Optimization of Agrobacterium-Mediated Genetic Transformation of Soybean Using Glufosinate as a Selective Agent.

Shaomian Yao

Louisiana State University and Agricultural & Mechanical College

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OPTIMIZATION OF AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION OF SOYBEAN USING GLUFOSINATE AS A SELECTIVE AGENT

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Agronomy

by

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May, 2001

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ABSTRACT

Soybean [Glycine max (L.) Merr] is one of the most important oil crops. Genetic transformation techniques can provide new tools for soybean improvement. The production of transgenic soybean has been limited. This research integrated a modified soybean regeneration system into a transformation protocol to optimize the production of transgenic soybean.

Soybean hypocotyls and cotyledonary nodes were cultured on Gamborg B5 medium containing thidiazuron (TDZ). TDZ at 0.16 μM was found superior to other concentrations for inducing multiple shoot formation. Explants isolated from seedlings germinated on medium containing 6-benzyl-aminopurine (BAP) greater than 7 μM produced more multiple shoots than when on lower concentrations of BAP. Elongation of shoots was achieved on Gamborg B5 medium containing 0.36 μM BAP for hypocotyl-derived, or containing 0.58 mg/l gibberellin acid (GA₃) and 0.67 mg/l indole-3-butyric acid (IBA) for cotyledonary node-derived multiple shoots. Plant recovery was achieved on medium consisting of Gamborg B5 with 0.58 mg/l GA₃, 0.67 mg/l IBA, 2.0 % sucrose, and 7 g/l phytagar.

Transgenic soybean plants were obtained with Agrobacterium-mediated transformation using glufosinate as a selective agent. Nodes with 1/3 cotyledons inoculated with Agrobacterium KYRT1 and subjected to vacuum infiltration during inoculation and 4 mg/l glufosinate in the selection medium, produced more glufosinate-resistant multiple shoots than other treatments. The optimal duration of vacuum infiltration was 10 minutes at 508 mm Hg. A glufosinate concentration of 1 to 1.2 mg/l was necessary to select transgenic shoots. Recovered plants were screened with a 0.3 ml/l solution of Liberty herbicide. PCR and southern hybridization analysis confirmed
transformation. Progeny tests using herbicide leaf painting assay, PCR, and RT-PCR analysis, indicated that the transgene was transmitted to and expressed in the next generation.

The effects of additional copies of \textit{vir}E and \textit{vir}G genes in \textit{Agrobacterium} and activation of \textit{vir} genes with acetylsyringone on plant transformation were also studied. A significant enhancement was observed when additional copies of \textit{vir}E and \textit{vir}G genes were included in the \textit{Agrobacterium} for transformation of \textit{Arabidopsis} but not for soybean. Activation of \textit{vir} genes with acetylsyringone (100\textmu M) increased the glufosinate-resistant multiple shoot formation rate in soybean, and reduced the rate in \textit{Arabidopsis}.
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Soybean \( [Glycine \textit{max} \text{ (L.) Merr.}] \) is one of the most important edible oil and protein crops in the world. During the past half century, soybean has become a major grain crop in the United States, ranked third in production after corn and wheat, and second in value after corn. The U.S. accounts for 43% of the world soybean production. In 1999, the total production was approximately 2643 million bushels (United Soybean Board 2000).

Genetic variation is the basis of plant improvement programs. The plant breeder’s task is to identify heritable variation and concentrate genes for desirable characteristics into a cultivar. The most widely used technique in creating genetic variation is to sexually cross genetically different parents. Through random gene recombination, the desired genes may be combined into a particular individual. Since the recombination involves thousands of genes, both desirable and undesirable, concentration of desirable genes into one plant is not easy in practice.

Nevertheless, soybean yields have increased at an annual rate of about 21 kg/ha with most of the increase (50%-80%) due to genetic improvement (Fehr 1984). A limitation of this technique is that it is based on the sexual hybridization of parents, which usually restricts the use of a gene source to within the same species. Plant genetic transformation can circumvent this limitation by enabling geneticists that have identified desirable traits and cloned specific genes from other organisms, to introduce these genes into different plant species. A few steps are essential for the production of
transgenic plants. First, foreign DNA needs to be delivered into plant cells. Second, the cells, in which the foreign DNA has been integrated, need to be identified. Finally, plants need to be regenerated from cells which have incorporated the foreign DNA.

Several methods have been used in the delivery of DNA into plants, including polyethylene glycol (PEG) treatment (Datta et al. 1990), electroporation (Potrykus et al. 1985, Fromm et al. 1985), particle bombardment (Klein et al. 1987, Stewart et al. 1996) and Agrobacterium tumefaciens-mediated transformation (Chilton et al. 1977). The Agrobacterium tumefaciens-mediated DNA transfer is the most widely used method for the introduction of new genes into plants. Transgenic plants have been obtained from a number of plant species, including both dicot and monocot species with this method (Gheysen et al. 1998).

Dissertation Overview

Dissertation Research Objectives

Generally, Agrobacterium based transformation methods require a series of tissue culture processes to regenerate a plant from the transformed cell. Transformation success can depend on the ability to regenerate transformed cells. This dissertation describes the production of transgenic soybean using an Agrobacterium-mediated gene delivery technique. The objectives include the improvement of the plant regeneration rate from cultured tissue, development of an efficient transformation and selection procedure, and the study of transgene stability.

Chapter Relationship

An efficient regeneration system is important for a successful transformation protocol. Chapter 2 describes the regeneration of intact plants from soybean hypocotyls and cotyledonary nodes (with 1/3 cotyledon attached). The cotyledonary node with 1/3
cotyledon attached was found to be more sensitive to glufosinate selection. This regeneration system was used in the transformation experiments using glufosinate as a selective agent to produce transgenic soybean (Chapter 3). The improvement of transformation efficiency by incorporating vacuum infiltration, choice of more virulent Agrobacterium strains, and optimization of the selection procedure will also be discussed in Chapter 3. The reliable transmission and expression of transgenes that have been introduced into plants are key requirements for utilization of transgenic plants. Inheritance and expression of the bar gene in transgenic soybean, produced with this transformation protocol, were studied using a herbicide (Liberty) leaf painting assay, the polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR), and are discussed in Chapter 3. The vir genes of the Agrobacterium Ti plasmid play key roles in transferring T-DNA into the plant cells. Chapter 4 describes the introduction of multiple copies of virG or virG/E genes into an Agrobacterium strain and the testing of its transformation capacity in Arabidopsis and soybean.

*Agrobacterium tumefaciens*-mediated DNA Transformation

Particle bombardment and *Agrobacterium tumefaciens*-mediated DNA transformation are two widely used plant transformation techniques. The major strength of particle bombardment is that the gene transfer is species and tissue-independent. The limitations of particle bombardment include low stable transformation rate, low regeneration capacity of target cells (due to tissue injury caused by bombardment) and high cost (reviewed by Barcelo and Lazzeri 1998).

*Agrobacterium tumefaciens*-mediated DNA transformation is a biological means to deliver a well-defined piece of foreign DNA into plant cells. DNA transfer via
*Agrobacterium* has the advantage that the length of the integrated DNA fragment is determined by the border sequences. Other advantages of *Agrobacterium*-mediated transformation are its simple procedure and low cost of equipment (Anna et al. 2000). It is now the most widely used method to transfer genes into plants.

T-DNA transfer from *Agrobacterium* to plant cells involves a series of complex processes. When *Agrobacterium tumefaciens* contacts a wounded plant cell, the virulent (*vir*) genes on the Ti plasmid are transcribed. The expression of the *vir* genes initiates the mobilization and leads to the transfer of the T-DNA into the plant cell. The T-DNA is then integrated into the plant chromosomal DNA. Apart from the border repeats, none of the DNA sequences are required for the process of the T-DNA transfer and integration. By replacing the T-DNA with a gene (or genes) of interest, genes can be transferred into plant cells. The transformed cells can then be developed into transgenic plants, in most cases through a tissue culture process. The choice of tissue and a reliable regeneration system becomes critical in such transformation protocols. Successful production of transformed plants is dependent on which cells are regenerated (Sato et al. 1993).

**The Binary Vector System**

Researchers have found that the T-DNA and the *vir* genes do not have to be in the same plasmid for transfer of T-DNA. This achievement has allowed development of a binary vector system (Figure 1.1) for the transfer of foreign DNA into plants. Two plasmids are used in the binary method, i.e. the Ti plasmid containing the *vir* genes with oncogenes eliminated, a so called 'disarmed' plasmid, and a genetically engineered T-DNA plasmid containing the desired genes (An et al. 1986).
Figure 1.1. Diagram of the binary vector system of Agrobacterium showing two plasmids in a cell: (1) disarmed Ti plasmid; (2) binary vector harboring the gene of interest.

The Roles of vir Genes and T-DNA Transfer

The understanding of the molecular mechanism of T-DNA transfer from Agrobacterium to the plant genome is far from complete. However, it is believed that the expression of the vir gene cluster on the Ti plasmid is one of the key processes during the T-DNA transfer, and 24 vir genes in 9 operons (virA, B, C, D, E, F, G, H, J) have been identified. The VirG protein is a positive transcription factor involved in the activation of all the other vir genes, while VirE is a ssDNA binding protein protecting the nicked T-DNA in the process of transfer to the plant genome (Steck et al. 1989, Han and Winans 1994, Sundberg et al. 1996) (Figure 1.2). Additional copies of the virG gene in Agrobacterium have been shown to enhance the transient transformation of celery, carrot, and rice (Liu et al. 1992).

Phenolic compounds released by wounded plant tissue can serve as a signal to activate or induce the expression of vir gene operons. Several reports have
VirE activates the other vir genes

Signal from a wounded plant cell

Figure 1.2. Schematic diagram of the Agrobacterium infection of a plant cell and the T-DNA transfer process.
demonstrated that acetosyringone induces the expression of vir genes, and enhances transformation efficiency (Ashby et al. 1987, Shaw et al. 1988).

Based on the findings of the key role of vir gene expression in T-DNA transfer, vectors have been made to provide constitutive expression of vir genes in order to enhance transformation efficiency (Hansen et al. 1994, Ishida et al. 1996). The functions of vir genes are listed in Table 1.1

Agrobacterium-mediated Transformation Methods

Regeneration-dependent Transformation

Although a de novo regeneration step is not absolutely required to generate transgenic plants, it forms the basis of the vast majority of transformation procedures. Target cells for transformation and regeneration can be protoplasts, suspension cultured cells, callus cells, or tissue explants. To be useful for Agrobacterium-mediated transformation, the cells must be competent to receive T-DNA, integrate it into chromosomal DNA, and be regenerated into transformed plants.

For many plant species, the lack of a reliable regeneration method is one of the main bottlenecks in developing a transformation protocol. Moreover, a regeneration method is often genotype dependent within the species. Regeneration can be negatively influenced by the presence of Agrobacterium, especially with high concentrations of bacteria and long co-cultivation periods. Furthermore, the antibiotics used to kill Agrobacterium can adversely affect regeneration capacity.

Regeneration-independent Transformation

Regeneration of plants from cells or tissues is labor and technique intensive. An alternative method of Agrobacterium tumefaciens-mediated transformation, termed in planta vacuum infiltration transformation, was proposed by Bechtold et al. (1993), and
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modified by several researchers (Bent et al. 1994) with Arabidopsis. This method is based on the in situ vacuum infiltration of flowering plants with a suspension of Agrobacterium cells containing a binary T-DNA vector. The plant, at flowering stage, is immersed into an Agrobacterium infiltration mixture and placed in a vacuum chamber. The chamber is drawn to a vacuum pressure of 500 mm Hg for 5-20 minutes. The plant is grown to maturity and the seeds are harvested. Bechtold et al. (1993) determined that the bacterium concentration in the infiltration mixture should not be lower than 0.8 OD$_{600}$. This method has only been successful in the transformation of Arabidopsis.

Feldmann and Marks (1987) co-cultivated germinating Arabidopsis seeds with Agrobacterium containing the nptII gene for 24 hours. Transformation was confirmed in the progenies of treated seeds. Seeds germinated for 12 hours before exposure to Agrobacterium gave the highest transformation rate (0.32%), and an imbibition time of less than 9 hours produced no transformants. A similar experiment was carried out by Chee et al. (1989) with soybean, and transgenic soybean was obtained.

Transgene Stability and Expression

A major requirement for the successful application of transgenic plants is the stability and normal Mendelian segregation of the transgenic phenotypes. Nevertheless, the loss of the transgenic phenotype is often observed (reviewed by Meyer 1998). This has been called gene silencing. Gene silencing does not necessarily occur in primary transgenic plants, but can develop in subsequent generations (Kilby et al. 1992, Assaad et al. 1993). Researchers have discovered that both transcriptional and post transcriptional inactivation can cause gene silencing in transgenic plants (Meyer 1998). The transgene silencing events are often associated with the presence of multiple
homologous copies, but single copy transgenes are not excluded from becoming inactive (Meyer 1998). Inactivation of single copy transgenes often follows an increase in DNA methylation (Amasino et al. 1984, Van Slogteren et al. 1984). Kilby et al. (1992) demonstrated that the loss of transgene resistance was associated with methylation of a promoter in *Arabidopsis* transformation. Methylation of cytosine residues located within essential transcription factor binding sites of a promoter can alter transcription levels. It has been shown that methylation of specific cytosine residues reduces or inhibits the promoter activity for several promoters (Muiznieks and Doerfler 1994).

**Agrobacterium-mediated Transformation of Soybean**

**Soybean Tissue Culture**

As mentioned previously, DNA must be introduced into cells that will regenerate into plants for transformation to be efficient and successful. The main factor that has limited transformation of soybean is the response of soybean to tissue culture manipulation. Soybean can be regenerated via two distinct processes: shoot morphogenesis and somatic embryogenesis. Shoot morphogenesis is the process where shoots develop from a source tissue, then are excised and rooted to obtain an intact plant. In embryogenesis, an embryo containing both shoot and root axes is developed from somatic tissue. An intact plant is obtained from germination of the embryo.

In an early regeneration study, Cheng et al. (1980) stimulated shoot formation from the cotyledonary nodes by removing the epicotyl and hypocotyl stem from germinating soybean seedlings which had been germinated on Gamborg B5 medium (Gamborg et al. 1968) containing 10-50 μM 6-benzyl-aminopurine (BAP). Soybean shoot morphogenesis was reported by Wright et al. (1986). They described a system
whereby shoots were obtained *de novo* by culturing cotyledonary nodes of soybean seedlings on MS based medium (Murashige and Skoog 1962) containing BAP. Dan and Reichert (1998) induced multiple-shoots from hypocotyls using medium containing 5 μM BAP. Intact plants were obtained by growth of the excised shoots.

Somatic embryogenesis in soybean was first reported by Christianson et al. (1983). They described a system in which embryogenic tissue was initially obtained from the zygotic embryo axis. Lippmann and Lippmann (1984) described the development of somatic embryos from the cotyledons of immature soybean embryos, but whole plants were not recovered. Recovery of intact plants from somatic embryos has been reported by others (Ranch et al. 1985, Barwale et al. 1986, Komatsuda et al. 1992).

**Agrobacterium-mediated Gene Transfer of Soybean**

Unlike most dicot plants, which are very susceptible to *Agrobacterium* inoculation (Owens and Cress 1985, Byrne et al. 1987). Although *Agrobacterium tumefaciens*-mediated transformation has been successfully used to obtain transgenic soybeans, genetic transformation efficiency remains low and therefore far from routine (Stewart et al. 1996, Meurer et al. 1998, Zhang et al. 1999). Large efforts have been made to develop an efficient *Agrobacterium tumefaciens*-mediated gene transformation system for the genetic improvement of soybean. Hinchee et al. (1988) inoculated cotyledonal nodes with disarmed *Agrobacterium tumefaciens* pTiT37-SE::pMON9749 (kanamycin resistance and GUS) and pTiT37-SE::pMON894 (kanamycin and glyphosate resistance). GUS, kanamycin resistance, and glyphosate tolerance were observed in transgenic plants. McKenzie and Cress (1992) co-cultivated *Agrobacterium tumefaciens* with soybean...
cotyledons from 10 South African varieties. GUS activity was observed in callus and regenerated plantlets. Unfortunately, no complete plant was obtained. Di et al. (1996) co-cultivated soybean cotyledonary nodes with an Agrobacterium tumefaciens suspension harboring the bean pod mottle virus gene. The cotyledonary nodes were transferred to tissue culture medium for plant regeneration. Five transformed plants were regenerated from 400 treated cotyledonary nodes. Three out of the five appeared to be germ line transformants. Transgene was detected in the T1 generation. Using the bar gene as a selectable marker, Zhang et al. (1999) reported that GUS-positive soybean plants were recovered at frequencies ranging from 0.0% to 0.5% under a lower selection regime (glufosinate concentration: 3.3 mg/l at initial selection, 1.7 mg/l during shoot elongation), and 0.0% to 3.0% under a higher selection regime (glufosinate concentration: 5.0 mg/l at initial selection, 2.0 mg/l during shoot elongation).

Chee et al. (1989) developed a transformation method by directly injecting the cotyledonary node area of germinating soybean seeds with Agrobacterium. Enzyme activity of the transferred gene (npt) was detected in both R0 and R1 plants (R0 denotes the first generation of transformation). The transformation rate was about 0.7%. This method did not involve a tissue culture phase.

Several parameters have been found to be important for Agrobacterium tumefaciens-mediated transformation. These include the strain of Agrobacterium tumefaciens, the bacteria concentration, the length of co-cultivation, and the degree of wounding on the plant tissue. In addition, susceptibility of a plant to Agrobacterium tumefaciens can limit the success. Susceptibility depends on the interaction between the plant genotypes and Agrobacterium tumefaciens strains. Some of the Agrobacterium tumefaciens strains found to be virulent to soybean are Z707 (Hepburn et al. 1985, Di et

In Agrobacterium-mediated transformation, wounding of the plant tissue is essential to induce the vir gene expression, which will result in the transfer of T-DNA into a plant cell. Several methods have been used to create a wound site, including injecting (Chee et al. 1989), shallow cutting (Di et al. 1996), and crushing (Parrott et al. 1989).

Tumorigenesis is an index to evaluate the susceptibility of a plant to Agrobacterium. Bailey et al. (1994) studied the tumorigenesis of different soybean cultivars, and concluded that tumorigenesis is a quantitative trait. Mauro et al. (1995) conducted a generation mean analysis of susceptibility, and obtained a similar result for soybean susceptibility to Agrobacterium tumefaciens. Some susceptible soybean genotypes are Peking, PI417138, Hutton, Impala, and Fayette.

**Selectable Markers Used in Soybean Transformation**

Strict selection is essential for an efficient transformation system as the fraction of stably transformed cells is usually small. For example, in a regeneration dependant transformation of Arabidopsis, none of the 172 plants regenerated on non-selective medium were transgenic (De Buck et al. 1998). In transformation of soybean, no transgenic plants were obtained without kanamycin selection when 100 shoots were analyzed (Hinchee et al. 1988). The choice of the selectable marker gene, the selective agent, and its concentration and timing of application are very important. Strict selection of transformed cells is required, however, regeneration should not be impeded. Selective agents include antibiotics, herbicides, and toxic levels of amino acids. A
commonly used selective agent in soybean transformation is kanamycin, though it is inefficient (Meurer et al. 1998, Di et al. 1996). An alternate selective agent used for soybean transformation is glufosinate. Transformation of intact soybean plants was reported by Zhang et al. (1999) using glufosinate as a selective agent.

**Glufosinate Selection and the \textit{bar} Gene**

The \textit{bar} gene was originally cloned from bacterium \textit{Streptomyces hygroscopius} (Murakami et al. 1986). It encodes for phosphinothricin acetyltransferase (PAT) (Thompson et al. 1987) that detoxifies phosphinothricin or glufosinate by acetylating it (Figure 1.3) (Murakami et al. 1986, Thompson et al. 1987). Glufosinate is the active ingredient of the herbicides Liberty and Basta (DeBlock et al. 1987). Therefore, plants expressing the \textit{bar} gene are tolerant to the herbicides Liberty and Basta. Dekeyser et al. (1989) suggested that the \textit{bar} gene is an effective selection marker in plant transformation. Transformation of plants using the \textit{bar} gene as a selectable marker has been studied in several plant species, including rice, maize, and soybean. (D’Halluin et al. 1992, Fromm et al. 1990, Zhang et al. 1999).

Over the last decade, genetic transformation techniques have become an important tool in crop improvement. The development of transformation techniques for soybean has been slow. As the progress in understanding the molecular mechanism of gene transfer and integration, soybean transformation will be improved through optimization of DNA transfer, plant regeneration and selection techniques.
Figure 1.3. The reaction mechanism catalysed by Phosphinothricin acetyltransferase (PAT), PPT is converted to Ac-PPT

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CHAPTER 2

OPTIMIZATION OF PLANT REGENERATION FROM SOYBEAN HYPOCHOTYLS AND COTYLEDONARY NODES

Introduction

One method of genetic transformation via Agrobacterium or bombardment is to regenerate plants from transformed cells or tissues through a tissue culture process. A successful transformation protocol largely depends on a reliable regeneration technique. In soybean genetic transformation protocols, cotyledonary nodes and immature cotyledons have been successfully used to generate transgenic soybean (Parrot et al. 1989, Di et al. 1996). Although the cotyledonary node transformation protocol provides a simple and repeatable method, the efficiency is low in obtaining transgenic soybean plants (Meurer et al. 1998). Improvement of the regeneration rate could be a possible way to increase the transformation efficiency.

Although Townsend and Thomas (1996) demonstrated that T-DNA could be delivered into hypocotyls, regeneration of an intact plant was not achieved. Dan and Reichert (1998) induced multiple shoots from hypocotyls using MSB medium [MS salt (Murashige and Skoog 1962) and Gamborg B5 vitamins (Gamborg et al. 1968)] containing 5 μM 6-benzyl-aminopurine (BAP). They reported that 1.0 to 5.0 shoots were obtained per cultured explant when 13 soybean genotypes were tested. This chapter presents results on improved regeneration rates from soybean cotyledonary nodes and hypocotyls. A concentration of 0.16 μM thidiazuron (TDZ) was found to be efficient for inducing multiple shoot formation from hypocotyls and cotyledonal...
nodes, and multiple shoots were easily recovered into intact plants. Seed germination and plant recovery media were optimized to increase the regeneration efficiency.

**Materials and Methods**

**Induction of Multiple Shoot Formation and Plantlet Recovery**

Soybean cultivar Peking was used as a model cultivar to conduct all investigations. Peking has been shown to be highly sensitive to *Agrobacterium* (Bailey et al. 1994). Seeds were sterilized by dipping in 70% ethanol for 1 min, transferred to 30% Clorox solution for 20 minutes, and then washed four times with sterile distilled water. About 20 seeds were placed in each 100 x 15 mm petri dish with 10 ml of MSB (MS salt + Gamborg B5 vitamins) liquid medium supplemented with BAP, and incubated at 28 °C in the dark.

**Culture of hypocotyls:** The hypocotyl segments were isolated by cutting below the cotyledonary nodes and leaving 3-5 mm of the hypocotyl from 6-7 day old seedlings (Dan and Reichert 1998) (Figure 2.1a, d). The segments were plated dorsal side down on multiple shoot induction medium consisting of Gamborg B5 basal nutrients, 0.59 g/l 2[N-morpholino] ethan esulmonic acid (MES), 30 g/l sucrose and 0.16 μM TDZ if not otherwise specified. The plates were cultured in a 28 °C incubator with fluorescent light (2300 Lux) for 3-4 weeks to induce multiple shoots (Figure 2.2 a,b). The multiple shoots were excised from the hypocotyls as clusters (not separated), and transferred to shoot elongation medium containing Gamborg B5 basal nutrients, 0.36 μM BAP, 30 g/l sucrose, solidified with 8 g/l agar, and adjusted to pH 5.8 (modified from Dan and Reichert 1998) (Figure 2.3 a). The number of shoots per hypocotyl was recorded after an additional 3 weeks of culture. Individual shoots were excised, and sub-cultured at 3-

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Figure 2.1. Preparation of soybean explants: (a) diagram of a soybean seedling, (b) cotyledonary nodes with entire cotyledon, bar = 0.7 cm, (c) cotyledonary nodes with 1/3 cotyledon, bar = 0.7 cm, (d) hypocotyls, bar = 0.5 cm
Figure 2.2. Multiple shoot formation from cultured soybean explants: bars = 1 cm: (a) multiple shoots on hypocotyls after 2 weeks in culture, (b) multiple shoots on hypocotyl after 4 weeks in culture; (c) multiple shoots (arrowhead) on cotyledonary node after 2 weeks in culture, (d) multiple shoots after 6 weeks of culture before separating and transfer to elongation medium.
Figure 2.3. Recovery of soybean plants from multiple shoots: (a) multiple shoots were excised from the explants and grown on elongation medium, (b, c) elongated multiple shoots were separated individually and plated on plant recovery medium, (d) plant recovery from elongated shoots in baby food jars.
4 week intervals on the plant recovery medium until plantlets had 2-3 triplet-leaves and healthy roots. The plant recovery medium consisted of Gamborg B5 salt and vitamins, 0.58 mg/l GA$_3$, 0.67 mg/l IBA, and sucrose (modified in this experiment, see experimental design), and solidified with gelling agents (modified in this experiment, see experimental design).

**Culture of cotyledonary nodes:** Cotyledonary nodes were prepared from 3-4 day old seedlings by conducting the three cuts as described below. Seedlings were first cut on the cotyledon to remove a 2/3 portion of the cotyledon, and then excised on the hypocotyls 3 mm below the nodes. Finally, the explants were bisected between the two cotyledons, and the original apical shoots were completely removed. This operation yield two identical explants as shown in Figure 2.1c. The cotyledonary nodes (10 explants per dish) were placed on multiple shoot induction medium containing 0 to 0.32 μM of TDZ, and cultured at 28 °C with fluorescent light (3050-3500 Lux). After 10 days, any shoots that were produced from the nodes were excised and discarded. The remaining cotyledonary nodes were transferred to a fresh dish containing the same medium and returned to the incubator to induce multiple shoot formation (Figure 2.2 c, d). This operation ensured the removal of all pre-existing shoots or buds from the node area. After about an additional 3 weeks of culture, multiple shoots produced from the nodes were excised, and transferred to plant recovery medium consisting of Gamborg B5 salt and vitamins, 0.58 mg/l GA$_3$, 0.67 mg/l IBA, and 2% sucrose. When the shoots became approximately 0.5 cm long, the individual shoots were separated from the cluster (Figure 2.3 b, c), and sub-cultured on the same medium at 3-4 weeks intervals in
100 x 65 mm petri dishes. Plantlets 2 cm in length were transferred to 170 ml baby food jars (98 mm height) each containing 50 ml plant recovery medium for further growth. Plantlets with 2-3 triplet leaves and healthy roots (Figure 2.3d) were transplanted into soil.

**Experimental Design and Treatments**

**Effect of germination medium on multiple shoot formation rate:** The concentration of BAP in seed germination medium was varied to determine its effect on multiple shoot formation. Seeds of cultivar Peking were sterilized as previously described, and germinated on medium containing BAP at 0, 1, 3, 5, 7, or 9 μM for 6 days. The hypocotyls were isolated and cultured on medium consisting of Gamborg B5 basal, 0.16 μM TDZ, 0.59 g/l MES, 30 g/l sucrose, 2 g/l gelrite (Gelrite is a registered trademark of Monsanto Company, and sold under the named Phytagel by Sigma) and the pH adjusted to 6.0 before autoclaving. The experiment was repeated three times on different days, and arranged in complete randomized block design (RBD) with days as blocks. Each treatment sample was represented by 20-25 hypocotyls in 3 blocks (i.e. 3 different days). The block was analyzed as a random variable with SAS proc GLM (SAS Institute, Cary, NC) for comparison of treatment (BAP) effects.

**Effects of TDZ on multiple shoot induction rate:** Seeds were germinated on medium containing 5 μM (for hypocotyls) or 9 μM (for cotyledonary nodes) of BAP. Hypocotyls and cotyledonary nodes isolated from seedlings were initially cultured on multiple shoot induction medium. The medium consisted of Gamborg B5 basal nutrient, 0.59 g/l MES, 30 g/l sucrose, various concentrations of TDZ from 0 to 0.32 μM, and solidified with 2 g/l gelrite (for hypocotyls), or 1 g/l gelrite plus 4 g/l phytagar
(for cotyledonary nodes). Medium (SI2) containing BAP at 5 μM (modified MS from Dan and Reichert 1998) was tested as a control for hypocotyls.

Ten explants were plated on each 100 x 15 mm petri dish. The treatments were replicated 5 times with dish as the experimental unit and replicate as block.

**Plantlet recovery from multiple shoots:** Elongated multiple shoots derived from hypocotyls were separated individually and placed on plant recovery medium for further shoot elongation and root development. To improve the plantlet recovery rate, a medium posted on the Internet by Dr. Glenn B. Collins’ Lab (University of Kentucky, Lexington, Kentucky, USA) (http://mars.cropsoil.uga.edu/homesoybean/cotprot.htm) for growth of cotyledonary node-derived shoots was used as a base. This medium consisted of Gamborg B5 basal nutrients, 0.59 g/l MES, 0.58 mg/l GA₃, 0.67 mg/l IBA, solidified with 2 g/l gelrite, and the pH was adjusted to 5.7 prior to autoclaving. The effect of gelling agents, [agar (Sigma), phytagar (Life Technologies), and gelrite (Sigma)], micronutrients, BAP and sucrose concentration on the plant recovery rate from multiple shoots induced from hypocotyls was evaluated. Ten petri dishes were initiated for each treatment with 8 to 10 shoots per dish. These were cultured at 28 °C in an incubator with continuous light (3050 Lux). After one month, the number of plantlets was recorded. A plantlet was defined as a shoot more than 2 cm in length with a root system. The experiment was arranged in a completely randomized design (CRD) with the dish as the experimental unit.

To study the post effect of TDZ on plant recovery, the multiple shoots derived from the cotyledonary node experiment described above with varying concentrations of TDZ were transferred directly to plant recovery medium consisting of Gamborg B5.
basal medium with 0.58 mg/l GA₃, 0.67 mg/l IBA, 2% sucrose and solidified with 7 g/l phytagar. This medium was found to be effective for both shoot elongation and rooting for cotyledonary node derived shoots. The number of plantlets was counted from each dish after one month of culture.

Results

Effects of BAP in Seed Germination Medium

BAP was found to be necessary in the germination of soybean seeds to obtain high rates of multiple shoot formation (Table 2.1). Hypocotyls from seedlings germinated on medium without BAP responded poorly with respect to both the percentage of hypocotyls forming multiple shoots and the number of shoots formed per hypocotyl. Addition of BAP in the germination medium significantly increased the

Table 2.1. Multiple shoot formation rate from cultured soybean hypocotyls germinated on medium with different concentrations of BAP.

<table>
<thead>
<tr>
<th>BAP concentration (μM)</th>
<th>Percentage of hypocotyls forming multiple shoots ± SE</th>
<th>No. of shoots formed per hypocotyl ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29 ± 11 b</td>
<td>4.5 ± 0.5 c</td>
</tr>
<tr>
<td>1</td>
<td>65 ± 8 a</td>
<td>6.3 ± 1.2 bc</td>
</tr>
<tr>
<td>3</td>
<td>67 ± 0 a</td>
<td>5.7 ± 0.7 c</td>
</tr>
<tr>
<td>5</td>
<td>73 ± 13 a</td>
<td>7.7 ± 1.7 b</td>
</tr>
<tr>
<td>7</td>
<td>72 ± 6 a</td>
<td>9.7 ±1.2 a</td>
</tr>
<tr>
<td>9</td>
<td>85 ± 8 a</td>
<td>10.0 ± 1.0 a</td>
</tr>
<tr>
<td>P value</td>
<td>0.0418</td>
<td>0.0007</td>
</tr>
<tr>
<td>LSD</td>
<td>28.6</td>
<td>1.9</td>
</tr>
</tbody>
</table>

BAP was added to MSB medium (MS salt + Gamborg B5 vitamins). Hypocotyls were isolated from 6-7 day old seedlings, and cultured on medium containing Gamborg B5 salts + 0.59 g/l MES + 0.16 μM TDZ + 30 g/l sucrose + 2 g/l gelrite. Means followed by different letters are significantly different at P ≤ 0.05 using LSD. Multiple shoot is defined as a shoot cluster with more than 5 shoots per explant.
percentage of hypocotyls forming shoots, and this tended to increase as BAP increased from 1 to 9 μM (Table 2.1). The hypocotyls germinated on BAP at 7 or 9 μM produced approximately 10 shoots per hypocotyl, which was significantly higher than other treatments (P< 0.01).

Effect of TDZ on Multiple Shoot Induction

Multiple shoots (>5 shoots per hypocotyl) appeared on hypocotyls after 2 weeks of culture, and became clearly visible at 4 weeks of culture (Figure 2.2 b). The small multiple shoots elongated when transferred to elongation medium (Figure 2.3 b c). Multiple shoots could be induced in medium with or without plant growth regulators. Addition of TDZ was beneficial for the production of multiple shoots in terms of the percentage of hypocotyls forming multiple shoots and the number of shoots per hypocotyl. The highest percentage of hypocotyls with multiple shoot was observed in medium containing 0.16 μM TDZ in which 73% hypocotyls produced multiple shoots (Table 2.2).

The average number of multiple shoots per hypocotyl ranged from 4 to 10 on (Table 2.2). Medium without plant growth regulators (TDZ or BAP) produced the least number of shoots per hypocotyl. The number of shoots tended to increase with increasing concentration of TDZ. TDZ at 0.16 μM stimulated the highest number of multiple shoots (10 shoots per hypocotyl), and also gave a more consistent. Statistical analysis indicated that TDZ at 0.16 μM produced significantly more shoots per hypocotyl than other concentrations. The medium (SI2), consisting of MS basal medium, B5 vitamins and 5 μM BAP (modified from Dan and Reichert 1998) produced
a comparable number of multiple shoots per hypocotyl to TDZ concentrations other than 0.16 μM, but significantly lower than medium containing 0.16 μM TDZ.

Table 2.2. Multiple shoot formation rate from cultured soybean hypocotyls on medium containing TDZ or BAP.

<table>
<thead>
<tr>
<th>Medium</th>
<th>% hypocotyls forming shoots ± SE</th>
<th>Number of shoots per hypocotyl ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN</td>
<td>63 ± 6 bc</td>
<td>4.4 ± 0.9 b</td>
</tr>
<tr>
<td>GN+ 0.005μM TDZ</td>
<td>72 ± 6 abc</td>
<td>5.8 ± 0.7 b</td>
</tr>
<tr>
<td>GN+ 0.01μM TDZ</td>
<td>82 ± 5 ab</td>
<td>6.8 ± 0.5 b</td>
</tr>
<tr>
<td>GN+ 0.02μM TDZ</td>
<td>85 ± 6 a</td>
<td>6.6 ± 0.7 b</td>
</tr>
<tr>
<td>GN+ 0.04μM TDZ</td>
<td>78 ± 7 abc</td>
<td>7.0 ± 1.6 b</td>
</tr>
<tr>
<td>GN+ 0.08μM TDZ</td>
<td>93 ± 5 a</td>
<td>6.6 ± 1.1 b</td>
</tr>
<tr>
<td>GN+ 0.16μM TDZ</td>
<td>89 ± 4 a</td>
<td>9.8 ± 0.6 a</td>
</tr>
<tr>
<td>GN+ 0.32μM TDZ</td>
<td>73 ± 14 abc</td>
<td>7.0 ± 1.4 b</td>
</tr>
<tr>
<td>*SI2 (5 μM BAP)</td>
<td>57 ± 7 c</td>
<td>7.0 ± 1.3 b</td>
</tr>
<tr>
<td>P value</td>
<td>0.0357</td>
<td>0.3115</td>
</tr>
<tr>
<td>LSD</td>
<td>22</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Hypocotyls were isolated from 6-7 day old seedlings. Multiple shoot is defined as a shoot cluster with more than 5 shoots per explant. GN medium: Gamborg B5 salts + 0.59 g/l MES + 30 g/l sucrose + 2 g/l gelrite SI2 was a medium modified from Dan and Reichert (1998) to induce multiple shoot formation in soybean hypocotyl culture. Means followed by different letters indicate significantly different at P<0.05 using LSD.

Cotyledonary nodes responded in a similar fashion to TDZ concentrations (Table 2.3). Multiple shoots appeared in the node area after about 2 weeks of culture. Shoots occurred in all treatments including the growth regulator free treatment, but multiple shoots (more than 5 shoots per node) were not observed unless the TDZ concentration was 0.04 μM or higher. TDZ concentrations greater than 0.08 μM significantly increase the percentage of nodes producing multiple shoots.
The average number of shoots per node was calculated by dividing the total number shoots by the total number of nodes that produced shoots. TDZ at a concentration of 0.16 or 0.32 μM produced significantly more shoots per cotyledonary node (Table 2.3).

Plant Recovery

The highest rate of plant recovery was observed on phytagar medium (53%) while the Sigma agar gave the lowest plant recovery rate (22%). The rate on gelrite (44%) was not significant different from phytagar, but was significantly higher than agar (Table 2.4). Some of the plantlets appeared hyperhydric in gelrite-solidified medium [hyperhydric is used in plant tissue culture to describe organs and tissue, particularly leaves, having an abnormal morphological appearance that are water-soaked and translucent (Debergh et al. 1992)]. Hyperhydric plants do not have wax on the surface of their leaves, and the plants are fragile. These plantlets do not survive transplanting to soil. Some of the soybean shoots were hyperhydric, and failed to grow into intact plants on gelrite-solidified medium.

Decrease of sucrose from 30 g/l to 20 g/l improved the plant recovery rate while increasing the iron concentration (2x the normal concentration) reduced the plant recovery rate (39%) as comparing to the normal iron concentration (69%). Increasing both iron and the other micro-nutrients to 2x the normal concentration had no effect on the plant recovery rate (53%) (Table 2.4). In medium containing BAP, root development was greatly inhibited, resulting in a low plant recovery rate (6%).

To evaluate the effects of TDZ induced multiple shoots on plant recovery rate, multiple shoots derived from cotyledonary nodes on various concentrations of TDZ...
Table 2.3. Multiple shoot formation rate from cultured Soybean cotyledonary nodes.

<table>
<thead>
<tr>
<th>TDZ (μM)</th>
<th>% Nodes &lt; 5 shoots</th>
<th>% Nodes &gt; 5 shoots</th>
<th>% Nodes forming shoots</th>
<th>Number of shoots per responding node</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33 ± 9 cde</td>
<td>0 c</td>
<td>33 ± 9 e</td>
<td>1.9 ± 0.1 c</td>
</tr>
<tr>
<td>0.005</td>
<td>67 ± 13 a</td>
<td>0 c</td>
<td>67 ± 13 bcd</td>
<td>2.5 ± 0.6 bc</td>
</tr>
<tr>
<td>0.01</td>
<td>60 ± 6 ab</td>
<td>0 c</td>
<td>60 ± 6 cd</td>
<td>2.5 ± 0.6 bc</td>
</tr>
<tr>
<td>0.02</td>
<td>77 ± 9 a</td>
<td>0 c</td>
<td>77 ± 9 abc</td>
<td>3.0 ± 0.3 bc</td>
</tr>
<tr>
<td>0.04</td>
<td>47 ± 3 bc</td>
<td>7 ± 7 c</td>
<td>53 ± 3 de</td>
<td>3.1 ± 0.3 bc</td>
</tr>
<tr>
<td>0.08</td>
<td>43 ± 7 bcd</td>
<td>43 ± 15 b</td>
<td>86 ± 9 ab</td>
<td>5.0 ± 0.8 b</td>
</tr>
<tr>
<td>0.16</td>
<td>27 ± 9 de</td>
<td>63 ± 3 a</td>
<td>90 ± 10 a</td>
<td>12.0 ± 1.5 a</td>
</tr>
<tr>
<td>0.32</td>
<td>17 ± 3 e</td>
<td>66 ± 3 a</td>
<td>83 ± 3 ab</td>
<td>10.0 ± 2.2 a</td>
</tr>
</tbody>
</table>

P value: 0.0002 <0.0001 0.0005 <0.0001

Cotyledonary nodes were isolated from 3-4 day old seedlings.

TDZ was added to medium containing Gamborg B5 salts + 0.59 g/l MES + 30 g/l sucrose + 1 g/l gelrite + 4 g/l phytagar

Responding node is defined as a cotyledonary node producing a minimum of 1 shoot.

Multiple shoot is defined as a shoot cluster with more than 5 shoots.

Means followed by different letters indicate significantly different at P<0.05 using LSD.
Table 2.4. Plant recovery rate using hypocotyl derived multiple shoots.

<table>
<thead>
<tr>
<th>Study</th>
<th>Medium</th>
<th>Total shoots tested</th>
<th>% Plant recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelling agents</td>
<td>TE + 30 g/l sucrose + 2 g/l Gelrite (control)</td>
<td>72</td>
<td>44 ± 7 b</td>
</tr>
<tr>
<td></td>
<td>TE + 30 g/l sucrose + 7 g/l Sigma agar</td>
<td>94</td>
<td>22 ± 8 c</td>
</tr>
<tr>
<td></td>
<td>TE + 30 g/l sucrose + 7 g/l phytagar</td>
<td>82</td>
<td>53 ± 4 ab</td>
</tr>
<tr>
<td>Sucrose concentration</td>
<td>TE + 20 g/l sucrose + 7 g/l phytagar</td>
<td>90</td>
<td>69 ± 6 a</td>
</tr>
<tr>
<td>Additional micro-nutrients</td>
<td>TE + 20 g/l sucrose + 7 g/l phytagar + 1x B5 [Fe^{2+}]</td>
<td>100</td>
<td>39 ± 6 b</td>
</tr>
<tr>
<td></td>
<td>TE + 20 g/l sucrose + 7 g/l phytagar + 1x B5 micronutrients</td>
<td>100</td>
<td>53 ± 5 ab</td>
</tr>
<tr>
<td>Additional BAP</td>
<td>TE + 20 g/l sucrose + 7 g/l phytagar + 0.08 mg/l BAP</td>
<td>62</td>
<td>6 ± 3 c</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LSD</td>
<td></td>
<td></td>
<td>17</td>
</tr>
</tbody>
</table>

Multiple shoots were derived from shoot induction medium containing 0.16 μM TDZ.
TE medium consisted of Gamborg B5 basal nutrients, 0.59 g/l MES, 0.58 mg/l GA₃ and 0.67 mg/l IBA, and pH was adjusted to 5.7 prior to autoclaving.
% Plant recovery is defined as number of intact plants regenerated from 100 shoots tested.
Means followed by different letters indicate significant difference at P<0.05 using LSD.
Sigma agar is composed of about 70% agarose and 30% agaropectin (Agarose: A neutral gelling fraction which consists of a linear polymer of alternating D-galactose and 3,6-anhydrogalactose units; Agaropectin: A non-gelling fraction which consists of β-1,3-glycosidically linked D-galactose units).
Phytager is produced by Life Technologies and is claimed containing the constituents and characteristics of agar that contribute to optimal growth of plant tissue.
Gelrite is a registered trademark of Monsanto Company, and sold as phytagel by Sigma. It is an agar substitute produced from a bacterial substrate composed of glucuronic acid, rhamnose and glucose.
medium were cultured onto the same plant recovery medium previously described in the Material and Methods. Shoots induced from lower concentrations of TDZ generally showed higher plant recovery rates. The shoots from 0.32 μM TDZ (the highest concentration tested) had the lowest plant recovery rate (Table 2.5). However, plant recovery rate itself may not reflect the efficiency of the culture condition. The total number of plants obtained from each cultured explant may reflect the efficiencies better. The regeneration efficiency was calculated by multiplying the number shoots per explant by the plant recovery rate. The regeneration efficiency reported in Table 2.5 reflects the average number of plants that can be obtained from one cultured node. For example, medium containing 0.16 μM TDZ produced 12 shoots per node (Table 2.3), and 65 % (plant recovery rate) of these shoots were recovered into intact plants (Table 2.5). On average, eight plants could be produced from each node initiated on medium containing 0.16 μM TDZ, which is significantly higher than the other treatments (Table 2.5).

Discussion

Wright et al. (1986) described a method to induce organogenesis from soybean cotyledonary nodes. They germinated seeds and cultured the nodes on MS based medium (Murashige and Skoog 1962) containing 5 μM BAP. Dan and Reichert (1998) reported a regeneration system from soybean hypocotyls. The hypocotyls were isolated from seedlings germinated on 5 μM BAP medium, and cultured on medium with various concentrations of BAP. Comparing the BAP concentration for multiple shoot induction, they suggested that the optimal concentration of BAP was 5 μM in MS basal medium.
Table 2.5. Plant recovery of multiple shoots derived from cotyledonary nodes cultured on different concentrations of TDZ.

<table>
<thead>
<tr>
<th>TDZ concentration in the induction medium</th>
<th>% Plant recovery from cultured shoots</th>
<th>Regeneration efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005μM</td>
<td>80 ± 15 a</td>
<td>2.3 ± 0.8 bc</td>
</tr>
<tr>
<td>0.01μM</td>
<td>66 ± 9 ab</td>
<td>1.8 ± 0.6 c</td>
</tr>
<tr>
<td>0.02μM</td>
<td>71 ± 2 a</td>
<td>2.1 ± 0.2 bc</td>
</tr>
<tr>
<td>0.04μM</td>
<td>67 ± 10 ab</td>
<td>2.2 ± 0.5 bc</td>
</tr>
<tr>
<td>0.08μM</td>
<td>75 ± 6 a</td>
<td>3.7 ± 0.6 bc</td>
</tr>
<tr>
<td>0.16μM</td>
<td>65 ± 8 ab</td>
<td>8.0 ± 1.4 a</td>
</tr>
<tr>
<td>0.32μM</td>
<td>42 ± 4 b</td>
<td>4.0 ± 0.9 b</td>
</tr>
<tr>
<td>P value</td>
<td>0.162</td>
<td>0.0020</td>
</tr>
<tr>
<td>LSD</td>
<td>27</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Multiple shoots were cultured on plant recovery medium consisting of Gamborg B5 salt, 0.59 g/l MES, 0.58 mg/l GA₃, 0.67 mg/l IBA, 20 g/l sucrose and 7 g/l phytagar.
% plant recovery is defined as the number of intact plants regenerated from 100 shoots tested.
Regeneration efficiency was defined as the average number of intact plants regenerated from one cotyledonary node.
Means followed by different letters indicate significant difference at P ≤ 0.05 using LSD.
Yasseen and Splittstoesser (1990) also cultured soybean cotyledonary nodes obtained from seedlings germinated on 5 μM BAP. These cotyledonary nodes produced over twice as many shoots as others germinated without BAP. We observed that hypocotyls isolated from seedlings germinated in BAP higher than 7 μM produced significantly more shoots than hypocotyls obtained from seedlings germinated in BAP less than 7 μM.

TDZ is primarily used as a cotton defoliant, and has shown strong cytokinin-like activity (Capelle et al. 1983, Thomas and Katterman 1986). It has recently received much attention in plant tissue culture. Reports have demonstrated that TDZ was more effective than other cytokinins, such as BAP, zeatin, kinetin, or 2-isopentenyladenine (2iP) in stimulating organogenesis, as well as embryogenesis of several plant species, including dicots and monocots (Visser et al. 1992, Bhagwat et al. 1996, Hutchinson and Saxena 1996, Hutchinson et al. 2000, Murthy et al. 1996, Hosokawa et al. 1996, Shan et al. 2000, Akasaka et al. 2000). For instance, Akasaka et al. (2000) tested commonly used cytokinins, BAP, 2iP, kinetin, TDZ and zeatin, for induction of bud primordia or shoots from peanut leaf segments. They reported that TDZ was the most efficient for inducing bud primordia. A high concentration of TDZ (10 mg/l) induced abnormal promordia during a short period of culture (less than 7 days of culture), and these promordia failed to grow into plantlets. Normal promordia were obtained in a lower concentration of TDZ (1 mg/l) during a long period of culture (21 days).

Information regarding the use of TDZ on soybean tissue culture is limited. Yasseen and Splittstoesser (1990) compared BAP and TDZ in stimulating shoot formation in soybean apexes, stem nodes and cotyledonary nodes on MS based
medium, and found that TDZ gave the highest rate of shoot regeneration. We have obtained similar responses with cotyledonary nodes and hypocotyls. In both cases, TDZ at 0.16 μM was optimal for the induction of multiple shoots as well as regeneration efficiency. TDZ concentrations greater than 0.32 μM reduced the normality of multiple shoots induced; therefore impairing the plant recovery rate of the shoots. In an experiment with cotyledonary nodes, Yasseen and Splittstoesser (1990) reported that 30% of the nodes produced shoots with an average of 10 shoots per node. Our research found that 63% of the nodes produced multiple shoots with an average of 12 shoots per node in medium containing 0.16 μM TDZ.

Pierik (1991) tested 20 different agars with three plant species (rose, lilac and Gerbera), and reported that the shoot number and the fresh weight of cultured explants were strongly influenced by the type of agar. Agars containing high levels of Ca, Na, Mg, I, Br, V and Cr produced poor growth while agars with low levels of N, Na, Br, V and Cr but high levels of Ca, Mn, Mg, Co and Fe promoted the growth of cultured tissue. Hyperhydricity is a serious problem in plant tissue culture since growth can be totally inhibited in a hyperhydric explant. Sato et al. (1993) noticed that gelrite induced hyperhydricity in carnation petal cultures. Hyperhydric shoots all died when they were cultured on rooting medium. Hyperhydric and swollen eggplant embryos were formed from cotyledons in a 0.2 % gelrite medium, and the embryos did not germinate normally (Saito and Nishimura 1994). Hidider and Desjardins (1993) have tested the agar hydrolysate anti-hyperhydricity agents EM1 and EM2 on micropropagated strawberry shoots, and observed that supplementation of EM1 or EM2 at 5 g/l resulted in the production of high quality shoots without hyperhydricity. In this study, we also

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observed gelrite-induced hyperhydricity in soybean tissue culture, and found that phytagar could prevent hyperhydricity.

Multiple shoots were induced from soybean hypocotyls and cotyledonary nodes. It was possible to obtain intact plants within 2 to 3 months. One hundred soybean genotypes have been screened with this protocol in an independent experiment to obtain regenerated plants from hypocotyls (Yao et al. 1999). Plants were regenerated from all genotypes. More genotypes responded positively to our protocol as compared to the protocol of Dan and Reichert (1998).

References


CHAPTER 3

PRODUCTION OF TRANSGENIC SOYBEAN USING GLUFOSINATE AS A SELECTIVE AGENT WITH AN IMPROVED AGROBACTERIUM-MEDIATED TRANSFORMATION PROTOCOL

Introduction

Genetic transformation offers novel approaches to crop improvement. In addition, genetic transformation provides new tools in understanding the functions of genes and the molecular mechanism of their relationships. Although Agrobacterium-mediated gene transfer is now well established for transferring genes into many crops, the transformation of soybean with this system remains inefficient. Only a handful of laboratories are able to produce transgenic soybean plants consistently.

Transformation efficiency is influenced by several factors, including Agrobacterium strain, additional of phenolic compounds (e.g., acetosyringone) to cocultivation medium, wounding treatment of target tissue (Godwin et al. 1991, Norelli et al. 1996), and appropriate selection of transformed cells or tissue from the majority of untransformed tissue. In the published protocols of Agrobacterium-mediated transformation of soybean, the more successful ones utilize cotyledonary nodes as explants, and kanamycin as the selective agent (Di et al. 1996, Meurer et al. 1998).

Selection of transformed cells, tissues, calli or plantlets is an essential and critical step toward obtaining transgenic plants in the transformation process. Absence of selection pressure resulted in the recovery of mostly wild type tissue (Christou and Ford 1995). Effective selection depends greatly on the selectable markers and the selection procedures employed in the transformation protocol. The kanamycin resistant gene is perhaps the most commonly used selectable marker in genetic transformation of plants.
including soybean. Effective selection of transformed cell or tissue is not easy with the kanamycin resistant marker for soybean because soybean has great innate resistance to kanamycin. Meurer et al. (1998) reported that only 1-2% of regenerated shoots were transformed when kanamycin was used as a selective agent in soybean transformation. Other researchers have also observed that kanamycin could not be efficiently used as a selective agent in the transformation of other crops (Escandon and Hahne, 1991).

The *bar* gene was originally cloned from the bacterium *Streptomyces hygroscopius*. It encodes for phosphinothricin acetyltransferase (PAT) (Thompson et al. 1987) that detoxifies phosphinothricin or glufosinate, the active ingredient of the herbicides Liberty and Basta (DeBlock et al. 1987). Therefore, plants expressing the *bar* gene are tolerant to the herbicides Liberty and Basta. Dekeyser et al. (1989) suggested that the *bar* gene may be an effective selection marker in plant transformation. Recently, transformation of plants using the *bar* gene as a selectable marker has been studied in several plant species, including rice, maize, and soybean (D’Hallein et al. 1992, Fromm et al. 1990, Zhang et al. 1999). The only report of production of transgenic soybean using glufosinate as a selective agent was by Zhang et al. (1999).

In this study, we attempted to improve the selection efficiency of glufosinate resistance in soybean transformation. Glufosinate sensitivity in both tissue cultured soybean explants and intact soybean plants was established. The *bar* gene and glufosinate were incorporated into the selection process with an improved transformation protocol for soybean. The concentrations of glufosinate for different stages of selection and screening are presented. The production of transgenic soybean expressing the *bar* gene was demonstrated.
Materials and Methods

Preparation of Cotyledonary Node Explants

Soybean cultivar Peking was used in the transformation experiments. Peking has been shown to be highly sensitive to *Agrobacterium* (Bailey et al. 1994). Although Peking is an old Chinese cultivar, it has some desired traits, for example, cyst nematode resistance (Mudge et al. 1997). Peking has been used as a donor parent to cross with other cultivars or lines (Concibido et al. 1997).

Seeds of the soybean cultivar Peking were sterilized by rinsing in 75% ethanol and soaking in a 30 % Clorox solution for 20 minutes, followed by four rinses with sterile distilled water. Seeds were germinated in MS basal medium (Murashige and Skoog 1962) containing 9 µM BAP (Table 3.1) for 3-4 days in petri dishes at 28 °C in a dark incubator. Each seedling was cut at 3 mm below the cotyledonary nodes, and then a 2/3 portion of the cotyledon above the nodes was either removed or kept intact according to the experimental design. The above explants were then bisected between the two cotyledons, and the apical meristem regions were removed and discarded. This operation yielded two identical explants from each seedling as shown in Figure 2.1 b, c.

Preparation of *Agrobacterium* Strains and Binary Vector

Four disarmed *Agrobacterium* strains were chosen for the study: KYRT1, EHA105, LAB4404 and GV3101 (Table 3.2), each containing a different Ti plasmid. KYRT1 was shown effective to delivery DNA into soybean (Torisky et al. 1997). EHA105 and LBA4404 have been previously used in soybean transformation (Zhang et al. 1999, Townsend et al 1996). GV3101 has been used to successfully transform some non-agronomically important plants (Muriel et al. 1997, Krasnyanski et al. 1999). The
## Table 3.1. Media used in this study

<table>
<thead>
<tr>
<th>Medium</th>
<th>Description</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterium growth</td>
<td>(1) LB, plus antibiotics 15 g/l Bacto agar (if solid plate), pH 7.0.</td>
<td>Growth of <em>E. Coli</em> and <em>Agrobacteria</em></td>
</tr>
<tr>
<td></td>
<td>(2) AB mineral plus antibiotics 0.5% sucrose, 15 g/l Bacto agar, pH 7.0.</td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis</em> Infiltration</td>
<td>2.2 g/l MS salt, Gamborg B5 vitamins, 0.5 g/l MES, 0.044 μM BAP, 50 g/l sucrose, 200 μl/l Silwet 77, pH 5.7</td>
<td>Suspension of <em>Agrobacterium</em> for infiltration of <em>Arabidopsis</em></td>
</tr>
<tr>
<td><em>Arabidopsis</em> selection</td>
<td>4.3 g/l MS salt, Gamborg B5 vitamins, 0.5 g/l MES, 10 g/l sucrose, 7g/l Sigma agar, 40 mg/l kanamycin, or 20 mg/l glufosinate, pH 5.7</td>
<td>Selection for transformed <em>Arabidopsis</em></td>
</tr>
<tr>
<td>Soybean germination</td>
<td>2.2 g/l MS salt, 1x Gamborg B5 vitamins, 9 μM BAP, 30 g/l sucrose, pH 5.8</td>
<td>Germination of soybean seeds for <em>in vitro</em> culture</td>
</tr>
<tr>
<td><em>Agrobacterium</em>-Soybean co-cultivation</td>
<td>Gamborg B5 salts and vitamins, 0.59 g/l MES, 0.16 μM TDZ, 30 g/l sucrose, 8 g/l phytagar (if solid), 100 μM acetylsyringone, pH 5.6</td>
<td>Suspension of <em>Agrobacterium</em>, <em>Agrobacterium</em>-Soybean co-cultivation</td>
</tr>
<tr>
<td>Soybean initial selection</td>
<td>Gamborg B5 salts and vitamins, 0.59 g/l MES, 0.16 μM TDZ, 30 g/l sucrose, 1 g/l gelrite, 4 mg/l glufosinate, 400 mg/l timentin, pH 6.0</td>
<td>Selection of <em>Agrobacterium</em> co-cultivated cotyledonal nodes for glufosinate-resistant multiple shoots</td>
</tr>
<tr>
<td>Soybean second selection</td>
<td>Gamborg B5 salts and vitamins 0.58 mg/l GA₃, 0.67 mg/l IBA, 2 % sucrose, 7 g/l phytagar, 200 mg/l timentin, glufosinate (0.6-1.2 mg/l), pH 5.8</td>
<td>Selection of multiple shoots for transgenic plants</td>
</tr>
<tr>
<td>Soybean plantlet recovery</td>
<td>Same as above but without glufosinate</td>
<td>Growth of soybean plantlets</td>
</tr>
</tbody>
</table>

All antibiotics were added to medium after autoclaving.
plasmid pBIMC-B is a binary vector containing the *bar* (driven by a CaMV 35S promoter) and *nptII* genes (driven by the Nos promoter) in its T-DNA region that confer resistance to glufosinate and kanamycin respectively, with a RK₂ origin of replication (Figure 3.1). This binary vector is a gift from Dr. Robin Buell formerly with the Department of Biological Sciences, Louisiana State University. No information regarding use of this vector in plant transformation was available at the time the vector was obtained.

The binary vector was introduced into *Agrobacterium* strains by electroporating a mixture of self prepared *Agrobacterium* competent cells (appendix 1) and pBIMC-B using *Cell Troporator* (Bio Rad Laboratories, Hercules CA. or Life Technology, Grand Island, NY.) following the procedures modified from the Bio Rad manufacturer’s protocol (appendix 1). The electroporated *Agrobacterium* cells were selected in Bacto agar solidified LB medium (Sambrook et al. 1989) containing appropriate antibiotics according to the nature of each *Agrobacterium* strain (Table 3.2).

Table 3.2. *Agrobacterium tumefaciens* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ti plasmid</th>
<th>Selective characteristics (mg/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>For Ti</td>
<td>Other</td>
</tr>
<tr>
<td>KYRT1</td>
<td>PTiChry5</td>
<td>Cb 100</td>
<td>Rif 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Km 200</td>
</tr>
<tr>
<td>EHA105</td>
<td>pTiBo 542</td>
<td>None</td>
<td>Rif 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Km 50</td>
</tr>
<tr>
<td>LBA 4404</td>
<td>pTiAch5</td>
<td>Str 40</td>
<td>Km 50</td>
</tr>
<tr>
<td>GV3101</td>
<td>PTiC58</td>
<td>Gm 40</td>
<td>Km 50</td>
</tr>
</tbody>
</table>

Cb=carbenicillin; Rif= rifampicin; Gm=gentamycin; Str= streptomycin, Km=kanamycin

Besides the Ti plasmid, KYRT1 also harbors a plasmid pHJI1, which contains a streptomycin resistant gene and the RK₂ origin of replication. Plasmid pHJI1 and the
Figure 3.1. Construct of the bar and nptII genes on binary vector pBIMC-B. Probe indicated was used in southern hybridization. 35S p=35S promoter; NOS p=NOS promoter; NOS t=NOS terminator; RB=right border; LB=left border. This binary vector was a gift from Dr. Robin Buell formerly with the Department of Biological Sciences, Louisiana State University.
binary vector pBIMC-B are incompatible since both of them have the same origin of replication (RK$_2$). Replacement of pHIJ1 with pBIMC-B is necessary. For transformation of KYRT1, the electroporated Agrobacterium cells were selected on Bacto agar solidified LB medium containing 100 mg/l carbenicillin, 100 mg/l rifampcin, and 200 mg/l kanamycin at 28 °C. Twenty clones were picked from the primary dish, and screened again in the medium containing the above antibiotics with and without 100 mg streptomycin. The clones grown in kanamycin, but not in streptomycin, indicated that pBIMC-B was introduced into the cells, and pPHIJ1 was replaced with pBIMC-B. The five clones that grew the fastest were chosen for further analysis to confirm transformation.

For transformation of EHA105, LAB4404 and GV3101, five clones of each strain that grew the fastest were picked directly from the primary selection dishes for further analysis. To further confirm the transformation of Agrobacterium cells, plasmids were isolated from each clone and digested with Hind III and Pst I. The digestion was separated by electrophoresis on a 1% agarose gel. The plasmid from putative transformed clones showed identical enzymatic digestion patterns in comparison to the pure pBIMC-B (Figure 3.2), indicating the successful transformation of the Agrobacteria with pBIMC-B. The transformed Agrobacteria were maintained in LB medium supplemented with appropriate antibiotics, and the stocks were stored at −80 °C for future use in plant transformation.

Transformation of Arabidopsis thaliana by Vacuum Infiltration

Since the pBIMC-B was a newly constructed binary vector with no information available concerning its effectiveness in plant transformation experiments, the vector was tested with a model plant transformation system. The Arabidopsis thaliana method
Figure 3.2. Enzymatic digestion of putatively transformed Agrobacterium clones to confirm the transformation of Agrobacteria. Agrobacterium strains were transformed with binary vector pBIMC-B. Plasmid was isolated from putatively transformed clones of Agrobacterium, and digested with Hind III or Pst I. MK=100 bp plus ladder (MBI). Within each strain, lane 1: undigested plasmid; lane 2: Hind III digestion; lane 3: Pst I digestion.
using vacuum infiltration (Bechtold et al. 1993) was conducted before applying the vector to soybean transformation.

**Vacuum infiltration**: *Arabidopsis thaliana* genotype ‘Columbia’ was grown in a 28 °C incubator with continuous light. The *Agrobacterium* strain used was GV3101 harboring pBIMC-B prepared as above. *Agrobacterium* was cultured at 28 °C on a shaker (150 rpm) in LB medium supplemented with appropriate antibiotics until an OD$_{600}$ of 1.5 was reached. The cells were collected by a 10 minute centrifugation at 4000 rpm, and then re-suspended in the infiltration medium (Table 3.1) which consisted of MS salts, 0.5 g/l MES, 50 g/l sucrose, 0.044 μM BAP, 200 μl/l Silwet77 (Lhele Seeds) and Gamborg B5 vitamins (Gamborg et al. 1968). The cell density of the *Agrobacterium* suspension was adjusted to 1.1 OD$_{600}$ at the time of infiltration.

*Arabidopsis* plants were grown in 3.5-inch pots until the primary inflorescences were 5-10 cm long. During the vacuum infiltration, the entire shoot of plants were completely immersed in an *Agrobacterium* suspension by inverting the pots over the container. The plants were placed into a vacuum chamber and subjected to vacuum pressure of 508 mm Hg for a 5 minute vacuum infiltration (Figure 3.3). After the infiltration, the plants were then grown to maturity, and $T_0$ putative transformed seeds (first generation transformants) were collected (Figure 3.4 a).

**Screening for transgenic *Arabidopsis***: $T_0$ seeds collected from infiltrated plants were sterilized and germinated on selection medium (Table 3.1) containing kanamycin (40 mg/l) under 18 hour cool white fluorescent light (3050-3500 Lux). After 1 week, germinated seeds and green seedlings were counted (Figure 3.4 b). The
Figure 3.3. Transformation of *Arabidopsis* using vacuum infiltration method: (a) *Arabidopsis* plants were grown in 3.5 inch pot, (b) vacuum infiltration of flowering plants by inverting a pot over an *Agrobacterium* suspension in a vacuum chamber.
Figure 3.4. Transformation of *Arabidopsis* using vacuum infiltration method: (a) after vacuum infiltration, *Arabidopsis* plants were grown to maturity to obtain *T₀* seeds; (b) *T₀* seeds were screened on medium containing 40 mg/l kanamycin for transgenic seedlings.
green seedlings were transferred to fresh medium for another week before transplanting into soil to obtain T₁ seeds (second generation transformants). T₁ seeds were germinated on medium containing kanamycin (40 mg/l) or glufosinate (Riedel-deHaen, Germany) (20 mg/l) (Akama et al. 1995) for approximately 10 days under the same conditions as T₀ seeds (Figure 3.5). Approximately 50 mg of leaf tissue was sampled from green T₁ seedlings derived from each T₀ plant for PCR analysis to confirm the present of nptII or bar genes.

Transformation of Soybean Cotyledonary Nodes

Agrobacterium co-cultivation: Agrobacteria carrying the binary vector pBIMC-B were used in the studies. The bacteria were grown on Bacto agar solidified AB mineral medium (Chilton et al. 1974) containing selective antibiotics for 3-4 days. The Agrobacterium cells were collected and suspended in liquid co-cultivation medium (Table 3.1) until they reached a density of 1.0-1.1 OD₆₀₀. The previously prepared cotyledonary nodes were transferred into a 125-ml flask along with 15-20 ml of Agrobacterium suspension. The flask was connected to a vacuum pump and subjected to a vacuum infiltration of 508 mm Hg of pressure for 10 minutes except for the time-course study of vacuum infiltration. After vacuum infiltration, the cotyledonary nodes were placed flat side up on phytagar solidified co-cultivation medium and incubated at 25 °C in darkness for 4 days (Figure 3.6 a).

Selection for glufosinate-resistant shoots and plantlets: After Agrobacterium co-cultivation, explants were washed 6 times with sterile distilled water. Cleaned cotyledonary nodes were blotted dry on a sterile paper towel, and cultured on the initial
Figure 3.5. Screening transformed *Arabidopsis*: (a) $T_0$ plants were grown to maturity to collect $T_1$ seeds; (b) $T_1$ seeds were germinated on medium containing kanamycin or glufosinate.
Figure 3.6. Transformation of soybean cotyledonary nodes with Agrobacterium-mediated gene transfer method: (a) co-cultivation of cotyledonary nodes and Agrobacterium on co-cultivation medium, (b) induction of glufosinate-resistant multiple shoots (arrowheads) on selection medium containing 4 mg/l glufosinate, (c) formation of glufosinate-resistant multiple shoots.
selection medium (Table 3.1) consisting of Gamborg B5 basal nutrients, 0.16 μM TDZ, 400 mg/l timentin (SmithKline Beecham Pharmaceuticals, Philadelphia, PA.), and 4 mg/l glufosinate. Cotyledonary nodes not inoculated with *Agrobacterium* were plated on the selection medium as a negative control. Timentin was included in the selection medium to suppress the *Agrobacterium*. The petri dishes were cultured at a temperature of 28 °C under an 18 h photoperiod (3050-3500 Lux light intensity) and sub-cultured every 3 weeks onto fresh medium to induce multiple shoot formation (Figure 3.6 b, c). The multiple shoots were excised from the cotyledonary node and transferred to a second selective medium (Table 3.1) containing 200 mg/l timentin, and supplemented with glufosinate at a designed concentration. The shoots that survived were sub-cultured every 3 weeks in the same medium with conditions stated above until shoots reached about 2 cm in length with roots (Figure 3.7). Plantlets were subsequently grown in 175 ml baby food jars containing the same medium but without further glufosinate selection. Plants were then transplanted into potting soil for analysis and seed collection (Figure 3.8).

**Confirmation of Transformation**

**Herbicide leaf painting assay:** Glufosinate sensitivity was tested using both seed-derived and regenerated plants with a leaf painting assay method to determine the appropriate concentration of glufosinate to distinguish transformed from untransformed plants. Plants were obtained by planting 'Peking' seeds or transplanting cotyledonary node derived regenerated plants into 1-gallon pots with potting soil. The upper-most fully expanded leaves of 3 week old plants (3 weeks after germination or transplanting) were painted with 0, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 ml/l ‘Liberty’ herbicide solution plus
Figure 3.7. Selection of glufosinate resistant multiple shoots on second selection medium containing glufosinate: (a) multiple shoots were excised from the nodes and cultured on the second selection medium, (b) shoots were sub-cultured every 3 weeks until formation of plantlets.
Figure 3.8. Growth of transgenic soybean plants: (a) putative transgenic plants ($T_0$) regenerated from glufosinate resistant shoots were transplanted into soil and subjected to a herbicide leaf painting assay. Black arrowhead identifies the susceptible leaf, and the white arrowhead identifies the resistant leaf; (b) fertile transgenic plant showing formation of pods.
0.1 ml/l Tween 20 using cotton swabs. Three triplet leaves on each plant were tested. The degree of damage of the painted leaves was assessed 5 days after painting, and the injury was classified into 4 levels as described in Table 3.3. The herbicide concentration determined in this experiment was used to screen the putatively transformed plants using the same leaf painting assay method or a spray in some cases.

Table 3.3. Classification of leaf injury levels 5 days after application of Liberty herbicide

<table>
<thead>
<tr>
<th>Degree of leaf injury</th>
<th>Symptom Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No symptoms or a little wrinkle on a leaf</td>
</tr>
<tr>
<td>1</td>
<td>Slight yellowing on the leaf of the treated area</td>
</tr>
<tr>
<td>2</td>
<td>Yellowing on the leaf of the treated area</td>
</tr>
<tr>
<td>3</td>
<td>Severe yellowing and necrosis on the leaf of the treated area</td>
</tr>
</tbody>
</table>

Polymerase Chain Reaction (PCR) analysis: DNA was isolated from leaves of regenerated plants using the ‘miniprep procedures’ of the Epicentre MasterPure Plant Leaf DNA Purification Kit (Epicentre Technologies, Madison WI.). The DNA concentration was determined at an optical reading of 260 nm. The DNA samples were tested for the presence of the T-DNA region using a pair of nptll specific primers (Table 3.4) to amplify the 770 bp nptll fragments. The samples were further screened for the presence of the bar gene by PCR using the bar gene specific primers (Table 3.4) chosen based on the nucleotide sequence of the bar gene (Thompson et al. 1987), which yields a 430 bp bar fragment. A pair of primers (designated as “121”) was selected from 2562 to 2796 bp down stream of the right board (RB) of the binary vector pBIMC-B for forward and reverse primers, respectively (both primers were located outside the T-DNA region of the vector). To ensure that the presence of the T-DNA is from the transformed plant cells, and not from residual Agrobacterium cells in the plant tissue, samples shown to be PCR positive for bar or nptll fragments were also tested with
'121' primers. Samples with PCR positive for T-DNA, but negative for "121" were counted as being PCR positive transformants.

For each given PCR reaction, 1 μg of genomic DNA was mixed with primers, Taq polymerase, dNTPs, and PCR buffer (contains MgCl₂) to obtain a reaction volume of 25 μl. For amplification of the bar fragment, 1 μl of 10x MasterAmp PCR enhancer (Epicentre Technologies, Madison WI.) was included in each reaction. All PCR reactions were conducted in a thermal cycler with denaturing at 94 °C for 45 seconds, annealing at 57 °C for 1 min, and extension at 72 °C for 2 min for 40 cycles. The PCR products were electrophoresed on a 1% agarose gel. Each gel was subsequently stained with ethidium bromide and viewed under UV light.

Table 3.4. Primer pairs used for PCR and RT-PCR in this study

<table>
<thead>
<tr>
<th>Name of primer*</th>
<th>Sequence</th>
<th>Fragment size (bp)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>nptII</td>
<td>5'-AGAACTCGT CAAGAAGGCGA-3' 5'-CTGAATGAACTGCAGGACGA-3'</td>
<td>770</td>
<td>Detect integration of nptII gene</td>
</tr>
<tr>
<td>bar</td>
<td>5'-CATCGTCAACCACTACATCGAG-3' 5'-CAGCTGCC AGAAACCCACGTCA-3'</td>
<td>430</td>
<td>Detect integration and expression of bar gene</td>
</tr>
<tr>
<td>121</td>
<td>5'-GGATACCTCGCGGAAAACTTGG-3' 5'-CCTCAAGTGTCAATACCACGCA-3'</td>
<td>234</td>
<td>Detect vector of pBIMC-B</td>
</tr>
<tr>
<td>Gm-actin</td>
<td>5'-TGGGATGAYATGGARAGATYTGG-3' 5'-ATCCACATCTGCTGGAGGTT-3'</td>
<td>830</td>
<td>Standard control of RT-PCR</td>
</tr>
</tbody>
</table>

* For each pair of primers, the first one is the forward primer, and the second one is the reverse primer

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Total RNA was extracted from 50 mg leaf samples with the Tri Reagent (Molecular Research Center, Inc. Cincinnati, OH), and treated with DNase I DNA-free kit (Ambion Inc. Austin, TX) to completely remove DNA contamination. The DNase I was then inactivated following the DNA-free kit protocol. The total RNA was quantitated at an
optical reading of 260 nm, and the RNA quality was determined using the ratio of OD 
260/280 to be between 1.8-2.1.

Reverse transcription (RT) was carried out by mixing 1 μg of RNA, first strand 
buffer, dNTPs, oligo dTs, 1,4-Dithiothreitol (DTT) and M-MLV reverse transcriptase 
(20 μl reaction volume) at 37 °C for 1 hour to generate the cDNA. PCR was carried out 
using the same conditions described previously. For each soybean RNA sample, 
amplifications of the bar gene fragment and the soybean actin gene cDNA fragment 
were attempted using bar and soybean actin gene specific primers, respectively. The 
soybean actin primers (Gm-actin) (Table 3.4) were chosen according to its cDNA 
sequence (Shah et al. 1982, Shimizu et al. 1999). The parallel amplification of the 
soybean actin cDNA fragment served as RT-PCR controls for the reaction and standard 
gene expression for the given sample. For a given PCR run, 3 μl of cDNA from the RT 
reaction was mixed with primers, Taq polymerase, dNTPs, PCR enhancer, and buffer to 
make a total volume of 25 μl. Following the PCR, 8 μl of RT-PCR product was 
fractionated on a 1% agarose gel stained with ethidium bromide to view the amplified 
DNA band. The net intensities of the correct bands were measured with Kodak Digital 
Science 1D image analysis software (Eastman Kodak Company, 1999). The expression 
levels of transgene were compared using relative intensity of bar RT-PCR product as 
normalized with actin expression (bar/actin ratio).

Southern Hybridization: The probe was prepared by digestion of pBIMC-B 
with Hind III and separated by electrophoresis on a 0.8 % agarose gel, which yielded a 
35S-bar fragment. The 35S-bar band was excised from the agarose gel, and purified 
using a Pre A Gene Kit (Bio Rad Laboratories, Hercules, CA.). The probe was then 
labeled with 32P-dCTP using a Random Primed Labeling Kit (Boehringer Mannheim
Corporation, Indianapolis, IN.) as described by the manufacturer. Genomic DNA was extracted from 2.5-3.0 g of young leaves using the Macroprep procedures of the Epicentre MasterPure Plant Leaf DNA Purification Kit (Epicentre Technologies, Madison WI.). DNA concentration was determined by using both DNA mass ladder (Life Technology, Grand Island, NY.) on a 1% agarose gel and by taking a spectrophotometer reading at 260 nm. Twenty μg of genomic DNA was digested with a 4 fold excess of restriction enzyme (Hind III) overnight in a 37 °C water bath (appendix 2). DNA fragments were fractionated by electrophoresis on a 0.8% agarose gel, and transferred onto a nylon membrane. Hybridization was carried out according to the Quickhib procedures (Stratagene, La Jolla, CA.), and then visualized by exposure to x-ray film at -80 °C.

Progeny test of transgenic soybean: The seeds of the first transgenic generation (T₁) line were harvested from individual primary transgenic plants (T₀) that were confirmed to have integration of the bar gene. The seeds were grown in a 28 °C light (2300 Lux) growth chamber with 5 to 6 plants in each pot. Plants 3-4 weeks old were subjected to the leaf painting assay or sprayed with 0.3 ml/l ‘Liberty’. Six to seven T₁ plants were randomly sampled from each line to conduct PCR and RT-PCR with the bar gene primers to determine the presence and the expression levels of the bar gene.

Results and Discussion

pBIMC-B as A Binary Vector in Transformation of Arabidopsis thaliana

Transgenic Arabidopsis thaliana plants were obtained with the tissue culture free, in planta vacuum infiltration method. All T₀ plants that survived the kanamycin selection (40mg/l) produced seeds (T₁). When T₁ seeds derived from each T₀ line were
germinated on medium containing kanamycin and glufosinate, without any other antibiotics, green seedlings were observed from all lines. PCR analysis showed that green T<sub>1</sub> seedlings contained the insertion of nptII and bar genes into their genome (Figure 3.9). Since the seedlings were Agrobacterium-free on selection medium (which contained no Agrobacterium inhibition agents), amplification of the bar gene fragment was from Arabidopsis cells, not from Agrobacterium cells.

Table 3.5. Transformation efficiency of Arabidopsis using pBIMC-B

<table>
<thead>
<tr>
<th></th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
<th>Rep 4</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total seedlings screened</td>
<td>606</td>
<td>616</td>
<td>1347</td>
<td>282</td>
<td></td>
</tr>
<tr>
<td>Resistant (green) seedlings</td>
<td>12</td>
<td>7</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Percentage of resistant seedlings</td>
<td>2.0</td>
<td>1.1</td>
<td>1.1</td>
<td>1.4</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

Seeds were harvested from plants inoculated with Agrobacterium GV3101/pBIMC-B using in planta vacuum infiltration.
Seeds were germinated on selection medium containing 40 mg/l kanamycin.

From the four trials conducted, the transformation rates varied from 1.1 to 2.0% (Table 3.5). The average transformation rate was 1.4 ± 0.4%. We expected a transformation rate of 1% based on the literature. The values observed from the above study were close to the expectation. Therefore, pBIMC-B can be used as a plant transformation vector. The inverted orientation of the 35S promoter on the vector did not affect the bar gene transfer and expression in plants.

Determination of Selection Conditions for Soybean Transformation

Glufosinate concentration in the first selection medium for shoot initiation:
The effects of glufosinate on growth and regeneration of untransformed soybean cotyledonary nodes was examined to determine the sensitivity of cotyledonary nodes to...
Figure 3.9. PCR analysis of transgenic *Arabidopsis* (T1) plants: (a) using nptII primers, lanes 1-10: putative transgenic plants, lane 11: pBIMC-B (positive control); lane 12: wild type (negative control). MK=1 kb ladder. (b) using bar primers; lanes 1-10: putative transgenic plants, lane 11: wild type (negative control); lane 12: pBIMC-B (positive control), MK=100 bp ladder. Cotyledonary nodes were selected on medium containing 4 mg/l glufosinate.
glufosinate. Explants were initially placed on *Agrobacterium* co-cultivation medium (with 100 μM acetosyringone) for 4 days to simulate the co-cultivation in the actual transformation system. The explants were then transferred onto medium containing glufosinate ranging from 2-5 mg/l. Approximately 10 cotyledonary nodes were placed in each dish as an experimental unit with 4 replications, and cultured in a 28 °C, 18 hour photoperiod, light incubator. After 6 weeks (subcultured at 3 week intervals), multiple shoots appearing on the nodes were counted, and the necrotic nodes were assessed. Two types of nodes (with entire or 1/3 cotyledon attached) were tested for their sensitivity to glufosinate. The nodes with 1/3 of the cotyledon attached were more sensitive to glufosinate than nodes with the entire cotyledon. A concentration of 3 mg/l glufosinate totally inhibited shoot formation from nodes with only 1/3 of the cotyledon while the same degree of inhibition for nodes with the entire cotyledon was 4 mg/l (Table 3.6) (Figure 3.10 b). The frequency of node death increased as the concentration of glufosinate increased. After 10 days of culture, the majority of the nodes with 1/3 cotyledon remained yellow and had necrotic areas whereas most nodes with the entire cotyledon became green (Figure 3.10 a). This was more obvious at higher concentrations of glufosinate. From these observations, it can be concluded that nodes with 1/3 of the cotyledon attached were more sensitive to glufosinate than nodes with the entire cotyledon. Thus, nodes with 1/3 of the cotyledon were used as explants for the subsequent transformation experiments. The first selection medium for shoot initiation should contain 3-5 mg/l glufosinate since 3 mg/l of glufosinate is the minimum concentration to completely inhibit the formation of multiple shoots from
Figure 3.10. Two types of cotyledonary nodes showed different sensitivities to glufosinate: (a) discoloration of nodes with 1/3 cotyledons vs. green nodes with entire cotyledons after 10 days of culture, (b) some nodes with entire cotyledons formed multiple shoots (arrowheads) whereas none of the nodes with 1/3 cotyledon formed shoots after 30 days of culture. (Explants were cultured on medium containing 3 mg/l glufosinate)
Table 3.6. Two types of cotyledonary nodes showed different glufosinate sensitivities

<table>
<thead>
<tr>
<th>Glufosinate concentration (mg/l)</th>
<th>Nodes with entire cotyledon</th>
<th>Nodes with 1/3 cotyledon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% node death</td>
<td>% nodes forming shoots</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>0</td>
</tr>
</tbody>
</table>

Cotyledonary nodes were pre-cultured on co-cultivation medium for 4 days, and then transferred to shoot induction medium containing various concentrations of glufosinate in 28 °C, 18 h photoperiod. Data were summarized from 4 replications. Node death is defined as occurrence of necrosis in cotyledonary node.

untransformed nodes. Glufosinate at 3 mg/l provides a low selection pressure, and 4-5 mg/l represents a high level of selection pressure for nodes with 1/3 of the cotyledon.

Glufosinate concentration in the second selection medium for putative transgenic plants: To determine the appropriate selection pressure for the multiple shoots, multiple shoots derived from untransformed cotyledonary nodes on shoot induction medium containing 0.16 μM TDZ (see Chapter 2) were excised from the nodes and placed on shoot elongation medium (10 shoots per dish) containing glufosinate varying from 0 to 1.2 mg/l at 0.2 increments. The number of elongated, rooted, dead shoots and the number of plantlets (shoot greater than 2 cm with roots) was counted in each dish after 6 weeks of culture.

The percentage of dead shoots increased as the glufosinate concentration increased in the culture medium from 0 to 1.2 mg/l. From the growth parameters measured (number of elongated, rooted shoots and number of plantlets formed), a majority of the shoots stopped further growth on medium with 0.4 mg/l of glufosinate.
About 90% of the cultured shoots died in medium containing 1.0 or 1.2 mg/l of glufosinate (Figure 3.11a). Shoot growth was completely inhibited when glufosinate was at a concentration of 1.0 mg/l or greater (Figure 3.11b, c). None of the shoots formed plantlets at glufosinate concentrations equal or greater than 1.0 mg/l (Figure 3.11d). Based on these observations, a glufosinate concentration of 1.0 mg/l or greater was determined to be the level for the effective selection of putatively transformed shoots.

**Injury of untransformed plants with herbicide leaf painting assay:** In order to screen the transgenic plants using herbicide under greenhouse-grown conditions, tissue culture regenerated plants and seed-derived plants were tested for their sensitivity to the commercial herbicide “Liberty” with a leaf painting assay as described in the Materials and Methods. Leaves grew normally when painted with a low concentration of herbicide (0.05 ml/l). Injury symptoms appeared when the concentration reached 0.1 ml/l, and became severe when a 0.3 ml/l Liberty solution was applied. The leaf sections treated with concentrations higher than 0.3 ml/l herbicide showed necrosis or death after 5 days of application (Table 3.7; Figure 3.12), suggesting that application or a spray of 0.3 ml/l Liberty (equivalent to 60.1 mg/l glufosinate) would provide an sufficient concentration for screening putative transgenic soybean plants. This concentration is approximately 1/10 of the recommended rate for weed control in the field.

**Production of Transgenic Soybean**

**Effect of different Agrobacterium strains on transformation efficiency of cotyledonary nodes:** Cotyledonary nodes were inoculated with *Agrobacterium* strains LBA 4404, KYRT1, EHA 105, and GV3101 harboring the same plasmid pBIMC-B. In four independent transformation experiments, a total of 76 to 104 explants were
Figure 3.11. Effects of glufosinate on the growth of untransformed shoots derived from cotyledonary nodes: (a) % dead shoots, (b) % rooted shoots, (c) % elongated shoots, (d) % plantlet formation. Multiple-shoots were induced from cotyledonary nodes with 1/3 cotyledon attached on medium containing 0.16 μM TDZ. Dead shoots, elongated shoots, rooted shoots and plantlets (elongated shoots with healthy roots) were counted after 6 weeks of culture. The vertical bars represent + standard error.
Figure 3.12. Leaf injury of untransformed plants in herbicide leaf painting assay. Leaves of untransformed plants regenerated from cotyledonary nodes were painted with Liberty herbicide varying from 0 to 0.5 ml/l with cotton swabs. The injury was assessed 5 days after application.

Table 3.7. Degree of injury of untransformed soybean in leaf painting assay

<table>
<thead>
<tr>
<th>Herbicide (Liberty) concentration (ml/l)</th>
<th>Equivalent to glufosinate (mg/l)</th>
<th>Degree of leaf injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>10.0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>20.0</td>
<td>1</td>
</tr>
<tr>
<td>0.2</td>
<td>40.1</td>
<td>2-3</td>
</tr>
<tr>
<td>0.3</td>
<td>60.1</td>
<td>3</td>
</tr>
<tr>
<td>0.4</td>
<td>80.2</td>
<td>3</td>
</tr>
<tr>
<td>0.5</td>
<td>100.2</td>
<td>3</td>
</tr>
</tbody>
</table>

Herbicide solution contains 0.1% Tween 20.
The degree of leaf injury was described in Table 3.3.
Leaf injury was assessed 5 days after application of herbicide solution.
inoculated with one of the four strains, and cultured on selective medium containing 4 mg/l glufosinate as previously stated to induce multiple shoot formation. The results (Table 3.8) showed that explants inoculated with KYRT1/pBIMC-B produced the highest rate (35%) of glufosinate-resistant multiple shoots, while explants inoculated with LBA 4404/pBIMC-B had the poorest response (9%). Statistical analysis indicated that KYRT1/pBIMC-B gave a significantly higher rate of glufosinate-resistant multiple shoot formation than EHA105/ pBIMC-B and LBA4404/ pBIMC-B, but not GV3101/ pBIMC-B. Based on these results, strain KYRT1 was chosen for the rest of the experiments.

Table 3.8. Glufosinate-resistant multiple shoot formation from cotyledonary nodes inoculated with various Agrobacterium strains

<table>
<thead>
<tr>
<th>Agrobacterium strain</th>
<th>Total No. of nodes treated</th>
<th>% nodes forming multiple shoots</th>
<th>Rep. 1</th>
<th>Rep. 2</th>
<th>Rep. 3</th>
<th>Rep. 4</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA4404</td>
<td>76</td>
<td></td>
<td>8</td>
<td>12</td>
<td>8</td>
<td></td>
<td>9.3 ± 1.3 c</td>
</tr>
<tr>
<td>KYRT1</td>
<td>104</td>
<td></td>
<td>31</td>
<td>29</td>
<td>50</td>
<td>30</td>
<td>35.0 ± 5.0 a</td>
</tr>
<tr>
<td>EHA105</td>
<td>95</td>
<td></td>
<td>33</td>
<td>12</td>
<td>15</td>
<td>21</td>
<td>20.3 ± 4.6 bc</td>
</tr>
<tr>
<td>GV3101</td>
<td>94</td>
<td></td>
<td>26</td>
<td>19</td>
<td>32</td>
<td>18</td>
<td>23.8 ± 3.3 ab</td>
</tr>
</tbody>
</table>

All Agrobacterium strains contain the binary vector pBIMC-B. Cotyledonary nodes were inoculated with an Agrobacterium suspension using a 10 minute vacuum infiltration. Cotyledonary nodes were selected on first selection medium containing 4 mg/l glufosinate. Means followed by different letters indicate significant difference at P≤0.05 using LSD, LSD=13. Rep=replication. SE=standard error.

Effect of vacuum infiltration on transformation efficiency of cotyledonary nodes: To study the effects of vacuum infiltration on transformation efficiency of cotyledonary nodes, explants were immersed in an Agrobacterium suspension (KYRT1/pBIMC-B) in flasks, and vacuum infiltrated at a pressure of 508 mm Hg was applied for durations of 0, 5, 10, or 15 minutes. After the vacuum infiltration, the
explants were transferred, as previously described in Materials and Methods, for induction of multiple shoot formation. Data for glufosinate-resistant multiple shoot formation from cotyledonary nodes are shown in Table 3.9.

Table 3.9. Effects of vacuum infiltration of cotyledonary nodes with *Agrobacterium* suspension on glufosinate-resistant shoot formation

<table>
<thead>
<tr>
<th>Vacuum duration (min.)</th>
<th>Total No. of explants tested</th>
<th>% nodes forming multiple shoots</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rep.1</td>
<td>Rep. 2</td>
</tr>
<tr>
<td>0</td>
<td>51</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>68</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>25</td>
<td>44</td>
</tr>
<tr>
<td>15</td>
<td>78</td>
<td>29</td>
<td>50</td>
</tr>
</tbody>
</table>

Cotyledonary nodes were inoculated with *Agrobacterium* strain KYRT1/pBIMC-B. Cotyledonary nodes were selected on medium containing 4 mg/l glufosinate. Means followed by different letters indicate significantly different at P<0.05 using LSD, LSD=19.

Vacuum infiltration increased the percentage of glufosinate-resistant multiple shoots formed from treated cotyledonary nodes (Table 3.9). The highest response was observed at 10 minute of infiltration. Statistical analysis showed all infiltration treatments except 5 minute were significantly better than no vacuum infiltration. The enhancement may be due to increased tissue injury or better delivery of the bacterium into the tissue during the infiltration. Bidney et al. (1992) showed that transformation rates could be significantly improved by enhancing the delivery of *Agrobacterium* to the tobacco leaf and sunflower apical meristem tissue.

Production of transgenic plants using glufosinate in the selection medium:

Cotyledonary nodes were inoculated with KYRT1 / pBIMC-B and multiple shoots were induced from inoculated cotyledonary nodes on selection medium containing 4 mg/l glufosinate. Putative transformed shoots were excised from cotyledonary nodes, and transferred to shoot selection medium supplemented with 0.6, 0.8, 1.0, or 1.2 mg/l glufosinate. Shoots that survived were sub-cultured every 3 weeks to obtain intact...
plants. Plant recovery rates were influenced by the glufosinate concentrations. Increasing glufosinate concentration in the medium reduced the plant recovery rate (Table 3.10).

Regenerated plants were screened by PCR using genomic DNA as a template for the presence of nptII and bar genes (Figure 3.13). The following patterns in PCR analysis were observed: 1) neither nptII nor bar fragment; 2) nptII fragment only; 3) bar fragment only; 4) both nptII and bar fragments. Pattern 1 suggested that the plants were not transformed. Patterns 2, 3, 4 (PCR-positive) indicated the presence of the transgene in the plant genome, where pattern 4 indicated the transformation of both the bar and nptII genes into plants. Among the PCR-positive plants, the majority of them contained both nptII and bar genes (Figure 3.13). The samples showing PCR positive for nptII and bar gene fragments were also subjected to PCR analysis with “121” primers to detect plasmid DNA. Positive PCR analysis with ‘121’ primers indicated that the samples contained DNA from Agrobacterium cells.

Table 3.11 presents the transformation efficiency based on PCR screening and herbicide leaf painting assay of putatively transgenic plants. PCR-positive refers to the presence of the bar fragment (pattern 3 and 4). These plants ranged from 5% to 33% of the total number of recovered plants under the different selection pressures. Only 5.3 % of the recovered plants showed PCR-positive at a low selection pressure (0.6 mg/l), whereas 33 % of the plants showed PCR positive at a high selection pressure (1.2 mg/l).

More ‘Liberty’ resistant plants were recovered when multiple shoots were selected on medium containing glufosinate at a concentration equal or greater than 1.0 mg/l. Of the 19 plants recovered from 0.6 mg/l glufosinate selection, only one showed
Table 3.10. Regeneration of putative transgenic plants from the selection of multiple shoots on medium containing glufosinate varying from 0.6 to 1.2 mg/l

<table>
<thead>
<tr>
<th>Glufosinate conc. (mg/l)</th>
<th>No. Shoots tested</th>
<th>No. Plants regenerated</th>
<th>% Regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>46</td>
<td>19</td>
<td>41 ± 9 a</td>
</tr>
<tr>
<td>0.8</td>
<td>95</td>
<td>37</td>
<td>37 ± 14 a</td>
</tr>
<tr>
<td>1.0</td>
<td>82</td>
<td>26</td>
<td>32 ± 5 a</td>
</tr>
<tr>
<td>1.2</td>
<td>69</td>
<td>12</td>
<td>17 ± 9 b</td>
</tr>
</tbody>
</table>

Plants reported were successfully transplanted into soil, and screened with herbicide. % Regeneration is defined as number of intact plants regenerated from 100 multiple shoots tested, and is the average of all replications. Means followed by different letters indicate significantly different at P≤0.05 using LSD, LSD=11.

Table 3.11. Screening of putative transgenic plants using PCR and herbicide leaf painting assay

<table>
<thead>
<tr>
<th>Glufosinate (mg/l)</th>
<th>Total plants screened</th>
<th>PCR positive plants</th>
<th>No. Herbicide resistant plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td>0.6</td>
<td>19</td>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>0.8</td>
<td>37</td>
<td>6</td>
<td>16.2</td>
</tr>
<tr>
<td>1.0</td>
<td>26</td>
<td>8</td>
<td>30.8</td>
</tr>
<tr>
<td>1.2</td>
<td>12</td>
<td>4</td>
<td>33.3</td>
</tr>
</tbody>
</table>

PCR positive refers to positive amplification of bar gene fragment. Resistant plants were defined as injury levels 0 and 1 as described in Table 3.3 with 0.3 ml/l 'Liberty' leaf painting assay.
Figure 3.13. PCR analysis of putative primary transgenic (T0) plants of soybean for presence of nptII and bar genes. (a) using nptII primers; (b) using bar primers; (c) using 121 primers to screen the PCR positive plants of nptII or bar fragments. For panels (a) and (b): lanes 1-20: putative transgenic plants; lane 21: Peking (negative control); lane 22: plasmid pBIMC-B (positive control). For panel (c): lane 1-10: putative transgenic plant showing PCR positive of nptII or bar fragments, lane 11: plasmid pBIMC-B (positive control). MK=100bp ladder.
resistance to the ‘Liberty’ herbicide whereas 7 out of 26 plants, and 4 out of 12 plants recovered from 1 mg/l and 1.2 mg/l selection, respectively, exhibited resistance (Table 3.11). Southern hybridization analysis confirmed the integration of the bar gene in the Liberty resistant plants (Figure 3.14). The results of the selection experiment indicated that glufosinate at a concentration less than 1.0 mg/l was not effective for the selection of transformed multiple shoots for the production of transgenic plants. A concentration of glufosinate greater than 1 mg/l should be used in shoot selection (second selection) medium to produce transgenic plants.

A comparison of PCR analysis and herbicide leaf painting assay for recovered plants (Table 3.11) demonstrated that not all of the PCR-positive plants possess herbicide resistance (0.3 ml/l Liberty leaf painting assay). Apparently, more plants derived from the lower selection (less than 1.0 mg/l glufosinate) were PCR positive, but was not herbicide resistant. However, most PCR-positive plants derived from a higher selection (more than 1.0 mg/l glufosinate) were herbicide resistant. One possible explanation could be that more transformed shoots with a low-level of expression of bar the gene survived in the lower selection pressure, and developed into intact plants. Several studies have reported that some transgenic plants show a variation in expression of the transgene (Raharjo et al. 1996). For example, Raharjo et al. (1996) noticed that transgenic cucumber plants with the chitinase gene varied in total chitinase activity levels.

Progeny Test of Transformed Herbicide Resistant Plants

Of the 15 primary transgenic plants (T₀) resistant to the herbicide, 9 of them successfully produced seeds. Seeds were germinated in the growth chamber and, morphologically, the plants (T₁) grew normally in comparison to their parent (Peking).
Figure 3.14. Southern hybridization analysis of putatively transformed soybean plants (T₀) showing Liberty resistance. Lane 1: Low DNA mass ladder (Life Technologies), lane 2: pBIMCB/Hind III (positive control), lanes 3-9: genomic DNA of putatively transformed soybean, lane 10: genomic DNA of untransformed Peking (negative control). Genomic DNAs were digested with Hind III overnight at 37 °C. Digested DNAs were transferred to nylon membrane, and hybridized with 35S-bar probe.
The inheritance and expression of the *bar* gene in the resulting T<sub>1</sub> plants were evaluated with the herbicide assay, PCR and RT-PCR.

In the herbicide assay, progeny plants were classified into resistant (damage levels 0 or 1) and susceptible (damage levels 2 or 3). The segregation ratio is given in Table 3.12. Of the 9 progeny lines tested, two lines were completely herbicide susceptible. Analysis of the progenies with PCR indicated that the transgene (*bar*) was not transmitted from T<sub>0</sub> to T<sub>1</sub> generation. Four lines conformed to a 3:1 segregation pattern, and one line to a 15:1 ratio, which could be explained as Mendelian inheritance of one or two integrated loci, respectively. The remaining two lines exhibited unusual segregation ratios. PCR and RT-PCR analysis of the progeny line 5(10.11) suggested the unusual segregation ratio of phenotype might be due to gene silencing (Figure 3.15).

Usually transgenes are inherited as simple dominant Mendelian traits (Spencer et al. 1992). However, various reports have shown exceptions to this simple pattern (Deroles and Gardner 1988, Akama et al. 1995) with transformants showing either complete loss of the trait in progenies, or non-Mendelian ratios of segregation. Inactivation of the introduced *bar* gene by methylation as suggested by Kilby et al. (1992) might be an explanation for the lines showing an unusual segregation.

Figures 3.16, 3.17 and 3.18 demonstrate the herbicide resistance, transgene inheritance and transgene expression levels among progenies of the transformed lines. Generally, the plants, which phenotypically were resistant to Liberty showed *bar* gene expression. In contrast, no gene expression was detected from plants susceptible to the herbicide. Gene silencing was observed among progenies of primary transgenic line 5(10.11). Transmission of the *bar* gene was seen in progeny plants from PCR analysis,
Table 3.12. Progeny test of transformed soybean plants showing the segregation ratio of herbicide (Liberty) resistant to susceptible

<table>
<thead>
<tr>
<th>$T_0$ plant ID</th>
<th>Number $T_1$ seedlings</th>
<th>Ratio (R:S)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Seedlings</td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td>5(4-12)</td>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>5(10-11)</td>
<td>13</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>6(1.5)</td>
<td>11</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>6(1.7)</td>
<td>31</td>
<td>26</td>
<td>5</td>
</tr>
<tr>
<td>6(2.5)</td>
<td>19</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>6(3.2)</td>
<td>22</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>6(3.5)</td>
<td>21</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>6(4.5)</td>
<td>21</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>6(5.7)</td>
<td>23</td>
<td>18</td>
<td>5</td>
</tr>
</tbody>
</table>

Plants were screened with 0.3 ml/L Liberty herbicide, and scored 5 days after application. The critical $\chi^2$ value is 3.84 for the test.

R = resistant; resistant is defined as an injury level of 0-1 as described in Table 3.3.

S = susceptible; susceptible is defined as an injury level of 2-3 as described in Table 3.3.
Figure 3.15. Progeny test of primary transgenic line 5(10.11) with herbicide leaf painting assay, PCR and RT-PCR. R= resistant, S=susceptible. Note that PCR-positive progenies did not express the bar gene from the RT-PCR. Leaves were painted with 0.3 ml/l Liberty for the herbicide leaf painting assay.
however, those plants did not show transgene (bar) expression based on RT-PCR analysis (Figure 3.15).

The bar gene expression levels of T₁ progenies were evaluated using the ratio of bar/actin (Figures 3.16-18) in RT-PCR. The ratio was calculated from the net intensity of bar and actin bands in ethidium bromide stained agarose gel for a given sample using Kodak Digital Science 1D image analysis software (Eastman Kodak Company, 1999). The ratio reflects the relative expression level of the bar gene because the actin gene is a fundamental structure gene. Soybean actin gene expresses at the same level in soybean plants for a given tissue (shoot or root) (Hightower and Meagher 1985).

Bar gene expression was not consistent among T₁ progeny plants within a given transgenic line. For example, within the transgenic line 6(3.5), progeny plants 1 and 2 showed significantly lower expression levels than the other plants (Figure 3.18). Similar expression patterns were observed in progenies of other transgenic lines tested.

Transformation of intact soybean plants was reported by Zhang et al (1999) using glufosinate as a selective agent in cotyledonary node transformation. Using a higher selection regime of 5 mg/l glufosinate during shoot initiation stage and 2 mg/l during the shoot elongation, they achieved 0-3% transformation.

This work has demonstrated the stable introduction of the bar gene into soybean and expression of the transgene in T₁ transgenic plants at both molecular and phenotypic levels (Figure 3.19). Compared to the published protocols of soybean cotyledonary node transformation, the most substantial difference in methodology is that we have used cotyledonary nodes with an attachment of only 1/3 of the cotyledon. The smaller portion of cotyledon attached to the node was more sensitive to glufosinate
Figure 3.16. Progeny test of primary transgenic line 6(1.5). (a) Leaf painting assay, PCR and RT-PCR. R=resistant, S=susceptible. (b) Relative expression levels of bar gene according to RT-PCR. Note the variation of bar gene expression among progenies. The vertical bars represent ± SE, and different letters indicate means that were significantly different at P< 0.05 using the Tukey t test. ne=no expression. Leaves were painted with 0.3 ml/l Liberty for the herbicide leaf painting assay.
Figure 3.17. Progeny test of primary transgenic line 6(1.7). (a) Leaf painting assay, PCR and RT-PCR. R=resistant, S=susceptible. Pek=Peking, +CTL= positive control (plasmid DNA pBIMC-B was used as a template. (b) Relative expression levels of bar gene according to RT-PCR. Note the variation of bar gene expression among progenies. The vertical bars represent + SE, and different letters indicate means that were significantly different at P< 0.05 using the Tukey t test. ne=no expression. Leaves were painted with 0.3 ml/l Liberty for the herbicide leaf painting assay.
Figure 3.18. Progeny test of primary transgenic line 6(3.5). (a) Leaf painting assay, PCR and RT-PCR. R= resistant, S= susceptible. (b) Relative expression levels of bar gene according to RT-PCR. Note the variation of bar gene expression among progenies. The vertical bars represent + SE, and different letters indicate means that were significantly different at P< 0.05 using the Tukey t test. ne= no expression. Leaves were painted with 0.3 ml/l Liberty for the herbicide leaf painting assay.
than the node with the entire cotyledon, and improved the selection efficiency. These experiments showed that about 30% of the regenerated plants were transgenic at optimum selection levels of 4 mg/l glufosinate at initial selection, and 1.2 mg/l glufosinate during shoot elongation. The overall transformation rate was approximately 3%. Zhang et al (1999) reported that GUS-positive soybean plants were recovered at frequencies ranging from 0.0 to 0.5% under a lower glufosinate selection regime (3.3 mg/l initial selection, 1.7 mg/l during shoot elongation), and a transformation rate of 0.0 to 3.0% under a higher glufosinate selection regime (5.0 mg/l initial selection, 2.0 mg/l during shoot elongation). Our results generally showed the same trend when using glufosinate as a selective agent.

In this study, production of transgenic soybean was based on the fact that glufosinate-resistant multiple shoots could be induced from *Agrobacterium* co-cultivated cotyledonary nodes with 1/3 of the cotyledon attached on a selection medium containing glufosinate. Several *Agrobacterium* strains (Table 3.2) harboring a binary vector were tested to inoculate the target explants. The highest rates of glufosinate-resistant multiple shoot formation were obtained from explants inoculated with the strain KYRT1. This result is consistent with the report by Torisky et al. (1997) when soybean cotyledonary nodes (with the entire cotyledons) were inoculated with the *Agrobacterium* strains EHA105, KYRT1 and GV3850. Torisky et al. (1997) reported that 26.9% the explants inoculated with KYRT1 showed GUS sectors whereas only 10-12% of the explants inoculated with EHA105 or GV3850 produced GUS sectors. They concluded that KYRT1 was more effective than EHA105 or GV3805 in a whole cotyledonary nodes transformation system.
Figure 3.19. Transgenic soybean plants ($T_1$) showed herbicide resistance as compared to untransformed plants (Pek = Peking): (a) 4 days after spraying with 0.3 ml/l Liberty herbicide, (b) 8 days after spraying with 0.3 ml/l Liberty herbicide.
*Agrobacterium* strain GV3101 has been used to successfully transform some non-agronomically important plants, for instance, *Papaver somniferum* (Muriel et al. 1997), peppermint (Krasnyanski et al. 1999) and *Ginkgo biloba* (Dupre et al. 2000). In this experiment, soybean explants inoculated with GV3101 also produced a high rate of glufosinate-resistant multiple shoots on the selection medium but were not significantly different from KYRT1.

Transformation efficiency could be enhanced by the improvement of explant wounding and delivery of *Agrobacterium* cells to the tissue (Norelli et al. 1996, Bidney et al. 1992). Vacuum infiltration was first used for *Agrobacterium*-mediated transformation of *Arabidopsis* in a tissue culture free protocol. We showed that vacuum infiltration of soybean cotyledonary nodes with an *Agrobacterium* suspension enhanced glufosinate-resistant multiple shoot formation. Vacuum infiltration may improve the delivery of *Agrobacterium* into the cotyledonary node tissue or create wounds in the tissues or cells. It has been shown that vacuum infiltration of apple pieces caused cellular damage that increased as the applied pressure increased from 59.9 to 9.3 kPa (Valle et al. 1998). In a bacterium inoculation experiment, Musson et al. (1995) demonstrated that introduction of bacterium strains into cotton plants could be accomplished by vacuum infiltration.

In our experiments in which we attempted to obtain transgenic soybean using hypocotyls as explants, we found that glufosinate-resistant multiple shoots produced from hypocotyls were difficult to regenerated into intact plants under glufosinate selection at concentrations needed to eliminate the untransformed shoots. Multiple shoots formed from hypocotyls were weaker, smaller and morphologically abnormal as compared to the shoots from cotyledonary nodes.
In summary, an improved *Agrobacterium* transformation system has been developed for the production of transgenic soybean. This research demonstrated that cotyledonary nodes with only 1/3 of cotyledons had increased sensitivity to glufosinate selection as compared to nodes with the entire cotyledons which had been used in earlier published transformation protocols. Vacuum infiltration of cotyledonary nodes in an *Agrobacterium* suspension with 508 mm Hg pressure for 10 or 15 minutes during the inoculation significantly increased the rate of glufosinate-resistant multiple shoot formation. *Agrobacterium* strain KYRT1 was found to be highly virulent to soybean, and to our knowledge, is the first report where it was used to produce intact transgenic soybean plants. Progeny test of transgenic soybean showed that the transgene was transmitted to the T1 generation. Transgenic soybean generated in this research has been given to Dr. Bobby Harville, the Louisianan State University soybean breeder, to be utilized as a *bar* gene donor for crossing with other genotypes to produce Liberty resistant cultivars.

**References**


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CHAPTER 4

EFFECTS OF ADDITIONAL COPIES OF \textit{virE} AND \textit{virG} GENES IN \textit{AGROBACTERIUM} ON PLANT TRANSFORMATION EFFICIENCY

Introduction

Numerous \textit{Agrobacterium}-mediated transformation protocols have been developed since the discovery of the natural ability of \textit{Agrobacterium} to transfer and integrate its T-DNA into a plant genome (Zaenen et al. 1974, Chilton et al. 1977). \textit{Agrobacterium}-mediated genetic transformation involves multiple processes. The combined functions of genes on both the chromosome and the Ti plasmid of \textit{Agrobacterium} are required for these processes (Reviewed by Zupan and Zambryski 1995, Gelvin 2000). Understanding of the molecular mechanism of T-DNA transfer from \textit{Agrobacterium} into a plant genome is far from complete. However, it is believed that expression of the virulent (\textit{vir}) gene cluster on the Ti plasmid is one of the key processes during T-DNA transfer and integration events. Twenty-four \textit{vir} genes in 9 operons (\textit{virA}, \textit{B}, \textit{C}, \textit{D}, \textit{E}, \textit{F}, \textit{G}, \textit{H}, \textit{J}) have been identified. Of the genes identified, the VirG protein is a positive transcription factor involved in the activation of all other \textit{vir} genes, while VirE is a ssDNA binding protein protecting the nicked T-DNA in the process of DNA transfer from \textit{Agrobacterium} into the plant genome (Steck et al. 1988, Han and Winans 1994, Gelvin 1998). Since \textit{vir} genes play such important roles in T-DNA transfer, it would be interesting to know if additional copies of \textit{vir} genes in \textit{Agrobacterium} could enhance the plant transformation capability. Liu et al. (1992) reported that addition of \textit{virG} gene in \textit{Agrobacterium} enhanced the transient transformation of celery, carrot and rice. Phenolic compounds and sugars released by
wounded plant tissue served as a signal to activate or induce the expression of vir operons. Several reports demonstrated that acetylsyringone induced the expression of vir genes, thus enhancing the transformation efficiency (Ashby et al. 1987, Shaw et al. 1988). Elucidation of these roles will promote the genetic manipulation of plants by Agrobacterium-mediated transformation.

Transgenic soybean was produced by incorporating the bar gene and glufosinate into the transformation system and by adding glufosinate to the selection medium (Chapter 3). The transformation was based on the induction of glufosinate-resistant multiple shoots from Agrobacterium co-cultivated cotyledonary nodes, and the glufosinate-resistant multiple shoots derived from the nodes were further selected on shoot elongation medium containing glufosinate to obtain transgenic plants. To enhance the production of transgenic soybean, it is necessary to improve the glufosinate-resistant multiple shoot formation rate, which reflects a transient or stable transformation rate.

Because of the important role of vir genes in T-DNA transfer, it was the objective of this study to determine if the increased copy number of virG and virE genes or activation of the expression of vir genes of Agrobacterium could increase the plant transformation efficiency using a model plant transformation system (Arabidopsis). The experiment was extended to the transformation of soybean.

Materials and Methods

Agrobacterium Strain and Plasmids

Agrobacterium GV3101 (Koncz and Shell 1986) was chosen from a preliminary transformation experiment. It was used as the host for all vectors and plasmids. pBIMC-B is a binary vector that contains the bar (driven by a CaMV 35S promoter) and nptII genes
(driven by the Nos promoter) in its T-DNA region (Figure 3.1). Those two genes confer resistance to glufosinate and kanamycin respectively. This binary vector was a gift from Dr. Robin Buell formerly with the Department of Biological Sciences, Louisiana State University. This vector has been tested for transformation of plants previously (Chapter 3).

The plasmids pCH30 and pCH32 were gifts from Cornell Research Foundation, Inc. Plasmid pCH30 contains one copy of **virG** from pTiBo542 and the plasmid pCH32 has one copy of **virG** from pTiBo542 plus the **virE** operon of *Agrobacterium* strain A6. Both plasmids have a wide host range origin of replication **pSa** (Figure 4.1), and are present at 5 to 10 copies per cell in *Agrobacterium* (Hamilton 1997). Both plasmids have tetracycline resistance as a selectable marker. The **virG** gene is the superactivator of all other **vir** genes (Chen et al. 1991) and the **virE** operon encodes the VirE2 ssDNA binding protein and the VirE1 protein that mediates the export of VirE2 protein (Sundberg et al. 1996).

Binary vector pBIMC-B was introduced into host GV3101 alone or with pCH30 or pCH32 using Cell Troporator (Bio Rad Laboratories, Hercules, CA. USA.) following the procedures modified from the manufacturer’s protocol (appendix 1). The electroporated *Agrobacterium* cells were selected on Bacto agar solidified LB medium (Sambrook et al. 1989) containing 40 mg/l gentamycin, and 50 mg/l kanamycin (for selection of pBIMC-B) or 40 mg/l gentamycin, 50 mg/l kanamycin, and 5 mg/l tetracycline (for selection of pBIMC-B + pCH30 or pCH32). Transformation of *Agrobacterium* was confirmed by enzymatic digestion of plasmids isolated from the putatively transformed *Agrobacterium* clones.
Figure 4.1. Plasmids pCH30 containing *vir G* and pCH32 containing *vir G* and *vir E* insertions used in this experiment. TetR = Gene encoding tetracycline resistant, and pSa is the origin of replication. The plasmids pCH30 and pCH32 were gifts from the Cornell Research Foundation, Inc.
Plant Materials

*Arabidopsis thaliana*: Genotype ‘Columbia’ was grown in a 28 °C incubator with continuous light. To synchronize the germination, the seeds were stored at -20 °C for 3 days prior to sowing. The plants were grown in 3.5-inch pots (8 plants in each pot) until the primary inflorescences were 5-10 cm long before inoculation with *Agrobacterium*.

*Soybean*: Sterilized seeds of the soybean cultivar Peking were germinated in petri dishes containing MS basal medium (Murashige and Skoog 1962) with 9 μM BAP (Table 2.1) for 3-4 days in petri dishes at 28 °C in a dark incubator. Each seedling was cut at 3 mm below the cotyledonary nodes, and then a 2/3 portion of the cotyledon above the nodes was removed. The explants were bisected between the two cotyledons, and the apical meristem regions were removed and discarded. This operation yielded 2 identical cotyledonary nodes as shown in Figure 2.1 c. The explants were temporarily stored on co-cultivation medium before being inoculated with *Agrobacterium*.

Transformation of *Arabidopsis*

*Agrobacterium* inoculation: *Agrobacterium* GV3101, harboring the binary vector or plasmids, was cultured on a 150 rpm shaker at 28 °C with LB medium plus appropriate antibiotics until an OD_{600} of 1.5 was reached. The cells were collected by a 10 minute centrifugation at 4000 rpm, and then re-suspended in the infiltration medium (Table 2.1). The density of *Agrobacterium* was adjusted to an OD_{600} of 1.1 with infiltration medium before inoculation. The shoots of *Arabidopsis* plants with 5-10 cm of the primary inflorescences were completely immersed into the *Agrobacterium* suspension by inverting the pot over the *Agrobacterium* suspension as shown in (Figure 3.3). The plants were placed in a vacuum chamber and subjected to 508 mm Hg of vacuum infiltration for 5
minutes (Figure 3.3). After the infiltration, the plants were grown to maturity to collect T₀ putative transformed seeds (first generation of transformation).

**Selection of Arabidopsis transformants:** T₀ seeds collected from infiltrated plants were screened as described previously (Chapter 3). Kanamycin resistant seedlings were transplanted to soil and grown to maturity to collect T₁ seeds. T₁ seeds harvested from individual T₀ plants were selected on medium containing 40 mg/l kanamycin under the same conditions for selection of T₀ seeds. All seeds were sterilized by an overnight exposure to Cl₂ gas in a desiccator prior to germination on medium. Cl₂ gas was generated by a mixture of 100 ml commercial Clorox with 4 ml HCl. Approximately 50 mg of leaf sample was collected from green seedlings of each T₁ line for genomic DNA isolation. Transformation was confirmed by PCR analysis of DNA from T₁ green seedlings showing tolerance to kanamycin. Seeds of wild type were germinated on selective medium as a control to guarantee effective selection.

**Segregation of transgenic progeny:** To assess the effects of multiple vir genes in *Agrobacterium* on the stability of the integrated T-DNA in transgenic plants, twenty-two primary transgenic plants (T₀) were sampled for the segregation study. T₁ seeds were harvested from each individual sampled plant, and germinated on medium containing either kanamycin (40 mg/l) or glufosinate (20 mg/l) with the procedures already described. The number of healthy green (resistant) and bleached yellow (sensitive) seedlings were recorded 10 days after germination.

**Transformation of Soybean Cotyledonary Nodes**

*Agrobacterium* co-cultivation: *Agrobacterium*-mediated transformation of soybean was carried out using the procedures optimized in Chapter 3. *Agrobacterium* was
prepared as stated previously. The bacterium was grown on Bacto agar solidified AB mineral medium containing 40 mg/l gentamycin, and 50 mg/l kanamycin (plus 5 mg/l tetracycline if the *Agrobacterium* contained either pCH30 or pCH32) for 3-4 days. *Agrobacterium* was collected and suspended in liquid co-cultivation medium at a OD_{600} of 1.0-1.1. The previously prepared cotyledonary nodes were transferred into a 125 ml flask along with 15-20 ml of *Agrobacterium* suspension. The flask was connected to a vacuum pump and subjected to a vacuum infiltration at 508 mm Hg for 10 minutes. After vacuum infiltration, the cotyledonary nodes were placed flat side up on phytagar solidified co-cultivation medium (Table 3.1) and incubated at 25 °C in darkness for 4 days.

**Selection for glufosinate-resistant multiple shoots:** After *Agrobacterium* co-cultivation, explants were washed 6 times with sterile distilled water to remove excessive *Agrobacterium*. Cleaned cotyledonary nodes were blotted dry on sterile paper towels, and cultured flat side down on the initial selection medium consisting of Gamborg B5 basal nutrients (Gamborg et al. 1968), 0.16 μM TDZ, 400 mg/l timentin (SmithKline Beecham Pharmaceuticals, Philadelphia, PA.), and 4 mg/l glufosinate (Table 3.1). Cotyledonary nodes not inoculated with *Agrobacterium* were plated on the same selection medium as a negative control. The petri dishes were cultured at a temperature of 28 °C under an 18 h photoperiod, and sub-cultured every 3 weeks onto fresh medium to induce multiple shoot formation.

**Polymerase Chain Reaction (PCR) Analysis:** Approximately 50 mg of shoot material was sampled for DNA isolation. DNA was isolated from *Arabidopsis* or soybean samples using ‘miniprep procedures’ of the Epicentre MasterPure Plant Leaf DNA Purification Kit (Epicentre Technologies, Madison WI, USA). The DNA concentration
was determined at an optical reading of 260 nm. The DNA samples were tested for the presence of the T-DNA region using a pair of \textit{nptII} specific primers (Table 3.4) to amplify the 770 bp \textit{nptII} fragment, and a pair of \textit{bar} gene primers to amplify the 430 bp \textit{bar} fragment (Table 3.4).

For each given PCR reaction, 1 \( \mu \)g of genomic DNA was mixed with primers, Taq polymerase, dNTPs, and PCR buffer (containing MgCl\(_2\)) to obtain a reaction volume of 25 ul. For amplification of the \textit{bar} gene, 1 \( \mu \)l of 10x MasterAmp PCR enhancer (Epicentre Technologies, Madison, WI, USA) was included in each reaction. All PCR reactions were conducted in a thermal cycler with denaturing at 94 °C for 45 seconds, annealing at 57 °C for 1 minute, and extension at 72 °C for 2 min for 40 cycles. The PCR products were electrophoresed on 1% agarose gel. Each gel was subsequently stained with ethidium bromide and viewed under UV light.

\textbf{Results and Discussion}

\textbf{Transformation Efficiency of \textit{Arabidopsis}}

\textit{Arabidopsis} plants were inoculated with \textit{Agrobacterium} containing additional \textit{vir} genes using the “\textit{in planta}” vacuum infiltration method (Bechtold et al. 1993). In parallel, \textit{Agrobacterium} without additional copies of the \textit{vir} genes were used as a control treatment. To evaluate the effect of pre-activation of \textit{vir} genes on transformation efficiency, \textit{Agrobacterium} cultures used for transformation were grown in medium with 100 \( \mu \)M acetoxyringone and compared with a no acetoxyringone treatment. Kanamycin resistant seedlings were observed from all treatments when \( T_0 \) seeds harvested from inoculated plants were screened on selective medium (Figure 4.2). Transformation was confirmed by PCR analysis of \( T_1 \) seedlings derived from \( T_0 \) plants.
The treatment effects were analyzed using Proc GLM of SAS (SAS Institute, Cary, NC). Addition of both \textit{virE} and \textit{virG} genes to \textit{Agrobacterium} and pre-activation of \textit{vir} genes by acetosyringone had significant effects on the transformation of \textit{Arabidopsis} (Table 4.1, Figure 4.2). Seeds from plants inoculated with \textit{Agrobacterium} containing additional \textit{virE} and \textit{virG} genes exhibited the highest transformation rate as reflected by kanamycin resistant seedlings. The transformation rate was approximately twice the rate of \textit{Agrobacterium} lacking any additional \textit{vir} genes. However, \textit{Agrobacterium} with addition of only the \textit{virG} gene did not enhance the transformation efficiency.

Several publications reported that increased copies of \textit{vir} genes enhance plant transformation efficiency (Liu et al. 1992, Richard et al. 1999). A two to five fold higher frequency of transient transformations was observed when \textit{Agrobacterium} harboring multiple copies of \textit{virG} gene was used to infect celery calli (Liu et al. 1992). The enhancement of transient transformation can also be influenced by the host \textit{Agrobacterium} type (agropine and octopine) and plant species (Liu et al. 1992). For example, no enhancement of the transient transformation of carrot calli was achieved by multiple copies of \textit{virG} genes in agropine type \textit{Agrobacterium} whereas a three-fold increase in the transformation of carrot calli was observed using a nopaline type \textit{Agrobacterium} harboring multiple copies of the \textit{virG} gene. In contrast, enhancement of transformation was obtained when multiple copies of \textit{virG} gene were present in agropine type \textit{Agrobacterium} but not in nopaline type \textit{Agrobacterium} strain in the transformation of rice (Liu et al. 1992). The host strain GV3101 used in our experiment is a nopaline type \textit{Agrobacterium}. The addition of \textit{virG} and \textit{virE} genes increased the transformation of \textit{Arabidopsis}. Increasing
Additional \textit{vir} genes

Figure 4.2. Effects of additional copies of \textit{vir} genes and pre-activation of \textit{vir} genes by acetosyringone on \textit{Arabidopsis} transformation efficiency (AS = acetosyringone). Acetosyringone was added to \textit{Agrobacterium} growth medium. Data shown are from 3 independent trials. Bars labeled with different letters indicate significant difference at $P \leq 0.05$. The vertical bars represent $\pm$ standard error. No add.$=$no addition of \textit{vir} gene.

Table 4.1. Statistical analysis of treatment effects of additional copies of \textit{vir} genes and pre-activation of \textit{vir} genes using acetosyringone on transformation of \textit{Arabidopsis}

<table>
<thead>
<tr>
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<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td>\textit{vir} gene</td>
<td>2</td>
<td>11.77</td>
<td>0.003</td>
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<tr>
<td>AS</td>
<td>1</td>
<td>21.71</td>
<td>0.001</td>
</tr>
<tr>
<td>Trials</td>
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</tr>
<tr>
<td>\textit{vir} gene x AS</td>
<td>2</td>
<td>2.73</td>
<td>0.12</td>
</tr>
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</table>

AS$=$ acetosyringone
**virG** gene copies alone had no significant effect on the transformation rate of *Arabidopsis* (Figure 4.2).

Addition of acetosyringone to bacterium growth medium reduced the transformation rate of *Arabidopsis* regardless of the copy number of **vir** genes in *Agrobacterium* (Figure 4.2). The decrease was significant when the *Agrobacterium* contained additional copies of the **vir** genes, where pre-activation treatments showed only half the transformation rate of the control (non pre-activation). The results are contrary to the other transformation studies with *Arabidopsis* (Pavingerova and Ondrej 1995).

**Inheritance of Transgenic *Arabidopsis***

A total of 22 T₀ plants were sampled for progeny testing. T₁ seeds were collected from each of the sampled plants, and germinated on selection medium containing kanamycin or glufosinate. Green resistant seedlings were easy to distinguished from the bleached sensitive seedlings after 10 days of germination (Figure 3.5). Out of 22 T₁ lines tested, none were completely sensitive to kanamycin or glufosinate selection. The segregation ratios were tested with Chi square goodness-of-fit test against the closest expected ratio (Table 4.2). The Mendelian segregation ratios of 3:1 and 15:1 were observed for most of the lines tested, which could be explained as one or two integrated loci respectively. Some lines did not exhibit a Mendelian segregation ratio.

Non-Mendelian segregation is often observed in transgenic plant progenies (Deroles and Gardner 1988, Heberle et al. 1988). It is not clear whether the non-Mendelian ratios in the first generation after transformation reflect chimaerism in the primary transformants, poor transmission of the inserts, transgene inactivation or a combination of these factors. *Comparing the **bar** (glufosinate-resistance) and nptII*
Table 4.2. Genetic analysis of transformed lines of *Arabidopsis*

<table>
<thead>
<tr>
<th>ID</th>
<th>Additional vir genes</th>
<th>T₀ plant</th>
<th>T₁ seedlings</th>
<th>Ratio (R/S)</th>
<th>χ²</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resistant Km</td>
<td>Observed Km</td>
<td>Expected Km</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glu</td>
<td>Susceptible Km</td>
<td>Glu</td>
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<tr>
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<td></td>
<td>125</td>
<td>89</td>
<td>35</td>
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<td>83</td>
<td>92</td>
<td>7</td>
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<td>105</td>
<td>135</td>
<td>6</td>
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<tr>
<td>A(3.7)1</td>
<td>virG</td>
<td></td>
<td>135</td>
<td>79</td>
<td>34</td>
</tr>
<tr>
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<td>virG</td>
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<td>113</td>
<td>92</td>
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<tr>
<td>A(3.7)3</td>
<td>virG</td>
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<td>15</td>
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<td>6</td>
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<td>17</td>
<td>24</td>
<td>9</td>
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<tr>
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<td>110</td>
<td>92</td>
<td>30</td>
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<tr>
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<td>virG/E</td>
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<td>73</td>
<td>77</td>
<td>29</td>
</tr>
<tr>
<td>A(1.13)3</td>
<td>virG/E</td>
<td></td>
<td>78</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>A(1.13)4</td>
<td>virG/E</td>
<td></td>
<td>88</td>
<td>72</td>
<td>35</td>
</tr>
<tr>
<td>A(1.15)1</td>
<td>virG/E</td>
<td></td>
<td>84</td>
<td>104</td>
<td>27</td>
</tr>
<tr>
<td>A(1.15)2</td>
<td>virG/E</td>
<td></td>
<td>70</td>
<td>51</td>
<td>33</td>
</tr>
<tr>
<td>A(3.15)1</td>
<td>virG/E</td>
<td></td>
<td>11</td>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td>A(1.15)4</td>
<td>virG/E</td>
<td></td>
<td>116</td>
<td>122</td>
<td>8</td>
</tr>
<tr>
<td>A(4.1)1</td>
<td>virG/E</td>
<td></td>
<td>105</td>
<td>96</td>
<td>32</td>
</tr>
<tr>
<td>A(4.1)2</td>
<td>virG/E</td>
<td></td>
<td>47</td>
<td>28</td>
<td>3</td>
</tr>
</tbody>
</table>

*Untrans = untransformed control, Km = kanamycin, Glu = glufosinate, R = resistant, S = susceptible.*

*Arabidopsis* seeds were germinated on medium containing 40 mg/l kanamycin or 20 mg/l glufosinate. The critical value for the chi square test is 3.841.
(kanamycin resistance) segregations, we found that transgenic lines showed irregular Mendelian segregation ratios for the *bar* gene but not for *nptII*. Three of the 22 lines showed abnormal segregation ratios for the glufosinate-resistant trait. The ratio of Resistant: Susceptible in all abnormal lines was approximately 1:1 (Table 4.2). The corresponding lines had regular Mendelian segregations (3:1) for the kanamycin resistant trait (*nptII* gene). These results might indicate the inactivation of the transgene (*bar*) in the progeny plants. Kilby et al. (1992) demonstrated that the loss of resistance is associated with methylation of a promoter in *Arabidopsis* transformation.

**Additional Copies of *vir* Genes in Agrobacterium and Soybean Transformation**

In the above experiment, additional copies of *vir* genes in *Agrobacterium* could enhance the transformation of *Arabidopsis*. A similar experiment was conducted with soybean to determine if transformation rates could be enhanced. The transformation protocol was based on the method previously described (Chapter 3). *Agrobacterium* containing multiple copies of *virG* or *virE* genes was used to inoculate soybean cotyledonary nodes. Cotyledonary nodes inoculated with *Agrobacterium* were co-cultivated on medium with or without supplemental acetosyringone to evaluate the effects of activation of *vir* genes on transformation of soybean. Glufosinate-resistant multiple shoots were obtained on medium containing 4 mg/l glufosinate. PCR analysis of the multiple shoots showed the amplification of *nptII* and *bar* fragments (Figure 4.3).

Additional copies of *vir* genes in *Agrobacterium* did not enhance the transformation rates of soybean (Figure 4.4, Table 4.3). Similar results were reported by Bond et al. (1996). Duplication of virulent genes *virB* and *virG* in wild type *Agrobacterium* strain...
Figure 4.3. PCR analysis of glufosinate-resistant multiple shoots derived from soybean cotyledonary nodes co-cultivated with *Agrobacterium*: (a) with npt II primers; (b) with bar primers. Lane 1-11: individual shoot samples; lane 12: Peking (negative control); lane 13: pBIMC-B (positive control), MK=100 bp ladder.
Additional vir genes

Figure 4.4. Effects of additional copies of vir genes and activation of vir genes by acetosyringone on soybean transformation efficiency (AS= acetosyringone). Acetosyringone was added to Agrobacterium soybean co-cultivated medium. Data shown are from 3 independent trials. The vertical bars represent + standard error. No add.=no addition of vir gene.

Table 4.3. Statistical analysis of treatment effects of additional copies of vir genes and activation of vir genes using acetosyringone on transformation of soybean

<table>
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<td>vir gene</td>
<td>2</td>
<td>0.23</td>
<td>0.80</td>
</tr>
<tr>
<td>AS</td>
<td>1</td>
<td>3.62</td>
<td>0.086</td>
</tr>
<tr>
<td>Trials</td>
<td>2</td>
<td>1.24</td>
<td>0.33</td>
</tr>
<tr>
<td>vir gene x AS</td>
<td>2</td>
<td>0.00</td>
<td>0.99</td>
</tr>
</tbody>
</table>

AS= acetosyringone
A208 and A281 failed to increase susceptibility to galling or increase gall size in cultivars Peking and Bragg.

Several alkylsyringamides have been shown to be powerful inducers of the vir genes. It was found that acetosyringone has high vir gene inducing activity (Bethelot et al. 1998). In this experiment, co-cultivation of cotyledonary nodes with Agrobacterium on the medium containing acetosyringone generally increased the multiple shoot frequency regardless of the number of copies of vir genes in Agrobacterium. Acetosyringone enhances vir gene function during transformation (Stachel et al. 1985), and has been shown to increase Agrobacterium-mediated transformation potential in several plant species (Van Wordragen and Dons 1992).

These experiments lead to the following conclusions: 1) the effect of additional copies of vir genes in Agrobacterium on plant transformation is species-dependent, and soybean cultivar ‘Peking’ is not sensitive to copies of vir genes in Agrobacterium, 2) addition of acetosyringone to co-cultivation medium increases the transformation efficiency of soybean regardless of the number of copies of vir genes in Agrobacterium.

References

Ashby AM, Watson MD and Shaw CH (1987). A Ti plasmid determined function is responsible for chemotaxis of A. tumefaciens towards the plant wound compound acetosyringone. FEMS Microbiology Letters 41: 189-192


Liu CN, Li XQ and Gelvin SB (1992). Multiple copies of *virG* enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. Plant Mol. Biol. 20: 1071-1087


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CHAPTER 5

CONCLUSIONS

Plant biotechnology has two important and interdependent components, tissue culture and molecular biology. With the development of efficient methods for the regeneration of plants from cultured cells or tissues, significant advances have been made in the production of transgenic plants with defined foreign genes. Several important discoveries in biological science have made significant contributions to these achievements. These include the discovery of DNA structure by Watson and Crick (1953), isolation of restriction enzymes and production of recombinant DNA (Chung and Cohen 1974), and the discovery of T-DNA transfer to plant cells by Agrobacterium (DeBlock et al. 1984, Horsch et al. 1985).

This dissertation research has integrated a modified regeneration system from soybean explants into a transformation protocol to produce transgenic soybean using glufosinate as a selective agent. Transgenic soybean plants showed the expression of the transgene at both the phenotypic and molecular levels. The effects of additional copies of virE and virG gene to Agrobacterium on transformation efficiency were evaluated.

Plant Regeneration from Soybean Explants

Protocols to regenerate plants from the culture of soybean hypocotyls and cotyledonary nodes (with 1/3 cotyledon attached) were developed. Soybean seeds were germinated in MS medium containing 6-benzylaminopurine (BAP). The concentration of BAP in the seed germination solution has a significant impact on multiple shoot induction. Hypocotyls obtained from seedlings germinated in medium containing BAP
7 μM or higher produced more multiple shoots than BAP below 7 μM. Yasseen and Splittstoesser (1990) reported that the cotyledonary nodes germinated with 5 μM BAP produced over twice as many shoots as others germinated without BAP.

Hypocotyls and cotyledonary nodes were cultured on Gamborg B5 basal medium (Gamborg et al. 1968) containing various concentrations of thidiazuron (TDZ) for 3-4 weeks. A concentration of TDZ at 0.16 μM was found effective for inducing multiple shoot formation. Elongation of the shoots was achieved on Gamborg B5 medium with 0.36 μM BAP for hypocotyl derived multiple shoots. Elongated shoots were separated into individuals and cultured on plant recovery medium consisting of Gamborg B5 and 0.58 mg/l GA₃ and 0.67 mg/l IBA for further growth and rooting. For cotyledonary node derived shoots, elongation and plant recovery were achieved on the same medium. When different gelling agents (agar, gelrite, phytagar) and sucrose levels in the plant recovery medium were compared, medium containing 2.0 % sucrose and solidified with phytagar gave a significantly better plant recovery rate than Sigma agar.

Based on these studies, an improved regeneration system for soybean hypocotyls and cotyledonary nodes has been developed. In average, approximately 8 plants can be produced from each cultured cotyledonary node. To my knowledge, this is the first report of the regeneration of soybean plants through the culture of cotyledonary nodes with attachment of only 1/3 of the cotyledon. DAN and Reichert obtained 1.0 to 5.0 shoots per cultured explant when 13 soybean genotypes were tested (Dan and Reichert 1998). We improved the average shoot number to 10 shoots per hypocotyl using soybean cultivar Peking.
Nodes with only 1/3 of the cotyledon are more sensitive than nodes with the entire cotyledon to glufosinate selection. This regeneration system was incorporated into an Agrobacterium-mediated genetic transformation protocol using glufosinate as a selective agent.

Production of Transgenic Soybean

Although Agrobacterium-mediated gene transfer has been established for routinely transferring genes into many crops, transformation of soybean has been inefficient with the Agrobacterium-mediated gene transfer system. Additionally, only a handful of laboratories in the world are able to produce transgenic soybean plants consistently. We have established techniques to improve the soybean transformation system.

Transgenic soybean plants were obtained with Agrobacterium-mediated genetic transformation, and incorporating the bar gene and glufosinate herbicide into the selection system. The binary vector pBIMC-B, containing the bar and nptII genes (driven by CaMV 35S and Nos promoters, respectively), was introduced into Agrobacterium strains EHA105, GV3101, KYRT1, and LBA4404. Cotyledonary nodes of soybean with 1/3 of the cotyledon attached were used as target explants. Since glufosinate at concentrations greater than 3.0 mg/l totally inhibited the formation of multiple shoots from untransformed explants, explants were selected on medium containing 4 mg/l glufosinate. KYRT1 produced more glufosinate-resistant multiple shoots than the other strains. Vacuum infiltration of the cotyledonary nodes with an Agrobacterium suspension at 508 mm Hg for 10 and 15 minutes during inoculation significantly increased the glufosinate-resistant multiple shoot formation rate.

Glufosinate-resistant multiple shoots were maintained on glufosinate containing
medium at 1-1.2 mg/l until the plantlets were more than 2 cm in length. This was necessary for the effective selection of transgenic shoots. Recovered plants were transplanted into soil, and screened with a 0.3 ml/l solution of Liberty herbicide applied to the leaves. PCR and southern hybridization analysis of Liberty resistant plants confirmed the successful transformation and integration of bar gene into the cultivar Peking. Previous reports using kanamycin selection found only 1-2 % of recovered shoots appeared transgenic (GUS-positive) (Meurer et al. 1998). Our research showed that 33 % of the recovered plants were transgenic using 1.2 mg/l glufosinate selection.

Transgenic soybean plants that produced seeds were subjected to progeny tests to evaluate transgene stability and expression using a herbicide (Liberty) leaf painting assay, PCR and RT-PCR analysis. The results indicated that most transgenic plants exhibited Mendelian segregation. Some transgenic plants did not follow Mendelian segregation patterns in the T₁ progeny. Gene silencing in some plants was shown with PCR and RT-PCR analysis of the progeny. Two of the nine primary transgenic plants tested failed to transmit the transgene to the next generation.

**Effect of Additional Copies of vir Genes in Agrobacterium on Plant Transformation**

One of the key processes of Agrobacterium-mediated genetic transformation is the expression of a series of virulent genes located on the Ti plasmid during the interaction between plant and Agrobacterium cells. The effects of additional copies of virE and virG genes in an Agrobacterium strain were studied for plant transformation with Arabidopsis and soybean.

Arabidopsis plants were inoculated with Agrobacterium harboring the binary vector pBIMC-B (carrying nptII and bar genes) with additional copies of virG or virG and virE genes using an “in planta” vacuum infiltration method, and then grown to

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maturity. Harvested seeds were screened on medium containing 40 mg/l kanamycin or 20 mg/l glufosinate to select for resistant seedlings. Progeny tests of the primary transformed plants showed that nptII and bar genes were passed to the second generation. Successful transformation was confirmed by PCR analysis of T1 plants. The transformation efficiency was influenced by the types of vir genes added. A 2 fold enhancement was observed when both virE and virG genes were included in the Agrobacterium. However, addition of only the virG gene in the Agrobacterium did not enhance the transformation rate. Pre-activation of vir genes of Agrobacterium with acetylsyringone reduced transformation rates.

Soybean cotyledonary nodes were co-cultivated with Agrobacterium harboring pBIMC-B with additional copies of virG or virG and virE genes, and then selected on medium containing 4 mg/l glufosinate. Glufosinate-resistant multiple shoot formation rates were not enhanced by additional copies of vir genes in Agrobacterium. Enhancement was observed when 100 μM acetylsyringone was added to Agrobacterium-cotyledonary node co-cultivation medium.

In conclusion, with the soybean cultivar Peking, we were able to get about 3.0% transformation rate by inoculation of the cotyledonary nodes with 1/3 cotyledon using strain KYRT1 and vacuum infiltration. The induction of glufosinate-resistant multiple shoots from the nodes was achieved on medium containing 0.16 μM TDZ and 4 mg/l glufosinate, and the plants were recovered by selection of the shoots on medium containing 1 to 1.2 mg/l glufosinate. This method, if applicable to other soybean genotypes, could greatly expand the ability to transform soybean and lead to new genetic improvements.
References


Yasseen YM and Splittstoesser WE (1990). Regeneration of soybean [*Glycine max* (L.) Merr.] from the seedling apex, stem node, cotyledonary node and cotyledons. PGRSA Quarterly 18: 203-210

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APPENDIX 1

PROCEDURE FOR ELECTRO-TRANSFORMATION OF
AGROBACTERIUM

(A) Preparation of Agrobacterium Competent Cells
Keep the cells as close to 0 °C as possible (in an ice/water bath) throughout the preparation.

1. Inoculate 1 liter of LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl) containing appropriate antibiotics with 1/100 volume of a fresh overnight culture of Agrobacterium.

2. Grow cells at 28 °C with vigorous shaking (200 rpm) to an OD$_{600}$ of approximately 0.6-0.8

3. To harvest, chill the culture on ice for 30 minutes, centrifuge cells in cold centrifuge bottles in a cold rotor at 4000 x g for 15 minutes.

4. Remove as much of the supernatant (medium) as possible.

5. Gently resuspend the pellets in a total of 1 liter of ice-cold 10% glycerol. Centrifuge as in steps 3 and 4.

6. Resuspend in 500 ml of ice-cold 10% glycerol. Centrifuge as in steps 3 and 4.

7. Resuspend in ~250 ml of ice-cold 10% glycerol. Centrifuge as in step 3 and 4.

8. Resuspend to a final volume of 2 to 3 ml in ice-cold 10% glycerol. The cell concentration should be about 1 - 3 x 10$^{10}$ cells/ml.

9. Store the suspension as aliquots (200 µl each tube) in 0.5 ml tubes at -70 °C for future use.

(B) Electro-transformation and Plating

1. Gently thaw the cells at room temperature and then immediately place them on ice.
2. Remove the sterile cuvettes from their pouches and place them on ice. Place the electroporation chamber in -20 °C.

3. In a cold, 1.5 ml polypropylene tube, mix 40-80 μl of the cell suspension with 10 ng of plasmid DNA (DNA should be in a low ionic strength buffer). Mix well and let sit on ice for 1 minute.

4. Set the Pulser apparatus to 2.50 kV using the 0.2 cm electroporation cuvettes.

5. Transfer the mixture of cells and DNA to a cold electroporation cuvette, and shake the suspension to the bottom. Place the cuvette in a cold safety chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber and pulse once.

6. Remove the cuvette from the chamber and immediately add 160 μl of SOC medium to the cuvette and quickly but gently resuspend the cells with a pasteur pipette. (This rapid addition of SOC after the pulse is very important in maximizing the recovery of transformants.)

7. Transfer the cell suspension to a glass test tube and incubate at 28 °C for 2 hours. (Shake the tubes at 200 rpm during this incubation).

8. Plate on selective medium consisting of LB and appropriate antibiotics.

9. Culture the plates in 28 °C for 2 days.

10. Isolate plasmid from the single putatively transformed clones and conduct the enzymatic digestion to confirm transformation.
APPENDIX 2

SOUTHERN HYBRIDIZATION

1. Determine the DNA concentration using DNA mass ladder and optical density OD_{260}.

2. Digest 20 µg of genomic DNA by using 4 times of regular dosage of restriction enzyme in 200 µl reaction volume in following manner.

3. Mix ½ of the total required enzyme to the DNA and stored in 4 °C for 1 hour. Then incubate at 37 °C for 3 hours.

4. Add the remaining ½ of enzyme to the reaction, and incubate for 20 hours.

5. Precipitate digested DNA with 2 volumes of ethanol (400µl) at −20 °C for 30 minutes.

   Centrifuge at > 10000 rpm for 5 minutes to collect DNA pellet.

6. Dissolve DNA pellet in 25 µl distilled water.

7. Load the digested DNA onto 0.8 % agarose gel and run the gel at 30 v.

8. Rinse gel with distilled water.

9. Rinse the gel twice for 15 minutes in each of the denaturing and neutralization solutions.

10. Transfer the DNA to nylon membrane in 20x SSC buffer overnight.

11. Hybridization of the membrane with *bar* gene probe follows the instruction manual of Stratagene QuickHyb Hybridization Solution (Stratagene Catalog # 201220).
## APPENDIX 3

### SOURCE OF CHEMICALS AND REAGENTS

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<td></td>
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<td>Random primed labeling kit</td>
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<td>Epicentre</td>
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VITA

Shaomian Yao was born in Guang-Zhou, Guangdong Province of People’s Republic of China, on October 20, 1963. In 1980, he graduated from Huaxi High School in Guiyang and entered the Agronomy Department of Guizhou Agriculture College, Guizhou University, for undergraduate training. He received his bachelor of science degree in 1984. Upon graduation, Mr. Yao was assigned to the Oil Crop Institute of Guizhou Academy of Agricultural Sciences as a plant breeder. He engaged in soybean breeding and oil crop germplasm research projects. He finished a project entitled Guizhou Small Oil Crop Germplasm Research, and received a merit award by the National Academy of Agricultural Science. In 1991, he entered the Central China Agricultural University for further non-degree training under Prof. D.T. Cai, specializing in the embryology of angiosperms.

In 1992, he came to the United States, and conducted research on soybean biotechnology as a visiting scholar under the guidance of Dr. Suzan S. Croughan at the Rice Research Station in Crowley. In the fall of 1995, Mr. Yao initiated his graduate study in the Department of Agronomy at Louisiana State University where he completed his master of science degree in 1997 with an emphasis in tissue and anther culture.

Upon graduation with master’s degree, he continued his graduate studies at Louisiana State University. At present, he is a candidate for the degree of Doctor of Philosophy with research area in molecular biology and genetic transformation.

Mr. Yao married Li Liu in 1992, and they have a son Lou Ray Yao, born on March 16, 1995, in Lafayette, Louisiana.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Shaomian Yao
Major Field: Agronomy
Title of Dissertation: Optimization of Agrobacterium-mediated Genetic Transformation of Soybean Using Glufosinate As A Selective Agent

Approved:

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

Bobby D. Harrell
Brad Kent
R. J. Constant
D. J. Stahl
James Osier

Date of Examination: March 16, 2001