker and 1-3 represents C-II-NtcP2-T-VSP fragment and 4-6 represents β-conglycinin-NtcP2-T-VSP cloned into the vector pSK with an expected fragment size of 575 bp.

3.8.3 Joining of KTi3-BBi and KTi1

The KTi1 sequence (Figure 3.17) was ligated to the KTi3-BBi sequence (Figure 3.15) to form one contagious sequence. For that, the KTi3-BBi conserved region (Figure 3.15) was cut from the vector pGEM-T Easy as a ClaI/XhoI fragment and cloned into the ClaI/XhoI restriction sites of the vector pSK. The resulting pSK-KTi3-BBi vector was then digested with BamHI and EcoRV so that KTi1 sequence could be cloned into the BamHI site of pSK-KTi3-BBi. In addition, the KTi1 PCR fragment (Figure 3.16) amplified with BglII KTi1 as a forward primer and BamH1KTiI as reverse primer was digested with BamHI and cloned into the BamHI site of the vector pSK-KTi3-BBi to join the three conserved regions (Figure 3.22).
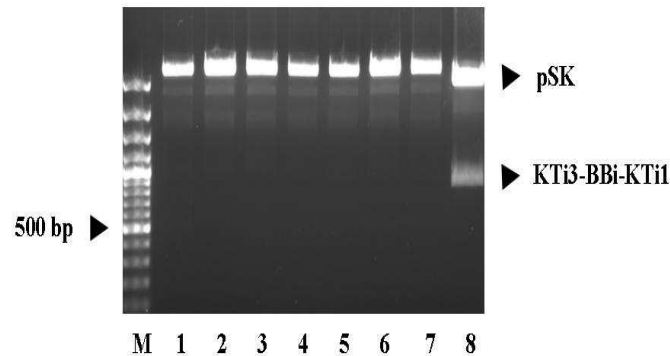
Figure 3.22: Detection of KTi3-BBi-KTi1 in vector pSK. M represents a DNA marker, lanes 1-7 no successful cloning was detected; lane 8 represents fragment detection of KTi3-BBi-KTi1 (809 bp) cloned into the vector pSK after digestion of the plasmid with the BamHI, which cuts at 3' of KTi1, and XhoI restriction enzyme which cuts at 5' of KTi3.

The three contagious conserved regions KTi3-BBi-KTi1 cloned into vector pSK-C-II/β-conglycinin-NtcP2-Tvsp) (Figure 3.23) were amplified using the following primers: forward primer (containing a restriction site for BamHI and SalI), IR (inverted repeat) primer 5′-CG*GGATCCGCTAGC*GTCACGCTTCATGCAGTTGAT-3′ (restriction sites are in italics and underlined) and the reverse (containing NheI and XhoI sites) IR-primer 5′-AA*CTGCAGGTCGAC*CTTTTGTGCCTTCACCACCT-3' to amplify IR KTi3-BBi-KTi (Figure 3.24, C). The amplified product was purified on an agarose gel and T/A-cloned into the vector pGEM-T Easy. This insert was then sequentially introduced into the customized vector pSK-C-II/β-conglycinin-KTI3-BBi-KTi1-NtcP2-T-VSP (Figure 3.24). For that, the three contagious conserved regions KTi3-BBi-KTi1 were cut out as a BamHI/XhoI fragment from the vector pGEM-T Easy and cloned into the

BamHI/XhoI sites of the two customized vectors (pSK-C-II/β-Conglycinin-NtcP2 intron-Tvsp and pSK-β-Conglycinin-NtcP2 intron-Tvsp) customized vectors. After confirming the presence of the insert KTi3-BBi-KTi1, the pGEM-T Easy vector was digested with SalI/NheI. The two restriction sites had been created by introducing Sal as a restriction site into the forward inverted repeat primer and NheI as a site into the reverse inverted repeat primer (Figure 3.24, A) to cut out the three contagious conserved regions KTi3-BBi-KTi1 from pGEM-T Easy into the SalI/NheI restriction sites of the customized vectors pSK-C-II/β-conglycinin-KTI3-BBi-KTi1-NtcP2-Tvsp to introduce the insert (antisense arm KTi3-BBi-KTi1) between the *NtCP2* intron and VSP terminator (Figure 3.24, B) to obtain a trypsin inhibitor silencing vector driven by two different promoters.

CTTTTGTGCCTTCACCACCTCATACCTACCTTCAGCCATCGCTGATTTCGTGCTCG
ATAATGAAGGTAACCCTCTTGAAAATGGTGGCACATATTATATCTTGTCAGACAT
AACAGCATTTGGTGGAATAAGAGCAGCCCCAACGGGAAATGAAAGATACCCTCT
CACTGTGGTGCAATCTCGCAATGAGCTCGACAAAGGGATTGGAACAATCATCTCG
TCCCCATATCGAATCCGTTTTATCGCCGAAGGCCATCCTTTGAGCCTTAAGTTCGA
TTCATTTGCAGTTATAATGCTGTGTGTTGGAATTCC***CGGCATGCCTACTGAGTGGG***
CTATTGTGGAGAGAGAGGGTCTACAAGCTGTTAAACTTGCTGCACGCGATACAGTA
GATGGTTGGTTTAATATTGAGAGAGTTTCCCGTGAATACAATGACTATAAGCTTGTG
TTCTGTCCACAGCAAGCTGAAGATAACAAATGCGAGGATATTGGGATTCAGATCGA
CGATGATGGAATCAGGCGTTTAGTGCTGTCTAAGAACAAACCATTAGTGGTTCAGT
TTCAGAAATTTAGATCATCAACTGCATGAAGCGTGA<u>TCAAATGATGATGAGTCTTC</u>
<u>AAAACCATGCTGTGATCAATGCGCATGCACAAAGTCAAACCCTCCTCAATGCCGC</u>
<u>TGTTCAGATATGAGGCTGAATTCGTGCCATTCAGCTTGCAAATCTTGTATTTGCGC</u>
<u>ATTATCGTATCCTGCACAGTGTTTTTGTGTTGACATAACCGATTTCTGCTATGAAC</u>
<u>CTTGCAAACCCAGTGAGGATGAC</u>

Figure 3.23: Gene sequence analysis of the 3 conserved regions KTi3-BBi-KTi1 cloned into the vector pSK-C-II/β-conglycinin-NtcP2 intron-T-VSP. Vector sequence in normal font style represents KTi3, sequence in italics represents BBi and the sequence underlined represents KTI1 region.
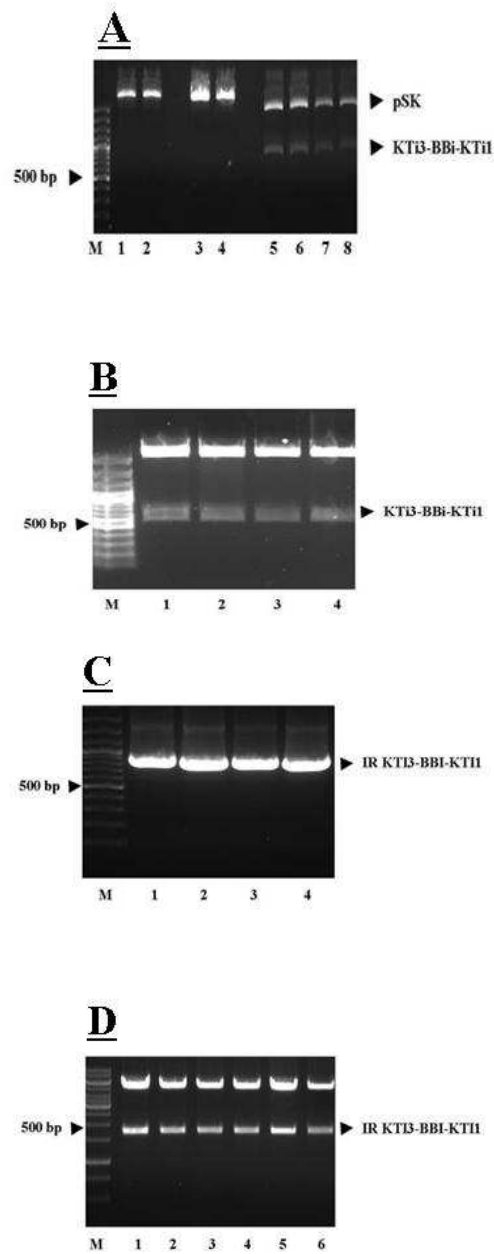
Figure 3.24: (A) Lanes 1- 4 represent C-II/β-conglycin-Ntcp2-VSP with an expected size of 2585 bp after digestion with XhoI and BamHI restriction enzymes and lanes 5-8 represent KTi3-BBi-KTi-1 insert in pSK with an expected size 809 bp after digestion Xho1 and BamH1 restriction enzymes. (B) Lanes 1-4 represent pSK-C-II/β-conglycin-Ntcp2-VSP-KTi3-BBi-KTi-1 with an expected fragment size of 809 bp after digesting pSK-C-II/β-conglycin-Ntcp2-VSP-KTi3-BBi-KTi-1 vector with

69

BamH1 and XhoI. (C) 1-4 represents the IR KTi3-BBi-KTi1 amplification product with an expected size 809 bp after amplification of the 3 domains (KTI3-BBi-KTi1) with inverted repeats primers. (D) 1-6 represents plasmid pSK- C-II/β-Conglycinin-KTi3-BBi-KTi -Ntcp2-IR KTi3-BBi-KTi-1-T-VSP with an expected fragment size of 809 bp after digesting the pSK- C-II/β-Conglycinin-KTi3-BBi-KTi -Ntcp2-IR KTi3-BBi-KTi-1-T-VSP with NHeI and SalI restriction enzymes.

3.9   Vector design for *gus* gene expression.

The *gus-intron* gene with the T35S terminator sequence was cut out of the vector pTF102 with the restriction enzymes BamHI and PstI (Figure 3.25A) and cloned into the BamHI/PstI sites of the vector pSK. In order to create the *gus-intron* sequence driven by a seed specific promoter, the vector pGEM-T Easy containing the C-II promoter sequence (Figure 3.8) was digested with NotI/BamHI and cloned in front of the *gus* gene as a NotI/BamHI fragment in pSK. Also, the β-conglycinin promoter was cut out of pGEM-T Easy (Figure 3.6) as a NheI/BamHI fragment and cloned in front of the *gus* gene into the XbaI/BamHI restriction sites.
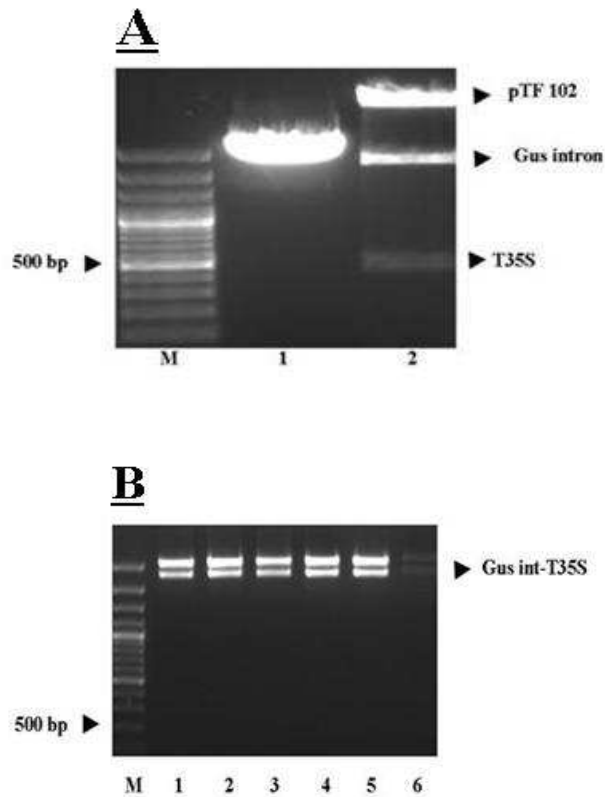
Figure 3.25: (A) M represents a DNA marker; lane 1 represents the vector pSK digested with BamHI and PstI and lane 2 represents the vector pTF102 vector digested with BamHI/PstI to release *gus* and T35S sequence. (B) Lanes 1-6 represent

gus-int-T35S sequence cloned into the BamHI/PstI restriction sites of vector pSK and an expected fragment of 2088 bp was detected after digestion of the plasmid with BamH1 and Pst1 restriction enzymes.

3.10   Cloning of silensing and Gus constructs into pTF101 binary vector

To release C-II/β-Conglycinin-trypsin inhibitor silencing construct from the pSK vector the pSK plasmid containing the C-II/β-conglycinin-trypsin inhibitor silencing construct cloned into the vector pSK was digested with NotI to release the β-Conglycinin-trypsin inhibitor silencing sequence from the pSK vector. The Conglycinin-trypsin inhibitor silencing construct was then purified from an agarose gel and blunt-ends were created by filling in DNA overhangs of the Conglycinin-trypsin inhibitor silencing sequence using *pfu polymerase*.

The C-II silenser fragment was also released from the vector pSK by NotI digestion, and blunt-ends were created in the C-II silencer fragment by the polymerase reaction. Further, the plasmid pTF101 (Figure 26A) was digested with the restriction enzyme SmaI to create blunt-ends and the cut plasmid was dephosphorylated using calf intestine alkaline phosphatase. The dephosphorylated plasmid was then ligated in a 3:1 ratio with either the β-conglycinin-trypsin inhibitor silencing or the C-II silencer sequence and *E. coli* cells were transformed with the ligation product. Resulting transformed *E. coli* colonies were screened for containing plasmids carrying the cloned C-II-silencer and β-Conglycinin-silencer sequence by digestion of plasmids with BamHI and visualizing the correct insert on an agarose gel containing ethidium bromide for DNA staining (Figure 3.26B).

3.10.1   Cloning Gus constructs into pTF101 binary vector.

Binary plasmid pTF101 vector containing the *bar* gene driven by CaMV 35S promoter was used for cloning the C-II/β-Conglycinin-*gus*-intron-T35S sequence. For that, the pSK–C-II/β-conglycinin–*gus*-int-T35S plasmids were digested with NotI and blunt-ends were created with *pfu* polymerase. The plasmids were then digested with EcoRI and the fragments containing C-II/β-conglycinin-*gus*-intron-T35S were purified after separation on an agarose gel. Further, plasmid pTF101 was digested with the restriction enzymes SmaI and EcoRI and then dephosphorylated with calf intestine alkaline phosphatase. The pSK–C-II/β-conglycinin–*gus* int-T35S fragments were ligated with cut plasmid pTF101 and the ligation product was used to transform *E. coli* cells and. cloned C-II/β-conglycinin–*gus*-int-T35S in pFT101 was confirmed by digestion of resulting plasmids derived from transformed colonies with BamHI (Figure 3.26).
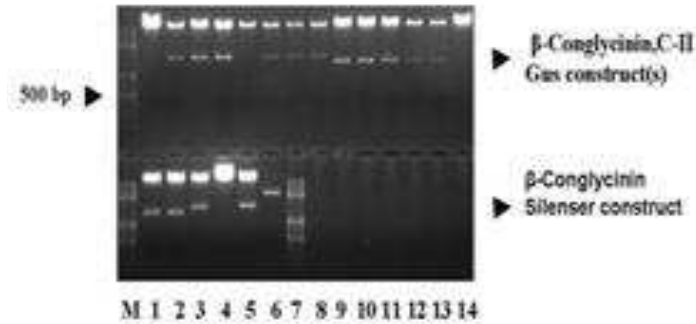
Figure 3.26: Detection of *gus* gene constructs cloned into the vector pTF101. M represents a DNA marker; lanes 2-7 represent C-II-*gus* gene construct cloned into EcorI/SmaI of pTF101; lanes 8-14 represent β-conglycinin-*gus* gene construct cloned into EcoRI/SmaI of pTF101. In the lower gel 3-5 represents β-conglycinin silencer cloned into SmaI site of pTF101 and 6 represents C-II silencer construct cloned into SmaI site of vector pTF101.

All constructs were finally used to transform the Agrobacterium strain *EHA 101* which was used for soybean transformation (Figure 3.28).

A
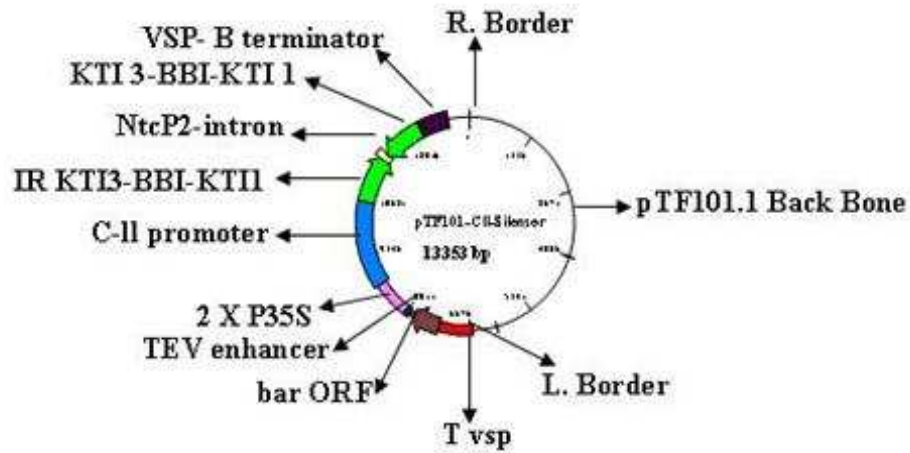
B
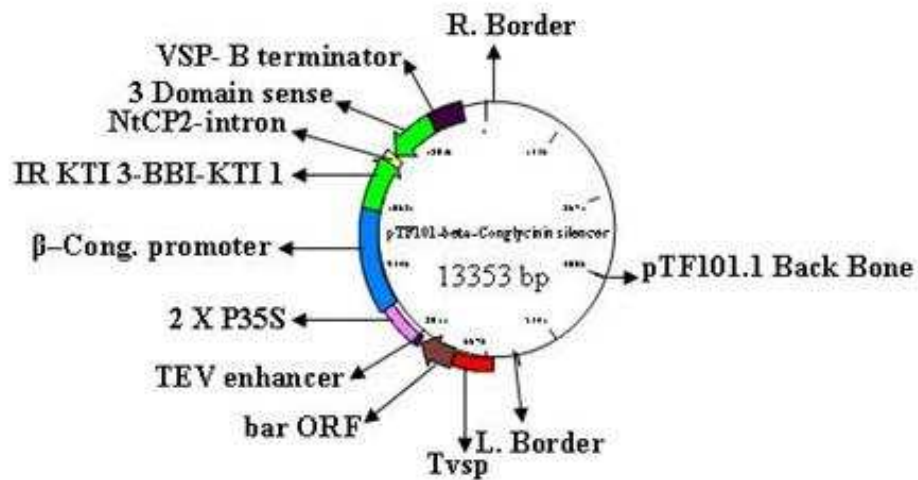
C
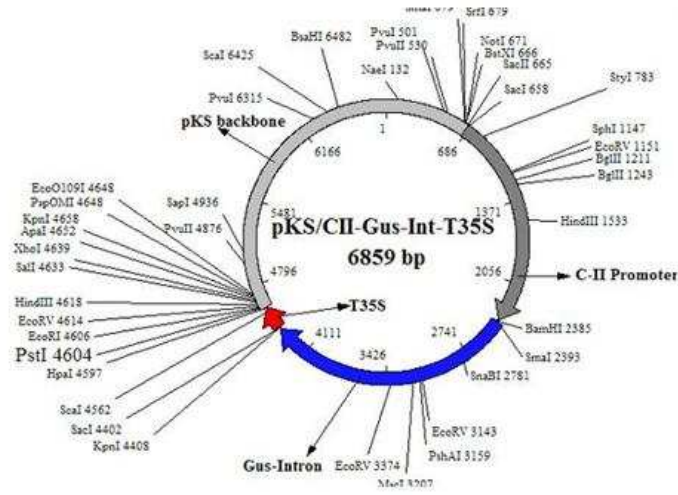
D
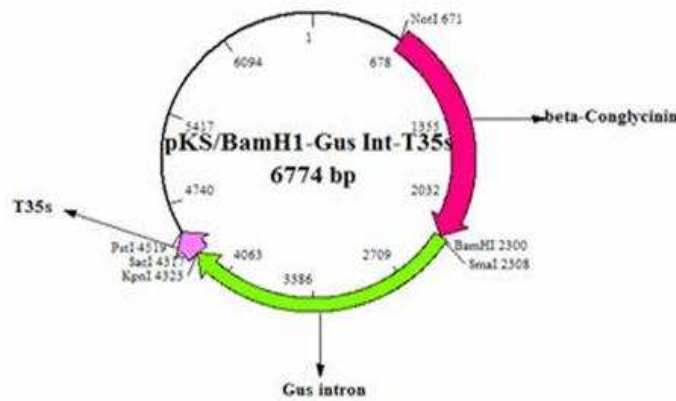
Figure 3.27: Different plasmids for transformation of Agrobacterium EHA 101. (A) C-II silencer construct; (B) β-Conglycinin silencer construct; (C) C-II promoter fused to the *gus*-intron sequence; (D) β-Conglycinin promoter fused to the *gus*-intron sequence.

3.10   Plant transformation

Figure 3.28 shows the different tissue culture steps during transformation of soybean. A major challenge experienced was preventing bacterial growth in particular during shoot regeneration, where Agrobacterium over-grows the explants resulting in death of the explant.
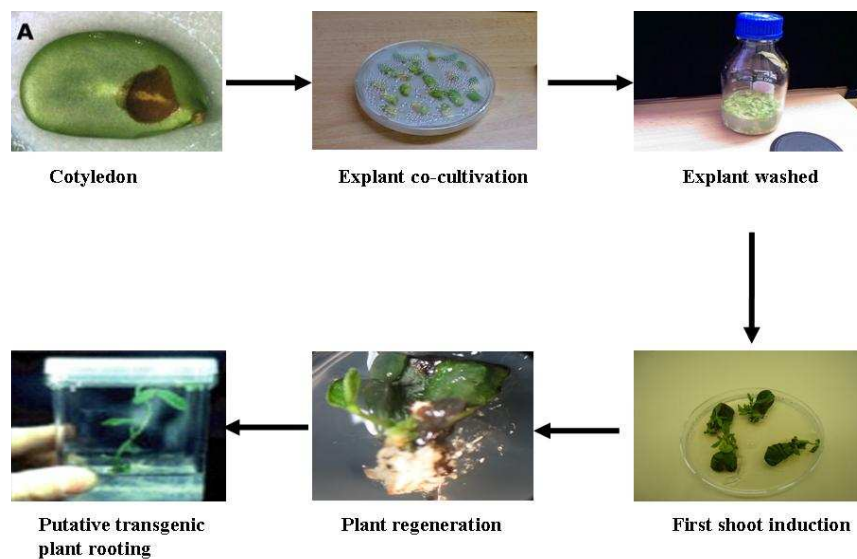
Figure 3.28: Different tissue culture steps involved in soybean transformation.

3.11   Detection of Gus expression

The histochemical assay of Gus activity was performed to analyse the activity of the two promoters (β-Conglycinin and C-II promoter). In contrast to the *gus* gene driven by the C-II promoter, which showed no transient Gus expression, the *gus* gene driven by the β-conglycinin promoter was expressed in cotyledons of the soybean cultivar Williams detectable as spots of transient Gus expression. (Figure 3.29). Further, the highest transient expression was obtained when an Agrobacterium solution with an optical density of 0.9 at 600 nm was used for infection (Figure 3.29).

Control	OD 0.7	OD 0.9

Zone where transient
expression of Gus occurs
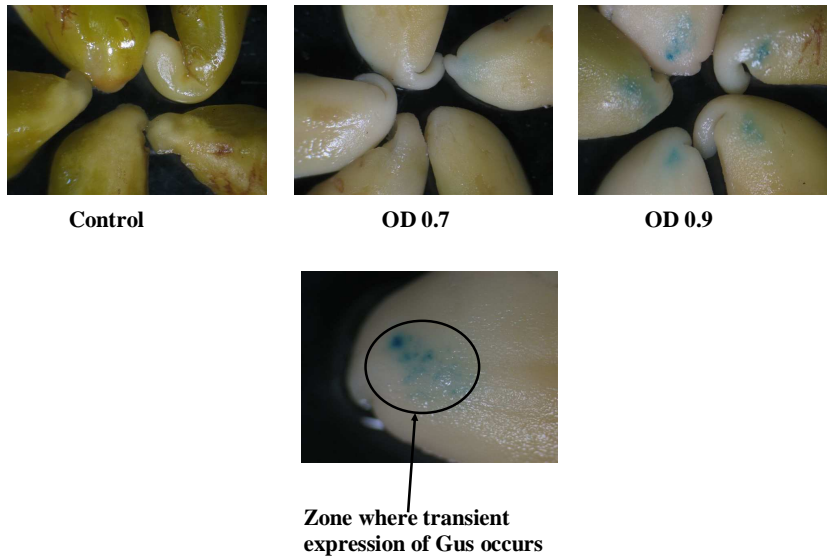
Figure 3.29: Transient Gus expression in soybean's cotyledons of the cultivar Williams after three days of co-cultivation with *Agrobacterium*. OD represents an optical density of Agrobacterium.

3.12   Screening of local cultivars

Different local soybean cultivars were evaluated against a commonly used cultivar, such as cultivar Peking, for Agrobacterium transformation. When the criteria of target tissue amenability to Agrobacterium infection and T-DNA uptake and transient Gus expression was used, Cultivar SNK 400 was determined to be more amenable to T-DNA transfer and expression. However, SNK 400 responded badly to the tissue culture process, because the target tissue has shown to be too sensitive to selection pressures of transformation and regeneration in earlier experiments. Therefore cultivar PAN 512 cultivar was selected for all subsequent transformation experiments due its regeneration potential although explants of this cultivar showed low transient Gus expression (Figure 3.30).

A

B
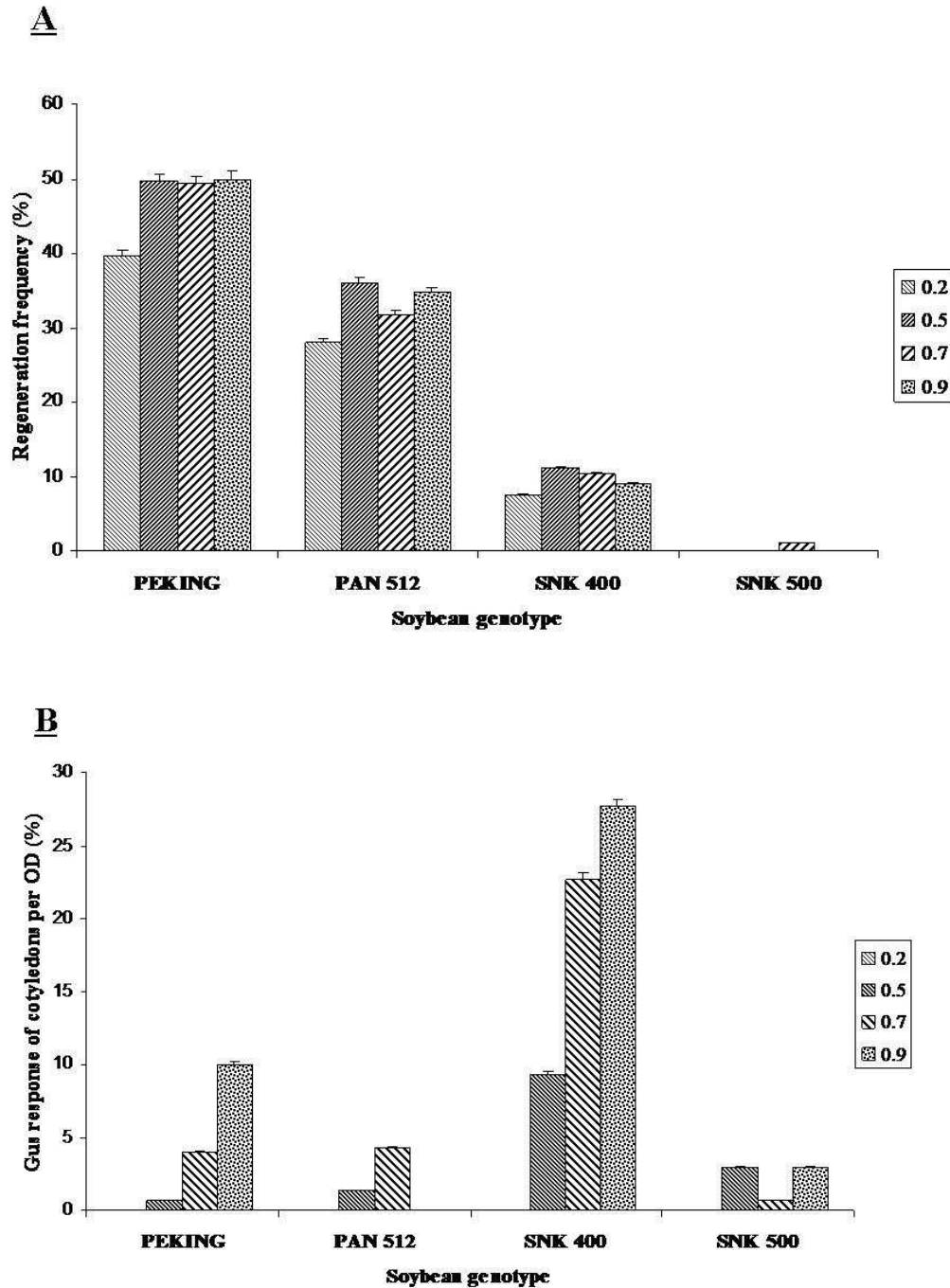
Figure 3.30: (A) Regeneration frequency of 4 different soybean genotypes transformed with C-II-Gus-T35S construct. (B) Number of Gus expression spots after co-cultivation of explants from four different soybean genotypes with an Agrobacterium solution using different optical densities (box on the right). Bars represent the mean (n=12) ±SE of the mean.

3.10   Detection of C-II-Gus-T35S construct into the soybean genome

Due to the challenges with Agrobacterium over-growing explants during the regeneration process only two putative transformed plants were obtained which were grown in the greenhouse for seed production (Figure 3.31). Integration of the C-II-*gus*-T35S into the plant genome of the two plants was confirmed by PCR analysis using a 20 bp primer covering the C-II promoter region.

A

B

Figure 3.31: Putative transformed plant and shoots that were analysed by PCR to detect the integration of C-II-*gus*-T35S into the soybean genome. (A) Putative

transformed plants and (B) shoots that were resistant to glufosinate addition to the tissue culture medium.

Figure 3.33 shows amplification of the C-II promoter region by PCR analysis using the genomic DNA isolated from the two putative plants. An amplification product with the expected size of 768 bp could be detected. Seeds obtained after self-fertilization of the two putative transformed plants were further analysed for *gus* gene expression using a histochemical assay. Some light blue regions could be detected in cotyledons of putative transformed seeds after 24 hrs incubation in a Gus buffer at 37°C (Figure 3.32 A). However, expression has to be confirmed in future with more sensitive enzymatic tests for Gus activity or Western blot analysis. As expected, leaf tissue of putative transformed plants did not show any Gus activity (Figure 3.32 C).

A

Gus positive seeds from
transformed plant

B

Gus negative seeds from
an untransformed plant

C

Gus negative leaf from
transformed plant

Figure 3.32: T1 seeds and leaf tissue that were analysed for Gus activity. (A) Gus-stained seeds from a putative transformed plant; (B) Gus-stained seeds from an untransformed plant; (C) Gus-stained leaf tissue from putative transformed plant.
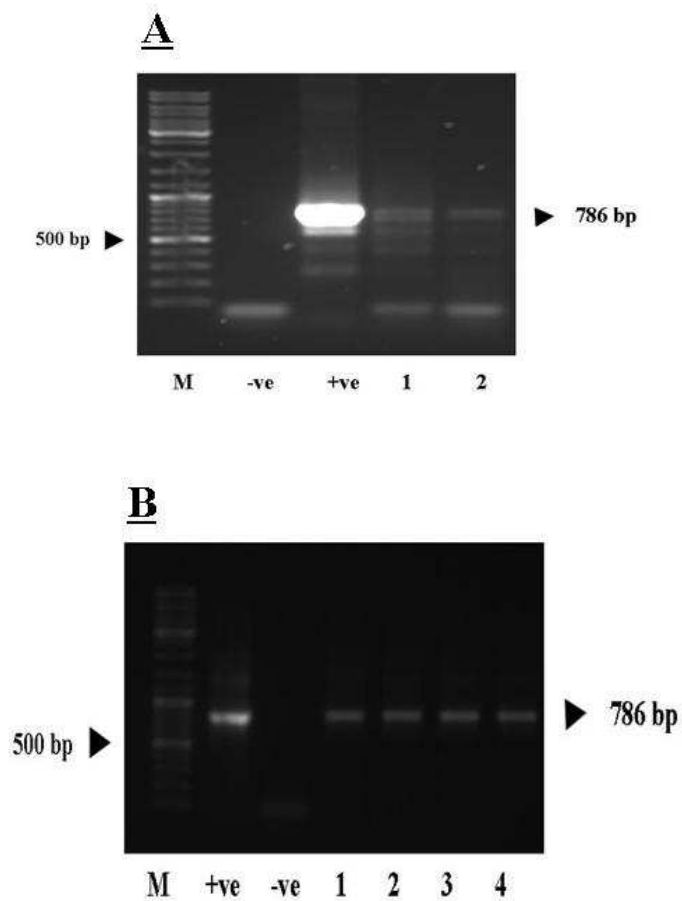
Figure 3.33: PCR analysis of the two T0 putative transformed plants grown in the green-house and shoots that were resistant on glufosinate-containing selection medium. (A) M represents a DNA marker; lane +ve represents positive C-II-*gus*-intron-T35S DNA plasmid control; lane -ve DNA represents control and lanes 1-2 represent C-II promoter coding region amplified fragments from genomic DNA with the expected fragment size of 786 bp. (B) M represents a DNA marker; lane +ve represents positive DNA plasmid control; lane -ve DNA represents control and lanes 1-2 represent C-II promoter coding region amplified fragments from genomic DNA with the expected fragment size of 786 bp.

Figure 3.34 shows four plants that were produced form seeds after self-fertilization of one of the two putative transformed plants (Figure 3.31).

Figure 3.34: Plants produced from seeds after self-fertilization of a putative transformed plant.

When a PCR analysis was performed utilising the following primers: 5'-TCGCGAGAGGAATGAAGAGT-3' as a forward primer and 5'-CGGCTTCAAATGGCGTATAG-3' to amplify the C-II promoter region two plants tested positive and a product of 768 bp could be amplified from isolated genomic DNA of these two plants (Figure 3.35). Furthermore, when a PCR analysis was performed with the following primers: 5'-GCAAATGCTGTGCCAGGCAGTTT-3' as a forward primer and 5'-CCTGTAAGTGCGCTTGCTGAGTT-3' as a reverse primer amplifying the *gus* gene these two plants also tested positive showing an amplification product for the *gus* gene of 1200 bp.

A

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Genomic DNA

B

500 bp ▶

768 bp ▶

M   +ve   -ve   1   2

C

500 bp ▶

1200 bp ▶

M   +ve   -ve   1   2
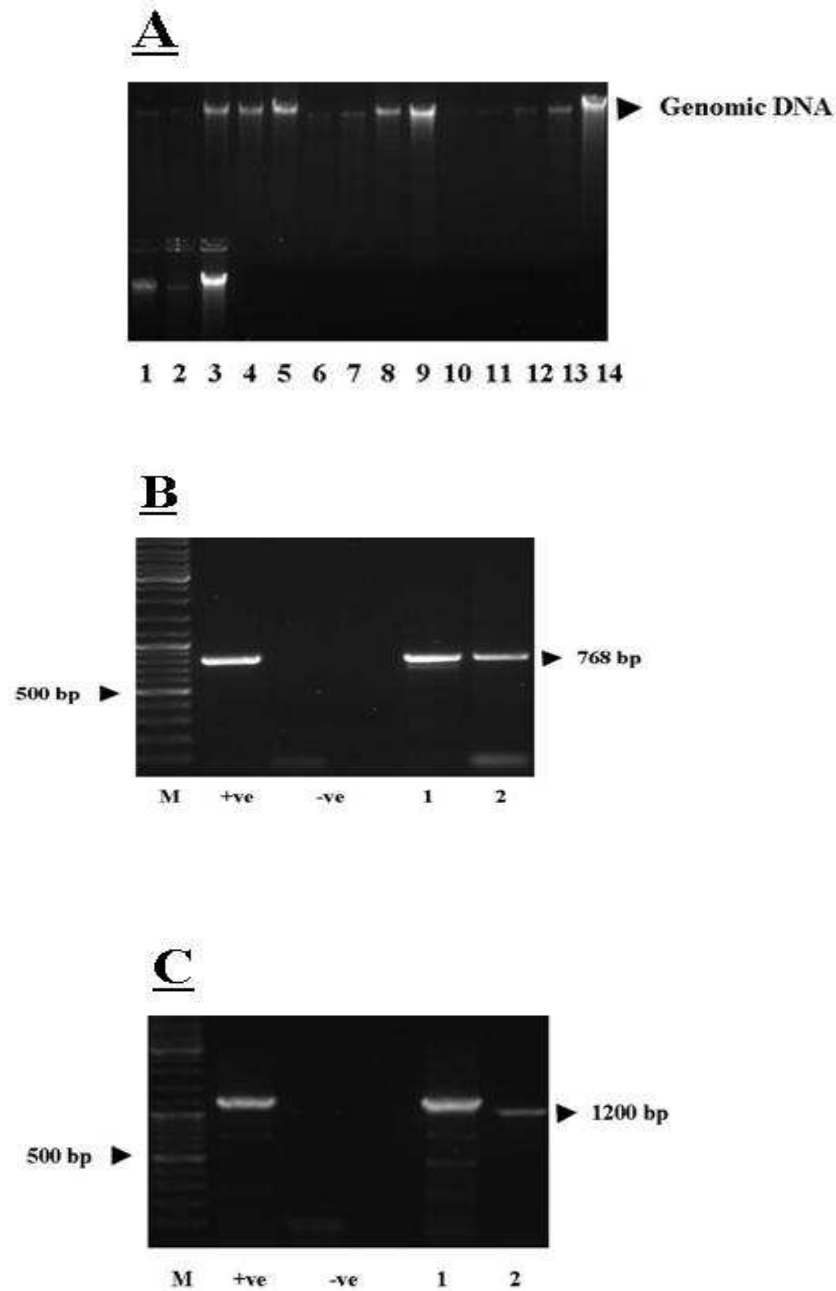
Figure 3.35: Amplification of the C-II promoter from genomic DNA isolated from leaf tissue of transformed plants. (A) Genomic DNA isolated from leaf tissue of transformed soybean; (B) PCR analysis of the two plants using C-II primers and (C) PCR analysis of the two plants using *gus* gene primers. M represents a DNA marker, lane +ve represents a positive DNA C-II-Gus-T35S plasmid control; lane -ve

represents a negative DNA control; lanes 1-2 represent C-II promoter and gus-intron

coding region amplified fragments from genomic DNA with the expected fragment

size of 786 bp and 1200 bp, respectively.

CHAPTER 4

Discussion

Soybean seeds are rich source of food for humans and animals having a protein concentration of up to 40%. However, 6% of the soluble proteins in the seeds are proteinase inhibitors and trypsin inhibitors function as storage of sulphur in the form of sulphur amino acids. In spite of the above mentioned advantage, trypsin inhibitors are anti-nutritional factors (Roebuck *et al.,* 1985; Liener and Hasdai, 1985) in the sense that they cause negative effects when raw grain or cake is consumed in substantial amounts. Using high levels of BBI Liener *et al.* (1982) found that BBI infusion into the human duodenum was associated with increased secretion of trypsin, chymotrypsin, and elastase. Further, a concentration of 0.5% caused a pancreatic enlargement and growth depression in birds and rats (Gallaher and Schneeman, 1986). This led to the hypothesis that BBI could be harmful to human health and nutritionists therefore considered BBI as an antinutrient (Liener *et al.*, 1988). Inactive BBI and Kunitz-type inhibitor genes were therefore designed in an effort to produce soybean varieties with no or low levels of BBI. To balance the loss of sulphur amino acids provided by the inhibitors, Jung *et al.* (1997) expressed an albumin protein from Brazil nut (*Berthollettia excelsa*), which is rich in sulfur amino acids but less than the amount found in BBI.

Genetic manipulation using antisense technology has been further suggested to limit BBI inhibitory activity (Domoney *et al.*, 1995) although there is no report about the application of the technology in soybean. In this study, the tools have been established to use the RNAi technology to decrease expression of serine protease inhibitors (Kunitz and Bowman-Birk-type) in soybean seeds which might add value to soybean

seed cake. Bioinformatic tools and public data bases were in particular used to facilitate the design and construction of appropriate constructs for the adoption of the RNAi approach.The conserved genetic region within members of the plant inhibitor families was targeted with the designed RNAi constructs (Waterhou *et al.*, 1998). The major trypsin inhibitors targeted were identified using the NCBI and EMBL databases and seed specific promoters were isolated and cloned that will be used to drive the expression of selected conserved regions of trypsin inhibitors hairpin constructs in seeds. Seed specific down-regulation of the target proteinase in soybean was selected to avoid interfering with possible essential physiological processes of the plant during the plant growth phases. Serine proteinases are known to be involved in various plant development processes (Laskowski and Kato, 1980). Reduction of proteinase inhibitors expression will be ultimately accomplished by introducing the RNAi silencing constructs designed and produced in this study into soybean through plant transformation.

Traditionally, inhibition of serine proteinase inhibitor activity has been addressed by heat treatment during food and feed processing. Heat does reduce proteinase inhibitor activity; however, the activity is restored over time and heat treatment is also expensive (Friedman and Brandon, 2000). Using a higher temperature destroying all inhibitor activity would denature essential amino acids, such as cysteine, methionine and aspartic acid. A different approach to lower inhibitor activity involves traditional breeding. This has resulted in the development of cultivars with lower serine proteinase inhibitor content (Maggo *et al.* (1999). However, to my knowledge there is no commercial soybean varieties available yet that are devoid of both types of serine proteinase inhibitors (Kunitz and Bowman-Birk-type).

However, a concern about lowering the expression of these inhibitors in food might be that nutritional biochemists have recently recognized that serine proteinase inhibitors might also be helpful for human health (Losso *et al.*, 2008). *In vitro* studies in human have demonstrated that Bowman-Birk trypsin inhibitors, despite being antinutrient, are also anticarcinogens having irreversible effects on cancer cells (Ekrami *et al.*, 1995, Kennedy, 1998, Koroma and de Juan, 1994). They further reduce the risk of coronary heart disease (Evans *et al.* 1992) and can also increase lifespan (Kennedy 1998). Since there is accumulating evidence that plant proteinase inhibitors are significant components of human food, the evidence that these inhibitors constitute mainly a hazard to human health might be presumptive and should be placed in perspective in relation to the level of total proteinase inhibitors consumed in the overall diet. A thorough future investigation about the advantages and disadvantages of engineered soybean would therefore be required in case seed cake would be available as an outcome of this study with lowered serine proteinase inhibitor content.

A further concern might be that resistance against in sects will be affected in soybean when the serine proteinase inhibitor amount has been lowered in seeds. It is well documented that proteinase inhibitors function in plants as a defence mechanism against seed-eating insects due to their antidigestive properties (Ryan 1990). They block protein digestion which results in starving of pests for essential amino acids (Broadway, 1996). *Helicoverpa armigera* is a polyphagous lepidopteron insect pest (Giri *et al.,* 1998) attacking soybean including developing soybean pods. A Kunitz-type trypsin inhibitor from chickpea, which is a representative of a novel protein sub-

family, has been recently identified to be active against *Helicoverpa armigera* (Srinivasan *et al.*, 2005). In addition, serine proteinase inhibitors are known to enhance the activity of the insect control protein derived from *Bacillus thuringiensis* (Bt). Genetically improved tobacco plants expressing a serine proteinase inhibitor fused to a truncated Bt protein showed Bt activity enhancement similar to those found with the purified proteinase inhibitor although the proteinase inhibitor had no insecticidal activity when tested alone (MacIntosh *et al.*, 1990). These studies clearly indicate that a thorough investigation would be required to evaluate any compromise in insect resistance when sufficient transformed soybean material will available with lowered serine proteinase inhibitor content.

To achieve the goal of silencing serine proteinase (Kunitz and Bowman Birk-type) inhibitors, in this study a soybean transformation protocol was established that could be used to routinely integrate genes into the soybean genome. Two major methods of DNA delivery are currently utilized to transform soybean. One is particle bombardment of embryogenic tissue (Tick *et al.*, 1998; Olhoft *et al.*, 2003; Droste, 2002). Other explants, such as proliferative embryogenic tissue, epical meristem and leaves, have also been used to transform soybean using the biolistic approach with high efficiency (Christou *et al.,* 1988; Rech *et al*., 2008). However, the biolistic technique often requires a prolonged tissue culture period to prepare target tissues and often results in complex insertion patterns of transgenes into the plant genome. A second often used method is *Agrobacterium*-mediated transformation of plant tissues (Olhoft *et al.*, 2003; Olhoft and Sommer, 2001). Agrobacterium-mediated transformation offers several advantages. This includes defined integration of transgenes, preferential integration into transcriptionally active chromosomal regions,

and potentially a single low copy number with relatively rare rearrangements. However, Agrobacterium strains differ with respect to their capacity to transform tissues. For example, the hyper-virulent strain EHA105 has been used for soybean transformation (Liu *et al*., 2004) and stable transformation of soybean using this strain has been reported (Paz *et al.,* 2006). Transformation using this strain has resulted in a significant increase in shoot production of cotyledonary node transformation. However, Agrobacterium strain EHA 101 was selected for transformation in this study. EHA 101, which is also hyper-virulent, has been previously used for soybean transformation by Paz *et al*. (2005) and the outlined protocol was exactly followed in this study.

Transformability using Agrobacterium is further generally defined using various criteria. These are independent of each other in scope and relevance. Two mostly used determinants are target tissues' ability to go through tissue culture and subsequently regenerate a plantlet(s) and target tissues amenability to Agrobacterium infection, T-DNA uptake into its genome, and its ability to express foreign genes. Other factors relate to the target tissues sensitivity to selection pressure especially at inititial stages of transformation when there are still few cells expressing introduced genes (Droste et al., 2002). In this study, the cultivar responses of elite varieties of soybean to *Agrobacterium* mediated transformation using the *gus* a marker gene were evaluated using two model soybean cultivars (Peking and Williams) and 6 local elite varieties PAN 512, PAN 421 R (high oil content), PAN522 R (high protein content), SNK440, SNK500 and 5409 RG which is a popular cultivar in South Africa. The genotype that was noted to be most amenable to *Agrobacterium* infection in this study was SNK 400. However, SNK 400 responded badly to the tissue culture process. The target

tissue was found to be very sensitive to selection pressures during transformation and regeneration in earlier experiments. Therefore, cultivar PAN 512 was selected for all subsequent transformation experiments in this study due its regeneration potential although explants of this cultivar showed low transient Gus expression.

The effect of the pH of the co-cultivation medium on transformation efficiency is further well documented (Mondal *et al.*, 2001). In this study, a pH of 5.4 for *Agrobacterium* co-cultivation was used. This pH value was previously reported to be the most efficient (Paz *et al*., 2005). Other researchers have also found that a pH of 5.5 is optimal for transforming soybean (Shrivasta *et al*., 2001; Husnain *et al.*, 1997). An acidic pH of 5.5 is generally considered to be suitable to induce the virulence genes (Shrivasta *et al.*., 2001; Husnain *et al.*, 1997). Further, acetosyringone, which was also used in this study, is known to activate the virulence genes of the Ti plasmid at a pH of 5.0-5.5 and to initiate the transfer of T-DNA.

Although high levels of antibiotics were used in the shoot regeneration process after Agrobacterium co-cultivation, Agrobacterium over-growth was a major problem in this study. Only two putative transgenic plants were ultimately recovered and confirmed to be transformed using PCR and GUS expression analyses. All other explants did not survive due to infection. The soybean transformation process developed so far clearly needs to be further optimized. Improvements of the transformation process might also include optimizing the co-cultivation temperature. In this study we used 22°C for co-cultivation. Optimizing the temperature has been shown to improve *Agrobacterium*-mediated gene transfer to plant cells. Sunilkumar *et al.* (2001) found that co-cultivation of cotton cotyledon discs at 21°C instead of 25°C

degrees consistently resulted in a higher transformation frequency. Dillen *et al.* (1997) also found that the temperature used in the transformation process has an important role in *Agrobacterium tumefaciens* transformation. In their study the best transformation efficiency was obtained at 22°C with both *Phaseolus acutifolius* and *Nicotiana tabacum* irrespectively of the type of Agrobacterium strain used. Further optimization of the transformation process also includes efficient rooting since several shoots could not be rooted and died.

Overall, the soybean bean transformation method reported in this study could potentially be used to make other modifications in soybean that are related to the improvement of grain quality or even plant performance attributes. The already transformed plants that were generated as part of this study might be already used a basis for preliminary investigations on the consequences of serine proteinase inhibitor down-regulation.

Media

Media

5g/L Yeast extract, 10g/L Peptone, 5g/L NaCl2, 12g/L Bacto-agar. pH to 7.0 with NaOH.

YEP Liquid medium

5g/L Yeast extract 10g/L Peptone, 5g/L Nacl2. Ph to 7.0 with NaOH.

Co-cultivation Medium

1/10X B5 major salts, 1/10 B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30g/L Sucrose, 3.9 g/L MES, and 4.25 g/L Noble agar (pH 5.4). filter sterilized 1X B5 vitamins, GA_3 (0.25mg/L), BAP (1.67mg/L), Cystein (400mg/L), Dithiothrietol (154.2mg/L), and 40mg/L acetosyringone are added to this medium after autoclaving. Pour into sterile 100x15mm plates. When solidified, overlay the co-cultivation medium with sterile filter paper to reduce bacterial overgrowth during co-cultivation (Whatman #1,70mm)

Infection Medium

1/10X B5 major salts, 1/10 B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30g/L Sucrose, 3.9 g/L MES (pH5.4) filter sterile 1X B5 vitamins, GA_3 (0.25mg/L), BAP (1.67mg/L), and 40mg/L acetosyringone are added to this medium after autoclaving.

Shoot induction Washing Medium

1/10X B5 major salts, 1/10 B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30g/L Sucrose, 3.9 g/L MES (pH5.4) filter sterile 1X B5 vitamins, BAP (1.11mg/L), Timentin (100mg/L), Cefotaxine (200mg/L), and Vancomycin (50mg/L) are added to this medium after autoclaving.

Shoot induction Medium I

1/10X B5 major salts, 1/10 B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30g/L Sucrose, 3.9 g/L MES (pH5.4) filter sterile 1X B5 vitamins, BAP (1.11mg/L), Timentin (100mg/L), Cefotaxine (200mg/L), and Vancomycin (50mg/L) are added to this medium after autoclaving. Pour into sterile 100x20 mm plates (26 plates/L).

Shoot induction Medium II

1/10X B5 major salts, 1/10 B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30g/L Sucrose, 3.9 g/L MES (pH5.4) filter sterile 1X B5 vitamins, BAP (1.11mg/L), Timentin (100mg/L), Cefotaxine (200mg/L), and Vancomycin (50mg/L) and Glufocinate 6mg/L) are added to this medium after autoclaving. Pour into sterile 100x20 mm plates (26 plates/L).

Shoot Elongation Medium

1⁄2 10X B5 major salts, 1⁄210 B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30g/L Sucrose, 0.59 g/L MES (pH5.7) filter sterile 1X B5 vitamins, BAP (1.11mg/L), Timentin (100mg/L), Cefotaxine (200mg/L), and Vancomycin (50mg/L) and Glufocinate (6mg/L) are added to this medium after autoclaving. Pour into sterile 100x20 mm plates (26 plates/L).

Rooting medium

1⁄2 10X B5 major salts, 1⁄210 B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30g/L Sucrose, 0.59 g/L MES (pH5.7) filter sterile 1X B5 vitamins, BAP (1.11mg/L),

Timentin (100mg/L), are added to this medium after autoclaving. Pour into sterile

magentas cups (26 plates/L).

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