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根毛農桿菌對黃豆之基因轉形研究

Agrobacterium rhizogenes-mediated

soybean gene transformation



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本論文係郭致均君 (R95B47104) 在國立臺灣大學微生物與生化學研究所完成之碩士學位論文，於民國九十七年七月九日承下列考試委員審查通過及口試及格，特此證明



口試委員：

(簽名)

(指導教授)

系主任、所長

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Abstract

Agrobacterium rhizogenes-mediated soybean multiple genes transformation was investigated in this study. Soybean Tainan #2 was selected as the material for the highest germination rate of sterile plants and high number of tissue segments for root induction. Two reporter genes were used for co-transformation study, *gfp* from pCAMBIA 1302 and *gus* from pCAMBIA 1201. Transformation efficiency of pCAMBIA 1201 and pCAMBIA 1302 was 5.5×10^5 and 8.25×10^5 for wild-type *A. rhizogenes*, and efficiency lowered to an average of 9.5×10^2 for transforming the second vector into pre-transformed *A. rhizogenes*. Co-transformation was carried out either by two binary vectors in one *A. rhizogenes* (2BV) or two binary vectors in separated *A. rhizogenes* and infecting soybean at the same time (2AR). Root induction rate from cotyledon was 48.0% for WT *A. rhizogenes*, 39.6% for 2BV, and 68.2% for 2AR, independently. We gained two hairy root clones after liquid culture, which both expressing GFP but not GUS. As a result, soybean hairy roots are successfully induced by *A. rhizogenes* transformants, and foreign gene is successfully transformed and expressed in hairy roots. Soybean multiple genes co-transformation is not yet fully constructed and still needs much effort to get a further understanding and for further applications.

Keywords: *A. rhizogenes*, soybean, hairy roots, GFP, GUS

中文摘要

本論文在進行根毛農桿菌對黃豆之多重基因共轉形之探討。黃豆臺南二號有最高之無菌植株發芽率且可用於農桿菌感染之組織材料數高而被選作後續研究材料。選用兩個廣泛使用之報導蛋白質進行黃豆之多基因共轉形：帶有綠色螢光蛋白質 (GFP) 之質體 pCAMBIA 1302 及帶有 β -葡萄糖酸苷酶 (GUS) 之質體 pCAMBIA 1201。首先探討對野生型農桿菌進行單一質體轉形時，pCAMBIA 1201 轉形效率為 5.5×10^5 ，pCAMBIA 1302 效率為 8.25×10^5 ；對已帶有一質體之農桿菌轉形平均轉形效率則降低至 9.5×10^2 。黃豆多基因共轉形操作分為單一菌體同時攜帶兩質體 (2BV) 或兩質體分別位於兩菌體來同時感染黃豆 (2AR)。黃豆子葉之根狀組織誘導率在野生型農桿菌及 2BV、2AR 組別分別為 48.0%、39.6% 及 68.2%。經過液態搖瓶培養後，得到的兩株毛狀根都會表現 GFP 而不表現 GUS。本研究已初步建立黃豆之毛狀根誘導系統、將外源基因送入黃豆染色體並在毛狀根中成功表現出異源蛋白質綠色螢光蛋白質。黃豆之多基因共轉形系統仍未完整建立，後續仍需要許多努力以對其有完整之了解並得以應用。

關鍵字：根毛農桿菌、黃豆、毛狀根、綠色螢光蛋白質、 β -葡萄糖酸苷酶

Abbreviations

<i>A. rhizogenes</i>	<i>Agrobacterium rhizogenes</i>
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
CaMV 35S	Cauliflower mosaic viral 35S
dNTP	Deoxyribonucleotide triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetraacetic acid
HPLC	High performance liquid chromatography
LB	Left Border
MS medium	Murashige and Skoog basal medium
<i>nos</i>	Nopaline synthase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RB	Right Border
Ri	Root inducing
SDS	Sodium dodecyl sulfate
T-DNA	Transfer DNA
Ti	Tumor-inducing
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

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<i>Agrobacterium rhizogenes</i>	根毛農桿菌
<i>Agrobacterium tumerfaciens</i>	根瘤農桿菌
callus	植物禦傷組織
CaMV 35S promoter	花椰菜嵌紋病毒 35S 啟動子
constitutive promoter	持續性啟動子
electrophoresis	電穿孔法
elicitor	誘引劑
GFP	綠色螢光蛋白質
GUS	β -葡萄糖酸苷酶
hairy roots	毛狀根
inducible promoter	誘導性啟動子
PCR	聚合酶連鎖反應
primer	引子
promoter	啟動子
reporter gene	報導基因
restriction enzyme	限制酶
Western blotting	西方墨漬法



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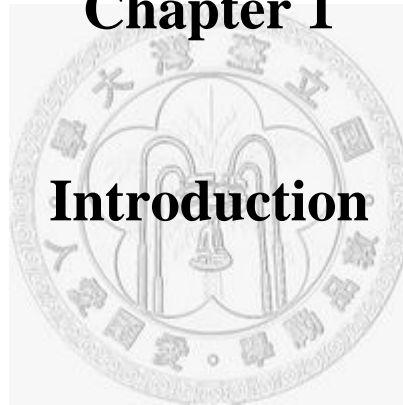


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Chapter 1

Introduction



1.1 Transgenic plant cell cultures

1.1.1 Plant cell cultures

Plants have been an important source of food for the past centuries, on top of that, plants are also sources of medical compounds. Many pharmaceuticals are derived from plant secondary metabolites, such as digitalis, L-DOPA, morphine, codeine, and reserpine. Anticancer drugs such as vincristine, vinblastine, and taxol are also derived from plants (Dicosmo and Misawa, 1995). The use of whole plants for the synthesis of recombinant proteins has received a great deal of attention because of advantages in economy, scalability and safety compared with traditional microbial and mammalian production systems. However, production systems that use whole plants have several disadvantages, including the needs of long periods and large areas for whole plant cultivation; the lack of several intrinsic benefits of cultured cells such as precise control over growth conditions, batch-to-batch product consistency, and the ability to produce recombinant proteins in compliance with good manufacturing practice (Hellwig et al., 2004).

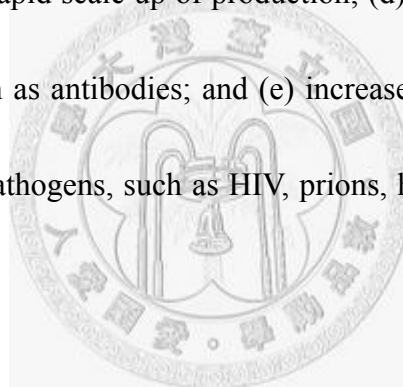
The term “plant cell cultures” means the propagation of any plant-derived cell tissue in gently agitated liquid culture, and they can be classified as unorganized cultures (e.g. callus, suspension, and the protoplast culture) and organized cultures (e.g. hairy roots or embryo cultures) depending on the tissue source and level of

differentiation (James and Lee, 2001). For the past three decades, significant effort was invested in producing high-value secondary metabolites such as shikonin (Yamamura et al., 2003), taxol (Hezari and Croteau, 1997; Hezari et al., 1997), and gensenoside (Palazon et al., 2003), via plant cell cultures.

In recent years, the number of recombinant proteins used for therapeutic applications has increased dramatically. These demands have driven the development of a variety of improvements in protein expression technology in a wide range of expression systems (Andersen and Krummen, 2002). Plant cells, like microbes, can be maintained in simple, synthetic media, but like animal cells they can synthesize complex multimeric proteins and glycoproteins. Plant cell cultures combine the merits of whole-plant systems with those of microbial and animal cell cultures, and already have an established track record for the production of valuable therapeutic secondary metabolites (Hellwig et al., 2004). The first recombinant protein produced in plant cells was reported nearly 30 years ago (Sijmons et al., 1990), since then, over 20 different recombinant proteins have been produced in plant cell cultures, including antibodies, enzymes, hormones, growth factors and cytokines (**Table 1**). Although most interest has been focused on microbial and animal cell cultures during the past 15 to 20 years, plants and plant cells are now considered as viable and competitive expression systems for

large-scale protein production (Doran, 2000), and >100 recombinant proteins have now been produced in a range of different species (Twyman et al., 2005).

Genetically engineered transgenic plant cell cultures have many advantages as sources of proteins compared with human or animal fluids/tissues, recombinant microbes, transfected animal cell lines, or transgenic animals. Among these are (a) production of raw material on an agricultural scale at low cost with the possibility of using the edible plant material directly; (b) reduced capitalization costs relative to fermentation methods; (c) rapid scale up of production; (d) correct eukaryotic assembly of multimeric proteins such as antibodies; and (e) increased safety, since plants do not serve as hosts for human pathogens, such as HIV, prions, hepatitis viruses, etc (Larrick et al., 2001).



1.1.2 *Agrobacterium*-mediated gene transformation

Plant transformation has become a core research tool in plant biology and a practical tool for cultivar improvement (Birch, 1997). There are two classes of plant transformation technologies: 'non-natural' or *in vitro* methods, and 'natural' methodologies. Among the *in vitro* technologies are microinjection, direct DNA uptake into protoplasts (with or without electroporation), and microprojectile (or particle) bombardment. 'Natural' technologies include the use of viral vectors (that will result in

transient but not stable transformation) and *Agrobacterium tumefaciens* T-DNA mediated transformation (Gelvin, 1998). The non-natural methods can normally express the transformed genes in as little as few hours post introduction, which are suitable methods for studying genetic regulatory networks such as promoter activity and RNA silencing (Hellens et al., 2005). These methods result in transient expressing and the foreign genes introduced are frequently unstable following propagation, leading to difficulties for gaining regenerated transgenic plants. For detailed examination of introduced genes expression pattern, stably transformed plants are needed. Hence, natural methods, typically *Agrobacterium*-mediated transformation, is required (Finnegan and McElroy, 1994).

Agrobacterium tumefaciens is a kind of Gram-negative soil bacterium which is also a plant pathogen. It genetically transforms its host by transferring a well-defined DNA segment from its tumor-inducing (Ti) plasmid to the host-cell genome. The integration and expression of T-DNA genes *ipt* (*tmr*) and *iaaM* (*tms*) result in the overproduction of auxin and cytokinin, leading to the tumor phenotype of crown gall disease (Binns, 2002).

Agrobacterium rhizogenes is closely related to *Agrobacterium tumefaciens* in terms of physiology and DNA homology, which also infect at plant wound sites and transfer T-DNA from the root-inducing (Ri) plasmid to plant genome. Integration and

expression of the root loci (*rol*) genes and the *aux* genes leads to the development of the hairy-root phenotype and synthesis of novel low-molecular-weight compounds called opines (Cho et al., 2000).

The *vir* region, located on the *Agrobacterium* Ti/Ri plasmid, encodes most of the bacterial virulence (Vir) proteins used by the bacterium to produce its T-DNA and to deliver it into the plant cell. The T-DNA segment is a specific element delimited by two 25-bp direct repeats called left border (LB) and right border (RB) on the Ti/Ri plasmid.

The genetic transformation process describes as follows. The transformation process begins with the bacterium–plant attachment (**Figure 1**; step 1) which is facilitated by chromosomal virulence protein A and B (ChvA, ChvB). Plant wound signals such as acetosyringon binds to the transmembrane VirA/VirG protein complex and triggers the expression of *vir* region on Ti/Ri plasmid (**Figure 1**; steps 2 and 3). A single-stranded (ss) T-DNA molecule (T-strand) (**Figure 1**; step 4) is then produced by the combined action of the bacterial VirC, VirD1, and VirD2 proteins. The ssT-DNA then exported into the host cell by a type IV secretion system formed by VirD4 and several proteins in the VirB family (**Figure 1**; step 5). The full length of the T-strand molecule is coated with numerous VirE2 molecules and nicked with VirD2 at both ends, these molecules confer to the T-DNA the structure and protection needed for its travel to the host-cell nucleus (**Figure 1**; step 6). It is mainly during the last steps of the transformation

process: transport through the cytoplasm (**Figure 1**; step 6), nuclear import (**Figure 1**; step 7), intranuclear transport (**Figure 1**; step 8), T-DNA uncoating (**Figure 1**; step 9) and integration (**Figure 1**; step 10) (Tzfira and Citovsky, 2006).

A. rhizogenes not only is able to transfer the T-DNA on its Ri plasmid *in cis*, but also able of transferring the T-DNA on a small, autonomous binary vectors *in trans*, thereby enabling the production of transgenic plants containing other foreign genes carried on a second plasmid. This method has been used to produce transgenic plants in several species (Christey, 1997).

1.1.3 Hairy root cultures

Hairy roots are formed from plants infected by *A. rhizogenes* at wound site. Hairy-root cultures grow rapidly, show plagiotropic root growth and are highly branched on hormone-free medium (Cho et al., 2000).

Many plant secondary metabolites of interest are accumulated in roots, and they may not be occurred in undifferentiated cell. Hairy root cultures, in the differentiation degree of tissue, have been proposed as an alternative method of producing plant secondary metabolites. Not only because they have genetic and biochemical stability, the capability to synthesize secondary products at levels comparable to that of the original plants, but also the capability to synthesize for a longer period compared to

original plant roots (Chang et al., 2005). A number of plant species including many medicinal plants have been successfully transformed with *A. rhizogenes* (Sevón and Oksman-Caldentey, 2002). Valuable metabolites were produced by hairy roots of several plant species (**Table 2**).

1.2 Multiple genes co-expression

Over the past few years, there has been a growing realization that metabolic pathways must be studied in the context of the whole cell rather than at the single pathway level, and that even the simplest modifications can send ripples throughout the entire system. Attention has therefore shifted away from reductionist, single-gene engineering strategies and towards more complex approaches involving the simultaneous overexpression and/or suppression of multiple genes (Capell and Christou, 2004). Therefore, the most important value of multiple genes co-expression system might be the use in metabolic engineering.

Multiple genes co-expression system can also be used in multiple proteins production. Heterologous gene expression can lead to the production of nonfunctional target proteins, this is often due to the absence of cofactors or post-translational modifications required for function, stability or folding. Coexpression of multiple genes in such as the members of a stable multiprotein complex or a protein with a chaperone,

can in many cases alleviate these problems (Bross et al., 1993; Li et al., 1997; Rivas et al., 2005). Co-expression system can also be used in expression of two functional related proteins, such as protein Der p 1 and Der p 2 which can be used in immunotherapy procedures (Taketomi et al., 2006).

1.3 Reporter proteins

For the study of multiple genes co-expression in plant, efficient and easy-to-use reporter proteins are required. Commonly used reporter proteins in plant gene transformation usually involve fluorescent and luminescent proteins; examples include the green fluorescent protein (GFP) and the enzyme luciferase, which catalyzes a reaction with a luciferin to produce light. Another common reporter used in plant is β -glucuronidase (GUS) which can be easily assayed within fresh cells or tissues. Our lab had successfully expressed both GFP and GUS in tobacco hairy roots (Liu, 2004; Chiang, 2006), the two reporter proteins were chosen for this study.

The green fluorescent protein (GFP) isolated from *Aequorea victoria mgfp5* has been used as a reporter protein in many studies. It is a protein of 238 amino acid residues with a molecular weight of 27 kDa. GFP has a major absorbance wavelength at 395 nm and a minor one at 475 nm. The major emission wavelength is at 508 nm when excited at 395 nm (Heim and Tsien, 1996). The intrinsic fluorescence of the GFP allows

non-destructive, in-vivo imaging of events within cells (Boevink et al., 1999). The mature purified protein is highly stable; remain fluorescent up to 65°C, pH 11, 1% sodium dodecyl sulphate (SDS) or 6M guanidinium chloride. GFP can also function as a protein tag since it tolerates both N- and C-terminal fusions to a variety of proteins and can be expressed in a broad range of host organisms (Cubitt et al., 1995).

The β -glucuronidase (GUS) is from *Escherichia coli gusA*, with a mutation at amino acid 358 from N to Q to avoid N-linked glycosylation. Furthermore, an intron was inserted inside the coding sequence to ensure that expression of glucuronidase activity is derived from eukaryotic cells, not from expression by residual *Agrobacterium* cells (Ohta et al., 1990). It is a protein with 620 amino acid residues with molecular weight of 68 kDa and appears to function as a tetramer. It is very stable, and will tolerate many detergents, widely varying ionic conditions, and general abuse. It is most active in the presence of reducing agents such as β -mercaptoethanol or DTT. It may be assayed at any physiological pH, with an optimum between 5.2 and 8.0 (Jefferson et al., 1987).

1.4 Soybean

The *Fabaceae* (or *Leguminosae*) commonly known as the bean family or pea family, is the third largest family of flowering plants with over 19,400 species, and

second only to *Poaceae* (grasses) in terms of agricultural and economic importance (Wojciechowski et al., 2006). They are important components of natural ecosystems and play an essential role in the global nitrogen cycle by establishing symbiotic interactions with soil bacteria of the *Rhizobiaceae* family to convert atmospheric nitrogen into ammonia and amino acids, which later can be used by other organisms. Legumes include a large number of domesticated species harvested as crops providing the world's largest source of vegetable protein as well as for oils, fiber, fuel, fertilizers, timber, medicinals, chemicals, and horticultural varieties. In addition, the family includes several species studied as genetic and genomic model systems (e.g., pea, *Pisum sativum*, barrel medic, *Medicago truncatula*, and trefoil, *Lotus corniculatus*).

Soybean (*Glycine max*) is one of the most important crop plants of the world market in oil crops and serving as an important source of protein for both human consumption and as fodder. The seed of soybean contains 35-40% protein, serving as a potential source of recombinant protein production.

Soybean contains the highest concentration of isoflavones among the foods consumed by human. These soy isoflavones (e.g., daidzein and genistein) are implicated in some health-enhancing properties such as the prevention of certain cancers (Cappelletti et al., 2000; Miura et al., 2002; Ravindranath et al., 2004), lowering the risk of cardiovascular diseases (Anthony et al., 1996; Goodman-Gruen and Kritz-Silverstein,

2001), and an improvement of bone health (Cotter and Cashman, 2003; Weaver and Cheong, 2005).

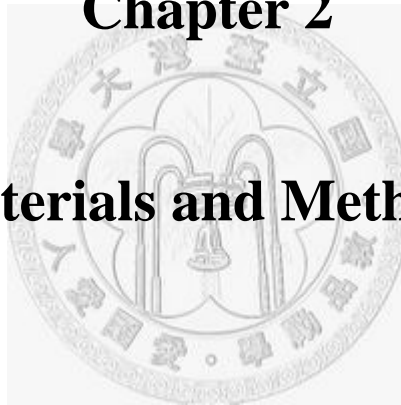
1.5 Research aim

With considerably high protein content, soybean is considered a suitable plant host for expressing recombinant proteins. Combining the capability of hairy roots to grow rapidly in a hormone free liquid culture, soybean hairy roots is thought to be an ideal system for metabolic engineering and a valuable material for specialty chemical production. Previous studies on soybean hairy roots focused on high efficiency hairy roots induction and using soybean hairy roots as a model for soybean root defense system (Cho et al., 2000; Xiang et al., 2005). A more recent study attempted to modify phenolic metabolic pathways in soybean hairy roots by transforming two key enzymes into soybean separately, it resulted in a down regulation due to homology-dependent gene silencing (Lozovaya et al., 2007).

In this study, we attempt to construct an *A. rhizogenes*-mediated soybean hairy roots induction system. Further, using soybean hairy roots as an expression system for studying the efficiency of multiple genes co-expression. This co-expression system is thought to be a useful tool for soybean isoflavone synthetic pathway research.

Chapter 2

Materials and Methods



2.1 Plasmid constructions

2.1.1 Binary vectors

Binary vectors pCAMBIA 1201, pCAMBIA 1301, and pCAMBIA 1302 were purchased from CAMBIA (Canberra, Australia). Vector pCAMBIA 1201 and pCAMBIA 1301 both have *gusA* in the T-DNA region, with chloramphenicol and kanamycin resistance genes on the backbone, respectively. Vector pCAMBIA 1302 contains *mgfp5* and kanamycin resistance gene (Figure 2).

For vector conservation and high-copy production of the vector, all vectors were transformed by heat shock into *E. coli* JM-109 competent cells (Yeastern Biotech, Taipei, Taiwan). Binary vectors were then purified from an over-night incubated *E. coli* cultures. Vectors were then digested with respective restriction enzyme, the digestion products were resolved on 1.2% agarose gel, and the digested fragments were confirmed after ethidium bromide (EtBr) staining.

Vector pCAMBIA 1201 and pCAMBIA 1302 were used directly without modification in *A. rhizogenes* transformation and then *A. rhizogenes*-mediated soybean co-transformation study. The two vectors were transformed into *A. rhizogenes* individually or simultaneously (the *A. rhizogenes* transformation method will be described later), resulting in *A. rhizogenes* containing each one, respectively, or both of the vectors. *A. rhizogenes* 1201 and *A. rhizogenes* 1302 were used together for

co-transformation of soybean (the soybean transformation method will be described later), this experiment construction was named 2AR for two strains of transformed *A. rhizogenes* inducing hairy roots simultaneously (**Figure 3**). The *A. rhizogenes* containing both pCAMBIA 1201 and pCAMBIA 1302 was also used in soybean co-transformation study and named as 2BV for one *A. rhizogenes* containing two binary vectors (**Figure 4**).

2.1.2 Two reporter genes in one T-DNA region

The *gfp* gene containing the upstream CaMV35S promoter and downstream *nos* terminator was cloned from binary vector pCAMBIA 1302. The forward primer contained a restriction site *Bam*HI, and the reverse primer contained a *Hind*III site (This primer-pair was designed according to NCBI nucleotide database AF234298). The 1679 base pairs target sequence was amplified by PCR reaction.

F-35SGFPNOS: 5'-TTTGGATCCTTTCCGCCTTCAGTTTAGCTTCATGGAGTCAAAGATTCA-3'

R-35SGFPNOS: 5'-TTTAAGCTTTTTTCTTTTCTCTTAGGTTTACCCGCCAATATATCCTGTC-3'

The composition of PCR reaction solution:

	Volume (μ l)	Final concentration
Template: pCAMBIA 1302	1.0	< 0.1 μ g
4 mM dNTP	2.5	400 μ M each
10X Reaction buffer	2.5	1X
10 μ M primers	2.5	1 μ M
Super-Therm Polymerase	0.5	2.5 U
Double distilled H ₂ O	13.5	-
Total volume	25.0	-

PCR amplification was performed for 30 cycles in a thermal cycler (PCR Px2 Thermal Cycler Personal, Thermo Fisher Scientific Inc., Waltham, MA, USA). The PCR program for *gfp* was: 95°C, 5 min for denaturation; 94°C, 1 min, 55°C, 1 min, 72°C, 2 min for 30 cycles; 72°C 15 min and paused at 4°C.

The PCR product and vector pCAMBIA 1301 were both digested by restriction enzyme *Bam*HI and *Hind*III, the two digested fragments were then ligated by T4 DNA ligase according to the user manual. The ligation product was resolved on 1.2% agarose gel, followed by EtBr staining to check the ligated vector. The ligated vector was transformed into *E. coli* JM-109 by heat shock method, then purified from an over-night incubated culture and sent for DNA sequencing (Tri-I Biotech Co., Taipei, Taiwan). This newly constructed binary vector was named plasmid 2RG for containing two reporter genes, *gfp* and *gus*, in a single T-DNA region (**Figure 5**).

2.1.3 Two T-DNA regions in one binary vector

The whole T-DNA region between the left-border and right-border of binary vector pCAMBIA 1302 was cloned by PCR amplification with primers containing restriction site *Sac*II (This primer pair was designed according to NCBI nucleotide database AF234298). The 4336 base pairs target sequence was amplified by ExSel High Fidelity DNA Polymerase (JRM Holdings, Kent, UK).

F-TDNAGFP: 5'-TTTTCCGCGGTGGCAGGATATATTGTGGTGTAACAAATTGACGCTTAG-3'

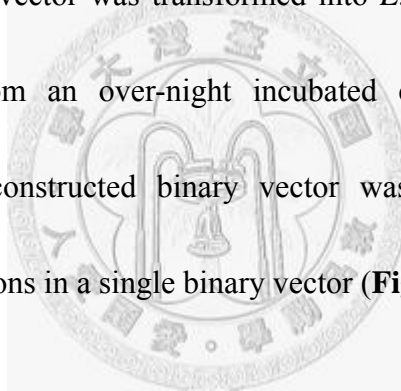
R-TDNAGFP: 5'-TTTTCCGCGGTAAACGCTCTTTTCTCTTAGGTTTACCCGCCAATATATC-3'

The composition of PCR reaction solution:

	Volume (μ l)	Final concentration
Template: pCAMBIA 1302	1.0	< 0.1 μ g
4 mM dNTP	2.5	400 μ M each
10X Reaction buffer	2.5	1X
10 μ M primers	1.0	0.4 μ M
ExSel Polymerase	1.0	5 U
Double distilled H ₂ O	16.0	-
Total volume	25.0	-

PCR amplification was performed for 30 cycles in a thermal cycler. The PCR program for the whole T-DNA region containing *gfp* was: 95°C, 5 min for denaturation; 94°C, 1 min, 65°C, 30 sec, 72°C, 5 min for 30 cycles; 72°C 15 min and paused at 4°C.

The PCR product and the vector pCAMBIA 1301 were digested by restriction enzyme *Sac*II, and then both dephosphorylated with alkaline phosphatase according to the user manual. The two fragments were then ligated with T4 DNA ligase, and the ligated vector was resolved on 1.2% agarose gel followed by EtBr staining to check the ligated vector. The ligated vector was transformed into *E. coli* JM-109 by heat shock method, then purified from an over-night incubated culture and sent for DNA sequencing. This newly constructed binary vector was named plasmid 2TD for containing two T-DNA regions in a single binary vector (**Figure 6**).



2.2 Bacteria transformation methods

2.2.1 Heat shock method for *E. coli* transformation

E. coli heat shock transformation method was carried out according to the user manual, describe as follows. Prepare Luria-Bertani (LB) plate containing antibiotics 50 $\mu\text{g ml}^{-1}$ kanamycin, and 50 $\mu\text{g ml}^{-1}$ chloramphenicol. Competent cells were thawed from -70°C storage by holding under running tap water for about 20 seconds until half thawed. Two μl of pre-chilled vector or ligation product was added to 100 μl competent

cells, mixed by vortex then placed the tube on ice for 5 minutes. Cells were heat shocked for 45 to 90 seconds in a water bath at exactly 42°C, then immediately placed back on ice for 10 minutes. Cell recovery was carried out by incubating the competent cells in 2 ml LB medium without antibiotics in an orbital shaker at 150 rpm, 37°C for 1 hour. Cells were plated on LB plate containing respective antibiotics and incubate at 37°C overnight. Finally, single colony was selected and conserved.

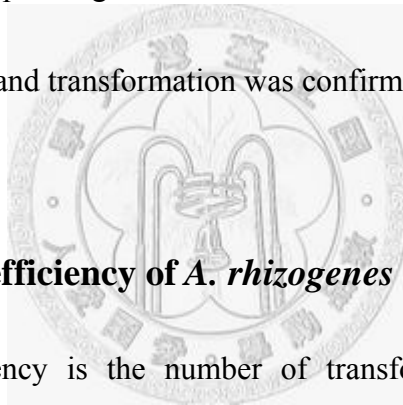
2.2.2 Electroporation for *A. rhizogenes* transformation

2.2.2.1 Preparation of *A. rhizogenes* cells

A. rhizogenes 1610 wild-type was cultured in a Hinton flask with 100 ml LB medium in an orbital shaker at 125 rpm, 30°C. *A. rhizogenes* was cultured until the cells grown to the middle-log phase with OD₆₀₀ at 0.6-0.8, the cells were collected by centrifugation at 1000x g, 4°C. The pellet was re-suspended by 100 ml pre-chilled sterile double distilled water, and then collected by centrifugation again. This step was repeated four times and decreasing the re-suspension volume by 50 ml, 25 ml, 10 ml, then 5 ml. The pellet was finally re-suspended in 1 ml pre-chilled sterile double distilled water, placed on ice, and the *A. rhizogenes* cells were prepared.

2.2.2.2 Electroporation of *A. rhizogenes*

Two micro liter purified binary vector (~200 ng) was mixed with 48 μ l *A. rhizogenes* cells, the mixture was then placed in a pre-chilled sterile Pulser Cuvette (Bridge Tech., Palo Alto, CA, USA) with a 1 mm electrode gap. The electroporation was performed at 25 μ F, 200 Ω , 2.5 kV with a Gene Pulser (Bio-Rad Laboratories Inc., Hercules, CA, USA). The transformed *A. rhizogenes* was then recovered by cultivating 1 hour in an orbital shaker at 100 rpm, 30°C. After cultivation, the cells were plated on LB plate containing corresponding antibiotics and incubated at 30°C for 48 hours. Single colony was selected and transformation was confirmed by PCR described later.



2.2.3 Transformation efficiency of *A. rhizogenes*

Transformation efficiency is the number of transformed cells (transformants) generated by 1 μ g of plasmid DNA in a transformation reaction. For wild-type *A. rhizogenes* single binary vector transformation, binary vector pCAMBIA 1201 was first transformed into wild-type *A. rhizogenes*. The transformed cells were series diluted and plated on LBC plate. After 48 hours of cultivation, colonies on each plate were counted and the transformation efficiency was calculated by dividing the number of colonies on plate by micro grams of plasmid DNA plated. For pCAMBIA 1302, the same process was carried out by using LBK plate instead of LBC plate.

Successfully transformed *A. rhizogenes* 1201 and *A. rhizogenes* 1302 were then used as recipient strains for the transformation efficiency test of the second binary vector. The transformed cells were planted on LBCK plate, and the transformation efficiency was calculated as mentioned above.

Finally, both pCAMBIA 1201 and pCAMBIA 1302 were transformed simultaneously into wild-type *A. rhizogenes* for the calculation of transformation efficiency of two plasmids. The transformed cells were planted on LBCK plate, and the transformation efficiency was calculated as mentioned above.

2.2.4 PCR confirmation

Transformants of *A. rhizogenes* were confirmed by colony PCR with respective primer pairs specific for *gfp* and *gus* (Primer pairs were designed according to NCBI nucleotide database AF234293 and AF234298).

F-*gfp*: 5'-GAGAGAACACGGGGGACTCTTGAC-3'

R-*gfp*: 5'-ACTTTATTGCCAAATGTTTGAACG-3'

F-*gus*: 5'-TGATGATGATAGTTACAGAACCGACGA-3'

R-*gus*: 5'-CAGTCAACAGACGCGTGGTTACAGTC-3'

The composition of PCR reaction solution:

	Volume (μ l)	Final concentration
<i>A. rhizogenes</i>	-	-
4 mM dNTP	2.5	400 μ M each
10X Reaction buffer	2.5	1X
10 μ M primers	2.5	1 μ M
Super-Therm Polymerase	0.5	2.5 U
Double distilled H ₂ O	14.5	-
Total volume	25	-

PCR amplification was performed for 30 cycles in PCR Px2 Thermal Cycler Personal (Thermo Fisher Scientific Inc., Waltham, MA, USA). The PCR program for both *gfp* and *gus* was: 95°C, 5 min for denaturation; 94°C, 1 min, 60°C, 30 sec, 72°C, 1 min for 30 cycles; 72°C 15 min and paused at 4°C.

PCR products were then resolved on a 1.2% agarose gel, followed by EtBr staining to check the 638 base pairs *gus* fragment and 833base pairs *gfp* fragment.

2.3 Establishment of hairy root clones

2.3.1 Soybean sterile plants germination

Six genotypes of soybean were used in this study to select the soybean as the suitable material for *A. rhizogenes*-mediated hairy roots induction. Soybean Tainan #2,

Tainan #5, Tainan #7, and Kaohsiung #5 were kindly provided by Tainan District Agricultural Research & Extension Station (Tainan, Taiwan). Soybean BS90T60 Minnesota was kindly provided by American Soybean Association IM (Taipei, Taiwan). In addition, one commercial non-transgenic soybean was also used (**Figure 8 (A)**).

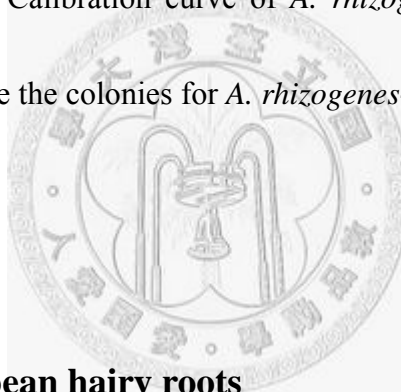
The surface-sterilization solution was prepared by adding 3 ml commercial hydrogen peroxide (~35%) into 21 ml commercial ethanol solution (~97%) in a sterile 50 ml tube. Soybean seeds were dipped in the solution and surface-sterilized by inverting the tube for 2 minutes. The seeds were washed with sterilized distilled water for 10 times, and then dipped in sterilized distilled water for 1 hour to remove residue detergents. Sterilized seeds were placed on sterilized vermiculite, and sterilized distilled water was added to moisturize the vermiculite. Seeds were germinated in the culture box with a cycle of illumination at 28°C for 12 hours, and at 25°C for another 12 hours in the dark.

2.3.2 Preparation of *A. rhizogenes* for induction of soybean hairy roots

A. rhizogenes harboring the binary vector was cultured overnight in LB medium containing corresponding antibiotics in an orbital shaker at 125 rpm, 30°C. Subsequently, 20 µl of the *A. rhizogenes* culture was inoculated to 2 ml fresh LB medium containing antibiotics, *A. rhizogenes* was cultured for 12 hours. Acetosyringon

was added to a final concentration of 200 μ M to enhance the virulence of *A. rhizogenes*. This culture was then cultured for another 12 hours, and then *A. rhizogenes* was ready for soybean infection.

Before using *A. rhizogenes* to induce soybean hairy roots, cells of *A. rhizogenes* strains were measured. Both strains 1201 and 1302 of *A. rhizogenes* underwent series dilution and their OD₆₀₀ was measured by spectrophotometer (Hitachi, Pleasanton, CA, USA). The same diluted series were also plated on LB plates, colonies were counted after overnight cultivation. Calibration curve of *A. rhizogenes* OD₆₀₀ versus colony number was used to estimate the colonies for *A. rhizogenes*-mediated soybean hairy root induction (**Figure 7**).



2.3.3 Induction of soybean hairy roots

One-week old soybean plants were about 8-10 cm in height and the cotyledons were green and expanded, soybean explants at this time are most suitable for *A. rhizogenes* infection (Kereszt et al., 2007). The soybean shoots were cut into ~2 cm segments, and cotyledons were cut at both ends to make wounded exposure. Subsequently, *A. rhizogenes* was applied to one end of the shoot or cotyledon, then the segments were placed on fresh 1/2 MS (Murashige and Skoog, 1962) plate with the

“clean” end. *A. rhizogenes* and soybean tissue were co-cultivated at dark, 22°C for hairy root induction.

2.3.4 Sterilization of hairy roots

After co-cultivation for 14 to 21 days, the induced roots were picked up individually and placed on fresh 1/2 MS plates containing 300 µg ml⁻¹ cefotaxime for sterilization of *A. rhizogenes*. After 7 days, roots were transferred to a fresh plate also containing cefotaxime and incubated for another 7 days. After two rounds of sterilization, roots were transferred to liquid cultures.

2.3.5 Liquid cultures of hairy roots

The roots were transferred individually to 50 ml 1/2 MS medium containing cefotaxime in 250 ml flasks and cultivated in an orbital shaker at 70 rpm, 25°C in the dark. Root clones were weighted at day 0, and weighted again after 21 days of cultivation. The root clones were then transferred to fresh 1/2 MS medium without cefotaxime and cultivated for another 21 days.

2.4 Confirmation of foreign DNA in root clones

2.4.1 Extraction of genomic DNA

Fresh root tissues were grounded into powder with liquid nitrogen, and genomic DNA was extracted using Wizard[®] Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the user manual with a few modifications: (1) Place 50 mg of root tissue powder in a 1.5 ml microcentrifuge tube, add 600 μ l of Nuclei Lysis Solution and mix by vortexing 3 seconds. (2) Incubate the tube at 65°C for 15 min and invert the tube every 3 to 5 minutes. (3) Add 3 μ l RNase Solution to cell lysate and mix by inverting the tube, incubate the tube at 37°C for 15 minutes. (4) After cooling the sample to room temperature, add 200 μ l Protein Precipitation Solution and vortex vigorously for 20 seconds. (5) Centrifuge for 10 minutes at 12,000 rpm and then carefully transfer the supernatant to a clean tube containing 600 μ l isopropanol. (6) Mix the solution by inversion until DNA form thread-like visible mass, centrifuge at 12,000 rpm for 3 minutes. (7) Carefully remove the supernatant then add 600 μ l 70% ethanol and wash the DNA by inverting the tube, centrifuge at 12,000 rpm for 3 minutes. (8) Carefully remove the supernatant, invert the tube on clean absorbent paper and air-dry the pellet for 15 minutes. (9) Add 100 μ l Rehydration Solution and incubate the tube at 65°C for 1 hour. (10) Store Genomic DNA at -30°C.

2.4.2 Confirmation of foreign genes with PCR

Genomic DNA extracted from transgenic root clones were used for PCR analysis.

For foreign genes confirmation, conditions of PCR reactions were the same as *A. rhizogenes*. To detect the hairy roots specific gene, *rolC* specific primers were used for PCR reaction. (Primers were designed according to the NCBI nucleotide database E03275.)

F-*rolC*: 5' - ATGGCGGAATTTGACCTATGTGCTCTCTTTTCC – 3'

B-*rolC*: 5' - CCTCACTCCATTCCAAATTTGCATTCGCCATGCC – 3'

The composition of PCR reaction solution:

	Volume (μl)	Final concentration
Genomic DNA extracts	2.5	< 1 μg
4 mM dNTP	2.5	400 μM each
10X Reaction buffer	2.5	1X
10 μM primers	2.5	1 μM
Super-Therm Polymerase	0.5	2.5 U
DMSO	1.0	5.0%
Double distilled H ₂ O	12	-
Total volume	25	-

PCR amplification was performed for 30 cycles in a thermal cycler. The PCR program for *rolC* was: 95°C, 5 min for denaturation; 94°C, 1 min, 56°C, 30 sec, 72°C, 40 sec for 30 cycles; 72°C, 10 min and paused at 4°C. The amplified fragment was resolved on a 1.2% agarose gel, followed by EtBr staining to check the PCR products.

2.5 Functional GFP and GUS confirmation

2.5.1 Direct observation of GFP

Green Fluorescence Protein (GFP) has two excitation peaks, a major one at 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm in the lower green portion of the visible spectrum. Fluorescence of roots was observed directly on 1/2 MS plate by fluorescence microscopy (Nikon E600, Tokyo, JP) combined with a digital camera. The fluorescence microscope has a fluorescence filter combination of excitation wavelength: 450-490 nm; dichroic mirror wavelength: 495 nm; barrier wavelength: 500-550 nm. The digital camera was Olympus C7070 wide-zoom (Olympus, Tokyo, Japan) set to full manual mode with aperture at 4.8, shutter at 30 sec, and ISO at 80. The image was taken with 1600 x 1200 pixels, and then resized without further modification of the image.

2.5.2 GUS activity assay

β -Glucuronidase (GUS) was detected by a histochemical assay using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) for qualitative assay (Jefferson et al., 1987). Fresh roots were washed several times with 50 mM NaH_2PO_4 (pH 7.0) to clean up culture medium, then transferred to 24 well plates. The 1 mM indigogenic substrate X-Gluc in 50 mM NaH_2PO_4 (pH 7.0) was added and incubate at 37°C overnight. After staining, roots were rinsed in 70% ethanol for 5 min, then roots were examined both bare eyes and under microscopy.

2.6 Protein quantification

2.6.1 Extraction of cytosol protein

Fresh roots were grounded into powder in liquid nitrogen, and 50 mg was taken for extraction of total intracellular protein by a homogenizer with 500 μL protein extraction buffer. After centrifugation at 12000x g for 10 min at 4°C, supernatant was collected, and then total intracellular protein extract was stored at -30°C.

2.6.2 Quantification of total protein

The total protein content was measured with Bradford's reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) with bovine serum albumin (BSA) as the standard.

2.6.3 Western blotting for detection of GFP

2.6.3.1 Gel electrophoresis

Protein extract was mixed with 2X loading buffer for SDS-PAGE analysis. After the sample was boiled at 95 °C for 10 min, it was loaded into the well of SDS-PAGE (resolving gel: 15.0%, stacking gel: 5.0%). The electrophoresis was performed in 150 V for 1 hour. For western blot analysis, the prestained molecular weight marker was utilized in SDS-PAGE in order to evaluate the efficiency of protein transfer.

2.6.3.2 Protein transfer

After electrophoresis, the gel was taken for protein transfer. It was transferred to Amersham Hybond polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) membrane in 80 V for 1 h by PowerPac HC Power Supply (Bio-Rad Laboratories Inc., Hercules, CA, USA). The presence of prestained molecular marker ensured that the protein transfer was successful.

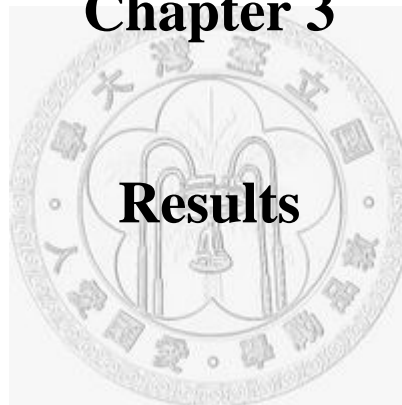
2.6.3.3 Immunoblotting

The membrane was blocked at room temperature for 1 h by blocking reagent buffer. Anti-penta His HRP conjugate was added on the membrane, incubated at room temperature for 1 h. The membrane was then washed with TBS-T buffer twice and TBS buffer once. Chemifluorescence reagent was used at a ratio of 1:1 for the chemilumogenic substrate and oxidizing reagent, and then analyzed by AutoChemii BioImaging System (UVP LLC, Upland, CA, USA).





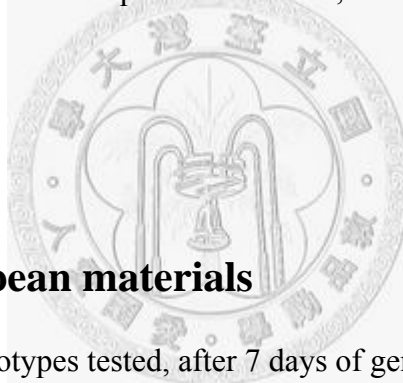
Chapter 3



Results

3.1 *A. rhizogenes* transformation

The transformation efficiency for transforming pCAMBIA 1201 into wild type *A. rhizogenes* (harboring only Ri plasmid) was 5.5×10^5 , and 8.25×10^5 for transforming pCAMBIA 1302 (**Table 3**). The transformation efficiency was 8.5×10^2 for transforming pCAMBIA 1201 into *A. rhizogenes* 1302 (harboring Ri plasmid and pCAMBIA 1302), and 1.05×10^3 for pCAMBIA 1302 into *A. rhizogenes* 1201 (harboring Ri plasmid and pCAMBIA 1201). The efficiency for transforming two binary vectors, pCAMBIA 1201 and pCAMBIA 1302, simultaneously into wild-type *A. rhizogenes* was 2.75×10^2 .



3.2 Selection of soybean materials

Among the six soybean genotypes tested, after 7 days of germination, soybean Tainan #2 and Tainan #7 showed the highest germination rate of 96.5% and 90.2%, average plant height of 3.05 cm and 4.35 cm, respectively (**Figure 8**)
(Utilization factor = total usable tissue fragments per seed planted)

Figure 8 (B)). Because of the need of cutting soybean plant into segments for *Agrobacterium* infection, the higher the plant is, the more usable materials are. We created a “utilization factor” here combining soybean germination rate and plant height,

which was calculated by dividing total usable fragments by total planted soybeans, resulting 2.62 and 2.64 for soybean Tainan #2 and Tainan #7.

Although soybean Tainan #7 showed slightly better results on calculation, but its plant shoot was much thinner than that of Tainan #2, considering this fact soybean Tainan #2 was the most suitable soybean material for fast and efficient obtaining materials for further studies.

3.3 Establishment and selection of transgenic root clones

After *A. rhizogenes* were applied to the wounded soybean tissues, roots were induced from tissue wound sites. The wild-type *A. rhizogenes*-mediated soybean Tainan #2 root induction rate was 48.0% on cotyledons and 15.4% on the lower-shoots (**Table 4**). Root induction rate of *A. rhizogenes* 2BV (harboring pCAMBIA 1201 and 1302) was 39.6% and 14.8% on cotyledons and lower-shoots, respectively. Root induction rate raised to 68.2% and 32.6% on cotyledons and lower-shoots respectively when infecting simultaneously with *A. rhizogenes* 1201 and *A. rhizogenes* 1302. The results above were calculated each with over 50 cotyledons and shoot segments in each construction. Most *A. rhizogenes* infected tissues induced more than one root. The fast elongating, branched roots were considered candidates for transgenic hairy roots (**Table 4**), these roots were removed from the soybean tissue and placed on plates with cefotaxime.

After detached from the original soybean tissues, several root clones didn't propagate anymore after two rounds of *A. rhizogenes* sterilization. Thirty candidate root clones were picked from each group according to the growth on solid 1/2 MS plate, transferred to liquid 1/2 MS medium containing antibiotic for mass cultivation.

3.4 Liquid cultures of transgenic root clones

Root clones induce by wild-type *A. rhizogenes* showed very poor growth after 21 days liquid cultivation in medium containing antibiotic, averaged in a 1.22 fold growth in fresh weight (**Figure 9**). The 2BV group root clones showed similar growth as WT root clones, with an average growth of 1.20 fold in fresh weight (**Figure 10**). The only exception after the first 21 days of cultivation was root line 2AR-C31-1, this line grew 8.09 fold and gained 790 mg in fresh weight, but the other lines in 2AR group showed poor growth with average growth at 1.32 fold (**Figure 11**). Our first assumption was that the antibiotic added for sterilization of *A. rhizogenes* suppressed root growth, so we transferred all 90 root clones into fresh medium containing no antibiotic.

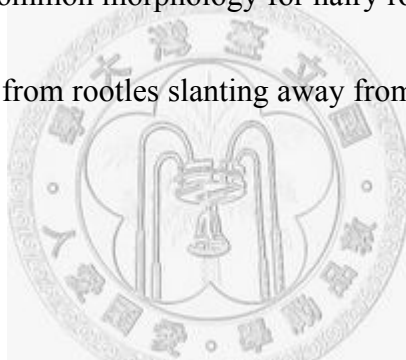
Wild-type roots grew better after 21 days of cultivation in antibiotic-free medium, averaged at 1.97 fold growth (**Figure 9**). On the other hand, most of the 2BV and 2AR root clones didn't show improvement after transferring to antibiotic free medium. One root line 2AR-C16-2 did result in rapid growth of 3.57 fold and gained 540 mg in fresh

weight. The previously rapid growing line 2AR-C31-1 grew 1.98 fold and gained 390 mg in fresh weight. Excluding these two lines, the average growth of 2BV and 2AR root were 1.15 fold and 1.16 fold in fresh weight, respectively (**Figure 10** and **Figure 11**).

We also calculated carbon source consumption by analyzing residue sugar content in the culture medium after the second cultivation using HPLC. The system was Shimadzu LC-3A (Shimadzu Science Co., Tokyo, Japan) combined with Bio-Rad Model 1770 Differential Refractometer (Bio-Rad Laboratories Inc., Hercules, CA, USA), using Thermo Hypersil® APS2 column (Thermo Scientific Inc., Waltham, MA, USA), and a mobile phase at 0.8 ml/min consist of 85% acetonitrile and 15% H₂O. The average carbon source consumption of WT root clones was 20.01% (**Figure 9**), which was quite low comparing to the two rapid growing 2AR root clones, C16-2 and C31-1, each with carbon source consumption at 61.11% and 58.77% (data not shown).

The morphology of root clones was quite variant between poor growing lines and rapid growing lines. All root clones showed fast extension on the taproot and low branching number, but the rootlets showed no further branching. The overall growth was considered fast while still attached to the original soybean tissue (14 to 21 days post induction, see **Figure 12** (A) and (B)). After detaching the original tissue and transferred to 1/2 MS plate containing antibiotic, growth of all root clones were dramatically lowered. For some root clones, after two rounds on plate for over 14 days,

the growth was not even visually identifiable. The chosen 30 root clones from each group were comparably faster growing clones on plates. However, after transferring to liquid medium and cultivate for two rounds of a total 42 days, most of the root clones remained the morphology with only little growth on the taproot and no visually identifiable growth on rootlets (**Figure 12** (D), (E), and (F)). On the other hand, the two fast growing root clones, 2AR-C16-2 and 2AR-C31-1, showed plagiotropic growth and were highly branched after culturing in liquid medium (**Figure 12** (G) and (H)). Plagiotropic growth is the common morphology for hairy roots, which means the longer axes of rootlets or branches from rootlets slanting away from the vertical line (Cho et al., 2000).



3.5 Transgenic root clones gene confirmation

The 90 root clones underwent 42 days of liquid cultivation were ground in liquid nitrogen and genomic DNA were extracted, DNA confirmation was then carried out by PCR amplification. For all candidate root clones, the first target in DNA confirmation is the 543 base pairs *rolC* sequence. In WT construction, *rolC* was not observed on gel for all thirty root clones (data not shown). For 2BV and 2AR construction, *rolC* was not observed either. Even the two root clones with the hairy roots like morphology did not

show *rolC* band as well (**Figure 13**). Because of the inexistence of *rolC* in all candidate root clones, we could not verify hairy roots and adventitious roots at this point.

The other two targets for 2BV and 2AR construction in DNA confirmation was *gfp* and *gus*, each sized 833 and 638 base pairs. For *gfp* confirmation, two of the root clones, 2BV-C30-2, and 2AR-C31-1 showed *gfp* band on gel (**Figure 13**). All root clones from these two constructions were then examined under fluorescent microscopy for the confirmation of functional GFP. For the two root clones that *gfp* was observed, GFP was functionally expressed and observed. In addition, root clone 2AR-C16-2 also showed GFP expressing (**Figure 14**).

For *gus* confirmation, all sixty candidate root clones from 2BV and 2AR construction did not show *gus* band on gel. With the experience of false estimating gene transcription of *gfp*, all root clones underwent examination of GUS activity. We found no root clones functionally expressing GUS.

The two fast growing root clones were the only ones that had sufficient material for protein extraction, and the 2BV-C16-2 clone expressing GFP was also extracted. We extracted total protein from the three root clones as mentioned above. After quantification, the protein extracts were further concentrated 5 fold because of the low protein content. SDS-PAGE was first carried out (**Figure 15**). According to the result on gel, both roots 2AR-C16-2 and 2AR-C31-1 had a 27 kD GFP band as expected. Root

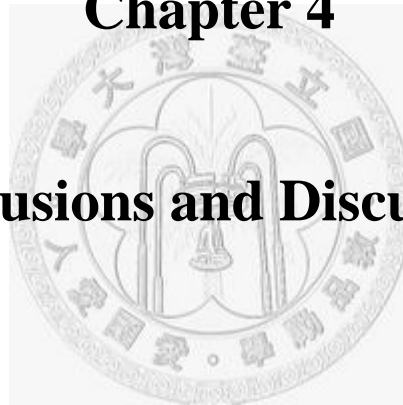
2BV-C16-2 had protein content too low for observing GFP band on gel. For GUS (68 kD), a light band of around 70 kD which might be GUS was observed for root 2AR-C31-1 and 2BV-C16-2. We then further examined the three protein extracts with Western blotting, using the 6x His tag on both GFP and GUS as target. We did not observe any band on the membrane, which might be due to the low protein content of root protein extracts (data not shown).

All of the gene confirmation results are summarized in **Table 5**.



Chapter 4

Conclusions and Discussions



4.1 *A. rhizogenes* transformation

In this study, we attempted to construct an *A. rhizogenes*-mediated multiple gene transformation system in soybeans. The strategies could be divided into two parts concerning the binary vector number being used. The first strategy is transforming two genes in separate binary vectors simultaneously, either harbored by one *A. rhizogenes* transformant or by two separate transformants. The second strategy is transforming two genes in a single binary vector, either in a single T-DNA region or in separate T-DNA regions. We have only managed to test the first strategy so far in this study.

Using the strategy of transforming genes in two separate binary vectors, we first have to understand the efficiency of constructing *A. rhizogenes* transformants. Transforming a single binary vector into wild-type *A. rhizogenes* had a transformation efficiency averaged at 6.88×10^5 , which had little difference depending on the vector transformed. This protocol is considered quite efficient of gaining *A. rhizogenes* transformants. Further transforming the second binary vector into transformed *A. rhizogenes* had a efficiency averaged at 9.5×10^2 . The protocol was still capable of gaining sufficient transformants but the transformation efficiency was a thousandth of transforming the first vector into *A. rhizogenes*. The transformation efficiency transforming two vectors simultaneously into wild-type *A. rhizogenes* was even lower at 2.75×10^2 . This decrease in the acceptability of *A. rhizogenes* might be related to

plasmid incompatibility due to the considerable similarity between the two binary vectors being transformed.

Plasmid incompatibility is due to the sharing of one or more element of the plasmid replication or partitioning systems (Novick, 1987). In this case, pCAMBIA 1201 and pCAMBIA 1302 share the backbone sequence which contains the replication origin and a hygromycin resistance gene, a total of 8737 base pairs (pCAMBIA 1201 and pCAMBIA 1302 are sized 12,001 and 10,549 base pairs, respectively). Vectorial plasmid incompatibility, in which case one plasmid is lost exclusively or with higher probability, might happened in this study. When transforming the second binary vector into a pre-transformed *A. rhizogenes*, most cells retained only the original vector harbored. Only 1×10^{-9} of the transformed cells lost the original vector and gained the newly transformed vector. Although plasmid incompatibility seems to played an important role affecting the transformation process, it is considered not to have its affect after the cells are cultured under selection stress, which is antibiotics in this case (Novick et al., 1976).

4.2 Soybean and transgenic soybean roots

We examined six genotypes of soybean for selecting the most suitable material for further soybean researches, and we found soybean Tainan #2 showed the highest

germination rate and a high utilization factor compared to the other five genotypes of soybean tested. The selection strategy was based on the germination rate and also considering the growth rate, plant height and shoot thickness at an early period of germination as the index of selecting the suitable soybean material. With this strategy, it seemed we have overlooked the difference of soybean genotype characteristics between the six soybean genotypes tested.

Using soybean Tainan #2 as the material for *A. rhizogenes* mediated gene transformation, we gained plenty candidate root clones which showed fast growth while still attached to the *A. rhizogenes* infected soybean tissue. However, from these candidate root clones we only gained two root clones which maintained fast growth on solid plate and in liquid medium after detaching the original tissue. Based on the morphology and the growth, these two clones had a high possibility being transgenic hairy roots, and the rest of the candidate root clones might be adventitious roots.

The PCR results for gene confirmation were not quite as we expected. For the poor growing root clones which were not expected to be hairy roots, *rolC* was not detected as expected. But for the fast growing clones which were expected to be hairy roots based on the morphology and growth, *rolC* was not found, either. Secondly, there was a root clone which had GFP functionally expressed but *gfp* not detected in the PCR confirmation. The two confusing results gave us a conclusion that the problem might

lies in PCR. We then tried many modifications with the PCR reaction mixture and the PCR sequence, but still gained the same results. As the PCR problem still not solved, for now we will make our judgments based mainly on the morphology and on the protein level. As a conclusion, we had gained two transgenic hairy root clones, 2AR-C16-2 and 2AR-C31-1, both expressing GFP but not GUS. We also gained one adventitious root 2BV-C30-2 expressing GFP, and we gained no wild-type hairy roots. As with the PCR problem, because of the massive numbers of gene within soybean genomic DNA pool and the unknown site of T-DNA insertion (Zambryski, 1988), it is somehow difficult to amplify some certain genes. To avoid this problem, reverse-transcription PCR can be carried out. Because not all genes in soybean genomic DNA are being transcript in hairy roots, RT-PCR can enhance the chance of finding the desired genes.

The two hairy root clones we gained is considered that both gained two of the three T-DNA we transformed, which in this case was a T-DNA region containing *rolC* and a T-DNA region containing *gfp*. Furthermore, the adventitious root expressing GFP can be considered which gained only one of the three T-DNA being transformed. From the above results, we suspect that *A. rhizogenes* might have a transformation limit for T-DNA numbers being transformed simultaneously into soybean. On the other hand as we mentioned above, plasmid incompatibility has a high probability of happening when

A. rhizogenes is cultured under no selection stress. After *A. rhizogenes* was applied to the wounded soybean tissue, *A. rhizogenes* and soybean were co-cultivated for a 14 to 21 days period on antibiotic free 1/2 MS plates. In this period, there was no selection stress for *A. rhizogenes* to retain the two nonessential extra-chromosomal binary vectors. So, plasmid incompatibility might be another reason causing some of the genes not being transformed into soybean.

This low efficiency of gaining soybean transgenic hairy roots, compared to previous studies in our lab of inducing tobacco hairy roots, might be related to the soybean characteristics. Soybean Tainan #2 is a crossbred genotype bred by the Tainan District Agricultural Research and Extension Station (Tainan, Taiwan) in 1993. This soybean genotype has a strong disease-resistant ability to soybean downy mildew caused by *Peronospora manshurica* and soybean rust caused by *Phakopsora pachyrhizi*, it also has a medium disease-resistant to bacterial leaf spots disease caused by *Xanthomonas axonopodis* and bacterial pustules caused by *Xanthomonas campestris* (連大進等, 1993). These disease-resistant abilities of soybean Tainan #2 might have an effect on the *A. rhizogenes*-mediated gene transformation. *Agrobacterium*, as mentioned above, is a soil born plant pathogen, so Tainan #2 might also have the ability to resist or partially-resist the infection of *A. rhizogenes*, which might be the reason that we were not able to gain sufficient transgenic hairy roots as expected.

Also, we suspected that the low efficiency of inducing soybean hairy roots might also have relation with the tissues used for induction. In this study, we used cotyledon and the lower shoot for induction of hairy roots. From our results, we suspected that many of the root clones inducted from lower shoot and cotyledon might just be soybean root which will grow even without *A. rhizogenes* induction. One member of our lab is currently studying *A. rhizogenes*-mediated soybean Tainan #5 gene transformation, most of the root clones inducted from the upper shoot of Tainan #5 showed hairy roots morphology on plates and in liquid cultures. We then tested the induction rate of soybean Tainan #2 upper shoot with wild-type *A. rhizogenes*. The induction rate was 51.4%, and 32.6% of the induced roots showed fast growth while still attached to the upper shoot tissues. Roots induced from upper shoot generally showed more branching than those inducted from lower shoot and cotyledon (**Figure 12 (C)**), more likely to be hairy roots. Although this assumption is not yet confirmed, it is a point that should be considered in the following studies.

4.3 Perspectives

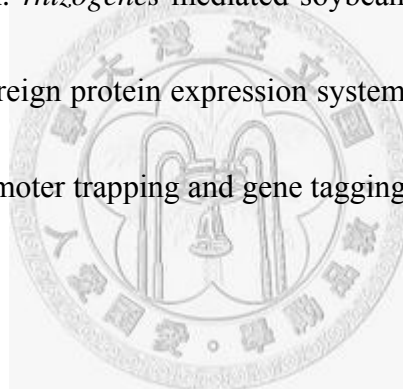
Our current strategy of transforming multiple genes into soybean is using multiple binary vectors (**Figure 3** and **Figure 4**), this strategy is thought to be easier to construct. But since plasmid incompatibility might have occurred and had a negative effect on the

transformation efficiency, other transformation strategies should be considered. The other strategy we constructed but not yet utilized in this study, transforming two genes in a single binary vector (**Figure 5** and **Figure 6**), is thought to be more suitable for multiple genes transformation. Construction of two genes in a single binary vector, either constructing the two genes in a single T-DNA region or in separate ones, can avoid plasmid incompatibility happening during the co-cultivation period of *Agrobacterium* and plant tissues. This will ensure the two genes both have its chance to be transformed into plant cells. So to transform soybean with the two constructions that use only one binary vector is the first step for the follow ups of this study. And the binary vector with the two genes in separate T-DNA regions can also be a model for the study to find out if there's a transform limit of T-DNA number for *A. rhizogenes*.

We had an assumption that soybean genotype characteristic and the tissue being induced might affect soybean susceptibility to *A. rhizogenes*-mediated transformation. This assumption needs a broad scanning of different tissues of plenty soybean genotypes, testing sterile plant germination rate, wild-type *A. rhizogenes* induction rate, and the percentage of hairy roots over induced roots. This test may take long to finally select the most suitable soybean tissue, but it should be an important step to construct a complete soybean gene transformation system.

The *A. rhizogenes*-mediated soybean multiple genes transformation is not yet fully constructed. Although this study leaves a lot of unsolved questions behind, it still give us some knowledge about *A. rhizogenes*-mediated soybean hairy roots induction system:

1. the *A. rhizogenes* our lab uses can successfully induce soybean hairy roots and the hairy roots can propagate under solid and liquid medium; 2. the foreign gene transformed is successfully inserted into soybean hairy roots genome; 3. the foreign gene transformed can be functionally expressed in soybean hairy roots. These knowledge assure us that *A. rhizogenes*-mediated soybean gene transformation system can be used to become a foreign protein expression system, and this system can also be used for Genomics like promoter trapping and gene tagging.





Tables and Figures

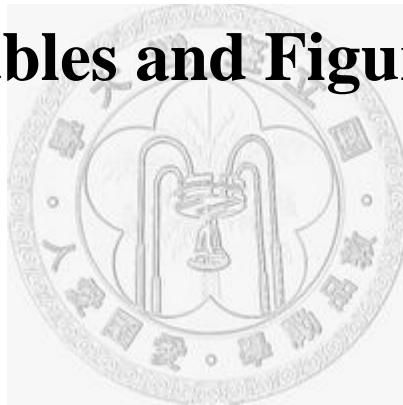


Table 1 Proteins of medical relevance produced in plant cell cultures

Expressed protein	Expression host	Promoter	Localization, yield
Human serum albumin	<i>N. tabacum suspension culture</i>	Modified 35S	Secretion/apoplast targeting, 0.25 µg mg ⁻¹ protein in supernatant
scFv antibody fragment	<i>N. tabacum suspension culture</i>	35S	Secretion, up to 0.5 µg l ⁻¹ up to 0.5% of TSP
Human erythropoietin	<i>N. tabacum cv BY-2 suspension culture</i>	35S	Secreted, 1 pg g ⁻¹ FW
Mouse monoclonal heavy-chain γ	<i>N. tabacum cv NT-1 suspension culture</i>	35 S	Native heavy-chain secretion signal, ca. 10 µg l ⁻¹ extracellular without, 350 µg l ⁻¹ with PVP
Mouse IgG2b/κ	<i>N. tabacum cv Petite Havana SR-1</i>	Enhanced 35S	15 µg g ⁻¹ FW, ~0.3% of TSP
Heavy chain mAb	<i>N. tabacum cv NT-1 suspension culture</i>	35S	Secreted up to 10 µg l ⁻¹ , with stabilization up to 350 µg l ⁻¹
Bryodin 1	<i>N. tabacum cv NT-1 suspension culture</i>	35S	Secreted up to 30 mg l ⁻¹
Human IL-2 & IL-4	<i>N. tabacum cv NT-1 suspension culture</i>	35S	Secreted (native signal peptides), 8–180 µg l ⁻¹ of culture broth
Recombinant ricin	<i>N. tabacum suspension culture</i>	35S	25–37.5 µg l ⁻¹
scFv antibody fragment	<i>Oryza sativa cv Bengal (rice) callus culture</i>	Maize ubiquitin	Apoplast targeting (optimized Ig leader peptides) and ER-retention, up to 3.8 µg g ⁻¹ callus FW
Full size IgG-2b/κ	<i>N. tabacum cv Petite Havana SR-1</i>	Enhanced 35S	0.3% of TSP or 15 µg/g wet weight
Human α1-antitrypsin	<i>O. sativa cv Taipei 309 suspension culture</i>	RAmy3D	Secreted, 85 mg l ⁻¹ in shake flask, 25 mg l ⁻¹ in bioreactor
HBsAg	<i>N. tabacum NT-1 suspension culture</i>	<i>A. thaliana ubq3</i>	Secreted, up to 10 µg l ⁻¹ of particulate HBsAg

(Hellwig et al., 2004)

Table 1 Proteins of medical relevance produced in plant cell cultures (Continue)

Expressed protein	Expression host	Promoter	Localization, yield
biscFv antibody fragment	<i>N. tabacum</i> cv <i>BY-2</i> suspension culture	Enhanced 35S	Cytosolic, apoplast-targeted (up to 0.0064% of TSP), ER-retained (up to 0.064% of TSP)
hGM-CSF	<i>N. tabacum</i> cv <i>NT-1</i> suspension culture	35S	Secreted/targeted to the apoplast ~250 µg l ⁻¹ extracellular, ~150 µg l ⁻¹ intracellular
scFv antibody fragment	<i>N. tabacum</i> suspension culture	35S	Apoplast targeting (sporamin secretion signal) 1 mg l ⁻¹ extracellular, 5 mg l ⁻¹ intracellular
Human α1-antitrypsin	<i>O. sativa</i> suspension culture	RAmy3D	Up to 200 mg l ⁻¹ (calli suspended to 40% (vol/vol)) cell density in induction medium
HBsAg	<i>Glycine max</i> cv <i>Williams 82</i> & <i>N. tabacum</i> <i>NT-1</i> suspension cultures	(ocs)3mas	Intracellular up to 22 mg l ⁻¹ in soybean ~2 mg l ⁻¹ in tobacco
hGM-CSF	<i>N. tabacum</i>	35S	1.6 to 6.6 µg ml ⁻¹ upon homogenizing the entire culture broth
Human lysozyme	<i>O. sativa</i> cv <i>Taipei 309</i> suspension culture	RAmy3D	Intracellular (although RAmy3D signal peptide was used), up to 3%–4% of TSP
IL-12	<i>N. tabacum</i> cv <i>Havana</i> suspension culture	Enhanced 35 S	Secreted, up to 800 µg l ⁻¹ of supernatant
hGM-CSF	<i>Lycopersicum esculentum</i> cv <i>Seokwang</i> suspension culture	Enhanced 35S	Secreted, up to 45 µg l ⁻¹ of supernatant
mAb against HBsAg	<i>N. tabacum</i> cv <i>BY-2</i> suspension culture	35S	Secreted, ~50/50 between supernatant and cells, total max ~15 mg l ⁻¹

(Hellwig et al., 2004)

Table 2 Recent reports on valuable metabolites produced by hairy roots

Plant species	Metabolites	Properties	Year
<i>Camptotheca acuminata</i>	Camptothecin	Anti-cancer, antiviral	2004
<i>Ginkgo biloba</i>	Ginkgolides	Aging disorders	2003
<i>Gmelina arborea</i>	Verbascoside	Stomach disorders, fevers, skin problems	2005
<i>Gynostemma pentaphyllum</i>	Gypenoside	Detergent	2005
<i>Linum flavum</i>	Coniferin	Anti-cancer	2003
<i>Papaver somniferum</i>	Morphine, sanguinarine, codeine	Sedative, analgesic	2004
<i>Pueraria phaseoloides</i>	Puerarin	Hypothermic, spasmolytic, hypotensive, anti-arrhythmic	2003
<i>Rauvolfia micrantha</i>	Ajmalicine, ajmaline	Anti-hypertensive	2003
<i>Saussurea medusa</i>	Jaceosidin	Anti-tumor	2004
<i>Solidago altissima</i>	Polyacetylene (cis dehydromatricaria ester)	Unknown	2003

(Guillon et al., 2006)

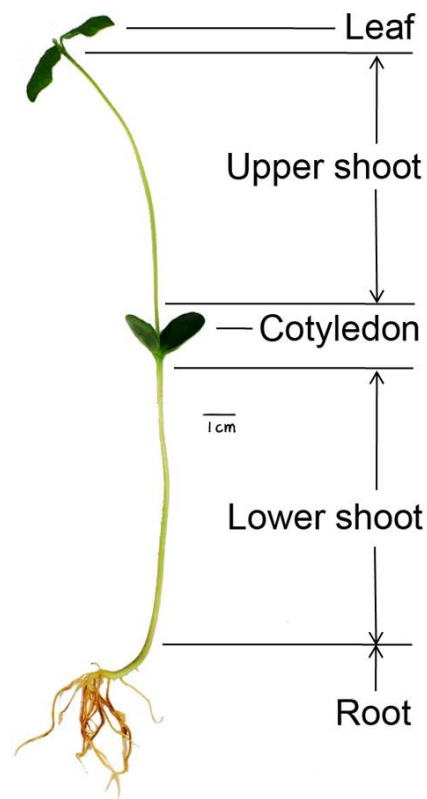
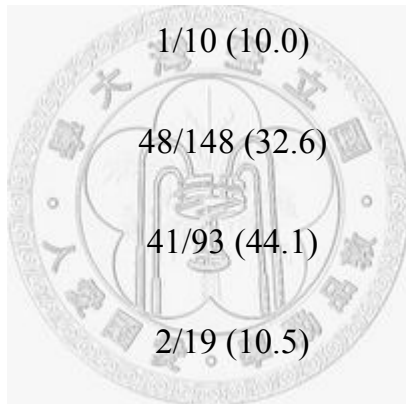
Table 3 *A. rhizogenes* transformation efficiency

Target <i>A. rhizogenes</i> (T-DNA)	Binary vector transforming		
	pCAMBIA1201	pCAMBIA1302	pCAMBIA1201 + pCAMBIA1302
WT (Ri)	5.5×10^5	8.25×10^5	2.75×10^2
1201 (Ri + pCAMBIA1201)	-	1.05×10^3	-
1302 (Ri + pCAMBIA1302)	8.5×10^2	-	-

Transformation efficiency (transformants/ μg) = (number of colonies on plate / ng of DNA plated) x 1000 ng/ μg

Table 4 Induction rate of soybean roots

Construction	Root induced tissues / Total infected tissues (%)	Fast growing roots / Total roots (%)*	Root clones picked
WT Cotyledon	24/50 (48.0)	30/74 (40.5)	30
WT Lower shoot	10/65 (15.4)	1/10 (10.0)	0
WT Upper shoot	55/107 (51.4)	48/148 (32.6)	-
2BV Cotyledon	40/101 (39.6)	41/93 (44.1)	29
2BV Lower shoot	16/108 (14.8)	2/19 (10.5)	1
2AR Cotyledon	45/66 (68.2)	43/75 (57.5)	30
2AR Lower shoot	21/65 (32.3)	0/21 (0)	0

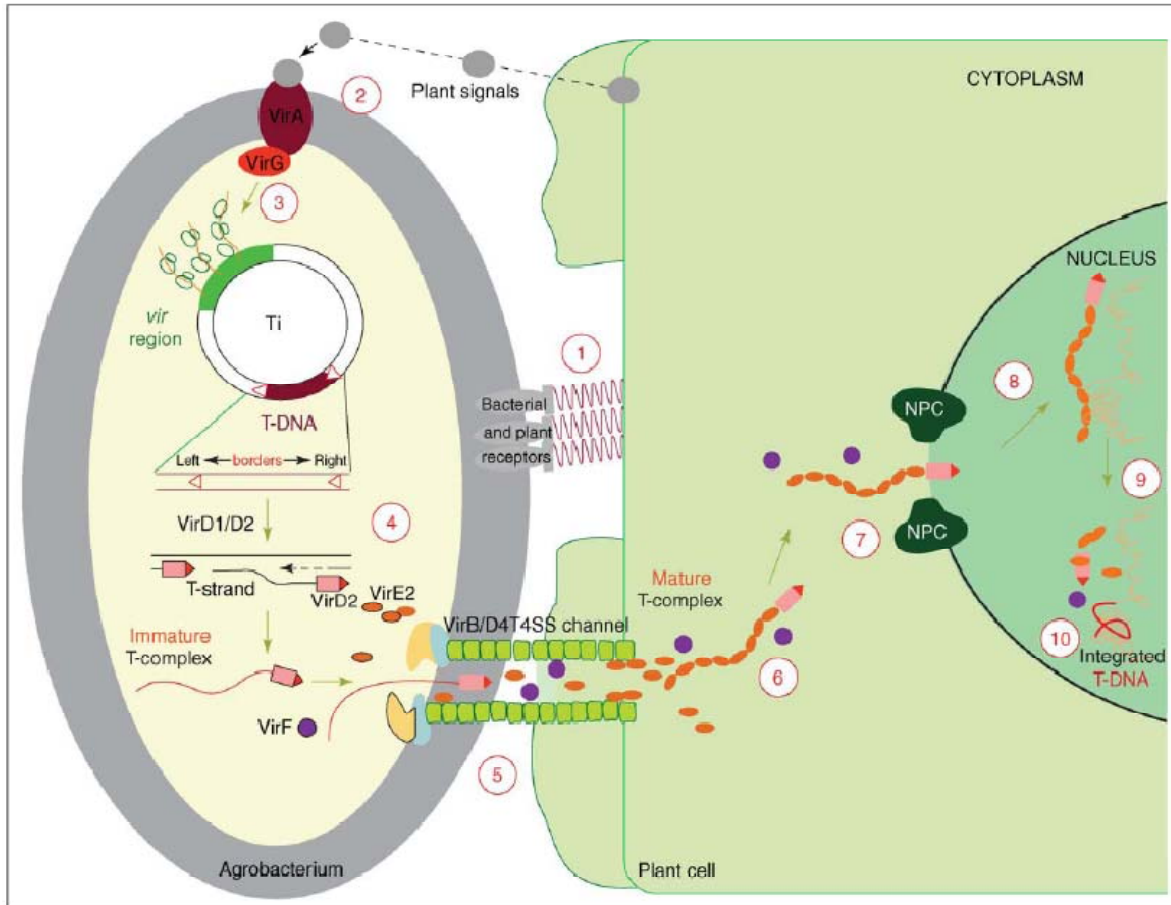


* Root induced tissues / Total infected tissues

** Roots were still attached to the soybean tissue from which the roots were induced

Table 5 Gene confirmation of 2BV and 2AR root clones
rolC, *gfp*, and *gus*: PCR confirmation of three foreign genes. HR: Hairy roots phenotypes. GFP and GUS: functional protein confirmation.

2BV	<i>rolC</i>	HR*	<i>gfp</i>	GFP	<i>gus</i>	GUS	2AR	<i>rolC</i>	HR*	<i>gfp</i>	GFP	<i>gus</i>	GUS
C04-1	-	-	-	-	-	-	C01-3	-	-	-	-	-	-
C05-1	-	-	-	-	-	-	C01-4	-	-	-	-	-	-
C06-1	-	-	-	-	-	-	C01-6	-	-	-	-	-	-
C06-2	-	-	-	-	-	-	C02-1	-	-	-	-	-	-
C10-1	-	-	-	-	-	-	C02-3	-	-	-	-	-	-
C12-1	-	-	-	-	-	-	C02-4	-	-	-	-	-	-
C13-2	-	-	-	-	-	-	C02-5	-	-	-	-	-	-
C15-1	-	-	-	-	-	-	C02-6	-	-	-	-	-	-
C16-1	-	-	-	-	-	-	C03-1	-	-	-	-	-	-
C16-2	-	-	-	-	-	-	C03-2	-	-	-	-	-	-
C17-1	-	-	-	-	-	-	C05-2	-	-	-	-	-	-
C18-1	-	-	-	-	-	-	C06-1	-	-	-	-	-	-
C19-1	-	-	-	-	-	-	C06-2	-	-	-	-	-	-
C19-2	-	-	-	-	-	-	C07-1	-	-	-	-	-	-
C19-3	-	-	-	-	-	-	C07-2	-	-	-	-	-	-
C20-1	-	-	-	-	-	-	C07-3	-	-	-	-	-	-
C21-1	-	-	-	-	-	-	C07-4	-	-	-	-	-	-
C22-1	-	-	-	-	-	-	C10-2	-	-	-	-	-	-
C22-2	-	-	-	-	-	-	C14-1	-	-	-	-	-	-
C25-1	-	-	-	-	-	-	C16-1	-	-	-	-	-	-
C26-1	-	-	-	-	-	-	C16-2	-	+	-	+	-	-
C29-1	-	-	-	-	-	-	C16-3	-	-	-	-	-	-
C30-2	-	-	+	+	-	-	C17-1	-	-	-	-	-	-
C30-3	-	-	-	-	-	-	C19-1	-	-	-	-	-	-
C35-1	-	-	-	-	-	-	C20-1	-	-	-	-	-	-
C36-1	-	-	-	-	-	-	C21-1	-	-	-	-	-	-
C36-2	-	-	-	-	-	-	C23-1	-	-	-	-	-	-
C37-1	-	-	-	-	-	-	C28-1	-	-	-	-	-	-
C38-1	-	-	-	-	-	-	C30-1	-	-	-	-	-	-
S02-1	-	-	-	-	-	-	C31-1	-	+	+	+	-	-



(Tzfira and Citovsky, 2006)

Figure 1 *Agrobacterium*-mediated genetic transformation model.

- ① Bacterium–plant attachment.
- ②-③ Transmembrane protein complex induction by plant signals and expression of *vir* region.
- ④ ssT-DNA production.
- ⑤-⑥-⑦ ssT-DNA export into host cell, transport through cytoplasm and then into nucleus.
- ⑧ Intranuclear transport.
- ⑨ T-DNA uncoating.
- ⑩ Integration.

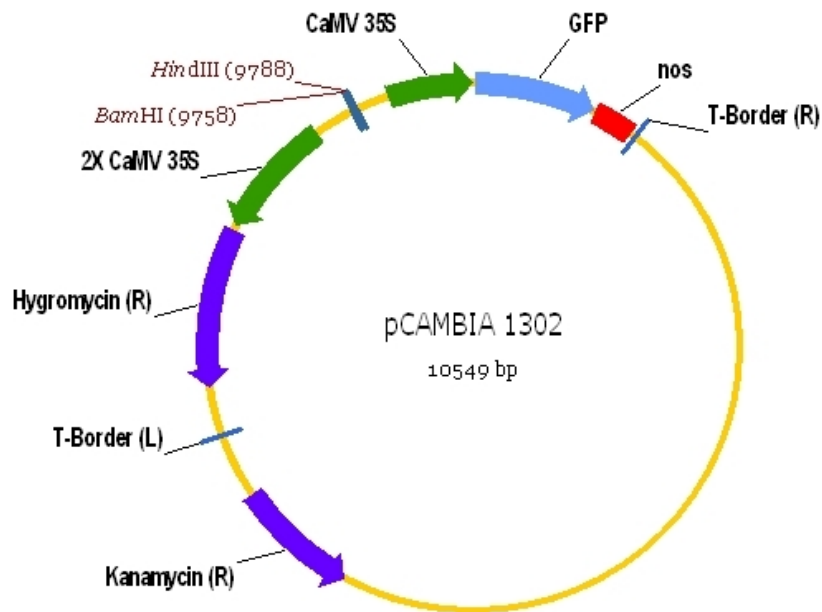
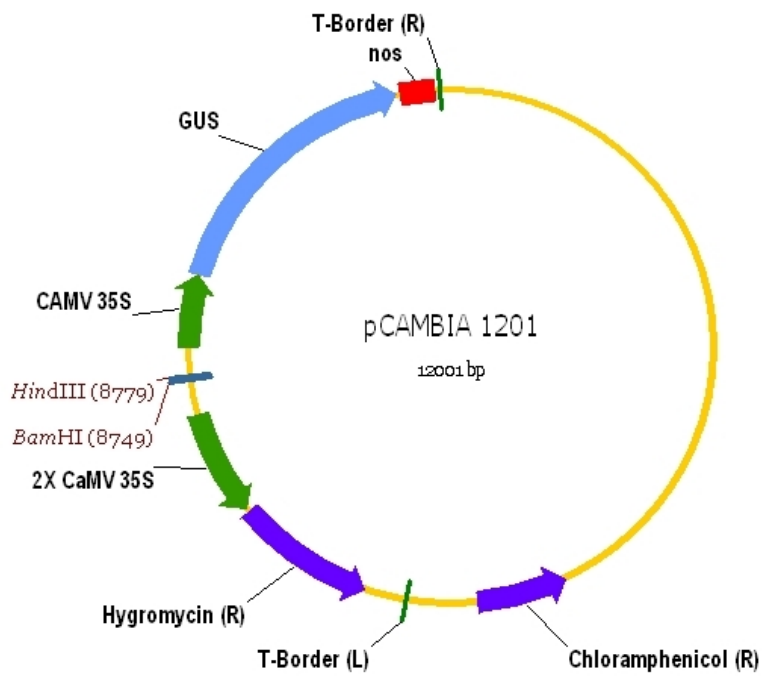


Figure 2 Binary vectors (A) pCAMBIA 1201 (B) pCAMBIA 1302

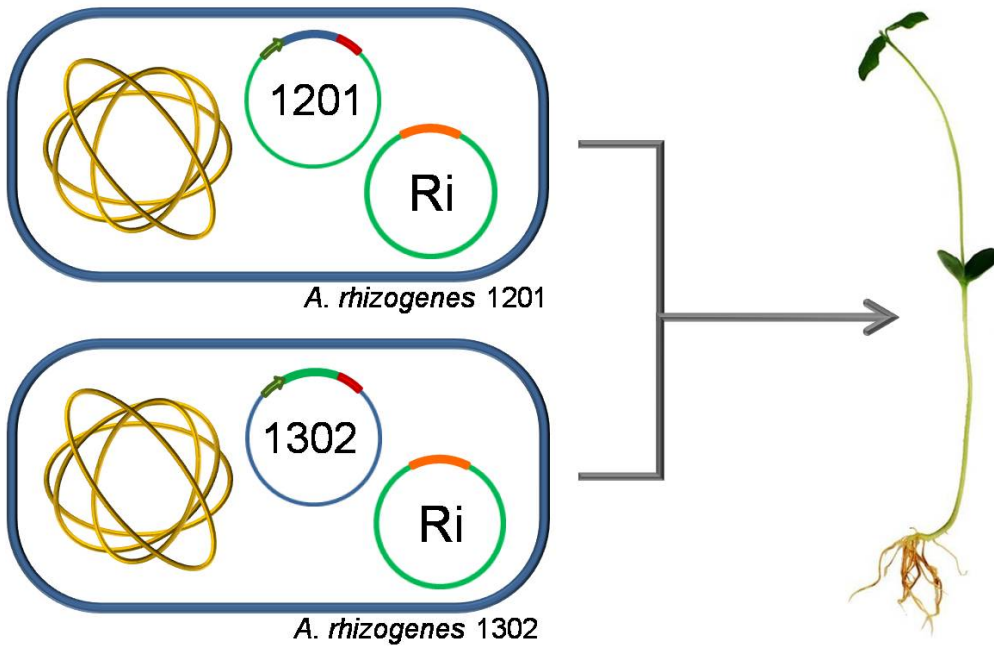


Figure 3 The two *A. rhizogenes* (2AR) construction

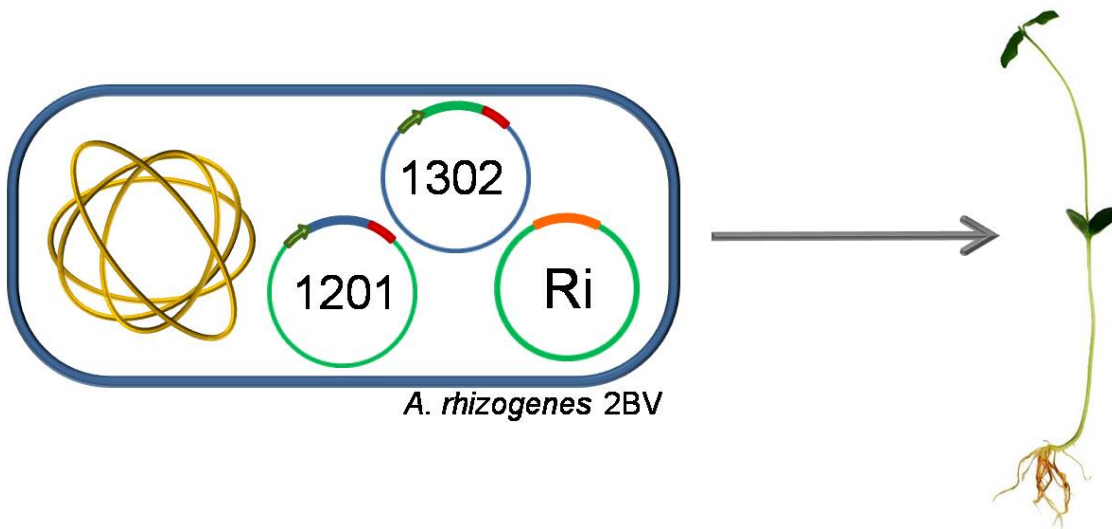


Figure 4 The two binary vector (2BV) construction

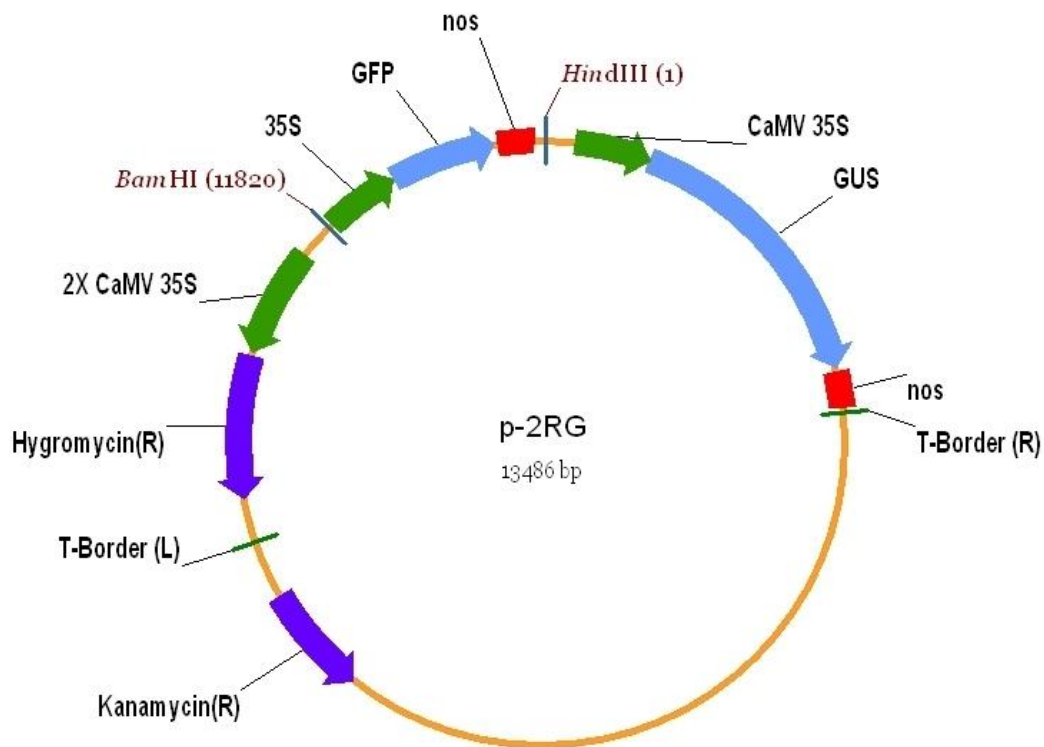


Figure 5 Binary vector p-2-Reporter-Geens (p-2RG)

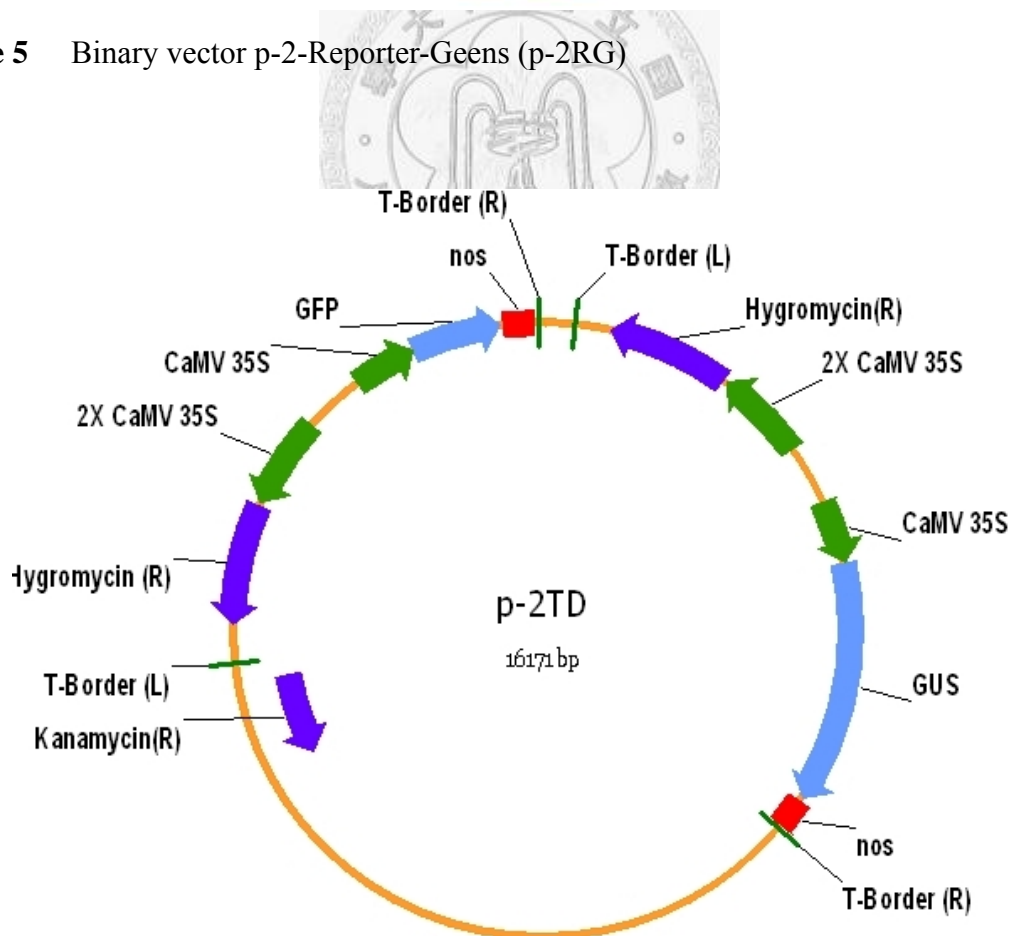
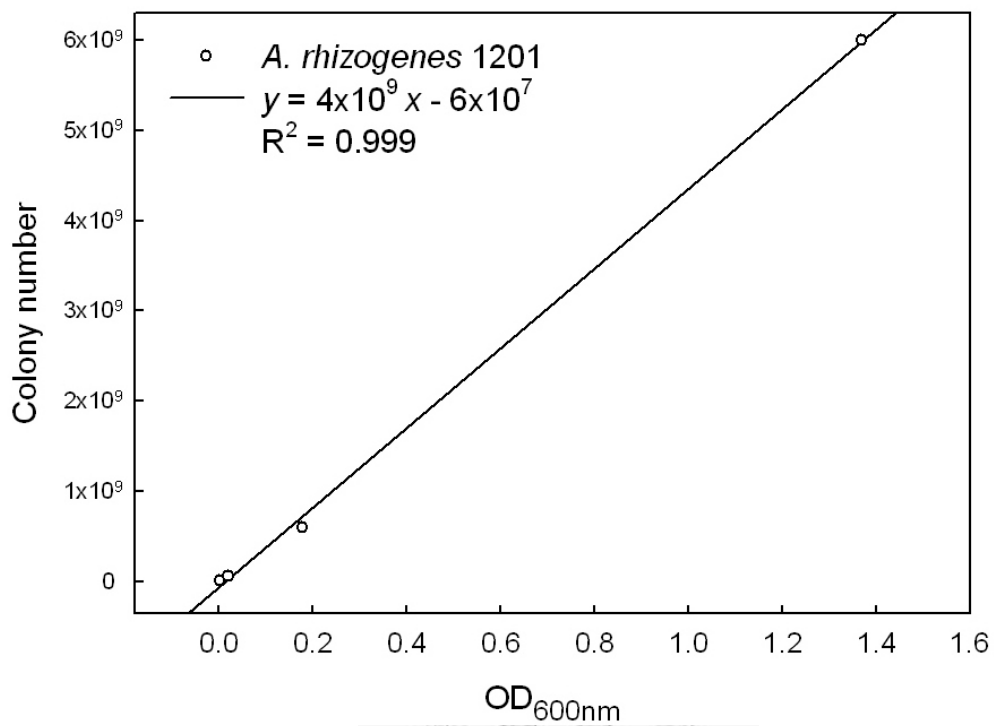
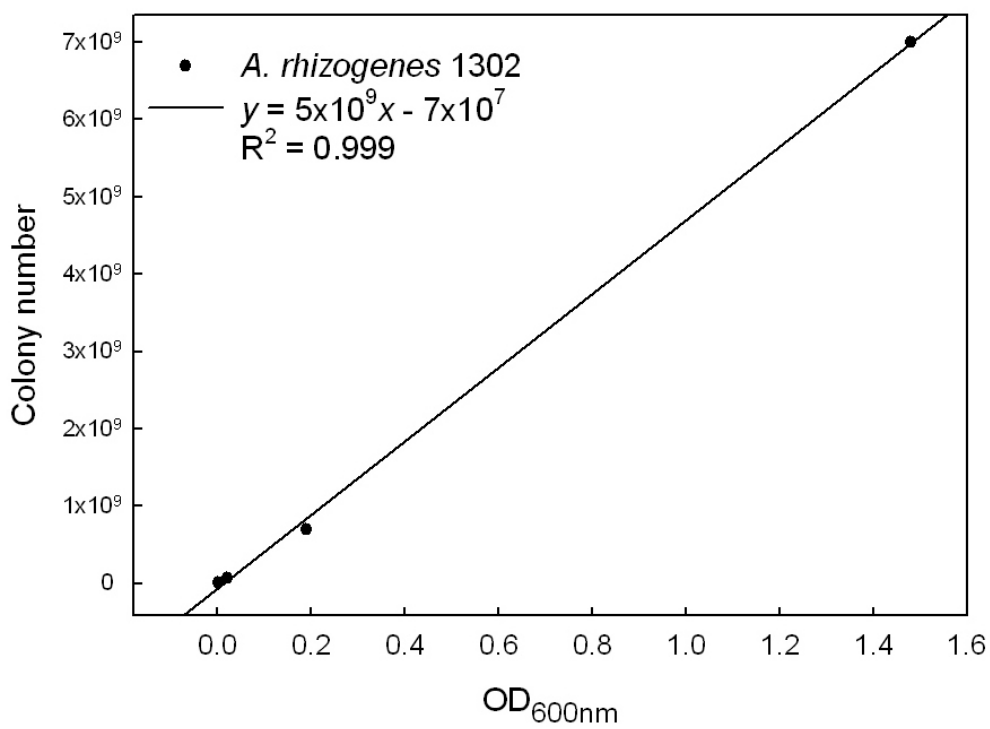


Figure 6 Binary vector p-2-T-DNA (p-2TD)



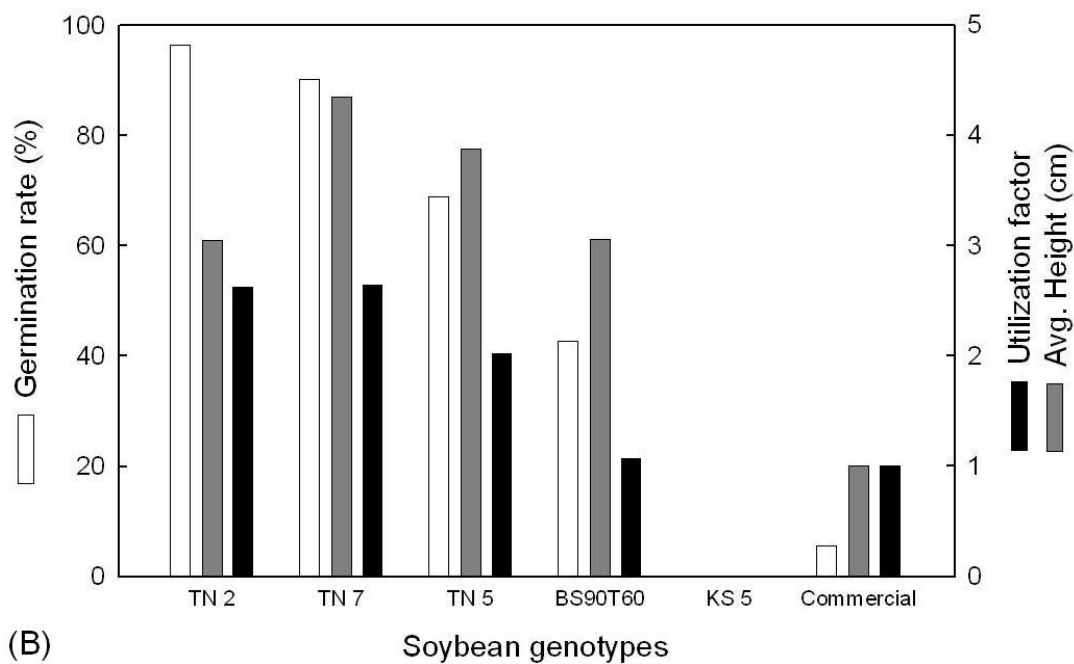
(A)



(B)

Figure 7 Calibration curve of *A. rhizogenes* OD₆₀₀ vs. colony number

(A) *A. rhizogenes* 1201 (B) *A. rhizogenes* 1302



(Utilization factor = total usable tissue fragments per seed planted)

Figure 8 Soybean germination tests. (A) Seeds of 6 genotypes of soybean. (B) Germination rate, average plant height (from the bottom of lower shoot to the top of cotyledon or leaf), and utilization factor 7 days after sterile plant germination.

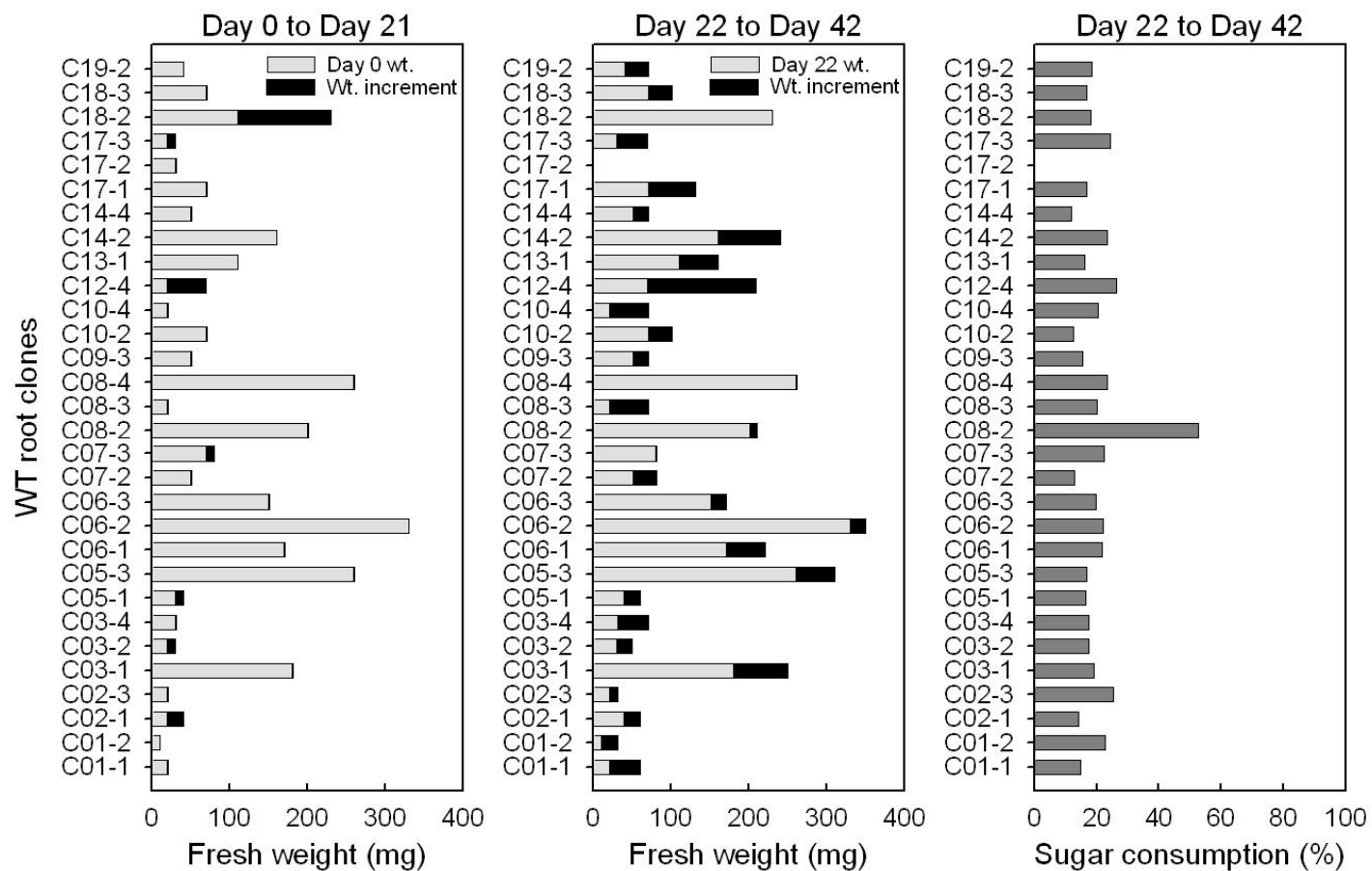


Figure 9 Growth of wild-type root clones. Left: growth from day 0 to 21 in 1/2 MS medium with cefotaxime; Center: growth from day 22 to 42 in 1/2 MS medium without cefotaxime; Right: Sugar consumption in the culture medium during cultivation from day 22 to 42.

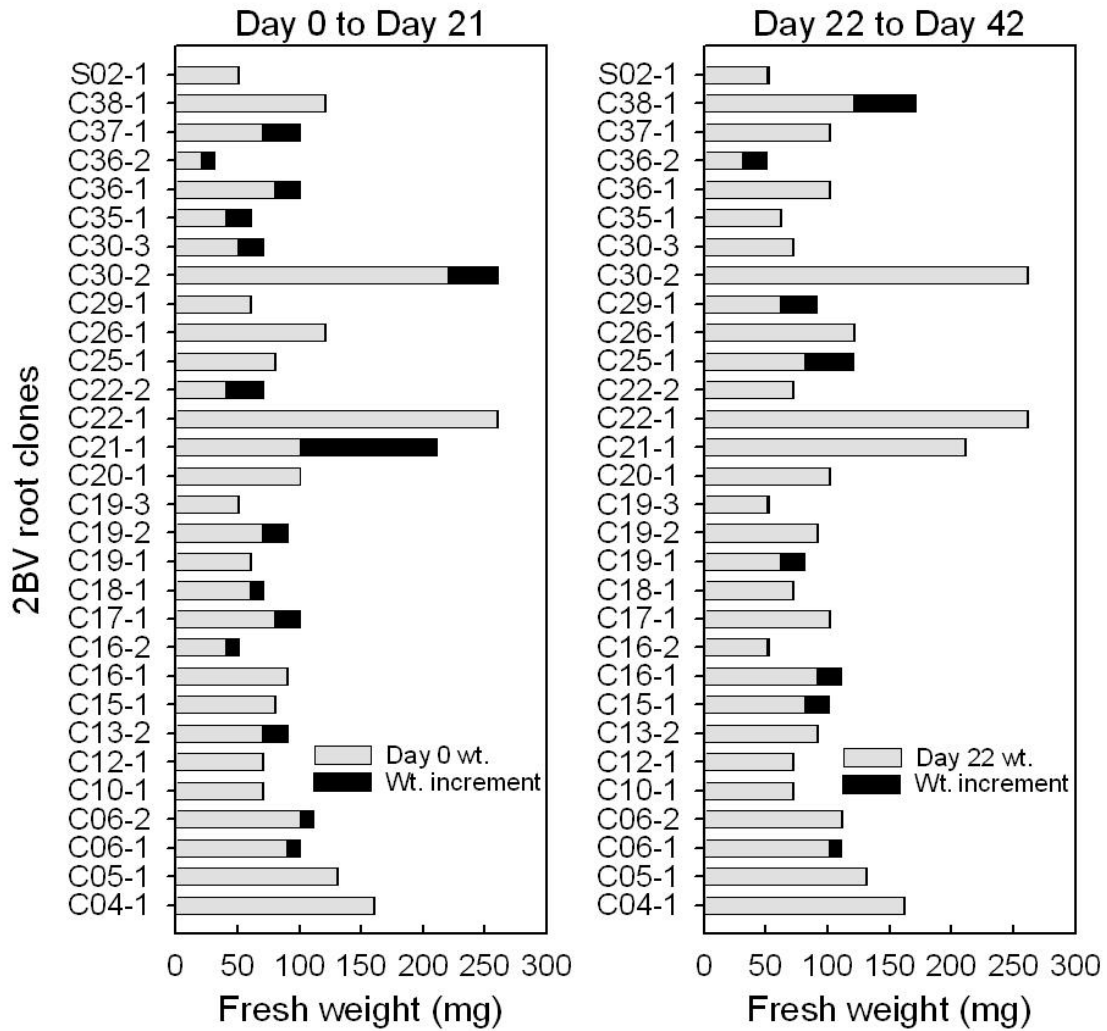


Figure 10 Growth of 2BV root clones. Left: growth from day 0 to 21 in 1/2 MS medium with cefotaxime; Right: growth from day 22 to 42 in 1/2 MS medium without cefotaxime.

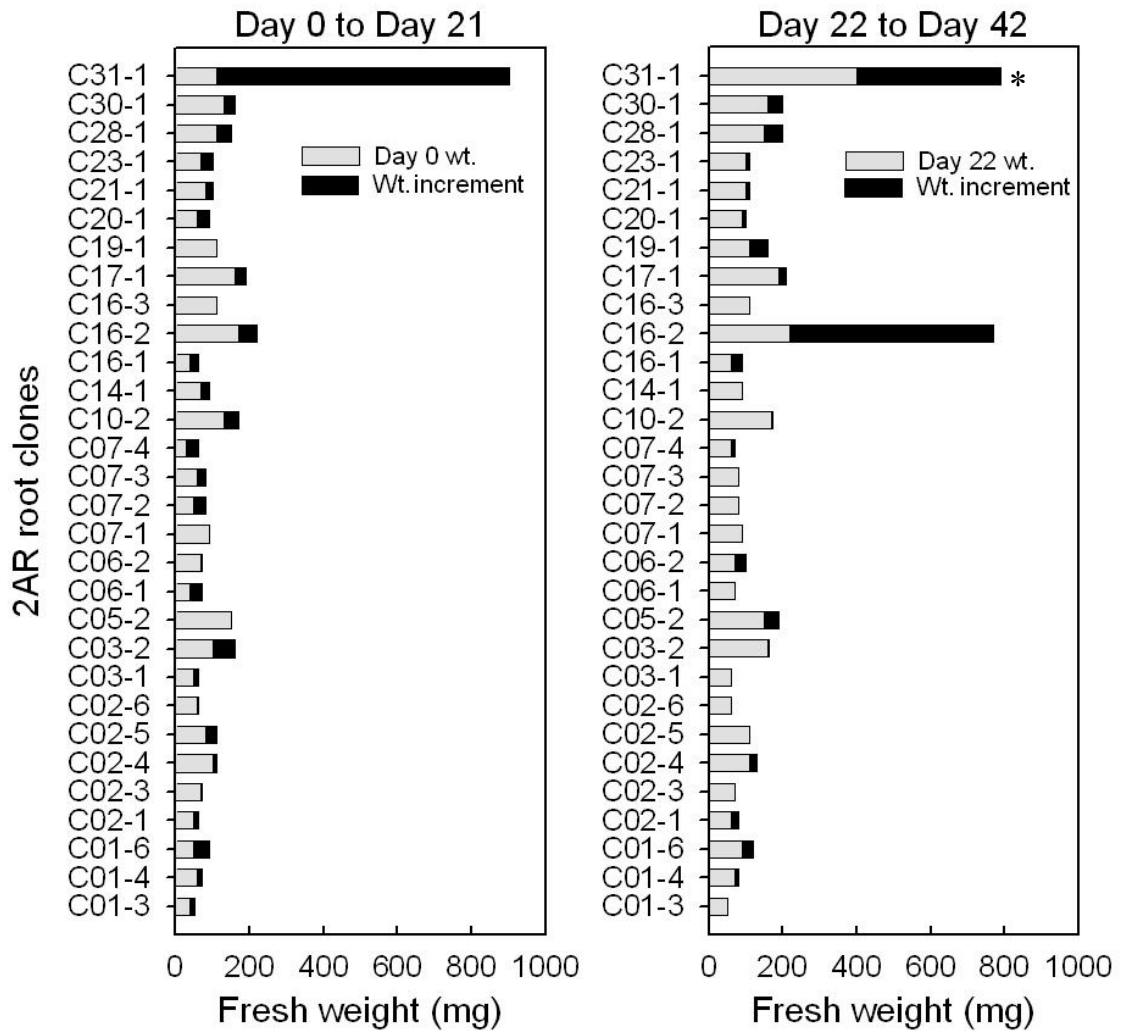


Figure 11 Growth of 2AR root clones. Left: growth from day 0 to 21 in 1/2 MS medium with cefotaxime; Right: growth from day 22 to 42 in 1/2 MS medium without cefotaxime.

*: 400 mg of 2AR-C31-1 was taken for experiments at day 22.



Figure 12 Morphology of transgenic roots (A) lower shoot 0 day after induction; (B) lower shoot 20 days after induction; (C) Upper shoot 20 days after induction; (D) Root WT-C01-01 after 42 days liquid culture; (E) Root 2BD-C13-2 after 42 days liquid culture; (F) Root 2AR-C05-2 after 42 days liquid culture; (G) Root 2AR-C16-2 after 42 days liquid culture; (H) Root 2AR-C31-1 after 42 days liquid culture.

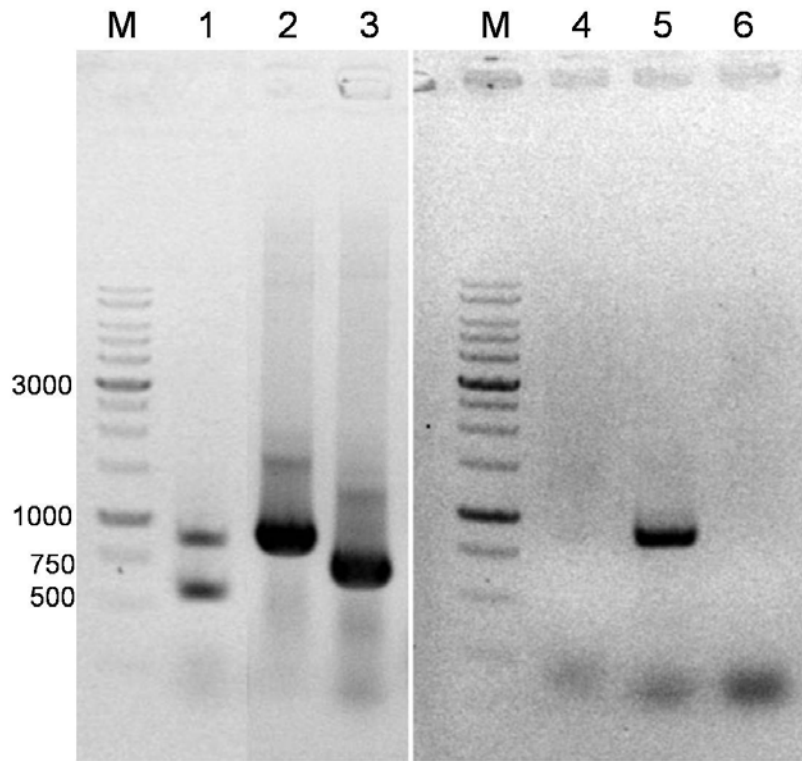


Figure 13 PCR confirmation of transgenic hairy roots

M: marker; 1: colony PCR of *A. rhizogenes* 1610 (*rolC* control ,543 bp); 2: colony PCR of *A. rhizogenes* 2BV (*gfp* control, 833 bp); 3: colony PCR of *A. rhizogenes* 2BV (*gus* control, 638 bp); 4: PCR of *rolC* in genomic DNA from 2AR-C31-1; 5: PCR of *gfp* in genomic DNA from 2AR-C31-1; 6: PCR of *gus* in genomic DNA from 2AR-C31-1

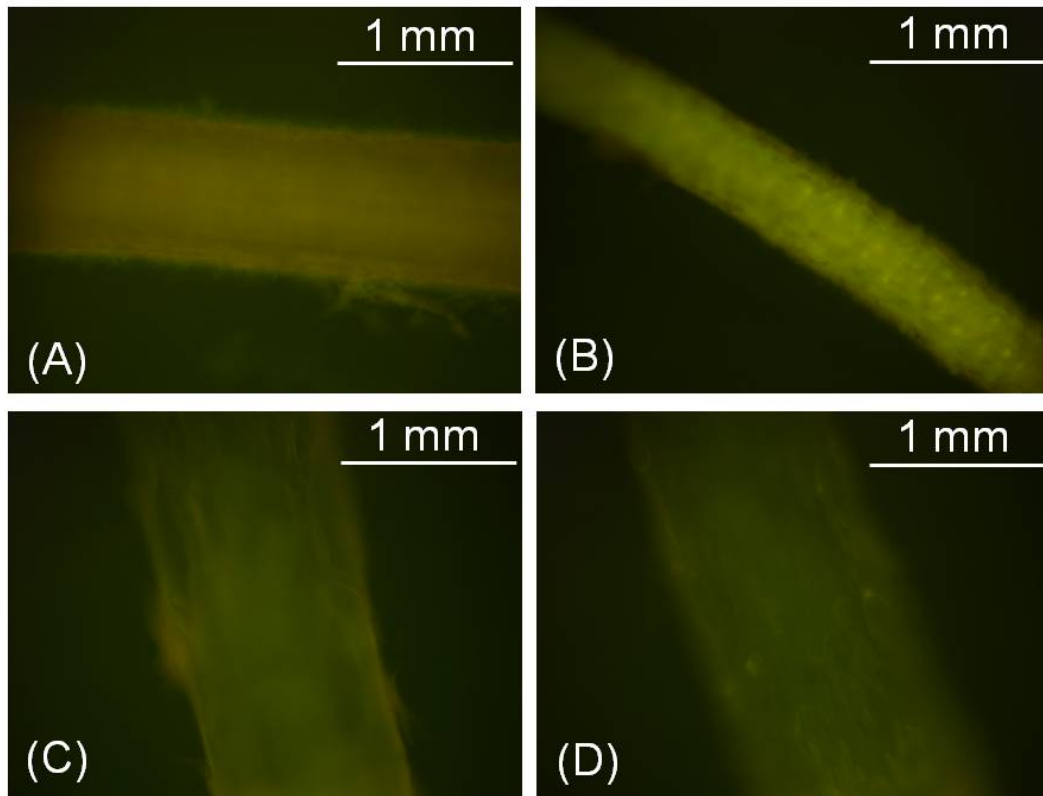


Figure 14 GFP confirmation under fluorescence microscope with Olympus C7070

WZ (aperture: 4.8, shutter: 30 sec, ISO: 80)

(A) wild-type root; (B) Root 2BV-C30-2;

(C) Root 2AR-C16-2; (D) Root 2AR-C31-1

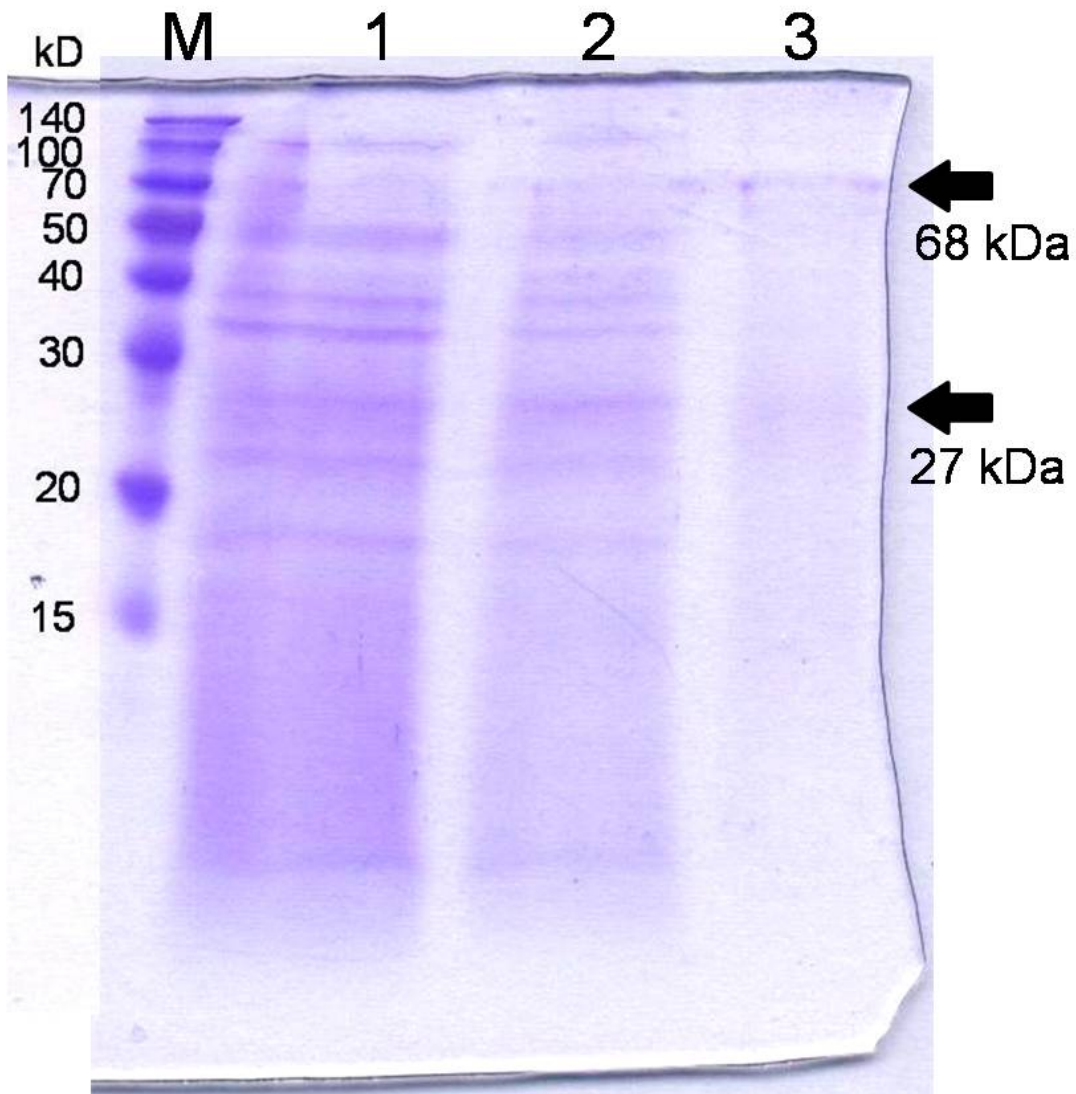
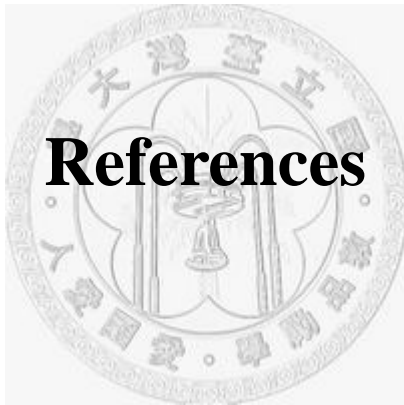


Figure 15 SDS-PAGE analysis of protein extracts from three root clones

M: marker; 1: 2AR-C16-2; 2: 2AR-C31-1; 3: 2BV-C16-2

Arrows indicate the putative position of GFP (27 kDa) and GUS (68 kDa)



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A.1 Molecular cloning

Vectors and Bateria

pCAMBIA 1201,1301, 1302 (CAMBIA, Canberra, Australia)

yT&A (Yeastern Biotech, Taipei, Taiwan)

E. coli JM 109 competent cell (ECOS 9-5) (Yeastern Biotech, Taipei, Taiwan)

PCR

Super-Therm DNA Polymerase (JRM Holdings, Kent, UK)

ExSel High Fidelity DNA Polymerase (JRM Holdings, Kent, UK)

dNTP (Viogene, Sunnyvale, CA, USA)

10x PCR reaction buffer (JRM Holdings, Kent, UK)

Primers (Blossom Biotech, Taipei, Taiwan)

Enzymes

*Bam*HI (TAKARA Bio Inc., Otsu, Shiga, Japan)

*Hind*III(TAKARA Bio Inc., Otsu, Shiga, Japan)

*Sac*II (TAKARA Bio Inc., Otsu, Shiga, Japan)

T4 DNA ligase (TAKARA Bio Inc., Otsu, Shiga, Japan)

Alkaline Phosphatase (TAKARA Bio Inc., Otsu, Shiga, Japan)

Kits

High-Speed Plasmid Mini Kit (Geneaid Biotech, Taipei, Taiwan)

Gel extraction kit (Geneaid Biotech, Taipei, Taiwan)

Wizard[®] Genomic DNA Purification Kit (Promega, Madison, WI, USA)

1.2% Agarose gel

Agarose	1.2 g
1X TAE buffer	180 mL

50X TAE buffer (per liter)

Tris Base	242 g
Acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL

* 1X TAE buffer is obtained by diluting in ddH₂O 50 times

A.2 Microbe culture

Medium

Luria Broth (LB medium) (Alpha Bioscience, Baltimore, MD, USA)

Bacto™ Agar (Bacton, Dickinson and Company, Sparks, MD, USA)

Antibiotics

Kanamycin (MD bio, Taipei, Taiwan)

Chloramphenicol (Sigma-Aldrich Co., St. Louis, MO, USA)

A.3 Plant culture

1/2 Murashige and Skoog medium (Sigma-Aldrich Co., St. Louis, MO, USA)

Content	(per liter)
MS basal salt	2.15 g
500X MS basal vitamin	1.0 ml
Sucrose	30 g
	pH 5.6 - 5.8

Cefotaxime (Yung Shin, Taichung, Taiwan)

Acetosyringon (Sigma-Aldrich Co., St. Louis, MO, USA)

A.4 Protein purification and detection

Protein Extraction

GFP extraction buffer

Phosphate buffer (pH 7.0)	50 mM
DTT	1 mM
EDTA	1 mM
SDS	0.1%

GUS extraction buffer

Phosphate buffer (pH 7.0)	50 mM
DTT	1 mM
EDTA	1 mM
Triton X-100	0.1%

Phosphate buffer (per liter)

Na ₂ HPO ₄	57.7 ml
NaH ₂ PO ₄	42.3 ml
dH ₂ O	900 ml

Protein quantification

Bradford (Bio-Rad, CA, USA)

GUS assay

5-bromo-4-chloro-3-indolyl glucuronide (Sigma-Aldrich Co., St. Louis, MO, USA)

GUS substrate	(per liter)
X-Gluc	5 mg
Dimethyl formamide	1 ml
50 mM phosphate buffer (pH 7.0)	9 ml

SDS-PAGE

15% running gel	(total volume: 10 ml)
A solution	5.0 ml
B solution	2.5 ml
10% SDS	0.1 ml
ddH ₂ O	2.35 ml
10% ammonium persulfate (APS)	0.05 ml

5% stacking gel	(total volume: 5 ml)
A solution	0.825 ml
C solution	1.55 ml
ddH ₂ O	2.53 ml
10% ammonium persulfate (APS)	0.1 ml

A solution	(per 50 ml)
Acrylamide	14.6 g
Bis	0.4 g

B solution	(per 100 ml)
Tris	18.2 g
TEMED	0.36 ml
pH	8.8

C solution	(per 100 ml)
Tris	0.6 g
TEMED	0.04 ml
pH	6.8
2X SDS sample buffer	(per 10 ml)
Tris Base	0.3 g
SDS	0.4 g
EDTA · 2Na	14.9 g
β-mercaptoethanol	1 ml
pH	6.8
Tracking dye	(per 10 ml)
Bromophenol blue	1 mg
1X TBE	5 ml
Glycerol	5 ml
5X SDS running buffer	(per 10 ml)
Tris Base	54.5 g
EDTA · 2Na	4.7 g
Boric acid,	24.8 g
SDS	0.5%
pH	8.4

Western blotting

Hybond polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA)

Western lightning-Chemical Reagent Plus (PerkinElmer Life And Analytical Sciences,
Waltham, MA, USA)

Blocking Reagent (QIAGEN, Hilden, Germany)

Anti-penta His HRP conjugate (QIAGEN, Hilden, Germany)

Transfer buffer

Glycine	192 mM
Tris	25 mM
Methanol	20%

10x TBS

Tris Base	0.5 M
NaCl	9%
pH	7.6

Blocking buffer

Blocking reagent	0.1 g
10X Blocking reagent buffer	2 ml
ddH ₂ O	18 ml

TBST

TBS	1X
Tween20	0.25 %