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Agrobacterium tumefaciens-mediated transformation of tobacco (Nicotiana tabacum L.) leaf disks: evaluation of the co-cultivation conditions to increase beta-glucuronidase gene activity

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AGROBACTERIUM TUMEFACIENS-MEDIATED
TRANSFORMATION OF TOBACCO
(NICOTIANA TABACUM L.) LEAF DISKS: EVALUATION OF THE
CO-CULTIVATION CONDITIONS TO INCREASE
β-GLUCURONIDASE GENE ACTIVITY

Thesis

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Louisiana State University and
Agricultural and Mechanical College
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By
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# TABLE OF CONTENTS

Acknowledgments ...........................................................................................................ii  

List of Tables ....................................................................................................................iv  

List of Figures ....................................................................................................................v  

List of Abbreviations .......................................................................................................vi  

Abstract ............................................................................................................................vii  

Chapter 1. Introduction ....................................................................................................1  

Chapter 2. Review of Literature .......................................................................................3  
  2.1 Molecular Mechanism of Agrobacterium-mediated DNA Transfer ...................3  
  2.2 Adoption for Plant Molecular Biology .................................................................10  
  2.3 Agrobacterium-mediated Plant Transformation Protocol Development ..........12  

Chapter 3. Materials and Methods ...............................................................................20  
  3.1 Materials ................................................................................................................20  
  3.2 Methods ..................................................................................................................22  

Chapter 4. Results ..........................................................................................................27  
  4.1 Effect of Temperature During the Co-cultivation ..............................................27  
  4.2 Comparison of Two \( \beta \)-glucuronidase Genes .................................................28  
  4.3 Effect of Surfactant, Vacuum Pressure Strength and Duration .......................32  
  4.4 Effect of Co-cultivation Durations and Bacteria Concentrations ...................39  
  4.5 Effect of Bacterial Pre-culture Conditions and Acetosyringone Concentrations 39  
  4.6 Effect of Wounding and Bacterial Removal .....................................................44  
  4.7 Timecourse Experiment .......................................................................................46  
  4.8 Evaluation of Antibiotic Selection Agents .......................................................46  

Chapter 5. Discussion .....................................................................................................49  

References .......................................................................................................................52  

Appendix. Phaseolin Promoter and Terminator Construction Scheme .....................60  

Vita .................................................................................................................................67
LIST OF TABLES

Table 1. Effect of Silwet L-77 concentrations and vacuum durations, separately or in combination, on callus formation and GUS-positive cells ..........................................................37

Table 2. Effect of co-cultivations duration and bacterial concentrations on callus formation and necrosis .............................................................................................................41
LIST OF FIGURES

Figure 1. Effect of different co-cultivation temperatures, 15°C, 18°C, 20°C, 22°C and 25°C on GUS activity ..............................................................29

Figure 2. Effect of co-cultivation temperature on GUS activity ........................................30

Figure 3. Histochemical staining of GUS activity was conducted after a four days co-cultivation at (a)15°C, (b)18°C, (c)20°C, (d)22°C and (e)25°C ..........................................................31

Figure 4. Comparison of two β-glucuronidase genes in two different protocols .................33

Figure 5. Effect of low and high vacuum strengths and durations on GUS activity .............34

Figure 6. Effect of Silwet L-77 concentrations and vacuum infiltration durations, separately (a) and (b), or in combination (c) and (d) on GUS activity ........................................35

Figure 7. Effect of the 0.001% Silwet L-77 and 50 mm Hg vacuum infiltration for 20 min, separately or in combination on callus formation (a) and fresh weight (b) .......................38

Figure 8. Effect of bacterial concentrations and days of the co-cultivation period on GUS activity ...................................................................................................................................40

Figure 9. Effect of the acetylsyringone (AS) and pre-culture conditions of Agrobacterium tumefaciens .........................................................................................42

Figure 10. Effect of the acetylsyringone (AS) concentrations during the four day co-cultivation ..43

Figure 11. Effect of wounding and bacterial removal on GUS activity .................................45

Figure 12. Timecourse of the increase in GUS activity after inoculation ............................47

Figure 13. Effect of antibiotics, kanamycin (a) and hygromycin (b) on plant necrosis ........48
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>Acetosyringone (3,5-dimethoxy-4-hydroxy Acetophenone)</td>
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<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl (COOH)-terminal</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
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<tr>
<td>F pilus</td>
<td>Flagella Pilus</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GUS</td>
<td>β-1,4-Glucuronidase</td>
</tr>
<tr>
<td>HDEL</td>
<td>Histidine(H), Aspartic acid(D), Glutamic acid(E), and Leucine(L) :ER retention signal</td>
</tr>
<tr>
<td>HPT</td>
<td>Hygromycin Phosphotransferase</td>
</tr>
<tr>
<td>NPTII</td>
<td>Neomycin Phosphotransferase II</td>
</tr>
<tr>
<td>Nos poly-(A)</td>
<td>Nopaline Synthase Poly (adenylation)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PIV2</td>
<td>Potato Intervening Sequence 2</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>Ti plasmid</td>
<td>Tumor-Inducing Plasmid</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer-DNA</td>
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<tr>
<td>T-Pilus</td>
<td>Transfer Pilus</td>
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<tr>
<td>Tra protein</td>
<td>T-DNA transfer protein</td>
</tr>
<tr>
<td>vir gene</td>
<td>virulence Gene</td>
</tr>
<tr>
<td>tml</td>
<td>tumor morphology large Locus of T-DNA of pTi 15955</td>
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ABSTRACT

Agrobacterium tumefaciens-mediated transformation is generally used for genetic transformation of higher plants. Several experimental factors important for the increase of β-glucuronidase (GUS) reporter gene activity were evaluated in this study using leaf disks of tobacco (Nicotiana tabacum L. cv. Xanthi). We found that co-cultivation temperature at 20°C is the most critical factor to obtain the reproducible enhancement of GUS activity. pCAMBIA 1305.01 resulted in higher GUS activity than the other two pCAMBIA vectors 1301 and 1305.02.

The highest GUS activity and transformation efficiency were achieved under the following experimental conditions: Agrobacterium tumefaciens strain LBA4404 containing pCAMBIA1305.01 was grown overnight at 28°C in liquid Agrobacterium media, and the concentration was adjusted to 3x10^7 cells/mL (0.3 A_600 units/mL). Tobacco leaf disks were inoculated with bacteria under 50 mm Hg vacuum infiltration for 20 min in the presence of 0.001% (w/v) Silwet L-77. Leaf disks were co-cultivated for four days under constant light at 20°C in MS shoot media containing 200 μM acetosyringone without antibiotics. Leaf disks were then transferred to MS shoot selection media containing 50 mg/L hygromycin and 500 mg/L carbenicillin, and grown for an additional 14 days under constant light at 25°C. β-Glucuronidase (GUS) activity was measured at the end of the growth period by quantitative GUS assay and GUS histochemical staining.
CHAPTER 1. INTRODUCTION

Agrobacterium tumefaciens-mediated transformation has been widely used for research in plant molecular biology and for genetic improvement of crops since 1983. The advantage of the method is the wide host-range of the bacterium, from microorganisms to higher animals and plants, including major crops such as soybean, cotton, rice, maize, sugarcane and wheat. The other merits include integration of the small copy number of T-DNA into plant chromosomes, and stable expression of transferred genes. However, even with those superior attributes, it is still difficult to achieve high reproducibility and consistency of transformation events that are prerequisites for large scale transformation experiments in plant biology.

Expression of virulence (vir) genes from the Ti plasmid of Agrobacterium tumefaciens are enhanced by several experimental factors, including phenolic compounds (Spencer and Towers, 1988), acetosyringone (AS) from 100 to 200 µM, sugars (Shimoda et al., 1990) and pH of co-cultivation media, ranging from pH 5.4 to 5.6 (Mondal et al., 2001). Temperature is also found to be an important factor in bacterial conjugation and vir gene expression. Fullner et al. (1996) reported that 19°C induced more pili formation than 28°C, and resulted in more efficient plasmid mobilization between the bacteria. Low temperature at 19°C significantly increased VirB protein that was the major subunit of the pilus of Agrobacterium (Lai and Kado, 1998; Baron et al., 2001). Baron et al. (2001) reported vigorous tumor formation after co-cultivation of wounded K. diagremontiana with Agrobacterium at 20°C.

Co-cultivation of host plants with Agrobacterium tumefaciens has been routinely conducted at room temperature, ranging from 24°C to 28°C, or under no precise temperature control at all. This was the case for transformation of tobacco (Krugel et al., 2002), soybean (Paz et al., 2006), rice (Terada et al., 2004), potato (Andersson et al., 2003), and cotton (Leelavathi et al., 2004). The effect of co-cultivation temperature on Agrobacterium-mediated plant transformation has not been well studied to date. Only a few experiments (Kapila et al., 1997; Dillen et al., 1997; Baron et al., 2001) tested lower co-cultivation temperatures compared to room temperature in Agrobacterium-mediated plant transformation and showed increased transformation efficiency, gene expression and tumor formation.

One of the major objectives of this study was to investigate the effects of temperatures lower than room temperature, and determine the specific temperature that is optimal for higher
expression of transferred gene activity after *Agrobacterium*-mediated transformation. We used tobacco leaf disks as a model system because tobacco is one of the most accessible species for plant transformation and gene expression experiments. We evaluated effects of co-cultivation temperature, not only by measuring GUS activity (quantification of GUS gene expression as a reporter gene for plant transformation), but also by detecting histochemical GUS expression and tissue viability (leaf necrosis) in this study.
CHAPTER 2. REVIEW OF LITERATURE

2.1 Molecular Mechanism of Agrobacterium-mediated DNA Transfer

2.1.1 Bacteriology, Host Range and Opines

The *Agrobacterium tumefaciens*-mediated transformation method is commonly used to create transgenic plants because it has several merits compared with direct gene transfer methods such as particle bombardment, electroporation, and silicon carbide fibers. The advantages are (1) stable gene expression because of the insertion of the foreign gene into the host plant chromosome; (2) low copy number of the transgene; and (3) large size DNA segments can be transferred. Agronomically and horticulturally important crops, flowers and trees have been genetically modified using this method (Ko and Korban, 2004; Lopez et al., 2004).

*Agrobacterium tumefaciens* is a gram-negative bacterium and soil phytopathogen that genetically transforms host plants and causes crown gall tumors at wound sites (Smith and Townsend, 1907). The interaction of *Agrobacterium* and eukaryotic cells is the only known mechanism for DNA transport between the different kingdoms in nature. *Agrobacterium* is usually classified by the disease symptomology (type of opine) and host range. *Agrobacterium* can transfer DNA to a broad group of organisms: angiosperms (dicots and monocots), gymnosperms and fungi, such as yeasts, ascomycetes, and basidiomycetes. Recently, it was found that *Agrobacterium* can transfer DNA to human cells (Gelvin, 2003). The genetic mechanism of host range determination is still obscure, but it was reported that several virulence (*vir*) genes on the Ti plasmid, *virC* (Yanofsky and Nester, 1986), *virF* (Regensburg-Tuink and Hooykaas, 1993), and *virH* (Jarchow et al., 1991) were involved in determination of the range of plant species. Hood et al. (1987) reported that the Ti plasmid in a natural host strain *A. tumefaciens* Bo542 can not transform leguminous plant species, but when it is placed in the C58 chromosomal background, pTi Bo542 causes tumors in soybeans and other legumes. Interaction of the Ti plasmid with a particular chromosomal background in bacteria may also control the host range. However, it appears that the plant host also plays a role in transformation, because many monocot plant species such as maize, rice, barley and wheat can not be genetically transformed by *Agrobacterium* without addition of chemicals, or these plants do not develop a crown gall tumor after transformation (Gelvin, 2003). Complex genetic mechanisms of the host plants are also necessary for transformation by *Agrobacterium*. 
Agrobacterium is an effective tool for plant genetic engineering, since a portion of the plasmid DNA from Agrobacterium is incorporated into higher plant cells and results in crown gall in the host plant (Chilton et al., 1977). Tumor induction is initiated by bacterial recognition of monosaccharides and phenolic compounds secreted by the plant wound site. “Activated” Agrobacterium transfers a particular gene segment, called transfer DNA (T-DNA), from the Ti plasmid, and T-DNA is stably integrated into the chromosomal DNA in nucleus of the host plant; the genes for opine synthesis and tumor inducing factors in T-DNA are transcribed in the infected cells. This expression of the foreign gene in the host plant results in neoplastic growth of the tumors, providing increased synthesis and secretion of opines for bacterial consumption (Nester et al., 1984). Opine is the condensation product of an amino acid with a keto acid or sugar, and is a major carbon and nitrogen source for Agrobacterium growth. Agrobacterium is classified based on the type of opine. Different Agrobacterium tumefaciens strains produce different opine phenotypes of crown gall tumors, because a particular opine expressed in the tumor is used for particular bacterial growth. Most common Agrobacterium strains produce an octopine or nopaline form of opines (Hooykaas and Beijersbergen, 1994). Octopine and nopaline are derivatives of arginine. Agropine was found in octopine-type tumors, and it is derived from glutamate (Guyon et al., 1980).

2.1.2 Tumor-inducing (Ti) Plasmid

Several components of Agrobacterium are necessary for transferring the piece of bacterial DNA into the plant cell. One component is the chromosomal virulence A (chvA) gene, which is on the Agrobacterium chromosome and activated by sugars. ChvA protein triggers bacteria to bind to the wounded plant tissue and to respond to a specific chemical (chemotaxis). The Ti plasmid in bacteria contains the other main components, which are generated or activated efficiently for causing crown gall in host plants after bacteria attach to the plant wound site. The first is T-DNA, which is actually integrated into the plant cell chromosome. The second is the 35-kb virulence (vir) region, which is composed of seven loci (virA, virB, virC, virD, virE, virG, and virH). Expression of vir genes is triggered by a phenolic compound, which is secreted from the wound site of the host plant. The main functions of Vir proteins are to mediate the T-DNA excision from the Ti plasmid, export of the T-DNA piece from the bacteria, and insert it into the host plant chromosome (Gelvin, 2003). These two components are essential for a successful gene transfer. The Ti plasmid also has other components, an opine catabolism region, a conjugal
transfer region, and a vegetative origin of replication of the Ti plasmid (oriV). Engler et al. (1981) found that these vir regions have sequence conservation between the octopine and nopaline Ti plasmids.

2.1.3 Transfer DNA (T-DNA) of Ti Plasmid

T-DNA is the DNA segment transferred into the plant cell. The T-DNA is present on the Ti-plasmid of the wild type Agrobacterium, and its size is an average of 25 kb, ranging from 10 to 30 kb. The T-DNA region is flanked and delineated by two 25bp direct repeats, known as the right border and left border (Sheng and Citovsky, 1996). These border sequences are highly homologous and are targets of the border-specific endonuclease (VirD1/VirD2). The excised single strand of T-DNA from the Ti plasmid is exported from the bacterial cell to the plant cell by the activity of the other Agrobacterium Vir proteins. Howard et al. (1992) showed that deletion of the right border leads to a reduction of virulence, whereas the left border does not. These data suggested that the right border is essential for Agrobacterium pathogenicity. They concluded that the transfer of T-DNA is directed from the right to left border by the polarity. Additional evidence, that the right border is more important than left border, is that the VirD2 protein can alone bind to the single stranded right border sequence and cleaves a single-stranded T-DNA. The VirD2 protein remained on the 5’ end (right border) of the resulting single stranded T-DNA molecule, termed the T-strand (Jasper et al., 1994). T-DNA of octopine-type Ti plasmid has an “overdrive” sequence near the right border, but not left border, which may enhance the functional polarity of right border and left border (Gelvin, 2003).

Wild type T-DNA also has genes that are involved in plant hormone synthesis in the host plant. They are the tml, tms, and tmr regions for leafy tumor, shooty tumor, and rooty tumor, respectively, in the plant wound site (Ream et al., 1983). The opine synthase region is also located within the T-DNA. Agrobacterium strains are classified based on opine type, and components of the T-DNA are different in different opine types. If the Ti plasmid is a nopaline type, all components on T-DNA are a contiguous stretch, whereas the octopine type T-DNA consists of three individual parts, left (13kb), central (1.5kb), and right (7.8kb) Each segment has T-DNA border repeat sequences (Sheng and Citovsky, 1996); therefore, these segments are transferred separately to the other organism due to the sequence. After T-DNA is integrated into the host plant, opine is synthesized, then secreted out of the cell and imported into Agrobacterium. The absorbed opine molecule is catabolized by a specific enzyme in
*Agrobacterium*. Opine is degraded into amino acid and the sugar moieties, which can be used as carbon and energy sources for bacterial growth.

2.1.4 Structure and Function of Virulence Genes in Ti Plasmid

2.1.4.1 Induction of *vir* Gene Expression

Matthysse (1986) reported that gene transfer is started from tight binding between *Agrobacterium* and the host cell. This process is caused by bacterial chromosomal proteins ChvA, ChvB and PscA. Expressions of these proteins are triggered by substances secreted from wound site. Characterized substances were sap with acidic pH (5.0 to 5.8) or phenolic compounds, such as acetosyringone (Winans, 1992), lignin or flavonoid precursors. Stachel et al. (1985) found that monocyclic phenolics, such as acetosyringone (AS), are the most effective *vir* gene inducers. Uninjured plants do not produce these phenolic compounds or produce them at low levels, but production is dramatically increased in the wounded plant. Addition of artificial phenolic compounds during bacterial infection of the plant increased gene transfer efficiency. Sugars also assist activation of the major phenolic-mediated, wound-signaling pathway when small amounts of phenolic compounds are secreted from wounded cells. Citovsky et al. (1992) reported when AS concentration is low or not detectable, *vir* gene expression was significantly increased by monosaccharides, such as glucose or galactose. Citovsky et al. (1989) found that non-metabolizable sugars also increased *vir* gene induction. Certain types of sugars can induce VirA protein activation with acetosyringone (AS) synergistically (Shimoda et al., 1990). After bacteria and plant cell binding, the protein in bacteria, which is activated by signal molecules and secreted from the plant wound, is VirA (Sheng and Citovsky, 1996). VirA can amplify the transformation system by detection of monosaccharide in the presence of low concentrations of phenolic compounds. Klee et al. (1983) identified the functions of five *vir* regions, from *vir*A through *vir*E, by transposon mutagenesis and genetic complementation. Vir proteins are involved in signal recognition, transcriptional activation, conjugal DNA metabolism, intercellular transport, nuclear import and probably T-DNA integration into the plant nucleus.

The major roles of the VirA and VirG proteins are activation of other *vir* genes. VirA activates VirG, which is a cytoplasmic DNA binding protein and works as a transcriptional activation factor to induce the expression of other *vir* genes. The autophosphorylated histidine residue of VirA by a signal molecule phosphorylates an aspartate residue of VirG. These two proteins initiate the process of T-DNA transport (Winans, 1992). The phosphorylated VirG
protein recognizes the *vir* genes containing a *vir* box, a conserved 12-bp sequence, and induces the expression of *vir* genes. This conserved region is located at a promoter region of the *vir* genes (Citovsky et al., 1992).

VirD1 and VirD2, a heterologous system, act like endonucleases that cut between the third and fourth base pairs of 24 bp right and left border repeats of the T-DNA bottom strand (Wang et al., 1987). A linear single-stranded copy of the T-DNA region, named T-strand, is generated in *vir*-induced *Agrobacterium* cells (Stachel et al., 1985). The T-strand is produced from the 5’ to 3’ direction, initiating at the right T-DNA border and terminating at the left border, by the endonuclease activity of the VirD protein (Citovsky et al., 1992). VirD2 covalently binds to the right border of the T-strand, and to the 5’ end of the remaining bottom strand of the Ti plasmid after the cleavage. The resulting single-stranded gap is repaired after the T-DNA strand is removed. VirD2 in the remaining strand may participate in ligating the left border nick (Sheng and Citovsky, 1996). Howard and Citovsky (1990) described a structural model of the T-strand when it is transferred out of the bacterium and into the plant cells, which is a protein-nucleic acid complex, called a T-complex. This T-DNA transport intermediate has at least three components: a T-strand, a VirD2, and VirE2 single strand DNA-binding protein. Citovsky et al. (1989) showed that VirE coats the single stranded DNA and forms a strong, stable, unfolded VirE2-ssDNA complex that is protected from external nucleolytic activity. The T-complex is 3600 nm long and 2 nm wide, and it seems to contain about 600 molecules of VirE2 and one molecule of VirD2. The predicted molecular mass is 50,000 kD. The structural model suggested that VirD2 and VirE proteins are transported to the plant cell with the T-strand (Citovsky et al., 1989). Another protein from the octopine Ti plasmid, VirC1, helps generate a T-strand when VirD1 and VirD2 are limiting.

2.1.4.2 Pili Formation

Several putative mechanisms of gene transfer from bacteria to plants have been proposed. One mechanism involves the conjugation machinery to transfer T-DNA into the plant cells. The T-DNA transfer system is similar to an inter-bacterial conjugative transfer system of broad-host-range plasmids (Lessl and Lanka, 1994). Eleven *vir*B genes in the Ti-plasmid make proteins that seem to be involved in T-DNA transfer (Lai et al., 2000). VirB proteins are primarily linked with the cytoplasmic and periplasmic membranes and are a part of putative trans-membrane pore or channel (Lai and Kado, 1998). The amino acid sequence of the VirB protein has high homology
to the Tra proteins in *Escherichia coli*. Tra proteins are directly involved in the synthesis and assembly of the flagella (F pilus), the way that the genetic material is transferred between individual bacterial cells during conjugation (Shirasu and Kado, 1993). Kado (1994) reported that the structure of the F pilus is mainly composed of TraA protein, and TraA has to be processed from 12.7-kDa propilin into 7.2-kDa pili to be a structural subunit of the F pilus. The VirB protein sequence has high homology with TraA. VirB2 also has to be processed from a 12.3-kDa protein into a 7.2-kDa protein. High amino acid sequence similarity, similar size of both proteins and post-processing into a small molecule strongly suggest that VirB2 is the propilin (Fullner and Nester, 1996). Fullner and Nester (1996) showed that pili are required for bacteria virulence, and the formation of pili in *Agrobacterium* required expression of the *vir* genes of the Ti-plasmid. Mutant experiments of *vir* genes demonstrated that *virA*, *virG*, *virB1* to *virB11*, and *virD4* are the only genes that are necessary for pili formation. Experiments with various loss or gain of function mutants of these genes for pili formation showed that T-DNA transfer between bacteria highly depends on the expression of these Vir proteins. This result implies that *Agrobacterium* pili are required for transferring DNA to plant cells in a process similar to that of conjugation of *E. coli*. Lai and Kado (1998) provided evidence that the VirB2 protein is the structural subunit of the conjugative pilus structure. Processed 7.2-kDa VirB2 protein was detected at the outside of the *Agrobacterium* cell consistently, but VirB2 protein was not detected in the *virB2* gene mutant or other *vir* gene mutations. VirB2 protein expression in the exocellular space of the *Agrobacterium* is directly correlated with pili formation. The correlation of high exocellular VirB2 protein expression and VirB2 filaments was determined by western blotting and electron microscopy. In the *virB2* or other *vir* gene mutants, VirB2 protein was not detected, and also pili were not detected. It was concluded that VirB2 is a major component of pili formation. Jones et al. (1996), Lai and Kado (1998), and Shirasu and Kado (1993) also confirmed that VirB2 is a major component of the *A. tumefaciens* transfer pilus (T pilus).

The main proteins for pili formation were identified, and they have been studied to find the mechanism for pili formation (Mushegian et al., 1996). Three VirB proteins (VirB1, VirB2, and VirB5) were proposed as the main pilus components. Mushegian et al. (1996) reported that VirB1 was highly homologous with bacterial transglycosylases in its N-terminal domain, and it may facilitate assembly of the pili by lysis of the cell wall. Chumakov and Kurbanova (1998)
reported that the C-terminal region of the VirB1 was localized in the extracellular space, and electron microscopy studies showed that VirB1-cross-reactive material was on the surface of *Agrobacterium*. Since VirB1, among other members of VirB protein family, has a weak similarity with TraA, which is major flagella component in *E. coli*, they concluded that VirB1 may have an important role in plant cell interaction. Schmidt-eisenlohr et al. (1999) reported that VirB1, VirB2 and VirB5 needed each other for the stability and extracellular localization. Although VirB1 is not related to VirB2 induction, the expression level of VirB5 was highly regulated by VirB1 and VirB2 expression. High amounts of VirB1 and VirB2 were detected when abundant pili formation was observed. It was concluded that VirB1 and VirB2 are major pili components. VirB5 also cofractionated with VirB2, and it was concluded that VirB5 is directly involved in the pili assembly as a minor component.

2.1.4.3 T-complex, an Intermediate of T-DNA Transfer

The T-complex (protein-nucleic acid complex), which is ssDNA bound with VirD2 and VirE2 proteins, is transported through the *Agrobacterium* channel, composed of the VirB protein family, into the cytoplasm of the host plant cell. The evidence for VirD2 and VirE2 involvement in T-strand transfer was reported by Howard et al. (1992) and Tinland et al. (1992). They showed direct evidence of VirE2 accumulation in the plant cell nucleus by β-glucuronidase reporter enzymes (GUS) and direct immunolocalization of VirD2. These results showed that the T-complex, T-strands along with VirD2 and VirE2, moves to the plant cell from the bacteria cell.

VirD2 and VirE2 are also known as the main factors for T-DNA insertion into the plant chromosome. These proteins have specific nuclear localization signals (NLS) to move into the nucleus. The common structure of the NLS is a bipartite signal that was first found in the *Xenopus parotein* nucleoplasm (Robbins et al., 1991). NLS is composed of a first active domain of two adjacent basic residues, linker, and second activator domain, containing at least three out of five basic amino acids. Howard et al. (1992) found the functional NLS in the VirD2 protein C-terminal region, which has the bipartite consensus motif. The direct relevance of the NLS for T-strand transfer to the host plant nucleus was confirmed by NLS deletion mutants of VirD2. It showed reduced T-DNA expression and tumor formation.

Citovsky et al. (1992) demonstrated nuclear localization of the VirE protein, using VirE2-GUS fusions. The VirE2 protein has two functional NLS in the central region. Sheng and Citovsky (1996) showed that fluorescent ssDNA (single stranded DNA), without VirE2, remains
in cytoplasm; however, the complex of ssDNA with VirE2 accumulated in the plant cell nucleus. The nuclear-imported *Agrobacterium* T-strand is integrated into the host plant cell chromosome. Both T-strand-associated proteins (VirD2 and VirE2) have been implicated in the integration process. A short amino acid sequence downstream of VirD2 NLS, designated the ω domain, is necessary for T-DNA integration (Narasimhulu et al., 1996). According to the analysis of the T-DNA integration junction, VirE2 is also required for integration at the 3’ end, but not the 5’ end, of the T-strand (Rossi et al., 1996). These results support a T-DNA complex model, in which VirD2 and VirE2 bind to the 5’ and 3’ ends specifically (Sheng and Citovsky, 1996). Regarding the conversion of ssDNA into dsDNA, there are two different models. Tinland (1996) proposed that double strand synthesis would be performed by the plant cell DNA repair machinery following T-strand integration. However, another study suggested that the T-strand was converted into a double strand form before integration (De la Riva et al., 1998).

The successful expression of the transgene depends on the position within the chromosome where the T-DNA integrates. T-DNA can be inserted near or far from transcriptional activating elements or enhancers, resulting in success or failure of activation of T-DNA-carried transgenes. The failure of transgene expression (gene silencing) can also be caused by methylation or post-transcriptional gene silencing of multiple copies of transgenes. RNAs from these transgene copies may interfere with each other and then be degraded. In this aspect, the *Agrobacterium*-mediated method is favored because fewer gene copies are integrated, compared to the direct gene transfer methods (e.g. polyethylene glycol-liposome-mediated transformation, electroporation, or particle bombardment) (De la Riva et al., 1998).

### 2.2 Adoption for Plant Molecular Biology

#### 2.2.1 Plant Transformation via *Agrobacterium tumefaciens*

*Agrobacterium tumefaciens* has been used for plant genetic engineering extensively. Transgenic plants have been released commercially by several companies, including Monsanto and Zeneca. Plants have been genetically engineered for the purpose of developing resistance to herbicides, insects or viruses; tolerance to drought, salt or cold; and increasing yield (Birch, 1997). Transgenic tomatoes do not express the gene for polygalacturonase, an enzyme that degrades pectin, leading to softening of fruit tissues. As a result, the tomatoes can accumulate flavor components for a longer period of time. Cotton, potato and maize were genetically engineered for insect resistance, and soybean, canola and cotton were genetically developed for
herbicide resistance. The Agrobacterium-mediated transformation method has not only been used for commercial purposes, but also for basic biology research to study gene regulation or protein function in transgenic plants (McIntosh et al., 2004). Although it has been possible to make a genetically modified plant in some plant species, it is still not easy to make transgenic plants of all plant species. This may due to the poor transformation event caused by improper environmental conditions during bacterial infection of the plant, poor plant regeneration frequency, gene silencing due to position effects or transgene copy number after stable integration into the plant chromosome. The Agrobacterium-mediated transformation method still requires improvement in these aspects.

2.2.2 Binary Vectors of Ti Plasmid

The Agrobacterium-mediated transformation method was improved by developing modern binary Ti vectors after the removal of all the genes for tumor induction and opine synthesis. Ti plasmids without the tumor-inducing function are called disarmed plasmids (non-oncogenic Ti plasmid). Ti plasmids have been engineered to separate T-DNA and vir regions into two distinct plasmids, resulting in a binary vector and a vir helper plasmid, respectively (Hoekema et al., 1983). Since disarmed binary plasmids, containing the T-DNA region, do not have the ability to move a T-DNA into the plant, they need the help of another separate plasmid containing the vir genes. Many Agrobacterium strains containing non-oncogenic vir helper plasmids (LBA 4404, GV3101 MP90, AGL0, EHA101, and its derivative strain EHA 105) have been developed (Gelvin, 2003).

Binary vector plasmids are small and easy to handle in E. coli and Agrobacterium when the wild type Ti plasmid is around 200 kb. The sizes of the processed binary vectors from wild type Ti plasmids have been reduced to less than 10 kb. The binary vector has a replication origin for both E. coli and Agrobacterium, an antibiotic selectable marker for bacteria and plants, a reporter gene and a T-DNA region containing a multi-cloning site for insertion of genes of interest. The binary vector is transformed to Agrobacterium harboring a disarmed Ti-plasmid, called the helper plasmid, providing vir gene functions. The T-DNA region from the binary vector is transferred to the plant by expression of the vir gene in the helper plasmid (Klee et al., 1983).

Selectable markers are a convenient method to distinguish between transformed and non-transformed tissues. Plants do not have an antibiotic resistance gene naturally. Transformed
tissues contain selectable markers and survive on selective media containing antibiotics, while non-transformed cells, which do not have an antibiotic resistance gene, are killed on the antibiotic media. Antibiotic-resistance genes, usually the kanamycin (neomycin phosphotransferase II) resistance gene or hygromycin (hygromycin phosphotransferase) resistance gene, have been used as selectable markers.

Reporter genes, such as β-glucuronidase (GUS), luciferase or green fluorescent protein (GFP), are important components of the T-DNA region. Expression of reporter genes is visualized in the transformed tissue through staining or auto-fluorescence. Transformed tissue can be distinguished from non-transformed tissue by reporter gene expression. β-glucuronidase (GUS) is a reporter gene isolated from E. coli and has been used extensively in plant molecular biology studies. Efforts to increase GUS gene expression have been conducted by adding an intron or optimizing codon efficiency. The pCAMBIA Company developed a GUS gene that has a catalase intron for preventing GUS expression in bacteria (Ohta et al., 1990), and they found that a GUS gene isolated from Staphylococcus resulted in 10 times higher expression in rice tissues than GUS from E. coli (Jefferson et al., 2003).

2.3 Agrobacterium-mediated Plant Transformation Protocol Development

2.3.1 Factors to Increase Gene Expression and Transformation Efficiency

Transformation efficiency can be increased by the manipulation of either the plant or bacteria for enhancing competency of plant tissue and vir gene expression, respectively (Henzi et al., 2000; Mondal et al., 2001; Lopez et al., 2004; Chakrabarty et al., 2002). Seedling age and pre-culturing of explants have been tested to increase the transformation efficiency. These trials were conducted to determine the best conditions for plant cell infection or increasing the number of dividing plant cells before bacterial infection (Amoah et al., 2001; Chakrabarty et al., 2002; Mets et al., 1995). To increase the virulence of bacteria by inducing the vir gene expression, temperature (Dillen et al., 1997; Chakrabarty et al., 2002; De Clercq et al., 2002), medium pH (Mondal et al., 2001; Holford et al., 1992; Godwin et al., 1991; De Clercq et al., 2002) and chemical inducers, such as acetosyringone (SomLeva et al., 2002; Lopez et al., 2004; Le et al., 2001; Chakrabarty et al., 2002; Henzi et al., 2000; Cervera et al., 1998; De Clercq et al., 2002; Stachel et al., 1985), have been tested. These factors likely enhance bacterial pili formation required for gene transfer between bacteria, as well as between the bacteria and plants. Manipulation of other factors, such as bacterial density, co-cultivation duration, surfactant, and
vacuum infiltration, have also increased transformation efficiency in many experiments (Mondal et al., 2001; Lopez et al., 2004; Cheng et al., 1997; Amoah et al., 2001). According to previous experiments, inducing \textit{vir} gene expression seems most important and effective for increasing plant transformation efficiency, regardless of the type of plant being studied. Although low temperature was very important to induce bacterial pili formation by inducing the \textit{vir} gene, optimization experiments were conducted at room temperature or over 25°C for co-cultivation.

2.3.1.1 Temperature Effect on Pili Formation

Temperature is an important environmental factor that affects T-pilus (transfer pilus) biogenesis in \textit{Agrobacterium}. Early studies demonstrated that crown gall tumor size increased with a decrease in temperature during bacterial infection, and tumor formation was inhibited at 28°C or above (Braun, 1947; Brown, 1942). Watson et al. (1975) found that Ti plasmids disappeared when \textit{A. tumefaciens} C58 was grown at high temperature for over 36 hours. Later, Tempe et al. (1977) reported that conjugation transfer of Ti plasmids from virulent to avirulent strains was also sensitive to temperature. Jin et al. (1993) studied the temperature effects on VirA and VirD proteins. They reported that high temperatures negatively affect VirA activity for phosphorylating VirG. A VirA autophosphorylation experiment was also conducted at 28, 32, and 37°C. VirA was not autophosphorylated at 37°C, whereas it was phosphorylated at 28 and 32°C. In the VirA mutant background, the VirG protein expression was not affected by elevated temperature. This result indicated that temperature is closely related to VirA induction. In this experiment, the VirB and VirA proteins decreased dramatically above 32°C. This result suggested that VirB, along with VirA protein, is affected by elevated temperatures. They also tested bacterial infection temperature using \textit{Kalanchoe daigremontiana}, and no tumor formation occurred at 32°C. Fullner and Nester (1996) tested the movement of the incompatibility group Q (IncQ) plasmid between the bacteria by the conjugal transfer machinery at various temperatures. When the mating was performed at 19°C, the highest plasmid transfer occurred between the bacteria; on the other hand, no transfer was detected in 28°C. However, this conjugation frequency was only induced in the presence of acetosyringone at 19°C on the pH 5.3 medium. They also reported a requirement of \textit{virB} gene expression for conjugal transfer, using various \textit{virB} gene mutants. Conjugal transfer efficiency of mutant \textit{virB} was not affected by low temperature, unlike wild type, which was affected. They concluded that \textit{virB} gene expression was affected by temperature directly, and pili required for inter-bacterial DNA transfer were
induced optimally at the low temperatures. Fullner et al. (1996) found that long bacterial pili were induced when wild type *Agrobacterium* was grown at 19°C in the presence of 200 μM acetosyringone, whereas pili were rare on bacteria grown on agar plates lacking acetosyringone or on bacteria containing no Ti-plasmid. *Agrobacterium* grown at 19°C in the presence of 200 μM acetosyringone produced three times more pili than *Agrobacterium* grown with acetosyringone at 28°C. Fullner and Nester (1996) showed exocellular VirB2 was produced abundantly at 19°C compared with production at 28°C. Schmidt-eisenlohr et al. (1999) reported that optimized virulence gene induction was detected from *Agrobacterium* grown on an agar plate at 20°C. Baron et al. (2001) monitored T-pilus assembly and virulence protein accumulation in *Agrobacterium tumefaciens* strain C58 at 20°C to 37°C by western blotting. Optimal T-pili formation was at the 20°C. They also showed that exocellular assembly of the major T-pilus, VirB2 and pilus associated VirB5 protein components were strongly inhibited at 28°C, but not completely inhibited at 26°C.

2.3.1.2 Temperature Effects on Plant Transformation

Temperature effects on plant transformation have been studied by a few groups (Dillen et al., 1997; Chakrabarty et al., 2002; De Clercq et al., 2002), after conjugal transfer of Ti plasmid was shown to be sensitive to temperature (Fullner et al., 1996). Dillen et al. (1997) tested the effect of temperature on transgene expression in two plant systems, *Phaseolus acutifolius* and *Nicotiana tabacum*. The optimal temperature for *Phaseolus* callus transformation was 22°C and for tobacco was 19 to 22°C. *Phaseolus* callus was incubated with bacteria at different temperatures from 15 to 29°C for 3 days. Expression of GUS at 25°C was lower than at 22°C. Very low levels of GUS expression were detected at 27°C and no expression at 29 and 15°C. They showed highest GUS expression at 22°C co-cultivation. They reported similar results with the tobacco experiment. In both studies, GUS expression was dramatically reduced when the temperature was increased from 22°C to 25°C. GUS expression was low at 27°C and undetectable at 29°C. Chakrabarty et al. (2002) evaluated a number of factors that increased the *Agrobacterium* mediated-transformation efficiency of hypocotyl explants of cauliflower. They showed co-cultivation at 22°C resulted in higher GUS expression than at 28°C. De Clercq et al. (2002) also reported that co-cultivation at 22°C resulted in higher GUS expression than temperatures above 22°C in *Phaseolus acutifolius*. 
2.3.1.3 Surfactant (Silwet L-77) Effect

Silwet L-77 is a silicon-based copolymer (octamethyl trisiloxane, MW 236.54). Use of Silwet L-77 was first reported by Whalen et al. (1991). Surfactant treatment increased the susceptibility of *Arabidopsis* leaves to *Agrobacterium* infection. Silwet L-77 reduced surface tension with low phytotoxicity. This allows aqueous droplets to spread evenly over the leaf surface and to penetrate the stomatal opening. Due to these characteristics, Silwet L-77 enhances entry of bacteria into relatively inaccessible plant tissues. Use of Silwet L-77 (0.001%) resulted in the development of disease phenotypes. Toxicity of L-77 was detected at concentrations above 0.1% in this experiment. Khatun et al. (1993) used a surfactant to stimulate shoot regeneration from jute cotyledons and found that surfactants were beneficial for the growth of plant cells. They reported that shoot regeneration from jute (*Corchorus capsularis* L.) cotyledons was increased 2-fold compared to untreated controls. They concluded that surfactant in the media enhanced cell viability and stimulated tissue growth.

Cheng et al. (1997) tested surfactants in *Agrobacterium*-mediated wheat calli transformation. Many factors were tested, such as different explant types, *Agrobacterium* cell density, co-culture media, surfactants in the inoculation medium, induction agents in the inoculation medium, leaf tissues from young seedling, immature inflorescences, freshly isolated immature embryos, pre-cultured immature embryos, and cells in suspension cultures. Surfactant was reported as a most critical factor for increasing GUS activity. GUS activity was enhanced with 0.01% Silwet L-77 treatment, and highest activity was detected at 0.05%, which resulted in 19 times higher GUS activity than the control. When the concentration was greater than 0.05%, most of the immature embryos could not survive; it was concluded that 0.01 to 0.02% of Silwet L-77 was optimal for wheat transformation. A surfactant effect has only been evaluated by Cheng et al. (1997) in the *Agrobacterium*-mediated plant transformation using tissue culture and plant regeneration. Silwet L-77 became important in *Arabidopsis* transformation after Clough and Bent (1998) reported that *Arabidopsis* transformation was successfully conducted with surfactant treatment in the floral dipping method. *Arabidopsis* flowers were dipped into the *Agrobacterium* inoculation medium containing sucrose (or glucose) and the surfactant, Silwet L-77. Clough and Bent (1998) also reported that plant developmental stage, sugar, and surfactant were critical to optimize transformation efficiency. Clough and Bent (1998) showed that Silwet L-77 (0.05%) treatment in the floral dipping method created more *Arabidopsis* transformants.
than the vacuum infiltration method. Simply adding Silwet L-77 to the *Agrobacterium* infection medium substituted for the labor-intensive vacuum infiltration method that is routinely used in *Arabidopsis* transformation.

Curtis and Nam (2001) reported that plant development stage and the use of surfactant were two of the most important factors for the optimization of transformation efficiency. Silwet L-77 (0.05%) resulted in optimum transformation efficiency in the floral-dip method for radish transformation. They also compared other surfactants, Pluronic F-68 and Tween-20, to Silwet L-77. Although treatment with Pluronic F-68 and Tween-20 was advantageous for transformation of radish, compared to controls that resulted in 0% transformed seeds, it was confirmed that 0.05% Silwet L-77 treatment was the most beneficial, with 1.4% transformation from 1110 seeds.

2.3.1.4 Vacuum Infiltration

Vacuum infiltration has been used mostly as an aid for efficient *Agrobacterium* inoculation for the flowering stage of *Arabidopsis* (Clough and Bent 1998), *Brassica napus* (Wang et al., 2003) or Chinese cabbage (Liu et al., 1998). However, there are many plants that can only produce transgenic plants efficiently through tissue culture and the regeneration process. A vacuum infiltration effect was tested in various plant transformation experiments involving a tissue culture and regeneration system (Kapila et al., 1997; Amoah et al., 2001; Mahmoudian et al., 2002; Wang et al., 2003; Leelavathi et al., 2004; Haq 2004; Spokevicius et al., 2005).

Kapila et al. (1997) reported that vacuum infiltration, ranging from 75 to 750 mm Hg for 20 min to *P. vulgaris* leaf transformation via *Agrobacterium tumefaciens*, resulted in high transient expression, and all infiltrated leaves showed large GUS expression sectors. Dillen et al. (1997) used the vacuum infiltration method on tobacco leaves and *P. vulgaris* leaf transformation to increase transformation efficiency. Amoah et al. (2001) tried to find optimal conditions for wheat inflorescence tissue transformation. They reported that GUS expression was not detectable without vacuum infiltration, but the numbers of GUS positive spots per explant was increased with vacuum treatment. GUS expression only increased after up to 60 min at 762 mm Hg vacuum infiltration. A vacuum infiltration effect was also tested for lentil (*Lens culinaris* M.) cotyledonary node transformation (Mahmoudian et al., 2002). Higher GUS transient expression was detected at 200 mm Hg for 20 min when it was compared to non-infiltrated tissues. Higher pressures for longer times (600 mm Hg, 30 min) decreased the transient expression. In cotton, vacuum infiltration at 1400 mm Hg for 10 min resulted in 47% transformation; on the other hand,
20% GUS activity was noted with the common non-infiltrated Agrobacterium-mediated transformation method using hypocotyl-derived embryogenic calli (Haq, 2004). Based on these results, although optimal conditions of vacuum pressure and duration varied depending on the genotype or different tissues, it was clear that the vacuum infiltration method was effective for the Agrobacterium-mediated transformation method using regenerable tissues.

Vacuum and Silwet L-77 combination effects were reported by Clough and Bent (1998), who concluded that the floral dipping method with surfactant can substitute for the vacuum infiltration method in Arabidopsis. They showed a possible synergistic effect of Silwet L-77 and vacuum infiltration on plant transformation.

2.3.1.5 Agrobacterium Concentration Effect

It has been considered in several experiments that transformation efficiency might be affected by bacterial growth phase and bacterial cell density. Different concentrations of the Agrobacterium have been used by different research groups and for different plant materials. In the standard protocol, cells are grown to the stationary phase (A<sub>600nm</sub> ≈ 2-2.4), pelleted and resuspended in inoculation medium to stationary or log or mid-log phase (A<sub>600nm</sub> ≈ 0.1-1.15). High concentrations of bacteria at the stationary phase have normally been used for rice, legume and tobacco transformation (Hood et al., 1987; Kapila et al., 1997; Dillen et al., 1997), and low concentrations of bacteria at the log or mid-log phase have been used for broccoli (Mets et al., 1995), cabbage (Henzi et al., 2000), wheat (Cheng et al., 1997), cottonwood (Han et al., 2000), and tobacco (Krugel et al., 2002). Clough and Bent (1998) reported that different bacterial concentrations, ranging from 0.15 to 1.75 of A<sub>600nm</sub>, resulted in different Arabidopsis transformation efficiencies. They reported that 0.8 A<sub>600nm</sub> was most effective for Arabidopsis transformation. Chakrabarty et al. (2002) developed a protocol for cauliflower transformation. They diluted 0.5 A<sub>600nm</sub> to 1:10 and 1:20 and found that a 1:20 dilution was effective. According to these results, it can be assumed that inoculum density can make a difference in the transformation efficiency.

2.3.1.6 Co-cultivation Duration Effect

Co-cultivation for 2 to 7 days has been normally used in Agrobacterium-mediated transformation under various co-cultivation temperatures (Han et al., 2000; Mondal et al., 2001; Cervera et al., 1998; SomLeva et al., 2002). Co-cultivation for 3 days resulted in high transformation efficiency, and transformation efficiency reached a maximum at day 5 in citrange.
(Citrus sinensis L. Osbeck x Poncirus trifoliate L. Raf.) (Cervera et al., 1998). They reported that more than 5 days caused bacterial overgrowth and decreased the transformation efficiency. Many transformation experiments in different plant species, such as tea (Camellia sinensis L.), cauliflower, white spruce (Picea glauca) and citrange, showed that 2 to 3 days of co-cultivation resulted in high transformation efficiency under room temperature co-cultivation conditions (Lopez et al., 2004; Chakrabarty et al., 2002; Le et al., 2001; Cervera et al., 1998). Therefore, 2 to 3 days co-cultivation has been routinely used in most transformation protocols, since longer co-cultivation causes bacterial overgrowth that covers the leaf tissue and causes toxicity under room temperature co-cultivation conditions. Cervera et al. (1998) reported that 5 days co-cultivation prevented callus formation and resulted in poor plant regeneration. The co-cultivation duration recommended from published protocols for tobacco leaf disk transformation varies from one research group to another. They are 2, 3 and 4 days at 26°C, 24°C, and room temperature co-cultivation conditions (Svab et al., 1995). Most previous experiments indicated that 2 to 3 days were optimal at 25°C co-cultivation conditions, regardless of plant species. No experiments have been conducted yet to find an optimal co-cultivation duration at lower than 25°C.

2.3.1.7 Bacterial Pre-culture and Phenolic Compound

The bacterial pre-culture effect has been tested by Clough and Bent (1998). They pre-induced vir genes by growing Agrobacterium in a standard vir-inducing liquid medium for 20 h at 19°C (Liu et al., 1998), and compared bacteria grown on a plate with bacteria grown in rich liquid medium. They reported that transformation efficiency of Arabidopsis did not change significantly. They also reported that growth of bacteria on solid vir-inducing CIB medium at 19°C led to a four-fold decrease in transformation. Bacteria for pili formation experiments in the microbiology era used to be grown on minimal media for 3 days under 19°C (Fullner et al., 1996). In this study, bacteria were pre-cultured for 3 days before inoculation.

Acetosyringone (AS) is a phenolic compound produced from wounded plant cells. Fullner et al. (1996) reported that Agrobacterium did not produce pili without 200 µM AS at both 19°C and 25°C. Results from a wheat inflorescence transformation experiment showed that T-DNA cannot be transformed to the plant tissue without AS (Amoah et al., 2001). These results indicated that AS is the main factor at the low temperatures that induces VirB protein, which is a subunit of pili. Transformation frequency was induced 2-fold with 100 µM AS in Carrizo citrange explants (Cervera et al., 1998), and different callus formation tendencies were detected.
with different concentrations of AS in genetic transformation of switchgrass (SomLeva et al., 2002). In the switchgrass experiment, high transformation efficiency was detected in the presence of 200 µM AS in both the inoculation suspension and co-cultivation medium during callus formation. They reported that switchgrass callus failed to regenerate a shoot, although callus was formed in the non-AS treatment. The optimal concentrations of AS depended on the plant species. But in most experiments, optimal AS concentrations for different plant species are in the range of 50 µM to 400 µM.
CHAPTER 3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratory Facilities and Equipment

This research was conducted in the Department of Plant Pathology and Crop Physiology, Life Sciences Building (LSB), Louisiana State University and LSU Agricultural Center, Baton Rouge, Louisiana. Biochemical experiments were carried out in LSB rooms 329, 333 and 360, and tissue culture work was done in LSB rooms 323 and 344A, and LSB Annex room A423.

Equipment used for the research is listed by manufacturer in alphabetical order:

Baker Company Inc. (Sanford, ME), Bio GARD® Hood and Edge GARD® Hood; Barnstead Thermolyne Co. (Dubuque, IA), Harvey Sterile-Max Steam Sterilizer (ST75925); Beckman Counter, Inc. (Palo Alto, CA), Induction-drive Centrifuge model J2-21M and Ultracentrifuge model L8-M, Spectrophotometer DU®7400 and Tube sealer; Bio-Rad Laboratories (Hercules, CA), Electrophoresis Kit (readyAgarose system, Sub-cell model 192 electrophoresis cell), Gel Doc 1000 and Electroporation Apparatus (E. coli pulser); Brinkman(Switzerland), Homogenizer (Polytron® PT3000); Eppendorf (Germany), Eppendorf Micro Centrifuge (5415C); GCA Corporation Scientific Group (Chicago, IL), Gravity Convection Incubator (Precision®); Jasco co. (Great Dunmow, UK), Jasco FP-6300 Spectrofluorometer; Millipore (Billerica, MA), Milli-Q™ Water System; Nalgene® Labwear (Rochester, NY), Nalgene desiccator with desiccator plate (Nalgene® 5312); New Brunswick Scientific Co., Inc. (Edison, NJ), GYROTORY® Water Bath Shaker model G-76, GYROTORY® Shaker model G-2, Incubator shaker model G-25 and Digital Illuminated Refrigerated Incubator Shaker Innova™ 4340; Northeastern Scientific Co. (Boston, MA), Electrophoresis Power Supply; ORION Research Inc. (Beverly, MA), ORION pH Meter (model 420A); Percival Scientific, Inc. (Perry, IA), Growth Chamber model Cu-36L; Puffer/Hubbard Mfg Co. (Minneapolis, MN), Ultra Low Temperature Freezer; Sargent-Welch Scientific Co. (Skokie, IL), Vacum Pump; Stratagene® (La Jolla, CA), Robocycler Gradient 96 PCR machine; Whatman Ltd. (Maidstone, England), Filer paper; Ultra-Violet Products Inc. (South Pasadena, CA), UV Lamp

3.1.2 Chemicals

Chemicals used for the research is listed according to the alphabetical order of suppliers: Aaper Alchohol and Chemical Co. (Shelbyville, KY), ethanol (EtOH); Amresco®
(Solon, OH), Agarose I, polyethylene glycol 8000 (PEG8000) and tris; Becton, Dickinson and Company (Sparks, MD), Bacto Trypton and Yeast Extract; Bio 101 Inc. (Vista, CA), Glass powder; Bio-Rad Laboratories (Hercules, CA); DC protein assay kit; Chlorox Company (Oakland, CA), Chlorox® Bleach; Silwet L-77; EM Science (Gibbstown, NJ), glucose, potassium chloride (KCl), and sodium hydroxide (NaOH); EMD Chemicals Inc. (Darmstadt, Germany), Bacto Agar and potassium phosphate dibasic (K2HPO4); Fisher Scientific (Fair Lawn, NJ), ammonium sulfate ((NH4)2SO4), chloroform, glycerol, mannitol, sodium carbonate (Na2CO3), and sodium chloride (NaCl); GibcoBRL (Grand Island, NY), sodium dodecyl sulfate (SDS); J. T. Baker Chemical Co. (Phillipsburg, NJ), potassium phosphate monobasic (KH2PO4) and sodium phosphate monobasic (NaH2PO4); Mallinckrodt Baker Inc. (Paris, KY), calcium chloride (CaCl2) and isoprophyl alcohol; Sigma-Aldrich (St. Louis, MO), acetylsyringone, ammonium chloride (NH4Cl), adenosine 5′-triphosphate (ATP), 6-benzylamino purine, carbenicillin, cesium chloride (CsCl), DL-dithiothreitol (DTT), deoxyribonucleotide triphosphate (dNTP), ethylenediaminetetraacetic acid (EDTA), ethidium bromide (EtBr), hygromycin, indole 3-acetic acid (IAA), kanamycin, lysozyme, 4-methyl umbelliferyl, β-glucuronide (MUG), β-mercaptoethanol, manganese (II) chloride (MnCl2·4H2O), magnesium sulfate (MgSO4), morpholine ethanesulfonic acid (MES), MS salt (Murashige & Skoog, 1962), myo-inositol, rifampicin, α-naphthalene acetic acid (NAA), nicotinic acid, pyridoxine, streptomycin, sucrose, sodium iodide (NaI), Tween-20, thiamine, Triton and zinc sulfate (FeSO4·7H2O).

3.1.3 Enzymes
Enzymes used for molecular biology work were purchased from GibcoBRL Products/Life Technologies (Rockville, MD) or New England Biolab Inc. (Beverly, MA). Restriction endonucleases are listed according to the alphabetical order; BamHI, BglII, EcoRI, HindIII, KpnI, NsiI, PstI, SphI, SacII, SalI, XbaI. T4 DNA ligase and Deep Vent Taq Polymerase were purchased from New England Biolab Inc.

3.1.4 Bacterial Strains
Escherichia coli strain (XL1 Blue) was used to amplify the plasmids, pBluescript KS-, pCAMBIA 1301, pLMNC94, and pTRA 321. The genotype of the strain XL1 Blue of Stratagene® (La Jolla, CA) is as follows: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacPZΔM15 Tn10 (TetR)].
**Agrobacterium tumefaciens** strain LBA4404 was obtained from Invitrogen (Carlsbad, CA)/Life Technology (Rockville, MD). EHA 105 was obtained from Stan Gelvin, Department of Biological Science, Purdue, University, West Lafayette, IN. The genotypes of the strains are as follows: LBA4404; TiAch5 rifampicin\(^r\) in chromosome, pAL4404 Ti plasmid with spectinomycin\(^r\) and streptomycin\(^r\), octopine (Hoekema et al., 1983); EHA105, C58 rifampicin\(^r\) in chromosome, pEHA 105 (pTiBo542ΔT-DNA) Ti plasmid, succinamopine (Hood et al., 1987).

3.1.5 Plasmid DNA and Oligonucleotides

Plasmids used for this research were obtained from suppliers as follows: pLMNC94 (CD3-419) of Arabidopsis Biological Resource Center (Columbus, OH) contains GFP coding sequence, pBluscript KS- of Strategene\(^\text{®} \) (La Jolla, CA), pCAMBIA 1301, 1305.01, 1305.02, and 2301 of CAMBIA (Canberra, Australia). pTRA321 (Zheng et al., 1995) plasmid has a phaseolin coding sequence in which new enzyme sites are to be incorporated by PCR. Primers used for phaseolin vector construction were ordered from Sigma (St. Louis, MO), as listed in Table A.1.

3.2 Methods

3.2.1 Bacterial Growth

3.2.1.1 *Escherichia coli*

Intact XL1 Blue strain for electroporation was grown in liquid LB medium (Luria-Bertani medium per L; 5.0 g yeast extract, 10.0 g trypton, 10.0 g NaCl, pH 7.2). Bacto Agar (1.5% (w/v)) was used as solid medium. LB medium was sterilized with a Harvey Sterile-Max Steam Sterilizer at 121°C, 18 Psi (lb/sq in) for 15 min. XL1 Blue strain, containing pTRA 321, pLMNC94, pBI121, or pBluscript KS-, was grown on LB medium supplemented with 50 mg/L ampicillin. XL1 Blue strain with pCAMBIA 1301 was grown in LB medium with 50 mg/L kanamycin (Burow et al., 1990).

3.2.1.2 *Agrobacterium tumefaciens*

Strain LBA 4404 was grown in *Agrobacterium* medium (0.16 g MgSO\(_4\)\cdot7H\(_2\)O, 0.011 g CaCl\(_2\), 0.005 g FeSO\(_4\)\cdot7H\(_2\)O, 10.9 g KH\(_2\)PO\(_4\), 0.002 g MnCl\(_2\)\cdot4H\(_2\)O, 2.0 g mannitol, 2.0 g (NH\(_4\))\(_2\)SO\(_4\), 5.0 g yeast extract per L). Bacto Agar (1.5% (w/v)) was used for solid media. *Agrobacterium* medium was prepared as follows: Ten-times (x10) salt solution (109.0 g KH\(_2\)PO\(_4\), 1.6 g MgSO\(_4\)\cdot7H\(_2\)O, 0.05 g FeSO\(_4\)\cdot7H\(_2\)O, 0.11 g CaCl\(_2\), 0.02 g MnCl\(_2\)\cdot4H\(_2\)O per L) was prepared and heated to boiling. The precipitate was filtered, and pH was adjusted to 7.0. Ten-times salts were mixed with mannitol, (NH\(_4\))\(_2\)SO\(_4\), and yeast extract (Burow et al., 1990). The volume was
adjusted to one liter and medium was sterilized as described above. Agrobacterium-containing pCAMBIA plasmids was grown on Agrobacterium medium with 50 mg/L kanamycin and 50 mg/L streptomycin.

3.2.2 Tobacco Transformation

3.2.2.1 Subculture in vitro of Tobacco Plantlets

Tobacco (Nicotiana tabacum L. cv. Xanthi) seeds were germinated and grown in vitro. About 100 tobacco seeds were placed on a Whatman filter paper (No.5:11 cm diameter), which was then made into a small pack. One pack of tobacco seeds was soaked in 95% (v/v) ethanol for 5 sec, and transferred using a sterile forceps to 50% (v/v) Clorox with one drop of Tween-20 for 20 min with constant stirring. Seeds were rinsed with sterile water five times and then dried on sterile Whatman filter papers (No.5:11 cm diameter). Ten seeds were planted per Petri dish on the T-media (per L; 0.1 g myo-inositol, 0.4 mg thiamine-HCl, 30.0 g sucrose, pH 5.6, and 10 g agar for solid medium and 4.4 g Sigma MS salts) (Burow et al., 1990). The MS (Murashige and Skoog, 1962) salts contain 1650 mg NH₄NO₃, 6.2 mg H₃BO₃, 332.2 mg CaCl₂·2H₂O, 0.025 mg CoCl₂·6H₂O, 0.025 mg CuSO₄·5H₂O, 37.26 mg NaFeEDTA, 27.80 mg FeSO₄·7H₂O, 180.70 mg MgSO₄·2H₂O, 16.90 mg MnSO₄·H₂O, 0.83 mg KI, 1900 mg KNO₃, 170 mg KH₂PO₄, 0.250 mg Na₂MoO₄·2H₂O, 8.6 mg ZnSO₄·7H₂O per L. Seeds were germinated and grown in the growth room under constant light with 61 µE m⁻² s⁻¹ at room temperature (Burow et al., 1990). Plantlets were subcultured every two to three weeks. Stems with one node and two leaves were cut out from grown plantlets and then transplanted to fresh T-medium.

3.2.2.2 Leaf Disk Co-cultivation and Selection

a. Bacteria inoculation

Plasmids pCAMBIA1301, pCAMBIA1305.01 and pCAMBIA1305.02 were transformed to LBA4404 using electroporation. Agrobacterium tumefaciens strains were grown on the Agrobacterium (Agro) agar medium overnight at 28°C to get a fresh colony. LBA 4404 strains containing pCAMABIA1301, pCAMBIA1305.01, or pCAMBIA1305.02 were selected on the Agro medium containing 50 mg/L streptomycin and 50 mg/L kanamycin. A single colony was picked and grown in 50 mL of Agro liquid medium (pH 7.0) overnight at 28°C, 250 rpm. The next day, cells were harvested by centrifugation for 20 min at room temperature at 5500 g and resuspended in MS induction liquid medium (4.4 g/L MS salts, 30 g/L sucrose, pH 5.5) containing 200 µM acetylsyringone(AS) until it reached to 0.3 A₆₀₀ units (method from Clough
and Bent, 1998 was modified). AS was prepared by dissolving AS powder in EtOH, and the appropriate amount was added to the cool medium after autoclaving. Ten mL of *Agrobacterium* inoculum was used for 45 leaf disks.

b. Infection and co-cultivation

Leaf disks were obtained from four week-old tobacco plantlets grown *in vitro*. Sterile tobacco leaves were cut from plantlets and soaked in MS liquid media in a sterile Petri dish to avoid de-hydration. One hundred leaf disks were cut-out using a cork borer with a 1-cm inner diameter, and placed in the liquid MS media (4.4 g/L MS salts, 30 g sucrose/L, pH 5.6) in a Petri dish at a time. Forty five leaf discs were placed into one Petri dish for bacterial inoculation. Leaf disks were soaked in 10 mL of *Agrobacterium tumefaciens* (0.3 A$_{600}$ units) containing 200 µM AS and Silwet L-77 at specific concentrations in the Petri dish. Three Petri dishes containing leaf disks and *Agrobacterium tumefaciens* were placed in a vacuum desiccator. Vacuum infiltration was performed using a vacuum pump (Sargent-Welch Scientific Co., Skokie, IL) for specified durations under 50 mm Hg, as indicated in the figures. Inoculated leaf disks were transferred to the Whatman filter papers (No.5:11 cm diameter) placed on the top of brown paper towels and then blotted thoroughly. Fifteen leaf disks were placed per Petri dish on the co-cultivation media containing per liter: 4.4 g MS basal salts, 1.0 mg nicotinic acid, 1.0 mg pyridoxine·HCl, 0.1 mg thiamine·HCl, 100 mg *myo*-inositol, 1.0 µg 6-benzylaminopurine (BA), 0.1 µg indole-3-acetic acid (IAA), 200 µM AS, 30.0 g sucrose, 10.0 g agar at pH 5.6 without antibiotic (Burow et al., 1990). Petri dishes were covered and sealed well with a strip of parafilm.

c. Selection

After leaf disks were co-cultivated with *Agrobacterium tumefaciens* for specified durations and temperatures in a Tissue Culture Chamber (model CU-36L, Percival Scientific, Inc., Perry, IA) under constant light with 56 µE m$^{-2}$ s$^{-1}$, leaf disks were collected and washed with MS liquid medium containing 500 mg/L carbenicillin four to five times. Leaf disks were blotted thoroughly on the filter paper. Ten leaf disks were placed per Petri dish on the shoot selection medium. The composition of the tobacco shoot selection medium is the same as the co-cultivation medium, except 1 mg/L naphthalene acetic acid was substituted for indole acetic acid. The shoot selection medium also contained 50 mg/L hygromycin and 500 mg/L carbenicillin. Leaf disks on selection medium were incubated at 25 ± 0.5°C under constant light for two weeks prior to the β-glucuronidase assay.
3.2.2.3 β-Glucuronidase Assay

a. Quantitative GUS Assay

β-Glucuronidase activity was measured by colorimetric assays (Zheng et al., 1993). Treated leaf disks were weighed and frozen at -80°C until they were assayed. Ice-cold seed extraction buffer (86.4 mM K$_2$HPO$_4$, 13.4 mM KH$_2$PO$_4$, and 1.0 mM DTT at pH 7.6) was added to a portion of leaf disks at a ratio of 100 mg tissue/mL buffer. Leaf disks were ground in seed extraction buffer using a drill (Robert Bosch Tool Co., Prospect, IL) at a low speed (level 1) for 2 min with a Kontes Pellet Pestle (Fisher Science Company, Fair Lawn, NJ). During homogenization, the sample tube was kept on the ice to prevent overheating. Samples were shaken with an Eppendorf mixer for 4 min at 10°C. Samples were centrifuged for 10 min at 4°C at 12,000 rpm in a Beckman refrigerated centrifuge (J2-21M). Supernatants were transferred to new Eppendorf tubes. Crude protein extracts were stored at -80°C for subsequent protein assays.

GUS assay buffer contains 1.2 mM 4-methyl umbelliferyl β-glucuronide (MUG), 50 mM Na$_2$HPO$_4$, 50 mM NaH$_2$PO$_4$, 10 mM β-mercaptoethanol, 10 mM EDTA (pH 8.0), 0.1% (w/v) SDS, and 0.1% (v/v) Triton X (pH 7.0). Four hundred ninety µL of GUS assay buffer in an Eppendorf tube were pre-warmed for 30 min at 37°C in a water bath. Frozen crude protein extracts were thawed on ice and mixed well. Ten µL of crude protein extract was added to the 490 µL GUS assay buffer and mixed well by vortexing. GUS assay mixtures were incubated at 37°C for 30 or 60 min in a water bath. After 30 or 60 min, 100 µL of this reaction mixture was added to 0.9 mL of GUS stop buffer (0.2 M Na$_2$CO$_3$) in an Eppendorf tube. GUS activity was measured with a Jasco FP-6300 Spectrofluorometer (Jasco Co., Great Dunmow, UK) in the laboratory of professor Marcia Newcomer, Department of Biological Sciences, LSU. The spectrofluorometer was warmed up for 30 min before measurement. The wavelength was set to 365 nm for excitation and 455 nm for emission. The spectrofluorometer was calibrated with GUS stop buffer using quartz cuvettes. A standard curve was prepared using 0.005, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 µM 4-methylumbelliferone solution (MU). Relative intensity of MU light emission in sample solutions read at 1, 15, 30, and 60 min. The amount of the GUS was calculated based on the established standard curve.

Protein concentration was determined by colorimetric assay using the DC protein assay kit (Bio Rad). The reagent package includes Reagent A, alkaline copper tartrate, and Reagent B, a
dilute Folin reagent. Three to five dilutions of bovine serum albumin from 0.2 mg/mL to 1.5 mg/mL were prepared for a standard curve. Five µL of standards and samples were distributed in a clean and dry microtiter plate. Twenty five µL of Reagent A was added to each well, and then 200 µL Regent B was added to each well. Absorbance was read by an ELISA reader at 630 nm after 15 min of reaction at room temperature.

b. GUS Histochemical Assay
Treated tobacco leaf disks were soaked in the phosphate buffer (50 mM NaPO₃, pH 6.8 with 0.1 % (v/v) Triton X-100) at 37°C for one hr. Meanwhile, 3.0 mg of X-gluc (5-Bromo-4--chloro-3-indoyl β-D-glucuronide sodium) was dissolved in 150 µL of dimethyl formamide. Eight hundred fifty µL of solution B (5.0 mM potassium ferricyanide, 5.0 mM potassium ferrocyanide, 0.1 M sodium phosphate, and 0.1% (v/v) Triton X-100) was added to 150 µL X-gluc solution. Sodium phosphate buffer was discarded after incubation for one hr and then Solution B with X-Gluc was added until leaf disks were submerged. They were incubated overnight at room temperature and destained with destaining solution [50% (v/v) ethanol, 10% (v/v) formaldehyde, and 5% (v/v) glacial acetic acid] overnight again (Zheng et al., 1993).

c. Statistical Analysis
Experiments for all different treatments described above were conducted using either two or three replications per treatment. Each replication consisted of a single Petri plate containing 15 leaf disks. All data were subjected to ANOVA, and means were compared using Fisher’s least significant difference (LSD) test (α =0.05).
CHAPTER 4. RESULTS

The primary objective of this research was to determine optimal experimental conditions to enhance the $\beta$-glucuronidase (GUS) activity after *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf disks. A co-cultivation temperature at 20$^\circ$C was the most important condition that increased GUS activity. Other factors examined in this study were surfactant (Silwet L-77) concentrations, vacuum infiltration, three pCAMBIA plasmids, co-cultivation durations and bacterial concentrations, bacterial pre-culture conditions, acetosyringone (AS) concentrations, a wounding effect, and antibiotic selection agents.

Unless otherwise specified, *Agrobacterium tumefaciens* strain LBA4404 containing pCAMBIA1305.01 was grown overnight at 28$^\circ$C in liquid media, and the concentration was adjusted to $3 \times 10^7$ cells/mL (0.3 $A_{600}$ units/mL). Tobacco leaf disks were inoculated with bacteria under 50 mm Hg vacuum infiltration for 20 min in the presence of the 0.001% (w/v) Silwet L-77. Leaf disks were co-cultivated in the presence of 200 $\mu$M of acetosyringone for four days under constant light at 20$^\circ$C, transferred to selection medium containing 50 mg/L hygromycin and 500 mg/L carbenicillin, and grown for an additional 14 days under constant light at 25$^\circ$C. $\beta$-Glucuronidase (GUS) activity was measured at the end of growth period by quantitative GUS assay and GUS histochemical staining to compare the effects of experimental variables.

4.1 Effect of Temperature During the Co-cultivation

Temperatures during four day co-cultivation period were compared, ranging from 15, 18, 20, 22 to 25$^\circ$C to find the optimal temperature for maximum GUS expression. Combinations of only three temperatures were examined simultaneously in four separate sets of experiments because only three identical growth chambers (model cu-36L5 from Percival Scientific) were available. These sets of experiments were 15, 18 and 20$^\circ$C (Figure 1(a)), 18, 20 and 25$^\circ$C (Figure 1(b)), 18, 20 and 22$^\circ$C (Figure 1(c)), and 20, 22 and 25$^\circ$C (Figure 1(d)). Results from four separate experiments were analyzed separately (Figure 1(a), (b), (c), and (d)) and in combination (Figure 2). Highest GUS activity was detected at 20$^\circ$C in three out of four experiments (Figure 1(a), (b) and (d)) and at 18$^\circ$C in one experiment (Figure 1(c)). Lowest GUS activity was detected at 25$^\circ$C in two experiments (Figure 1(b) and (d)), and at 15$^\circ$C in one experiment (Figure 1(a)). GUS activity at 20$^\circ$C co-cultivation was significantly higher than that at 15$^\circ$C (Figure 1(a)) and
at 25°C (Figure 1(b) and (d)). GUS activity at 18°C co-cultivation was significantly higher than that at 15°C (Figure 1(a)), at 22°C (Figure 1(c)) and at 25°C (Figure 1(b)).

All GUS activity values were pooled from the four separate experiments and plotted as dots to show the range of activity at each temperature (Figure 2). Values were distributed from 20 to 2300 (pmol/µg protein/h). As before, highest average of GUS activity was obtained at 20°C followed by at 18 and 22°C. There was no statistical difference among the GUS activities at these three temperatures. However, the histological GUS assay showed that callus formation was more pronounced at 20°C than at 18°C (Figure 3). The lowest average of GUS activity was observed at 25°C co-cultivation treatment. The GUS activity at 15°C was an intermediate between the highest and lowest activities. We concluded that co-cultivation at 20°C is the most effective condition for the enhanced GUS activity after transformation of tobacco leaf disks.

4.2 Comparison of Two β-glucuronidase Genes

Three pCAMBIA plasmids, 1301, 1305.01, and 1305.02 were compared for expression of the β-glucuronidase activity (Figure 4(a)). pCAMBIA 1301, 1305.01, and 1305.02 have the hygromycin phosphotransferase II gene (HPTII, 1025 bp), under the Cauliflower Mosaic Virus (CaMV) 35S promoter, as an antibiotic resistance marker for selecting transformed cells. pCAMBIA 1305.01 and 1305.02 vectors have the same structural components as pCAMBIA 1301, except for the GUS coding sequence. The GUS coding sequence (1868 bp) of pCAMBIA 1301 originated from *E. coli*, while the GUS coding sequence (1861 bp) of pCAMBIA 1305.01 and 1305.02 (GUSplus), was from *Staphylococcus* sp. The codon usage of both GUS coding sequences was adjusted to increase the GUS expression in higher plants (Jefferson et al., 2003). An intron (189 bp) from the castor bean catalase gene is located in between the first and second exon of the GUS gene. The insertion of the intron was necessary to ensure that the observed GUS activity occurs in plant cells, but not in bacteria, since the intron is spliced out by plant splicesome machinery. Intron insertion also appears to increase the GUS expression (Marillonnet et al., 2005). pCAMBIA 1305.02 is distinguished from pCAMBIA 1305.01 by insertion of a signal peptide from the rice glycin-rich protein sequence (79 bp) in front of the first exon of the GUS coding sequence. This signal peptide targeted the mature protein for extracellular secretion (Lei and Wu, 1991). It enables GUS to be detected rapidly and by *in vivo* assays.
Figure 1. Effect of different co-cultivation temperatures, 15°C, 18°C, 20°C, 22°C and 25°C on GUS activity. Four separate experiments were conducted since only three identical growth chambers were available. Leaf disks were inoculated with bacteria (0.3 A_{600} units) under 50 mm Hg vacuum infiltration for 20 min in the presence of 0.001% Silwet L-77. Co-cultivation was conducted for four days at 20°C with 200 μM AS. GUS activity was detected after two weeks selection at 25°C. Bars represent the standard deviation. Means followed by the same letter are not significantly different at the \( \alpha = 0.05 \) level. The experiments were conducted from 10/18/05 to 11/25/05.
Figure 2. Effect of co-cultivation temperature on GUS activity. All values from four different experiments (Figure 1) were pooled and plotted as dots. Averages of GUS activity at each temperature are represented as horizontal bars, and vertical bars represent the standard deviation. Leaf disks were inoculated with bacteria (0.3 $A_{600}$ units) under 50 mm Hg vacuum infiltration for 20 min in the presence of 0.001% Silwet L-77. GUS activity was measured after four day co-cultivation with 200 µM AS at 20ºC and a 14-day selection at 25ºC. Means followed by the same letter are not significantly different at the $\alpha = 0.05$ level. The experiment was conducted from 10/18/05 to 11/25/05.
Co-cultivation temperature | Hygromycin selection for two weeks

(a) 15°C

(b) 18°C

(c) 20°C

(d) 22°C

(e) 25°C

Figure 3. Histochemical staining of GUS activity was conducted after four days co-cultivation at (a)15°C, (b)18°C, (c)20°C, (d)22°C and (e)25°C, followed by selection for additional 14 days at 25°C. A representative set of plants is shown. The experiment was repeated three times.
The size of the GUS coding sequence of pCAMBIA 1301 is 2057 bp, and 1305.01 is 2050 bp, and pCAMBIA 1305.02 is 2129 bp due to the addition of a signal peptide sequence.

Different plasmids were tested in two inoculation methods: by soaking or with Silwet L-77 and vacuum infiltration. In the former condition, leaf disks were inoculated with bacteria for 20 min. This was compared with leaf disks that were vacuum-infiltrated with bacteria for 20 min in the presence of 0.001% (v/v) Silwet L-77.

In both conditions, pCAMBIA 1305.01 showed the highest GUS activity among the three plasmids (Figure 4(b)). When pCAMBIA1305.01 and pCAMBIA1305.02 were compared, two and four times higher GUS activity was obtained with pCAMBIA 1305.01 plasmid than with pCAMBIA 1305.02 in both treatments. We concluded that pCAMBIA 1305.01 was the most effective vector for detecting GUS activity after tobacco transformation under these conditions (Figure 4(b)).

4.3 Effect of Surfactant, Vacuum Pressure Strength and Duration

Low and high vacuum pressures and various vacuum durations were tested with the pCAMBIA1305.01 plasmid in the absence of Silwet L-77 (Figure 5). The vacuum durations ranging from 0.5 to 20 min were used at 50 mm Hg vacuum. The vacuum durations ranging from 0.25 to 6 min were tested at 600 mm Hg vacuum. Using low vacuum at 50 mm Hg, GUS activity increased with the longer durations from 0.5 min to 8 min (Figure 5). However, the durations longer than 8 min up to 20 min did not further enhance the effectiveness of vacuum infiltration on GUS activity (Figure 5). At the higher vacuum of 600 mm Hg, the shortest duration of 0.25 min yielded the highest average GUS activity, but results were highly variable. Leaves were necrotic in all treatments at 600 mm Hg. The results showed that the low vacuum pressure at 50mm Hg for 8 to 20 min was most effective for the increased GUS activity.

The next experiment was designed to find optimal interaction between the Silwet L-77 concentration and vacuum duration at the initial inoculation step with Agrobacterium. Silwet L-77 was added at the concentrations ranging from 0.001% to 0.1%, to the bacterial inoculum prior to the co-cultivation. Different vacuum durations were tested at 50 mm Hg vacuum, ranging from 1.5 to 40 min (Figure 6).
(a) Structure of the β-glucuronidase gene in three pCAMBIA binary vectors

pCAMBIA1301

35S promoter

GUS First Exon

Catalase Intron

Escherichia coli

Nos polyA

GUS Second Exon

pCAMBIA1305.01

35S promoter

GUSplus First Exon

Catalase Intron

Staphylococcus sp.

Nos polyA

GUSplus Second Exon

pCAMBIA1305.02

35S promoter

GUSplus First Exon

Catalase Intron

Staphylococcus sp.

Nos polyA

GRP signal

(b) GUS activity

![GUS activity graph]

Figure 4. Comparison of two β-glucuronidase genes in two different protocols (a) Structure of the β-glucuronidase genes in three pCAMBIA binary vectors (b) GUS activity from the different plasmids in two different protocols. Soaking: leaf disks were submerged with bacterial inoculum (0.3 A₆₀₀ units) for 20 min; no vacuum or Silwet L-77 was applied for this treatment. Vacuum and Silwet L-77: leaf disks were inoculated with bacterial inoculum (0.3 A₆₀₀ units) under vacuum infiltration for 20 min at 50 mm Hg pressure in the presence of 0.001% Silwet L-77. GUS activity was measure after a four days co-cultivation at 20°C in the presence of 200 µM AS and 14 day selection at 25°C. Means followed by the same letter (upper/lower case) are not significantly different at the α =0.05 level.
Figure 5. Effect of low and high vacuum strengths and durations on GUS activity. Low and high vacuum was applied at 50 and 600 mmHg, respectively. Leaf disks were inoculated with bacterial inoculum (0.3 A₆₀₀ units) at 50 mm Hg for 0.5, 2, 4, 8, 15 and 20 min and at 600 mm Hg for 0.25, 0.5, 2, 4 and 6 min. This experiment was conducted in the absence of Silwet L-77. After inoculation, leaf disks were co-cultivated in the presence of 200 µM AS at 20°C for four days. GUS activity was measured from treated leaf disks after two weeks on antibiotic selection media at 25°C. Bars represent the SD. Means followed by the same letter are not significantly different at the α =0.05 level. The experiment was conducted from 08/10/05 to 08/28/05.
Figure 6. Effects of Silwet L-77 concentrations and vacuum infiltration durations, separately (a) and (b), or in combination (c) and (d) on GUS activity. Silwet L-77 was added to the bacteria suspension just before inoculation of leaf disks. Co-cultivation was conducted for four days at 20°C in the presence of 200 µM AS followed by selection for 14 days at 25°C on shooting media containing 50 mg/L hygromycin prior to GUS assay. The experiment was conducted from 10/10/05 to 10/28/05. Bars represent standard deviation. Means followed by the same letter are not significantly different at the $\alpha$ =0.05 level.
The GUS activity increased at 0.005% to 0.05% Silwet L-77 concentrations, and declined sharply at 0.1% (Figure 6(a)). In the absence of Silwet L-77, the highest average GUS activity was detected after the longest duration of 40 min of vacuum infiltration at 50 mm Hg (Figure 6(b)). The GUS activity values at 0.005 and 0.05% Silwet L-77, or with 40 min vacuum infiltration were statistically significantly higher than the untreated controls (Figure 6(a) and (b)). In the presence of either 0.001 or 0.005% Silwet L-77, the average GUS activity increased with the longer vacuum durations from 1.5 to 40 min (Figure 6(c) and (d)). The highest GUS activity values obtained after a 40 min treatment were statistically higher than those in the controls without vacuum (Figure 6(c) and (d)). The results showed that the combination of 0.001 or 0.005% Silwet L-77 with 40 min vacuum infiltration was optimal for the increased GUS activity.

The results from the quantitative GUS assay were in agreement with visual observation of callus formation and histochemical staining of GUS-positive cells (Table 1). The higher concentrations of Silwet L-77 increased the number of necrotic leaf disks without any positive effect on the number of GUS-positive stains (Table 1(a)). The vacuum durations for 20 and 40 min dramatically increased the GUS-positive stains in the absence or presence of 0.001 or 0.005% Silwet (Table 1(b), (c) and (d)). However, size of the GUS-positive stains was smaller as the vacuum duration increased. The highest percentages of leaf disks producing callus and the third highest number of GUS-positive stains per leaf disk were observed after treatment with 40 min vacuum infiltration in the presence of 0.005% Silwet L-77. However, four out of 48 leaf discs were necrotic in this treatment. In contrast, there were no necrotic leaf disks observed after treatment with 20 min vacuum infiltration in the presence of 0.001% Silwet L-77. The percentage of callus formation was second highest, and the average number of GUS-positive stains per leaf disk was fifth highest in this condition. Thus, We concluded that the treatment with 20 min vacuum duration in the presence of 0.001% Silwet L-77 was the optimum condition for the overall increased GUS activity.

This conclusion was corroborated further by visual observation of shape and size of calli formed on leaf disks. Figure 7(a)-A showed that only two leaf disks produced a few number of calli at the edge after soaking alone, while leaf disk size remained larger than after any other treatments. After 0.001% Silwet L-77 treatment, more calli at edges of more leaf disks were formed (Figure 7(a)-B). Fresh weights of the leaf disks remained the same in the presence or absence of 0.001% Silwet L-77 (Figure 7(b)). After 20 min vacuum infiltration, a smaller-sized
Table 1. Effect of Silwet L-77 concentrations and vacuum durations, separately or in combination, on callus formation and GUS-positive cells. Vacuum pressure at 50 mm Hg was used for this experiment. GUS histochemical staining was conducted after four days of co-cultivation at 20°C with 200 μM AS, followed by 14 days of selection at 25°C on shooting media containing 50 mg/L hygromycin and 500 mg/L carbenicillin. Experiments were performed twice with two replications. Each replication contains 12 leaf disks.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number of leaf disks formed</th>
<th>Number of leaf disks died</th>
<th>Average number of the GUS positive calli per leaf disk</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Silwet L-77 Concentration without Vacuum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>4/48 (8%)</td>
<td>0/48 (0%)</td>
<td>3</td>
</tr>
<tr>
<td>0.001%</td>
<td>10/48 (21%)</td>
<td>2/48 (4%)</td>
<td>7</td>
</tr>
<tr>
<td>0.005%</td>
<td>16/48 (33%)</td>
<td>14/48 (29%)</td>
<td>10</td>
</tr>
<tr>
<td>0.05%</td>
<td>14/48 (29%)</td>
<td>10/48 (21%)</td>
<td>11</td>
</tr>
<tr>
<td>0.1%</td>
<td>8/48 (17%)</td>
<td>12/48 (25%)</td>
<td>16</td>
</tr>
<tr>
<td>(b) Without Silwet L-77 and Vacuum Duration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 min</td>
<td>20/48 (42%)</td>
<td>0/48 (0%)</td>
<td>26</td>
</tr>
<tr>
<td>4 min</td>
<td>18/48 (39%)</td>
<td>4/48 (8%)</td>
<td>26</td>
</tr>
<tr>
<td>20 min</td>
<td>14/48 (29%)</td>
<td>0/48 (0%)</td>
<td>186</td>
</tr>
<tr>
<td>40 min</td>
<td>20/48 (42%)</td>
<td>12/48 (25%)</td>
<td>134</td>
</tr>
<tr>
<td>(c) 0.001% Silwet L-77 with Vacuum Duration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 min</td>
<td>32/48 (67%)</td>
<td>10/48 (21%)</td>
<td>38</td>
</tr>
<tr>
<td>4 min</td>
<td>20/48 (42%)</td>
<td>0/48 (0%)</td>
<td>32</td>
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<td>20 min</td>
<td>36/48 (75%)</td>
<td>0/48 (0%)</td>
<td>78</td>
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<tr>
<td>40 min</td>
<td>32/48 (67%)</td>
<td>8/48 (17%)</td>
<td>66</td>
</tr>
<tr>
<td>(d) 0.005% Silwet L-77 with Vacuum Duration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 min</td>
<td>26/48 (54%)</td>
<td>0/48 (0%)</td>
<td>22</td>
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<td>4 min</td>
<td>22/48 (46%)</td>
<td>2/48 (4%)</td>
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<td>32/48 (67%)</td>
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</tr>
<tr>
<td>40 min</td>
<td>38/48 (79%)</td>
<td>4/48 (8%)</td>
<td>125</td>
</tr>
</tbody>
</table>
(a) Histochemical staining of GUS activity

<table>
<thead>
<tr>
<th>A. Soaking</th>
<th>B. 0.001% Silwet L-77</th>
<th>C. 20 min Vacuum</th>
<th>D. 0.001% Silwet L-77 and 20 min Vacuum</th>
</tr>
</thead>
</table>

(b) Fresh weight

Figure 7. Effect of the 0.001% Silwet L-77 and 50 mm Hg vacuum infiltration for 20 min, separately or in combination on callus formation (a) and fresh weight (b). GUS activity was detected by histochemical staining. A; soaking without Silwet L-77 and vacuum infiltration; B; 0.001% Silwet L-77; C; 20 min vacuum infiltration; D; 0.001% Silwet L-77 with 20 min vacuum infiltration. GUS staining and fresh weight measurements were conducted after four days of co-cultivation at 20°C in the presence of 200 µM AS, followed by 14 days of selection at 25°C on shooting media containing 50 mg/L hygromycin and 500 mg/L carbenicillin. Means followed by the same letter are not significantly different at the α = 0.05 level.
but higher number of calli were formed all over the surface of leaf disks than after 0.001% Silwet L-77 treatment (Figure 7(a)-C). Vacuum infiltration for 20 min reduced the size and fresh weight of leaf disks dramatically in the absence or presence of 0.001% Silwet L-77 (Figure 7(b)). The larger-sized calli were formed all over the surface of most of the leaf disks after treatment for 20 min vacuum infiltration in the presence of 0.001% Silwet L-77 (Figure 7(a)-D).

4.4 Effect of Co-cultivation Durations and Bacteria Concentrations

Co-cultivation periods from two, four to six days were tested at four different bacterial concentrations, $3 \times 10^5$/mL (0.003 $A_{600}$ units/mL), $3 \times 10^6$ (0.03 $A_{600}$ units), $3 \times 10^7$ (0.3 $A_{600}$ units) and $10^8$ cells (1 $A_{600}$ unit). As shown in Figure 8 GUS activity values after four or six days co-cultivation were statistically higher than those after two days co-cultivation at most bacteria concentrations. The highest average GUS activity was observed after six days co-cultivation at $3 \times 10^7$ cells (Figure 8).

The higher number of callus-forming leaf disks and lower number of necrotic disks were observed after four or six days co-cultivation at all bacteria concentrations than those after two days co-cultivation, based on the results from visual observation of leaf disks (Table 2). No necrotic leaf discs were evident, and all or almost all leaf disks formed calli after four days co-cultivation at $3 \times 10^7$ and $10^8$ cells/mL. We concluded that this would be an optimal condition for testing for the increased GUS activity.

4.5 Effect of Bacterial Pre-culture Conditions and Acetosyringone Concentrations

Prior to the co-cultivation, *Agrobacterium tumefaciens* was grown either in liquid medium at 28°C or on solid plate medium at 20°C, in the presence or absence of 200 µM acetosyringone (AS). The results in Figure 9 showed that GUS activity in the leaf disks inoculated with plate-cultured bacteria at 20°C with AS was significantly higher than without AS, and also was significantly higher than leaf disks inoculated with liquid-cultured bacteria at 28°C in the presence or absence of 200 µM of AS.

Concentrations of AS ranging from 0 to 400 µM were tested during the co-cultivation at 20°C for four days. The GUS activity was compared after two weeks of culture on selection media (Figure 10). GUS activity was not detectable in the absence or presence of 100 µM of AS. GUS activity increased dramatically in the presence of 200 and 400 µM AS. The means of GUS activity were not significantly different between 200 and 400 µM AS. These results showed that 200 µM or 400 µM of AS is the optimal amount during the four days co-cultivation at 20°C.
Figure 8. Effect of bacterial concentrations and days of the co-cultivation period on GUS activity. Leaf disks were inoculated at four different bacteria concentrations, ranging from $10^8$ to $3 \times 10^5$ cells per mL which corresponds to 0.003 to 1 $A_{600}$ units per mL. Forty five leaf disks were inoculated with 10mL each of four different bacterial concentrations. After 50 mm Hg vacuum infiltration for 20 min, inoculated leaf disks were co-cultivated for two, four or six days at 20°C in the presence of 200 µM AS. Treated tissues were selected at 25°C on shooting media containing 50 mg/L hygromycin and 500 mg/L carbenicillin for 16, 14, or 12 days, respectively. Values are mean ± SD. Means followed by the same letter are not significantly different at the $\alpha = 0.05$ level. The experiment was conducted from 10/19/05 to 11/06/05.
Table 2. Effect of co-cultivation durations and bacterial concentrations on callus formation and necrosis. Visual inspection was conducted after co-cultivation and selection on shoot selection media containing 50 mg/L hygromycin and 500 mg/L carbenicillin. Experiments were performed two times with two replications. Each replication contains 15 leaf disks.

<table>
<thead>
<tr>
<th>Co-cultivation Period</th>
<th>Bacteria concentration per mL</th>
<th>Average number of leaf disks producing callus (percentage)</th>
<th>Average number of necrotic leaves (percentage)</th>
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</thead>
<tbody>
<tr>
<td>Two days</td>
<td>3x10^5</td>
<td>0/60 (0%)</td>
<td>40/60 (67%)</td>
</tr>
<tr>
<td></td>
<td>3x10^6</td>
<td>0/60 (0%)</td>
<td>20/60 (33%)</td>
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<tr>
<td></td>
<td>3x10^7</td>
<td>10/60 (17%)</td>
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<tr>
<td></td>
<td>1x10^8</td>
<td>5/60 (8%)</td>
<td>30/60 (50%)</td>
</tr>
<tr>
<td>Four days</td>
<td>3x10^7</td>
<td>30/60 (50%)</td>
<td>0/60 (0%)</td>
</tr>
<tr>
<td></td>
<td>3x10^8</td>
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<td>0/60 (0%)</td>
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<tr>
<td></td>
<td>3x10^9</td>
<td>60/60 (100%)</td>
<td>0/60 (0%)</td>
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<tr>
<td></td>
<td>1x10^10</td>
<td>50/60 (83%)</td>
<td>0/60 (0%)</td>
</tr>
<tr>
<td>Six days</td>
<td>3x10^7</td>
<td>45/60 (75%)</td>
<td>25/60 (22%)</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>3x10^9</td>
<td>55/60 (92%)</td>
<td>0/60 (6%)</td>
</tr>
<tr>
<td></td>
<td>1x10^10</td>
<td>25/60 (42%)</td>
<td>5/60 (8%)</td>
</tr>
</tbody>
</table>
Figure 9. Effect of the acetosyringone (AS) and pre-culture conditions of *Agrobacterium tumefaciens*. Bacteria were grown either on solid plate Agro media for three days at 20 ºC with or without 200 µM AS, or in liquid culture at 28 ºC with or without 200 µM AS. Bacteria were washed with 1 mL of MS liquid media (4.4 g/L MS salt, 30g/L sucrose, pH 5.5) containing 200 µM AS using an L-shaped glass rod. Bacterial concentration was adjusted to 0.3 A₆₀₀ units/mL with MS liquid media. Leaf disks were inoculated with *Agrobacterium* with 50 mm Hg vacuum infiltration for 20 min in the presence of 0.001% Silwet L-77. Inoculated leaf disks were co-cultivated for four days at 20 ºC on a medium containing 200 µM AS. Leaf disks were selected on selection media containing 50 mg/L hygromycin and 500 mg/L carbenicillin for 14 days at 25 ºC. Bars represent the SD. Means followed by the same letter are not significantly different at the α =0.05 level. The experiment was conducted from 05/30/05 to 06/18/05.
Figure 10. Effect of the acetosyringone (AS) concentrations during the four day co-cultivation. Leaf disks were inoculated with bacteria in Agro liquid medium in the presence of 0.001% Silwet L-77 with 20 min vacuum infiltration under 50 mm Hg. Leaf disks were co-cultivated for four days at 20°C on MS co-cultivation medium containing 0, 100, 200, or 400 µM AS. Bars represent the SD. Means followed by the same letter are not significantly different at the $\alpha=0.05$ level. The experiment was conducted from 05/30/05 to 06/18/05.
4.6 Effect of Wounding and Bacterial Removal

This set of experiments was designed to test the timing of wounding introduction to the intact tobacco leaf before or after bacterial inoculation, or before or after co-cultivation at 20°C. The effect of removal of bacteria by washing the inoculated leaf disks at the end of the four days co-cultivation was also tested. Intact leaves without introduction of any wounding were inoculated with $3 \times 10^7$ cells/mL (0.3 $A_{600}$ units/mL) bacteria for 20 min under 50 mm Hg vacuum infiltration in the presence of 0.001% Silwet L-55, co-cultivated at 20°C for four days on media containing 200 µM AS, and followed by 14 days of selection. GUS activity was not detectable after this treatment (Treatment 1, Figure 11). In Treatment 2, intact leaves were inoculated in the same manner as Treatment 1, and leaf disks were cut from the inoculated intact leaf with a cork borer. These leaf disks were co-cultivated at 20°C for four days, followed by 14 days selection. In Treatments 3 and 4, small disks were first cut from intact leaves, and inoculated with $3 \times 10^7$/mL bacteria. After bacterial inoculation, leaf discs were either not washed (Treatment 3) or washed (Treatment 4) to remove bacteria.

Figure 11 shows that the GUS activity was significantly higher in Treatments 3 and 4 than the control in Treatment 1. The activity in Treatment 2 was not statistically different from that of either Treatment 1 or Treatments 3 and 4. This result indicated that wounding is prerequisite for the increased GUS activity after *Agrobacterium*-mediated transformation. The amount of the GUS activity with washing (Treatment 4) or without washing (Treatment 3) was not distinguishable statistically. The results from Figure 11 demonstrate the importance of timing of wounding for the increased GUS activity and indirectly for transformation.
Figure 11. Effect of wounding and bacterial removal on GUS activity. Treatment 1, the intact leaf was inoculated with *Agrobacterium tumefaciens* without any wounding, co-cultivated for four days, and selected for 14 days. Treatment 2, the intact leaf was inoculated first, wounded by cutting the intact leaf to disks with a cork borer, then leaf disks were co-cultivated for four days and selected for 14 days. Treatment 3, wounding was introduced by cutting the intact leaf into disks, and then the disks were inoculated with bacteria. Inoculated leaf disks were co-cultivated for four days and selected for 14 days. Treatment 4 was the same as Treatment 3, except that bacteria were removed by washing leaf disks with MS liquid media right after bacterial infection. Washed leaf disks were co-cultivated for four days and selected for 14 days. In common, leaf disks were inoculated with *Agrobacterium* with 50 mm Hg vacuum infiltration for 20 min in the presence of 0.001% Silwet L-77. Inoculated leaf disks were co-cultivated for four days at 20°C on a medium containing 200 μM AS. Leaf disks were selected on selection media containing 50 mg/L hygromycin and 500 mg/L carbenicillin for 14 days at 25°C. Bars represent standard deviation. Means followed by the same letter are not significantly different at the $\alpha=0.05$ level. The experiment was conducted from 09/06/05 to 10/04/05.
4.7 Timecourse Experiment

GUS activity was measured every two days after inoculation with *Agrobacterium tumefaciens* (0.3 A_{600} units/mL) for 20 min under 50 mm Hg vacuum infiltration in the presence of 0.001% Silwet L-77. No GUS activity was detectable during the four days co-cultivation period and even four days after transferring to the hygromycin selection media. GUS activity increased gradually from four to nine days on selection media. A dramatic increase of GUS activity was detected 12 days after co-cultivation (16 days after infection) (Figure 12).

4.8 Evaluation of Antibiotic Selection Agents

The optimal concentrations of the antibiotic selection agents, hygromycin and kanamycin, were determined on leaf disks of tobacco. The tested concentrations of hygromycin ranged from 50 to 200 mg/L, and kanamycin from 500 to 4000 mg/L (Figure 13). All leaf disks on hygromycin (100, 150 and 200 mg/L) turned white and necrotic completely within a week, while the edge of the leaf disks on 50 mg/L hygromycin showed signs of necrosis after the first week of selection. All leaf disks at all four concentrations of hygromycin were necrotic after two weeks. With kanamycin, all leaf disks were not necrotic at 500, 2000 and even at 4000 mg/L after one week of selection. After two weeks, all leaf disks turned white completely at 2000 and 4000 mg/L kanamycin. All leaf disks were still green at 500 mg/L kanamycin even after two weeks of selection.
Figure 12. Timecourse of the increase in GUS activity after inoculation. Leaf disks were inoculated with bacteria (0.3 A_{600} units) with 50 mm Hg vacuum infiltration for 20 min in the presence of 0.001% Silwet L-77. Leaf disks were co-cultivated for four days co-cultivation at 20 °C in the presence of 200 µM AS. GUS activity was measured for 16 days from the leaf disks incubated on selection media at 25 °C in the presence of 50 mg/L hygromycin and 500 mg/L carbenicillin.
Figure 13. Effect of antibiotics, kanamycin (a) and hygromycin (b) on plant necrosis. Tobacco leaf disks without bacterial infection were incubated on MS media containing different antibiotics of different concentrations. Leaf disks were incubated at 25°C for 2 weeks.
CHAPTER 5. DISCUSSION

We found that a co-cultivation temperature at 20°C was the most critical factor to achieve reproducibility and consistency of GUS activity expression after *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf disks. Other experimental conditions examined using GUS gene reporter were plasmids, duration of co-cultivation, bacterial concentrations, surfactant, vacuum infiltration, bacterial pre-culture conditions, AS concentrations, wound timing, and selection agents.

Among the five temperatures tested from 15, 18, 20, 22 to 25°C, co-cultivation at 18, 20 and 20°C resulted in statistically higher GUS activity than that at 25°C. Co-cultivation at 15°C resulted in a statistically intermediate value between the highest and lowest GUS activities. This result is different from those reported previously. Transient expression of quantitative GUS activity in *Phaseolus acutifolius* embryogenic calli had a peak at 22°C over a range of co-cultivation temperatures from 15, 19, 22, 25, 27 to 29°C. Histochemical staining of GUS activity was also the highest at 22°C for both *Phaseolus* calli and *Nicotiana tabacum* (Dillen et al., 1997). For transformation of hypocotyl explants of cauliflower, 22°C co-cultivation resulted in the highest GUS expression, and no GUS expression was detected at 28°C (Chakrabarty et al., 2002). GUS expression in a *Phaseolus acutifolius* transformation experiment also showed that 22°C co-cultivation was the optimal condition to increase transformation efficiency (De Clercq et al., 2002). In an agreement with the present study, all previous experiments reported that GUS expression was reduced dramatically by co-cultivation temperatures at or above 25°C.

The lower optimal co-cultivation temperature in this study may be partly accounted for by the differences between stable vs transient expression, and also the difference in co-cultivation and selection conditions used. We assayed for quantitative GUS activity of presumably stable transformants, and applied a more physiologically appropriate condition during the co-cultivation and selection period using three identical tissue culture chambers, Model CU-36L5 of Percival Scientific. Leaf disks were placed for four days on MS shoot media in a Petri dish under constant light (56 µE m⁻² s⁻¹) with the temperature variation of ± 0.7°C. Selection for transformed cells was conducted for 14 days on MS shoot media containing hygromycin and carbenicillin in a Petri dish under constant light at 25°C ± 0.7°C. In comparison, Dillen at al. (1997) used less than physiologically appropriate conditions during the co-
cultivation, no selection of tissues and a shorter culture period before GUS assay for transient expression. Leaves of *Nicotiana tabacum* cv SR1 Petit Havana were infiltrated with 0.8 A$_{600}$ unit of *Agrobacterium tumefaciens* under reduced pressure for 20 min, placed on a filter paper in a Petri dish that was sealed in a plastic bag, and submerged for three days under water in a water bath. Calli of *Phaseolus acutifolius* were placed in a glass jar that was submerged under water in a water bath. After two days co-cultivation, treated calli were cultured on non-selected medium for four days prior to GUS assay. Consequently, the reported GUS activity of Dillen et al. (1997) was significantly lower than this study and had no standard deviation indicated. We assumed a minimum effect exerted by the difference in binary vectors, helper plasmids or strains of *Agrobacterium tumefaciens* used in this and previous experiments.

Riker (1926) made an initial observation of important role of temperature on the formation of crown gall tumors in tomato and other plants. The size of tumors was largest at the optimal temperature of 22°C and gradually decreased as the incubation temperature increased up to 28°C. No tumors were formed at or above 30°C. Braun (1947) used this thermo-inactivation treatment to distinguish two phases of crown gall tumor formation between the temperature-sensitive inception phase and the temperature-insensitive development phase. The autonomous neoplastic growth of transformed cells was achieved only after plant cells reached the second development phase. The inception phase was further divided into two processes, the thermo-sensitive induction process and the thermo-insensitive conditioning process. The conditioning of plant cells was initiated by wounding, took place either below or above 30°C, and improved during the 48 hr period after wounding up to the time the first cell division was observed. Thus, Braun (1947) identified the thermo-sensitivity of the induction process in which a Tumor Inducing Principal (TIP) generated by *Agrobacterium tumefaciens* transfers from the bacteria to plant cells. The nature of Braun’s TIP was identified as T-DNA of the Ti-plasmid in *Agrobacterium tumefaciens*, and the components of T-DNA transfer machinery are being elucidated more recently.

The thermo-sensitivity of conjugative transfer of the Ti plasmid from the virulent to cured avirulent strains of *Agrobacterium tumefaciens* was noted by Tempe et al. (1977). The frequency of plasmid transfer was 4.0 and 3.5 x 10$^{-2}$ at 23 and 27°C, respectively, reduced by four-fold to 0.9 x 10$^{-2}$ at 30°C, and went below 10$^{-6}$ at 33 and 36°C. The IncQ group plasmid RSF1010 is a non-conjugative plasmid, but can utilize the transfer machinery of the conjugative Ti plasmid of *Agrobacterium tumefaciens*. Conjugal transfer of the IncQ plasmid mediated by
Agrobacterium tumefaciens was thermo-sensitive. The frequency of conjugal transfer was the highest \((2.6 \times 10^{-3})\) at 19°C, gradually declined to \(1.1 \times 10^{-3}\) at 22°C, \(5.3 \times 10^{-4}\) at 25°C, and \(2.0 \times 10^{-4}\) at 15°C, and then was drastically reduced by over 1,000-fold at 28 and 31°C. The temperature profile of conjugal transfer of the IncQ plasmid is more similar to that of crown gall tumor formation than that of conjugal transfer of the Ti plasmid. The similarity of the thermo-sensitivity profile between crown gall formation and conjugal transfer of IncQ and Ti-plasmid suggests that the same machinery may be involved in transfer of T-DNA from the bacteria to plant cells, and in conjugal transfer of these plasmids between bacteria.

The thermo-sensitivity of bacteria pilus assembly was observed by Fullner et al. (1996). The induction of \(\text{vir}\) genes by 200 \(\mu\)M acetosyringone was essential for the pilus assembly. The pilated cells constituted 10 to 20% of cells observed under the electron microscope at 19°C and were over ten-fold more abundant than those at 28°C. Loss and gain of function experiments demonstrated that the same collection of \(\text{vir}\) genes (\(\text{virA, virG, virB1 through virB11, virD4}\)) was required for pilus production and conjugal transfer of the plasmid. The additional \(\text{vir}\) gene, \(\text{virE1}\) required for transport of \(\text{virE2}\), was necessary for tumor induction on leaves of Kalanchoe daigremontiana. The results suggest that the assembly of the pilus on Agrobacterium tumefaciens is essential for both processes, transfer of T-DNA from the bacteria to plant cells and conjugal transfer of the plasmid between bacteria.

Distinct profiles of thermo-sensitivity were evident between the induction of \(\text{vir}B\) gene activity and conjugal transfer frequency of the IncQ plasmid. The induction of \(\text{virB}\) genes was optimal at 25°C, while the conjugal transfer of the plasmid was optimal at 19°C. The induction of \(\text{vir}B\) gene expression at 25°C declined gradually to 98 % at 22 °C, 76% at 19°C, and 3 % at 15°C as the incubation temperature decreased, and 70% at 28°C, 47% at 31°C, and 0.5% at 34°C as temperature increased from 25°C. The gradual decline of the \(\text{vir}\) gene induction at temperatures above 30°C is not consistent with over 1,000-fold reduction of conjugal transfer frequency of the IncQ plasmid, and with inactivation of crown gall tumor formation.

Baron et al. (2001) reported that highest amounts of pili and the pili component VirB were detected at 20°C, and small amounts of pili were detected at 26°C, while pili formation at 28°C was strongly inhibited. They also reported that tumor formation was induced at 20°C in wounded K. diagremontiana. It can be concluded that 20°C is the optimal temperature to induce vigorous pili formation and to maintain plant tissue viability.
REFERENCES


Brown NA (1942) The effect of certain chemicals, some of which produce chromosome doubling, on plant tumors. Phytopathology 32:25-45


53


This experiment has been designed to find out the phaseolin gene regulatory system using a reporter gene. The phaseolin promoter sequence was amplified by PCR from plasmid pTRA 321 (Zheng et al., 1995) using primers listed in Table A.1. pTRA 321 contains a 783bp phaseolin 5’-upstream promoter region, a 1990bp phaseolin coding region, and a 1100bp of 3’-downstream region. The PCR product should include 295bp of proximal promoter sequence, 77bp of 5’untranslated region and 72bp (24 amino acids) of N-terminal codons of phaseolin. Twenty-four codons of N-terminal of phaseolin coding sequence from the start codon was added to the GFP and GUS coding sequence as a signal sequence, which was deduced by the SignalP (www.cbs.dtu.dk/index.html) neural network method. The SignalP neural network method was developed by the Center for Biological Sequence Analysis at the Technical University of Denmark (Nielsen et al., 1997). A 31 amino acid sequence (MMRARVPLLLGLFLASLSASFTATSLREEE) from the start codon of phaseolin coding sequences was analyzed (Figure A.1). The SignalP program determines the signal peptide cleavage site based on changes of the values of C-score and S-core. The C-score of the cleavage site score is expected to be the highest at the amino acid position immediately after the cleavage site (+1) than all other positions. The C-score from the 31 amino acid of phaseolin was highest at position 25 (alanine), which is 0.75. The S-score or signal peptide score complements the results of C-score to determine the accurate cleavage site. The S-score is generally higher within the deduced signal peptide than outside of signal peptide. The S-score from the phaseolin signal peptide sequence analysis showed a higher score from position 1 to 24 and declined by three fold at position 25. Based on the C-score and S-score in SignalP analysis, the cleavage site of the signal sequence of phaseolin is expected to be in between position 24 (alanine) and 25 (thereonine) (Figure A.1). The deduced signal sequence of phaseolin contains a hydrophobic sequence characterized by a stretch of four consecutive leucines, which are surrounded by two additional leucines, two arginines, isoleucine, and valine. The deduced signal sequence of phaseolin was incorporated into the GUS and GFP coding sequence.

Restriction enzyme sites for *XbaI* and *HindIII* were incorporated into the forward primer of phaseolin promoter, and the *BamHI* site was incorporated into a reverse primer amplifying the 444bp phaseolin promoter region (Table 1, Table 1). All primers that were used in this experiment
were ordered from the Sigma. When the phaseolin promoter of 444bp was amplified by PCR from pTRA321, the resulting PCR product of 469bp would contain XbaI and HindIII sites at the end of coding strand and BamHI restriction sites at non-coding strand. This PCR product of phaseolin was digested with XbaI and BamHI restriction enzymes and then ligated into the XbaI/BamHI site of pBluscript KS- (Strategene®) (Figure A.2). The PCR was conducted using a Robocycler gradient 96 (Stratagene®). Plasmid DNA (20 ng) was mixed with PCR buffer (10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1 % Triton X-100, GibcoBRL)), 5 μM dNTPs (Sigma), 100 pM primers (Sigma), and 1 unit of Deep Vent Taq polymerase (BioLAB). The PCR reaction was conducted under conditions of pre-denaturation at 95 °C for 5 min for 1 cycle, and 30 cycles for denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min 30 sec, and elongation at 72 °C for 1 min. Restriction enzymes used in the DNA construction were purchased from NEB.

The terminator region of the phaseolin gene was also amplified by PCR from pTRA321. The 132 bp of terminator region was amplified from the nucleotide that immediately follows the stop codon to the end of poly A (A₃UA₂) sequence for transcription termination. The PstI restriction enzyme site was added to the forward primer of the phaseolin terminator, and KpnI, SpeI, and BglII sites were incorporated into the reverse primer (Table A.1,©). The resulting 166bp product was digested with PstI and KpnI and ligated into PstI and KpnI sites of pBluscript KS- (Figure A.2).

The GUS coding sequence of 1824bp from the start codon to stop codon was amplified by PCR from pBI121 (Chen et al., 2003). The forward primer of the GUS coding sequence contains a BglII restriction site. KDEL peptide sequences, the E.R. retention signal, which are not present in the original GUS coding sequence, were added in front of the termination codon of GUS by a reverse primer containing a NsiI restriction site (Table A.1,®). The resulting 1846bp PCR product has new BglII and NsiI restriction sites added. The PCR product was digested with BglII and NsiI, and then ligated to the BamHI and PstI sites of pBluscript KS- to obtain the construct phas promoter/GUS/phas ter (Figure A.2). Restriction enzyme sites BamHI and PstI were eliminated as a result of ligation because of sequence alternation (Figure A.2).

The GFP coding sequence, 726bp, also was amplified by PCR from the GFP419 vector (pLMNC94) (Mankin and Thompson, 2001). The HDEL peptide sequence of GFP, which was added to the original GFP coding sequence, was modified to KDEL. Histidine (H), which is
encoded by CAT in the original sequence, was converted to lysine (K) encoded by AAA. Restriction enzyme sites for \textit{Bgl}II and \textit{Nsi}I were incorporated into the forward and reverse primers, respectively (Table A.1,\textsuperscript{®}). The resulting 1203bp PCR product contains the GFP coding sequence, and the KDEL sequence was digested with \textit{Bgl}II and \textit{Nsi}I, and ligated to \textit{Bam}HI and \textit{Pst}I sites at pBluscript KS- containing the phaseolin promoter and terminator. \textit{Bam}HI and \textit{Pst}I sites in the KS- vector were also eliminated in the ligation step (Figure A.2).

Meanwhile, \textit{Sma}I, \textit{Pst}I, \textit{Eco}RI, and \textit{Eco}RV restriction sites were eliminated from the pBluscript KS- plasmid. KS- was cut by \textit{Sma}I and \textit{Eco}RV, and then blunt ends were re-ligated (Figure A.2). The construct phaseolin promoter/GFP/ter was then digested with \textit{Hind}III and \textit{Kpn}I, and then the \textit{Hind}III/\textit{Kpn}I fragment was ligated to \textit{Hind}III and \textit{Kpn}I sites of the KS-/-S~E vector (Figure A.2). The construct phaseolin promoter/GUS/terminator was digested with \textit{Xba}I and \textit{Bgl}II, and then the 2kbp \textit{Xba}I/\textit{Bgl}II fragment was ligated to \textit{Xba}I and \textit{Bam}HI sites of KS-/-S~E (Figure A.2).

NPTII with \textit{tml} promoter and terminator, 987bp, was also amplified by PCR to be incorporated with GFP and GUS cassette in KS-/-S~E. The promoter region for NPTII was selected, and the terminator region was selected up to 21bp after the $A_3U_A_2$ poly A region. Restriction enzyme sites for \textit{Sac}II and \textit{Sal}I were added to the forward primer of the NPTII gene, and an \textit{Xba}I site was added to the reverse primer, respectively (Table A.1,\textsuperscript{®}). The resulting 1014bp PCR product was digested with \textit{Sac}II and \textit{Xba}I, and the \textit{Sac}II/\textit{Xba}I fragment was ligated to \textit{Sac}II and \textit{Xba}I sites of KS-/-S~E with a GUS or GFP cassette to obtain KS-/-S~E containing NPTII, GUS cassette and GFP cassette (Figure A.2). The construct NPTII/GUS/GFP was then digested with \textit{Sal}I and \textit{Spe}I, and the 4642bp pg \textit{Sal}I/\textit{Spe}I fragment was ligated into \textit{Sal}I and \textit{Spe}I sites of the pCAMBIA 1380 vector (Figure A.2). In this construct, NPTII is adjacent to the left border, so that selection of transformed plant tissues for kanamycin resistance will ensure the transfer of the T-DNA all the way from the right to the left border. The GFP coding sequence was cloned into the pCAMBIA 1301 vector for the first step of this experiment (Figure A.3).
Figure A.1. Signal peptide analysis using signal P, neural network method (www.cbs.dtu.dk/index.html).

<table>
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Table A.1. Primer list for gene cloning

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<td><strong>1</strong> Phaseolin Promoter</td>
<td>5’CTAG TCTAGA AAGCTT GATCAAGATCGCCGCG3’&lt;br&gt;5’CGC GGATCC GCGAAATGAGGCAGAA3’&lt;br&gt;5’CTAG TCTAGA AAGCTT GATCAAGATCGCCGCG3’&lt;br&gt;5’CGC GGATCC GCGAAATGAGGCAGAA3’</td>
<td>phs 5’-promoter&lt;br&gt;forward primer&lt;br&gt;phs 3’-promoter&lt;br&gt;reverse primer</td>
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<td><strong>2</strong> Phasolin Terminator</td>
<td>5’ GAAAAA CTGCAG ATAAATGAACTAAAATGCA3’&lt;br&gt;5’ CCGGC GGTACC ACTAGT AGATCT KpnI SpeI BglII ATCATACATCCCTTTGTTTA</td>
<td>phs 5’-terminator&lt;br&gt;forward Primer&lt;br&gt;phs 3’-terminator&lt;br&gt;reverse primer</td>
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<tr>
<td><strong>3</strong> GUS coding sequence</td>
<td>5’GCCGA AGATCT ATGGTTACGTCTTGAGAA3’&lt;br&gt;5’CGCCA ATGCAT NsiI TCAAAGCTCATCTT TTGTGTGCTCCCTGCT3’&lt;br&gt;5’GCCGA AGATCT ATGGTTACGTCTTGAGAA3’&lt;br&gt;5’CGCCA ATGCAT NsiI TCAAAGCTCATCTT TTGTGTGCTCCCTGCT3’</td>
<td>gus 5’-coding&lt;br&gt;forward primer&lt;br&gt;gus 3’-coding&lt;br&gt;reverse primer</td>
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<td><strong>4</strong> GFP coding sequence</td>
<td>5’GCCGA AGATCT AGTAAAGGAGAAGAACTT3’&lt;br&gt;5’CGCCA ATGCAT NsiI TTAAAGCTCATTT TTGTATAGTTCTCCGCT3’&lt;br&gt;5’GCCGA AGATCT ATGGTTACGTCTTGAGAA3’&lt;br&gt;5’CGCCA ATGCAT NsiI TCAAAGCTCATCTT TTGTGTGCTCCCTGCT3’</td>
<td>gfp 5’-coding&lt;br&gt;forward primer&lt;br&gt;gfp 3’-coding&lt;br&gt;reverse primer</td>
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<td><strong>5</strong> NPTII gene (tml promoter and terminator)</td>
<td>5’GCTCC CCGCGG GTCGAC SacII SalI AATTATGAGCAGATTTTTGG3’&lt;br&gt;5’GCCGA AGATCT ATGGTTACGTCTTGAGAA3’&lt;br&gt;5’GCCGA AGATCT ATGGTTACGTCTTGAGAA3’&lt;br&gt;5’GCCGA AGATCT ATGGTTACGTCTTGAGAA3’</td>
<td>tml promoter&lt;br&gt;forward primer&lt;br&gt;tml terminator&lt;br&gt;reverse primer</td>
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Figure A.2. Scheme of phaseolin promoter and terminator gene cassette construction
Figure A.3. Schematic representation of the binary plasmid vector pCAMBIA1301 (A) and pCMABIA1301 with GFP419 (B)
VITA

Sunjung Park was born in Chon-Ju, Korea, on March 1, 1973. Her hometown is renowned for delicious food and famous scholars. She graduated from Seoul Women’s University, the Department of Horticultural Science, with a Bachelor of Science degree in agriculture in 1997. She enrolled in the Department of Horticultural Science, Graduate School of Natural Resources, Korea University, in March 1997. She worked as a teaching assistant for a year while working toward the Master of Science degree. She graduated with the master’s degree in horticultural science in 1999. The master’s thesis is entitled “The Effect of Chitosan on Regeneration and Transformation of Chrysanthemum (Dendranthema grandiflora).

After the master’s degree, she worked as a research scientist at Natural Resources Institute, Korea University from December 1998 to August 1999, at Life and Environment Science Laboratory of Kumho Petrochemical Co., Ltd., from February to July 2000, and in the Laboratory of Agricultural Postharvest Preservation of the Biosystech Research Institute, Biosystech Co., Ltd. from July 2000 to July 2001. The topics of her research were cDNA sequence from leaves of hibiscus (Hibiscus syriacus, National Flower of Korea), overexpression of the Pra2 gene in E. coli, and postharvest physiology to prolong the vase life of cut flowers by the MA (modified atmosphere) package method, respectively.

Sunjung enrolled at Louisiana State University as a non-maticulating student in August 2001 and took several biochemistry courses. She advanced to the LSU Graduate School in January 2002 and pursued the master and doctoral degrees in the Department of Plant Pathology and Crop Physiology. She worked as a graduate research assistant in the laboratory of Dr. Norimoto Murai.